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*J Immunol* 2003; 171:3142-3147;

doi: 10.4049/jimmunol.171.6.3142

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IL-10 Prevents Liver Necrosis During Murine Infection with Trichinella spiralis

Susan K. Bliss, Ana Alcaraz, and Judith A. Appleton

Infection with Trichinella spiralis rarely leads to significant morbidity. In this study, we show that IL-10 knockout mice infected with this parasite develop extensive areas of coagulative necrosis in the liver, and newborn larvae are required for lesion formation. Histopathological examination revealed that the hepatic inflammatory infiltrate was mixed but dominated by eosinophils. Accordingly, infected IL-10 knockout mice displayed a marked eosinophilia. IL-10 was expressed during infection in mesenteric lymph node populations and liver tissue. Analysis of cytokine profiles revealed a codominant expression of type 1 and 2 mediators that was enhanced in the absence of IL-10. Additionally, CD11c+ MHC class II+ cells were increased in mesenteric lymph nodes of IL-10 knockout mice, suggesting a possible link between IL-10 and dendritic cell trafficking. Nevertheless, there were no significant differences in mortality or parasite burdens between the strains of mice, indicating that IL-10 is necessary to control the host’s inflammatory response but does not impact establishment of the parasite. Expression of IL-10 appears to be an adaptation used by the liver to protect itself from damage caused by migrating newborn larvae.

Materials and Methods

Mice

C57BL/6 (wild-type) and B6.129P2-Iliomt1cr/+ (IL-10 knockout) female mice (6–12 wk of age) were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were housed under specific pathogen-free conditions in a Bioclean isolation unit (Lab Products, Seaford, DE) at the James A. Baker Institute for Animal Health in a facility that is accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were given autoclaved feed and acidified water.

Parasites and infections

T. spiralis infectious first stage larvae (L1) were recovered from muscle tissue of irradiated AO strain rats by digestion with 1% pepsin in acidified water, as described (7). For oral infections, mice were administered 600 L1 by gavage. To determine intestinal worm burdens, mice were euthanized on the indicated days, and small intestines were isolated. The intestinal tissue was cut longitudinally and incubated in sterile saline. After 4–5 h at 37°C, worms that had migrated into the saline were counted. In some studies, wild-type and IL-10 knockout mice were given either neomycin sulfate at 2.4 mg/ml diluted in drinking water or 0.05% thiabendazole mixed in feed starting 1 day before infection. Mice that received neomycin sulfate in drinking water were also given 0.3 ml by gavage every other day to ensure consumption. Semiquantitative aerobic bacterial cultures of intestinal contents revealed a decrease, or in some animals a complete elimination, of bacterial growth upon neomycin administration. To determine muscle burden, mice were euthanized, and carcasses were digested with 1% pepsin in acidified water. After washing the recovered worms, three to five aliquots from each mouse sample were counted. Results are expressed as the mean number of worms per mouse. Preparations of excretory-secretory (ES) proteins from L1 were made, as described previously (8).

Cell culture conditions

Mesenteric lymph node cells were obtained on the indicated days, and single cell suspensions were cultured in complete DMEM consisting of 10% FCS, 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Invitrogen), 0.1 mg/ml streptomycin (Invitrogen), and 5 × 10⁻⁵ M 2-ME at a concentration of 5 × 10⁶/ml in triplicate in 96-well plates for 3 days. Cells were stimulated with medium alone, ES proteins at 3.2 µg/

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well, or plate-bound anti-CD1 (BD PharMingen, San Diego, CA) at 0.54 

µg/well. Supernatants were harvested and stored at −20°C until used.

Cell depletion
To determine the cellular source of IL-10, cells were depleted of selected subsets with specific Abs and complement. Briefly, single cell suspensions of mesenteric lymph node cells (2 × 10^5 cells/sample) from wild-type mice were incubated on ice for 45 min with medium or hybridoma supernatants containing anti-CD4, anti-CD8, or anti-B220 (clones GK1.5, 3.155, and RA3-3A16/1.1, respectively). After washing in medium, cells were incubated at 37°C with rabbit serum as a source of complement (Accurate Chemical and Scientific, Westbury, NY). This treatment was repeated once. Cells from the control group (medium treated) were counted and adjusted to 5 × 10^5 cells/ml, and cell suspensions were plated. To directly compare the cytokine response in depleted groups with the control group in vitro, the depleted populations were adjusted to the same volume as the control group. This process was repeated on cells collected on days 5, 10, 14, and 20. Flow cytometry confirmed that a minimum of 95% of CD4^+ cells, 95% of CD8^+ cells, or 92% of B220^- cells was depleted. Cells were cultured with ES proteins, as described above. Supernatants were collected, and IL-10 concentrations were determined by ELISA.

Cytokine ELISAs
IL-4, IL-5, IL-10, IFN-γ, and TNF-α were measured in supernatants by ELISA. To measure IL-4, plates were coated overnight at 4°C with mAb 11B11 (BD PharMingen) at 2 µg/ml diluted in PBS. Following three washes in PBS containing 0.05% Tween (PBST), plates were blocked with 1% BSA in PBS for 2 h at 37°C. After washing in PBST, sample supernatants or a recombinant standard were added. Plates were incubated overnight at 4°C. After washing, biotinylated mAb BVD6-24G2 (BD PharMingen) was added, and plates were incubated for 90 min at 37°C. HRP-conjugated streptavidin (BD PharMingen) was added, and plates were incubated for 1 h. Finally, tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) substrate was added, and absorbance was read at 450 nm on a Bio Kinetics Microplate Reader (Bio-Tek Instruments, Winooski, VT). The same basic protocol was used to measure IL-5 and IL-10 using the mAb pairs, TRFK4 and TRFK5 (for IL-5) and JES5-2A5 and SXC-1 (for IL-10). IFN-γ concentrations were determined, as previously described (9). TNF-α levels were measured with an ELISA kit (BD PharMingen), according to manufacturer’s instructions.

Flow cytometry
Mesenteric lymph node cells (prepared as described above) were suspended in PBS containing 10% normal mouse serum. After 20 min on ice, cells were washed and similarly incubated with saturating concentrations of anti-CD11c (clone HL3; BD PharMingen) or anti-MHC class II (M5/114.15.2; BD PharMingen). After washing, cells were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA) and a FACS caliber flow cytometer.

RNA isolation and RT-PCR
Livers were collected from wild-type and IL-10 knockout mice on days 0, 7, and 13 of infection and homogenized in TRIzol (Invitrogen). RNA isolation and reverse transcription were performed, as described previously (10). A master mix containing 2.5 mM dNTP, 10× PCR buffer with 1.5 mM MgCl2 (Sigma-Aldrich, St. Louis, MO), 0.2 µM primers, and 5 U/ml of Taq polymerase (Invitrogen) was used to amplify the cDNA. The nucleotide sequences for sense and antisense primers, respectively, were: hypoxanthine phosphoribosyltransferase, GTP GGA TAC AGG CCA GAC TTT GGT G and GAT TCA TCG GCT CAT CTT AGG C; IL-10, CGG GAA GAC AAT AAC TG and CAT TTC CGA TAA GGC TTG G. Ten microliters of cDNA were added to 40 µl of master mix and then subjected to PCR amplification (10). Products were resolved in 2% agarose gels, and cDNA was stained with ethidium bromide (Sigma-Aldrich). Product migrations were compared with m.w. standards to ensure that each product was of the predicted size.

Blood eosinophil counts
Blood was collected by cardiac puncture into EDTA-containing tubes on days 0, 5, 10, 14, and 20. White blood cell counts were performed, and blood smears were made from each sample and stained with Diff-Quik (American Scientific Products, McGraw Park, IL). A total of 300–500 white blood cells was counted, and the percentage of eosinophils was calculated. Results are expressed as the mean of three to four mice ± SD at each time point.

Histology
Tissues were fixed in 10% (w/v) buffered formaldehyde and embedded in paraffin, according to standard laboratory procedures. Sections (6 µm) were stained with H&E for microscopic examination.

Statistical analysis
Each experiment was performed two to five times. Means of replicates were compared by the Student’s t test or ANOVA. Probability values <0.05 were considered significant.

Results
IL-10 knockout mice develop hepatic lesions during the acute stage of infection but heal during the chronic stage
To evaluate the regulatory role of IL-10 during infection, we infected wild-type and IL-10 knockout mice orally with 600 muscle stage larvae. The mice were euthanized on days 0, 12 (during the intestinal, or acute, stage), and 28 (during the chronic stage). As shown in Fig. 1, A and B, no hepatic lesions were detected in

FIGURE 1. T. spiralis-infected IL-10 knockout mice develop hepatic lesions. C57BL/6 mice and IL-10 knockout mice were orally infected with 600 larvae on day 0, and on day 12 (C–F) or day 28 (G–H), livers were fixed, sectioned, and stained with H&E. Uninfected tissue displayed normal architecture (A and B). No lesions were detected in livers from day 12 infected wild-type mice (C). D, Represents a typical lesion in a knockout animal. E and F, Higher magnifications of the inflammatory infiltrate surrounding the lesion taken from the area indicated by the box in D. Many eosinophils were present. By day 28, the large necrotic areas in knockout mice had resolved, leaving only small multifocal areas of inflammation (H), while no lesions were present in the livers of infected wild-type mice (G). Bars represent 300 (A–D), 30 (E, G, and H), or 10 µm (F).
suggesting an initial insult with subsequent expansion of necrosis throughout the liver. The lesions were similar in appearance, developed multifocal to locally extensive areas of coagulative necrosis from uninfected mice. However, by day 12, all IL-10 knockout mice had developed multifocal to locally extensive areas of coagulative necrosis throughout the liver. The lesions were similar in appearance, suggesting an initial insult with subsequent expansion of inflammation and necrosis (Fig. 1D). Indeed, serial samples obtained earlier in infection revealed continuous development of lesions from small inflammatory foci to large areas with central necrosis surrounded by inflammatory cells (data not shown). Newborn larvae, the migratory stage of the parasite, were not detected in tissue samples. Cellular infiltrates contained mononuclear and polymorphonuclear cells but were dominated by eosinophils (Fig. 1, E and F). In contrast, no lesions appeared when thiabendazole was administered. Intestinal worm counts were performed on all mice, and results are expressed as means ± SD. No statistically significant differences were noted. Bars represent 300 μm.

FIGURE 2. Migrating newborn larvae induce liver lesions. Wild-type (A, C, and E) and IL-10 knockout (B, D, and F) mice were infected with 600 muscle larvae orally. Neomycin sulfate was administered in drinking water (C and D), thiabendazole was administered in feed (E and F), or control mice (A and B) were provided unmodified feed and water, as described in Materials and Methods. After 12 days, livers were obtained and prepared for staining with H&E. Lesions were present in livers from control and neomycin-treated IL-10 knockout mice. In contrast, no lesions developed when thiabendazole was administered. Intestinal worm counts were performed on all mice, and results are expressed as means ± SD. No statistically significant differences were noted. Bars represent 300 μm.

FIGURE 3. Blood eosinophil response in C57BL/6 and IL-10 knockout mice. Mice (four animals/group) were infected orally with 600 larvae. At the indicated times, blood eosinophil numbers were measured. Values shown represent means ± SD. The asterisks indicate a statistically significant difference between wild-type and knockout values at each time point.

Migrating parasites elicit hepatic lesions

Adult *T. spiralis* causes extensive damage to the intestinal epithelium, providing an opportunity for normal intestinal bacteria to reach the liver via the portal system. Newborn larvae, released by adult females in the intestine, also travel via the portal system (11). To discern between these two potential causes of liver damage, we treated IL-10 knockout mice with either a broad spectrum antibiotic (neomycin sulfate) or an anthelmintic. Thiabendazole inhibits the production and release of newborn larvae from adult females, but does not eliminate worms from the intestine (12). Lesions were found in livers collected 12 days postinfection from untreated and neomycin sulfate-treated IL-10 knockout mice, but not from mice treated with thiabendazole (Fig. 2), demonstrating that newborn larvae caused the damage observed.

**IL-10 knockout mice develop an exaggerated eosinophilia**

Because the hepatic lesions noted in IL-10 knockout mice were characterized by an eosinophilic infiltrate, we measured blood eosinophil levels in our experimental animals. Although both groups of mice developed a leukocytosis with eosinophilia, a more pronounced eosinophilia (~20% by day 20) arose in knockout mice (Fig. 3).

**IL-10 suppresses type 1 and 2 mediator production during infection**

The exaggerated eosinophilia in IL-10 knockout mice suggested elevated expression of type 2 cytokines such as IL-5. Because immune responses are readily detected in intestinal tissue during infection and there exists potential for induction of intestinal responses to influence hepatic responses, we evaluated cytokine synthesis by mesenteric lymph node cells. In response to anti-CD3 stimulation, cultured wild-type cells produced IL-10 with concentrations peaking by ~10 days postinfection. Furthermore, cells derived from knockout mice consistently produced more of each cytokine tested, namely IL-4, IL-5, IFN-γ, and TNF-α. Similar trends were observed when cells collected 10 days postinfection were stimulated with ES proteins (Fig. 4B). Thus, mesenteric lymph node cells were more responsive to both specific and nonspecific stimuli in the absence of IL-10.

**CD4+ T cells are required for IL-10 production**

To determine whether IL-10 synthesis is dependent on lymphocytes, we depleted CD4+ T cells, CD8+ T cells, and B cells with...
specific Abs and complement. Resultant populations were stimulated with ES proteins in vitro for 3 days. Fig. 5 demonstrates that IL-10 synthesis depended upon CD4\(^+\) T cells. Moreover, IL-10 concentrations were highest in cultures established on day 5, indicating different kinetics compared with cells stimulated with anti-CD3 (Fig. 4).

**T. spiralis**-infected mice express IL-10 in liver

Because hepatic lesions developed only in the absence of IL-10, we sought to determine whether IL-10 was expressed in the liver upon infection. RT-PCR analysis demonstrated detectable levels of IL-10 mRNA in wild-type mice 7 and 13 days postinfection with *T. spiralis* (Fig. 6). No IL-10 message was detected in knockout mice. Whether local IL-10 was derived from resident or infiltrating cells was not determined.

**Dendritic cells are increased in mesenteric lymph nodes of IL-10 knockout mice**

We speculated that one outcome of IL-10 deficiency might be increased Ag presentation by dendritic cells, leading to enhanced cytokine production. Flow cytometric analysis revealed that lymph nodes from uninfected knockout mice possessed approximately twice as many dendritic cells (CD11c\(^+\) MHC class II\(^+\)) compared with uninfected wild-type mice. Furthermore, infection induced a greater expansion of these cells in knockout mice compared with wild-type mice (Fig. 7).

**IL-10 does not impact parasite survival**

To determine whether IL-10 affects parasite survival in the intestine or establishment in muscle, intestinal worm counts were performed on days 5, 10, 14, and 20, and muscle burdens were measured on day 28 (Fig. 8). No differences were detected between the two groups of mice, indicating that IL-10 does not affect survival or fecundity.

**Discussion**

*T. spiralis* rarely causes liver disease (13). In our experiments, mice deficient in IL-10 developed large areas of multifocal coagulative necrosis in the liver upon infection, suggesting a critical role for this cytokine in hepatoprotection (Fig. 1). Lesions were first grossly visible by day 7 and reached their maximum volume...
by approximately day 14. Our data support the hypothesis that intestinally derived, bloodborne newborn larvae are required for lesion formation (Fig. 2). The lesions we observed may represent areas in which individual larvae died, inciting an intense inflammatory response that is normally prevented or modulated by IL-10. Alternatively, newborn larvae may release substances that are hepatotoxic when not counteracted by the effects of IL-10. These processes would be extremely important in natural infections in which over 97% of newborn larvae directly enter capillaries in the intestine and travel to the liver via the portal vein before spreading to the rest of the body (11). To date, newborn *T. spiralis* larvae and their secreted products have not been well characterized.

We have observed hepatic lesions in knockout mice only when they have been infected orally. Lesions do not form when the mice are injected with newborn larvae by the i.p. or i.v. routes. This suggests that hematogenous movement from the intestine to the liver is necessary. Alternatively, the presence of the parasite within the intestine may induce an immune response that subsequently affects the liver. Thus, it is possible that primed immune cells could be generated in situ in the liver via antigenic activation by hepatic APCs or they could develop in gut-associated lymphoid tissue and then migrate to the liver (14). The liver is required to defend against infections, toxins, and tumor cells at the same time it must limit unwarranted responses to dietary Ags. The inherent tolerogenicity of the liver, including its potential role in oral tolerance, poses important questions about how immune defense in the liver is regulated. Future studies will be centered on the liver-specific immune response and how IL-10 promotes the hyporesponsive state during infection.

Hepatic lesions in *T. spiralis*-infected IL-10 knockout mice resolved by the chronic stage of infection (Fig. 1). Similar to our model, IL-10 knockout mice infected with *Schistosoma mansoni* display an enhanced acute inflammatory response to parasite eggs in the liver (15). Inflammation is eventually down-modulated via unknown mechanisms; however, lesions persist in *S. mansoni*-infected mice presumably because eggs are not destroyed. *T. spiralis* larvae that elicit hepatic lesions do not persist. We have speculated that TGF-β may be playing a role in the down-modulation of inflammation in the later phases of *T. spiralis* infection. In preliminary experiments, we have found that TGF-β1 expression is up-regulated in the liver upon infection in both knockout and wild-type mice (data not shown).

Schopf et al. (16) recently demonstrated increased susceptibility to *Trichuris muris* infection by IL-10 knockout mice. Infected
knockout mice displayed heightened morbidity and mortality when compared with control animals. Additionally, mesenteric lymph node cell populations from *T. muris*-infected knockout mice expressed an enhanced type 1 and suppressed type 2 cytokine profile. Increased susceptibility was attributed to the lack of type 2 cytokine production (16). In contrast, we noted no differences in host survival or parasite burden in *T. spiralis*-infected mice (Fig. 8). The differences in outcome may be due to the longer duration of intestinal infection by *T. muris*, the initial mucosal penetration of larval stages of *T. muris*, or the intestinal location of each parasite. Given that a Th2 response is essential for protection against *T. spiralis* infection, we think it likely that wild-type and knockout mice are equally resistant because both strains produce adequate amounts of type 2 mediators (Fig. 4) (17). Moreover, the increased IL-5 levels observed provide a reasonable explanation for the enhanced eosinophilia in knockout mice (Fig. 3). Clearly, the influence of IL-10 on disease varies, even between closely related nematode species such as *T. spiralis* and *T. muris*.

IL-10 mediates many different processes that could contribute to the cytokine alterations we observed. For example, IL-10 inhibits Ag presentation by down-regulating MHC class II expression (4). Furthermore, Demangel et al. (18) have shown increased migration of bone marrow-derived dendritic cells to the draining lymph node in IL-10 knockout mice that were infected with *Mycobacterium bovis* bacillus Calmette-Guerin. We found increased dendritic cell numbers in mesenteric lymph nodes from IL-10 knockout mice, suggesting that IL-10 may affect trafficking. In this context, it will be important to elucidate the influence of IL-10 on synthesis of chemokines known to facilitate dendritic cell movement such as MIP-3β.

IL-10 is produced during infection with *T. spiralis*. We found protein in cultures derived from mesenteric lymph nodes and increased mRNA levels in the livers of wild-type mice (Figs. 4 and 6). However, we only observed differences in lesion development (19). There were no quantitative or qualitative differences in increased eosinophilia in knockout mice (Fig. 3). Clearly, the influence of IL-10 on disease varies, even between closely related nematode species such as *T. spiralis* and *T. muris*.

Toxoplasma gondii infection leads to impaired immunity and mortality in IL-10 knockout mice (20). Additionally, mesenteric lymph node cell populations from *T. gondii*-infected knockout mice exhibit heightened morbidity and mortality when compared with control animals. Moreover, mesenteric lymph node cells indicated that CD4+ T cells were necessary for IL-10 depletion during *T. spiralis* infection. In this work, we provide new information about the role of IL-10 during infection with *T. spiralis*. This study provides evidence that IL-10 is critical in the liver during the acute stage in preventing widespread necrosis. Expression of IL-10 appears to be an adaptation by the host to protect the liver against the damaging inflammation induced by migrating newborn larvae. Future work will be aimed at determining the nature of the immune response in the liver itself and the specific role(s) of IL-10 in this organ during infection.

Acknowledgments

We thank D. Beiting for critical review of the manuscript, and E. Denkers for providing the anti-B220 Ab-secreting hybridoma.

References


