Published in final edited form as:

Vaccine. 2009 January 22; 27(4): 597–605. doi:10.1016/j.vaccine.2008.10.079.

Cellular and humoral immunity are synergistic in protection against types A and B *Francisella tularensis*

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Abstract

Herein we report studies with a novel combination vaccine that, when administered to mice, conferred protection against highly virulent strains of *Francisella tularensis* by stimulating both arms of the immune system. Our earlier studies with *Ft.LVS::wbtA*, an O-polysaccharide (OPS)–negative mutant derived from the available live vaccine strain of *F. tularensis* (*Ft.LVS*), elucidated the role of antibodies to the OPS—a key virulence determinant—in protection against virulent type A organisms. However, when expressed on the organism, the OPS enhances virulence. In contrast, in purified form, the OPS is completely benign. We hypothesized that a novel combination vaccine containing both a component that induces humoral immunity and a component that induces cellular immunity to this intracellular microbe would have an enhanced protective capacity over either component alone and would be much safer than the LVS vaccine. Thus we developed a combination vaccine containing both OPS (supplied in an OPS–tetanus toxoid glycoconjugate) to induce a humoral antibody response and strain *Ft.LVS::wbtA* (which is markedly attenuated by its lack of OPS) to induce a cell-mediated protective response. This vaccine protected mice against otherwise-lethal intranasal and intradermal challenge with wild-type *F. tularensis* strains Schu S4 (type A) and FSC 108 (type B). These results represent a significant advance in our understanding of immunity to *F. tularensis* and provide important insight into the development of a safer vaccine effective against infections caused by clinical type A and B strains of *F. tularensis*.

Keywords

Francisella tularensis; Combination vaccination; protective immunity

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1. Introduction

Given the potential of *Francisella tularensis* as an agent of biological warfare and bioterrorism, the development of a fully protective and safe vaccine for the prevention of tularemia has become the main focus of several research laboratories. Unfortunately, the precise mechanisms of host protective immunity to this category A pathogen are still poorly defined. Because *F. tularensis* is an intracellular pathogen, some investigators have thought it likely that immunity to this organism would prove to rely mainly on cell-mediated mechanisms [1]. However, studies from several laboratories have demonstrated that humoral immunity also plays a role [2–7]. An empirically derived, still-unlicensed vaccine strain of *F. tularensis* (live vaccine strain, or LVS) was created more than 50 years ago by exhaustive *in vitro* passage of a type B clinical isolate and is now available as an investigational new drug for at-risk individuals [8]. Unfortunately, routine use of *F. tularensis* LVS (*Ft.LVS*) as a vaccine is complicated by several issues, including adverse side effects, incomplete immunity, and the unknown derivation and undefined immunogenic properties of the strain [8–10]. These issues have prompted investigation of a new generation of live attenuated and subunit vaccines [11–14].

The nontoxic nature of the lipopolysaccharide (LPS) of *Ft.LVS* and the documented transfer of some degree of immunity by specific antibodies to the LPS make this molecule an attractive candidate for inclusion in a subunit vaccine [4]. Our recent studies suggest an important role for O-polysaccharide (OPS)-specific antibodies in conferring protection against type A strains [11]. In immunization studies, an OPS-bovine serum albumin glycoconjugate (OPS-BSA) completely protected mice against intradermal challenge with *Ft.LVS* and partially protected the animals against aerosol challenge [5]. In addition, this vaccine provided partial protection against intradermal challenge with an *F. tularensis* type A strain. However, the glycoconjugate failed to protect mice against aerosol challenge with the type A strain [5]. These studies indicate that the development of a fully protective and safe vaccine against tularemia may depend on a combination containing both the surface polysaccharide and immunodominant *F. tularensis* proteins that are recognized by T cells and that elicit effective local and systemic immune responses. It is important to note however, that when presented on the organism, the OPS contributes substantially to the virulence of the microbe. In this study, we used a novel combination vaccine containing both OPS (provided by an OPS-tetanus toxoid [OPS-TT] glycoconjugate) and immunodominant protein antigens of *Ft.LVS* (provided by *Ft.LVS::wbtA*—a live, severely attenuated OPS-negative mutant). We hypothesized (1) that the use of *Ft.LVS::wbtA* in a combination vaccine would allow the cellular immune system to respond to *F. tularensis* protein antigens and (2) that the use of an OPS-TT glycoconjugate would generate a robust specific antibody response to OPS. Indeed, the combination vaccine displayed protective efficacy comparable to that obtained with LVS, along with markedly reduced toxicity.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

F. tularensis subspecies *holarctica* strain LVS was provided by Karen Elkins (U.S. Food and Drug Administration, Rockville, MD). *Ft.LVS* was grown at 37°C in modified Mueller-Hinton broth (Difco) supplemented with glucose (0.1%), ferric pyrophosphate (0.025%), and Isovitalex (2%) or on cysteine heart agar (Difco) supplemented with 1% hemoglobin solution (VWR, <http://www.vwrsp.com>). *F. tularensis* subspecies *tularensis* type A wild-type strain Schu S4 (FSC237) was obtained from the *Francisella* Strain Collection (FSC) of the Swedish Defense Research Agency (Umea). *F. tularensis* type B wild-type strain FSC108 was provided by Dr. John Cherwonogrodsky (Defense Research and Development Canada, Alberta, Canada). The two wild-type strains were grown in Mueller-Hinton broth,

harvested, and frozen at -70°C in 1-mL aliquots (10^{10} cfu/mL) in the presence of 10% (w/v) sucrose, as described previously.

2.2. Adoptive transfer studies

The CD3^{+} T cell population was enriched from whole splenocytes harvested 2 weeks after intradermal immunization of BALB/c mice ($n = 8$) with $\sim 10^8$ cfu of *Ft.LVS::wbtA* or 10^4 cfu of the parent LVS strain. For enrichment, the Pan T cell isolation kit was used according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA). Adoptive transfer studies were performed as previously described. In brief, groups of 8 mice received an intraperitoneal injection of 0.1 mL of cell suspension containing 1×10^7 CD3^{+} T cells. The mice were challenged 2 h after cell transfer with ~ 8 times the intraperitoneal LD_{50} of *Ft.LVS*. Survival was monitored and mean time to death determined over a 28-day observation period.

2.3. Ft.LVS OPS purification

For production of OPS, *Ft.LVS* from a fresh cysteine heart agar plate was inoculated into five 250-mL vented-cap erlenmeyer flasks, each containing 100 mL of tryptic soy broth with 0.1% cysteine, 0.025% ferric pyrophosphate, and 0.1% Antifoam 204 (T soy with additives). After incubation overnight at 37°C and aeration at 200 rpm, the cultures were used to inoculate five 3-L vented-cap erlenmeyer flasks containing 2 L of T-soy with additives, which were then incubated as described above for 72 h. Cells were harvested by centrifugation and frozen at -80°C until used for OPS purification.

OPS was purified by the addition of a hot solution of fresh 50% phenol to thawed *Ft.LVS* cells (at a ratio of 1 g of cells to 10 mL of phenol solution), with subsequent mixing for 2 h at 68°C with use of sterile glass beads and an overhead mixer. After further mixing overnight at 4°C , cell debris and phenol were removed by centrifugation ($6K \times g$ for 20 min at 4°C) in Teflon FEP centrifuge bottles (Nalgene). After the top aqueous phase was removed, additional phenol was removed by dilution with one volume of water and two volumes of ether. The solution was mixed vigorously for 10 min in a separatory funnel and allowed to separate overnight at room temperature. The bottom aqueous phase was decanted, and residual ether was removed with a rotary evaporator. The sample was lyophilized to reduce its volume before dialysis against water and enzyme treatment. Nucleic acid and protein were degraded by sequential treatment with DNase, RNase, and pronase (Seward Ltd., Worthington, UK). The solution was then clarified by low-speed centrifugation ($5K \times g$ overnight at 4°C). LPS was sedimented and washed three times with water by ultracentrifugation ($60K \times g$ overnight at 4°C). Lipid A was hydrolyzed by treatment with acetic acid (6%, v/v) at 100°C for 2 h. The solution was cooled to room temperature before centrifugation ($15K \times g$ for 10 min at 4°C) to sediment the cleared lipid. OPS was further purified by gel filtration. NMR and wavelength scanning at 206, 260, and 280 nm were performed to establish purity.

2.4. Preparation of the OPS-TT glycoconjugate

OPS was conjugated to TT as previously described [5]. In brief, OPS was suspended in water and activated with cyanogen bromide at pH 10.5 and then with adipic dihydrazide at pH 8.5 and 4°C , with subsequent dialysis and lyophilization. For conjugation, activated OPS and monomeric TT (in a ratio of 1.5:1) were suspended in saline and allowed to react with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl at pH 5.5 in an ice bath. The conjugate was then passed over a Superdex 200 column, and void-volume fractions were pooled and dialyzed against saline with thimerosal. The conjugate ratio of OPS to TT was 1:2.5.

2.5. Immunization and Ft.LVS challenge studies

Male BALB/cByJ mice (6–8 weeks old; Jackson Laboratory, Bar Harbor, ME) were caged in a microisolator in a pathogen-free environment in the animal facility at Harvard Medical School. For these experiments, there were seven mice per group. Mice received two doses (2 weeks apart) of mutant strain *Ft.LVS::wbtA* ($\sim 10^7$ cfu, 50 μ L per mouse, given intranasally) and OPS-TT (8 μ g of OPS, 500 μ L per mouse, given subcutaneously with an 18-gauge needle). The glycoconjugate was emulsified with either complete Freund's adjuvant (first dose) or incomplete Freund's adjuvant (second dose) in a 1:1 ratio. Mice immunized with an equivalent dose of *Ft.LVS::wbtA* alone, OPS-TT alone, or adjuvant alone served as controls. After a 4-week rest period, all mice were challenged intranasally on day 42 with 3.7×10^5 *Ft.LVS* organisms (~ 370 times the intranasal LD₅₀). Survival was monitored for 28 days after challenge, at which point the survivors were humanely sacrificed.

2.6. Immunization and wild-type challenge studies

Specific pathogen-free female BALB/c mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada) and were used in experiments starting at 8–10 weeks of age. Mice were maintained and used in accordance with the recommendations of the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care. For immunization with the combination, mice were inoculated twice (with doses 2 weeks apart), as described above, with 50 μ L of an *Ft.LVS::wbtA* suspension (1.5×10^7 cfu per mouse, given intranasally) and 0.1 mL of an emulsified suspension of OPS-TT (8 μ g of OPS) and incomplete Freund's adjuvant at a ratio of 1:1 (given subcutaneously). Challenge with virulent type A strain FSC237 (Schu S4) or type B strain FSC 108 took place in a federally certified and Select Agent-approved small-animal level 3 biocontainment facility. Mice that survived the challenge for 21 days were killed; their tissues were removed, homogenized, and plated onto cysteine heart agar supplemented with 1% hemoglobin solution (CHAH). Actual bacterial cfu counts in the inocula and the challenge dose were determined by plating of 10-fold serial dilutions onto CHAH.

2.7. Dissemination studies

To determine LVS bacterial burden, tissues from mice were first hand-mashed and then homogenized with a Stomacher^R 80-paddle action blender (Seward). Serial dilutions were prepared with sterile 1X PBS supplemented with 2% fetal bovine serum. A 10- μ L volume of each dilution was streaked onto CHAH plates, and the plates were incubated at 37°C in the presence of 5% CO₂ until colonies became visible. To determine bacterial burdens of FSC 108 and Schu S4, organs were minced with scissors and homogenized in aerosol-proof homogenizers. Serial dilutions of homogenates were plated as described above.

2.8. Histopathology

Spleen and liver tissues were harvested from each group and fixed in Bouin's fixative solution for histopathologic assessment. Fixed tissues were embedded in paraffin, and sections were subjected to hematoxylin and eosin staining. All histopathology slides were evaluated and scored by a veterinary pathologist in a blinded fashion.

2.9. Statistical analysis

Kaplan-Meier survival analysis and the log-rank test to determine *P* values for survival curves were performed with GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA).

3. Results

3.1. Induction of both cellular and humoral immunity is required for an effective tularemia vaccine

Previous studies from our laboratory have demonstrated that *Ft.LVS::wbtA*, an OPS-negative mutant of *Ft.LVS*, is highly attenuated ($\sim 10^7$ times less virulent in terms of mouse lethality). Despite this significant attenuation, the *Ft.LVS::wbtA* mutant remains immunogenic and confers protective immunity to mice against challenge with an ordinarily lethal intradermal dose of *Ft.LVS* or a fully virulent clinical type B isolate of *F. tularensis*. Because *Ft.LVS::wbtA* lacks OPS expression, we hypothesized that the protective efficacy of this mutant might be due to the induction of an effective cellular immune response by bacterial protein antigens. To examine the role of immune cells (i.e., cell-mediated immunity), we performed adoptive transfer experiments with a splenic CD3⁺ T cell-enriched population from immunized mice. In brief, 2 weeks after primary immunization of mice with mutant strain *Ft.LVS::wbtA* or parent strain *Ft.LVS*, 1×10^7 splenic CD3⁺ T cells were transferred to naïve mice by the intraperitoneal route (see Materials and Methods). Two hours after the initial cell transfer, recipient mice were challenged by the intraperitoneal route with 8 times the intraperitoneal LD₅₀ of *Ft.LVS*, and survival was monitored. As expected, all mice that had received *Ft.LVS*-primed CD3⁺ T cells survived homologous challenge (Fig. 1A, *, $P = 0.0001$). The level of protection was also significant among mice that received *Ft.LVS::wbtA*-primed CD3⁺ T cells: 5 of 8 mice in this group survived otherwise-lethal challenge with the parent LVS strain (Fig. 1A, **, $P = 0.0001$). In contrast, all mice that received naïve CD3⁺ T cells succumbed to infection 5–7 days after a similar challenge dose.

Our previous immunization/challenge studies indicate that active immunization with *Ft.LVS*—but not with *Ft.LVS::wbtA*—protects mice against a low but otherwise lethal intradermal challenge dose of a fully virulent type A strain [11]. The major difference between the two immunizing strains lies in the complete lack of surface OPS expression in the *wbtA* mutant strain; thus antibodies to OPS may play an important role in delaying the progression of disease due to virulent type A *F. tularensis*. In passive serum transfer studies, all mice receiving *Ft.LVS::wbtA* immune serum succumbed to infection, as did control mice that received normal rabbit serum. However, all mice receiving *Ft.LVS* polyclonal immune serum survived this otherwise-lethal *Ft.LVS* challenge (Fig. 1B, ***, $P < 0.0001$). These studies, which are consistent with our previous passive serum transfer studies, suggest that antibodies (most likely those to OPS) are important in conferring protection, as inferred by comparison of the protective capacities of anti-*Ft.LVS* polyclonal immune serum and *Ft.LVS::wbtA* immune serum. Taken together, these adoptive cell transfer and passive serum transfer studies strengthen the evidence indicating that both cellular and humoral immunity are important for an effective tularemia vaccine.

3.2. Development of the OPS-TT glycoconjugate

Despite the greater protection observed after *Ft.LVS* immunization than after *Ft.LVS::wbtA* immunization, *Ft.LVS*-based vaccine has met with limited acceptance both because of the lack of knowledge about the mechanism of its attenuation and because of the toxicity associated with its use. In an attempt to incorporate all the immunogenic components of *Ft.LVS* vaccine while eliminating potential adverse effects, we developed a novel approach using an OPS-TT glycoconjugate in combination with attenuated mutant strain *Ft.LVS::wbtA*. High-molecular-weight OPS from *Ft.LVS* was purified and conjugated to TT as previously described [5]. Silver staining and immunoblot analysis using α -TT and an *Ft.LVS* OPS-specific monoclonal antibody (mAb2033) confirmed the presence of the glycoconjugate (data not shown). Our initial studies of vaccine potential indicated that mice

immunized with the glycoconjugate subcutaneously were fully protected against an otherwise-lethal intradermal *Ft.LVS* challenge (15 times the intradermal LD₅₀). In contrast, only a delay in time to death was recorded in another group of glycoconjugate-immunized mice challenged via the intranasal route with 20 times the intranasal LD₅₀ (data not shown). Quantitative studies of the bacterial burden in reticuloendothelial tissues yielded data consistent with the results of survival studies (data not shown).

3.3 The combination vaccine provides enhanced protection against lethal intranasal challenge with *Ft.LVS*

Mice received two doses (2 weeks apart) of both *Ft.LVS::wbtA* (via the intranasal route) and the OPS-TT glycoconjugate (via the subcutaneous route). Four weeks after the second immunization, animals were challenged via the intranasal route with a lethal dose of *Ft.LVS* (370 times the intranasal LD₅₀) and survival monitored (Fig. 2A). Similar to those in the adjuvant control group, mice immunized with either the glycoconjugate or the *Ft.LVS::wbtA* strain alone showed obvious signs of infection and succumbed to intranasal *Ft.LVS* challenge (Fig. 2B). In contrast, mice given the combination vaccine remained asymptomatic and survived this high-dose intranasal challenge (Fig. 2B).

The efficacy of the combination vaccine was further assessed by determination of the bacterial burden in reticuloendothelial tissues (lung, liver, and spleen) at days 5, 8, and 28 after intranasal challenge. By day 5 (Fig. 2C), mice given the combination vaccine harbored ~3–4 logs fewer bacteria in the spleen and liver than those in the adjuvant control group. Although significant bacterial loads were found in the lung tissue of combination-vaccine recipients, counts were nevertheless ~1 log lower than those in the adjuvant group. On day 8 (Fig. 2D), the bacterial loads in all tissues of mice receiving the combination vaccine were significantly lower than those of the other vaccinated groups. The organ-specific bacterial burden in the liver and spleen of combination-vaccine immunized mice was below the level of detection at day 28 after challenge (Fig. 2E). The bacterial load was reduced by ~4 logs in lung tissue from this group at day 28.

3.4. Mice given the combination vaccine exhibit less severe pathology after *Ft.LVS* challenge

Histopathologic studies of liver tissue from mice vaccinated with the glycoconjugate or with mutant strain *Ft.LVS::wbtA* (Figs. 3A and 3D, respectively) showed obvious signs of inflammation and severe necrosis, with several foci of neutrophilic infiltration on day 7 after intranasal challenge. In stark contrast, liver tissue from mice given the combination vaccine contained only minor sporadic infiltrations, which were most likely due to the low numbers of bacteria in these tissues (Fig. 3G). Histopathologic examination of splenic tissue from glycoconjugate- or *wbtA*-vaccinated mice showed massive infiltration by neutrophils and macrophages (pyogranuloma) on day 7 after challenge (Figs. 3B and 3E), while normal architecture with only mild infiltration by polymorphonuclear leukocytes was evident in spleen tissue from mice immunized with the combination vaccine (Fig. 3H). In contrast to the findings on spleen and liver histology, histopathologic analysis of lung tissue from mice given the combination vaccine (Fig. 3I) revealed obvious signs of inflammation (bronchopneumonic lesions) and necrotic cellular debris similar to that found in lung tissue from mice vaccinated with the glycoconjugate (Fig. 3C) or the *wbtA* strain (Fig. 3F). On day 28, however, histopathologic examination of liver, spleen, and lung tissues from combination-vaccine recipients demonstrated only minor sporadic infiltration, with a histology closely resembling that of normal tissue (Figs. 3J, 3K, and 3L).

The combination vaccine confers protection against intranasal challenge with wild-type *F. tularensis*

The efficacy of protective immunity generated by the combination vaccine was evaluated after an otherwise-lethal intranasal challenge with wild-type organisms of subspecies type A (strain Schu S4) or type B (strain FSC 108) (Fig. 4A). As shown in Fig. 4B, the time to death after a low but lethal type A challenge was significantly longer among mice given the combination vaccine (median survival time: 15 days) than among adjuvant control mice (median survival time: 7 days). Complete protection was documented when combination-vaccinated mice were challenged intranasally with ~10 cfu of virulent type B strain FSC 108 (Fig. 4C). Moreover, a high level of protection was noted when combination-vaccine recipients were challenged intranasally with ~100 cfu of virulent type B organisms 6 weeks after second immunization dose (Fig. 4D). Determination of the bacterial load in reticuloendothelial tissues after type A challenge indicated a 2-log bacterial reduction in spleen and liver tissues from combination-vaccinated mice (Table 1) and the presence of a similar bacterial burden in lung tissues from both groups. The bacterial burden documented in blood samples from the vaccinated group was 3 logs lower than that in blood from control mice after type A intranasal challenge (Table 1). Consistent with the complete protection observed after a low-dose intranasal challenge with type B organisms, all reticuloendothelial tissues from the vaccinated group exhibited a cfu reduction of ~4–5 logs from counts in the adjuvant control group (Table 1). Remarkably, a 10⁶-fold reduction in bacterial burden was documented when blood samples from the vaccinated group were examined after type B challenge (Table 1).

3.5. The combination vaccine confers protection against high-dose intradermal challenge with wild-type *F. tularensis*

Six weeks after the second combination vaccine immunization dose, mice were challenged intradermally with a high dose (1000 cfu) of wild-type *F. tularensis* type A (strain Schu S4). A high level of protection was observed, with 60% survival (Fig. 5A). Complete protection of immunized recipients was documented after intradermal challenge with the same dose of *F. tularensis* type B (strain FSC 108) (Fig. 5B). Although combination-immunized mice showed no overt signs of infection, bacteriologic studies of survivors on day 28 demonstrated $\sim 2.256 \pm 0.568$ cfu per organ of the type B strain and $\sim 3.174 \pm 0.540$ cfu per organ of the type A strain in splenic tissue.

4. Discussion

The pivotal role of T cell-mediated events in the control of tularemia has been well established [6,15–20]. However, studies from several laboratories have shown that humoral immunity also plays a critical role, especially in the early phase of type B *F. tularensis* infection [21,22]. Using B cell-deficient mice, Stenmark *et al* demonstrated the important role of humoral antibodies in protection against a type B strain of *F. tularensis* [23]. Drabick *et al* found that pooled immune sera from human LVS recipients protected mice fully against challenge with 10,000 times the LD₅₀ of the LVS strain [24]. Fulop *et al* demonstrated the role of antibodies to LPS in protection against LVS in T cell-depleted mice [2,4]. Our recent studies indicate that protective antibodies directed specifically toward the OPS of *Ft.*LVS LPS are an important component of humoral immunity [11]. On the basis of these findings, it seems reasonable to conclude that an efficacious tularemia vaccine should effectively elicit both humoral components (OPS-specific antibodies) and cell-mediated components (protein antigen[s]). *Ft.*LVS fulfills these criteria but has the potential to cause adverse side effects. The identification of key virulence factors and candidate protein antigens has been challenging because of a lack of sequenced genomes and of well-defined genetic manipulation techniques [8]. Recently, however, these impediments have

been overcome, and significant progress has been made toward both genetic manipulation of *F. tularensis* and identification of candidate antigens [25]. Although several putative candidate protein antigens that are immunogenic and promote T cell proliferation have been identified, none have been shown to induce protective immunity against type A challenge in the mouse model [2,26–29].

The currently available, empirically derived, still-unlicensed vaccine strain of *F. tularensis* is effective, but its use is complicated by significant undesirable side effects. To elucidate the role of immunodominant protein antigens and to address the need for a safe tularemia vaccine, we have used a novel combination containing the OPS-deficient mutant strain *Ft.LVS::wbtA* as a source of protein antigens (to induce cellular immunity) and an OPS-TT glycoconjugate as an inducer of a robust OPS antibody response (to elicit humoral immunity). Use of the *Ft.LVS::wbtA* strain is an attractive option for two reasons. First, this strain is markedly attenuated compared with LVS [11,30]. Second, the lack of an immunodominant surface polysaccharide in the *wbtA* strain may permit better exposure of other antigens that may be critical for protective immunity. Furthermore, use of OPS from *Ft.LVS* is expected to facilitate cross protection against type A strain, since the chemical structure of OPS from *F. tularensis* subspecies *tularensis* (Schu S4) and *F. tularensis* subspecies *holarctica* (LVS) has been demonstrated to be identical [31,32].

Pneumonic tularemia—the most potent form of the disease—is thought to result from inhalation of highly infectious type A *F. tularensis* via the aerosol route [1,8,29,33]. After aerosol exposure, the bacterium proliferates exponentially in the lung. Once the organ load in the lung reaches a certain threshold, infection disseminates and becomes systemic; proliferation of organisms in spleen and liver tissues ultimately leads to organ failure and death. An ideal tularemia vaccine thus should contain both the proliferation of the pathogen at the portal of entry (the respiratory tract) and its dissemination to reticuloendothelial tissues. Compelling evidence from a growing body of literature has indicated the importance of aerosol and/or intranasal immunization for optimal priming of mucosal immunity [34]. In addition, the control of *F. tularensis*-mediated respiratory infections (especially those initiated by type A organisms) is enhanced after intranasal immunization [10,35,36]. Aerosol but not intradermal immunization with LVS protects mice against subsequent aerosol challenge with a highly virulent type A strain by an $\alpha\beta$ T cell and interferon gamma dependent mechanism. It is important to note, however, that mice are much more susceptible to exposure to *F. tularensis* LVS via the intranasal route (LD_{50} = 1000 cfu) than via the intradermal route (LD_{50} = 10^6 cfu) [35,37]. Thus intranasal immunization with *Ft.LVS* often results in infection and sometimes causes death. In humans, aerosol vaccination with effective doses of LVS can also result in overt infection [10]. To ensure the safety of the immunization regimen, we have used *Ft.LVS::wbtA*, a highly attenuated OPS mutant of *Ft.LVS*. Our previous studies had shown that this mutant, although significantly attenuated, retains its ability to confer protective immunity to mice against challenge with an otherwise-lethal dose of either *Ft.LVS* or a fully virulent clinical isolate of *F. tularensis* type B [11]. The importance of LPS/OPS antibodies in protective immunity is well documented [4,11]. To compensate for the lack of OPS in the *wbtA* vaccine strain, we purified the core OPS from *Ft.LVS* and chemically conjugated it to a tetanus toxoid carrier protein, thereby generating a T cell-dependent OPS-TT glycoconjugate as previously described [5]. We envisaged that use of the OPS mutant strain in combination with the OPS-TT glycoconjugate would yield a safe immunization regimen that retains all the immunogenic components of *Ft.LVS*. We administered the *wbtA* vaccine strain by the intranasal route and the OPS-TT glycoconjugate by the subcutaneous route.

This combination vaccine afforded complete protection against high-dose *Ft.LVS* intranasal challenge (~400 times the intranasal LD_{50}). The combination prevented the proliferation of

Ft.LVS; immunization was followed by the resolution of *Ft.LVS*-initiated respiratory tularemia. Histopathologic study of liver and spleen tissues from mice receiving the combination vaccine revealed only minor sporadic infiltrations that most likely were due to the low numbers of bacteria in these tissues. Although a significant level of infiltration and a higher bacterial count were observed in lung tissue from these mice during the initial stages of infection, the combination vaccine appeared to control the bacterial burden. By day 28 after challenge, the pneumonic lesions had resolved and the lung-tissue architecture was nearly normal, with only mild infiltrates. This approach was not associated with specific side effects like those that typically follow immunization with *Ft.LVS* (especially via the intranasal route) in the murine tularemia model.

Likewise, high-level protection was conferred when combination-immunized mice were challenged intranasally with both a low dose (~10 cfu) and a high dose (~100 cfu) of wild-type organisms of type B strain FSC 108. Moreover, time to death was significantly longer when similarly immunized mice were challenged intranasally with a low dose (~10 cfu) of type A strain Schu S4. The combination vaccine effectively protected mice against intradermal challenge with a high dose (~1000 cfu) of wild-type organisms of type A (Schu S4) or type B (FSC 108). The ability of this combination vaccine to control proliferation and dissemination more effectively for wild-type organisms of type B than for wild-type organisms of type A was further evidenced by the significantly greater reduction in the number of cfu in the blood and in organ cultures of immunized mice after type B challenge.

Although immunization with the combination vaccine conferred only partial protection against intranasal challenge with wild-type strain Schu S4 (type A), the overall level of protection was significantly greater than in previous glycoconjugate immunization studies [5]. Additionally, the overall protection observed here following a combination vaccine was far superior to the previously observed protection following single immunization with *Ft.LVS::wbtA* vaccine strain [11]. In those investigations, only partial protection was noted when mice were subjected to low-dose aerosol challenge with wild-type organisms of type B or to intradermal challenge with Schu S4, and no protection was observed against aerosol challenge with a highly virulent type A strain [5].

The exact reason for the inability of the combination regimen to completely control respiratory tularemia initiated by type A *F. tularensis* is not clear. It is important to note that comparative genomic analyses have indicated the existence of genetic differences between the *F.tularensis* subspecies [38]. This and other studies have indicated that more than 20 additional genes exist which are unique to subspecies *tularensis* and are missing from subspecies *holaractica* [13,38]. The *Ft.LVS::wbtA* vaccine strain used in this study is a *holaractica* (type B) derivative; one likely explanation for the inability of the current combination vaccine to completely protect against a type A intranasal challenge is that the *wbtA* vaccine strain may lack the critical type A-specific antigen(s) that are instrumental in controlling type A-induced respiratory tularemia [38]. This possibility is supported by the findings of Wu *et al.*, who have shown that mice primed with LVS raise a stronger type A-specific protective immune response upon receipt of a boost with type A—but not with the LVS strain—before type A challenge [35]. Further studies using a similar OPS-negative live attenuated strain with a type A background are warranted to address these issues. Nevertheless, the results of this study represent a significant advance toward the provision of a safer vaccine to combat tularemia due to wild-type strains of *F. tularensis* type A and type B.

Acknowledgments

The authors thank Dr. R. T Bronson for expert contributions and Hua Shen for studies employing virulent isolates of *F. tularensis*. This work was supported by the New England Regional Center of Excellence for Biodefense and Emerging Infectious Diseases (AI057157).and grant AI48474 to Dr. Wayne Conlan.

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Fig. 1.

Adoptive transfer studies: (A) Splenic CD3⁺ T cells were enriched after a 2-week prime with either *Ft.LVS* or *Ft.LVS::wbtA*. CD3⁺ T cells enriched from splenocytes of age- and sex-matched naïve mice served as controls. Approximately 1×10^7 CD3⁺ T cells of each population [*Ft.LVS*-primed (■), *Ft.LVS::wbtA*-primed (▲), and naïve (●)] were adoptively transferred via the intraperitoneal route to naïve male BALB/c mice. All groups were challenged 2 h after adoptive transfer with an ordinarily lethal dose of *Ft.LVS* (8 times the intraperitoneal LD₅₀). Mice were monitored for 28 days after challenge. The survival curves for groups receiving *Ft.LVS*-primed and *Ft.LVS::wbtA*-primed CD3⁺ T cells, respectively, differed significantly from that for mice given naïve CD3⁺ T cells (*, **, $P = 0.0001$); (B)

Passive serum transfer studies. Mice (8 per group) received 100 μ L of polyclonal rabbit serum [*Ft.LVS* polyclonal serum (■), *Ft.LVS::wbtA* polyclonal serum (▲), or normal rabbit polyclonal serum (●)] via the intracardiac route and were challenged after 24 h with *Ft.LVS* (30 times the intradermal LD₅₀). The survival curve for the *Ft.LVS* polyclonal serum group differed significantly from those for the other two groups (***, $P < 0.0001$).

Fig. 2.

Survival after an ordinarily lethal intranasal challenge with *Ft.LVS* in a representative study: (A) Immunization schedule. BALB/c mice received two immunizing doses (2 weeks apart) and were challenged intranasally after a 4-week rest period with *Ft.LVS* (370 times the intranasal LD₅₀). Animals were monitored daily for 28 days after challenge; (B) Survival of mice (7 per group) after immunization with the TT-OPS glycoconjugate alone (■), with *Ft.LVS::wbtA* (▼), with the combination vaccine (●), or with adjuvant alone (▲); (C and D) Bacterial burden in spleen, lung, and liver tissues on days 5 and 8, respectively, after an ordinarily lethal intranasal challenge with *Ft.LVS* in mice previously immunized with adjuvant alone (■), TT-OPS alone (●), *Ft.LVS::wbtA* alone (▲), or the combination vaccine (□); (E) Bacterial burden on day 28 after an otherwise-lethal intranasal challenge with *Ft.LVS* in mice receiving the combination vaccine.

Fig. 3. Histopathologic features: Tissues from mice vaccinated with TT-OPS alone (day 7, A–C), *Ft.LVS::wbtA* alone (day 7, D–F), or the combination vaccine (day 7, G–I; day 28, J–L) (hematoxylin and eosin). Asterisks indicate sites of massive infiltration in tissues; arrows indicate areas of severe necrosis in lung tissue.

Fig. 4. Survival of mice (5 per group) after lethal intranasal challenge with a wild-type strain of *F. tularensis*: (A) Immunization schedule. BALB/c mice received two immunizing doses (2 weeks apart) and were challenged intranasally after a 4-week (panel B and C) or 6-week (panel D) rest period with an ordinarily lethal dose of a wild-type strain; (B) Survival after intranasal challenge with ~10 cfu of virulent type A strain Schu S4, (FSC 237); (C) Survival after intranasal challenge with ~10 cfu of virulent type B strain (FSC 108); (D) Survival after intranasal challenge with ~100 cfu of virulent type B strain (FSC 108).

Fig. 5. Survival of BALB/c mice (5 per group) after high dose lethal intradermal challenge with wild-type strain of *F. tularensis* following 6-week post immunization: (A) Survival after challenge with ~1000 times the LD₅₀ of *F. tularensis* wild-type type A strain Schu S4 (FSC 237); (B) Survival after challenge with ~1000 times the LD₅₀ of *F. tularensis* wild-type type B strain (FSC 108).

Table 1

Bacterial burdens in mock immunized and immunized mice challenged intranasally with type A or type B *F. tularensis*.

Mice	Challenge strain	Day of infection	Log10 CFU $\pm$ SD / organ (proportion infected)				Blood /mL
			liver	spleen	lungs		
Mock immunized	SCHU S4	5	6.87 $\pm$ 1.46 ^a	7.55 $\pm$ 1.53 ^a	6.26 $\pm$ 1.54		5.56 $\pm$ 1.0 ^a (4/5)
Combination vaccine			4.88 $\pm$ 1.18	5.10 $\pm$ 1.64	6.30 $\pm$ 0.89		2.48 $\pm$ 0.67 (2/5)
Mock immunized	FSC 108	8	8.76 $\pm$ 2.02 ^a	8.51 $\pm$ 1.90 ^a	8.25 $\pm$ 1.68 ^a		8.00 $\pm$ 2.05 ^a (4/5)
Combination vaccine			2.92 $\pm$ 0.86	3.97 $\pm$ 0.62 (3/5)	3.57 $\pm$ 1.07		2.15 $\pm$ 0.22 (2/5)

Mice were challenged IN with 6 or 7 CFU of type A strain, SCHU S4 or type B strain, FSC 108 respectively, four weeks post-vaccination. The former mice were killed on day 5, and the latter mice on day 8 post-challenge.

^aSignificantly higher burden ($P<0.05$) than in immunized mice by two tailed t test.