Regulation of Liver Inflammatory Injury by Signal Transducer and Activator of Transcription-6

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Liver injury induced by hepatic ischemia/reperfusion is characterized by activation of the transcription factor NF- κ B, increased production of tumor necrosis factor- α (TNF α), liver neutrophil accumulation, and hepatocellular damage. Exogenous administration of interleukin-4 (IL-4) or IL-13 was recently shown to regulate this inflammatory injury in association with activation of signal transducer and activator of transcription-6 (STAT6). The objective of the present study was to determine whether STAT6 was required for the regulation of liver inflammation by IL-4 and IL-13. Wild-type and STAT6 knockout mice underwent 90 minutes of hepatic ischemia followed by 8 hours of reperfusion. Hepatic ischemia/reperfusion in wild-type and STAT6 knockout mice significantly increased ($P < 0.05$) NF- κ B activation, serum levels of TNF α , liver accumulation of neutrophils [measured by myeloperoxidase (MPO) content], and hepatocellular damage [measured by serum alanine aminotransferase (ALT)] compared to sham controls. In wild-type mice, activation of STAT6 was not observed after ischemia/reperfusion. Administration of 1 μ g of IL-4 or IL-13 at reperfusion reduced serum TNF α , liver neutrophil accumulation, and hepatocellular injury in wild-type mice. Treatment with IL-4 or IL-13 had no effect on liver NF- κ B activation but significantly increased activation of STAT6. In STAT6 knockout mice, neither IL-4 nor IL-13 had any effect on TNF α , MPO, or ALT values, the regulatory effects of these cytokines being completely abolished. The data suggest that activation of STAT6 may regulate liver inflammatory injury. (*Am J Pathol* 2000, 157:297–302)

Acute inflammation of the liver, as may occur during infection, trauma, or ischemia/reperfusion injury associated with liver resectional surgery or transplantation of the liver, is characterized by an elaborate cascade of proinflammatory mediators. Experimental models of liver injury induced by ischemia/reperfusion have further delineated the events initiating and propagating the hepatic inflammatory response. These studies have demonstrated critical roles for proinflammatory cytokines,^{1,2}

CXC chemokines,^{3,4} and vascular cell adhesion molecules^{5,6} in the liver recruitment of neutrophils and subsequent hepatocellular injury. The transcription factor NF- κ B is a major regulator of the gene expression of all of these mediators.⁷ Recent reports have suggested that activation of NF- κ B may be an essential component in the development of the hepatic inflammatory response.⁸ Other studies have shown that blockade of NF- κ B activation may result in reduced proinflammatory mediator production and reduced liver inflammation *in vivo*.^{9,10}

The Th2 cytokines, interleukin-4 (IL-4) and IL-13, are known to modulate inflammatory responses in part by down-regulating the production of proinflammatory mediators. Consistent with these functions, we recently demonstrated that IL-4 and IL-13 protect against liver inflammatory injury induced by ischemia/reperfusion by preventing production of proinflammatory mediators, including tumor necrosis factor- α (TNF α) and macrophage inflammatory protein-2 (MIP-2).^{11,12} Gene expression of TNF α and MIP-2 is controlled, at least in part, by NF- κ B.⁷ However, in previous studies we found that IL-4- and IL-13-mediated reductions in hepatic production of TNF α and MIP-2 mRNA and protein occurred without suppression of NF- κ B nuclear translocation or DNA binding.^{11,12} These findings suggested that the hepatoprotective properties of IL-4 and IL-13 may be unrelated to effects on NF- κ B activation.

Signal transducer and activator of transcription-6 (STAT6) is another rapidly activated transcription factor that is known to be critical for the immunoregulatory functions of IL-4 and IL-13 on macrophages *in vitro*.^{13,14} STAT6 is present in the cytoplasm in an unphosphorylated, monomeric form. Binding of IL-4 or IL-13 to their cognate receptors results in phosphorylation of STAT6 by receptor-associated members of the Janus kinase family.¹⁵ Phosphorylated STAT6 proteins dimerize and are then able to translocate into the nucleus and bind specific target regions of DNA. We found previously that treatment with IL-4 or IL-13 results in rapid activation (nuclear translocation) of STAT6 in liver during inflammation induced by ischemia/reperfusion.^{11,12} In the present

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studies, we sought to determine the role of STAT6 in the regulation of the acute inflammatory response in liver.

Materials and Methods

Model of Hepatic Ischemia and Reperfusion Injury

Male BALB/c (wild-type controls) and BALB/c-Stat6^{tm1Gru} (STAT6 $-/-$) mice (6–8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Partial hepatic ischemia was induced as described previously.⁴ Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal). A midline laparotomy was performed, and an atraumatic clip was used to interrupt the blood supply to the cephalad lobes of the liver. After 90 minutes of partial hepatic ischemia, mice received either sterile saline or 1 μ g recombinant murine IL-4 or IL-13 (R&D Systems, Minneapolis, MN) via the lateral tail vein, and the vascular clip was removed, initiating hepatic reperfusion. Sham control mice underwent the same protocol, but without vascular occlusion. Mice were sacrificed after 1, 4, or 8 hours of reperfusion, and blood and liver tissues were taken for analysis. This project was approved by the University of Louisville Animal Care and Use Committee and was in compliance with the National Institutes of Health guidelines.

Electrophoretic Mobility Shift Assay

Nuclear extracts of liver tissue were prepared by the method of Deryckere and Gannon¹⁶ and analyzed by electrophoretic mobility shift assay (EMSA). Briefly, double-stranded NF- κ B consensus oligonucleotide (Promega, Madison, WI) or STAT6 consensus oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, CA) was end-labeled with [γ -³²P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham, Arlington Heights, IL). Binding reactions containing equal amounts of nuclear protein extract (20 μ g) 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 μ l. Reaction products were separated in a native 4% polyacrylamide gel and analyzed by autoradiography. Autoradiographs were digitized, and activation of NF- κ B and STAT6 was quantitated with image analysis software (Adobe Systems, San Jose, CA).

Reverse Transcription-Polymerase Chain Reaction

Total RNA from liver tissue was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA). RNA (1 μ g) was reverse transcribed to cDNA, using random hexamers. cDNA products were coamplified by polymerase chain reaction (PCR) (30 cycles; 95°C for 60 seconds, 59°C for 90 seconds, and 72°C for 10 seconds). Primers for TNF α (446-bp product) and β -actin (245-bp product) have been described elsewhere.⁹ PCR products were electro-

phoresed in a 2% agarose gel, stained with ethidium bromide, and photographed. Digitized photographs were assessed using image analysis software (Adobe Systems). Relative TNF α mRNA expression was determined from the ratio of band intensities and presented graphically as percentage β -actin.

Myeloperoxidase Assay

Liver myeloperoxidase (MPO) content was assessed by methods similar to those of Schierwagen et al.¹⁷ Liver tissue (100 mg) was homogenized in 2 ml of buffer A (3.4 mmol/L KH₂HPO₄, 16 mmol/L Na₂HPO₄, pH 7.4). After centrifugation for 20 minutes at 10,000 $\times g$, the pellet was resuspended in 10 volumes of buffer B (43.2 mmol/L KH₂HPO₄, 6.5 mmol/L Na₂HPO₄, 10 mmol/L EDTA, 0.5% hexadecyltrimethylammonium, pH 6.0) and sonicated for 10 seconds. After heating for 2 hours at 60°C, the supernatant was reacted with 3,3',3,5'-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO), and the optical density was determined at 655 nm.

Blood and Tissue Analysis

At the time of sacrifice, blood was obtained from the inferior vena cava for analysis of serum alanine aminotransferase (ALT) as an index of hepatocellular injury, using a commercially available diagnostic kit (Sigma Chemical Co.). Serum levels of TNF α were measured by sandwich enzyme-linked immunosorbent assay with antibodies to mouse TNF α purchased from R & D Systems. Liver and lung edema was determined by organ wet-to-dry weight ratios.

Statistical Analysis

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

Results

Modulation of STAT6 Activation by IL-4 and IL-13 during Hepatic Ischemia/Reperfusion Injury

To assess the effects of IL-4 and IL-13 on the activation of STAT6 and NF- κ B in liver after hepatic ischemia and reperfusion in BALB/c mice, either saline, IL-4, or IL-13 was administered intravenously just before reperfusion. Liver nuclear extracts were obtained over an 8-hour time course of ischemia/reperfusion injury and analyzed by EMSA. Nuclear translocation of STAT6 was quantitated by image analysis of autoradiographs. Activation of STAT6 in livers from saline-treated mice was not observed at any time (Figure 1). However, treatment with either IL-4 or IL-13 resulted in significant increases in STAT6 activation within 1 hour of reperfusion. These effects persisted throughout the 8-hour time course stud-

Figure 1. Effects of IL-4 and IL-13 on activation of STAT6 in liver during hepatic ischemia/reperfusion injury. **A:** EMSA analysis of STAT6 in liver nuclear extracts from sham controls, 90 minutes of ischemia, and 1, 4, or 8 hours reperfusion from mice treated with saline, IL-4, or IL-13. **B:** Autoradiographs were digitized and quantitated by image analysis.

Figure 2. Effects of IL-4 and IL-13 on the activation of NF-κB in liver during hepatic ischemia/reperfusion injury. **A:** EMSA analysis of NF-κB in liver nuclear extracts from sham controls, 90 minutes of ischemia, and 1, 4, or 8 hours reperfusion from mice treated with saline, IL-4, or IL-13. **B:** Autoradiographs were digitized and quantitated by image analysis.

ied. Similar to our earlier reports,^{11,12} NF-κB activation was markedly increased 1, 4, and 8 hours after reperfusion in mice treated with saline (Figure 2). Treatment with IL-4 or IL-13 had no effect on nuclear translocation or DNA binding of NF-κB.

STAT6 Is Required for Suppression of Hepatic TNFα Expression by IL-4 and IL-13

Because both IL-4 and IL-13 reduce TNFα production *in vitro* and *in vivo*,^{11,12,18} and because we have recently shown that suppressed TNFα production in hepatic ischemia/reperfusion injury by IL-4 or IL-13 is associated with increased STAT6 activation,^{11,12} we assessed whether

STAT6 was required for IL-4- and IL-13-mediated suppression of TNFα production in this model. Liver RNA extracts from wild-type mice and STAT6 ^{-/-} mice were analyzed by reverse transcriptase-polymerase chain reaction for expression of TNFα mRNA. As shown in Figure 3, TNFα mRNA expression was greatly increased in both wild-type and STAT6 ^{-/-} mice after hepatic ischemia and 8 hours of reperfusion ($P < 0.001$ compared to sham controls). Treatment with IL-4 or IL-13 significantly reduced the expression of TNFα mRNA in wild-type mice. However, in STAT6 ^{-/-} mice, neither IL-4 nor IL-13 had any effect on hepatic ischemia/reperfusion-induced TNFα mRNA expression. Similar results were obtained when serum levels of TNFα were measured by ELISA. In

Figure 3. Effects of IL-4 and IL-13 on TNFα mRNA expression in wild-type and STAT6^{-/-} mice. Liver RNA extracts were obtained from sham control mice and mice undergoing ischemia and 8 hours of reperfusion treated with saline, IL-4, or IL-13. **A:** Liver RNA extracts were assessed by reverse transcriptase-polymerase chain reaction. **B:** Ethidium bromide-stained gels were digitized and analyzed using computer software. TNFα mRNA expression is presented as percentage β-actin. Values are mean ± SEM with *n* = 3–6 per group.

both wild-type mice and STAT6^{-/-} mice, hepatic ischemia/reperfusion caused a significant increase in the circulating levels of TNFα (*P* < 0.001 compared to sham controls; Figure 4). In wild-type mice, treatment with IL-4 or IL-13 caused significant reductions in serum levels of TNFα. In contrast, neither IL-4 nor IL-13 had any effect on serum TNFα in STAT6^{-/-} mice.

Protective Effects of IL-4 and IL-13 Are Absent in STAT6^{-/-} Mice

We have recently shown that IL-4 and IL-13 protect against hepatic ischemia/reperfusion injury in association with STAT6 activation in liver.^{11,12} To determine whether STAT6 was required for the protective effects of IL-4 and IL-13, we assessed the ability of IL-4 and IL-13 to suppress liver neutrophil recruitment and hepatocellular injury in wild-type and STAT6^{-/-} mice. Neutrophil recruitment into liver was determined by the content of myeloperoxidase (MPO) in liver homogenates. In both wild-type and STAT6^{-/-} mice, ischemia and 8 hours of reperfusion resulted in significant increases in liver MPO content compared to sham controls (*P* < 0.001; Figure 5). Treatment of wild-type mice with IL-4 or IL-13 greatly reduced the amount of liver neutrophil accumulation. However, in STAT6^{-/-} mice, treatment with IL-4 or IL-13 had no effect on the hepatic recruitment of neutrophils.

Figure 4. Effects of IL-4 and IL-13 on plasma levels of TNFα in wild-type and STAT6^{-/-} mice. Serum samples were obtained from sham control mice and mice undergoing ischemia and 8 hours of reperfusion treated with saline (I/R), IL-4 (I/R + IL-4), or IL-13 (I/R + IL-13). Serum samples were analyzed by enzyme-linked immunosorbent assay. Values are mean ± SEM with *n* = 5–10 per group.

Hepatocellular injury was assessed by measuring serum levels of ALT. In both wild-type and STAT6^{-/-} mice, hepatic ischemia/reperfusion induced significant hepatocellular injury (*P* < 0.001 compared to sham controls; Figure 6A). In wild-type mice, administration of IL-4 or IL-13 caused marked reductions in serum ALT. In contrast, in STAT6^{-/-} mice, IL-4, and IL-13 had no effect on hepatocellular injury. Similar data were obtained when liver wet-to-dry weight ratios were measured. Both wild-type and STAT6^{-/-} mice had significantly greater ratios than sham controls (*P* < 0.001; Figure 6B). Treatment of wild-type mice with IL-4 or IL-13 greatly attenuated the increase in liver wet-to-dry weight ratios, whereas treatment of STAT6^{-/-} mice with IL-4 or IL-13 had no effect.

Discussion

While a great deal has been learned about the cellular and molecular mechanisms leading to the induction and propagation of acute liver inflammation after ischemia and reperfusion, much less is known about the factors involved in the regulation of this process. Our previous studies have shown that endogenous mediators, including IL-10 and secretory leukocyte protease inhibitor, regulate the acute hepatic inflammatory response by suppressing activation of NF-κB.^{9,19} Other studies have shown that IL-4 and IL-13 prevent liver inflammatory injury by an NF-κB-independent mechanism.^{11,12} The present studies provide evidence that activation of the transcription factor STAT6 is required for the regulation of liver inflammation by IL-4 and IL-13.

In wild-type mice, administration of IL-4 or IL-13 rapidly activated STAT6 in liver and suppressed TNFα mRNA and protein expression. These effects were completely lost in STAT6 knockout mice. Because the gene for TNFα

Figure 5. Effects of IL-4 and IL-13 on liver neutrophil accumulation in wild-type and STAT6^{-/-} mice. Liver myeloperoxidase (MPO) content was measured in sham control mice and mice undergoing ischemia and 8 hours of reperfusion treated with saline (I/R), IL-4 (I/R + IL-4), or IL-13 (I/R + IL-13). Values are mean $\pm$ SEM with $n = 6-8$ per group.

lacks a STAT6 binding site, it is unlikely that STAT6 directly represses TNF α gene transcription. However, STAT6 binds to the transcriptional coactivator p300, which is required for transcriptional activation by STAT6.^{20,21} p300 also binds to the RelA (p65) subunit of NF- κ B and has been shown to be necessary for NF- κ B-mediated gene transcription.^{22,23} p300 is present in the nucleus in limited quantities,²⁴ and thus it is possible that STAT6 competes with NF- κ B for p300 as a potential mechanism of transcriptional deactivation of NF- κ B. In this scenario NF- κ B, but not STAT6, would translocate to the nucleus of liver cells shortly after hepatic reperfusion in untreated mice. The NF- κ B binds to p300 and begins transcription of target genes, such as TNF α . However, when mice are treated with IL-4 or IL-13, there is nuclear translocation of STAT6, which can compete with NF- κ B for p300. Decreased availability of p300 to NF- κ B may result in decreased transcriptional activation. Our attempts to investigate the competitive interactions between p300 and NF- κ B or STAT6 have been limited by an inability of commercially available antibodies to immunoprecipitate murine p300. However, the role of p300 in the regulation of NF- κ B transcriptional activation has been demonstrated in studies involving the tumor suppressor p53.^{25,26} These reports showed that p53 inhibited the transcriptional activation of NF- κ B by outcompeting NF- κ B for binding to p300. Our current data suggest, but do not prove, that a similar relationship may exist between STAT6 and NF- κ B.

The fact that STAT6 was not appreciably activated by ischemia/reperfusion may suggest that STAT6 is not involved in the endogenous mechanisms that serve to regulate acute liver inflammation. In addition, STAT6 knock-out mice were indistinguishable from wild-type mice in their response to hepatic ischemia/reperfusion; there were no differences in TNF α expression, neutrophil ac-

Figure 6. Effects of IL-4 and IL-13 on liver injury in wild-type mice and STAT6^{-/-} mice. **A:** Serum levels of alanine aminotransferase (ALT) were measured in sham control mice and mice undergoing ischemia and 8 hours of reperfusion treated with saline (I/R), IL-4 (I/R + IL-4), or IL-13 (I/R + IL-13). Values are mean $\pm$ SEM with $n = 4-7$ per group. **B:** Liver edema was quantitated by liver wet-to-dry weight ratios. Values are mean $\pm$ SEM with $n = 5-10$ per group.

cumulation, or hepatocellular injury. Whether the expression of IL-4 and/or IL-13 is up-regulated during hepatic ischemia/reperfusion injury is currently under investigation. Based on the present data demonstrating that STAT6 is not activated after ischemia/reperfusion, it would seem unlikely that endogenous production of IL-4 or IL-13 is a relevant component of the hepatic regulatory machinery.

Functional knowledge of STAT6 has primarily been associated with Th2 cell development and immunoglobulin class switching to IgE.^{27,28} More recent studies have suggested that activation of STAT6 may be involved in the suppression of proinflammatory mediator expression in macrophages.²⁹⁻³⁰ Our *in vivo* data demonstrating a requirement for STAT6 for IL-4- and IL-13-mediated suppression of TNF α expression and development of liver

inflammatory injury identify a novel function for STAT6 as a regulator of the acute inflammatory response. The precise mechanism by which STAT6 confers these effects requires further investigation, but the present findings suggest that development of pharmacological agents that activate STAT6, or use of IL-4 and/or IL-13, may represent plausible therapeutic strategies for the treatment of inflammatory liver disease.

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