Intrahepatic STAT-3 activation and acute phase gene expression predict outcome after CLP sepsis in the rat

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Interleukin-6 (IL-6) regulates hepatic acute phase responses by activating the transcription factor signal transducer and activator of transcription (STAT)-3. IL-6 also may modulate septic pathophysiology. We hypothesize that 1) STAT-3 activation and transcription of α₂-macroglobulin (A2M) correlate with recovery from sepsis and 2) STAT-3 activation and A2M transcription reflect intrahepatic and not serum IL-6. Nonlethal sepsis was induced in rats by single puncture cecal ligation and puncture (CLP) and lethal sepsis via double-puncture CLP. STAT-3 activation and A2M transcription were detected at 3–72 h and intrahepatic IL-6 at 24–72 h following single-puncture CLP. All were detected only at 3–16 h following double-puncture CLP and at lower levels than following single-puncture CLP. Loss of serum and intrahepatic IL-6 activity after double-puncture CLP correlated with mortality. Neither intrahepatic nor serum IL-6 levels correlated with intrahepatic IL-6 activity. STAT-3 activation following single-puncture CLP inversely correlated with altered transcription of gluconeogenic, ketogenic, and ureagenic genes. IL-6 may have both beneficial and detrimental effects in sepsis. Fulminant sepsis may decrease the ability of hepatocytes to respond to IL-6.

Clinical data, however, are inconclusive. One study has demonstrated a link between serum IL-6 levels and the development of SIRS/MODS. In most investigations in sepsis indicate highly variable IL-6 levels. In the absence of single-puncture CLP experimental intra-abdominal sepsis, IL-6 is essential for recovery and liver regeneration following partial hepatectomy. This cytokine might also be important in the prevention of or recovery from sepsis-induced hepatic dysfunction.

The lack of correlation between serum IL-6 levels and outcome from SIRS/MODS may reflect the fact that cytokines can affect target cells via three mechanisms: 1) autocrine, affecting the secreting cell; 2) paracrine, affecting a nearby cell; and 3) endocrine, affecting a remote cell. In a nonlethal animal model of sepsis, we have recently shown that tumor necrosis factor-α (TNF-α)-dependent transcription factor activation and acute phase gene transcription did not correlate with serum TNF-α levels. Rather, intrahepatic processes appeared to be mediated by Kupffer-endothelial cell-derived TNF-α (1) and correlated most directly with activation of a TNF-linked transcription factor, nuclear factor-κB, and transcription of the TNF-linked acute phase reactant α₂-acid glycoprotein. Thus to determine the exact role played by IL-6 in sepsis-induced hepatic dysfunction it would be useful to examine activation of STAT-3 and transcription of A2M.

In this investigation we examine the role of IL-6 in single- and double-puncture CLP. We postulate that following the nonlethal insult of single-puncture CLP, activation of STAT-3 and transcription of A2M will persist. In contrast, these processes will decrease following double-puncture CLP (fulminant sepsis), a disorder characterized by progressive hepatic dysfunction and death. Furthermore, we hypothesize that intrahepatic IL-6 abundance will be more predictive of STAT-3 activation and A2M transcription than serum levels.

METHODS

Induction of sepsis. All animal studies conform to National Institutes of Health standards for the use of laboratory animals. Overnight-fasted male Sprague-Dawley rats (Charles River, Boston, MA) weighing between 250 and 270 g were used in all experiments. The induction of nonfulminant sepsis was performed under methoxyflurane anesthesia using cecal
ligation with a single, 18-gauge puncture (CLP) as previously described (14). Fulminant sepsis similarly was induced by ligation of the cecum and then puncturing it twice (3, 31). Sham-operated controls were anesthetized and underwent laparotomy with cephalic manipulation but without cecal ligation or puncture. After surgery, animals were fluid resuscitated with 40 ml/kg of subcutaneously administered sterile saline and were given free access to water but not food. At 0, 3, 6, 16, 24, 48, and 72 h following single-puncture CLP or sham operation and at 0, 3, 6, 16, 24, and 48 h after double-puncture CLP, animals were reanesthetized with a 50 mg/kg intraperitoneal injection of pentobarbital. Vena caval blood was obtained, and liver tissue was either harvested for nuclear protein or nuclei, or the entire liver was perfusion fixed with 2% paraformaldehyde.

Isolation and preparation of nuclear extracts. Isolation of nuclear proteins was performed as previously described (14, 15). All procedures were performed at 4°C, and all buffers contained protease inhibitors and phosphatase inhibitors. Briefly, liver tissue was homogenized, nuclei were separated by ultracentrifugation, the nuclear pellet was lysed, and protein was isolated. Protein concentrations were determined using the Bradford method.

Nuclear extraction. Livers were perfused via the portal vein with PBS (pH 7.3) for 5 min to remove red cells. This was followed by perfusion for 5 min with 2% paraformaldehyde in PBS (pH 7.3) at a flow rate of 20 ml/min. The perfused liver was excised, cut into slices, and fixed in the paraformaldehyde solution for 2 h at 4°C. Slices were paraffin embedded and cut into 7-µm sections. Sections were adhered to poly-L-lysine-coated glass slides and dried overnight at 37°C.

Electrophoretic mobility shift assay and supershift analysis. Hepatic nuclear extraction and binding reactions were performed as previously described with minor modifications (11, 14, 15). Briefly, a preannealed HPLC-purified oligonucleotide from the serum-inducible-factor binding element in the c-fos promoter was end-labeled with [γ-32P]ATP and used as a probe for STAT-3 binding activity. An excess of probe was incubated with 2.5 µg of nuclear extract plus 1 µg polydodeoxyinosinic-deoxytidylic acid, a nonspecific DNA competitor, for 15 min at room temperature. Other samples were supershifted by the addition of 3 µl of STAT-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h after the addition of labeled oligonucleotide. In cold competition experiments, unlabeled oligonucleotide was incubated with nuclear extracts for 15 min before the addition of radiolabeled probe. Each sample was subjected to electrophoresis on a 5% nondenaturing polyacrylamide gel. Gels were then dried, and autoradiography was performed. Radiographic density of gels containing samples following sham operation, single-puncture CLP, and double-puncture CLP was determined via densitometry. Values at time 0 were arbitrarily set at unity, and other results were normalized to this value.

Transcription elongation analysis (nuclear run-on). Nuclei were isolated, and nuclear run-on was performed using a modification of a previously reported method (2). All procedures were performed at 4°C. Briefly, liver tissue was harvested, minced, homogenized in 10 mM Tris·HCl, pH 8.0, 0.3 M sucrose, 3 mM CaCl2, 2 mM magnesium acetate, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, filtered, and layered onto a cushion buffer containing 50 mM Tris·HCl, pH 8.0, 2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. Ultracentrifugation was performed at 100,000 g for 30 min. The pellet was resuspended in 25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, and 5 mM dithiothreitol, and stored at −120°C until use. Nitrocellulose-immobilized targets were prepared using 10 µg each of plasmids containing A2M and ATP synthase (positive control) cDNAs. Plasmids without inserts served as negative controls. Two hundred fifty microliters of 4× reaction mix (100 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM dithiothreitol, 300 mM KCl, and 20% glycerol) were combined with 1 mCi [32P]UTP, 125 µl 8× triphosphat mix (2.8 mM each ATP, GTP, and CTP; Boehringer Mannheim, Indianapolis, IN) and diluted to a volume of 500 µl to yield the 2× reaction mixture. For each reaction, 100 µl of thawed nuclei were added to an equal volume of 2× reaction mixture and incubated at room temperature for 30 min. The reaction was stopped by adding 2 µl of Dnase 1 (10,000 U/ml in 50% glycerol; Boehringer Mannheim) and incubated at 37°C for 30 min. Stop buffer (600 µl; 2% SDS, 7 M urea, 350 mM LiCl, 1 mM EDTA, and 10 mM Tris, pH 8.0), 1,000 µl/ml of proteinase K, and 100 µl of tRNA were added and the mixture was incubated at 40°C for 1 h. The mixture was phenol-chloroform extracted, trichloroacetic acid precipitated, and ethanol washed. The pellet was resuspended in 100 µl TES (10 mM Tris, 1 mM EDTA, and 0.5% SDS), added to 5 ml of hybridization solution (see above), and hybridized with the targets at 42°C for 48 h. Targets were washed with 2× saline sodium citrate (SSC; 0.03 M sodium citrate, pH 7.3, and 0.3 M NaCl) at 65°C for 1 h and then incubated with 10 mg/ml RNase A in 8× SSC at 37°C for 30 min. Targets were then washed with 2× SSC at 37°C for 1 h. Targets were autoradiographed for ~5 days at −80°C, and quantitative laser densitometry (Molecular Dynamics) was performed. Radiographic density was normalized to the density of ATP synthase. The normalized density at time 0 was arbitrarily set at unity, and means ± SD were determined.

Determination of IL-6 levels in vena caval blood. Determinations of IL-6 serum concentrations were performed using an ELISA kit specific for rat IL-6 (Biosource, Camarillo, CA). Plasma samples were assayed in duplicate. Immunohistochemistry of IL-6 on rat liver sections. Tissue fixation and preparation were performed as previously described (1). The primary incubation was for 2 h at room temperature using a goat anti-rat polyclonal antibody for IL-6 (Santa Cruz Biotechnology). The secondary incubation, also at room temperature, used a biotinylated anti-goat antibody (Vector Laboratories, Burlingame, CA) for 1 h. Chromogenic staining was performed with metal-enhanced diaminobenzidine substrate (Pierce, Rockford, IL) using immunoperoxidase methodology (New England Nuclear, Wilmington, DE).

Statistics. Statistical significance (P < 0.05) was determined using ANOVA for repeated measures with the Bonferroni test of post hoc significance.

RESULTS

Outcome. The clinical response differed among sham operation, single-puncture CLP, and double-puncture CLP. Sham-operated animals recovered from surgery uneventfully. After single-puncture CLP, rats developed mild signs of sepsis as previously described (2, 14). These included lethargy, decreases in spontaneous movement, and poor grooming that resolved by 48 h. In contrast, 8–10 h following double-puncture CLP, animals became severely ill (14). Water intake decreased, there was no spontaneous movement observed, eyes became encrusted, and grooming was absent. By 16 h animals were extremely lethargic and had diarrhea, piloerection, and tachypnea. These signs are characteristic of severe late sepsis.

Outcome.
There were no deaths following sham operation or single-puncture CLP but a significant number of animals died following double-puncture CLP. Six animals were killed following an overnight fast, 36 animals underwent sham operation, 36 underwent single-puncture CLP, and 40 underwent double-puncture CLP. The intention was to kill six animals in each group at each time point (3, 6, 16, 24, 48, and 72 h). No double-puncture animals died before 16 h, but mortality was 50% at 24 h, only three animals survived to 48 h, and none survived to 72 h. Overall mortality in the double-puncture CLP group was 70%. Four extra animals were needed in the double-puncture CLP group so that three animals could be studied at the 48-h time point.

STAT-3 activation in liver nuclear extracts via electrophoretic mobility shift assay. Activation of STAT-3, which is believed to correlate directly with intrahepatic IL-6 activity (11), was measured following sham operation, single-puncture CLP, and double-puncture CLP (Fig. 1). No STAT-3 DNA binding activity could be detected in time 0 samples. Low levels of STAT-3 binding (<10% of that detected following single-puncture CLP and <20% of that following double-puncture CLP) were detected 3 h following sham operation. Maximal STAT-3 DNA binding activity was detected 3 h after single-puncture CLP and double-puncture CLP but levels following single-puncture CLP were nearly twice those observed following double-puncture CLP. At all other time points the differences were equally more pronounced. At 6 and 16 h following double-puncture CLP, levels of STAT-3 activity reached a plateau, whereas levels following single-puncture CLP continued to decline. However, levels were 2.5-fold

![STAT-3 activation in liver nuclear extracts via electrophoretic mobility shift assay.](image-url)

**Fig. 1.** A: representative electrophoretic mobility shift assay (EMSA) detailing STAT-3 DNA binding activity following sham operation, single-puncture cecal ligation and puncture (CLP), and double-puncture CLP. DNA binding activity was determined by EMSA. T and subscript indicate time (in h) following sham operation, single-puncture CLP, or double-puncture CLP. In cold competition (second panel from left) either 10-fold (10 X) or 100-fold (100 X) excess of unlabeled oligonucleotide was added. In “supershift” analysis (third panel from left) an antibody specific for STAT-3 was added. **B:** quantification of relative activity of STAT-3 following sham operation, single-puncture CLP, and double-puncture CLP. Each data point represents mean ± SD of values from 3 different animals. Mean value at time 0 arbitrarily set equal to unity; values at other time points normalized to this. Time following intervention is shown on x-axis. *Significantly different from time 0. †Significantly different from sham operation value at same time point. #Double-puncture CLP value significantly different from single-puncture CLP value at same time point. DNA binding activity was determined using EMSA.
greater in single-puncture CLP than in double-puncture CLP at these time points. No STAT-3 binding activity was detected 24 and 48 h after double-puncture CLP. These data demonstrate that IL-6 activity was more pronounced following single-puncture CLP than following double-puncture CLP, but a sustained elevation was noted following double-puncture CLP.

A2M transcription in rat liver. Transcription of A2M also provides an indication of intraparenchymal IL-6 activity (4). Nuclear run-on was used to determine time-dependent changes in A2M transcription following sham operation, single-puncture CLP, and double-puncture CLP (Fig. 2). After sham operation, we detected a twofold elevation of A2M transcription at 6 h. A2M transcription increased fivefold 3 h after both single- and double-puncture CLP. This mild increase was sustained up to 16 h following double-puncture CLP. Transcription of A2M rose even further following single-puncture CLP, with a maximal 16-fold increase observed at 24 h. Levels then declined. This differed from double-puncture CLP, in which no A2M transcription could be detected at 16, 24, and 48 h. As in previous studies, transcription of ATP synthase was unchanged after single- or double-puncture CLP (1, 14). Although there was no correlation between maximal STAT-3 binding activity and A2M transcription following single-puncture CLP, we detected a temporal correlation between the loss of both STAT-3 binding activity and A2M transcription 24 h after double-puncture CLP.

Immunohistochemical detection of IL-6 in liver tissue. Because we have previously shown that intraparenchymal TNF-α abundance correlated with activation of a TNF-dependent transcription factor and expression of a TNF-dependent acute phase reactant (1), we used immunohistochemistry to see whether a similar correlation existed among intraparenchymal IL-6 abundance, STAT-3 activation, and A2M transcription (Fig. 3). No IL-6 was detected in the liver at any time point after sham operation. Intrahepatic IL-6 was detected in Kupffer and endothelial cells (Fig. 3, black arrows) at 24, 48, and 72 h following single-puncture CLP. More IL-6 staining was detected at 48 and 72 h than at 24 h after single-puncture CLP. After double-puncture CLP, IL-6 was first detected in liver samples 6 and 16 h after the procedure. There was no IL-6 staining at later time points. We detected no correlation between IL-6 abundance and the magnitude of either STAT-3 activation or A2M transcription in the liver after single-puncture CLP. However, the loss of detectable IL-6 in the liver following double-puncture CLP correlated with the loss of STAT-3 binding activity and A2M transcription.

IL-6 serum concentrations. Because previous study after single-puncture CLP has indicated that serum levels of TNF-α do not correlate with intraparenchymal levels or activity, we examined IL-6 serum concentrations in vena caval blood (Fig. 4). Low levels were detectable at time 0 and following sham operation. Serum levels after single-puncture CLP were statistically elevated over time 0 and over sham operation only at 16 h. In contrast, peak serum levels following double-puncture CLP were statistically increased 6 and 16 h after the insult. Peak levels after double-puncture

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Fig. 2. A: representative transcript elongation assay of α2-macroglobulin (A2M) and ATP synthase (ATPS) transcription following sham operation, single-puncture CLP, and double-puncture CLP. B: quantification of relative rates of transcription of A2M. Radiographic density from individual elongation analysis divided by ATP synthase density from same animal. Each data point represents mean ± SD of values from 3 different animals. Mean value at time 0 arbitrarily set equal to unity; values at other time points normalized to this. Time following intervention is shown on x-axis. *Significantly different from time 0. †Significantly different from sham operation value at same time point. ††Double-puncture CLP value significantly different from single-puncture CLP value at same time point.
CLP were eight times greater than time 0, sham operation, and single-puncture CLP. Levels decreased to baseline 24 h after double-puncture CLP and were statistically lower than values for sham operation and single-puncture CLP at 48 h. Serum IL-6 levels following single-puncture CLP did not correlate with STAT-3 activity, A2M transcription, or intrahepatic IL-6 abundance. Although no correlation was evident at 3, 6, and 16 h, the abrupt decrease in serum IL-6 levels 24 h after double-puncture CLP correlated with the loss of STAT-3 binding activity, A2M transcription, and intrahepatic IL-6 abundance.

**DISCUSSION**

In this study we demonstrate that loss of STAT-3 activity, transcription of A2M, intrahepatic IL-6 abundance, and immunoreactive IL-6 in the serum correlate with mortality in sepsis. We further show that neither serum IL-6 levels nor intrahepatic IL-6 abundance correlate in magnitude or time with two reliable indicators of intrahepatic IL-6 activity, STAT-3 activity, or A2M transcription (4, 11), except in the terminal phase of fulminant sepsis.

This study focuses on the role of IL-6 in hepatic dysfunction during sepsis/SIRS/MODS. Regel et al. (25) have found that the liver is the second most commonly affected organ in MODS, surpassed only by the lung. We have previously characterized early hepatic dysfunction by examining the transcription of a series of liver-specific genes in animal models of sepsis (2, 14). These genes are involved in essential metabolic processes such as gluconeogenesis, ketogenesis, and ureagenesis. In mild sepsis (single-puncture CLP), transcription of these genes decreases and then recovers as the insult resolves. However, transcription of the gluconeogenic enzyme glucose 6-phosphatase does not return to normal in the face of a fulminant, highly lethal septic insult, double-puncture CLP (14). Thus single-puncture CLP is associated with mild, reversible hepatic dysfunction, whereas the alteration associated with double-puncture CLP is irreversible.

Our data indicate that IL-6 is important in mediating two distinct, opposing effects in the liver in sepsis. After single-puncture CLP, IL-6 activity, as measured by STAT-3 activation and A2M transcription, inversely correlates with the sepsis-induced decrease in the transcription of hepatic gluconeogenic, ketogenic, and ureagenic genes. Specifically, the increase in STAT-3 activity and A2M transcription parallel, in magnitude and in time, the decrease in transcription of phospho-
endpyruvate carboxykinase, glucose 6-phosphatase, carnitine palmitoyltransferase, acetyl-CoA acyltransferase, and ornithine transcarbamylase seen after single-puncture CLP (2). Studies in cultured cells and nonseptic animals have demonstrated that IL-6 reduces transcription of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase (9, 21). However, because a STAT binding domain has not been identified in the promoters of any of these genes, it is unlikely that STAT-3 directly mediates the decrease in transcription.

IL-6, via STAT-3 activation, might also mediate hepatic dysfunction by activating a pathway that culminates in cell death, either via necrosis or apoptosis. It is well known that the double puncture model of CLP is associated with the late appearance of hepatic transaminases in the blood, indicative of necrosis (3, 31). Transaminases are not elevated following single-puncture CLP. We have examined apoptosis following both forms of CLP and found that it is indeed increased (L. Bellin, K. M. Andrejko, J. Chen, and C. S. Deutschman, unpublished data).

The correlation between decreased transcription of gluconeogenic, ketogenic, and ureagenic genes and increased IL-6 activity supports the hypothesis that the effects of IL-6 in sepsis are detrimental. However, a second line of evidence indicates that IL-6 activity is beneficial. Between 16 and 24 h following double-puncture CLP, there is a loss of STAT-3 activation, A2M transcription, detectable IL-6 abundance in the liver, and immunoreactive IL-6 in the blood. At this point in time, outcome following single and double-puncture CLP diverges. In previous work, we found that double-puncture CLP persistently decreases the transcription of glucose 6-phosphatase (14). The effect of single-puncture CLP is not persistent, and the difference between the two forms of sepsis becomes apparent 16 h after the original insult (14). Therefore, IL-6 may function as a “recovery factor” from sepsis and modulate the reversal of hepatic dysfunction. Although the mechanism is unknown, IL-6 is essential in hepatic regeneration (11, 32). A similar effect could contribute to recovery from sepsis.

Double-puncture CLP seems to attenuate the hepatic response to IL-6. At 6 and 16 h following this fulminant insult, STAT-3 activation and A2M transcription are less than those observed after single-puncture CLP despite higher levels of both intrahepatic and circulating IL-6. This may reflect a change in an intracellular, IL-6-linked pathway or that circulating cytokines do not have access to hepatocytes. Alternatively, the detection of IL-6 by immunohistochemistry may have no functional importance, indicating nothing more than the accumulation of IL-6 in Kupffer and endothelial cells, or normalization of STAT-3 density to values at time 0 may be invalid. The abundance of IL-6 in nonparenchymal cells at 6 and 16 h following double-puncture CLP also could reflect an inability of these cells to secrete cytokines.

Our data also demonstrate a discrepancy between the time course of STAT-3 activation and A2M transcription following single-puncture CLP. Cytokines such as TNF and IL-1 are known to enhance IL-6-induced A2M transcription and activate transcription factors other than STAT-3 (4, 19, 23). Intrahepatic TNF-IL-1 activity peaks later than IL-6 activity (1, 15). The enhancement afforded by this increase could explain increased A2M transcription despite declining STAT-3 activity. Alternatively, Wen et al. (30) have shown that both serine and tyrosine phosphorylation are required for maximal STAT-3 transcriptional activity (30). Thus there may be a difference between maximal DNA binding activity and maximal transcriptional activation.

Another important ramification of this study involves the role of serum levels of IL-6 (and other cytokines) in sepsis, SIRS, or MODS. In septic shock, death results from cardiovascular collapse secondary to vasodilatation. During clinical septic shock or following direct experimental insults, serum TNF, IL-1, and IL-6 levels may correlate with pathophysiological changes. However, the mechanism of death in patients with SIRS/MODS is not shock but progressive dysfunction in multiple organ systems (5, 13). We have previously shown that TNF-dependent processes correlate with intrahepatic TNF-α abundance and not with serum TNF-α levels (1). Neither serum nor intrahepatic IL-6 correlates with two markers of IL-6 activity in sepsis. This emphasizes that strategies designed to eliminate or block circulating cytokines are unlikely to be successful.

Perhaps most importantly of all, these data highlight the complex biology of cytokines such as IL-6 in sepsis. Levels of IL-6, TNF-α, and IL-1 in the circulation during clinical sepsis are highly variable. Clinical trials designed to neutralize circulating TNF-α or IL-1 have not altered the outcome from SIRS/MODS (10). We have demonstrated that intrahepatic IL-6 activity is associated with both detrimental (decreased transcription of gluconeogenic, ketogenic, and ureagenic genes) and beneficial (improved outcome) effects during sepsis induced by CLP. In another cell population in a different setting, similarly divergent effects have been shown for TNF-α (6, 28). In view of this a better understanding of the complex nature of cytokine-mediated responses will be essential to develop successful therapeutic approaches to sepsis.

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