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Short Communication

IL-13 Activates STAT6 and Inhibits Liver Injury Induced by Ischemia/Reperfusion

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Hepatic ischemia/reperfusion injury is initiated by the activation of Kupffer cells and their subsequent release of proinflammatory mediators, including tumor necrosis factor-α (TNFα). These mediators stimulate a cascade of events including up-regulation of CXC chemokines and vascular endothelial adhesion molecules, leading to hepatic neutrophil recruitment and tissue injury. Interleukin-13 (IL-13) is a cytokine that has been shown to suppress macrophage production of proinflammatory mediators. The objective of the current study was to determine whether IL-13 could regulate the liver inflammatory injury induced by ischemia and reperfusion. C57BL/6 mice underwent 90 minutes of partial hepatic ischemia followed by reperfusion with or without intravenous administration of recombinant murine IL-13. Hepatic ischemia/reperfusion increased expression of TNFα and macrophage inflammatory protein-2 (MIP-2), leading to hepatic neutrophil recruitment, hepatocellular injury, and liver edema. Administration of IL-13 reduced the production of TNFα and MIP-2 mRNA and protein. IL-13 suppressed liver neutrophil recruitment by up to 72% and hepatocellular injury and liver edema were each reduced by >60%. Administration of IL-13 had no effect on liver NFκB activation, but greatly increased the activation of STAT6. The data suggest that the hepatoprotective effects of IL-13 may be a result of STAT6 activation. ***(Am J Pathol 1999, 155:1059–1064)***

Liver injury caused by hepatic ischemia and reperfusion is a complication of hepatic resectional surgery, liver transplantation, and hemorrhagic shock with fluid resuscitation.[1–4] Experimental models of hepatic ischemia/reperfusion (I/R) injury have defined two distinct phases during the development of organ injury. During the initial phase, Kupffer cells are activated and release reactive oxygen species and proinflammatory cytokines, including tumor necrosis factor-α (TNFα).[5–7] The later phase of injury is dependent upon neutrophils. Enhanced hepatic production of TNFα plays a critical role in the initiation of a mediator cascade responsible for hepatic recruitment of neutrophils and subsequent liver injury. TNFα up-regulates intercellular adhesion molecule-1 (ICAM-1) expression on the hepatic vascular endothelium.[8,9] In addition, TNFα induces the hepatic expression of CXC chemokines, including macrophage inflammatory protein-2 (MIP-2) and epithelial neutrophil-activating protein-78 (ENA-78).[10,11] The combined effects of ICAM-1 and CXC chemokines result in adhesion and transmigration of neutrophils from the intravascular space to liver parenchyma. Neutrophil accumulation in hepatic sinusoids obstructs blood flow and contributes to hepatic hypoperfusion.[12] The ensuing liver injury is caused by neutrophil-derived oxidants and proteases, which directly damage endothelial cells and hepatocytes.[13]

Interleukin-13 (IL-13) was originally identified as a product of activated Th2 cells which suppressed *in vitro* monocyte production of proinflammatory cytokines, including TNFα, IL-1, IL-6, IL-8, and MIP-1α.[14–17] *In vivo*, IL-13 prevents lipopolysaccharide-induced lethality and IgG immune complex-induced lung injury.[18–20] The mechanism(s) of the anti-inflammatory effects of IL-13 have been linked with inhibition of the transcription factor nuclear factor κB (NFκB).[21,22] However, *in vitro*, IL-13 potently activates the transcription factor, signal transducer and activator of transcription 6 (STAT6).[23,24] In the current studies, we evaluated the effects of IL-13 on the activation state of NFκB and STAT6 in liver during I/R injury and assessed whether these effects of IL-13 precluded development of liver inflammatory injury induced by hepatic ischemia and reperfusion.

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Materials and Methods

Hepatic Ischemia/Reperfusion Injury Model

Male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) weighing 22–28 g were used in all experiments. Partial hepatic ischemia was induced as described previously.[10] Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.). Mice received either sterile saline or recombinant murine IL-13 (1 μg; R&D Systems Inc., Minneapolis, MN) via the lateral tail vein before the induction of ischemia. A midline laparotomy was performed and an atraumatic clip was used to interrupt blood supply to the cephalad lobes of the liver. After 90 minutes of partial hepatic ischemia, mice again received either sterile saline or IL-13 (1 μg) via the lateral tail vein, and the clip was removed, initiating hepatic reperfusion. Sham control mice underwent the same protocol, but without vascular occlusion. Mice were sacrificed after 4 hours of reperfusion, and liver tissues and blood samples were taken for analysis. This project was approved by the University of Louisville Animal Care and Use Committee and was in compliance with National Institutes of Health guidelines.

Electrophoretic Mobility Shift Assay

Nuclear extracts of liver tissue were prepared by the method of Deryckere and Gannon[25] and analyzed by electrophoretic mobility shift assay (EMSA). Briefly, double-stranded NFκB consensus oligonucleotide (Promega, Madison, WI) or STAT6 consensus oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA) were end-labeled with $\gamma[^{32}$ P] ATP (3,000 Ci/mmol at 10 mCi/ml, Amersham, Arlington Heights, IL). Binding reactions containing equal amounts of nuclear protein extract (10 μg) and 35 fmol ($\sim$50,000 cpm, Cherenkov counting) of oligonucleotide were incubated at room temperature for 30 minutes. Reaction volumes were held constant at 15 μl. For competition and supershift assays of STAT6 binding, unlabeled NFκB or STAT6 oligonucleotide (50-molar excess) or antibodies to STAT4 or STAT6 (Santa Cruz Biotechnology) were added to the reaction mixtures. Binding reaction products were separated in a 4% polyacrylamide gel and analyzed by autoradiography.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from liver tissue was extracted using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). RNA (1 μg) was reverse transcribed to cDNA using random hexamers. cDNA products were coamplified by PCR (30 cycles of 95°C for 60 seconds, 59°C for 90 seconds, and 72°C for 10 seconds). Primers for TNFα (446-bp product), MIP-2 (205-bp product), and β-actin (245-bp product) have been described elsewhere.[26] PCR products were electrophoresed in a 3.5% agarose gel, stained with ethidium bromide, and photographed.

Liver Neutrophil Accumulation

Liver myeloperoxidase (MPO) content was determined by methods described elsewhere.[10] Briefly, liver tissue (50 mg) was homogenized, sonicated, and centrifuged for 20 minutes at 10,000 $\times$ *g*. Supernatants were reacted with 3,3′, 3,5′-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) and read at 655 nm.

Liver neutrophil morphometric analysis was performed on frozen liver sections fixed in 0.5% glutaraldehyde and stained with hematoxylin and eosin. The number of neutrophils were counted in 5 separate high-power fields for each liver section. The results are expressed as neutrophils/high-power field.

Liver Edema

The extent of liver edema was measured by tissue wet-to-dry weight ratios. After dissection, liver samples were weighed and placed in a drying oven at 55°C until a constant weight was obtained. In this determination, liver edema is represented by an increase in the wet-to-dry weight ratios.

Blood Analyses

Blood was obtained by cardiac puncture at the time of sacrifice. Serum was analyzed for TNFα and MIP-2 by enzyme-linked immunosorbent assays (ELISA) according to manufacturer's instructions (BioSource International, Camarillo, CA). Serum was also analyzed for alanine aminotransferase (ALT) as an indicator of hepatocellular injury. Measurements of serum ALT were made using a diagnostic kit from Sigma Chemical Co.

Statistical Analyses

All data are expressed as mean $\pm$ SE. Data were analyzed with a one-way analysis of variance with subsequent Student-Newman-Keuls test. Differences were considered significant when $P < 0.05$. To calculate percentage change, negative control values were subtracted from positive control and treatment group values.

Results

Effects of IL-13 on the Activation of NFκB and STAT6 in Liver

Because IL-13 is known to suppress activation of NFκB,[21] and because IL-13-induced activation of STAT6 is associated with anti-inflammatory effects of this cytokine,[24] we sought to determine whether IL-13 altered activation of NFκB and/or STAT6 in the liver during I/R injury. Nuclear extracts from liver tissue obtained from sham controls and livers undergoing ischemia and 1 or 4 hours of reperfusion were analyzed by EMSA. NFκB activation was greatly increased in liver after hepatic ischemia and 1 or 4 hours of reperfusion, compared to that in

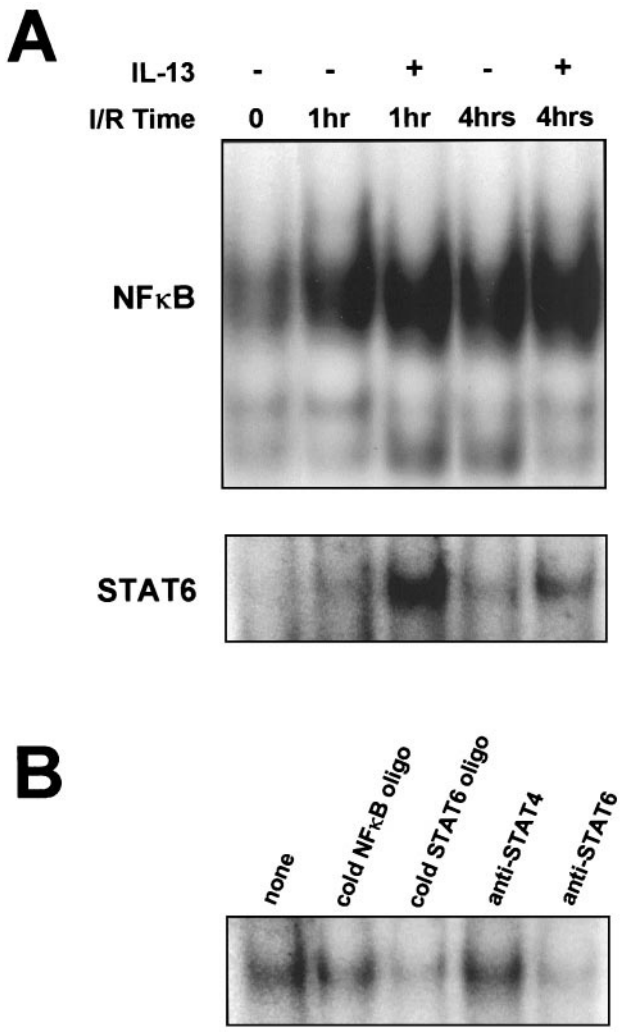

Figure 1. Effects of IL-13 on activation of NFκB and STAT6 in liver during hepatic ischemia/reperfusion (I/R) injury. A: EMSA analysis of NFκB and STAT6 in whole liver nuclear extracts from sham controls (I/R time: 0), ischemia and 1 hour reperfusion, and ischemia and 4 hours reperfusion in the presence or absence of IL-13. B: Competition and supershift assays were performed to verify the specificity of the STAT6 consensus oligonucleotide probe. Whole liver extracts obtained from mice treated with IL-13 and undergoing ischemia and 1 hour of reperfusion were incubated with ^{32}P-labeled STAT6 oligonucleotide in the absence (none) or presence of unlabeled (cold) NFκB oligonucleotide, unlabeled (cold) STAT6 oligonucleotide, or antibodies to STAT4 or STAT6.

liver from sham controls (Figure 1A). The administration of IL-13 had no effect on NFκB activation at either time point. In contrast, hepatic ischemia and reperfusion did not result in detectable increases in STAT6 activation (Figure 1A). However, intravenous treatment with IL-13 resulted in a large increase in activation of STAT6 after 1 hour of reperfusion. IL-13-induced STAT6 activation was also present after 4 hours of reperfusion.

The specificity of the STAT6 consensus oligonucleotide probe was confirmed by experiments using nuclear extracts from liver undergoing ischemia and 1 hour of reperfusion (Figure 1B). In DNA binding reactions, liver nuclear extracts that were incubated with only ^{32}P-labeled STAT6 consensus oligonucleotide probe showed typical binding to the labeled oligonucleotide (Figure 1B). Competition with 50-fold molar excess of unlabeled NFκB oligonucleotide had no effect on DNA binding to the STAT6 oligonucleotide. Competition with excess unlabeled STAT6 oligonucleotide completely abolished DNA binding to the labeled STAT6 oligonucleotide. Addition of polyclonal rabbit anti-STAT6, but not anti-STAT4, resulted in almost complete disappearance of the STAT6 band. These data demonstrate that IL-13 administration causes hepatic activation of STAT6.

Effects of IL-13 on Production of TNFα and MIP-2

To further investigate the effects of IL-13, including activation of STAT6, on the development of liver injury induced by I/R, we assessed whether IL-13 administration altered the generation of TNFα and MIP-2 mRNA in liver tissue. Liver RNA extracts were analyzed by RT-PCR. Hepatic ischemia and 4 hours of reperfusion resulted in large increases in the amount of TNFα (Figure 2A) and MIP-2 mRNA (Figure 2B). Administration of IL-13 greatly reduced the generation of TNFα and MIP-2 mRNA in liver tissue. Results shown are representative of three independent experiments.

In this model, serum levels of TNFα and MIP-2 have been shown to be closely associated with the hepatic production of these mediators.[6,7,10] ELISA analysis of serum demonstrated that TNFα was significantly increased after ischemia and 4 hours of reperfusion (Figure 2C). Administration of IL-13 reduced the amount of TNFα in serum by 75% ($P < 0.001$). Serum levels of MIP-2 were also increased after hepatic ischemia and reperfusion (Figure 2D). In the presence of IL-13, serum levels of MIP-2 were decreased by 45% ($P = 0.011$).

Effects of IL-13 on Hepatic Neutrophil Accumulation and Inflammatory Injury

Because TNFα and MIP-2 have been shown to be critical mediators for the hepatic recruitment of neutrophils, we assessed whether the suppressive effects of IL-13 on TNFα and MIP-2 production were associated with reduced liver accumulation of neutrophils. Hepatic neutrophil accumulation was measured by liver MPO content. After hepatic ischemia and 4 hours of reperfusion there was a significant increase in liver MPO content compared to the sham controls (Figure 3A). In the presence of IL-13, liver MPO content was reduced by 72% ($P = 0.019$). Similarly, hepatic I/R caused a significant increase in the number of neutrophils in liver sections assessed by morphometric analysis (Figure 3B). The administration of IL-13 resulted in a 62% decrease in the number of neutrophils observed in liver sections ($P = 0.003$).

To investigate the effects of IL-13 on liver injury induced by hepatic ischemia/reperfusion, we assessed hepatocellular injury and liver edema in the presence or absence of IL-13. Hepatocellular injury was quantified by measuring serum levels of ALT and liver edema was determined by measuring liver wet-to-dry weight ratios.

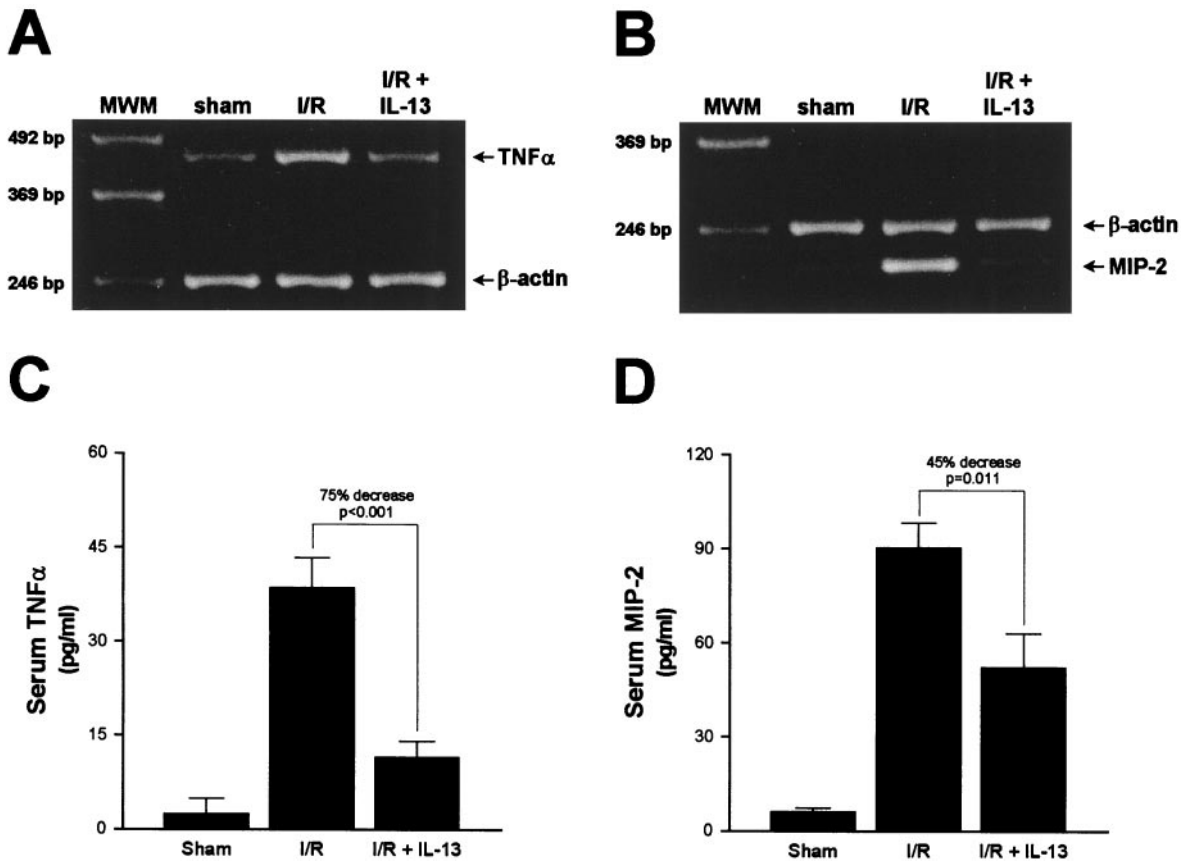

Figure 2. Effects of IL-13 on mRNA expression for TNFα (A) and MIP-2 (B) as well as serum levels of TNFα (C) and MIP-2 (D) protein. Liver RNA extracts and serum samples were obtained from sham control mice and mice undergoing ischemia and 4 hours of reperfusion treated with saline (I/R) or IL-13 (I/R + IL-13). Liver RNA extracts were assessed by RT-PCR (A and B). Results are representative of three independent experiments. Serum samples were analyzed by ELISA (C and D). Values represent mean ± SE with $n = 10$ per group.

After hepatic ischemia and 4 hours of reperfusion there were significant increases in hepatocellular injury compared to the sham controls (Figure 3C). In the presence of IL-13, serum ALT levels were decreased by 60% ($P = 0.002$). Significant liver edema was induced by hepatic ischemia and reperfusion (Figure 3D). Treatment with IL-13 reduced liver edema by 66% ($P = 0.004$).

Discussion

The pathogenesis of hepatic I/R injury begins with activation of Kupffer cells during ischemia and results in the subsequent release of proinflammatory cytokines.[5–7] These proinflammatory cytokines, including TNFα, then cause the up-regulation of CXC chemokines and adhesion molecules, leading to hepatic recruitment of neutrophils and ensuing inflammatory tissue injury.[7,9,11] The administration of IL-13 reduced I/R-induced production of TNFα and MIP-2. Reduction of these mediators resulted in reduced accumulation of neutrophils in the liver and limited the development of liver injury. To determine the mechanism(s) of the protective effects of IL-13, we assessed the effects of IL-13 on the activation of NFκB and STAT6 in the liver.

Although a distinct role for STAT6 in the inflammatory response has not been defined, it has recently been shown that STAT6 activation by IL-13 enhances the production of IL-1 receptor antagonist production in hepatocytes.[27] Furthermore, the anti-inflammatory activity of IL-13 is compromised in macrophages from STAT6-deficient mice.[24] In the current studies, hepatic ischemia and up to 4 hours of reperfusion did not result in detectable activation of STAT6 in liver. Similarly, others have reported that in a murine model of partial hepatectomy STAT6 is not activated in liver during or after liver resection.[28] However, we show that administration of IL-13 caused substantial activation of STAT6 within 1 hour after hepatic reperfusion. This activation was associated with greatly reduced production of TNFα and MIP-2, suppressed neutrophil accumulation, and decreased liver injury. Although our data provide evidence suggesting an anti-inflammatory role for STAT6, little is known about the manner in which this transcription factor may exert these effects. Activation of STAT6 has been reported to suppress TNFα-induced adhesion molecule expression by antagonizing the DNA-binding of NFκB.[29] More recently, it has been shown that STAT6 regulates the expression of FcεRI in mast cells[30] as well as an inhibitor of cyclin-

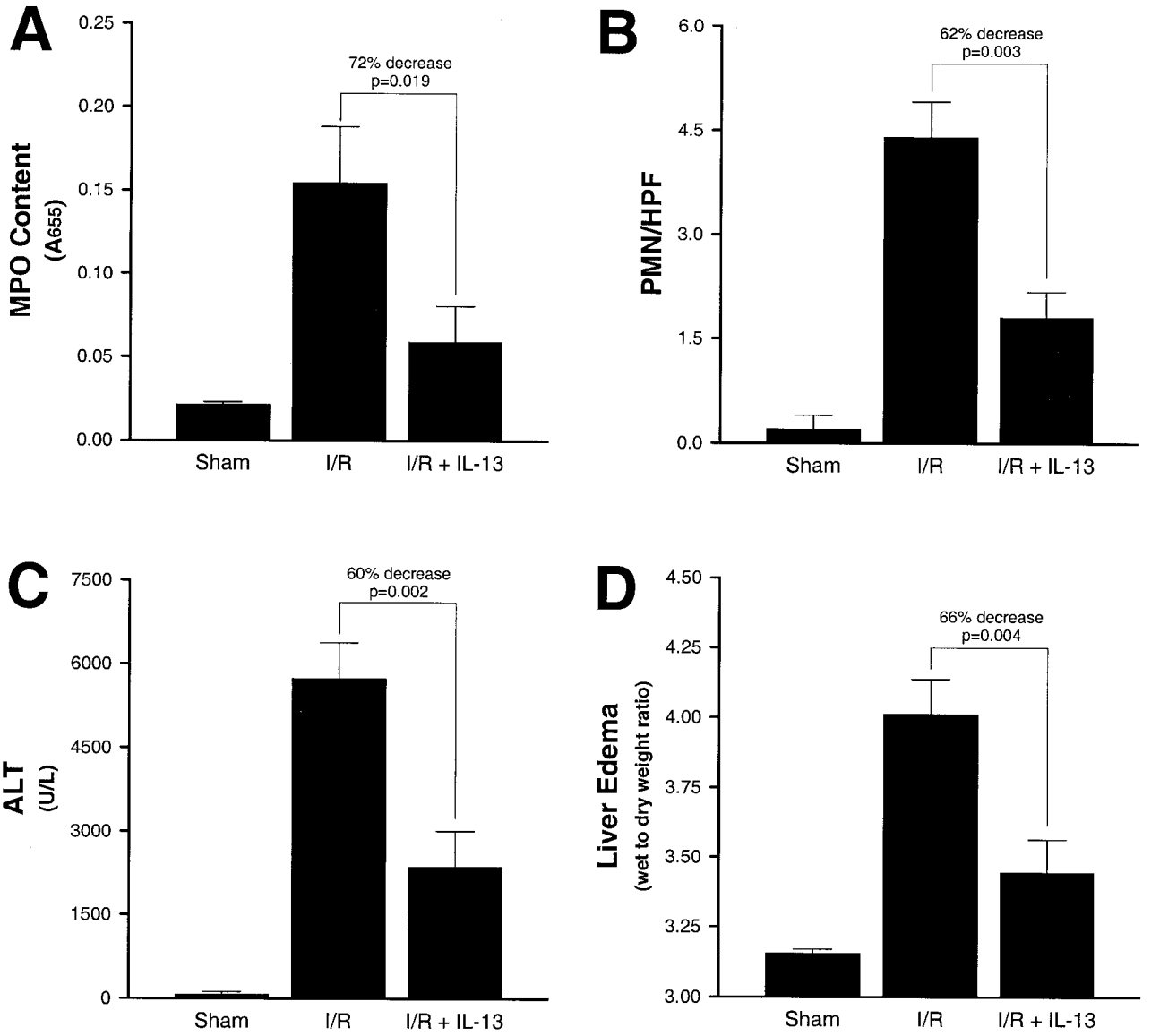

Figure 3. Effects of IL-13 on liver neutrophil accumulation (A and B), hepatocellular injury (C), and liver edema (D) in sham control mice and mice undergoing ischemia and 4 hours of reperfusion treated with saline (I/R) or IL-13 (I/R + IL-13). Liver neutrophil accumulation was determined by measuring liver myeloperoxidase (MPO) content (A), as well as by liver tissue neutrophil morphometrics (PMN/high power field; B). Hepatocellular injury was assessed by determining serum levels of alanine aminotransferase (ALT). Liver edema was quantitated by liver wet-to-dry weight ratios. Values represent mean ± SE with n = 10 per group.

dependent kinase, p27Kip1.[31] Thus, there is evidence that STAT6 may function as a negative regulator of transcription. Furthermore, a recent report demonstrated that STAT6 activation was required for IL-4-induced suppression of TNFα production in macrophages.[32] The precise mechanism by which STAT6 may suppress the production of proinflammatory mediators, such as TNFα and MIP-2, is currently unknown.

We have previously shown that IL-13 suppresses NFκB activation by preventing the degradation of the NFκB-inhibiting protein IκBα.[21] However, it was recently demonstrated that liver activation of NFκB during hepatic ischemia/reperfusion occurs without degradation of IκBα.[33] Furthermore, those studies showed that tyrosine phosphorylation of IκBα occurred, a mechanism of NFκB activation that does not require IκBα degradation.[34] Our current studies suggest that the protective effects of IL-13 in liver injury induced by I/R are unrelated to its effects on NFκB. The administration of IL-13 greatly suppressed proinflammatory cytokine and chemokine production, liver neutrophil recruitment and development of liver injury induced by ischemia and reperfusion. These data suggest that IL-13 may represent a potential therapeutic strategy for the treatment of inflammatory liver disease. The protective effects of IL-13 were preceded by activation of STAT6 in liver, suggesting that STAT6 may operate as a negative regulator of proinflammatory mediator production. Combined with the recent findings

of others,[29–32] these studies provide evidence for an antiinflammatory role for STAT6 *in vivo*. Further studies of the function of STAT6 should provide a better understanding of the role of this transcription factor in the regulation of acute inflammatory responses.

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