Combinatorial Effects of Interleukin 10 and Interleukin 4 Determine the Progression of Hepatic Inflammation Following Murine Enteric Parasitic Infection

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Mice lacking the immunoregulatory cytokine interleukin 10 (IL-10) develop necrotizing hepatitis after infection with *Trichinella spiralis*, and inflammation is dependent on the migration of intestinally activated $CD4^+$ T cells into the liver. Hepatic production of IL-4 is elevated in these mice, and we hypothesized that it plays a role in the development of hepatic pathology. Wild-type (WT), IL-10 knockout (KO), IL-4 KO, and IL-10/IL-4 KO mice were orally infected, and disease progression was followed by histological examination, alanine aminotransferase assays, and flow cytometric analysis of hepatocellular content. Both IL-10 KO and IL-10/IL-4 KO mice experienced hepatocellular injury, but only IL-10 KO mice advanced to a necrotic phase. Hepatic $CD4^+$ T cells were the major source of IL-4, and IL-10 regulated the number of intestinally-derived $CD4^+IL\text{-}4^+$ cells. Sequestration of activated neutrophils in the liver required IL-4, and neutrophil depletion prevented progression to overt necrosis. Adoptive transfer of intestinal WT $CD4^+$ T cells inhibited neutrophil accumulation and inflammation, but their regulatory effects did not require IL-10 signaling. *Conclusion:* The absence of IL-10 led to hepatocyte injury during infection, but IL-4 was necessary for the development of neutrophil-dependent necrosis. These studies provide new insight into the combinatorial role of these cytokines and their targets in the generation and progression of hepatic inflammation. (HEPATOLOGY 2010;51:2162-2171)

The liver performs many crucial metabolic activities, but it also functions in immune defense.[1] Its constant exposure to harmless and potentially harmful antigens requires effective discrimination and the generation of appropriate responses. Failure can lead to inadequate immunity and tissue injury. Inflammatory hepatic diseases, which can be distinguished by heterogeneous etiologies, often share common mechanisms of tissue injury.[2,3] For example, T cell–mediated hepatopathies include viral hepatitis and autoimmune hepatitis, diseases that are instigated by different antigens.[4,5] Moreover, immune-mediated damage can be facilitated by cells such as granulocytes and macrophages that become activated and migrate into the liver in an antigen-nonspecific manner.[6,7] The cytokine environment shapes their activities, and a better appreciation of the interactions between cytokine-producing cells and their targets will shed light on key aspects of the pathogenesis of inflammatory diseases of the liver.[2]

Previously, we established an infection model to investigate the role of interleukin 10 (IL-10) in the liver.[8,9] In contrast to wild-type (WT) animals, we found that IL-10 knockout (KO) mice developed severe multifocal hepatic inflammation and necrosis after oral infection with the parasitic nematode *Trichinella spiralis*. This pathogen matures and reproduces in the intestine. The juvenile form, or newborn larva, enters the mesenteric vasculature and is carried to the liver by the portal vein. Beyond the liver, newborn larvae circulate systemically and may penetrate skeletal

Abbreviations: ALT, alanine aminotransferase; CCR9, chemokine (C-C motif) receptor 9; GALT, gut-associated lymphoid tissue; IgG, immunoglobulin G; IL, interleukin; KO, knockout; Ly6-G, lymphocyte antigen 6 complex locus G; NK, natural killer; PBS, phosphate-buffered saline; WT, wild type.

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muscle cells, where they grow and await ingestion of the host to renew the life cycle. In our current studies, we have begun to investigate the mechanisms underlying hepatocyte injury and death. Our results demonstrated that the progression of initial hepatocyte damage into organized regions of necrosis was controlled by the prevailing cytokine environment. Although the absence of IL-10 led to cellular injury during infection, IL-4 was required for the evolution to necrotizing hepatitis. These results support a critical role for IL-4 in controlling the progression of hepatic inflammation after enteric parasitic infection, and they illustrate the importance of the enterohepatic cytokine balance for appropriate hepatic immune function.

Materials and Methods

Mice. C57BL/6 and IL-10 KO (on a C57BL/6 background) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-4 KO and IL-10/IL-4 KO mice were a generous gift from Dr. Tom Wynn at the National Institutes of Health. PHIL (eosinophil deficient) mice were provided by Dr. Jamie Lee at the Mayo Clinic. These mice were bred onto an IL-10 KO background, and transgenic mice were identified as described.[10] Disruption of the IL-10 locus was confirmed by polymerase chain reaction with primer sequences previously listed.[8] Animals were bred and housed at Cornell University, a facility accredited by the American Association for Accreditation of Laboratory Animal Care. Studies were approved by the Institutional Animal Care and Use Committee.

Parasites and Infections. *T. spiralis* first-stage larvae were recovered from the muscles of irradiated Albino Oxford rats by digestion with 1% pepsin in acidified water as described previously.[11] Experimental mice were administered 600 first-stage larvae by gavage.

Neutrophil Depletion. In some experiments, mice were given a control rat immunoglobulin G (IgG) or were rendered neutropenic by the injection of an α-granulocyte receptor-1 (Gr-1) antibody (clone RB6-8C5), as described previously,[12] or clone NIMP-R14, a kind gift from Dr. Fabienne Tacchini-Cottier.[13] RB6.8C5 recognizes Gr-1, which is expressed by other cell types in addition to neutrophils, albeit at lower levels.[14] NIMP-R14 recognizes a 25- to 30-kDa protein present on the neutrophil surface and is reported to be specific.[13] To confirm neutropenia, differential cell counts were performed on blood smears obtained from individual mice at the time of euthanasia.

Isolation of Hepatic Leukocytes. Hepatic leukocytes were recovered as described previously.[9] Cells were analyzed by flow cytometry, were cultured for cytokine determination, or were centrifuged onto glass slides with a Shandon Cytospin 2 (Thermo Fisher Scientific, Waltham, MA) for differential cell counting (300 cells per sample counted).

Flow Cytometry. Cells were restimulated ex vivo, stained, and analyzed as described.[9,12] The employed antibodies were specific for CD4 (clone RM4-5), CD62L (clone MEL-14), CD11b (clone M1/70), chemokine (C-C motif) receptor 9 (CCR9; clone CD-1.2; eBioscience, San Diego, CA), α4β7 (clone DATK32), lymphocyte antigen 6 complex locus G (Ly6-G; clone 1A8), IL-4 (clone 11B11; BD Pharmingen, San Jose, CA), and F4/80 (clone BM8; Caltag, Carlsbad, CA). Appropriate isotype-matched clones served as controls and were used to set analysis gates.

Cytokine Enzyme-Linked Immunosorbent Assay. Hepatic leukocytes were restimulated in vitro with medium or somatic larval antigens at 10 μg per well. After 3 days, supernatants were collected, and IL-4 levels were determined by enzyme-linked immunosorbent assay as described.[9]

Measurement of Alanine Aminotransferase (ALT) Activity. ALT activity was measured in individual serum samples with a commercially available kit from Pointe Scientific (Canton, MI).

Histology. Liver tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Six-micrometer sections were stained with hematoxylin and eosin for microscopic examination. Photomicrographs were created with a BX51 microscope and a DP12 digital camera system from Olympus (Center Valley, PA).

Adoptive Transfer of CD4$^+$ T Cells. Mesenteric lymph nodes from WT mice were obtained 5 days after oral infection. CD4$^+$ T cells were purified by negative selection with the CD4$^+$ T cell isolation kit from Miltenyi Biotec (Auburn, CA). The percentage of CD4$^+$ cells was determined to be ≥95% by flow cytometry. Cells (2×10^6) in 0.5 mL of phosphate-buffered saline (PBS) or PBS alone were injected intraperitoneally into IL-10 KO recipients 1 day prior to their infection. In some groups of recipients, a control IgG or α-IL-10R (clone 1B1.3a) antibody (300 μg intraperitoneally every other day beginning 1 day before infection) was administered. Other groups included mice that were given cells and PBS or PBS only. To aid in the interpretation of the effects of the α-IL-10R treatment, we also included a group of WT recipients that were given the same dose and regimen as the IL-

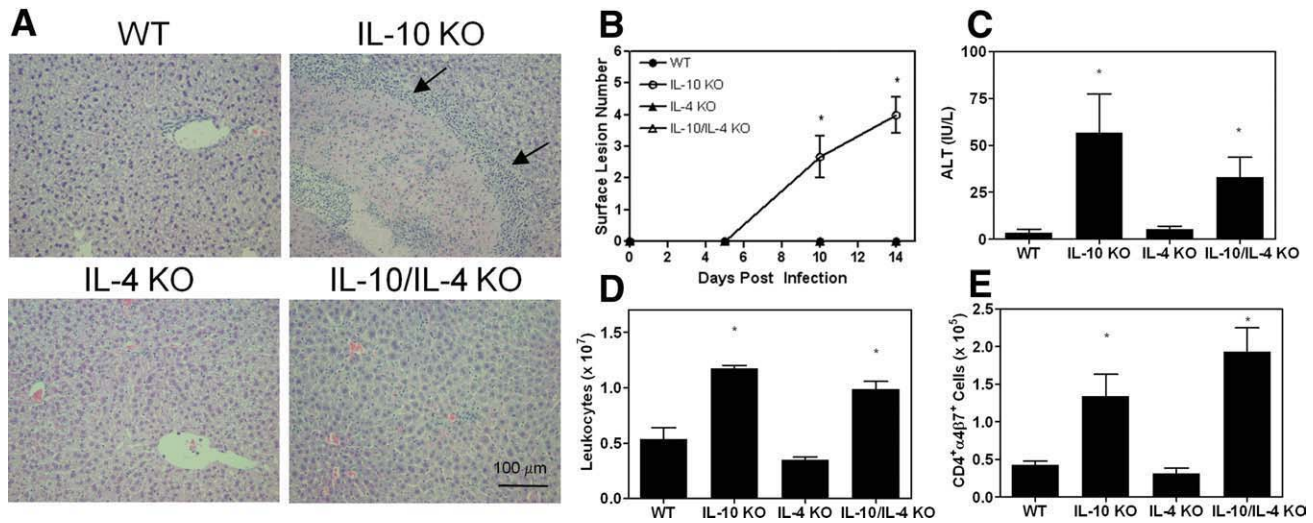

Fig. 1. IL-4 is required for the development of hepatic necrosis in infected IL-10 KO mice. (A) Representative liver sections obtained 12 days after oral infection. Arrows distinguish the margins of a lesion in an IL-10 KO mouse. (B) Number of surface lesions. (C) ALT activity. (D) Number of intrahepatic leukocytes. (E) Number of $CD4^+\alpha4\beta7^+$ cells. Asterisks indicate a statistically significant difference with respect to WT values.

10 KO mice. Twelve days later, mice were evaluated for ALT activity, liver histology, hepatic leukocyte content (total, $CD4^+\alpha4\beta7^+$ cells, and Ly6-G^+F4/80^- cells), and cytokine production.

Statistical Analysis. Each experiment was performed three to five times, and each group contained three to five mice. Means and standard deviations were calculated from values obtained from individual mice in a treatment group. Means were compared by the Student t test or analysis of variance followed by an appropriate posttest with GraphPad Prism software (San Diego, CA). Significance was assessed at $P < 0.05$.

Results

The Capacity to Produce IL-4 Correlates with Disease Severity. In previous studies, we found that oral infection resulted in hepatic inflammation and necrosis in IL-10 KO mice.[8] Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling staining and histopathological examination of liver tissue indicated that cell death was necrotic and not apoptotic in nature. Hepatic injury was mediated by intestinally-derived $CD4^+$ T cells during infection and could be mitigated by blocking their entry into the liver.[9] Moreover, transfer of these cells from IL-10 KO mice to recombination activation gene 2 KO animals reproduced the disease, and this suggested that this lymphocyte subset alone was sufficient for inciting injury. Type 2 cytokine production, particularly IL-4 synthesis, was prominent in the infected IL-10 KO liver.[9] Therefore, we hypothesized that IL-4 promoted inflammation and necrosis during infection in IL-10 KO mice. The infection of singly and doubly deficient animals revealed that lesion development was dependent on IL-4 in IL-10 KO mice (Fig. 1A). While multifocal lesions were grossly and histologically visible in IL-10 KO animals, they were completely absent in IL-10/IL-4 KO mice. Lesions were characterized by central necrosis that was surrounded by mononuclear and polymorphonuclear cells. Although liver tissue from infected IL-10/IL-4 KO mice appeared to contain more leukocytes than that from WT animals, areas of hepatocellular necrosis were not detected. Neither WT nor IL-4 KO mice acquired hepatic lesions (Fig. 1B). As we reported previously, serum ALT activity at 12 days post-infection was significantly greater in infected IL-10 KO mice compared to WT mice (Fig. 1C).[9] Infection did not lead to an increase in ALT values in IL-4 KO mice, and this indicated a lack of hepatocyte damage. In contrast, ALT levels in infected IL-10/IL-4 KO mice rose significantly above WT levels but were not different from those in IL-10 KO mice. When considered with the histological evidence, the results suggested that initial hepatocyte injury occurred in the absence of IL-10, but the evolution of organized necrotic lesions required IL-4. We considered, however, that differences in parasite burden between IL-10 KO and IL-10/IL-4 KO mice might affect lesion development. Accordingly, we counted intestinal worm numbers as an indication of the load

that the liver received during the acute phase of infection and found no differences, suggesting that the disparity in hepatic response was due not to parasite burden but rather reflected differences in immunity (data not shown). Immune-mediated hepatic injury is the result of effector leukocyte recruitment and activity, and we find enumeration of hepatic leukocytes to be a sensitive indicator of inflammation. Both infected IL-10 KO and IL-10/IL-4 KO mice had elevated numbers of hepatic leukocytes in comparison with WT mice, implying that IL-10 regulated the total leukocyte content within the liver independently of IL-4 (Fig. 1D). Additionally, the number of $CD4^+\alpha4\beta7^+$ cells in the liver was increased in both IL-10 KO and IL-10/IL-4 KO mice, and this suggested that IL-10 also controlled intestinally-derived $CD4^+$ cell infiltration of the liver in a manner unaffected by IL-4 (Fig. 1E). Comparison of other lymphocyte subsets between IL-10 KO and IL-10/IL-4 KO mice revealed only a slight and variable decrease in $CD8^+$ T cell numbers in IL-10 KO animals (data not shown). Overall, the data supported the contention that IL-10 prevented hepatocyte injury and accumulation of intestinally-derived $CD4^+$ cells, whereas IL-4 was required for the development of hepatic necrosis.

$CD4^+$ T Cells Are the Major Source of IL-4 in the Liver During Infection. To investigate further the role of IL-4 in the liver during infection, we sought to determine which cell type(s) produced it. The majority of $IL\text{-}4^+$ cells were $CD4^+$; however, the percentage of $CD4^+IL\text{-}4^+$ cells in IL-10 KO mice was approximately twice that in WT mice (Fig. 2A). Most $CD4^+$ cells in the liver are conventional $CD4^+$ T cells, but some classical natural killer (NK) T cells also express CD4. To distinguish between contributions from these two cell types, we stained cells for CD4, IL-4, and NK1.1. $IL\text{-}4^+$ cells were gated, and the percentages of IL-4 expressing conventional $CD4^+$ T cells versus NK T cells are shown in Fig. 2B. Almost all of the $IL\text{-}4^+$ cells colocalized with the $CD4^+NK1.1^-$ population. Thus, $CD4^+$ T cells were the major source of IL-4. Additionally, this population was expanded in IL-10 KO animals in comparison with WT mice. Because we previously discovered that an intestinal immune response was a prerequisite for hepatic inflammation, we asked if any $CD4^+NK1.1^-IL\text{-}4^+$ cells were gut-derived. CCR9, like $\alpha4\beta7$, is up-regulated on lymphocytes after activation within gut-associated lymphoid tissue (GALT) and is used as a marker of intestinal origin.[15] Infected IL-10 KO animals had significantly more $IL\text{-}4^+$, intestinally derived $CD4^+$ T cells than WT mice (Fig. 2C).

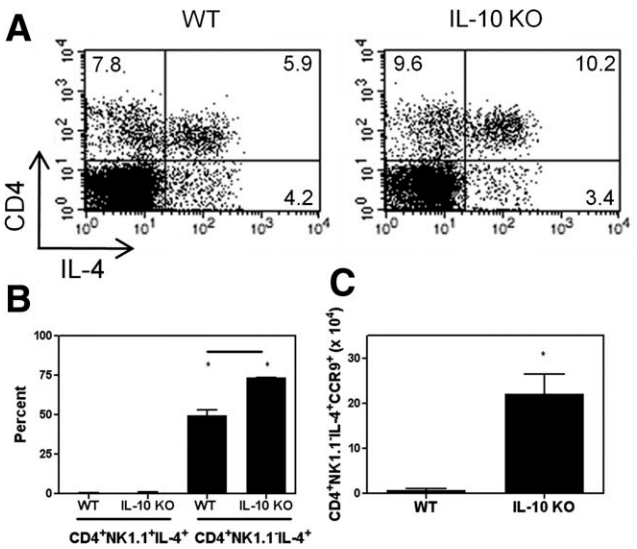

Fig. 2. IL-10 suppresses the accumulation of $CD4^+IL\text{-}4^+$ T cells in the liver. Twelve days post-infection, livers were obtained for flow cytometric analysis. (A) Hepatic CD4 and IL-4 expression are shown for representative animals. (B) $IL\text{-}4^+$ cells were gated, and the percentages of $CD4^+NK1.1^+IL\text{-}4^+$ and $CD4^+NK1.1^-IL\text{-}4^+$ cells per strain are shown. Asterisks denote significant differences between cell types per strain, and the line indicates a difference between WT and IL-10 KO values. (C) Numbers of hepatic $CD4^+NK1.1^-IL\text{-}4^+CCR9^+$ cells. The asterisk designates a significant difference between WT and IL-10 KO values.

Infected Eosinophil-Deficient IL-10 KO Mice Develop Hepatic Inflammation and Necrosis. Previously, we noted that lesions in infected IL-10 KO mice contained an abundance of granulocytes, including neutrophils and eosinophils. IL-4 promotes eosinophil proliferation, recruitment, and effector functions, and its expression is elevated by *T. spiralis* infection.[16] This led us to ask if eosinophils were involved in the development of hepatic necrosis. We compared eosinophil infiltration in singly and doubly deficient mice after infection (Fig. 3A). As expected, IL-4 KO mice displayed reduced eosinophilia in comparison with WT animals. In contrast, eosinophil numbers were higher in infected IL-10 KO mice compared to WT animals. The hepatic eosinophil content in IL-10/IL-4 KO mice was similar to that in WT mice. Hence, eosinophil accumulation in the liver was inhibited by IL-10 and promoted by IL-4. We tested whether eosinophils were essential in the development of hepatic necrosis by mating IL-10 KO animals to eosinophil-deficient (PHIL) mice to generate mice lacking both IL-10 and eosinophils. Subsequent infection revealed that both IL-10 KO and IL-10 KO/PHIL mice formed hepatic lesions (Fig. 3B). Eosinophils were abundant in areas juxtaposed to lesions in IL-10 KO animals; however,

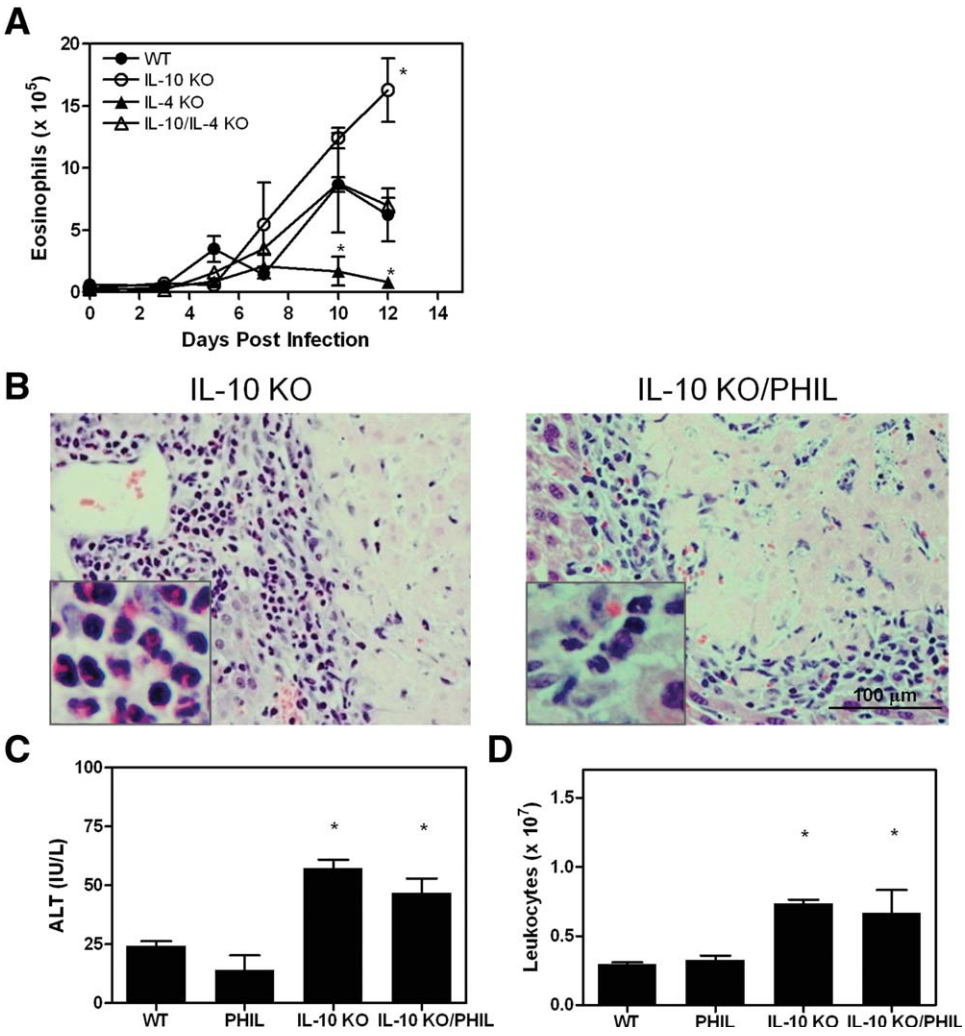

Fig. 3. Eosinophils do not mediate hepatic necrosis. Leukocytes were isolated at the indicated times by density gradient centrifugation. The total number of cells was enumerated, and an aliquot from each mouse was spun onto a glass slide and stained for differential cell counting. (A) The average number of eosinophils and standard deviation per group per time point are shown. Asterisks indicate a significant difference with respect to WT values at the same time point. (B) Representative liver sections from infected IL-10 KO and IL-10 KO/PHIL mice on day 12. The inset in the left panel is a higher magnification of an area around the lesion showing eosinophils. A similar inset is shown for an IL-10 KO/PHIL mouse to demonstrate the absence of eosinophils. (C) ALT activity. (D) Hepatic leukocytes. Asterisks indicate significant differences from WT values.

they were absent in IL-10 KO/PHIL mice, demonstrating that eosinophils were not critical in the development of hepatic necrosis. Results of ALT activity assays and hepatic leukocyte counts corroborated this interpretation (Fig. 3C,D).

IL-10 Suppresses Hepatic Infiltration of Neutrophils. During infection, neutrophils were significantly increased in the livers of IL-10 KO animals in comparison with WT mice, with peak numbers (day 10) occurring just prior to the time of maximal lesion size (days 12-14; Fig. 4A). Numbers remained low in both WT and IL-4 KO mice, whereas those in IL-10/IL-4 KO animals initially rose but then fell, never achieving the values observed in IL-10 KO mice. This was confirmed by flow cytometric analysis of hepatic leukocytes (data not shown). Additionally, the prevalence of neutrophils in the liver was suppressed by IL-10. For example, neutrophils represented 14% ± 1.7% of total leukocytes in IL-10 KO livers on day 12 versus 9% ± 1% in WT animals ($P < 0.05$). Loss of endogenous IL-4 decreased the prevalence to 2.7% ± 1.5% in IL-10/IL-4 KO mice. Activated neutrophils can release cytotoxic mediators, suggesting their potential participation in lesion development. We used expression of CD11b and CD62L to determine if IL-10 and IL-4 influenced the neutrophil activation state in the liver. Infection resulted in a significant increase in the number of Ly6-G$^+$F4/80$^-$ cells (markers of neutrophils) with an activated CD11b$^+$CD62L^{lo} phenotype in WT and IL-10 KO mice in comparison with naive animals

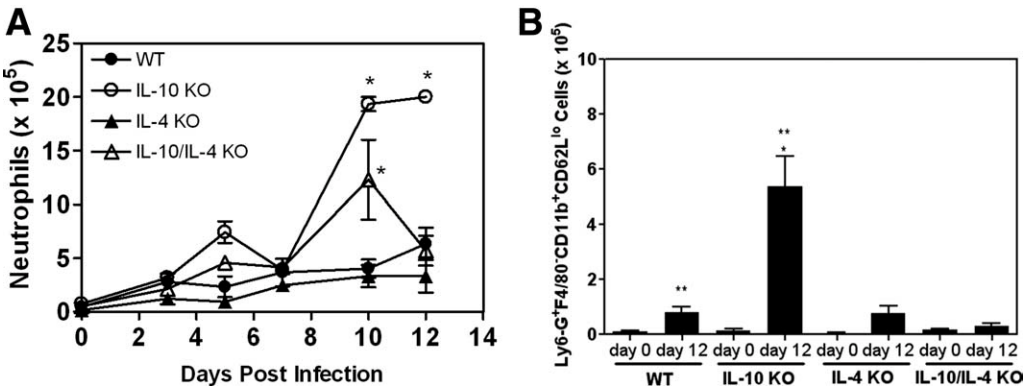

Fig. 4. The capacity to produce IL-10 is associated with reduced infiltration of activated neutrophils. (A) At indicated times, hepatic leukocytes were recovered, and the number of neutrophils was determined. Asterisks indicate significant differences with respect to WT mice. (B) Hepatic leukocytes obtained 12 days post-infection were stained for expression of Ly6-G, F4/80, CD62L, and CD11b. The average number of Ly6-G$^+$F4/80$^-$CD11b$^+$CD62L^{lo} cells and standard deviation per liver are displayed. The single asterisk denotes a significant difference between day 12 WT and IL-10 KO values, whereas the double asterisks indicate significant differences between naive and infected mice.

(Fig. 4B). Upon infection, only the number of activated neutrophils in IL-10 KO mice differed significantly from that in WT animals. Taken together, the data suggested that IL-10 and IL-4 may have differential effects on neutrophil trafficking and activation state.

Neutrophils Mediate Hepatic Necrosis but Are Not Involved in Initiating Hepatic Injury. To determine whether neutrophils played a role in initial hepatocyte injury and/or subsequent development of hepatic necrosis, we depleted mice of neutrophils with one of two monoclonal antibodies. Figure 5A shows the effect of antibody administration on peripheral neutrophils. Both depleting antibodies reduced the prevalence of neutrophils to less than 2%. Control antibody-treated and neutropenic IL-10 KO mice had greater ALT values and hepatic leukocyte numbers than WT mice after infection (Fig. 5C,D). However, only control antibody-treated IL-10 KO mice developed hepatic necrosis (Fig. 5B). The number of CD4$^+\alpha4\beta7^+$ cells was significantly increased in both control and depleted IL-10 KO mice in comparison with WT mice, corresponding to the elevated serum ALT activity in these animals (Fig. 5C,D). Thus, in the absence of IL-10 and in the presence of IL-4, neutrophils were necessary for the development of hepatic necrosis but were not required for the initiation of hepatocyte injury.

CD4$^+$ T Cells Generated in an IL-10 Sufficient Environment Do Not Require IL-10 to Protect Mice. Our previous studies showed that infection of recombination activation gene 2 KO mice, which are deficient in lymphocytes but possess neutrophils, did not lead to hepatocyte injury or necrosis, but transfer of intestinal CD4$^+$ T cells from infected IL-10 KO animals induced necrotizing hepatitis upon infection.[9] Moreover, neutrophil or CD4$^+$ cell depletion prevented necrosis in infected IL-10 KO mice[9] (Fig. 5). Thus, our data support a model in which, in the absence of IL-10, CD4$^+$ T cells activated within GALT migrate to the liver and elaborate cytokines that regulate both neutrophil accumulation and the state of activation. In support of this, we reported that adoptive transfer of intestinal CD4$^+$ T cells from infected IL-10 KO mice to WT mice led to a mild hepatitis upon infection, whereas the transfer of WT cells to IL-10 KO recipients was protective.[9] To determine whether IL-10 was required for protective activity, we transferred WT CD4$^+$ T cells into IL-10 KO mice that had received PBS, an irrelevant antibody, or α-IL-10R antibody. Animals that received WT CD4$^+$ T cells had decreased ALT activity and hepatic leukocyte content (total and intestinally-derived CD4$^+$ cells) in comparison with IL-10 KO mice that did not receive cells (Fig. 6). Additionally, the development of necrotic lesions was suppressed in IL-10 KO recipients that received cells in comparison with those given PBS (data not shown). Interestingly, cultured hepatic leukocytes from adoptively transferred mice released less IL-4, and this suggested that the transferred WT CD4$^+$ T cells controlled IL-4 production (Fig. 6D). In vivo blockade of the IL-10R did not compromise protection, indicating that IL-10 was important during T cell activation in GALT rather than for T cell function in the liver. Because neutrophil depletion blocked the development of hepatic necrosis, we hypothesized that

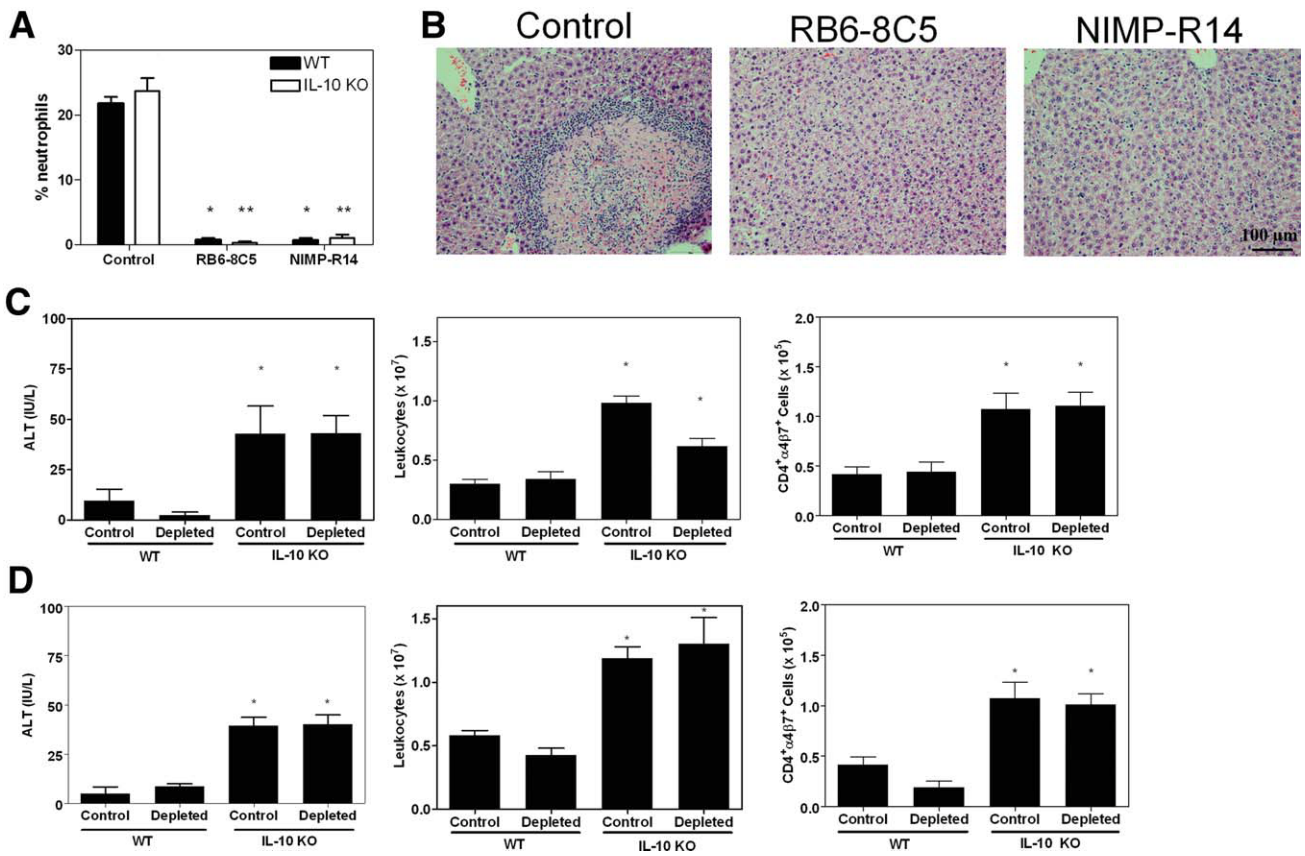

Fig. 5. Neutrophil-depleted mice are protected from hepatic necrosis. Mice (five mice per group) either were given a control IgG antibody or were depleted of neutrophils with (C) clone RB6.8C5 or (D) clone NIMP-R14. (A) Percentages of peripheral blood neutrophils. Single asterisks designate significant differences between control and depleted WT values, and double asterisks denote differences between control and depleted IL-10 KO numbers. (B) Representative photomicrographs of control and depleted IL-10 KO liver tissue (day 12). (C,D) ALT activity and cell numbers. Asterisks indicate significant differences with respect to WT values.

the transfer of intestinal $CD4^+$ T cells from WT mice would reduce neutrophil numbers and decrease hepatic necrosis. Indeed, IL-10 KO recipients accumulated significantly fewer $Ly6\text{-}G^+F4/80^-$ cells in the liver (Fig. 6E). Furthermore, blockade of IL-10 signaling did not reverse this effect. To aid in the interpretation of these results, we included a group of WT recipients that were administered α-IL-10R antibodies. These animals experienced hepatocellular damage and an influx of $CD4^+\alpha4\beta7^+$ cells similar to those experienced by IL-10 KO mice. Hepatic IL-4 levels were greater in WT mice versus IL-10 KO mice that received cells but less than those in PBS-injected IL-10 KO animals. Additionally, two-thirds of WT mice developed small necrotic foci (data not shown). Thus, the α-IL-10R antibody preparation antagonized the effects of IL-10. Overall, our data indicate that intestinally derived $CD4^+$ T cells, activated in an IL-10 sufficient environment, can protect the liver against hepatic injury and necrosis by regulating effector cell trafficking and function.

Discussion

Clinically significant liver disease may result from a multitude of insults, including infection, alcohol, drugs, and ischemia/reperfusion. Inflammation is a common feature of most forms of liver disease, is a normal process that may rid the host of an insulting agent, and often is a necessary precursor to regenerative tissue repair. However, if the inflammatory response is severe and prolonged, hepatic necrosis may eventually lead to extensive loss of parenchyma and irreversible tissue fibrosis.

Neutrophils are capable of migrating rapidly to foci of infection or inflammation. Infiltration and contact with inflammatory mediators can reprogram cells to alter effector responses. Chakravarti et al.[17] recently described a subset of human blood neutrophils that became long-lived, expressed human leukocyte antigen DR, CD80, and CD49d de novo, and alternatively produced leukotrienes, superoxide anions, and cytokines upon exposure to granulocyte-macrophage

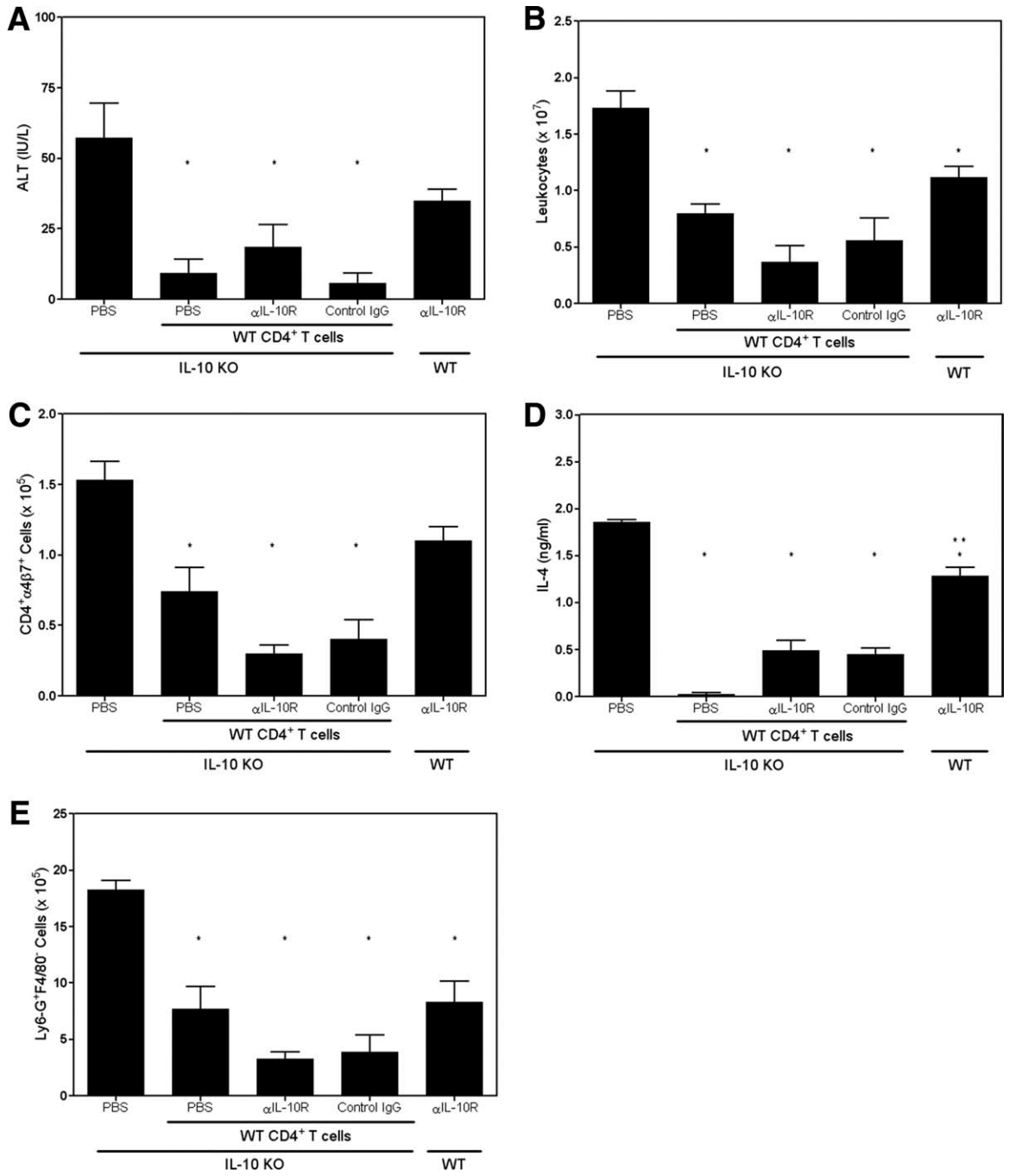

Fig. 6. $CD4^+$ T cells generated in an IL-10 sufficient environment do not require IL-10 to protect mice. $CD4^+$ T cells from infected WT mice were transferred to IL-10 KO recipients. The recipients received WT cells plus PBS, a control rat IgG, or an antibody that blocks IL-10 from binding to its receptor. Additionally, one group received only PBS as a positive control. Another group consisted of WT mice given α-IL-10R antibodies. All mice were infected with 600 first-stage larvae and were evaluated on day 12. (A) ALT activity. (B) Hepatic leukocytes. (C) $CD4^+\alpha4\beta7^+$ cell numbers. (D) IL-4 was measured in supernatants from cultured hepatic leukocytes that were restimulated with parasite antigens. No IL-4 was detected from cells cultured only in medium. (E) Number of $Ly6\text{-}G^+F4/80^-$ cells. Single asterisks indicate significant differences with respect to the positive control group, and the double asterisk signifies a difference between the indicated group and all others that received donor cells.

colony-stimulating factor, tumor necrosis factor α, and IL-4. Thus, the microenvironment can reprogram cells that traditionally have been thought to be terminally differentiated, and this can affect disease progression. Here, we show that IL-4 was necessary for the full development of hepatic necrosis in infected IL-10 KO mice, and $CD4^+$ T cells, a proportion of which were activated within GALT, constituted a major source of IL-4 in the liver. Furthermore, our data indicated that neutrophils played a critical role in the progression

from hepatocellular injury to necrosis. The accumulation of neutrophils was inhibited in the absence of IL-4 concomitantly with altered expression of key activation molecules, highlighting a role for this cytokine in the management of neutrophil function. These data define a critical balance between IL-10 and IL-4 in the hepatic response to enteric infection and suggest a role for $CD4^+$ T cells and IL-4 in regulation of neutrophil activity during hepatic injury. Our results also demonstrated the utility of this in vivo system not only for the investigation of the specific roles of IL-10 and IL-4 in the hepatic response to infection with this parasite but also for broader inquiry into the coordination of enteric and hepatic immune mechanisms.

In several experimental models of liver injury, IL-4 has been shown alternatively to be protective or deleterious. For example, IL-4 protects mice from damage induced by ischemia/reperfusion, but it promotes hepatitis after concanavalin-A injection.[18,19] Although IL-4 and neutrophils are known to participate in the pathogenesis of certain liver diseases, very little is established about how IL-4 directly or indirectly influences neutrophil activity. Interestingly, Huang et al.[20] recently reported that IL-4 stimulated the expression of chemokine (C-X-C motif) ligand 8, CD62E, vascular endothelial growth factor, and inducible nitric oxide synthase by equine pulmonary artery endothelial cells, resulting in neutrophil migration in vitro. In our studies, the capacity to produce IL-4 influenced expression of neutrophil adhesion molecules and sequestration in the liver. Activated neutrophils may release proteases and oxidants that further damage hepatic cells, leading to necrosis. Elucidation of the mechanisms by which this cytokine modulates neutrophil function in the liver and the means of communication between T cells and neutrophils currently are centers of effort in our laboratory.

IL-10 is hepatoprotective in conditions such as alcoholic hepatitis and fatty liver disease.[21,22] Moreover, the importance of IL-10 in protecting against pathogen-induced liver damage has been demonstrated in several models.[23,24] Our previous work with *T. spiralis*–infected mice revealed that IL-10 abrogated liver injury, and experiments showed that it was necessary during T cell activation in GALT to prevent the development of a subset of $CD4^+$ T cells that migrated to the liver to induce damage.[9] Here, we have provided evidence that IL-10 was not required for control of hepatic inflammation when WT $CD4^+$ T cells were transferred to IL-10 KO recipients. Taken together, these results strongly implicated intestinal $CD4^+$ T cells in the initial hepatocellular injury that occurs after infection in the context of IL-10 deficiency. Whether this early hepatic damage is caused directly by these cells or $CD4^+$ T cell–dependent injury is mediated indirectly through a secondary nonlymphocyte effector cell remains unclear. Interestingly, Alford et al.[25] reported that crosslinking of CD46 on the surface of $CD4^+$ T cells resulted in their ability to mediate cytotoxicity through perforin and granzyme B. Following initial injury, the manner in which T cells regulate neutrophil activity in our system has not been discovered. We have considered the potential effects of IL-10 and IL-4 on IL-17 production. Although we did not find a significant difference in IL-17A production by $CD4^+$ T cells between mouse strains in preliminary studies, the possible influence of this cytokine requires further testing. Clarification of the functional differences between $CD4^+$ T cells primed in the intestine in the presence and absence of IL-10 and their effects on the liver would constitute a significant advance in our understanding of enterohepatic immune regulation. Furthermore, given the dominance of neutrophils in many inflammatory liver diseases, a greater understanding of how the microenvironment alters neutrophil phenotype and function would likely advance the development of targeted disease interventions.

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