



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



available at www.sciencedirect.com



journal homepage: www.elsevier.com/locate/vaccine



Relative immunogenicity and protection potential of candidate *Yersinia Pestis* antigens against lethal mucosal plague challenge in Balb/C mice

Shixia Wang^a, Swati Joshi^a, Innocent Mboudjeka^a, Fangjun Liu^a,
Tzufan Ling^a, Jon D. Goguen^b, Shan Lu^{a,*}

^a Laboratory of Nucleic Acid Vaccines, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2397, United States

^b Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655, United States

Received 7 November 2007; received in revised form 3 January 2008; accepted 11 January 2008
Available online 4 February 2008

KEYWORDS

Y. pestis antigens;
Immunogenicity;
DNA vaccine

Summary *Yersinia Pestis* outer proteins, plasminogen activator protease and Yop secretion protein F are necessary for the full virulence of *Yersinia pestis* and have been proposed as potential protective antigens for vaccines against plague. In the current study, we used DNA immunization as a tool to study the relative protective immunity of these proteins with a standardized intranasal challenge system in mice. While the natural full-length gene sequences for most of these *Y. pestis* proteins did not display a good level of protein expression *in vitro* when delivered by a DNA vaccine vector, the overall immunogenicity of these wild type gene DNA vaccines was low in eliciting antigen-specific antibody responses and gene sequence modifications improved both of these parameters. However, even modified YopD, YopO and YscF antigens were only able to partially protect immunized mice at various levels against lethal challenge with *Y. pestis* KIM 1001 strain while no protection was observed with either the YopB or Pla antigens. These results demonstrate that DNA immunization is effective in screening, optimizing and comparing optimal antigen designs and immunogenicity of candidate antigens for the development of a subunit-based plague vaccine.

© 2008 Elsevier Ltd. All rights reserved.

Introduction

Plague is one of the most feared infectious diseases in humans. It has killed over 200 million humans throughout history and still remains endemic in certain areas of the world. The causative agent of plague, *Yersinia pestis* (*Y.*

* Corresponding author. Tel.: +1 508 856 6791;
fax: +1 508 856 6751.

E-mail address: shan.lu@umassmed.edu (S. Lu).

pestis), is also a concern for its potential use as an agent of biological warfare and biological terrorism. Although killed whole-cell vaccines are available for human use in certain countries, poor efficacy has limited their use for prevention of natural or human-inflicted outbreaks [1–3]. Up to this point, the effort to develop next generation plague vaccines has focused mainly on the use of two well-studied protective antigens, F1 and V (LcrV), in the form of subunit-based recombinant protein vaccines [4–8]. However, as shown in a recent study conducted in humans with subunit-based plague vaccines, the immunogenicity of the current vaccine composition is quite weak [9]. Therefore, the importance of identifying additional protective antigens is clear, especially for the highly virulent pneumonic plague.

Previous studies have shown that antibodies to some of the *Yersinia* outer proteins (Yops) are present in convalescence sera from patients infected by *Y. pestis* and in mice that survived experimental challenge [10,11]. Therefore, it was hypothesized that these proteins may serve as protective antigens. Along with F1 and LcrV, Yops and the plasminogen activator protease (Pla) are necessary for the full virulence of *Y. pestis* [12,13] and are encoded by the virulence plasmids [13–15]. Among the 12 secreted Yops, YopB and YopD are known as translocators in the Ysc-Yop type III secretion system involved in the translocation of the Yop effectors across the eukaryotic cell plasma membrane into the target cells [16,17]. The type III secretion apparatus of *Y. pestis* is a conserved mechanism to deliver virulent factors into mammalian host cells [15,18]. Both YopB and YopD are transmembrane proteins and interact with LcrV to form the pore of the Type III secretion apparatus through which the Yop effectors are translocated [13,19,20]. YopO/YpkA is a 81.7kDa protein kinase with the ability to catalyze autophosphorylation on a serine residue, as shown by *in vitro* experiments, and is targeted to the inner surface of the plasma membrane inside the cells [21].

Recent reports have demonstrated that mice immunized with recombinant YscF, which is a surface localized protein, can produce YscF-specific antibody responses and partial protective immunity against *Y. pestis* by intravenous or subcutaneous challenge [22,23]. These particular results suggest that the antibody responses induced by YscF protein immunization are useful for protection against bubonic plague although the protection mechanism is not quite clear. There is no evidence whether YscF-specific antibody responses are protective for mucosal infection including the pneumonic plague.

Pla is an integral outer membrane protein and a surface protease. It belongs to the omptin family of enterobacterial surface proteins and derives its name from the fact that it can activate the mammalian plasma proenzyme plasminogen into plasmin [24–26]. The importance of Pla for bacterial virulence has been demonstrated *in vivo*. The LD50 for a Pla-negative mutant strain was close to 10⁷ bacteria, as compared to less than 50 bacteria for the isogenic Pla-positive strain [12]. This protein is responsible for the highly efficient invasion of plague bacterium from the subcutaneous infection site into circulation [24]. Furthermore, recent data have shown that intranasal infection with a *Y. pestis* strain lacking Pla resulted in the development of a deadly plague infection after 7 days in only 50% of the infected mice when compared to a 100% fatal-

ity rate less than 4 days post-inoculation in the wild type strain indicating that Pla has a significant involvement in the fatal outcome following respiratory infection with *Y. pestis* [27].

The immunogenicity and potential for protection have not been well characterized for the Yop and Pla proteins. In one study using recombinant Yop proteins expressed in *Escherichia coli* (*E. coli*) and purified by the His-tag approach, the recombinant YopO protein significantly prolonged the mean survival time but did not increase the overall survival of mice that had received a subcutaneous challenge of a virulent, non-encapsulated isogenic variant of *Y. pestis* [28]. In this same study, YopD was also shown to be partially protective against death in mice challenged with the non-encapsulated strain but not the wild-type encapsulated *Y. pestis* [28]. No detailed data were provided regarding the antibody responses to individual Yop proteins. The authors also noted that several Yop proteins were denatured before their use as immunogens which may have contributed to the poor protection with Yop proteins.

In recent years, the DNA vaccine approach has been used as an effective tool to study the immunogenicity of candidate antigens against a variety of infectious agents due to its potential to express protective antigens *in vivo*. For example, DNA immunization was used to quickly confirm the Spike protein as the protective antigen against the Severe Acute Respiratory Syndrome – associated coronavirus (SARS-CoV) and to map the protective domains on this Spike protein [29,30]. DNA immunization was also used to effectively identify individual protective antigens in order to formulate subunit-based vaccines against smallpox infection [31]. In a previous study from our laboratory, we successfully established a mouse intranasal challenge model coupled with DNA immunization technology to test the protection efficacy of modified *Y. pestis* antigens against lethal plague challenge [32]. This system provided a standard assay process to screen and score the relative effectiveness of known or novel *Y. pestis* antigens.

In the current study, a molecular antigen engineering approach was used to improve the expression and immunogenicity of Yops, YscF and Pla proteins. Our previous studies, which used DNA vaccines against plague and other infectious agents, have demonstrated that a proper leader sequence is important in eliciting a high level of antibody responses and protective immunity [30,32,33]. Since *yopB*, *yopD* and *yopO* genes do not encode typical signal peptides, the tPA leader sequence was engineered with each of these *yop* genes individually to produce modified *yop* gene inserts for DNA immunization. In addition, certain intragene hydrophobic regions were removed to further improve the immunogenicity of Yop antigens. Our results demonstrate that antigen engineering was effective in improving the immunogenicity of the above Yop, Pla and YscF antigens and that these modified antigens in general induced higher antibody responses than the wild-type gene inserts. In addition, modified YopD, YopO and YscF proteins improved the level of protection. Overall, the protection efficacies with these *Y. pestis* proteins were still low and incomplete when compared to the known protective antigen LcrV, indicating that a search for additional protective antigens against *Y. pestis* is needed.

Materials and methods

Bacterial strains

Y. pestis strain KIM 1001, fully virulent and containing all known virulent factors [12] was prepared by growing inocula for 18 h at 37 °C on Tryptose Blood Agar Base (Difco) supplemented with 2.5 mM CaCl₂ 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 (OD₆₀₀). The number of bacteria in the final inocula was confirmed by colony counts.

Construction of plague DNA vaccines

Y. pestis genes (*yopB*, *yopD*, *yopO*, *pla* and *yscF*) were first amplified with pfu polymerase (Stratagene, CA) from plasmids pCD1 or pPCP1. The *yopB*, *yopD* and *yopO/yopA* are encoded by the 70 kb plasmid, pCD1 [13] and the *pla* is encoded by the 9.5 kb plasmid, pPCP1 [34–35]. Primer pairs are shown in Table 1. Two DNA vaccine constructs for each primer pair were made for expressing either YopB, YopD or YopO and three constructs for Pla.

For YopB constructs, the full-length *yopB* (*wt.yopB*) gene was amplified with primers YopB-1 and YopB-2. The truncated *yopB* (*yopB.dTM*) gene, following removal of the hydrophobic regions (aa128–aa258), was first amplified as two gene fragments: the 5' *yopB* gene and the 3' *yopB* gene fragments with primer pairs YopB-1/YopB-4 and YopB-3/YopB-2 respectively and these two fragments were ligated together by inducing an HindIII site in the junction.

For YopD DNA vaccine constructs, the full-length *yopD* (*wt.yopD*) gene was amplified with primers YopD-1 and YopD-2. The truncated *yopD* (*yopD.dTM*) gene, following removal

of the hydrophobic regions (aa128–aa149), was first amplified as two gene fragments: the 5' *yopD* gene and the 3' *yopD* gene fragments with primer pairs YopD-1/YopD-4 and YopD-3/YopD-2 respectively, and these two fragments were ligated together by inducing a PstI site in the junction.

While there is a hydrophobic region at the N-terminus of the Pla protein, it does not represent a natural signal peptide. Several versions of Pla DNA vaccines were constructed both with and without the removal of this N-terminal hydrophobic region. The full-length *pla* gene and *d-pla* gene, following removal of the hydrophobic region at N-terminus of Pla, were amplified with primer pairs Pla-1/Pla-2 and Pla-3/Pla-2, respectively. Each of the above gene inserts was directly sub-cloned into the previously described DNA vaccine vector, pJW4303, immediately downstream of the tPA leader sequence [32,33], which is in-frame with such sub-cloned inserts.

The full-length *yopO* gene was amplified with primers YopO-1 and YopO-2 and cloned into pJW4303 with or without tPA leaders sequence and produced wt.YopO and tPA.YopO DNA vaccines, respectively.

Three YscF DNA vaccines were constructed: single copy of *yscF* gene with and without tPA leader sequence, and two copies of *yscF* gene in tandem. The wild type full-length *yscF* gene was amplified with primers YscF-1 and YscF-2. The fragments of two-copy *yscF* tandem gene insert were amplified with primer pairs YscF1/YscF-4 and YscF-5/YscF-2, respectively, and these two fragments were ligated together by inducing a SpeI site and a linker (Ala-Ala-Thr-Ser-Thr-Ser-His-Val-Asp) in the junction. The wild type *yscF* and two-copy *yscF* tandem inserts were subcloned into pJW4303 vector at HindIII and BamHI sites without using any leader sequence. The *tPA-yscF* insert was amplified with primers YscF-3 and YscF-2 and subcloned into the pJW4303 vector immediately downstream of tPA leader sequence. Sufficient amounts of the DNA vaccines were

Table 1 Primers used in the study

Primers	Oligonucleotide sequence ^a	Description
YopB-1	gtcgctcc <i>GCTAGC</i> _{NheI} ATGAGTGC GTTGATAACCCATGAC	<i>yopB</i> , sense
YopB-2	agtcac <i>GGATCC</i> _{BamHI} TTAACAGTATGGGGTCTGCC	<i>yopB</i> , anti-sense
YopB-3	gtcgctcc <i>GCTAGC</i> _{NheI} <i>AAGCTT</i> _{HindIII} GGTGCTAACACCGCAAG	<i>yopB</i> , sense
YopB-4	agtcac <i>GGATCC</i> _{BamHI} TCA <i>AAGCTT</i> _{HindIII} TAGTGCGATGCCGGATTG	<i>yopB</i> , anti-sense
YopD-1	gtcgctcc <i>GCTAGC</i> _{NheI} ATGACAATAATATCAAGACAGAC	<i>yopD</i> , sense
YopD-2	agtcac <i>GGATCC</i> _{BamHI} TCAGACAACACAAAAGCGGC	<i>yopD</i> , anti-sense
YopD-3	acaa <i>CTG CAG</i> _{PstI} TCTATAGCGAAAGAGGTGAAAATAG	<i>yopD</i> , sense
YopD-4	agtcac <i>GGATCC</i> _{BamHI} TCA <i>CTGCAG</i> _{PstI} TTTTGACCCGTGACCATC	<i>yopD</i> , anti-sense
YopO-1	<i>p-CATGAAAAGCGTGAAAATCATGGG</i>	<i>yopO</i> , sense
YopO-2	agtcac <i>TGATCA</i> _{BclI} TCACATCCATCCCCGCTCCAACCG	<i>yopO</i> , anti-sense
Pla-1	gtcgctcc <i>AAGCTT</i> _{HindIII} <i>GCTAGC</i> _{NheI} ATGAAGAAAAGTTCTATTGTGGC	<i>pla</i> , sense
Pla-2	gagc <i>AGGCC</i> <i>T</i> _{StuI} CAGAAGCGATATTCAGACCCGC	<i>pla</i> , anti-sense
Pla-3	gtcgctcc <i>GCTAGC</i> _{NheI} TCCGGGAGTGCTAATGCAGCATC	<i>pla</i> , sense
YscF-1	gtcgctcc <i>AAGCTT</i> _{HindIII} ATGAGTAACCTCTCTGGATTACG	<i>yscF</i> , sense
YscF-2	agtcac <i>GGATCC</i> _{BamHI} TTA TGGGAACCTCTGTAGGATGCC	<i>yscF</i> , anti-sense
YscF-3	gtcgctcc <i>GCTAGC</i> _{NheI} AGTAACCTCTCTGGATTACG	<i>yscF</i> , sense
YscF-4	atgg <i>ACTAGT</i> _{SpeI} <i>ggcggc</i> TGGGAACCTCTGTAGGATGCC	<i>yscF</i> , anti-sense
YscF-5	atgg <i>ACTAGT</i> _{SpeI} <i>ggcggc</i> AGTAACCTCTCTGGATTACG	<i>yscF</i> , sense

^a The lower case letters indicate the non-specific sequences; the upper case letters indicate the specific sequences matching the target genes. The italicized sequences are the restriction enzyme sites.

prepared by the Qiagen Mega plasmid purification kit (Valencia, CA) for *in vitro* transfection and animal immunization applications.

DNA vaccines for plague antigens, V and F1, were as constructed as previously reported [32] and were used as a positive control in the intranasal challenge study as they have been previously shown to provide protection in a mucosal challenge model in mice [32].

DNA immunization of Balb/C mice

Six to eight week old female Balb/C mice (9–10 mice/group) 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), using helium gas pressure at 300 psi according to manufacturer's instruction. Each mouse received four monthly immunizations with six DNA shots of 1 µg each at each immunization. Blood samples were collected periorbitally prior to each immunization and 2 weeks after the last immunization.

In vitro expression of *Y. pestis* antigens and Western-blot analysis

The expression of the YopB, YopD, YopO, Pla and YscF DNA vaccines were tested in transiently transfected 293T cells by a calcium phosphate co-precipitation method using 10 µg of plasmid DNA for 2×10^6 cells on a 60-mm dish. The cells were harvested 72 h after transfection. The transiently expressed YopB, YopD, YopO, Pla or YscF antigens were analyzed by SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto PVDF membranes, 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 group pooled mouse sera immunized with corresponding DNA vaccines. The mouse sera against YopB, YopD, YopO, Pla or YscF were generated by YopB.dTM, YopD.dTM, tPA.YopO, tPA.dPla or YscF-2 DNA vaccine, respectively. 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).

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed to measure the titers of specific IgG against each individual *Y. pestis* antigens in pooled mouse sera of each group (equal amount of serum from each mouse in a group were first pooled before further dilution and testing). The YopB, YopD or YopO antigens used for ELISA were supernatants from 293T cells transfected with YopB.dTM, YopD.dTM or tPA.YopO DNA vaccines, respectively. Non-purified Pla or YscF antigen used for ELISA was from 293T cell lysate transfected with tPA-Pla or YscF-2 DNA vaccine, respectively. Ninety-six-well microtiter plates were coated with 100 µl of one of the above antigens harvested from

transfected 293T cells at 1 µg/ml and incubated overnight at 4°C. Serially diluted mouse sera were added to the plates and assayed in duplicate. The plates were incubated with biotinylated anti-mouse IgG diluted at 1:1000, followed by horseradish peroxidase-conjugated streptavidin diluted at 1:2000 and finally, developed with 3,3',5,5'-tetramethylbenzidine (TMB) solution. The reactions were stopped by adding 25 µl of 2 M H₂SO₄ and the plates were read at OD₄₅₀.

Intranasal challenge with lethal doses of *Y. pestis*

Challenge was performed by an intranasal instillation of 50 µl saline containing lethal doses of *Y. pestis* (KIM 1001 strain) to ketamine-anesthetized mice. KIM 1001 is a fully virulent *Y. pestis* strain and contains all known virulent factors [12]. This challenge method leads to a rapid infection and is lethal to 100% of non-immunized mice within 3–4 days [32]. The LD₅₀ (median lethal dose) of this challenge model was determined by a previous study: 5000 cfu is equal to approximately 15 LD₅₀ [32]. Individual mice were challenged 1 week after the fourth immunization and observed twice daily to monitor both morbidity and mortality. Studies were conducted in a Biosafety Level 3 containment facility at the Department of Animal Medicine, UMMS.

Statistical analyses

Fisher's exact test was used to compare the protection efficacies against *Y. pestis* infection between different vaccination groups.

Results

Construction and expression of DNA vaccines expressing YopB, YopD, YopO, Pla and YscF antigens

For *Y. pestis* antigens YopB and YopD, two versions of the DNA vaccine inserts were designed (Figs. 1A and 2A, respectively). Both Yop proteins contain at least one hydrophobic region, a potential transmembrane (TM) segment, in the middle of polypeptide sequences. Therefore, one modified version of the DNA insert ("dTM") had the hydrophobic region(s) removed while the wild type version (*wt.yopB* and *wt.yopD*) retained the full-length sequence. A human tissue plasminogen activator leader sequence (tPA) was included in both versions of the inserts to facilitate the expression of DNA vaccine antigens in mammalian cells, as previously reported [36]. For the YopO, which does not contain any significant hydrophobic segments, only the tPA leader was added to the full-length gene insert and a control *yopO* gene insert was produced with the wild type sequence without any identifiable leader sequences (Fig. 3A). These *yop* gene inserts were individually cloned into the DNA vaccine vector, pJW4303, which is driven by a cytomegalovirus (CMV) promoter, as previous reported [37] and was used effectively for a LcrV DNA vaccine [32].

Mammalian cell expression of the above Yop DNA vaccines was confirmed by using a transiently transfected 293T cell

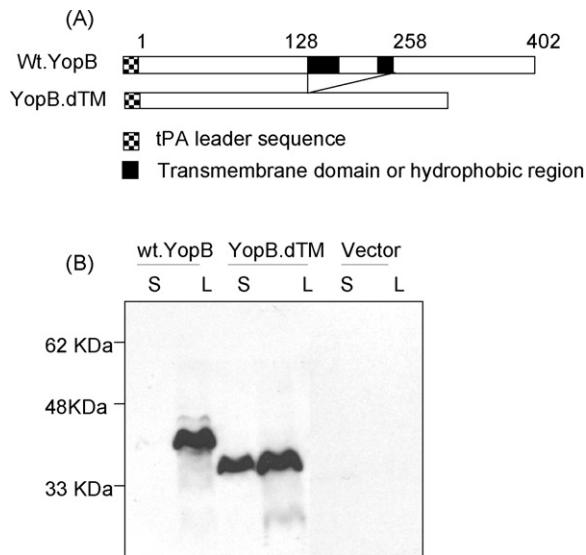


Figure 1 (A) Schematic diagram of *yopB* gene inserts in DNA vaccine constructs. Weaved and solid boxes represent the added tPA-leader sequence and hydrophobic domains, respectively. The numbers indicate the amino acid positions. (B) Western-blot analysis on the expression of either the full-length YopB protein or the YopB protein without the hydrophobic domains coded in two YopB DNA vaccines, plus empty vector control, in supernatant (S) and lysate (L) of transiently transfected 293T cells. Pooled sera from mice immunized with YopB.dTM DNA vaccine was used at 1:200 dilution as the detecting antibody.

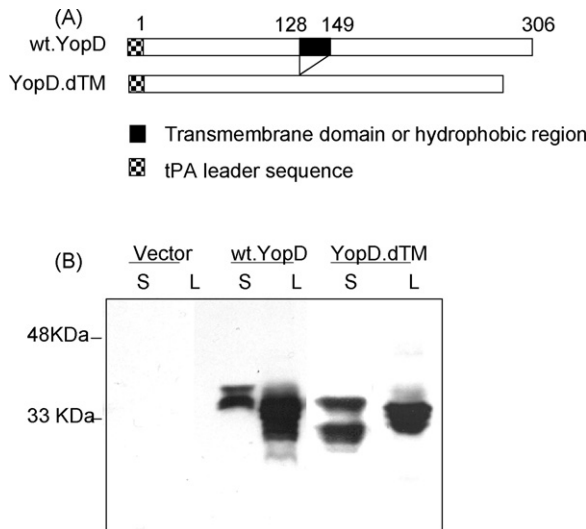


Figure 2 (A) Schematic diagram of *yopD* gene inserts in DNA vaccine constructs. Weaved and solid boxes indicate the tPA-leader sequence and hydrophobic domains, respectively. The numbers indicate the amino acid positions. (B) Western-blot analysis of YopD protein expression by DNA vaccines encoding either the full-length YopD protein or the YopD protein without the hydrophobic domain, plus empty vector, in supernatant (S) and lysate (L) of transiently transfected 293T cells. Pooled sera from mice immunized with YopD.dTM DNA vaccine was used at 1:200 dilution as the detecting antibody.

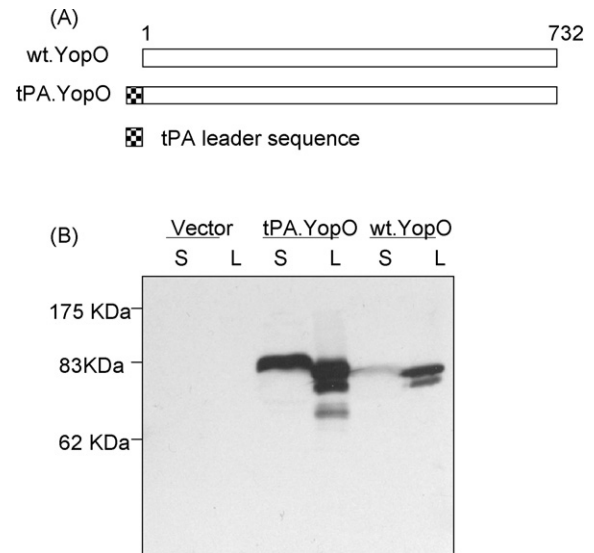


Figure 3 (A) Schematic diagram of *yopO* gene inserts in DNA vaccine constructs. Weaved box represents the tPA-leader sequence. The numbers indicate the amino acid positions. (B) Western-blot analysis of expression of the wild type YopO protein or the YopO protein with a new tPA leader, plus empty vector, in supernatant (S) and lysate (L) of transiently transfected 293T cells. Pooled sera from mice immunized with the tPA.YopO DNA vaccine was used at 1:200 dilution as the detecting antibody.

system *in vitro* [32]. Both cell lysate and culture supernatant were collected after the transfection and examined by Western-blot analysis. YopB antigen expression was observed for both full-length *yopB* and modified *yopB* gene inserts (Fig. 1B) but only the modified *yopB* insert, with the removal of hydrophobic regions, displayed a good level of expression in both cell lysate and supernatant. Therefore, the removal of 130 aa in the middle of *yopB* gene greatly enhanced the secretion of YopB protein (Fig. 1B). On the other hand, the impact of removing a much smaller 21 aa hydrophobic sequence on the expression of YopD was limited. Both full-length YopD and modified YopD DNA vaccines were able to express comparable levels of antigen in both cell lysate and supernatant (Fig. 2B).

The role of the leader sequence in the expression of YopO protein was significant. Compared to the YopO DNA vaccine without leader sequence (wt.YopO), the YopO construct with the tPA leader (tPA.YopO) was able to produce a larger amount of YopO protein in the supernatant, supporting the utility of tPA leader for expressing secreted proteins (Fig. 3B). Interestingly, the addition of a tPA leader also led to an overall higher level of expression for YopO protein even though the exact mechanism for such a role has not been well elucidated.

Our previous data demonstrated that DNA immunization with the *pla* gene in its wild type sequence was not immunogenic despite the possibility of there being a putative leader sequence with 21 hydrophobic amino acid residues at the N-terminus of Pla. Although the addition of a tPA leader to the wild type *pla* sequences was able to achieve better expression of Pla protein in transiently transfected 293T cells, it was still not immunogenic [32]. In the current study, another

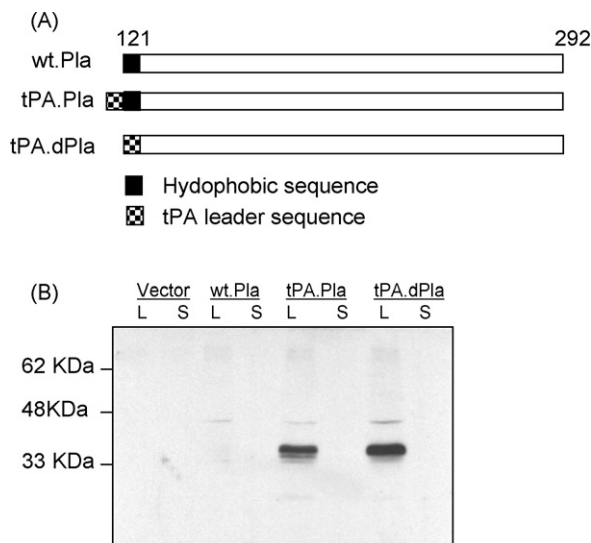


Figure 4 (A) Schematic diagram of *pla* gene inserts in DNA vaccine constructs. The wt.Pla, tPA.Pla and tPA.dPla, respectively, represent the constructs with full-length *pla* gene alone, full-length *pla* gene with addition of tPA leader sequence, and *pla* gene with deletion of the N-terminal hydrophobic sequences and addition of the tPA leader sequence. Weaved and solid boxes indicate the added tPA-leader sequence and hydrophobic domains, respectfully. The numbers indicate the amino acid positions. (B) Western-blot analysis of the Pla protein expression by DNA vaccines encoding wt.Pla, tPA.Pla, tPA.dPla proteins, plus empty vector, in supernatant (S) and lysate (L) of transiently transfected 293T cells. Pooled sera from mice immunized with the tPA-dPla DNA vaccine was used at 1:200 dilution as the detecting antibody.

modified Pla DNA vaccine was constructed by removing the natural hydrophobic sequence while keeping the tPA leader (Fig. 4A). Transient expression of Pla was confirmed with this new DNA vaccine plasmid. Both versions of Pla DNA inserts were able to express Pla antigen only in cell lysate (Fig. 4B).

Three versions of DNA vaccine inserts were designed for YscF antigen (Fig. 5A). YscF is a small protein without any leader sequence. Therefore, one construct with full-length wild type YscF (wt.YscF) and another modified YscF with tPA leader sequence (tPA-YscF) were made. To improve the expression and immunogenicity of YscF, the third YscF DNA vaccine with two-copy tandem YscF antigen (YscF-2) was designed and constructed. These *yscF* gene inserts were individually cloned into the DNA vaccine vector pJW4303. The transient expression of three YscF DNA vaccine constructs in 293T cells demonstrated that both wt.YscF and tPA.YscF did not induce detectable YscF antigen expression (Fig. 5B). Although the addition of the tPA leader sequence could improve the overall expression of many other antigens, it did not improve the YscF expression in the current tPA.YscF DNA vaccine design. Interestingly, YscF-2 antigen was well expressed as a dimeric YscF protein with expected size (16 kDa) (Fig. 5b). There is a non-specific band just above the 25 kDa Marker across all transfected samples including vector control, which was not seen in other vector control samples (Figs. 1–4). Depending on the cell conditions, we

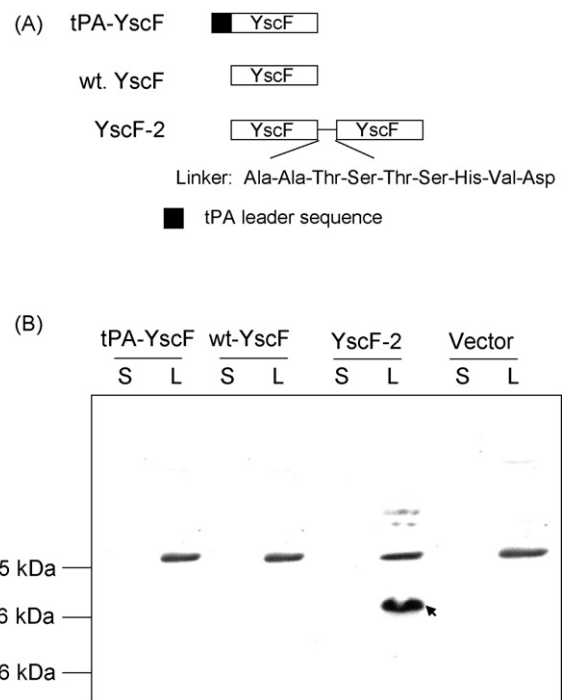


Figure 5 (A) Schematic diagram of *yscF* gene inserts in DNA vaccine constructs: wt-YscF, tPA-YscF and YscF-2, respectively, represent the full-length *yscF* gene alone, full-length *yscF* gene with addition of tPA leader sequence, and the two-copy *yscF* gene in tandem. (B) Western-blot analysis of the YscF protein expression by DNA vaccines encoding wt-YscF, tPA-YscF and YscF-2 proteins, plus empty vector, in supernatant (S) and lysate (L) of transiently transfected 293T cells. Pooled sera from mice immunized with the YscF-2 DNA vaccine was used at 1:200 dilution as the detecting antibody. Arrow points to the dimeric form of YscF.

have observed that mouse sera sometimes may show non-specific reactivity against cell lysate samples (unpublished observation).

Serum IgG responses in mice immunized with individual YopB, YopD, YopO, Pla and YscF DNA vaccines

Y. pestis antigen-specific antibody responses were measured by ELISA using pooled mice sera from each immunization group (9–10 mice/group), at 2 weeks after the third monthly DNA vaccination. While the YopB.dTM DNA vaccine induced positive anti-YopB IgG responses, the full-length YopB DNA vaccine did not induce detectable antibody responses (Fig. 6A). Anti-YopD IgG responses were elicited with both the full-length and the modified (i.e., removal of the hydrophobic domain) DNA vaccines. However, the latter was more immunogenic as indicated by the elicitation of higher anti-YopD IgG when compared to the full-length YopD insert (Fig. 6B). Therefore, the deletion of hydrophobic regions improved the immunogenicity of YopB and YopD DNA vaccines. YopO DNA vaccine with the tPA leader (tPA.YopO) was also able to induce higher titer anti-YopO antibody responses than the wild type YopO DNA

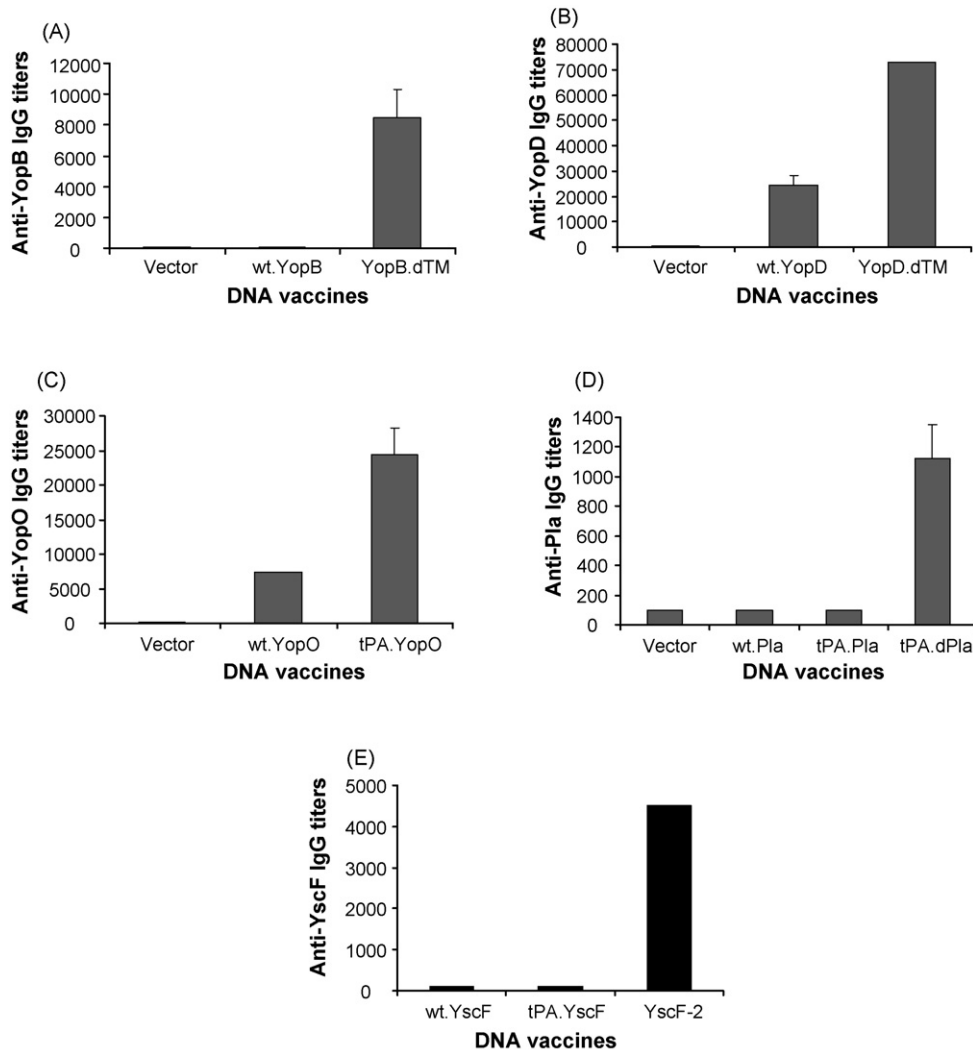


Figure 6 Antigen-specific IgG responses induced by different YopB, YopD, YopO, Pla and YscF DNA vaccines in Balb/C mice after the third DNA immunization. The levels of antibody responses were measured by ELISA against autologous antigens expressed from 293T cells transfected with the DNA vaccines YopB.dTM, YopD.dTM, tPA.YopO, tPA-Pla and YscF-2 for Panels A, B, C, D and E, respectively. The bars show the average antibody titers (the reciprocal of sera dilutions) of three duplicated assays using pooled mouse sera from each group as indicated (9 mice/group for Panels A, B and C and 10 mice per group for Panels D and E). Standard error bars are shown if applicable.

vaccine (wt.YopO) (Fig. 6C). Based on the reciprocal end-titration titers, modified YopD and YopO constructs were able to induce higher levels of antibody responses (in the range of 1:20,000–1:70,000) than the modified YopB DNA vaccine (~1:8000).

The tPA-dPla DNA vaccine, which removed the N-terminal hydrophobic region, was clearly able to induce positive anti-Pla IgG responses (Fig. 6D) even though the final titers were still relatively low (~1:900). This low immunogenicity of the Pla DNA vaccine was not completely unexpected given that none of the modified Pla DNA vaccine constructs were able to induce a significant level of secreted Pla protein in supernatant fractions from transfected 293T cells (Fig. 4b). No significant anti-Pla IgG antibody responses were detected from mice immunized with either wt-Pla or tPA-Pla DNA vaccines, as previously reported [32]. It is interesting to note that while the tPA-dPla construct was more immunogenic

than the tPA-Pla construct, they both showed a similar pattern of Pla protein expression. Indeed, this would imply that the N-terminal hydrophobic region had a negative effect on the immunogenicity of Pla protein when delivered via DNA vaccine vector.

YscF-specific antibody responses were produced in mice that received the YscF-2 DNA vaccine (Fig. 6E) but not in those that received the wt.YscF or tPA.YscF DNA vaccines. The results are consistent with the finding that only YscF-2 DNA vaccine had detectable YscF protein expression (Fig. 5B). The YscF-specific IgG titers were 1:4000–1:5000 after three monthly DNA immunizations in YscF-2 immunized mice.

In the current study, both IgG1 and IgG2a subclasses are present in the above sera analysis with the ratio of IgG2a/IgG1 less than 1 (data not shown), as expected based on well-established information in literature that gene gun

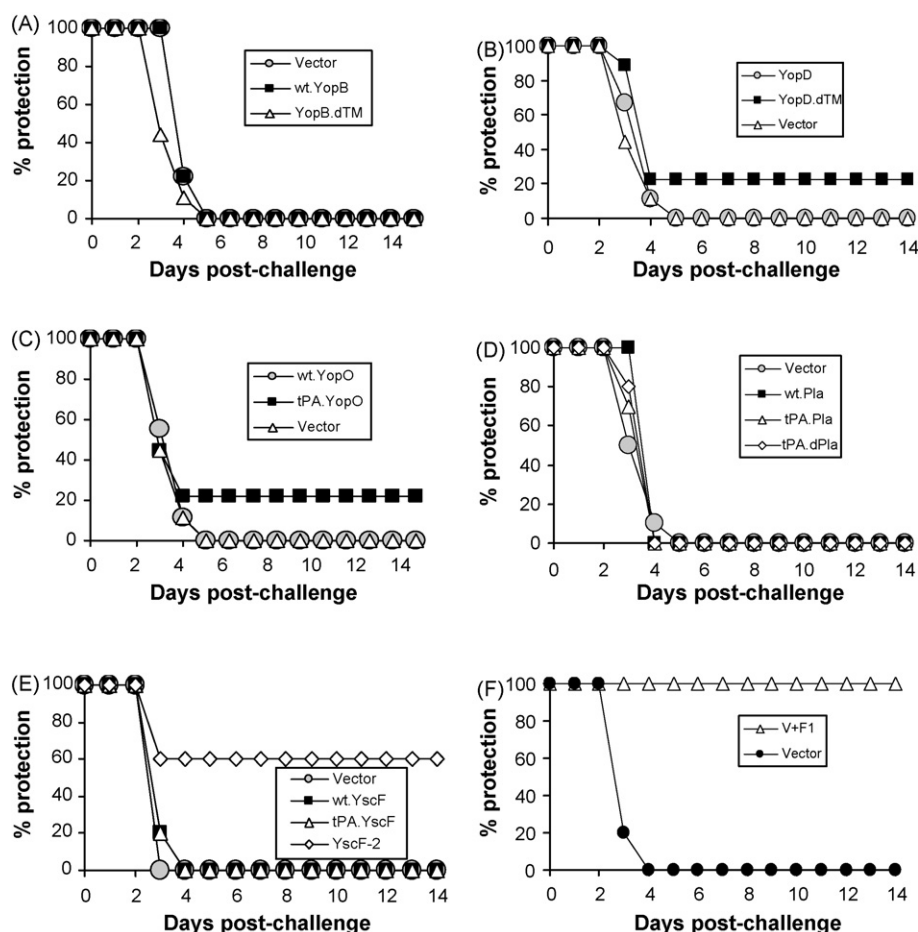


Figure 7 *In vivo* protection of mice immunized with different DNA vaccines, as indicated. Balb/C mice were challenged with a lethal dose of 5000 cfu *Y. pestis* (KIM 1001 strain) by intranasal inoculation 1 week after the 4th DNA immunization. Cumulative survival curves were plotted to show the protection for each group (9 mice/group for Panels A–C and 10 mice/group for Panel D–F). A vector group was included in each plot as the negative control.

mediated DNA immunization mainly induces a Th2 type of antibody responses (IgG2a/IgG1 ratio < 1) [32].

Protection of immunized mice against intranasal challenge with lethal *Y. pestis*

Mice (9–10 mice/group) received another boost with the same DNA vaccines in week 12, and 1 week later, they were challenged with a lethal dose of the KIM 1001 strain of *Y. pestis* at 5000 cfu (~15 LD₅₀), delivered intranasally. Only three DNA vaccines with modified *Y. pestis* gene inserts, YopD.dTM, tPA.YopO and YscF-2, were able to provide partial protection, as measured by survival during 2 weeks' observation (Fig. 7). In YopD.dTM and tPA.YopO immunized groups, 22.2% of mice survived the intranasal challenge. No statistical difference was observed by Fisher's exact test possibly because there was not enough statistical power (9 mice/group) to compare an observed 20–25% difference. In the YscF-2 immunized group, 60% of mice survived the intranasal challenge, which was significant as compared to either wt.YscF or tPA.YscF DNA vaccines according to Fisher's exact test ($p < 0.05$). No protection was observed with the wt.YopD, wt.YscF or tPA.YscF DNA vaccines or any

of the YopB and Pla DNA vaccines (Fig. 7) although wt.YopO, wt.YopD and YopB.dTM DNA vaccines induced good levels of antibody responses (Fig. 6).

The mock DNA immunization group (received empty DNA vector without any *Y. pestis* antigen insert) quickly developed easily observable signs of sickness (e.g., rough coats, hunched or huddled posture, shivering, labored breathing and lethargy) within the first 24–36 h post-challenge and all of them died within 5 days (Fig. 7). Mice immunized with any of the YopB or Pla DNA vaccines did not show any improvement over the mock (empty vector control) DNA immunization group (Fig. 7). The positive control group immunized with the combination of V and F1 DNA vaccines achieved 100% protection (Fig. 7).

Discussion

The current study compared the relative immunogenicity of wild type and modified antigens for three Yop antigens, the Pla antigen and the YscF antigen to demonstrate that DNA vaccines can be used as an effective tool to study the relative immunogenicity of candidate protective antigens, to optimize the design of such antigens and to screen for new

antigens against *Y. pestis*. Overall, YopB, YopD, YopO, Pla and YscF DNA vaccines were not very immunogenic when the wild type gene sequences were delivered by DNA immunization. Production of these antigens in the mammalian expression system is limited by the lack of proper leader sequences and/or by the presence of hydrophobic segments in the middle of their coding sequences. Antigen engineering approaches, such as the use of tPA leader sequences and the removal of intra-polypeptide hydrophobic regions, were effective in improving the expression of coded Yop antigen, resulting in higher levels of antigen expression, with some in secreted form of antigenic proteins.

In general, modified Yop DNA inserts led to better immunogenicity when compared to their wild type counterparts. For YopB, a 41.8 kDa protein with two putative hydrophobic domains and YopD, a 33.3 kDa protein with a 31-amino acid hydrophobic region in the middle of the protein, removal of the hydrophobic regions clearly improved the immunogenicity of these proteins although the modified YopD insert had a similar level of antigen expression as the full-length YopD insert. Modified DNA vaccine inserts also became better protective antigens for YopD and YopO proteins.

Information on the immunogenicity and protection potential of Yop antigens is limited in the literature. Studies that have been conducted show that YopD and YopO are only partially protective against mostly non-capsulated *Y. pestis* strains in a subcutaneous challenge model [28]. In certain cases, the effect of protection can be shown only by extended length of survival but not improved percentage of survival [28]. The current study attempts to link antigen expression, immunogenicity of specific antibody induction and final protection of animals immunized against plague in a mucosal challenge model. Our results demonstrate that antigen engineering is effective in improving antigen expression, immunogenicity and final protection against the Yop proteins that were included in this study. The quality of protection induced by modified YopD or YopO antigens was better than that previously reported as these antigens provided better survival than their wild type antigens against mucosal challenge with a highly virulent *Y. pestis* KIM 1001 strain. However, the current designs are still not sufficient to elicit full protection when used alone.

To our knowledge, this is the first study showing that anti-Pla antibody responses could be induced by DNA immunization. This effect was achieved by removing the N-terminal hydrophobic region, making this strategy different from two previous designs of Pla DNA vaccines that were not immunogenic at all [32]. These results suggest that the N-terminal hydrophobic region for bacterial proteins may not function as a signal peptide leader sequence as it does for many viral proteins. This information is important for the design of bacterial vaccines that are using gene-based (i.e. DNA or viral vector) vaccination approaches for human applications.

YscF is an 7 kDa surface localized protein encoded by the 70 kb plasmid pCD1. The polymerized YscF has been shown to form a needle structure (referred to as an 'injectosome') on the type III secretion system [20,38]. At least six Yop effectors: YopH, YopE, YopM, YpkA, YopK, and YopJ, are delivered by YscF injectosome into target eukaryotic cells. Based on the fact that YscF is a surface-expressed protein

and is required for virulence in *Y. pestis*, it was assumed that YscF might be a candidate as a protective antigen. Recent studies have showed that immunization with a purified YscF protein could induce limited protective immunity against experimental plague infection [22,23]. However, more conventional YscF DNA vaccines with the wild type gene insert and even with the addition of a tPA leader sequence could not induce detectable antibody responses or protective immunity against lethal *Y. pestis* challenge. Interestingly, the YscF-2 DNA vaccine, constructed to produce a YscF dimer protein, induced YscF-specific antibody responses and a significant level of protection (60%). It is known that the natural polymerization of YscF protein forms the injectosome of the type III secretion system [20]. The dimeric designed YscF-2 DNA vaccine might produce antibody responses against the polymerized YscF conformation, which may be critical for the induction of protective immunity by the YscF-DNA vaccine, in addition to overall improved YscF protein expression and immunogenicity. In the current study, we did not test the design with both tPA leader and dimeric YscF-2 components because putting two changes together will be hard to see whether they may interfere with each other. But future studies can include such design to further optimize the immunogenicity of YscF antigen. Results from this current report suggest that antigen conformation is critical for any candidate protective antigen, including those against *Y. pestis*. These results also provide important information to aid in the design of other forms of subunit-based vaccines, including recombinant protein vaccines.

Among the five *Y. pestis* antigens that were included in the current analysis, three antigens (YopD, YopO and YscF) were able to provide only partial protection and induce significant levels of antigen-specific antibody responses above a titer of 1:1000 when optimally designed. Although the modified YopB DNA vaccine induced a high level of antibody responses, it did not protect the mice against intranasal *Y. pestis* challenge. Furthermore, the Pla DNA vaccine induced only low levels of antibody responses and also did not provide any protection against lethal *Y. pestis* challenge. Given the need to identify new protective antigens for improved plague vaccines, the antigen optimization strategy and testing system reported here offers an efficient and reproducible platform to screen for new protective *Y. pestis* antigens. The use of DNA immunization to modify and optimize the immunogenicity of candidate *Y. pestis* antigens can be used to accelerate such an effort. In addition, optimized DNA vaccines expressing *Y. pestis* antigens can also be used as part of a DNA prime-protein boost regimen that may be more immunogenic than the protein alone approach in eliciting high quality protective antibody responses as shown in HIV vaccine studies [39].

Acknowledgements

This work was supported in part by the NIH grant U01 AI 05636 (S Lu). The project also used core facility resources at the University of Massachusetts Medical School supported by NIH grant 5P30DK32520 from the NIDDK. The authors thank Dr. Jill M. Grimes-Serrano for critical reading of the manuscript.

References

- [1] Marshall Jr JD, Cavanaugh DC, Bartelloni PJ, Meyer KF. Plague immunization. 3. Serologic response to multiple inoculations of vaccine. *J Infect Dis* 1974;129(May (Suppl.)):S26–9.
- [2] Marshall Jr JD, Bartelloni PJ, Cavanaugh DC, Kadull PJ, Meyer KF. Plague immunization. II. Relation of adverse clinical reactions to multiple immunizations with killed vaccine. *J Infect Dis* 1974;129(May (Suppl.)):S19–25.
- [3] Williamson ED, Eley SM, Stagg AJ, Green M, Russell P, Titball RW. A sub-unit vaccine elicits IgG in serum, spleen cell cultures and bronchial washings and protects immunized animals against pneumonic plague. *Vaccine* 1997;15(July):1079–84.
- [4] Powell BS, Andrews GP, Enama JT, Jendrek S, Bolt C, Worsham P, et al. Design and testing for a nontagged F1-V fusion protein as vaccine antigen against bubonic and pneumonic plague. *Biotechnol Prog* 2005;21(September–October):1490–510.
- [5] Anderson Jr GW, Leary SE, Williamson ED, Titball RW, Welkos SL, Worsham PL, et al. Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and -negative strains of *Yersinia pestis*. *Infect Immun* 1996;64(November):4580–5.
- [6] Williamson ED, Eley SM, Stagg AJ, Green M, Russell P, Titball RW. A single dose sub-unit vaccine protects against pneumonic plague. *Vaccine* 2000;19(October (4–5)):566–71.
- [7] Elvin SJ, Williamson ED. The F1 and V subunit vaccine protects against plague in the absence of IL-4 driven immune responses. *Microb Pathog* 2000;29(October (4)):223–30.
- [8] Titball RW, Williamson ED. Vaccination against bubonic and pneumonic plague. *Vaccine* 2001;19(July (30)):4175–84.
- [9] Williamson ED, Flick-Smith HC, Lebutt C, Rowland CA, Jones SM, Waters EL, et al. Human immune response to a plague vaccine comprising recombinant F1 and V antigens. *Infect Immun* 2005;73(June (6)):3598–608.
- [10] Benner GE, Andrews GP, Byrne WR, Strachan SD, Sample AK, Heath DG, et al. Immune response to *Yersinia* outer proteins and other *Yersinia pestis* antigens after experimental plague infection in mice. *Infect Immun* 1999;67(4):1922–8.
- [11] Mazza G, Karu AE, Kingsbury DT. Immune response to plasmid- and chromosome-encoded *Yersinia* antigens. *Infect Immun* 1985;48(June (3)):676–85.
- [12] Sodeinde OA, Subrahmanyam YV, Stark K, Quan T, Bao Y, Goguen JD. A surface protease and the invasive character of plague. *Science* 1992;258(November (5084)):1004–7.
- [13] Cornelis GR, Boland A, Boyd AP, Geuijen C, Iriarte M, Neyt C, et al. The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol Mol Biol Rev* 1998;62(December (4)):1315–52.
- [14] Cornelis GR. The *Yersinia* Yop virulon, a bacterial system to subvert cells of the primary host defense. *Folia Microbiol (Praha)* 1998;43(3):253–61.
- [15] Perry RD, Fetherston JD. *Yersinia pestis*—etiologic agent of plague. *Clin Microbiol Rev* 1997;10(1):35–66.
- [16] Hakansson S, Schesser K, Persson C, Galyov EE, Rosqvist R, Homble F, et al. The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *EMBO J* 1996;15(November (21)):5812–23.
- [17] Boland A, Sory MP, Iriarte M, Kerbourch C, Wattiau P, Cornelis GR. Status of YopM and YopN in the *Yersinia* Yop virulon: YopM of *Y. enterocolitica* is internalized inside the cytosol of PU5-1.8 macrophages by the YopB, D, N delivery apparatus. *EMBO J* 1996;15(October (19)):5191–201.
- [18] Torruellas J, Jackson MW, Pennock JW, Plano GV. The *Yersinia pestis* type III secretion needle plays a role in the regulation of Yop secretion. *Mol Microbiol* 2005;57(September (6)):1719–33.
- [19] Hakansson S, Bergman T, Vanootehem JC, Cornelis G, Wolf-Watz H. YopB and YopD constitute a novel class of *Yersinia* Yop proteins. *Infect Immun* 1993;61(January (1)):71–80.
- [20] Cornelis GR. The *Yersinia* Ysc-Yop virulence apparatus. *Int J Med Microbiol* 2002;291(February (6–7)):455–62.
- [21] Hakansson S, Galyov EE, Rosqvist R, Wolf-Watz H. The *Yersinia* YpkA Ser/Thr kinase is translocated and subsequently targeted to the inner surface of the HeLa cell plasma membrane. *Mol Microbiol* 1996;20(May (3)):593–603.
- [22] Swietnicki W, Powell BS, Goodin J. *Yersinia pestis* Yop secretion protein F: purification, characterization, and protective efficacy against bubonic plague. *Protein Expr Purif* 2005;42(July (1)):166–72.
- [23] Matson JS, Durick KA, Bradley DS, Nilles ML. Immunization of mice with YscF provides protection from *Yersinia pestis* infections. *BMC Microbiol* 2005;5(1):38.
- [24] Lahteenmaki K, Kuusela P, Korhonen TK. Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev* 2001;25(December (5)):531–52.
- [25] Lahteenmaki K, Kukkonen M, Korhonen TK. The Pla surface protease/adhesin of *Yersinia pestis* mediates bacterial invasion into human endothelial cells. *FEBS Lett* 2001;504(August (1–2)):69–72.
- [26] Kukkonen M, Korhonen TK. The omptin family of enterobacterial surface proteases/adhesins: from housekeeping in *Escherichia coli* to systemic spread of *Yersinia pestis*. *Int J Med Microbiol* 2004;294(July (1)):7–14.
- [27] Lathem WW, Price PA, Miller VL, Goldman WE. A plasminogen-activating protease specifically controls the development of primary pneumonic plague. *Science (New York, NY)* 2007;315(Jan (5811)):509–13.
- [28] Andrews GP, Strachan ST, Benner GE, Sample AK, Anderson Jr GW, Adamovicz JJ, et al. Protective efficacy of recombinant *Yersinia* outer proteins against bubonic plague caused by encapsulated and nonencapsulated *Yersinia pestis*. *Infect Immun* 1999;67(March (3)):1533–7.
- [29] Yang ZY, Kong WP, Huang Y, Roberts A, Murphy BR, Subbarao K, et al. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. *Nature* 2004;428(April (6982)):561–4.
- [30] Wang S, Chou TH, Sakhatskyy PV, Huang S, Lawrence JM, Cao H, et al. Identification of two neutralizing regions on the severe acute respiratory syndrome coronavirus spike glycoprotein produced from the mammalian expression system. *J Virol* 2005;79(February (3)):1906–10.
- [31] Hooper JW, Thompson E, Wilhelmson C, Zimmerman M, Ichou MA, Steffen SE, et al. Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. *J Virol* 2004;78(May (9)):4433–43.
- [32] Wang S, Heilman D, Liu F, Giehl T, Joshi S, Huang X, et al. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague. *Vaccine* 2004;22(September (25–26)):3348–57.
- [33] Lu S, Wyatt R, Richmond JF, Mustafa F, Wang S, Weng J, et al. Immunogenicity of DNA vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with and without deletions in the V1/2 and V3 regions. *AIDS Res Hum Retroviruses* 1998;14(January (2)):151–5.
- [34] Sodeinde OA, Sample AK, Brubaker RR, Goguen JD. Plasminogen activator/coagulase gene of *Yersinia pestis* is responsible for degradation of plasmid-encoded outer membrane proteins. *Infect Immun* 1988;56(October (10)):2749–52.
- [35] Sodeinde OA, Goguen JD. Genetic analysis of the 9.5-kilobase virulence plasmid of *Yersinia pestis*. *Infect Immun* 1988;56(October (10)):2743–8.
- [36] Wang S, Farfan-Arribas DJ, Shen S, Chou TH, Hirsch A, He F, et al. Relative contributions of codon usage, promoter efficiency

- and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. *Vaccine* 2006;24(May (21)):4531–40.
- [37] Lu S, Manning S, Arthos J. Antigen engineering in DNA immunization. *Methods Mol Med* 1998;29:355–74.
- [38] Swietnicki W, O'Brien S, Holman K, Cherry S, Brueggemann E, Tropea JE, et al. Novel protein-protein interactions of the *Yersinia pestis* type III secretion system elucidated with a matrix analysis by surface plasmon resonance and mass spectrometry. *J Biol Chem* 2004;279(September (37)):38693–700.
- [39] Wang S, Arthos J, Lawrence JM, Van Ryk D, Mboudjeka I, Shen S, et al. Enhanced immunogenicity of gp120 protein when combined with recombinant DNA priming to generate antibodies that neutralize the JR-FL primary isolate of human immunodeficiency virus type 1. *J Virol* 2005;79(June (12)):7933–7.