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# IL-10 Regulates Movement of Intestinally Derived CD4<sup>+</sup> T Cells to the Liver<sup>1</sup>

Susan K. Bliss,<sup>2\*</sup> Stuart P. Bliss,<sup>†</sup> Daniel P. Beiting,<sup>\*</sup> Ana Alcaraz,<sup>†</sup> and Judith A. Appleton<sup>\*</sup>

Diseases that affect the intestine may have hepatic manifestations, but the mechanisms involved in establishing hepatic disease secondarily remain poorly understood. We previously reported that IL-10 knockout (KO) mice developed severe necrotizing hepatitis following oral infection with *Trichinella spiralis*. In this study, we used this model of intestinal inflammation to further examine the role of IL-10 in regulating hepatic injury. Hepatic damage was induced by migrating newborn larvae. By delivering the parasite directly into the portal vein, we demonstrated that an ongoing intestinal immune response was necessary for the development of hepatitis. Intestinally derived CD4<sup>+</sup> cells increased in the livers of IL-10 KO mice, and Ab-mediated blockade of MAdCAM-1 inhibited the accumulation of CD4<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup> cells in the liver. Moreover, adoptive transfer of intestinally primed CD4<sup>+</sup> T cells from IL-10 KO mice caused hepatitis in infected immunodeficient animals. Conversely, transfer of wild-type donor cells reduced the severity of hepatic inflammation in IL-10 KO recipients, demonstrating regulatory activity. Our results revealed that IL-10 prevented migration of intestinal T cells to the liver and inhibited the development of hepatitis. *The Journal of Immunology*, 2007, 178: 7974–7983.

In mammals, the liver and intestine are anatomically and functionally interconnected. During embryogenesis, the primordial liver develops as an endodermal outgrowth from the ventral aspect of the primitive foregut and, throughout the life of the organism, maintains direct afferent and efferent links with the intestine through the portal venous tributaries and the biliary tree, respectively (1). In the fetus, the intestine is invaded by lymphocyte precursors that are derived from the developing liver, and some evidence suggests that these two organs retain a coordinated immunological connection postnatally (2). For example, debilitating inflammatory diseases of the intestine such as Crohn's and ulcerative colitis, conditions subsumed under the general heading of inflammatory bowel disease, may be concurrent with hepatic diseases like primary sclerosing cholangitis and autoimmune hepatitis. Some studies suggest the intriguing possibility that these anatomically distinct diseases may share considerable overlap with respect to their immunopathogenesis (3). MAdCAM-1, an addressin important in mucosal homing, has been shown to be expressed in the livers of patients with some chronic inflammatory intestinal diseases (4). Such overlap in the expression of specific addressins suggests a mechanism for cell recirculation that may be important in the context of certain enterohepatic inflammatory diseases.

The intestine represents the largest mucosal site in the body and interfaces with the external milieu. It is continuously exposed to a vast array of foreign Ags, and efficient discrimination between innocuous and potentially pathogenic Ags is the basis of effective intestinal immune function. Under normal conditions, dietary Ags elicit an immune response characterized by tolerance (i.e., termed

oral tolerance) (5). The mechanisms underlying this phenomenon include anergy, deletion, and regulation (5). Recent work has greatly broadened our understanding of many aspects of these basic mechanisms; however, much remains unclear. In particular, the role of the liver in the induction of oral tolerance is largely undefined. Several observations indicate that the liver participates in the development of oral tolerance. For example, diversion of blood from the portal system to the systemic circulation via experimentally created portosystemic shunts attenuates induction of oral tolerance (6). Moreover, patients with naturally occurring portosystemic shunts have higher Ab titers against intestinal bacteria (7). In addition, when the venous drainage from experimentally placed small bowel allografts is channeled directly into the systemic circulation of the recipient, the grafts undergo rapid and acute rejection, whereas direction of graft outflow into the portal circulation delays rejection (8). Several potential explanations for the liver's role in oral tolerance have been offered, including Ag presentation by sinusoidal endothelial cells or hepatic cytokine production (9, 10). However, to date, insufficient data have been reported upon which to base a unifying hypothesis regarding the liver's contribution to this phenomenon.

IL-10 is a cytokine that exerts regulatory activity over many different cell types and influences a number of immunological functions such as Ag presentation and cytokine production with consequent effects on the initiation and maintenance of inflammation (11). IL-10 has been implicated in several models of oral tolerance. Mucosal administration of IL-10 promotes the induction of oral tolerance in experimental autoimmune encephalomyelitis (12), and T cell clones purified from mice tolerized to myelin basic protein produce IL-10 (13). In addition to maintaining immune homeostasis, IL-10 can modulate ongoing inflammation in the intestine and liver. For example, IL-10 limits hepatic injury due to Con A administration (14). Moreover, Asseman et al. (15) established that IL-10-dependent type 1 regulatory T (Tr1)<sup>3</sup> cells suppress intestinal inflammation in the CD45RB<sup>high</sup>CD4<sup>+</sup> T cell

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<sup>3</sup> Abbreviations used in this paper: Tr1, type 1 regulatory T; ALT, alanine aminotransferase; ES, excretory-secretory; KO, knockout; L1, first-stage larvae; NBL, newborn larvae; WT, wild type.

transfer model of inflammatory bowel disease. To further investigate the immunoregulatory role of IL-10 within the intestine and liver, we have used a model of hepatitis wherein an enteric parasite incites hepatic inflammation in the absence of IL-10.

*Trichinella spiralis* is a parasitic nematode that has proven to be a valuable model for the study of intestinal immunity. Rodents, pigs, and humans are natural hosts, and infection is initiated by the ingestion of first-stage larvae (L1). In the small intestine, larvae molt, become adults, and mate. Eggs hatch in utero, and adult female worms release newborn larvae (NBL), which enter mesenteric tributaries that ultimately discharge into the portal vein (16). NBL enter the liver through portal venules and traverse the hepatic sinusoids, leaving the liver via central venules. It is notable that, despite migration through the liver, hepatic disease is not a prominent feature of *T. spiralis* infection (17). Beyond the liver, NBL circulate systemically and may penetrate skeletal muscle cells to form a unique structure called the Nurse cell. L1 develop in the Nurse cell and establish chronic infection. This parasite-host cell complex can survive for many years (18).

In previous studies, we found that mice lacking IL-10 developed necrotizing hepatitis following oral infection with *T. spiralis* (19). We report here that CD4<sup>+</sup> T cells primed within the intestinal environment promote hepatic inflammation in the absence of IL-10. IL-10 limits the accumulation of leukocytes in the liver as well as their cytokine production. These data define a novel role for IL-10 in modulating the hepatic immune response to an intestinally derived pathogen and shed new light on the complex immunological relationship between the liver and gut.

## Materials and Methods

### Mice

Male and female C57BL/10SgSnAi (wild-type (WT)) mice were purchased from Taconic Farms. C57BL/10SgSnAi-[KO]IL-10 (IL-10 knockout (KO)), C57BL/10SgSnAi-[KO]RAG2 (RAG2 KO), and C57BL/10SgSnAi-[KO]IL-10RAG2 (IL-10/RAG2 KO) mice were bred at the Transgenic Mouse Core Facility at the College of Veterinary Medicine at Cornell University. Animals were housed under specific pathogen-free conditions at the James A. Baker Institute for Animal Health, a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Studies were approved by the Institutional Animal Care and Use Committee. Mice were maintained on autoclaved feed and acidified water.

### Parasites and infections

*T. spiralis* L1 were recovered from the muscle tissue of irradiated AO strain rats by digestion with 1% pepsin in acidified water as described previously (20). Excretory-secretory (ES) proteins from L1 were prepared as described previously (21). For oral infections, mice were administered 600 L1 by gavage. To obtain NBL for portal vein injection, we recovered adults from the intestines of infected donor rats. Briefly, donor rats were infected orally with 5000–6000 L1, and intestines were harvested 6 days later. Intestinal tissue was flushed with saline, opened longitudinally, and incubated in sterile saline containing 200 IU/ml penicillin, 200 µg/ml streptomycin, and 50 µg/ml gentamicin (Invitrogen Life Technologies) for 2 h. Adults were collected on a sterile 200 mesh sieve, washed, and incubated in MEM (Invitrogen Life Technologies) containing 30% FBS and antibiotics for 24 h. Released NBL were subsequently separated from adults by a 200 mesh sieve, washed, and resuspended in MEM. For portal vein injections, mice were anesthetized with isoflurane, and a ventral abdominal midline celiotomy was performed to access the portal vein. NBL (3000), suspended in 100 µl of MEM or MEM alone, were injected into the portal vein through a 30-gauge needle. The body wall and skin were closed routinely with suture. Pain was managed with butorphanol (0.1 mg/kg s.c.). As additional controls, groups of mice were orally infected 4 days before surgery while being maintained on feed containing 0.05% thiabendazole to prevent the release of NBL (22). They were injected with either 3000 NBL or MEM into the portal vein. Preliminary studies confirmed that thiabendazole administration did not alter the viability or infectivity of NBL. Additionally, diaphragms from all experimental mice were collected at the time of euthanasia, and sections were examined microscopically for the pres-

ence of NBL. All mice that were injected with NBL possessed developing L1 in muscle (data not shown).

### In vivo Ab administration

In some experiments, WT and IL-10 KO mice were depleted of CD4<sup>+</sup> cells with a mAb (clone GK1.5) administered at 500 µg/mouse i.p. every other day for 13 days, beginning 1 day before infection. Alternatively, mice were injected with a control rat IgG (Accurate Chemical and Scientific) at the same dose. To assess the efficacy of the depletion, mesenteric lymph node cells were obtained from individual mice, stained with anti-CD4 (clone RM4-5), and examined by flow cytometry. In mice treated with GK1.5, CD4<sup>+</sup> cells were reduced by 98–100% (data not shown). NK1.1<sup>+</sup> cells were depleted through the use of clone PK136 (200 µg/mouse i.p., same regimen as above). Hepatic leukocytes were stained with anti-NK1.1 to determine the efficiency of depletion. In mice treated with PK136, NK1.1<sup>+</sup> cells were reduced by 90–93% (data not shown). In some experiments, mice were administered an Ab that blocks binding to MAdCAM-1 (clone MECA-367; 200 µg i.p., same regimen as above) or a control rat IgG.

### Histology

Mice were euthanized by CO<sub>2</sub> inhalation at the indicated times. Liver tissue was fixed in 10% (w/v) neutral-buffered formalin and embedded in paraffin, and 6-µm sections were stained with H&E for microscopic examination. Photomicrographs were obtained using an Olympus BX51 microscope and Olympus DP12 microscope digital camera system.

### Measurement of alanine aminotransferase (ALT) activity

Blood was collected by cardiac puncture and separated from serum. The liver transaminase, ALT, was measured in individual serum samples, using a commercially available kit (Pointe Scientific). Results are expressed as the mean IU/L ± SD.

### Hepatic leukocyte and mesenteric lymph node cell isolation

Before liver excision, the caudal vena cava was transected, and the organ was perfused with PBS through the portal vein. Subsequently, livers were removed and pressed through a steel mesh. Cell suspensions were washed twice in PBS, and erythrocytes were lysed with ammonium chloride. Cells were then washed and either stained with Abs for flow cytometry, or leukocytes were separated from parenchymal cells by Percoll (Amersham Biosciences) sedimentation. Liver cells were pelleted and resuspended in 4 ml of 28% Percoll. This was layered onto 4 ml of 80% Percoll and spun for 30 min at 900 × g. Leukocytes at the interface were collected, washed, and counted. In some experiments, hepatic leukocytes were cultured (see below). Mesenteric lymph node cells were prepared as described previously (19).

### Flow cytometry

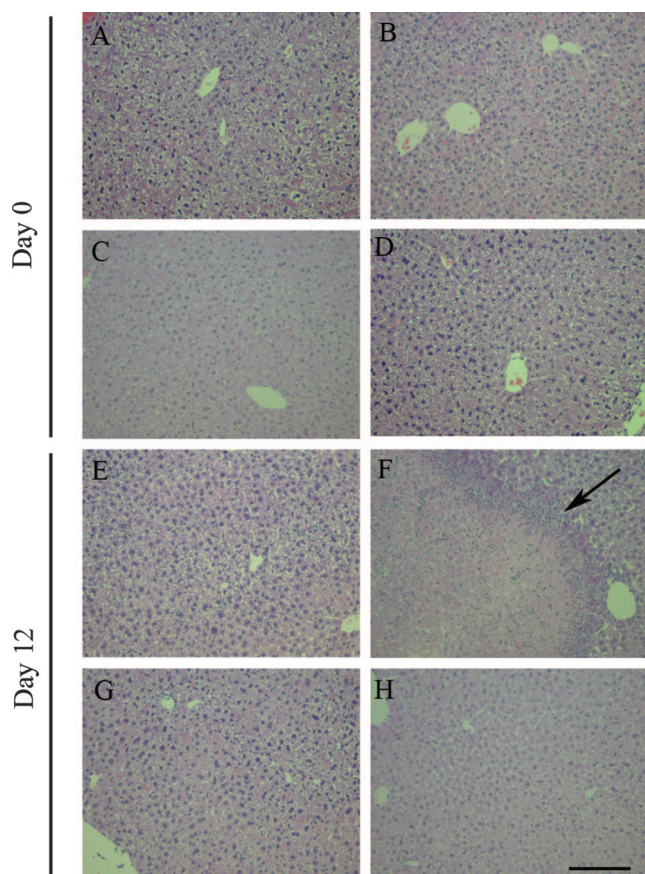
Isolated liver cells were suspended in PBS containing 10% normal mouse serum. After 20 min on ice, cells were washed and incubated with fluorochrome-conjugated Abs against CD4 (clones GK1.5 and RM4-5; eBioscience), CD44 (clone IM7; eBioscience), CD62L (clone MEL-14; eBioscience), NK1.1 (clone PK136; BD Pharmingen), CCR9 (clone CW-1.2; eBioscience), CD69 (clone H1.2F3; eBioscience), or α<sub>4</sub>β<sub>7</sub> (clone DATK32; BD Pharmingen). After washing, events were collected on a FACSCalibur flow cytometer and analyzed with CellQuest software (BD Immunocytometry Systems).

### Cytokine ELISA

Hepatic leukocytes and mesenteric lymph node cells from individual mice were cultured at concentrations of 4–6 × 10<sup>5</sup> and 5 × 10<sup>6</sup> cells/ml, respectively, in complete DMEM (Invitrogen Life Technologies) consisting of 10% FBS, 0.1 mM nonessential amino acids (Invitrogen Life Technologies), and 5 × 10<sup>-5</sup> M 2-ME. Cells were stimulated with medium, ES proteins at 3 µg/well, or plate-bound anti-CD3 (BD Pharmingen) at 0.5 µg/well. After 72 h, supernatants were collected, and IL-4, IL-5, IL-10, IL-13, and IFN-γ concentrations were measured by ELISA as described previously (19). IL-13 levels were determined following the same protocol but using clone 38213, a mAb, and a biotinylated polyclonal goat anti-mouse IL-13 Ab (both from R&D Systems). Replicate values for each mouse were used to calculate the mean ± SD for each treatment group.

### Adoptive transfers

Single-cell suspensions from mesenteric lymph nodes of WT and IL-10 KO mice were obtained 5 days following oral infection. CD4<sup>+</sup> T cells were purified by negative selection using the CD4<sup>+</sup> T cell isolation kit from



**FIGURE 1.** Lymphocytes are required for the development of hepatitis in infected mice. Representative liver sections were stained with H&E from day 0 (A–D) and day 12 following oral infection with 600 L1 (E–H). Sections from WT (A and E), IL-10 KO (B and F), RAG2 KO (C and G), and IL-10/RAG2 KO (D and H) mice are included. The arrow indicates periphery of the lesion in F. Bar, 100  $\mu$ m;  $n = 3$ –5 mice/group.

Miltenyi Biotec. The percentage of CD4<sup>+</sup> cells was determined to be  $\geq 95\%$  by flow cytometry. CD4<sup>+</sup> T cells ( $2 \times 10^6$ ) in 0.5 ml of PBS, or PBS alone, were injected i.p. 1 day before infection with 600 L1. Recipient mice included WT, IL-10 KO, RAG2 KO, and IL-10/RAG2 KO animals. Twelve days later, mice were euthanized. Serum samples from individual mice were collected for ALT measurement, and liver tissue was obtained for histology and flow cytometry.

### Statistical analysis

Each experiment was performed a minimum of three times, and each group contained at least three mice. All means were calculated from values obtained from individual mice in a treatment group. Means of replicates were compared by the Student *t* test (see Fig. 4B) or ANOVA (see Figs. 2, 3, 4A, 5, 6, C–F, 7, and 8, B and D), followed by Fisher's posttest using Minitab Software. Statistical significance was assessed at  $p < 0.05$ .

## Results

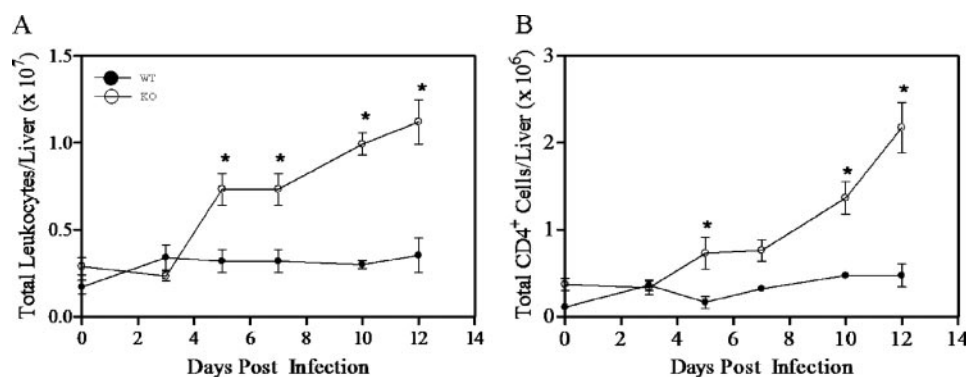
### *Lymphocytes mediate hepatitis in infected IL-10 KO mice*

Previously, we demonstrated that IL-10 KO mice developed hepatitis when orally infected with *T. spiralis* (19). The liver lesions were multifocal and characterized by a central area of coagulative necrosis that was surrounded by inflammatory cells, including mononuclear and polymorphonuclear (mainly eosinophil) cells. Conversely, no evidence of inflammation in the livers of WT mice was observed. To better understand the role of IL-10 in the pathogenesis of hepatitis, we investigated the phenotypes of infiltrating cells. Because the cellular infiltrate around the liver lesions contained many mononuclear cells that appeared to be lymphocytes, we asked whether they mediated hepatic inflammation by infecting lymphocyte deficient mice. WT, IL-10 KO, RAG2 KO, and IL-10/RAG2 KO mice were infected with 600 L1. From prior experiments, we established that this dose did not cause mortality but led to the development of hepatitis in all the IL-10 KO animals (19). After 12 days, a time of maximal inflammation in IL-10 KO mice, liver tissue was obtained for histological examination. We found that IL-10/RAG2 KO mice did not develop inflammatory lesions while IL-10 KO mice did, indicating that lymphocytes were required for the development of hepatitis (Fig. 1).

### *IL-10 regulates hepatic leukocyte trafficking*

The histological appearance of hepatic lesions in orally infected IL-10 KO mice suggested leukocytic infiltration. This prompted us to hypothesize that IL-10 regulated cell trafficking in the liver during infection. Leukocyte numbers in the liver increased after day 3 in both strains but were significantly greater in IL-10 KO mice (Fig. 2A). By day 12, leukocyte numbers in IL-10 KO animals were  $\sim 3$ -fold greater than in WT mice. Indeed, WT mice experienced a very mild increase of leukocytes during infection.

The lack of hepatitis in infected lymphocyte-deficient mice implied the pathogenic importance of this cell type. CD4<sup>+</sup> T cells are critical in coordinating host protective immunity to *T. spiralis*. During infection, activated CD4<sup>+</sup> T cells invade intestinal tissue and release cytokines, promote smooth muscle hypercontractility,



**FIGURE 2.** Infection induces an increase in leukocytes and CD4<sup>+</sup> cells in the liver. A, Livers from individual mice were processed, and leukocytes were counted as described in *Materials and Methods*. B, At the indicated times, intrahepatic leukocytes were isolated and subjected to immunofluorescent staining and flow cytometry using Abs specific to CD4. Cell numbers were determined by multiplying the total number of leukocytes with the percentage of CD4<sup>+</sup> cells in the leukocyte gate. Data represent the mean value  $\pm$  SD of three mice per group, and asterisks indicate statistically significant differences between WT and IL-10 KO values.

Table I. Percentage of activated CD4<sup>+</sup> cells in the liver<sup>a</sup>

	Day 0	Day 12
WT	53.4 (2.5)	74.2 (1.4)
IL-10 KO	52.9 (5.5)	77.0 (1.6)

<sup>a</sup> Liver cells from uninfected and day 12-infected WT and IL-10 KO mice were stained with Abs against CD4, CD44, and CD62L. CD4<sup>+</sup> cells were gated, and expression of CD44<sup>high</sup>CD62L<sup>low</sup> cells as a percentage is shown.

and facilitate parasite expulsion (23–25). Additionally, adoptive transfer of activated CD4<sup>+</sup> T cells from infected animals protects against a primary infection in naive recipients (26). We asked whether the CD4<sup>+</sup> subset increased in the liver during infection. Within leukocyte populations, we observed an increase in CD4<sup>+</sup> cells that was again more substantial in IL-10 KO mice (Fig. 2B). Livers from IL-10 KO animals possessed approximately four times the number of CD4<sup>+</sup> cells by day 12, indicating that IL-10 controlled trafficking of this subset in the liver.

The majority of T cells in the normal liver possesses an activated phenotype (27–29). To determine the activation status of CD4<sup>+</sup> cells in the liver, we examined the expression of the activation markers, CD62L and CD44, on hepatic cells (30). In uninfected mice, slightly more than half were activated (Table I). Infection with *T. spiralis* invoked an equivalent increase in activated T cells in both strains of animals. Additionally, we determined that there were no significant differences in CD69 expression by WT and IL-10 KO hepatic CD4<sup>+</sup> cells (data not shown). Hence, infection caused a greater influx of CD4<sup>+</sup> cells in IL-10 KO mice, but the proportion of these cells that was activated was similar in WT mice.

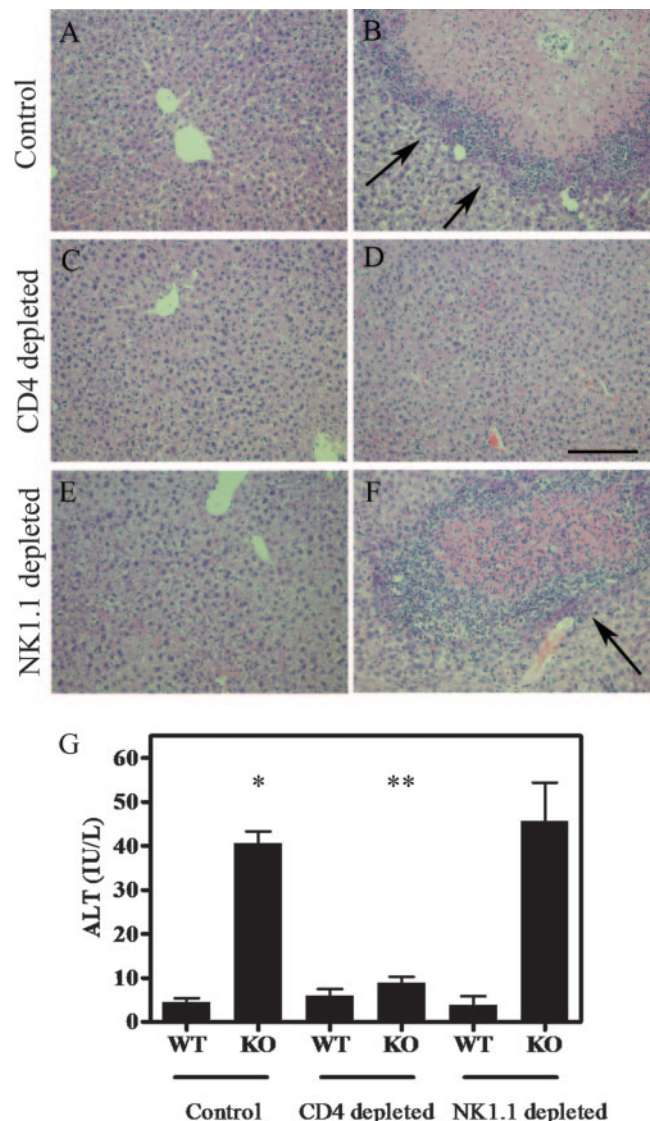
#### CD4<sup>+</sup> T cells induce liver injury

The significant increase of hepatic CD4<sup>+</sup> cells in IL-10 KO mice suggested a functional role in the development of liver inflammation. To determine whether CD4<sup>+</sup> cells contributed to the hepatic damage observed in IL-10 KO mice, we depleted WT and IL-10 KO animals of CD4<sup>+</sup> cells with specific mAbs. In depleted mice, CD4<sup>+</sup> cells were reduced by 98–100% as determined by flow cytometric analysis of mesenteric lymph node populations. IL-10 KO mice that received a control Ab developed hepatic inflammation and necrosis after infection (Fig. 3B). However, depleted IL-10 KO mice did not develop hepatitis (Fig. 3D). Moreover, we found significantly higher serum ALT activity, a measure of hepatocyte damage, in control IL-10 KO mice compared with WT animals after infection (Fig. 3G). ALT values in CD4-depleted IL-10 KO mice did not increase above WT levels. Thus, CD4<sup>+</sup> cells were essential in the immunopathogenesis of liver inflammation that developed in the absence of IL-10.

Most CD4<sup>+</sup> cells in the liver are conventional CD4<sup>+</sup> T cells. However, some classical NK T cells also express CD4 (31). Although there is no universal marker for the latter cell type that can be used for Ab-mediated depletion, Benlagha et al. (32) have shown that most classical NK T cells in the liver are NK1.1<sup>+</sup>. We depleted WT and IL-10 KO mice of NK1.1<sup>+</sup> cells with a specific mAb and infected them with 600 L1. After 12 days, IL-10 KO mice depleted of NK1.1<sup>+</sup> cells developed hepatitis (Fig. 3F), and ALT activity was equivalent to that in control animals (Fig. 3E), suggesting that NK T cells do not contribute to hepatic impairment. Thus, conventional CD4<sup>+</sup> T cells were most likely responsible for mediating liver injury.

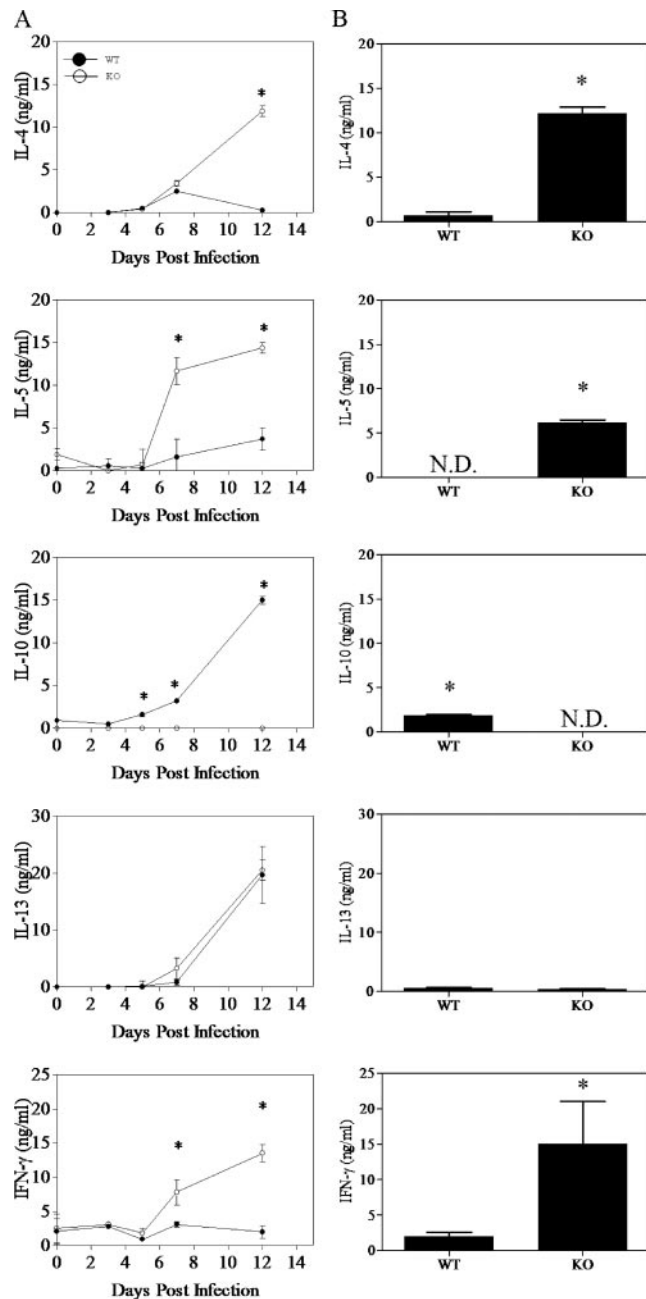
#### IL-10 suppresses hepatic leukocyte cytokine production

We previously reported that mesenteric lymph node cells from IL-10 KO animals released greater amounts of IL-4, IL-5, and



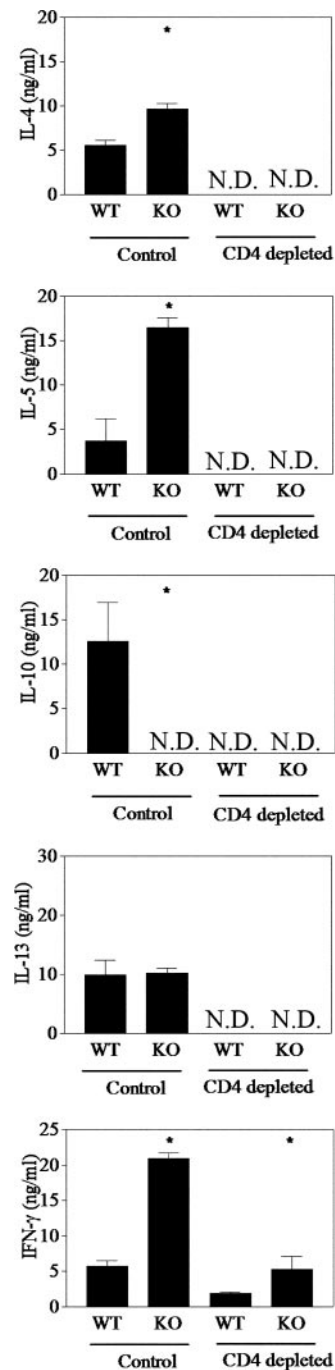
**FIGURE 3.** CD4<sup>+</sup> T cells are essential in the pathogenesis of hepatitis. WT (A, C, and E) and IL-10 KO (B, D, and F) mice were given a control Ab (A and B), a depleting Ab against CD4 (C and D), or a depleting Ab against NK1.1 (E and F) and infected with 600 L1 orally. Livers were obtained on day 12 and fixed, sectioned, and stained with H&E. Arrows indicate periphery of lesions in B and F. Bar, 100  $\mu$ m. G, Blood was collected at the time of euthanasia, and ALT activity was measured. Data represent the mean ALT value  $\pm$  SD of three mice per group. The single asterisk indicates a statistically significant difference between control WT and IL-10 KO values, whereas the double asterisk signals a significant difference between CD4 depleted and control IL-10 KO values.

IFN- $\gamma$  (19). To ascertain whether hepatic leukocytes behaved comparably, we isolated hepatic cells at various time points throughout the acute phase of infection and stimulated them with anti-CD3. After 3 days in culture, cytokine concentrations in supernatants were determined (Fig. 4A). Significantly higher amounts of IL-4, IL-5, and IFN- $\gamma$  were measured in cultures from IL-10 KO mice; however, cells from both strains produced equivalent amounts of IL-13. As expected, WT cells elaborated IL-10, while none was detected in the cultures from IL-10 KO mice. It is important to note that cytokines were not detected before day 5, which correlates with the first release of NBL into the vasculature. To determine whether hepatic leukocytes could respond to parasite-specific Ags, we also stimulated cells with a preparation of ES Ags derived from



**FIGURE 4.** Cytokine production by hepatic leukocytes. Leukocytes were purified from the livers of WT and IL-10 KO mice at various indicated times (A) or on day 12 (B) after infection and cultured with anti-CD3 (A) or ES proteins (B) for 72 h. Supernatants were collected, and cytokine levels were determined by ELISA. No cytokines were detected when the cells were cultured with medium (data not shown). Data represent the mean value  $\pm$  SD of three mice per group. The asterisks indicate a statistically significant difference between WT and IL-10 KO values. N.D., None detected.

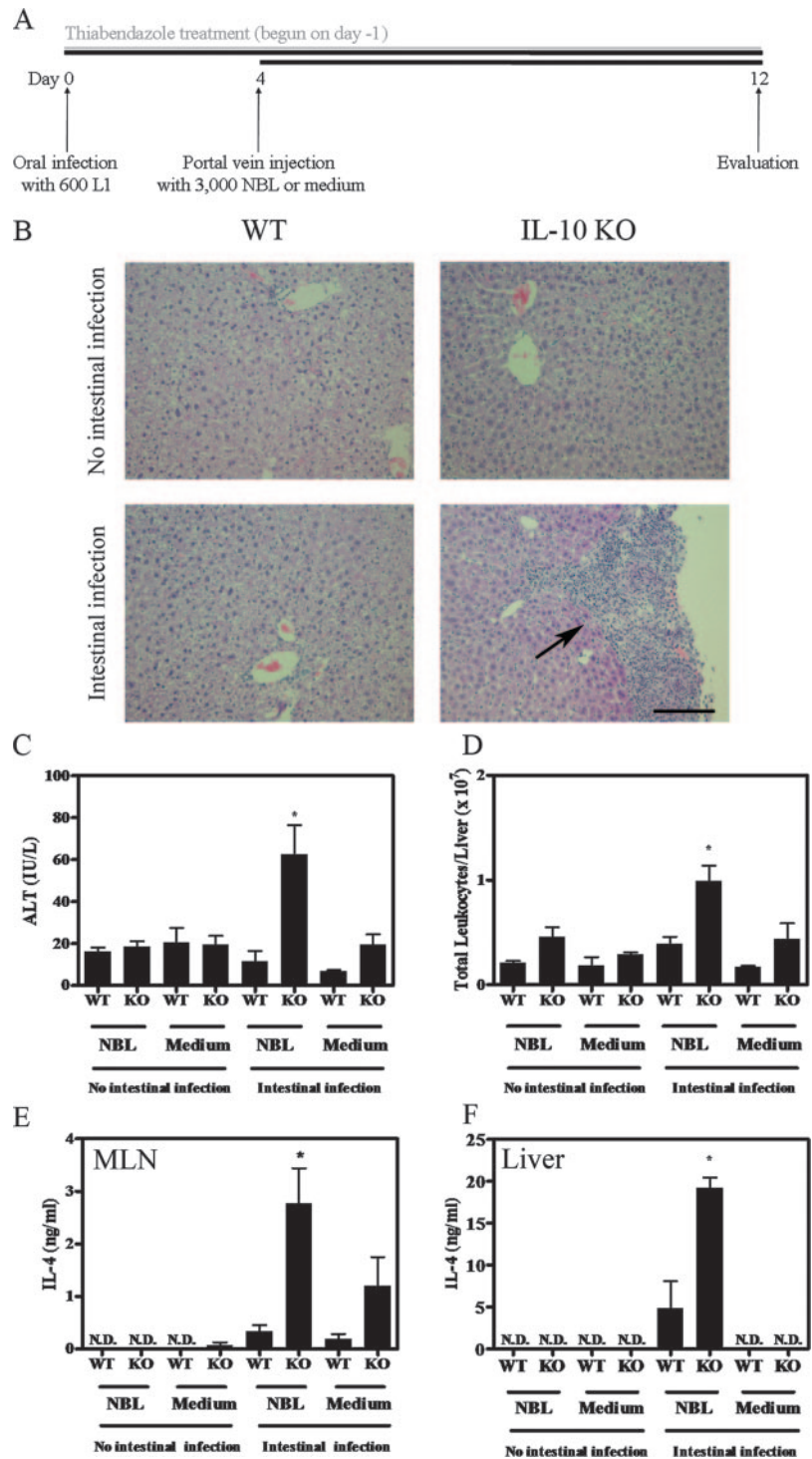
L1 (Fig. 4B). Similar results were obtained, but levels were consistently lower than what was detected after anti-CD3 triggering. Because the differences in mediator production were generally greatest at 12 days after infection, we used this time point to determine whether CD4<sup>+</sup> cells were responsible for some or all of the released cytokines. When CD4<sup>+</sup> cells were depleted in vivo, production of IL-4, IL-5, IL-10, and IL-13 was abolished (Fig. 5). IFN- $\gamma$  was detected in cultures from depleted animals, indicating that a non-CD4<sup>+</sup> cell contributed to the response.



**FIGURE 5.** CD4<sup>+</sup> cells mediate cytokine production in the liver. WT and IL-10 KO mice were administered either anti-CD4 or a control Ab and infected with 600 L1 orally. Livers were obtained on day 12, and purified leukocytes were restimulated with anti-CD3 for 72 h. Cytokine levels were determined by ELISA. Data represent the mean value  $\pm$  SD of three mice per group. The asterisks indicate a statistically significant difference between WT and IL-10 KO values. N.D., None detected.

#### *An intestinal immune response is necessary for the development of hepatitis*

During infection with *T. spiralis*, cytokine production by mesenteric lymph node cells can be detected by day 2 (33); yet, production by hepatic leukocytes lags by several days (Fig. 4). This suggested to us that the intestinal immune response may be influencing the hepatic response. To ask whether intestinal immunity during infection impacts the liver's response, we omitted the



**FIGURE 6.** Introduction of NBL into the liver via the portal vein without a prior intestinal response is insufficient to induce hepatitis. **A**, Experimental design. **B**, Representative histological sections from parenterally infected WT and IL-10 KO mice that either received an oral infection on day 0 or not. The arrow indicates lesion periphery. Bar, 100  $\mu$ m. **C**, ALT activity was measured in all recipients. The asterisk indicates a statistically significant difference. **D**, The number of intrahepatic leukocytes was determined in all recipients, and the statistically significant difference is shown by the asterisk. **E** and **F**, IL-4 production by mesenteric lymph node cells (**E**) or hepatic leukocytes (**F**) was determined by ELISA after a 3-day incubation with anti-CD3. The asterisks indicate a statistically significant difference between IL-10 KO and WT values. Data represent the mean value  $\pm$  SD of three mice per group.

intestinal infection and injected NBL directly into the portal vein. We also used a method to chemically sterilize adult parasites in the intestine with thiabendazole (22). This allowed the intestinal phase to proceed, but the migratory phase of infection was prevented. NBL are not affected by thiabendazole (data not shown). Thus, we were able to infect mice orally, induce intestinal immune responses, and determine the effect of that response on the liver in the presence or absence of NBL that we delivered to the portal vein by injection. Introduction of NBL into the portal veins of naive IL-10 KO mice, mimicking their natural route of entry, did not cause hepatitis or elevated ALT activity (Fig. 6). Orally infected, thiabendazole-treated IL-10 KO mice that received NBL parenter-

ally developed hepatic lesions, elevated ALT activity, and leukocyte accumulation (Fig. 6). In contrast, sham injections with medium did not induce hepatitis in IL-10 KO mice, nor in any of the WT mice. These data indicated that an intestinal infection and migrating NBL were essential for inciting hepatic inflammation.

It is well established that enteric *T. spiralis* infection results in the release of IL-4 within the intestinal immune environment (19, 24, 34). We used this cytokine as a marker to indicate that an intestinal response was generated. Fig. 6E shows that minimal to no IL-4 was detected in cultures of mesenteric lymph node cells derived from mice that received portal vein injections only. In contrast, IL-4 was present in cell cultures from WT and IL-10 KO

mice that were orally infected. Cells from IL-10 KO mice produced more IL-4, consistent with our previous results (19). Only when mice were enterally and parenterally infected did we detect IL-4 in cultures of hepatic leukocytes, with levels being greater in those from IL-10 KO mice (Fig. 6F). Hence, an intestinal immune response and circulation of NBL were necessary to elicit IL-4 production in the liver, and IL-10 suppressed this response.

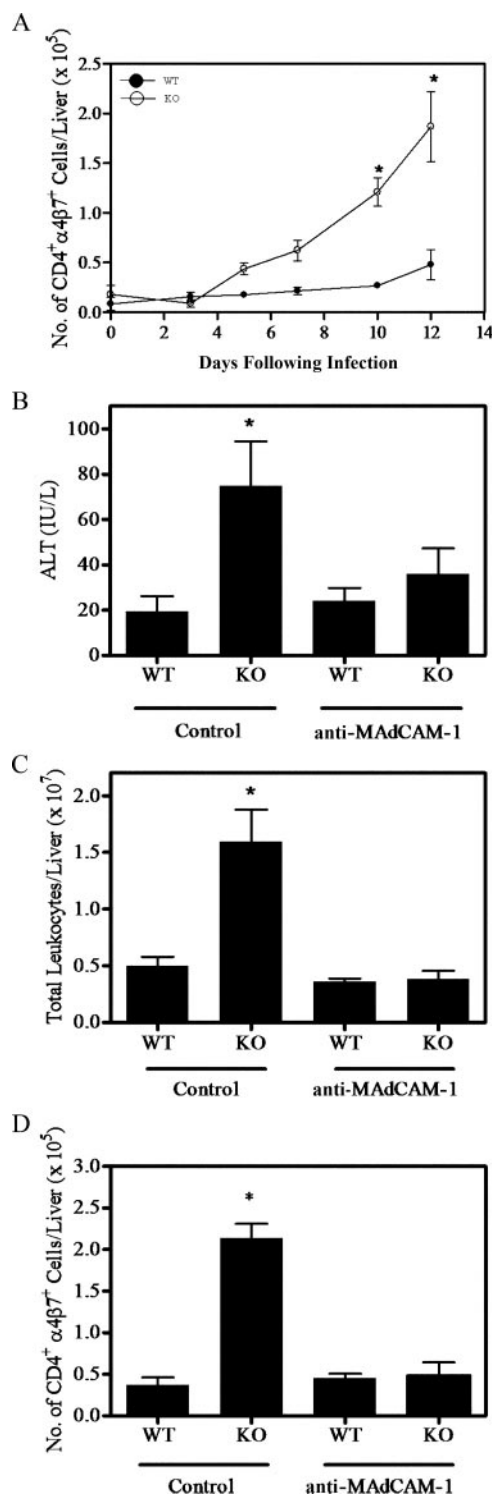
#### *Infection incites an increase of CD4<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup> cells in the liver*

Lymphocytes primed within the GALT express the integrin, α<sub>4</sub>β<sub>7</sub> (35). The dependence of hepatitis on an intestinal immune response suggested that intestinally primed cells migrated to the liver. Therefore, we examined expression of α<sub>4</sub>β<sub>7</sub> by CD4<sup>+</sup> cells in the livers of WT and IL-10 KO mice. We recovered low numbers of CD4<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup> cells in uninfected mice. Infection induced an increase of cells in both strains, but this was significantly greater in IL-10 KO animals (Fig. 7A). Indeed, this increase was apparent as early as day 5 after infection. We also examined hepatic CD4<sup>+</sup> cells for expression of CCR9, another molecule induced on intestinally primed cells. We found a similar representation of CCR9<sup>+</sup> cells in WT and IL-10 KO CD4<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup> cells (WT-16 ± 5.5% vs IL-10 KO-17 ± 2.0%).

α<sub>4</sub>β<sub>7</sub> binds to MAdCAM-1. To determine whether interactions between these two molecules mediated cell entry into the liver, we administered an Ab specific for MAdCAM-1 that blocks binding of α<sub>4</sub>β<sub>7</sub>. Infected IL-10 KO mice that received anti-MAdCAM-1 displayed less hepatic inflammation with no development of mature lesions (data not shown). ALT activity and hepatic leukocyte counts from this group were not significantly different from control WT values (Fig. 7, B and C). Additionally, anti-MAdCAM-1 administration reduced the number of CD4<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup> cells in the livers of infected IL-10 KO mice (Fig. 7D). Furthermore, we performed immunohistochemistry on liver sections from WT and IL-10 KO mice and detected positive staining for MAdCAM-1 only in the livers of infected IL-10 KO animals (data not shown). Overall, these data support a migratory route for T cells between the intestine and liver that can be increased by infection, regulated by IL-10, and mediated by α<sub>4</sub>β<sub>7</sub>-MAdCAM-1 interactions.

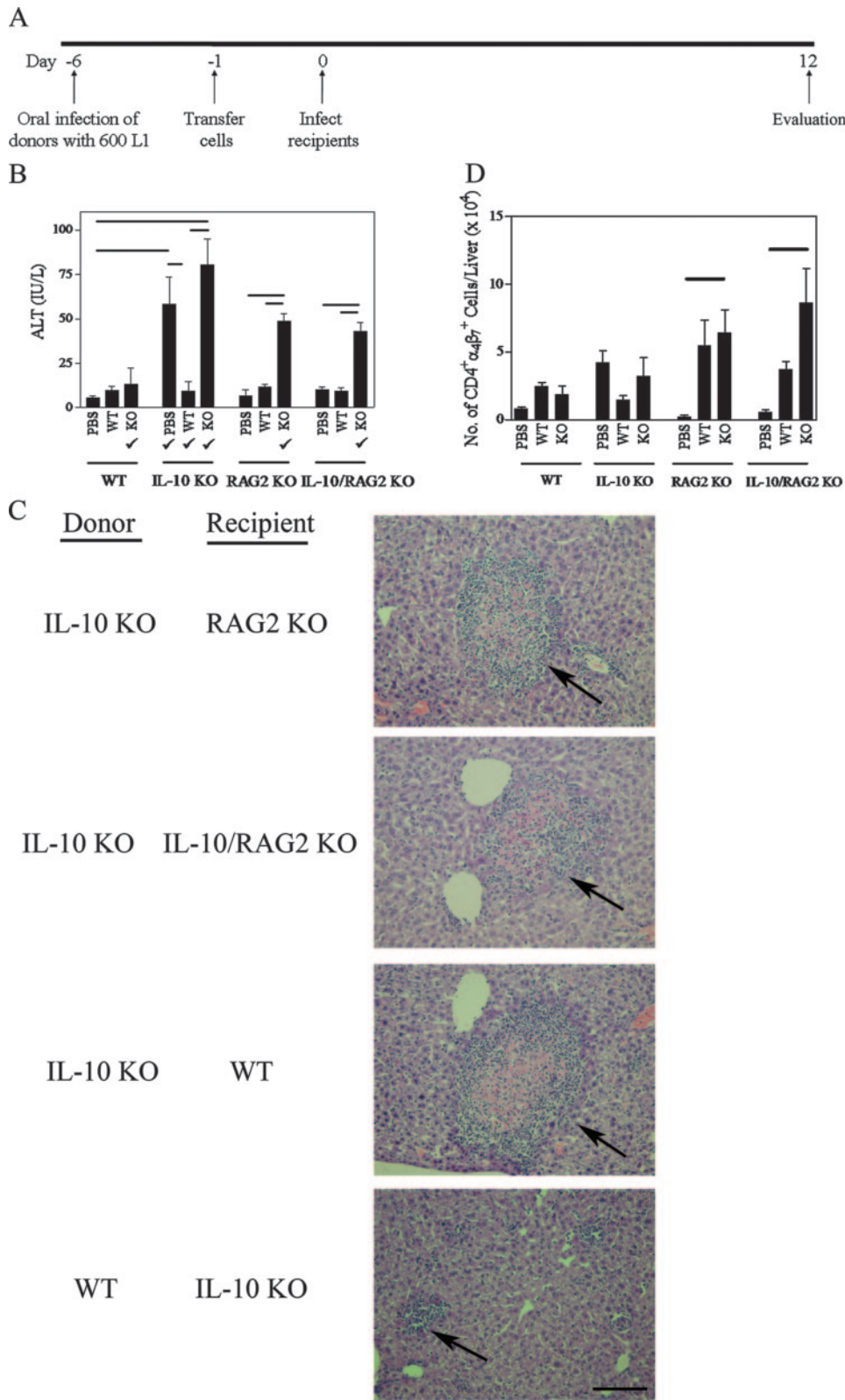
#### *Intestinally derived CD4<sup>+</sup> T cells from infected IL-10 KO mice induce hepatic inflammation while cells from infected WT mice protect*

Because our data indicated that CD4<sup>+</sup> T cells were required (Fig. 3) and some hepatic CD4<sup>+</sup> T cells were activated in GALT (Fig. 7), we sought to induce hepatitis by transferring intestinally primed CD4<sup>+</sup> T cells. To achieve this, CD4<sup>+</sup> T cells were purified from the mesenteric lymph nodes of WT or IL-10 KO mice 5 days following oral infection. Cells were transferred 1 day before oral infection of WT, IL-10 KO, RAG2 KO, and IL-10/RAG2 KO mice (Fig. 8A). Fig. 8 shows that when RAG2 and IL-10/RAG2 KO mice were given IL-10 KO cells, they developed hepatic lesions. Serum ALT activity was significantly higher in these mice compared with RAG2 KO or IL-10/RAG2 KO mice injected with WT cells or PBS. Thus, IL-10 KO CD4<sup>+</sup> T cells induced lesions in RAG2 KO mice and were not controlled by nonlymphoid cell-driven IL-10. Predictably, IL-10 KO recipients that received PBS or IL-10 KO cells released more ALT compared with PBS-injected WT mice. In contrast with the damaging effects of IL-10 KO CD4<sup>+</sup> T cells, transfer of WT cells into IL-10 KO mice inhibited lesion development in the majority of animals. Although some inflammation was evident, we detected a significant diminution in ALT release in this group (Fig. 8B). Similarly, when WT recipients received donor IL-10 KO CD4<sup>+</sup> T cells, ALT activity remained



**FIGURE 7.** CD4<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup> cells accumulate in the liver after infection. A, WT and IL-10 KO mice were orally infected with 600 L1. At the indicated times, hepatic cells were isolated from individual mice (*n* = 3 mice/group), stained for expression of CD4 and α<sub>4</sub>β<sub>7</sub>, and analyzed by flow cytometry. The number of double-positive cells/liver is shown. The asterisks indicate a statistically significant difference between IL-10 KO and WT values. Mice were given an Ab that blocks binding to MAdCAM-1 or a control rat IgG and infected. On day 12, mice were euthanized, and ALT activity (B), hepatic leukocyte numbers (C), and CD4<sup>+</sup>α<sub>4</sub>β<sub>7</sub><sup>+</sup> numbers (D) were determined. Asterisks denote statistically significant differences.

low (Fig. 7B), although occasional lesions were present as shown in Fig. 8C. Thus, WT cells protected mice from damage induced by IL-10 KO cells. To ask whether the transferred intestinally



**FIGURE 8.** Adoptive transfer of intestinally derived CD4<sup>+</sup> T cells from infected IL-10 KO mice induces hepatic inflammation in infected recipients whereas cells from infected WT mice are protective. *A*, Experimental design (*n* = 3 mice/group). *B*, ALT activity was measured. The checks indicate the groups of mice that developed gross hepatic lesions. The lines start and stop at groups that were statistically significantly different. *C*, Histological sections from representative animals. Arrows indicate periphery of the lesions. Bar, 100 μm. *D*, On day 12, livers were collected and processed for flow cytometry. The average number of CD4<sup>+</sup>α4β7<sup>+</sup> cells per liver ± SD is shown. The lines start and stop at groups that were statistically significantly different.

primed CD4<sup>+</sup> T cells entered the liver, we isolated hepatic leukocytes at the time of euthanasia and determined the number of CD4<sup>+</sup>α4β7<sup>+</sup> cells by flow cytometry. Compared with PBS-injected animals, RAG2 and IL-10/RAG2 KO mice had significantly more CD4<sup>+</sup>α4β7<sup>+</sup> cells when IL-10 KO donor cells were transferred. When donor cells were from WT mice, there was an increase in cells in the recipients compared with PBS-injected con-

trols, but the difference was not statistically significant. Taken together, these results indicate that intestinally primed CD4<sup>+</sup> T cells could enter the hepatic parenchyma and suggest that IL-10 regulated the circulation of T cells between the intestine and liver. Transferred intestinal CD4<sup>+</sup> T cells from IL-10 KO mice induced hepatitis while cells from WT mice protected against its development.

## Discussion

This study is the first to demonstrate that IL-10 regulates trafficking of intestinal CD4<sup>+</sup> T cells to the liver. The gastrointestinal tract serves as a portal of entry into the body. Any Ag that gains access to the mesenteric venous blood will reach the liver through the portal vein. Thus, the liver may be regarded as a second line of defense against harmful Ags such as pathogens. Nevertheless, many innocuous Ags like nutrients derived from food enter the liver. These latter Ags must be recognized as posing no threat to the host and incite a tolerogenic immune response.

From an evolutionary point of view, a coordinated immune defense between the liver and intestine would seem to provide a reasonable strategy by which appropriate responses to common Ags may be regulated. Evidence for such coordination comes from several observations. Although oral tolerance is established in GALT, the liver contributes to the tolerogenic response (36). In a landmark study in 1967, Cantor and Dumont (37) demonstrated that oral tolerance to 1-chloro-2,4-dinitrobenzene is blocked by the surgical creation of a portosystemic shunt, indicating that passage of oral Ags through the liver modifies specific immune responses. More recently, Li et al. (38) performed orthotopic liver transplantation using OVA-fed or nonfed mice as donors. Recipients were tested for tolerance induction by delayed-type hypersensitivity challenge. Successful tolerance induction was achieved when livers from OVA-fed mice were transferred to syngeneic recipients, implicating the liver as a key element in oral tolerance. Importantly, the authors isolated and adoptively transferred hepatic non-parenchymal cells and determined that cells derived from OVA-fed mice could transfer tolerance. In addition to a role in promoting tolerance to dietary and commensal Ags, the liver can respond to hematogenous pathogens derived from the intestine. To investigate the immunological relationship between the liver and intestine, we used a parasite that establishes enteric infection but also migrates to the liver.

Hepatic disease is not a prominent feature of human trichinellosis (17). In mice as in humans, oral infection with L1 leads to the development of adult *T. spiralis* and release of NBL, which transit the liver. Minimal injury and inflammation were noted in WT mice, either histologically or by serum enzyme activities. In uninfected mice, we found a small but reproducible population of cells in the hepatic parenchyma that expressed  $\alpha_4\beta_7$ , indicating that they were of intestinal origin. We hypothesize that these cells represent a component of the host's intestinal immune system that provides surveillance in the liver. No significant differences in CD4<sup>+</sup> $\alpha_4\beta_7$ <sup>+</sup> cell numbers were noted between WT and IL-10 KO mice, implying that enterohepatic circulation in the resting state is IL-10 independent. Oral infection of WT mice led to a small increase of this population in the liver that contrasted with the substantial accumulation observed in IL-10 KO mice, suggesting that IL-10 regulated trafficking of these intestinally primed cells to the liver during infection. Indeed, MADCAM-1 was expressed only in the livers of infected IL-10 KO mice, and Ab-mediated blockade of MADCAM-1 inhibited hepatic inflammation in this group. These results imply a mechanism for T cell entry into the liver that is regulated by IL-10.

A number of studies have revealed a crucial role for CD4<sup>+</sup> T cells in oral tolerance. Depletion of CD4<sup>+</sup> cells at the time of Ag feeding prevents tolerance induction to OVA (39, 40). Cong et al. (41) reported that adoptively transferred OVA-specific CD4<sup>+</sup> T cells from WT mice induced tolerance to subsequent Ag exposure, but importantly, if the donor cells were derived from IL-10 KO mice, tolerance did not develop. The authors concluded that oral tolerance required early production of IL-10 by CD4<sup>+</sup> T cells.

Although our system does not involve dietary Ags and thus is not a model for oral tolerance, we found that CD4<sup>+</sup> T cells from IL-10-sufficient donors inhibited the development of hepatitis in infected IL-10 KO recipients. Conversely, adoptive transfer of IL-10 KO CD4<sup>+</sup> T cells into immunodeficient animals reproduced the hepatic lesions. This was true whether or not the recipients could produce IL-10 from a nonlymphoid source. Strikingly, we also detected lesions in WT recipients, demonstrating the potent abilities of intestinal CD4<sup>+</sup> T cells from IL-10 KO mice to damage the liver. Although CD4<sup>+</sup> T cells secrete immunoregulatory cytokines, they also can have cytolytic activity that may be important in mediating hepatocyte necrosis (42). Alternatively, necrosis could be due to an indirect function of parasite-specific CD4<sup>+</sup> T cells on other cells present in the inflammatory infiltrate. Regardless of their specific role, it was clear that transfer of IL-10 KO CD4<sup>+</sup> T cells could fully reproduce hepatic lesions, thereby indicating that IL-10 was important in the education of T cells at the level of the intestine. It is also possible that IL-10 release by CD4<sup>+</sup> T cells in the liver itself contributed to regulation. In support of this, WT CD4<sup>+</sup> T cells displayed regulatory activities, leading to the reduction of hepatic inflammation in IL-10 KO recipients. The specific functional differences between these two populations of CD4<sup>+</sup> T cells are the subject of ongoing investigation in our laboratory.

A common occurrence among animal pathogens is the development of strategies to evade deleterious immune responses by promoting immune regulation (43). It has been shown that such regulation benefits the host because excessive inflammation leads to bystander tissue damage, and effective memory responses require persistence of the Ag(s) (44). Pursuant to this, many pathogens have evolved the means to exploit host regulatory immune mechanisms. One such mechanism is to induce IL-10 production from APCs and/or T cells (45). An IL-10-rich environment promotes the development of tolerogenic APCs and Tr1 cells. Whether this regulatory response is advantageous (e.g., inhibition of tissue damage) or disadvantageous (e.g., pathogen persistence) to the host depends on many factors. In our hands, endogenous IL-10 due to *T. spiralis* infection does not alter parasite burden but does prevent hepatic injury (19).

In addition to promoting the development of tolerogenic APCs and Tr1 cells, IL-10 influences functions such as cytokine production (11). In previous experiments, we found that IL-10 suppressed IL-4, IL-5, and IFN- $\gamma$  production by mesenteric lymph node cells during intestinal infection with *T. spiralis* (19). Cytokine production in the liver mirrored that in the mesenteric lymph node with one exception. Cytokine release lagged in the liver by several days, prompting us to consider whether T cells were first activated in GALT and then migrated to the liver or whether in situ hepatic T cell activation occurred. To address this question, we injected NBL into the portal vein to create hepatic infection in the absence of intestinal infection. Only when mice were infected intestinally and injected subsequently with NBL did hepatitis develop. This finding indicated that the arrival of NBL caused intestinal T cells to be diverted to the liver, and we hypothesize that the damage done by NBL as they passed through the liver was essential for initiating this process, mediated at least in part by the induction of addressin expression.

Overall, our data revealed a role for IL-10 in enterohepatic immune cell circulation. IL-10 regulated cell trafficking and the function of intestinal CD4<sup>+</sup> T cells during infection. The capacity to produce IL-10 supported the development of intestinal CD4<sup>+</sup> T cells with regulatory activity. They entered the liver and could inhibit injury induced by migrating NBL. We think it unlikely that nonlymphoid IL-10 contributed to local inhibition because transfer

of IL-10 KO cells to RAG2 and IL-10/RAG2 KO mice led to hepatitis. It is possible that CD4<sup>+</sup> T cells were the main source of IL-10, or alternatively, that WT CD4<sup>+</sup> T cells may induce IL-10 expression by other cell types. Certainly, IL-10 was instructive in GALT, leading to the generation of CD4<sup>+</sup> T cells with the ability to down-modulate hepatic inflammation. Obligate parasites need to preserve their host to ensure their own survival. Our results document a mechanism by which a highly adapted, intestine-dwelling parasite may co-opt a process of tolerance induction to minimize host tissue damage, thereby promoting its own continued existence in the host population.

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## Disclosures

The authors have no financial conflict of interest.

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