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# A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague

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## Abstract

There is an urgent need to develop effective vaccines against pneumonic plague, a highly lethal and contagious disease caused by the Gram-negative bacterium *Yersinia pestis*. Here we demonstrate that a novel DNA vaccine expressing a modified V antigen (LcrV) of *Y. pestis*, with a human tissue plasminogen activator (tPA) signal sequence, elicited strong V-specific antibody responses in BALB/c mice. This tPA-V DNA vaccine protected mice from intranasal challenge with lethal doses of *Y. pestis*. In comparison, a DNA vaccine expressing the wild type V antigen was much less effective. Only tPA-V formed oligomers spontaneously, and elicited a higher IgG2a anti-V antibody response in immunized mice, suggesting increased T<sub>H</sub>1 type cellular immune response. Our data indicate that antigen engineering is effective in inducing high quality protective immune responses against conformationally sensitive antigens. These results support that optimized DNA vaccines have the potential to protect against bacterial pathogens than is generally recognized.

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**Keywords:** *Yersinia pestis*; LcrV antigen; DNA vaccine; tPA leader

## 1. Introduction

The Gram-negative bacterium *Yersinia pestis* (*Y. pestis*), the causative agent of plague, is among the agents of greatest concern with respect to illegitimate use in bioterrorism or biological warfare and is currently the target of several vaccine development efforts. In nature, *Y. pestis* is endemic in rodent populations in many parts of the world. It is transmitted primarily via the bite of infected fleas, causing bubonic plague, a disease characterized by grossly swollen lymph nodes (buboes). While *Y. pestis*-based weapons used during the World War II employed flea-based transmission, direct infection of humans via aerosols is thought to be the most likely and most dangerous mode of delivery which will produce the pneumonic plague. In contrast to bubonic plague, pneumonic plague progresses more rapidly, is highly transmissible via droplets to mucosal surface, and requires extremely prompt therapeutic intervention. Unless appropriate antimicrobial therapy is instituted with 24 h of the onset of

symptoms, this disease is uniformly fatal. These features of pneumonic plague, coupled with the potential use of antibiotic resistant *Y. pestis*, argue strongly for the development of vaccines effective in preventing mucosal transmission of *Y. pestis* as a tool in biodefense.

Currently there is no known effective clinical plague vaccine against pneumonic plague. There is a killed whole cell (KWC) plague vaccine licensed in certain parts of the world [1], but other discontinued KWC plague vaccine was shown not effective in protecting against pneumonic plague in small animal studies [2,3]. Certain recombinant protein vaccines in advanced stages of development have been demonstrated to provide various levels of protection against pneumonic plague in animal models. These vaccines utilize one or both of two known protective antigens, Fraction 1 (F1) and LcrV (V). The F1 protein forms a capsule [4] and is believed to confer resistance to phagocytosis, possibly by forming aqueous pores in the membranes of phagocytic cells [5] or by interfering with complement-mediated opsonization [6]. F1 has been shown to be an effective vaccine component [7–9]. Since mutant *Y. pestis* strains unable to produce F1 antigen remain highly virulent, reliance on F1 as a protective vac-

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cine antigen may be unwise. Identification of new protective antigens is needed to make improved multi-component plague vaccines.

LcrV is an intracellular protein, which is secreted from the *Y. pestis* under appropriate conditions and has been observed on the bacterial surface [10]. It is believed to participate in the type III secretion system by facilitating the translocation of many Yersinia outer proteins (Yop's) into the host cell to achieve anti-host properties [11,12]. It was shown to be a potent immunogen because the recombinant V protein as a sub-unit vaccine was able to induce protective IgG in the mouse model either on its own or in combination with the recombinant F1 protein [8,13].

There are active searches for new protective antigens against *Y. pestis*. For instance, the *Y. pestis* protein, Pla protease, plays an important role in determining the invasive nature of *Y. pestis* [14]. When delivered subcutaneously, various Pla mutants produce a sustained local infection without causing systemic disease. They remain highly virulent if given intravenously. Pla is an integral outer membrane protein with its active site exposed on the cell surface, and thus, *a priori*, has potential as an attractive vaccine component.

DNA vaccine technology offers particular utility in searching for safe and effective vaccine against emerging infectious disease and bioterrorism, when timely development of vaccines to new or genetically modified pathogens is of great value. The necessary plasmid constructs can be quickly produced and no purification of protein is required since the antigens encoded by the DNA vaccines are expressed *in vivo*. This allows rapid screening and optimization of many alternative antigens based on immunogenicity and protection data collected from animal studies. Significant improvements have been made in recent years in DNA vaccine technology and several DNA-based vaccines have entered into early phase clinical trials [15–19]. However, major effort in DNA vaccine studies so far has been directed against pathogenic viruses. Like actual viral infections, the proteins encoded by DNA vaccines are expressed within host cells and hence are available for presentation via the Class I Major Histocompatibility Complex (MHC) pathway to elicit viral antigen-specific cytotoxic T lymphocyte (CTL) responses. The development of DNA vaccines against bacterial pathogens has been explored less thoroughly, perhaps because protective immunity to most bacterial infections is thought to be heavily dependent on humoral immune responses. Although the ability of DNA vaccines to generate specific antibody is well documented, the potential of DNA vaccines to induce protective levels of antibody responses against acute bacterial infections has just begun to be recognized [20].

Previous attempts to develop a DNA vaccine based on *Y. pestis* V antigen met with limited success, eliciting only low level V-specific antibody titers [21] and providing poor protection against challenge with *Y. pestis* [22]. Given that protein-based LcrV formulations can provide good protection, this failure has cast doubt on the role of DNA-based

approaches. Our previous DNA vaccine studies against other infectious agents have demonstrated that a proper leader sequence was important in inducing high level antibody responses by increasing soluble antigen production in mammalian cell systems [23–26]. In particular, we have described a molecular approach to enhance the secretion and thus the immunogenicity of the HIV-1 gp120 antigen by linking the human tissue plasminogen activator (tPA) leader sequence to the N terminus of the gp120 protein [23,24,27]. Since LcrV gene does not encode a typical signal peptide, the focus of this study was to determine if the immunogenicity of an LcrV-based DNA vaccine could be improved when engineered to include the signal sequence from tPA. We report here that this vaccine design was able to elicit high level anti-V antibody responses protecting mice from lethal doses of *Y. pestis* in an intranasal challenge model. Interestingly, this tPA-V antigen had a strong tendency to form oligomers as examined by the *in vitro* expression system. Data presented in this study confirmed the importance to include LcrV component in a multigene plague DNA formulation. Our study further demonstrated that the F1 DNA vaccine provided much lower protection and the Pla DNA vaccine was not protective in its current designs.

## 2. Materials and methods

### 2.1. Bacteria

*Y. pestis* strain KIM 1001 [14] was prepared by growing inocula for 18 h at 37 °C on Tryptose Blood Agar Base (Difco) supplemented with 2.5 mM CaCl<sub>2</sub> but without the addition of blood. Bacteria were removed from the plate with an inoculating loop and resuspended in injection-grade PBS. The bacteria count in the suspension was correlated to its optical density (OD<sub>600</sub>). The number of bacteria in the final inocula was confirmed by colony counts.

### 2.2. Construction of plague DNA vaccines

V, F1 and Pla genes were amplified with pfu polymerase (Stratagene, CA) from the three virulence plasmids of *Y. pestis* KIM5: pCD1, pMT1 and pPCP1 respectively [28] using the following primer pairs. For V gene: V-1 (5'-GTCGCTCCAAGCTTGCTAGCATGATTAGAGCCTACGAACAAAACCC-3') and V-2 (5'-AGTCACGGATCCTCATTTACCAGACGTGTCATCTAGC-3'), for F1 gene: F1-1 (5'-GTCGCTCCAAGCTTGCTAGCATGAAAAAATCATTTCCGTTATCGCC-3') and F1-2 (5'-AGTCACGGATCCTTATTGGTTAGATACGGTTACGG-3'), for Pla gene: Pla-1 (5'-GTCGCTCCAAGCTTGCTAGCATGAAGAAAAGTTCTATTGTGGC-3') and Pla-2 (5'-GAGCAGGCCTCAGAGCGATATTGCAGACCCGC-3'). For the wild type inserts, the PCR amplified plague DNA sequences were directly subcloned into the DNA vaccine vector pJW4303 immediately after the CMV promoter [27]. For the inserts

with an additional tPA leader sequence, the PCR amplified *Y. pestis* gene sequences were subcloned into the same pJW4303 vector but downstream of the tPA leader sequence which was already included in the vector [27]. The leader sequence is in frame with such subcloned plague gene inserts. The final plague DNA vaccines were prepared by using the Mega purification kit made by Qiagen (Valencia, CA) prior to animal immunization.

### 2.3. DNA immunization of BALB/c mice

Six to eight weeks old female BALB/c mice were purchased from Taconic Farms (Germantown, NY) and housed in the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS) in accordance with IACUC approved protocols. The animals were immunized with a Helios gene gun (Bio-Rad) at the shaved abdominal skin as previously reported [29]. Each mouse first received three monthly immunizations with six DNA shots of 1 µg each per immunization. The blood samples were collected peri-orbitally prior to the first immunization and 4 weeks after each immunization for analyses of plague-specific antibody responses. A fourth DNA immunization with the same dose was given at 2 weeks prior to the challenge (see Results section).

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

*Y. pestis* antigen-specific IgG responses in immunized mice were measured by ELISA using individual or pooled mouse sera from each animal group. ELISA plates were coated with 100 µl of the antigens at 1 µg/ml harvested from 293T cells transiently transfected with the plague DNA vaccine plasmids and incubated overnight at 4 °C. Serially diluted mouse sera (100 µl) were added to each well and assayed in duplicate after the blocking. The plates were incubated with biotinylated anti-mouse IgG diluted at 1:1000 (100 µl per well), followed by horseradish peroxidase-conjugated streptavidin diluted at 1:2000 and finally developed with 3,3',5,5'-tetramethylbenzidine solution (100 µl per well). The reactions were stopped by adding 25 µl of 2 M H<sub>2</sub>SO<sub>4</sub>, and the plates were read at OD<sub>450</sub>. IgG1 or IgG2 isotype-specific ELISA was conducted as described above, except using horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG1 or IgG2 (Southern Technology Associates, AL) at 1:2000 dilution. The concentrations for V-specific mouse IgG1 or IgG2a were calculated from the standard curve using known amount of purified mouse IgG.

### 2.5. Intranasal challenges of immunized mice

Mice were challenged two weeks after the fourth immunization and observed twice daily for morbidity and mortality. An intranasal instillation of 50 µl saline solution con-

taining lethal doses of *Y. pestis* (KIM strain) was administered into the nostril of ketamine-anesthetized mice. This method leads to rapid infections and is lethal to 100% of non-immunized mice in 3–4 days. The LD<sub>50</sub> of this challenge model was determined to be at about 333 cfu by a pilot challenge study. Therefore the challenge dose in current study ranged from ~15 LD<sub>50</sub> (5000 cfu) to ~240 LD<sub>50</sub> (80,000 cfu). The challenge studies were conducted in a Biosafety Level 3 containment facility at the Department of Animal Medicine, UMMS. The statistical analyses were performed for animal survival using Fisher's exact test. Significance was considered when *P*-values were <0.05.

### 2.6. Western blot analysis of *in vitro* expressed V antigens

The 293T cells were transiently transfected by a calcium phosphate co-precipitation method using 10 µg of plasmid DNA for 2 × 10<sup>6</sup> cells in a 60-mm dish, and were harvested 72 h later. Same amount of transiently expressed V antigens (10 ng of protein) were loaded for the SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto PVDF membranes (Bio-Rad, Hercules, CA), and blocked overnight at 4 °C in blocking buffer (0.2% I-block, 0.1% Tween-20 in 1X PBS). Membranes were incubated with a 1:200 dilution of mouse sera immunized with corresponding DNA vaccines. After being washed, blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG at 1:5000 dilution, and signals were detected using a chemiluminescence Western-Light Kit (Tropix, Bedford, MA, USA). Some of the tPA-V samples were prepared in the presence of 4M urea. For the glycosylation study, Peptide *N*-Glycosidase F, PNGaseF (New England BioLab, Beverly, MA, USA) was added to the denatured samples prepared from the supernatants of transiently transfected 293T cells according to manufacturer's instruction. After incubating overnight at 37 °C, samples were loaded onto SDS-PAGE, and analyzed by Western blot as described above.

## 3. Results

### 3.1. Construction of DNA vaccines expressing *Y. pestis* antigens

Two versions of DNA vaccines were designed for each of the three *Y. pestis* antigens, LcrV, F1 and Pla (Fig. 1). One encoded the wild type (wt) protein, while the other encoded a modified version with an added tissue plasminogen activator (tPA) leader sequence. LcrV and Pla do not have a definitive leader sequences but Pla has a hydrophobic region near the N-terminus (aa 6–14) which was left unchanged in the current designs. The F1 gene includes a natural leader sequence (aa 1–22) which was also unchanged to keep the two F1 gene designs compatible with those for V and Pla. *Y. pestis* gene inserts were incorporated individually into the

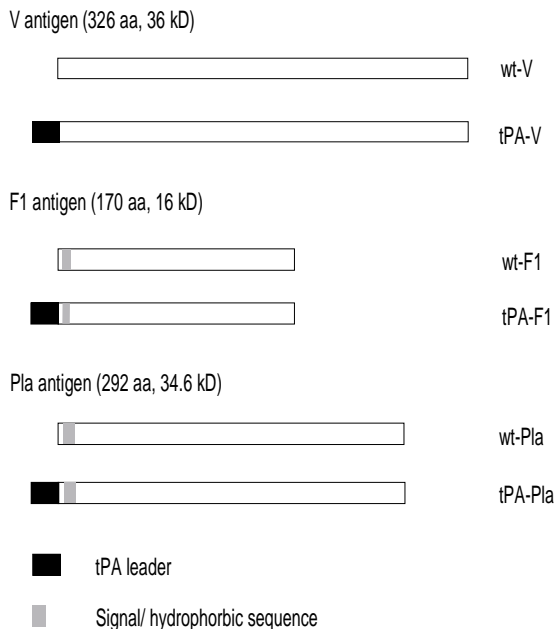


Fig. 1. Schematic diagram of the *Y. pestis* antigen inserts used in the DNA vaccine constructs. The wild-type genes (wt) and the genes with an additional tissue plasminogen activator (tPA) leader sequence were individually cloned into the DNA vector pJW4303. The natural leader sequence or the hydrophobic regions at the N-terminal region of each gene is marked.

DNA vaccine vector pJW4303 which has been widely used in a very broad range of DNA vaccine studies [23–26]. It contains the CMV immediate early promoter, an Intron A sequence, and the bovine growth hormone polyA tail as previously reported [27].

### 3.2. High level anti-plague antibody responses in mice immunized with V or F1 DNA vaccines

*Y. pestis* antigen-specific antibody responses in immunized mice were measured four weeks after the third monthly DNA vaccination. Both LcrV and F1 DNA vaccines induced high titer IgG antibody responses against their respective antigens as detected by ELISA (Fig. 2a). LcrV DNA vaccines induced about one log higher antibody responses than the F1 DNA vaccines. The LcrV and F1 DNA vaccines with the tPA leader sequence gave higher antibody responses than their wild type counterparts, but the improvement was only a few fold (Fig. 2a). The rises of anti-V antibody responses with time were further analyzed (Fig. 2b). The tPA-V DNA vaccine was able to induce a sharp increase in antibody response following a single immunization while the wt-V DNA vaccine required at least two immunizations to produce a response of the similar level (Fig. 2b). No significant anti-Pla IgG antibody responses were detected from animals immunized with either wt-Pla or tPA-Pla DNA vaccines (Fig. 2a). The poor immunogenicity of Pla DNA vaccines may be the result of low level expression of Pla. It

was barely detected when 293T cells were transiently transfected with either of the Pla constructs and tested by ELISA or Western blot using known positive rabbit anti-Pla sera (data not shown).

### 3.3. Protection of immunized mice against lethal *Y. pestis* challenges by intranasal route

The DNA vaccinated mice as described above received the fourth DNA immunization at Week 32 and were challenged 2 weeks later. The long delay between the third and the fourth immunizations was due to the unavailability of BL-3 animal room for plague challenge. Each animal was inoculated with a lethal challenge of the Kim strain of *Y. pestis* at 5000 cfu (~15 LD<sub>50</sub>) delivered by the intranasal route. Animals that received vector control DNA or Pla DNA plasmids quickly developed easily observable signs of sickness, such as rough coats, hunched or huddled posture, shivering, labored breathing and lethargy, within the first 24–36 h post-challenge and most of them died within three to four days (Fig. 3). Three DNA vaccines, wt-F1, tPA-F1 and wt-V, provided partial protection with 50–75% animal surviving after 2 weeks' observation (Fig. 3). Animals survived in these partially protected groups usually had only transient and minor signs of sickness, with decreased grooming which lasted for 3–5 days. On the other hand, the tPA-V DNA vaccine provided 100% protection in this initial study (Fig. 3). Most significantly, animals in this group showed no sign of sickness and remained active during the entire two weeks.

The superior protection provided by the tPA-V DNA vaccine was further confirmed in a subsequent study by using dose escalating challenges with increased size of animal groups. Two groups of 30 mice each received three monthly immunizations of either the wt-V or the tPA-V DNA vaccines following the same immunization procedure as described in Materials and Methods. After confirming the positive anti-V antibody responses by ELISA in these DNA immunized animals (data not shown), the fourth DNA immunization is given at week 16 and the animals were challenged two weeks later. Three intranasal challenge doses were given to both vaccine groups. As shown in Fig. 4, survival was substantially better for the tPA-V vaccinated mice at all three challenge dose levels, 90%, 80% and 70% for challenge doses of 5000 cfu (15 LD<sub>50</sub>), 20,000 cfu (60 LD<sub>50</sub>), and 80,000 cfu (240 LD<sub>50</sub>) respectively, than the animals received the wt-V DNA vaccines (20% survival for all three challenge doses). Table 1 summarized the results for both challenge studies. The differences in survival between tPA-V and wt-V were statistically significant ( $P = 0.0055$  and  $P = 0.023$  for the first two challenge doses (5000 cfu and 20,000 cfu), based on the Fisher's exact test). The third challenge does (80,000 cfu) was less significant ( $P = 0.0698$ ) due to the relatively small sampling sizes. Combining the data from all three challenge doses, the survival rate for tPA-V immunized animals was 80%



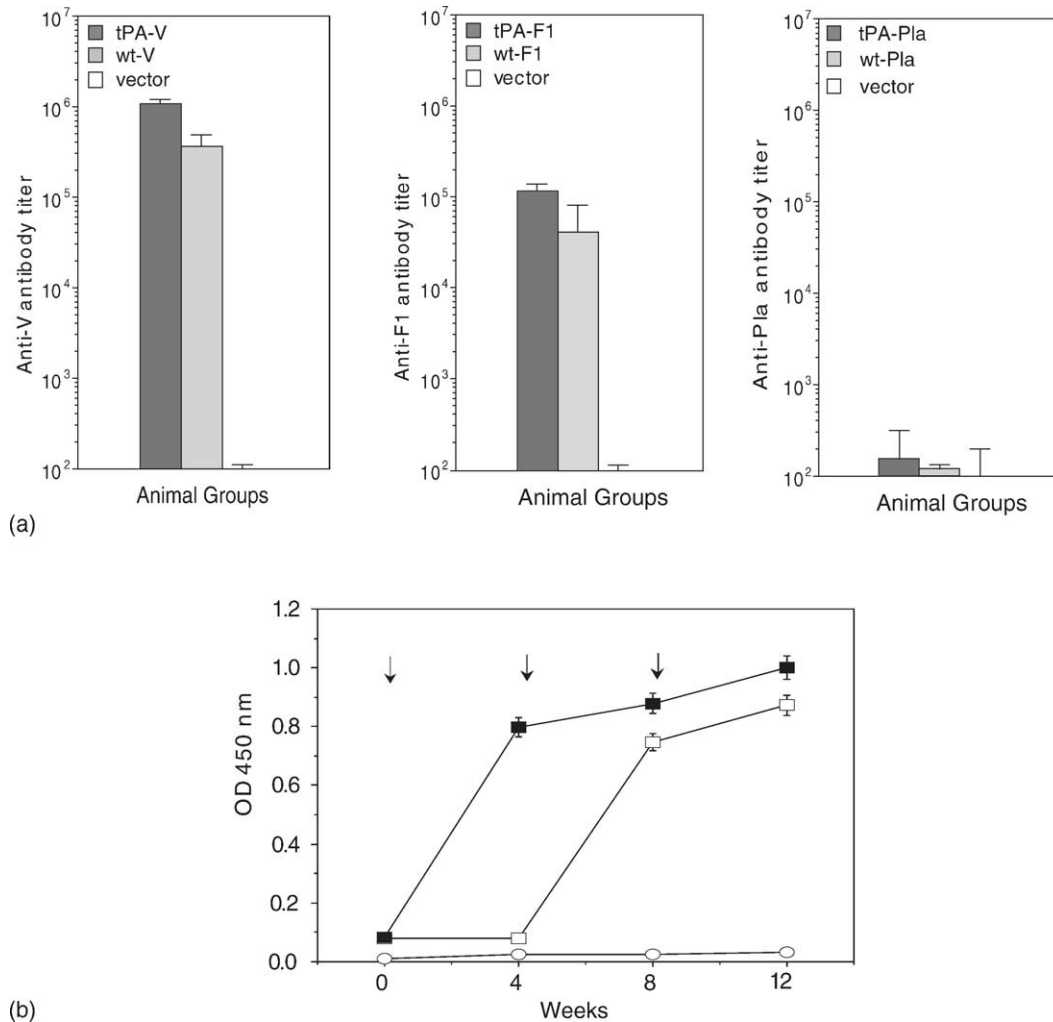


Fig. 2. *Y. pestis* antigen-specific IgG responses in sera of DNA vaccine immunized BALB/c mice as measured by ELISA. Same amounts of transiently expressed *Y. pestis* proteins were used as the coating antigens. (a) Results are shown as the geometric mean titers with pooled mouse sera in duplicates from each group (6 mice per group) as indicated after the third DNA immunization. (b) Temporal mouse sera anti-V IgG responses measured by ELISA OD value in the pre-bleed and 4 weeks following each DNA immunization as indicated by arrows. Pooled sera from each group of 6 mice were used. Sera dilution was at 1:500. Solid square: mouse group immunized with the tPA-V DNA vaccine; open square: mouse group immunized with the wt-V DNA vaccine; and open circle: mouse group received empty vector DNA.

compared with 20% survived for wt-V DNA immunized animals. Again, none of the control animals survived even at the lowest challenge dose of 5000 cfu (Fig. 4 and Table 1).

### 3.4. tPA-V has a strong tendency to form oligomers but is not N-glycosylated

Supernatant and cell lysate collected from 293T cells transiently transfected with either the wt-V or tPA-V DNA vaccines were analyzed by Western blot analysis. Both tPA-V and wt-V DNA constructs displayed good levels of V antigen expression (Fig. 5a). However, the tPA-V protein showed higher tendency to form dimers and tetramers in addition to monomers while wt-V protein was mainly in the monomer form as revealed by the SDS-PAGE analysis (Fig. 5a). When transiently expressed V antigens were heat treated in the presence of urea, the oligomerized V protein can be con-

verted to monomers (Fig. 5b), suggesting that the conventional SDS-PAGE is not efficient in completely denaturing certain proteins, such as tPA-V, which have a high tendency to form oligomers.

Because the tPA leader can lead to expression of secreted proteins and many such proteins are glycosylated as part of their intracellular processing, one would question whether the LcrV protein expressed from the tPA-V construct is glycosylated which may further affect the structure and function of V antigen. Based on the amino acid sequence of V protein, there are no putative N-glycosylation sites. We confirmed this prediction by comparing the molecular weights of tPA-V protein before and after the treatment with Peptide N-glycosidase F (PNGaseF). PNGaseF is an amidase which cleaves the link between the amino acid asparagine and the innermost GlcNAc of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins. There was no

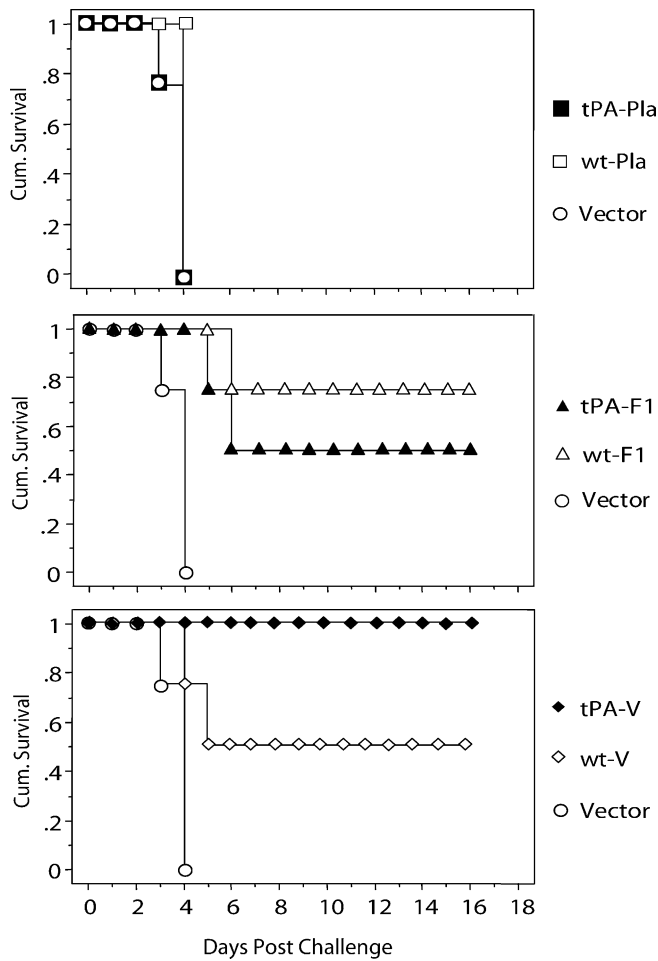


Fig. 3. Protection of mice immunized with different plague DNA vaccines expressing V, F1 or Pla antigens. The BALB/c mice received four DNA immunizations (Weeks 0, 4, 8 and 32) prior to the intranasal challenge at Week 34 with a dose of 5000 cfu (15 LD<sub>50</sub>) *Y. pestis* Kim strain. There were 6 mice per group in this first challenge study. Animals were followed for 2 weeks after the challenge. Accumulated survivals were plotted to compare the efficacy of each vaccine pair (the wild type and tPA-) to the vector alone as indicated.

change on the apparent molecular weight among the PNGaseF treated, untreated or mock treated tPA-V antigens (Fig. 6). As a positive control in this assay, the spike glycoprotein of Severe Acute Respiratory Syndrome associated Coronavirus (SARS-CoV), did show reduced molecular weight after PNGase F treatment (Fig. 6). In summary, our data ruled out *N*-glycosylation as a contributing factor to the oligomer structure and better protection induced by the tPA-V antigen.

### 3.5. Enhanced IgG2a responses induced by the tPA-V vaccine

Given the fact that tPA-V induced only slightly higher anti-V IgG responses than the wt-V DNA construct (Fig. 2a), enhanced production and secretion of V antigen by tPA-V

DNA construct did not appear to significantly improve the immunogenicity of V antigen. Therefore, the more effective protection afforded by tPA-V may be related to the quality of tPA-V induced immune responses to which the LcrV oligomers may play important roles as recently reported [30].

The qualitative difference in immune responses induced between tPA-V and wt-V DNA vaccines were further demonstrated when the levels of isotypes of anti-V IgG responses in animal sera immunized with these two LcrV-based vaccines were compared. Both antigens induced predominantly IgG1 antibody responses (Fig. 7) as

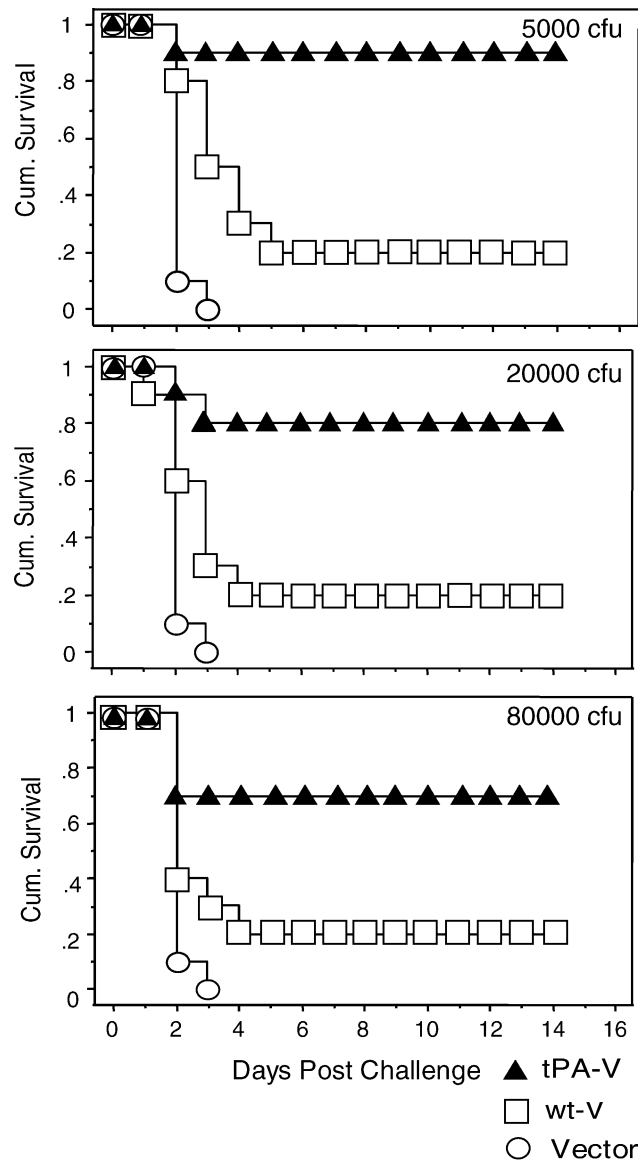


Fig. 4. Protection of the tPA-V or wt-V DNA vaccine immunized mice against intranasal challenge of *Y. pestis* Kim strain at one of the three escalating doses: 5000 cfu (15 LD<sub>50</sub>), 20,000 cfu (60 LD<sub>50</sub>) or 80,000 cfu (240 LD<sub>50</sub>). There were 10 mice per group in this expanded challenge study. Mice received 4 DNA immunizations at Weeks 0, 4, 8, 16 and the lethal challenge was delivered at Week 18.

Table 1  
Survival of Mice Immunized with tPA-V or wt-V DNA vaccines

DNA vaccine	Challenge dose (cfu)	Number of survived	Total number of animal	Survival (%)	P-value
vector	5,000	0	5	0	
tPA V	5,000	9	10	90	0.0055
wt-V	5,000	2	10	20	
tPA V	20,000	8	10	80	0.0230
wt-V	20,000	2	10	20	
tPA V	80,000	7	10	70	0.0698
wt-V	80,000	2	10	20	

Animals were monitored for 2 weeks after the intranasal challenge.

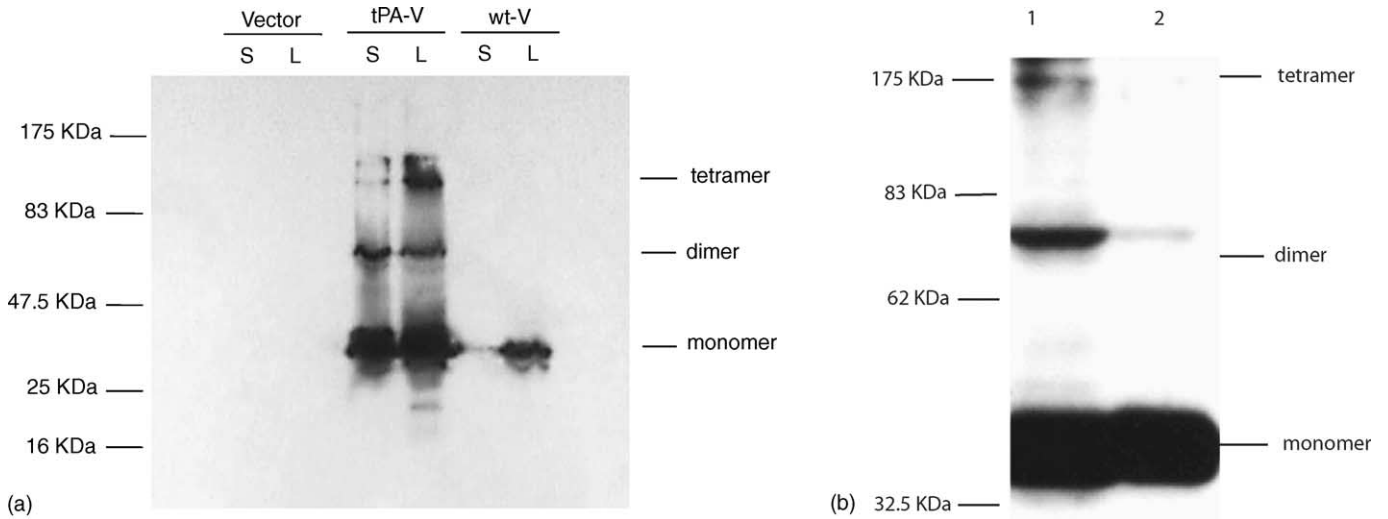


Fig. 5. Comparison of the wt-V and tPA-V antigen expression in vitro. The 293T cells were transiently transfected with either wt-V or tPA-V DNA vaccines. The cells were harvested 72 h later and the expressions of V antigen were examined by Western blot analysis. For each lane, 10 ng of samples were loaded. The mouse sera immunized with the tPA-V DNA vaccine were used to detect *Y. pestis* V antigen. (a) The conventional Western blot results; L: cell lysates and S: the supernatant of transfected 293T cells. (b) Urea (4M at final concentration) was added to the transiently expressed tPA-V samples from supernatant of transfected 293T cells and then the samples were either heat treated (Lane 2) or untreated (Lane 1) before subjected to Western blot analysis.

expected from gene gun delivered DNA immunizations [31,32]. However, the tPA-V DNA vaccine group displayed a large increase (nearly 10-fold) in IgG2a titer relative to the wt-V DNA vaccine group (Fig. 7). In mice, increased IgG2a levels are indicative of a  $T_H$  1 type immune response

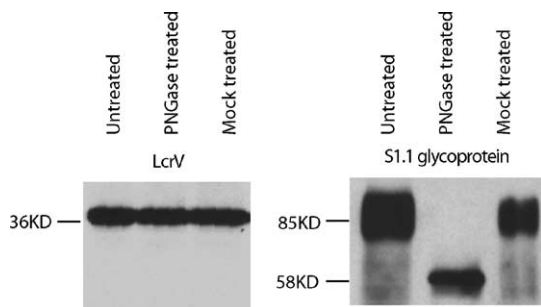


Fig. 6. The tPA-V antigen was not glycosylated. Fully denatured tPA-V protein transiently expressed from 293T cells was subjected to the treatment of PNGase F and then was analyzed by Western blot. Untreated or mock treated tPA-V samples were included as the negative controls. PNGase F treatment of S1.1 fragment of SARS-CoV spike protein was used as the positive control.

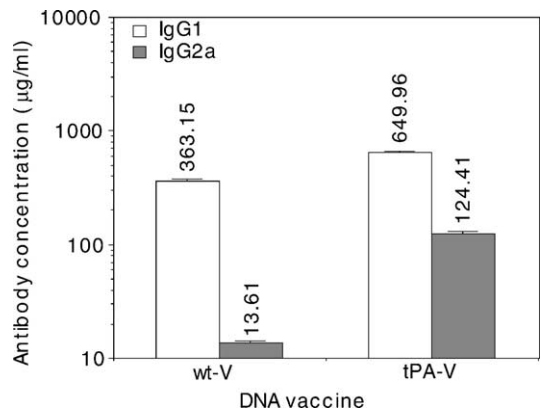


Fig. 7. Subtypes of anti-V IgG in mouse sera raised by the wt-V or tPA-V DNA vaccines as measured by the quantitative ELISA. The assay is similar to the regular ELISA as in Fig. 2 except the second antibodies used in this assay were purified anti-IgG1 or anti-IgG2a antibodies. The ELISA OD values were then converted to the concentration for each IgG subtypes in mouse sera by using the standard curves established with known concentration of IgG1 or IgG2a.



which might play critical roles for the better protection provided by the tPA-V DNA vaccine.

#### 4. Discussion

While it is well known that DNA vaccines can be highly effective in the induction of cell-mediated responses [23,33,34], our data argue strongly that DNA immunization is also effective in the induction of protective antibody responses critical for the development of vaccines against bacterial infections. The level of antibody produced by the tPA-V construct was comparable to that reported for the most effective protein vaccine formulations containing LcrV [35]. Moreover, the protection afforded by the tPA-V DNA vaccine is comparable to that reported for LcrV protein-based vaccines [35,36], and only moderately inferior to that reported for two-component F1+V protein formulations tested against *Y. pestis* in mouse respiratory infection models. Previous study using LcrV protein alone reported 80% protection at 590 LD<sub>50</sub> [35] and an LcrV-F1 fusion protein induced 100% protection at the same challenge dose [35], compared to the 70% protection observed with tPA-V at our highest challenge dose of 240 LD<sub>50</sub>. With a two-component LcrV+F1 formulations, 100% protection was achieved against 100 LD<sub>50</sub> [2], and another study reported 100% protection against 10<sup>4</sup> LD<sub>50</sub> with LcrV+F1 subunit vaccine [3], reflecting the variation in different experimental systems. While aerosol infections were used for pneumonic plaque in these studies, we used the intranasal route. Both routes could yield acute pneumonia and rapid development of systemic infection, and they appeared to produce compatible LD<sub>50</sub> values in a given mouse strain with similar mean time to death. Given these similarities in the pattern of disease, it is unlikely that the challenging route has a major influence on the outcome of these protection studies.

The addition of the tPA leader sequence to the LcrV gene played a crucial role in achieving good protection, proving that antigen engineering affords strong potential for the development of effective DNA vaccines against bacteria, including those—like *Y. pestis*—are primarily extracellular pathogens. As shown in our results, the tPA leader in the tPA-V construct enhanced protein production, and resulted LcrV antigen with modified structure features as indicated by its tendency to form oligomers. Oligomers of LcrV antigen have been associated with enhanced immunogenicity, and was observed in protein-based V formulations [30]. Further studies are needed to elucidate the mechanism of why tPA-V construct had a stronger tendency to form oligomeric V than the wt-V construct, but the leader sequence may affect the folding of a secreted protein as previously reported [37].

In addition, tPA-V induced a 10-fold greater level of IgG2a responses than wt-V, indicating an enhanced T<sub>H</sub>1-type preference. LcrV was recently reported to in-

duce high levels of IL-10 production through a mechanism that involved direct interaction of LcrV and TLR-2 [38]. The importance of this effect in vivo has yet to be firmly established, and there is no data concerning its effect on the development of LcrV-specific immunity. Given the fact that IL-10 inhibits T<sub>H</sub>1-type immune responses, it is likely that tPA-V may be less effective than wt-V in inducing IL-10—perhaps due to a reduced ability to interact with TLR-2—resulting in a bias toward a more effective T<sub>H</sub>1-type response against *Y. pestis*. While the basis for the enhanced immunogenicity and protection afforded by tPA-V is not the focus of the current study, mechanisms mentioned above may contribute in concert and further study is needed to examine the exact process that tPA-V induced better protection.

DNA vaccines expressing F1 antigen yielded only partial protection against the lethal mucosal challenge, confirming results from other vaccine studies using the recombinant F1 protein as candidate vaccine [3,39–42]. Addition of the tPA leader to F1 did not improve the levels of protection. This is not surprising given the fact that the F1 native signal sequence supports secretion, and our finding is consistent with other experiments in which addition of an extra signal sequence to the wild type F1 gene did not improve the immunogenicity of F1 DNA vaccines [32]. In our study, DNA vaccines expressing Pla antigen did not provide any protection. The Pla gene has an unusual hydrophobic sequence near its N-terminus that may be responsible for the poor expression of soluble Pla antigens, and thus the poor immunogenicity of Pla DNA vaccines. It will be interesting to see if the Pla antigen can achieve a better protection when the N-terminal hydrophobic sequence is removed.

Our results indicate that properly designed DNA vaccines can offer effective protection against highly virulent extracellular bacterial pathogens. Such vaccines can be used not only alone, but also as a component in a DNA prime plus protein boost formulation. Such a combination can offer many advantages over more conventional single modality vaccines because DNA and protein are individually effective in inducing different sets of immune responses. Protein-based vaccines are effective in inducing high antibody responses, but multiple inoculations and strong adjuvants are usually required. Using DNA components in the priming phase can significantly reduce the amount of protein needed for the boost, or decrease the numbers of protein immunizations required to induce the same level of protective immune responses. DNA is also more stable and easier to manufacture than the recombinant proteins. DNA vaccines have been proven to be extremely safe in all the clinical studies conducted to date. The ease of modification of the encoded antigens and expression of conformation-sensitive antigens in vivo, circumventing the lengthy and expensive process of in vitro protein production, are important advantages offered by DNA vaccine technology. Particularly in light of emerging infectious diseases and the threat of bioterrorism, where the rapid development of robust vaccines may become a fre-

quent requirement, the potential of DNA vaccines against bacterial pathogens warrants more thorough investigation.

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