Divergent functions of CD4\(^+\) T lymphocytes in acute liver inflammation and injury after ischemia-reperfusion

Charles C. Caldwell,* Tomohisa Okaya,* Andre Martignoni, Thomas Husted, Rebecca Schuster, and Alex B. Lentsch

The Laboratory of Trauma, Sepsis & Inflammation Research, Department of Surgery, University of Cincinnati, Cincinnati, Ohio

Submitted 17 May 2005; accepted in final form 1 July 2005

Divergent functions of CD4\(^+\) T lymphocytes in acute liver inflammation and injury after ischemia-reperfusion. Am J Physiol Gastrointest Liver Physiol 289: G969–G976, 2005. First published July 7, 2005; doi:10.1152/ajpgi.00223.2005.—Hepatic ischemia-reperfusion results in an acute inflammatory response culminating in the recruitment of activated neutrophils that directly injure hepatocytes. Recent evidence suggests that CD4\(^+\) lymphocytes may regulate this neutrophil-dependent injury, but the mechanisms by which this occurs remain to be elucidated. In the present study, we sought to determine the type of CD4\(^+\) lymphocytes recruited to the liver after ischemia-reperfusion and the manner in which these cells regulated neutrophil recruitment and tissue injury. Wild-type and CD4 knockout (CD4\(^{-/-}\)) mice were subjected to hepatic ischemia-reperfusion. CD4\(^+\) lymphocytes were recruited in the liver within 1 h of reperfusion and remained for at least 4 h. These cells were comprised of conventional (\(\alpha\beta\)TCR-expressing), unconventional (\(\gamma\delta\)TCR-expressing), and natural killer T cells. CD4\(^{-/-}\) mice were then used to determine the functional role of CD4\(^+\) lymphocytes in hepatic ischemia-reperfusion injury. Compared with wild-type mice, CD4\(^{-/-}\) mice had significantly greater liver injury, yet far less neutrophil accumulation. Adoptive transfer of CD4\(^+\) lymphocytes to CD4\(^{-/-}\) mice recapitulated the wild-type response. In wild-type mice, neutralization of interleukin (IL)-17, a cytokine released by activated CD4\(^+\) lymphocytes, significantly reduced neutrophil recruitment in association with suppression of MIP-2 expression. Finally, oxidative burst activity of liver-recruited neutrophils was higher in CD4\(^{-/-}\) mice compared with those from wild-type mice. These data suggest that CD4\(^+\) lymphocytes are rapidly recruited to the liver after ischemia-reperfusion and facilitate subsequent neutrophil recruitment via an IL-17-dependent mechanism. However, these cells also appear to attenuate neutrophil activation. Thus the data suggest that CD4\(^+\) lymphocytes have dual, opposing roles in the hepatic inflammatory response to ischemia-reperfusion.

liver injury; cell trafficking; neutrophils; T cells; interleukin-17

HEPATIC ISCHEMIA-REPERFUSION injury is a complication of trauma surgery, liver transplantation, and resectional surgery that can lead to liver dysfunction and failure as well as remote organ injury (8, 16, 17, 29). The liver injury induced by ischemia-reperfusion has two distinct phases. The initial phase of this response is characterized by activation of Kupffer cells and their production of reactive oxygen species, leading to mild hepatocellular injury. The generation of oxidants during this phase is also thought to activate redox-sensitive transcription factors, such as nuclear factor-\(\kappa\)B and activator protein-1, which control the expression of proinflammatory mediators, such as interleukin (IL)-12 and tumor necrosis factor (TNF)-\(\alpha\) (6, 12, 19, 23, 34). The expression of these mediators leads to a later phase of liver injury characterized by the induction of secondary mediators, including neutrophil-attracting CXC chemokines and endothelial cell adhesion molecules, which facilitate the adhesion and transmigration of neutrophils from the vascular space into the hepatic parenchyma (4, 5, 9, 18). Accumulated neutrophils release oxidants and proteases that directly injure hepatocytes and vascular endothelial cells and may also obstruct hepatic sinusoids, resulting in hepatic hypoperfusion (11).

Although the mechanisms for neutrophil recruitment in the liver after ischemia-reperfusion are known to involve cytokines, chemokines, and adhesion molecules, a previous study provided evidence that neutrophil accumulation in liver tissue was also dependent on CD4\(^+\) T lymphocytes (33). That study showed that depletion of CD4\(^+\) T lymphocytes with antibody diminished neutrophil recruitment and injury after hepatic ischemia-reperfusion. Studies in a model of intestinal ischemia-reperfusion injury have suggested a similar role for lymphocytes in neutrophil recruitment and tissue injury (7). However, the mechanism(s) by which CD4\(^+\) T lymphocytes regulate neutrophil recruitment and the subsequent injury has not been elucidated. The objective of the present study was to determine how CD4\(^+\) T lymphocytes regulate liver neutrophil recruitment and injury after ischemia-reperfusion.

MATERIALS AND METHODS

Hepatic ischemia-reperfusion injury. Male C57BL/6 B cell-deficient and CD4 knockout (CD4\(^{-/-}\)) mice (10–12 wk of age) were obtained from Jackson Laboratories (Bar Harbor, ME). This project was approved by the University of Cincinnati Animal Care and Use Committee and conformed to National Institutes of Health guidelines. Partial hepatic ischemia was induced as described previously (18). Briefly, mice were anesthetized with pentobarbital sodium (60 mg/kg ip). A midline laparotomy was performed, and an atraumatic clip was used to interrupt blood supply to the left lateral and median lobes of the liver. After 90 min of partial hepatic ischemia, the clip was used to initiate hepatic reperfusion. Sham control mice underwent the same protocol without vascular occlusion. Mice were killed after the indicated periods of reperfusion, and blood and liver samples were taken for analysis.

Adoptive transfer of CD4\(^+\) lymphocytes. CD4\(^+\) cells were isolated from the spleens of male wild-type mice by negative selection using fluorescein-conjugated monoclonal antibody to B220, CD8, and FcRII

* C. Caldwell and T. Okaya contributed equally to this study.

Address for reprint requests and other correspondence: A. B. Lentsch, Univ. of Cincinnati College of Medicine, Dept. of Surgery, 231 Albert Sabin Way, Cincinnati, OH 45267-0558 (e-mail: alex.lentsch@uc.edu).

http://www.ajpgi.org 0193-1857/05 $8.00 Copyright © 2005 the American Physiological Society
CD4+ LYMPHOCYTES AND HEPATIC I/R INJURY

Fig. 1. Hepatic recruitment of CD4+ lymphocytes after ischemia-reperfusion (I/R). Liver mononuclear cells were isolated as described in MATERIALS AND METHODS. CD4+ lymphocytes were analyzed and enumerated using flow cytometry. Data are means ± SE with n = 4–6 mice/group. *P < 0.05 compared with the sham group.

(BD Pharmingen, San Diego, CA) followed by incubation with magnetic beads coated with anti-FITC antibodies and separation on an AutoMACS, as described by the manufacturer (Miltenyi Biotec, Auburn, CA). The purity of the CD4+ cells was determined to be 60–70% by flow cytometry, with >90% of contaminating cells identified as B cells. Purified CD4+ cells were washed extensively and resuspended in sterile PBS. CD4+ cells (1×10⁶) were injected in CD4−/− mice via the penile vein 18 h before liver ischemia. This results in total liver CD4+ cell counts of 1.2×10⁴ ± 2,000 and total blood CD4+ cells of 1.9×10⁵ ± 7.5×10⁴.

Isolation and flow cytometric analysis of liver lymphocytes. Livers were flushed through the portal vein with saline before being processed through a 200-gauge stainless steel wire mesh and collected in media. The dispersed liver suspension was transferred to a 50-ml conical centrifuge tube and centrifuged at 450 g for 10 min. The pellet was resuspended in media, carefully layered on Histopaque-1083 (Sigma Chemical, St. Louis, MO), and centrifuged at 450 g for 30 min at room temperature. Mononuclear cells at and above the opaque interface were collected and centrifuged at 450 g for 10 min at room temperature. Cell viability was >95% in all cases, as determined by trypan blue exclusion.

Analysis of cell surface antigen expression in situ was performed as described elsewhere (3). Phycoerythrin-conjugated anti-natural killer (NK) 1.1 and anti-γδ T cell receptor (TCR) and anti-CD8, Alexa-488-conjugated anti-CD4, allophycocyanin-conjugated anti-T cell receptor β-chain (TCRB), and anti-CD11b antibodies were purchased from Caltag Laboratories (Burlingame, CA). Mononuclear cells (0.5–1.5×10⁶ cells/tube) were placed in polystyrene tubes containing 1 μg anti-CD16/32 and 5% rat serum for 10 min to block nonspecific antibody labeling. Labeling antibodies (0.5–1 μg antibody/tube) were then added and incubated at 4°C for 20 min. Cells were washed two times with cold fluorescence-activated cell sorter buffer (PBS with 1% BSA and 0.1% azide) and fixed with 250 μl of 1% paraformaldehyde. Samples were analyzed with an LSR flow cytometer and Cell Quest Pro software (Becton-Dickinson, San Diego, CA).

Neutrophil oxidative burst. Neutrophils from peripheral blood and from liver were isolated for measurement of hydrogen peroxide production by oxidation of dihydrorhodamine to fluorescent rhodamine. Cells were washed two times with ice-cold bovine albumin (0.5%) containing PBS. Cells were then incubated with 10⁻⁵ M dihydrorhodamine for 5 min at room temperature. Cells were then stimulated with nothing or with 10⁻⁵ M formyl-methionyl-leucyl-phenylalanine (FMLP) for 15 min at 37°C. Activation was stopped by putting cells on ice. FcγII/III receptors were blocked with 1 μg anti-mouse CD16/CD32 and 5% rat serum. Cells were then incubated with phycocerythrin-anti-mouse Ly6G (GR-1, 0.5 mg/ml) and allophycocyanin (APC)-anti-mouse CD11b (0.5 mg/ml) for 15 min. After being washed with PBS, cells were subjected to flow cytometry. Neutrophils were identified by dual staining for GR-1 and CD11b. Hydrogen peroxide production was determined by the fluorescence intensity of rhodamine. Appropriate compensation was set on fluorescence channels to avoid signal overlap. All data were collected in the log amplified mode, and readings were linearized using CellQuest ProSoftware (Becton-Dickinson).

Myeloperoxidase assay. Liver myeloperoxidase (MPO) content was assessed by methods similar to Schierwagen et al. (26). Liver tissue (100 mg) was homogenized in 2 ml of 3.4 mM KH₂HPO₄ and 16 mM Na₂HPO₄, pH 7.4. After centrifugation for 20 min at 10,000 g, the pellet was resuspended in 10 vol of 43.2 mM KH₂HPO₄, 6.5 mM Na₂HPO₄, 10 mM EDTA, and 0.5% hexadecyltrimethylammonium, pH 6.0, and sonicated for 10 s. After being heated for 2 h at 60°C, the supernatant was reacted with 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical), and optical density was determined at 655 nm.

Blood and tissue analysis. Blood was obtained by cardiac puncture at the time of death for analysis of serum alanine aminotransferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnostic kit (Biotron Diagnostics, Hemet, CA). Serum samples were also analyzed for TNF-α and macrophage inflammatory protein-2 (MIP-2) by sandwich ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Liver tissue was fixed in buffered formalin before embedding in paraffin. Livers were sectioned and stained with hematoxylin and eosin.

Statistical analysis. All data are expressed as means ± SE. Data were analyzed with a one-way ANOVA with subsequent Student-Newman-Keul’s test. Differences were considered significant at P < 0.05.

RESULTS

Phenotypic analysis of liver resident and recruited CD4+ lymphocytes during ischemia-reperfusion. We first examined the temporal nature of CD4+ lymphocyte recruitment after ischemia-reperfusion to assess the rapidity and longevity of this response. After ischemia-reperfusion, we found that CD4+ lymphocytes were recruited very rapidly, with maximal recruitment occurring within 1 h of reperfusion (Fig. 1). Increased numbers of liver CD4+ lymphocytes were observed up to 4 h later, indicating trapping and/or infiltration of these cells in the liver (Fig. 1). Using similar methods, we did not find any evidence for CD8+ lymphocyte recruitment at any time point (data not shown).

We next assessed the phenotypes of liver-resident and recruited CD4+ lymphocytes to determine the subsets present under normal conditions and those recruited after ischemia-

Table 1. Phenotypic analysis of liver resident and infiltrating lymphocytes (×10⁵)

<table>
<thead>
<tr>
<th>Group</th>
<th>Conventional CD4</th>
<th>NK T</th>
<th>CD4δδ</th>
<th>NK</th>
<th>DN γδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT sham</td>
<td>2.2±0.5</td>
<td>1.0±0.8</td>
<td>0.8±0.4</td>
<td>5.7±2.1</td>
<td>9.8±2.0</td>
</tr>
<tr>
<td>WT I/R</td>
<td>5.0±1.5*</td>
<td>3.4±1.7*</td>
<td>4.1±1.6*</td>
<td>7.0±4.8</td>
<td>12.9±6.4</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE with n = 4–7 mice/group. NK, natural killer; T, cell; I/R, ischemia-reperfusion. Conventional CD4+, CD4+; TCRβ⁺; NK T, CD4−; NK 1.1⁺; TCRβ−; CD4δδ, CD4−; γδTCR−; NK, CD4−; NK 1.1⁺; TCRβ−; double negative (DN) γδ, CD4−, CD8−, γδTCR−. *P < 0.05 compared with wild-type (WT) mice.
CD4⁺ T lymphocytes and have a deficit in helper T cell
activity (24). To establish that these mice were indeed an
appropriate model for our studies, we examined the number
of liver CD4⁺ T lymphocytes in wild-type and CD4⁻/⁻ mice.
After sham surgery or ischemia-reperfusion, CD4⁺ T lympho-
cyte numbers in CD4⁻/⁻ mice were virtually nil (Fig. 2).

To determine whether hepatic ischemia-reperfusion injury is
dependent on CD4⁺ T lymphocytes, we compared the re-
sponses of wild-type and CD4⁻/⁻ mice. After hepatic ischemia
and 8 h of reperfusion, CD4⁻/⁻ mice had significantly more
liver injury, as measured by serum ALT levels, than wild-type
mice (Fig. 3A). Interestingly, CD4⁻/⁻ mice had far less evidence
of neutrophil accumulation, as determined by liver MPO
content, than wild-type mice (Fig. 3B). An examination of liver
histology supported both biochemical findings. Wild-type and
CD4⁻/⁻ sham controls had normal liver architecture (Fig. 3, C
and E, respectively). After ischemia-reperfusion, wild-type
mice showed focal areas of oncocytic necrosis and marked
accumulation of neutrophils (Fig. 3D). In contrast, liver sec-
tions from CD4⁻/⁻ mice undergoing ischemia-reperfusion
showed widespread areas of hemorrhagic necrosis and paren-
chymal cell destruction, with little evidence of neutrophilic
infiltrates (Fig. 3F).

We next examined whether the changes in liver injury and
neutrophil accumulation observed in CD4⁻/⁻ mice were a
result of altered production of proinflammatory cytokines.
TNF-α is a primary cytokine that has been shown to drive this
inflammatory response (6). Serum levels of TNF-α were ele-
vated after ischemia-reperfusion in both wild-type and CD4⁻/⁻
mice but were not different between these groups (Fig. 4, top).
The CXC chemokine MIP-2 plays a critical role in the hepatic
inflammatory response to ischemia-reperfusion by facilitating
neutrophil recruitment (18). Serum levels of MIP-2 were

CD4⁺ lymphocytes and have a deficit in helper T cell activity (24). To establish that these mice were indeed an appropriate model for our studies, we examined the number of liver CD4⁺ T lymphocytes in wild-type and CD4⁻/⁻ mice. After sham surgery or ischemia-reperfusion, CD4⁺ T lymphocyte numbers in CD4⁻/⁻ mice were virtually nil (Fig. 2).

To determine whether hepatic ischemia-reperfusion injury is dependent on CD4⁺ T lymphocytes, we compared the responses of wild-type and CD4⁻/⁻ mice. After hepatic ischemia and 8 h of reperfusion, CD4⁻/⁻ mice had significantly more liver injury, as measured by serum ALT levels, than wild-type mice (Fig. 3A). Interestingly, CD4⁻/⁻ mice had far less evidence of neutrophil accumulation, as determined by liver MPO content, than wild-type mice (Fig. 3B). An examination of liver histology supported both biochemical findings. Wild-type and CD4⁻/⁻ sham controls had normal liver architecture (Fig. 3, C and E, respectively). After ischemia-reperfusion, wild-type mice showed focal areas of oncocytic necrosis and marked accumulation of neutrophils (Fig. 3D). In contrast, liver sections from CD4⁻/⁻ mice undergoing ischemia-reperfusion showed widespread areas of hemorrhagic necrosis and parenchymal cell destruction, with little evidence of neutrophilic infiltrates (Fig. 3F).

We next examined whether the changes in liver injury and neutrophil accumulation observed in CD4⁻/⁻ mice were a result of altered production of proinflammatory cytokines. TNF-α is a primary cytokine that has been shown to drive this inflammatory response (6). Serum levels of TNF-α were elevated after ischemia-reperfusion in both wild-type and CD4⁻/⁻ mice but were not different between these groups (Fig. 4, top). The CXC chemokine MIP-2 plays a critical role in the hepatic inflammatory response to ischemia-reperfusion by facilitating neutrophil recruitment (18). Serum levels of MIP-2 were

CD4⁺ lymphocytes and have a deficit in helper T cell activity (24). To establish that these mice were indeed an appropriate model for our studies, we examined the number of liver CD4⁺ T lymphocytes in wild-type and CD4⁻/⁻ mice. After sham surgery or ischemia-reperfusion, CD4⁺ T lymphocyte numbers in CD4⁻/⁻ mice were virtually nil (Fig. 2).

To determine whether hepatic ischemia-reperfusion injury is dependent on CD4⁺ T lymphocytes, we compared the responses of wild-type and CD4⁻/⁻ mice. After hepatic ischemia and 8 h of reperfusion, CD4⁻/⁻ mice had significantly more liver injury, as measured by serum ALT levels, than wild-type mice (Fig. 3A). Interestingly, CD4⁻/⁻ mice had far less evidence of neutrophil accumulation, as determined by liver MPO content, than wild-type mice (Fig. 3B). An examination of liver histology supported both biochemical findings. Wild-type and CD4⁻/⁻ sham controls had normal liver architecture (Fig. 3, C and E, respectively). After ischemia-reperfusion, wild-type mice showed focal areas of oncocytic necrosis and marked accumulation of neutrophils (Fig. 3D). In contrast, liver sections from CD4⁻/⁻ mice undergoing ischemia-reperfusion showed widespread areas of hemorrhagic necrosis and parenchymal cell destruction, with little evidence of neutrophilic infiltrates (Fig. 3F).

We next examined whether the changes in liver injury and neutrophil accumulation observed in CD4⁻/⁻ mice were a result of altered production of proinflammatory cytokines. TNF-α is a primary cytokine that has been shown to drive this inflammatory response (6). Serum levels of TNF-α were elevated after ischemia-reperfusion in both wild-type and CD4⁻/⁻ mice but were not different between these groups (Fig. 4, top). The CXC chemokine MIP-2 plays a critical role in the hepatic inflammatory response to ischemia-reperfusion by facilitating neutrophil recruitment (18). Serum levels of MIP-2 were
production of MIP-2 (Figs. 3 and 4), we next assessed the potential mechanism of this CD4⁺ lymphocyte-dependent effect. IL-17 is a cytokine released almost exclusively by activated CD4⁺ lymphocytes and has immunomodulatory effects (31). To determine if IL-17 was involved in the regulation of neutrophil recruitment and injury by CD4⁺ lymphocytes, we conducted a set of neutralization studies. A dose response of neutralizing antibodies to murine IL-17 was performed examining MIP-2 expression, neutrophil recruitment, and liver injury. Administration of anti-IL-17 resulted in a dose-dependent decrease in ischemia-reperfusion-induced MIP-2 (Fig. 6, top). Similarly, the extent of neutrophil recruitment to the liver, measured by tissue MPO content, was also significantly reduced by anti-IL-17 in a dose-dependent fashion (Fig. 6, middle). However, despite reductions in neutrophil recruitment, neutralization of IL-17 had no measurable effect on the degree of liver injury after ischemia-reperfusion (Fig. 6, bottom). These data suggest that the regulation of neutrophil recruitment by CD4⁺ lymphocytes is mediated by IL-17, which appears to modulate the production of MIP-2.

Adoptive transfer of CD4⁺ lymphocytes in CD4⁻/⁻ mice restores the wild-type phenotype. To demonstrate that CD4⁺ lymphocytes were indeed responsible for the alterations in chemokine (MIP-2) expression, neutrophil recruitment, and liver injury, we conducted adoptive transfer experiments. CD4⁺ lymphocytes were isolated from spleens of wild-type mice, and 1×10⁷ cells were injected in CD4⁻/⁻ mice 18 h before sham surgery or hepatic ischemia-reperfusion. There was no effect of the cells in sham-operated mice; MIP-2, MPO, and ALT values were identical to vehicle-treated mice (Table 2). In vehicle-treated mice undergoing ischemia-reperfusion, the expected increases in MIP-2 expression, neutrophil recruitment, and liver injury were observed (Table 2). However, with adoptive transfer of CD4⁺ lymphocytes, MIP-2 expression and neutrophil recruitment were significantly increased, and liver injury was significantly reduced (Table 2).

Liver histology was consistent with the observed changes in MIP-2, MPO, and ALT values. Adoptive transfer of CD4⁺ lymphocytes regulates hepatic neutrophil recruitment. Because our data suggest that CD4⁺ lymphocytes regulate neutrophil recruitment in a manner paralleling the greatly elevated in wild-type mice after ischemia-reperfusion (Fig. 4, bottom). However, in CD4⁻/⁻ mice undergoing ischemia-reperfusion, MIP-2 levels were reduced by >50% compared with wild-type mice (Fig. 4, bottom). These latter data are consistent with the reduction in neutrophil recruitment observed in CD4⁻/⁻ mice (Fig. 3).

Increased activation of liver-recruited neutrophils from CD4⁻/⁻ mice. One puzzling aspect of our data is the finding that there is increased liver injury after ischemia-reperfusion, yet reduced neutrophil accumulation. To begin to assess the possible causes for the increased injury, we analyzed circulating and liver-recruited neutrophils for their oxidative burst activity. Neutrophils isolated from blood and liver tissue were stained with dihydrorhodamine and then analyzed by flow cytometry. As shown in Fig. 5, there were no differences in oxidative burst activity in unstimulated or fMLP-stimulated neutrophils isolated from the blood of wild-type and CD4⁻/⁻ mice. However, in liver-recruited neutrophils, there were significant increases in the oxidative burst in unstimulated and fMLP-stimulated neutrophils from CD4⁻/⁻ mice compared with wild-type mice (Fig. 5). These data suggest that the presence of CD4⁺ T cells in the liver may suppress activation of recruited neutrophils.

IL-17 from CD4⁺ lymphocytes regulates hepatic neutrophil recruitment. Because our data suggest that CD4⁺ lymphocytes regulate neutrophil recruitment in a manner paralleling the
cells in sham-operated CD4^-/- mice resulted in no alteration of liver histology compared with sham-operated mice that received vehicle (Fig. 7, A and B). CD4^-/- mice undergoing ischemia-reperfusion and receiving vehicle had severe hemorrhagic necrosis and parenchymal cell destruction, with little evidence of neutrophilic infiltrates (Fig. 7C). Adoptive transfer of CD4^+ cells in mice undergoing ischemia-reperfusion resulted in a histological profile similar to the wild-type phenotype, with areas of somewhat normal architecture and neutrophilic infiltrates (Fig. 7D). To exclude the possibility that contaminating B cells contributed to the protective phenotype, we examined the response to ischemia-reperfusion in B cell-deficient mice. We found no significant difference between wild-type and B cell-deficient mice in the extent of liver injury.

Fig. 6. Effects of anti-interleukin (IL)-17 on liver neutrophil accumulation, chemokine production, and liver injury after ischemia and 8 h of reperfusion. Wild-type mice were treated with a dose range of anti-IL-17. Liver neutrophil accumulation was determined by liver content of MPO (top), serum levels of MIP-2 were determined by ELISA (middle), and liver injury was determined by serum levels of ALT (bottom). Data are expressed as means ± SE, with n = 4 mice/group. *P < 0.05 compared with wild-type mice.
or neutrophil accumulation (data not shown). Combined with the biochemical data presented in Table 2, these data demonstrate that adoptive transfer of \( \text{CD}4^+ \) lymphocytes to \( \text{CD}4^{-/-} \) mice recapitulates the wild-type phenotype of hepatic ischemia-reperfusion.

### DISCUSSION

The studies presented herein suggest that \( \text{CD}4^+ \) T cells have divergent effects that both positively and negatively regulate the hepatic inflammatory response to ischemia-reperfusion. We found that \( \text{CD}4^+ \) lymphocytes are recruited rapidly after hepatic reperfusion. These findings were consistent with the report by Zwacka et al. (33), which showed that \( \text{CD}4^+ \) lymphocytes were recruited within 1 h of reperfusion. However, contrary to their findings, we found that these recruited cells remained sequestered in the liver for at least 4 h after reperfusion. There have been no previous attempts to phenotype the lymphocytes that are recruited in the liver after ischemia-reperfusion. We show here that the \( \text{CD}4^+ \) cells infiltrating the liver are comprised of conventional (\( \alpha\beta\text{TCR-expressing} \)), unconventional (\( \gamma\delta\text{TCR-expressing} \)), and NK T cells. Studies are ongoing to determine if the incoming conventional T cells are naïve or non-naïve. However, it seems unlikely that these cells are naïve, because it has not been shown that naïve \( \text{CD}4^+ \) T cells can infiltrate the liver because of a lack of expression of the requisite adhesion molecules (14). Non-naïve conventional T cells have different surface expression of adhesion molecules and chemokine receptors, which may make them more likely to infiltrate the liver (14). These cells can respond to a large variety of antigens in the context of a major histocompatibility complex because of a large repertoire of TCRs. Possible antigens could be liver cell proteins that have been altered because of the ischemic insult and are no longer recognized as self. Others have recently demonstrated the existence of a natural antigen in models of intestinal and skeletal muscle ischemia-reperfusion that contributes to the induction of the inflammatory response (2, 32). It is unclear if a similar antigen(s) is present in the liver after ischemia-reperfusion or if it will interact with the infiltrating \( \text{CD}4^+ \) T cells. It is also not understood if the process by which \( \text{CD}4^+ \) lymphocytes are recruited and activated in the liver is driven by antigen presentation or by paracrine signaling. It has been reported that non-naïve and unconventional T cells can functionally respond to inflammatory cytokines in a short time period (15, 20, 27). Furthermore, it has been shown that, during hepatic ischemia-reperfusion, inflammatory cytokines such as IL-12 and IL-18 are elevated (19, 28). Studies are underway in our laboratory to determine whether the \( \text{CD}4^+ \) lymphocyte actions are driven by antigen or the inflammatory milieu.

Our data demonstrate that \( \text{CD}4^+ \) lymphocytes are recruited long before any appreciable neutrophil accumulation. Furthermore, we observed reduced liver recruitment of neutrophils in \( \text{CD}4^{-/-} \) mice. To explore the potential mechanism of this effect, we focused on the cytokine IL-17. Our data provide strong evidence that \( \text{CD}4^+ \) lymphocytes contribute to the liver recruitment of neutrophils by producing the cytokine IL-17. When animals were treated with neutralizing antibodies to IL-17, liver neutrophil accumulation was reduced. IL-17 is preferentially expressed and secreted by activated \( \text{CD}4^+ \) lymphocytes (31). In a model of peritoneal inflammation, IL-17 was found to mediate neutrophil recruitment by increasing the production of the chemokines MIP-2 and keratinocyte-derived cytokine by the peritoneal mesothelium (30). IL-17 has also been shown to induce CXC chemokine production by other cell types, including epithelial cells, fibroblasts, osteoblasts, and endothelial cells (13, 21, 25). We found that production of the CXC chemokine MIP-2 was decreased in \( \text{CD}4^{-/-} \) mice and in wild-type mice treated with anti-IL-17 antibodies. Moreover, adoptive transfer of \( \text{CD}4^+ \) lymphocytes in \( \text{CD}4^{-/-} \) mice recapitulated the wild-type phenotype. In these experiments, mice receiving \( \text{CD}4^+ \) lymphocytes had dramatic increases in the expression of MIP-2 and the degree of liver neutrophil recruitment. Thus it would appear that \( \text{CD}4^+ \) lymphocytes are an important regulator of hepatic neutrophil recruitment after ischemia-reperfusion and that this occurs via their release of IL-17.

Although not addressed by these studies, the primary stimulus for IL-17 production has been linked to IL-23. Stimulation

### Table 2. Effects of adoptive transfer of \( \text{CD}4^+ \) lymphocytes on hepatic I/R injury in \( \text{CD}4^{-/-} \) mice

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT, IU/l</th>
<th>MPO, U/g</th>
<th>MIP-2, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + vehicle</td>
<td>18.0±1.0</td>
<td>11.3±7.9</td>
<td>2.5±0.1</td>
</tr>
<tr>
<td>Sham + ( \text{CD}4^+ ) cells</td>
<td>21.5±2.5</td>
<td>12.9±1.2</td>
<td>9.4±3.3</td>
</tr>
<tr>
<td>I/R + vehicle</td>
<td>9.296±143</td>
<td>178.1±17.4</td>
<td>149.5±19.0</td>
</tr>
<tr>
<td>I/R + ( \text{CD}4^+ ) cells</td>
<td>8.791±167*</td>
<td>325.6±47.1*</td>
<td>211.7±20.4*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE with \( n = 4–7 \) mice/group. ALT, alanine aminotransferase; MPO, myeloperoxidase; MIP-2, macrophage inflammatory protein-2. * \( P < 0.05 \) compared with I/R + vehicle group.
of CD4+ lymphocytes with IL-23 results in their activation and secretion of IL-17 (1). IL-23 is a heterodimer that shares one component, the p40 subunit, with IL-12 (22). The other component is the p19 subunit, which is specific for IL-23. However, stimulation of CD4+ lymphocytes with IL-12 does not result in production of IL-17 (1). We have previously shown that IL-12 is expressed during the ischemic insult and is required for the subsequent production of TNF-α and the ensuing inflammatory response (19). These studies were performed before the discovery of IL-23 and used anti-p40 antibodies and p40-knockout mice. Thus it is possible that the actions ascribed to IL-12 may have actually been a result of IL-23 expression and function. We are currently investigating this possibility.

The current studies show that CD4+ lymphocytes positively regulate liver neutrophil accumulation, which could be characterized as a proinflammatory process. Somewhat contradictory to these findings were our observations that, in CD4−/− mice, which had far less liver neutrophil recruitment, liver injury was greatly augmented. There is clear evidence that the majority of the liver injury occurring as a result of ischemia-reperfusion is the result of neutrophil-dependent killing of hepatocytes (10, 11). Because we show that the recruitment of CD4+ lymphocytes occurs before neutrophil recruitment and that these CD4+ lymphocytes are present within the liver at the time of active neutrophil recruitment, it is possible that they may directly alter the function of neutrophils entering the liver. Our data support this concept because neutrophils from CD4−/− mice had a higher oxidative burst activity. However, the manner in which CD4+ lymphocytes may directly or indirectly affect the activation state of neutrophils remains to be elucidated.

In conclusion, the present study demonstrates that CD4+ lymphocytes are recruited to the liver rapidly after ischemia-reperfusion and positively regulate the subsequent inflammatory response by facilitating neutrophil recruitment. This latter effect appears to occur through their production of IL-17, which modulates the expression of the neutrophil chemokine MIP-2. At the same time, liver-recruited CD4+ lymphocytes appear to suppress the respiratory burst activity of recruited neutrophils. Further study of the role of CD4+ lymphocytes in the acute hepatic inflammatory response to ischemia-reperfusion may yield clues to the interplay of innate and adaptive immune effector cells in the development of liver injury.

GRANTS

This work was supported by National Institutes of Health Grants DK-56029, AG-025881, and HL-72552.

REFERENCES


