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Intranasal Vaccination with a Defined Attenuated *Francisella novicida* Strain Induces Gamma Interferon-Dependent Antibody-Mediated Protection against Tularemia

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***Francisella tularensis* is an intracellular gram-negative bacterium that is the causative agent of tularemia and a potential bioweapon. We have characterized the efficacy of a defined *F. novicida* mutant (Δ*iglC*) as a live attenuated vaccine against subsequent intranasal challenge with the wild-type organism. Animals primed with the *F. novicida* Δ*iglC* (KKF24) mutant induced robust splenic gamma interferon (IFN-γ) and interleukin-12 (IL-12) recall responses with negligible IL-4 production as well as the production of antigen-specific serum immunoglobulin G1 (IgG1) and IgG2a antibodies. BALB/c mice vaccinated intranasally (i.n.) with KKF24 and subsequently challenged with wild-type *F. novicida* (100 and 1,000 50% lethal doses) were highly protected (83% and 50% survival, respectively) from the lethal challenges. The protection conferred by KKF24 vaccination was shown to be highly dependent on endogenous IFN-γ production and also was mediated by antibodies that could be adoptively transferred to naive B-cell-deficient mice by inoculation of immune sera. Collectively, the results demonstrate that i.n. vaccination with KKF24 induces a vigorous Th1-type cytokine and antibody response that is protective against subsequent i.n. challenge with the wild-type strain. This is the first report of a defined live attenuated strain providing protection against the inhalation of *F. novicida*.**

Francisella tularensis is an intracellular gram-negative bacterium that can cause pneumonic tularemia in humans (20, 48). Humans infected by *F. tularensis* usually acquire the disease by contact with infected animals or vectors (ticks), exposure to contaminated food and water, or aerosol exposure (21, 48). *F. tularensis* can be classified into several subspecies, including those relevant to human disease (*F. tularensis* subsp. *tularensis* [type A] and *F. tularensis* subsp. *holarctica* [type B]) (50). An additional species, *F. novicida*, has low virulence for humans but shares a high degree of antigenic and genetic similarities with *F. tularensis* types A and B (22) and maintains high virulence in mice (33, 40), thus making *F. novicida* infections of mice an attractive model for tularemia vaccine development.

One of the arguments supporting the use of *F. novicida* infections as a model for *F. tularensis* subsp. *tularensis* infections is that *F. novicida* exhibits the same behaviors of evasion of phagolysosome fusion and phagosome escape within infected human-derived macrophages as *F. tularensis* subsp. *tularensis* (7, 44). Also, *F. novicida* has proven to be much more amenable to genetic manipulation than the *F. tularensis* subspecies, allowing for the identification of a number of attenuating mutations that might be suitable for a live vaccine strain. However, the lipopolysaccharide (LPS) of *F. novicida* differs from those of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* and is more stimulatory to the production of proinflammatory cytokines, which might affect the relative immune responses to the different (sub)species (11, 29, 53). Other notable differences from *F. novicida* include the ability of the type A and type B strains of *Francisella* to undergo phase variation (11), which can alter the LPS phenotype. Given the pressing need to identify potential tularemia vaccine candidates, important information on the nature of protective immunity to *F. tularensis* inhalation can be derived by utilizing defined mutant *F. novicida* infections of mice. Specific attenuating mutations can then be compared to infections with the same mutant strains of *F. tularensis* subsp. *tularensis* when genetic manipulation of this subspecies is achieved.

To date, the *F. tularensis* live vaccine strain (LVS), which is derived from *F. tularensis* subsp. *holarctica* (type B), has been the only vaccine candidate for tularemia. In humans (45) and animals (14), vaccination with LVS has demonstrated various degrees of protection against aerosolized and parenteral *F. tularensis* subsp. *tularensis* challenges. However, the basis of attenuation of the LVS strain is unknown, making its use in humans somewhat questionable given the uncertain probability of reversion to virulence of the vaccine strain. Still, the efficacy of this live vaccine approach suggests that an effective vaccine could be derived from defined attenuated *F. tularensis* strains.

Several genes essential for intramacrophage growth have been identified in *F. tularensis* (3, 25). Among these, the *iglC* gene, which encodes a 23-kDa protein specifically upregulated during intramacrophage growth, was identified by two different laboratories as being important for intramacrophage survival and growth of *F. novicida* and LVS (24, 25). Moreover, *iglC* mutants of *F. novicida* and LVS are highly attenuated for virulence in mice and growth within amoebae (24, 33). IglC is important for the inhibition of phagosome-lysosome fusion (43), escape from the phagosome (36, 43), and induction of apoptosis in infected macrophages (31). IglC also may play a role in the downregulation of Toll-like receptor-mediated signaling (49).

In this study, we have demonstrated the efficacy of using a defined *F. novicida* Δ*iglC* mutant as a live attenuated vaccine

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candidate against subsequent intranasal wild-type challenge. Pulmonary *F. novicida* infections in mice were used as a model of *F. tularensis* subsp. *tularensis* human infections. Vaccination with the *F. novicida* Δ*iglC* mutant induced a robust (gamma interferon [IFN-γ] and interleukin-12 [IL-12]) cytokine response with concurrent increases in antigen-specific serum immunoglobulin G2a (IgG2a) antibody levels. Moreover, mice vaccinated intranasally (i.n.) with the Δ*iglC* mutant strain were highly protected against subsequent pulmonary challenge with wild-type *F. novicida*. This protection was highly dependent on endogenous IFN-γ production and was mediated by antibodies.

MATERIALS AND METHODS

Bacteria. *F. novicida* U112 was kindly provided by Francis Nano (University of Victoria, Canada). Construction of the isogenic strain KKF24 (*F. novicida* Δ*iglC*::*ermC*) was described previously (32). Strains were grown at 37°C in Trypticase soy broth supplemented with 0.1% cysteine.

Mice. Six- to 8-week-old female BALB/c mice were obtained from the National Cancer Institute (Bethesda, MD). BALB/c IFN-$\gamma^{-/-}$ mice, C57BL/6 μMT (B-cell-deficient) mice, and wild-type animals were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee guidelines.

Intranasal immunization and pulmonary challenge. Mice were first anesthetized with 3% isoflurane using a rodent anesthesia system (Harvard Apparatus, Holliston, MA) (38, 40) and then inoculated intranasally with 10^6 CFU of KKF24 in 25 μl of phosphate-buffered saline (PBS). Mock-vaccinated animals were treated with PBS alone. All animals were then challenged 4 weeks later i.n., as described above, with escalating CFU (100 50% lethal doses [LD_{50}] to 10,000 LD_{50}) of U112 (the LD_{50} of U112 administered i.n. has been calculated to be ~10 CFU [33]). The actual CFU administered in each experiment was determined by serial dilution of inocula and plating on Trypticase soy agar (TSA) supplemented with 0.1% cysteine. Animals were monitored daily for morbidity and mortality. Sera were prepared by collection of blood from the orbital plexus.

Spleen and lymph node cell culture for cytokine profiles. Spleens and cervical lymph nodes were collected from mice 10 days following i.n. vaccination with 10^6 CFU of KKF24 or PBS (mock-vaccinated animals). Single-cell suspensions were prepared (1×10^6 cells/well for spleen cells and 2×10^5 cells/well for lymph node cells) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (Mediatech, Fairfax, VA), with or without increasing concentrations (10^3 CFU to 10^5 CFU) of UV-inactivated KKF24 for 72 h. Cells were also cultured with the unrelated antigen hen egg lysozyme (HEL). Culture supernatants were removed for IFN-γ, IL-12, and IL-4 analysis using BD OptEIA kits (BD Pharmingen, San Diego, CA) according to the manufacturer's instructions and as described previously (40). Briefly, culture supernatants or recombinant murine standards were incubated for 2 h in 96-well plates that were precoated overnight with 100 μl of anti-IFN-γ, anti-IL-12, or anti-IL-4 capture antibody. Following washing, the wells were blocked with 10% fetal calf serum in PBS. The plates were subsequently washed and incubated for 1 h at room temperature with 100 μl of biotinylated anti-IFN-γ, anti-IL-12, or anti-IL-4 monoclonal antibody plus streptavidin-horseradish peroxidase conjugate. The plates were again washed and incubated with tetramethylbenzidine substrate to obtain color development. The absorbance values were measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (μ-quant; Bio-Tek Instruments, Winooski, VT), and results were calculated by comparison of test samples against standards. Lymph nodes and spleens from vaccinated mice were simultaneously evaluated for viable bacteria. No viable bacteria were recovered from the examined tissues at 10 days postinoculation.

Detection of antibody and isotype levels by ELISA. Microtiter plates were coated overnight with 10^6 CFU of UV-inactivated KKF24 in sodium bicarbonate buffer (pH 9.5), washed with PBS containing 0.3% Brij 35 (Sigma), and blocked for 1 h at room temperature with PBS containing 2% bovine serum albumin (BSA; EM Science, Gibbstown, NJ). Serial dilutions of serum were added to wells and incubated at room temperature for 2 h. The plates were then washed and incubated for an additional 1 h with goat anti-mouse total Ig, IgG1, and IgG2a conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After incubation for 1 h, the plates were washed, and *p*-nitrophenyl phosphate substrate was added for color development. The absorbance at 405 nm was measured using an ELISA microplate reader (Biotek Instruments). The reciprocal serum dilutions corresponding to 50% maximal binding were used to obtain titers. No binding of immune sera was observed when the plates were coated with the unrelated antigen HEL.

Opsonophagocytosis assays. To examine the opsonic potential of the immune sera, an opsonophagocytosis assay was established, using chamber slides (Lab-Tek, Nunc, Naperville, IL) that were seeded overnight with 1×10^5 J774A.1 cells (macrophage cell line; American Type Culture Collection, Manassas, VA) overnight. Wild-type *F. novicida* U112 (10^5 CFU) was incubated with various concentrations of heat-inactivated immune (collected 30 days after i.n. vaccination) or normal mouse serum in Eppendorf tubes for 30 min at 37°C with end-over-end rotation. The opsonized bacteria were then incubated for an additional 1 h at 37°C with the J774A.1 macrophages in the chamber slides. Following incubation, the solutions containing the bacteria were removed, and macrophages were incubated with Dulbecco's modified Eagle's medium plus 10 μg/ml of gentamicin to eliminate extracellular bacteria. The macrophages were then washed three times with PBS and fixed with a 2% paraformaldehyde solution overnight at 4°C. The macrophages were subsequently washed, treated with 1% saponin (Sigma) for 30 min at room temperature, incubated with 3% BSA (EM Science), and subsequently stained for 1 h at 37°C with R-phycoerythrin-conjugated rat anti-mouse CD11b (BD Bioscience), *F. novicida* LPS monoclonal antibody 8.2 conjugated to Alexa 488, and bisbenzimide H 33258 (Sigma) for nuclear staining. Cells were washed, and images were acquired using an Axiocam digital camera (Zeiss, Thornwood, NY) connected to a Zeiss Axioskop 2 Plus research microscope. Random fields were imaged, and the numbers of cells containing bacteria were counted. The percentage of macrophages containing fluorescent bacteria was used as a measure of phagocytic activity.

Adoptive transfer studies. Immune sera were prepared by collection from 10 C57BL/6 mice 4 weeks after i.n. vaccination with 10^6 CFU of KKF24. Normal mouse sera were prepared from unvaccinated animals. Naive C57BL/6 μMT (B-cell-deficient) recipient mice were injected intraperitoneally with 200 μl of a 1:3 dilution of pooled immune or normal sera 8 h before i.n. challenge with 100 LD_{50} of *F. novicida*. All animals were also injected with similar amounts of either immune or normal mouse sera 24 h, 48 h, and 72 h after bacterial challenge. Animals were monitored daily for morbidity and mortality.

Histology and immunofluorescence staining. Lungs were removed 3 days and 60 days after primary immunization, embedded in optimal-cutting-temperature resin, and snap frozen. Serial horizontal cryosections of 5 μm were prepared and placed on silane-coated slides (VWR International, West Chester, PA). All slides were dried overnight and fixed in fresh acetone for 20 s at room temperature. Some sections were also fixed with formalin for 10 min and stained by hematoxylin and eosin. For immunofluorescence staining, slides were blocked with 3% BSA for 5 min, followed by incubation with 10% normal rat serum (Sigma) for 30 min. Tissue sections were subsequently incubated with R-phycoerythrin-conjugated rat anti-mouse CD11b (BD Biosciences, San Diego, CA) for 40 min. Some sections were also stained with anti-*F. novicida* LPS monoclonal antibody 8.2 (ImmunoPrecise Antibodies Ltd., Victoria, Canada) conjugated to Alexa 488 (BD Biosciences). Sections were then washed and mounted using Fluorsave reagent (Calbiochem, La Jolla, CA) containing Hoescht stain for nuclear staining. Images were acquired using an Axiocam digital camera (Zeiss, Thornwood, NY) connected to a Zeiss Axioskop 2 Plus research microscope.

Statistical analysis. Survival data were analyzed by the Mann-Whitney rank sum test, and the antibody titers and cytokine analyses were evaluated by Student's *t* test using the statistical software program SigmaStat. The data are presented as means ± standard deviations. The number of repetitions of each experiment is indicated in the figure legends. Each experiment was repeated at least twice.

RESULTS

Intranasal vaccination with the *F. novicida* Δ*iglC* mutant is highly efficacious against intranasal challenge with the wild-type strain. IglC has been shown to be necessary for *F. tularensis* to survive and replicate within macrophages as well as amoebae (33). Moreover, the *F. novicida* Δ*iglC* mutant strain KKF24 is highly attenuated in mice, exhibiting an LD_{50} of $>9.4 \times 10^7$ CFU by the intranasal route, which is $>10^6$-fold higher than the LD_{50} (~10 CFU) of the wild-type U112 strain by the same route (33). To directly assess the efficacy of the *F. novicida* Δ*iglC* mutant to function as a vaccine, BALB/c mice were vaccinated i.n. with 10^6 CFU of KKF24. Vaccinated mice exhibited no signs of morbidity at this dosage and were chal-

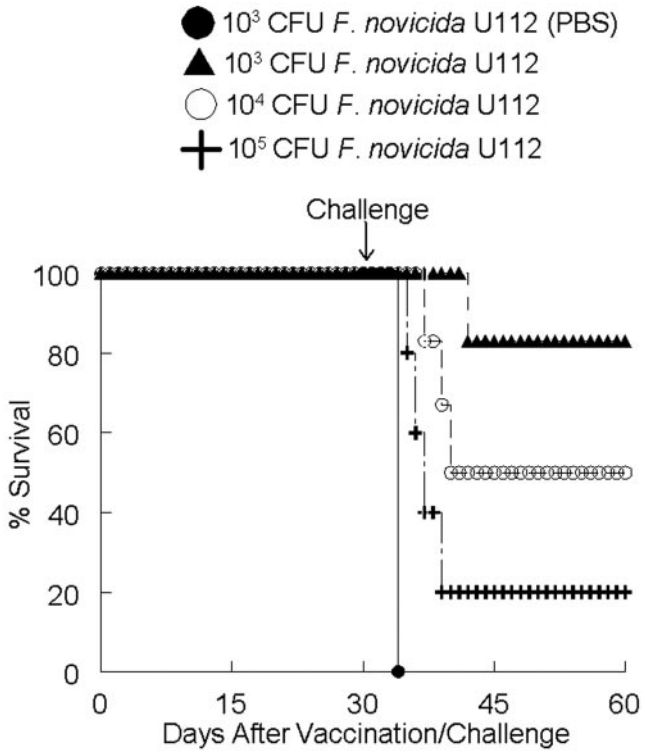

FIG. 1. Efficacy of intranasal vaccination with KKF24. BALB/c mice (6 mice/group) were anesthetized with 3% isoflurane and vaccinated immediately i.n. with KKF24 (10^6 CFU) in 25 μl of sterile PBS. Unvaccinated mice received only PBS. All animals were challenged after 30 days with escalating inocula (100, 1,000, and 10,000 LD_{50}) of wild-type *F. novicida* U112. All animals were monitored daily for survival. Differences in survival between KKF24-vaccinated and mock-vaccinated mice were significant, with *P* values of <0.001. Results are representative of two independent experiments.

lenged 30 days later i.n. with escalating inocula of the wild-type *F. novicida* U112 strain. As shown in Fig. 1, vaccinated animals challenged with 10^3 CFU (100 LD_{50}) of U112 were highly protected (82% survival), with minimal losses of body weight (data not shown). When the challenge inoculum was increased to 10^4 CFU (1,000 LD_{50}) of U112, the survival rate decreased to 50%. Increasing the challenge inoculum further to 10^5 CFU (10,000 LD_{50}) of U112 resulted in 20% survival. There was no survival of any unvaccinated animals at the challenge doses tested, indicating that all three inocula (10^3, 10^4, and 10^5 CFU) of U112 were lethal doses, as expected.

Histological analyses were performed on vaccinated and challenged mice, as shown in Fig. 2. Lung sections from mice vaccinated i.n. with 10^6 CFU KKF24 at 3 days (Fig. 2A) or 60 days (Fig. 2B) postvaccination revealed open air spaces with normal pulmonary architecture and no obvious evidence of histopathological changes, similar to the case for mock (PBS)-treated and unchallenged mice (Fig. 2E). Moreover, the lung tissues of vaccinated mice that were challenged i.n. with 10^3 CFU of the wild-type U112 strain 30 days following challenge (Fig. 2C) appeared similar to the lung tissues of vaccinated unchallenged mice (Fig. 2B). In contrast, the lungs of mice that were mock vaccinated with PBS and then challenged with 10^3 CFU of the wild-type U112 strain displayed severe consolidation and polymorphonuclear cell infiltration 3 days after challenge (Fig. 2D). In situ immunohistochemistry (Fig. 3) was performed on lung tissues from vaccinated and challenged mice with anti-CD11b (red; stains macrophages) and anti-*F. novicida* LPS (green; stains bacteria). Mice vaccinated i.n. with 10^6 CFU KKF24 had very few bacteria within the lung sections 3 (Fig. 3A) or 60 (Fig. 3B) days after vaccination and showed a modest to no influx of macrophages in comparison to mock-treated and challenged mice (Fig. 3D). Moreover, mice vaccinated i.n. with KKF24 and then challenged i.n. with 10^3 CFU of the wild-type U112 strain had no detectable bacteria within the lungs at 30 days postchallenge (Fig. 3C), whereas abundant macrophages and bacteria could be detected in the lungs of mock-vaccinated mice challenged i.n. with 10^3 CFU of the wild-type U112 strain as early as 3 days postchallenge (Fig. 3D). As expected, there were few macrophages and no detectable bacteria in mock (PBS)-treated and unchallenged mice (Fig. 3E). In addition, parallel lung tissues were also evaluated for bacterial loads. The mice vaccinated i.n. with KKF24 exhibited no viable bacteria within the lungs at 3 days postinoculation and no recoverable bacteria 60 days after immunization, which illustrates the high degree of attenuation of this strain. These results obtained with *F. novicida* suggest that an *F. tularensis* Δ*iglC* mutant strain may be a viable candidate for a live attenuated vaccine against pneumonic tularemia.

Vaccination with the *F. novicida* Δ*iglC* mutant induces a Th1-type immune response. It has previously been reported that cell-mediated immunity is a crucial component of protective immunity against *F. tularensis* (16). Thus, we examined whether vaccination with the KKF24 strain induces antigen-specific cell-mediated responses. Mice were vaccinated with KKF24 (10^6 CFU), and 10 days later, spleen and lymph node cells were tested for *F. novicida*-induced cytokine recall responses. As shown in Fig. 4A, the draining cervical lymph node and spleen cells stimulated with UV-inactivated KKF24 induced an appreciable IFN-γ response in a dose-dependent manner in culture. Similarly, there was potent IL-12 secretion (Fig. 4B) from the stimulated cells upon i.n. vaccination with KKF24. There was negligible IL-4 induction in stimulated cultures (data not shown). Cells from mock-vaccinated (PBS) animals had no cytokine responses upon recall with KKF24. In addition, there was no recall response in cells from animals vaccinated with the unrelated control antigen HEL.

Sera from KKF24-vaccinated mice were analyzed for antibody profiles 30 days after initial vaccination as well as 30 days following challenge with the wild-type U112 strain. Intranasal immunization with 10^6 CFU KKF24 induced a robust primary antibody response that included the induction of *F. novicida*-specific total, IgG1, and IgG2a antibodies (Fig. 5). Following i.n. challenge of vaccinated animals with 10^3 CFU of the wild-type strain, surviving animals displayed higher *F. novicida*-specific IgG2a antibody titers than IgG1 titers. No binding of immune sera was observed with the unrelated antigen HEL. The antibodies induced were specific, and the antigenic determinants of *F. novicida* that stimulated the reactivities of the antibodies are currently under investigation. Collectively, the results demonstrate that i.n. vaccination with KKF24 induces a robust Th1-type cytokine and antibody response.

IFN-γ is required for *F. novicida* Δ*iglC* mutant-mediated protection. IFN-γ is a known isotype switch factor for IgG2a

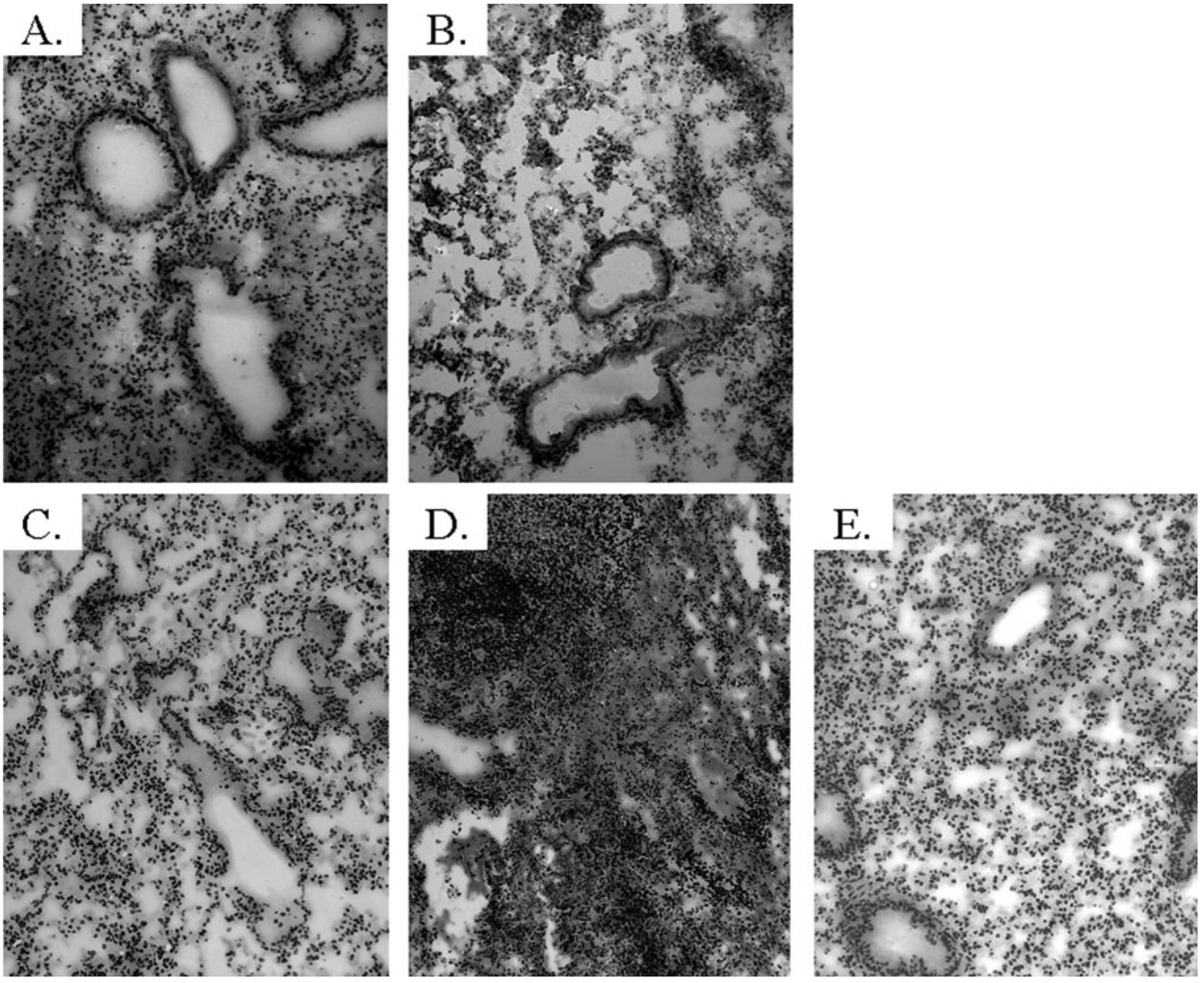

FIG. 2. Intranasal vaccination with KKF24 induces minimal pulmonary histopathological changes. Lungs were harvested from vaccinated animals, prepared for histological study, and stained with hematoxylin and eosin. (A) KKF24-primed lung on day 3; (B) KKF24-primed lung on day 60; (C) KKF24-primed and wild-type-challenged lung on day 60; (D) PBS mock-treated and wild-type-challenged lung on day 3; (E) PBS mock-treated lung. Magnification, ×10. Results are representative of two independent experiments.

production (8) and has been shown to be important for protective immunity against *F. tularensis* (16). To determine the contribution of IFN-γ to protection mediated by the Δ*iglC* mutant strain, BALB/c IFN-$\gamma^{-/-}$ and IFN-$\gamma^{+/+}$ mice were immunized i.n. with 10^6 CFU of the *F. novicida* KKF24 strain. Notably, the vaccinated IFN-$\gamma^{-/-}$ mice all survived the infection with KKF24, with no overt symptoms of disease, indicating the highly attenuated nature of the Δ*iglC* mutant strain. Vaccinated mice were challenged i.n. 30 days later with either 50 LD_{50} (500 CFU) (Fig. 6A) or 100 LD_{50} (1,000 CFU) (Fig. 6B) of the wild-type *F. novicida* strain U112. It was found that all of the IFN-$\gamma^{-/-}$ vaccinated mice quickly succumbed within 4 to 5 days to the pulmonary challenge with the wild-type strain, whereas 100% of the IFN-$\gamma^{+/+}$ mice were completely protected against both challenge doses. As expected, mock-vaccinated (PBS) IFN-$\gamma^{+/+}$ and IFN-$\gamma^{-/-}$ mice were highly susceptible to the lethal challenges. These results indicate that IFN-γ-dependent mechanisms play a pivotal role during vaccination with KKF24 in shaping the protective immune response against i.n. challenge with the wild-type strain.

Antibodies contribute to the protection conferred by i.n. vaccination with the *F. novicida* Δ*iglC* mutant. Intranasal vaccination with KKF24 induced a humoral response and enhanced the production of IgG2a, which is the major murine isotype involved in opsonization and phagocytosis of bacteria (39, 52). To test the functional ability of such antibodies to mediate phagocytic uptake of *F. novicida*, we utilized a complement-independent opsonophagocytic assay using the J774A.1 murine macrophage cell line (Fig. 7). Sera prepared from mice vaccinated with KKF24 and collected at 30 days efficiently mediated phagocytosis of the wild-type *F. novicida* strain U112 in a concentration-dependent manner, whereas normal mouse serum did not. Similar results were observed with primary bone marrow-derived macrophages (data not shown).

To further elucidate the role of humoral immunity in protection, B-cell-deficient (μMT) and wild-type mice were vac-

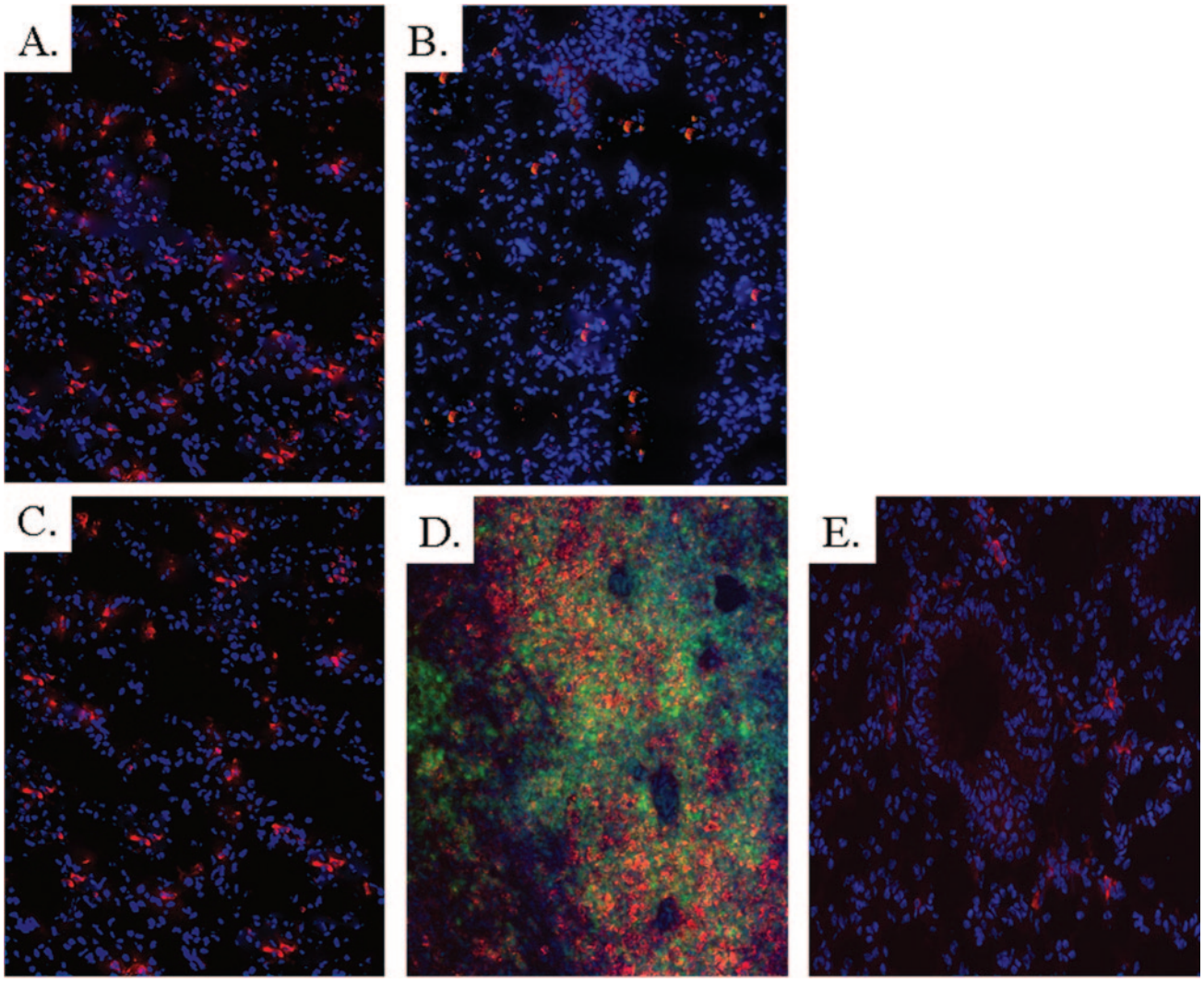

FIG. 3. Immunofluorescence staining of *F. novicida* following pulmonary challenge. Lungs were harvested from vaccinated animals, and sections were stained with R-phycoerythrin-conjugated rat anti-mouse CD11b (red) and anti-*F. novicida* LPS monoclonal antibody 8.2 (green). Nuclear staining (blue) was visualized with bisbenzimide H 33258. (A) KKF24-primed lung on day 3; (B) KKF24-primed lung on day 60; (C) KKF24-primed and wild-type-challenged lung on day 60; (D) PBS mock-treated and wild-type-challenged lung on day 3; (E) PBS mock-treated lung. Magnification, ×20. Results are representative of two independent experiments.

cinated with 10^6 CFU KKF24 and then challenged 30 days later with 100 LD_{50} of the *F. novicida* wild-type strain U112. As shown in Fig. 8A, vaccinated B-cell-deficient mice were found to be highly susceptible (20% survival) to the lethal wild-type *F. novicida* challenge compared to similarly vaccinated wild-type (80% survival) mice. These results demonstrate a role for B cells in the protective immune response of Δ*iglC* mutant-vaccinated mice. To determine whether antibodies could reconstitute protection in B-cell-deficient animals, adoptive transfer studies using immune or normal serum were performed. The passive transfer regimen was based on previously published methods for protection in B-cell-deficient mice against another intracellular organism, *Chlamydia trachomatis* (37). Adoptive transfer of immune serum from KKF24-vaccinated mice to naive B-cell-deficient mice afforded a high level of protection (80% survival of mice over the 30-day observation period) against i.n. lethal challenge with 100 LD_{50} of the *F. novicida* wild-type strain U112 compared to 0% survival by day 6 in the same animals receiving normal mouse serum (Fig. 8B). These results indicate that antibodies contribute to the protection afforded by i.n. vaccination with the *F. novicida* Δ*iglC* mutant.

DISCUSSION

F. tularensis subsp. *tularensis* is classified as one of the most infectious pathogenic bacteria because inhalation of only a few organisms will cause disease and significant mortality (45). *F. tularensis* is also considered a potential biological weapon and has been developed as a germ warfare agent by several government programs (6, 12, 26). In this regard, the respiratory tract and lungs are major portals of entry for inhalation exposure and serve as primary sites of infection before systemic spread.

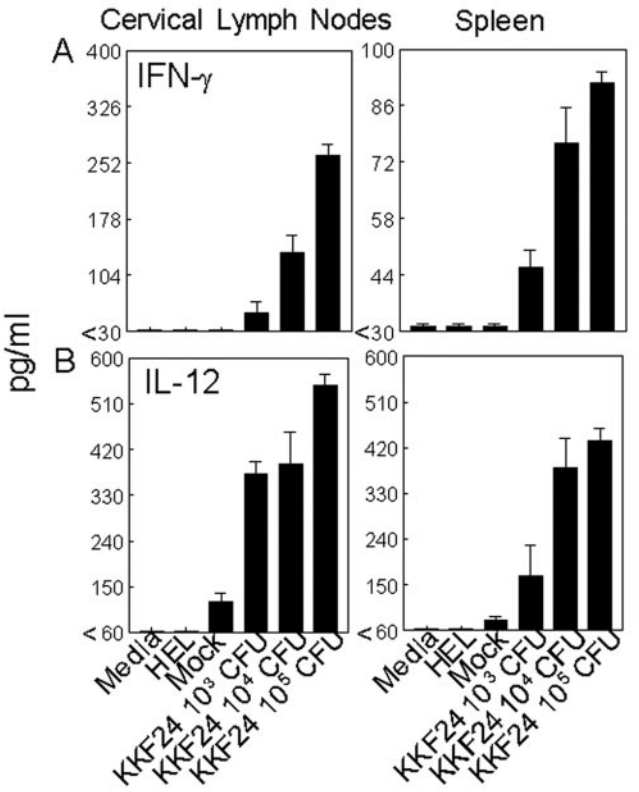

FIG. 4. Cytokine recall responses after vaccination with KKF24. BALB/c mice (3 mice/group) were anesthetized with 3% isoflurane and vaccinated i.n. with KKF24 (10^6 CFU) in 25 μl of sterile PBS. On day 10, spleen and lymph node cells were tested for KKF24-induced IFN-γ (A) and IL-12 (B) secretion by ELISA. Differences in IFN-γ and IL-12 secretion between cells exposed to KKF24 and cells alone were significant, with P values of <0.005. Results are representative of three independent experiments.

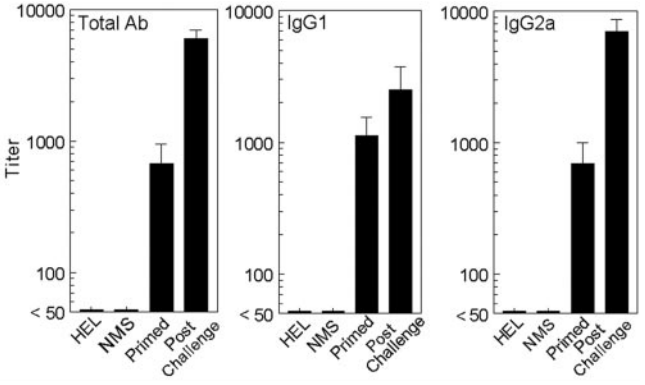

FIG. 5. Serum antibody profiles after vaccination i.n. with KKF24. BALB/c mice were first anesthetized with 3% isoflurane and primed i.n. with KKF24 (10^6 CFU) in 25 μl of sterile PBS. All animals were bled on day 30 and subsequently challenged with 100 LD_{50} of wild-type *F. novicida* U112. Surviving animals were bled on day 60, and sera were analyzed by isotype-specific ELISAs using UV-inactivated KKF24-coated microtiter plates. The results are reported as 50% end-point titers. Differences in antibody binding between immune sera and normal mouse sera (NMS) for total antibody (Ab), IgG1, and IgG2a were significant, with P values of <0.005. Results are representative of three independent experiments.

Aerosol exposure to *F. tularensis* leads to high levels of morbidity and mortality, yet there currently is no tularemia vaccine approved for human usage in the United States. Because *F. tularensis* is an intracellular pathogen, a live attenuated strain would be predicted to be a potentially effective means of vaccination. While LVS vaccination has been shown to provide protection against aerosol challenge with *F. tularensis* subsp. *tularensis* in mice and humans (14, 45, 56), the basis for attenuation of the LVS strain is unknown, thus bringing its safety for humans into question. Still, the protective efficacy of LVS indicates that defined attenuated *F. tularensis* strains can be identified which can function as vaccines to induce protection against pulmonary exposure to this organism. Utilizing an *F. novicida* intranasal infection model in mice, we have evaluated the efficacy of a Δ*iglC* mutant strain to protect against intranasal challenge with the wild-type *F. novicida* strain. Vaccination with the Δ*iglC* mutant strain proved to be efficacious against lethal pulmonary challenge, and protection was shown to be dependent on endogenous IFN-γ production and mediated by antibodies. These results are the first description of a defined attenuated live *F. tularensis* vaccine strain that provides protection against *F. novicida*-induced pneumonic disease.

Intranasal vaccination strategies have the advantage of directly targeting the respiratory compartment and hence inducing protective immunity at the primary site of infection. Protection against pulmonary challenge with *F. tularensis* will be a critical

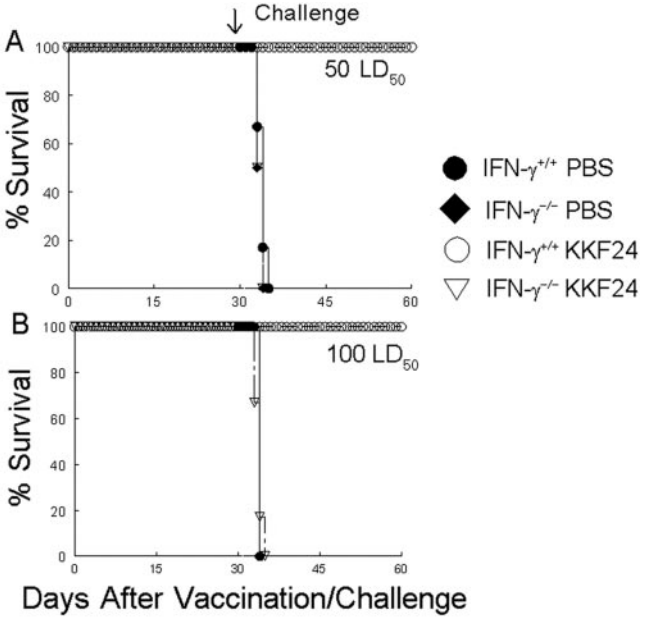

FIG. 6. IFN-γ-dependent KKF24-mediated protection. BALB/c IFN-$\gamma^{-/-}$ mice and IFN-$\gamma^{+/+}$ mice (6 mice/group) were anesthetized with 3% isoflurane and vaccinated i.n. with KKF24 (10^6 CFU) in 25 μl of sterile PBS. Animals were challenged after 30 days with 50 LD_{50} (A) or 100 LD_{50} (B) of wild-type *F. novicida* U112. All animals were monitored daily for survival. Differences in survival between vaccinated IFN-$\gamma^{+/+}$ mice and IFN-$\gamma^{-/-}$ mice were significant, with P values of <0.001. Results are representative of two independent experiments.

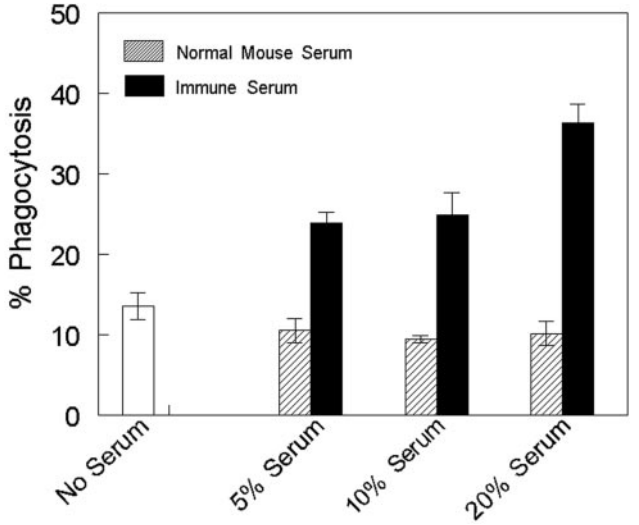

FIG. 7. Enhanced antibody-dependent phagocytic activity against *F. novicida*. *F. novicida* U112 was opsonized with various concentrations of normal mouse serum or immune sera obtained from KKF24-vaccinated BALB/c mice. Phagocytosis was performed with the J774A.1 macrophage cell line (multiplicity of infection, 1:1 [bacterium-to-macrophage ratio]). Cells were stained with anti-*F. novicida* LPS monoclonal antibody 8.2 and anti-mouse CD11b and visualized by fluorescence microscopy. The percentage of macrophages containing bacteria was used as a measure of phagocytic activity. Results are shown as mean percentages of macrophages containing fluorescent bacteria ± standard errors of the means. For differences between 5% serum and normal mouse serum, $P < 0.004$; for differences between 10% serum and normal mouse serum, $P < 0.005$; and for differences between 20% serum and normal mouse serum, $P < 0.001$. Results are representative of two independent experiments.

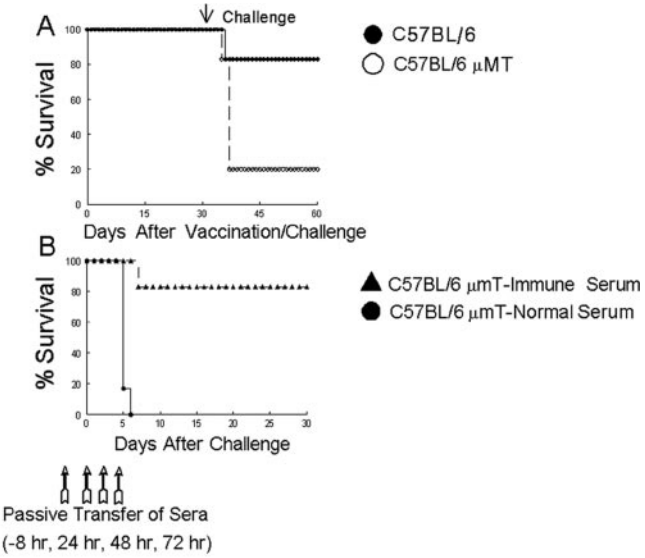

FIG. 8. Contribution of antibodies to KKF24-mediated protection. (A) C57BL/6 μMT (B-cell-deficient) and C57BL/6 wild-type mice (6 mice/group) were anesthetized with 3% isoflurane and vaccinated i.n. with KKF24 (10^6 CFU) in 25 μl of sterile PBS. Animals were challenged after 30 days with 100 LD_{50} of wild-type *F. novicida* U112. (B) Adoptive transfer of immune or normal serum to naive C57BL/6 μMT animals. Immune sera were prepared from C57BL/6 mice vaccinated i.n. with KKF24. Normal sera were collected from unvaccinated C57BL/6 mice. Naive C57BL/6 μMT animals (6 mice/group) were injected intraperitoneally with 200 μl of either pooled immune or normal sera (1:3 dilution) 8 h before i.n. challenge with 100 LD_{50} of *F. novicida*. All animals were also injected with similar amounts of either immune or normal mouse sera 24 h, 48 h, and 72 h after bacterial challenge. Animals were monitored daily for morbidity and mortality. Results are representative of two independent experiments.

component of an effective vaccine against the illicit use of this organism. In this regard, the route of vaccine delivery is an important issue for consideration. Since parenteral immunization strategies are only partially effective in inducing optimal respiratory immunity (54), vaccination regimens, such as that utilized in this study, that directly target the respiratory mucosa may induce optimal local immunity against inhalation exposure to *F. tularensis* and reduce the dissemination of the organisms systemically. Our results show that the Δ*iglC* mutant strain KKF24 efficiently primes cell-mediated and humoral responses and confers protective immunity against pulmonary challenge with *F. novicida* (82% protection against 100 LD_{50} and 50% protection against 1,000 LD_{50}). Previous studies (27) have shown that aerogenic immunization with LVS in both mice and humans afforded greater protective capacity than conventional intradermal methods against aerosol challenges with *F. tularensis*. More recently, Lyons and colleagues (56) have shown the feasibility of i.n. vaccination with LVS for protection against the virulent type A strain. These results, collectively with the present study, demonstrate the efficiency of priming local defenses in the respiratory compartment to generate optimal protection against tularemia acquired through the pulmonary route. Clearly, the safety considerations of the vaccination strategy have to be evaluated in the future, but similar vaccination strategies, such as the licensed live attenuated FluMist vaccine for influenza, have been shown to be highly efficacious and safe by this route of inoculation (28).

Intranasal vaccination with the Δ*iglC* mutant strain induced a biased Th1-type cytokine response with enhanced IFN-γ and IL-12 production. IFN-γ has a variety of immunoregulatory functions, which include induction of Th1 cell differentiation, activation of NK cells (51), and induction of isotype switching to IgG2a antibody production (8). Additionally, IFN-γ may limit the excessive inflammatory pathology that is associated with intracellular bacterial infections in a nitric oxide-dependent manner (10). The pivotal contribution of IFN-γ to Δ*iglC* mutant vaccine-mediated protection is evident in that all vaccinated IFN-$\gamma^{-/-}$ animals quickly succumbed to challenge with the wild-type *F. tularensis* strain. The IFN-γ produced during the adaptive phase of the immune response is presumably from memory T cells, as indicated by the susceptibility of the mock-vaccinated IFN-$\gamma^{+/+}$ mice compared to the vaccinated IFN-$\gamma^{+/+}$ animals. Previous studies (2, 9, 18, 19, 34, 46) have demonstrated that protective immunity to *F. tularensis* is primarily mediated by IFN-γ and tumor necrosis factor alpha (TNF-α). Naive mice treated with IFN-γ- and TNF-α-depleting antibodies and IFN-$\gamma^{-/-}$ mice are highly susceptible to normally sublethal challenges with LVS (19, 46). $CD4^+$ T cells and $CD8^+$ T cells have also been shown to be important for the resolution of a primary *F. tularensis* LVS challenge and for secondary protection (9, 57). Additionally, there is evidence that B cells play an important early role in protective immunity against *F. tularensis* (4, 15, 17).

Our studies also demonstrated a role for B cells in conferring protection after i.n. vaccination with KKF24. Specifically, vaccinated B-cell-deficient animals were shown to be highly susceptible to i.n. challenge with the wild-type *F. novicida* strain. Moreover, passive transfer of immune serum to naive B-cell-deficient mice reconstituted protection in these animals, indicating a role for antibodies in protection. In other experiments using different doses of challenge inoculum, immune serum was found to be 300-fold more protective than normal serum, indicating that the protection afforded by immune serum was highly titratable (M. A. Pammit and B. P. Arulanandam, unpublished observations). In previous studies, the contribution of antibodies to host protection against *Francisella* has been somewhat ambiguous. Passively transferred antibodies have been shown to confer protection against *F. tularensis* subsp. *holarctica* wild-type and LVS strains (23, 42, 47) but not against *F. tularensis* subsp. *tularensis* strains (1, 48). Intracellular bacteria are thought to be inaccessible to serum proteins and, consequently, would be protected from the neutralizing activities of antibodies. However, there is now convincing evidence to suggest that antibodies may confer significant protection against intracellular bacteria (5), including *Listeria monocytogenes* (13) and *Ehrlichia chaffeensis* (35, 55). Antibodies may directly influence the phagocytic activity of Fc receptor-bearing cells, such as macrophages and NK cells, to enhance phagocytosis of intracellular pathogens (30, 41). Consistent with this observation, sera from Δ*iglC* mutant-vaccinated animals exhibited enhanced antibody-mediated phagocytosis of *F. novicida* in both an established macrophage cell line (J774A.1) and primary bone marrow-derived macrophages. Antibodies may have an important underappreciated role in eliciting optimal protective immunity against *F. tularensis*.

The studies reported here utilized i.n. challenges of mice with *F. novicida* as a model for human pneumonic tularemia. While no animal infection model accurately reflects all aspects of human disease, *F. novicida* infections are a reasonable model for tularemia because *F. novicida* has an LD_{50} similar to that of the *F. tularensis* subsp. *tularensis* strain via the i.n. route in mice, and the hallmarks of disease appear similar during infections caused by the two species (22, 33, 40). Furthermore, *F. novicida* behaves similarly to *F. tularensis* subsp. *tularensis* within human macrophages (7, 44), suggesting that the underlying pathogenic mechanisms utilized by both species are similar. The cause of the difference in host tropism exhibited by the two species is currently unclear, but the protective efficacy of an *F. novicida* Δ*iglC* mutant strain against wild-type *F. novicida* challenge suggests that an *F. tularensis* subsp. *tularensis* Δ*iglC* mutant strain may be protective against *F. tularensis* subsp. *tularensis* challenge. Genetic techniques have been established for the manipulation of *F. novicida* strains (3, 25, 32), but as yet there have been no published reports of a successful defined mutation introduced into an *F. tularensis* subsp. *tularensis* strain. Given that genetic manipulation of *F. tularensis* subsp. *tularensis* strains is achievable, construction of a defined *iglC* mutant of this organism may provide a potent vaccine candidate against virulent *F. tularensis* in humans.

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