Evaluation of the Role of LcrV–Toll-Like Receptor 2-Mediated Immunomodulation in the Virulence of *Yersinia pestis*

Kimberly Pouliot, Ning Pan, Shixia Wang, Shan Lu, Egil Lien, and Jon D. Goguen

Department of Molecular Genetics and Microbiology and Division of Infectious Diseases, Department of Medicine, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655

Received 11 October 2006/Returned for modification 26 November 2006/Accepted 30 March 2007

Pathogenic members of the *Yersinia* genus require the translocator protein LcrV for proper function of the type III secretion apparatus, which is crucial for virulence. LcrV has also been reported to play an independent immunosuppressive role via the induction of interleukin-10 (IL-10) through stimulation of Toll-like receptor 2 (TLR2). To investigate the LcrV–TLR2 interaction in vitro, His-tagged recombinant LcrV (rLcrV) from *Yersinia pestis* was cloned and expressed in *Escherichia coli* and purified through Ni-nitrilotriacetic acid column chromatography. High concentrations (5 μg/ml) of rLcrV stimulated TLR2 in vitro. Fractionation of rLcrV preparations via gel filtration revealed that only a minor component consisting of high-molecular-weight multimers or aggregates has TLR2 stimulating activity. Dimer and tetramer forms of rLcrV, which constitute the bulk of the material, do not have this activity. To investigate the potential role of LcrV/TLR2 in plague pathogenesis, we infected wild-type and TLR2−/− mice with virulent *Y. pestis*. No discernible difference between the two mouse strains in severity of disease or kinetics of survival after subcutaneous challenge was observed. IL-6, tumor necrosis factor, and IL-10 levels from spleen homogenates; bacterial load; and the extent of inflammation observed in organs from mice infected intravenously were also indistinguishable in both mouse strains. Taken together, our data indicate that the most abundant molecular species of *Y. pestis* LcrV do not efficiently activate TLR2-signaling and that TLR2-mediated immunomodulation is unlikely to play a significant role in plague.

Three members of the *Yersinia* genus are pathogenic for humans. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* cause self-limiting mesenteric lymphadenitis or ileitis. In contrast, *Yersinia pestis* infection results in the highly invasive and often fatal systemic infection known as plague. All three species elaborate a type III contact-dependent secretion system (TTSS) that is essential for virulence and is encoded on closely related plasmids. This apparatus allows delivery of effector molecules directly into host cell cytosol. These intracellular effectors, termed Yops, inhibit phagocytosis, induce apoptosis, and inhibit cytokine induction (4, 29). Three translocator proteins, YopB, YopD, and LcrV, are required for efficient intracellular delivery of the Yops (4). LcrV (V antigen) is a multifunctional protein essential for virulence. It comprises the tip of the injection needle of the TTSS (6, 11, 17) and along with LcrG has a regulatory role in Yop secretion (14). In addition, LcrV has been shown to be one of only two proteins to serve as highly effective vaccine antigens against *Yersinia pestis* (9, 30, 32).

An immunomodulatory role for LcrV has also been proposed (3). In vivo studies have shown that a recombinant *Y. pseudotuberculosis* LcrV-protein A fusion, produced in *Escherichia coli*, suppressed induction of tumor necrosis factor (TNF) and gamma interferon induced by injection of lipopolysaccharide (LPS) into Swiss Webster mice. It also enhanced the severity of disease and the bacterial burden following infection of treated mice with attenuated *Y. pestis*, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* (18). Further studies utilizing recombinant *Y. enterocolitica* LcrV (rLcrV), also produced in *E. coli*, showed suppression of zymosan-induced TNF production in C57BL/6 macrophages (24). It has been proposed that rLcrV interacts with both Toll-like receptor 2 (TLR2) and CD14 to induce interleukin-10 (IL-10) in transfected HEK293 cells (25). Candidate residues responsible for this interaction with TLR2 were identified in the N-terminal region of *Y. enterocolitica* LcrV based on the activity of cognate synthetic peptides (23, 25). Lower activity was detected in similar peptides based on *Y. pestis* and *Y. pseudotuberculosis* LcrV sequences (23). More recently, Overheim et al. (22) showed that His-tagged rLcrV derived from *Y. pestis* and purified from *E. coli* induced IL-10 from murine macrophages and human monocytes. It also suppressed induction of TNF from murine macrophages. That group also showed that deletion of either of two domains within LcrV eliminated stimulation of IL-10 secretion but that deletion of only one of these, near the C terminus of the protein, eliminated both induction of IL-10 secretion and suppression of TNF. Neither of these domains corresponds with, or overlaps the sequence of, the active peptide identified by Sing et al. in the LcrV of *Y. enterocolitica* (23, 25). Overheim et al. (22) also presented evidence that a fragment of LcrV lacking the C terminus, which includes the domain responsible for suppression of TNF, was an effective vaccine against *Y. pestis* at lower doses than unmodified rLcrV.

TLRs participate in many aspects of the host defense against infections (2). Stimulation of TLR2 results in the induction of proinflammatory cytokines such as TNF and IL-6. Like TLR4, the receptor for LPS, TLR2 is also responsible for the release of anti-inflammatory cytokines such as IL-10 and IL-4, al-

---

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655. Phone: (508) 856-2490. Fax: (508) 856-3355. E-mail: jon.goguen@umassmed.edu.

† Published ahead of print on 16 April 2007.
through these are usually detected at a later time than proinflammatory mediators induced simultaneously via the same pathway (1, 21). Curiously, TLR2 has been associated with immunosuppression in microorganisms in addition to Y. enterocolitica (7, 20), suggesting that exploitation of this pathway as a means to evade innate immunity may be a common strategy among pathogens. However, no clear mechanistic basis for a predominantly immunosuppressive effect of TLR2 stimulation has been established.

Here we report that Yersinia pestis-derived rLcrV, purified from E. coli through Ni-nitrilotriacetic acid chromatography, activates TLR2, as has been reported for Yersinia enterocolitica LcrV. However, further purification through gel filtration indicates that only a very high-molecular-weight multimer or aggregate, which comprises a small proportion of total rLcrV, has stimulatory activity. Stimulation with Yersinia pestis LcrV-derived peptides corresponding to stimulatory peptides derived from Yersinia enterocolitica LcrV failed to activate TLR2. In infection experiments, TLR2 deficiency in mice had no significant influence on the course of disease, levels of IL-10, or degree of inflammation in infected tissue. Taken together, these results strongly suggest that TLR2-mediated induction of IL-10 does not contribute significantly to the virulence of Yersinia pestis.

MATERIALS AND METHODS

Bacterial strains, plasmids, cell lines, and reagents. Virulent Y. pestis strain KIM1001 (27), biotype mediasi, was grown and purified on solid medium (TB) containing 10 g Bacto-trypose, 5 g NaCl, 3 g beef extract (paste form; Difco catalog no. 212610), and 15 g agar per liter and supplemented with 2.5 mM CaCl2. Although this composition is identical to that given for precompounded TB (containing 10 g Bacto-tryptose, 5 g NaCl, 3 g beef extract (paste form; Difco), 5 g CaCl2, 2.5 g NaH2PO4, 300 mM NaCl, and 10 mM imidazole, pH 8), Endotoxin-free PBS (Cambrex) was used for column equilibration and the following size exclusion chromatography on G25 Sephadex to remove Triton X-114 phase separation and the following size exclusion chromatography on G25 Sephadex to remove Triton X-114 phase separation and the following size exclusion chromatography on G25 Sephadex to remove Triton X-114 phase separation and the following size exclusion chromatography on G25 Sephadex to remove Triton X-114 phase separation.

Analysis of purified rLcrV treated with 5 mM dithiothreitol (DTT) by liquid chromatography-mass spectroscopy yielded a mass consistent with removal of the signal sequence; no material corresponding to the full-length precursor form was detected.

(ii) Expression and purification from plasmid prLcrV. Plasmid prLcrV was transformed into BL21(D3E) and protein production was induced, and the rLcrV was extracted and purified as described by Overheim et al. with the following modifications: (i) rather than sonication, a French pressure cell was used to disrupt the bacteria as described above, and (ii) Triton X-114 phase separation and the following size exclusion chromatography on G25 Sephadex to remove Triton X-114 phase separation and the following size exclusion chromatography on G25 Sephadex to remove Triton X-114 phase separation and the following size exclusion chromatography on G25 Sephadex to remove Triton X-114 phase separation and the following size exclusion chromatography on G25 Sephadex to remove Triton X-114 phase separation.

Mice. Female C57BL/6 mice (6 to 8 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME). TLR2−/− mice were originally a generous gift of S. Akira (28) and have now been back-bred to C57BL/6 for 11 generations. All mice were bred under specific-pathogen-free conditions. All animal studies were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee, and all relevant policies regarding animal care, biosafety, and security were followed.

Experimental infection of mice. To prepare inocula for experimental infection, Yersinia pestis KIM1001 was inoculated heavily onto TB plates from suspensions stored at ~70°C in TB (no agar) plus 5% glycerol and incubated at 37°C for 24 h. Bacteria were harvested from the plate with a loop and resuspended in sterile distilled water with a vortex mixer to match a turbidity standard of an OD600 of 0.3. It was important to use a low-ionic-strength medium for this initial suspension step to achieve good dispersion of the cells. This initial high-density suspension was subsequently diluted as required using endotoxin-free injection-grade PBS for survival experiments, 10 age- and sex-matched mice per treatment group were infected with Yersinia pestis KIM1001 by subcutaneous (s.c.) injection of 50 μl on the nape of the neck. For intravenous (i.v.) infection control groups, five mice per group were injected with 500 μl of inoculum containing the indicated doses in the tail vein. Survival was monitored every 12 h for up to 21 days. All TLR2−/− mice were individually genotyped and bore uniquely numbered ear tags. For collection of organs, mice were sacrificed at 48 h following i.v. infection by pentobarbital overdose followed by cervical dislocation. To deter-
mine bacterial load, spleens were homogenized in 1 ml of PBS and titers of bacteria in the resulting suspension were determined by serial dilution and plating. Differences in survival were analyzed by Kaplan-Meyer survival analysis and the log rank test.

Histopathology. Livers were fixed for in neutral buffered 4% formalin, and sections were stained with hematoxylin-eosin.

Cytokine determination. (i) Cytokine determination from spleens. Spleens were homogenized in 1 ml of PBS. Following removal of a sample for determination of bacterial titer, ciprofloxacin (100-μg/ml final concentration) and protease inhibitor cocktail (Roche catalog no. 11-873-580; 1× final concentration) were added. The suspension was centrifuged at 10,000 × g for 1 min. The resulting supernatant was filtered to remove bacteria (0.2-μm pore, polyvinylidene difluoride; Pall Acrodisc). Cytokines in the resulting supernatant were measured using enzyme-linked immunosorbent assay (ELISA) kits from BD Pharmingen (moIL-10) or R&D Systems (moTNFa and moIL-6) according to the manufacturer’s directions. IL-10 levels were determined twice, each time in triplicate from the same set of tissue samples.

(ii) Cytokine determination from tissue culture. Culture medium was removed from the wells and diluted as required with fresh medium for use in the assays. IL-8 was determined with a kit from R&D Systems (hulIL-8). All cytokine assays were performed in triplicate. The significance of observed differences in median cytokine concentrations were analyzed by the nonparametric Pittman exact test.

NF-κB luciferase reporter assays. Cells were transfected with the NF-κB luciferase plasmid (a gift of Katherine Fitzgerald, University of Massachusetts Medical School) using Genejuice (Promega) as described by the manufacturer. Following stimulation and incubation as indicated, cells were lysed with passive lysis buffer (Promega), and luciferase activity was measured by luminometry following addition of luciferase substrate.

RESULTS

Preparation of Y. pestis rLcrV. The lcrV gene from Yersinia pestis strain KIM1001 was cloned into the expression vector pBADgIIIB (Invitrogen), resulting in pLcrVYp. (See Materials and Methods for a detailed description of the protein produced by this construct.) rLcrV was expressed and purified by nickel chelation chromatography as described in Material and Methods. This method of purification yields primarily dimeric rLcrV, as shown by native PAGE, SDS-PAGE, and gel filtration chromatography. To determine if similar rLcrV preparations derived from the Yersinia pestis rLcrV gene are also able to stimulate TLR2, we transfected HEK293 cells with TLR2 in either the presence or absence of cotransfected CD14 and stimulated them for 18 h with rLcrV purified as described above. As a measure of TLR2-stimulating activity, cell supernatants were assayed by capture ELISA for the presence of IL-8 as an indicator of TLR2 activation. (C) The same cells as for panel B were transfected with an NF-κB luciferase reporter and stimulated with P3C, MALP2, or rLcrV at the indicated concentrations for 18 h. Relative luciferase activity is shown. Data shown are means from triplicate assays with error bars indicating ranges and are representative of at least three experiments.

Yersinia pestis rLcrV and stimulation of TLR2. His-tagged rLcrV derived from Y. enterocolitica (rLcrVYe) has been reported to activate TLR2 in a CD14-dependent manner (25). In these reports, LcrV from Y. enterocolitica was cloned and expressed in E. coli with an N-terminal His6 tag in the vector pQE30 (QIAGEN), and purified by Ni2+ chelation chromatography. To determine if similar rLcrV preparations derived from the Yersinia pestis rLcrV gene are also able to stimulate TLR2, we transfected HEK293 cells with TLR2 in either the presence or absence of cotransfected CD14 and stimulated them for 18 h with rLcrV purified as described above. As a measure of TLR2-stimulating activity, cell supernatants were assayed for IL-8 by capture ELISA. As shown in Fig. 1A, TLR2 is required for induction of IL-8 by rLcrV, and this induction is enhanced by, but not dependent upon, the coexpression of CD14. These observations are similar to the results reported by Sing et al. (25) for rLcrVYe, except that we find enhancement by, rather than a strict requirement for, CD14.

Because Sing et al. relied primarily on an NF-κB reporter construct to indicate TLR2 activation in the HEK293/TLR2/CD14 system, we also examined activation with a similar reporter construct. The results (Fig. 1C) were consistent with observations made using IL-8 release.
Yersinia pestis LcrV synthetic peptides fail to stimulate TLR2. Peptides identical to specific domains of Y. enterocolitica LcrV and capable of stimulating TLR2 have been previously described (23, 25). These are derived from the N-terminal globular portion preceding alpha helix 1 in the LcrV structure (6). A peptide comprising residues 31 to 49, designated V7, was most active. We tested the ability of the synthetic cognate peptide based on the Y. pestis sequence, which differs at single residue (K35→N) from the homologous region in Y. enterocolitica, to activate TLR2. This peptide has identical residues at all of the positions established by Sing et al. (23) to be required for TLR2 activation. As shown in Fig. 1B, this peptide is unable to stimulate HEK293 cells stably transfected with both TLR2 and CD14. Sing et al. (23) observed only weak activity with a longer synthetic peptide comprising residues 31 to 66 of Y. pestis LcrV (23). A peptide comprising residues 27 through 43, corresponding to the inactive peptide designated V5 by Sing et al. (25), was also found to be inactive.

Multimers of rLcrV in purified preparations. It has been previously observed that in solution, rLcrV is not present in significant amounts in monomer form but instead exists primarily as a mixture of dimers and tetramers (29). Dimers can form through disulfide bonds between the single cysteine residue (C273) in each rLcrV molecule (6). To determine the form(s) present in our rLcrV preparation, we analyzed samples by electrophoresis, gel filtration, and immunoblotting. As shown in Fig. 2, the major band of rLcrV seen by PAGE under native conditions migrates at a rate expected of rLcrV tetramer, and the preparation also contains higher-molecular-weight forms. The addition of a reducing agent results in migration consistent with a dimer. Under combined reducing and denaturing conditions, migration is consistent with a monomer.

Analysis of an alternative rLcrV construct. Virtually all studies of the immunosuppressive properties of LcrV are based on recombinant constructs with somewhat different structures. For example, the parent rLcrV fusion protein utilized by Overheim et al. (22) differs from ours in two significant ways: it has an amino-terminal decahistidyl domain as opposed to the carboxy-terminal hexahistidyl domain in our construct,
and it includes the native *Y. pestis* LcrV sequence with no other additions. In contrast, our mature construct has three additional amino-terminal residues (TMV) that precede the native LcrV domain and two additional carboxy-terminal residues (VD) which precede the added polyhistidine sequence. To determine if our finding that TLR2 stimulation is due to high-molecular-weight forms was robust to such differences, we purified and analyzed rLcrV produced by the construct used by Overheim et al. (22). The results of these experiments are shown in Fig. 4. As was found with our construct, all of the TLR2-stimulating activity of this rLcrV form was associated with fractions having much shorter elution times than those for dimers and tetramers. Also, in agreement with results obtained with our construct, almost all of the rLcrV was in the form of dimers and tetramers. No monomer was present. In fact, the preparation analyzed in Fig. 4 contained less high-molecular-
compared the diseases produced in wild-type and TLR2-deficient Y. pestis mice. A dose of 1,000 CFU of Y. pestis strain KIM1001 was uniformly lethal for both C57BL/6 and C57BL/6 TLR2−/− mice following s.c. infection with virulent Y. pestis. A dose of 1,000 CFU of Y. pestis strain KIM1001 was uniformly lethal for both C57BL/6 and C57BL/6 TLR2−/− mice, and no significant difference in mean time of survival was observed (Fig. 5A). Subtle differences in resistance are more readily observed at doses that do not cause complete mortality. Accordingly, we conducted an additional comparison using a dose of 100 CFU, again delivered s.c. (Fig. 5B). This dose yielded 80% mortality in both wild-type and TLR2−/− animals. Again, no significant difference in mean time of survival between mouse genotypes was observed.

Bacterial load and cytokine levels in mice infected with Y. pestis. To determine if the bacterial load and/or the induction of selected cytokines during infection was influenced by the TLR2 status of the mice, we measured the numbers of bacteria and the levels of IL-10, TNF, and IL-6 in the spleens of five mice of each genotype at 2 days following i.v. infection with 1,000 CFU of KIM1001. The i.v. route was used in this experiment because it results in essentially simultaneous infection of internal organs; with s.c. infection, the time of dissemination of the bacteria sometimes varies among the animals. The observed differences in mean bacterial load and IL-6 titers were not statistically significant (Fig. 6C and D). IL-10 levels were about twofold greater (1.07 versus 0.58 ng/ml) in TLR2−/− mice (Fig. 6A), a difference that was statistically significant (P = 0.04) but which is inverse to the result expected if interaction of LcrV with TLR2 elevates IL-10 levels. Despite the enhanced IL-10 levels in TLR2−/− mice, their mean TNF levels were also slightly but significantly higher than those observed in the wild-type controls (34% [621 versus 462 pg/ml]; P = 0.02) (Fig. 6B).

Inflammation at foci of infection. If TLR2-dependent stimulation of IL-10 production makes an important contribution to inhibition of local inflammation, we would expect enhanced inflammation at foci of infection in TLR2−/− mice. This should be readily observable in the livers of infected mice, because there is little inflammatory response to wild-type Y. pestis in this tissue (18, 27) and any enhancement of the inflammation can be readily detected. Accordingly, we harvested livers from the mice at 2 days after infection and examined liver sections to determine the state of inflammation at foci of infection. As
predicted that TLR2<sup>−/−</sup> mice will show (i) enhanced resistance to Y. pestis, (ii) enhanced inflammation at foci of infection, and (iii) decreased IL-10 and enhanced proinflammatory cytokine production during Y. pestis infection. The results of our experiments with TLR2<sup>−/−</sup> mice did not confirm these predictions. We observed no significant differences in survival, mean time to death, or bacterial load; no evidence of enhanced inflammation at foci of infection; and an increase, rather than a reduction, in IL-10 titers. We did observe a slight increase in TNF titer, but this occurred in the presence of enhanced rather than reduced IL-10 levels. The other proinflammatory cytokine measured, IL-6, showed no change. In comparison with survival experiments, small numbers of animals were utilized for bacterial titer and cytokine measurements, and we may have failed to observe modest differences. For example, for bacterial titers and IL-6 titers, the two measurements for which no significant differences were observed, power analysis using a sample size of five and the measured variances of our observations yields an 80% probability of detecting differences of about twofold relative to the mean values, with a correspondingly lower probability of detecting smaller differences. Another limitation of these experiments is that measurements were made at a single time point, 2 days postinfection, and we cannot be certain that our results reflect conditions pertaining at earlier stages of infection. However, both absolute survival and survival kinetics were remarkably consistent at both doses tested, indicating that any influence of TLR2 on the course of infection must be minor. It should be noted that the virulent Y. pestis-mouse infection model is a very sensitive one, in that specific genetic modifications of bacteria or mice often have large effects on virulence. For example, otherwise virulent mutants lacking the Pla protease (27, 31) and mutants with defects in iron acquisition (31) show an increase in 50% lethal dose of several orders of magnitude, as do strains modified to produce highly stimulatory LPS (15). TLR4-deficient mice are highly susceptible to a strain producing stimulatory LPS, while wild-type mice are highly resistant (15). In both of these instances, reduced virulence (or enhanced resistance) was also associated with marked enhancement of inflammation at foci of infection. Thus, it is clear that experimental manipulations in this system do indeed have very large effects when they are related to functional differences in the interaction between the bacteria and host defenses. Our failure to observe any indication of enhanced resistance or improved inflammatory response in TLR2-deficient mice must therefore be regarded as strong evidence that this receptor does not play a significant role in interactions contributing to virulence of Y. pestis during infection. Because we did not carefully examine the progress of infection at multiple time points, it remains possible that there are subtle differences in the development of infection between the genotypes that do not affect survival or time to death.

The literature regarding the induction of IL-10 by the LcrV of the yersiniae presents a somewhat confusing picture. Sing et al. reported that specific residues are required for induction of IL-10 by LcrV of Yersinia enterocolitica and that peptides containing these residues are effective inducers (23, 25). However, they also found that a peptide from the cognate region of Y. pestis and Y. pseudotuberculosis LcrV has little activity (23). On the other hand, Overheim et al. reported that regions of LcrV entirely distinct from that defined by Sing et al. are required...
for IL-10 induction by *Y. pestis* LcrV (22). While Sing et al. provided evidence from a well-established in vivo model supporting a role for the TLR2-LcrV interaction during *Y. enterocolitica* infection (23, 25), these results are unfortunately dependent on the mouse strain employed (26). No similar evidence has been published previously regarding *Y. pestis*. For this species, currently available in vivo data are indirect in that all the relevant experiments involve injection of mice with various forms of rLcrV, followed by measurement of cytokine levels and/or challenge with LPS, attenuated *Y. pestis*, or other unrelated pathogens (18, 19). While the differences in IL-10 levels that we observe are not consistent with the LcrV-TLR2 model (levels were not reduced in TLR2-deficient animals), IL-10 is clearly elevated in mice with well-developed *Y. pestis* infection. Thus, we cannot rule out the possibility that immunosuppression due to elevated IL-10 levels induced by a TLR2-independent mechanism plays an important role in plague. Indeed, it is possible that the unusual combination of proinflammatory stimuli presented by *Y. pestis*, which as we have shown previously activates TLR4 very poorly (15), results in an aberrant cytokine profile that may compromise innate defenses.

A variety of fusion proteins have been used to demonstrate the immunosuppressive properties of LcrV. For example, Overheim et al. utilized an N-terminal decahistidyl tag (22). Motin et al. fused a 34-kDa fragment of protein A to the N terminus of a truncated LcrV lacking the first 67 residues (16). The three-dimensional structure of LcrV was determined from a fusion protein containing five residues fused to an LcrV N terminus beginning at residue 28 and a C-terminal addition of four residues plus a hexahistidyl tag (6). This structure shows that both the N and C termini are very flexible and are located near each other, external to one of the LcrV globular domains. The flexibility and location of these termini are consistent with tolerance for additions and deletions. Thus, it is unlikely that the three-residue N-terminal and the eight residue C-terminal additions (the latter including a six-residue His tag) present in our LcrV construct are less reflective of the properties of native LcrV than those employed by others. Moreover, we have shown directly that the rLcrV protein of Overheim et al. (22) behaves similarly to our own. It should be noted that the cytokine-inducing properties of native LcrV purified from *Y. pestis* or any other *Yersinia* species have not been studied.

Our results with His-tagged *Y. pestis* rLcrV are consistent with those of others in that we do observe stimulation of TLR2 in vitro. However, we also show that the ability of this material to stimulate TLR2 is unexpectedly complex at the biochemical level. The major forms of rLcrV in our preparations, dimer and tetramer, have no TLR2-stimulating activity. Such activity is detected only in high-molecular-weight multimers or aggregates. Although the data from experimental infections discussed above argue strongly against a role for this activity in *Y. pestis* virulence, we consider three alternative hypotheses regarding TLR2 stimulation by LcrV, one of which implies physiological significance.

First, the stimulatory activity may result from the presence of a potent TLR2-activating contaminant (e.g., a lipoprotein, lipopeptide, peptidoglycan, etc.) constituting a small proportion of the aggregate and not from rLcrV per se. Such contamination is both common in material purified from whole-cell extracts and notoriously difficult to exclude. TLR2-stimulating activity initially attributed to what were thought to be highly purified materials has later been shown to result from such contamination (for example, see references 10, 12, and 33). There is no general method to ensure freedom from such contaminants in protein preparations. Note that vulnerability to such contaminants is greatly increased when cells expressing a variety of TLRs, such as mouse macrophages, are the targets of stimulation.

A second possibility is that the stimulatory activity is indeed due to rLcrV in the aggregates/multimers but that this material is nonphysiological and is an artifact of overexpression and purification techniques. The tendency of recombinant proteins expressed at high levels in *E. coli* to form high-molecular-weight aggregates is well established. Exposure of mature rLcrV to cell extracts, as occurs during purification, could also be critical to formation of the stimulatory material. In *Y. pestis* the level of LcrV expression is much lower, and exposure of mature LcrV to concentrated cell extracts does not occur. In this view, the stimulatory aggregates/multimers are purely an in vitro artifact and have no physiologic relevance.

A third possibility is that the stimulatory fractions contain structures that are, or resemble, physiological multimeric species that are normally detected by TLR2 as an indicator of pathogens with TTSS machinery. Micrographs of LcrV arranged at the tips of secretion needles (17) suggest to us an octamer composed of four dimers. Perhaps such structures are occasionally released from bacteria in vivo and elicit proinflammatory responses via TLR2. However, there is no evidence to support this idea from infection experiments with *yersiniae*. Also, PerV, a related protein from *Pseudomonas aeruginosa*, does not stimulate TLR2 (25).

The association of TLR2 stimulation with aggregates/multimers of rLcrV suggests that individual rLcrV molecules may interact weakly with TLR2 and that activation results from clustering of the receptor. This would explain the failure of low-molecular-weight forms to cause activation. Other groups have not reported the molecular sizes of the active fractions in their rLcrV preparations under nondenaturing conditions. Consequently, the presence of a similar stimulatory high-molecular-weight aggregate in their experiments cannot be excluded. The specific activity of our preparations is similar to that reported by others; if the activity that they observe is not due to aggregates, which we find to constitute a small fraction of total protein, then the activity of rLcrV in their preparations on a per-molecule basis must be correspondingly low. The clustering hypothesis suggests that rather than disrupting a specific TLR2-LcrV interaction, mutations which give rise to inactive rLcrV preparations may interfere with formation of aggregates capable of activating TLR2.

Overheim et al. (22) have presented data suggesting that LcrV deletion mutants failing to stimulate IL-10 production are more effective immunogens, presumably due to the elimination of immunosuppressive activity. Our results show that highly purified LcrV preparations containing only dimers and/or tetramers lack TLR2-stimulating activity and hence may also have improved performance in vaccine applications. However, we have previously shown that with DNA vaccines, production of LcrV multimers in vivo was critical to providing an effective protective response and also biased the response
toward TH1 compared with the response produce by constructs producing only rLcrV monomers (30). While we do not
know the extent of multimerization occurring in vivo, the association of multimerization with TLR2 stimulation sug-
gests the possibility that large multimers form in vivo and provide adjuvant activity in the context of the live vaccine
through stimulation of TLR2, rather than immunosuppres-
sion. This adjuvant effect is more consistent with current understanding of TLR2 function than is an immunosuppres-
sive effect.

In summary, our investigation provides no support for the hypothesis that activation of TLR2 by LcrV contributes to the
virulence of *Y. pestis* via immunomodulation. In a sensitive infection model using virulent *Y. pestis*, elimination of TLR2
has no effect on the course of disease and little on cytokine
levels observed in vivo. The bulk of rLcrV protein has no
TLR2-stimulating activity in vitro, and such activity is re-
stricted to high-molecular-weight aggregates/multimers which
contain LcrV but are of undetermined composition. Given
the well-established sensitivity of the *Y. pestis* mouse infection
model, its lack of response to TLR2 deficiency must be re-
garded as strong evidence that TLR2-induced immunomodu-
lation does not have a significant role in plague. The early
observations suggesting a direct immunosuppressive role for
LcrV were based on direct injection of rLcrV preparations into
mice, resulting in immunosuppression and elevated levels of
IL-10 (19). A TLR2-independent mechanism of IL-10 induc-
tion would be consistent with these early observations.

Detailed infection experiments have also been conducted with
*Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by Victoria Auerbuch and Ralph Isberg (2a). They also observed
no differences in the course or pattern of disease, or in cyto-
kine levels, between TLR2-sufficient and -deficient mice. The
conflicting results of Sing et al. imply that LcrV-TLR2-mediat-
ed immunosuppression may operate under certain limited
circumstances (i.e., with specific combinations of *Y. enteroco-
litica* strains and mouse strains), but, given the present
weight of evidence, it is unlikely to be a phenomenon of general
importance to virulence in the yersiniae.

**ACKNOWLEDGMENTS**

We thank Nancy Deitemeyer and Chrono Lee for general technical
assistance and Neil Silverman and Ebbie Lutz for advice regarding
purification of protein with minimal contamination by TLR-activating
receptors as an escape mechanism from the host defense. Trends Microbiol.

*N. T. Perna, D. J. Rose, B. Mau, S. Zhou, D. C. Schwartz, J. D. Fetherston,
immunity to yersiniae mediated by anti-recombinant V antigen and protein

*Mueller, C. A., P. Broz, S. A. Muller, P. Ringer, F. Erno-Brande, I. Sorg,
M. Kuhn, A. Engel, and G. R. Cornelis. 2005. The V-antigen of Yersinia
forms a distinct structure at the tip of injectisome needles. Science 310:
674–676.

*Nakajima, R., V. L. Motin, and R. R. Brubaker. 1995. Suppression of cyto-
kines in mice by protein A-V antigen fusion peptide and restoration of

 polysaccharide mediated by the Yersinia pestis V antigen-polyhistidine fusion

*Netea, M. G., R. Sutmuller, C. Hermann, C. A. A. Van der Graaf, J. W. M.
Van der Meer, J. H. Van Kreiken, T. Hartung, G. Adema, and B. J.
Kullberg. 2004. Toll-like receptor 2 suppressed immunity against Candida
albicans through induction of IL-10 and regulatory T-cells. J. Immunol.

receptors as an escape mechanism from the host defense. Trends Microbiol.

plague vaccine with altered immunomodulatory properties. Infect. Immun.

*Sing, A., D. Reitmeier-Rost, K. Granfors, J. Hill, A. Roggenkamp, and
determines Toll-like receptor 2-mediated IL-10 induction and mouse viru-

enterocolitica evasion of the host innate immune response by V-antigen-
induced IL-10 production of macrophages is abrogated in IL-10 deficient

*Sing, A., J. D. Rost, N. Vardovskaiia, P. Roggenkamp, A. Wiedemann, C. J.
Kirschning, M. Aepfelbacher, and J. Heesemann. 2002. Yersinia V-antigen
exploits Toll-like receptor 2 and CD14 for interleukin 10-mediated immu-

*Sing, A., N. Vardovskaiia, D. Rost, C. J. Kirschning, H. Wagner, and
J. Heesemann. 2003. Contribution of toll-like receptors 2 and 4 to the host
response to *Yersinia enterocolitica* mouse infection model. Int. J. Med.

*Sodeinde, O. A., Y. V. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J. D.
Boggs. 1992. A surface protein on *Yersinia enterocolitica* induces the

*Takeuchi, O., K. Hoshino, T. Kawai, H. Sanio, T. H., T. Ogawa, K. Takeda,
and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of


