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CXCR3⁺CD4⁺ T Cells Mediate Innate Immune Function in the Pathophysiology of Liver Ischemia/Reperfusion Injury

Yuan Zhai,* Xiu-da Shen,* Wayne W. Hancock,§ Feng Gao,* Bo Qiao,* Charles Lassman,† John A. Belperio,‡ Robert M. Strieter,‡ Ronald W. Busuttil,* and Jerzy W. Kupiec-Weglinski2* 

Ischemia-reperfusion injury (IRI), an innate immune-dominated inflammatory response, develops in the absence of exogenous Ags. The recently highlighted role of T cells in IRI raises a question as to how T lymphocytes interact with the innate immune system and function with no Ag stimulation. This study dissected the mechanism of innate immune-induced T cell recruitment and activation in rat syngeneic orthotopic liver transplantation (OLT) model. Liver IRI was induced after cold storage (24–36 h) at 4°C in University of Wisconsin solution. Gene products contributing to IRI were identified by cDNA microarray at 4-h post-transplant. IRI triggered increased intrahepatic expression of CXCL10, along with CXCL9 and 11. The significance of CXCR3 ligand induction was documented by the ability of neutralizing anti-CXCR3 Ab treatment to ameliorate hepatocellular damage and improve 14-day survival of 30-h cold-stored OLTs (95 vs 40% in controls; p < 0.01). Immunohistology analysis confirmed reduced CXCR3⁺ and CD4⁺ T cell infiltration in OLTs after treatment. Interestingly, anti-CXCR3 Ab did not suppress innate immune activation in the liver, as evidenced by increased levels of IL-1β, IL-6, inducible NO synthase, and multiple neutrophil/monokine-targeted chemokine programs. In conclusion, this study demonstrates a novel mechanism of T cell recruitment and function in the absence of exogenous Ag stimulation. By documenting that the execution of innate immune function requires CXCR3⁺CD4⁺ T cells, it highlights the critical role of CXCR3 chemokine biology for the continuum of innate to adaptive immunity in the pathophysiology of liver IRI. The Journal of Immunology, 2006, 176: 6313–6322.

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Abbreviations used in this paper: IR, ischemia-reperfusion; IRI, IR injury; OLT, orthotopic liver transplantation; MPO, myeloperoxidase; iNOS, inducible NO synthase; sALT, serum alanine aminotransferase.

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facility under specific pathogen-free conditions, and received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institute of Health (National Institutes of Health publication 86-23, revised 1985).

Cold ischemia and syngeneic OLT

Livers were harvested from LEW rats, stored for 0, 24, 30, or 36 h at 4°C in University of Wisconsin (UW) solution, and then transplanted into syngeneic LEW recipients, with revascularization without hepatic artery re-connection (13, 14). In the treatment group, LEW livers were stored at 4°C in UW solution for 30 h before transplantation into syngenic rats that were treated with polyclonal rabbit-anti-murine CXCR3 or normal rabbit serum (24 h before and at the time of OLT; 1 ml i.v.). The production, characterization and in vivo efficacy of this anti-CXCR3 serum in mouse models have been described (13). However, this antiserum cross-reacts with rat CXCR3. Indeed, the sequence of murine peptide (N-terminal 16-mer of CXCR3: PYDYGENSEDFSDDSPP) used to generate polyclonal Ab is identical with that of the rat CXCR3 peptide. Hence, we assume that the infused serum binds rat and mouse CXCR3 with similar specificity. OLT recipients were followed for survival and serum alanine aminotransferase (ALT) levels, an indicator of hepatocellular injury. Separate groups of rats were sacrificed at 4 or 24 h, and OLT samples were collected for RNA/protein isolation, as well as for histology/immunohistochemistry evaluation.

Immunopatohology

For histological examination, portions of each liver were fixed in formalin, paraformaldehyde and stained by H&E. For immunohistology, cryostat sections of paraffin-embedded samples were stained using mouse anti-rabbit Ab directed against T cell subsets (BD Pharmingen), and polyclonal Abs to rat IFN-γ-inducible protein-10 (IP-10) and CXCR3 (Santa Cruz Biotechnology). Labeling was detected using an Envision immunoperoxidase kit (DakoCytomation) and sections were counterstained with hematoxylin. Control sections were incubated with isotype-matched mAbs, purified rabbit or goat IgG, and polyclonal Abs preabsorbed overnight using corresponding mAb. Quantitation of CD4+, CD8+, and CXCR3+ cells was performed blindly, using 10 consecutive high power fields of each graft. Sections were cut from two different levels per graft and three grafts per group per time point were evaluated; data were expressed as mean ± SD cells per field.

cDNA microarray and data analysis

Total tissue RNA was used prior to reverse transcription (Invitrogen Life Technologies). Sample preparation for cDNA microarray and microarray processing was performed using the UCLAMicroarray Core, using Rat Genome U34A gene chips from Affymetrix. The raw data was analyzed using the GeneChip Analysis Suite (Affymetrix), and expression data were imported into Microsoft Excel 2000 for further analysis and plotting. Microarray data were normalized to the housekeeping gene (GAPDH). To identify the genes significantly increased by IR, genes with “absent” call in two different levels per graft and three grafts per group per time point were evaluated; data were expressed as mean ± SD cells per field.

Quantitative RT-PCR

Five micrograms of RNA was reverse-transcribed into cDNA using random hexamers and Omniscript reverse transcriptase (Qiagen). Quantitative PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research). In a final reaction volume of 25 μl, the following were added: 1 X SuperMix (Platinum SYBR Green PCR kit; Invitrogen Life Technologies), cDNA and 0.5 mM of each primer. Amplification conditions were: 50°C (2 min), 95°C (5 min) followed by 50 cycles of 95°C (15 s), 60°C (30 s). Primers used to amplify a specific fragment of β-actin, CXCL9, 10, 11, IL-1β, inducible NO synthase (iNOS), RANTES, CD86, MCP-1, and MIP-2 are listed (Table I).

Western blots

Protein was extracted from liver samples with PBS/TDS buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100 (pH 7.2)). Proteins (30 μg/sample) in SDS-loading buffer (50 mM Tris, (pH 7.6), 10% glycerol, 1% SDS) were subjected to 20% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The gel was stained with Coomassie blue to document equal protein loading. Membranes were blocked with 5% dry milk and 0.1% Tween 20 (USB) in PBS and incubated with rabbit-anti-rat IP-10 (Cell Science) or β-actin Ab (Santa Cruz Biotechnology). Membranes were then washed and incubated with HRP-conjugated donkey anti-rabbit IgG (Amersham Biosciences).

Myeloperoxidase (MPO) assay

The presence of MPO, an enzyme specific for neutrophils, was used as an index of neutrophil accumulation in the liver (14). Briefly, the frozen tissue was thawed and placed in 4 ml of iced 0.5% hexadecyltrimethyl-ammonium bromide and 50 mM potassium phosphate buffer solution with the pH adjusted to 5. Each sample was homogenized for 30 s and centrifuged at 15,000 rpm for 15 min at 4°C. Supernatants were mixed with hydrogen peroxide-sodium acetate and tetramethyl-benzidine solutions. The change in absorbance was measured spectrophotometrically at 655 nm. One unit of MPO activity was defined as the quantity of enzyme degrading 1 μmol peroxide/min/g of tissue at 25°C.

Statistical analysis

All values are expressed as mean ± SD; data were analyzed with an unpaired two-tailed Student’s t test, and p < 0.05 was considered statistically significant.

Results

The development of IRI in syngeneic OLT

Transplanted organs experience “warm” ischemia during organ procurement and surgery, and “cold” ischemia during organ storage and transport. Moreover, transplantation itself triggers a certain stress, which may alter gene expression profiles. Thus, we first set-up a series of rat OLT groups in which LEW livers were harvested and stored in UW solution at 4°C for varying periods (0, 24, 30, 36 h) before being transplanted into syngeneic LEW recipients (15, 16). One hundred percent of recipients receiving 0 or 24 h cold-preserved OLTs survived long-term (>50 days; n = 6; data not shown). However, livers stored for 36 h suffered irreversible and lethal injury, as evidenced by 100% recipient death within 48 h (n = 6). The recipients received 30-h cold-preserved livers had a 40% survival rate (n = 12), and was used as a “treatment group.”

Histological analysis showed that the extent and severity of liver parenchyma damage correlated with cold preservation time, and that the full development of liver IRI was observed after 24-h reperfusion (Fig. 1). At 4 h of reperfusion, 24-h cold-stored OLTs (group no. 24-4) revealed minimal early necrosis with moderate sinusoidal congestion. There were scattered, small hepatocyte

Table I. Quantitative PCR primers

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<thead>
<tr>
<th>Gene</th>
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<tr>
<td>RANTES</td>
<td>S: ctacccctctcaacctctgtg</td>
<td>S: gagttggtccagcagcata</td>
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</tbody>
</table>

*S, sense primer or 5’ primer; AS, antisense primer or 3’ primer.*
samples per group for each time point. /H11003

Posttransplant following 24, 30, or 36 h of cold ischemia. FIGURE 1. Histology of rat IRI in OLT samples harvested at 4 or 24 h posttransplant following 24, 30, or 36 h of cold ischemia. a and b, Twenty-four hour cold-preserved OLTs after 4 h of reperfusion (24-4) with minimal necrosis and moderate sinusoidal congestion, scattered, small hepatocyte clusters with hypereosinophilic cytoplasm and nuclear hypochromasia (Fig. 1, a and b). However, the same livers after 24-h reperfusion (group no. 24-24) developed moderate/severe necrosis with multiple small foci of necrosis composed of clusters of hepatocytes with loss of cellular detail, generally midzonal involving fewer than half of the lobules. There were few large foci of necrosis extending across multiple lobules with periporal sparing (Fig. 1c). Although largely well-preserved at 4-h post-OLT, the 30-h cold-preserved livers after 24 h of reperfusion (group no. 30-24) developed large foci of necrosis extending across multiple lobules (Fig. 1d). In contrast, livers after 36 h of cold storage developed parenchyma perivenular necrosis involving ~50% of the lobules as early as 4 h of reperfusion (group no. 36-4; Fig. 1, e and f). Interestingly, the areas of necrosis were associated with increased numbers of inflammatory cells in sinusoids. sALT levels increased in all three OLT groups, as compared with those from native or liver grafts with no cold ischemia (group no. 0-4), indicating that hepatocellular damage did occur already after 4-h reperfusion and in the absence of histological manifestation of liver parenchyma damage (e.g., sALT = 514 ± 186 vs 197 ± 42 U/ml in 24-4 and 0-4 groups, respectively; p < 0.01, n = 6/group; data not shown).

Cold ischemia induces intrahepatic CXCL10 expression

To identify gene products involved in the initiation of IRI, we used cDNA microarray to analyze 4-h reperfused OLT samples, which were at the early stage of IRI as shown above; native livers and 0-h cold-preserved OLT (group no. 0–4) served as controls. Microarray data were uploaded to (www.GeneSifter.Net), and pairwise analysis of expression data from 24- vs 36-h preserved OLT was performed to identify differentially expressed gene products, particularly those related to immune activation. Data from each liver sample were normalized to its own global mean expression levels. Four hundred and forty genes were found to have significant changes (≥2-fold) between these two groups. KEGG (Kyoto Encyclopedia Genes and Genomics) pathway analysis of this gene list revealed two molecular signaling pathways affected the most by z-score: MAP signaling, and TLR signaling, which is consistent with our recent finding that TLR4 signaling is critical in triggering liver IRI cascade (7). There were six chemotaxis-related genes identified by gene ontology analysis. CXCL10 (IP-10, IFN-inducible protein 10) was the most up-regulated one (10-fold increase) associated with T cell chemotaxis. As warm ischemia and surgical procedure itself did not trigger its up-regulation, the cold ischemia was the key factor in CXCL10 induction (group no. 0–4 vs group no. 24-4, Fig. 2a). The extent of CXCL10 gene induction was correlated with the length of cold ischemia time (group no. 24-4 vs group no. 30-4 vs group no. 36-4). We confirmed IR-mediated intracellular induction of CXCL10 by using quantitative RT-PCR (see Fig. 4) and immunohistology analyses (Fig. 2b). Among other chemotaxis or immune-related genes associated with lethal liver IRI after 36 h of cold storage, IL-6, CXCL1 (KC, MIP-2), CCL2 (MCP-1), LIX, iNOS and IL-1β were also significantly up-regulated (Fig. 3). CXCL1, in particular, was expressed in livers at very high levels, similar to CXCL10. However, unlike CXCL10, its induction was initiated by warm and further increased by cold ischemia (group no. 0–4 vs group no. 24-4, Fig. 3).

Anti-CXCR3 Ab ameliorates liver IRI

CXCL10 signals through a G-protein-coupled receptor, CXCR3 expressed on activated T cells, and is capable to initiate T cell chemotaxis. There are two other CXCR3 ligands, CXCL9 (monokine induced by IFN-γ) and CXCL11 (IFN-inducible T cell α chemotactrant), which may also affect CXCR3+ T cell recruitment. As rat U34 array does not provide their expression data, we...
undertook quantitative RT-PCR to assess CXCL 9 and 11 levels in our model. As shown in Fig. 4, cold ischemia induced expression of both genes, similarly to CXCL10. However, the extent of CXCL11 induction did not correlate well with the cold preservation time. Thus, to inhibit T cell recruitment into livers, we reasoned that targeting their shared CXCR3 could be more effective than neutralizing any single CXCR3 chemokine alone. A rabbit anti-CXCR3 serum (13) was administered to LEW rats of 30-h cold-preserved OLTs. As shown in Fig. 5, 40% of untreated or normal rabbit serum-treated OLT recipients survived long-term (n = 6–10 rats/group). The CXCR3 blockade improved OLT survival to 95% (p < 0.01). Histological analysis of liver samples harvested at 4- and 24-h posttransplant showed that Ab treatment prevented development of severe hepatic necrosis. There was only mild necrosis with multiple small foci involving clusters of 10–20 cells, generally midzonal in approximately one-third of the lobules (Fig. 1, g and h).

To examine in vivo effects of anti-CXCR3 serum on rat peripheral lymphocytes, blood samples were collected before, as well as 4- and 24-h postserum administration, i.e., the time frame of liver IRI. A commercial rabbit anti-rat CXCR3 polyclonal Ab was used to analyze cell surface CXCR3 expression on circulating lymphocytes by FACS. As shown in Fig. 6a, our anti-CXCR3 serum and the commercial anti-CXCR3 Ab had very similar in vitro staining pattern of peripheral PBLs. Furthermore, anti-CXCR3 serum used in this study bound to lymphocytes in vivo at 24 h postinfusion. To determine whether CXCR3-targeted therapy depleted peripheral lymphocytes, we analyzed CD4 and CD8 T cell frequencies in total PBLs. Interestingly, although transient decrease of total CD4 and CXCR3+CD4 was noted at 4-h postserum injection, both frequencies returned to normal by 24 h (Fig. 6b). The marked decrease of CXCR3+CD8 T cells was observed at 4 and 24 h, and of total CD8 T cells at 24 h. Thus, the binding to CXCR3 Ag rather than target cell depletion represents the major mechanism by which anti-CXCR3 sera exerts its early acute in vivo effect in rat recipients.

We performed immunohistology examination of 30-h cold-preserved syngeneic OLTs that were harvested at various time points. Hepatocyte expression of CXCL10 was observed at 4-h posttransplant in the case of both IgG and anti-CXCR3 groups (Fig. 7, a and b). However, whereas isografts from IgG-treated rats showed a moderate and diffusely distributed population of CD4 and smaller numbers of CD8 T cells (Fig. 7, d and j), along with prominent CXCR3 expression (Fig. 7g), corresponding samples from anti-CXCR3 Ab-treated recipients showed 50% reduction in CD4 (p < 0.01) and CD8+ T cells (p < 0.01), and no expression of CXCR3 (p < 0.001, Fig. 7, e, h, and k) (Table II). Analysis of grafts from anti-CXCR3 Ab-treated rats 7 days later showed negligible CXCL10 expression and small numbers of CD4 and CD8 T cells (Fig. 7, c, f, and l), comparable to 4-h samples from anti-CXCR3 Ab group or baseline controls, and no CXCR3 expression (Fig. 7i) (Table II). Hence, anti-CXCR3 Ab prevented local accumulation of CXCR3+ T cells, critical to facilitate the hepatocellular damage in this model.

CXCR3 blockade does not inhibit early intrahepatic innate immune activation

To investigate the molecular mechanism of the CXCR3-targeted therapy in ameliorating liver IRI, we applied microarray analysis

FIGURE 2. IP-10 expression in OLT groups by (a) microarray data: average ± SD (n = 2/group) after normalized to global means; b, immunohistology staining (anti-IP-10 Ab and control Ig). OLTs were harvested at 4 h posttransplant after hepatic cold ischemia ranging from 0 to 36 h.
to identify genes altered by the treatment in the 30-h cold-preserved OLTs. By pairwise comparison of the treated (group no. 30-4T) and control (group no. 30-4) livers, 172 genes were identified to have at least 2-fold changes between the two groups. KEGG pathway analysis of this gene list by $z$-score revealed that three molecular/cellular pathways were affected the most: cytokine-cytokine receptor interaction, apoptosis, and TLR-signaling pathway. Bcl2-like 11 was down-regulated by 2-fold, and wild-type p53-induced gene 1/H11003 was down-regulated by 10-fold. CCL3 and CCL4, which bind to CCR1, CCR5 and are associated with wide-range lymphocyte chemotaxis, were up-regulated by the treatment (Fig. 3). IL-1β and IL-6 were also up-regulated. Interestingly, some of the genes associated with lethal IRI, including CXCL10, CXCL1, CCL2, and LIX, were all up-regulated by the treatment (Fig. 3), indicating a fully activated status of innate immunity in the liver despite CXCR3 Ab treatment. To further investigate the effects of CXCR3-targeted therapy on innate immune activation, we measured innate immune activation gene products at both 4 h and 24 h after reperfusion in OLTs by quantitative RT-PCR. As shown in Fig. 8, innate immune activation products were induced most significantly at 4 h, and all but CD86 declined by 24 h. CXCR3 Ab treatment did not down-regulate their induction seen otherwise during untreated IRI. Additionally, MPO assay, which reflects neutrophil infiltration, showed increased enzymatic activity at 4 h in both control and treated livers ($p < 0.01$, vs native livers); there was a decline in MPO activity at 24 h in the treated OLTs (Fig. 9).

**Discussion**

In this study, we aimed to determine the mechanism responsible for the recruitment and activation of T cells in the pathophysiology of liver IRI in the absence of exogenous Ags. A systemic cDNA

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**FIGURE 3.** Cytokine/chemokine expression in OLT groups by microarray analysis. Gene expression by average ± SD ($n = 2/group$) after normalized to global means were plotted using the Genesifter.net tool at [www.Genesifter.net](http://www.Genesifter.net)
microarray was performed in a rat syngeneic OLT model to identify gene products altered early after reperfusion (4 h), in particular genes encoding T cell-targeted innate immune activation products. Our results demonstrate increased intrahepatic expression of CXCR3 ligands, including CXCL9, 10, 11, in response to the extended cold ischemia. The functional significance of IRI-induced chemokines was documented by the blockade of their shared receptor, CXCR3, which not only ameliorated cardinal features of IRI, but also improved OLT survival from 40 to 95%. Concomitant immunohistology analysis has confirmed that there was a reduction in the number of OLT infiltrating CD4+ T and CXCR3+ cells. Interestingly, CXCR3-targeted therapy failed to suppress innate immune activation early during liver IRI, as manifested by increased levels of IL-6, IL-1β, iNOS, and neutrophil/monokine-targeted chemokine programs. Together, these results indicate that liver IR induced-activation of innate immunity results in the production of T cell-targeting CXCR3 ligands, which are critical for the selective recruitment of CXCR3+CD4+ T cells. These T cells are required for the execution of innate immune function, leading to the ultimate hepatocellular damage.

The interlocked roles of proinflammatory cytokines in IRI have been extensively studied. Increased TNF-α and IL-1 plasma levels were observed as early as 5 min of liver reperfusion (17). Their functional role was confirmed by neutralization experiments in which IL-1 or TNF-α blockade ameliorated the severity of IRI (18, 19). Because IRI develops in the absence of exogenous Ags, most attention in chemokine studies has been focused on those targeting Ag nonspecific leukocytes, in particular macrophages and neutrophils (20). IL-8 (CXCL8 or CINC in rodents), LIX, and MIP-2, are all involved in neutrophil trafficking, and their neutralization was protective in lung, kidney, and myocardial IRI models (21–24). Recently, small molecule-mediated blockade of CXCR1/CXCR2, the neutrophil chemokine receptors that bind IL-8/MIP-2, reduced granulocyte infiltration and mitigated rat kidney and liver IRI (25, 26). Similar effects were recorded in myocardial IRI model by using CXCR2-deficient mice (27). Chemokines targeted to monocytes, such as MCP-1, have also proven to be critical in renal IRI, as evidenced by tubular cytoprotection in CCR2 (binds MCP-1) deficient mice (28). Despite confirmed roles of these proinflammatory cytokines/chemokines in IRI, the regulation of their induction during IRI and mechanisms of their action other than effects on lymphocyte infiltration have not been fully addressed. In particular, the cascade of proinflammatory chemokine/cytokine

FIGURE 4. CXCR3 ligand expression in OLT groups by quantitative RT-PCR. Gene expression by ratios of target gene/β-actin (average ± SD; n = 2/group) were plotted with Microsoft Excel.

FIGURE 5. OLT recipient survival. LEW livers were stored at 4°C in UW solution for 30 h before transplantation into rat syngeneic recipients. Recipients were treated with rabbit anti-rat CXCR3 serum or normal rabbit serum twice: 24 h prior and at the time of liver transplantation (1 ml/injection). Rat survival was monitored daily; n = 6–12 rats/group.
FIGURE 6. Impact of anti-CXCR3 serum treatment on peripheral lymphocytes in vivo. 

a, PBLS were harvested from normal LEW rats, and stained with anti-murine CXCR3 serum (first panel), or a commercial anti-CXCR3 (third panel) rabbit polyclonal Ab. PBLS were harvested 24 h after i.v. infusion of 1 ml of anti-CXCR3 serum, and stained with a FITC-labeled anti-rabbit Ig (second panel). The frequency of CD4 expression was screened in parallel. 

b, PBLS were harvested before as well as 4 h, and 24 h after i.v. administration of anti-CXCR3 serum. Cells were stained with anti-CD4, anti-CD8, and commercial anti-CXCR3 polyclonal Abs. Lymphocytes were gated for the analysis of total CD4, CD4CXCR3⁺, and total CD8, CD8 CXCR3⁺ ratios. Results are representative of three different experiments.

FIGURE 7. Immunopathology of rat liver IRI after anti-CXCR3 Ab treatment. Gene expression was analyzed in OLT samples harvested at 4 h or 7 days posttransplant following 30 h of cold ischemia. 

a–c, Anti-CXCR3 Ab therapy did not abolish IP-10 induction by hepatocytes at 4 h; it had essentially fallen to trace levels by 7 days of follow-up. d–f, Levels of CD4⁺ T cells were higher in IgG-treated rats than in those receiving anti-CXCR3 Ab. g–i, Numbers of CXCR3⁺ leukocytes were markedly increased in the IgG-treated group. j–l, Numbers of CD8⁺ T cells were comparable between groups. No staining was seen using control IgG or after peptide absorption of polyclonal Abs; data for IP-10 controls are shown as insets in each panel. (Cryostat sections labeled by immunoperoxidase and counterstained with hematoxylin; original magnification ×150). Representative of three to four samples per group per time point.
programs during IR, and the inflammation status altered by the absence of any of these chemokines or their receptors has not been studied in a comprehensive manner.

Our study is the first that used a systemic approach, i.e., microarray, to identify not only genes induced during liver IRI, but also those altered by anti-CXCR3 Ab treatment. Results show that IR-induced hepatic induction of IL-1β/H9252 did not correlate with the severity of liver injury. Interestingly, CXCR3-targeted therapy did not decrease, but actually increased IL-1β/H9252 levels without causing hepatocellular damage. In parallel, IR-induced neutrophil and monocyte targeted chemokines, such as CCL-2, CCL-3, CCL-4, CXCL1 or LIX were readily detectable in our model, and anti-CXCR3 Ab treatment did not inhibit their expression. Consistent with the chemokine expression data, we did find increased neutrophil infiltration (assessed by hepatic MPO activity) at 4-h post-transplant despite anti-CXCR3 Ab treatment. These results indicate that depletion of CXCR3+/H11001 cells blocked the development of inflammation response without affecting the initial innate immune activation by liver IRI. This implies that the execution of IR-activated innate immune function requires CXCR3+/H11001 T cells, which may regulate either the responsiveness of neutrophil/monocytes to their chemokines, or the functions of subsequently recruited and activated innate immune cells. Indeed, in the absence of CXCR3+/H11001 T cells, IR failed to trigger hepatocellular damage despite activation of the innate immune system.

T cells have been shown to play a key role in the mechanism of IRI. Indeed, in the absence of T cells, in particular CD4 T cells, both livers and kidneys are largely protected from IRI (9, 10). Interestingly, concomitant neutrophil infiltration was also reduced in the ischemic livers (9). The question arises how Ag-specific T cells are being recruited into this, by definition Ag-independent inflammatory immune reaction? Our finding that liver IR induced CXCR3 ligands very early (4 h) after reperfusion, provides a clue to this question. The reduction of CD4 and CD8 infiltration after CXCR3 blockade further supports the idea that CXCR3 ligands are responsible for local T cell recruitment in OLTs. Furthermore, our results suggest that intrahepatic accumulation of CXCR3+/H11001 T cells plays a key role in the pathogenesis of liver IRI. CXCR3 is being expressed mostly on preactivated T cells (memory), particularly

### Table II. Quantitation of CD4+, CD8+, and CXCR3+ T cells in 30-h cold-preserved syngeneic OLT

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<tr>
<th>Marker</th>
<th>IgG treated (4 h)</th>
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* Mean ± SD cells/high power field, with quantitation as detailed in Materials and Methods: *, p < 0.01 and **, p < 0.001 vs IgG-treated group.

![Figure 8](http://www.jimmunol.org/)

**Figure 8.** Gene expression of innate immune activation products in OLT groups by quantitative RT-PCR. Gene expression by ratios of target gene/β-actin (average ± SD; n = 2/group) were plotted with Microsoft Excel.
Th1, and some NK and B cells (29, 30). Our data that CXCR3+ cells are critical in the mechanism of liver IRI is consistent with previously shown cytoprotection against hepatic and renal IRI in Stat4- or Th1-type deficient mice (31, 32). Although type-I proinflammatory T cells produce IL-2, IFN-γ, and TNF-α, and support macrophage activation (33), we failed to detect the induction of these cytokines in our OLT model (data not shown). This might be due to the lack of sufficient T cell stimulation during liver IRI, in which no specific Ags are present or potent enough to provide the first signal for full T cell activation. This raises an intriguing question as to how T cells function to promote IRI without being fully activated? Our preliminary data suggest a role of TRAIL, which may be expressed constitutively on activated T cells (data not shown). Additionally, we have shown that CD154 costimulatory pathway is critical for the development of IRI in a mouse liver ischemia model (12). Indeed, CD154 expressed on activated CD4 T cells, may also be involved in the activation of liver DCs and/or macrophages (Kupffer cells) via CD40.

One obvious question is which mechanism triggers intrahepatic induction of CXCR3 ligands in the absence of any detectable IFN-γ in livers early during IR. Although this question remained beyond the scope of this study, our recent data in a mouse liver ischemia model indicate that TLR4 activation might represent the initiating event (7). We have also shown that MyD88-independent signaling downstream of TLR4 mediated by IRF3 is critical for the development of IR-induced hepaticcellular damage. IFN-β, one of the major players along this pathway (34), can then stimulate multiple liver cell types to elaborate CXCR3 ligands (data not shown).

In conclusion, this study demonstrates a novel mechanism of T cell recruitment and function in the absence of exogenous Ag stimulation. By documenting that the execution of innate immune function requires CXCR3+CD4+ T cells, it highlights the critical role of CXCR3 chemokine biology for the continuum of innate to adaptive immunity in the pathophysiology of liver IRI.

Disclosures

The authors have no financial conflict of interest.

References


