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An IL-12 DNA vaccine co-expressing *Yersinia pestis* antigens protects against pneumonic plague

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Abstract

Pneumonic plague remains problematic in endemic areas, and because it can be readily transmitted and has high mortality, the development of efficacious vaccines is warranted. To test whether stimulation of cell-mediated immunity with IL-12 will improve protective immunity against plague, we constructed two IL-12 DNA vaccines using a bicistronic plasmid encoding the protective plague epitopes, capsular (F1) antigen and virulence antigen (V-Ag) as F1-V fusion protein and V-Ag only, respectively. When applied intramuscularly, antibody responses to F1- and V-Ag were detectable beginning at week 6 after three weekly doses, and F1-Ag protein boosts were required to induce elevated Ab responses. These Ab responses were supported by mixed Th cell responses, and the IL-12/V-Ag DNA vaccine showed greater cell-mediated immune bias than IL-12/F1-V DNA vaccine. Following pneumonic challenge, both IL-12 DNA vaccines showed similar efficacy despite differences in Th cells simulated. These results show that IL-12 can be used as a molecular adjuvant to enhance protective immunity against pneumonic plague.

Keywords

IL-12; Pneumonic plague; DNA vaccine

1. Introduction

Plague is a zoonotic disease caused by *Yersinia pestis* and assumes three forms of the disease in humans, bubonic, pneumonic and septicemic. Bubonic and septicemic plagues arise from bites from fleas that previously were feeding on infected animals [1,2]. 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 [3]. In the past, three major pandemics of plague disease have resulted in an estimated 200 million deaths, and plague still remains endemic in regions of Africa, Asia, and North and South America [1,2]. Therefore, development of vaccines for plague is desirable.

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At present, there are no licensed plague vaccines in the United States. For development of a subunit vaccine to plague, efforts have focused on two primary *Y. pestis* antigens (Ags), the outer capsule protein (F1-Ag), which is believed to help avoid phagocytosis [4,5], and the low calcium response protein (lcrV) or V-Ag, which has been suggested to mediate a suppressive effect upon Th1 cells via the stimulation of IL-10 [6]. These individual vaccines effectively protect against bubonic and pneumonic plague [7,8], and when these Ags are applied in combination or fusion form, enhanced protection is obtained [9–12]. On the other hand, while the observed protective immunity is largely Ab-dependent, *Y. pestis* is an intracellular pathogen, and new data suggest that cellular immunity can contribute to effective protection against plague [13–15].

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 (APCs), such as dendritic cells (DCs) and macrophages; these cells often produce IL-12 in response to a number of bacterial products [16–18]. IL-12 has a central function in initiating and regulating cellular immune responses by stimulating IFN-γ production by both NK cells and T helper (Th) cells via binding its receptor comprised of two subunits, IL-12Rβ1 and IL-12Rβ2 [17,19]. Thus, we hypothesize 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 constructed IL-12 DNA vaccines using bicistronic plasmids that encode either V-Ag or F1-V fusion protein and assessed their vaccine efficacy against pneumonic plague challenge. Although only partial protection against pneumonic plague was obtained, these DNA vaccines did effectively prime with subsequent F1-Ag protein boosts. Thus, the IL-12 DNA vaccine can be used as a primary vaccine for protection to pneumonic plague.

2. Materials and methods

2.1. Plasmids

To develop the IL-12 DNA vaccines [20], cDNA fragments for *Y. pestis* V and F1-V Ags were amplified by polymerase chain reaction (PCR) from a synthetic gene (GenScript, Piscataway, NJ) optimized for mouse codon usage, as previously described [20], into pBudCE4.1 vector (Invitrogen Corp., Carlsbad, CA). The DNA fragment for IL-12 is referred to pGT146-mIL-12 vector (Invivogen, SanDiego, CA) with sequences cloned from the *Sal* I site at the 5′-teminus to the *Sca* I site at the 3′-terminus that contain a linker sequence between p35 and p40, Val-Pro-Gly-Val-Gly-Val-Pro-Gly-Val-Gly. Each of the amplified DNA fragments for the plague antigens contains sequences for the *Not* I site at the 5′-terminus and for the *Kpn* I site at the 3′-terminus; for the F1-V fusion protein, residues contain a linker sequence, Pro-Gly-Gly, between F1 and V-Ag. Following sequence confirmation of the TA cloned (TOPO cloning kit, Invitrogen) PCR products, each of the fragments was digested and sequentially inserted into the vectors, resulting in pBud-IL-12/V and IL-12/F1-V with the expression of IL-12 under the CMV promoter and expression of the plague Ags under EF-1α promoter [20]. These DNA plasmids were purified with a commercially available plasmid purification kit (Qiagen, Inc., Valencia, CA) and resuspended with DNase-free water.

2.2. Transfection

To evaluate the expression of IL-12, V-Ag, and F1-V-Ag fusion protein, 293A cells (ATCC, Manassas, VA) were transfected with each DNA plasmid using Lipofectamine LTX (Invitrogen). The 293A cells were cultured in complete medium (CM): RPMI-1640 (Invitrogen) containing 10% FBS (Atlanta Biologicals, GA), 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml

streptomycin. The cell culture supernatants and lysates were subjected to immunoblotting and ELISA 2 days after transfection, respectively, as described below.

2.3. Immunoblotting

Transfected 293A cells were lysed in Milli-Q water; 30 μg of total protein were electrophoresed on a 12% SDS-polyacrylamide gel, and then transferred onto a nitrocellulose membrane (Bio-Rad Lab., Hercules, CA). The membrane was incubated with anti-V-Ag rabbit serum [21] overnight at 4 °C and then with HRP-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) for 90 min at room temperature. The signals were visualized using the substrate 4-chloro-1-naphtol chromogen and H_2O_2 (Sigma-Aldrich, St. Louis, MO).

2.4. 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.

At 8-10 wks of age, mice were immunized intramuscularly with each DNA vaccine on wks 0, 1, and 2. For each immunization, 100 μg DNA were administered with a needle into the tibialis anterior muscles of the two hind legs. On wks 8 and 9, mice were nasally boosted with 25 μg of F1-Ag protein plus 2.5 μg of cholera toxin (CT; List Biological Laboratories, Campbell, CA) adjuvant. A final boost of DNA vaccine (100 μg) intramuscularly and F1-Ag protein (25 μg) plus CT adjuvant intranasally was given on week 12.

To test the efficacy of the DNA vaccines, immunized mice were nasally challenged with 100 LD_{50} of *Y. pestis* Madagascar strain (MG05) 44 days after the last immunization, as previously described [20,21]. All mouse care and procedures were in accordance with institutional policies for animal health and well-being, and all challenges adhered to Biosafety Level 3 practices, as previously done [20,21].

2.5. Measurement of anti-F1- and V-Ag Abs titers by ELISA

Blood was collected from the saphenous vein, and nasal washes were performed at the termination of the study, as previously described [20]. After microcentrifugation, sera and nasal washes were collected and frozen at −30 °C until assay. Serum and nasal wash Ab titers were determined by ELISA. Briefly, recombinant F1- or V-Ag [20,21] in sterile PBS was coated onto Maxisorp Immunoplate II microtiter plates (Nunc, Roskilde, Denmark) at 50 μl/well. After overnight incubation at room temperature, wells were blocked with PBS containing 1% BSA for 1 hour at 37 °C; individual wells were loaded with serially diluted mouse serum or nasal wash in ELISA buffer (PBS containing 0.5 % BSA and 0.5 % Tween 20) overnight at 4 °C. Ag-specific Abs were reacted with HRP-conjugated goat anti-mouse IgG, IgA, IgG1, IgG2a, or IgG2b Abs (Southern Biotechnology Associates) for 90 minutes at 37 °C. The specific reactions were detected with soluble enzyme substrate, 50 μl of ABTS (Moss, Inc., Pasadena, CA), and absorbance was measured at 415 nm after 1 hour 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 OD above negative controls after 1 hour at room temperature.

2.6. Lymphocyte isolation

Lymphocytes were isolated from nasal passages (NP), head and neck lymph nodes (HNLNs), spleens, lumbar lymph nodes (LLNs), sciatic lymph nodes (SLNs), and popliteal lymph nodes (PopLNs). HNLN, splenic, LLN, SLN, and PopLN mononuclear cells were isolated by

conventional methods using Dounce homogenization [20,21]. To isolate the mononuclear cells from NPs, 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 [20]. After incubation, the digestion mixtures were passed through Nitex mesh (FairviewFabrics, 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. Greater than 95 % viability was obtained for all lymphocytes isolated from each tissue, as determined by Trypan blue exclusion.

2.7. Ab ELISPOT

On wk 14, sets of studies were terminated to collect NP, HNLN, splenic, LLN, and PopLN mononuclear cells from immunized mice. Ag-specific Ab-forming cell (AFC) responses by the ELISPOT method were detected, using mixed cellulose ester membrane-bottom microtiter plates (MultiScreen-HA; Millipore, Bedford, MA) by coating with 5 μg/ml F1- or V-Ag in sterile PBS, as previously described [20,21]. For total IgA or IgG AFC responses, wells were coated with 5 μg/ml goat anti-mouse IgA or IgG Abs (Southern Biotechnology Associates) in sterile PBS.

2.8. Cytokine ELISA and ELISPOT assays

To measure IL-12 expression, cell culture supernatants from transfected 293A cells were collected, and IL-12 was measured by a sandwich ELISA, as previously described [22]; serially diluted recombinant murine IL-12 (R&D Systems, MN) was used to generate a standard curve. Cell supernatants were collected 2 days after transfection of 293 cells, as described above.

To determine Th cell responses to F1- and V-Ags, on wk 14, groups of immunized mice were used. Total lymphocytes from spleens, HNLNs, and peripheral lymph node (PLNs), containing LLNs, SLNs and PopLNs, were resuspended in CM. These lymphocytes were restimulated with 10 μg of recombinant F1, V-Ag, or with media as control in the presence of 10 U/ml human IL-2 (PeproTech) for 2 days at 37 °C in a humidified 5 % CO_2 incubator. After incubation, cell supernatants were collected by centrifugation and stored at 4 °C until use. The supernatants were prepared as duplicate sets of samples. These supernatants were used for measurement of IFN-γ, IL-6, IL-10, IL-17, and TGF-β by sandwich ELISA, as described previously [22,23].

For cytokine ELISPOT assays, likewise on wk 14, total mononuclear cells from spleens, HNLNs, and peripheral lymph nodes (PLNs), containing LLNs, SLNs, and PopLNs, were resuspended in CM. Mononuclear cells were restimulated with 10 μg of recombinant F1, V-Ag, or with media as control in the presence of 10 U/ml human IL-2 (PeproTech) for 2 days at 37 °C in a humidified 5 % CO_2 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 [20,21].

2.9. Statistical analysis

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

3. Results

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

To verify the expression of IL-12, V-Ag, and F1-V-Ag fusion proteins, replicate cultures of 293A cells were transfected with one of these DNA vaccines, and cell culture supernatants and lysates were collected (Fig. 1A, B). IL-12 could readily be detected in each of the cell supernatants from the transfected 293A cells when compared to supernatants from DNA plasmids lacking IL-12 (Fig. 1A). To detect the expression of V-Ag and F1-V fusion proteins, cell lysates were used for immunoblotting. The V-Ag and the F1-V could be detected using the anti-V-Ag rabbit serum (Fig. 1B). The F1-V protein migrated with an apparent MW of 54 kDa, which represents the expected molecular mass for F1-Ag (17 kDa) plus V-Ag (37 kDa).

3.2. Ab responses to F1- and V-Ag

Groups of BALB/c mice were immunized via the intramuscular (i.m.) route on days 0, 7, and 14 with 100 μg IL-12/V or IL-12/F1-V DNA vaccine. To evaluate the relative immunogenicity of the IL-12 DNA vaccines, samples were collected at 6 wks post-primary immunization and subsequently at two wk intervals. Past studies with other DNA vaccines show that Ab responses are delayed and peak between 8 and 10 wks post-primary immunization [24]. Ag-specific Ab titers in sera were measured by ELISA using F1- or V-Ag coated wells (Fig. 2A). Mice immunized with the IL-12/F1-V DNA vaccine showed induced serum IgG Ab titers between 6 – 8 wks post-primary immunization to F1- and V-Ags (Fig. 2A). While serum IgG anti-V-Ag Abs steadily increased with time, serum IgG anti-F1-Ag Abs did not. Thus, to enhance these Ab responses, mice were boosted nasally with 25 μg of recombinant F1-Ag protein plus CT on wks 8 and 9, resulting in robust serum IgG Ab titers against both F1- and V-Ags by wk 12 (Fig. 2A). A final i.m. boost with DNA vaccine, as well as nasally with recombinant F1-Ag plus CT, was given on wk 12.

Mucosal Ab responses in nasal washes were also evaluated. Mice vaccinated with either DNA vaccine showed no differences in nasal IgA anti-V-Ag Ab titers (Fig. 2C). However, while both groups showed elevated nasal IgA Abs to F1-Ag, IL-12/V-Ag-immunized mice showed significantly greater Ab titers (Fig. 2C).

3.3. IgG subclasses responses with IL-12 DNA vaccines

IgG subclass responses were also determined using serum samples collected on wk 12 (Fig. 2B). The IL-12/V DNA vaccine stimulated greater IgG1 than IgG2a or IgG2b anti-F1-Ag Ab responses, and equivalent IgG1 and IgG2a, but less IgG2b anti-V-Ag Ab titers. The IL-12/F1-V DNA vaccine induced greater IgG2a anti-F1- and -V-Ag Abs than IgG1 or IgG2b Ab responses.

3.4. Distribution of immune B cells following immunization

To assess the magnitude and distribution of Ab-forming cell (AFC) responses induced by IL-12 DNA vaccines, a B cell ELISPOT was performed using lymphocytes from spleens, HNLNs, NPs, LLNs, and PopLNs at 14 wks post-primary immunization (Fig. 3). F1- and V-Ag-specific IgA and IgG AFC responses were significantly elevated from both IL-12 DNA-immunized groups than those mice given F1-Ag protein only. Furthermore, NP IgA and IgG AFC responses from IL-12 DNA vaccines immunized mice were also significantly greater than F1-Ag protein-immunized mice. These results suggest that IL-12 DNA vaccine primed Ag-specific B lymphocytes into both systemic and mucosal lymphoid compartments.

3.5. IL-12 DNA vaccines elicis a mixed Th cell response

To assess the types of Th cell responses elicited by the DNA vaccines, cytokine-forming cell (CFC) responses were measured at 14 wks post-primary immunization by cytokine-specific ELISPOT. To compare these responses between the two IL-12 DNA-vaccinated groups, lymphocytes from spleens, HNLNs, and PLNs, containing LLNs, SLNs, and PopLNs, were restimulated with F1-Ag, V-Ag, or media for 2 days (Fig. 4). By cytokine ELISPOT (Fig. 4A), production of IFN-γ, IL-4, and IL-10 by spleen, HNLN, and PLN was significantly enhanced by both vaccines. In addition, IL-6, IL-10, IL-17, IFN-γ, and TGF-β were measured in cell supernatants from lymphocytes restimulated with F1- or V-Ag by ELISA (Fig. 4B). Although TGF-β was not detected (data not shown), Ag-specific IL-6 and IL-17 were significantly enhanced as were IFN-γ and IL-10, particularly by the IL-12/V-Ag-immunized group. These results suggest that both IL-12 DNA vaccines stimulated Ag-specific Th1-, Th2-, and Th17-type cytokines.

3.6. Challenge study

To determine the IL-12 DNA vaccines' efficacies against pneumonic plague, immunized mice were challenged nasally with 100 LD_{50} *Y. pestis* Madagascar strain 44 days after the final boost, and the mean survival rates were determined (Fig. 5). All mice dosed with PBS died within 3 days after challenge. Mice immunized with the IL-12/V-Ag or IL-12/F1-V-Ag DNA vaccine showed partial protection (50% survival; $P < 0.05$ and $P < 0.001$, respectively). These efficacies were better than those mice immunized with F1-Ag protein only (20% survival; $P < 0.001$). These results suggest that the IL-12 DNA vaccines can contribute to protective immunity against pneumonic plague.

4. Discussion

Previous reports suggest that a plague vaccine capable of inducing cell-mediated and humoral immunity would be effective against pneumonic plague [13–15]. Therefore, in this current study, DNA vaccines were constructed encoding plague V-Ag or a F1-V fusion protein in combination with IL-12 as a molecular adjuvant. IL-12 is produced by APCs, indicating its crucial role for protection against intracellular pathogens through induction of natural killer (NK) cells and Th1 cell responses [17,19]. IL-12 has also been adapted as a molecular adjuvant for development of vaccines against intracellular pathogens, such as human immunodeficiency virus (HIV) [25,26] and *Mycobacterium* [27], and these reports suggest that protective efficacies are associated with activation of proliferating T cells and IFN-γ production upon Ag-specific stimulation. In this study, although IL-12 DNA vaccinations showed only partial protection against pneumonic plague, IL-12 DNA vaccinations induced significantly greater IgG1 and IgG2a subclass responses to F1- and V-Ag, as well as production of IFN-γ. These results show that IL-12 can be used as a molecular adjuvant for vaccines to enhance protective immunity against pneumonic plague.

In this study, i.m. IL-12 DNA vaccinations induced significant Ab responses to F1- and V-Ags by week 6, but while the IgG anti-V-Ag titers continued to increase, serum IgG anti-F1-Ag Ab titers did not. Boosting nasally with F1-Ag protein was required to induce robust IgG responses. These results were consistent with previous observations that DNA immunization effectively primes the host [26,28], and the combination of DNA and Ag immunizations represents one means to effect optimal immunity to plague. Indeed, our results also showed that IL-12 DNA vaccination with nasal boosting with F1-Ag protein afforded greater protection than immunization with F1-Ag protein only. Thus, IL-12 DNA vaccines provided effective priming that ultimately led to protective immunity against plague.

To obtain more effective protection against pneumonic plague, induction of immune responses in the respiratory tract may be required. Indeed, our previous study showed that oral administration of *Salmonella* vaccine resulted in stimulation of distal mucosal sites, afforded effective protection, and > 80 % survival against pneumonic plague [21]. In this study, i.m. IL-12 DNA vaccinations also induced significant Ab responses at mucosal sites, including NP. However, the protective efficacies conferred by i.m. IL-12 DNA vaccinations were less than those conferred by our oral *Salmonella* vaccine [20] but better than the same IL-12 DNA vaccine given nasally [20]. To obtain better protective immunity, enhancing the IL-12-based DNA vaccines may be required. Others have shown that the immunogenicity of DNA vaccines for plague varies depending on the type of eukaryotic promoter/enhancer used or mode of Ag expression, e.g., polymeric form, [29,30]. Previous reports have also shown that DNA immunization with a gene gun induces greater Ab responses and more effective protection against plague than by conventional injection methods [29–31]. Additional studies using IL-12 DNA vaccine platform to enhance protective immunity against pneumonic plague are warranted.

Our results also showed that IL-12 DNA vaccination induced significantly elevated IgG1 responses, as well as IgG2a Ab responses. A previous report suggests that elevated IgG1 Abs to F1- and V-Ags responses are deemed important since enhanced IgG1 subclass titers to F1- and V-Ag correlated with protection against plague [32]. In this study, mice were primed with IL-12 DNA vaccine and subsequently boosted with F1-Ag protein plus CT. CT is a mucosal adjuvant capable of inducing Th2-type responses [33,34]. Using this vaccination regimen, a mixed Th cell response was induced, as evidenced in our IgG subclass responses and cytokine analyses. Depending upon immunogen used, cytokine responses can vary [15,21]. Immunization with live yersiniae has been shown to stimulate elevated IFN-γ responses by immune T cells, and adoptive transfer of these T cells could confer protective immunity against pneumonic plague [15]. Although other Th cell cytokines may have also been produced, these were not reported [15], and this further suggests that IFN-γ-producing T cells can contribute to protection to plague. On the other hand, using an attenuated *Salmonella* vaccine vector, it has been shown that a mixed Th cell response is obtained against V- and F1-Ags despite the presumed bias imparted by *Salmonella* [21]. It has been suggested that the expression of capsular F1-Ag on the cell surface of *Salmonella* may redirect host responses to passenger Ags, thus, eliciting the mixed Th cell phenotype [21]. Nonetheless, the described IL-12 DNA vaccines also impart a mixed phenotype, which may be, in part, contributed by the nasal co-delivery of F1-Ag with CT adjuvant, a known potent Th2 cell-promoting adjuvant [33]. Thus, the combination of IL-12 with CT may temper the IL-12-driven Th1 cell responses. An important note is that the amount of IL-12 produced by the DNA vaccines, at best, is in the nanomolar range, whereas, typically soluble IL-12 is given in the micromolar range [35,36], resulting in enhanced Th1 cell responses, but also greater toxicity to the host [36]. Each of these variables could account for the observed mixed Th cell phenotype obtained with the described IL-12 DNA vaccines.

On the other hand, Brandler et al. reports variable Ab responses among different inbred mouse strains, as well as among outbred mice, immunized with plague DNA vaccines, and suggests caution be used in interpreting DNA immunization studies that rely on data obtained from a single mouse strain [37]. However, BALB/c mice, as in our study, are responsive to DNA vaccines when boosted with proteins, suggesting that the combination of DNA and a protein vaccine approach is required to induce optimal promotion in both humoral and cellular immunity in all mice strains [37]. Outbred Swiss-Webster mice are unresponsive to any DNA vaccinations [37]. Our study also showed that the combination of DNA vaccination priming followed by protein boosts induces optimal immune responses against plague.

In this study, priming with IL-12/V-Ag or IL-12/F1-V conferred similar protection against pneumonic plague challenge as long as F1-Ag protein boosts were included in the vaccination regimen. Although these results are discrepant with previous observations that a combination or fusion of F1- and V-Ag has an additive protective effect when used to immunized mice against plague [9–12], vaccination with each protein alone also indicates sufficient protection against both bubonic and pneumonic plague [7,8]. Our results also showed that Ab responses to F1- and V-Ag in sera and nasal washes from mice immunized with IL-12/V DNA vaccine were elevated or better than mice immunized with IL-12/F1-V DNA vaccine.

In summary, by using a bicistronic plasmid encoding the molecular adjuvant, IL-12, plus the vaccine encoding V- or F1-V-Ag, we showed effective priming using the IL-12 DNA vaccine followed by booster immunizations with recombinant F1-Ag protein. Although these vaccines provided partial protection against pneumonic plague, Th1, Th2, and Th17 cell responses were induced locally, as well as systemically. These results suggest that IL-12 can be used as a molecular adjuvant to allow inclusion of a cell-mediated component to enhance protective immunity against pneumonic plague.

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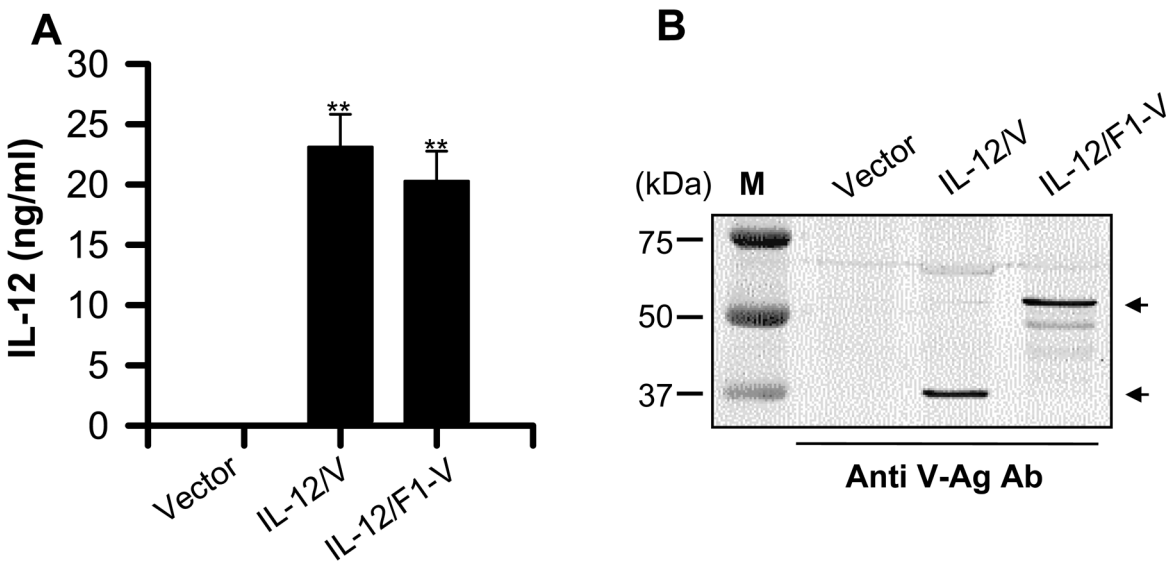

Fig. 1.

Plasmid expression determinations for the bicistronic IL-12 DNA vaccines. (A) Expression of IL-12 was determined by IL-12-specific ELISA using supernatants from 293A cells transfected with the IL-12 DNA vaccines. Data were represented by mean ± SEM of three experiments and analyzed using the Mann-Whitney U-test: **, $P < 0.01$. (B) To detect V- or F1-V-Ag expression by IL-12 DNA vaccines, cell lysates from transfected 293A were subjected to Western immunoblot analysis using rabbit anti-V-Ag sera.

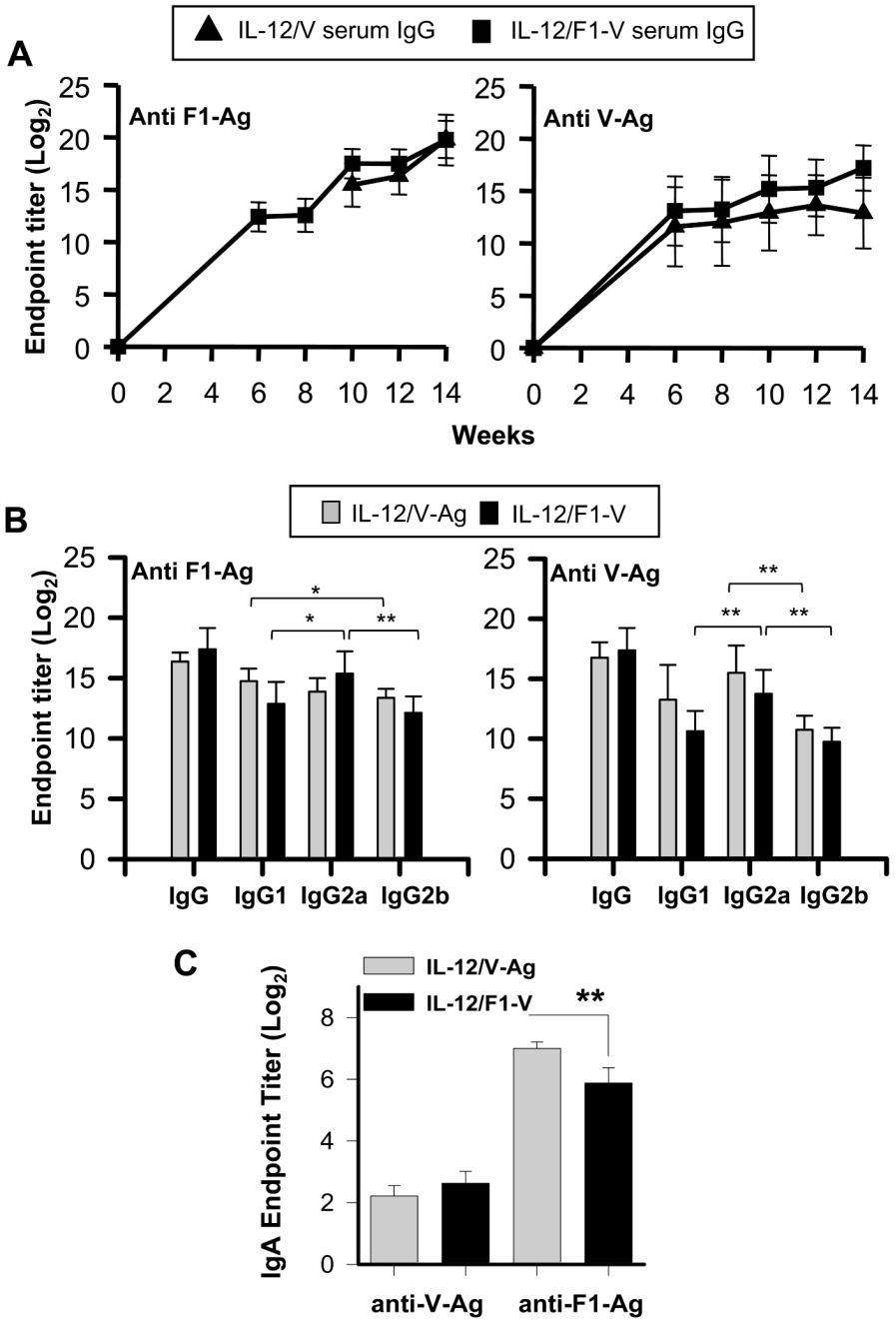

Fig. 2. I.m. immunization with IL-12 DNA vaccines induces elevated serum IgG and mucosal IgA Abs to F1-Ag and V-Ag. I.m. immunizations of BALB/c mice with the IL-12 DNA vaccines were given on wks 0, 1, and 2 followed by nasal boosts with recombinant F1-Ag plus 2.5 μg CT on wks 8 and 9, plus a final immunization was given on wk 12 with the DNA vaccine and F1-Ag plus CT

(A) A kinetics analysis was performed on the IL-12/V DNA and IL-12/F1-V DNA vaccines (34 mice/group). Serum IgG Ab titers to F1- and V-Ags were followed for 14 wks. (B) IgG subclass responses to F1- and V-Ag in sera from IL-12 DNA-vaccinated mice (8 mice/group). Endpoint titer depicted as the mean ± SD. * P <0.05 and ** P <0.01 are significant differences in IgG subclass endpoint titers. (C) Nasal washes from IL-12 DNA-vaccinated mice (8–24 mice/group) were performed on wk 14, and IgA anti-F1-Ag and anti-V-Ag Ab titers were

measured. **P = 0.022 depicts significant difference between IL-12/V-Ag- and IL-12/F1-V-vaccinated groups.

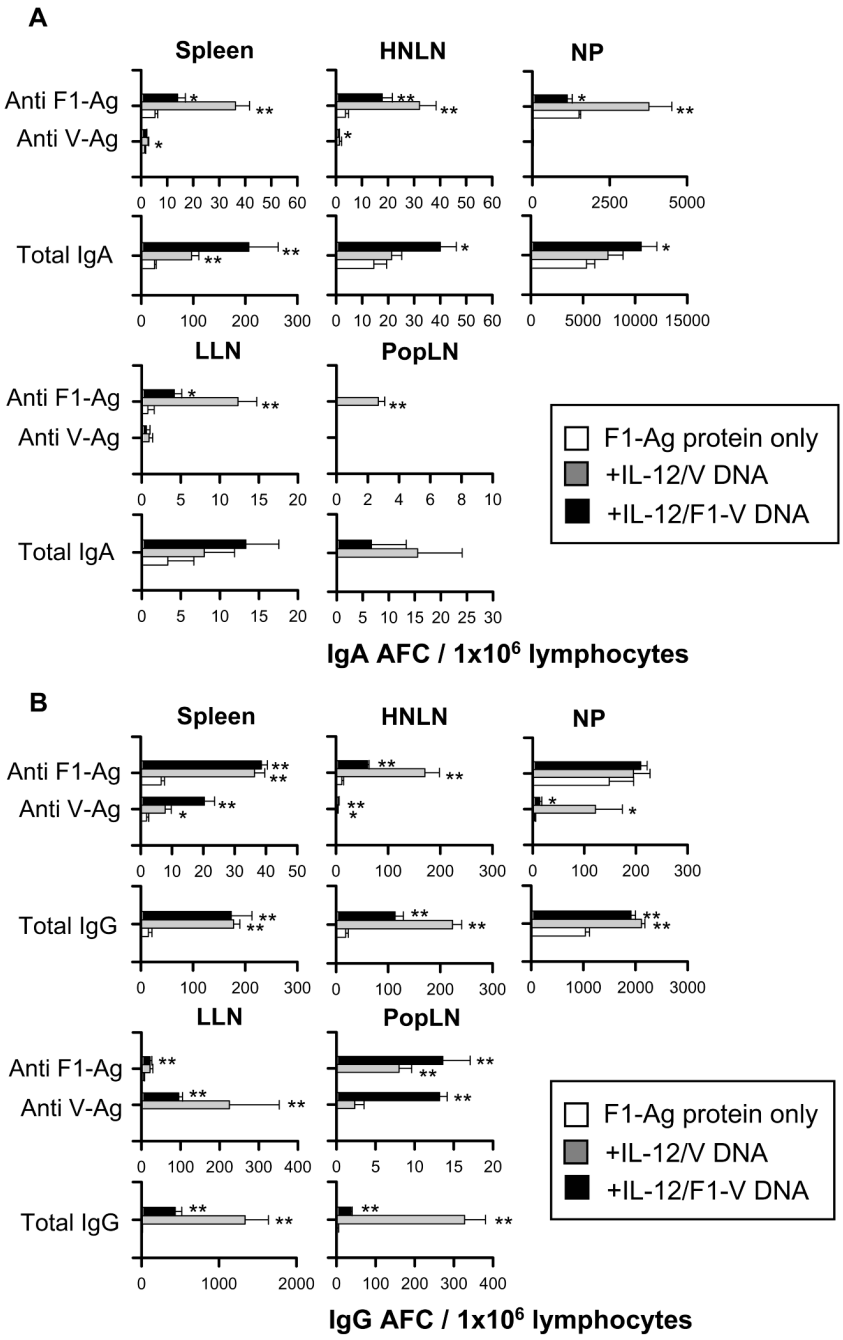

Fig. 3.

IgA (A) and IgG (B) Ab-forming cell (AFC) responses by mice vaccinated with IL-12/V DNA and IL-12/F1-V DNA vaccines. Mice were dosed, as described in Fig. 2, and lymphoid tissues were isolated on wk 14. Total splenic, head and neck lymph node (HNLN), nasal passage (NP), lumbar lymph node (LLN) and popliteal lymph node (PopLN) mononuclear cells were isolated from each DNA vaccine group (5 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. Depicted is the mean ± SEM of AFC responses taken from two experiments. Significant differences compared with mice only immunized F1-Ag protein: $^{*}P < 0.05$ and $^{**}P < 0.01$.

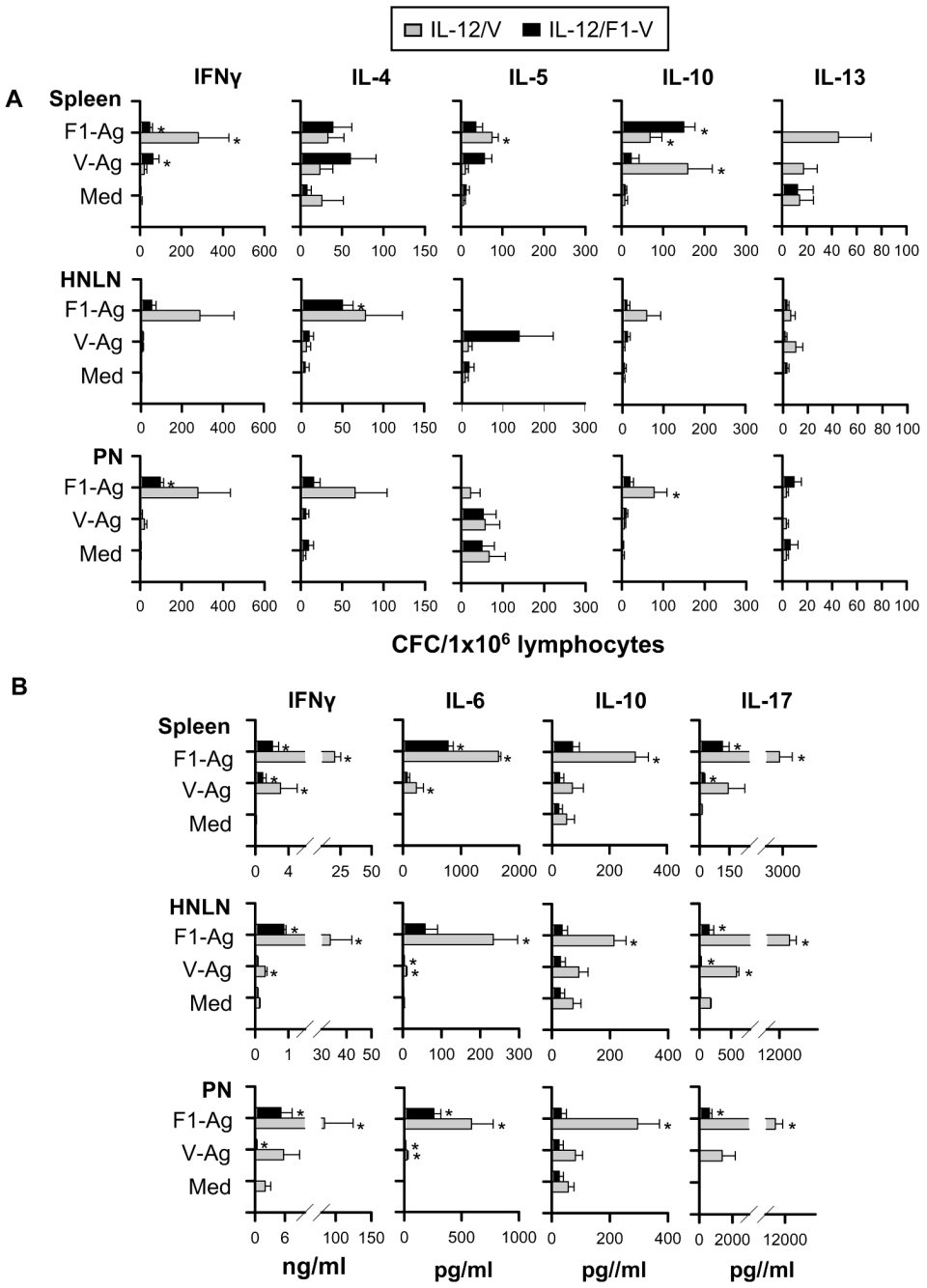

Fig. 4.

Cytokine responses by IL-12 DNA primed-mice show enhanced Th1-, Th2-, and Th17-type cell responses. BALB/c mice were dosed, as described in Fig. 2, and lymphoid tissues were isolated on wk 14. Total lymphocytes were isolated from spleens, HNLNs, and PLNs and were Ag-pulsed for 2 days; and (A) CFC responses and (B) cytokine responses were measured by cytokine-specific ELISPOT and sandwich ELISA, respectively. Depicted is the mean ± SEM of two experiments (16 mice/group). Significant differences in the different tissues were detected by comparing them with the group treated with media (Med) (*$P < 0.05$).

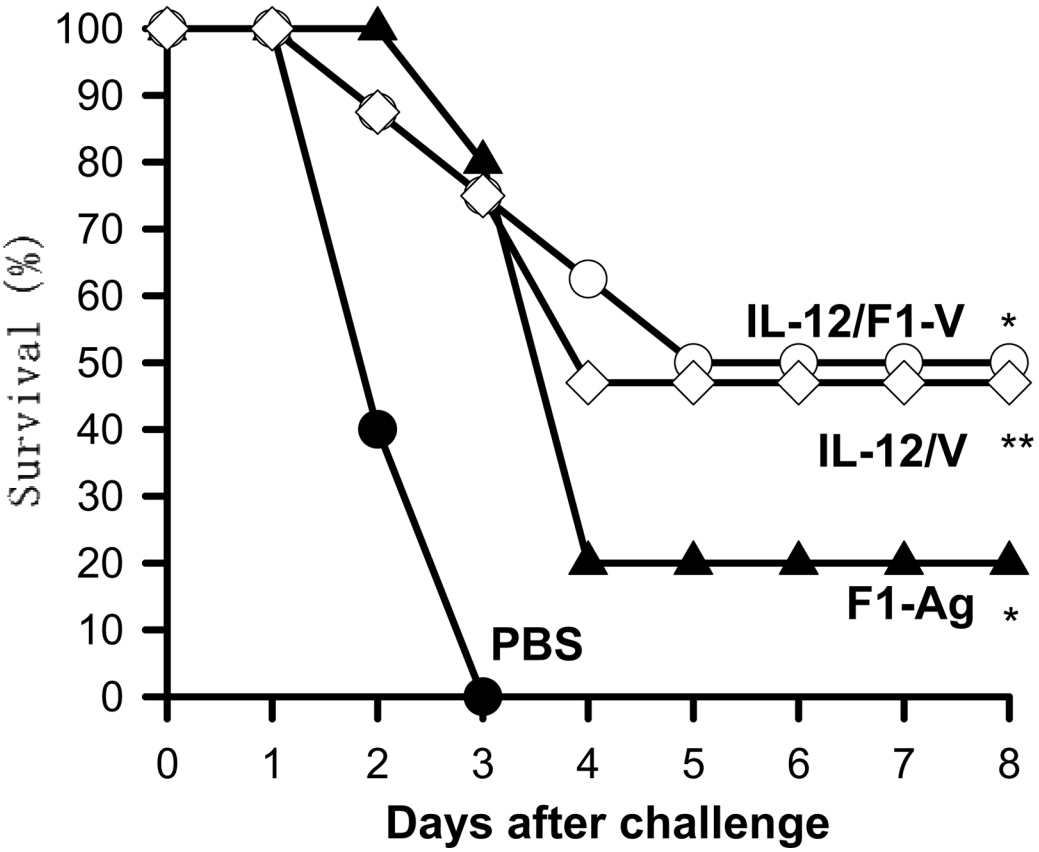

Fig. 5.

IL-12/DNA vaccine confers partial protection against pneumonic plague. Mice were dosed three times at wks 0, 1, 2, and 12 with the IL-12/DNA vaccines: V-Ag (n = 8), or F1-V-Ag (n = 8) and boosted on wks 8, 9, and 12 with recombinant F1-Ag. An additional group received recombinant F1-Ag (n = 5) only on wks 8, 9, and 12; a negative control group received PBS only (n = 5). All mice were nasally challenged 44 days after the last immunization with 100 LD50 MG05. Survival fractions obtained from vaccinated-mice were compared to PBS-dosed mice, and significance was determined: **P* < 0.001, ***P* < 0.05.