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CXC Chemokine Receptor-1 is Expressed by Hepatocytes and Regulates Liver Recovery after Hepatic Ischemia/Reperfusion Injury

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Abstract

CXC chemokines mediate hepatic inflammation and injury following ischemia/reperfusion (I/R). More recently, signaling through CXC chemokine receptor-2 (CXCR2) was shown to delay liver recovery and repair after I/R injury. The chemokine receptor, CXCR1 shares ligands with CXCR2, yet nothing is known about its potential role in liver pathology. In the present study, we examined the role of CXCR1 in the injury and recovery responses to I/R using a murine model. CXCR1 expression was undetectable in livers of sham-operated mice. However, after ischemia CXCR1 expression increased 24 hours of reperfusion and was maximal after 96 hours of reperfusion. CXCR1 expression was localized largely to hepatocytes. In order to assess the function of CXCR1, CXCR2^{-/-} mice were treated with the CXCR1/CXCR2 antagonist, repertaxin. Prophylactic treatment with repertaxin had no effect on acute inflammation or liver injury. However, when repertaxin was administered 24 hours post-reperfusion there was a significant increase in hepatocellular injury and a delay in recovery compared to control-treated mice. CXCR1^{-/-} mice also demonstrated delayed recovery and regeneration after I/R when compared to wild-type mice. In vitro, hepatocytes from CXCR2^{-/-} mice that were stimulated to express CXCR1 showed increased proliferation in response to ligand. Hepatocyte proliferation was decreased in CXCR1^{-/-} mice in vivo.

Conclusion—This is the first report to show that CXCR1 expression is induced in hepatocytes after injury. Furthermore, the data suggest that CXCR1 has divergent effects from CXCR2 and appears to facilitate repair and regenerative responses after I/R injury.

Keywords

chemokines; chemokine receptors; liver repair; hepatocyte proliferation; regeneration

Ischemia/reperfusion (I/R) of the liver often occurs as a result of liver resection surgery, transplantation, and trauma and is a primary cause of subsequent liver dysfunction (1-3). The recovery and regeneration of the liver after I/R is associated with the temporal expression of cell cycle control proteins (4). Recent data from our laboratory has demonstrated that this process is regulated by signaling through CXC chemokine receptor-2 (CXCR2) (5). These studies demonstrated that deletion or pharmacological blockade of CXCR2 increased hepatocyte proliferation and liver regeneration in association with increased activation of the transcription factors, nuclear factor- κ B (NF- κ B) and signal

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transducer and activator of transcription-3 (STAT3). Furthermore, we showed that ligands of CXCR2 have direct, dose-dependent effects on hepatocytes to regulate cell death or proliferation (5).

The ligands for CXCR2 are comprised of a subclass of CXC chemokines which possess the amino acid sequence Glu-Leu-Arg (ELR motif) in the amino terminus (6,7). However, CXCR2 is not the only receptor for ELR⁺ CXC chemokines. In humans, CXC chemokine receptor-1 (CXCR1) also binds many of these ligands and has both overlapping and independent functions to CXCR2 (8-13). The murine homolog of human CXCR1 remained elusive for many years, but the gene for murine CXCR1 was recently cloned and characterized (14,15). Murine CXCR1 shares 64% and 89% homology at the amino acid level with human CXCR1 and murine CXCR2, respectively (15). Murine CXCR1 has been shown to bind to multiple CXC chemokines (16), but it is unknown if the receptor is expressed in the liver during injury. In the present study, we sought to determine if CXCR1 plays a functional role in the injury and recovery from hepatic I/R.

Experimental Procedures

Hepatic I/R Injury Model

Male wild-type (BALB/c and C57Bl/6), CXCR2^{-/-} mice on a BALB/c background, and CXCR1^{-/-} mice on a C57Bl/6 background (Jackson Laboratory, Bar Harbor, ME) weighing 22-28 g were used in these experiments. This project was approved by the University of Cincinnati Animal Care and Use Committee and was in compliance with the National Institutes of Health guidelines. The animals underwent sham surgery or I/R. Partial hepatic ischemia was induced as described previously (17). Briefly, mice were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). 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. The caudal lobes retained intact portal and arterial inflow and venous outflow, preventing intestinal venous congestion. After 90 minutes of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion. Sham control mice underwent the same protocol without vascular occlusion. Some CXCR2^{-/-} mice were injected intra-peritoneally with saline (vehicle control) or 30 mg/kg repertaxin (Sigma Chemical Co., St. Louis, MO), a noncompetitive allosteric inhibitor of CXCR1 and CXCR2 (18), dissolved in saline 2 hours prior to induction of ischemia, after 2 and 12 hours of reperfusion. Other CXCR2^{-/-} mice were treated with saline (vehicle control) or 30 mg/kg repertaxin after 24 hours of reperfusion and every 12 hours thereafter. Mice were sacrificed after the indicated periods of reperfusion, and blood and samples of ischemic lobes and non-ischemic lobes of the liver were taken for analysis.

Western Blot Analyses

Liver samples were homogenized in lysis buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.6% NP-40, 0.5 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 1 µg/ml pepstatin). Isolated hepatocytes were resuspended with RIPA buffer containing 0.5 mM PMSF, 1 mM sodium orthovanadate, and 1 µl/ml protein inhibitor cocktail (1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, 1 mg/ml pepstatin). Samples were then sonicated and incubated for 30 minutes on ice. Cellular debris was removed by centrifugation at 10,000 rpm. Protein concentrations of each sample were determined. Samples containing equal amounts of protein in equal volumes of sample buffer were separated in a denaturing 10% polyacrylamide gel and transferred to a 0.1 µm pore nitrocellulose membrane. Nonspecific binding sites were blocked with tris-buffered saline (TBS; 40 mM Tris, pH 7.6, 300 mM NaCl) containing 5% non-fat dry milk for 1 hour at room temperature. Membranes were then incubated with

antibodies to CXCR1 (RDI, Concord, MA) or β -actin (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS with 0.1% Tween 20 (TBST). Membranes were washed and incubated with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence. Lysates from human neutrophils were used as positive controls for CXCR1.

Blood and Tissue Analysis

Blood was obtained via cardiac puncture for analysis of serum alanine aminotransferase (ALT) as an index of hepatocellular injury. Measurements of serum ALT were made using a diagnosis kit via bioassay (Wiener Laboratories, Rosario, Argentina). Liver tissues were fixed in 10% neutral-buffered formalin, processed and then embedded in paraffin for light microscopy. Sections were stained with hematoxylin and eosin for histological examination. Quantitative morphometric analysis of hepatocellular necrosis was performed in a blinded fashion with histologic sections at low power (10 $\times$) using Photoshop image analysis software (Adobe Systems, Inc., San Jose, CA). Necrotic area was expressed as percentage of total area examined. Liver neutrophil accumulation was determined via myeloperoxidase (MPO) content using methods described elsewhere (19).

Proliferating Cell Nuclear Antigen Staining

Immunohistochemical staining for proliferating cell nuclear antigen staining (PCNA) was performed on paraffin-embedded liver tissue with anti-PCNA antibody using a DakoCytomation ARK kit (Dako, Copenhagen, Denmark). Briefly, a three-step peroxidase method was performed according to the manufacturer's instructions. PC-10 monoclonal antibody (Santa Cruz Biotechnology) was used at a dilution of 1:50, for 15 minutes at room temperature. The sections were counterstained with hematoxylin. Evaluation of PCNA immunostaining was performed based on the percentage of positive nuclei of 400-600 hepatocytes from 4-6 highest positive fields at high power (400 $\times$), and was expressed as PCNA labeling index.

Immunofluorescence staining for CXCR1

Liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin prior to sectioning. Tissue sections were then deparaffinized and washed twice in phosphate-buffered saline (PBS) (pH 7.4) and blocked with Image-iT FX signal enhancer (Molecular Probes, Eugene, Oregon) for 30 minutes. Following a wash in PBS, tissue sections were incubated with rabbit polyclonal CXCR1 antibody (RDI). Sections were washed and then incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Molecular Probes) for two hours at room temperature protected from light. After a final PBS wash, sections were examined and images acquired on a Nikon PCM 2000 laser confocal scanning microscope.

Primary hepatocyte studies

Primary murine hepatocytes were isolated as described elsewhere (20). Isolated hepatocytes were distributed onto 24-well flat-bottomed plates (Trasadingen, Switzerland) at a concentration of 2.0×10^5 cells/500 μ l/well, and incubated overnight to allow cell adherence. Some hepatocytes were stimulated with 20 ng/ml TNF α for 3 hours and then media was changed to normal Williams media without TNF α to allow recovery from cellular stress. Hepatocytes were harvested at the indicated periods and cell lysates were prepared for Western blot analysis. Primary hepatocytes were also distributed onto 96-well flat-bottomed plates (Trasadingen) at a concentration of 1.5×10^4 cells/200 μ l/well, and incubated overnight to allow cell adherence. Cells were reincubated for 24 hours in Williams media with human recombinant IL-8 (Peprotech) at 1-10,000 ng/ml. In some experiments, hepatocytes were stimulated with TNF α for 3 hours prior to IL-8 treatment to

induce CXCR1. Cytotoxicity induced by IL-8 was determined by lactate dehydrogenase (LDH) assay according to the manufacturer's instructions (Roche, Mannheim, Germany), and percentage death of cultured hepatocytes was calculated. DNA incorporation of 5-bromo-2'-deoxyuridine (BrdU) after stimulation with IL-8 was examined to evaluate hepatocyte proliferation, and data were normalized by the amount of viable cells and expressed as a ratio compared with medium alone. The Biotrak™ cell proliferation ELISA system (GE Healthcare, Buckingham, UK) was used for this assay. Data are expressed as a ratio compared with 0 ng/ml.

Neutrophil Activation and Phagocytosis

Liver neutrophils were isolated using a GentleMACS according to the manufacturer's instructions (Miltinyi Biotec, Auburn, CA). CD11b expression and phagocytosis were determined as described (21,22). Briefly, CD11b (Clone: M1/70, Invitrogen, Carlsbad, CA) expression acquisition and analysis was performed on an LSR II using FACS Diva software (BD Biosciences, Mountain View, CA). For phagocytosis, liver neutrophils were mixed with fluorescently labeled (Alexa Fluor 488) *E. coli* (Invitrogen) for 10 minutes at 37 °C, and then fixed with 4% paraformaldehyde. After labeling with antibodies toward surface antigens, samples were run on a Becton Dickinson LSR II to determine the mean fluorescence intensity (MFI), a measure of the number of bacteria taken up per cell. To quench the fluorescence of adherent bacteria, trypan blue was added after the first acquisition by FACS scan and 1 min before the second acquisition. Quenching with trypan blue reduced the FITC fluorescence of adherent bacteria by abrogating excitation energy transfer (23,24).

Statistical Analysis

All data are expressed as the mean $\pm$ standard error of the mean (SEM). Data were analyzed with a one-way analysis of variance with subsequent Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$.

Results

CXCR1 is expressed in the liver after ischemia/reperfusion

Because other studies have shown that normal liver does not express CXCR1 (14,16), we examined whether expression of CXCR1 was induced in the liver during I/R injury in wild-type and CXCR2^{-/-} mice. Consistent with previous reports, no hepatic CXCR1 expression was observed in sham-operated mice by Western blot (Figure 1A) or immunohistochemical staining (Figure 1B). After ischemia and 12 hours of reperfusion, CXCR1 expression was detectable, but at very low levels in both wild-type and CXCR2^{-/-} mice (Figure 1A and B). However, after ischemia and 24, 48 or 96 hours of reperfusion CXCR1 expression was markedly increased (Figure 1A and B). Interestingly, CXCR2^{-/-} mice had more CXCR1 expression compared to wild-type mice after 24 and 48 hours of reperfusion (Figures 1A and B). The immunohistochemical staining showed CXCR1 expression on hepatocytes and vascular endothelial cells (Figure 1B). To verify that hepatocytes did indeed stain positive for CXCR1, hepatocytes were isolated from wild-type mice after sham operation or ischemia and 48 hours of reperfusion and subjected to Western blot for CXCR1. As shown in Figure 1C, hepatocytes stained positive for CXCR1 after I/R.

Development of an in vitro model to directly assess function of CXCR1

Because TNF α is a central regulator of the hepatic inflammatory response to I/R and because TNF α is a potent stimulator of hepatocytes (25,26), we evaluated whether TNF α was responsible for upregulation of CXCR1 expression in hepatocytes. Hepatocytes were

isolated from wild-type and CXCR2^{-/-} mice and stimulated with 20 ng/ml TNF α for 3 hours (Figure 2A). Media was then changed to Williams media without TNF α to allow recovery from cellular stress. Hepatocytes were harvested at the indicated periods and cell lysates were prepared for Western blot analysis. Similar to our in vivo studies (Figure 1), no CXCR1 expression was detected in unstimulated (sham) hepatocytes from either wild-type and CXCR2^{-/-} mice (Figure 2B). Interestingly, hepatocytes from wild-type mice did not express CXCR1 at any time point examined. In contrast, hepatocytes from CXCR2^{-/-} mice had marked expression of CXCR1 after 24 hours of recovery from TNF α stimulation (Figure 2B). This provides an in vitro model to study the functional role of CXCR1 on hepatocyte proliferation and cell death.

CXCR1 signaling promotes hepatocyte proliferation in vitro

Although several studies have found that murine CXCR1 signaling induces chemotactic effects on lymphoid cells or neural stem cells (16,27), no data exists on the function of CXCR1 signaling in hepatocytes. Because we have previously reported that signaling through CXCR2 has effects on both hepatocyte cell death and proliferation (5), we evaluated the effects of CXCR1 signaling on the same parameters. Since human IL-8 has been shown to be the most potent agonist of murine CXCR1 (16), we used this ligand to stimulate CXCR1 on murine hepatocytes. Primary hepatocytes from CXCR2^{-/-} mice were treated with TNF α for 3 hours to induce CXCR1 expression as shown in Figure 2. We then stimulated the cells with different concentrations of human IL-8 for 24 hours. There were no effects of IL-8 stimulation on hepatocyte cell death, except at the highest dose tested which induced a modest level of toxicity (Figure 3A). In contrast, IL-8 stimulated marked hepatocyte proliferation at doses as low as 10 ng/ml and a maximal response was observed at doses of 1,000 and 10,000 ng/ml (Figure 3B).

CXCR1 is not involved in the acute inflammatory injury induced by hepatic I/R

CXC chemokines are known to mediate hepatic I/R injury through the recruitment of neutrophils in the initial 24 hours after reperfusion (17,28). Our recent studies suggested that much of this recruitment was dependent on CXCR2 (5). Because we found that CXCR1 expression was increased in the liver between 12 to 24 hours of reperfusion (Figure 1), we examined whether CXCR1 contributes to acute inflammatory liver injury after I/R. To do this, wild-type and CXCR2^{-/-} mice were treated with 30 mg/kg repertaxin, an antagonist of CXCR1 and CXCR2, and liver injury and neutrophil accumulation were determined after ischemia and 24 hours of reperfusion. In wild-type mice, treatment with repertaxin significantly reduced liver injury and neutrophil accumulation, probably via inhibition of CXCR2 (Figure 4A). However, treatment of CXCR2^{-/-} mice with repertaxin had no effect on liver injury or neutrophil infiltration compared to those receiving the vehicle control (Figure 4A). We next investigated the acute injury response in wild-type and CXCR1^{-/-} mice. Similar to our results with repertaxin in CXCR2^{-/-} mice, we found no differences between wild-type and CXCR1^{-/-} mice in liver injury or neutrophil accumulation after ischemia and 24 hours of reperfusion (Figure 4B). These data suggest that CXCR1 plays no role in the acute inflammation and injury induced by I/R.

CXCR1 promotes recovery from hepatic I/R injury

Since we found no evidence that CXCR1 was involved in the acute injury induced by I/R (Figure 4), and the temporal pattern of CXCR1 expression in the liver after I/R (Figure 1) suggested that it may play a role in the recovery from I/R injury, we examined whether CXCR1 was involved in the recovery/reparative response. CXCR2^{-/-} mice were injected intraperitoneally with saline (control) or 30 mg/kg repertaxin 24 hours after reperfusion and every 12 hours thereafter. Since the injury induced by I/R peaks within the first 24 hours of reperfusion (5), administration of repertaxin at this time allows evaluation of CXCR1 in the

recovery response. Treatment with repertaxin had no effect on the degree of neutrophil accumulation after 48 or 96 hours of reperfusion (Figure 5A). However, after 48 hours of reperfusion, liver injury (as measured by serum ALT) was significantly higher in repertaxin-treated mice compared to mice treated with vehicle control (Figure 5A). After 96 hours of reperfusion, serum levels of ALT had returned to baseline in both groups. Parallel experiments conducted with wild-type and CXCR1^{-/-} mice showed no differences between the groups for either MPO or ALT (Figure 5B). However, evaluation of liver histology provided strong evidence that CXCR1 regulates the recovery response after I/R. Control mice undergoing sham surgery had normal hepatic architecture (Figure 6A and B). After 48 hours of reperfusion, livers from CXCR2^{-/-} mice treated with vehicle had the usual degree of hepatocellular necrosis, whereas livers from repertaxin-treated mice had significantly more hepatocellular necrosis (Figure 6A). After 96 hours of reperfusion, livers from CXCR2^{-/-} mice treated with vehicle had markedly improved, whereas significantly larger necrotic areas remained in the livers from CXCR2^{-/-} mice treated with repertaxin (Figure 6A). Similar results were observed between wild-type and CXCR1^{-/-} mice (Figure 6B), however after morphometric analysis, there were no statistical differences between these groups (Figure 6B).

To determine if the decreased hepatocellular recovery observed in repertaxin-treated CXCR2^{-/-} mice and CXCR1^{-/-} mice might be due to altered production of hepatocyte growth factor (HGF) and/or subsequent effects on hepatocyte proliferation, we measured liver expression of HGF and stained liver sections for PCNA, a marker of proliferating hepatocytes. CXCR1 blockade with repertaxin in CXCR2^{-/-} mice increased liver HGF expression at both 48 and 96 hours of reperfusion, but had no significant effect on hepatocyte proliferation (Figure 7A). However, mice nullizygous for CXCR1 had increased HGF expression at 96 hours of reperfusion, and had reduced hepatocyte proliferation at 96 hours of reperfusion compared to their wild-type counterparts (Figure 7B).

Lack of CXCR1 does not alter activation or phagocytosis in liver-recruited neutrophils

In order to determine if the delayed liver recovery/regeneration observed in mice with blockade or deletion of CXCR1 might be due to a defect in clearance of dead hepatocytes by recruited phagocytes, we evaluated activation and phagocytosis of neutrophils from wild-type and CXCR1^{-/-} mice. Neutrophils were isolated from liver after ischemia and 24 hours of reperfusion and activation state (surface expression of CD11b) and phagocytosis were determined. Gene deletion of CXCR1 had no effect on the activation state or phagocytosis of liver-recruited neutrophils after I/R (Figure 8).

Discussion

While the CXCR1 receptor has been widely studied in myeloid cells (29,30), the present study is the first to document the expression and function of CXCR1 in murine liver during I/R injury. In concurrence with previous studies (14-16), we found that under normal conditions, CXCR1 is not expressed in the liver. However, we found that after I/R, CXCR1 expression in the liver is upregulated on hepatocytes. Interestingly, hepatic expression of CXCR1 peaked earlier in CXCR2^{-/-} mice after I/R, suggesting that the presence of CXCR2 may regulate expression of CXCR1. In vitro, we found that stimulation of hepatocytes with TNF α only resulted in CXCR1 expression in CXCR2^{-/-} cells. Collectively, these data suggest that CXCR2 prevents CXCR1 expression in a cross-regulative fashion and is further suggestive of the divergent roles these receptors play in hepatocyte responses to injury.

In neutrophils, it is well-known that increasing concentrations of chemokine ligands results in receptor downregulation and subsequent desensitization. A number of studies have demonstrated that for any given concentration of chemokine, CXCR2 will be down-

regulated more than CXCR1 (9,31,32). The biological significance of the differential ligand sensitivity and receptor downregulation is not completely understood, but one hypothesis that is plausible for neutrophils is that the two receptors might transduce preferentially at different points of an inflammatory response. At lower concentrations of ligands, as might occur at the periphery of an inflammatory locus, CXCR2 might be used preferentially for chemotaxis towards the inflammatory site. As the cell moves towards the nidus of the inflammation and ligand concentrations increase, CXCR2 expression may become saturated, leading to receptor downregulation, allowing CXCR1 to bind ligand and signal to induce respiratory burst. How and whether similar regulation may occur in hepatocytes has yet to be determined.

Previous studies have demonstrated that pharmacologic blockade of CXCR1 and CXCR2 receptor with repertaxin resulted in decreased neutrophil recruitment and necrosis in rat livers after I/R (33), however they failed to distinguish between the role of CXCR1 and CXCR2 signaling. Our present data, together with our previous study (5), suggest that CXCR1 and CXCR2 may have quite different functions in the liver during recovery from I/R injury. CXCR2 signaling appears to be a critical mediator of acute liver injury and early neutrophil recruitment after hepatic I/R, as well as a negative regulator of hepatocyte recovery during the reparative process after I/R injury (5). In contrast, signaling through CXCR1 does not contribute to acute injury or neutrophil recruitment, but appears to facilitate liver repair and recovery after I/R. However, CXCR1 seems to have less impact on liver repair than CXCR2. While we found that ligand binding to CXCR1 in hepatocytes devoid of CXCR2 induces robust proliferation in vitro, blockade of CXCR1 in CXCR2^{-/-} mice in vivo had no effect on hepatocyte proliferation assessed by PCNA staining. Conversely, evaluation of CXCR1^{-/-} mice demonstrated reduced hepatocyte proliferation 96 hours after reperfusion. Interestingly, CXCR2^{-/-} mice treated with repertaxin had increased hepatocellular necrosis, whereas no significant difference in hepatocellular necrosis was observed between wild-type and CXCR1^{-/-} mice. Combined, the data suggest that CXCR1 is an important regulator of liver repair and recovery after I/R injury, but that there may be some receptor crosstalk with CXCR2 that alters the expression and function of CXCR1.

There are a number of possible explanations for the observed effects of CXCR1 in liver repair after I/R injury. We have excluded the possibility that altered neutrophil activation and/or phagocytosis could be responsible as liver-recruited neutrophils from CXCR1^{-/-} mice were no different from wild-type neutrophils in these parameters. As previously mentioned, in neutrophils it is known that CXCR2 is more susceptible to downregulation than CXCR1 in vitro (9,31,32). In vivo studies have shown that high expression of CXC chemokines cause marked desensitization of CXCR2 (34). Thus, one plausible explanation could be that the high concentrations of CXC chemokines known to exist in the liver microenvironment after I/R (5), cause selective downregulation or desensitization of CXCR2 within the initial 24-48 hours after reperfusion. This would coincide with the induction of CXCR1 in the liver which would contribute to liver repair and recovery. Alternatively, CXCR1 may compete with CXCR2 for available ligands and therefore, in the absence of any downregulation or desensitization of CXCR2, CXCR1 may effectively lower the ligand availability for binding to CXCR2 and thus limit the hepatotoxic effect of CXCR2.

In summary, the present study is the first to examine the expression and function of CXCR1 in liver injury and repair after I/R. We document that while CXCR1 is not constitutively expressed by hepatocytes, the receptor is induced within 24 hours after reperfusion. Our data suggest that CXCR2 suppresses CXCR1 expression in vitro and in vivo. We demonstrate that CXCR1 plays no role in the acute injury response but that it plays a pro-reparative function by facilitating liver recovery after injury. Future studies examining the precise signaling pathways utilized by CXCR1 and CXCR2 and the nature of their cross-talk may

provide important insights into therapeutic targets for manipulation of liver injury and repair.

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Abbreviations

I/R	ischemia/reperfusion
NF-κB	nuclear factor κB
IL	interleukin
TNFα	tumor necrosis factor-α
MIP-2	macrophage inflammatory protein-2
KC	keratinocyte chemokine
CXCR1	CXC chemokine receptor-1
CXCR2	CXC chemokine receptor-2
LPS	lipopolysaccharide
TBS	tris-buffered saline
ALT	alanine amino transferase
MPO	myeloperoxidase
PCNA	Proliferating Cell Nuclear Antigen
PBS	phosphate-buffered saline
LDH	lactate dehydrogenase
BrdU	5-bromo-2'-deoxyuridine
SEM	standard error of the mean
STAT3	signal transducer and activator of transcription-3

Figure 1.

Hepatic expression of CXCR1 during ischemia/reperfusion (I/R) injury. (A) Liver lysates were assessed for CXCR1 protein expression by Western blot. Lysates from human neutrophils served as a positive control and β-actin staining served as a loading control. Chemiluminescence films were quantitated by image analysis. Data are mean ± SEM with $n = 3$ per group. $*P < 0.05$ compared to wild-type mice. (B) CXCR1 protein expression in liver sections after hepatic I/R. CXCR1 was visualized using immunofluorescence staining. (C) Hepatocyte expression of CXCR1 after ischemia and 1 hour of reperfusion. Protein extracts from hepatocytes isolated from wild-type mice undergoing either sham operation or I/R were subjected to Western blot. Lysates from human neutrophils served as a positive control.

Figure 2. Hepatocyte CXCR1 expression after $\text{TNF}\alpha$ stimulation *in vitro*. (A) Experimental protocol for hepatocyte stimulation with $\text{TNF}\alpha$. (B) Hepatocyte expression of CXCR1 after $\text{TNF}\alpha$ stimulation. Protein extracts from wild-type or CXCR2^{-/-} hepatocytes stimulated with $\text{TNF}\alpha$ were subjected to Western blot. Lysates from human neutrophils served as a positive control.

Figure 3.

Effects of CXCR1 signaling on hepatocyte cell death and proliferation. Primary mouse hepatocytes from CXCR2^{-/-} mice stimulated with TNF α were treated with IL-8 (0-10,000 ng/mL) for 24 hours. (A) Cell death was determined via release of lactate dehydrogenase. (B) Cell proliferation was determined by DNA incorporation of BrdU assay. Data are expressed as the mean $\pm$ SEM with $n = 6-8$ per group. * $P < 0.05$ compared to 0 ng/ml.

Figure 4.

Effects of CXCR1 signaling on acute inflammation and injury after hepatic ischemia/reperfusion. A) Wild-type and CXCR2^{-/-} mice were injected intraperitoneally with saline (vehicle) or 30 mg/kg repertaxin 2 hours prior to induction of ischemia. Twenty-four hours after reperfusion liver injury was measured by serum levels of ALT and neutrophil accumulation was determined by liver content of MPO. Data are mean $\pm$ SEM with $n = 4$ per group. (B) Wild-type and CXCR1^{-/-} mice were subjected to hepatic I/R. Twenty-four hours after reperfusion liver injury was measured by serum levels of ALT and neutrophil accumulation was determined by liver content of MPO. Data are mean $\pm$ SEM with $n = 5$ per group.

Figure 5.

Effects of delayed CXCR1 blockade or CXCR1-knockout on liver neutrophil accumulation and late phase injury after I/R. (A) Mice were injected intraperitoneally with saline (vehicle) or 30 mg/kg repertaxin after 24 hours of reperfusion and every 12 hours thereafter. Neutrophil accumulation was determined by liver content of MPO and liver injury was measured by serum levels of ALT. Data are mean $\pm$ SEM with $n = 4-6$ per group. * $P < 0.05$ compared to control mice. (B) Wild-type and CXCR1^{-/-} mice were subjected to hepatic I/R. Neutrophil accumulation was determined by liver content of MPO and liver injury was measured by serum levels of ALT. Data are mean $\pm$ SEM with $n = 5$ per group. * $P < 0.05$ compared to wild-type mice.

Figure 6.

Effects of delayed CXCR1 blockade or CXCR1-knockout on histologic evidence of liver injury and repair/recovery following I/R. (A) CXCR2^{-/-} mice were injected intraperitoneally with saline (vehicle) or 30 mg/kg repertaxin after 24 hours of reperfusion and every 12 hours thereafter. Sham-operated mice displayed normal hepatic architecture. After 48 hours of reperfusion, livers from control mice had the usual degree of hepatocellular necrosis, whereas livers from mice treated with repertaxin had larger areas of hepatocellular necrosis. After 96 hours of reperfusion, control mice had significantly improved livers with small necrotic areas. However, large necrotic areas remained in the livers from mice treated with repertaxin. Original magnification was 50 $\times$. Morphometric quantitation demonstrated significantly increased necrosis in repertaxin-treated mice after 48 and 96 hours of reperfusion. Data are mean $\pm$ SEM with n = 5 per group. * P < 0.05 compared to vehicle-treated mice. (B) Sham-operated wild-type and CXCR1^{-/-} mice displayed normal hepatic architecture. After 48 hours of reperfusion, livers from both wild-type and CXCR1^{-/-} mice had large areas of necrosis. After 96 hours of reperfusion, wild-type mice seemed to have smaller areas of necrosis compared to CXCR1^{-/-} mice, but quantitative morphometric analysis showed no significant difference in overall necrotic area between wild-type and CXCR1^{-/-} mice after 48 or 96 hours of reperfusion. Data are mean $\pm$ SEM with n = 5 per group.

Figure 7. Effects of delayed CXCR1 blockade or CXCR1-knockout on hepatocyte growth factor (HGF) expression and hepatocyte proliferation after I/R. (A) CXCR2^{-/-} mice were injected intraperitoneally with saline (vehicle) or 30 mg/kg repertaxin after 24 hours of reperfusion and every 12 hours thereafter. (B) Wild-type and CXCR1^{-/-} mice were subjected to hepatic I/R. Liver HGF levels were measured using tissue ELISA. Hepatocyte proliferation in ischemic liver was determined by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and quantitative analysis of PCNA labeling was performed. Data are mean $\pm$ SEM with $n = 4-12$ per group. * $P < 0.05$ compared to control or wild-type mice.

Figure 8.

Effects of CXCR1-knockout on activation and phagocytosis in liver-recruited neutrophils. Wild-type and CXCR1^{-/-} mice were subjected to hepatic I/R. Twenty-four hours after reperfusion, neutrophils from liver were isolated. (A) Neutrophil activation was determined by cell surface expression of CD11b. (B) Phagocytosis of fluorescently labeled *E. coli*. Data are mean $\pm$ SEM with n =5 per group.