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Yersinia enterocolitica Evasion of the Host Innate Immune Response by V Antigen-Induced IL-10 Production of Macrophages Is Abrogated in IL-10-Deficient Mice¹

Andreas Sing, Andreas Roggenkamp, Anna M. Geiger, and Jürgen Heesemann²

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-α production in zymosan A-stimulated mouse macrophages and the human monocytic Mono-Mac-6 cell line. The underlying mechanism of TNF-α 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-α suppression, and 3) TNF-α 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); 2) the surface-exposed adhesin YadA; 3) antihost 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-κB 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-α and IFN-γ 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 cytoplasmic 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.
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 monocye-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 ACC TTA CAT AAG AGC GAT) and yopH2 (CTC GAG CAT CTC AGA TAA ACC TCA ACT AAT) (29) as well as pcrV1 (ATA GGA TCC ATG GAA GTC AGA AAC C) and pcrV2 (ATA AAG CTG CTA GAT CCG CTC GAG AAT GCT GAA AAT C) 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 bicinchoninic acid protein assay (Pierce, Freiburg, Germany). rLcrV, rH, and rPcrV were virtually LPS free as measured by the bicinchoninic acid protein assay (Pierce, Freiburg, Germany). rLcrV, rH, and rPcrV were virtually LPS free as measured by the bicinchoninic acid protein assay (Pierce, Freiburg, Germany).

**Preparation of Abs against rLcrV and rH**

Antiserum against rLcrV (anti-rLcrV) was produced in rabbits as described previously (28). After the animals were sacrificed, the sera were collected and stored until tested.

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Antiserum against rLcrV (anti-rLcrV) was produced in rabbits as described previously (28). After the animals were sacrificed, the sera were collected and stored until tested.

**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 (Bioehrom), 100 U/ml penicillin (Bioehrom), and 10% heat-inactivated FCS (Life Technologies, Karlsruhe, Germany). Cells (1 x 10⁶/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% CO₂ 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 Salmonella 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 antisera 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 rLcrV 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 x 10⁶ cells/ml were used for experiments testing responsiveness to yersiniae and Pseudomonas V Ags. Cells (2 x 10⁶/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) using a capture ELISA using rat anti-mouse TNF-α mAb (G281-2626) and biotin-labeled anti-TNF-α mAb (MPSX73) 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 (mA1) 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 solutions containing 1 x 10⁸ CFU. Survival was monitored for 14 days.

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

Mice were injected i.p. with 1 x 10⁴ 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). Yersiniae 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-α-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-α inducer in macrophages (34). TNF-α in culture supernatants was measured by ELISA. Pretreatment with rLcrV impaired the ability of C3H/HeJ macrophages to produce TNF-α in a dose-dependent manner, whereas pretreatment with rH or with *Pseudomonas* rPcrV did not result in TNF-α suppression (Fig. 1). A similar dose-dependent immunomodulating effect of rLcrV was also seen in PPMs of the LPS nonresponder C57BL/10SCr and BALB/c LPSd mice as well as in PPMs from the LPS responder mouse strains BALB/c and C57BL6 (data not shown).

To rule out that a possible LPS contamination of rLcrV might be responsible for the observed TNF-α 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-α suppression completely in LPS nonresponder C3H/HeJ macrophages (Fig. 1); and 2) proteolytic degradation of rLcrV by preincubation with proteinase K inhibited the TNF-α-suppressive effect of rLcrV in a similar manner (Fig. 1).

Specific removal of rLcrV using anti-rLcrV abolishes TNF-α suppression

To show that the TNF-α suppression was specifically attributable to V Ag, rLcrV was removed from the preparation after the 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-α 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-α suppression by rLcrV is abolished by anti-IL-10 Abs

To test whether the TNF-α 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-β Abs, respectively. After zymosan A restimulation, no TNF-α suppression was found in anti-IL-10-treated macrophage cultures suggesting a role for IL-10 in the TNF-α-suppressive effect (Fig. 2). Anti-IL-4 and anti-TGF-β Abs were not able to abolish the rLcrV-induced TNF-α suppression.

**rLcrV, but not rPcrV, elicits IL-10 production in peritoneal macrophages which is essential for TNF-α 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-α suppression, PPMs from IL-10−/− mice were pretreated for 3 h with rLcrV and subsequently stimulated with zymosan A. No TNF-α 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-α suppression in macrophages derived from wild-type C57BL/6 mice (Fig. 4).

**rLcrV, but not rPcrV, causes TNF-α 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-α suppression, whereas boiling of rLcrV completely abolished the cytokine-suppressive effect (Fig. 5A). 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. 5B). The *P. aeruginosa* homolog rPcrV was not able either to suppress TNF-α production or to induce IL-10. Additionally, these data indicate that TNF-α suppression by rLcrV does not require the participation of a cell type other than macrophages.
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 × 10⁶ 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...
claimed to suppress TNF-α production in a murine peritoneal macrophage model (34). In this study, YopB purified from culture supernatants of induced pYY 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-α suppression was due to V Ag possibly bound to YopB.

In our study using recombinant rLcrV, we could demonstrate V Ag-caused TNF-α suppression in a macrophage cell culture model. rLcrV was able to reduce TNF-α 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-α suppression as a rLcrV-specific effect and differentiate it from LPS tolerance. Denaturation of the protein component in the rLcrV preparation by boiling or protease K degradation and the use of PPMs of three LPS nonresponder mouse strains allowed attribution of the TNF-α 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-α induction, because it had been shown that LPS pretreatment of PPMs of LPS responder C3H/HeSlc mice did not affect zymosan-induced TNF-α production (34).

The TNF-α-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-α suppression completely in PPMs of C3H/HeJ mice. Moreover, the capacity of V Ag to down-modulate a TNF-α response is not restricted to the murine host, because rLcrV was able to suppress TNA-α 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-α expression in macrophages can be inhibited by a number of factors. Of TNF-α 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 (V₈) (24). Our data underline the decisive role of IL-10 in the rLcrV-caused TNF-α 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-α suppression could be achieved by showing that rLcrV-caused TNF-α suppression was absent in PPMs derived from IL-10⁻/⁻ mice.

Inhibition of TNF-α 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-α 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-α suppression have not yet completely elucidated. Schmidt et al. showed that activated T cells are strictly required for V Ag-caused TNF-α suppression upon LPS-IFN-γ 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-α 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-α in our two cell models, it is tempting to speculate that rLcrV-induced IL-10 leads to TNF-α 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-α suppression, it may be concluded that *V. Ag*-released by yersiniae supports evasion of the innate immune effector TNF-α in *Y. enterocolitica* infection by stimulating IL-10 production, thus exploiting the TNF-α down-regulating capacity of endogenous host IL-10. This conclusion is supported by the finding that i.p. injection of V₈ 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-α 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. *Mycoplasma 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-α-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 monocyteic 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 proinflammatorycytome 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 translactory 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.

References


