Enhancement of Antibody Responses to *Bacillus anthracis* Protective Antigen Domain IV by Use of Calreticulin as a Chimeric Molecular Adjuvant

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The generation of protective humoral immune responses against the receptor-binding domain (domain IV) of protective antigen [PA(dIV)] of *Bacillus anthracis* represents a plausible approach against anthrax toxin. In the current study, we have developed a naked DNA vaccine encoding calreticulin (CRT) linked to PA(dIV) of *Bacillus anthracis* [CRT/PA(dIV)]. We transfected a human embryonic kidney cell line (HEK 293) with CRT/PA(dIV) DNA and performed Western blotting and confocal microscopy analysis. We found that linkage of CRT to PA(dIV) targets PA(dIV) to the endoplasmic reticulum, resulting in secretion of the chimeric CRT/PA(dIV) protein. We then evaluated the ability of CRT/PA(dIV) DNA to generate PA(dIV)-specific antibody responses and protective immunity against lethal anthrax toxin (PA plus lethal factor) challenge. We found that mice immunized with CRT/PA(dIV) DNA were capable of rapidly inducing significantly higher PA(dIV)-specific antibody responses than mice immunized with PA(dIV) DNA alone. Furthermore, we observed that this enhanced antibody response generated by CRT/PA(dIV) DNA was CD4 dependent, since CD4 knockout mice demonstrated a significant reduction in antibody responses. In addition, analysis of the titers and avidity maturation of the induced PA-specific antibodies revealed that vaccination with CRT/PA(dIV) DNA vaccine accelerated the avidity maturation of antibodies to PA(dIV) compared to vaccination with PA(dIV) DNA. Importantly, the enhanced antibody responses correlated to protective immunity against lethal anthrax toxin challenge. Thus, DNA vaccines encoding CRT linked to PA(dIV) may dramatically enhance PA-specific protective antibody responses. Our results have significant clinical applications for biodefense against anthrax toxin.

Anthrax is caused by *Bacillus anthracis*, a gram-positive, endospore-forming, rod-shaped bacterium, which primarily infects farm animals such as cattle and sheep. Anthrax is rare in human beings but can occasionally be caused through contact with contaminated farm animals and animal products (23). Recently, it was reported that two postal workers who handled envelopes contaminated with anthrax spores died of inhalational anthrax in the United States. Thus, it is clear that anthrax is a deadly disease, and it has also been reported that 50 kg of anthrax spores could infect hundreds of thousands of people if released into well-populated cities (14).

The pathogenesis of anthrax is known to be caused by two independent self-replicating plasmids, the 184-kb plasmid pXO1 and the 90-kb plasmid pXO2 (5, 27, 36). The pXO1 plasmid carries the genes necessary for the secreted toxins. The pXO2 plasmid carries the genes necessary for formation of a capsule composed of a homopolymer of poly-D-glutamate that prevents phagocytosis. Both plasmids are essential for virulence. Plasmid pXO1 instructs the expression of three virulence factors, protective antigen (PA), edema factor (EF), and lethal factor (LF) (5). PA first binds its cellular receptor, on which LF and EF are then assembled. After the complex is assembled, it is internalized by receptor-mediated endocytosis (36), resulting in cellular dysfunction. LF is a protease, and EF is a calmodulin-activated adenylate cyclase. Many reports have shown that the pathogenicity of anthrax disappears when the receptor-binding sites of PA are mutated or the binding of the PA receptor is blocked, which is a rate-limiting step in the pathogenesis of anthrax (4, 15). For these reasons, PA has been used as a target antigen for the development of vaccines against anthrax.

In the current study we aimed to elicit immune responses against the receptor-binding domain (domain IV) of PA [PA(dIV)] using intradermal administration of DNA vaccines via gene gun. The use of DNA vaccines has emerged as a favorable approach for antigen-specific immunotherapy because of their safety, stability, and ease of production (12, 34). Gene gun administration represents a needle-free method for gene delivery. Several other strategies have also been developed using needle-free devices (3, 11). Intradermal administration of DNA vaccines using a gene gun represents an effective means of delivering DNA directly into the professional antigen-presenting cells and thus allows the application of...
strategies to modify the properties of antigen-presenting cells to enhance DNA vaccine potency (for reviews, see references 18 and 35).

One strategy to enhance DNA vaccine potency is to improve the major histocompatibility complex class I and class II presentation of model antigens using an intracellular targeting strategy (for reviews, see references 18, 26, and 35). This strategy involves the employment of molecules such as calreticulin (CRT), HSP110, HSP70, and CD9 to enhance the antigen-specific immune responses, including humoral and cellular immunity (9, 25, 33). CRT is a 46-kDa calcium-binding endoplasmic reticulum (ER)-resident protein that associates with CD91 on the surface of T cells and is implicated in a diverse number of functions (2, 22). Recent studies have shown that the binding of CRT to CD91 leads to the induction of phagocytosis and proinflammatory responses (2, 13) and induces cross-presentation in dendritic cells (16). We have previously shown that linkage of CRT with a target antigen significantly enhances the antigen-specific cell-mediated and humoral immune responses in vaccinated mice (8, 17, 21). Furthermore, mice vaccinated with DNA vaccines encoding CRT linked to a target antigen have demonstrated significant protective antitumor effects (8, 17, 20, 21, 28–30). Thus, we have chosen CRT as an immune adjuvant and have engineered a DNA vaccine encoding CRT linked to a model antigen, PA(dIV), in order to elicit antigen-specific immune responses against anthrax.

**MATERIALS AND METHODS**

**Plasmid DNA constructs and DNA preparation.** In the current study we used the mammalian expression vector pcDNA3.1 (−) (Invitrogen, Carlsbad, CA) for our DNA vaccine studies. For the generation of pcDNA3-PA(dIV), the DNA fragment encoding PA(dIV) was amplified with PCR using primers 5′-CCGAAA TTCTATGGAAATCTGAAACTAAC-3′ and 5′-GGGCTCAGTATTCTC ATTCCTAATGCTTT-3′ and pET-wt-PA (1) as a template, which was a gift from John Collier (Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA). The amplified product was further cloned into the EcoRI/HindIII sites of the pcDNA3.1(−) vector. For the generation of pcDNA3-CRT/PA(dIV), the amplified PA(dIV) was cloned into the EcoRI/KpnI sites of pcDNA3-CRT (8). The accuracy of these constructs was confirmed by DNA sequencing. Plasmid DNA with a CRT/PA(dIV) or PA(dIV) gene insert and the “empty” plasmid vector were transfected into subcloning-efficient DH5α (Life Technologies, Rockville, MD) according to the vendor’s manual. At 24 h after transfection, cells were lysed with protein extraction reagent (Pierce, Rockford, IL). The DNA concentration was determined by absorbance measured at 260 nm. The presence of the inserted Pa(dIV) fragment was confirmed by restriction enzyme digestion and gel electrophoresis.

**Western blot analysis.** The expression of PA(dIV) and CRT/PA(dIV) in HEK 293 cells (a human embryonic kidney cell line) transfected with the plasmids encoding no insert, CRT, PA(dIV), or CRT/PA(dIV) was characterized by Western blot analysis. The cells were grown in Dulbecco modified Eagle medium supplemented with 10% vol/vol fetal bovine serum, 50 units/ml penicillin-streptomycin, 2 mm l-glutamine, 1 mM sodium pyruvate, and 2 mM nonessential amino acids at 37°C in a 5% CO2-air incubator. Twenty micrograms of each plasmid was transfected into 5 × 106 HEK 293 cells using Lipofectamine 2000 (Life Technologies, Rockville, MD) according to the vendor’s manual. At 24 h after transfection, cells were lysed with protein extraction reagent (Pierce, Rockford, IL). Equal amounts of proteins (50 μg) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel. The gels were electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Blots were blocked with phosphate-buffered saline (PBS)-0.05% Tween 20 (PBST) containing 5% nonfat milk for 2 h at room temperature. Membranes were probed with mouse anti-PA(dIV) sera, generated by immunization with a mixture of a recombinant PA (List Biological Laboratories, West Grove, PA) and Freund’s complete adjuvant (Sigma), at a 1:1,000 dilution in PBST for 2 h, washed four times with PBST, and then incubated with rabbit anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Zymed, San Francisco, CA) at a 1:1,000 dilution in PBST containing 5% nonfat milk. Membranes were washed four times with PBST and developed using enhanced Hyperfilm-enhanced chemiluminescence (Amersham, Piscataway, NJ).

**DNA vaccination.** DNA-coated gold particles were prepared according to a previously described protocol (7). DNA-coated gold particles were delivered to the shaved abdominal regions of mice using a helium-driven gene gun (Bio-Rad, Hercules, CA) with a discharge pressure of 400 lb/in2. C57BL/6 or BALB/c mice were immunized with 2 μg of the plasmid encoding no insert or CRT, PA(dIV), or CRT/PA(dIV) protein. The mice received two boosters with the same dose at a 1-week interval.

**ELISA.** The presence of PA-specific antibodies in the sera from DNA-vaccinated mice was determined by enzyme-linked immunosorbent assay (ELISA) using microwell plates coated with bacterially derived recombinant PA protein (List Biological Laboratory, Inc.). PA protein was diluted to 1 μg/ml with 0.05 M carbonate buffer (pH 9.6), and 0.1 ml/well was added to 96-well microtiter plates. The plates were incubated overnight at 4°C, washed with PBST, incubated with biotinylated mouse serum albumin (0.1 ml/well) for 60 min at 37°C, and washed again with PBST. Serial dilutions of the tested sera were added (0.1 ml/well), and the plates were incubated for 60 min at 37°C. The plates were washed with PBST and incubated with peroxidase-conjugated rabbit anti-mouse IgG (0.1 ml/well) (Zymed, San Francisco, CA) for 30 min at 37°C. For subtyping the PA-specific antibody, rabbit anti-mouse IgG1 and IgG2a antibodies conjugated with peroxidase were used. The plates were washed with PBST and incubated with peroxidase-conjugated rabbit anti-mouse IgG (0.1 ml/well) (Zymed, San Francisco, CA) for 30 min at 37°C. The plates were read on a MicroELISA reader at a wavelength of 450 nm. Readings higher than threefold of the negative control value were scored as positive reactions.

**Avidity of the anti-PA IgG antibody.** Serum samples were obtained from mice immunized with DNA encoding PA(dIV) or CRT/PA(dIV) 21 days after immunization and diluted to 1:100 in PBS. Different concentrations of the chaotropic agent sodium thiocyanate (NaSCN) in PBS were added to final concentrations of 0.5, 0.5, 1, 1.5, 2, 2.5, and 3.0 μg/ml. Plates were incubated for 30 min at 37°C and then washed six times with PBST–2% bovine serum albumin. Subsequent steps were performed as described for the ELISA.

**Immunofluorescence staining.** Immunofluorescence staining was performed using a protocol similar to that described previously (10). Briefly, 1 × 106 HEK 293 cells were cultured in 60-mm culture chamber slides (Nalge Nunc International, Naperville, IL) until they reached 50% confluence. The cells were transfected with DNA encoding no insert, CRT, PA(dIV), or CRT/PA(dIV). After 24 h of infection, cells were fixed and permeabilized with Cytofix/Cytoperm (Pharmingen) for 30 min. Mouse polyclonal anti-CRT/PA(dIV) serum was added into the chamber at a dilution of 1:100 and incubated for 30 min. Diluted fluorescein isothiocyanate–goat anti-mouse IgG (4 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) was added and incubated for 30 min. To confirm that the CRT/PA(dIV) chimera digested to the ER, we performed immunofluorescent staining of the transfected cells using rabbit polyclonal anticalcinexin (Stressgen), a well-characterized marker for the ER. Diluted Cy3–goat anti-rabbit IgG (10 μg/ml; Jackson ImmunoResearch Laboratories) was added and incubated for 30 min to detect the bound calcinein-IgG. The stained cells were examined under a confocal laser scanning microscope using a protocol described previously (8).

**Secretion of CRT/PA(dIV).** Culture supernatants of the transfected HEK 293 cells with DNA encoding no insert, CRT, PA(dIV), or CRT/PA(dIV) were prepared by removing serum-containing Dulbecco modified Eagle medium from transfected cells 4 h after transfection and incubating cells with 5 ml of serum-free Opti-MEM for 48 h. Four-milliliter samples of culture supernatants were collected, centrifuged to remove any cellular debris for 15 min at 10,000 × g, and concentrated to 0.2 ml using Amicon ultracentrifugal filter devices. The presence of PA(dIV) and CRT/PA(dIV) in the concentrated supernatants (5, 10, and 20 μl) was characterized by Western blot analysis.

**In vivo toxin challenge.** Naive or immunized mice (five per group) were challenged by tail vein injection of a mixture of PA (12.5, 25, 50, or 100 μg)
between individual data points were made using a Student t test. Challenge experiments were evaluated by analysis of variance. Comparisons between individual data points were made using a Student t test.

**Statistical analysis.** All data were expressed as means ± standard deviations (SD) and are representative of at least two different experiments. Data for intracellular cytokine staining with flow cytometry analysis and in vivo viral challenge experiments were evaluated by analysis of variance. Comparisons between individual data points were made using a Student t test.

**RESULTS**

Linkage of CRT to PA(dIV) targets PA(dIV) to the ER, resulting in secretion of the chimeric CRT/PA(dIV) protein. PA consists of four domains. PA(dI) is responsible for binding to EF and LF, PA(dII) and PA(dIII) are responsible for translocation of EF and LF, and PA(dIV) is responsible for binding to the anthrax toxin receptor (for a review, see reference 37). Figure 1 shows a schematic diagram of the different domains of PA. In order to characterize the intracellular localization and expression of PA(dIV) and chimeric CRT/PA(dIV), we transfected HEK 293 cells with various DNA constructs, including no insert, CRT, PA(dIV), or CRT/PA(dIV). Three days after transfection, we performed confocal fluorescence microscopy and Western blotting using the media of the various transfectants. As shown in Fig. 2A, we observed the colocalization of CRT/PA(dIV) with the ER resident protein calnexin. This indicates that the linkage of CRT to PA(dIV) targets PA(dIV) to the ER. We also characterized the secretion of CRT/PA(dIV) by Western blotting using the media of the various transfectants. We observed that the linkage of CRT to PA(dIV) led to the secretion of the chimeric CRT/PA(dIV) protein (Fig. 2B). In comparison, the cells transfected with DNA encoding PA(dIV) alone did not lead to the secretion of the PA(dIV) protein, as no bands were visible (data not shown). Taken together, these results indicate that the linkage of CRT to PA(dIV) targets PA(dIV) to the ER, resulting in secretion of the chimeric CRT/PA(dIV) protein.

Vaccination with CRT/PA(dIV) DNA rapidly induces significantly higher levels of PA(dIV)-specific antibody responses than vaccination with PA(dIV) DNA alone. In order to evaluate the PA-specific antibody responses generated by vaccination with the various DNA constructs, BALB/c and C57BL/6 mice (five per group) were vaccinated intradermally via gene gun with plasmid DNAs encoding no insert, CRT, PA(dIV), or CRT/PA(dIV). The mice received two boosters with the same dose at a 1-week interval. Sera were collected from the retroorbital plexuses in the vaccinated mice 1 week after the last vaccination under anesthetization, and the titer of PA-specific antibody in sera was assessed using ELISA as described in Materials and Methods. As shown in Fig. 3A, mice that were immunized with CRT/PA(dIV) DNA had significantly higher titers of anti-PA IgG than mice immunized with PA(dIV) DNA alone. In contrast, mice immunized with no insert and CRT plasmid did not show significant antibody responses against PA. This indicates that vaccination with CRT/PA(dIV) induces significantly higher levels of PA(dIV)-specific antibody responses than vaccination with PA(dIV) DNA alone. Furthermore, comparable levels of antibody responses were observed in BALB/c mice (data not shown).

In order to determine the isotypes of the IgG antibody responses generated by vaccination with the various DNA constructs, levels of IgG isotypes (IgG1 and IgG2a) were determined using ELISA. As shown in Fig. 3B, we found that the titers of PA-specific IgG1 were higher than those of PA-specific IgG2a in C57BL/6 mice vaccinated with either PA(dIV) or CRT/PA(dIV) DNA. These data suggest that vaccination with PA(dIV) or CRT/PA(dIV) DNA induces PA(dIV)-specific antibody responses more consistent with the Th2 type of responses. Comparable levels of antibody responses were observed in BALB/c mice (data not shown).

In order to investigate the kinetics of the PA-specific antibody responses following vaccination with the various DNA constructs, we carried out an ELISA using serum samples obtained from immunized mice at various time points after immunization. As shown in Fig. 3C and D, we demonstrated that mice immunized with CRT/PA(dIV) DNA rapidly induced PA-specific antibody responses and had significantly higher titers at 10 days after the initial vaccination than mice immunized with PA(dIV) alone. This was observed in both BALB/c and C57BL/6 mice. These data indicate that vaccination with CRT/PA(dIV) DNA rapidly induces significantly higher levels of PA(dIV)-specific antibody responses than vaccination with PA(dIV) DNA alone.

Enhanced antibody responses generated by vaccination with CRT/PA(dIV) DNA are CD4+ T-cell dependent. In order to determine whether CD4+ T cells are important for the observed enhancement of PA-specific antibody responses gener-
ated by vaccination with CRT/PA(dIV) DNA in C57BL/6 mice, we measured the titers of PA-specific IgG and IgM antibodies in C57BL/6 CD4KO mice vaccinated with various DNAs, including those encoding no insert, CRT, PA(dIV), or CRT/PA(dIV). As shown in Fig. 4A, we observed that the titers of PA-specific IgG antibody responses were significantly reduced in CD4KO mice vaccinated with CRT/PA(dIV) or PA(dIV) compared to vaccinated wild-type C57BL/6 mice.

FIG. 2. Intracellular localization and secretion of PA(dIV) and CRT/PA(dIV) after in vitro transfection. HEK 293 cells were transfected with various DNA constructs, including no insert, CRT, PA(dIV), or CRT/PA(dIV). (A) Confocal fluorescence microscopy to demonstrate the expression and distribution of PA(dIV) and chimeric CRT/PA(dIV) proteins. Immunofluorescent staining was performed as described in Materials and Methods, and colocalization of PA(dIV) or its chimeric protein, CRT/PA(dIV), with calnexin was demonstrated by the yellow color in the merge image. (B) Western blot analysis demonstrating the secretion of the CRT/PA(dIV) protein. Four-milliliter samples of culture supernatants were collected, centrifuged to remove any cellular debris for 15 min at 10,000 × g, and concentrated to 0.2 ml using Amicon ultracentrifugal filter devices. Different volumes (5, 10, and 20 µl) of concentrated supernatants were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel before blotting. The presence of PA(dIV) protein was detected by probing with mouse anti-PA(dIV) sera.
However, the levels of PA-specific IgG antibody responses in the CD4KO mice vaccinated with CRT/PA(dIV) were still significantly higher than those in the control groups (mice vaccinated with CRT alone or no insert). Taken together, these data indicate that CD4^+^ T cells are important for the antibody responses generated by vaccination with either CRT/PA(dIV) or PA(dIV) DNA but that there are non-CD4-dependent mechanisms that also contribute to the observed PA-specific antibody responses. Similarly, the titers of PA-specific IgM antibody responses were also significantly reduced in CD4KO mice vaccinated with CRT/PA(dIV) compared to vaccinated wild-type C57BL/6 mice. However, the titers of PA-specific IgM antibody responses were similar in CD4KO mice and wild-type mice vaccinated with PA(dIV). Thus, our results indicate that CD4^+^ T cells are also important for the observed enhanced PA-specific IgG and IgM immune responses generated by vaccination with CRT/PA(dIV) DNA.

**Vaccination with CRT/PA(dIV) DNA results in enhanced avidity maturation of anti-PA antibody compared to vaccination with PA(dIV) DNA.** In order to compare the avidity of the antibodies generated by vaccination with CRT/PA(dIV) DNA and PA(dIV) DNA, we performed NaSCN displacement
ELISAs. The avidities of the specific antibodies to PA(dIV) were compared by using graded concentrations of NaSCN, a chaotropic agent, which disrupts antigen-antibody interaction (31). The binding of antibodies with less avidity to the antigen is disrupted at lower concentrations of NaSCN compared to antibodies with greater avidity to the antigen. As shown in Fig. 5A and B, the avidity of the antibody generated by vaccination with CRT/PA(dIV) DNA was significantly higher than that of the antibody generated by vaccination with PA(dIV) DNA alone. The effective concentrations of NaSCN required to release 50% of antiserum (optical density at 450 nm) collected at 3 weeks after vaccination with CRT/PA(dIV) or PA(dIV) were \( \approx 1.0 \text{ M} \) and \( \approx 0.5 \text{ M} \), respectively, in BALB/c mice and \( \approx 1.1 \text{ M} \) and \( \approx 0.4 \text{ M} \), respectively, in C57BL/6 mice. These results indicate that the antibody from CRT/PA(dIV) DNA-vaccinated mice had undergone more rapid avidity maturation than the antibody from PA(dIV) DNA-vaccinated mice in both BALB/c and C57BL/6 mice.

Anthrax toxin-challenged mice immunized with CRT/PA(dIV) show prolonged survival. In order to determine whether mice challenged with anthrax toxin were protected against anthrax toxin challenge by immunization with CRT/PA(dIV), we performed an in vivo protection experiment. The optimized conditions were set up to determine LD50. It was observed that the LD50s were different for the BALB/c mice and the C57BL/6 mice. We speculate that this difference may be due to the different genetic backgrounds. The dose for anthrax toxin challenge was determined. BALB/c or C57BL/6 mice (five per group) were immunized intradermally via a gene gun with the various DNA constructs, including DNA encoding no insert, CRT, PA(dIV), or CRT/PA(dIV). The mice received two boosters with the same dose at a 1-week interval. One week after the last immunization, BALB/c mice were challenged by tail vein injection with the mixture of PA at 50 \( \mu \text{g/mouse} \) and LF at 25 \( \mu \text{g/mouse} \), and C57BL/6 mice were challenged by tail vein injection with the mixture of PA at 100 \( \mu \text{g/mouse} \) and LF at 50 \( \mu \text{g/mouse} \). As shown in Fig. 6, prolonged survival was observed in both BALB/c and C57BL/6 mice vaccinated with CRT/PA(dIV) DNA compared to mice vaccinated with PA(dIV) DNA, CRT DNA, or no-insert DNA.
These data suggest that vaccination with CRT/(dIV) DNA is capable of generating protection against lethal challenge with anthrax toxin compared to vaccination with PA(dIV) DNA and other control DNA vaccines.

**DISCUSSION**

In the current study, we have demonstrated that linkage of domain IV of PA to CRT can significantly increase the potency of a PA-expressing DNA vaccine. Vaccination with CRT/PA(dIV) DNA generated a potent PA-specific humoral immune responses and significant protective effects against anthrax toxin challenge compared to vaccination with PA(dIV) DNA in vaccinated mice. Furthermore, vaccination with CRT/PA(dIV) rapidly induced the production of PA-specific antibodies and generated enhanced avidity maturation of PA-specific antibodies compared to vaccination with PA(dIV) DNA. These data are consistent with our previous observations. Previously, we have shown that antigen-specific immune responses can be significantly enhanced by linkage of the target antigen to CRT in the context of DNA vaccines using several antigenic systems, such as human papillomavirus (HPV) E6 or E7 antigen and severe acute respiratory syndrome coronavirus nucleocapsid (N) protein (8, 9, 17, 21). Therefore, DNA vaccines encoding CRT may potentially be employed in several antigenic systems and may serve as a platform for DNA vaccine development.

In the current study, we targeted domain IV of the PA protein because the PA(dIV) region is involved in binding to the cell receptor. Previous studies using monoclonal antibodies targeting the PA(dIV) region showed blocking the interaction of PA with the cell receptor, leading to protection from toxin challenge (6, 24). However, several reports have shown that antibodies against other regions of the PA protein that are capable of protecting the host against anthrax may not be directly involved in the blocking of the interaction of PA with the cell receptor (6, 32). These and other similar monoclonal antibodies have shown success in protection of the host against anthrax.

In the current study, we observed that the linkage of CRT to PA(dIV) targeted PA(dIV) to the ER, resulting in secretion of the chimeric CRT/PA(dIV) protein (Fig. 2). The released chimeric CRT/PA(dIV) protein can directly serve as a source of antigen to trigger a PA-specific humoral immune response. Furthermore, in our study we found that depletion of CD4+ T cells led to a significant reduction in the PA-specific antibody responses, indicating that CD4+ T cells are important for the generation of PA-specific antibody responses (Fig. 4). The characterization of the immunoglobulin isotypes generated by vaccination with CRT/PA(dIV) also showed that IgG1 is the major isotype of the humoral immune responses, implying the induction of Th2 immune responses (Fig. 3B). In general, Th2 immune responses are involved in the induction of humoral immunity. Taken together, these data suggest that the chimeric CRT/PA(dIV) protein leads to the activation of CD4+ T-cell-mediated humoral immune responses. Thus, our study indicates that PA-specific humoral immune responses generated by vaccination with CRT/PA(dIV) are contributed mainly by CD4-dependent mechanisms, although CD4-independent mechanisms also contribute to the observed immune responses.

It is important to identify the most potent vaccination strategy against anthrax toxin for eventual clinical translation. In our previous studies using different antigenic systems, we have observed that the potency of DNA vaccines can be maximally enhanced by combining various strategies, such as intracellular targeting, prolonging dendritic cell life, and induction of CD4 T help. For example, we have observed that the potency of HPV type 16 E7 DNA vaccines employing intracellular targeting strategies, such as CRT with a strategy to prolong the life of dendritic cells.
(Bel-1L DNA), can be further enhanced by coadministration of DNA vaccines with DNA encoding the invariant chain linked to the pan HLA-DR epitope (Il-PADRE), which leads to the induction of CD4+ T-cell help (19). Thus, it is conceivable that the future clinical translation of DNA vaccines would require a combination of strategies that are capable of enhancing DNA vaccines through different mechanisms in order to generate the most potent immune responses.

The current study has employed intradermal administration of antigen using a gene gun device. For this approach to be useful in the clinical arena, it would require a gene gun that is suitable for clinical usage. A proprietary clinical grade ND-10 gene gun device has recently been developed by PowderMed/Pfizer and will soon be employed in clinical trials of a DNA vaccine targeting HPV type 16 E7 antigen in patients with stage IB1 cervical cancer (Ronald Alvarez and Cornelia Trimble, personal communication). The same device can also be used in the formulation of the current DNA vaccine targeting the PA antigen of anthrax toxin for clinical trials. Thus, the current approach has great potential for future clinical translation.

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