A Nasal Interleukin-12 DNA Vaccine Coexpressing *Yersinia pestis* F1-V Fusion Protein Confers Protection against Pneumonic Plague[▽]

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Previous studies have shown that mucosal application of interleukin-12 (IL-12) can stimulate elevated secretory immunoglobulin A (IgA) responses. Since possible exposure to plague is via *Yersinia pestis*-laden aerosols that results in pneumonic plague, arming both the mucosal and systemic immune systems may offer an added benefit for protective immunity. Two bicistronic plasmids were constructed that encoded the protective plague epitopes, capsular antigen (F1-Ag) and virulence antigen (V-Ag) as a F1-V fusion protein but differed in the amounts of IL-12 produced. When applied nasally, serum IgG and mucosal IgA anti-F1-Ag and anti-V-Ag titers were detectable beginning at week 6 after three weekly doses, and recombinant F1-Ag boosts were required to elevate the F1-Ag-specific antibody (Ab) titers. Following pneumonic challenge, the best efficacy was obtained in mice primed with IL-12(Low)/F1-V vaccine with 80% survival compared to mice immunized with IL-12(Low)/F1, IL-12(Low)/V, or IL-12(Low) vector DNA vaccines. Improved expression of IL-12 resulted in lost efficacy when using the IL-12(High)/F1-V DNA vaccine. Despite differences in the amount of IL-12 produced by the two F1-V DNA vaccines, Ab responses and Th cell responses to F1- and V-Ags were similar. These results show that IL-12 can be used as a molecular adjuvant to enhance protective immunity against pneumonic plague, but in a dose-dependent fashion.

Plague is a zoonotic disease caused by *Yersinia pestis*. Throughout history, three major pandemics of plague disease have resulted in an estimated 200 million deaths. Plague still remains endemic in regions of Africa, Asia, and North and South America (2, 26). Plague assumes three forms of disease in humans: bubonic, pneumonic, and septicemic. Bubonic and septicemic plague arise following bites from fleas, which have been infected via feeding on infected animals (2, 26). The most feared form is pneumonic plague because this form can be readily transmitted from person to person via inhalation of contaminated airborne droplets, and because of its rapid disease progression, there is a high mortality rate (15).

At present, there are no licensed plague vaccines in the United States. To enable development of a subunit vaccine to plague, efforts have focused on two primary *Y. pestis* antigens (Ags), the outer capsule protein antigen (F1-Ag), which is believed to help avoid phagocytosis (3, 35), and the low calcium response (LcrV) protein or V-Ag, which has been suggested to mediate a suppressive effect upon Th1 cells via the stimulation of interleukin-10 (IL-10) (28). When given in combination, these vaccines effectively protect against bubonic and pneumonic plague (16, 20). While the observed protective im-

munity is largely antibody (Ab) dependent, *Y. pestis* is an intracellular pathogen, and new data have shown that cellular immunity can contribute to protection against plague (23, 24, 27).

IL-12 is a heterodimeric cytokine composed of two disulfide-linked peptides, p35 and p40. A major source of IL-12 is the antigen-presenting cells, such as dendritic cells and macrophages; these cells often produce IL-12 in response to bacterial products (8, 10, 13). IL-12 has a central function in initiating and regulating cellular immune responses by stimulating gamma interferon (IFN- γ) production in both natural killer (NK) cells and helper T cells via binding its receptor comprised of two subunits, IL-12 receptor β 1 (IL-12R β 1) and IL-12R β 2 (1, 10). Thus, we hypothesized that IL-12 can enhance vaccine efficacy, since *Y. pestis* is an intracellular pathogen.

In the present study, to develop an effective vaccine against pneumonic plague, we used bicistronic DNA vaccines that coexpress IL-12 and F1-V fusion protein, using two different bicistronic eukaryotic expression vectors, and assessed their vaccine efficacy against pneumonic plague challenge. This is the first example of using a nasal immunization approach with DNA vaccines for plague. These DNA vaccines did effectively prime and, with subsequent F1-Ag protein boosts, were able to confer protection against pneumonic plague. Thus, the IL-12(Low)/F1-V DNA vaccine can be used as a primary vaccine for protection to pneumonic plague.

MATERIALS AND METHODS

Plasmids. Eukaryotic expression plasmids used in this study are summarized in Table 1. To develop the IL-12(Low) DNA vaccines, cDNA fragments for *Y. pestis* of F1-Ag, V-Ag, and F1-V Ag were amplified by PCR from a synthetic gene

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TABLE 1. DNA vaccine plasmids used in this study

Plasmid	Size (kb)	F1-Ag	V-Ag	Ag promoter	IL-12 promoter
pGT146-mIL-12/ β gal	10.3	None	None	hEF1-HTLV ^b	hCMV-1A ^c
pGT146-mIL-12/F1	7.75	Present	None	hEF1-HTLV	hCMV-1A
pGT146-mIL-12/V	8.23	None	Present	hEF1-HTLV	hCMV-1A
pGT146-mIL-12/F1-V	8.75	Present ^a	Present ^a	hEF1-HTLV	hCMV-1A
pBud-mIL-12	6.22	None	None	EF1- α	hCMV
pBud-mIL-12/F1-V	7.73	Present ^a	Present ^a	EF1- α	hCMV

^a F1-V was expressed as a fusion protein.

^b Hybrid (h) promoter with elongation factor 1 α (EF1- α) promoter and the 5' untranslated region of human T-cell leukemia virus (HTLV).

^c The human cytomegalovirus (hCMV) immediate-early gene 1 promoter/enhancer containing intron A of the cytomegalovirus immediate-early gene.

(GenScript, Piscataway, NJ) optimized for mouse codon usage, similar to that previously described (17), and the F1-Ag lacking its leader sequence, was cloned into pGT146-mIL-12 vector (Invivogen, San Diego, CA). IL-12 is expressed as a single polypeptide chain with a linker sequence between the p35 and p40 subunits, Val-Pro-Gly-Val-Gly-Val-Pro-Gly-Val-Gly. Each of the amplified DNA fragments for the plague proteins contained sequences for the BamHI site at the 5' terminus and for the NheI site at the 3' terminus; for the F1-V fusion protein, residues contained a linker sequence, Pro-Gly-Gly, between F1-Ag and V-Ag. To develop the IL-12(High)/F1-V DNA vaccine, the pBudCE4.1 vector (Invitrogen Corp, Carlsbad, CA) was used. The DNA fragment for IL-12 was obtained from pGT146-mIL-12 with sequences cloned from the SalI site at the 5' terminus to the ScaI site at the 3' terminus. The fragment of F1-V fusion protein was amplified by PCR using restriction sites for NotI at the 5' terminus and KpnI at the 3' terminus. Following sequence confirmation of the TA-cloned (Topo TA cloning kit; Invitrogen) PCR products, each of the fragments was digested and sequentially inserted into the vectors, resulting in pGT146 IL-12/F1, IL-12/V, IL-12/F1-V, and pBud-IL-12/F1-V. These DNA plasmids were purified with a commercially available plasmid purification kit (Qiagen, Inc., Valencia, CA) and resuspended with DNase-free water.

Transfection. To evaluate the expression of IL-12, F1-Ag, V-Ag, and F1-V fusion proteins, we used supernatants and lysates of 293A cells (ATCC, Manassas, VA) that were transfected with each DNA plasmid using Lipofectamine LTX (Invitrogen). The 293A cells were cultured in complete medium (CM), which consisted of RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals, GA), 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cell culture supernatants and lysates were subjected to immunoblotting and enzyme-linked immunosorbent assays (ELISAs) 2 days after transfection, respectively, as described below.

F1-V fusion protein for bacterial expression. F1-Ag cDNA was amplified with primers containing an EcoRI site and a SmaI site at the 5' and 3' termini, respectively. The amplification product was gel purified, Topo cloned, and cut with EcoRI and SmaI. This intermediate step was performed, since many restriction enzymes inefficiently cut PCR products when the restriction sites are proximal to the ends of the fragment. Such EcoRI/SmaI fragment was gel purified and cloned into pUC19 cut with EcoRI and SmaI. Successful cloning was determined by standard molecular procedures. The resulting construct was cut with BamHI and SalI and used as a backbone for cloning a V-antigen-coding region that had been amplified with BamHI and SalI primers, Topo cloned, and retrieved with BamHI and SalI, a procedure similar to that used for F1-Ag. Primers were designed to maintain an open reading frame between F1 and V and to produce a Pro-Gly linker between the two components and to delete the V-antigen ATG start codon. The whole sequence was verified by DNA sequencing. In order to express the F1-V fusion in *Escherichia coli*, the fusion product was transformed with two primers containing EcoRI sites at both ends. The 5' primer was designed so as to delete the F1 leader peptide and to frame the whole PCR product into a pGEX1 glutathione S-transferase expression vector (Amersham Biosciences, Pittsburgh, PA). Upon ligation and transformation, the correct orientation was determined by DNA digestion followed by sequencing. The construct was then induced, and protein production was tested by Western blot and probing with either rabbit anti-F1-Ag or anti-V-Ag Abs.

Immunoblotting. Transfected 293A cells were lysed in Milli-Q water; 30 μ g of total protein was electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated with anti-F1- or anti-V-Ag rabbit serum (36) overnight at 4°C and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates) for 90 min at

room temperature. The signals were visualized using the substrate 4-chloro-1-naphthol chromogen and H₂O₂ (Sigma-Aldrich, St. Louis, MO).

Immunizations and challenge. Female BALB/c mice were obtained from the National Cancer Institute (Frederick Cancer Research Facility, Frederick, MD). Mice were maintained at Montana State University Animal Resources Center under pathogen-free conditions in individual ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water ad libitum.

When the mice were 8 to 10 weeks old, they were nasally immunized with each DNA vaccine (80 μ g/dose) on weeks 0, 1, and 2 with each dose administered over a 2-day period. On weeks 8 and 9, mice were nasally boosted with 25 μ g of F1-Ag protein plus 2.5 μ g of cholera toxin (CT) (List Biological Laboratories, Campbell, CA) adjuvant. A final boost of DNA vaccine (100 μ g) and F1-Ag protein (25 μ g) plus CT adjuvant was given on week 12.

To test the efficacy of the DNA vaccines, immunized mice were nasally challenged with 100 50% lethal doses of *Y. pestis* Madagascar strain (MG05) 44 days after the last immunization as previously described (36). All mouse care and procedures were in accordance with institutional policies for animal health and well-being.

Collection of serum and mucosal samples. Blood was collected from the saphenous vein. Fresh fecal pellets from individual mice were solubilized in sterile phosphate-buffered saline (PBS) containing 50 μ g/ml of soybean trypsin inhibitor (Sigma-Aldrich) by vortexing for 10 min at 4°C. After microcentrifugation, supernatants were collected and frozen at -30°C until assayed. Nasal washes were collected when mice were euthanized to collect various lymph nodes. Nasal washes were performed at the termination of the study as previously described (17).

Measurement of anti-F1-Ag and V-Ag Ab titers by ELISAs. Serum, fecal, or nasal wash Ab titers were determined by ELISAs. Briefly, recombinant F1-Ag or V-Ag (36) in sterile PBS was used to coat the wells on Maxisorp Immunoplate II microtiter plates (Nunc, Roskilde, Denmark) at 50 μ l/well. After overnight incubation at room temperature, wells were blocked with PBS containing 1% bovine serum albumin for 1 h at 37°C; individual wells were loaded with serially diluted mouse serum, fecal, or nasal samples in ELISA buffer (PBS containing 0.5% bovine serum albumin and 0.5% Tween 20) overnight at 4°C. Ag-specific Abs were reacted with horseradish peroxidase-conjugated goat anti-mouse IgG, IgA, IgG1, IgG2a, or IgG2b Abs (Southern Biotechnology Associates, Birmingham, AL) for 90 min at 37°C. The specific reactions were detected with soluble enzyme substrate, 50 μ l of 2,2'-azino-bis(3-ethylbenzothiazolinesulfonic acid) (ABTS) (Moss, Inc., Pasadena, CA), and absorbance was measured at 415 nm after 1-h incubation at room temperature using Bio-Tek Instruments ELx808 microtiter plate reader (Winooski, VT). Endpoint titers were determined to be an absorbance of 0.1 optical density unit above negative controls after 1 h at room temperature.

Lymphocyte isolation. Lymphocytes were isolated from nasal mucosa-associated lymphoid tissues (NALT), nasal passages (NPs), head and neck lymph nodes (HNLNs), submaxillary glands (SMGs), spleens, small intestinal lamina propria (iLP), and Peyer's patches (PPs). HNLN, splenic, and PP mononuclear cells were isolated by conventional methods using Dounce homogenization (17, 36). To isolate the mononuclear cells from NALT, NPs, SMGs, and iLP, the tissues were minced and digested using 300 units/ml of *Clostridium histolyticum* type IV collagenase (Worthington, Freehold, NJ) for 30 min at 37°C in spinner flasks (17). After incubation, the digestion mixtures were passed through Nitex mesh (Fairview Fabrics, Hercules, CA) to remove undigested tissues. Mononuclear cells were separated by Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation and cells interfaced between 40% and 60% Percoll. A greater than 95% yield was obtained for all lymphocytes isolated from each tissue, as determined by trypan blue exclusion.

Ab ELISPOT assay. On week 14, sets of studies were terminated to collect NALT, NP, HNLN, SMG, splenic, iLP, and PP mononuclear cells from immunized mice. Ag-specific Ab-forming cell (AFC) responses by the enzyme-linked immunospot (ELISPOT) method were detected, using mixed cellulose ester membrane-bottom microtiter plates (MultiScreen-HA; Millipore, Bedford, MA) by coating with 5 μ g/ml F1- or V-Ag in sterile PBS as previously described (17). For total IgA or IgG AFC responses, wells were coated with 5 μ g/ml goat anti-mouse IgA or IgG Abs (Southern Biotechnology Associates) in sterile PBS.

Cytokine detection assays. On week 7, groups of immunized mice were evaluated for cytokine responses to F1- and V-Ags. Total mononuclear cells from spleens, HNLNs, and PPs were resuspended in CM. Mononuclear cells were restimulated with 10 μ g of recombinant F1, V-Ag, or with medium as a control in the presence of 10 U/ml human IL-2 (PeproTech) for 2 days at 37°C in a humidified 5% CO₂ incubator. Cells were washed and resuspended in CM, and then these stimulated lymphocytes were evaluated by IFN- γ , IL-4, IL-5, IL-10, and IL-13-specific ELISPOT assays as described previously (17, 36). Supernatants from these restimulation cultures were also assayed for the presence of IFN- γ , IL-6, IL-10, IL-17, and TGF- β by sandwich ELISAs as described previously (21, 25).

To measure IL-12p70 and IL-12p40 expression in the collected cell supernatants from transfected 293A cells, sandwich ELISAs were performed as previously described (25). Cell supernatants were collected 2 days after transfection as described above. To determine the amount of IL-12 present in these supernatants, serially diluted recombinant murine IL-12 (R&D Systems, MN) was used to generate a standard curve.

Statistical analysis. Analysis of variance followed by Tukey's method was used to evaluate differences in expression of IL-12 Ab titers; the Mann-Whitney U-test was used to evaluate differences in AFC and cytokine-forming cell (CFC) responses. The Kaplan-Meier method (GraphPad Prism; GraphPad Software, Inc., San Diego, CA) was applied to obtain the fraction of surviving mice following nasal *Y. pestis* challenges of nasally immunized mice. Using the Mantel-Haenszel log rank test, the *P* value for statistical differences between the mice surviving plague challenges and the vaccinated groups of mice or the mice dosed with PBS was discerned at the 95% confidence interval.

RESULTS

Expression of IL-12, F1-Ag, V-Ag, and F1-V fusion proteins.

Since *Y. pestis* is an intracellular pathogen, we questioned whether IL-12 could be used as a mucosal molecular adjuvant to facilitate protective immunity to pneumonic plague. The approach used was to incorporate the cDNA for F1-Ag, V-Ag, or F1-V fusion proteins into a bicistronic DNA plasmid (pGT146-based vector) that carried the murine IL-12p70 as a molecular adjuvant and referred to as IL-12(Low)/^{*} vaccines (Table 1; Fig. 1A). Furthermore, a previous study showed that the DNA vaccine's immunogenicity for plague could be manipulated by using different promoters and enhancers (9). Thus, to address the potential issue of IL-12 dosage, a second plasmid (pBud-based vector) that contained a stronger cytomegalovirus promoter to enhance IL-12 expression was developed (Table 1; Fig. 1A). This latter construct is referred to as IL-12(High)/^{*} vaccine.

To verify the expression of IL-12, F1-Ag, V-Ag, and F1-V fusion proteins, replicate cultures of 293A cells were transfected with one of these DNA vaccines, and cell culture supernatants and lysates were collected (Fig. 1B to E). IL-12p70 could be readily detected in each of the cell supernatants from the transfected 293A cells compared to supernatants from DNA plasmids lacking IL-12 (Fig. 1B and D). The amount of IL-12 expressed by the IL-12(High)/F1-V was 10-fold greater than that detected for the IL-12(Low)/F1-V (Fig. 1D). The ratio of IL-12p40/IL-12p70 was similar for each IL-12 DNA vaccine, suggesting that no free IL-12p40 was being synthesized (data not shown). To detect expression of F1-Ag, V-Ag, and F1-V fusion proteins, cell lysates were used for immuno-

blotting. The F1-Ag could not be detected using the rabbit anti-F1-Ag serum (Fig. 1C); however, this antiserum could detect F1-Ag in the recombinant F1-V fusion protein, suggesting that the expressed F1-Ag following DVA vaccination was being degraded. These results are similar with those previously reported in that F1-Ag has a low molecular weight and is rapidly eliminated from transfected eukaryotic cells disallowing detection of eukaryote expressed F1-Ag (11). The V-Ag and the F1-V could be detected using rabbit anti-V-Ag serum (Fig. 1C and E). The F1-V protein migrated with an apparent molecular mass of 53 kDa, which represents the expected molecular mass for F1-Ag (17 kDa) plus V-Ag (37 kDa). There were no differences in the amounts of F1-V-Ag expressed between the two plasmids (Fig. 1E).

Nasal immunization with IL-12(Low)/F1-V DNA vaccine shows the best efficacy despite similar Ab levels induced with the single IL-12(Low) vaccines. To evaluate the relative immunogenicity of the IL-12(Low) DNA vaccines, BALB/c mice were selected, since these mice have been used extensively to evaluate efficacies of plague vaccines (6, 9, 11, 30–32, 34). Samples were collected at 6 weeks after primary immunization and subsequently at 2-week intervals. Past studies with other DNA vaccines show that Ab responses are delayed and peak between 8 and 10 weeks after primary immunization (37). Ag-specific Ab titers in sera and fecal extracts were measured by ELISAs using F1-Ag- or V-Ag-coated wells (Fig. 2 and 3). No differences in Ab titers between 6 and 8 weeks after primary immunization to F1-Ag were detected using either the single vaccine (Fig. 2A) or the F1-V DNA vaccine (Fig. 2A and 3A). On the other hand, Ab responses to V-Ag appeared delayed (Fig. 2A and 3A). Thus, to enhance anti-F1-Ag immunity, mice were boosted nasally with 25 μ g of recombinant F1-Ag plus CT on weeks 8 and 9, resulting in robust mucosal IgA (Fig. 3A and B) and serum IgG titers against both F1-Ag and V-Ag by week 12 (Fig. 2A and 3A). A final boost with DNA vaccine, as well as with recombinant F1-Ag plus CT, was given on week 12.

To test the efficacy of these DNA plague vaccines, IL-12(Low) DNA-immunized mice were challenged nasally with 100 50% lethal doses of *Y. pestis* Madagascar strain 44 days after the final boost, and the mean survival rates were determined (Fig. 4A and B). All the PBS-dosed control mice (*n* = 10) died within 2 days of challenge. The mice vaccinated with IL-12(Low)/V-Ag and IL-12/F1-Ag DNA showed partial protection with 60% (*P* = 0.005) and 40% (*P* < 0.001) survival, respectively. An adjuvant effect for IL-12 was observed in IL-12(Low)/ β -galactosidase (β -Gal) DNA vaccine plus F1-Ag protein-dosed mice in which efficacy was improved to a 40% survival (*P* = 0.005) more than with F1-Ag protein-only immunization (20% survival; *P* < 0.001) (Fig. 4A). These results suggest that IL-12 contributed to protection against plague and enhanced vaccine efficacy. The most effective protection was achieved by mice vaccinated with IL-12(Low)/F1-V DNA vaccine (Fig. 4B), showing 80% survival (*P* < 0.001). Not surprisingly, protection against plague required immunity to both F1- and V-Ags, but DNA vaccination for F1-Ag required additional protein boosts to elicit elevated anti-F1-Ag Ab titers.

Nasal immunization with IL-12(High)/F1-V DNA vaccine showed poor efficacy despite similar Ab levels induced with the IL-12(Low)/F1-V vaccine. To determine whether increased

FIG. 1. Plasmid maps and expression determinations for the bicistronic IL-12(Low) and IL-12(High) DNA vaccines. (A) Circular plasmid maps for IL-12(Low)/F1-V (pGT146-mIL-12) DNA and IL-12(High)/F1-V (pBud-IL-12) DNA vaccines. hEF1-HTLV prom, hybrid elongation factor 1 α -human T-cell leukemia virus promoter; EMCV IRES, encephalomyocarditis virus internal ribosome entry site; hCMV-IA prom, human cytomegalovirus IA promoter; Ori and ori, origin; BGH PA, bovine growth hormone and polyadenylation signal; PEF1- α , promoter of human elongation factor 1- α ; Zeo-R, zeomycin resistance; SV40 PA, simian virus polyadenylation signal. (B and D) Expression of IL-12 was determined by IL-12-specific ELISAs using supernatants from 293A cells transfected with the IL-12(Low)/F1-V (B) or IL-12(High)/F1-V (D) DNA vaccine. Data are the means plus standard errors of the means (error bars) of two or three experiments and analyzed using the Mann-Whitney U-test. Values that were significantly different from vector alone are indicated by the brackets and asterisks as follows: *, $P < 0.05$; **, $P < 0.01$. (C and E) To detect F1-Ag or V-Ag expression by the single IL-12(Low)/F1-V DNA vaccines (C) or the IL-12(Low)/F1-V DNA and IL-12(High)/F1-V DNA vaccines (E), cell lysates from transfected 293A cells were subjected to Western immunoblot analysis using rabbit anti-F1-Ag or rabbit anti-V-Ag sera. Expression of F1-Ag could not be detected for any of the vaccines, but expression of V-Ag was detected. F1-V expression by both IL-12 DNA vaccines was equivalent. The positions of molecular mass markers (M) (in kilodaltons) are indicated to the left of the immunoblots.

production of IL-12 could improve DNA vaccine efficacy, the IL-12(High)/F1-V DNA vaccine was tested. Since the single IL-12(Low)/F1- and V-Ag DNA vaccines were less protective than the F1-V fusion DNA vaccine, only the IL-12(High)/F1-V DNA vaccine was made (Fig. 1A) and tested. Ab responses in

mice immunized with IL-12(High)/F1-V DNA vaccine were not significantly different in their anti-F1- or anti-V-Ag Ab titers compared to mice immunized with IL-12(Low)/F1-V DNA vaccine (Fig. 3A). IgA titers against F1-Ag and V-Ag in nasal washes were also evaluated on week 12 (Fig. 3B), and

FIG. 2. Ab titers from mice dosed with the IL-12(Low)/F1-V DNA vaccine were equivalent to those induced with the single IL-12(Low)/F1-Ag or IL-12(Low)/V-Ag DNA vaccine. BALB/c mice (five mice/group) were first nasally dosed with DNA vaccines on weeks 0, 1, and 2 followed by nasal boosts with recombinant F1-Ag plus 2.5 μ g CT on weeks 8 and 9. (A) Serum anti-F1-Ag and (B) anti-V-Ag Ab titers were determined by ELISAs at 6 and 12 weeks (wk) after primary immunization (p.i.). nd, not detected.

these showed no significant differences between high- and low-dose IL-12 DNA vaccines.

Despite similar anti-F1-Ag and anti-V-Ag Ab titers between the high- and low-dose IL-12-immunized mice, those immunized with IL-12(High)/F1-V DNA vaccine showed only 28.6% survival ($P = 0.02$; Fig. 4B). Despite equivalent expression of the F1-V fusion protein between the two IL-12 DNA vaccines, only the IL-12(Low)/F1-V DNA vaccine showed protective efficacy. Thus, we queried whether these differences in IgG subclass responses or the differences in Th cell responses could account for the observed differences obtained with these IL-12 DNA vaccines.

IgG subclass responses with high- and low-dose IL-12 DNA vaccinations. To assess possible differences in IgG subclass responses, a comparison was made between mice vaccinated with high- and low-dose IL-12 DNA vaccines. No differences in induced IgG1, IgG2a, and IgG2b to F1- and V-Ags could be detected when either IL-12(Low)/F1-V or IL-12(High)/F1-V DNA vaccine was used (Fig. 5). Both vaccines induced greater IgG1 anti-F1-Ag responses than IgG2a or IgG2b responses. While the IL-12(High)/F1-V DNA vaccine produced greater IgG1 than IgG2a anti-V-Ag Ab responses, the IL-12(Low)/F1-V DNA vaccine produced equivalent amounts of IgG1, IgG2a, and IgG2b anti-V-Ag Ab responses (Fig. 5). These results show that despite differences in the amount of IL-12 expressed, a bias in IgG subclass responses was not evident and did not account for the observed differences in protective immunity.

FIG. 3. Increased expression of IL-12 has no effect upon serum IgG or mucosal IgA Ab titers. BALB/c mice (10 mice/group) were nasally immunized, as described in the legend to Fig. 2, plus a final immunization was given on week 12 with the DNA vaccine and F1-Ag plus CT. A kinetics analysis was performed on the IL-12(Low)/F1-V DNA and IL-12(High)/F1-V DNA vaccines. (A) Serum IgG and fecal IgA Ab titers to F1- and V-Ags were monitored for 14 weeks. The values are shown as means $\pm$ standard deviations (error bars). (B) Nasal washes were performed at week 14, and mucosal IgA Ab titers were measured. The values are shown as means plus standard deviations (error bars).

Distribution of immune B cells following immunization. To compare the magnitude and distribution of AFC responses induced by the two IL-12 DNA vaccines, a B-cell ELISPOT assay was performed using lymphocytes of various lymphoid

FIG. 4. IL-12(Low)/F1-V DNA vaccine confers optimal protection against pneumonic plague. Single Ag DNA vaccines (A) were less effective than the IL-12(Low)/F1-V DNA vaccine (B). (A) Mice were dosed four times at weeks 0, 1, 2, and 12 with the IL-12(Low)/F1-V DNA vaccine (F1-Ag ($n = 5$), V-Ag ($n = 5$), or β -Gal ($n = 5$)) and boosted on weeks 8, 9, and 12 with recombinant F1-Ag. An additional group received recombinant F1-Ag ($n = 5$) only on weeks 8, 9, and 12; a negative-control group received PBS only ($n = 5$). All mice were challenged 44 days after the last immunization. Mice dosed with IL-12(Low)/V-Ag plus F1-Ag protein boost showed the best efficacy at 60%. The fraction of mice that survived obtained from vaccinated mice were compared to PBS-dosed mice, and significance was determined as follows: *, $P \leq 0.001$; **, $P \leq 0.005$. (B) Similar immunization regimen using DNA vaccine plus recombinant F1-Ag in panel A was applied to mice dosed with IL-12(Low)/F1-V ($n = 5$) or IL-12(High)/F1-V ($n = 5$), and the additional controls were given F1-Ag only ($n = 5$) or PBS only ($n = 5$). The fraction of mice that survived of the vaccinated mice were compared to PBS-dosed mice, and significance was determined as follows: *, $P \leq 0.001$; **, $P = 0.006$; ***, $P = 0.02$. The values for the two IL-12/F1-V vaccines were significantly different ($P = 0.007$) (#).

FIG. 5. IgG subclass responses to F1- and V-Ag in sera from IL-12(Low)/F1-V DNA- and IL-12(High)/F1-V DNA-vaccinated mice. The mouse sera evaluated were shown in Fig. 3. The endpoint titers are depicted as the means plus standard deviations (error bars) for 10 mice/group. IgG1 and other IgG subclass endpoint titers that were significantly different are indicated by the brackets and asterisks as follows: *, $P < 0.05$; **, $P < 0.01$.

tissues at 14 weeks after primary immunization. F1- and V-Ag-specific IgA and IgG AFC responses were detected in the spleens, HNLNs, NALT, NPs, SMGs, and PPs from both IL-12 DNA-immunized groups (Fig. 6). Although serum IgG and mucosal IgA titers were similar between the two DNA-vaccinated groups, there were some notable differences in total and Ag-specific IgA and IgG AFC responses. The NALT and SMG IgA anti-F1-Ag and PP anti-V-Ag responses from IL-12(Low)/F1-V DNA-vaccinated mice were significantly greater than those from IL-12(High)/F1-V DNA-vaccinated mice (Fig. 6A). Between the two IL-12 DNA-vaccinated groups, F1- and V-Ag-specific IgG AFC responses were not different except for V-Ag-specific IgG responses in the spleen (Fig. 6B). These

FIG. 6. IgA (A) and IgG (B) AFC responses by mice nasally vaccinated with IL-12(Low)/F1-V DNA and IL-12(High)/F1-V DNA vaccines. Mice were nasally dosed, as described in the legend to Fig. 3, and lymphoid tissues were isolated on week 14. Total splenic, head and neck lymph node (HNLN), nasal mucosa-associated lymphoid tissues (NALT), nasal passage (NP), submaxillary gland (SMG), small intestinal lamina propria (iLP), and Peyer's patch (PP) mononuclear cells were isolated from each DNA vaccine group (five mice/group/experiment) and evaluated in a B-cell ELISPOT assay to assess F1-Ag- and V-Ag-specific (A) IgA and (B) IgG AFCs, as well as total (A) IgA and (B) IgG AFCs. Values are the means plus standard errors of the means (error bars) of AFC responses taken from two experiments. In some instances, significant differences between the high-dose and low-dose IL-12 vaccines in the different tissues were detected: #, $P < 0.05$; ##, $P < 0.01$.

FIG. 7. Cytokine responses by mice primed with IL-12(Low)/F1-V DNA and IL-12(High)/F1-V DNA vaccines. BALB/c mice were nasally dosed with the DNA vaccines on weeks 0, 1, and 2, and on week 7, total lymphocytes were isolated from spleens, HNLNs, and PPs. Lymphocytes were Ag pulsed for 2 days, and IFN- γ , IL-4, IL-5, IL-10, and TGF- β CFC responses (A) and secreted IFN- γ , IL-6, IL-10, and IL-17 (B) were measured by cytokine-specific ELISPOT assay and sandwich ELISA, respectively. Values are shown as means plus standard errors of the means (error bars) for two experiments (16 mice/group). In some instances, significant differences ($P < 0.05$) in the different tissues in the two IL-12 DNA vaccine groups (#) or versus medium (Med) (*) were detected.

results suggest that the differences in lymphoid tissues between high- and low-dose IL-12 DNA vaccines may contribute to protection.

Nasal DNA priming elicits a mixed Th cell response. To assess the types of Th cell responses elicited by the DNA priming, CFC responses were measured at 7 weeks after primary immunization by a cytokine-specific ELISPOT assay. To compare these responses between the two IL-12 DNA-vaccinated groups, lymphocytes from spleens, HNLNs, and PPs were restimulated with F1-Ag, V-Ag, or medium for 2 days

(Fig. 7). By cytokine ELISPOT assays (Fig. 7A), production of IFN- γ , IL-4, and IL-10 by spleens, HNLNs, and PPs, as well as PP IL-5, was significantly enhanced by both vaccines. In addition, IL-6, IL-17, and transforming growth factor β (TGF- β) were measured in cell supernatants from lymphocytes restimulated with F1- or V-Ag by sandwich ELISA, as well as IFN- γ and IL-10 (Fig. 7B). Although TGF- β was not detected (data not shown), Ag-specific IL-6 and IL-17 were significantly enhanced as IFN- γ and IL-10. For the most part, there were no significant differences in the cytokine responses induced by

either IL-12 DNA vaccine in any of the tissues examined, except for PP IFN- γ CFC responses induced by IL-12(Low)/F1-V DNA vaccine following restimulation with F1-Ag. These results suggest that both IL-12 DNA vaccines primed Ag-specific Th1- and Th2-type cytokines in both systemic and mucosal compartments.

DISCUSSION

In this study, to obtain an effective DNA vaccine against pneumonic plague, several constructs expressing the individual plague Ags or coexpressing them as a F1-V fusion protein in combination with IL-12 DNA as a molecular adjuvant were generated. Since *Y. pestis* is an intracellular pathogen, M. A. Parent et al. and Philipovskiy and Smiley suggested that plague vaccines should be designed to maximally prime both cellular and humoral immunity for effective protection (23, 24, 27). IL-12 was selected as a molecular adjuvant because past studies have shown that IL-12 could exhibit both Th1- and Th2-type properties and enhance IgA production when applied mucosally (4, 18). IL-12 is produced by antigen-presenting cells, indicating its crucial role for protection against intracellular pathogens through the induction of NK cell activity and Th1 cell responses (1, 10). IL-12 has also been adapted as a mucosal adjuvant for development of mucosal vaccines against intracellular pathogens, such as human immunodeficiency virus (5, 7) and *Mycobacterium tuberculosis* (38). For the development of an effective plague vaccine, we tested IL-12 as a mucosal adjuvant against *Y. pestis*. Interestingly, our results showed that mice nasally immunized with the IL-12 DNA vaccine, without encoding F1- and V-Ags, but with a F1-Ag protein boost, showed better survival against pneumonic plague than those mice immunized with F1-Ag protein (plus CT) only. These results show that IL-12 can be used as a mucosal adjuvant for vaccines to enhance protective immunity.

Ab responses in mice immunized with IL-12(Low)/F1-Ag, IL-12(Low)/V-Ag, or IL-12/F1-V began to increase by week 6. Although three DNA immunizations were insufficient to elevate the anti-F1-Ag and anti-V-Ag Ab responses, robust Ag-specific responses were induced in mice nasally boosted with F1-Ag protein. These results were consistent with previous observations that DNA immunization effectively primes the host (7, 22), and the combination of DNA and Ag immunizations represents one means to effect optimal immunity to plague. Other studies have shown the effectiveness of DNA vaccines to plague, but these were all applied parenterally, either via intramuscular injection (9, 11) or with a gene gun (9, 11, 30). As with our nasal immunization, multiple deliveries were required. These studies also showed that the immunogenicity of DNA vaccines for plague varied depending on the mode of Ag expression, e.g., polymeric form (30). Such an approach may be required to enhance F1-Ag's immunogenicity. However, none of these studies evaluated different molecular adjuvants as described in this current study. Thus, our results showed that mucosal IL-12 DNA vaccines provide sufficient priming that leads to protection, and nasal application of recombinant F1-Ag alone is insufficient to confer protection. This priming effect was partly enhanced by IL-12 alone, since priming mice with IL-12(Low)/ β -Gal was as effective in conferring protection as immunizing mice with IL-12(Low)/F1-Ag.

As anticipated, immunity to pneumonic plague also requires anti-V-Ag immunity. Immunizing mice with IL-12(Low)/V-Ag and boosting with F1-Ag protein were not as effective as immunizing mice with IL-12(Low)/F1-V DNA vaccine and boosting with F1-Ag protein, again suggesting that the DNA-encoded F1-Ag must be priming the host to improve protective immunity.

Our results showed that IL-12 DNA vaccination induces higher IgG1 Ab titers to F1-Ag than the other IgG subclasses. In this study, mice were primed with IL-12 DNA vaccines and subsequently boosted with F1-Ag protein plus CT; such adjuvant combinations have been previously tested (18). CT, a well-known mucosal adjuvant that induces Th2-type responses (19, 29), has been used mucosally in combination with recombinant IL-12 (18). Such combinations induce significant amounts of IgG2a, as well as IgG1 and IgG2b Ab titers, suggesting that IL-12 is an effective mucosal adjuvant to induce Th1 responses (18); however, such Th1 cell bias was not evident in our study, and it was not evident when F1- and V-Ags were delivered by a *Salmonella* vaccine vector (36). This finding suggests that the potency obtained with the IL-12(Low)/F1-V DNA vaccine is less than when using the recombinant IL-12 (4, 18). Nonetheless, elevated IgG1 Abs to F1- and V-Ags were induced, which has been previously deemed important, since enhanced IgG1 subclass titers to F1- and V-Ag correlates with protection against plague (34). Given our findings, the IL-12(Low)/F1-V DNA vaccine mediates a mixed Th cell phenotype, as evidenced in our CFC analyses, and may further push this bias using Th2 cell-promoting adjuvants, as with booster immunizations using F1-Ag protein plus CT, to enable protection against pneumonic plague. On the other hand, Brandler et al. reported variable Ab responses between different inbred mouse strains, as well as outbred mice, immunized with plague DNA vaccines, and suggested caution be used in interpreting DNA immunization studies that rely on data obtained from a single mouse strain (6). However, BALB/c mice, as in our study, were responsive to DNA vaccines when boosted with protein, suggesting that the combination of DNA and protein vaccine approach was required to induce optimal promotion in both humoral and cellular immunity in all mouse strains (6). Outbred Swiss-Webster mice were unresponsive to any DNA vaccination (6). Our study also showed that the combination of DNA vaccination priming followed by protein boosts induced optimal immune responses against plague.

Our results showed that IL-12 DNA(Low) vector encoding F1-Ag plus V-Ag induces greater protection than those encoding only F1-Ag or V-Ag. These results are consistent with previous observations that a combination or fusion of these Ags has an additive protective effect when used to immunized mice against plague (12, 31–33). The F1-Ag and V-Ag are considered the most effective candidates for vaccines against plague, and vaccination with each protein alone is sufficient for protecting mice against both bubonic and pneumonic plague (16, 20).

In this study, two IL-12 DNA vaccines encoding F1-V fusion protein differed in the amounts of IL-12 produced by 10-fold. Since IL-12p40 has both antagonistic and agonistic effects via binding IL-12R β 1 (14), IL-12p40/p70 expression ratios were determined, and no significant differences were noted, suggest-

ing all of the polypeptide was intact IL-12p70. When the efficacies of these vaccines were compared, it was anticipated that the IL-12(High)/F1-V DNA vaccine would show improved protection, but instead the efficacy was lost compared to protection conferred by the IL-12(Low)/F1-V DNA vaccine. Thus, the best protection was obtained using the DNA vaccine for F1-V fusion protein in combination with the low-dose IL-12. It was unclear why the IL-12(High)/F1-V DNA vaccine was less protective, since similar Ab and Th cell responses were induced by both vaccines. Subtle differences were evident in the distribution of IgA AFCs, particularly, in the NALT, SMGs, and PPs, which may contribute to enhanced protection. In addition, perhaps differences in innate immune responses may have contributed to the observed differences in protection.

In summary, this is the first description of a nasal DNA immunization regimen that applies DNA vaccines for pneumonic plague. Using a bicistronic plasmid encoding the molecular adjuvant, IL-12, plus the vaccine encoding F1-V-Ag, we show effective priming using the IL-12(Low)/F1-V DNA vaccine followed by booster immunizations with recombinant F1-Ag protein resulting in protection against pneumonic plague. Both Th1 and Th2 cell responses were induced locally as well as systemically. Although a definitive correlate of protective efficacy to discriminate between the IL-12(Low)/F1-V DNA and IL-12(High)/F1-V DNA vaccines could not be defined, these results suggest that IL-12 can be used as a mucosal adjuvant to allow inclusion of a cell-mediated component to enhance protective immunity against pneumonic plague, albeit the amount of IL-12 is dose dependent.

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