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VACCINES

Monophosphoryl Lipid A Enhances Efficacy of a *Francisella tularensis* LVS-Catanionic Nanoparticle Subunit Vaccine against *F. tularensis* Schu S4 Challenge by Augmenting both Humoral and Cellular Immunity

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ABSTRACT *Francisella tularensis*, a bacterial biothreat agent, has no approved vaccine in the United States. Previously, we showed that incorporating lysates from partially attenuated *F. tularensis* LVS or fully virulent *F. tularensis* Schu S4 strains into catanionic surfactant vesicle (V) nanoparticles (LVS-V and Schu S4-V, respectively) protected fully against *F. tularensis* LVS intraperitoneal (i.p.) challenge in mice. However, we achieved only partial protection against *F. tularensis* Schu S4 intranasal (i.n.) challenge, even when employing heterologous prime-boost immunization strategies. We now extend these findings to show that both LVS-V and Schu S4-V immunization (i.p./i.p.) elicited similarly high titers of anti-*F. tularensis* IgG and that the titers could be further increased by adding monophosphoryl lipid A (MPL), a nontoxic Toll-like receptor 4 (TLR4) adjuvant that is included in several U.S. FDA-approved vaccines. LVS-V+MPL immune sera also detected more *F. tularensis* antigens than LVS-V immune sera and, after passive transfer to naive mice, significantly delayed the time to death against *F. tularensis* Schu S4 subcutaneous (s.c.) but not i.n. challenge. Active immunization with LVS-V+MPL (i.p./i.p.) also increased the frequency of gamma interferon (IFN-γ)-secreting activated helper T cells, IFN-γ production, and the ability of splenocytes to control intramacrophage *F. tularensis* LVS replication *ex vivo*. Active LVS-V+MPL immunization via heterologous routes (i.p./i.n.) significantly elevated IgA and IgG levels in bronchoalveolar lavage fluid and significantly enhanced protection against i.n. *F. tularensis* Schu S4 challenge (to ~60%). These data represent a significant step in the development of a subunit vaccine against the highly virulent type A strains.

KEYWORDS *Francisella tularensis*, LVS, Schu S4, catanionic surfactant, immunization, mice, monophosphoryl lipid A, nanoparticle, subunit vaccine, tularemia

Francisella tularensis, the causative agent of tularemia, is a Gram-negative coccobacillus. The infectious dose for *F. tularensis* can be extremely small (<10 organisms), depending on the strain and route of infection (1–3). Type A strains, such as *F. tularensis* Schu S4, are associated with high mortality and morbidity in mammals (1–3). *F. tularensis* is an excellent model organism for immune-evasive pathogens because of its broad host range, including rodents, and because of its rapid progression through the stages of infection. Clinical presentation of tularemia depends on the route of infection,

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of which the pneumonic form is the most severe (4, 5). *F. tularensis* expresses a lipopolysaccharide (LPS) that has an atypical, tetraacylated structure that fails to stimulate Toll-like receptor 4 (TLR4) (6–8). However, components of live *F. tularensis* are recognized via TLR2, the absent in melanoma 2 (AIM2) inflammasome, and stimulator of interferon genes (STING) that leads to the induction of type I interferon (9–18). *F. tularensis* replicates intracellularly, preferentially in macrophages (19, 20). *F. tularensis* infection of macrophages causes them to switch from a proinflammatory M1 differentiation state to an anti-inflammatory M2 phenotype (21), which is accompanied by enhanced bacterial replication. To date, neither a subunit nor attenuated vaccine has been licensed in the United States for this tier 1 agent.

Catanionic surfactant vesicles (V) are self-assembling nanostructures that form spontaneously from a mixture of cationic and anionic surfactants to produce small (~100- to 150-nm), unilamellar vesicles (22). These can be "functionalized" by incorporation of lipid-conjugated molecules (23, 24) or bacterial lysates (25). Thus, V represent a potentially effective vehicle for vaccine delivery, with significant advantages over those composed of phospholipids or nonionic surfactants, liposomes and niosomes, e.g., low cost, ease of formation, and high stability (22, 26).

In our earlier study utilizing V as a vaccine carrier for *F. tularensis* antigens, we derived the antigenic components from whole-cell lysates of either *F. tularensis* LVS or *F. tularensis* Schu S4 strains (LVS-V and Schu S4-V, respectively). Two successive immunizations by the intraperitoneal route (i.p./i.p.) with LVS-V or passive administration of immune sera from such immunized mice to naive mice conferred protection against lethal *F. tularensis* LVS i.p. challenge. We also previously reported that immunization (i.p./i.p. or intranasal [i.n.]/i.n.) with Schu S4-V was only minimally protective against *F. tularensis* Schu S4 i.n. challenge. In contrast, immunization of mice with Schu S4-V, administered by a heterologous prime-boost regimen (i.p./i.n.), resulted in a significant delay in time to death compared to that of unimmunized mice, with ~25% survivors in mice challenged i.n. with *F. tularensis* (25). Anti-LVS-V and anti-Schu S4-V antisera cross-reacted with the other strain in Western blot analysis, as well as with clinical isolates spanning all major clades of *F. tularensis* (25).

Monophosphoryl lipid A (MPL) is a well-characterized TLR4 agonist that has been utilized as a vaccine adjuvant in some vaccines that are U.S. FDA approved for human use (27–29). A synthetic form of MPL used in the present study contains a single species of MPL and differs slightly in fatty acid chain length from the major structure approved for human use (30).

The aim of the present study was to improve the protective efficacy of LVS-V against *F. tularensis* Schu S4 i.n. challenge by increasing both the humoral and cellular responses. To this end, mice vaccinated with LVS-V+MPL as an exogenous adjuvant exhibited enhanced anti-*F. tularensis* antibody titers and antigenic diversity. When passively administered, immune sera from LVS-V+MPL-immunized mice significantly extended the mean time to death of naive mice challenged with *F. tularensis* Schu S4 by a subcutaneous (s.c.) route but not an i.n. route. Active immunization of mice (i.p./i.p.) with LVS-V+MPL enhanced T cell activation, as demonstrated by increased numbers of gamma interferon (IFN-γ)-producing T cells, the enhanced ability of splenocytes to control intramacrophage replication of *F. tularensis* LVS, and their increased production of IFN-γ *ex vivo*. Active immunization of mice via heterologous routes (i.p./i.n.) also elicited an elevated immunoglobulin A (IgA) response in bronchoalveolar lavage fluid (BALf) and conferred 60% protection against i.n. challenge with *F. tularensis* Schu S4. Collectively, these data provide evidence that inclusion of MPL in the vaccine preparation further improved the efficacy of the LVS-V nanoparticle vaccine by enhancing both humoral and cellular immune responses.

RESULTS

Immunization of mice with LVS-V elicits serum IgG responses similar to those elicited by Schu S4-V. Previously, Crane et al. and Ireland et al. reported a broadly immune-suppressive effect specific to the *F. tularensis* Schu S4 strain, but not the

attenuated *F. tularensis* LVS strain, that was attributed to the presence of *F. tularensis* Schu S4 lipids (31, 32). Since the catanionic vaccine carrier, V, is composed of surfactants with long hydrophobic tails, bacterial lipids, as well as bacterial proteins, present in the bacterial lysates are likely incorporated into our "functionalized" V preparations. Therefore, we compared levels of anti-*F. tularensis* IgG produced after i.p. immunization of mice with LVS-V or Schu S4-V (35 μg total protein/injection). Sera were harvested from C57BL/6 mice 2 weeks after primary and secondary immunizations with LVS-V or Schu S4-V. As expected, anti-*F. tularensis* IgG titers were significantly elevated after the first immunization and increased more than 10-fold after the second immunization with both formulations (see Fig. S1 in the supplemental material). However, the primary IgG response to LVS-V immunization was, on average, only ~2-fold higher than the response to Schu S4-V and not significantly different. After the second immunization, the IgG responses to both vaccines were increased, essentially to the same antibody titer (Fig. S1). This suggests that any immune inhibitory effect of Schu S4-V is not reflected at the level of vaccine-induced IgG.

Immunization of mice with LVS-V also protects against i.n. challenge with *F. tularensis* LVS. In our previous studies, all *F. tularensis* LVS challenge infections were i.p. While immunization with LVS-V protected fully and was associated with high serum titers of IgG, significant partial protection was gained through immune stimulation with the empty V, despite an absence of IgG and cytokine production. This suggested that the V possess intrinsic innate immune stimulatory activity (25). To determine the efficacy of LVS-V nanoparticles to protect against respiratory tularemia, which has a more severe clinical presentation, we tested this vaccine in an i.n. challenge model in mice. LVS-V (35 μg protein per injection) was administered by either i.p. or s.c. routes to mice on days 0 and 14. To test the effect of exogenous adjuvant on vaccine-induced immunity, half of the immunized mice from each experimental condition also received 100 μg of the synthetic TLR4 agonist monophosphoryl lipid A (MPL; Avanti Polar Lipids). MPL was admixed with the immunogen (LVS-V) just prior to immunization. On day 28, all mice were challenged i.n. with ~5,000 CFU of *F. tularensis* LVS. Clinical symptoms and survival were monitored for 2 weeks after the live challenge, as described previously (25). While the majority of naive control mice succumbed to tularemia within 6 to 8 days following challenge, all LVS-V-immunized mice (with or without MPL) survived challenge with minimal weight loss and only mild clinical symptoms (score of 1 [see Materials and Methods for a description of the clinical scores]) (Fig. S2). Thus, i.n. challenge with *F. tularensis* LVS is not sufficiently sensitive to assess increased efficacy of vaccine preparations. Similar to the i.p. challenge studies with *F. tularensis* LVS we reported earlier (25), 8 of 10 mice that received the empty carrier (V) with MPL prior to i.n. *F. tularensis* LVS challenge also recovered from their tularemia, despite the fact that their onset of clinical symptoms was nearly as fast as that for unimmunized control mice and reached a clinical score of 3 (Fig. S2).

MPL enhances LVS-V-induced IgG responses. Despite the fact that lethal i.n. infection with *F. tularensis* LVS failed to discriminate between mice that had been vaccinated with preparations without adjuvant (LVS-V) or with adjuvant (LVS-V+MPL) through i.p. or s.c. routes of immunization (i.e., there was 100% survival under each protocol [Fig. S2]), immunization with LVS-V+MPL increased *F. tularensis*-specific pre-challenge IgG titers by ~10-fold (Fig. 1A). Empty V+MPL failed to elicit detectable anti-*F. tularensis* IgG (Fig. 1A). In contrast, titers of *F. tularensis*-specific IgA were undetectable in sera of all treatment groups of mice immunized by the i.p. or s.c. routes (data not shown).

We previously showed that *F. tularensis* antigens detected by LVS-V- or Schu S4-V immune sera in Western blot analyses are highly conserved across the major evolutionary clades of *F. tularensis* strains (25). We next asked whether the addition of MPL adjuvant enhanced the detection of either the same or additional *F. tularensis* antigens. Bacterial lysates of *F. tularensis* strains LVS and Schu S4, as well as lysates from clinical strains MA00 (type A) and OR96 (type B), were separated by SDS-PAGE on a 12% gel and

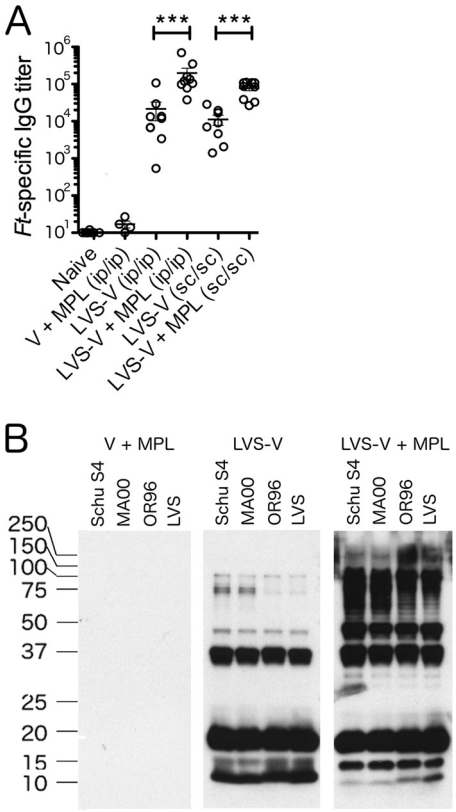

FIG 1 Serum anti-*F. tularensis* IgG is enhanced by addition of MPL adjuvant to LVS-V by multiple immunization routes. C57BL/6 mice were immunized on days 0 and 14 with saline, LVS-V alone, or LVS-V+MPL by either i.p. or s.c. routes, as indicated. (A) Sera harvested on day 28 were analyzed for *F. tularensis*-specific IgG antibody titers by ELISA. The combined results from two independent experiments of 4 mice per treatment group per experiment are shown. Statistical analysis was carried out on log-transformed data using one-way ANOVA, $P < 0.0001$, and Bonferroni's multiple-comparison *post hoc* test with the following *P* values: LVS-V versus LVS-V+MPL by i.p. route, $P < 0.0001$ (***); LVS-V versus LVS-V+MPL by s.c. route, $P < 0.0001$ (***). (B) Bacterial lysates of *F. tularensis* strains Schu S4 (type A), MA00 (type A), OR96 (type B), and LVS (type B) were separated by SDS-PAGE (12% gel) in triplicate and transferred to a nitrocellulose membrane. Western analysis of cut sections of the same membrane were probed using day 28 pooled sera (1:100,000 dilution) from mice immunized twice (i.p./i.p.) with empty V+MPL (left), LVS-V (center), or LVS-V+MPL (right). Pooled HRP-conjugated anti-mouse IgG1, IgG2b, IgG2c, and IgG3 antibodies were used to detect *F. tularensis*-specific antigens. This Western blot is representative of two independent experiments. *Ft*, *F. tularensis*.

transferred to a nitrocellulose membrane. The membrane was subjected to Western analysis using pooled sera from mice immunized by the i.p./i.p. route with V+MPL, LVS-V, or LVS-V+MPL and detected with pooled horseradish peroxidase (HRP)-conjugated secondary antibodies directed against all IgG subtypes. As expected, control sera from V+MPL-immunized mice failed to detect any *F. tularensis* antigens, while immunoblotting with anti-LVS-V immune sera revealed ~10 prominent *F. tularensis* antigens, as we previously reported (25). Anti-LVS-V+MPL immune sera revealed additional *F. tularensis* antigens, especially in the 50- to 250-kDa range, and expression of these antigens was largely conserved among the four *F. tularensis* strains tested (Fig. 1B). Careful inspection of the blot developed with sera from mice immunized with LVS-V+MPL suggests a "ladder" that is commonly associated with LPS of differing O-antigen chain lengths. It was previously reported that different antigens were expressed when *F. tularensis* was grown in different types of media (33); in our hands, however, antigens detected by our LVS-V immune sera were no different whether *F. tularensis* LVS was grown in Mueller-Hinton broth (MHB) or trypticase soy broth (TSB) medium (data not shown).

Effects of passive transfer of LVS-V or LVS-V+MPL immune sera against s.c. and i.n. *F. tularensis* Schu S4 challenge. The role of humoral immunity in protection

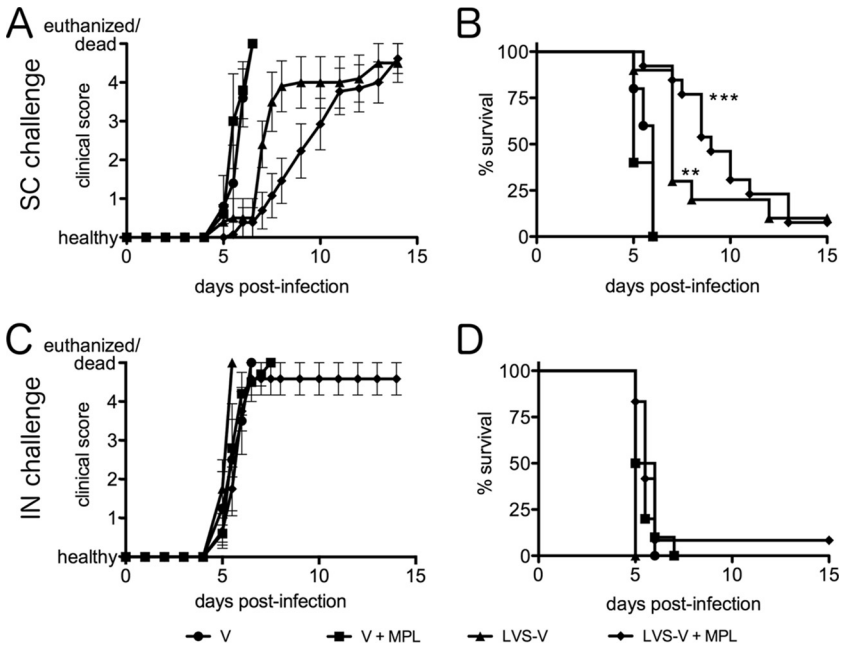

FIG 2 Passive transfer of serum from LVS-V+MPL-immunized mice partially protects against s.c. but not i.n. challenge with *F. tularensis* Schu S4. Mice were immunized as described in the legend to Fig. 1. Immune sera from each group were pooled and administered i.v. to naive mice (50 μl/mouse). (A and B) One day following the passive transfer of serum, mice were challenged with ~50 CFU of *F. tularensis* Schu S4 by the s.c. route. (C and D) One day following the passive transfer of serum, mice were challenged with ~20 CFU of *F. tularensis* Schu S4 by the i.n. route. All mice were followed for 2 weeks and euthanized at the first sign of disease. (A and C) Clinical symptoms; (B and D) survival. Combined data from two independent experiments with 3 to 7 mice per group per experiment are shown. Survival curves were analyzed by log rank (Mantel-Cox) test: for the s.c. challenge, overall $P < 0.0001$, and pairwise log rank posttests of LVS-V versus V, $P = 0.0031$ (**), and of LVS-V+MPL versus V, $P = 0.0002$ (***); for the i.n. challenge, overall differences were nonsignificant.

against *F. tularensis* is controversial, as evidenced by our data showing that immunization with bare V resulted in significant protection against *F. tularensis* LVS challenge (25) (Fig. S2) but not *F. tularensis* Schu S4 challenge (25). Therefore, we sought to examine the performance of sera from MPL-immunized mice in passive transfer experiments. Fifty microliters of pooled sera from mice immunized with saline only (not shown), V only, V+MPL, LVS-V, or LVS-V+MPL by the i.p./i.p. route (as described in the legend to Fig. 1) was administered i.v. to naive C57BL/6J mice 1 day prior to s.c. challenge with ~50 CFU of the highly lethal *F. tularensis* Schu S4 strain, which mimics infectious transmission through a tick or insect vector, the most common way to contract tularemia. All control mice that received sera from either saline-, V-, or V+MPL-immunized mice became symptomatic 5 to 6 days after challenge and were euthanized (same as saline-immunized mice), in accordance with institutional regulations. Adoptive transfer of sera from LVS-V-immunized mice conferred significant delays in the onset of symptoms after s.c. *F. tularensis* Schu S4 challenge ($P = 0.0031$, log rank test) and, consequently, in the time of euthanasia. Serum transfer from LVS-V+MPL-immunized mice conferred an ~2-day delay in onset of symptoms ($P = 0.0002$, log rank test) and time of euthanasia (Fig. 2A and B). Despite these statistically significant extensions of mean time to death, only 1 of 10 mice from each of the groups that received sera from LVS-V- or LVS-V+MPL-immunized mice survived lethal challenge, and this trend failed to reach statistical significance.

In contrast to s.c. challenge models, i.n. challenge models are more relevant to aerosolized *F. tularensis*. Therefore, in a parallel series of experiments, passive transfers of the same donor serum pools were followed by i.n. challenge with ~20 CFU of *F. tularensis* Schu S4 (Fig. 2C and D). However, none of the sera, including the anti-*F. tularensis* IgG-rich sera from LVS-V+MPL-immunized mice, conferred passive protection

in mice challenged i.n. with *F. tularensis* Schu S4. All mice showed clinical signs of tularemia within 4 to 7 days and were euthanized, with no significant differences in time to onset of symptoms between treatment groups. These data indicate that protection against i.n. challenge with *F. tularensis* Schu S4 likely requires other immune mechanisms or soluble factors that are not transferrable with immune serum.

Analysis of BALf IgA in response to i.p./i.n. immunization with LVS-V or LVS-V+MPL. We previously reported that immunization of mice with Schu S4-V by homologous routes (e.g., i.p./i.p., i.n./i.n., or s.c./s.c.) was poorly protective against i.n. challenge with *F. tularensis* Schu S4 (25). We also determined that active immunization with Schu S4-V using a heterologous prime-boost i.p./i.n. regimen increased protection to ~25% survival in mice challenged i.n. with *F. tularensis* Schu S4-V (25). In an effort to enhance protection further, mice were immunized with saline, V+MPL, LVS-V, or LVS-V+MPL, first by the i.p. route and then by the i.n. route 2 weeks later. Two weeks following the second immunization, sera and BALf were collected and *F. tularensis* LVS-specific IgG and IgA titers were measured by enzyme-linked immunosorbent assay (ELISA). In contrast to results from i.p./i.p. or s.c./s.c. immunization regimens, serum IgG titers were not significantly increased by inclusion of MPL in the LVS-V vaccine (Fig. 3A). However, a small fraction of the IgG response, <1% of serum IgG titers, was detected in the BALf of these mice (Fig. 3B). Differences in amount of IgG reaching the lungs that can be attributed to using MPL as an adjuvant with LVS-V were only ~3-fold but were found to be statistically significant ($P = 0.0015$ for LVS-V versus LVS-V+MPL). In addition, ~65% (9 of 14) of the LVS-V-immunized mice showed a detectable *F. tularensis*-specific IgA response in BALf, while ~90% (11 of 12) of LVS-V+MPL-immunized mice exhibited detectable *F. tularensis*-specific IgA. LVS-V+MPL IgA titers were ~8-fold higher than LVS-V IgA titers (Fig. 3C; $P = 0.0003$ for LVS-V compared to LVS-V+MPL). BALf from naive and V+MPL-immunized (control) mice contained extremely low titers of *F. tularensis*-specific IgG or IgA (Fig. 3B and C, respectively).

Western analyses developed with secondary anti-IgA or anti-IgG antibodies of pooled BALf from both the LVS-V- and LVS-V+MPL-immunized groups detected bands at ~20 kDa, ~37 kDa (detected more strongly by LVS-V sera), ~45 kDa, and ~100 kDa (detected more strongly by LVS-V+MPL sera) (Fig. 3D, left blots). In blots developed using sera from mice immunized with LVS-V alone, few additional antigens were detected after overnight exposure (data not shown). In contrast, secondary anti-IgG antibodies detected several additional *F. tularensis* antigens when BALf from LVS-V+MPL-immunized mice was used as a source of primary antibody (Fig. 3D, center blots). BALf IgG from LVS-V-immunized mice failed to detect purified *F. tularensis* LPS, even after overnight exposure (Fig. 3D, right blots, and data not shown), while BALf IgG from LVS-V+MPL-immunized mice detected a ladder formed by electrophoresis of purified *F. tularensis* LVS LPS (Fig. 3D, right blots). In our hands, BALf IgA from any immunization group failed to detect purified *F. tularensis* LPS (data not shown).

Enhanced cellular response to *F. tularensis* after LVS-V+MPL immunization. Cellular immune responses have been shown to be critical for controlling *F. tularensis* infection. In particular, the ability of T cells to produce IFN-γ has been reported to be a strong correlate of protection in mice (34–37). We measured IFN-γ production in four separate experiments, two in which the splenocyte donor mice had been immunized by a homologous (i.p./i.p.) route of immunization, as described in the legend to Fig. 1, and two in which the splenocyte donor mice had been immunized by a heterologous (i.p./i.n.) route of immunization, as described in the legend to Fig. 3. Two weeks after final immunization, splenocytes were harvested from naive mice or mice that had been immunized with V+MPL, LVS-V, or LVS-V+MPL, and these cells were cocultured with *F. tularensis* LVS-infected bone marrow-derived macrophages (BMDM), as described previously (35, 38). After 72 h of coculture, the nonadherent cells were analyzed by flow cytometry, using a gating strategy for discriminating subsets of T cells (Fig. S3). Activated helper T cells (TCRβ^+ CD4$^+$ CD8$^-$ CD44^{hi} [39]) from mice immunized with LVS-V+MPL showed a significant increase in the frequency of IFN-γ-producing (IFN-γ^+)

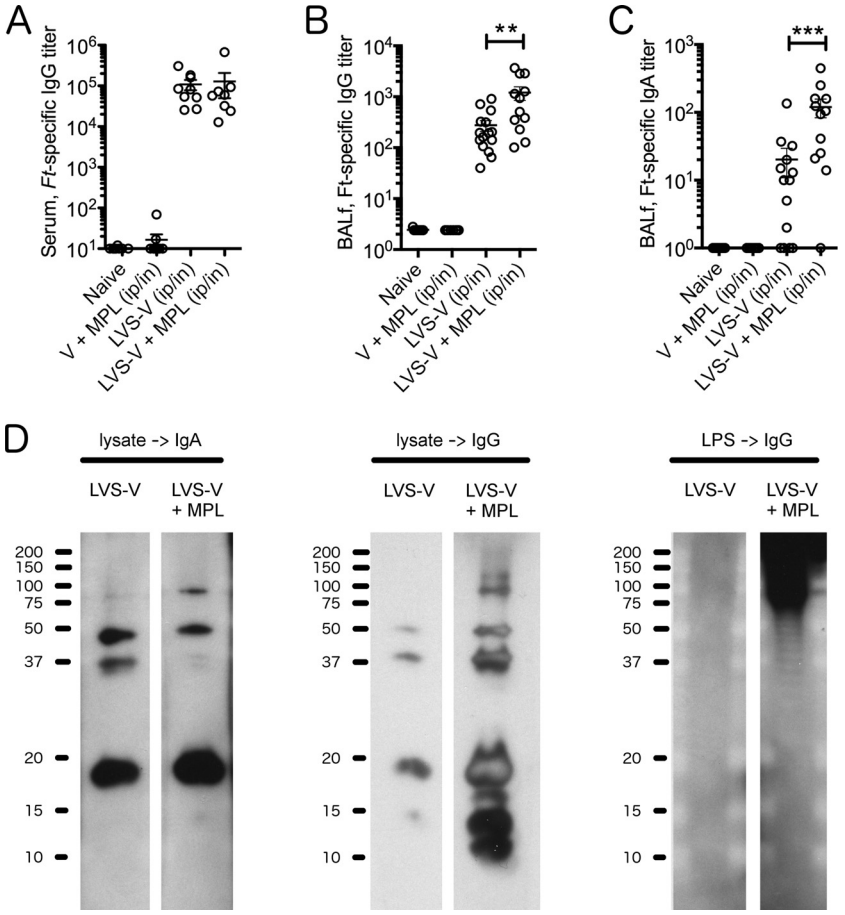

FIG 3 BALf IgG and IgA are enhanced after i.p./i.n. immunization with LVS-V in the presence of MPL. Mice were immunized with empty V or LVS-V without or with MPL, first by the i.p. route (day 0) and then by the i.n. route (day 14). Sera (A) and BALf (B and C) were obtained on day 28 and were analyzed by ELISA for *F. tularensis*-specific IgG titers (A and B) and IgA titers (C). Presented are the combined results from 3 independent experiments with 4 to 5 mice per treatment group per experiment. Statistical analysis was carried out on log-transformed data using one-way ANOVA and Bonferroni multiple-comparison *post hoc* tests: (A) serum IgG, overall $P < 0.0001$, LVS-V versus LVS-V+MPL, nonsignificant; (B) BALf IgG, overall $P < 0.0001$, LVS-V versus LVS-V+MPL, $P = 0.0015$ (**); (C) BALf IgA, overall $P < 0.0001$, LVS-V versus LVS-V+MPL, $P = 0.0003$ (***). (D) Western analysis of *F. tularensis* LVS lysates (left and center panels) or purified LPS from *F. tularensis* LVS (right panels), separated by SDS-PAGE on a 12% Tris-glycine gel, and detected using pooled BALf from 9 mice immunized with LVS-V (i.p./i.n.) and 11 mice immunized with LVS-V+MPL (i.p./i.n.), as indicated at the top of each blot (the mice with a positive IgA ELISA titer, identified in panel C) and developed using a secondary HRP-conjugated anti-mouse IgA antibody (left panels) or all four of the IgG subtype-specific antibodies (center and right panels).

cells, from ~ 5% in splenocytes from naive or V+MPL- or LVS-V-immunized mice to ~11% (Fig. 4A; $P = 0.025$). In contrast, there was essentially no difference in the percentages of IFN-γ^+ CD8$^+$ T cells after coculture (data not shown). Cell culture supernatants from these same cocultures were analyzed by ELISA for secreted IFN-γ, which confirmed the observation that MPL inclusion in the LVS-V significantly increased secretion of IFN-γ during coculture (Fig. 4B; $P = 0.0008$).

Previous studies have shown that treatment of macrophages with IFN-γ results in macrophage activation and killing of intracellular organisms (35, 38). Therefore, in the same coculture system, the ability of the splenocytes to control intramacrophage replication of *F. tularensis* LVS was analyzed. Cocultures with splenocytes derived from naive or V+MPL-immunized mice were included as negative controls. Splenocytes from LVS-V-immunized mice failed to control *F. tularensis* LVS intramacrophage replication (Fig. 4C), consistent with the relatively low number of activated helper T cells that produced IFN-γ shown in Fig. 4A and the amount of secreted IFN-γ (Fig. 4B). However,

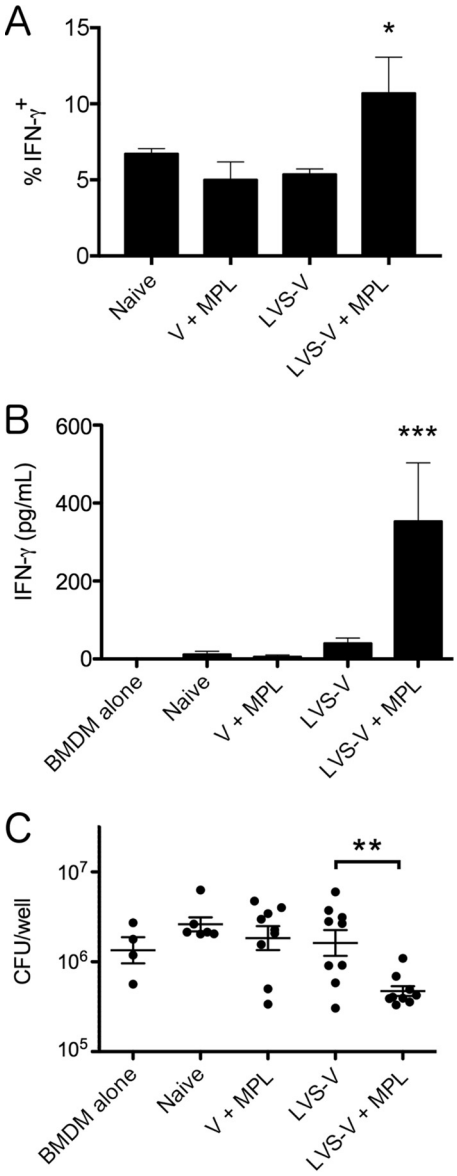

FIG 4 Cellular responses to LVS-V are increased in the presence of MPL adjuvant. Mice were immunized as described in the legend to Fig. 3. Splenocytes were isolated 2 weeks after the second immunization and cocultured with *F. tularensis* LVS-infected BMDM (MOI, 0.05). After 72 h of coculture, nonadherent cells were analyzed by flow cytometry and adherent cells were analyzed for internal *F. tularensis* LVS. (A) Frequency of IFN-γ^+ TCRβ^+ CD4$^+$ CD8$^-$ CD44$^+$ T cells after 72 h of coculture. The results are representative of 2 independent experiments using i.p/i.p. immunization and 2 independent experiments using i.p./i.n. immunization. Data were analyzed by the nonparametric Kruskal-Wallis test, $P = 0.025$, and LVS-V versus LVS-V+MPL data were compared by Dunn's multiple-comparison *post hoc* test, $P = 0.025$ (*). (B) Mouse IFN-γ from coculture supernatants harvested at 72 h, detected by ELISA. Significance was determined using one-way ANOVA ($P = 0.0009$), and LVS-V and LVS-V+MPL data were compared by Bonferroni's multiple-comparison *post hoc* test, $P = 0.0008$ (***). (C) Average results for 3 wells/mouse of *F. tularensis* LVS plate counts from adherent BMDM after 72 h of coculture. Data are combined from two independent experiments. Each symbol represents one mouse. Data were log transformed, and significance was determined using one-way ANOVA ($P = 0.0010$), and LVS-V and LVS-V+MPL data were compared by Bonferroni's multiple-comparison *post hoc* test, $P = 0.0015$ (**).

splenocytes from LVS-V+MPL-immunized mice reduced the number of *F. tularensis* LVS CFU ~5-fold (Fig. 4C; $P = 0.0015$). Similar profiles were detected when the splenocyte donors were immunized s.c./s.c. or i.p./i.p. (data not shown).

Effects of LVS-V or LVS-V+MPL immunization against i.n. *F. tularensis* Schu S4 challenge. We previously elicited partial protection against i.n. challenge with *F.*

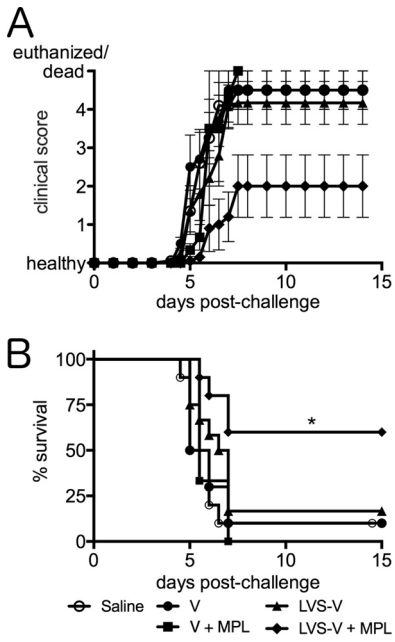

FIG 5 LVS-V+MPL confers significant partial protection against i.n. challenge with *F. tularensis* Schu S4. Mice were immunized as described in the legend to Fig. 3. Mice were challenged on day 28 with ~20 CFU of *F. tularensis* Schu S4 by the i.n. route. Mice were monitored for 2 weeks (twice daily) and euthanized at the first sign of disease. (A) Clinical scores; (B) survival. Combined data from four independent experiments with 3 to 7 mice per group per experiment are shown (two experiments with the saline, V+MPL, LVS-V, and LVS-V+MPL groups and two experiments with the saline, V, and LVS-V groups). Data were analyzed by log rank (Mantel-Cox) test, $P = 0.017$. Pairwise log rank posttests revealed that only the results for the LVS-V+MPL group were significantly different from the results for the rest, with a P of 0.038 for LVS-V versus LVS-V+MPL (*).

tularensis Schu S4 by using a heterologous i.p./i.n. immunization regimen (25). Therefore, we next sought to assess whether protection gained by immunizing mice by these heterologous routes would be further increased by using MPL as an adjuvant with the LVS-V vaccine. Mice were immunized i.p./i.n., as described above. On day 28, mice were challenged i.n. with ~20 CFU of *F. tularensis* Schu S4. Consistent with our previous studies, 9 of 10 mice that were immunized with sterile saline or V alone, 3 of 3 mice that received V+MPL, and 10 of 12 mice immunized with LVS-V only had to be euthanized within 4 to 7 days of infection. In contrast, 6 of 10 mice immunized with LVS-V+MPL by the i.p./i.n. heterologous protocol remained symptom-free for the entire observation period and survived (Fig. 5A and B; LVS-V versus LVS-V+MPL, $P = 0.038$). Of the remaining 4 of 10 LVS-V+MPL-immunized mice that required euthanasia, there was an ~1-day delay in the onset of symptoms (Fig. 5A). Considering all these results, LVS-V+MPL, administered i.p./i.n., significantly increased protection against i.n. *F. tularensis* Schu S4 challenge.

DISCUSSION

Our previous study served as a starting point for the continued development of an *F. tularensis* subunit vaccine that is protective against highly virulent type A strains such as *F. tularensis* Schu S4. By modifying the route of administration, and by comparing LVS-V and Schu S4-V vaccine formulations, we found that enhanced protection against lethal i.n. infection with *F. tularensis* Schu S4 was observed when a prime-boost regimen (i.p./i.n. or i.n./i.p.) was utilized. At best, however, we found that immunization of mice with Schu S4-V by heterologous prime-boost delivery routes (i.p./i.n. or i.n./i.p.) resulted in only ~25% survival of intranasally challenged mice (25). In contrast, i.n./i.n. immunization with Schu S4-V resulted in 0% survival and no significant delay in time to death. Therefore, we sought to improve the efficacy of our subunit vaccine.

To this end, we first sought to determine if immunization of mice with LVS-V versus

Schu S4-V by the same routes would lead to differences in antibody titers elicited. *F. tularensis* Schu S4 possesses immunosuppressive lipids not found in *F. tularensis* LVS (31, 32). Since we observed no differences in anti-*F. tularensis* IgG titers between LVS-V- and Schu S4-V-immunized mice, the immune-suppressive lipid is either not sufficiently stable to contribute significantly to the antigenicity of Schu S4-V or fails to be incorporated into the vesicles or the immune inhibition does not affect the mice at the level of vaccine-induced IgG serum titers.

Since LVS-V performed no worse than Schu S4-V and can be produced in a biosafety level 2 (BSL2) laboratory, we next focused on increasing the efficacy of the LVS-V vaccine by incorporating MPL into the vaccine. MPL is a nontoxic lipid A analog that was found by the Ribi laboratory to greatly increase immune responses to antigens (40). Preparations of MPL are already being included in the vaccine formulation of some human vaccines that are approved by the U.S. FDA (27–29). In the present studies, we used a synthetic MPL preparation to avoid confounding results due to differentially efficacious lipid A substructures found in natural preparations. The endogenous LPS produced by *F. tularensis* is not a TLR4 agonist (6–8). We and others have shown previously that antibodies to this endogenous LPS are produced, making up the majority of the anti-*F. tularensis* IgM response and a small fraction of the IgG response in LVS-V-immunized mice (25, 41–43). However, *F. tularensis* LPS-mediated protection is effective only in a narrow 2-day window prior to challenge and does not yield lasting protection against systemic or aerosol challenge, although partial protection against intradermal challenge and low-virulence strains was observed in previous studies (13, 41–45). Inclusion of synthetic MPL in the LVS-V vaccine augmented IgG titers significantly (Fig. 1A). Importantly, the number of antigenic bands detected on Western blots using antisera derived from LVS-V with MPL as an adjuvant was greatly increased, suggesting that not only was the titer increased but also the spectrum of antibody specificities being generated. Most of these antigens fall into the high-molecular-mass range (>50 kDa), and some display LPS-like laddering. Previous studies have shown a decrease in anti-*F. tularensis* LPS antibodies when MPL was administered along with immunization with *F. tularensis* glycolipid preparations (41). Since no further *F. tularensis* antigens are supplied in the LVS-V+MPL vaccine formulation, yet additional bands are detected by Western blotting (Fig. 1B), we speculate that the addition of MPL to LVS-V enhances antigen recognition by the innate activation of cells in the responding milieu.

F. tularensis replicates intracellularly, and thus the role of humoral immunity against this pathogen has been controversial; however, significant contributions of the humoral response in *F. tularensis* infection are supported by a growing body of literature (46–52). The fact that passive administration of LVS-V+MPL immune sera fully protected mice against challenge with *F. tularensis* LVS (data not shown) and extended the mean time to death significantly in mice challenged with *F. tularensis* Schu S4 by the s.c. route, but not by the i.n. route, suggests the need to increase mucosal immunity while also eliciting a cellular immune response to this largely intracellular pathogen. Of note, the requirement for IgA to protect against i.n. challenge with *F. tularensis* LVS has been described previously (51, 52).

To increase mucosal IgA, we tested the efficacy of LVS-V+MPL, again using the heterologous prime-boost strategy (i.p./i.n.). Indeed, the IgA levels in BALf were significantly enhanced in mice immunized with LVS-V+MPL by this route. Importantly, we found that immunization with LVS-V+MPL increased cellular immunity as well; not only did it increase the number of IFN-γ-producing T cells and the level of secreted IFN-γ, but it also enhanced the ability of splenic T cells from LVS-V+MPL-immunized mice to activate macrophages *in vitro* to kill or inhibit the intracellular replication of *F. tularensis*. The assay using splenocytes to control intramacrophage growth of *F. tularensis* has been shown by others to represent an excellent correlate of cell-mediated immunity (35).

Finally, when mice were immunized using LVS-V+MPL by the i.p./i.n. route, and challenged i.n. with an otherwise lethal dose of *F. tularensis* Schu S4 (~20 CFU), survival

was increased to ~60% of the mice, and none of the survivors exhibited overt clinical signs of tularemia at any point during the entire 2-week observation period. Since *F. tularensis* Schu S4-infected mice are euthanized at the time they become symptomatic, as required by our animal protocol, it is unclear whether the immunized mice could have recovered from *F. tularensis* Schu S4 infection. Historically, killed whole-cell and subunit vaccines, although generally considered to be safer than live attenuated vaccines, have not been effective against *F. tularensis* Schu S4 (53, 54). Early studies with nanoparticles, i.e., immunostimulatory complexes containing Quil A (ISCOMs) displaying Tul4 protein, were only partially protective even against challenge with *F. tularensis* LVS (55). However, the problem may have been the choice of antigen, since other studies focusing on immunodominant antigens also lacked efficacy (56, 57). Vaccines that combine diverse *F. tularensis* antigens have conferred significant protection against *F. tularensis* LVS, but protection against *F. tularensis* Schu S4 has not been reported (58, 59). In a subunit vaccine comprised of Tul4, DnaK, and a non-TLR4 adjuvant, mice infected with *F. tularensis* LVS were protected, while mice infected with *F. tularensis* Schu S4 were not (S. Michalek, personal communication). However, the best results derived from subunit vaccines thus far have been through multivalent presentation of *F. tularensis* membranes. Consistent with our best protection (~60%), Huntley and colleagues recently reported 50% protection against *F. tularensis* Schu S4 using native outer membrane vesicles emulsified with Freund's adjuvant system (60). A different type of outer membrane vesicle using *Escherichia coli* membranes expressing the *F. tularensis* O polysaccharide was immunogenic but only extended the time to death in *F. tularensis* Schu S4 challenge (61). Our LVS-V and Schu S4-V vaccines are structurally similar to *F. tularensis* outer membrane vesicles but have several important advantages, including high batch to batch consistency (25), high stability, and ease of production. Furthermore, LVS-V and Schu S4-V are likely to associate with bacterial DNA and other structures that may activate additional innate immune pattern recognition receptors.

Although it was not the primary goal of our study to compare mouse models of tularemia, this study also revealed other possible differences based on animal protocols and challenge strains used. Most notably, we only observed the intrinsic adjuvant activity of V in *F. tularensis* LVS challenge by using an animal model that euthanizes only moribund mice, thus allowing time for sick mice to recover. The *F. tularensis* Schu S4 challenge model, however, is more stringent and is better for assessing vaccine efficacy.

In conclusion, *F. tularensis* is a highly virulent pathogen for which no licensed vaccine exists in the United States. Effective subunit vaccines against this pathogen are difficult to achieve (53, 54). The LVS-V+MPL subunit vaccine, which enhances both B and T cell adaptive immune responses and confers 60% survival after i.n. challenge with *F. tularensis* Schu S4, represents a significant step forward in the development of a safe subunit vaccine for protecting against this potential biothreat agent. We will continue to optimize this vaccine in other animal models of tularemia with the goal of providing full protection against this important pathogen.

MATERIALS AND METHODS

Reagents and vaccines. Synthetic MPL was purchased from Avanti Polar Lipids (Alabaster, AL). Self-assembling catanionic surfactant vesicles as empty carriers (V) or containing components of *F. tularensis* LVS (LVS-V) were produced and purified by size exclusion chromatography as previously described (25). Briefly, *F. tularensis* LVS cultures are pelleted by centrifugation (3,000 rpm) and lysed with 7.07 mg/ml sodium dodecyl benzenesulfonate (SDBS; Tokyo Chemical Company, Ltd., Tokyo, Japan) in endotoxin-free water (Quality Biological, Gaithersburg, MD). Dispersal is facilitated by gently pipetting the mixture up and down and then incubating it at room temperature with a sterile magnetic stir bar for 1 h prior to the addition of recrystallized cetyltrimethylammonium tosylate (CTAT; Sigma, St. Louis, MO) to a final concentration of 3 mg/ml to induce vesicle formation. Resulting vesicles were purified from excess surfactants by chromatography over Sephadex G-100 columns (Sigma). Protein content in the vaccine batches was analyzed by DC protein assay with reagent "S" (Bio-Rad, Hercules, CA). Where indicated, MPL was admixed with LVS-V within 30 min of vaccine administration (see below).

Bacteria. *F. tularensis* subsp. *holarctica* LVS (ATCC 29684), which has been tested for bacterial viability, phenotypic profile, and virulence, was a generous gift from Karen Elkins (CIBR, FDA). Frozen aliquots were prepared as described previously (62). All *F. tularensis* LVS bacteria were grown in

Mueller-Hinton broth (MHB; BD Microbiology Systems, Franklin Lakes, NJ) supplemented with 2% IsoVitaleX (BD Biosciences, Franklin Lakes, NJ), 1.0% glucose, and 0.25% ferric PP_i (Sigma-Aldrich, St. Louis, MO); Mueller-Hinton agar (MHA) with 5% sheep blood (Teknova, Hollister, CA) was used as solid culture medium. Since there exist reports of loss of structural integrity of *F. tularensis* grown in MHB medium (63), we periodically return aliquots of our frozen stocks to Karen Elkins' laboratory (FDA) to verify that virulence is maintained *in vivo*.

F. tularensis subsp. *tularensis* Schu S4 was obtained from the Centers for Disease Control, Fort Collins, CO, and handled in accordance with CDC-approved biosafety protocols at the University of Virginia (UVA). After mice were infected with this strain, either by the s.c. or i.n. route (for details, see "Immunization and challenge" below), serial dilutions of infectious material were plated on MHA to calculate the infectious dose.

Mice. Female C57BL/6J mice, 6 to 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed and treated according to approved IACUC protocols at both the University of Maryland, Baltimore (UMB), and UVA. It should be noted that male mice are considerably more sensitive than female mice to *F. tularensis* infection (64), and therefore we purposefully decided to optimize our vaccine in female mice first.

Immunizations and challenges. Mice were immunized twice, 2 weeks apart (day 0 and day 14), by i.p. or s.c. injection with empty vesicles (V) or vesicles in which *F. tularensis* LVS components were incorporated (LVS-V; 35 μg protein/mouse), without or with the addition of 100 μg MPL to the LVS-V immediately prior to immunization. For the i.n. booster vaccine, V or LVS-V (10 μg protein/mouse), without or with the addition of 10 μg/mouse of MPL, was administered in a split volume of 25 μl in each nare. Prechallenge sera were collected 1 day prior to bacterial challenge and frozen until further use. Mice were challenged i.n. with live *F. tularensis* LVS (~5,000 CFU/mouse) or with *F. tularensis* Schu S4 (~20 CFU/mouse), diluted in 25 μl sterile saline. Mice were observed twice daily for tularemia symptoms for at least 2 weeks following bacterial challenge. All *F. tularensis* LVS challenges were carried out at UMB under an IACUC protocol that allowed us to observe the mice with early tularemia symptoms to see if they would recover, and moribund mice were euthanized. All *F. tularensis* Schu S4 challenges were carried out in the UVA animal BSL-3 (ABSL-3) facility, and since disease progression in *F. tularensis* Schu S4-infected mice can proceed rapidly and recovery is quite rare, the IACUC protocol for these challenges requires euthanization of mice at the first clinical signs of tularemia disease.

For passive immunization experiments, prechallenge sera from each group of immunized mice were pooled and 50 μl was administered to naive 6- to 8-week old female C57BL/6J mice by i.v. injection 1 day prior to challenge. Mice were challenged by the s.c. route with *F. tularensis* Schu S4 (~50 CFU/mouse) or by the i.n. route with *F. tularensis* Schu S4 (~20 CFU/mouse), diluted in 25 μl sterile saline. Mice were observed twice daily for symptoms for 2 weeks following bacterial challenge.

Clinical scores were assigned as follows: 0, healthy appearance; 1, reduced activity and/or weight loss; 2, symptoms of a score of 1 plus piloerection; 3, symptoms of a score of 2 plus hunched posture; 4, symptoms of a score of 3 plus severely reduced activity. A score of 5 was used for animals that were moribund and had to be euthanized or were found dead. Mice in the ABSL-3 facility are euthanized at the first sign of disease, typically with a clinical score of 1.

ELISA and Western blot analysis. Sera were harvested from mice immunized by various protocols at the indicated time points. Bronchoalveolar lavage fluid (BALf) was harvested in 1 ml sterile phosphate-buffered saline (PBS) from euthanized mice on day 7 after i.p./i.n. immunizations. *F. tularensis*-specific antibody titers were determined by ELISA on plates coated with whole *F. tularensis* LVS, using HRP-conjugated secondary antibodies (goat anti-mouse IgA, goat anti-mouse IgM, or a pool of goat anti-mouse IgG1, IgG2b, IgG2c, and IgG3; all human adsorbed [Southern Biotech, Birmingham, AL]) and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma) reaction in 0.05 M phosphate-citrate buffer (Sigma) for detection as previously described (25, 34, 65). IFN-γ ELISA on cell culture supernatants was carried out by the Cytokine Core Facility at UMB.

Sera from V+MPL-, LVS-V-, and LVS-V+MPL-immunized mice were also analyzed by Western blotting as previously described (25). Briefly, to approximate equal loading per lane, overnight cultures of *F. tularensis* strains Schu S4, MA00, OR96, and LVS were diluted to an optical density at 600 nm (OD_{600}) of 0.30 and then 20 ml of bacteria was pelleted. Bacterial pellets were lysed in 1 ml Laemmli sample buffer. These lysates were boiled for 10 min and separated by SDS-PAGE (10 μl/well) on 12% Tris-glycine (TGX) gels (Bio-Rad, Hercules, CA) in triplicate and transferred to a membrane (Millipore, Billerica, MA). Cut sections of the membrane were subjected to Western analysis with pooled sera (1:100,000 dilution) from immunized mice 2 weeks after the second immunization (i.e., prechallenge). Detection was achieved using the same secondary antibodies as those for the ELISA, followed by reaction with ECL Plus (Pierce, Rockford, IL). Western blots were imaged on X-ray film (GE Healthcare Limited, Little Chalfont, United Kingdom).

Coculture of macrophages and splenocytes. Functional assays utilizing coculture of *F. tularensis* infected BMDM and splenocytes from immunized mice were performed as previously described (35, 38). Briefly, bone marrow was harvested from immunologically naive mice and cultured for 1 week in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% conditioned medium from the LADMAC cell line as a source of macrophage colony-stimulating factor (M-CSF), as well as 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), 200 mM L-glutamine (Gibco, Grand Island, NY), and 10 mM HEPES buffer (Sigma). Confluent monolayers of BMDM were infected with *F. tularensis* LVS at a multiplicity of infection (MOI) of ~0.05 (1 CFU per 20 cells) for 2 h, followed by gentamicin treatment (50 μg/ml for 40 min) to kill any extracellular bacteria. Infected BMDM were switched to antibiotic-free medium, and splenocytes from naive and immunized mice (see detailed description

above) were added at a final concentration of 5×10^5 cells/well. Cocultures were incubated at 37°C with 5% CO_2 for 72 h. After nonadherent cells were harvested for further analysis, the adherent cells were lysed in ice-cold 0.02% SDS and serial dilutions were plated on MHB blood plates to quantify intracellular *F. tularensis* LVS growth, in comparison to uptake in the same assay determined immediately following gentamicin treatment.

T cell intracellular staining. Spleens were harvested from immunized mice, euthanized 2 weeks after the second and final i.p. immunization with V+MPL, LVS-V, or LVS-V+MPL. Splenocytes were recovered by manually grinding spleens in DMEM enriched with 10% FBS, 200 mM L-glutamine, 1× minimal essential medium (MEM) nonessential amino acids, and 10 mM HEPES buffer (10% FBS–DMEM) and treatment with ACK lysis buffer (Quality Biological, Gaithersburg, MD) for 1 min at room temperature. Single-cell suspensions of splenocytes were split for direct staining and cell culture with *F. tularensis*-infected BMDM (see coculture assay above). Nonadherent cells harvested after 72 h of coculture were also subjected to staining. Staining protocols were carried out essentially as previously described (39, 66, 67). Briefly, cells were incubated on ice in 2% FBS–PBS. FcγR was blocked using anti-CD32/CD16 unconjugated antibodies (BioLegend, San Diego, CA). Live/Dead stain (Molecular Probes, Eugene, OR) and fluorescent dye-conjugated antibodies for surface markers to identify T cell subpopulations (TCRβ-fluorescein isothiocyanate [BD Pharmingen, San Jose, CA], CD4-Pacific Blue [Biolegend], and CD8b-phycoerythrin [PE]-Cy7 and CD44-PE-Cy5 [both from eBioscience, San Diego, CA]) were added to cells for 30 min. After washing in 2% FBS–PBS, cell permeabilization was achieved using a CytoPerm/CytoFix kit (BD) according to the manufacturer's instructions. FcγR was reblocked, and permeabilized cells were stained with PE-conjugated anti-IFN-γ (BD). After extensive washing in CytoPerm/CytoWash buffer, cells were postfixed in 2% paraformaldehyde (PFA) and stored at 4°C until analysis. Flow cytometry was performed at the UMB Flow Cytometry Core facility on an LSR II flow cytometer (BD), and data were further analyzed using FlowJo analysis software (Tree Star, Ashland, OR).

The following gating strategy was employed (see Fig. S3 in the supplemental material). Single lymphocytes were first selected by forward and side scatter profiles. Dead cells were excluded based on live/dead staining with Live/Dead stain (Molecular Probes, ThermoFisher). Next, T cells were included based on TCRβ expression. T cells were categorized into $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ populations. $CD4^+$ $CD8^-$ T cells were further classified as memory based on CD44 expression. The frequency of IFN-γ-positive cells was assessed in these populations.

Statistical analysis. Survival curves were analyzed by log rank test (Mantel-Cox test), with pairwise log rank posttests using the Bonferroni method. Data from ELISA and bacterial colony counts were log transformed to normalize data sets and analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison *post hoc* analyses. Frequencies of activated T cells were analyzed to check for Gaussian distribution by the D'Agostino and Pearson normality test. Then nonnormal data were analyzed by the Kruskal-Wallis test (one-way analysis of ranks for nonparametric data) and by Dunn's multiple-comparison test as a posttest to compare results for immunization groups. All statistical analyses were performed using Prism 7 software with its settings to match SPSS and SAS software (GraphPad Software, Inc., La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00574-16.

TEXT S1, PDF file, 1.1 MB.

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We declare no conflicts of interest.

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