Tularemia is an extremely infectious zoonotic bacterial disease caused by Francisella tularensis. The CDC has classified it as a category A bioterrorism agent with high potential to be used by bioterrorists (4, 5, 7). A live attenuated vaccine, derived from the multiple passage of a fully virulent strain of F. tularensis subspecies holarctica, was previously used as an investigational new drug in the United States. However, this vaccine is not fully licensed and does not offer a high level of protection against respiratory challenge. In addition, cultures of live vaccine strain (LVS) under some conditions can result in poorly immunogenic variants (8), and there have been reports of human tularemia arising from incorrect administration. Therefore, there is a need to move forward with developing a safe and improved vaccine against F. tularensis.

Strategies for development of a new generation of tularemia vaccines include identification of individual components of F. tularensis, such as the lipopolysaccharide or various other outer membrane proteins, as potential vaccine antigens. A 17-kDa membrane protein, Tul4 (of unknown function), has been previously characterized as a potential immunogen (16). Although Tul4 protein immunization has been shown to be ineffective at protecting against low-virulence strains, it reduces liver and spleen colonization by F. tularensis (13) and elicits strong humoral and T-cell proliferative activity (25, 26). Therefore, Tul4 may warrant use of another approach as a vaccine target. In this study, Tul4 was delivered as an adenovirus-vectored genetic vaccine.

Replication-incompetent adenoviruses are currently available as efficient gene transfer vehicles for both in vitro and in vivo approaches (2, 24, 31). A replication-incompetent adenovirus is ideal as a vaccine vector because it can infect a broad range of human cells, causes only mild symptoms in humans, and can accommodate up to 7.5 kbp of DNA (29). Adenovirus-vectored recombinant vaccines expressing a wide array of antigens have previously been constructed, and protective immunity against different pathogens has been demonstrated in animal models (19, 28, 30, 33, 34). In this study, we constructed a replication-incompetent adenovirus carrying the Tul4 gene, Ad/opt-Tul4, and demonstrated the efficacy of using Ad/opt-Tul4 for genetic vaccination against tularemia in a murine model.

MATERIALS AND METHODS

Construction of adenoviral vector encoding codon-optimized Tul4 of F. tularensis. The adenoviral vector used in this study was derived from human adenovirus serotype 5 that was rendered replication incompetent by deletion of the E1 and E3 regions. Replication-incompetent recombinant adenoviral vectors were constructed using the AdEasy System (Stratagene, La Jolla, CA). The codon-optimized gene encoding Tul4 of F. tularensis was synthesized by GenScript (Piscataway, NJ). The signal peptide of human tissue plasminogen activator (PLAT) (amino acids 1 to 25; GenBank accession no. BC002793) plus two serine residues were added upstream of the Tul4 sequence. The human codon-optimized sequence of tul4, along with the native gene and protein sequences of Tul4, are shown in Table 1.

The synthesized DNA was subsequently cloned into a shuttle vector, pShuttle-CMV (Stratagene, La Jolla, CA), at its SalI site. The DNA sequence of the synthesized gene was further confirmed by DNA sequencing analysis. The adenoviral vector was then constructed according to the standard procedures as described previously (15, 32). Similarly, the Ad/
Null vector without transgene was also constructed as a negative control. Adenoviruses isolated from single plaques were then propagated in AD293 cells (Stratagene, La Jolla, CA), purified by CsCl gradient purification, and dialyzed with adenovirus storage buffer (10 mM Tris, pH 7.5, 135 mM NaCl, 5 mM KCl, and 1 mM MgCl₂). The purified adenovirus was stored in 1.0 M sucrose at −86°C until use. Viral titers (PFU/ml) were determined by plaque assay.

Animal vaccination, sample collection, and challenge. Six- to 8-week-old female BALB/c mice were purchased from Taconic Farms (Hudson, NY) and housed with 5 animals per cage. They were maintained in a controlled environment (22 ± 2°C; cycles of 12 h light and 12 h dark) in accordance with the U.S. Public Health Service and the *Guide for the Care and Use of Laboratory Animals* (20). The animals were provided Laboratory Rodent Diet 5001 with access to food and water *ad libitum*. The research was conducted in compliance with the Animal Welfare Act and other federal and state statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (20).

Mice were allotted into different groups comprised of 5 to 10 mice per group. They were vaccinated on week 0 as follows: with the Ad/opt-Tul4 vaccine or the control vector Ad/Null via injection intramuscularly (i.m.; 10⁵ PFU/mouse) to the hind-leg quadriceps or intradermally (i.d.; 10⁷ PFU/mouse) to the back of the neck or tail.

### TABLE 1 Native and codon-optimized *tul4* DNA sequences along with the protein sequences

| Sequence     | Position | Sequence                  |
|--------------|----------|---------------------------|
| Native       | 1        | ATGAAAAAAAAATAAAGCTTAAGCTTTATCTTTATCAATGGCAGTTAGCTGTTCTACTCTA |
| Optimized    | 1        | ATGAAAGAGACTCTAAAGCTTCGCTCTGCTCTCTCTCTCTGCTCTGCTCTGCTACTCTG |
| Protein      | 1        | M K K I I K L S L L S L S I A G L A S C S T L |
| Native       | 70       | GGTTAGTTGCGCTCTGATGATGCAAAAGCTTCAGCTAAAAGATACGCTGCTGCTCAAGACGACTGACTG |
| Optimized    | 70       | GGCCTGGGCGCTCCGAGACGCAGAAAGCTCCCCAGACGCACAGCGCTGCTGCTGCTGCTGCTGCTG |
| Protein      | 24       | G L G S D D A K A S A K D T A A A Q T A T T |
| Native       | 139      | GAGCAAGCTCTGCTGACTCAAGCCAACATGAAATAGATGACATAGATGACATAGATGACATAGATGAC |
| Optimized    | 139      | GACGCAAGCCCGCCGGCTCGTCAAGCAACAGCCAGGAAAGATGACATAGATGACATAGATGACATAGATGAC |
| Protein      | 47       | E Q A A A V S K P T A K V S L N K L G Q D K I |
| Native       | 208      | AAAGCAACTGTATATAACAGATACAAATAATACCCCAAACAGGAAGTGGAAGATTCTGACGAGGAGCTC |
| Optimized    | 208      | AAGGCAACTGTATATAACAGATACAAATAATACCCCAAACAGGAAGTGGAAGATTCTGACGAGGAGCTC |
| Protein      | 70       | K A T V T A Y N N N P Q G S V R L Q W Q A P |
| Native       | 277      | GAAGTTCTCTAGTCGCAATGATAGATGACATAGATGACATAGATGACATAGATGACATAGATGAC |
| Optimized    | 277      | AAGGATCTCAAAGCGAGACACCTCTCTCCACACCCACAGGAAGTGGAAGATTCTGACGAGGAGCTC |
| Protein      | 93       | E G S K C H D T S F P I T K Y A E K N D K T W |
| Native       | 346      | GCAACTGTAACAGTAAAGCAAGGTAGTAAGGCTCTGCTAGCAGCTGAGTGGACGACGTAATAGTATAGT |
| Optimized    | 346      | GCCCACTGACGAAAGGAAAGGAAACAGGACTCTGCTCTGCTAGGGAACGGCCCAAGATGCGATAGCAG |
| Protein      | 116      | A T V T V K Q G N N F C S G K W T A N V V Y D |
| Native       | 415      | AAAGAAGTTATGCGCCTCTGATTTCAATAATATTAA |
| Optimized    | 415      | AAGAAATGTAGTCGCTCTGAGATTATCAACATCAGT |
| Protein      | 139      | K E V I A S D S I N I * |

* Nucleotide changes are in red.
PFU/mouse), with recombinant Tul4 protein (i.m.; 5 µg/mouse), or with *F. tularensis* LVS (i.d.; 105 CFU). Various mouse strains have been shown to survive i.d. challenge with LVS ≥ 105 CFU (23); thus, we deemed an i.d. dose of 105 CFU appropriate as a positive control for our vaccination studies. Some groups of Ad/opt-Tul4-, Ad/Null-, or recombinant Tul4 vaccinated mice were administered booster doses on week 2 and week 4, while other groups were not. Mice vaccinated once with *F. tularensis* LVS did not receive additional doses.

Animal sera were obtained by retro-orbital bleeding every 2 weeks (week 0, 2, 4, and 6) and stored at −20°C. All animals were challenged via intraperitoneal (i.p.) injection of *F. tularensis* LVS (210 to 400 CFU) after 7 weeks of vaccination. It has been previously shown that 100% of BALB/c mice perish following i.p. administration of 100 CFU LVS by 7 days post-inoculation (12). The challenged mice in this study were monitored for 10 days. They were observed four times a day for 1 week and then twice a day thereafter. The number of deaths for each group was recorded as the endpoint.

**ELISA for determination of antibody concentration.** Serum anti-Tul4 IgG and subtypes IgG1 and IgG2a antibody concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) quantization kit (Bethyl Laboratories, Inc., Montgomery, TX) using a modified procedure. Ninety-six-well flat-bottom immunoplates (Nunc) were coated with 100 ng/well of His-tagged Tul4 recombinant protein (produced in BL21 Star DE3 *Escherichia coli*) at 4°C overnight. For the standard curve, plates were coated with capture antibodies as follows: goat anti-mouse IgG, IgG1, or IgG2a affinity purified (Bethyl) in 100 µl coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). Plates were washed 5 times with phosphate-buffered saline (PBS) buffer containing 0.05% Tween and then blocked with 200 µl PBS (pH 7.4) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. After the plates were washed, 1:100 dilutions of mouse serum samples in PBS (pH 7.4) containing 0.05% Tween 20 and 1% BSA were added and incubated at 37°C for 2 h. For the standard curve, 100-µl serial dilutions of reference serum containing given amounts of mouse antibodies were added. The plates were washed five times and incubated with 100 µl/well of 1:1,000 dilution of goat anti-mouse IgG, IgG1, or IgG2a conjugated to alkaline phosphatase for 1 h at room temperature. After the plates were washed 5 times, the bound antibody was developed with p-nitrophenyl phosphate phosphatase substrate system (KPL., Gaithersburg, MD) for 20 min. The color reaction was stopped by adding 100 µl 0.5 M EDTA, and the absorbance values were obtained using a Dynatech MR4000 model microplate reader at 405 nm. A standard curve was generated for each set of samples, and serum antibody concentrations were calculated in accordance with the standard curve.

**Statistical analyses.** All the statistical analyses were performed on GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). Serum antibody titers from different time points in vaccinated groups were compared and analyzed using a two-tailed, unpaired *t* test; those with *P* values of <0.05 and <0.01 were considered to be significant and very significant, respectively. A paired *t* test was used to analyze the subclass of IgG titer within the same group. The log-rank (Mantel-Cox) survival test was used to compare the survival statistics between two groups.

**Nucleotide sequence accession number.** The nucleotide sequence of the synthesized Tul4 gene has been deposited in GenBank under accession number JQ629934.

**RESULTS**

**Protective immunity elicited by Ad/opt-Tul4.** To explore whether a Tul4-based adenovirus-vectored vaccine, Ad/opt-Tul4, could protect against tularemia disease, vaccinated mice were i.p. challenged with 210 CFU of *F. tularensis* LVS 3 weeks after the third dose of vaccination. The results showed that i.m. vaccination with Ad/opt-Tul4 protected 60% of the mice against challenge, while the negative control Ad/Null was not protective (Fig. 1). Additionally, the positive-control mouse group, vaccinated i.d. with *F. tularensis* LVS, resulted in 100% protection. Finally, a vaccination with recombinant Tul4 protein and adjuvant conferred only 20% protection against challenge (Fig. 1). The log-rank (Mantel-Cox) survival test shows a significant difference between the Ad/opt-Tul4 group and the recombinant Tul4 protein group (*P* < 0.05).

Increasing challenge doses of *F. tularensis* LVS decreased the survival rate in Ad/opt-Tul4 (Table 2). The route of immunization resulted in different protection rates. An i.d. vaccination with 3 doses of Ad/opt-Tul4 provided only 12.5% protection against challenge, which is significantly different (*P* < 0.05) than i.m. vaccination with Ad/opt-Tul4 (Fig. 1). Additionally, antibody ti-

**TABLE 2 Comparison of challenge doses of *F. tularensis* LVS and survival rates of mice in different vaccination groups**

| Dose of LVS (CFU) used for challenge (i.p.) | Ad/opt-Tul4 (i.m.) | Ad/Null (i.m.) | LVS (i.d.) |
|-------------------------------------------|-------------------|---------------|-----------|
| 210                                       | 60 (6/10)         | 0             | NA*       |
| 300                                       | 40 (2/5)          | 0             | 100 (5/5) |
| 400                                       | 20 (1/5)          | 0             | 100 (5/5) |

*NA, not applicable; the challenge of 210 CFU LVS was not performed with mice vaccinated i.d. with *F. tularensis* LVS.*
Recombinant Tul4 protein vaccine elicited a high level of Tul4-specific IgG antibody but did not protect against challenge. The total serum IgG antibody levels (anti-Tul4) were determined for the groups Ad/Null, F. tularensis LVS, Ad/opt-Tul4, and recombinant Tul4. Mice vaccinated with recombinant Tul4 protein showed a significantly higher antibody response (2,000 ng/ml) than mice vaccinated with Ad/opt-Tul4 (Fig. 3). However, the higher antibody response did not correlate with protection against challenge.

Tul4-specific IgG antibody titers at different time points in the vaccination schedule of Ad/opt-Tul4 were assayed, and groups with booster immunizations and without were compared. After the second week, antibody titers showed significant differences (P < 0.01) from week to week (Fig. 4A). Regardless of whether the mice received booster doses of Ad/opt-Tul4 or not, there were no significant differences in antibody titer. However, the protection rate was higher in mice vaccinated with booster shots of Ad/opt-Tul4 (Fig. 4B).

Intramuscular vaccination with Ad/opt-Tul4 elicited a Th1-biased antibody response. The proportion of Tul4-specific serum IgG1 and IgG2a antibody subclasses in mice i.m. vaccinated with Ad/opt-Tul4 was also assessed by ELISA. IgG2a antibody levels specific to Tul4 protein were three times higher than IgG1 antibody levels (Fig. 5).

DISCUSSION

A new generation of adenovirus-vectored tularemia vaccine encoding a Tul4 lipoprotein sequence, Ad/opt-Tul4, has been assessed in this study. A murine model was used to test serum Tul4-specific antibody levels, and protection against challenge with F. tularensis LVS was observed for mice immunized with Ad/opt-Tul4. The sequence for Tul4 was human codon optimized, and the adenovirus vector containing the optimized insert elicited a more robust immune response than that with a native insert (R. Kaur and M. Zeng, unpublished data); this effect has also been observed in other studies (1). The ease of genetic manipulation provides some advantages for Ad/opt-Tul4 over a live attenuated strain of F. tularensis. Although F. tularensis LVS can protect against challenge with the same strain, the efficacy of LVS derived from F. tularensis subspecies holarctica may be compromised if a different subspecies strain were to be used in the event of a bioterrorist attack. An adenovirus-vectored vaccine can be easily adjusted to code for subspecies-specific, codon-optimized sequences. Thus, the vaccine’s flexible design makes it an appealing candidate.

Ad/opt-Tul4 may also be an attractive candidate vaccine due to its ability to provide dose-dependent protection against F. tularensis LVS. When mice were challenged with 210 CFU F. tularensis LVS via the i.p. route, we observed a 60% protection rate in mice immunized three times with Ad/opt-Tul4 (i.m.). Without the booster shots protection dropped to 12.5%. Similarly, when mice were vaccinated with recombinant Tul4 protein (three times), protection hovered at 20%. Both vaccines elicited a robust antibody titer, but protection was observed only in mice immunized three times with Ad/opt-Tul4. This high vaccine dose activated humoral immunity and, potentially, cell-mediated immunity. However, more studies are needed to assess whether there is a high enough dose of Ad/opt-Tul4 to elicit the most potent immune response.

Comparing the Tul4-specific antibody titers between mice immunized with Ad/opt-Tul4 with and without booster shots, both groups were observed to have antibody titers at around 1,400 ng/ml at 6 weeks after immunization. A closer look at the booster group’s IgG antibodies revealed that subclass IgG2a was predominantly produced at levels three times higher than IgG1. While the predominance of IgG2a to IgG1 has been observed previously in mice immunized i.d. with 1,000 CFU LVS (21), we are the first to report this observation using a Tul4-based vaccine. Thus, a strong and predominant IgG2a response may signal protection against a lethal tularemia challenge in mice. The more significant IgG2a response also indicates a bias toward the Th1 immune response, and because high IgG antibody titers alone are not associated with protection against a lethal dose of LSV, this implies that cell-mediated immunity may be responsible for the additional clearance of tularemia pathogen and survival of mice. In fact, while passive transfer of high-titer anti-LSV IgG protects against i.p. challenge with 3,000 LD50 in normal BALB/c mice, transfer of anti-LSV IgG alone does not protect athymic nu/nu+ mice from challenge (21). However, once nu/nu+ mice are reconstituted with T cells from normal mice, passive transfer of anti-LSV IgG does
protect against challenge with LSV. Furthermore, gamma interferon (IFN-γ) may provide additional protection, as anti-LSV IgG passive transfer to normal and *nu/nu* mice reconstituted with T cells did not survive LSV challenge in the presence of anti-IFN-γ (21). Moreover, it has also been demonstrated that T-cell immunity is essential for long-term survival of mice challenged with tularemia (9). In humans, a recent study has demonstrated that cellular immunity persists for 30 years after vaccination with LVS (10) and highlights the importance of cell-mediated immunity to tularemia for long-term protection. Thus, humoral responses alone may not be enough to protect against challenge with tularemia. Therefore, future studies should also characterize cellular immunity rendered via immunization with Ad,opt-Tul4 vaccine with and without booster shots.

The role of humoral immunity to intracellular pathogens has been equivocal. Typically, antibodies are thought to work against extracellular pathogens, whereas cell-mediated immunity has a more important role in the clearance of intracellular pathogens such as *F. tularensis*. However, it has been shown that antibodies are more complex than we previously understood, enhancing innate immunity and regulating the inflammatory response, and are active in clearing intracellular pathogens such as *Cryptococcus neoformans* and *Mycobacterium tuberculosis* (3). Without an appropriate antibody response, the immune system may not be able to clear a tularemia infection. Strong early protection against colonization is highly dependent on B-cell activity (6), and *F. tularensis* has been shown to spread from the lungs via the hematogenous route to systemic organs (11). We observed a low antibody titer (about 250 ng/ml IgG) in mice immunized with *F. tularensis* LVS. Therefore, from this study and others (9, 21), it can be inferred that B-cell activity is required for full protection. Additionally, a highly virulent strain of *F. tularensis* may completely shut down the inflammatory responses (17); thus, circulating anti-Tul4 antibodies raised in response to the Ad/opt-Tul4 vaccine may be especially important in the early stages of preventing high colonization rates of *F. tularensis*. Our Ad/opt-Tul4 vaccine has been shown to elicit sufficient B-cell activity and provides a 60% protection rate against LVS i.p. challenge.

Mucosal antibody responses producing IgA antibodies have previously been observed following vaccination with *F. tularensis* LVS (18). Although not evaluated in this study, IgA antibodies in the mucosal lining of respiratory tracts can prohibit adhesion and motility of *F. tularensis*. Our Ad/opt-Tul4 vaccine is an attractive candidate because of the innate ability of adenovirus to elicit a mucosal response. Further experimentation involving intranasal (i.n.) immunization may provide exciting results. Theoretically, challenge with *F. tularensis* LVS via the i.n. route may show that our vaccine can provide better protection due to the strong humoral response compared with that for LVS. A bioterrorist attack would conceivably be directed toward the inhalation route, and the spread of the bacteria may be halted before vital organs are reached. In this study, Ad/opt-Tul4’s efficacy has been assessed via the i.m. and i.d. routes. Although some studies have observed that adenovirus-based vaccines against melanoma are optimal when given through the i.d. route (14), we observed that the i.d. route is ineffective for an adenovirus-vectored vaccine against tularemia. This raises the question of the efficacy of additional routes; perhaps an i.n. or oral route could elicit a more varied and robust immunogenic response. Therefore, additional experimentation is also needed to assess these possibilities.

We reiterate the need for further experimentation to assess the T-cell activity that Ad/opt-Tul4 induces to provide a more complete profile for the vaccine. In an effort to develop a safe next-generation subunit vaccine, a multitude of membrane proteins have been identified in *F. tularensis* LVS that can stimulate T cells from LVS-primed individuals (22, 26, 27). Recombinant Tul4 protein has been shown to be recognized by T cells, and the responding T cells produced IL-2 and IFN-γ. Our Ad/opt-Tul4 vac-

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FIG 4 Effect of booster immunization. (A) Anti-Tul4 IgG antibody response after 0, 2, 4, and 6 weeks of immunization with Ad/opt-Tul4 without booster and with booster. The difference between the titers after 2 to 6 weeks and 4 to 6 weeks of primary immunization was very significant, with a *P* value < 0.001 (***) and a *P* value < 0.01 (**), respectively. (B) Survival curve of Ad/opt-Tul4 mice with and without booster. As a control, the survival rate of Ad/Null group is also shown.

FIG 5 Anti-Tul4 IgG subtype IgG1/IgG2a comparison. Anti-Tul4 response in vaccinated mice with Ad/opt-Tul4 immunization (i.m.). IgG1 and IgG2a concentrations were measured by ELISA in the serum samples collected after 6 weeks of vaccination. The values shown in the column are means ± standard errors of the mean (*n* = 8).
cine utilizes Tu4, but mechanisms of protection against tularemia challenge must be further elucidated. Given the efficacy of the Ad/opt-Tu4 vaccine and its ability to elicit a strong humoral response, our next-generation vaccine candidate shows potential pending additional studies.

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