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Recent evidence suggests that IL-17A regulates neutrophil-dependent organ injury. Accordingly, the purpose of this study was to determine the role of IL-17A in neutrophil recruitment after ischemia–reperfusion (I/R) and in subsequent liver injury. Two mouse models including wild-type and IL-17A knockout mice were evaluated for I/R injury. The medial largest lobe of the liver was clamped for 90 min. In another set of experiments, recombinant mouse (rm)IL-17A homodimer or rmIL-17A/F heterodimer were administered to knockout mice before I/R, and liver injury was investigated. Isolated Kupffer cells were incubated with rmIL-17A or rmIL-17F, and production of TNF-α was measured. Studies evaluating the extent of liver injury as measured by serum transaminase levels demonstrated similar levels in the acute phase (6 h) in these two models. In contrast, in the subacute phase (20 h) after I/R, both serum transaminase levels and percent of hepatic necrosis were significantly reduced in the knockout mice compared with the wild-type mice. This reduction in liver injury seen in the knockout mice was associated with suppression of chemokine and adhesion molecule expression and reduction in infiltration of neutrophils into the liver. Administration of rmIL-17A homodimer, but not IL-17A/F heterodimer, increased liver injury in the subacute phase of I/R in KO mice. TNF-α production by isolated Kupffer cells increased significantly in the cells incubated with rmIL-17A compared with rmIL-17F. These results indicate that IL-17A is a key regulator in initiating neutrophil-induced inflammatory responses and hepatic injury in the subacute phase after reperfusion. The Journal of Immunology, 2011, 187: 4818–4825.

Hepatic ischemia–reperfusion (I/R) injury is a complication of trauma, transplantation, and resectional surgery, which leads to liver dysfunction and failure, resulting in multiple organ failure (1–3). The liver injury induced by I/R has two distinct phases. The acute phase of this response is characterized by activation of the 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 NF-κB and activator protein-1, which control the expression of proinflammatory mediators, such as IL-12 and TNF-α (4–6). The expression of these mediators leads to the subacute phase of liver injury characterized by the induction of secondary mediators, including neutrophil-attracting CXC chemokines and endothelial adhesion molecules, which facilitate the adhesion and transmigration of neutrophils from the vascular space into the hepatic parenchyma (7–9). Accumulated neutrophils release oxidants and proteases that directly injure hepatocytes and vascular endothelial cells and may also obstruct hepatic sinusoids, resulting in hepatic hypoperfusion (10).

Murine IL-17A is a 21-kDa glycoprotein with 147 aa and 63% aa homology with human IL-17A (155 aa) (11). Recently, five additional related cytokines were identified (IL-17B, IL-17C, IL-17D, IL-17E also called IL-25, and IL-17F) with 16–50% aa identity with IL-17A. IL-17A induces intercellular cell adhesion IL-1 and TNF-α in macrophages. Overexpression of IL-17A and IL-17F in the lungs leads to increased proinflammatory cytokine and chemokine expression, causing inflammation associated with neutrophil infiltration (12–14). These observations suggest that these cytokines have similar biological functions. Furthermore, IL-17A and IL-17F can be secreted as both homodimers and heterodimers (15, 16). Recent studies showed that the IL-17A/F heterodimer is more potent than IL-17F homodimer but less potent than IL-17A homodimer in inducing chemokine expression (15, 16). Although the mechanisms for neutrophil recruitment into the liver after I/R involve cytokines, chemokines, and adhesion molecules, a previous study reported evidence that neutrophil accumulation in liver tissue is also dependent on CD4+ T lymphocytes (17). That study showed that depletion of CD4+ T lymphocytes with Ab diminishes neutrophil recruitment and injury after hepatic I/R. Furthermore, in studies with CD4 knockout mice, hepatic I/R injury also diminished neutrophil recruitment and injury after hepatic I/R (18). Moreover, neutralization of IL-17, a cytokine released by activated CD4+ T lymphocytes, significantly reduces neutrophil recruitment in association with suppression of CXCL-2 expression (18). These results support the hypothesis that IL-17A will be involved in the mechanism of hepatic I/R injury. The specific purpose of the current study was to investigate the role of IL-17A in I/R injury in the liver using IL-17A knockout mice.

Materials and Methods

Partial hepatic warm I/R injury model

Male wild-type mice (C57BL/6, 10–12 wk of age; obtained from The Jackson Laboratory, Bar Harbor, ME) and IL-17A knockout mice (backcrossed onto C57BL/6) were housed in a clean, temperature-controlled environment with a 12-h light–dark cycle and were given free access to...
regular laboratory chow diet and water for several days. IL-17A knockout mice were generated, as previously described, using E14.1 ES cells (19). All animals received humane care, and the study protocols were approved by the Committee of Laboratory Animals at University of Yamanashi according to institutional guidelines.

Partial hepatic ischemia (70%) was induced as described previously (20). Briefly, mice were anesthetized with pentobarbital sodium (60 mg/kg, injected i.p.). A midline laparotomy was performed, and an atrumatic clip was placed to interrupt blood supply to the left lateral and median lobes of the liver. After 90 min of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham control mice underwent the same protocol without vascular occlusion.

To investigate role of the spleen in I/R, splenectomy was performed just before I/R, and serum and liver tissues were collected at designated times after reperfusion for further analysis.

**Treatment with recombinant mouse IL-17A homodimer or IL-17A/F heterodimer in IL-17A knockout mice**

Recombinant mouse (rm)IL-17A homodimer (R&D Systems, Minneapolis, MN) or rmIL-17A/F heterodimer (R&D Systems) were diluted in sterile endotoxin-free PBS and administered i.p. at a dose of 0.5 μg/mouse in a total volume of 100 μl 6 h before I/R (n = 6). The concentration of rmIL-17A homodimer or rmIL-17A/F heterodimer to be applied was determined in preliminary dose-response experiments, which showed that 0.5 μg IL-17A consistently produced a significant influx of neutrophils (21).

**Collection of samples and measurements**

Blood samples were collected via the aorta at designated times after I/R or sham operation, centrifuged, and stored at −80°C until assay. Blood samples were also collected at designated times after I/R or sham operation and were stored at −80°C for further analysis. Samples were fixed in formalin, embedded in paraffin, and serially sectioned. Some sections were stained with H&E to assess inflammation and necrosis.

**Pathological evaluation**

The extent of liver damage as indicated by hepatocellular necrosis was confirmed by blinded morphometric assessment in paraffin sections (4 μm) using a Leica Digital Module R image analysis system to determine the percent necrotic area in liver sections. In these studies, the area of necrosis was traced by computer-assisted morphometry and the percentage of necrosis calculated from the total micrometer square area of the tissue section. In total, four sections were analyzed for each ischemic lobe tissue sample (n = 6 animals for each experimental point). Nonischemic lobes, which demonstrated no significant necrosis except for infrequent focal areas at the surface of the liver because of surgical manipulation, were not included in this analysis.

**Measurements of serum aspartate aminotransferase and ALT**

Blood was obtained via the aorta at the designated time points for further analysis of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as an index of hepatocellular injury (n = 6). Serum AST and ALT levels were measured to assess hepatic parenchymal damage using Fuji DRI-CHEM analyzers (Fujifilm, Tokyo, Japan).

**Measurement of serum cytokines**

Blood samples were collected via the inferior vena cava at designated time points after I/R (n = 6 for each time point). The samples were centrifuged at 1200 × g for 10 min at 4°C, and serum was stored at −80°C until the assays. Serum TNF-α and IL-17A levels were measured using ELISA kits (R&D Systems, Minneapolis, MN).

**Immunohistochemical staining for neutrophils or IL-17A**

Immunohistochemical staining for neutrophils was performed on frozen sections fixed in 4% paraformaldehyde for 10 min, followed by postfixation in methanol for 10 min at −20°C. Sections were permeabilized with 0.1% Triton X-100 before blocking and incubation with a 1/500 dilution of rat anti-mouse neutrophil Ab (Caltag Laboratories, South San Francisco, CA). Morphometric analysis of immunoreactive neutrophils in liver sections was performed by quantifying the total numbers of immunoreactive cells in five random (×400) fields (six independent animals were analyzed for each experimental group). The number of cells per 0.25 mm² of necrotic area in the liver was assessed.

For immunohistochemical staining of IL-17A in the liver and spleen, formalin-fixed, paraffin-embedded tissue specimens were cut into 4-μm sections. Each section was mounted on a silane-coated glass slide, deparaffinized, and soaked for 15 min at room temperature in 0.3% H₂O₂/methanol to block endogenous peroxidase. Anti–IL-17A mAbs (Santa Cruz Biotechnology Santa Cruz, CA) were applied for 12 h at 4°C. Peroxidase-linked secondary Abs and diaminobenzidine (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) were used to detect specific binding. Peroxidase-linked secondary Abs and diaminobenzidine (Vectastain Elite ABC Kit; Vector Laboratories) were used to detect specific binding. Sections were counterstained with hematoxylin as described elsewhere (22). Values were expressed as the number of positive cells per 400 hepatocytes in the liver (n = 6 in each group).

**Measurement of tissue CXCL-2 and ICAM-1 levels by ELISA**

Liver tissues were homogenized in cold PBS using a Polytron-type homogenizer. Tissue homogenate was then centrifuged at 20,000 rpm for 20 min at 4°C to obtain the supernatant. Total protein concentrations of the tissue supernatant and whole-cell lysate were measured using a Bio-Rad protein assay kit (Bio-Rad) for calibration, and protein concentrations of CXCL-2 and ICAM-1 in the tissue homogenate and culture media were determined using ELISA kits (CXCL-2, Immuno-Biological Laboratories, Gunma, Japan; and ICAM-1, Abcam, Cambridge, U.K.), according to the manufacturer’s instructions.

**Measurement of myeloperoxidase activity**

Tissue myeloperoxidase (MPO) activity was determined by a standard enzymatic procedure as previously described by Krawisz et al. (23) with slight modifications. Briefly, each tissue specimen (−100 mg) was homogenized in buffer (0.5% hexadecyltrimethylammonium bromide in 50 mmol/l potassium phosphate buffer [pH 6]) for 90 s on ice. Then, tissue homogenate was sonicated for 10 s and underwent three cycles of freeze-thaw (−70°C/37°C). Samples were centrifuged at 20,000 rpm for 20 min at 4°C, and the supernatant was collected. Samples (100 μl) were added to 2.9 ml 50 mmol/l phosphate buffer (pH 6) containing 0.167 mg/ml O-dianisidine hydrochloride and 0.0005% hydrogen peroxide, and the kinetics of absorbance at 460 nm was measured using a spectrophotometer at 25°C. Protein concentration of the supernatant was determined using a Bradford assay kit (Bio-Rad, Hercules, CA) for calibration, and values were standardized using MPO purified from human leukocytes (Sigma-Aldrich, St. Louis, MO).

**Isolation of CD4⁺ lymphocytes in the liver and spleen**

Six hours after I/R, liver tissues were flushed through the portal vein with saline. Then, liver and spleen tissues were pressed through a 200-gauge stainless steel wire mesh and collected in media (18). The dispersed tissue suspension was transferred to a 50-ml conical centrifuge tube and centrifuged at 300 × g for 10 min. The pellet was resuspended in media.
Five to twenty million Kupffer cells were obtained from each mouse. B (370 mg/l KCl, 210 mg/l MgCl$_2$-6 H$_2$O, 70 mg/l MgSO$_4$-7 H$_2$O, 150 mg/l NaHCO$_3$, 190 mg/l EDTA, 900 mg/l glucose, and 6 mg/l phenol red [pH 7.4] at 37˚C) containing 0.06% collagenase type IV (Sigma-Aldrich) for an additional 15 min. After perfusion, the liver was removed, cut into small pieces, and homogenized. After passing through a gauze filter (mesh size 60 m), cells were washed twice with warm Gey’s balanced salt solution B, and resuspended at 400 × g for 30 min at room temperature. Cell viability was >95% in all cases, as determined by trypan blue exclusion.

Measurement of intracellular cytokine staining for IL-17A in CD4$^+$ lymphocytes

Detection of IL-17–positive CD4$^+$ lymphocytes was determined by intracellular cytokine staining (24). Before fixation and permeabilization, cells were stained with allophycocyanin-conjugated anti–CD4 Abs (eBioscience, San Diego, CA) to detect cell surface Ags. Then, cells were fixed and permeabilized with the Fixation/Permeabilization buffer (eBioscience) for 30 min at 4˚C before intracellular staining. Then, cells were stained with allophycocyanin-conjugated anti–IL-17A Abs (eBioscience). Flow cytometric analysis was performed with a Cytomics FC500 (Beckman Coulter, Fullerton, CA).

Isolation of the Kupffer cells and assessment of TNF-α production

Kupffer cells were isolated as detailed elsewhere with minor modifications (25). Briefly, mice were anesthetized with sodium pentobarbital (80 mg/kg body weight, i.p.), the abdomen was opened, and the portal vein was cannulated. The liver was perfused in situ for 5 min with Ca$^{2+}$/Mg$^{2+}$-free liver perfusion medium (LPM)-1 (8000 mg/l NaCl, 400 mg/l KCl, 88.17 mg/l NaH$_2$PO$_4$·2 H$_2$O, 120.45 mg/l Na$_2$HPO$_4$, 2380 mg/l HEPES, 350 mg/l NaHCO$_3$, 190 mg/l EDTA, 900 mg/l glucose, and 6 mg/l phenol red [pH 7.4]) at 37˚C) and was then perfused with complete LPM-2: same as LPM-1 except without EDTA and glucose but with 560 mg/l CaCl$_2$-2 H$_2$O [pH 7.4] at 37˚C) containing 0.06% collagenase type IV (Sigma-Aldrich) for an additional 15 min. After perfusion, the liver was removed, cut into small pieces, and homogenized. After passing through a gauze filter (mesh size ~60 μm), cells were washed twice with warm Gey’s balanced salt solution B (370 mg/l KCl, 210 mg/l MgCl$_2$·6 H$_2$O, 70 mg/l MgSO$_4$·7 H$_2$O, 150 mg/l NaH$_2$PO$_4$·2 H$_2$O, 30 mg/l KH$_2$PO$_4$, 1090 mg/l glucose, 227 mg/l NaHCO$_3$, 225 mg/l CaCl$_2$·2 H$_2$O, 6 mg/l phenol red, 8000 mg/l NaCl, 100 U/l streptomycin, and 10$^5$ IU/l penicillin G [pH 7.4]) and centrifuged over 16% (w/v) Nycodenz (Axis Shields, Oslo, Norway) gradient for 20 min at 1900 × g at 4˚C. Kupffer cells were collected from under the interface, washed with Gey’s balanced salt solution B, and resuspended at a concentration of 1 × 10$^6$ cells/ml in DMEM (Invitrogen, Carlsbad, CA). Isolated Kupffer cells were seeded (1 × 10$^6$ cells/well) in 24-well dishes and cultured in DMEM for 12 h. After changing the media to remove the nonadherent cells, cultures were maintained for an additional 24 h, and the media were collected and stored at −80˚C. Viability of the cells was confirmed by trypan blue exclusion and was >95% in all experiments. Five to twenty million Kupffer cells were obtained from each mouse.

TNF-α production from Kupffer cells into the medium was assayed using ELISA kits, according to the manufacturer’s protocol (BioSource International, Camarillo, CA). In all experiments, Kupffer cells were isolated from four different mice in each group.

Statistical analysis

Data were expressed as mean ± SEM. Statistical differences between mean values were analyzed by ANOVA with Bonferroni’s post hoc test. $p < 0.05$ was considered significant.
FIGURE 4. Tissue CXCL-2 and ICAM-1 expression in the liver after I/R. Expression of CXCL-2 (A) and ICAM-1 (B) in the liver tissue 6 and 20 h after I/R was determined as described in Materials and Methods. Data represent mean ± SEM (five groups, n = 6 independent animals for each group). *p < 0.05 compared with wild-type mice received sham operation; **p < 0.05 compared with wild-type mice that underwent I/R; #p < 0.05 compared with knockout mice that underwent I/R by ANOVA with Bonferroni’s post hoc test. KO, IL-17A knockout mice; sham, sham operation; WT, wild-type mice.

Results

Pathological findings and evaluation of liver injury after I/R

In the sham groups, no pathological changes were observed (Fig. 1A–C). In contrast, hepatocellular necrosis was observed 6 h after I/R in the wild-type and the knockout mice (Fig. 1D–F). There were no significant differences between the two groups. In the wild-type mice, pathological changes were more severe 20 h after I/R than 6 h after I/R (Fig. 1G). However, these pathological changes were not observed in the knockout mice (Fig. 1H). In the knockout mice administered rmIL-17, liver injury was similar to that seen in the wild-type mice at 6 h and 20 h after I/R (Fig. 1I, 1J).

To evaluate hepatic injury after I/R, the mean percent of hepatocellular necrosis was assessed (Fig. 2A). No hepatocellular necrosis was observed in the sham groups. In contrast, hepatocellular necrosis was observed in both the wild-type (26%) and the knockout mice (22%) 6 h after I/R. Twenty hours after I/R, the necrotic area increased to 58% in the wild-type mice; however, the mean percent of the necrotic area did not change in the knockout mice. There were significant differences between the two groups. In the knockout mice administrated rmIL-17, the mean percent area of hepatocellular necrosis was similar to that in the wild-type mice at both 6 and 20 h after I/R.

Serum AST and ALT levels in the wild-type mice that underwent 90 min of ischemia demonstrated a biphasic pattern of injury that peaked at 6 and 20 h, representing early acute and subacute damage, respectively (Fig. 2B, 2C). In comparison, the knockout mice after 90 min of ischemia demonstrated a complete lack of subacute-mediated liver injury (20 h after reperfusion), suggesting that the inflammatory response after I/R injury in IL-17A–deficient mice was impaired. In the knockout mice administrated rmIL-17A homodimer, serum AST and ALT demonstrated a biphasic pattern of injury that peaked at 6 and 20 h after I/R. These effects were not observed in animals treated with rmIL-17A/F (data not shown).

Serum cytokine levels after I/R

In the sham group, serum TNF-α levels were minimal in both groups (data not shown). Serum TNF-α concentrations in the wild-type mice that underwent 90 min of ischemia demonstrated a biphasic pattern, which peaked at 3 and 20 h after reperfusion (Fig. 3A). In comparison, the knockout mice that underwent 90 min of ischemia demonstrated a biphasic pattern with a complete lack of subacute-mediated liver injury (20 h after reperfusion). Serum IL-17A was not detected, as expected, in the knockout mice that underwent sham operation or 90 min of ischemia. In the wild-type mice, in the sham groups, serum IL-17A levels were minimal (data not shown). Serum IL-17A concentrations in the wild-type mice that underwent 90 min of ischemia demonstrated an early peak at 6 h after reperfusion and then gradually increased up to 36 h after reperfusion (Fig. 3B). Importantly, serum IL-17A levels were significantly blunted in splenectomized animals 20 h after I/R (Fig. 3C).

Tissue CXCL-2 and ICAM-1 concentrations in the liver

In the sham group, tissue CXCL-2 concentrations were minimal in both the wild-type mice and the knockout mice (Fig. 4A). In contrast, CXCL-2 concentrations increased significantly in the wild-type mice 6 h after I/R. This increase was blunted in the knockout mice. There were significant differences between the two groups. In the knockout mice administrated rmIL-17A homodimer, tissue CXCL-2 concentration increased to levels similar to those observed in the wild-type mice. These effects were not
observed in animals treated with rmIL-17A/F heterodimer (data not shown).

In the sham group, tissue ICAM-1 concentrations were minimal in both the wild-type mice and the knockout mice (Fig. 4B). In contrast, ICAM-1 concentrations increased significantly in the wild-type mice 6 h after I/R. This increase was blunted in the knockout mice. There were significant differences between the two groups. In the knockout mice administrated rmIL-17, tissue ICAM-1 concentration increased to levels similar to those observed in the wild-type mice. These effects were not observed in animals treated with rmIL-17A/F heterodimer (data not shown).

Investigation of population and distribution of neutrophils in the liver

To evaluate the population and distribution of neutrophils in the liver, immunohistochemical staining was performed (Fig. 5). Six hours after I/R, the wild-type mice showed marked accumulation of neutrophils (Fig. 5A). In contrast, accumulation of neutrophils was minimal in the knockout mice (Fig. 5B). Furthermore, 20 h after I/R, accumulation of neutrophils increased significantly in the wild-type mice (Fig. 5D). At the same time point, the knockout mice showed little evidence of infiltration of neutrophils into the liver (Fig. 5E). Furthermore, in the knockout mice administered rmIL-17A homodimer, accumulation of neutrophils was similar to that in the wild-type mice at 20 h after I/R (Fig. 5F). These effects were not observed in animals treated with rmIL-17A/F heterodimer (data not shown).

To evaluate the number of infiltrating neutrophils was assessed. In the sham groups, the mean number of neutrophils was minimal (Fig. 6A). In contrast, the mean number increased to ∼120/0.25 mm² necrotic area at 6 h after I/R in the wild-type mice. In the knockout mice, the number was ∼70/0.25 mm² necrotic area at 6 h after I/R. Furthermore, at 20 h after I/R, the mean number of neutrophils increased to 19 in the wild-type mice. In contrast, the mean number decreased significantly to 7 in the knockout mice. In the knockout mice administered rmIL-17A homodimer, accumulation of neutrophils was similar to that in the wild-type mice at both 6 and 20 h after I/R. These effects were not observed in animals

Table I. Analysis of CD4⁺ lymphocytes and IL-17A–positive CD4⁺ lymphocytes in the liver and spleen

<table>
<thead>
<tr>
<th>Organs</th>
<th>CD4⁺ Lymphocytes and IL-17A–Positive CD4⁺ Lymphocytes</th>
<th>Sham</th>
<th>6 h after I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>No. of CD4⁺ lymphocytes</td>
<td>2.8 ± 0.2 × 10⁶</td>
<td>5.2 ± 0.2 × 10⁷*</td>
</tr>
<tr>
<td></td>
<td>Ratio of IL-17A–positive CD4⁺ lymphocytes/CD4⁺ lymphocytes (%)</td>
<td>0.22 ± 0.03</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>Spleen</td>
<td>No. of CD4⁺ lymphocytes</td>
<td>3.8 ± 0.5 × 10⁷</td>
<td>4.2 ± 0.2 × 10⁷*</td>
</tr>
<tr>
<td></td>
<td>Ratio of IL-17A–positive CD4⁺ lymphocytes/CD4⁺ lymphocytes (%)</td>
<td>0.24 ± 0.05</td>
<td>2.11 ± 0.24**</td>
</tr>
</tbody>
</table>

CD4⁺ lymphocytes and IL-17A–positive CD4⁺ lymphocytes were determined as described in Materials and Methods. Data represent mean ± SEM (n = 4). I/R, ischemia–reperfusion. Sham, sham operation. *p < 0.05 compared with the number of hepatic CD4⁺ lymphocytes isolated from sham group; **p < 0.05 compared with ratio of IL-17A–positive CD4⁺ lymphocytes/CD4⁺ lymphocytes ratio in sham group; #p < 0.05 compared with ratio of IL-17A–positive CD4⁺ lymphocytes/CD4⁺ lymphocytes in the liver by two-way ANOVA using Bonferroni’s post hoc test.
that underwent sham operation (Table I). The number of CD4+ lymphocytes increased significantly in the liver 6 h after I/R. There were no significant differences in this ratio between the liver and the spleen.

**Population of CD4+ lymphocytes in the liver and spleen after I/R and analysis of IL-17A–producing cells by immunohistochemistry and intracellular cytokine staining**

In the wild-type mice, the number of CD4+ lymphocytes was 2.8 ± 0.2 × 10^7 in the liver and 3.8 ± 0.5 × 10^7 in the spleen in animals that underwent sham operation (Table I). The number of CD4+ lymphocytes increased significantly in the liver 6 h after I/R, consistent with the previous reports (18). In contrast, the number of CD4+ lymphocytes did change in the spleen after I/R.

In the wild-type mice, IL-17A–positive cells were not detected in the liver 6 h after I/R by immunohistochemistry (data not shown). In contrast, IL-17A–positive cells were detected in the white pulp of the spleen 6 h after I/R (Fig. 7A). The number of IL-17A–positive cells increased markedly 20 h after I/R (Fig. 7C). As expected, IL-17A–positive cells were not detected in the spleen from the knockout mice at either time point (Fig. 7B, 7D).

In addition to immunohistochemistry, intracellular cytokine staining for IL-17A was performed by flow cytometrical analysis to detect IL-17A–producing cells in the liver and spleen. As a result, a percentage of IL-17A–positive cells to CD4+ lymphocytes was 0.29 ± 0.05% 6 h after I/R in the liver (Fig. 8). In contrast, it was 2.11 ± 0.24% in the spleen 6 h after I/R. There were significant differences in this ratio between the liver and the spleen.

**Production of TNF-α by isolated Kupffer cells**

Production of TNF-α was minimal in Kupffer cells incubated with saline (vehicle [VEH] + VEH) (Fig. 9). In contrast, the production increased significantly in cells incubated with LPS (VEH + LPS). The production was also minimal in IL-17F–treated cells incubated with saline. In contrast, the production increased significantly in IL-17A–treated cells incubated with saline compared with in IL-17F–treated cells incubated with saline. Alternatively, the TNF-α production increased significantly in IL-17A–treated Kupffer cells incubated with LPS compared with in IL-17F–treated cells incubated with LPS. There were significant differences in the production at all treatments studied.

**FIGURE 7.** Immunohistochemical staining using anti–IL-17A Abs in the spleen after I/R. Experimental groups were compared for the extent of IL-17A–positive cells within ischemic lobes at 6 and 20 h after I/R (four groups, n = 6 independent animals for each group). A, Spleen from wild-type mice 6 h after I/R; B, spleen from knockout mice 6 h after I/R; C, spleen from wild-type mice 20 h after I/R; and D, spleen from knockout mice 20 h after I/R. Original magnification ×400. Representative photomicrographs. KO, IL-17A knockout mouse; WT, wild-type mouse.

**FIGURE 8.** Intracellular cytokine staining for IL-17A. Hepatic mononuclear cells were isolated, and IL-17A–positive CD4+ lymphocytes was determined by intracellular cytokine staining described in Materials and Methods (four groups, n = 4 in each group). Representative data are shown.

**FIGURE 9.** Production of TNF-α by isolated Kupffer cells incubated with rmIL-17A or IL-17F. Kupffer cells were isolated from wild-type mice, and production of TNF-α was determined as described in Materials and Methods (10 groups, n = 4 in each group). *p < 0.05 compared with cells incubated with a saline VEH; #p < 0.05 compared with cells incubated with 10 pg/ml rm IL-17F and a saline VEH; **p < 0.05 compared with cells incubated with 1 pg/ml rm IL-17F and LPS; ***p < 0.05 compared with cells incubated with 10 pg/ml rm IL-17F and LPS; $p < 0.05$ compared with cells incubated with 100 pg/ml rm IL-17F and LPS by ANOVA with Bonferroni’s post hoc test.
Discussion

Role of IL-17A in hepatic ischemia reperfusion injury

The mechanism of ischemic damage in the liver associated with reoxygenation is of great clinical relevance in understanding and preventing early graft failure in orthotopic liver transplants and hepatocellular damage after liver resection. In addition to free radical-induced hepatic injury in the acute phase, inflammatory responses in the subacute phase play a major role in the liver injury after I/R, which leads to a decline of liver function and potentially increased organ immunogenicity. In the current study, the analysis of genetically defined IL-17A--deficient mice provided an animal model for studying the immunology of subacute inflammatory responses after I/R in the liver. Findings that demonstrate a substantially reduced subacute inflammatory-mediated I/R injury response in the IL-17A knockout mice compared with the wild-type mice suggest that IL-17A is required for activation of subacute inflammatory responses after I/R. The pathophysiological changes including serum transaminase levels, percentage of hepatocellular necrosis (Fig. 2), and infiltration of neutrophils (Fig. 6) were all reduced in the knockout mice compared with the wild-type mice. Furthermore, the involvement of IL-17A in mediating the subacute phase of I/R injury was supported by experiments of administration of rmIL-17A to the knockout mice, which increased both serum transaminase levels and neutrophil influx to damaged tissue in the subacute phase after I/R (20 h) (Figs. 1, 2, 6). Moreover, these effects were not observed in animals treated with rmIL-17A/F heterodimer (data not shown). Thus, IL-17A homodimer plays an important role in the subacute phase of I/R.

Role of IL-17A in neutrophil recruitment after I/R

Neutrophils accumulate in the hepatic sinusoid in response to the exposure to inflammatory mediators such as TNF-α, IL-1β, CXC chemokines (e.g., IL-8, keratinocyte-derived cytokine, CXCL-2, cytokine-induced neutrophil chemoattractant), activated complement factors (e.g., C5a), platelet-activating factor, and others (10, 26, 27). Previous studies evaluated the increased adherent characteristics of neutrophils to the sinusoidal endothelial cells after I/R. Indeed, in ICAM-1--deficient mice, the important role of this adhesion molecule in the recruitment of neutrophils was demonstrated in an LPS-induced liver injury model (28). Those studies demonstrated protection against leukocytosis in the liver of ICAM-1--deficient mice treated with LPS. Furthermore, neutralization of IL-17 significantly reduced neutrophil recruitment in association with suppression of CXCL-2 expression (18). In the current study, the role of CXCL-2 and ICAM-1 in the recruitment of neutrophils was also demonstrated. Indeed, expression of CXCL-2 and ICAM-1 (Fig. 4) and recruitment of neutrophils into the liver (Figs. 5, 6) were dramatically reduced in the knockout mice. Furthermore, administration of rmIL-17A to the knockout mice increased CXCL-2 and ICAM-1 expressions (Fig. 4) and neutrophil recruitment to the liver in the subacute phase of I/R (Figs. 5, 6), suggesting that IL-17A play a key on neutrophil recruitment after I/R. TNF-α has been produced by Kupffer cells within the early phases of ischemic liver injury before reperfusion and implicated in amplification of subsequent neutrophil inflammatory responses (4, 8, 29). In the current study, serum TNF-α concentrations in both the wild-type mice and the knockout mice that underwent 90 min of ischemia demonstrated a biphasic pattern, which peaked at 3 and 20 h (Fig. 3A). In the acute phase, these levels were not different between the two groups, suggesting that IL-17A does not involve the expression of TNF-α. In contrast, in the subacute phase, serum TNF-α levels were greater in the wild-type mice than the knockout mice (Fig. 3A). The initial peak of serum IL-17A was at 6 h after I/R, which was earlier than the second peak of serum TNF-α (Fig. 3B). Furthermore, treatment with rmIL-17A but not rmIL-17F increased production of TNF-α by isolated Kupffer cells in vitro (Fig. 9). These results supported the hypothesis that IL-17A may be involved in the expression of TNF-α in the subacute phase. In contrast, serum IL-17A levels increased continuously up 36 h after I/R; however, serum TNF-α levels decreased by subacute phase after I/R. Recently, it was reported that γδT cells have been implicated as the major producer of IL-17 during sepsis (30). Furthermore, treatment of IL-7, which stabilizes the number of T cell and function, accelerates neutrophil recruitment through γδ T cell IL-17 production in a murine model of sepsis (31). On the basis of these two reports, γδ T cell could be involved in production of IL-17A in late phase after I/R. Therefore, further investigation is needed to clarify this important issue.

Role of Kupffer cells and the splenic lymphocytes in hepatic I/R injury

The question is whether the Th17 cell involvement lies proximal or distal to the activation of Kupffer cell, which has been demonstrated to play a major role in neutrophil recruitment. If the Th17 cell involvement is proximal to activation of Kupffer cells, this would imply that circulating Th17 cells within the liver are directly activated by ischemia and reperfusion (i.e., changes in the cellular or extracellular redox state) and are potentially involved in activation of Kupffer cells. Alternatively, T cells may be critical in amplifying primary Kupffer cell cytokine responses within the initial phases of I/R injury. This hypothesis is based on the fact that secretion of TNF-α by Kupffer cells has been demonstrated to occur before reperfusion. However, because Th17 cells are also resident in the liver before reperfusion, it is impossible to rule out primary inciting activation of Kupffer cells by Th17 cells during the ischemic period. In addition, T cell-mediated effects could be amplified by other ectopic sites such as the spleen. In support of this hypothesis, a recent report by Okuaki et al. (32) has demonstrated reduction of neutrophil infiltration and attenuation of I/R injury in splenectomized animals compared with controls. In the context of this present study, data suggest that Th17 cells at distant sites to the liver such as the spleen may be involved in the inflammatory process. Indeed, in the current study, IL-17A expression increased 20 h after I/R in the spleen, suggesting that the splenic lymphocyte may play a key role in the systemic expression of IL-17A in the subacute phase of I/R (Fig. 7). Indeed, the number of IL-17A--positive cells in the liver was relatively few compared with that in the spleen after I/R (Fig. 8), and serum IL-17A levels were significantly blunt in splenectomized animals (Fig. 3C). Furthermore, the number of infiltrating neutrophils into the liver was significantly blunt in splenectomized animals compared with nonsplenectomized animals 20 h after I/R. These results indicated that this action could occur through soluble mediators derived from the spleen.

In the current study, serum TNF-α concentrations in both the wild-type mice and the knockout mice that underwent 90 min of ischemia demonstrated a biphasic pattern, which peaked at 3 and 20 h (Fig. 3A). In the acute phase, these levels were not different between the two groups; however, the levels were significantly greater in the wild-type mice compared with the knockout mice in the subacute phase. The acute response is characterized by activation of the Kupffer cells and their production of reactive oxygen species, leading to hepatocellular injury. The expression of inflammatory mediators derived from the Kupffer cells leads to the subacute phase of liver injury characterized by the induction of secondary mediators, which facilitate the adhesion and trans-
migration of neutrophils from the vascular space into the hepatic parenchyma (7–9). In the current study, an initial peak of serum IL-17A concentrations was at 6 h after reperfusion in the wild-type mice that underwent 90 min of ischemia. Although serum IL-17A levels were not detected in the knockout mice, there were no significant differences in liver injury, suggesting that the Kupffer cell may play a key role in the acute response of I/R. The IL-17A–positive cells in the spleen (Figs. 7, 8) and infiltrating neutrophils in the liver increased markedly after I/R (Figs. 5, 6), suggesting mice that underwent 90 min of ischemia. Although serum IL-17A concentrations was at 6 h after reperfusion in the wild-type migration of neutrophils from the vascular space into the hepatic

Disclosures

The authors have no financial conflicts of interest.

References