INFECTION AND IMMUNITY, Aug. 2008, p. 3664–3671 Vol. 76, No. 8
0019-9567/08/$08.00+0 doi:10.1128/IAI.00374-08

Native Outer Membrane Proteins Protect Mice against Pulmonary Challenge with Virulent Type A *Francisella tularensis*▽

Jason F. Huntley,[1] Patrick G. Conley,[1] David A. Rasko,[1] Kayla E. Hagman,[1] Michael A. Apicella,[2] and Michael V. Norgard[1]*

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390,[1] and Department of Microbiology, Roy J. and Lucille A. Carver School of Medicine, University of Iowa, Iowa City, Iowa 52242[2]

Received 24 March 2008/Returned for modification 30 April 2008/Accepted 21 May 2008

***Francisella tularensis* is a gram-negative intracellular bacterium and the causative agent of the zoonotic disease tularemia. *F. tularensis* is a category A select agent and thus a potential agent of bioterrorism. Whereas an *F. tularensis* live, attenuated vaccine strain (LVS) is the basis of an investigational vaccine, this vaccine is not licensed for human use because of efficacy and safety concerns. In the present study, we immunized mice with isolated native outer membrane proteins (OMPs), ethanol-inactivated LVS (iLVS), or purified LVS lipopolysaccharide (LPS) and assessed the ability of each vaccine preparation to protect mice against pulmonary challenge with the virulent type A *F. tularensis* strain SchuS4. Antibody isotyping indicated that both Th1 and Th2 antibody responses were generated in mice after immunization with OMPs or iLVS, whereas LPS immunization resulted in only immunoglobulin A production. In survival studies, OMP immunization provided the greatest level of protection (50% survival at 20 days after infection with SchuS4), and there were associated 3-log reductions in the spleen and liver bacterial burdens (compared to nonvaccinated mice). Cytokine quantitation for the sera of SchuS4-challenged mice indicated that OMP and iLVS immunizations induced high levels of tumor necrosis factor alpha and interleukin-2 (IL-2) production, whereas only OMP immunization induced high levels of IL-10 production. By comparison, high levels of proinflammatory cytokines, including RANTES, granulocyte colony-stimulating factor, IL-6, IL-1α, IL-12p40, and KC, in nonvaccinated mice indicated that these cytokines may facilitate disease progression. Taken together, the results of this study demonstrate the potential utility of an OMP subunit (acellular) vaccine for protecting mammals against type A *F. tularensis*.**

Francisella tularensis, the causative agent of tularemia, is a gram-negative intracellular coccobacillus that is capable of causing acute infection following exposure to fewer than 10 organisms (38). *F. tularensis* is considered to be one of the most infectious bacterial pathogens known because of its ease of aerosolization, high infectivity, and propensity for causing severe morbidity and mortality in a number of mammalian species, including humans. Given these considerations, *F. tularensis* long has been recognized as a potential bioweapon and thus has been designated a category A select agent (30). Reports that Soviet scientists engineered antibiotic- and vaccine-resistant strains of *F. tularensis* in the 1990s are a further cause for concern (38).

Currently, no vaccine is approved for human use to prevent *F. tularensis* infection. The attenuated *F. tularensis* subsp. *holarctica* (type B) live vaccine strain (LVS) was originally developed and tested in the 1950s and 1960s, but its status is still considered investigational by the Food and Drug Administration (12). Indeed, it is not certain whether LVS will ever obtain approval and be licensed for widespread human use, as little is known about the genetic mutations that contribute to its attenuation. Safety and efficacy issues for human LVS immunization also need to be addressed more completely. Whereas scarification is the conventional delivery method for current LVS immunizations, a 1966 study demonstrated that aerosol LVS immunization of humans provided stronger protection against type A (*F. tularensis* subsp. *tularensis*) pulmonary challenge than scarification. However, mild to severe reactions, including lymph node inflammation and chronic flulike symptoms, were noted in aerosol-immunized humans (23).

In order to circumvent the obvious ethical constraints associated with experimental vaccine development and virulent *F. tularensis* challenge studies with humans, more recent LVS experiments and refinement of the delivery method have been conducted using murine infection models. In BALB/c and C3H/HeN mice, LVS immunization provides at least partial protection against virulent type A *F. tularensis* aerosol challenge (13, 37, 46). While aerosol LVS immunization appears to provide the greatest level of protection against type A aerosol challenge, LVS infections of mice are inconsistent, often resulting in death (13, 46). Oral LVS inoculation has been proposed as another potential immunization method, as orally immunized BALB/c mice were protected from respiratory challenge with either virulent type A or B *F. tularensis* (27). However, oral immunization is equally problematic, as comparably immunized C57BL/6 mice were not protected from aerosol challenge, and only a 2-log difference in inoculation separated a nonprotective immune response from immunization-induced death (27).

The use of an inactivated (subunit) *F. tularensis* vaccine has obvious potential safety advantages over the use of live, attenuated vaccines. However, various nonviable formulations have

* Corresponding author. Mailing address: Department of Microbiology, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, TX 75390. Phone: (214) 648-5900. Fax: (214) 648-5905. E-mail: michael.norgard@utsouthwestern.edu.

▽ Published ahead of print on 27 May 2008.

provided only limited protection against aerosol challenge with virulent strains of *F. tularensis*, including type A *F. tularensis* subsp. *tularensis* strains (12). In a recent study, irradiation-inactivated LVS mixed with immune-stimulating complexes and CpG oligonucleotides protected mice from virulent type B aerosol challenge but not from virulent type A aerosol challenge (17). Little is known about *F. tularensis* virulence factors, making the development of a subunit vaccine problematic. To date, the only antigen that has provided any appreciable degree of protection has been lipopolysaccharide (LPS). While LPS alone was not able to protect mice from either virulent type A or type B aerosol challenge (11), a bovine serum albumin-LPS conjugate provided limited protection against virulent type B aerosol challenge (10).

Rational vaccine design should take into consideration the correlates of protective immunity. Unfortunately, little is known about the mechanisms or effector molecules that protect against virulent *F. tularensis* strains. Given that *F. tularensis* is an intracellular pathogen, it is not surprising that cell-mediated immunity (CMI) has been implicated as a factor that plays a major role in containing tularemia (1, 14, 28). However, despite the intracellular lifestyle of *F. tularensis*, there also is a well-documented role for humoral immunity in protection against tularemia (15, 19, 26, 34). This is not entirely surprising, as antibodies have been shown to protect against other respiratory pathogens and can even enter host target cells to inhibit intracellular bacterial growth (6, 35).

We previously described a new method for the isolation of outer membrane proteins (OMPs) from *F. tularensis* (24). OMPs often are strategic for facilitating bacterial host invasion, intracellular survival, virulence, and immune evasion (20, 22, 36). Their significance also is highlighted by the fact that they can serve as protective vaccines for a number of bacterial diseases (7, 31, 44). In the present study, we examined the potential ability of OMPs to protect against pulmonary type A *F. tularensis* infection in mice. OMP immunization provided significant protection against *F. tularensis* challenge and reduced spleen and liver bacterial burdens in mice. Antibody isotype analyses indicated that OMP immunization induced the production of high titers of immunoglobulin A (IgA), IgG1, IgG2a, IgG3, and IgM. Further, cytokine analyses at day 5 postinfection indicated that OMP immunization induced the production of high levels of the regulatory cytokine interleukin-10 (IL-10), which may have resulted in the suppression of at least six proinflammatory cytokines. The results of this study demonstrate, for the first time, that a subunit acellular vaccine was able to protect animals against pulmonary challenge with a virulent type A strain of *F. tularensis*.

MATERIALS AND METHODS

Bacterial strains and culture. *F. tularensis* type A strain SchuS4 was obtained from the Centers for Disease Control and Prevention (Fort Collins, CO) in accordance with all federal and institutional select agent regulations. All manipulations of SchuS4 were conducted under strict biosafety level 3 (BSL3) containment conditions. *F. tularensis* type B LVS was obtained from Karen Elkins (Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD) and was manipulated under BSL2 containment conditions. *F. tularensis* cultures were grown as previously described on modified Mueller-Hinton (MH+) medium (24).

***F. tularensis* LPS.** LPS was extracted from *F. tularensis* LVS using the hot phenol method previously described (33). The resulting highly purified LPS preparation was found to contain less than 0.5% protein or nucleic acid and was lyophilized for storage.

***F. tularensis* LVS inactivation.** *F. tularensis* LVS was grown for 48 h, cells were harvested and transferred into phosphate-buffered saline (PBS), and the resulting cell suspension was diluted to obtain an optical density at 600 nm (OD_{600}) of 0.4, which corresponded to approximately 10^8 CFU/ml. The cells were washed twice in an equal volume of PBS, suspended in an equal volume of 70% ethanol, and incubated at 4°C with gentle rocking for 48 h. Following ethanol inactivation, the cells were pelleted, washed twice with PBS, and suspended in an equal volume of PBS. To confirm sterility, 10% of the final suspension volume was plated onto MH+ agar and incubated at 37°C for 72 h. Aliquots of the resulting ethanol-inactivated LVS (iLVS) preparation were stored at −80°C until they were used.

***F. tularensis* OM isolation.** The method used for isolation of *F. tularensis* outer membranes (OMs) and their associated OMPs was described previously (24). OMP-containing fractions (densities, 1.17 to 1.20 g/ml) were pooled and dialyzed in 3- to 12-ml, 5,000-Da-molecular-weight-cutoff, Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL) against PBS for 12 to 16 h. Dialyzed fractions were concentrated using Amicon Ultra-4 centrifugal filter units (Millipore, Billerica, MA) and stored at −80°C until use.

Antigen preparation and comparison. The total amounts of protein in iLVS and OMP preparations were quantitated using the DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of LPS, iLVS, and OMP preparations were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and either visualized by silver staining (Silver Stain Plus; Bio-Rad) or transferred to nitrocellulose for immunoblot analysis as previously described (24). For comparison of the antigen preparations by immunoblotting, the relative amount of LPS in each antigen preparation was visualized using mouse monoclonal antibody FB11 to *F. tularensis* LPS (Abcam, Cambridge, MA).

Mouse immunization and challenge. A preliminary comparison of mouse strains C3H/HeN and BALB/c demonstrated that these two strains were equally susceptible to intranasal infection by the virulent type A strain SchuS4 (data not shown). Further, in our hands, C3H/HeN mice were more consistently protected from SchuS4 intranasal challenge than BALB/c mice following intranasal immunization with LVS (data not shown). These results led us to use the C3H/HeN strain of mice and are consistent with results reported previously by other workers (13, 37, 46).

The UT Southwestern Medical Center Institutional Animal Care and Use Committee and the Biological and Chemical Safety Advisory Committee approved all animal procedures. During the immunization period, mice were housed in microisolator cages at the UT Southwestern Animal Resource Center and were fed irradiated food and water ad libitum. Female C3H/HeN mice that were 4 to 6 weeks old (Charles River Laboratories, Wilmington, MA) were separated into three groups of 16 mice and were intraperitoneally (i.p.) inoculated with 20 μg of LPS, iLVS, or OMPs (in 100 μl of PBS, emulsified with an equal volume of complete Freund's adjuvant [Sigma, St. Louis, MO]). Additionally, two groups of control mice (16 mice infected with SchuS4 and 16 mice that served as noninfected, negative controls) were sham immunized by i.p. injection of 100 μl of PBS. At weeks 3 and 5, mice were boosted i.p. with a similar amount of antigen preparation emulsified in incomplete Freund's adjuvant (Sigma) or PBS. At week 7, test bleeding was performed, and sera were collected for immunoblot and antibody isotyping analyses. Following test bleeding, immunized mice were transferred to the UT Southwestern BSL3 animal facility and were housed in an individually ventilated cage system with HEPA filtration (Techniplast, Exton, PA). In week 8, immunized and control mice were anesthetized with ketamine, xylazine, and acepromazine and were inoculated intransally (i.n.) with 20 μl (10 μl per naris) of either live SchuS4 (40 CFU; determined by quadruplet plating onto MH+ agar) or PBS (negative control group). Following inoculation, mice were monitored daily for signs of morbidity and mortality. On day 5 postinfection, eight mice from each group were anesthetized and exsanguinated, and the lungs, livers, and spleens were aseptically harvested for bacterial enumeration. Organs were individually weighed and transferred to Whirl-pack bags (Nasco, Fort Atkinson, WI), 25 μl of PBS per mg of tissue was added, and tissues were homogenized for 1 min in a stomacher (Seward, Worthing, West Sussex, United Kingdom). Organ homogenates were 10-fold serially diluted in PBS, and 100 μl of each dilution was plated in duplicate onto MH+ agar. After 72 h of incubation, the number of CFU per plate was determined for each dilution, and the average number of CFU/mg of tissue was calculated. Blood from each mouse was allowed to clot for 4 h at room temperature and centrifuged at 1,500 × *g* for 15 min, and the serum was removed, filter sterilized, and transferred to new tubes. To confirm serum sterility, 10% of each serum sample was plated onto MH+ agar and incubated for 72 h before removal from the BSL3 facility. Sera were stored at −80°C until use. The time to death

was monitored for the remaining eight mice in each group until day 20 postinfection, at which point any surviving mice were euthanized. Immunization and challenge experiments were performed twice to confirm reproducibility.

Antibody isotyping ELISAs. Following immunization with PBS, LPS, iLVS, or OMPs, mouse antiserum isotype responses were characterized by enzyme-linked immunosorbent assays (ELISAs). *F. tularensis* LVS was grown on MH+ agar for 48 h, and cells were harvested and washed twice in PBS before they were suspended in carbonate buffer (50 mM $NaHCO_3$, 50 mM Na_2CO_3; pH 9.4) to an OD_{600} of 1.35. Wells of MaxiSorp 96-well plates (Nalge Nunc, Rochester, NY) were coated with 100 μl of the bacterial suspension by incubation at 37°C for 2 h, followed by overnight incubation at 4°C. The plates were washed three times with wash buffer (PBS containing 0.1% Tween 20), blocked at 37°C for 30 min with PBS containing 10% fetal calf serum (HyClone, Logan, UT), washed three times with wash buffer, and stored at 4°C until they were used. Test bleed sera were 10-fold serially diluted (from 1:10 to 1:100,000) in diluent buffer (PBS containing 10% fetal calf serum and 0.05% Tween 20), and 100 μl of each dilution was added in duplicate to microtiter wells and incubated at 37°C for 90 min. The plates were washed three times with wash buffer, and 100 μl of secondary biotinylated rat anti-mouse IgG1, IgG2a, IgG2b, and IgM (diluted 1:10,000 in diluent buffer; Caltag Invitrogen, Carlsbad, CA) or rabbit anti-mouse IgG3 (diluted 1:2,000 in diluent buffer; United States Biological, Swampscott, MA) and IgA (diluted 1:10,000 in diluent buffer; United States Biological) were added to each well and incubated at 37°C for 1 h. The plates were washed three times with wash buffer, and 100 μl of a 1:10,000 dilution of streptavidin-horseradish peroxidase conjugate (BioSource Invitrogen, Carlsbad, CA) was added to each well and incubated at 37°C for 20 min. The plates were washed three times with wash buffer, 100 μl of a 3,3′,5,5′-tetramethylbenzidine peroxidase substrate solution (KPL, Gaithersburg, MD) was added to each well, the plates were incubated at 37°C for 20 min, and the colorimetric reaction was stopped by addition of 100 μl of 1.8 N H_2SO_4. The OD_{450} was determined using a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Endpoint antibody titers were calculated for each antibody isotype by linear regression analysis and were expressed as the reciprocal of the highest antiserum dilution that gave an OD_{450} above the cutoff. The cutoff was defined as the mean OD_{450} for the PBS-immunized control mice plus 2.0 standard deviations.

Cytokine quantitation. Sera were collected from PBS-, LPS-, iLVS-, and OMP-immunized mice on day 5 after infection with SchuS4. Sera were also collected from age-matched, negative control mice that were neither immunized nor challenged with SchuS4. Serum cytokine concentrations were determined using the Bio-Plex mouse cytokine assay (Bio-Rad). The Bio-Plex assay was performed by using the conditions indicated in the manufacturer's instructions. Mouse cytokine panel A (IL-1β, IL-2, IL-4, IL-5, IL-10, granulocyte-macrophage colony-stimulating factor, gamma interferon, and tumor necrosis factor alpha [TNF-α]) and panel B (IL-1α, IL-3, IL-6, IL-12p40, IL-12p70, IL-17, granulocyte colony-stimulating factor [G-CSF], KC, MIP-1α, and RANTES) were analyzed for each immunization group.

Statistics. All statistical analyses were performed using the GraphPad Prism 4 software (GraphPad, San Diego, CA). Survival data were analyzed by the Mantel-Cox log rank test. Bacterial burden data for spleens, livers, and lungs were $\log_{10}$ transformed to justify the assumption of normality. Data for antibody isotype titers, bacterial burdens, and cytokine quantitation were expressed as means ± standard errors of the means, and groups were compared by using one-way analysis of variance. P values of <0.05 were considered statistically significant.

RESULTS

Antigen assessment and quantitation. Equal quantities of LPS, iLVS, and OMP preparations were separated by SDS-PAGE and silver stained to compare the amounts of protein in vaccine preparations (Fig. 1A). We were not able to detect any contaminating proteins in the LPS preparation by silver stain analysis. The initial comparison of the iLVS and OMP preparations, including enhanced staining of a subset of enriched OMPs, indicated that more protein was present in the OMP preparation. However, more precise examination of the two vaccine preparations revealed that there was a greater number of individual proteins in the iLVS preparation than in the OMP preparation.

Identical amounts of vaccine preparations were analyzed by immunoblot analysis using a monoclonal antibody against LPS from LVS (Fig. 1B). LPS was detected in all three preparations, albeit in different amounts. As expected, the LPS preparation was essentially pure. By comparison, very little LPS reactivity was observed in the iLVS preparation, likely due to some LPS shedding during overnight inactivation in 70% ethanol. Finally, a moderate amount of LPS was detected in the OMP preparation, indicating that our OM extraction procedure predictably left LPS within the isolated OM vesicles.

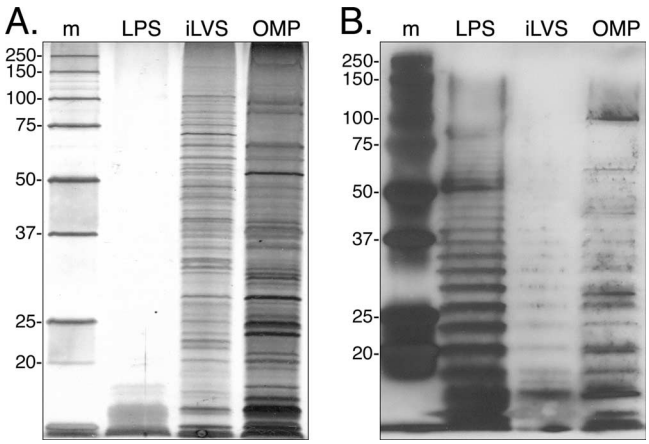

FIG. 1. Comparison of *F. tularensis* LVS antigen preparations used to immunize mice. LPS from LVS was phenol extracted as described in Materials and Methods. iLVS was prepared by overnight incubation in 70% ethanol. OMPs were isolated as described in Materials and Methods. Antigen preparations (20 μg each) were separated by SDS-PAGE and analyzed by (A) silver staining and (B) immunoblotting using an anti-LPS monoclonal antibody. Molecular masses (in kDa) are indicated on the left for each gel. Lane m contained markers.

Antigen preparations induce distinct mouse antibody responses. Groups of 16 mice were immunized i.p. at weeks 0, 3, and 5 with PBS, LPS, iLVS, or OMPs, each emulsified with Freund's adjuvant. At week 7, mice were test bled, and antisera were analyzed by immunoblot analysis for reactivity against whole-cell lysates of LVS and SchuS4 (Fig. 2A). Sera from PBS-immunized mice did not react against LVS or SchuS4 lysates (data not shown). In general, the antibody responses to LPS immunization were the weakest of the responses to the three vaccine preparations. Sera from LPS-immunized mice did not display a typical LPS ladderlike pattern of reactivity (10); rather, six to eight reactive bands were observed for both LVS and SchuS4 lysates. Despite the poor overall serum reactivity following LPS immunization, there was notable reactivity against an unknown 45-kDa protein. By comparison, sera from iLVS-immunized mice reacted with a number of *F. tularensis* proteins, including LPS-like reactivity against LVS lysates but not SchuS4 lysates. These results suggest that ethanol inactivation may have exposed a cryptic LPS epitope that is LVS specific. Whereas LVS and SchuS4 have identical O-antigen structures, differences in lipid A substitutions between the two strains have been reported (32, 43). The antiserum reactivity profiles varied widely within the iLVS-immunized group (Fig. 2A and data not shown). We cannot account for this variability given that a homogeneous antigen preparation was administered multiple times to this group of mice.

Although preliminary antigen assessment demonstrated that

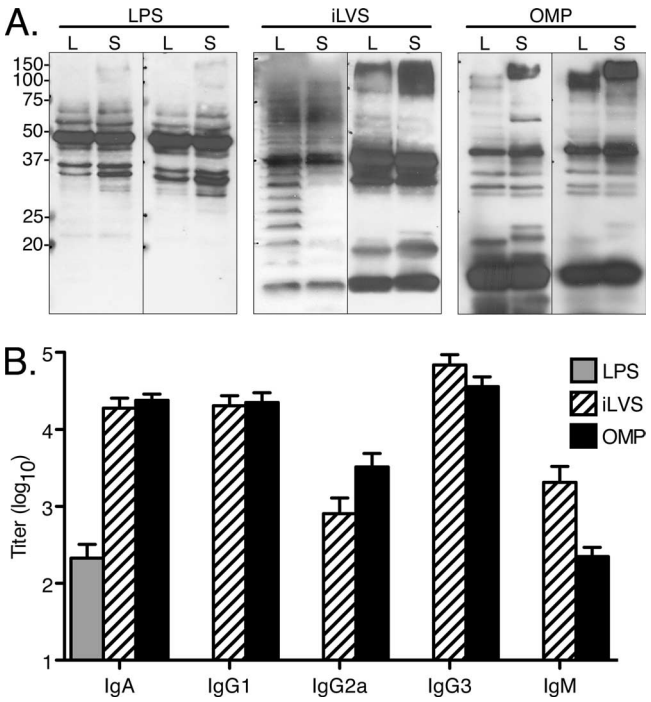

FIG. 2. Characterization of mouse antibodies induced by immunization. Groups of 16 mice were immunized at week 0 and boosted at weeks 3 and 5 with 20 μg of LPS, iLVS, or OMPs, each complexed with Freund's adjuvant. At week 7, mice were test bled and sera were collected. (A) Immunoblot analysis was performed to determine antiserum reactivities against whole-cell lysates of LVS (lanes L) and SchuS4 (lanes S). Molecular masses (in kDa) are indicated on the left. Representative immunoblots are shown. (B) Antibody isotype titers against LVS were determined by performing isotype-specific ELISAs. Mean log_{10} titers and standard errors of the means (error bars) were calculated for each group.

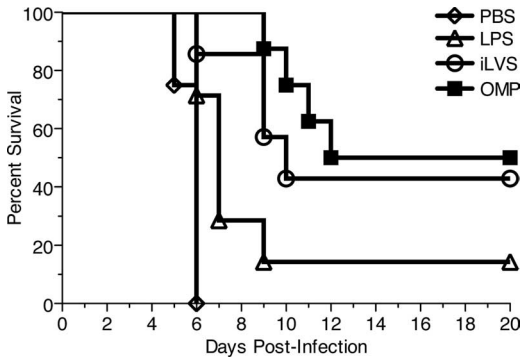

FIG. 3. Survival of immunized mice following i.n. *F. tularensis* challenge. Groups of mice were immunized and boosted with PBS, LVS LPS, iLVS, or LVS OMPs. At week 7, mice were i.n. challenged with 40 CFU of the virulent type A *F. tularensis* strain SchuS4, and survival was monitored through day 20 postinfection. Experiments were performed twice to confirm reproducibility.

there were larger amounts of LPS in the OMP vaccine preparation (compared to iLVS) (Fig. 1B), sera from OMP-immunized mice did not react against LPS from LVS or SchuS4 (Fig. 2A). Sera from OMP-immunized mice reacted against similar proteins in both LVS and SchuS4 lysates. Most notably, strong reactivities against 15-, 39-, and 150-kDa proteins were observed. This cross-reactivity verifies the high degree of homology between the two *Francisella* subspecies and supports the use of LVS proteins as immunogens against SchuS4 challenge.

As an additional means to characterize the antibody response following immunization, antibody isotype titers were determined by ELISAs (Fig. 2B). The antiserum reactivity of PBS-immunized mice against LVS was used as the baseline for each antibody isotype. With the exception of IgG2b (data not shown), all isotypes were induced at least 100-fold by immunization with the three vaccine preparations. At this time, we cannot account for the low levels of IgG2b across all immunization groups in this experiment. Of the three immunization groups, LPS immunization generated the weakest overall antibody titer response, and only IgA was detected at levels higher (200-fold) than those in control mice. These results correlated with the limited LPS-antiserum immunoblot reactivity noted above. Our finding that only IgA was induced following LPS immunization conflicts with previous reports that *F. tularensis* LPS induces primarily IgM and IgG1 responses (10, 16, 19). It is possible, however, that our inclusion of Freund's adjuvant with LPS biased the antibody response, as Freund's adjuvant can induce various antigen-dependent immune responses (41).

By comparison, iLVS and OMPs induced IgA, IgG1, IgG2a, IgG3, and IgM antibody titers that were between 200- and 70,000-fold higher than control mouse titers (Fig. 2B). The predominant antibody isotype induced by iLVS and OMP immunization was IgG3, indicating that there was a Th1-biased response (39). OMP and iLVS immunization induced similar amounts of IgA and IgG1 antibodies. Whereas IgA is well known to protect mucosal surfaces from infection by viruses and bacteria (45), IgG1 has been associated with Th2 responses (40). Different antibody isotype titers were observed for IgG2a and IgM when iLVS- and OMP-immunized mice were compared, but these titers were not statistically different for different immunization groups. OMP immunization induced larger amounts of IgG2a, which tends to be a Th1-biased antibody response (18). By comparison, iLVS immunization induced larger amounts of IgM. IgM is the single most potent activator of complement-mediated bacterial neutralization and has been associated with Th2 responses (4). Taken together, these antibody isotype data demonstrate that i.p. immunization with iLVS and OMPs induced a wide range of Th1 and Th2 antibody responses, with possible mucosal immunity involvement (IgA production). By comparison, LPS immunization induced only a low-titer IgA response, consistent with previous reports that *F. tularensis* LPS is a weak inducer of antibody (8, 11, 16).

OMPs and iLVS protect mice from i.n. challenge with virulent type A *F. tularensis*. Two weeks following the final antigen boost, immunized mice were challenged i.n. with approximately 40 CFU of the *F. tularensis* type A virulent strain SchuS4. The time to death was monitored for each immunization group (Fig. 3). All PBS-immunized mice (8/8 mice) succumbed to SchuS4 infection within 6 days, with a mean time to death of 5.9 days. LPS-immunized mice had a mean time to death of 7.3 days following i.n. SchuS4 infection, with 71% of the mice (5/7 mice) surviving through day 6, 29% (2/7 mice)

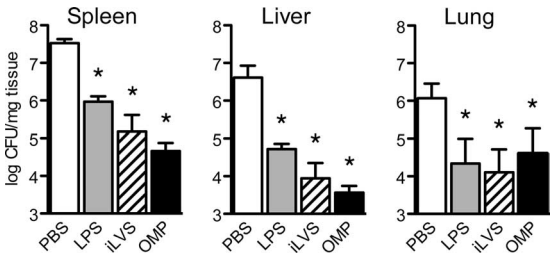

FIG. 4. Bacterial tissue burdens for immunized mice following i.n. *F. tularensis* challenge. Groups of mice were immunized with PBS, LVS LPS, iLVS, or LVS OMPs and subsequently i.n. challenged with 40 CFU of the virulent type A *F. tularensis* strain SchuS4. At day 5 postinfection, spleens, livers, and lungs were harvested from mice and plated for bacterial enumeration (CFU per mg of tissue). Means and standard errors of the means (error bars) were calculated for each tissue and immunization group, and statistically significant differences are indicated by an asterisk ($P < 0.05$). Experiments were performed twice to confirm reproducibility.

surviving through day 7, and 14% (1/7 mice) surviving through the end of the study. These results confirmed previous reports that LPS immunization does not protect against challenge with type A virulent strains but rather delays the time to death by 24 to 64 h (11, 19). iLVS-immunized mice had a mean time to death of 9.1 days following i.n. SchuS4 challenge, with 57% of the mice (4/7 mice) surviving through day 9 and 43% (3/7 mice) surviving through the end of the study. OMP-immunized mice had a mean time to death of 11.3 days following i.n. SchuS4 challenge, with 88% of the mice (7/8 mice) surviving through day 9, 63% (5/8 mice) surviving through day 11, and 50% (4/8 mice) surviving through the end of the study (day 20). Mantel-Cox log rank survival analysis was performed to compare the ratios of deaths at each time point for different immunization groups. Compared to PBS-immunized mice, LPS-immunized mice ($P = 0.0043$), iLVS-immunized mice ($P = 0.0016$), and OMP-immunized mice ($P = 0.0002$) were significantly protected from i.n. SchuS4 challenge. When antigen-immunized groups were compared, the differences in survival between iLVS- and OMP-immunized mice and between LPS- and iLVS-immunized mice were not statistically significant. However, OMP immunization provided significantly better protection ($P = 0.0185$) than LPS immunization. Whereas immunization with LPS, immunization with iLVS, and immunization with OMPs each provided statistically significant protection from i.n. SchuS4 infection (PBS-immunized mice), the mean time to death (11.3 days) and increased overall survival rate (50%) for the OMP-immunized group were most striking.

Immunization reduces mouse tissue bacterial burdens. On day 5 postinfection, eight mice from each immunization group were sacrificed, and lungs, livers, and spleens were collected for enumeration of bacteria. Day 5 was selected because previous studies in our laboratory demonstrated that naïve C3H/HeN mice show clinical signs of disease (hunching, slow movement, and ruffled fur) by day 5 after infection with SchuS4 (data not shown). In the present study, all PBS-immunized mice were severely moribund by day 5. Significant reductions in bacterial burdens were observed in the spleens, livers, and lungs of antigen-immunized mice following SchuS4 challenge (Fig. 4). Compared to PBS-immunized mice, LPS immunization resulted in a 1.6-log reduction in the number of SchuS4 CFU in the spleens of infected mice ($P = 0.0018$). By comparison, the spleens of iLVS-immunized mice harbored 2.4-log-fewer bacteria ($P < 0.0001$) and the spleens of OMP-immunized mice harbored 2.9-log-fewer bacteria ($P < 0.0001$) than the spleens of PBS-immunized mice. When the spleen CFU for immunization groups were compared, only LPS- and OMP-immunized mice were significantly different ($P = 0.02$). In the livers, a 1.9-log reduction ($P = 0.0003$) in the number of CFU was observed in LPS-immunized mice, a 2.7-log reduction ($P < 0.0001$) in the number of CFU was observed in iLVS-immunized mice, and a 3.1-log reduction ($P < 0.0001$) in the number of CFU was observed in OMP-immunized mice. When differences in the number of liver CFU for immunization groups were examined, only LPS- and OMP-immunized mice were significantly different ($P = 0.03$). In the lungs, LPS, iLVS, and OMP immunization resulted in 1.5- to 2.0-log decreases in the bacterial burdens ($P < 0.005$ for each group). For the three organs examined in this study, the lung bacterial burdens were the most variable, and differences among immunization groups were not significant. In general, the markedly reduced bacterial burdens directly correlated with the survival data described above, with the OMP-immunized mice having the greatest overall reductions in the bacterial burdens in their spleens and livers.

Cytokine analyses. To gain a better understanding of the immunological factors that culminated in reduced bacterial burdens and promoted survival following i.n. type A *F. tularensis* challenge, mouse sera were collected at day 5 postinfection. Using an ELISA-based luminescent mouse cytokine assay, 18 different cytokines were quantitated, and the responses of PBS-immunized mice and mice immunized with LPS, iLVS, or OMPs were compared (Fig. 5). Notably, IL-10 production was increased fourfold ($P < 0.001$) in OMP-immunized mice compared to mice in other immunization groups. The levels of TNF-α and IL-2 were significantly increased in mice immunized with iLVS or OMPs compared to PBS-immunized mice. Dramatic increases in TNF-α production were quantitated, and there was a 38-fold ($P < 0.001$) increase in the TNF-α level in iLVS-immunized mice and a 26-fold ($P < 0.001$) increase in the TNF-α level in OMP-immunized mice. IL-2 production was increased nearly 10-fold ($P < 0.05$) by both iLVS and OMP immunization. By comparison, statistically significant higher levels of RANTES, G-CSF, IL-6, IL-1α, IL-12p40, and KC were detected in PBS-immunized mice but not in LPS-, iLVS-, or OMP-immunized mice. No statistically significant differences were found for gamma interferon, IL-1β, IL-3, IL-4, IL-5, IL-12p70, IL-17, granulocyte-macrophage colony-stimulating factor, or MIP-1α (data not shown). Together with the tissue bacterial burdens and survival data, these results suggest that there are two distinct type A *F. tularensis*-induced cytokine profiles: a survival profile consisting of IL-10 (induced only by OMP immunization), TNF-α, and IL-2 and a death profile that includes RANTES, G-CSF, IL-6, IL-1α, IL-12p40, and KC.

DISCUSSION

Because of the low infectious dose, high morbidity, and mortality rates following infection, as well as the potential use

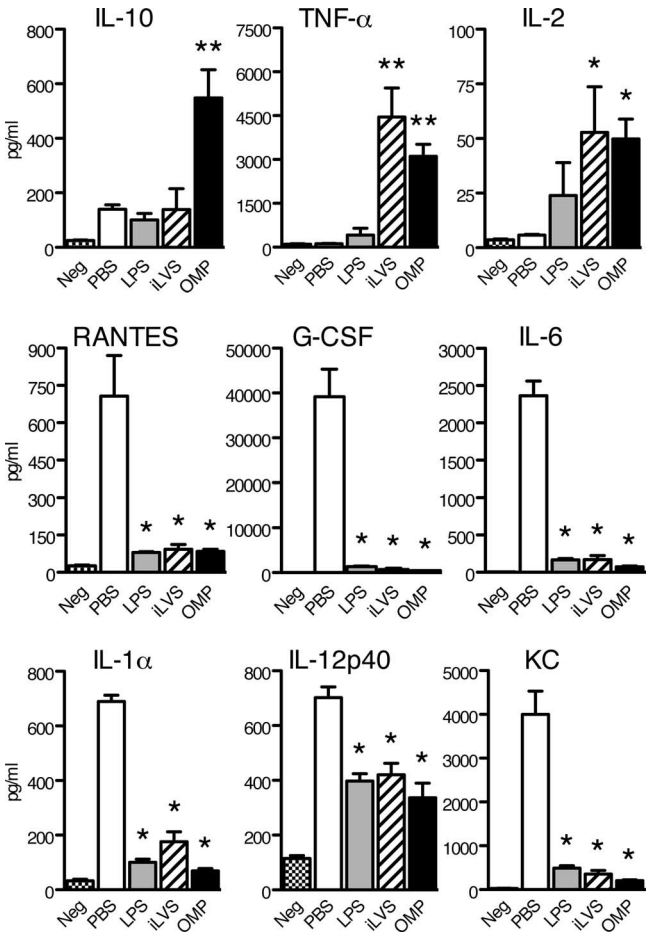

FIG. 5. Serum cytokine analyses for immunized mice following i.n. *F. tularensis* challenge. Groups of mice were immunized with PBS, LVS LPS, iLVS, or LVS OMPs and subsequently i.n. challenged with 40 CFU of the virulent type A *F. tularensis* strain SchuS4. Age-matched, negative control mice (Neg) were not immunized or challenged with SchuS4. The mean cytokine production and standard error of the mean (error bars) were calculated for each group. Significant differences between PBS-immunized mice and mice immunized with LPS, iLVS, or OMPs are indicated by asterisks (one asterisk, $P < 0.05$; two asterisks, $P < 0.001$).

of *F. tularensis* as a biological weapon, the need for a safe and efficacious tularemia vaccine is unquestionable. LVS was originally developed in the 1950s, yet it still has investigational drug status and is administered only to a restricted group of researchers and military personnel (12). Indeed, it is doubtful that LVS will ever be approved for human use, as concerns about the nature of its attenuation, immunization-induced reactions, and efficacy are unlikely to be resolved. In an effort to advance the field, this study investigated the vaccinogenic potential of three antigen (acellular) preparations for protecting mice against pulmonary challenge with the virulent type A *F. tularensis* strain SchuS4. It is worth noting that conventional wisdom within both the intracellular pathogen and tularemia research communities advocates the use of live attenuated vaccines; therefore, there is no precedent for testing a subunit vaccine against type A *F. tularensis*.

Each of the three *F. tularensis* antigen preparations examined in this study contained some level of LPS. *F. tularensis* LPS is unusual in that it is relatively inert (2, 21), has a limited ability to induce an antibody response (8, 11, 14), and does not protect against type A *F. tularensis* challenge (11, 19). Here, immunization with LVS LPS alone induced very weak serum antibody responses (IgA only). A previous study reported that IgA, a hallmark of mucosal immunity, was necessary for survival after respiratory LVS infection (3). However, in our study, LPS induction of IgA was able to protect only one of seven mice from SchuS4 respiratory challenge. Further, LPS immunization did not promote significant production of any of the 18 cytokines analyzed following SchuS4 challenge. Despite this, the possibility that LPS may have conferred subtle immunomodulatory properties that enhanced anti-*Francisella* responses in the iLVS and OMP vaccine preparations cannot be ruled out. Finally, protection could not be attributed to Freund's adjuvant, as all mice immunized with Freund's adjuvant alone succumbed to SchuS4 challenge within 6 days after infection (data not shown).

Although both OMP immunization and iLVS immunization provided significant protection from i.n. SchuS4 challenge, the cumulative results of this study suggest that OMP immunization has an advantage. First, the use of an inactivated *F. tularensis* vaccine in humans is not likely to be a feasible approach given previously reported adverse reactions to immunization and poor protection against virulent aerosol infection (25). Second, we found that antibody responses, tissue bacterial burdens, and cytokine responses were highly variable in the iLVS-immunized group. At this time, we cannot provide a logical explanation for this observed variability. Third, OMP-immunized mice had an increased mean time to death (2.1 days) and a substantially increased overall survival rate (50%) following SchuS4 challenge (compared to iLVS-immunized mice). Whereas these survival differences between OMP-immunized mice and iLVS-immunized mice were not statistically significant, they are not trivial, and with alterations in the immunization route and/or adjuvant (discussed below), OMP immunization could be even more efficacious. Fourth, OMP immunization resulted in the most dramatic reductions in the numbers of spleen and liver CFU following SchuS4 challenge. The importance of enhanced liver and spleen bactericidal activity is highlighted by a previous mouse infection study that noted that overwhelming bacterial replication and severe pathological changes occurred in the livers and spleens, but not in the lungs, of naïve mice infected with virulent *F. tularensis* strains (9). Fifth, only OMP immunization induced a significant increase in IL-10 production following SchuS4 challenge. IL-10, generally accepted to be a Th2 cytokine, has been shown to regulate T-cell activation, as well as suppress inflammatory cytokine production by a number of cell types (42). Further, it is well documented that IL-10 plays a critical regulatory role in mediating protection against other pathogens while simultaneously limiting disease pathology (29). Little is known about the role of IL-10 in controlling pulmonary *F. tularensis* infection; however, a previous study noted that pulmonary SchuS4 infection did not induce IL-10 production in mice (5). Here, the OMP-induced increase in IL-10 may have resulted in the observed suppression of the proinflammatory cytokines RANTES, G-CSF, IL-6, IL-1α, IL-12p40, and KC in OMP-immunized mice. Indeed, IL-10 can inhibit secretion of all of

the cytokines mentioned above (29). Whereas proinflammatory cytokine production is generally believed to have protective antimicrobial effects, cytokine overexpression ("cytokine storm") can cause immune dysregulation, tissue damage, and anergy (42). In the context of this study, our cumulative results suggest that OMP immunization induced tight regulation of the cytokine cascade, which ultimately led to enhanced bacterial clearance and survival following SchuS4 pulmonary challenge. Simultaneous decreases in levels of proinflammatory cytokines were observed in both LPS- and iLVS-immunized mice, suggesting that factors other than IL-10 may be important. At this time, we do not fully understand the potential regulatory and protective roles of IL-10 production during SchuS4 infection; however, the potential implications warrant further investigation.

CMI appears to play a pivotal role in protection against *F. tularensis* infection (1, 14). Moreover, the importance of antibody in controlling tularemia has gained increasing popularity in recent years (3, 26). However, most previous immunological studies focused primarily on using avirulent *Francisella* strains in ex vivo/in vitro models. Here, we examined the immunological responses to pulmonary type A *F. tularensis* infection in both naïve and immunized mice and correlated protection with both CMI and humoral responses. Our results suggest that immunological protection against type A pulmonary challenge is complex, as OMP-induced protection correlated with high titers of Th1 and Th2 antibody isotypes, IgA production, and production of proinflammatory cytokines. We propose here for the first time, however, that protection against type A *F. tularensis* also may be associated with suppression of a subset of cytokines, presumably mediated through the regulatory action of IL-10. At this time, we cannot speculate on the possible correlation between high titers of serum IgA and mucosal IgA levels, but it is quite possible that mucosal IgA (from serum exudates) helped to limit dissemination of *F. tularensis* from the lungs to other organs. Experiments to examine local mucosal IgA production (via bronchoalveolar lavage) in the lungs of OMP-immunized mice are warranted, but such experiments are very difficult to conduct with mice.

The results of this study demonstrated that purified OMPs can serve as a protective subunit (acellular) vaccine against pulmonary challenge with virulent type A *F. tularensis*. This observation is largely counter to the current dogma that a live attenuated vaccine is necessary to achieve immunity against this intracellular pathogen. Whereas native LVS OMPs did not protect all mice from *F. tularensis* challenge, this study demonstrated, at the very least, proof of principle that a subunit vaccine can induce substantial protective immunity against pulmonary *F. tularensis* infection. For the purposes of this study, it was advantageous to use LVS OMPs for mouse immunizations, but the future implications of employing OMP vaccine preparations from the type A strain are clear. In this regard, we believe that further enhancements in protection are likely, and thus, experiments are currently under way to investigate whether i.n. immunization with type A-derived OMPs or recombinant counterparts of these OMPs can provide even greater protection. Evaluations of the use of more clinically relevant adjuvants, such as immune-stimulating complexes and CpG, also are highly warranted.

ACKNOWLEDGMENTS

We thank Jon Blevins for a critical review of the manuscript, Christine Ingle for technical assistance, and Laura Brulé (UT Southwestern Internal Medicine Mouse Metabolic Phenotyping Core) for assistance with the mouse cytokine assay.

This work was supported by National Institutes of Health National Institute of Allergy and Infectious Diseases grants P01-AI055637 and T32-AI070116 (to J.F.H.).

REFERENCES

1. **Anthony, L. S., and P. A. Kongshavn.** 1988. H-2 restriction in acquired cell-mediated immunity to infection with *Francisella tularensis* LVS. Infect. Immun. **56:**452–456.
2. **Barker, J. H., J. Weiss, M. A. Apicella, and W. M. Nauseef.** 2006. Basis for the failure of *Francisella tularensis* lipopolysaccharide to prime human polymorphonuclear leukocytes. Infect. Immun. **74:**3277–3284.
3. **Baron, S. D., R. Singh, and D. W. Metzger.** 2007. Inactivated *Francisella tularensis* live vaccine strain protects against respiratory tularemia by intranasal vaccination in an immunoglobulin A-dependent fashion. Infect. Immun. **75:**2152–2162.
4. **Boes, M., A. P. Prodeus, T. Schmidt, M. C. Carroll, and J. Chen.** 1998. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. J. Exp. Med. **188:**2381–2386.
5. **Bosio, C. M., H. Bielefeldt-Ohmann, and J. T. Belisle.** 2007. Active suppression of the pulmonary immune response by *Francisella tularensis* Schu4. J. Immunol. **178:**4538–4547.
6. **Casadevall, A.** 2003. Antibody-mediated immunity against intracellular pathogens: two-dimensional thinking comes full circle. Infect. Immun. **71:**4225–4228.
7. **Cassataro, J., S. M. Estein, K. A. Pasquevich, C. A. Velikovsky, S. de la Barrera, R. Bowden, C. A. Fossati, and G. H. Giambartolomei.** 2005. Vaccination with the recombinant *Brucella* outer membrane protein 31 or a derived 27-amino-acid synthetic peptide elicits a $CD4^+$ T helper 1 response that protects against *Brucella melitensis* infection. Infect. Immun. **73:**8079–8088.
8. **Cole, L. E., K. L. Elkins, S. M. Michalek, N. Qureshi, L. J. Eaton, P. Rallabhandi, N. Cuesta, and S. N. Vogel.** 2006. Immunologic consequences of *Francisella tularensis* live vaccine strain infection: role of the innate immune response in infection and immunity. J. Immunol. **176:**6888–6899.
9. **Conlan, J. W., W. Chen, H. Shen, A. Webb, and R. KuoLee.** 2003. Experimental tularemia in mice challenged by aerosol or intradermally with virulent strains of *Francisella tularensis*: bacteriologic and histopathologic studies. Microb. Pathog. **34:**239–248.
10. **Conlan, J. W., H. Shen, A. Webb, and M. B. Perry.** 2002. Mice vaccinated with the O-antigen of *Francisella tularensis* LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. Vaccine **20:**3465–3471.
11. **Conlan, J. W., E. Vinogradov, M. A. Monteiro, and M. B. Perry.** 2003. Mice intradermally-inoculated with the intact lipopolysaccharide, but not the lipid A or O-chain, from *Francisella tularensis* LVS rapidly acquire varying degrees of enhanced resistance against systemic or aerogenic challenge with virulent strains of the pathogen. Microb. Pathog. **34:**39–45.
12. **Conlan, J. W., and P. C. Oyston.** 2007. Vaccines against *Francisella tularensis*. Ann. N. Y. Acad. Sci. **1105:**325–350.
13. **Conlan, J. W., H. Shen, R. Kuolee, X. Zhao, and W. Chen.** 2005. Aerosol-, but not intradermal-immunization with the live vaccine strain of *Francisella tularensis* protects mice against subsequent aerosol challenge with a highly virulent type A strain of the pathogen by an αβ T cell- and interferon gamma-dependent mechanism. Vaccine **23:**2477–2485.
14. **Cowley, S. C., and K. L. Elkins.** 2003. Multiple T cell subsets control *Francisella tularensis* LVS intracellular growth without stimulation through macrophage interferon gamma receptors. J. Exp. Med. **198:**379–389.
15. **Drabick, J. J., R. B. Narayanan, J. C. Williams, J. W. Leduc, and C. A. Nacy.** 1994. Passive protection of mice against lethal *Francisella tularensis* (live tularemia vaccine strain) infection by the sera of human recipients of the live tularemia vaccine. Am. J. Med. Sci. **308:**83–87.
16. **Dreisbach, V. C., S. Cowley, and K. L. Elkins.** 2000. Purified lipopolysaccharide from *Francisella tularensis* live vaccine strain (LVS) induces protective immunity against LVS infection that requires B cells and gamma interferon. Infect. Immun. **68:**1988–1996.
17. **Eyles, J. E., M. G. Hartley, T. R. Laws, P. C. Oyston, K. F. Griffin, and R. W. Titball.** 2008. Protection afforded against aerosol challenge by systemic immunisation with inactivated *Francisella tularensis* live vaccine strain (LVS). Microb. Pathog. **44:**164–168.
18. **Finkelman, F. D., I. M. Katona, T. R. Mosmann, and R. L. Coffman.** 1988. IFN-γ regulates the isotypes of Ig secreted during in vivo humoral immune responses. J. Immunol. **140:**1022–1027.
19. **Fulop, M., P. Mastroeni, M. Green, and R. W. Titball.** 2001. Role of anti-

body to lipopolysaccharide in protection against low- and high-virulence strains of *Francisella tularensis*. Vaccine **19:**4465–4472.
20. **Haake, D. A.** 2000. Spirochetal lipoproteins and pathogenesis. Microbiology **146:**1491–1504.
21. **Hajjar, A. M., M. D. Harvey, S. A. Shaffer, D. R. Goodlett, A. Sjostedt, H. Edebro, M. Forsman, M. Bystrom, M. Pelletier, C. B. Wilson, S. I. Miller, S. J. Skerrett, and R. K. Ernst.** 2006. Lack of in vitro and in vivo recognition of *Francisella tularensis* subspecies lipopolysaccharide by Toll-like receptors. Infect. Immun. **74:**6730–6738.
22. **Hancock, R. E., R. Siehnel, and N. Martin.** 1990. Outer membrane proteins of *Pseudomonas*. Mol. Microbiol. **4:**1069–1075.
23. **Hornick, R. B., and H. T. Eigelsbach.** 1966. Aerogenic immunization of man with live tularemia vaccine. Bacteriol. Rev. **30:**532–538.
24. **Huntley, J. F., P. G. Conley, K. E. Hagman, and M. V. Norgard.** 2007. Characterization of *Francisella tularensis* outer membrane proteins. J. Bacteriol. **189:**561–574.
25. **Kadull, P. J., H. R. Reames, L. L. Coriell, and L. Foshay.** 1950. Studies on tularemia. V. Immunization of man. J. Immunol. **65:**425–435.
26. **Kirimanjeswara, G. S., J. M. Golden, C. S. Bakshi, and D. W. Metzger.** 2007. Prophylactic and therapeutic use of antibodies for protection against respiratory infection with *Francisella tularensis*. J. Immunol. **179:**532–539.
27. **KuoLee, R., G. Harris, J. W. Conlan, and W. Chen.** 2007. Oral immunization of mice with the live vaccine strain (LVS) of *Francisella tularensis* protects mice against respiratory challenge with virulent type A *F. tularensis*. Vaccine **25:**3781–3791.
28. **Lopez, M. C., N. S. Duckett, S. D. Baron, and D. W. Metzger.** 2004. Early activation of NK cells after lung infection with the intracellular bacterium, Francisella tularensis LVS. Cell. Immunol. **232:**75–85.
29. **Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra.** 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. **19:**683–765.
30. **Oyston, P. C., A. Sjostedt, and R. W. Titball.** 2004. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat. Rev. Microbiol. **2:**967–978.
31. **Pal, S., E. M. Peterson, and L. M. de la Maza.** 2005. Vaccination with the *Chlamydia trachomatis* major outer membrane protein can elicit an immune response as protective as that resulting from inoculation with live bacteria. Infect. Immun. **73:**8153–8160.
32. **Phillips, N. J., B. Schilling, M. K. McLendon, M. A. Apicella, and B. W. Gibson.** 2004. Novel modification of lipid A of *Francisella tularensis*. Infect. Immun. **72:**5340–5348.
33. **Rahhal, R. M., T. J. Vanden Bush, M. K. McLendon, M. A. Apicella, and G. A. Bishop.** 2007. Differential effects of *Francisella tularensis* lipopolysaccharide on B lymphocytes. J. Leukoc. Biol. **82:**813–820.
34. **Rhinehart-Jones, T. R., A. H. Fortier, and K. L. Elkins.** 1994. Transfer of immunity against lethal murine *Francisella* infection by specific antibody depends on host gamma-interferon and T cells. Infect. Immun. **62:**3129–3137.
35. **Robbins, J. B., R. Schneerson, and S. C. Szu.** 1995. Perspective: hypothesis: serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. J. Infect. Dis. **171:**1387–1398.
36. **Schuch, R., and A. T. Maurelli.** 1999. The Mxi-Spa type III secretory pathway of *Shigella flexneri* requires an outer membrane lipoprotein, MxiM, for invasin translocation. Infect. Immun. **67:**1982–1991.
37. **Shen, H., W. Chen, and J. W. Conlan.** 2004. Susceptibility of various mouse strains to systemically- or aerosol-initiated tularemia by virulent type A *Francisella tularensis* before and after immunization with the attenuated live vaccine strain of the pathogen. Vaccine **22:**2116–2121.
38. **Sjostedt, A.** 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann. N. Y. Acad. Sci. **1105:**1–29.
39. **Snapper, C. M., T. M. McIntyre, R. Mandler, L. M. Pecanha, F. D. Finkelman, A. Lees, and J. J. Mond.** 1992. Induction of IgG3 secretion by interferon γ: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. J. Exp. Med. **175:**1367–1371.
40. **Snapper, C. M., and W. E. Paul.** 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science **236:**944–947.
41. **Stills, H. F., Jr.** 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. ILAR J. **46:**280–293.
42. **Taylor, A., J. Verhagen, K. Blaser, M. Akdis, and C. A. Akdis.** 2006. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-β: the role of T regulatory cells. Immunology **117:**433–442.
43. **Wang, X., A. A. Ribeiro, Z. Guan, S. C. McGrath, R. J. Cotter, and C. R. Raetz.** 2006. Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in *Francisella tularensis* subsp. *novicida.* Biochemistry **45:**14427–14440.
44. **Winslow, G. M., E. Yager, K. Shilo, E. Volk, A. Reilly, and F. K. Chu.** 2000. Antibody-mediated elimination of the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis* during active infection. Infect. Immun. **68:**2187–2195.
45. **Woof, J. M., and J. Mestecky.** 2005. Mucosal immunoglobulins. Immunol. Rev. **206:**64–82.
46. **Wu, T. H., J. A. Hutt, K. A. Garrison, L. S. Berliba, Y. Zhou, and C. R. Lyons.** 2005. Intranasal vaccination induces protective immunity against intranasal infection with virulent *Francisella tularensis* biovar A. Infect. Immun. **73:**2644–2654.

Editor: J. B. Bliska