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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*

Wei Sun [a,*], Roy Curtiss III [a,b]

[a] Center for Infectious Disease and Vaccinology, The Biodesign Institute, Arizona State University, P.O. Box 875401, 1001 S. McAllister Avenue, Tempe, AZ 85287-5401, USA
[b] Life Sciences, Arizona State University, 1001 S. McAllister Avenue, Tempe, AZ 85287-5401, USA

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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-alpha (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*.

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1. Introduction

Yersinia 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 these plasmids facilitate the ability of Yersiniae 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 trigged 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

Abbreviations: Ap, ampicillin; Ap[r], ampicillin resistance; Cam, chloramphenicol; Kan, kanamycin; Tet, tetracycline.

* Corresponding author. Tel.: +1 480 727 0497; fax: +1 480 727 0599.
E-mail address: wei.sun.1@asu.edu (W. Sun).

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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 $DEEI_{203-205}$) capable of recognizing TLR2 as well as human IFN-γ bound to its receptor, IFN-γR, 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 cannot 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].

2. Materials and methods

2.1. 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. *Escherichia 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 μg ml^{-1}; chloramphenicol (Cm), 20 μg ml^{-1}; tetracycline (Tc), 10 μg ml^{-1}

2.2. 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 lcrV2 and cloned into the *Nco*I and *Hin*dIII sites of plasmid pBAD-HisB to form plasmid pYA4836 and pYA4837, respectively. Primer sets lcrV-3/lcrV-4 and lcrV5/lcrV-6 were used for amplifying the '*lcrR-lcrG*' (upstream of the *lcrV* gene) and '*lcrH-*

Table 1
Bacterial strains and plasmids used in this study.

Strains	Relevant genotype or annotation	Source or derivation
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr–hsdRMS–mcrBC*) φ80 *lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara–leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen, Carlsbad, CA
Y. pestis KIM6	Pgm$^-$, pMT1, pPCP1, cured of pCD1	[47,48]
Y. pestis KIM6 (pCD1Ap)	Pgm$^-$, pMT1, pPCP1, pCD1Ap	[36]
Y. pestis KIM6+	Pgm$^+$, pMT1, pPCP1, cured of pCD1	[24]
Y. pestis KIM6+ (pCD1Ap)	Pgm$^+$, pMT1, pPCP1, pCD1Ap	[28]
Y. pestis KIM6+ (pCD1-S2)	Pgm$^+$, pMT1, pPCP1, pCD1-S2	This study
χ10045	Δ*pla-525 Y. pestis* KIM6	This study
χ10045 (pCD1Ap)	Δ*pla-525 Y. pestis* KIM6, pCD1Ap	This study
χ10045 (pCD1Ap-S2)	Δ*pla-525 Y. pestis* KIM6, pCD1-S2	This study
Plasmid		Source
pUC19	For cloning and sequencing	Lab collection
pBAD-HisB	Expressing vector	Lab collection
pACYC184	*E. coli* plasmid with p15A origin, *cam, tet*	Lab collection
pKD46	λ Red recombinase expression plasmid, Ap^r	[49]
pKD4	Template for amplifying *kan* cassette gene	[49]
pCD1Ap	70.5-kb pCD1 with *bla* cassette inserted into *yadA'*; 71.7-kb Lcr$^+$ Ap^r	[24]
pYA4373	The *cat-sacB* cassette in the *Pst*I and *Sac*I sites of pUC18.	[27]
pYA4665	pYA3620 *lcrV5214* with five amino acid replacements (E33Q, E34Q, K42Q, E204Q and E205Q)	[40]
pCD1-S1	The *kan* cassette replaced *lcrV* ORF in pCD1Ap; LcrV$^-$ Ap^r Kan^r	pCD1Ap
pCD1-S2	The *lcrV2345* gene in pCD1Ap; Ap^r	pCD1Ap
pYA4768	The *tet* gene cassette replacing part of ampicillin-resistance gene in pKD46	pKD46
pYA4769	The *lcrR'-lcrG-lcrH-yopB'* fragment ligated by overlapping PCR cloned into *Eco*RI and *Hin*dIII sites of pUC19.	pUC19
pYA4770	The *kan* cassette cloned into pYA4769 between the *lcrR'-lcrG* and *lcrH-yopB'* fragments	pYA4769
pYA4836	The *lcrV* gene encoding a C-terminal 6×His sequence amplified from pCD1Ap and cloned into the *Nco*I and *Hin*dIII sites of plasmid pBAD-HisB	pBAD-HisB
pYA4837	The *lcrV2345* gene encoding a C-terminal 6×His sequence amplified from pYA4665 and cloned into the *Nco*I and *Hin*dIII sites of plasmid pBAD-HisB	pBAD-HisB
pYA4838	The *lcrR'-lcrG-lcrV2345* fragment ligated by overlapping PCR cloned into the *Hin*dIII and *Pst*I sites of pYA4769.	pYA4769
pYA4839	The *cat-sacB* cassette from pYA4373 ligated into the *Pst*I and *Sac*I sites of pYA4838	pYA4838
pYA4840	The *lcrV2345'-lcrH-yopB'* fragment ligated by overlapping PCR cloned into the *Cla*I and *Eco*RI sites of pYA4838	pYA4838