Growth of *Yersinia pseudotuberculosis* in Mice Occurs Independently of Toll-Like Receptor 2 Expression and Induction of Interleukin-10[∇]

Victoria Auerbuch² and Ralph R. Isberg^{1,2*}

Howard Hughes Medical Institute¹ and Department of Molecular Biology and Microbiology,²
Tufts University School of Medicine, 150 Harrison Avenue, Boston, Massachusetts 02111

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Pathogenic *Yersinia* translocates effector proteins into target cells via a type III secretion system (TTSS), modulating the host immune response. A component of the TTSS translocon, LcrV, has been implicated in preventing inflammation through Toll-like receptor 2 (TLR2) by inducing expression of the anti-inflammatory cytokine interleukin-10 (IL-10). TLR2^{-/-} mice were reported to be less susceptible to the enteropathogen *Yersinia enterocolitica*. To determine whether TLR2 also plays a role in recognition of the enteropathogen *Yersinia pseudotuberculosis* and whether this results in an immune response that is detrimental to the host, we evaluated the macrophage cytokine response to live *Y. pseudotuberculosis* and analyzed the susceptibility of TLR2^{-/-} mice to enteropathogenic *Yersinia*. We find that *Yersinia* induction of macrophage IL-10 occurs independently of TLR2 and is blocked by the TTSS. In particular, the TTSS effector protein YopJ, which inhibits production of the inflammatory cytokine tumor necrosis factor alpha (TNF-α), also inhibits IL-10 expression. Consistent with these results, IL-10 is undetectable in *Y. pseudotuberculosis*-infected mouse tissues until advanced stages of infection. In addition, we find that TLR2^{-/-} mice (derived independently from those used in previous studies) do not display altered susceptibility to enteropathogenic *Yersinia* compared to wild-type mice. Tissue levels of IL-10, as well as the inflammatory cytokines TNF-α, IL-6, and gamma interferon and the chemokine macrophage chemotactic protein 1, are similar in TLR2^{+/+} and TLR2^{-/-} mice during enteropathogenic *Yersinia* infection. Therefore, the absence of TLR2 alone does not affect the cytokine response of macrophages to, or the in vivo growth and survival of, enteropathogenic *Yersinia*.

Yersinia pestis, the causative agent of bubonic plague, has caused extensive human mortality, killing an estimated 200 million people (43). The related food-borne pathogens *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* express many of the same virulence factors as *Y. pestis* yet cause gastroenteritis and lymphadenitis (37, 58). These shared virulence factors include a type III secretion system (TTSS) encoded by a plasmid (called pYV in enteropathogenic *Yersinia*). The components of the TTSS apparatus as well as its effector Yops (*Yersinia* outer membrane proteins) have been linked to immunomodulation of the mammalian immune system (10). Numerous studies have shown that effector Yops interfere with innate immune functions such as phagocytosis and cytokine release by cultured cells in vitro (37, 58). In addition, LcrV, a component of the TTSS translocon, has been reported to inhibit inflammation both in vivo and in vitro (10, 36, 41, 49).

LcrV, also known as V antigen, is important in controlling the expression levels of TTSS components (7, 46). In addition, LcrV is thought to cooperate with two other proteins at the tip of the TTSS needle to facilitate the translocation of effector Yops into host cells, allowing these effectors access to their mammalian targets (13, 34, 39, 44). Consistent with its essential role in *Yersinia* virulence, LcrV represents a major protective antigen against *Yersinia* infection. Injection of a purified chimeric protein derived from the 259-amino-acid C-terminal

portion of *Y. pseudotuberculosis* LcrV induces passive immunity to *Y. pestis* or *Y. pseudotuberculosis* (33). This same recombinant immunogen was also shown to inhibit the production of inflammatory cytokines when injected into mice infected with plasmid-free (pYV⁻) *Yersinia* (36). More recently, purified LcrV from *Y. pestis* was shown to induce expression of the anti-inflammatory cytokine interleukin-10 (IL-10) in cultured macrophages (41). Deletions within either the C terminus or the central region, but not the N terminus, of *Y. pestis* LcrV were associated with reduced IL-10 secretion. In contrast, a separate study demonstrated that a moiety within the *Y. enterocolitica* LcrV N terminus, which is missing in the *Y. pseudotuberculosis* LcrV immunogen, elicits IL-10 production dependent upon the presence of the innate immune sensor Toll-like receptor 2 (TLR2) (48, 50).

TLR signaling is used largely by cells of the innate immune system to detect the presence of microbes. Thus far, 13 mammalian TLRs have been described, with each TLR recognizing a discrete set of molecules produced by bacteria, viruses, fungi, or parasites that are typically conserved components necessary for microbial life (16). TLR2 recognizes a number of microbial molecules, including lipoproteins, lipoteichoic acid (LTA), peptidoglycan, and the yeast cell wall polysaccharide zymosan, among others (60). Controversy over the nature of TLR2 ligands remains, primarily because lipoproteins and LTA are ligands for TLR2 and can easily contaminate preparations of other molecules (56). However, a recent study demonstrated that peptidoglycan from *Staphylococcus aureus* can activate TLR2 in the absence of lipoprotein or LTA contaminants (11).

IL-10 plays a crucial role in limiting inflammatory responses while promoting other immune processes (2). IL-10 inhibits

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111. Phone: (617) 636-3993. Fax: (617) 636-0337. E-mail: ralph.isberg@tufts.edu.

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macrophage inflammation and cell-mediated immunity yet facilitates the humoral immune response by promoting B-cell function. One major source of IL-10 *in vivo* is the macrophage, and IL-10 can be expressed by macrophages downstream of TLR ligands such as lipopolysaccharide (LPS) (1, 18). This may serve to temper the inflammatory effects of other TLR-induced cytokines such as tumor necrosis factor alpha (TNF- α).

Sing et al. (50, 51) reported that treatment of macrophages with recombinant *Y. enterocolitica* LcrV activates the transcription factor NF- κ B and induces the expression of IL-10 in a TLR2-dependent manner. The induction of IL-10 was linked to a single residue within the LcrV N terminus (K42) (48). In line with these *in vitro* results, mice lacking TLR2 were reported to produce less splenic IL-10 and more of the inflammatory cytokine TNF- α than wild-type mice during infection with *Y. enterocolitica*, predicting that these mice should have enhanced resistance to this pathogen resulting from increased inflammation. In fact, TLR2^{-/-} mice were shown to be more resistant to infection with *Y. enterocolitica* serogroup O:8 strain WA-314 than TLR2^{+/+} mice (50). Those authors explained the intriguing resistance of TLR2^{-/-} mice, which was observed in either the C57BL/6 or 129 murine genetic background, by proposing a model in which LcrV specifically induces IL-10 expression via TLR2, thereby dampening any inflammatory cytokine response. The significance of this result is unclear, however, because the resistance phenotype was lost after backcrossing of the TLR2^{-/-} mutation into the C3H background (51). In addition, although LcrV from *Y. pseudotuberculosis* contains the putative TLR2-activating K42 residue, recombinant *Y. pseudotuberculosis* LcrV does not induce IL-10 *in vitro* (48).

In order to assess the significance of TLR2 and IL-10 during *Y. pseudotuberculosis* infection, we examined the cytokine response of primary cultured macrophages to live *Y. pseudotuberculosis* and the susceptibility of mice to enteropathogenic *Yersinia* in the presence or absence of TLR2. Modulation of IL-10 was found to occur independently of both TLR2 and LcrV during infection of macrophages. Furthermore, wild-type and TLR2^{-/-} mice derived independently from mice used in previous studies showed a similar level of susceptibility to enteropathogenic *Yersinia* infection.

MATERIALS AND METHODS

Animals. TLR2^{-/-} mice were a kind gift from S. Akira and were originally described by Takeuchi et al. (54). Mice were bred into the C57BL/6 background for 10 generations and were housed under sterile conditions. Age- and sex-matched C57BL/6 control as well as IL-10^{-/-} mice were obtained from The Jackson Laboratory.

Bacterial strains and LcrV sequencing. *Y. pseudotuberculosis* strains used in this study were derived from the serogroup III clinical isolate IP2666 (8), while the *Y. enterocolitica* strain was serogroup O:8 strain 8081 (45). JMB111, referred to here as the pYV⁻ strain, is a plasmid-cured derivative of IP2666 (5). IP2666pIB1 is *Y. pseudotuberculosis* IP2666 cured of its endogenous virulence plasmid and instead carries pYV from the well-characterized *Y. pseudotuberculosis* YPIII strain (pIB1) (8). MLF36 (14), referred to here as the $\Delta yopJ$ mutant, is an IP2666pIB1 derivative that harbors an in-frame deletion of *yopJ* and a single point mutation within *YopH* (20), creating a novel NdeI site. We have found that this point mutation does not affect *YopH* function, based on virulence in the mouse model and an assay for cytokine signaling in cell culture (*yopH*^{Nde}) (data not shown). To sequence the N-terminal portion of LcrV from *Y. enterocolitica* 8081 and *Y. pseudotuberculosis* IP2666pIB1, single-colony PCR was performed using *Taq* polymerase (Invitrogen) and the primer pair LcrV1F (5'-ACTTATC

CGAGCAGGTGGTG-3') and LcrV1R (5'-TGAAATCACTCTTGGTGGG-3'). The resulting products were used as templates for sequencing with primers LcrV1F/R, described previously (48).

Cell and bacterial culture. Bone marrow-derived macrophages (BMDMs) were prepared as previously described (3) and determined to be free of *Mycoplasma* contamination by using the MycoSensor PCR assay kit (Stratagene). Freshly harvested BMDMs were used for *in vitro* infections, and excess BMDMs were frozen in L-cell conditioned medium supplemented with 10% dimethyl sulfoxide and 10% extra fetal bovine serum (HyClone) for future use. Results were similar when fresh or frozen BMDMs were used (data not shown). *Y. pseudotuberculosis* strains were grown in 2 $\times$ YT overnight at 26°C with agitation for incubation with cultured macrophages. The overnight cultures were diluted 1:40 into 2 $\times$ YT containing 20 mM sodium oxalate and 20 mM MgCl₂, grown at 26°C for 2 h with agitation, and transferred to 37°C for an additional 2 h with agitation to induce the expression of the type III secretion system (29). For mouse inoculation studies, *Y. pseudotuberculosis* and *Y. enterocolitica* strains were grown in L broth at 26°C with agitation for approximately 17 h. The presence of the virulence plasmid in *Y. pseudotuberculosis* L broth cultures was verified by the presence of YadA-dependent cell clumping after growth at 37°C in RPMI as visualized under $\times 40$ magnification. Bacterial cultures were diluted in phosphate-buffered saline (PBS) to achieve the concentrations described below before infection of mice.

Analysis of BMDM response to *Y. pseudotuberculosis*. Fresh or previously frozen BMDMs from TLR2^{+/+} and TLR2^{-/-} mice were seeded onto tissue culture-treated 24-well plates at 5 $\times$ 10⁵ cells per ml in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum and L-glutamine (Gibco). Cell monolayers were incubated overnight in a humidified 37°C incubator. Bacteria were prepared as described above to maximize Yop expression, added to BMDMs at a multiplicity of infection of 5, and spun onto the monolayers at 1,000 rpm for 5 min. Monolayers were incubated at 37°C with 5% CO₂ for 2 h. Alternatively, BMDMs were incubated with the synthetic lipopeptide Pam₃Cys-SKKKK (1- μ g/ml final concentration; EMC Microcollections) for 4 h to verify the presence of TLR2 signaling in TLR2^{+/+} BMDMs and the loss of this signaling in TLR2^{-/-} BMDMs (see Fig. 5C). Supernatants for enzyme-linked immunosorbent assay (ELISA) were collected and frozen at -80°C before analysis.

Animal inoculation studies. Seven- to 9-week-old C57BL/6, TLR2^{+/+}, and TLR2^{-/-} mice were used for intraperitoneal (i.p.) and oral infections. For i.p. infections, bacteria were prepared as described above and inoculation doses of 10⁴ *Y. enterocolitica* and 2 to 6 $\times$ 10³ *Y. pseudotuberculosis* organisms in a 100- μ l volume of PBS were used. Mice were euthanized at 4 to 5 days postinoculation for *Y. pseudotuberculosis* and at 4 to 8 days postinoculation for *Y. enterocolitica*. For oral infections, mice were starved overnight (water was given *ad libitum*) and 100 μ l 5% sodium bicarbonate was orally administered. Mice were then orogastrically inoculated with 4 $\times$ 10⁷ *Y. enterocolitica* organisms in a 200- μ l volume, using a feeding needle. Mice were again given food and water *ad libitum* and were euthanized at 5 days postinoculation. Portions of isolated spleens and livers were homogenized for 30 s in PBS for determination of CFU and for 20 s in 1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} (Sigma) in PBS for cytokine analysis using an OMNI International homogenizer. Tissue samples for cytokine analysis were frozen at -80°C. For one of the three *Y. pseudotuberculosis* growth curve experiments (Fig. 1A), organs for CFU determination were homogenized in 0.2% NP-40 instead of PBS. Homogenizing in PBS or NP-40 did not lead to significant differences in CFU (data not shown). Samples for CFU determination were diluted appropriately and plated onto LB plates supplemented with 1 μ g/ml irgasan, which selects for *Yersinia* species. Plates were incubated at room temperature for 48 h and CFU enumerated using a ProtoCOL SR colony counter (Microbiology International). The remaining CFU samples were mixed 1:1 with 30% glycerol and frozen at -80°C. If no CFU were detected for a specific organ homogenate (i.e., were below the limit of detection by this method), samples were thawed and 300 to 400 μ l was added to LB supplemented with 1 μ g/ml irgasan and incubated at 26°C overnight with agitation. Cultures were plated onto LB supplemented with 1 μ g/ml irgasan and incubated at room temperature for 48 h to determine the presence or complete absence of any irgasan-resistant bacteria.

Determination of cytokine production by ELISA. BMDM supernatants and organ homogenates were thawed on ice and centrifuged at 13,000 rpm for 1 min. The mouse inflammatory cytometric bead array kit (BD Biosciences) was used to detect IL-12p70, TNF- α , gamma interferon (IFN- γ), macrophage chemotactic protein 1 (MCP-1), IL-10, and IL-6 according to the manufacturer's instructions, except only 4 μ l of each antibody-conjugated bead, 20 μ l of sample or standard, and 20 μ l of phycoerythrin-conjugated detection reagent were used per reaction. Data were acquired using a FACSCalibur flow cytometer and CellQuest software

FIG. 1. Characterization of *Y. pseudotuberculosis* IP2666pIB1 infection in vivo after i.p. inoculation of 2×10^3 to 6×10^3 bacteria. (A) Average CFU per g tissue $\pm$ SEM. Data represent three independent experiments ($n = 10$ mice for each time point). (B and C) Average pg cytokine/g spleen (B) and liver (C) $\pm$ SEM. Data represent two independent experiments ($n = 14$ [spleen] and $n = 12$ [liver] for uninfected mice; $n = 6$ for each infected-mouse time point). (D) Average pg cytokine/ml serum $\pm$ SEM. Data represents two independent experiments ($n = 1$ for uninfected mice; $n = 7$ for each infected-mouse time point).

(BD Immunocytometry System). Data analysis was performed using BD analysis software. For confirmation of IL-10 results, the IL-10 Quantikine kit (R&D Systems) was used according to the manufacturer's instructions.

Statistics. Kaleidagraph (Synergy Software) was used to calculate *P* values using the Wilcoxon-Mann-Whitney rank sum test.

RESULTS

***Y. pseudotuberculosis* infection induces TNF- α , IFN- γ , MCP-1, and IL-6 production in vivo, but IL-10 is detectable only during advanced stages of infection.** The innate immune response to *Y. pseudotuberculosis* during disseminated disease in the mouse has not been well characterized. In order to investigate this response, we inoculated wild-type C57BL/6 mice i.p. with *Y. pseudotuberculosis* IP2666pIB1 and monitored CFU and cytokine levels in the spleen, liver, and serum at different times postinoculation (Fig. 1). Robust growth was detected in both the spleen and liver during the time course, with an especially rapid CFU increase observed in the spleen between 48 and 72 h postinoculation (Fig. 1A). During this time interval, we observed a sharp increase in the levels of

IL-6, MCP-1, IFN- γ , and TNF- α in the spleen (Fig. 1B). Lower overall levels of these four cytokines were detected in the liver during *Yersinia* infection (Fig. 1C). The kinetics of serum cytokine production resembled that in the liver (Fig. 1D). In contrast to previously published data with *Y. enterocolitica* serogroup O:8 strain WA-314 (51), there was no notable increase in IL-10 throughout the *Y. pseudotuberculosis* time course. These results are in accordance, however, with the reported lack of IL-10 in response to *Y. pestis* infection in the lung (23).

At 72 h postinoculation, there appeared to be a small increase in IL-10 production in the liver (153 pg per gram tissue) (Fig. 1C). However, this corresponded to less than 10 pg/ml of organ homogenate. This value is below the level required to obtain an accurate reading of cytokine production, as it falls within a nonlinear portion of the standard curve used for normalizing ELISA readings to cytokine concentrations (data not shown). Furthermore, an IL-10 reading of 160 ± 80 pg per gram liver was observed in IL-10^{-/-} mice (data not shown), indicating that values in this low range are not above background detection. Addition of protease inhibitors to organ

FIG. 2. TLR2^{-/-} mice are not resistant to enteropathogenic *Yersinia*. (A) Analysis of *Y. pseudotuberculosis* infection at 4 days after i.p. inoculation of 2×10^3 to 3×10^3 *Y. pseudotuberculosis* IP2666pIB1 bacteria. Individual closed diamonds represent data from one organ. Dashes represent the geometric mean. (B) Analysis of *Y. enterocolitica* infection at 4 days after i.p. inoculation of 10^4 *Y. enterocolitica* serogroup O:8 strain 8081 bacteria. Open diamonds indicate no CFU detected upon overnight culturing of organ homogenates. *, CFU present (as determined by culturing homogenates) but below the limit of regular plating detection (see Materials and Methods). (C) Analysis of *Y. enterocolitica* infection at 5 days after oral inoculation of 4×10^7 *Y. enterocolitica* 8081 bacteria. Each graph represents data from two independent experiments.

homogenates did not uncover any detectable IL-10 (data not shown). We also obtained similar, negative results for IL-10 using the same detection system as in a previous study (51) that demonstrated IL-10 production during *Y. enterocolitica* infection of mice (data not shown) (see Materials and Methods).

In order to determine whether IL-10 is induced by *Y. pseudotuberculosis* at later time points, the infection was allowed to progress until the animals were moribund (100 to 123 h postinoculation). Under these conditions, IL-10 was indeed detectable, with a concurrent further increase in the levels of TNF- α , IFN- γ , MCP-1, and IL-6 (data not shown). We conclude that IL-10 can be detected only starting at 101 h postinoculation in terminally ill animals, more than 24 h after levels of other cytokines increased dramatically (Fig. 1B and C).

Efficient growth of enteropathogenic *Yersinia* in a mouse strain lacking TLR2. *Y. enterocolitica* LcrV has been reported to induce IL-10 expression in a TLR2-dependent manner, and the *Tlr2*^{-/-} genotype has been described as resulting in reduced *Y. enterocolitica* growth in mouse tissues (50). In order to determine the susceptibility of TLR2^{-/-} mice to *Y. pseudotuberculosis*, we performed i.p. infections of TLR2^{-/-} mice that were derived from a different *Tlr2* construction than that used in the previous studies (48, 50, 51) and which had been completely backcrossed into the C57BL/6J background (54). Surprisingly, these animals had levels of *Y. pseudotuberculosis* CFU similar to those in TLR2^{+/+} mice in both the liver and spleen at 4 days after i.p. inoculation (Fig. 2A) spleen, $P = 0.78$; liver, $P = 0.61$).

To determine whether this lack of resistance of TLR2^{-/-} mice to *Y. pseudotuberculosis* represented a difference between the two enteropathogenic *Yersinia* species, we performed infections of mice with the *Y. enterocolitica* serogroup O:8 strain 8081. While *Y. pseudotuberculosis*-infected mice occasionally had small, visible liver lesions, *Y. enterocolitica*-infected mice had more prominent abscesses throughout their peritoneal cavities (unpublished observations). In addition, spleens and livers of *Y. pseudotuberculosis*-infected mice decreased somewhat in weight over the course of the infection, while *Y. enterocolitica*-infected spleens became increasingly large, reaching almost three times the weight of uninfected spleens eight

days postinfection (data not shown), consistent with a previous report (4).

Even though there are distinct differences in infection with the two enteropathogenic *Yersinia* species, wild-type and TLR2^{-/-} mice also showed no difference in resistance to *Y. enterocolitica* i.p. or oral infection (Fig. 2B and C). In fact, there was a slight, but not statistically significant, trend towards susceptibility of TLR2^{-/-} mice to *Y. enterocolitica* via the i.p. route of infection (Fig. 2B and C) (i.p. infection and spleen, $P = 0.13$; i.p. infection and liver, $P = 0.08$; oral infection and spleen, $P = 0.47$; oral infection and liver $P = 0.1$). This result is not a consequence of our *Y. enterocolitica* strain 8081 harboring a nonactivating *lcrV* allele, because the DNA sequence of the *lcrV* gene from this strain encodes the putative TLR2-activating K42 residue (data not shown).

We measured cytokine levels in the tissues of the infected animals from Fig. 2. As we observed during the *Y. pseudotuberculosis* growth curve (Fig. 1), TNF- α , IFN- γ , MCP-1, and IL-6 were produced in both the spleens and livers of control and TLR2^{-/-} mice infected by the i.p. route with *Y. pseudotuberculosis* (Fig. 3A and D). Interestingly, while the CFU ranges of *Y. pseudotuberculosis* and *Y. enterocolitica* per gram of tissue were not statistically different after i.p. infection (Fig. 2A and B) (spleen, $P = 0.96$; liver, $P = 0.12$ [for wild-type mice]), we detected significantly less TNF- α , IFN- γ , MCP-1, and IL-6 in *Y. enterocolitica*-infected tissues, with very low but significant levels of TNF- α and IFN- γ in the spleen (Fig. 3B) ($P = 0.02$ and $P = 0.006$, respectively, for wild-type mice compared to uninfected tissues) and no TNF- α or IFN- γ detected in the liver (Fig. 3E) ($P = 0.9$ and $P = 0.3$, respectively, for wild-type mice). In addition, the lowest levels of MCP-1 and IL-6 were detected during oral infection with *Y. enterocolitica* (Fig. 3C, F), which correlated with lower bacterial burden (Fig. 2C). Notably, there were no significant differences in TNF- α levels of wild-type and TLR2^{-/-} mice during all three enteropathogenic *Yersinia* infections. Furthermore, no IL-10 or IL-12p70 was detected in any of the conditions tested (Fig. 3). The only observed statistically significant difference between the wild-type and TLR2^{-/-} animals described here was an 11-fold increase in IFN- γ levels in the spleens, but not the

FIG. 3. TNF- α , IFN- γ , MCP-1, and IL-6 are produced in enteropathogenic *Yersinia*-infected tissues. The spleens (A to C) and livers (D to F) of uninfected C57BL/6 or TLR2^{-/-} mice or of mice infected with *Y. pseudotuberculosis* IP2666pIB1 (A and D) or *Y. enterocolitica* 8081 (B, C, E, and F) from Fig. 2 were assayed for cytokines. For each graph, data from two independent experiments was pooled and the average pg/g tissue $\pm$ SEM is shown. (A to C) $n = 14$ for uninfected C57BL/6 mice; $n = 4$ for uninfected TLR2^{-/-} mice. (D to F) $n = 12$ for uninfected C57BL/6 mice; $n = 4$ for uninfected TLR2^{-/-} mice. (A, C, D, and F) $n = 7$ for infected C57BL/6 mice; $n = 8$ for infected TLR2^{-/-} mice. (B and E) $n = 8$ for infected mice. Note that each graph has a different y axis scale.

livers, of TLR2^{-/-} animals during oral infection with *Y. enterocolitica* (Fig. 3C) ($P = 0.006$).

To verify that TLR2 does not alter susceptibility of mice to *Y. pseudotuberculosis* under conditions of very advanced infection (when IL-10 is produced), we infected wild-type and TLR2^{-/-} mice with *Y. pseudotuberculosis* and let the infection progress for just over 100 h. By this time, the

majority of mice in both groups showed symptoms of progressed disease (unpublished observations). The bacterial burdens in the spleen and liver remained similar between wild-type and TLR2^{-/-} animals (Fig. 4A) (spleen, $P = 0.4$; liver, $P = 1$). Importantly, IL-10 levels were identical in the presence or absence of TLR2 (Fig. 4B) (spleen, $P = 0.4$; liver, $P = 0.7$).

FIG. 4. IL-10 is produced in *Y. pseudotuberculosis*-infected tissues regardless of TLR2 expression during late stages of infection. Results are for 101 h after i.p. inoculation of 4.5×10^3 *Y. pseudotuberculosis* IP2666pIB1 bacteria. (A) Individual diamonds represent CFU data from one organ. Dashes represent the geometric mean. (B) Spleen and liver homogenates from the mice from panel A were assayed for cytokines. Individual diamonds represent pg IL-10 per g tissue from one organ. Dashes represent average pg/g tissue.

FIG. 5. *Y. pseudotuberculosis* lacking LcrV induces macrophage IL-10 independently of TLR2, and this IL-10 expression is suppressed by YopJ. TLR2^{+/-} (A to C) or TLR2^{-/-} (C) BMDMs were infected for 2 hours with *Y. pseudotuberculosis* and grown under Yop-inducing conditions. Data represent averages of five (A and B) or three (C) independent experiments $\pm$ SEM. Supernatant was assayed for levels of TNF- α (A) and IL-10 (B and C). Confidence values: $P < 0.008$ compared to IP2666pIB1 (A and B, *), $P < 0.1$ compared to pYV⁻ (A, **), and $P = 0.03$ compared to pYV⁻ (B, **). (D) Results are for 4 days after i.p. inoculation of 2×10^3 to 4×10^3 *Y. pseudotuberculosis* bacteria expressing YopJ [IP2666(pIB1-yopH^{Ndc})] or lacking YopJ (Δ yopJ). Individual diamonds represent data from one organ. Dashes represent the geometric mean. Data represent two (+YopJ) or three (-YopJ) independent experiments.

YopJ inhibits IL-10 expression during *Y. pseudotuberculosis* infection of macrophages. Previously published studies describing LcrV as a TLR2 ligand capable of inducing IL-10 and thereby suppressing TNF- α have used purified LcrV protein or peptide (48, 50). In addition, the TTSS effector protein YopJ has been shown to suppress TNF- α directly (i.e., not through IL-10). In order to assess TNF- α and IL-10 expression during infection of macrophages with live *Yersinia*, we exposed TLR2^{+/-} primary BMDMs to wild-type *Y. pseudotuberculosis* as well as *Y. pseudotuberculosis* lacking the virulence plasmid (pYV⁻) or lacking only YopJ (Δ yopJ) for 2 hours and measured cytokine levels in the culture supernatant. As previously reported (9, 42), TNF- α expression was suppressed by YopJ (Fig. 5A). In the absence of YopJ, during infection with either the *Y. pseudotuberculosis* pYV⁻ or Δ yopJ strain, macrophages produced significant levels of both TNF- α and IL-10 (Fig. 5A and B). IL-10 expression occurred independently of LcrV,

since the pYV⁻ strain lacks the *lcrV* gene. Heat-killed *Y. pseudotuberculosis* also induced macrophage TNF- α and IL-10 production (data not shown). These data suggest that IL-10 induction during infection of macrophages with live *Yersinia* results from activation of the TLR pathway by general TLR ligands such as LPS.

We also observed that wild-type *Y. pseudotuberculosis* induced five- and threefold less IL-10 than *Y. pseudotuberculosis* pYV⁻ and Δ yopJ, respectively (Fig. 5B), indicating that the TTSS effector protein YopJ normally suppresses IL-10 production. *Y. pseudotuberculosis* pYV⁻ induced significantly more IL-10 than the Δ yopJ mutant ($P = 0.03$), perhaps because multiple plasmid-encoded determinants inhibit IL-10 expression. In regard to TNF- α , the pYV⁻ and Δ yopJ strains showed the opposite relationship. Both strains demonstrated the known alleviation of TNF- α suppression seen when YopJ is absent. However, macrophages produced more TNF- α in re-

TABLE 1. Expression of YopJ does not alter in vivo cytokine production in response to *Y. pseudotuberculosis*

Infection ^a	Mean pg/g tissue $\pm$ SEM ^b			
	TNF- α		IL-10	
	Spleen	Liver	Spleen	Liver
None	720 $\pm$ 70	30 $\pm$ 10	30 $\pm$ 20	10 $\pm$ 10
IP2666(pIB1-yopH ^{Nde})	6,480 $\pm$ 3,430	420 $\pm$ 140	160 $\pm$ 110	80 $\pm$ 30
Δ yopJ	9,450 $\pm$ 5,420	380 $\pm$ 130	140 $\pm$ 130	60 $\pm$ 20

^a C57BL/6 mice were infected with *Y. pseudotuberculosis* IP2666(pIB1-yopH^{Nde}) or with *Y. pseudotuberculosis* lacking YopJ.

^b Data represent two independent experiments ($n = 7$) for IP2666(pIB1-yopH^{Nde}) and three independent experiments ($n = 10$) for Δ yopJ.

sponse to the Δ yopJ mutant than the pYV⁻ strain (Fig. 5A) ($P = 0.095$). This may be due to lower IL-10 levels in the supernatants of macrophages infected with the Δ yopJ strain (Fig. 5B), leading to a decrease in IL-10-mediated suppression of TNF- α .

We next compared TNF- α and IL-10 levels in the supernatants of TLR2^{+/-} and TLR2^{-/-} macrophages exposed to *Y. pseudotuberculosis* or the synthetic TLR2 ligand Pam₃CysK₄. While the expression of IL-10 in response to Pam₃CysK₄ was dependent on TLR2, IL-10 expression after exposure of macrophages to *Y. pseudotuberculosis* occurred efficiently in TLR2^{-/-} macrophages (Fig. 5C). Collectively, these results demonstrate that (i) *Yersinia* lacking LcrV induces IL-10, (ii) this induction is TLR2 independent, (iii) fully virulent *Yersinia* actively inhibits IL-10 expression, and (iv) this IL-10 suppression is at least partially dependent upon the type III secreted effector protein YopJ.

The presence or absence of YopJ does not affect *Y. pseudotuberculosis* burden or cytokine levels in mouse tissues after i.p. infection. To determine whether *Yersinia* lacking YopJ induces IL-10 in mouse tissues, we infected wild-type C56BL/6 mice via the i.p. route with *Y. pseudotuberculosis* Δ yopJ. We observed no significant defect in *Y. pseudotuberculosis* growth in the spleens or livers of mice at 4 days postinoculation (Fig. 5D). While all mice infected with wild-type *Y. pseudotuberculosis* survived up to 4 days postinoculation (7/7), only 71% survived infection with *Y. pseudotuberculosis* Δ yopJ (10/14). However, there was no overall difference between the wild-type and Δ yopJ strains in TNF- α , IL-10, IL-12p70, IL-6, MCP-1, or IFN- γ levels in either the spleen or liver at this time point (Table 1 and data not shown). The lack of IL-10 in *Y. pseudotuberculosis* Δ yopJ-infected tissues may indicate that additional plasmid-associated factors distinct from YopJ, which contribute to IL-10 suppression in culture (Fig. 5B), may be sufficient for IL-10 suppression in mouse tissues.

DISCUSSION

Human pathogenic *Yersinia* species manipulate target cell signaling pathways in order to facilitate growth in a variety of tissue sites (10). One current model suggests that the engagement of the innate immune sensor TLR2 by the TTSS component LcrV leads to such misregulation and that this facilitates growth of the microorganism within host tissues (10, 17, 22, 50). Specifically, LcrV binding to TLR2 leads to activation of the anti-inflammatory cytokine IL-10, resulting in lowered

production of the inflammatory cytokine TNF- α and thus increased bacterial replication (50). In support of this model, it has been reported that two out of three mouse strains tested that lack TLR2 are resistant to *Y. enterocolitica* infection (50, 51). However, several aspects of this proposed pathway are inconsistent with the data presented here. A TLR2^{-/-} mouse strain, which was derived from a different parent strain than that used in previous *Yersinia* infection studies, did not demonstrate enhanced resistance to two enteropathogenic *Yersinia* species after either oral or i.p. inoculation. Moreover, expression of IL-10 and TNF- α was similar between TLR2^{-/-} and TLR2^{+/-} mice during enteropathogenic *Yersinia* infection. In fact, translocated substrates of the *Yersinia* TTSS appeared to actively suppress macrophage IL-10. Furthermore, we observed that *Y. pseudotuberculosis* induced macrophage IL-10 expression independently of TLR2 and LcrV in the absence of TTSS translocated effectors.

The pleiotropic effects of LcrV on *Yersinia* virulence make it challenging to tease apart its direct and indirect effects on the host and make it impossible to test an *lcrV* null mutant to address specific contributions to virulence. A mutant *Yersinia* strain lacking LcrV misregulates effector Yops and is incapable of translocating the Yops that are produced into target host cells (13, 34, 39, 44). In addition, purified *Y. enterocolitica* LcrV (or presumably LcrV secreted from bacterial cells during an infection) can stimulate cultured cells in a TLR2-dependent manner (50). However, in the presence of live *Yersinia*, which produces a number of other TLR ligands and harbors the immunomodulatory TTSS, the LcrV-TLR2 interaction may not have a strong or straightforward effect on the innate immune system.

Enhanced pathogen resistance of mice lacking TLR2 has rarely been reported, as most studies indicate that TLR2^{-/-} mice either behave similarly to the wild type in this regard or are more susceptible to a number of different pathogens (21, 28, 53, 55). Indeed, TLR2^{-/-} and TLR2^{+/-} mice are equally susceptible to *Y. pestis* (K. Pouliot and J. Goguen [University of Massachusetts, Worcester], personal communication). One notable exception is a report that showed decreased susceptibility of TLR2^{-/-} mice to the fungal pathogen *Candida albicans* (38). In contrast, two other publications demonstrated that TLR2^{-/-} mice derived from a different *Thr2* knockout construction either were more susceptible to *C. albicans* infection (59) or were unaffected (6) compared to wild-type control animals. This discrepancy is strikingly similar to the results found here and to those previously reported for *Y. enterocolitica* strain WA-314 (50). In fact, the mouse knockout line that shows decreased susceptibility to *C. albicans* is identical to the strain used in the previous *Y. enterocolitica* studies showing a similar phenotype (50). This indicates that either the nature of the genetic construction or the genetic background harboring the TLR2^{-/-} genotype could affect the results obtained with these knockout lines. Consistent with this proposition, a previous report indicates that backcrossing the *Thr2* mutation into the C3H murine genetic background reverses the *Y. enterocolitica* susceptibility properties of the mouse (51). Furthermore, the fact that TLR2^{-/-} C3H mice no longer restrict *Y. enterocolitica* growth but still display decreased splenic IL-10 compared to wild-type control animals (51) indicates that de-

creased IL-10 levels do not necessarily lead to enhanced resistance to *Yersinia*.

IL-10 is a major anti-inflammatory cytokine, and mice lacking IL-10 have been reported to be less susceptible than wild-type animals to a number of different pathogens (32), including *Y. enterocolitica* (49). However, IL-10^{-/-} mice have an increased incidence of inflammatory diseases such as inflammatory bowel disease (2), so increased resistance to pathogens seems to come at the price of aberrant inflammation. IL-10 expression is induced by the TLR pathway upon recognition of TLR ligands, such as LPS, along with inflammatory cytokines, such as TNF- α (1, 18). This may be a mechanism by which inflammation downstream of TLRs is kept in check, since IL-10 could serve to dampen further expression of inflammatory cytokines, such as TNF- α . Our findings that *Y. pseudotuberculosis* lacking LcrV induces TLR2-independent IL-10 expression in vitro and that IL-10 is produced in both wild-type and TLR2^{-/-} mice at late stages of *Y. pseudotuberculosis* infection suggest that other TLRs (such as TLR4) recognize general TLR ligands on *Yersinia* (such as LPS), leading to a general TLR response which includes IL-10.

The possibility remains that, in contrast to the case for *Y. pseudotuberculosis*, the late stages of *Y. enterocolitica* infection could result in IL-10 levels that are dependent on TLR2, leading to a less restrictive environment to *Y. enterocolitica* growth when TLR2 is present. However, we could not detect IL-10 in the tissues of *Y. enterocolitica*-infected C57BL/6 mice as late as 8 days after i.p. inoculation (data not shown). In addition, we observed a trend towards enhanced susceptibility to *Y. enterocolitica* of TLR2^{-/-} mice compared to TLR2^{+/+} mice at 4 days postinoculation (Fig. 2B), as did Sing et al. when infections were performed in the C3H mouse background (51). Another possibility is that some strains of *Y. enterocolitica* (such as WA-314) may induce IL-10 earlier during infection of mice. Further studies comparing different *Y. enterocolitica* strains are needed to address this possibility.

The lack of IL-10 in *Y. pseudotuberculosis*-infected tissues until very late stages of infection is consistent with the clinical manifestations of *Yersinia*-induced enterocolitis in humans. Enteropathogenic *Yersinia* causes inflammatory diarrhea which can be associated with fever and swelling of gastric tissues and lymph nodes (19). These disease manifestations do not suggest a major role for induction of anti-inflammatory cytokines as a virulence mechanism of enteropathogenic *Yersinia*.

Two previously published reports support our data showing that the TTSS effector protein YopJ can inhibit macrophage IL-10 expression. Erfurth et al. (12) showed that dendritic cells produced IL-10 upon exposure to *Y. enterocolitica* either lacking the virulence plasmid or lacking only the *yopJ* homolog, *yopP*, but not upon exposure to wild-type *Y. enterocolitica* strain WA-314. In addition, Zhang et al. (61) reported that IL-10 was differentially expressed by macrophages after exposure to *Y. pseudotuberculosis* encoding either catalytically active or dead YopJ. Although the exact nature of the catalytic activity of YopJ/P is controversial (30, 35, 40, 62), the action of the protein clearly leads to inhibition of the NF- κ B and mitogen-activated protein kinase (MAPK) pathways (9, 42, 47). While IL-10 expression is independent of NF- κ B, MAPKs are thought to play a role (25–27). Inhibition of the MAPK pathway by YopJ may explain the ability of this effector protein to

suppress IL-10 expression. In addition to YopJ, other plasmid-encoded determinants contribute to IL-10 suppression in vitro (Fig. 5B) and may be sufficient for IL-10 suppression in vivo (Table 1). Whether IL-10 suppression is an important virulence mechanism for enteropathogenic *Yersinia* remains to be determined and awaits discovery of these other putative factors that suppress IL-10 secretion.

The contribution of the TTSS effector protein YopJ to virulence seems to vary depending on the *Yersinia* species and route of entry. *Y. pestis* lacking *yopJ* has been reported to possess no virulence defect, whereas a *Y. enterocolitica yopJ* mutation was shown to lower virulence via both the oral and intravenous routes of infection (24, 52, 57). The role of YopJ in *Y. pseudotuberculosis* infection has been studied via the oral route, but the published results are conflicting (15, 31). In this study we showed that a *yopJ* mutant was identical to *Y. pseudotuberculosis* expressing YopJ in terms of bacterial burden following i.p. infection. However, we did observe increased mortality in the mouse groups that received the $\Delta yopJ$ strain compared to those that received *Y. pseudotuberculosis* expressing YopJ. This was not a result of increased inflammation in the mice infected with the $\Delta yopJ$ mutant, because TNF- α , IFN- γ , IL-6, and MCP-1 levels were similar between both groups of mice (Table 1 and data not shown), even though the $\Delta yopJ$ mutant induced threefold more TNF- α than wild-type bacteria in cell culture (Fig. 5B). Whether TNF- α suppression by YopJ is an important virulence mechanism in vivo remains to be determined.

Further studies will be required to determine which innate immune sensors participate in detecting and mounting an efficient immune response to *Yersinia*. In addition, understanding how these immune responses are manipulated by the *Yersinia* TTSS and how this relates to virulence in vivo will be important in deciphering this complex host-microbe interaction.

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