A hypervariable N-terminal region of *Yersinia* LcrV determines Toll-like receptor 2-mediated IL-10 induction and mouse virulence

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The virulence antigen LcrV of *Yersinia enterocolitica* O:8 induces IL-10 in macrophages via Toll-like receptor 2 (TLR2). The TLR2-active region of LcrV is localized within its N-terminal amino acids (aa) 31–57. Sequencing of codons 25–92 of the *lcrV* gene from 59 strains of the three pathogenic *Yersinia* species revealed a hypervariable hotspot within aa 40–61. According to these sequence differences, seven LcrV groups were identified, with *Y. pestis* and *Y. pseudotuberculosis* represented in group I and the other six distributed within *Y. enterocolitica*. By testing LcrV sequence-derived synthetic oligopeptides of all seven LcrV groups in CD14/TLR2-transfected human embryonic kidney 293 cells, we found the highest TLR2 activity with a peptide derived from group IV comprising exclusively *Y. enterocolitica* O:8 strains. These findings were verified in murine peritoneal macrophages by using recombinant LcrV truncates representing aa 1–130 from different *Yersinia* spp. By systematically replacing charged aa residues by glutamine in synthetic oligopeptides, we show that the K42Q substitution leads to abrogation of TLR2 activity in both *in vitro* cell systems. This K42Q substitution was introduced in the *lcrV* gene from *Y. enterocolitica* O:8 WA-C(pYV), resulting in WA-C(pYVLcrV$_{K42Q}$), which turned out to be less virulent for C57BL/6 mice than the parental strain. This difference in virulence was not observed in TLR2$^{-/-}$ or IL-10$^{-/-}$ mice, proving that LcrV contributes to virulence by TLR2-mediated IL-10 induction. LcrV is a defined bacterial virulence factor shown to target the TLR system for evasion of the host's immune response.

pathogenesis

The virulence antigen (V-antigen, LcrV) was originally described as the major virulence marker of *Yersinia pestis*, the etiologic agent of plague (1). Later it was demonstrated that the encoding gene *lcrV* is located on the virulence plasmid of *Yersinia* (pYV) common for *Y. pestis* and the related enteropathogenic *Y. pseudotuberculosis* and *Y. enterocolitica*. Additionally to LcrV, pYV encodes the type III protein secretion system (TTSS) and the *Yersinia* outer proteins (Yops), which are translocated as anti-host effector proteins into host cells by the TTSS (2, 3). LcrV is described to be involved in several functions such as regulation of Yops production and TTSS-dependent translocation of Yops into host cells (2–6). Moreover, LcrV probably acts as an extrabacterial chaperone for the YopB/YopD translocation pore (7) and is released into the environment after YopB/YopD pore formation. Previously, LcrV has been demonstrated to be also an immunomodulator (TNF-α and IFN-γ down-regulation and IL-10 induction) both *in vivo* and *in vitro* (4, 8–13). The decisive pathogenic role of LcrV is underlined by the observation that LcrV is protective as a vaccine in different forms of *Yersinia* infection, including plague (8, 14–16).

Recently, we demonstrated that recombinant LcrV of *Y. enterocolitica* O:8 (LcrVO:8) causes TNF-α suppression in macrophages in a CD14- and Toll-like receptor 2 (TLR2)-dependent manner by inducing IL-10 (10–12). This activity is similar to signaling by bacterial lipoprotein, another pathogen-associated molecular pattern (PAMP) sensed by TLR2 (12). Interestingly, despite sharing several functional features with LcrV, such as participation in TTSS regulation and effector protein translocation, the LcrV-homolog protein PcrV from the opportunistic pathogen *Pseudomonas aeruginosa* neither elicits a comparable immunosuppressive capacity in macrophages nor signals via CD14/TLR2 (10, 11). By comparing the amino acid (aa) sequences of LcrV and PcrV, an N-terminal region present in LcrV and lacking in PcrV, was identified. Using synthetic peptides derived from this region [named V7 and V9, (11)], the TLR2-activating region was localized within the N-terminal aa residues 31–57 of LcrVO:8. This region corresponds to the α1-helix, the β-1 strand, and the first half part of a disordered hairpin loop within the recently resolved dumbbell-like structure of *Y. pestis* LcrV (17). A potential role for this TLR2-dependent immunomodulating mechanism in pathogenicity of *Y. enterocolitica* has been suggested, because mice deficient for IL-10 (IL-10$^{-/-}$) or TLR2 (TLR2$^{-/-}$) are resistant to *Y. enterocolitica* infection when compared with wild-type C57BL/6 mice (10, 11). However, because of the multifunctional character of LcrV, the key role of this proposed LcrV-dependent virulence mechanism exploiting both TLR2 and endogenous IL-10 for immune evasion in *Y. enterocolitica* infection cannot be demonstrated by simple deletion of the *lcrV* gene, because this procedure leads to impairment of Yop translocation and consequential loss of virulence. Therefore, a mutagenized *lcrV* gene is required that encodes a LcrV derivative with abrogated TLR2 signaling but maintenance of all other LcrV functions. Here we report on the construction of a *Y. enterocolitica* O:8 mutant encoding a selectively TLR2-inactive LcrV by replacing the invariant lysine residue 42 with glutamine. By comparing the mouse virulence of this mutant with its parental strain in different mouse infection models, we finally prove that the N-terminal region of LcrV, and in particular lysine residue 42, is decisive for TLR2- and IL-10-dependent *Y. enterocolitica* pathogenicity in mice.

Materials and Methods

Bacterial Strains. The *Yersinia* strains analyzed in this study are summarized in Table 2, which is published as supporting informa-

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Abbreviations: aa, amino acid; CFU, colony-forming unit; HEK 293 cells, human embryonic kidney 293 cells; PAMP, pathogen-associated molecular pattern; pYV, virulence plasmid of *Yersinia*; TLR, Toll-like receptor; TTSS, type III secretion system; Yop, *Yersinia* outer protein.

Data deposition: The 59 *Yersinia* LcrV sequences (codons 25–92) reported in this paper have been deposited in the GenBank database (accession nos. DQ016435–DQ016493).

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Table 1. Oligopeptides used in this study

Peptide	Sequence
V7	31 VLEELVQLVKDKKIDISIK 49
V71	31 VLE**Q**LVQLVKDKKIDISIK 49
V72	31 VLEELVQLV**Q**DKKIDISIK 49
V73	31 VLEELVQLVKD**Q**KIDISIK 49
V74	31 VLEELVQLVKDK**Q**IDISIK 49
V75	31 VLEELVQLVKDKKIDISI**Q** 49
I	31 VLEELVQLV**K**DK**N**IDISIKYDP**R**KDSEVFA**N**RVITD 66
II	31 VLEELVQLV**K**DK**K**IDISIKYDP**K**KDSEVFA**N**RVITD 66
III	31 VLEELVQLV**K**DK**K**IDISIKYDP**K**KDSEVFA**D**RVITD 66
IV	31 VLEELVQLV**K**DK**K**IDISIKYDP**K**KDSEVFA**E**RVITD 66
V	31 VLEELVQLV**Q**DK**K**IDISIKYDP**Q**KDSEVFA**D**RVITD 66
VI	31 VLEELVQLV**Q**DK**K**IDISIKYDP**K**KDSEVFA**D**RVITD 66
VII	31 VLEELVQLV**Q**DK**K**IDISIKYDP**Q**KDSEVFA**N**RVITD 66

Sequence differences are indicated with bold letters.

tion on the PNAS web site. The Finnish *Yersinia* strains were isolated at the Department of Medical Microbiology, University of Turku (Turku, Finland). All *Yersinia* strains have been characterized by routine biochemical and serological testing.

Mice. IL-$10^{-/-}$ and C57/BL6 mice were purchased from The Jackson Laboratory. $TLR2^{-/-}$ mice were provided by Tularik (South San Francisco, CA) (11). All mice were bred under specific-pathogen-free conditions. Female mice were used at 6–8 weeks of age. The studies have been reviewed and approved by an institutional review committee.

Recombinant Proteins. rLcrV of *Y. enterocolitica* O:8 (strain WA-314; rLcrVO:8) and *Y. enterocolitica* O:3 (strain Y-108-P; rLcrVO:3) were prepared by using the QIAexpress histidine-tagged protein expression and purification system (Qiagen) as described in refs. 10 and 11. For generation of LcrVO:8 derivatives carrying the substitution K40Q and K42Q, the *lcrV* gene was subjected to site-specific mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene). The recombinant rLcrVO:8 derivatives rLcrVO:8 (K40Q) and rLcrVO:8 (K42Q) bearing an aa exchange at aa position 40 (K40Q) and 42 (K42Q), respectively, and recombinant truncates comprising the 130 N-terminal aa of different LcrVs (LcrVO:8 and LcrVO:3 from *Y. enterocolitica* and LcrV from *Y. pseudotuberculosis*; abbreviated as rLcrVO:$8_{1–130}$, rLcrVO:$3_{1–130}$, and rLcrVpstb$_{1–130}$) were similarly generated as rLcrVO:8 (10, 11). All recombinant proteins were found to be virtually LPS-free as measured by limulus amebocyte assay (Pyroquant, Walldorf, Germany).

Peptides. Synthetic peptides were either provided by Dieter Palm (Physiologische Chemie I, Würzburg, Germany) (20-mer) or purchased from Genosphere Biotechnologies (Paris) (36-mer) (Table 1).

Sequence Analysis. Codons 25–92 of *lcrV* genes from different *Yersinia* strains were sequenced by using primers LcrVF (ATGATTAGAGCCTACGAACAA) and LcrVR (GTTGTCATAATGACCGCCTTTAAG) as described in ref. 16.

Transfections. Cells of a subclone of the human embryonic kidney 293 (HEK 293) cell line (Tularik) were transiently transfected with DNA constructs for CD14, FLAG-tagged TLR2, NF-κB-dependent endothelial cell-leukocyte adhesion molecule 1 luciferase (11), and Rous sarcoma virus-β-gal (for normalizing transfection efficiencies), as described in ref. 11. For more information, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Macrophage Experiments. Proteose peptone elicited peritoneal macrophages from C57BL/6 were prepared as described in refs. 10 and 11. Briefly, 1×10^6 cells per ml were treated for 2 h with the respective recombinant LcrV constructs as indicated. Supernatants were collected for IL-10 measurements by ELISA (R & D Systems).

Construction of $lcrV_{K42Q}$ and $lcrV_{WT}$. The wild-type *lcrV* gene of *Y. enterocolitica* strain WA-C(pYV) was replaced by the mutated *lcrVK42Q* gene (substitution of lysine residue 42 by glutamine residue by using site-specific mutagenesis) by reverse genetics as has been described for the *yadA* gene of the pYV plasmid in ref. 18. Briefly, as a first step, the *lcrV* gene of pYV was disrupted by insertion of a spectinomycin resistance cassette ($Spc^R\Omega$ fragment) by using a derivative of the suicide plasmid pGP704 (denoted as pGP-G2), which carries a chloramphenicol-resistance cassette for positive selection, and the *sacB* gene (sucrose sensitivity for counter selection) (19), resulting in WA-C(pYV*lcrV*::$Spc^R\Omega$). The *lcrV* wild-type gene was mutagenized by site-specific mutagenesis (QuikChange Site-Directed Mutagenesis Kit), resulting in $lcrV_{K42Q}$. Suicide plasmid pGP-G2 (20), carrying the SalI/SacI fragment of the *lcrGVHyopBD* operon and the $lcrGV_{K42Q}H$ fragment, respectively, was constructed and introduced for allelic exchange into WA-C(pYV*lcrV*::$Spc^R\Omega$). By appropriate selection, we obtained the revertant WA-C(pYV*lcrV*) (denoted as strain $lcrV_{WT}$), corresponding to the wild-type strain WA-C(pYV) and the mutant WA-C(pYV$lcrV_{K42Q}$) (denoted as strain $lcrV_{K42Q}$). The resulting strains were checked by PCR and sequencing for the presence of the relevant *lcrV* region. Subsequently, both engineered strains were phenotypically characterized with respect to Yop secretion, phagocytosis resistance in a HeLa cell culture system [with the secretion-deficient *yscV/lcrD* mutant WA-C, named *lcrD*, as positive control for a *Yersinia* strain sensitive to phagocytosis (12, 21)], and induction of apoptosis in the macrophage cell line J774A.1, respectively, as described in refs. 22–24.

Experimental Infection of Mice. i.p. and peroral infection of mice was performed as described in refs. 10 and 11. In survival experiments, mice were observed for 14 days. For IL-10 measurement in organs, spleens and Peyer's patches were dissected and homogenized in HBSS (Invitrogen) containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma) as described in ref. 13. The resulting preparations were centrifuged for clearing and stored at −80°C before IL-10 measurement by ELISA (R & D Systems).

Determination of the Number of Yersiniae in Organs. Spleens and Peyer's patches were dissected and homogenized as described in ref. 10 and 11.

Statistical Analysis. *In vitro* experiments were performed three to eight times. Results are presented as means ± SD. Animal experiments were performed at least twice by using 5–10 animals per group. Statistical analysis was performed by using Student's two-sided *t* test. Differences were considered statistically significant at P values < 0.05.

Results

Sequencing of *lcrV* from Different *Yersinia* Strains Reveals Hotspots of aa Sequence Polymorphism. Initially, we sequenced the *lcrV* genes comprising the codons for the N-terminal aa 25–92 ($lcrV_{25–92}$) from a set of 59 different *Yersinia* strains of the three pathogenic *Yersinia* species to check aa sequence variation in LcrV (Table 2). A comparison of the obtained aa sequences with each other and additionally with published sequences available in the GenBank database (Table 2) revealed that $LcrV_{25–92}$ from six *Y. pestis* strains of the three known biovars (antiqua, medievalis, and orientalis) and

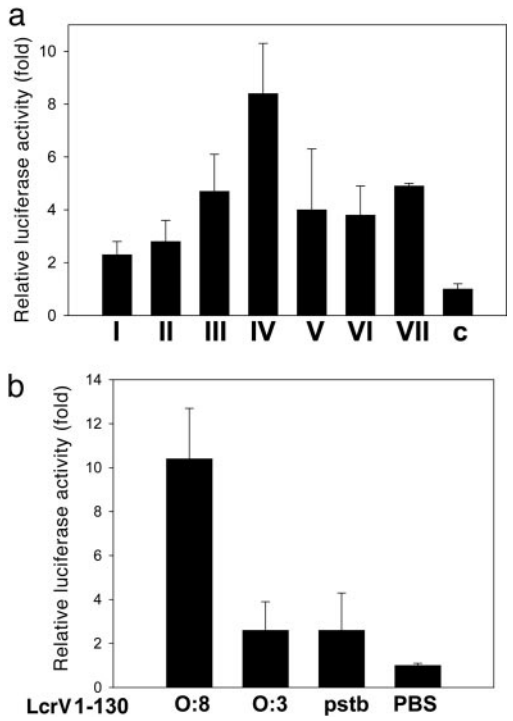

Fig. 1. Variable TLR2-activity in the N-terminal LcrV of different *Yersinia* strains. (*a*) NF-κB-dependent luciferase reporter activity of 36-meric synthetic peptides (20 μg/ml; 5 μM) representing the N-terminal aa 31–66 of LcrV, derived from different *Yersinia* strains in CD14/TLR2-transfected HEK 293 cells. I–VII indicate the respective LcrV groups listed in Table 1, c (control) indicates unstimulated cells. (*b*) NF-κB-dependent luciferase reporter activity in CD14/TLR2-transfected HEK 293 cells after treatment with recombinant proteins comprising the 130 N-terminal aa from *Y. enterocolitica* O:8, O:3, and *Y. pseudotuberculosis* (pstb) (1.9 μg/ml; 135 nM).

eight *Y. pseudotuberculosis* strains of five different serotypes were completely identical. Surprisingly, there were also two *Y. enterocolitica* strains of serotype O:9 carrying a $lcrV_{25–92}$ sequence identical to that from *Y. pestis*/*Y. pseudotuberculosis*. In contrast, significant sequence variations were found among $lcrV_{25–92}$ from different serotypes and biotypes of *Y. enterocolitica*. The hotspots of aa sequence polymorphism were found for aa residues 40, 43, 53, and 61. In summary, seven sequence types of $LcrV_{25–92}$ could be grouped (LcrV I–VII, Table 2).

Variable TLR2 Activity in the N-Terminal LcrV of Different *Yersinia* Strains. Considering this sequence polymorphism, we designed synthetic 36-meric peptides comprising aa 31–66 from the seven groups of LcrV and tested their CD14/TLR2-dependent NF-κB activation capacity in HEK 293 cells transiently transfected with TLR2, CD14, and a NF-κB-dependent luciferase reporter (Fig. 1*a*). Interestingly, the synthetic peptides representing group IV LcrV (LcrV IV) exclusively found in *Y. enterocolitica* O:8 strains induced CD14/TLR2-dependent NF-κB activation to the highest degree, whereas the corresponding peptides from the *Y. enterocolitica* LcrV groups II, III, and V–VII or, in particular, from *Y. pestis*/*Y. pseudotuberculosis* (LcrV I), were significantly less active. In a next step, we tested the CD14/TLR2-dependent NF-κB activation by recombinant-truncated LcrV proteins comprising the 130 N-terminal aa residues of LcrV from *Y. enterocolitica* strain WA-314 (serotype O:8; $rLcrVO:8_{1–130}$), Y-108-P (serotype O:3; $rLcrVO:3_{1–130}$), and *Y. pseudotuberculosis* ($rLcrVpstb_{1–130}$), respectively. $rLcrVO:8_{1–130}$ was significantly more active regarding CD14/TLR2 signaling than $rLcrVO:3_{1–130}$ or $rLcrVpstb_{1–130}$ (Fig. 1*b*). In conclusion, the *lcrV* sequence data and the TLR2-signaling results

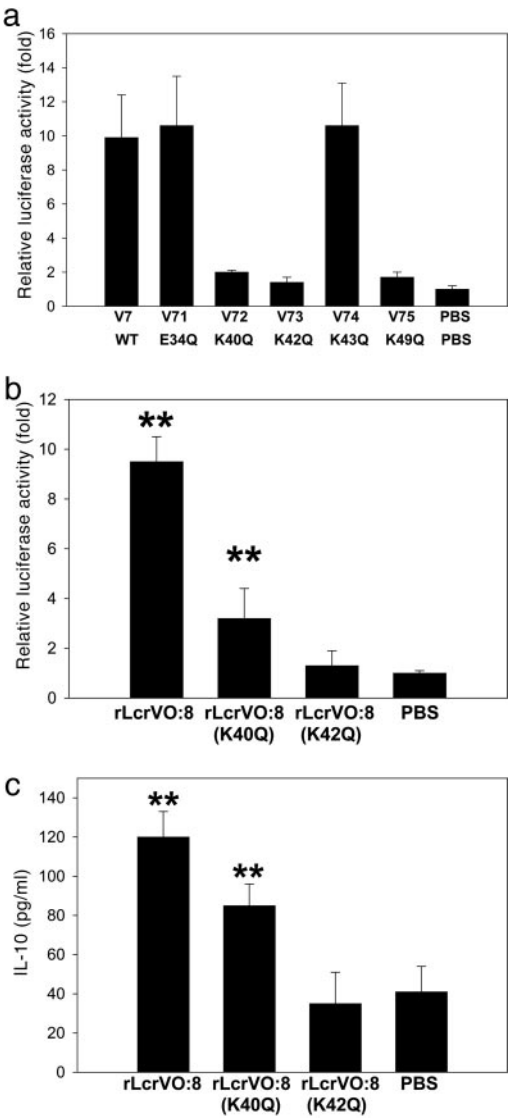

Fig. 2. A single point mutation in the N terminus of LcrV abolishes its TLR2-activity. (*a*) NF-κB-dependent luciferase reporter activity of 20-meric synthetic peptides (10 μg/ml; 5 μM) representing the N-terminal aa 31–49 of *Y. enterocolitica* O:8 strain WA-314 LcrV without or with single aa mutations. (*b* and *c*) NF-κB-dependent luciferase reporter activity in CD14/TLR2-transfected HEK 293 cells (*b*) and IL-10-production by C57BL/6 proteose peptone (*c*) elicited peritoneal macrophages after treatment with recombinant proteins (5 μg/ml; 135 nM), representing wild-type LcrV or LcrV derivates bearing a single point mutation. **, $P < 0.01$ compared with negative controls (PBS).

demonstrate that the immunomodulating capacity of LcrV varies within the group of pathogenic *Yersinia* species and is highest with LcrV of *Y. enterocolitica* O:8.

A Single Point Mutation in the N Terminus of LcrV Abolishes Its TLR2 Activity. Because the synthetic peptide V7 corresponding to aa 31–49 of LcrVO:8 was found to activate a NF-κB-luciferase reporter in a CD14/TLR2-dependent manner (11), we generated related peptides with single substitutions of charged aa residues within aa 31–49 by glutamine (Q) and tested these peptides for their CD14/TLR2-dependent NF-κB activation capacity (Fig. 2*a*). V71 (E34Q) and V74 (K43Q) peptides were as active as the V7 peptide of the wild-type sequence. However, substitution of lysine K40, K42, or K49 with Q strongly attenuated or completely abolished CD14/TLR2 signaling activity. These results prompted us to substitute the

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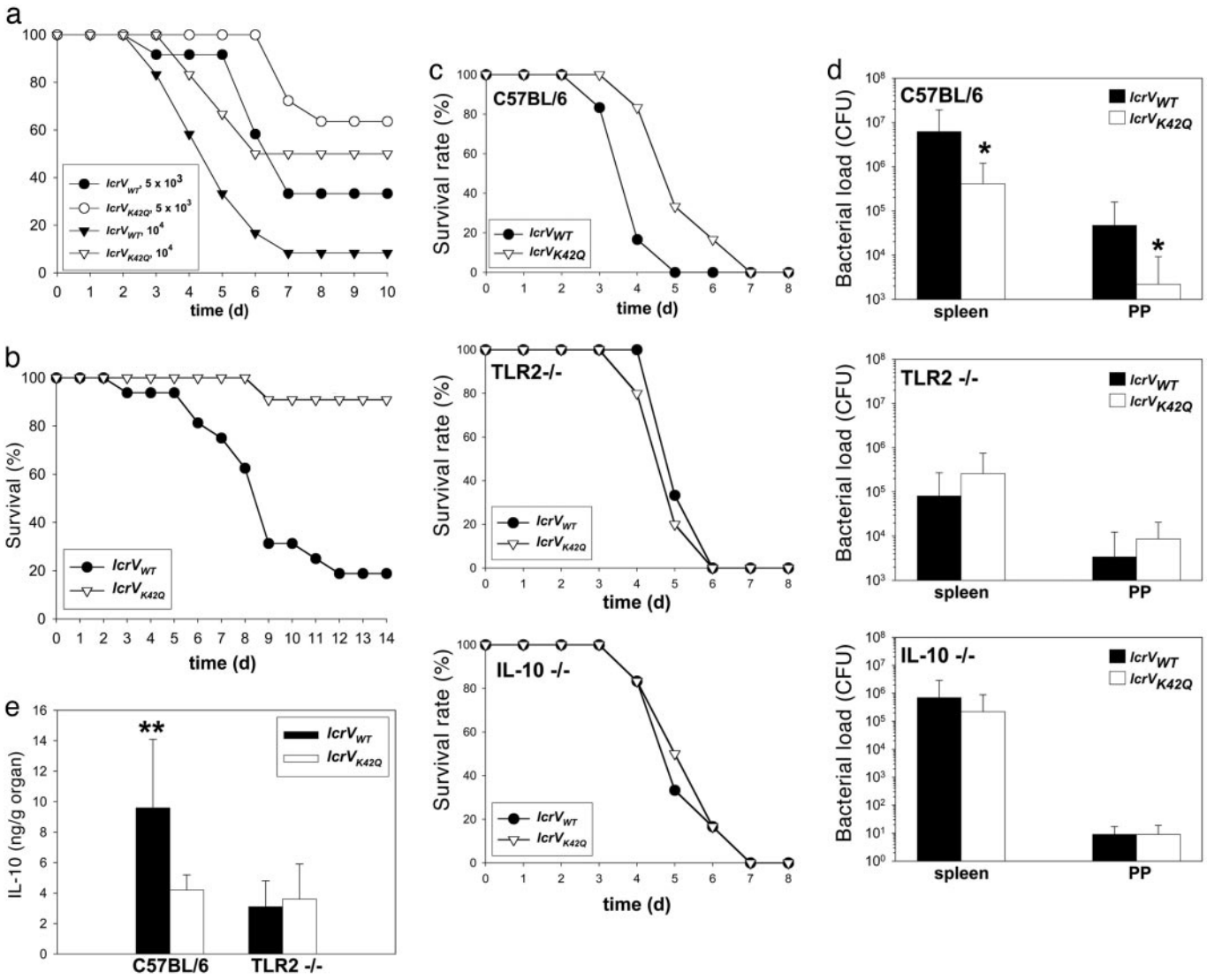

Fig. 3. The K42Q mutation impairs the TLR2-dependent pathogenicity of *Y. enterocolitica*. Survival curves of C57BL/6 mice infected i.p. (5×10^3 and 10^4 CFU; $n = 11$ to 12 mice) (*a*) or orally (10^7 CFU; $n = 16$ mice for $lcrV_{WT}$, $n = 11$ mice for $lcrV_{K42Q}$) (*b*) with $lcrV_{WT}$ or $lcrV_{K42Q}$, respectively. (*c*) Survival curves of C57BL/6 ($n = 12$ per group), TLR2$^{-/-}$ ($n = 10$ per group), and IL-10$^{-/-}$ ($n = 12$ per group) mice infected i.p. (10^5 CFU) with $lcrV_{WT}$ or $lcrV_{K42Q}$, respectively. (*d*) Bacterial load in spleen and Peyer's patches (PP) of C57BL/6 ($n = 21$ per group), TLR2$^{-/-}$ ($n = 11$ per group), and IL-10$^{-/-}$ ($n = 10$ per group) mice 7 days after peroral infection (5×10^8 CFU) with $lcrV_{WT}$ or $lcrV_{K42Q}$, respectively. *, $P < 0.05$ when comparing $lcrV_{WT}$- vs. $lcrV_{K42Q}$-infected mice of the indicated strain. (*e*) Splenic IL-10 production in C57BL/6 ($n = 12$ per group) and TLR2$^{-/-}$ ($n = 10$ per group) mice 7 days after peroral infection (5×10^8 CFU) with $lcrV_{WT}$ or $lcrV_{K42Q}$, respectively. **, $P < 0.01$ when comparing $lcrV_{WT}$- vs. $lcrV_{K42Q}$-infected mice of the indicated strain.

codon for K40 and the invariant K42 of *lcrVO:8*, respectively, by a codon for Q by using site-specific mutagenesis to produce rLcrVO:8 (K40Q) and rLcrVO:8 (K42Q), respectively. As shown in Fig. 2 *b* and *c*, rLcrVO:8 (K40Q) was still able to both signal via CD14/TLR2 in transfected HEK 293 cells and to induce IL-10 in murine C57BL/6 proteose peptone elicited peritoneal macrophages, albeit to a significantly lower degree than wild-type rLcrVO:8, whereas rLcrVO:8 (K42Q) was practically inactive in both assays.

The K42Q Substitution in LcrV Does Not Influence TTSS-Related Effects on Yop Secretion or Translocation. To test the relevance of the K42Q substitution for virulence, we replaced the wild-type *lcrV* by $lcrV_{K42Q}$ in *Y. enterocolitica* O:8 strain WA-C(pYV*lcrV*::Spc$^{R}\Omega$) by reverse genetics, resulting in the $lcrV_{K42Q}$ mutant, and compared this mutant with an analogously constructed *Y. enterocolitica* O:8 revertant expressing wild-type *lcrV* (denoted as $lcrV_{WT}$ strain) in a murine infection model. To rule out that differences in virulence of $lcrV_{K42Q}$ and $lcrV_{WT}$ strains could be assigned to differences in LcrV production and/or TTSS-related functions of the two respective LcrVs, we analyzed LcrV and Yop secretion of $lcrV_{WT}$ and $lcrV_{K42Q}$ by using SDS/PAGE and immunoblotting with anti-LcrV (Fig. 5 *a* and *b*, which is published as supporting information on the PNAS web site) as well as Yop translocation indirectly by examining apoptosis induction in J774A.1 macrophages due to YopP translocation ($lcrV_{WT}$: 40 ± 3% apoptotic cells after 4 h; $lcrV_{K42Q}$: 42 ± 3% apoptotic cells after 4 h) and phagocytosis resistance of $lcrV_{K42Q}$ and $lcrV_{WT}$ with respect to HeLa cells due to YopE and YopH translocation (Fig. 5*c*). In all settings tested, $lcrV_{K42Q}$ and $lcrV_{WT}$ strains were similarly active, suggesting that Yop secretion and Yop translocation are obviously not influenced by the K42Q mutation.

The K42Q Mutation Impairs the TLR2-Dependent Pathogenicity of *Y. enterocolitica*. In a first set of experiments, we compared the virulence of $lcrV_{K42Q}$ and $lcrV_{WT}$ strains in wild-type C57BL/6 mice

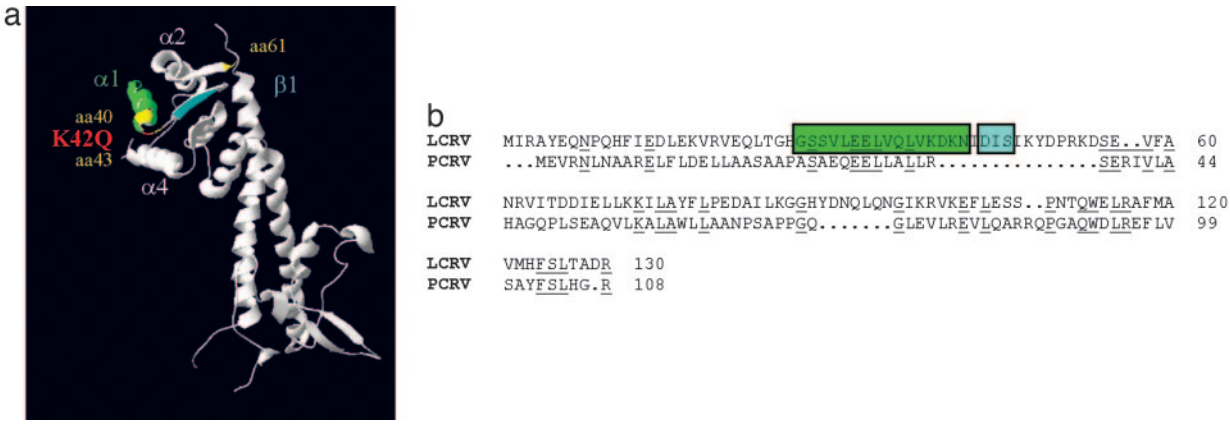

Fig. 4. 3D structure and sequence of *Y. pestis* LcrV. (*a*) The image shows a 3D model of *Y. pestis* LcrV as reported in ref. 17. The α1-helix (green), the β1-strand (turquoise), the site for the K42Q mutation (red), and the hotspots at aa positions 40, 43, and 61 (yellow) are highlighted. The hotspot at aa 53 cannot be shown, because this region revealed no interpretable crystallographic structure. The ribbon diagram was generated with DEEPVIEW PDB. (*b*) Sequence alignment of the N terminus from *Y. pestis* LcrV and *P. aeruginosa* PcrV. The α1-helix and the β1-strand of LcrV according to ref. 17 are colored in green and turquoise, respectively. The sequence alignment was performed with DNAMAN.

after i.p. (i.p. infection dose: 5×10^3 and 10^4 CFU) or peroral (oral infection dose: 10^7 CFU) infection, respectively. Both i.p. and orally $lcrV_{WT}$-infected mice demonstrated substantially increased lethality of more rapid onset than $lcrV_{K42Q}$-infected mice (Fig. 3 *a* and *b*). This result indicates that LcrV harbors a pathogenetically relevant region in its N terminus.

According to the concept that LcrV mediates IL-10 induction in a TLR2-dependent manner, thus protecting yersinae from host defense, one would expect that $lcrV_{WT}$ and $lcrV_{K42Q}$ are equally virulent in TLR2$^{-/-}$ and IL-10$^{-/-}$ mice. TLR2$^{-/-}$ and IL-10$^{-/-}$ mice were found to be less susceptible to *Y. enterocolitica* O:8 infection when compared with isogenic C57BL/6 mice. Correspondingly, both $lcrV_{WT}$ and $lcrV_{K42Q}$ strains did not colonize spleen and liver or cause significant disease in both TLR2$^{-/-}$ and IL-10$^{-/-}$ mice after i.p. (10^4 CFU) or oral (10^7 CFU) infection, respectively. Therefore, we applied higher infection doses (i.p.: 10^5 CFU and oral: 5×10^8) for comparison of mouse virulence of $lcrV_{WT}$ and $lcrV_{K42Q}$ strains in IL10$^{-/-}$, TLR2$^{-/-}$, and C57BL/6 mice. Survival of C57BL/6 mice infected i.p. with $lcrV_{K42Q}$ was significantly extended when compared with $lcrV_{WT}$-infected mice, whereas survival rate curves for both strains did not differ in either TLR2$^{-/-}$ or IL-10$^{-/-}$ mice (Fig. 3*c*). Similarly, orally $lcrV_{WT}$-infected mice exhibited higher bacterial loads in spleen and Peyer's patches than $lcrV_{K42Q}$-infected C57BL/6 mice (Fig. 3*d*); in contrast, bacterial loads did not differ significantly between $lcrV_{WT}$- and $lcrV_{K42Q}$-infected TLR2$^{-/-}$ or IL-10$^{-/-}$ mice, respectively (Fig. 3*d*), thus demonstrating that the attenuating effect of the K42Q substitution in LcrV seen in C57BL/6 mice is absent in both TLR2$^{-/-}$ and IL-10$^{-/-}$ mice.

Finally, we compared the IL-10 content of spleens from TLR2$^{-/-}$ and wild-type C57BL/6 mice infected with $lcrV_{WT}$ or $lcrV_{K42Q}$, respectively. As expected, IL-10 spleen levels were significantly lower in $lcrV_{K42Q}$- than in $lcrV_{WT}$-infected C57BL/6 mice. In contrast, splenic IL-10 levels did not differ between $lcrV_{K42Q}$- and $lcrV_{WT}$-infected TLR2$^{-/-}$ mice (Fig. 3*e*). From this finding, we conclude that the TLR2-active domain of LcrVO:8 is also involved in IL-10-induction and that this effect contributes significantly to mouse virulence of *Y. enterocolitica* O:8.

Discussion

The innate immune system is endowed with a set of TLRs, which are capable to sense different classes of PAMP molecules such as LPS by TLR4 or lipoprotein by TLR2. PAMP sensing by macrophages via TLR4 or TLR2 results in the production and release of pro- and antiinflammatory cytokines. To unravel the role for PAMPs for pathogenicity of a microbe *in vivo*, comparative experimental infections have to be performed by using mutant and isogenic wild-type organisms of both the host (deficient or wild-type for a given TLR) and the microbe (expressing a modified or wild-type PAMP sensed by the respective TLR).

Using this straightforward approach, we verified in this study that LcrV of *Y. enterocolitica* O:8 contributes to mouse pathogenicity via TLR2-mediated IL-10 induction by comparing the $lcrV_{K42Q}$ mutant with strain $lcrV_{WT}$ expressing *lcrV* of the parental strain in two mouse infection models (peroral and parenteral infection).

Because the crystal structure of *Y. pestis* LcrV is now available (17), some interesting structure–function relationships can be discussed with respect to TLR2 signaling (Fig. 4). (*i*) The N-terminal domain of LcrV, which is required for TLR2 signaling, consists of an antiparallel five-helix bundle, a pair of short parallel β-strands (β-1, aa residues 45–47; β-2, aa residues 111–114), and an antiparallel apically disordered hairpin (aa residues 48–65) protruding between helices α-1 (aa residues 28–43) and α-2 (aa 67–79); this region forms a globular structure (17). (*ii*) Previously, we could localize the TLR2-active region to aa 31–57 of LcrV (11), which corresponds to the surface exposed helix α-1, the β-1 strand, and the succeeding first half of the disordered hairpin loop (17). In *Pseudomonas* PcrV, this region is truncated and consists only of the N-terminal portion of helix α-1, which thus may explain the failure of PcrV to be sensed by TLR2. (*iii*) Extending our previous findings on LcrV polymorphisms in different humanpathogenic *Yersinia* spp. and strains (16), we found a polymorphic "hot spot" in the N-terminal region of aa 40–61 (aa 40, 43, 53, and 61), which results in different TLR2-activities. Moreover, peptides corresponding to this N-terminal region from low mouse virulent *Y. enterocolitica* strains of serotype O:3 and O:9 and from highly mouse virulent *Y. pestis*/*Y. pseudotuberculosis* showed less TLR2-activity than those derived from highly mouse virulent *Y. enterocolitica* O:8 strains, which are found mainly in Northern America (New World strains) (25). The LcrV sequence difference might reflect different pathogen-host coevolutionary pathways, because *Y. enterocolitica* O:8 strains are phylogenetically distinct with respect to 16S rRNA sequence (26) and the presence of the high pathogenicity island (HPI) (27). As previously shown, the dominant mouse virulence determinant of *Yersinia* is assigned to the HPI, which is localized on the chromosome and encodes for the yersiniabactin siderophore biosynthesis and uptake system (28, 29). Thus, the polymorphic N-terminal region of LcrV can be considered a immunomodulating factor with moderate contribution to *Yersinia* virulence. One might speculate that differences in the N-terminal aa sequence may lead

to structural changes of helix α-1 and the hairpin, thus explaining the differences in TLR2-activity. (*iv*) Our identification of a TLR2-active region of LcrV within the N terminus fits very well with the crystal structure of *Y. pestis* LcrV, proposing a surface exposed area in this part of the protein (30). (*v*) For crystallization of *Y. pestis* LcrV, it was necessary to substitute aa 40–42 by alanine; thus, the structure of the TLR2-active region of LcrV remains ambiguous (17). Strikingly, in our assays, peptides with the mutation K40Q or K42Q were basically TLR2 inactive. Moreover, the K42Q mutation, replacing an invariant lysine present in all LcrVs from different pathogenic *Yersinia* spp. analyzed so far, leads to a significant attenuation of virulence of *Y. enterocolitica* O:8. These results might have some interesting implications for vaccine design against yersiniae by using recombinant LcrV. Interestingly, a protective antigenic region located between aa 2 and 135 of *Y. pestis* LcrV had previously been identified by vaccination experiments (31). (*vi*) Finally, we found that the N terminus of *Y. pestis*/*Y. pseudotuberculosis* is less TLR2-active than that of *Y. enterocolitica* O:8 strains. Taking into account that a major protective region is situated between aa 135 and 275 in *Y. pestis* LcrV (31), one might speculate that the N-terminal TLR2-interacting domain is less important or active in *Y. pestis*/*Y. pseudotuberculosis* with respect to TLR2-signaling than in *Y. enterocolitica* O:8 strains. The finding of an IL-10-inducing activity in a truncated *Y. pestis* LcrV lacking the 67 N-terminal aa might indicate that different IL-10-inducing regions could exist in LcrV (9). This assumption is supported by the recent study of Overheim *et al.* (32), showing various degrees of IL-10 inducing or TNF-α suppressing capacity in different recombinant LcrV truncates. Recently, an IL-10-independent protective mechanism was identified for anti-LcrV antibodies in murine plague (33). Therefore, anti-LcrV antiserum could have different effects: (*i*) inhibition of IL-10 induction by LcrV, (*ii*) affecting Yop translocation into host cells due to impairing YopB/YopD pore formation (7), and (*iii*) opsonization activity, because LcrV has also been localized to the surface of yersiniae (15).

Taken together, the data of this study verify our previous hypothesis that *Y. enterocolitica* O:8 may use LcrV for IL-10 induction via TLR2, an effect that contributes to *Yersinia* mouse virulence. By constructing the *Y. enterocolitica* mutant $lcrV_{K42Q}$ bearing a mutated LcrV with preserved TTSS regulatory functions, but impaired TLR2 activity, we were able to dissect the importance of indirect TTSS-dependent and direct immunomodulating effects of LcrV. The fact that this *lcrV* mutated strain is attenuated in wild-type C57BL/6, but not in $TLR2^{-/-}$ or $IL\text{-}10^{-/-}$ mice, finally proves that TLR2-dependent IL-10 induction via LcrV is an important pathogenicity mechanism of *Y. enterocolitica* O:8. LcrV is a defined bacterial virulence factor shown to target the TLR system for evasion of the host's immune response.

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