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Amino acid substitutions in LcrV at putative sites of interaction with toll-like receptor 2 do not affect the virulence of *Yersinia pestis*

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Abstract

LcrV, a component of the type III secretion system (T3SS) translocon in *Yersinia pestis*, has been concerned in suppressing inflammation through Toll-like receptor 2 (TLR2) by inducing expression of the anti-inflammatory cytokine interleukin-10 (IL-10). Previous studies have reported that LcrV aa E33, E34, K42 and/or E204 and E205 were important for interactions with TLR2 in vitro. While, recently there have been conflicting reports doubting this interaction and its importance in vivo. To further investigate the role of these residues, we replaced the wild-type *lcrV* gene on the pCD1Ap virulence plasmid of *Y. pestis* with *lcrV2345* gene, which encodes a mutant protein by substituting all five of the amino acid residues with glutamine. The characteristics of the wild-type LcrV and mutant LcrV2345 were evaluated in tissue culture and mice. When purified protein was incubated with HEK293 cells synthesizing human TLR2 with or without CD14, LcrV2345 induced higher levels of IL-8 than wild-type LcrV, indicating that the LcrV2345 was not impaired in its ability to interact with TLR2. LcrV2345 stimulated higher levels of tumor necrosis factor- α (TNF- α) production than LcrV in J774A.1 cells, while neither protein elicited significant levels of IL-10. We also found there was no statistically significant difference in virulence between strains with wild-type LcrV and with mutated LcrV2345 administered by either subcutaneous or intranasal route in mice. Additionally, there were no discernible differences in survival kinetics. Serum levels of cytokines, such as IL-10 and TNF- α , bacterial burden, and the extent of organ inflammation were also indistinguishable in both strains. Our data confirm that immunomodulation mediated by LcrV/TLR2 interactions does not play a significant role in the pathogenicity of *Y. pestis*.

Keywords

LcrV; virulence and *Yersinia pestis*; pathogenicity

Introduction

Y. pestis is the causative agent of bubonic and pneumonic plague [1]. There is a common 70-kb conserved virulence plasmid in *Y. pestis* (designated pCD1) and the enteropathogenic species *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (designated pYV). Genes on

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these plasmids facilitate the ability of *Yersinia* to overwhelm its mammalian host during systemic growth by evading phagocytosis and inhibiting the inflammatory response [2]. One of them, LcrV is a multifunctional virulence protein encoded on these 70-kb plasmids, which also encode a set of virulent effectors called Yops and the Ysc type III secretion system (T3SS) [2, 3]. In early studies, LcrV was observed to be required by *Y. pestis* to resist phagocytosis [4]. Further, researches show that LcrV plays a role involving in translocation of Yops into host cells through the Ysc type III injection system [2, 3]. LcrV also interacts with the Ysc gate protein LcrG [2, 5] and cooperates with YopB and D for delivering Yops into eukaryotic cells [6]. Additionally, LcrV has immunomodulatory features such as injecting mice with recombinant LcrV results in suppression of TNF- α and interferon gamma (IFN- γ) production and increase of IL-10 level in spleen homogenates [7, 8], and may raise IL-10 production in multiple cell types [9]. IL-10 increase triggered by LcrV also has been demonstrated with a monocyte/macrophage cell lines observed in vitro [10]. It has been observed that recombinant LcrV can inhibit chemotaxis of polymorphonuclear neutrophils (PMNs) [11], and alter host cytokine production as an immunosuppressive agent [12, 13]. Subsequently, these cell-poor lesions spread over the entire liver and spleen, causing organ damage. However, when the mice are immunized with LcrV, inflammatory cells migrate into sites of infection to form protective granulomas and then the bacteria are cleared [12]. Although the detailed immunomodulatory mechanisms of LcrV, its timing during the course of infection, and its relative importance in pathogenesis of plague are not known, there were evidences that the protective capacity of LcrV as a vaccine is based on the fact that anti-LcrV antibodies play roles to neutralize the immunosuppressive effect and/or inhibit Yop translocation [7, 8, 14].

Sing *et al.* demonstrated that a recombinant his-tagged LcrV derived from *Y. enterocolitica* O:8 (LcrVO:8) can interact with TLR2/CD14 to induce IL-10 production which causes TNF- α suppression in macrophages [10, 15, 16]. Short deletions within LcrV of *Y. pestis* [17] and replacement of the invariant lysine residue 42 with glutamine in LcrVO:8 [18] can reduce its immunosuppressive properties. Abramov *et al.* reported that LcrV possessed two non-cooperative binding domains (LEEL_{32–35} and DEEL_{203–205}) capable of recognizing TLR2 as well as human IFN- γ bound to its receptor, IFN- γ R, and demonstrated that both binding domains of LcrV were related with up-regulation of IL-10 and down-regulation of LPS-induced TNF- α [19]. DePaolo *et al.* showed that LcrV can utilize the TLR2/6 pathway to stimulate IL-10 production, which obstructs host protective inflammatory responses [20]. Additionally, report from Khan *et al.* also showed that two LcrV peptides (37–57 and 271–285) stimulated high levels of IL-10 production [21].

However, other studies provided contrary evidences that *Y. pestis* LcrV could not efficiently activate TLR2-signaling and that TLR2-mediated immunomodulation did not play a major role in pathogenesis of plague [22, 23]. The paradoxical results can't be explained well. Additionally, all in vitro experiments performed in those studies use LcrV peptides or purified LcrV, and therefore may not be the real scenario of LcrV in the *Y. pestis* infected host. To attempt to shed some light on this controversy, we tried to investigate the effect of altering the amino acids reported to be important in TLR2 signaling. In this study, we altered the *lcrV* gene of pCD1Ap [24], the *Y. pestis* KIM5+ plasmid pCD1 derivative, in which the codons for glutamic acid residues 33 and 34, the invariant lysine residue 42 and the glutamic acid residues 204 and 205 were replaced with glutamines (E33Q, E34Q, K42Q, E204Q, E205Q) and then evaluated the effects in vivo. Our results showed that the mutant and wild-type strains had similar virulence attributes which further support previous results indicating that the LcrV/TLR2 interactions do not play a critical role in plague [22, 23].

Materials and methods

Bacterial strains, culture conditions and plasmids

All bacterial strains and plasmids used in this study are listed in Table 1. All strains were stored at -70°C in phosphate-buffered glycerol. *E. coli* strains were grown at 37°C in LB broth [25] or LB solidified with 1.2% Agar (Difco) for plasmid construction and replication. Plasmid pCD1Ap was replicate in *E. coli* TOP10 [24]. *Y. pestis* was cultured routinely in heart infusion broth (HIB) or on tryptose-blood agar (TBA) at 26°C [26]. The chemical defined medium PMH2 was used evaluating Yops secretion profiles [24]. Antibiotics were necessarily used at the following concentrations: Ampicilin (AP), $100\text{ }\mu\text{g ml}^{-1}$; chloramphenicol (Cm), $20\text{ }\mu\text{g ml}^{-1}$; tetracycline (Tc), $10\text{ }\mu\text{g ml}^{-1}$.

Plasmid construction

All primers used in this paper are listed in Table 2. The *tet* (tetracycline resistance gene) cassette was cut from pACYC184 using *Ava*I and *Xba*I restriction endonucleases, and blunted by T4 DNA polymerase. Plasmid pKD46 was *Ahd*I and *Pvu*I-digested, blunted by T4 DNA polymerase and dephosphorylated with shrimp alkaline phosphatase (SAP). The two fragments were ligated to form plasmid pYA4678. The wild-type *lcrV* and *lcrV2345* encoding a C-terminal 6×His were amplified from pCD1Ap and pYA4665 using primers *lcrV*-1 and *lcrV*-2 and cloned into the *Nco*I and *Hind*III sites of plasmid pBAD-HisB to form plasmid pYA4836 and pYA4837, respectively. Primer sets *lcrV*-3/*lcrV*-4 and *lcrV*-5/*lcrV*-6 were used for amplifying the '*lcrR-lcrG*' (upstream of the *lcrV* gene) and '*lcrH-yopB*' (downstream of the *lcrV* gene) fragments from plasmid pCD1Ap, respectively. Bold lettering indicates complementarity between primers *lcrV*-4 and *lcrV*-5. The '*lcrR-lcrG*' and '*lcrH-yopB*' fragments were fused by overlapping PCR using primers *lcrV*-3 and *lcrV*-6. The resulting PCR product was digested with *Eco*RI and *Hind*III and ligated with pUC19 digested with the same enzymes to construct the plasmid pYA4769. The *kan* (kanamycin-resistance gene) cassette was cut from pKD4 using the *Xba*I restriction endonuclease, and blunted using T4 DNA polymerase. Plasmid pYA4769 was *Pst*I and *Sac*I-digested, blunted by T4 DNA polymerase and dephosphorylated with SAP. The two fragments were ligated to form plasmid pYA4770. Primer sets *lcrV*-3/*lcrV*-7 and *lcrV*-8/*lcrV*-9 were used for amplifying the '*lcrR-lcrG*' (upstream of the *lcrV* gene) fragment from pCD1Ap and the *lcrV2345* fragment from pYA4665, respectively. Complementarity between primers *lcrV*-7 and *lcrV*-8 are indicated by bold lettering. The '*lcrR-lcrG*' and *lcrV2345* fragments were fused by overlapping PCR using primers *lcrV*-3 and *lcrV*-9. The resulting PCR product was digested with *Hind*III and *Pst*I and ligated with pYA4769 digested with the same enzymes to construct the plasmid pYA4838. The *cat-sacB* fragment was cut from pYA4373 using *Pst*I and *Sac*I restriction endonucleases and ligated to pYA4838 digested with the same enzymes to form plasmid pYA4839. To obtain linear DNA fragments of '*mlcrV-lcrH-yopB*' without any exogenous intervening sequences, primer sets *lcrV*-10/*lcrV*-11 and *lcrV*-12/*lcrV*-6 were used for amplifying the '*lcrV2345*' (partial *lcrV2345* gene) and '*lcrH-yopB*' fragments, respectively. Complementarity between primers *lcrV*-11 and *lcrV*-12 are indicated by bold lettering. The '*lcrV2345*' and '*lcrH-yopB*' fragments were fused by overlapping PCR using primers *lcrV*-6 and *lcrV*-10. The resulting PCR product was digested with *Cl*aI and *Eco*RI and ligated with pYA4838 digested with the same enzymes to construct plasmid pYA4840. DNA sequences were confirmed by nucleotide sequencing at the DNA lab at Arizona State University.

Construction of mutant strains

Replacement of *lcrV* with the *lcrV2345* allele in plasmid pCD1Ap was accomplished using the two-step recombination method [27]. The procedure was as follows: *Y. pestis* KIM5(pCD1Ap) was electroporated with pKD46 derivative pYA4768, cultured at 26°C

with 0.2% arabinose for competent cell preparation. *Y. pestis* KIM5(pCD1Ap, pYA4768) was electroporated with the linear '*lcrR-lcrG-kan-lcrH-yopB*' fragment excised from plasmid pYA4770 using *EcoRI* and *HindIII*. Electroporants were selected on TBA + Kan + Tet plates and verified by PCR. Colonies with the correct PCR profile were made into competent cells, which were electroporated with the linear '*lcrR-lcrG-mlcrV-cat-sacB-lcrH-yopB*' fragment excised from plasmid pYA4839 using *EcoRI* and *HindIII*. Electroporants were selected on TBA + Cam + Tet plates and verified by PCR. Colonies with the correct PCR profile were streaked onto TBA + Cam + sucrose plates to verify sucrose sensitivity. To remove the *cat-sacB* cassette or to construct a *lcrV* deletion, electrocompetent cells were prepared from a sucrose-sensitive isolate and electroporated with approximately 1 µg of a linear DNA ('*lcrV2345-lcrH-yopB*') fragment cut from pYA4840 using *ClaI* and *EcoRI* or a linear DNA '*lcrR-lcrG-lcrH-yopB*' fragment cut from pYA4769. Electroporants were selected on TBA + 5% sucrose plates incubated at 26°C. Colonies were tested using PCR to validate that the *cat-sacB* cassette was eliminated. Plasmid pYA4768 was cured from a single colony isolate of a sucrose-resistant, chloramphenicol-sensitive strain by shifting temperature from 26°C to 37°C. The *lcrR-lcrG-lcrV2345-lcrH-yopB* operon in plasmid pCD1-S2 was confirmed by sequencing.

T3SS analysis and assessment of apoptosis assays

Under BSL2 containment, plasmids pCD1Ap and pCD1-S2 were then introduced by electroporation into χ 10045 (Δ *pla-525* Pgm[−]) for T3SS analysis and *Y. pestis* KIM6 (Pgm[−]) for tissue culture. The procedures for analyzing secreted virulence factors were described previously [28]. BALB/c murine macrophage J774A.1 cells were used for assessment of apoptosis and phagocytosis. Apoptotic cells were detected and quantified by an assay based on the detection of phosphatidylserine exposed on the outer leaflet of apoptotic cells. Cells were stained with fluorescein-conjugated annexin V (Invitrogen, Carlsbad, CA) for the detection of phosphatidylserine. Propidium iodide (PI) (Sigma), the DNA stain, was employed simultaneously to distinguish apoptotic from necrotic cells [29]. Cell apoptosis was examined by fluorescence microscopy and the percentage of apoptotic cells was determined by flow cytometry according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Experiments were performed three times.

Synthesis and purification of LcrV and LcrV2345

Escherichia coli Top10 (Invitrogen, Carlsbad, CA) carrying pYA4836 or pYA4837 was grown overnight at 37°C in LB broth supplemented with 100 µg/ml ampicillin. Bacteria were then diluted into 500 ml of fresh medium to an optical density at 600 nm (OD₆₀₀) of 0.1 and grown at 37°C in a 2 L flask (agitation at 200 rpm) to an OD₆₀₀ of 0.5. Arabinose (0.1%) was then added to induce production of LcrV or LcrV2345, and incubation was continued for an additional 3 h. Cells were harvested by centrifugation at 8,000 × *g* for 10 min, resuspended in 50 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, and broken using an ultra-sonicator on ice. The extract was centrifuged at 12,000 × *g* for 20 min, and the soluble fraction was applied to a nickel nitrilotriacetic acid (Ni-NTA) column (1-ml bed volume) pre-equilibrated with 20 ml column buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 40 mM imidazole, pH 8.0. The column was washed with 15 ml of the same buffer. Bound protein was eluted in buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, and 250 mM imidazole, pH 8.0. Purified His-tagged LcrV or LcrV2345 protein isolated from the nickel column was subjected to Sephadex 200 (Amersham, NJ) gel filtration chromatography to remove contaminating proteins. Endotoxin was removed using a Detoxi-Gel endotoxin removal gel kit (Thermo Scientific, Pittsburgh, PA). Both proteins were determined to be free of endotoxin using the Endotoxin Assay Kit (Genscript, Piscataway, NJ). Protein concentrations were determined by a bicinchoninic acid assay kit (Sigma, St. Louis, MO). Proteins were frozen at −80°C for future use.

Cell stimulation

HEK293 cells stably expressing human TLR2, TLR2/human CD14 TLR2/6 cells were purchased from InvivoGen (San Diego, CA). HEK293-TLR2/6/human CD14 cells were generated by stably expressing human CD14 from the vector pCEP4 [22], selecting for hygromycin resistance and verifying by western blotting. The HEK293 cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 100 IU/ml penicillin and 100 µg/ml streptomycin, with addition of blasticidin (10 µg/ml) for HEK293-TLR2 or blasticidin plus hygromycin (50 µg/ml) for HEK293-TLR2 / human CD14 or TLR2/6/human CD14. The transcription of *tlr2*, *tlr6* and *cd14* was confirmed by RT-PCR (Figure S2 and Table S1). Cells were seeded at 3×10^4 per well in 96-well tissue culture plates (Costar, Washington, DC) and stimulated in triplicate with LPS (100 ng/ml), LcrV and LcrV2345 (5 or 10 µg/ml). Culture supernatants were collected after 18 h of incubation and analyzed by human IL-8 Ready-SET-Go kits (ebioscience, San Diego, CA). LPS, from *E. coli* strain 0111:B4, was purchased from Sigma and was subjected to two rounds of phenol re-extraction to remove contaminating TLR2-stimulating lipoproteins [30].

BALB/c murine macrophage cells (J774A.1, ATCC TIB-67) were cultured in DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, and incubated at 37°C in 5% CO₂. J774A.1 cells were cultured at a density of 5×10^5 cells/ml and stimulated in triplicate with LPS (100 ng/ml), LcrV or LcrV2345 (5 or 10 µg/ml). Culture supernatants were collected after 18 h of incubation and analyzed by IL-10 and TNF-α Ready-SET-Go kits (ebioscience, San Diego, CA).

Virulence studies in mice

Under BSL3 containment, plasmids pCD1Ap and pCD1-S2 were then introduced by electroporation into *Y. pestis* KIM6+ (Pgm+) for animal experiments. Single colonies of each strain were used to inoculate HIB cultures and grown at 26°C overnight. To select for plasmid pCD1Ap and its derivatives, ampicillin was added to the medium at a concentration of 25 µg/ml. Bacteria were diluted into 10 ml of fresh HIB enriched with 0.2% xylose and 2.5mM CaCl₂ to attain an OD₆₂₀ of 0.1 and incubated at 26°C for s.c. infections (bubonic plague) or at 37°C for i.n. infections (pneumonic plague). Both cultures were grown to an OD₆₂₀ of 0.6. The cells were then harvested and the pellet resuspended in 1 ml of isotonic PBS.

All animal procedures were approved by the Arizona State University Animal Care and Use Committee. Female 7-week-old Swiss Webster mice from Charles River Laboratories were inoculated by s.c. injection with 100µl of bacterial suspension or by i.n. administration with 20 µl of bacterial suspension. Actual numbers of colony-forming units (CFU) inoculated were determined by plating serial dilutions onto TBA agar. To determine 50% lethal dose (LD₅₀), groups of six mice were infected with serial dilutions of the bacterial suspension. Mice were monitored twice daily for 21 days, and the LD₅₀ was calculated as described [31].

For colonization/dissemination analysis, 3 mice per time point were infected by s.c. injection in the front of the neck or by i.n. inoculation. At the indicated times after infection, mice were euthanized, and samples of lungs, spleen and liver were removed. The bacterial load for each organ was determined by plating dilutions of homogenized tissues onto TBA with ampicillin plates and reported as CFU per gram of tissue. Infections were performed in at least two independent experiments.

Histopathology

Spleens and lungs were fixed in neutral buffered 10% formalin, and sections were stained with hematoxylin-eosin.

In vivo cytokine analysis

Mice in groups of three were euthanized at intervals after infection by submandibular vein piercing under anesthesia. Blood was allowed to clot overnight at 4°C, and serum was separated by centrifugation at 10,000 x g for 10 min. Sera were filtered once through a 0.22 µm filter, cultured on TBA to confirm that bacteria had been removed and stored at -70°C prior to assay for cytokines. IL-10 and TNF-α concentrations were determined using the BioPlex multiplex assay (Bio-Rad, Hercules, CA). Assays of sera were performed three times.

Statistical analysis

The log rank test was used for analysis of the survival curves. Data are expressed as means ± SD. Log-rank test for trend was used for LD₅₀ analysis. Cytokine values were compared using two-way ANOVA. **P* value of <0.05, ** *P* value of <0.01. *P*<0.05 was considered significant.

Results

The amino acid (aa) substitutions in LcrV do not affect T3SS-related functions of Yops secretion

To determine the relevance of the E33Q, E34Q, K42Q, E204Q, E205Q substitutions for virulence, we replaced the wild-type *lcrV* with *lcrV2345* in pCD1Ap of *Y. pestis* to yield pCD1-S2 (Fig. 1A). Since LcrV is secreted via a T3SS in *Y. pestis* during infection, and mutations that abrogate the bacterial expression of LcrV or the type III secretion machinery are avirulent [4, 32], we anticipated that LcrV2345 in pCD1-S2 retains all other LcrV functions except possible interaction with TLR2. Therefore, we analyzed LcrV content in cells and LcrV and Yops secretion by *Y. pestis* strains carrying either pCD1Ap or pCD1-S2 (Fig. 1B). Results showed that the expression and secretion level of wild-type LcrV and mutant LcrV2345, and the Yop proteins were not significantly different in vitro.

The tissue culture experiments were done for further determining T3SS-related functions. Yops secreted via T3SS are known to cause apoptosis in macrophages [33]. The murine macrophage cell line J774A.1 was infected with KIM6 (pCD1Ap), KIM6 (pCD1-S2) (*lcrV2345*) and a nonpathogenic, virulence-plasmid-cured *Y. pestis* KIM6 strain at a ratio of 50 bacteria per cell. Morphological changes of J774A.1 macrophages were analyzed by phase contrast microscopy following 4 h of incubation. There was no observable difference in morphology between untreated cells (Fig. 2AI), and cells infected with the non-virulent strain KIM6 (Fig. 2BI). In contrast, cells infected with KIM6 (pCD1Ap) or KIM6 (pCD1-S2) were shrunken and condensed, the characteristics of cells undergoing apoptosis (Fig. 2CI, DI). Further, we labeled macrophages with fluorescein-conjugated annexin V, which binds with high affinity to phosphatidylserine exposed on the outer membranes of cells undergoing apoptosis. Cells infected with KIM6 (pCD1Ap) and KIM6 (pCD1-S2) were Annexin V positive (green fluorescence) (Fig. 2CII and DII), indicating that they were undergoing apoptosis. In addition, *Y. pestis*-induced apoptosis of J774A.1 macrophages was examined indirectly by flow cytometry. The *Y. pestis* strain producing LcrV induced 28.2% apoptotic cells and the *Y. pestis* strain producing LcrV2345 induced 28.0% apoptotic cells at 4 h post-infection (Figure S1). Both of these assays suggest that mutant *lcrV2345* and wild-type *lcrV* strains seem to be similarly active and no significant difference of Yop secretion

and Yop translocation are observed as a consequence of the amino acid substitutions in LcrV2345.

The aa substitutions in LcrV improve its ability to interact with TLR2 in vitro

Recombinant LcrV and LcrV2345 were synthesized, purified, and contaminating endotoxin removed as described in Material and Methods. The purity of the recombinant proteins was evaluated by SDS-PAGE and appeared to be free of contaminating proteins (Fig. 3A). Previous reports showed that LcrV could interact with TLR2/CD14 [15]. We thus hypothesized that the aa substitutions in mutant protein LcrV2345 would abrogate this interaction. To test this hypothesis, we compared the ability of LcrV and LcrV2345 to stimulate HEK293 cells stably expressing human TLR2 or TLR2/human CD14. Our results indicate that LcrV could only weakly interact with TLR2 or TLR2/CD14 and stimulate low levels of IL-8 production (Fig. 3B), which is similar to results reported by Reithmeier-Rost *et al.* [23]. In contrast, LcrV2345 strongly stimulated TLR2 or TLR2/CD14, eliciting high levels of IL-8. In both cases, stimulation of IL-8 production was TLR2-dependent (Fig. 3B). For LcrV, this stimulation was enhanced by, but not dependent upon co-expression of CD14. At low concentrations, LcrV2345-mediated stimulation of IL-8 production was enhanced by the presence of CD14, but was CD14-independent at higher concentrations.

DePaolo *et al.* reported that LcrV specifically hijacks the TLR2/6 pathway to stimulate IL-10 production in vivo, which blocks host protective inflammatory responses [20]. This suggested that TLR6 might play a role in LcrV-TLR2/6 interaction. Thus, we measured the ability of LcrV and LcrV2345 to stimulate HEK293 cells with human TLR2/6 or TLR2/6/human CD14 wondering whether similar results happened in vitro. The profile of IL-8 production was similar to that of HEK293 with TLR2 or TLR2/CD14 shown in Fig. 3B (data not shown). These results suggest that TLR6 at least does not play a significant role in stimulating IL-8 production in vitro.

To test whether LcrV or LcrV2345 can activate IL-10 secretion in mammalian cells, purified recombinant proteins were added to J774A.1 cells which can express TLR2, TLR6 or CD14 [34] and incubated for 18 h. Cytokine productions were analyzed by ELISA in cell supernatants. LPS served as a positive control and stimulated significantly greater IL-10 production than PBS-treated cells, whereas the level of IL-10 production by cells stimulated with LcrV or LcrV2345 was similar to the PBS controls (Fig. 3C). In contrast, LcrV2345 induced high levels of TNF- α in J774A.1 cells, similar to LPS controls (Fig. 3D), while the wild-type *Y. pestis* LcrV protein elicited only very low, but statistically significant levels of TNF- α compared with PBS-treated controls. These in vitro results inferred that IL-10 production might be not mediated through *Y. pestis* LcrV/TLR2 or TLR6 interactions in vivo.

Virulence of *lcrV2345 Y. pestis* in mice

To investigate the impact of LcrV2345 on *Y. pestis* virulence, we infected Swiss Webster mice subcutaneously or intranasally with *Y. pestis* KIM6+(pCD1Ap) or *Y. pestis* KIM6+(pCD1-S2). The LD₅₀s of *Y. pestis* KIM6+(pCD1Ap) by s.c. infection was <10 CFU, consistent with previous results [35], and the LD₅₀ of *Y. pestis* KIM6+(pCD1Ap) by i.n. infection was ~100 CFU [36]. Our results indicated that the LD₅₀s of *Y. pestis* KIM6+(pCD1-S2) were similar with that of the wild-type strain by either route of inoculation. Mice inoculated with either *Y. pestis* KIM6+(pCD1-S2) or KIM6+(pCD1Ap) by s.c. or i.n. route succumbed to the infection in a highly synchronous manner, indicating that the mutant protein did not affect the survival kinetics (Fig. 4).

To further evaluate the ability of *Y. pestis* to disseminate to internal organs, we monitored the growth of both *Y. pestis* KIM6+(pCD1Ap) and *Y. pestis* KIM6+(pCD1-S2) in the lungs, spleens and livers of infected mice at various times after s.c. or i.n. infection. We observed that the kinetics of colonization were similar for both strains (Fig. S3). In addition, we compared histopathological changes between mice infected with the *Y. pestis* KIM6+(pCD1Ap) and *Y. pestis* KIM6+(pCD1-S2) mutant. Spleens from mice by s.c. infection at 6 days post-infection (d p.i.) and lungs from mice by i.n. infection at 48 hours post-infection (h p.i.) were removed for analysis. Additionally, *Y. pestis* KIM6+(pCD1Ap) and *Y. pestis* KIM6+(pCD1-S2) induced very similar pathological patterns in lungs and spleen (Fig. S4). By 6 d p.i., mice inoculated s.c. by either strain had severe lymphoid depletion in the white pulp of the spleen, with congestion, edema and fibrin and cellular loss in the red pulp. The spleen was also infiltrated with inflammatory cells. For i.n. inoculation at 48 h p.i., the lungs of mice infected by either strain were inflamed along with extensive hemorrhage, edema, large numbers of neutrophils tightly packed and severe lesions in the lung tissue. These host responses are typical of *Y. pestis* infections [37, 38].

Induction of cytokines by *Y. pestis* KIM6+(pCD1Ap) and *Y. pestis* KIM6+(pCD1-S2) in vivo

Cytokines are critical to the development and functioning of both the innate and adaptive immune responses. They are secreted by immune cells that have encountered pathogens, thereby activating and recruiting additional immune cells to the site of infection. Since in vitro experiments showed that there were some differences between LcrV and LcrV2345 mediated cytokine production (Fig. 3), we compared production of IL-10 and TNF- α in mice infected with *Y. pestis* strains KIM6+(pCD1Ap) or KIM6+(pCD1-S2). For this experiment, groups of three Swiss-Webster mice were inoculated s.c. with 1.35×10^3 CFU of *Y. pestis* KIM6+(pCD1Ap) or 2.16×10^3 CFU of *Y. pestis* KIM6+(pCD1-S2) or i.n. with 1.67×10^4 CFU of *Y. pestis* KIM6+(pCD1Ap) or 2.0×10^4 CFU of *Y. pestis* KIM6+(pCD1-S2). A group of uninfected mice served as controls. Blood was collected via cardiac puncture at 2, 4 and 6 days after s.c. infection or 12, 24 and 48 h after i.n. infection for cytokine analysis. Measurements indicated that levels of IL-10 were increased over time regardless of infection route and the serum IL-10 levels were essentially identical in animals infected with either strain (Fig. S5A, C). Production of the pro-inflammatory cytokine TNF- α remained constant at early time points and did not increase until late in infection (Fig. S5B, D). No differences in TNF- α production were observed in mice inoculated with either strain.

Discussion

Y. pestis has evolved multiple strategies aimed at blocking immune defense mechanisms, eventually leading to the rapid death of the host. One of them, LcrV inducing IL-10 production through interactions with TLR2 [17, 18, 39] and CD14 [15] was proposed to suppress the induction of protective inflammatory responses. The five amino acids (E33, E34, K42, E204 and E205) in LcrV were considered to be related with TLR2 interaction [18, 19]. Here, to examine the potential role of these residues in infection, we modified five of these residues in otherwise intact LcrV to examine their role in vitro, using purified protein to stimulate cells in tissue culture and in vivo using an otherwise *Y. pestis* strain synthesizing the mutant protein, LcrV2345, in mice. While the amino acid substitutions in the mutant protein resulted in a small difference in the isoelectric points (pI) between LcrV (5.58) and LcrV2345 (6.02), our results indicated that the amino acid substitutions in LcrV2345 did not affect its ability to function in *Y. pestis*, since strain carries wild type *lcrV* and mutated *lcrV2345* produced similar amount of LcrV and Yops secretion in vitro (Figure 1B).

There have been conflicting reports concerning the effects of LcrV/TLR2 interactions. Specific residues are required for induction of IL-10 by *Y. enterocolitica* LcrV and peptides containing these residues are effective inducers [15, 18]. Overheim *et al.* reported that regions of *Y. pestis* LcrV (amino acids 181 to 210 and 271 to 300) are important for IL-10 induction [17]. In contrast, Sing *et al.* found that a peptide from the cognate region (amino acids 31 to 66) of *Y. pestis* and *Y. pseudotuberculosis* LcrV has little activity [18]. In our experiments, LcrV did not induce IL-8 production through TLR2 or TLR2/TLR6 in vitro, while LcrV2345 was quite active (Figure 3B). In addition, our results showed that both LcrV and LcrV2345 were poor inducers of IL-10 in J774.1 cells (Fig. 3C), similar to results reported by Reithmeier-Rost *et al.* that LcrV from *Y. pestis* stimulated low level IL-10 in the supernatant of Bone-marrow derived macrophages (BMDM) from C57BL/6. Surprisingly, TNF- α induction by *Y. pestis* LcrV in J774.1 cells was dramatically lower than that achieved by LcrV2345 (Figure 3D). Both proteins were determined to be free of endotoxin, so it is unlikely that the results we obtained with LcrV2345 were due to endotoxin contamination. Furthermore, purified LcrV2345 did not stimulate IL-10 in J774A.1 cells, in contrast to purified LPS (Fig 3C). Similar results were obtained in Human THP-1 cells (ATCC TIB-202), a human monocytic cell line (data not shown). The phenomenon happened in vitro experiments need to be further investigated.

Our animal experiments also demonstrated that the strain synthesizing LcrV2345 exhibited wild-type characteristics with respect to virulence, bacterial dissemination, histopathological changes and cytokine induction. This was interesting for several reasons. First, if the LcrV2345 protein resulted in a strain deficient in IL-10 induction, as we originally hypothesized, then we would have expected a reduction in virulence or other measures of pathology. Since no changes were observed, all animal results support our in vitro experiments indicating that LcrV is not directly mediating immunosuppression, as judged by IL-10 production (Fig. 3C). Second, the ability of LcrV2345 to induce production of proinflammatory cytokines in vitro was apparently unable to overcome *Y. pestis* pathogenesis in vivo (Fig. 3D), which one might expect to result in a loss of virulence. In our previous study, we constructed a similar *lcrV* mutant gene, *lcrV5214*. This gene was codon-optimized for expression in *Salmonella* but encoded a protein with the same 5 aa substitutions as *lcrV2345* (E33Q, E34Q, K42Q, E204Q E205Q). Mice immunized with a recombinant attenuated *Salmonella typhimurium* strain expressing *lcrV5214* produced high titers of serum antibodies against LcrV [40], similar to titers we obtained using an attenuated *Salmonella* vector to deliver a codon-optimized native LcrV (unpublished results). Immunized mice were partially protected against intranasal, but not subcutaneous challenge with virulent *Y. pestis* CO92 [40]. The reason was unknown, this result suggests one or more of the 5 aa residues in LcrV may play a role in pathogenesis, but are unlikely to be involved in immunosuppression. These results also indicate that the capacity of *Y. pestis* to suppress the host immune system is profound and is not mediated by the residues we modified in LcrV and, most likely not by LcrV at all.

Our results are in agreement with previous studies showing that IL-10 induction in macrophages occurs independently of LcrV/TLR2 interaction [41]. However, it is clear that IL-10-deficient mice are resistant to virulent *Y. pestis* [42], indicating an important role for IL-10 induction in pathogenesis. Recent reports showed that TLR2 recognized bacterial lipoproteins [43, 44] which could induce inflammatory (IL-6 and IL-1 β) and anti-inflammatory (IL-10) cytokines [45], suggesting that there may be certain *Y. pestis* lipoproteins that mediate IL-10 production. Alternatively, the YopJ protein, but not the LcrV protein, of *Y. pseudotuberculosis* can induce immunosuppressive IL-10 [41]. *Y. pestis* YopJ (100% homological to *Y. pseudotuberculosis* YopJ) which has capability to suppress production of TNF- α , also appears to be important for mediating immunosuppression [46], but currently no report shown that whether *Y. pestis* YopJ could induce immunosuppressive

IL-10. Therefore, it is likely that one or more other proteins, but not LcrV, are responsible for mediating immunosuppressive IL-10 production in *Y. pestis*-infected hosts. Further studies will be needed to explore the mechanism(s).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ap	ampicillin
Ap^r	ampicillin resistance
Cam	chloramphenicol
Kan	kanamycin
Tet	tetracycline

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Highlights

- We construct of LcrV mutant protein, LcrV2345 (E33Q, E34Q, K42Q, E204Q E205Q), in which five amino acid residues demonstrated or postulated to be important for interactions between LcrV and TLR2 were replaced with glutamine.
- We replace the wild-type *lcrV* gene on the pCD1Ap virulence plasmid of *Y. pestis* with *lcrV2345* gene, which encodes a mutant protein by substituting all five of the amino acid residues with glutamine.
- Both wild type LcrV and LcrV2345 could not elicit significant levels of IL-10 in J774A.1 cell lines.
- In vitro and in vivo experiments indicate that immunomodulation mediated by LcrV/TLR2 interactions does not play a significant role in the pathogenicity of *Y. pestis*.

Fig. 1.

Schematic plasmid structure of pCD1-S2 and virulence factor synthesis and secretion in *Y. pestis* by western blot. (A) Schematic map of pCD1-S2; (B) Plasmids pCD1Ap and pCD1-S2 were then introduced by electroporation into $\chi 10045$ ($\Delta pla-525$ Pgm⁻) for T3SS analysis under BSL2 containment. Whole cell lysates and supernatant fractions were separated by SDS-PAGE and detected by western blots probed with antiserum against LcrV, YopM, or YopH. For each sample, equivalent amounts of protein were loaded.

Fig. 2.

Morphology and surface phosphatidylserine exposure of J774A.1 macrophages infected with different *Y. pestis* strains. J774A.1 cells were either uninfected (A), infected with the virulence-plasmid-cured strain KIM6 (B), strain KIM6 (pCD1Ap) (C), or strain KIM6 (pCD1-S2) (*lcrV2345*) (D). After 4 h, cells were washed and labeled with FITC annexin V. Cells were analyzed by phase-contrast microscopy for morphology (I) and by fluorescence microscopy for surface phosphatidylserine exposure (II).

Fig. 3.

Protein expression and cell stimulation in vitro

(A) SDS-PAGE of purified LcrV and LcrV2345 (~2 μg each protein was loaded). (B) Stimulation of TLR2 by rLcrV. HEK 293 cells stably transfected with TLR2 alone, or both TLR2 and CD14 were stimulated with PBS, 100 ng/ml LPS, various concentrations of LcrV (2 μg/ml, 4 μg/ml, 5 μg/ml or 10 μg/ml), or LcrV2345 (2 μg/ml, 4 μg/ml, 5 μg/ml or 10 μg/ml), as indicated for 18 h. Supernatants were assayed by capture ELISA for the presence of IL-8 as an indicator of TLR2 activation. Data shown are means from triplicate assays with error bars indicating ranges and are representative of at least three experiments. (C) Induction of IL-10 expression from murine macrophages (J774.A) stimulated with LcrV and LcrV2345. Murine macrophages were incubated with PBS, LPS, LcrV or LcrV2345, and IL-10 levels in supernatant were determined by ELISA after 18 h of incubation. Data were collected from three independent experiments. (D) Induction of TNF-α expression from murine macrophages (J774.A) stimulated with LcrV and LcrV2345. The release of TNF-α after 18 h was determined by ELISA and compared to mock (PBS)-stimulated macrophages. Data were collected from three independent experiments. *, the *P* value less than 0.05, **, the *P* value less than 0.01.

Fig. 4.

Survival of Swiss Webster mice (3 mice per strain) infected either s.c. with KIM6+(pCD1Ap) (*IcrV*) (black circles), KIM6+(pCD1-S2) (*IcrV2345*) (open squares), or i.n. with KIM6+(pCD1Ap) (*IcrV*) (black triangle), KIM6+(pCD1-S2) (*IcrV2345*) (open triangle). The experiment was performed twice with similar results.

TABLE 1

Bacterial strains and plasmids used in this study.

Strains	Relevant genotype or annotation	Source or derivation
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80/ <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>) 7697 <i>galU</i> <i>galK</i> <i>tpsL</i> <i>endA1</i> <i>nupG</i>	Invitrogen, Carlsbad, CA
<i>Y. pestis</i> KIM6	Pgm ⁻ , pMT1, pPCP1, cured of pCD1	[47, 48]
<i>Y. pestis</i> KIM6(pCD1Ap)	Pgm ⁻ , pMT1, pPCP1, pCD1Ap	[36]
<i>Y. pestis</i> KIM6+	Pgm ⁺ , pMT1, pPCP1, cured of pCD1	[24]
<i>Y. pestis</i> KIM6+(pCD1Ap)	Pgm ⁺ , pMT1, pPCP1, pCD1Ap	[28]
<i>Y. pestis</i> KIM6+(pCD1-S2)	Pgm ⁺ , pMT1, pPCP1, pCD1-S2	This study
χ10045	Δ <i>pla</i> -525 <i>Y. pestis</i> KIM6	This study
χ10045 (pCD1Ap)	Δ <i>pla</i> -525 <i>Y. pestis</i> KIM6, pCD1Ap	This study
χ10045 (pCD1Ap-S2)	Δ <i>pla</i> -525 <i>Y. pestis</i> KIM6, pCD1-S2	This study
Plasmid		Source
pUC19	For cloning and sequencing	Lab collection
pBAD-HisB	Expressing vector	Lab collection
pACYC184	<i>E. coli</i> plasmid with p15A origin, <i>cam</i> , <i>tet</i>	Lab collection
pKD46	λ Red recombinase expression plasmid, Ap ^r	[49]
pKD4	Template for amplifying <i>kan</i> cassette gene	[49]
pCD1Ap	70.5-kb pCD1 with <i>bla</i> cassette inserted into <i>yadA</i> ' ; 71.7-kb Lcr ⁺ Ap ^r	[24]
pYA4373	The <i>cat-sacB</i> cassette in the <i>Pst</i> I and <i>Sac</i> I sites of pUC18.	[27]
pYA4665	pYA3620 <i>lcrV</i> 5214 with five amino acid replacements (E33Q, E34Q, K42Q, E204Q and E205Q)	[40]
pCD1-S1	The <i>kan</i> cassette replaced <i>lcrV</i> ORF in pCD1Ap; LcrV ⁻ Ap ^r Kan ^r	pCD1Ap
pCD1-S2	The <i>lcrV</i> 2345 gene in pCD1Ap; Ap ^r	pCD1Ap
pYA4768	The <i>tet</i> gene cassette replacing part of ampicillin resistance gene in pKD46	pKD46
pYA4769	The <i>lcrR</i> '- <i>lcrG</i> - <i>lcrH-yopB</i> ' fragment ligated by overlapping PCR cloned into <i>Eco</i> RI and <i>Hind</i> III sites of pUC19.	pUC19
pYA4770	The <i>kan</i> cassette cloned into pYA4769 between the <i>lcrR</i> '- <i>lcrG</i> and <i>lcrH-yopB</i> ' fragments	pYA4769
pYA4836	The <i>lcrV</i> gene encoding a C-terminal 6×His sequence amplified from pCD1Ap and cloned into the <i>Nco</i> I and <i>Hind</i> III sites of plasmid pBAD-HisB	pBAD-HisB
pYA4837	The <i>lcrV</i> 2345 gene encoding a C-terminal 6×His sequence amplified from pYA4665 and cloned into the <i>Nco</i> I and <i>Hind</i> III sites of plasmid pBAD-HisB	pBAD-HisB
pYA4838	The <i>lcrR</i> '- <i>lcrG</i> - <i>lcrV</i> 2345 fragment ligated by overlapping PCR cloned into the <i>Hind</i> III and <i>Pst</i> I sites of pYA4769.	pYA4769
pYA4839	The <i>cat-sacB</i> cassette from pYA4373 ligated into the <i>Pst</i> I and <i>Sac</i> I sites of pYA4838	pYA4838
pYA4840	The <i>lcrV</i> 2345'- <i>lcrH-yopB</i> ' fragment ligated by overlapping PCR cloned into the <i>Cl</i> aI and <i>Eco</i> RI sites of pYA4838	pYA4838

TABLE 2

Oligonucleotides used in this work

Name	Sequence
lcrV-1	<u>cggccatgggcatgattagagctacgaaca</u> (<i>NcoI</i>)
lcrV-2	<u>Cggaagcttt</u> caatgatgatgatggtgtttaccagacgtgtcatctag (<i>HindIII</i>)
lcrV-3 ^a	5' <u>ctcaagcttgcctggcgctagaaattg</u> 3' (<i>HindIII</i>)
lcrV-4 ^b	5' gtggagctcggcagcctcag attaaataattgcctcgcacatcgttgg 3'
lcrV-5 ^b	5' aatctgcaggctgccagctcc acgaggttaattgcaacaagaga 3'
lcrV-6 ^a	5' <u>cgggaattcagttctccgcgactgttgggt</u> 3' (<i>EcoRI</i>)
lcrV-7 ^b	5' gtgggttttgttcgtaggcgcgaatcatattaaataattgcctcgcacatcgttgg 3'
lcrV-8 ^b	5' caacgatgatgcgagggcaaattatttaatatgattcgcgcctacgaacaaaaccac 3'
lcrV-9 ^a	5' <u>agcctgcagtcatttaccagacgtgtcatcgagca</u> 3' (<i>PstI</i>)
lcrV-10	5' <u>accgccgatcgtatcgatgatatttga</u> 3' (<i>Clal</i>)
lcrV-11	5' tcttgtgcataattacctcgtgtcatttaccagacgtgtcatcgag 3'
lcrV-12	5' ctc gatgacacgtctggtaaatgacacgaggttaattatgcaacaaga 3'
lcrV-13	5' ggcttacccaacatggcttggttgcca 3'
lcrV-14	5' ggctggcaaccactgctagtgtgca 3'
Cam-V	5' gttgtccatattggccagttta3'
SacB-V	5' gcagaagagatatttttaattgtggacg 3'

^athe restriction endonuclease sites are underlined

^bthe bold letters show the reverse complementary region