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Interleukin-6-Deficient Mice Are Highly Susceptible to *Listeria monocytogenes* Infection: Correlation with Inefficient Neutrophilia

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We have produced interleukin-6 (IL-6)-deficient mice to examine, in vivo, the wide variety of biological activities attributed to this multifunctional cytokine. To investigate the role of IL-6 during infectious disease, IL-6-deficient mice were challenged with sublethal doses of *Listeria monocytogenes*, a facultative intracellular bacterium. While normal control animals were able to clear the infection, mutant animals exhibited a high mortality rate and showed uncontrolled replication of the bacteria in the spleen and liver at 2 and 3 days postinfection. Sections of infected tissues showed an increase in the number and severity of inflammatory foci. All aspects of this phenotype in the mutant animals were completely reverted upon administration of recombinant murine IL-6 (rIL-6). Various parameters of natural killer (NK) cell and macrophage function were unaffected in the challenge of the mutant animals. However, IL-6-deficient animals failed to mount peripheral blood neutrophilia in response to listeriosis, whereas control animals displayed a prominent neutrophilia in the blood at 24 and 48 h postinfection. Additionally, we analyzed the efficacy of rIL-6 in protecting animals devoid of lymphocytes or devoid of neutrophils during listeriosis. Administration of rIL-6 was protective to animals devoid of lymphocytes, suggesting that the rIL-6 protective effect was not mediated through lymphocytes. In contrast, control and mutant animals depleted of neutrophils were refractory to the rIL-6 protective effect. These data suggest that IL-6 is critical early during listeriosis, perhaps acting by stimulating neutrophils either directly or indirectly. Additionally, these data show a promising therapeutic potential for rIL-6 administration during opportunistic infection.

Interleukin-6 (IL-6) is a cytokine having multiple biological activities. Although originally cloned on the basis of its biological activity on B cells, IL-6 has been shown to stimulate a variety of cell types (13, 14, 33). High levels of IL-6 are detected in serum during microbial infection, tissue damage, and other inflammatory conditions (8). Many studies have used anti-IL-6 antibodies to study the role of this cytokine activity in murine disease models. However, two independent reports have shown surprisingly high levels of IL-6 in the sera of animals that received anti-IL-6 neutralizing antibodies, indicating possible "chaperoning" effects (12, 29). Therefore, mice with a targeted mutation may provide a clearer system to evaluate the role of IL-6 in vivo. We have created IL-6-deficient mice, by standard homologous recombination techniques, to investigate the role of IL-6 in vivo normally and during infectious disease states. One such model of an infectious disease and inflammatory state is listeriosis, in which IL-6 expression is known to correlate with disease severity (11).

Listeria monocytogenes is a facultative intracellular bacterium which primarily infects macrophages and hepatocytes in the mouse. Numerous cytokines have been shown to influence resistance to *L. monocytogenes*. Mice treated with a gamma interferon (INF-γ)-neutralizing antibody show a higher mortality rate and an increased bacterial load (3). Mice lacking the 55-kDa tumor necrosis factor receptor (TNFR p55), generated by homologous recombination, succumb to *L. monocytogenes* at doses that are sublethal for wild-type controls (22). A similar result was obtained by using a neutralizing antibody to tumor necrosis factor alpha (20). Treatment of mice with a neutralizing antibody against IL-1 renders them more sensitive to *L. monocytogenes* (23), while exogenous IL-1 enhances resistance to *L. monocytogenes* (5). IL-6 also enhances resistance to *L. monocytogenes* when administered exogenously (17).

Aspects of both innate and adaptive immunity have been shown to function in antilisterial immunity. The macrophage, a cell type infected by the intracellular bacterium, exhibits antilisterial activity in vitro (7), as well as probably playing a central role in an integrated immune response to the infection in vivo. Neutrophils have recently been shown to be very important in the early phases of *L. monocytogenes* infection. Animals depleted in vivo of neutrophils by use of the antibody RB6-8C5 are extremely sensitive to infection (4, 6, 25). Additionally, mice immunized with virulent *L. monocytogenes* are immune to rechallenge (19), and dominant T-cell epitopes of *L. monocytogenes* proteins have been identified (21).

In this study we characterized the increased susceptibility of IL-6-deficient mice to listeriosis by mortality rates, bacterial accumulation, and histopathology. Recombinant murine IL-6 (rIL-6) administration reverted this susceptibility to levels of resistance above that of control animals. Furthermore, we investigated cell types known to be important in antilisterial immunity, to determine if the absence of IL-6 may have altered their function.

MATERIALS AND METHODS

Generation of IL-6-deficient mice. The IL-6 targeting vector was constructed by using BALB/c genomic DNA and the plasmid ptkneoA$^+$. A 5.1-kb *Eco*RI fragment containing exons I, II, and III was inserted upstream of the neomycin resistance gene, and a 0.9-kb *Stu*I fragment consisting mostly of the intron sequence between exons IV and V was inserted downstream of the neomycin resistance gene (30). Additionally, a herpes simplex virus thymidine kinase gene

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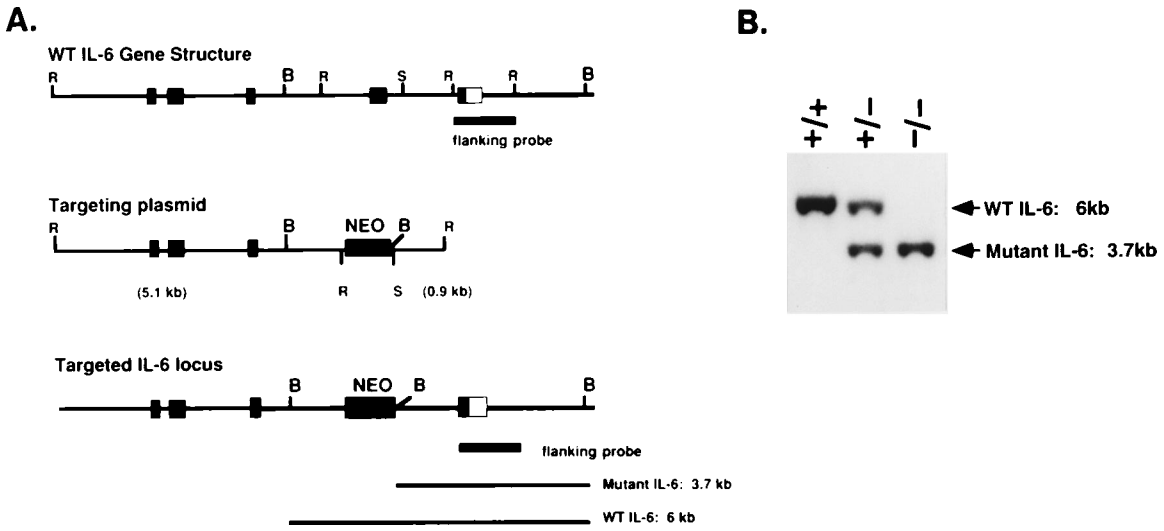

FIG. 1. Generation of IL-6-deficient mice. (A) Restriction map of the wild-type (WT) IL-6 genomic locus, with exons represented by boxes. The targeting construct and predicted structure of the targeted IL-6 locus are shown in the middle and bottom panels, respectively. The targeting construct contains 6 kb of homology, flanking the neomycin (NEO) resistance gene. Additionally, a herpes simplex virus thymidine kinase gene was positioned in the construct outside the region of homology (not shown). Homologous recombination between the targeting construct and the IL-6 genomic locus results in the deletion of 95% of exon 4. The flanking probe as well as the expected *Bam*HI fragments for wild-type and homologous recombinants is shown. R, *Eco*RI; B, *Bam*HI; S, *Stu*I. (B) Southern blot of tail DNA from an F_1 intercross, showing results for a wild-type animal (+/+), a heterozygous animal (+/−), and an animal homozygous for the IL-6 mutation (−/−). DNAs were digested with *Bam*HI, and the blot was probed with the flanking probe shown in panel A.

was located upstream of the 5.1-kb *Eco*RI fragment. Upon homologous recombination, this construct resulted in the replacement of 95% of exon IV with the neomycin resistance gene (Fig. 1).

E14.1 embryonic stem (ES) cells were transfected with 20 μg of linearized IL-6 targeting construct per 2×10^7 cells by electroporation. Electroporation was performed in a Bio-Rad Gene Pulser at 240 V and 500 μF. Cells were then plated out onto neomycin-resistant mouse embryo fibroblasts in ES medium (Dulbecco modified Eagle medium supplemented with 15% fetal calf serum, leukemia inhibitory factor, L-glutamine, penicillin-streptomycin, sodium pyruvate, nonessential amino acids, and β-mercaptoethanol) and allowed to recover overnight before being placed in G418 (330 μg/ml) and ganciclovir (2 μM). Medium was changed daily. Resistant colonies were obtained 6 to 12 days after selection. Clones were picked into 48-well dishes and subsequently expanded to yield cells for freezing and DNA preparation. Clones were screened by Southern blot hybridization using *Hin*dIII-digested DNA and a 0.9-kb *Stu*I-*Eco*RI internal probe and confirmed by using *Bam*HI-digested DNA and a 1.1-kb *Eco*RI flanking probe. Chimeric mice were produced by microinjecting clones containing the IL-6 mutation into C57BL/6 blastocysts with subsequent transfer into the uterine horns of pseudopregnant surrogates [(C57BL/6 × DBA/2)F_1]. Male chimeras were then bred with C57BL/6 females, and germ line transmission was determined by coat color. Germ line-positive pups were then screened by Southern blotting using DNA prepared from the distal portion of the tail, and animals heterozygous for the IL-6 mutation were intercrossed to yield mice homozygous for the mutation.

ELISA. Enzyme-linked immunosorbent assays (ELISA) for IL-6 were performed with tissue culture supernatants or serum samples by using rat monoclonal antibodies 20F3 for coating and 32C11 for detecting as described previously (1, 28). All cell culture experiments used Dulbecco modified Eagle medium and 10% fetal calf serum. Tissue culture supernatants were generated from adherent stromal cells from primary thymic cultures expanded for 2 weeks in vitro. To initiate cultures, thymi were disrupted and adherent cells were passaged. A total of 2×10^6 cells were stimulated with 10 μg of lipopolysaccharide (LPS) per ml overnight for supernatants. ELISA for IFN-γ were performed with tissue culture supernatants generated by incubating splenocytes, isolated from infected animals and uninfected controls, overnight in the presence of antibiotics, which were added 8 h after initiation of cultures. Splenocytes were obtained by teasing the spleens to single cells and removing erythrocytes by hypotonic lysis. Antibody XMG1.2 (supplied by Verax) was used for coating, and polyclonal rabbit anti-mouse IFN-γ was used for detection.

***L. monocytogenes* infection and analysis.** Mice were injected intravenously with 10^4 or 4×10^4 live *L. monocytogenes* organisms (kindly provided by H. Rogers, K. Murphy, and E. Unanue, St. Louis, Mo.). For the death curves, each group contained 10 animals, which were monitored daily for survival. rIL-6 (R&D) was administered at day −1, day 0, and in some experiments, day 1 of infection (12.5 μg; subcutaneously). Monoclonal antibody RB6-8C5 (a gift from R. Coffman, Palo Alto, Calif.) was administered at day −1 and at day 0 of infection (0.25 mg; intraperitoneally). Animals were shown to be depleted of neutrophils by peripheral blood differentials. For bacterial plating, animals were sacrificed either 2 or 3 days postinfection. The livers and spleens were removed and weighed, and cells were lysed by Dounce homogenization in phosphate-buffered saline. Tissue lysates were plated out in 10-fold dilutions onto triplicate Trypticase soy agar plates (BBL), incubated at 37°C overnight, and enumerated. For cytospins, single cell suspensions from portions of spleens were prepared, and erythrocytes were lysed by hypotonic shock. Cells were resuspended at 5×10^5/ml, and 100-μl portions of these suspensions were spun at 1,000 rpm for 5 min in a Cytospin3 (Shandon). For blood counts, blood was analyzed on a Serano 9010 blood analyzer. For differential blood counts, animals were bled through the tail vein and smears were made, fixed in 100% ethanol, stained with Wright's and Giemsa stains (Sigma), and analyzed on a Nikon Optiphot at a magnification of ×400. For tissue sections, portions of livers were fixed in formalin, cut into blocks, paraffin embedded, and step and serial sectioned. Serial sections were stained with hematoxylin and eosin or were Gram stained. Sections were analyzed on a Nikon Optiphot at ×200 and ×1,000.

NK cell staining and cytolytic assay. Spleen suspensions from infected and uninfected animals were prepared as described in the section above. For staining we used the antibody DX5 (a gift from L. Lanier, Palo Alto, Calif.), which recognizes NK cells from a variety of mouse strains, including 129. For cytolytic assays, YAC-1 cells (2×10^6 to 4×10^6) were labeled with 200 μCi of ^{51}Cr (Amersham) by incubation at 37°C for 2 h. Cells were then washed and used as targets at 10^4 per well on a 96-well microtiter plate, and effector cells were added at various ratios. Cytotoxic activity against YAC-1 was measured in a standard 4-h ^{51}Cr release assay (16).

Macrophage activation assays. For fluorescence-activated cell sorter (FACS) analysis, animals were infected intraperitoneally, and peritoneal cells were isolated at 3 days postinfection, stained with purified fluorescein isothiocyanate-F4/80 and phycoerythrin-I-A^b (7-16.17), and analyzed on a FACScan machine. Cells were pretreated with normal mouse serum and the Fc receptor-blocking antibody 2.4G2 such that isotype controls showed no positive staining.

RESULTS

Generation of IL-6-deficient mice. IL-6 deficient-mice were generated by standard homologous recombination techniques. The mutation was created in E14.1 ES cells by positive negative selection using the vector shown (Fig. 1A). Mutant clones were injected into blastocysts, yielding chimeras which transmitted the mutation through the germ line. These F_1 progeny were intercrossed, and homozygous IL-6-deficient mice were obtained in normal Mendelian ratios. Figure 1B shows a typical Southern blot identifying a homozygous mutant. Mice lacking a functional IL-6 gene appeared normal and healthy

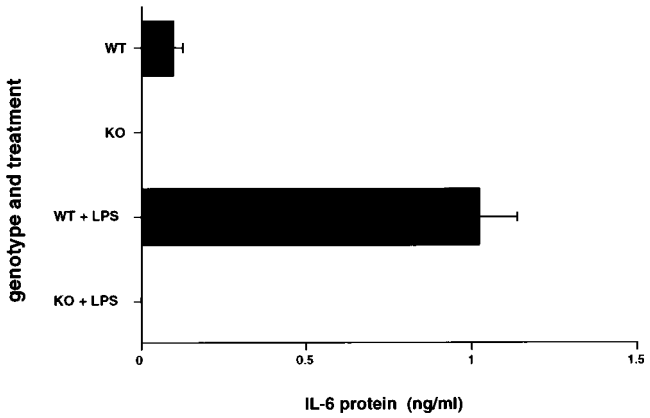

FIG. 2. IL-6-deficient mice do not produce detectable levels of IL-6. Representative IL-6 ELISA of supernatants of thymic stromal cells with and without LPS stimulation. Note that IL-6 was not detectable in IL-6-deficient (KO) stroma, even upon LPS stimulation, which resulted in significant production of IL-6 by control animals (WT).

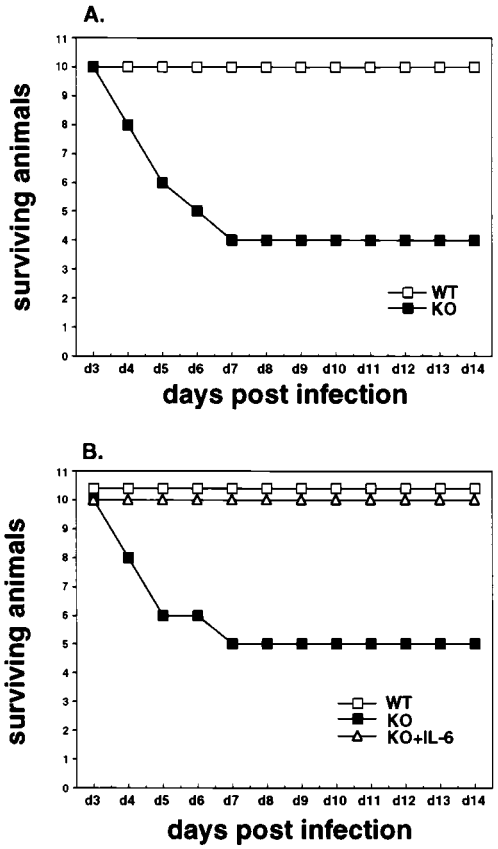

FIG. 3. IL-6-deficient mice succumbed to sublethal doses of *L. monocytogenes*. (A) Death curves for 10 IL-6-deficient (KO) and 10 control (WT) animals infected intravenously with 10^4 live *L. monocytogenes* organisms. (B) A repeat experiment which included a group of 10 KO animals that received 12.5 μg of mouse recombinant IL-6 subcutaneously, at day −1, day 0, and day 1 of *L. monocytogenes* infection, was performed.

when unchallenged, and both sexes were fertile. IL-6 was not detectable by ELISA in mutant animals, either in vitro with LPS-stimulated stromal cells or in vivo by analyzing sera from animals stimulated with LPS, while IL-6 levels of greater than 1 ng/ml were detectable in littermate control animals (Fig. 2). The sensitivity of the ELISA was less than 20 pg/ml. Additionally, bioassays using the B9 cell line were also performed on the above samples with similar results, and the specificity of the assay was confirmed with an IL-6-neutralizing antibody (data not shown).

We examined the cellular compositions of various tissues of IL-6-deficient mice by using antibodies against cell surface markers and FACS analysis. Although there was a slight increase in overall bone marrow cellularity, all other tissues, including spleen, thymus, and lymph nodes, and blood were normal. We observed no statistically significant differences in total numbers or subtypes of B cells, T cells, NK cells, macrophages, or neutrophils (data not shown).

IL-6-deficient mice succumbed to sublethal doses of *L. monocytogenes*. We investigated the role of IL-6 during listeriosis by infecting IL-6-deficient mice and control animals with various titers of *L. monocytogenes*. The IL-6 deficient animals displayed a 50 to 60% mortality rate within a week of inoculation of 10^4 bacteria, a dose which was sublethal in wild-type animals (Fig. 3A). Mice consistently began to die at day 4 postinfection. In a repeat experiment, an additional group of 10 IL-6-deficient mice were treated prophylactically (day −1 and day 0 of infection) with rIL-6 and then infected along with a group of 10 control and 10 IL-6-deficient mice. The sensitivity in the IL-6-deficient group was completely reverted by the exogenous administration of rIL-6 (Fig. 3B). The nature of the prophylactic treatment suggests that IL-6 works very early in the antilisterial response.

***L. monocytogenes* replicated extensively in the spleens and livers of IL-6-deficient animals.** We examined the growth of *L. monocytogenes* in the spleens and livers of infected IL-6-deficient and control animals. Infected animals were examined at day 3 postinoculation, since IL-6-deficient animals consistently began to die 4 days after infection (Fig. 3). As expected from the mortality data, the numbers of CFU of *L. monocytogenes* in the spleens and livers of the IL-6-deficient animals were significantly higher than those in the spleens and livers of the control animals. At day 3 postinfection the increase in bacteria in the livers of mutant animals was approximately 300-fold, whereas the increase in the spleens was 10-fold (Table 1). These data suggest that an essential defense mechanism in IL-6-deficient animals was suboptimal to account for such dramatic bacterial growth. The total numbers of colonies were greatly reduced in both groups when treated with rIL-6. The levels of absolute protection produced by using rIL-6 were similar for both groups and exceeded that for the untreated control animals, showing that IL-6 confers additional protection when administered exogenously. The protection provided by rIL-6 administration in vivo was expressed as a relative protective index (PI) (Table 1). Whereas control animals were protected at least 20-fold at day 3 postinfection, IL-6-deficient animals displayed a striking protective index of greater than 2,000, showing that early administration of this cytokine can rescue animals from a completely IL-6-deficient state.

Examination of tissue sections from infected animals supported the data presented above. Liver sections from infected animals showed the presence of microabscesses containing many neutrophils. Wild-type controls displayed few such foci (1.6 ± 1.7 per section; $n = 12$), whereas the IL-6-deficient animals contained significantly ($P < 0.0001$) more foci (20 ± 12.5 per section; $n = 19$). Additionally, the foci in the IL-6-deficient sections were significantly larger, showed considerable necrosis, and contained live bacteria, whereas the control

TABLE 1. *L. monocytogenes* CFU in IL-6-deficient and wild-type mice either replete with or depleted of neutrophils

Animal group[a]	No. of CFU in[b]:				rIL-6 PI[c]
	Spleen tissue		Liver tissue		
	− rIL-6	+ rIL-6	− rIL-6	+ rIL-6	
Neutrophil containing					
WT	13 (0.2)	1.3 (1.3)	1.9 (0.3)	<0.1	>19
KO	126 (9.2)	3.6 (1.0)	>666	0.3 (0.1)	>2,220
Neutrophil depleted					
WT			41,000 (7,000)	74,000 (9,000)	0.55
KO			47,000 (8,000)	150,000 (2,000)	0.3

[a] IL-6-deficient (KO) or wild-type (WT) animals were infected with *L. monocytogenes* and treated with rIL-6 and/or neutrophil depleted as described in Materials and Methods. Four animals per group were used. Experiments with neutrophil-containing animals were repeated four times, and experiments with neutrophil-depleted animals were repeated three times. Shown are results of a representative experiment. In all experiments, the magnitudes of difference between the groups were similar.

[b] *L. monocytogenes* CFU are presented as (bacterial colony numbers × 10^2)/100 mg of tissue, with standard deviations shown in parentheses.

[c] rIL-6 PIs were calculated by dividing the bacterial load in the livers of untreated animals by the bacterial load in the livers of animals that received rIL-6 treatment.

animals contained foci that appeared more resolved, with no visible bacteria (Fig. 4).

Natural killer cell activation, IFN-γ production, and macrophage activation phenotypes were normal in IL-6-deficient mice during listeriosis. To examine the basis of this difference in immunity, we evaluated several cell types known to be involved in innate immunity. We analyzed the IL-6-deficient animals for their capability to induce NK cell activity upon *L. monocytogenes* infection. We observed no differences either in the numbers of NK cells, as determined by FACS analysis, from infected IL-6-deficient animals and control animals (data not shown) or in the cytolytic activities of these NK cells when assayed in vitro by using NK cell-sensitive YAC-1 targets (Fig. 5A).

IFN-γ is a critical molecule in many cell-mediated immune responses and is thought to be a key component of resistance to *L. monocytogenes* (3). NK cells have been shown to be potent producers of IFN-γ during listeriosis (9). Additionally, IL-2 induces NK cells to produce large amounts of IFN-γ (10). Therefore, we investigated whether the increased susceptibility of IL-6-deficient mice during listeriosis was correlated with altered patterns of IFN-γ expression. Splenocytes isolated from uninfected and infected control or IL-6-deficient animals were cultured in the absence of exogenous stimuli for 24 to 48 h. The supernatants were then analyzed for IFN-γ by ELISA. Similar amounts of IFN-γ from splenocytes of infected control and mutant animals were detected, whereas splenocytes isolated from uninfected animals produced no detectable IFN-γ (Fig. 5B). The addition of IL-2 greatly enhanced IFN-γ production from splenocyte cultures derived from infected control and mutant animals, whereas IL-2 did not stimulate uninfected cultures to produce IFN-γ (Fig. 5C). Therefore, IL-6 was not necessary for normal levels of IFN-γ production during listeriosis.

We examined macrophage activation in IL-6-deficient mice during listeriosis in two ways. First, we used a FACS to analyze the expression of major histocompatibility complex class II and F4/80. The IL-6-deficient animals were able to upregulate major histocompatibility complex class II and downregulate F4/80 (Fig. 6), a well-established parameter of macrophage activation (25). In these experiments, we observed an approximately twofold variation from animal to animal in the populations of cells defined by the percentage of cells in each quadrant of Fig. 6. Six individual animals from each of the four groups uninfected wild type, uninfected IL-6 deficient, infected wild type, and infected IL-6 deficient, were analyzed. Collectively, there

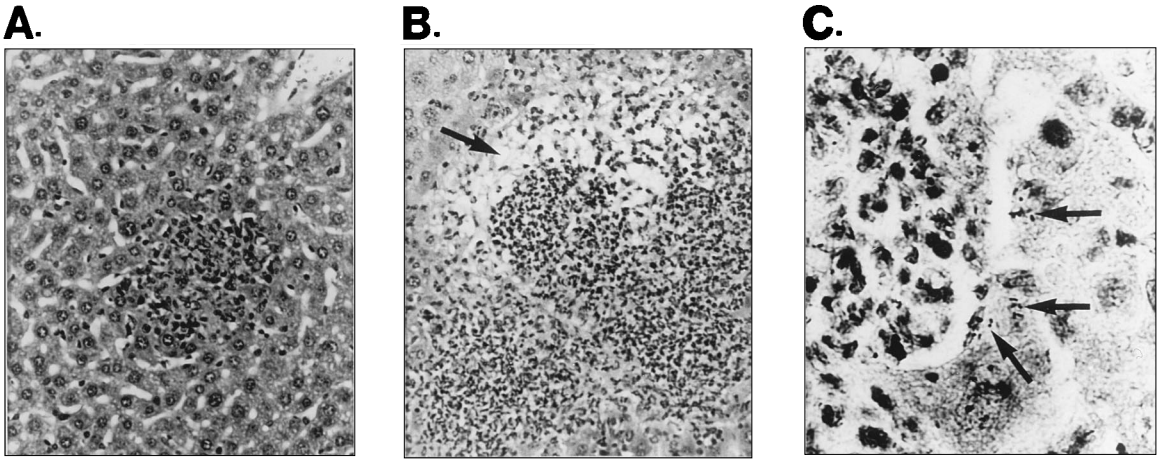

FIG. 4. Infected IL-6-deficient animals displayed uncontrolled bacterial proliferation in affected tissues. (A) Hemotoxylin and eosin-stained section through the liver of a representative control animal at 3 days post-*L. monocytogenes* infection. Shown is a typical control inflammatory focus (magnification, ×200). Note that the lesion appears fairly resolved. (B) Hemotoxylin and eosin-stained section through the liver of a similarly infected representative IL-6-deficient animal. Shown is a typical IL-6-deficient inflammatory focus. Note the large size and necrotic areas (arrow) (magnification, ×200). (C) Section through the liver of an infected IL-6-deficient animal, Gram stained for bacteria. Note the presence of gram-positive bacteria in the IL-6-deficient focus (arrows) (magnification, ×1,000). These bacteria were rarely observed in sections of control infected animals (data not shown).

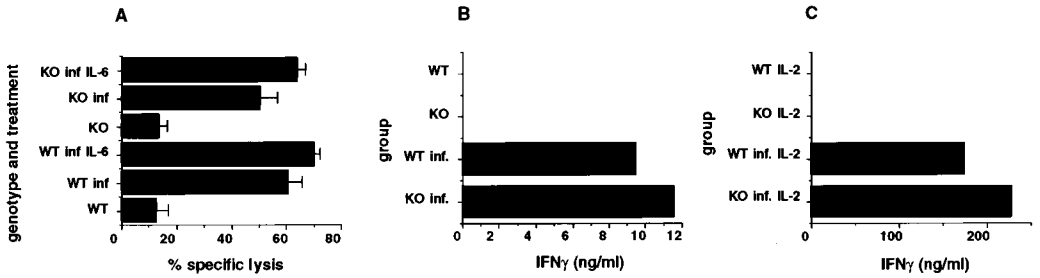

FIG. 5. NK cell function and IFN-γ production occured normally in infected IL-6-deficient mice. (A) Percent specific splenic NK cell lysis of YAC-1 target cells by control animals (WT), infected control animals (WT inf), and infected control animals treated with rIL-6 (WT inf IL-6) and by similar groups of IL-6-deficient (KO) animals. Shown are values for an effector/target ratio of 100:1. (B) IFN-γ production from splenocytes isolated from uninfected and infected WT or KO animals. Abbreviations are as for panel A. The sensitivity of the assay was less than 1 ng/ml. (C) IFN-γ production from IL-2-stimulated splenocytes isolated from uninfected and infected WT or KO animals. Abbreviations are as for panel A, and the sensitivity was the same as that for panel B.

was no difference in the loss of F4/80 staining or the acquisition of phycoerythrin-I-A^b staining between infected control and mutant animals, other than the typical variations in populations mentioned above.

We then evaluated nitric oxide (NO) production by macrophages isolated directly from infected or uninfected mutant and control animals. Macrophages were isolated from the different groups by Percoll gradient centrifugation from splenocytes and cultured in different experiments from 8 h to overnight. Supernatants from cultures of infected control and mutant animals were found to produce similar increased levels of NO compared with uninfected counterparts when analyzed by the Griess reagent protocol (15) (data not shown).

IL-6-deficient mice were unable to mount peripheral blood neutrophilia in response to listeriosis. To evaluate the induction of neutrophilia during the course of *L. monocytogenes* infection, control and IL-6-deficient mice were infected and blood was analyzed at various time intervals postinfection. Absolute numbers of neutrophils were determined from total and differential white cell counts. Normal animals displayed a prominent neutrophilia in the blood which peaked at 24 h postinfection, whereas IL-6-deficient animals did not mount this neutrophilia. IL-6-deficient mice displayed both lower total neutrophil counts and lower percentages of neutrophils in differential counts (Fig. 7). In multiple experiments, 24 h postinfection consistently showed the most pronounced difference in neutrophilia. However, at 48 h postinfection, control

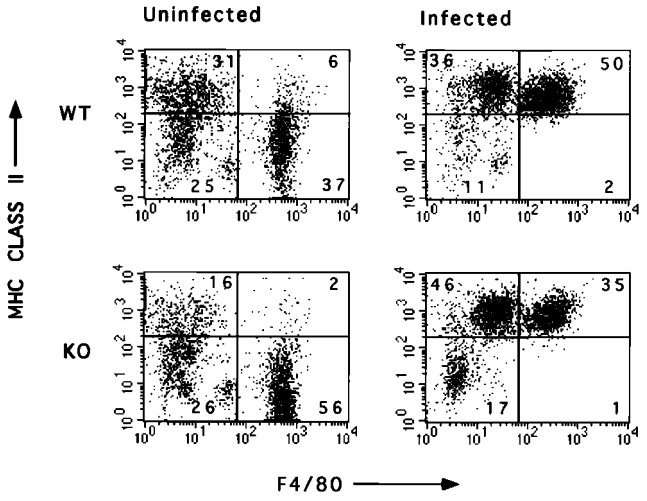

FIG. 6. Macrophage activation occured normally in IL-6-deficient mice. FACS analysis of uninfected wild-type (WT) (upper left), uninfected IL-6-deficient (KO) (lower left), infected WT (upper right), and infected KO (lower right) cells double stained with antibodies against major histocompatibility complex (MHC) class II (I-A^b) and the macrophage marker F4/80. Percentages of cells in each quadrant are shown.

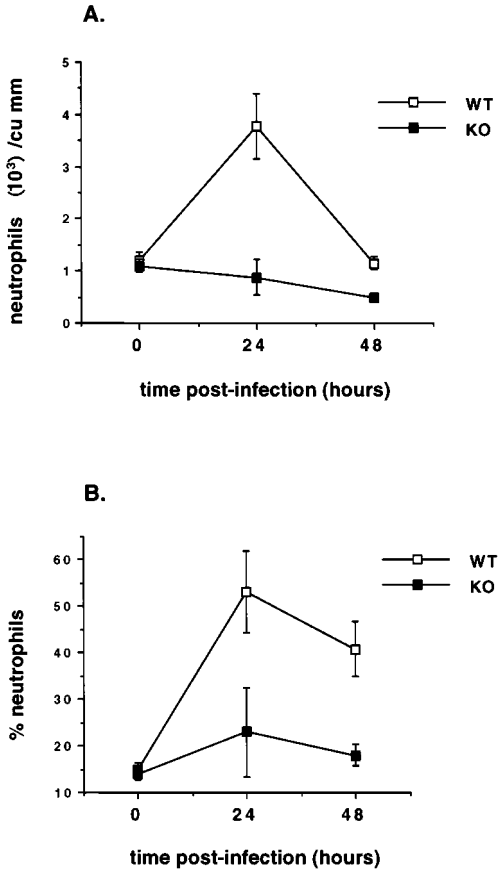

FIG. 7. IL-6-deficient mice failed to mount peripheral blood neutrophilia in response to listeriosis. (A) Absolute numbers of peripheral blood neutrophils per mm^3 (cu mm) were determined by tail bleeds at various time points from five mice per group. Differences at 24 and 48 h postinfection were statistically significant ($P < 0.0001$ for both time points). (B) Percentages of neutrophils in the peripheral blood were calculated from differential counts at the same time points as for panel A. Differences at the 24- and 48-h time points were statistically significant ($P = 0.001$ at 24 h; $P < 0.0001$ at 48 h). WT, wild type; KO, IL-6 deficient.

animals still retained a high percentage of neutrophils (Fig. 7B), which was masked in the total neutrophil counts (Fig. 7A) by generalized leukopenia (data not shown).

The IL-6 protective effect was dependent on the presence of neutrophils but not on the presence of lymphocytes. To further evaluate the potential role of IL-6 in neutrophil-mediated immunity, we treated IL-6-deficient and control animals with RB6-8C5, a monoclonal antibody known to deplete neutrophils in vivo (4, 6, 25, 31). By infecting neutrophil-depleted animals, we could determine if IL-6-deficient mice maintain their increased susceptibility to bacterial growth in the absence of neutrophils. Neutrophil-depleted control and IL-6-deficient animals showed similar levels of bacterial growth in the liver at day 2 postinfection (Table 1). Additional groups of control and IL-6-deficient animals in this experiment were neutrophil depleted and treated with rIL-6, allowing us to determine whether rIL-6 could provide protection in the complete absence of mature neutrophils. Administration of rIL-6 provided no protection to neutrophil-depleted control or mutant animals, as indicated by PIs of less than 1 (Table 1). This result was in sharp contrast to the dramatic IL-6 PIs for neutrophil-containing animals presented in Table 1.

Finally, we addressed the potential role of lymphocytes as an effector cell population in IL-6-mediated immunity to listeriosis. Since rIL-6 provides additional protection in normal animals (17) (Table 1), we investigated the protective effect of rIL-6 in animals lacking lymphocytes. Rag-2-deficient mice are devoid of lymphocytes but have, presumably, an intact innate immune system (27). Rag-2 mutant mice were either treated with rIL-6 or not treated and then infected with *L. monocytogenes*. The numbers of *L. monocytogenes* CFU from Rag-2-deficient (lymphoid-deficient) animals (calculated as [bacterial colony number $\times 10^2$]/100 mg of tissue) were as follows, with standard deviations in parentheses: 454 (44.1) for liver tissue of untreated mice and 6.2 (5.2) for liver tissue of mice treated with rIL-6. The rIL-6 PI was 73. Treatment of animals was as described in Table 1, and the rIL-6 PI was calculated as described in Table 1. Four animals per group were used. The Rag-2 mutant mice treated with rIL-6 were highly protected against accumulation of viable bacteria in the liver, suggesting that the rIL-6 protective effect was independent of lymphocytes.

DISCUSSION

Our results demonstrate that IL-6-deficient mice are less efficient than control animals at resolving *L. monocytogenes* infection, resulting in a significantly higher mortality rate. Mutant animals had greatly increased bacterial loads in the liver and spleen. Liver sections showed large increases in the number and severity of microabscesses in the IL-6-deficient animals. Additionally, the lesions in the IL-6-deficient animals contained many visible gram-positive bacteria (Fig. 4B), whereas the smaller lesions in the control animals showed few bacteria.

The bacterial accumulation data that we presented in Table 1 agree well with a report from Kopf et al. (15), who independently produced IL-6-deficient mice and reported on the increase of bacterial load in the tissues of these mutant animals infected with *L. monocytogenes*. In addition to describing a similar observation in this report, we have characterized mortality rates, tissue pathology, and reversion of the phenotype by rIL-6 administration and investigated in vivo cellular function in relation to IL-6 protection of the mutant animals undergoing this challenge. This more detailed analysis helps to elucidate a possible mechanism responsible for susceptibility phenotypes in IL-6-deficient mice.

Since IFN-γ is thought to play a central role in antilisterial defense, we tested and found no difference in the levels of IFN-γ produced throughout the disease. Therefore, the IL-6-deficient immunodeficiency does not simply affect IFN-γ levels. Liu et al. have recently suggested that the rIL-6 protective effect is due to IL-6 activation of T cells to produce IFN-γ (18). Although IL-6 and IFN-γ may both be necessary for optimal resistance, our data clearly show that IFN-γ expression is independent of IL-6 and that exogenous rIL-6 can provide protection to lymphocyte-deficient Rag-2 mutant animals.

The macrophage, a main target cell for *L. monocytogenes*, is an important cell type in antilisterial immunity. Our data indicate that IL-6 does not play an obvious role in macrophage function during listeriosis, at least as measured by activation phenotype (upregulation of major histocompatibility complex class II and downregulation of F4/80) or NO production from macrophages isolated directly from infected animals (data not shown). However, this does not rule out other potential interactions of the macrophage and IL-6 that may not be obvious from our analysis.

We have shown that prophylactic treatment with rIL-6 was able to provide significant levels of protection to the mutant animals. The absolute levels of protection observed were similar to that of rIL-6-treated control animals, showing that the lack of endogenous IL-6 can be completely compensated for by exogenous IL-6. rIL-6 also provided protection to Rag-2-deficient animals, showing that lymphocytes are not the main effector population responsible for the increased resistance. However, lymphocytes may be producers of IL-6 during the challenge.

Neutrophilia is a hallmark for many systemic bacterial infections, and neutrophil parameters have been used to successfully predict clinical classifications in severe septic disease states (26). As the importance of neutrophils in antilisterial immunity has been well established, we examined the initial neutrophil response in the IL-6-deficient animals. IL-6-deficient mice were unable to induce peripheral blood neutrophilia, which was prominent in the control animals. The inability of IL-6 mutants to induce neutrophilia is consistent with studies in which rIL-6 protein, administered to normal animals, was able to increase peripheral blood neutrophil numbers, possibly by demargination of peripheral pools of cells (32). Therefore, we suggest that in normal animals, one of the functions of IL-6 during listeriosis is to rapidly induce peripheral blood neutrophilia. Additionally, IL-1 has recently been shown to be necessary for the induction of peripheral blood neutrophilia and recruitment of neutrophils to the peritoneum during listeriosis (24). As IL-1 is known to induce IL-6, it will be of interest to see if the IL-1- and IL-6-induced neutrophilias are mechanistically related.

The numbers of steady-state neutrophils in IL-6-deficient animals appear normal, suggesting that IL-6 is not necessary for normal neutrophil development. The numbers of neutrophils in the spleens of mutant animals at 3 days postinfection were similar to those in the spleens of control animals, as determined by FACS analysis (data not shown). Whether these neutrophils fail to function properly once they infiltrate the tissues or simply arrive too late to combat the infection, perhaps because of the inefficient initial neutrophilia, remains to be elucidated. IL-6 has also been reported to influence the oxidative burst and degranulation capacity of human neutrophils (2), suggesting the possibility that neutrophil effector function may be suboptimal in IL-6-deficient mice, although we have not directly addressed this issue.

Neutrophil-depleted control and mutant animals displayed the same levels of sensitivity to *L. monocytogenes* infection. Thus, the endogenous production of IL-6 in the controls was unable to influence immunity in the absence of neutrophils. Similarly, rIL-6 administration to the same neutrophil-depleted animals was unable to cause the dramatic PI seen with neutrophil-containing animals. These data are consistent with the IL-6 protective effect being linked with the neutrophil lineage.

The description of immunodeficiency in the IL-6-deficient animals presented here suggests that these mice will be useful for other models of infectious disease and in many other situations in which changes in IL-6 levels have been correlated with certain pathologies. Data from these models will likely lead to a better understanding of the complex mechanisms involved in response to virulent organisms and show that certain cytokines may be useful therapeutically under conditions in which chances of opportunistic infection are increased.

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