# *Yersinia enterocolitica* Evasion of the Host Innate Immune Response by V Antigen-Induced IL-10 Production of Macrophages Is Abrogated in IL-10-Deficient Mice[1]

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**The virulence-associated V Ag (LcrV) of pathogenic *Yersinia* species is part of the translocation apparatus, required to deliver antihost effector proteins (*Yersinia* outer proteins) into host cells. An orthologous protein (denoted as PcrV) has also been identified in the ExoS regulon of *Pseudomonas aeruginosa*. Additionally, it is known that LcrV is released by yersiniae into the environment and that LcrV causes an immunosuppressive effect when injected into mice. In this study, we demonstrate for the first time that rLcrV, but not PcrV, is capable of suppressing TNF-$\alpha$ production in zymosan A-stimulated mouse macrophages and the human monocytic Mono-Mac-6 cell line. The underlying mechanism of TNF-$\alpha$ suppression could be assigned to LcrV-mediated IL (IL)-10 production, because 1) LcrV induces IL-10 release in macrophages, 2) anti-IL-10 Ab treatment completely abrogated TNF-$\alpha$ suppression, and 3) TNF-$\alpha$ suppression was absent in LcrV-treated macrophages of IL-10-deficient (IL-10$^{-/-}$) mice. The relevance of LcrV-mediated immunosuppression for the pathogenicity of yersiniae became evident by experimental infection of mice; in contrast to wild-type mice, IL-10$^{-/-}$ mice were highly resistant against *Yersinia* infection, as shown by lower bacterial load in spleen and liver, absent abscess formation in these organs, and survival.** ***The Journal of Immunology*****, 2002, 168: 1315–1321.**

The genus *Yersinia* includes three human pathogenic species: *Yersinia pestis*, the etiologic agent of plague; and the enteropathogenic species *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. The pathogenicity of these three species is dependent on the presence of a 70-kb conserved virulence plasmid (pYV). Four groups of components required for virulence are encoded by pYV: 1) a type III protein secretion/translocation apparatus (TTS)[3]; 2) the surface-exposed adhesin YadA, 3) anti-host effector proteins, which are injected into the cytosol of target cells by the TTS apparatus; and 4) proteins released into the environment (1, 2). The injected proteins are known as *Yersinia* outer proteins (Yops), which inhibit or modulate signaling pathways of *Yersinia*-contacted host cells resulting in inhibition of phagocytosis and release of proinflammatory cytokines. YopH acts as a protein tyrosine phosphatase on focal adhesion and TCR complex formation (3, 4); YopE and YopT inactivate the small GTPases Rac and Rho, respectively (5–7); and YopP/YopJ inhibit activation of NF-$\kappa$B and mitogen-activated protein (MAP) kinase activities (8–10).

Because these Yops are injected directly into the host cell cytoplasm by a needle-like apparatus (11), they are not accessible to the Ab immune response. However, protection in mice against yersiniae can be achieved by treatment with Abs against YadA of *Y. enterocolitica* (12) or against the V Ag (LcrV) of *Y. enterocolitica* and *Y. pestis*, respectively (13–16). The V Ag is one of the virulence-associated Ags of *Y. pestis* recognized as early as 50 years ago. LcrV is a secreted protein encoded in the *lcrGVHYopBD* operon located on pYV (17, 18) and thus belongs to the common virulence-associated Ags of the three *Yersinia* spp. Although LcrV has been studied for many decades, it remains one of the most enigmatic proteins involved in *Yersinia* virulence. LcrV appears to be a multifunctional protein in that 1) it is required for regulation of Yop production, 2) it is required for translocation of Yops into host cells (together with YopB and D), and 3) it is capable of forming channels in artificial membranes (19–21). Moreover, it has been demonstrated that treatment of mice with a staphylococcal protein A-*Y. pestis* V Ag fusion peptide results in suppression of TNF-$\alpha$ and IFN-$\gamma$ and in amplification of IL-10 in spleen homogenates (22–24). Evidence is accumulating that V Ag is on one hand associated with the Yop translocation complex that connects the target cytoplasm membrane with the needle apparatus of yersiniae and on the other is released into the environment where it presumably affects cells of the immune system (17, 18). Considering translocated Yops and released V Ag, we would expect a two-process strategy by which yersiniae suppress the immune response: 1) suppression of contacted cells by injected Yops (short distance effect); and 2) suppression of bystander cells by released V Ag (long distance effect). This model would also explain why active or passive immunization with V Ag or anti-V Abs, respectively, has such a high protective effect.

Recently, it has been demonstrated that the opportunistic pathogen *Pseudomonas aeruginosa* is also equipped with a TTS apparatus for secretion/translocation of anti-host effector proteins (25). It is also striking that the TTS apparatus of *P. aeruginosa* is closely related to that of *Yersinia* spp. including a LcrV homolog, denoted PcrV. Moreover, it has been shown that active and passive immunization of mice with PcrV and anti-PcrV, respectively, protects against *P. aeruginosa* infection (26) and that PcrV has also the

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[3] Abbreviations used in this paper: TTS, type III protein secretion/translocation apparatus; Yop, *Yersinia* outer protein; rH, recombinant YopH; MAP, mitogen-activated protein; PPM, proteose peptone-elicited peritoneal macrophage; TLR, Toll-like receptor.

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capacity to form channels (21). However, it is still unknown whether PcrV is also capable to suppress proinflammatory cytokine production or to induce IL-10 release in spleen tissue as it is known for LcrV.

For these reasons, the major aim of this study was to elucidate comparatively the immunomodulatory function of *Y. enterocolitica* O8 and *P. aeruginosa* V Ags in murine peritoneal macrophages and human cells of the monocyte-macrophage line Mono-Mac-6 by analyzing their role in TNF-α suppression and IL-10 induction. When TNF-α suppression by V Ag of *Y. enterocolitica* was found to be dependent on IL-10 induction, we were prompted to check whether IL-10-deficient mice are resistant to *Y. enterocolitica* infection because of subversion of the infection strategy of the pathogen.

## Materials and Methods

### *Mice*

BALB/c and LPS nonresponder C3H/HeJ mice were purchased from Charles River Wiga (Sulzfeld, Germany). LPS nonresponder mice (C57BL/10ScCR and BALB/c LPSd) (27), a gift from M. A. Freudenberg and C. Galanos, were bred under specific-pathogen-free conditions at the Max Planck Institut für Immunbiologie (Freiburg, Germany). IL-10-deficient (IL-$10^{-/-}$) mice on a C57BL/6 background and C57BL/6 mice, serving as wild-type control animals, were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice were used at 6–8 wk of age.

### *Preparation of recombinant proteins*

Expression, production, and purification of recombinant V Ag (rLcrV) derived from *Y. enterocolitica* O8 strain WA-314 (28) was performed as described previously (16). For construction of the recombinant YopH (rH) and rPcrV, the *P. aeruginosa* strain PAO1 homolog of the *Yersinia* V Ag, the QIAexpress histidine-tagged protein expression and purification system (Qiagen, Hilden, Germany) was used as described previously for rLcrV (16). To amplify *yopH* and *pcrV*, the primer pairs yopH1 (CTC GGA TCC ATG AAC TTA TCA TTA AGC GAT) and yopH2 (CTC GAG CTC AGC AGA TAA ACC TCA ACT AAT) (29) as well as pcrV1 (ATA GGA TCC ATG GAA GTC AGA AAC C) and pcrV2 (ATA AAG CTT CTA GAT CGC GCT GAG AAT GTC) were used, respectively. Protein concentrations were measured by the bicinchoninic acid protein assay (Pierce, Freiburg, Germany). rLcrV, rH, and rPcrV were virtually LPS free as measured by the *Limulus* amebocyte assay (Pyroquant, Walldorf, Germany). The purity of the recombinant proteins was also checked by SDS-PAGE (data not shown). Aliquots of the preparations were stored at −80°C, thawed immediately before the experiments, and used only once to avoid repeated freezing and thawing cycles.

### *Preparation of Abs against rLcrV and rH*

Antiserum against rLcrV (anti-rLcrV) was produced in rabbits as described previously (16). Polyclonal anti-rH serum was prepared similarly. Briefly, 2-month-old New Zealand rabbits were immunized at four different times with 150 μg rH in 1 ml PBS mixed with lyophilized adjuvant (ABM adjuvant; Sebak, Aidenbach, Germany). After the animals were sacrificed, blood was obtained by heart puncture, and serum was collected after clotting. The serum (anti-rH) was tested in immunoblots using different Yops and rH and reacted specifically with a 51-kDa protein at a serum dilution of $1/10^4$. The Ig fraction was enriched by ammonium sulfate precipitation and subsequent extensive dialysis against PBS as described (16).

### *Preparation and stimulation of murine peritoneal macrophages in vitro*

Proteose peptone-elicited peritoneal macrophages (PPMs) were prepared as follows. Peritoneal exudate cells were obtained from mice that had received an i.p. injection of 1 ml 10% proteose peptone (Difco Laboratories, Detroit, MI) 3 days before. The cells were washed three times and resuspended in ice-cold RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine (Biochrom), 10 mM HEPES (Biochrom), 100 μg/ml streptomycin (Biochrom), 100 U/ml penicillin (Biochrom), and 10% heat-inactivated FCS (Life Technologies, Karlsruhe, Germany). Cells ($1 \times 10^6$/ml) were plated in 24-well tissue culture plates (Nunc, Roskilde, Denmark). After the cells had been incubated at 37°C in a humidified 5% $CO_2$ atmosphere for 2 h, nonadherent cells were removed by vigorous washing with sterile endotoxin-free PBS. Macrophage monolayers were pretreated for 3 h with rLcrV, rH, rPcrV, or LPS from S*almonella abortus equi* (Sigma, Deisenhofen, Germany) as control. Denaturation and degradation of LcrV were achieved by 1 h boiling or digestion with 20 μg/ml proteinase K (Sigma) for 30 min, respectively. To remove rLcrV specifically, the rLcrV-containing solution was treated with purified antiserum against rLcrV, and the immune complexes were subsequently separated using protein A-Sepharose beads (Pharmacia, Upsala, Sweden). As control, solubilized rLcrV was incubated similarly with anti-rH.

After the 3-h pretreatment period of macrophages with the mentioned proteins or LPS, cells were stimulated with 1 mg/ml zymosan A (Sigma) for 18 h. Supernatants were collected, and levels of TNF-α were determined. For IL-10 measurements, macrophage supernatants were collected after rLcrV treatment for 2 h and stored until tested.

To analyze the influence of IL-10 on TNF-α production, inhibiting rat anti-murine IL-10 Abs (JES5-2A5) were purchased from BD PharMingen (Hamburg, Germany). Inhibiting anti-TGF-β (R&D Systems, Minneapolis, MN) and IL-4 (R&D Systems) Abs served as controls. Abs and rLcrV were coincubated with PPMs for 3 h. Thereafter, PPMs were stimulated with 1 mg/ml zymosan A for 18 h for TNF-α production.

### *Stimulation of Mono-Mac-6 cells*

Cells of the human monocytic cell line Mono-Mac-6 (31) grown in RPMI 1640 supplemented with 10% FCS for 3 days at a cell density of $2 \times 10^5$ cells/ml were used for experiments testing responsiveness to yersiniae and *Pseudomonas* V Ag. Cells ($2 \times 10^5$/ml) were plated in 24-well tissue culture plates and pretreated for 18 h with different amounts of rLcrV, rLcrV boiled for 1 h, or rPcrV. Unpretreated cells served as control. After pretreatment, supernatants were collected for measurement of IL-10. For TNF-α production cells pretreated as indicated above were stimulated with 1 mg/ml zymosan A. After 6 h incubation, supernatants were investigated for TNF-α levels.

### *Assays for murine and human TNF-α and IL-10*

Murine TNF-α levels were measured in Nunc-Immuno plates (Nunc) by using a capture ELISA using rat anti-mouse TNF-α mAb (G281-2626) and biotin-labeled anti-TNF-α mAb (MP6XT3) as described previously (30). Murine IL-10 was determined by a commercial ELISA (R&D Systems) according to the manufacturer's recommendations. Human TNF-α levels were measured by a capture ELISA using mouse anti-human TNF-α mAb (mAb1) and biotin-labeled anti-TNF-α mAb (mAb11) as recommended by the manufacturer (BD PharMingen). Similarly, human IL-10 was determined by a capture ELISA based on the mAbs JES3-19F1 and JES3-12G8 (BD PharMingen).

### *Experimental infection of mice.*

*Y. enterocolitica* O8 strain WA-314 carrying the virulence plasmid pYVO8 (28) was grown in Luria-Bertani medium at 27°C overnight, sedimented, resuspended in 20% glycerol, and frozen at −80°C. For infection of mice, aliquots of glycerol stock cultures were thawed, washed in sterile PBS (pH 7.4), and diluted to the appropriate bacterial concentrations (16). Mice were given i.p. injections of 0.2 ml bacterial solution containing $1 \times 10^4$ CFU. Survival was monitored for 14 days.

### *Determination of the number of yersiniae in spleen and liver*

Mice were injected i.p. with $1 \times 10^4$ CFU *Y. enterocolitica* O8 strain WA-314. After 4 days, the mice were sacrificed. Spleens and livers were dissected and homogenized as described previously (16). *Yersinia*e CFU were determined by plating serial dilutions on *Yersinia* selective agar (CIN agar; BD Biosciences, Heidelberg, Germany) and counting the CFU after an incubation period of 40 h at 27°C.

## Results

### *TNF-α production in murine peritoneal macrophages is suppressed by rLcrV, but not by rPcrV, in a dose-dependent manner*

TNF-α plays an important role as a defense effector in innate immunity (32). Therefore, it may be speculated that for bacteria the suppression of macrophage-dependent TNF-α production might be important, especially in the initial phase of infection when mainly macrophages build the first line of defense. A similar phenomenon is seen in LPS tolerance. To differentiate V Ag-induced immunomodulation from LPS tolerance, we chose LPS nonresponder mice

for our experiments. For testing of the TNF-$\alpha$-suppressing capacity of rLcrV, PPMs from the LPS nonresponder mouse strain C3H/HeJ were pretreated for 3 h with rLcrV at different concentrations (33). Macrophages were subsequently stimulated for 18 h with zymosan A, because zymosan A is known to be a potent TNF-$\alpha$ inducer in macrophages (34). TNF-$\alpha$ in culture supernatants was measured by ELISA. Pretreatment with rLcrV impaired the ability of C3H/HeJ macrophages to produce TNF-$\alpha$ in a dose-dependent manner, whereas pretreatment with rH or with *Pseudomonas* rPcrV did not result in TNF-$\alpha$ suppression (Fig. 1). A similar dose-dependent immunomodulating effect of rLcrV was also seen in PPMs of the LPS nonresponder C57BL/10ScCR and BALB/c LPSd mice as well as in PPMs from the LPS responder mouse strains BALB/c and C57/BL6 (data not shown).

To rule out that a possible LPS contamination of rLcrV might be responsible for the observed TNF-$\alpha$ suppression, two additional control approaches were chosen besides the use of LPS nonresponder macrophages: 1) boiling of our rLcrV preparation which was expected to inactivate LcrV, but not LPS, abolished the TNF-$\alpha$ suppression completely in LPS nonresponder C3H/HeJ macrophages (Fig. 1); and 2) proteolytic degradation of rLcrV by preincubation with proteinase K inhibited the TNF-$\alpha$-suppressive effect of rLcrV in a similar manner (Fig. 1).

### *Specific removal of rLcrV using anti-rLcrV abolishes TNF-$\alpha$ suppression*

To show that the TNF-$\alpha$ suppression was specifically attributable to V Ag, rLcrV was removed from the preparation after incubation with anti-rLcrV using protein A-Sepharose beads. Specific binding of anti-rLcrV to rLcrV was shown previously (16). After removal of rLcrV, no TNF-$\alpha$ suppression could be observed in C3H/HeJ peritoneal macrophages (Fig. 1). A similar treatment using anti-rH Abs did not influence the immunosuppressive capacity of rLcrV (data not shown).

### *TNF-$\alpha$ suppression by rLcrV is abolished by anti-IL-10 Abs*

To test whether the TNF-$\alpha$ suppression could be attributed to IL-10, C3H/HeJ peritoneal macrophages were pretreated with rLcrV and anti-IL-10, anti-IL-4, or anti-TGF-$\beta$ Abs, respectively. After zymosan A restimulation, no TNF-$\alpha$ suppression was found in anti-IL-10-treated macrophage cultures suggesting a role for IL-10 in the TNF-$\alpha$-suppressive effect (Fig. 2). Anti-IL-4 and anti-TGF-$\beta$ Abs were not able to abolish the rLcrV-induced TNF-$\alpha$ suppression.

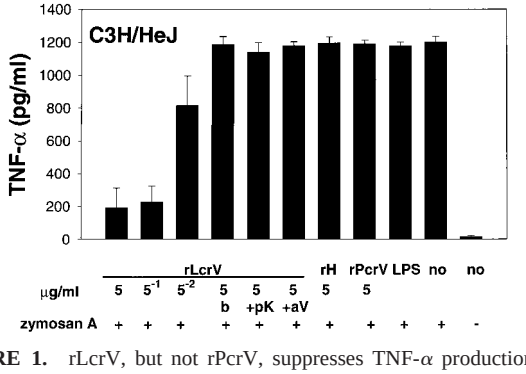


**FIGURE 1.** rLcrV, but not rPcrV, suppresses TNF-$\alpha$ production in a dose-dependent manner. PPMs ($1 \times 10^6$ cells/ml) of LPS nonresponder C3H/HeJ mice were pretreated for 3 h with rLcrV, rH, or rPcrV in the indicated concentrations. Several controls were included: 1) rLcrV (5 $\mu$g/ml) was either boiled (b) for 1 h or preincubated with proteinase K (20 $\mu$g/ml; +pK) for 30 min before use; 2) the rLcrV preparation was used after removal of rLcrV (5 $\mu$g/ml) with anti-rLcrV (75 $\mu$g/ml) and protein A Sepharose beads (+aV) as described in *Materials and Methods*; and 3) cells were pretreated with 1 ng/ml LPS. TNF-$\alpha$ production on stimulation with zymosan A (1 mg/ml) for 18 h was determined in the cell supernatants by ELISA. Unpretreated cells only stimulated with zymosan A served as positive control, whereas unpretreated cells without subsequent zymosan A stimulation were used as negative controls. Data represent mean values $\pm$ SD of eight independent experiments.

### *rLcrV, but not rPcrV, elicits IL-10 production in peritoneal macrophages which is essential for TNF-$\alpha$ suppression*

Proteose-peptone elicited C3H/HeJ peritoneal macrophages were stimulated with rLcrV; boiled rLcrV; rLcrV after incubation with anti-rLcrV using protein A-Sepharose beads, rH, or rPcrV; or remained untreated. After 2 h cultivation, macrophage supernatants were collected for IL-10 measurement by ELISA. IL-10 was found in supernatants of rLcrV-treated macrophages, whereas incubation with rH or rPcrV did not result in IL-10 induction (Fig. 3). Furthermore, both boiling and removal of rLcrV by the anti-rLcrV-protein A-Sepharose strategy impaired the IL-10 induction (Fig. 3). To investigate whether IL-10 is sufficient and essential for rLcrV-caused TNF-$\alpha$ suppression, PPMs from IL-$10^{-/-}$ mice were pretreated for 3 h with rLcrV and subsequently stimulated with zymosan A. No TNF-$\alpha$ suppression upon zymosan A stimulation could be observed in rLcrV-pretreated macrophages from IL-10-deficient mice when compared with PBS-pretreated macrophages (Fig. 4). In contrast, rLcrV caused marked TNF-$\alpha$ suppression in macrophages derived from wild-type C57BL/6 mice (Fig. 4).

### *rLcrV, but not rPcrV, causes TNF-$\alpha$ suppression and elicits IL-10 production in human monocytic Mono-Mac-6 cells*

To analyze whether the immunomodulatory capacity of *Yersinia* V Ag not only is restricted to mouse macrophages but can also be found in the human system, the human monocytic cell line Mono-Mac-6 was used. Pretreatment of Mono-Mac-6 cells with rLcrV resulted in TNF-$\alpha$ suppression, whereas boiling of rLcrV completely abolished the cytokine-suppressive effect (Fig. 5*A*). Accordingly, IL-10 could be detected after 18 h pretreatment of Mono-Mac-6 cells with rLcrV, whereas boiled rLcrV did not induce IL-10 production (Fig. 5*B*). The *P. aeruginosa* homolog rPcrV was not able either to suppress TNF-$\alpha$ production or to induce IL-10. Additionally, these data indicate that TNF-$\alpha$ suppression by rLcrV does not require the participation of a cell type other than macrophages.

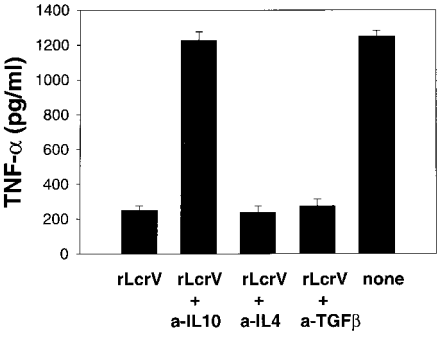


**FIGURE 2.** Anti (a)-IL-10 Abs inhibit rLcrV-caused TNF-$\alpha$ suppression. Proteose peptone-elicited peritoneal C3H/HeJ macrophages ($1 \times 10^6$ cells/ml) were pretreated for 3 h with rLcrV (5 $\mu$g/ml) with or without simultaneous addition of anti-IL-10 (10 $\mu$g/ml), anti-IL-4 (10 $\mu$g/ml), or anti-TGF$\beta$ (10 $\mu$g/ml). TNF-$\alpha$ levels after zymosan A (1 mg/ml) stimulation for 18 h were measured in the cell supernatants and represent mean values $\pm$ SD of five independent experiments.

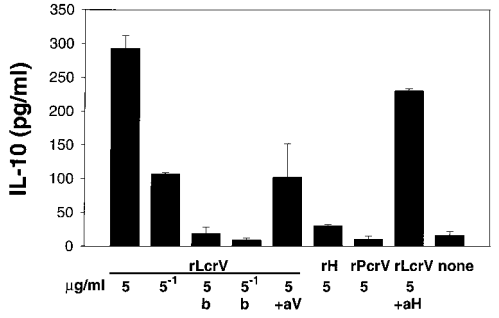


**FIGURE 3.** rLcrV elicits IL-10 production in peritoneal macrophages. Proteose peptone-elicited peritoneal C3H/HeJ macrophages ($1 \times 10^6$ cells/ml) were stimulated for 2 h with the indicated amounts of rLcrV. rLcrV was used untreated, after boiling (b) for 1 h or after removal of rLcrV with anti-rLcrV (75 $\mu$g/ml) and protein A-Sepharose beads (+aV). rH (5 $\mu$g/ml), rPcrV (5 $\mu$g/ml), as well as rLcrV (5 $\mu$g/ml) pretreated with anti-rH (75 $\mu$g/ml) and protein A-Sepharose beads (+aH) served as controls. IL-10 in supernatants was measured by ELISA. Data represent mean values ± SD of five experiments.

*Mice lacking IL-10 are highly resistant to infection with* Y. enterocolitica

To evaluate the in vivo role of IL-10 in *Y. enterocolitica* infection, wild-type and IL-$10^{-/-}$ mice were infected i.p. with $1 \times 10^4$ CFU *Y. enterocolitica*, and their survival was monitored for 14 days. Although >80% of IL-$10^{-/-}$ mice survived for 14 days, all wild-type mice were dead on day 10 postinfection, with 50% of wild-type mice succumbing to *Y. enterocolitica* within 7 days (Fig. 6). The high resistance of IL-$10^{-/-}$ mice to *Y. enterocolitica* was paralleled by the absence of macroabscesses in spleen after 4 days of infection, whereas the spleens of wild-type mice showed impressive macroabscesses. Accordingly, bacterial numbers in spleens and livers of IL-$10^{-/-}$ mice obtained 4 days after i.p. infection with *Y. enterocolitica* were significantly lower than in those from wild-type mice, consistent with low morbidity and mortality of IL-$10^{-/-}$ mice after *Y. enterocolitica* infection (Fig. 7).

## Discussion

TNF-α is a proinflammatory cytokine which is primarily released by activated macrophages and plays a crucial role in limiting the severity of bacterial infections (32). To evade the host immune defense, the inhibition of TNF-α would be a powerful strategy that a pathogen might develop. Two ways of TNF-α suppression have been described in *Yersinia* infection: one is dependent on the injection of the effector proteins YopP in *Y. enterocolitica* (8, 10) or YopJ in *Y. pseudotuberculosis* into target cells (9); the other has been attributed to secreted *Yersinia* V Ag and YopB. Although the molecular mechanism of TNF-α suppression by YopP/YopJ could be assigned to inhibition of NF-κB and of the extracellular signal-regulated kinase 2, c-Jun N-terminal kinase, and p38 MAP kinase activities (8, 9), the underlying mechanisms of YopB- and V Ag-caused immunomodulation have not been studied yet.

A role for V Ag in TNF-α and IFN-γ suppression was shown in spleens of infected mice (22) and in mixed macrophage-T cell cultures (33). Besides V Ag, YopB of *Y. enterocolitica* has been

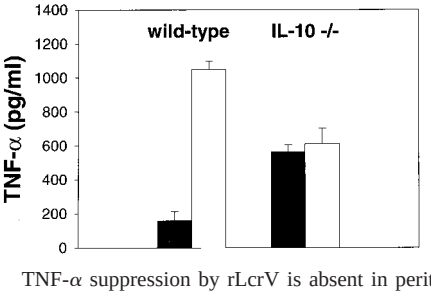


**FIGURE 4.** TNF-α suppression by rLcrV is absent in peritoneal macrophages from IL-$10^{-/-}$ mice. Proteose peptone-elicited peritoneal macrophages ($1 \times 10^6$ cells/ml) of wild-type C57BL/6 and IL-$10^{-/-}$ mice were pretreated for 3 h with 5 $\mu$g/ml rLcrV (■) or PBS (□). TNF-α levels after 18 h stimulation with zymosan A (1 mg/ml) were measured in the cell supernatants. Data show mean values ± SD of three separate experiments.

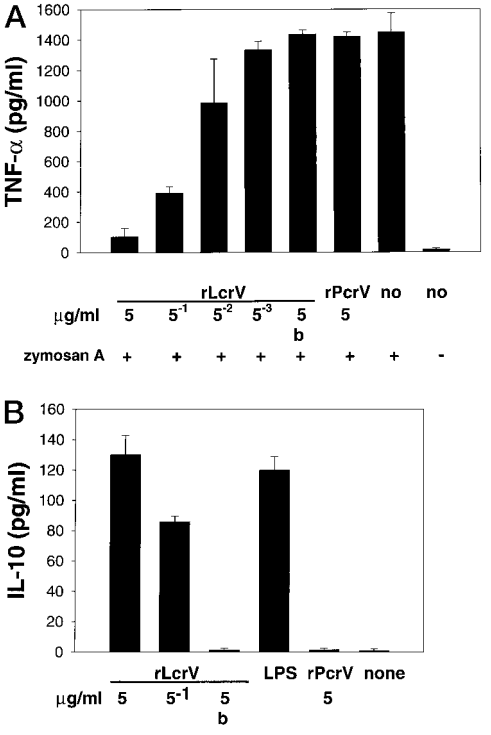


**FIGURE 5.** rLcrV, but not rPcrV, causes TNF-α suppression and IL-10 induction in Mono-Mac-6 cells. *A*, Mono-Mac-6 cells ($2 \times 10^5$ cells/ml) were pretreated with the indicated amounts of rLcrV, rLcrV boiled (b) for 1 h (as negative control), or rPcrV for 18 h. TNF-α production after stimulation with zymosan A (1 mg/ml) for 6 h was determined in the cell supernatants by ELISA. Unpretreated Mono-Mac-6 cells only stimulated with zymosan A served as positive control, whereas unpretreated cells without subsequent zymosan A stimulation were used as negative controls. Data represent mean values ± SD of six independent experiments. *B*, Mono-Mac-6 cells ($2 \times 10^5$ cells/ml) were stimulated with the indicated amounts of rLcrV, boiled rLcrV or rPcrV for 18 h. Cells pretreated with 1 ng/ml LPS and unpretreated cells served as controls. Supernatants were tested for IL-10 by ELISA. Results represent mean values ± SD of six independent experiments.

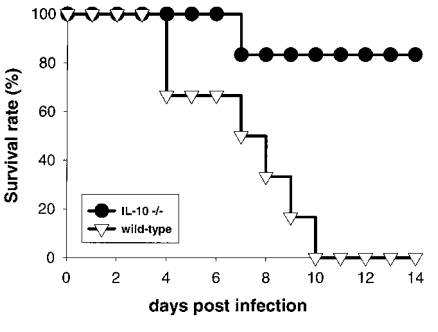


**FIGURE 6.** Survival of IL-$10^{-/-}$ ($n = 6$; ●) and wild-type mice ($n = 6$; ▽) after i.p. infection of $1 \times 10^4$ CFU *Y. enterocolitica* (WA-314).

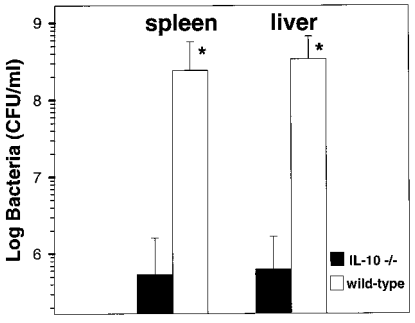


**FIGURE 7.** Numbers of live *Y. enterocolitica* in spleens and livers of IL-$10^{-/-}$ ($n = 8$, ■) and wild-type ($n = 8$, □) mice after i.p. infection of $1 \times 10^4$ CFU of *Y. enterocolitica* (WA-314). Mice were sacrificed on day 4 postinfection, and the organs were homogenized. Bacterial counts were determined by serial dilutions. Data represent the mean number of CFU ± SD. *, $p < 0.05$ by Student's *t* test.

claimed to suppress TNF-$\alpha$ production in a murine peritoneal macrophage model (34). In this study, YopB purified from culture supernatants of induced pYV plasmid-bearing *Y. enterocolitica* was used, but no recombinant YopB (35). V Ag, however, has been shown to bind to YopB (36). Therefore, it cannot be ruled out that the observed TNF-$\alpha$ suppression was due to V Ag possibly bound to YopB.

In our study using recombinant rLcrV, we could demonstrate V Ag-caused TNF-$\alpha$ suppression in a macrophage cell culture model. rLcrV was able to reduce TNF-$\alpha$ production induced by zymosan A in LPS nonresponder peritoneal macrophages in a dose-dependent manner.

One aim of this study was to clearly establish TNF-$\alpha$ suppression as a rLcrV-specific effect and differentiate it from LPS tolerance. Denaturation of the protein component in the rLcrV preparation by boiling or proteinase K degradation and the use of PPMs of three LPS nonresponder mouse strains allowed attribution of the TNF-$\alpha$ suppression to the protein component of rLcrV and exclusion of a LPS-induced tolerance phenomenon due to contaminating LPS. Additionally, the latter is corroborated by the use of zymosan A for TNF-$\alpha$ induction, because it had been shown that LPS pretreatment of PPMs of LPS responder C3H/HeSlc mice did not affect zymosan-induced TNF-$\alpha$ production (34).

The TNF-$\alpha$-suppressive effect observed by us could clearly be attributed to rLcrV, because removal of rLcrV from the preparation after incubation with anti-rLcrV using protein A-Sepharose beads abolished the TNF-$\alpha$ suppression completely in PPMs of C3H/HeJ mice. Moreover, the capacity of V Ag to down-modulate a TNF-$\alpha$ response is not restricted to the murine host, because rLcrV was able to suppress TNA-$\alpha$ production in the human monocytic cell line Mono-Mac-6.

As control, we used similarly produced recombinant YopH which was nonactive in any of the murine and human experimental cell models presented here. Surprisingly, we were unable to detect any LcrV-like immunomodulating activity for the *P. aeruginosa* homolog PcrV. This lack of immunosuppressive effects of rPcrV in the macrophage models tested is especially striking, because other functions of rLcrV such as participation in translocation of type III effectors and channel formation in lipid bilayer membranes are shared by PcrV (26).

TNF-$\alpha$ expression in macrophages can be inhibited by a number of factors. Of TNF-$\alpha$ down-regulating cytokines produced by macrophages, the anti-inflammatory IL-10 has been shown to be increased in spleens of BALB/c mice after injection of *Y. pestis* V Ag-polyhistidine fusion peptide ($V_h$) (24). Our data underline the decisive role of IL-10 in the rLcrV-caused TNF-$\alpha$ suppression, because anti-IL-10-Abs completely abolished the cytokine-suppressive effect of rLcrV in murine PPMs. Furthermore, it could be shown that rLcrV induces IL-10 directly in murine PPMs and in human Mono-Mac-6 cells. The final confirmation that IL-10 induction is the key mechanism by which rLcrV establishes TNF-$\alpha$ suppression could be achieved by showing that rLcrV-caused TNF-$\alpha$ suppression was absent in PPMs derived from IL-$10^{-/-}$ mice.

Inhibition of TNF-$\alpha$ production has been described in several bacterial infections (37–41). In most of these cases, however, the underlying cytokine-suppressive mechanism has not yet been elucidated. IL-10 induction by a bacterial protein leading to TNF-$\alpha$ suppression has thus far been found only for V Ag of *Yersinia* spp., as shown in the present study.

The cell type(s) involved in V Ag-caused TNF-$\alpha$ suppression have not yet completely elucidated. Schmidt et al. showed that activated T cells are strictly required for V Ag-caused TNF-$\alpha$ suppression upon LPS-IFN-$\gamma$ stimulation in a peritoneal exudate cell model (33). Our findings using the Mono-Mac-6 cells, however, suggest that a rather direct effect of V Ag on cells of the macrophage-monocytic lineage seems to be sufficient to cause TNF-$\alpha$ suppression on zymosan A stimulation without involving other cell types. Because cells of the macrophage-monocytic lineage are the main or the only possible source of both IL-10 and TNF-$\alpha$ in our two cell models, it is tempting to speculate that rLcrV-induced IL-10 leads to TNF-$\alpha$ suppression by "silencing" macrophages in a paracrine or autocrine loop.

The importance of IL-10 for sustaining a *Y. enterocolitica* infection was highlighted by in vivo experiments showing that IL-10-deficient mice were highly resistant to i.p. *Y. enterocolitica* infection compared with wild-type mice. Because rLcrV was shown in vitro to induce IL-10 leading to TNF-$\alpha$ suppression, it may be concluded that V-Ag released by yersiniae supports evasion of the innate immunity effector TNF-$\alpha$ in *Y. enterocolitica* infection by stimulating IL-10 production, thus exploiting the TNF-$\alpha$ down-regulating capacity of endogenous host IL-10. This conclusion is supported by the finding that i.p. injection of $V_h$ into BALB/c mice resulted in an early increase of IL-10 in spleens (24).

The role of IL-10 in microbial infection has been investigated for several bacterial and parasitic pathogens using IL-10-deficient mice (for a review, see Ref. 42). After infection with *Toxoplasma gondii* (43) or *Trypanosoma cruzi* (44), IL-10-deficient mice died rapidly due to overproduction of proinflammatory cytokines from $CD4^+$ T cells. Similarly, in an in vivo model of acute endotoxic shock, the high mortality rate of LPS-challenged IL-10-deficient mice was accompanied by an uncontrolled TNF-$\alpha$ production (45). In contrast, up-regulation of proinflammatory type 1 cytokine responses resulted in increased innate and acquired immunity in IL-10-deficient mice when challenged with sublethal doses of *Listeria monocytogenes* (46). In the early state of experimental i.p. *Mycobacterium bovis* bacillus Calmette-Guérin infection (47), IL-10-deficient mice exhibited a significantly lower bacteria burden in spleen and liver, which was explained to be a macrophage- rather than a T cell-dependent phenomenon. Only one study thus far using IL-10-deficient mice allowed the attribution of an IL-10-dependent pathomechanism to a "microbial" virulence factor; mice lacking IL-10 showed decreased lesion development and reduced parasite burdens after local *Leishmania major* infection which was linked to the absence of IL-12- and TNF-$\alpha$-down-regulating IL-10 that is normally induced by host IgG bound to the *Leishmania* amastigote surface in wild-type mice (48).

The striking similarity between V Ag-induced innate immunity modulation and LPS tolerance might prompt one to assume that V Ag and LPS share a common Toll-like receptor (TLR) and a corresponding signal transduction pathway. However, from our study, it can be concluded that TLR 4 which has been found to transmit LPS effects is not involved in V Ag-induced cellular responses, because TNF-α suppression could be achieved in PPMs from mice with a missense mutation in the *Tlr4* gene (C3H/HeJ, BALB/c LPSd) and a null mutation of *Tlr4* (C57BL/10ScCr), respectively (49). It is tempting to speculate that, in analogy to other bacterial products, V Ag uses a different TLR to exhibit its immunomodulating features on monocytic cells. Experiments to identify V Ag-signaling receptors are currently undertaken in our laboratory.

In conclusion, we propose that yersiniae have established two strategies for suppression of the proinflammatory cytokine TNF-α: 1) direct suppression of TNF-α via translocated YopP/YopJ (short distance effect); and 1) indirect suppression of TNF-α by released V Ag via IL-10 induction in bystander macrophages (long distance effect). The exploitation of endogenous IL-10 by microbes has thus far been described only for intracellular pathogens (50); in the present study, we show for the first time that also an extracellular bacterium is able to use this immunomodulating strategy. Moreover, the differences between LcrV and PcrV regarding their IL-10-inducing and TNF-α-down-regulating capacity might explain why *Yersinia* spp. act as pathogenic bacteria, whereas *P. aeruginosa* must be considered an opportunistic microorganism.

The perturbation of cytokine networks is increasingly recognized as a pathogenicity mechanism of bacteria (for a review, see Ref. 51). For the bacterial proteins responsible for these effects, the term bacteriokine has been coined. Besides its regulatory and translocatory features, V Ag causes immunomodulation by interfering with the host's TNF-α response. Therefore, V Ag may belong to this newly recognized group of bacterial proteins.

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