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Protective Role of Interleukin-6 during *Yersinia enterocolitica* Infection Is Mediated through the Modulation of Inflammatory Cytokines

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***Yersinia enterocolitica* is a gram-negative enteric pathogen responsible for a number of gastrointestinal disorders. A striking feature of the pathology of a *Y. enterocolitica* infection is inflammation. Recently, we demonstrated a role for interleukin-1α (IL-1α) in the establishment of intestinal inflammation in response to a *Y. enterocolitica* infection. A cytokine directly affected by IL-1 levels is IL-6. A previous report suggested that IL-6 plays an anti-inflammatory role during *Y. enterocolitica* infection, and in other systems IL-6 has been shown to be proinflammatory. Therefore, a closer examination of the roles of IL-6 and inflammatory cytokines in the control of *Y. enterocolitica* infection in IL-6$^{-/-}$ mice was undertaken. *Y. enterocolitica* organisms were more virulent in the IL-6$^{-/-}$ mice (60-fold decreased 50% lethal dose) and colonized systemic tissues more rapidly and to a higher level than in the wild-type mice. One role of IL-6 during a *Y. enterocolitica* infection may be the downmodulation of the inflammatory response. The IL-6$^{-/-}$ mice have a more robust T_H1 T-cell response, as well as hyperinflammatory pathologies. These phenotypes appear to be due to the misregulation of tumor necrosis factor alpha, monocyte chemotactic protein 1, IL-10, transforming growth factor β1, and gamma interferon in the IL-6$^{-/-}$ mouse. These data provide further insight into the intricate cytokine signaling pathways involved in the regulation of inflammatory responses and the control of bacterial infections.**

There are three species within the genus *Yersinia* that are pathogenic for humans. These include the two enteric pathogens *Yersinia enterocolitica* and *Y. pseudotuberculosis*, as well as *Y. pestis* the plague bacillus. The diseases caused by these bacteria are varied, ranging from gastroenteritis and lymphadenitis to both bubonic and pneumonic plague (6, 25). Infection of healthy individuals with *Y. enterocolitica* is usually self-limiting, resulting in gastrointestinal disease (31). A typical *Y. enterocolitica* infection is the result of consuming contaminated food or water. The bacteria are capable of surviving the harsh environment of the stomach and travel to the small intestine, where they attach to and invade the lymphoid follicles of the small intestine called Peyer's patches (PP). Once inside the PP the bacteria are mainly extracellular pathogens capable of replicating to high numbers (10^9 CFU/g of tissue). Subsequently, if systemic disease is established, the bacteria can disseminate to deeper tissues, such as the mesenteric lymph nodes (MLN), spleen, liver, and lung. A consequence of *Y. enterocolitica* infection is acute inflammation.

The murine model of *Y. enterocolitica* infection has proven extremely valuable for gaining a greater understanding of the cytokine responses to bacterial pathogens. This model of yersiniosis mimics all aspects of disease observed in humans, including systemic disease (8–11). To date, a role during the host response to *Y. enterocolitica* has been demonstrated in the murine model for interleukin-1 (IL-1), IL-1 receptor antagonist (IL-1ra), IL-6, IL-10, IL-12, IL-18, monocyte chemotactic protein 1 (MCP-1), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) (2, 3, 5, 14, 15, 17, 29).

Using *Y. enterocolitica* as a model for studying yersiniosis in vivo, we recently demonstrated that IL-1α is important for initiating early intestinal inflammatory responses (14). During these experiments and a subsequent analysis of the host response by genome scale transcriptional profiling (GeneChips), a role for IL-6 in the regulation of the inflammatory response was suggested that was more involved than previously reported (17; S. A. Handley et al., unpublished data).

IL-6 is a multifunctional cytokine that has been described to have both pro- and anti-inflammatory effects, as well as being involved in a variety of immune responses. Like IL-1 and TNF-α, IL-6 is an inflammatory cytokine (21). IL-6 plays a major role in the elaboration of the acute-phase responses (21, 22). In addition, IL-6 is involved in B-cell proliferation (20, 30). In the context of a *Y. enterocolitica* infection, IL-6 has an anti-inflammatory function through feedback inhibition of the IL-1 signaling pathway via the induction of IL-1ra (17, 27). Recent evidence demonstrates that IL-6, in conjunction with soluble IL-6 receptor, mediates the switch from the early neutrophilic inflammatory response to the sustained monocytic response (16). In addition, recent data have demonstrated a role for IL-6 in the cellular immune response by inhibiting the differentiation of activated CD4$^+$ T cells to the T_H1 phenotype (13).

IL-6 provides protection during infection with the intracellular bacterial pathogens *Listeria monocytogenes* and *Mycobacterium tuberculosis* (22, 23), but the exact mechanism for the protective role of IL-6 during a bacterial infection is not fully understood. We provide here evidence for a protective role of IL-6 during *Y. enterocolitica* infection. IL-6 protection appears to be mediated through the action of several immunomodulatory cytokines (IL-10, MCP-1, TNF-α, IFN-γ, and transform-

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ing growth factor β1 [TGF-β1]), in addition to the established role for IL-1ra (17). Our data suggest that the in vivo misregulation of these cytokines in the IL-6$^{-/-}$ mouse leads to a defect in the regulation of inflammatory responses and, ultimately, to a hyperinflammatory phenotype, severe sepsis, and increased mortality.

MATERIALS AND METHODS

Mice. Female C57BL/6j and C57BL/6j–IL-6^{tm1Kopf} mice (hereafter referred to as IL-6$^{-/-}$ mice) 6 to 8 weeks of age were purchased from Jackson Laboratory and maintained in the barrier facility at Washington University School of Medicine. Mice were given free access to food and water throughout all experiments. Animals were sacrificed by carbon dioxide asphyxiation. The Washington University committee on animal studies approved all animal experiments.

Bacteria. The *Y. enterocolitica* strain used in the present study (JB580v) is a virulent derivative of the serogroup O8 strain 8081 (19). Bacteria were grown overnight in Luria-Bertani (LB) broth at 26°C. Actual numbers of CFU were determined by serial dilutions of the overnight culture, followed by plating on LB agar.

LD_{50} (50% lethal dose) and kinetics analysis. Bacteria were grown 16 to 18 h in LB at 26°C. Five groups of six mice were infected orally with successive 10-fold dilutions of the bacterial suspension (10^4-10^9 bacteria). The mice were monitored twice daily for 14 days. This analysis was done in duplicate. The LD_{50} values were determined according to the method of Reed and Muench (26). Kinetics of infection analysis was done by orally infecting mice with the indicated dose of strain JB580v (19). At various times postinfection (1, 3, or 7 days), mice from each strain were sacrificed and dissected. Bacterial loads recovered from the infected organs were determined by plating dilutions of the macerated tissues on LB plates containing 20 μg of nalidixic acid/ml to select for *Y. enterocolitica* and are reported as CFU per gram of tissue. This analysis was done in duplicate.

Peritoneal macrophages. C57BL/6j or IL-6$^{-/-}$ mice were injected intraperitoneally with 3 ml of a sterile lipopolysaccharide (LPS)-free 2% starch solution. All materials used in these studies for tissue culture or in vivo were certified to be "low endotoxin" by the vendor. After 4 days the mice were sacrificed, and the peritoneal cavities were washed with Dulbecco modified Eagle medium containing 10% fetal calf serum. Peritoneal exudate cells were collected and plated in appropriate culture dishes at a cellular concentration appropriate for the given assay. After 4 h, the cells were extensively washed with phosphate-buffered saline (PBS) to remove the nonadherent cells and then maintained in Dulbecco modified Eagle medium–10% fetal calf serum until use.

Histopathology. Mice of the specified genotype were infected orally with the indicated amounts of strain JB580v. On days 1, 3, and 7 postinfection the mice were sacrificed, and the small intestines, MLN, spleens, and livers were removed. The lumen of the intestine was flushed with PBS, and then tissues were fixed in 10% neutral buffered formaldehyde prior to being embedded in paraffin and stained with hematoxylin and eosin. Slides were investigated in a blind fashion by two independent investigators. Tissues were scored on a scale of 0 to 4, with 0 being a pathology consistent with the mock-infected control tissues and 4 being the most severe pathology observed. Dead mice were excluded from analysis.

ELISA and CBA. Splenocytes from infected mice, at 10^5 cells/well, from the indicated mouse genotype were prepared as described and plated in 96-well plates. Briefly, mice were infected orally with *Y. enterocolitica* strain JB580v, and the infection was allowed to proceed for the indicated amount of time before spleens and sera were harvested. Single-cell splenocyte cultures were plated at the indicated cell densities. Assay plates were then centrifuged at $1,000 \times g$ for 5 min, and the culture supernatant was removed and frozen at −80°C until use. TGF-β1, IFN-γ, IL-6, and IL-10 capture enzyme-linked immunosorbent assay (ELISA) kits were purchased from Pharmingen, and experiments were performed according to the manufacturer's instructions. The results are the average of four assays done in duplicate. For assays done on serum, mice were bled at the indicated times after infection, and the serum was frozen at −80°C until use. Cytometric bead array (CBA) analysis (mouse inflammation arrays [Pharmingen] specific for IL-6, IL-10, MCP-1, TNF-α, IFN-γ, and IL-12p70) was done on serum, and splenocyte supernatants were prepared as described for the ELISA analysis according to the manufacturer's instructions.

For assays in which cytokine levels were determined directly from spleen homogenates, mice were infected and spleens were harvested as described above. Single-cell splenocyte suspensions were then subjected to four rounds of freeze-thaw, and the suspensions cleared by centrifugation. Cleared supernatants were used in CBA analysis to determine cytokine levels as described above. The results in Table 3 are the average of two experiments. Five mice per treatment group were used per experiment.

IL-6$^{-/-}$ phenotype rescue. The IL-6 reconstitution experiments were done as described by Diehl et al. (12). IL-6$^{-/-}$ mice infected with *Y. enterocolitica* (10^8 CFU) were injected subcutaneously (s.c.) with 1 μg of recombinant human IL-6 (Peprotech) for 5 days. The splenocytes were then harvested and used in ELISA or CBA assays. Control C57BL/6j and IL-6$^{-/-}$ mice were injected with a mock injection of PBS. Both the recombinant human IL-6 and the PBS vector were certified to be endotoxin free by the manufacturer of the product (i.e., with <0.1 ng of endotoxin/μg). To determine whether the hyperinflammatory phenotype could be rescued, mice given the indicated treatment were orally infected with 10^9 CFU of *Y. enterocolitica*. IL-6$^{-/-}$ mice (10 per experimental group) were also treated with rIL-6 as described above for 3 days and then sacrificed for histopathologic analysis. Tissues were prepared as described above and scored in a blind manner by two independent investigators. Spleens from C57BL/6j mice, IL-6$^{-/-}$ mice, and IL-6$^{-/-}$ mice that received 1 μg of recombinant human IL-6 s.c. were scored for the inflammatory lesions.

Cytotoxicity assays. Nonradioactive cytotoxicity assays were performed by using the CYTO-TOX 96 kit from Promega. Assays and controls were performed as recommended by the manufacturer's instructions. Briefly, peritoneal macrophages served as antigen-presenting cells (APC) and were plated in 96-well culture dishes at a density of 10^5 cells/well. APC (target cells) were loaded with antigen (10 ng of heat-killed *Y. enterocolitica*) overnight as previously described (4) and then washed prior to the addition of 10^5 splenocytes to the well. The cytotoxicity of the target cells was determined spectrophotometrically by enzymatic analysis of lactate dehydrogenase release. Specific lysis was calculated as follows: % cytotoxicity = $(EE_{sp} - Ta_{sp})/Ta_{max} - Ta_{sp})$, where EE_{sp} is spontaneous experimental effector, Ta_{sp} is spontaneous target, and Ta_{max} is target maximum. The results are the average of four assays done in duplicate.

Statistical analysis. Data were analyzed by using the Mann-Whitney test (a two-tailed nonparametric analysis of variance [ANOVA]). This test assigns a statistically significant P value if P is ≤0.02. Statistically significant comparisons are indicated where appropriate.

RESULTS

IL-6 is protective during murine yersiniosis. Previous evidence suggested that IL-6 has a protective role during bacterial infection (22, 23). Thus, the ability of IL-6 to confer protection against a *Y. enterocolitica* infection was directly assessed by using 50% lethal dose (LD_{50}) analysis. IL-6$^{-/-}$ mice and the congenic C57BL/6j strain of mice were infected with various doses of *Y. enterocolitica*, and the LD_{50} values were determined. IL-6$^{-/-}$ mice had an LD_{50} 60-fold lower than the C57BL/6j mice: 5×10^5 CFU for the IL-6$^{-/-}$ mice versus 3×10^7 CFU for the C57BL/6j mice (Fig. 1A). These data suggest that the IL-6$^{-/-}$ mice have a defect in their ability to control a *Y. enterocolitica* infection.

A kinetic analysis of *Y. enterocolitica* infection also demonstrated a defect in the ability of IL-6$^{-/-}$ mice to control *Y. enterocolitica* infection (Fig. 1B). IL-6$^{-/-}$ mice and C57BL/6j mice were infected orally with 10^9 CFU of *Y. enterocolitica*. The mice were then sacrificed at the indicated time, and the extent of bacterial colonization was determined in the PP, MLN, and spleen of each animal. After infection, there was consistent colonization of the PP and MLN in both mouse strains. There was a statistically significant difference in the day 3 PP and the day 7 MLN ($P = 0.02$). The IL-6$^{-/-}$ mice showed a more pronounced systemic spread by day 3 compared to the C57BL/6j mice. This is most clearly demonstrated by the early and consistent colonization of the spleen by the bacteria. It should be noted that many of the IL-6$^{-/-}$ mice destined for the day 7 time point died prior to day 7 (7 of 12 IL-6$^{-/-}$ mice died prior to day 7 compared to 2 of 12 C57BL/6j mice). Death and dissemination of the bacteria to the spleen are signs of systemic yersiniosis that is more severe in the IL-6$^{-/-}$ mice. This more rapid colonization phenotype was also observed in IL-6$^{-/-}$

A)

LD_{50} Analysis

Mouse	LD_{50}
C57BL/6j	3.1 X 10^7 CFU
IL-6$^{-/-}$	5.4 X 10^5 CFU

B)

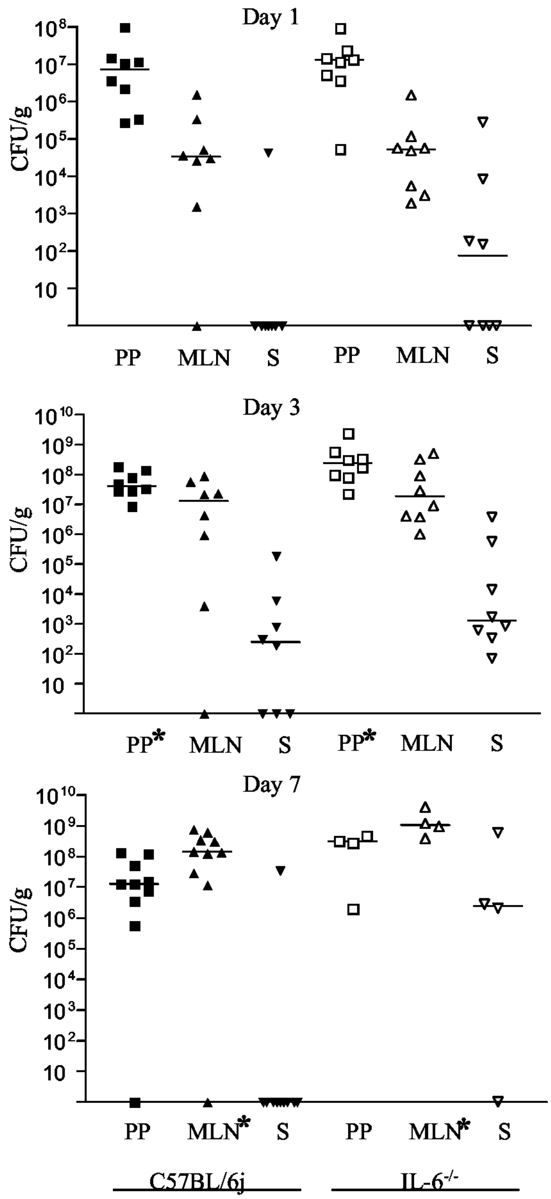

FIG. 1. IL-6$^{-/-}$ mice are deficient in the control of a *Y. enterocolitica* infection. (A) C57BL/6j or IL-6$^{-/-}$ mice (five mice per dose) were infected orally with a 10-fold serial dilutions (10^9 to 10^4 CFU) of *Y. enterocolitica* and then monitored for death over a 14-day period. The LD_{50} was calculated by the method of Reed and Muench (26). (B) Kinetic analysis of infection. IL-6$^{-/-}$ or C57BL/6j mice were infected orally with 10^9 CFU of *Y. enterocolitica*. The infection was allowed to proceed for the indicated amount of time. Mice were then sacrificed, and the indicated organs were harvested. The bacterial load was determined by enumerating the number of viable bacteria in each organ. Each point on the scatter plot represents data from an individual mouse. Points on the *x* axis represent tissues from mice that survived to that time point but did not have recoverable bacteria. The median for each data set is indicated with a horizontal bar, and asterisks represent statistically significant comparisons as determined by a Mann-Whitney nonparametric two-tailed ANOVA (day 3 PP, P = 0.02; day 7 MLN, P = 0.008). The results are the average of two independent experiments.

mice inoculated with 10-fold-fewer bacteria than the C57BL/6j mice (data not shown).

IL-6$^{-/-}$ mice have a hyperinflammatory phenotype. The most striking pathological phenotype observed during acute yersiniosis due to infection with the enteropathogenic yersiniae is acute inflammation. Inflammation is part of the normal response to infection and is usually an effective part of the innate immune response to *Y. enterocolitica* infection. The inflammatory response to *Y. enterocolitica* infection is mediated by inflammatory cytokines (IL-1 and TNF-α) (2, 5, 14). During infection of C57BL/6j mice with *Y. enterocolitica*, there is an increase in IL-6 mRNA, as determined by quantitative real-time PCR and microarray analysis, as well as an increase in IL-6 levels in serum (Handley et al., unpublished) (Table 2). Because IL-6 has been suggested to have an anti-inflammatory role during *Y. enterocolitica* infection (17), it was hypothesized that infection would lead to a strong inflammatory response in the *Y. enterocolitica*-infected IL-6$^{-/-}$ mouse. Consistent with this hypothesis, gross examination suggested that the IL-6$^{-/-}$ mice had a more severe inflammatory pathology after infection with *Y. enterocolitica*. IL-6$^{-/-}$ mice infected with *Y. enterocolitica* had more pronounced visible abscesses of the PP, MLN, spleen, and liver earlier and at a greater frequency than did the C57BL/6j mice (data not shown). Gastrointestinal bleeding was common with the IL-6$^{-/-}$, mice as were intestinal adhesions. These are uncommon findings with the C57BL/6j mouse (data not shown) at the time points examined.

To investigate the pathology further, a detailed histopathologic analysis was done that compared IL-6$^{-/-}$ and C57BL/6j mice infected with *Y. enterocolitica*. Both strains of mice were infected orally with 10^9 CFU of *Y. enterocolitica*. The mice were then sacrificed on the indicated day, and tissues were removed for histological analysis. Two independent investigators examined the slides in a blind fashion. Consistent with the results obtained during the kinetic analysis, the microscopic pathology of PP did not show any significant differences (data not shown), both strains of mice had severe inflammatory pathologies in the PP. However, examination of the deeper tissues (MLN, spleen, and liver) revealed several important differences. The IL-6$^{-/-}$ mice had inflammatory pathologies more frequently than the C57BL/6j mice. It should also be noted that there was a higher mortality rate for the IL-6$^{-/-}$ mice. Many of the mice destined for the day 7 time point died prior to day 7 (60% for the IL-6$^{-/-}$ mice compared to 20% for the C57BL/6j mice). Presumably, these mice had significant pathology, however, these mice were excluded from examination, since the exact time of death could not always be determined.

Pathological evidence of early systemic yersiniosis was frequently observed in the IL-6$^{-/-}$ mice. Fibrin thrombi with hepatic ischemia were evident as early as day 3 in the IL-6$^{-/-}$ mice (Fig. 2B). Fibrin thrombi in the liver were not observed until day 7 in the wild-type mice (Fig. 2C). Fibrin thrombi were

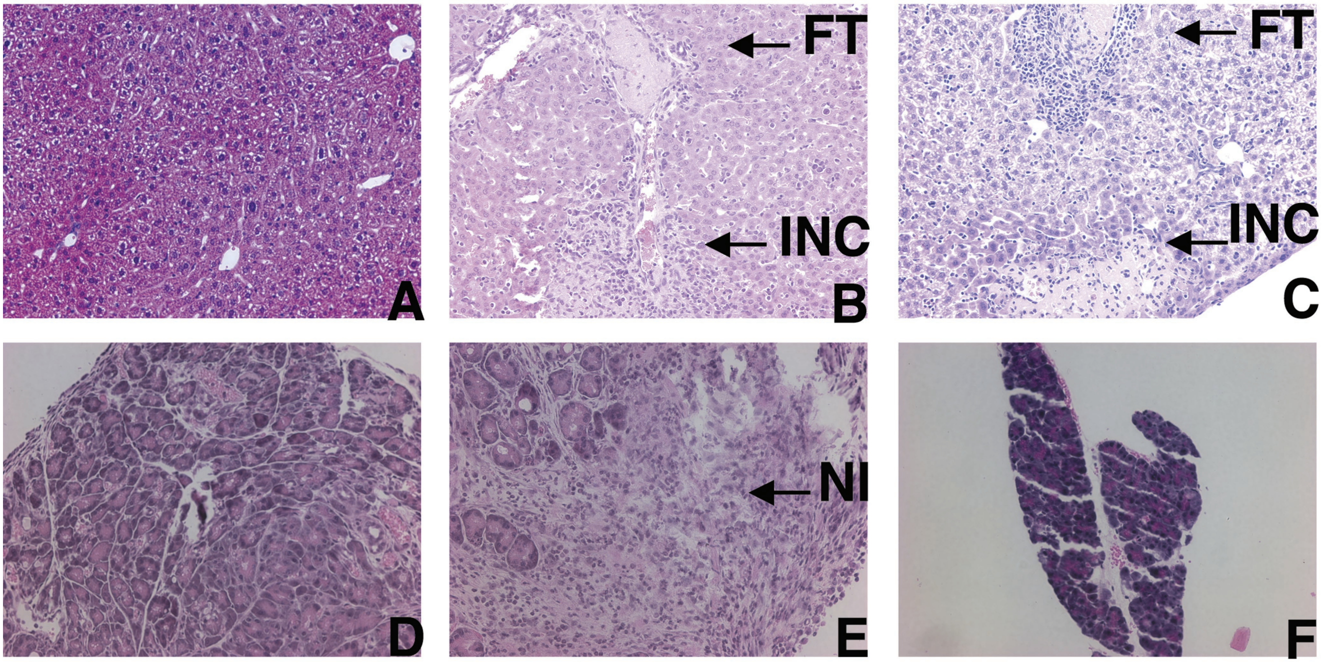

FIG. 2. IL-6$^{-/-}$ mice have a hyperinflammatory response to *Y. enterocolitica* infection. C57BL/6j and IL-6$^{-/-}$ mice were infected orally with 10^9 *Y. enterocolitica*. Infection was allowed to proceed for the indicated amount of time, and then tissues were prepared for histological analysis. Tissue sections were stained with hematoxylin and eosin. (A) Image of an uninfected liver from a C57BL/6j mouse (magnification, ×200). (B) High-power image (magnification, ×200) of a liver from an IL-6$^{-/-}$ mouse infected for 3 days. Note the fibrin thrombus (FT) and the resulting ischemic necrosis (INC). (C) High-power image (magnification, ×200) of a liver from a C57BL/6j mouse infected for 7 days. Note the fibrin thrombus (FT) and the resulting ischemic necrosis (INC) compare to panel B. (D) Normal pancreas of a C57BL/6j mouse (magnification, ×200). (E) Pancreas of an IL-6$^{-/-}$ mouse infected for 3 days. Note the neutrophilic inflammatory lesion (NI). (F) High-power image (magnification, ×200) of a pancreas of a C57BL/6j mouse infected for 3 days.

frequently observed in the spleens of the IL-6$^{-/-}$ mice starting at day 3 but were never observed in the spleens of the wild-type mice at the times examined.

The inflammatory lesions of the IL-6$^{-/-}$ mice were predominantly neutrophilic, whereas the inflammatory lesions of C57BL/6j mice were largely neutrophilic but also contained macrophages. The neutrophilic nature of the response was best observed in the microabscesses (infiltrates of inflammatory cells without visible bacterial colonization [Fig. 2E]) and the microcolonies (infiltrates of inflammatory cells with visible bacterial colonization) of the IL-6$^{-/-}$ mice. The neutrophilic nature of the inflammatory lesions is consistent with the hypothesis that IL-6 mediates the transition from neutrophilic to monocytic inflammatory lesions. In addition, these mice developed these pathologies at earlier time points, more frequently, and to a greater extent than the C57BL/6j mice. At early time points the infiltrates are well defined and clearly neutrophilic. As disease progresses, the infiltrates contain increasing amounts of cellular debris and *Y. enterocolitica* becomes microscopically evident. At latter time points both lymphocytes and macrophages are present at sites of inflammation, although the lesions remain mostly neutrophilic. Furthermore, these pathologies were present in organs from the IL-6$^{-/-}$ mice not usually affected during infection of the C57BL/6j mouse such as the pancreas (Fig. 2E), suggesting a broader systemic spread. Altogether, these data suggest that the IL-6$^{-/-}$ mice have a defect in the control of the inflammatory response to *Y. enterocolitica* in conjunction with an inability to control the bacterial infection.

IL-6$^{-/-}$ mice infected with *Y. enterocolitica* have a stronger inflammatory cytokine response than do C57BL/6j mice. The resolution of a *Y. enterocolitica* infection ultimately depends on the establishment of an adequate T_H1 T-cell response (4). The most significant cytokine in establishing a T_H1 type T-cell response is IFN-γ. IFN-γ is involved in the activation of inflammatory cells, and it has been well documented that IFN-γ has a critical role in the control of *Y. enterocolitica* infection (1, 2). Due to the histopathology of *Y. enterocolitica*-infected IL-6$^{-/-}$ mice, it was of interest to examine IFN-γ levels from the tissues of infected mice. IL-6$^{-/-}$ mice and C57BL/6j mice were infected with *Y. enterocolitica*, and the infection was allowed to progress for 5 days. Mice were then sacrificed, and spleens were removed. Splenocytes were prepared and subsequently, ELISA and CBA determined the amount of IFN-γ secreted into the culture supernatant. IL-6$^{-/-}$ mice had a more robust IFN-γ response than did the C57BL/6j mice, with 2.5-fold more IFN-γ secreted into the culture supernatant (Fig. 3A). When splenocytes were preincubated with heat-killed *Y. enterocolitica* (HK Ye), the splenocytes from the C57BL/6j mice showed a 13-fold increase in the amount of IFN-γ produced, whereas the splenocytes from the IL-6$^{-/-}$ mice stimulated with HK Ye showed a 5-fold change compared to the IL-6$^{-/-}$ untreated group. Treatment of the splenocytes from the C57BL/6j or IL-6$^{-/-}$ mice with concanavalin A (ConA) led to 10- and 5-fold increases in IFN-γ, respectively, compared to the untreated group. Increased levels of IFN-γ secreted by splenocytes treated with HK Ye or ConA demonstrate that the cells

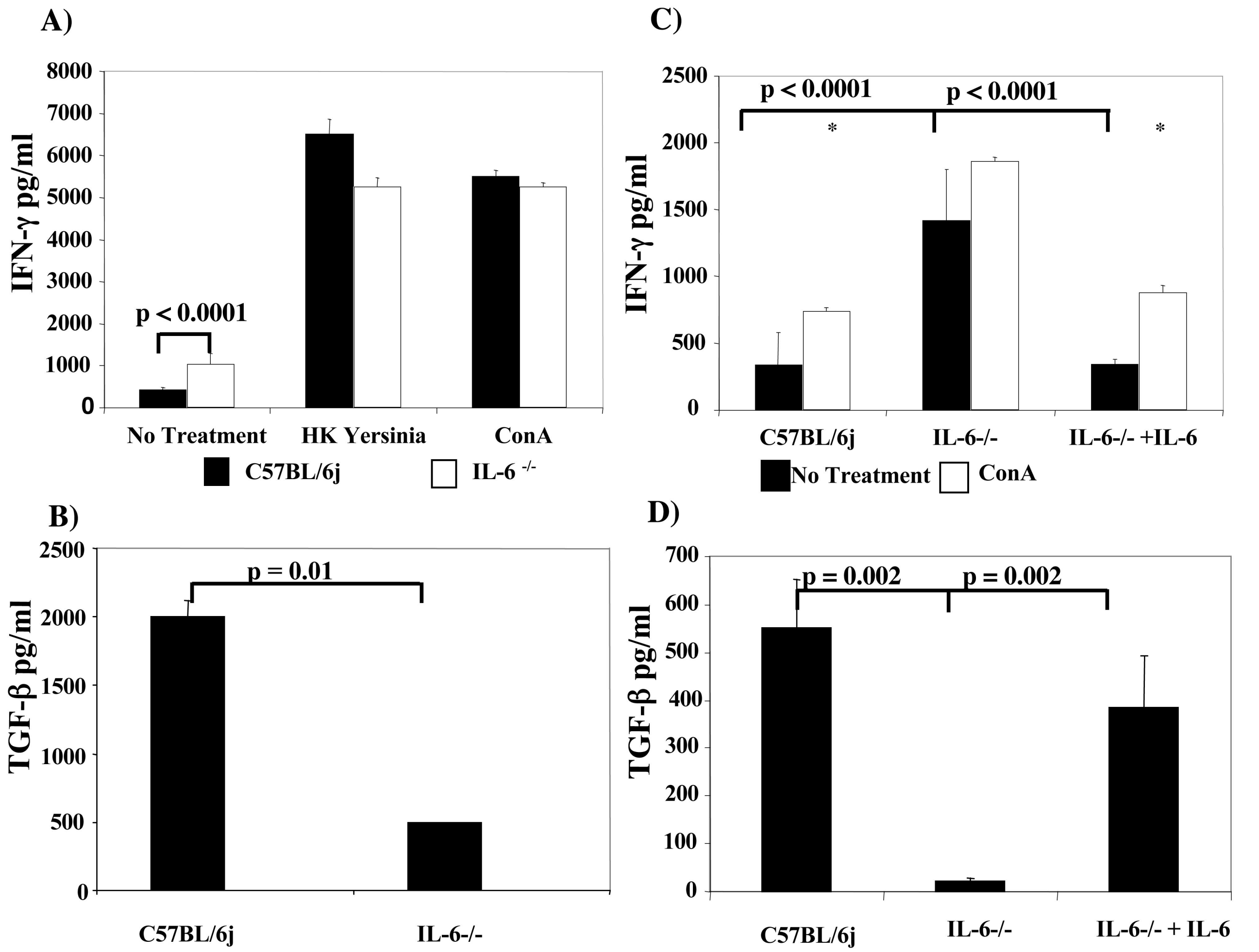

FIG. 3. Analysis of cytokine production by ELISA. (A) IFN-γ ELISA from the supernatant of splenocytes (10^5) of mice infected orally with *Y. enterocolitica*. Splenocytes received the indicated treatment: no further treatment, 10 μg of heat-killed *Y. enterocolitica*/ml, or 5 μg of ConA/ml for 3 days. The concentration of IFN-γ secreted into the culture supernatant was determined by sandwich ELISA according to the manufacturer's instructions. Splenocytes from C57BL/6j mice are represented by black bars, and those from IL-$6^{-/-}$ mice are represented by white bars. (B) TGF-β1 ELISA from the supernatant of splenocytes (10^5 cells) of mice orally infected with *Y. enterocolitica*. The concentration of TGF-β1 secreted into the culture supernatant was determined by sandwich ELISA according to the manufacturer's instructions. (C) IFN-γ ELISA. Splenocytes from infected mice (C57BL/6j mice, IL-$6^{-/-}$ mice, and IL-$6^{-/-}$ mice treated with 1 μg of rIL-6 SC every day for 5 days) were used for ELISA. Splenocytes (10^5 cells) received the indicated treatment, i.e., no further treatment or 5 μg of ConA/ml. The concentration of IFN-γ secreted into the culture supernatant was determined by sandwich ELISA according to the manufacturer's instructions. The asterisks indicate comparisons that were not statistically significant. (D) TGF-β1 ELISA. Mice were treated as described for panel C except that ConA was not added. Subsequently, splenocytes (10^5) from these mice were cultured for 3 days. The concentration of TGF-β1 secreted into the culture supernatant was determined by sandwich ELISA according to the manufacturer's instructions. The results are the average of four assays done in duplicate. The error bars represent the standard deviation, and statistically significant comparisons (as determined by a Mann-Whitney nonparametric two-tailed ANOVA) are indicated by brackets.

are capable of responding to specific antigenic stimulation (HK Ye) and a nonspecific mitogen (ConA). These data suggest a specific response and argue against LPS tolerance as a reason for the differences in cytokine levels. Differences in cytokine levels due to differences in bacterial colonization of the splenocytes were ruled out by enumerating the numbers of viable bacteria in the spleens prior to use in the ELISAs. C57BL/6j mice had between 3×10^8 and 7×10^8 CFU/g of spleen tissue, whereas the IL-$6^{-/-}$ mice had between 2.5×10^8 and 5×10^8 CFU/g of tissue, suggesting that the spleens had a similar bacterial load.

The histopathologic examination suggested that the IL-$6^{-/-}$ mice have a defect in the downregulation of the inflammatory response to *Y. enterocolitica*. One cytokine that is involved in the regulation of inflammatory responses and has recently been shown to be involved in the downregulation of the inflammatory pathology of intestinal toxoplasmosis is TGF-β (7). Interestingly, it was observed that the administration of high concentrations of recombinant TGF-β1 provided limited protection to mice infected with *Y. enterocolitica* (3). To further examine the molecular determinants of the inflammatory response to *Y. enterocolitica* infection, single-cell splenocyte suspensions were made from infected mice and cultured as described above and then ELISA determined TGF-β1 levels (Fig. 3B). Interestingly, the splenocytes from C57BL/6j mice showed a fourfold increased level of TGF-β1 compared to

TABLE 1. Cytokine levels in supernatants of splenocytes isolated from infected mice

Cytokine	Mean cytokine level (pg/ml) ± SEM[a] in: C57BL/6j mice			IL-6$^{-/-}$ mice			IL-6$^{-/-}$ mice + rIL-6		
	No Tx	HK	ConA	No Tx	HK	ConA	No Tx	HK	ConA
TNF-α	710 ± 51*	3,297 ± 320	892 ± 69	1,311 ± 49*	6,450 ± 229	2,029 ± 40	266 ± 5	1,538 ± 51	567 ± 26
IFN-γ	3 ± 0.5*	1,250 ± 377	1,034 ± 100	17 ± 2*	374 ± 21	145 ± 21	2 ± 0.7	21 ± 3	403 ± 24
MCP-1	254 ± 33*	1,767 ± 167	1,325 ± 359	1,263 ± 62*	2,727 ± 180	2,151 ± 29	75 ± 12	532 ± 35	350 ± 22
IL-10	15 ± 7*	2,309 ± 612	1,325 ± 360	721 ± 43*	4,900 ± 869	1,893 ± 171	14 ± 8	231 ± 24	52 ± 3
IL-6	42 ± 10	691 ± 116	254 ± 59	0	0	0	0	0	0
IL-12p70	0	0	0	0	0	0	0	0	0

[a] IL-6$^{-/-}$ mice were treated with 1 μg of rIL-6/day through the course of infection. No Tx, cultures that received no further treatment; HK, cultures that were treated with 10 μg of heat-killed *Y. enterocolitica*/ml; ConA, cultures that received 1 μg of ConA/ml. An asterisk indicates a statistically significant comparison of at least P = 0.02 as determined by Mann-Whitney ANOVA.

splenocytes from the IL-6$^{-/-}$ mice. The levels of IFN-γ and TGF-β1 appeared to be inversely related, suggesting a relationship. We were able to show a direct connection between IL-6 and the levels of TGF-β and IFN-γ by treating mice during the course of infection with rIL-6 (Fig. 3C and D; see also Tables 2 and 3). When IL-6$^{-/-}$ mice were treated with recombinant IL-6 (rIL-6) during the course of infection they displayed a cytokine secretion profile that was most similar to that of the wild-type mice. This suggests that the differences in cytokine levels are due to the lack of IL-6 and not some other defect in these animals. Taken together, high levels of IFN-γ and low levels of TGF-β could account for the hyper-inflammatory phenotype observed in the IL-6$^{-/-}$ mice infected with *Y. enterocolitica*.

Levels of the immunomodulatory cytokines IL-10, TNF-α, and MCP-1 are different in the IL-6$^{-/-}$ mouse. Although several lines of evidence implicated IFN-γ, TGF-β1, and IL-1ra in the IL-6 response to *Yersinia* infection, several other inflammatory cytokines are known to be involved in the resolution of infection. Expression of these cytokines (IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70) in splenocyte cultures from *Y. enterocolitica*-infected IL-6$^{-/-}$ and C57BL/6j mice were investigated. Mice were infected, and then serum and splenocyte supernatants were harvested as described for the ELISA experiments. Splenocytes cultured from infected tissues were then subjected to CBA analysis to determine levels of cytokines (Tables 1 and 2). As described for the ELISA experiments, significantly higher levels of IFN-γ were observed in the splenocyte cultures from IL-6$^{-/-}$ mice compared to C57BL/6j mice.

Levels of TNF-α were significantly elevated in the serum and splenocyte supernatants of *Y. enterocolitica*-infected IL-6$^{-/-}$ mice. The IL-6$^{-/-}$ mice had between 7- and 17-fold more TNF-α in the serum and threefold more in splenocyte supernatants. This is in good agreement with in vitro data with human monocytes that demonstrated an IL-6-dependent suppression of TNF-α (27). High levels of TNF-α in serum correlate with endotoxic shock, which is consistent with the hyperinflammatory phenotype and histopathologic observations of the IL-6$^{-/-}$ mice.

MCP-1 is a CC chemokine that was recently shown to be upregulated in response to the Invasin protein of *Yersinia* in HeLa cells (18). Levels of MCP-1 were significantly increased in the serum and splenocyte supernatants of *Y. enterocolitica*-infected IL-6$^{-/-}$ mice. IL-6$^{-/-}$ mice had between 7- and 46-fold more MCP-1 in the serum and 5-fold more MCP-1 in splenocyte supernatants. Levels of both TNF-α and MCP-1 in serum increase over the course of infection and were present at much higher levels in the sera of the IL-6$^{-/-}$ mice.

Recent data suggest that IL-10 may have a role in the response to *Y. enterocolitica* infection (29). Splenocyte cultures from both C57BL/6j mice and IL-6$^{-/-}$ mice produced detectable levels of IL-10, but the IL-6$^{-/-}$ mice made significantly more IL-10, and only in the sera of these mice was IL-10 detectable. The IL-6$^{-/-}$ mice had 52-fold more IL-10 in splenocyte supernatants. Splenocytes from both mouse strains responded to stimulation by HK *Yersinia* or ConA by producing more MCP-1, IL-10, TNF-α, and IFN-γ, and, in the case of C57BL/6j mice, IL-6, suggesting that differences in cytokine levels were not due to LPS tolerance. In none of the assays performed was IL-12p70 detectable.

Levels of MCP-1, IL-10, TGF-β1, and IFN-γ are related to the presence of IL-6. To gain further insight into the relation-

TABLE 2. Cytokine levels in sera of infected mice

Cytokine	Mean cytokine level (pg/ml) ± SEM[a] at: Day 0		Day 1		Day 3		Day 5			Day 7	
	C57BL/6	IL-6$^{-/-}$	C57BL/6	IL-6$^{-/-}$	C57BL/6	IL-6$^{-/-}$	C57BL/6	IL-6$^{-/-}$	IL-6$^{-/-}$ + rIL-6	C57BL/6	IL-6$^{-/-}$
TNF-α	9	8	17 ± 2*	9 ± 0.8*	11 ± 1*	76 ± 3*	13 ± 4*	197 ± 16*	92 ± 28	25 ± 4*	590 ± 92*
IFN-γ	0	1.5	17 ± 3*	6 ± 1*	3 ± 0.5*	16 ± 5*	7 ± 2*	31 ± 2*	14 ± 5	13 ± 0.8*	40 ± 9*
MCP-1	19	40	140 ± 40	109 ± 15	85 ± 19*	642 ± 70*	113 ± 14*	887 ± 95*	288 ± 82	72 ± 2*	3,643 ± 1200*
IL-10	0	0	0	69 ± 3	0	0	0	2,147 ± 1320	811 ± 475	0	154 ± 66
IL-6	0	0	120 ± 21	0	134 ± 17	0	135 ± 11	0	0	213 ± 34	0
IL-12p70	0	0	0	0	0	0	0	0	0	0	0

[a] IL-6$^{-/-}$ mice were treated with 1 μg of rIL-6/day through the course of infection. An asterisk indicates a statistically significant comparison of at least P = 0.02 as determined by Mann-Whitney ANOVA.

TABLE 3. Cytokine levels in spleen homogenates

Cytokine	Mean cytokine level (pg/ml) ± SD[a] in:		
	C57BL/6j mice	IL-6$^{-/-}$ mice	IL-6$^{-/-}$ mice + rIL-6
TNF-α	16 ± 15	60 ± 89	22 ± 16
IFN-γ	83 ± 23*	168 ± 41	65 ± 26
MCP-1	35 ± 12*	130 ± 81	46 ± 34
IL-10	29 ± 4	19 ± 17	22 ± 5
IL-6	58 ± 18	ND	ND
IL-12p70	ND	ND	ND

[a] Cytokine levels present in the spleen homogenate of infected mice were determined by CBA. ND, no detectable cytokine. Asterisks indicate a statistically significant result when C57BL/6j mice are compared to IL-6$^{-/-}$ mice. Samples were compared by using a Mann-Whitney test ($P = 0.007$).

ship between IL-6 and MCP-1, IL-10, TNF-α, IFN-γ, and TGF-β1, IL-6$^{-/-}$ mice were concurrently infected with *Y. enterocolitica* and injected with rIL-6. Subsequently, splenocytes and sera were harvested, and cytokine levels were determined by ELISA or CBA. Treatment of IL-6$^{-/-}$ mice with rIL-6 resulted in a cytokine secretion profile that was most similar to that of the C57BL/6j control mice (Fig. 3C and D and Tables 1 to 3). The rIL-6-treated IL-6$^{-/-}$ mice had lower levels of IFN-γ, MCP-1, IL-10, and TNF-α and higher levels of TGF-β1 than the mock-treated IL-6$^{-/-}$ mice. Surprisingly, IL-6 was not detectable in the sera of IL-6$^{-/-}$ mice treated with rIL-6; this could be due to a multitude of factors affecting the stability or persistence of this cytokine in the serum or the fact that rHIL-6 was used to complement the IL-6$^{-/-}$ mice and the ELISA and CBA utilizes mouse monoclonal antibodies for detection. This experiment links the expression of IFN-γ, MCP-1, IL-10, TNF-α, and TGF-β1 with that of IL-6. Altogether, these data suggested that IL-6 modulates the levels of several immunomodulatory cytokines in response to *Y. enterocolitica* infection in vivo.

Although the levels of cytokines in serum are a good indication of systemic responses, they may not accurately represent cytokine levels in microenvironments such as spleen tissue. To determine whether there were differences between the cytokine levels observed in the serum or in splenocyte culture and those observed directly from spleen homogenates, we measured the levels of IL-6, MCP-1, IL-12p70, IFN-γ, and TNF-α directly from spleen homogenates (Table 3). As expected, the concentration of cytokines was not identical but the trends observed when cytokine levels were measured in the spleen homogenates were the same as those observed from the serum or splenocyte cultures. In the spleen homogenates, the IL-6$^{-/-}$ mice had statistically significant ($P = 0.007$) fourfold more MCP-1 and twofold more IFN-γ than homogenates from the C57BL/6j spleens. In this assay the differences in IL-10 and TNF-α were determined to not be statistically significant. Whether cytokines are measured from splenocyte cultures, serum, or spleen homogenates, it appears that the IL-6$^{-/-}$ mice have a higher concentration of inflammatory cytokines than the C57BL/6j mice. Furthermore, if mice are treated with rIL-6 during the course of infection the cytokine profile more closely resembles that of the C57BL/6j mouse. Altogether, these data suggest that IL-6 plays a key role in balancing the levels of pro- and anti-inflammatory cytokines during a *Y. enterocolitica* infection.

To determine whether the hyperinflammatory pathology observed in the IL-6$^{-/-}$ mice could be rescued by the administration of rIL-6 during the course of infection, C57BL/6j mice, IL-6$^{-/-}$ mice, or IL-6$^{-/-}$ mice treated with rIL-6 were infected orally with 10^9 CFU of *Y. enterocolitica*, and the infection was allowed to proceed for 3 days. The mice were then sacrificed, and tissues were prepared for pathological analysis as described above. Spleens were scored for inflammatory lesions in a blind manner by two investigators (Table 4). When the inflammatory lesions of the spleens were examined, the IL-6$^{-/-}$ mice treated with rIL-6 had lesions most similar to the lesions observed in the C57BL/6j mice (Table 4). The IL-6$^{-/-}$ had more severe inflammatory lesions than either the IL-6$^{-/-}$ mice treated with rIL-6 or the C57BL/6j mice. Twenty percent of the C57BL/6j mice and the IL-6$^{-/-}$ mice treated with rIL-6 had observable inflammatory changes in the spleen, whereas sixty percent of the IL-6$^{-/-}$ mice had inflammatory changes in the spleen. Thus, based on spleen pathology, the addition of exogenous rIL-6 to IL-6$^{-/-}$ mice was sufficient to rescue the hyperinflammatory phenotype observed in the IL-6$^{-/-}$ mice. We did not attempt to rescue the lethality observed in the IL-6$^{-/-}$ mice by treating them with rIL-6, but this was successfully done in an *L. monocytogenes* infection of IL-6$^{-/-}$ mice (12).

IL-6$^{-/-}$ mice show a stronger cytotoxic response to *Y. enterocolitica*. The high levels of IFN-γ observed in the splenocytes of *Y. enterocolitica*-infected IL-6$^{-/-}$ mice are suggestive of a stronger T_H1 T-cell response. A stronger T_H1 T-cell response should lead to a more robust cytotoxic-T-lymphocyte (CTL) response to *Y. enterocolitica*. To investigate the CTL response in C57BL/6j and IL-6$^{-/-}$ mice, the animals were infected orally, and the infection was allowed to progress for 5

TABLE 4. Rescue of inflammatory pathologies in an IL-6$^{-/-}$ mouse with rIL-6

Animal[a]	Tissue score for:				% Spleens with pathology
	Microabscess[b]	Microcolony[c]	Inflammatory cell infiltrate[d]	Necrosis	
C57BL/6j mice	1 (0.5)	NA	2 (1.0)	NA	20
IL-6$^{-/-}$ mice	2.8 (0.83)	3 (0.5)	3 (1.0)	2 (0.16)	60
IL-6$^{-/-}$ mice + rIL-6[e]	2.5 (1.0)	NA	2 (1.0)	NA	20

[a] The genetic background of the animals used is indicated. Animals were infected for 3 days with wild-type *Y. enterocolitica*, and then spleens were harvested for investigation of the inflammatory pathologies.

[b] Microabscess refers to a defined inflammatory lesion without microscopically visible bacterial colonization. Lesions were scored as described in Materials and Methods, and average scores for the spleens that had inflammatory pathology are reported. In parentheses is the fraction of inflamed spleens with the indicated pathology.

[c] Microcolony refers to a defined inflammatory lesion with microscopically visible bacterial colonization.

[d] Inflammatory cell infiltrate refers to increased numbers of neutrophils and macrophages in the spleen compared to uninfected mice.

[e] To rescue the inflammatory pathology, IL-6$^{-/-}$ mice received daily injections of 1 μg of recombinant human IL-6 s.c.

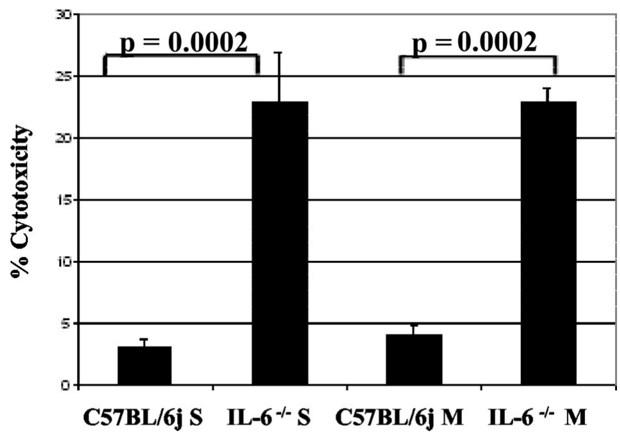

FIG. 4. Cytotoxicity assays. Single-cell suspensions were prepared from the spleens and MLN of C57BL/6j and IL-6$^{-/-}$ mice infected orally with *Y. enterocolitica* for 5 days. Columns labeled with an "S" represent the results from spleen cells, and columns labeled with and "M" represent cells from the MLN. The cell suspensions (10^5 cells) were cocultured with APCs (10^5 peritoneal macrophages) loaded with 10 μg of heat-killed *Y. enterocolitica* for 4 h, and the amount of cytotoxicity determined according to the manufacturer's instructions. The results are the averages of four assays done in duplicate. The error bars represent the standard error of the mean, and statistically significant comparisons (as determined by a Mann-Whitney nonparametric two-tailed ANOVA) are indicated by brackets.

days. Mice were then sacrificed, and single-cell suspensions were made from the spleens and MLN. Cell suspensions were then added to peritoneal macrophages that were preincubated with heat-killed *Y. enterocolitica*, and the percentage of cytotoxicity was determined enzymatically by measuring the release of lactate dehydrogenase from the macrophage monolayer. IL-6$^{-/-}$ splenocytes or lymph node cells from IL-6$^{-/-}$ mice were four- to eightfold more cytotoxic than those of the C57BL/6j mice (Fig. 4). When this experiment was performed with cells from mice infected for 7 days, the same observations were made. However, at day 7 the cells from the C57BL/6j mice showed 30 to 40% cytotoxicity, whereas the IL-6$^{-/-}$ cells showed 75 to 95% cytotoxicity (data not shown).

DISCUSSION

The three pathogenic bacteria of the genus *Yersinia*, *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* all cause significant disease in humans. In order to understand the pathogenesis of *Y. enterocolitica* infection, it is important to understand the nature of the host response. We recently demonstrated a key role for IL-1α in the initiation of intestinal inflammation in response to *Y. enterocolitica* infection (14). Other researchers have demonstrated a role for IL-1, IL-1ra, IL-6, IL-10, IL-12, IL-18, TNF-α, and IFN-γ in the resolution of *Y. enterocolitica* infection or in in vitro models (2, 3, 5, 14, 15, 17, 29). Interestingly, all of the cytokines identified to date function predominantly as enhancers of the inflammatory responses. With the exception of IL-6, IL-1ra, and IL-10, no cytokines have yet been identified that are involved in the downregulation of the inflammatory response to *Y. enterocolitica* infection, and the data presented here suggest that, in the context of an IL-6 deficiency, IL-10 is insufficient to decrease inflammatory responses. We have extended our investigation of the host inflammatory response to *Y. enterocolitica* infection to reexamine the multifunctional cytokine IL-6 that appears to have a function in downregulating the inflammatory response to *Y. enterocolitica* through the modulation of several pro- and anti-inflammatory cytokines.

When the first IL-6$^{-/-}$ mice were produced, it was known that the mice were immunocompromised and could not handle bacterial or viral challenge as well as the wild-type controls (22). IL-6 provides protection against the intracellular bacterial pathogens *L. monocytogenes* and *M. tuberculosis*, and several theories have been proposed to explain these deficiencies (12, 23). Most theories relating to the role of IL-6 in the control of bacterial infection have evolved around the functioning of neutrophils. It is clear from the data presented here that IL-6$^{-/-}$ mice are defective in the control of a *Y. enterocolitica* infection as well, and it is probable that there is a defect in the neutrophil response to *Y. enterocolitica* since these mice suffer from a rapid and overwhelming sepsis.

The pathological analysis suggests that inflammatory cells are getting to the appropriate tissues in the infected IL-6$^{-/-}$ mice. However, control of the infection is inadequate, as demonstrated by both LD_{50} and kinetic analyses. The IL-6$^{-/-}$ mice show early signs of severe sepsis. The combination of fibrin thrombi in multiple organs and gastrointestinal bleeding suggests that the mice are suffering from disseminated intravascular coagulation a sign of severe sepsis. Interestingly, much of the pathology that is observed in the IL-6$^{-/-}$ mice is seen at earlier time points than what is observed in the C57BL/6j mice. For example, IL-6$^{-/-}$ mice show rapid dissemination of *Y. enterocolitica* to the liver and spleen, suggesting again a defect in innate immune responses. The exact role of IL-6 in innate immunity remains unclear but, as previously demonstrated, neutrophils have been shown to be the main source of IL-1ra in response to *Y. enterocolitica* infection (17), and the downregulation of the IL-1-mediated inflammatory responses at the site of infection may be a key role of IL-6.

Like IL-6, TGF-β1 is a multifunctional cytokine that has an important role in the regulation of inflammatory responses (24). In fact, when TGF-β1-deficient mice were produced it was noted that these mice died 2 to 4 weeks after birth and that they died of severe inflammatory responses (28). The severe inflammatory response and early mortality make the direct analysis of *Y. enterocolitica* infection in the TGF-β1$^{-/-}$ mice impractical. More recently, it was noted that TGF-β1 has a role in the regulation of responses to infectious agents. The administration of rTGF-β1 to mice infected with *Y. enterocolitica* provides limited protection (3). Furthermore, in the murine model of intestinal toxoplasmosis, TGF-β1 controls intestinal inflammatory responses to this pathogen by controlling IFN-γ levels (7). The observed decrease in TGF-β1 in IL-6$^{-/-}$ mice is consistent with the pathology we observed in these mice, suggesting a role for TGF-β1 in modulation of the inflammatory cells during a *Y. enterocolitica* infection. In agreement with the data on intestinal toxoplasmosis, the severe inflammatory pathology appears to be related to the misregulation of inflammatory cytokines, including IFN-γ.

Splenocytes from infected C57BL/6j and IL-6$^{-/-}$ mice had different cytokine secretion profiles that appear to be inversely related. The IL-6$^{-/-}$ splenocytes had high levels of IFN-γ, TNF-α, MCP-1, and IL-10 and low levels of TGF-β1, whereas

the C57BL/6j splenocytes showed lower levels of IFN-γ, TNF-α, MCP-1, IL-10, and higher levels of IL-6 and TGF-β1. The cytokine secretion profile and the hyperinflammatory phenotype of the IL-$6^{-/-}$ mouse could be restored to that of the wild-type control mice by treating the IL-$6^{-/-}$ mice with rIL-6. Consistent with the hypothesis that the cytokine secretion profile is responsible for the hyperinflammatory phenotype, antigen-stimulated splenocytes from IL-$6^{-/-}$ mice were significantly more cytotoxic than antigen-stimulated splenocytes from C57BL/6j mice.

Much of the work investigating the role of IL-6 during bacterial infection has focused on the innate immune response. However, it is also clear that there is a role for IL-6 in the cellular immune response. Many of the pathologies observed in the IL-$6^{-/-}$ mice could also be accounted for by the overly robust T_H1 response. IFN-γ not only plays an important role in polarizing $CD4^+$-T-cell responses, but it is also a key cytokine for the activation of macrophages. The increase in IFN-γ in IL-$6^{-/-}$ mice can be explained by the recent observation that IL-6 inhibits the T_H1 polarization of $CD4^+$ T cells (13). Because there is no IL-6 in these mice, this inhibition is lost, leading to more IFN-γ and a more robust T_H1 T-cell response and probably a greater macrophage and CTL response. All of these observations could account for the hyperinflammatory response of the IL-$6^{-/-}$ mice to *Y. enterocolitica* infection.

The inflammatory response to bacterial pathogens is clearly an attempt by the host to resolve the infection before serious disease can result. However, when the inflammatory response is left unchecked the results are dire for the host. We provide evidence in the present study that IL-6 has a protective role during a *Y. enterocolitica* infection. The role of IL-6 in the control of bacterial infections is most likely multifactorial, involving both the innate and the cellular immune responses. Because IL-$6^{-/-}$ mice still have acute sepsis and are unable to control the *Y. enterocolitica* infection we cannot rule out a defect in the early innate responses, as suggested by other investigators. However, we further suggest that a molecular reason for the observed pathology of this infection involves the modulation of IL-1ra (17), TNF-α, MCP-1, IL-10, TGF-β1, and IFN-γ (the present study) in an IL-6-dependent fashion. We have established a direct link between these cytokines and IL-6 in vivo; these cytokines appear to be working in concert to control the *Y. enterocolitica* infection and/or the inflammation that results from the infection. It is the misregulation of these cytokines in the IL-$6^{-/-}$ mouse that leads to the hyperinflammatory phenotype observed in these mice. These data provide further insight into the intricate nature of the host response to bacterial pathogens and the complex nature of the immunomodulatory cytokines involved in the control of the infection. Altogether, these data suggest that IL-6 may be a negative regulator of the inflammatory response to *Y. enterocolitica* infection.

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