Role of IL-10 in regulating proinflammatory cytokine release by Kupffer cells following trauma-hemorrhage

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CIRCULATING PROINFLAMMATORY cytokines, such as IL-6 and TNF-α, increase dramatically following trauma-hemorrhage (T-H) (2, 35). Studies have shown that hemorrhagic shock induces profound immunosuppression (3, 30) and hepatic dysfunction (34), which is associated with elevated plasma levels of proinflammatory cytokines. Kupffer cells are resident hepatic macrophages and have been shown to be one of the major sources of proinflammatory cytokine released in response to T-H stress (22). The production of IL-6 and TNF-α from Kupffer cells is significantly increased at both the gene expression (37) and protein levels (1) at an early stage following hemorrhagic shock. In this regard, the reduction of Kupffer cell numbers by administration of gadolinium chloride significantly reduced plasma IL-6 levels following T-H (22). Therefore, modulating the Kupffer cell-mediated cytokine release could be a potential therapeutic option in controlling morbidity and mortality.

IL-10 is a major anti-inflammatory cytokine produced in response to various stresses (19). It was initially identified as cytokine synthesis inhibitory factor that inhibited cytokine synthesis by Th1 cells (20). IL-10 is not only produced by T cells but also by B cells, macrophages/monocytes, and keratinocytes, and it alters the production of cytokines by various immune cells. Thus IL-10 modulates a variety of immunologic reactions under stressful conditions (29).

Several models have been used to elucidate the role of IL-10 in inhibiting the production of proinflammatory cytokines such as IL-6 and TNF-α by macrophage/monocytes (28, 32). Human monocytes activated by LPS produce high levels of IL-10 in a dose-dependent manner and inhibit the production of IL-6 and TNF-α in an autocrine fashion (32). Alveolar macrophages produce IL-10 following sepsis (25), hemorrhagic shock (28), and chronic lung disease (15), and this cytokine profoundly inhibits the release of proinflammatory cytokines.

In the liver, Kupffer cells produce IL-10 in response to LPS challenge and downregulate the release of IL-6 and TNF-α (10). Following partial hepatectomy, Kupffer cell-mediated IL-10 production is an important counterregulatory mechanism for TNF-α synthesis during liver regeneration (24). These results imply that the Kupffer cells could be a major source of IL-10 and may play an important role in regulating inflammatory responses under hepatic stress. However, the role of IL-10 in a T-H model has not yet been completely elucidated. Furthermore, the interaction between IL-10 and proinflammatory cytokines under the condition of severe T-H is unclear. Therefore, the aim of this study was to determine whether IL-10 production by Kupffer cells is enhanced following T-H. Additionally, the role of IL-10 in regulating the proinflammatory cytokines IL-6 and TNF-α was studied by using isolated Kupffer cells. Furthermore, recombinant IL-6 and TNF-α, as well as anti-IL-6 and anti-TNF-α MAbs, were used to elucidate whether there is any effect of IL-6 or TNF-α on the Kupffer cell-mediated IL-10 production.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River Labs, Wilmington, MA) weighing 250–350 g were used for these experiments. All procedures were performed in accordance with National Institutes of Health guidelines under a protocol approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

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**T-H procedure.** The rats were fasted overnight but were allowed free access to water before the experiments. The rats were anesthetized by isoflurane inhalation (Attane; Minrad, Bethlehem, PA) followed by a 5-cm midline laparotomy to produce soft tissue trauma. The abdominal wound was closed in layers, and polyethylene tubes (PE-50; Becton Dickinson, Sparks, MD) were placed in both femoral arteries and the right femoral vein. The groin wounds were bathed in 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) throughout the procedure to minimize the wound pain. The rats were then allowed to awaken, after which they were rapidly bled to a mean arterial pressure (MAP) of 40 mmHg and maintained at that pressure for ~90 min. This hypotension was maintained until the animals could no longer maintain MAP of 35 mmHg unless some fluid in the form of Ringer’s lactate was administered. This time was defined as the point of maximum bleedout. Following the maximum bleedout, MAP was maintained between 35 and 40 mmHg until 40% of the maximum bleedout volume was returned in the form of Ringer’s lactate. Thereafter, the animals were resuscitated with four times the volume of the withdrawn blood over 60 min with Ringer’s lactate. Following resuscitation, the catheters were removed and the wounds were closed. Sham-operated animals were subjected to the same midline laparotomy, groin incision, and vessel ligation but were not subjected to hemorrhage or resuscitation.

**Plasma collection.** The rats from the T-H or sham group were anesthetized by isoflurane inhalation, and blood samples were collected from the abdominal aorta with heparinized syringe. Plasma was then separated by centrifugation at 4°C and stored at ~80°C until being assayed. Blood samples from the T-H group were collected at different time points (2, 5, and 24 h) after the completion of fluid resuscitation.

**Isolated liver perfusion.** The isolated liver perfusion system was used to determine whether the production of IL-10 from the liver is upregulated following T-H. At 2 h after T-H and resuscitation or sham operation, animals were anesthetized with isoflurane and the livers were isolated and perfused in situ. The liver was exposed through a wide transverse incision, and the portal vein was isolated. The portal vein was cannulated with a PE-240 catheter, and the liver was perfused with warmed Krebs-Henseleit-bicarbonate buffer (in mM: 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 0.1 EDTA, 2.5 CaCl2, pH 7.4). The perfusate was pumped from a reservoir in which buffer was oxygenated by bubbling with gas containing 95% O2-5% CO2. The temperature of the perfusate was maintained at 36-37°C by warming the reservoir in a water bath. After the washout period, when the effluent from the liver became clear, a cannula was placed in the thoracic aortic cava outlet to return the perfusate to the reservoir (recirculating perfusion). The flow was maintained at a rate of 30 ml/min. The liver was further perfused for 60 min. The total volume of the perfusate during the recirculating perfusion was 150 ml. A small amount of perfusate (~500 µl) was collected before starting the recirculating perfusion (0 min) and after 30 and 60 min of recirculating perfusion for the measurement of IL-10 levels.

**Kupffer cell isolation.** In a separate experiment, Kupffer cells were isolated to determine whether the production of IL-10 from Kupffer cells is enhanced following T-H. Furthermore, an in vitro study was performed using anti-IL-10 antibodies (Abs) to elucidate the role of IL-10 in regulating proinflammatory cytokine release from the Kupffer cells. At 2 h after the completion of resuscitation or sham operation, Kupffer cells were obtained by an in situ collagenase digestion method. Briefly, the liver was perfused with oxygenized HBSS (GIBCO-BRL, Gaithersburg, MD) for 10 min to wash out the blood and was perfused with 0.03% collagenase (Sigma, St. Louis, MO) for 5 min. After the digestion and mechanical disruption of the liver, the cell suspension was filtered through a sieve and centrifuged at 50 g for 3 min to separate parenchymal from nonparenchymal cells. Nonparenchymal cells were collected and centrifuged over 18% metrizamide (Accurate Chemical, Westbury, NY) for 45 min (2,000 g; 4°C). The cells at the interface were collected and washed twice by centrifugation with HBSS (450 g, 10 min, 4°C). The pellet was then resuspended in William’s E medium (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL). After the cell numbers were counted and the cell viability was assessed by Trypan blue exclusion (average viability >95%), the concentration was adjusted to 1×10⁶ cells/ml in William’s E medium and 200 µl of cell suspension was added per well of a 96-well culture dish. After being incubated for 2 h, the plates were washed with warm HBSS to remove nonadherent cells and 200 µl William’s E medium was added. The adherent Kupffer cells were incubated with different concentrations of anti-rat IL-10 Ab (Research Diagnostics, Flanders, NJ). Irrelevant monoclonal IgG was used as control. After 16 h of incubation, IL-6 and TNF-α levels were measured by ELISA. In a separate experiment, anti-rat IL-6 and anti-rat TNF-α MAbs as well as recombinant rat IL-6 and TNF-α were used to study the effects of proinflammatory cytokines on the Kupffer cell IL-10 production. The concentrations of Abs and recombinant cytokines used in this study followed supplier’s recommendations to yield appropriate biological activity.

**Assessment of proinflammatory cytokine release.** IL-10, IL-6, and TNF-α levels in the plasma, perfusate collected from the isolated liver perfusion, and supernatant from the Kupffer cell culture were determined by ELISA methods. The assay was performed according to the manufacturer’s protocol (Pharmingen, San Diego, CA), except for a minor modification of incubating the samples overnight at 4°C.

**Statistical analysis.** There were six animals in each group. The results were presented as means ± SE. Student’s t-test was used for the comparison of two groups. One-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons was used to determine the significant differences among the experimental groups. When criteria for parametric testing were violated, the appropriate nonparametric test (Mann-Whitney U-test) was used. A P value <0.05 was considered to indicate a significant difference.

**RESULTS**

**Plasma IL-10 levels.** Plasma IL-10 levels were markedly elevated at 2 h following T-H compared with sham (Fig. 1). Since our preliminary studies indicated that plasma IL-10 levels were not different at different intervals following sham operation, only the 2-h sham animals were used. The levels of plasma IL-10 were also elevated at 5 h following T-H rats compared with shams. However, they returned to sham levels...
at 24 h, suggesting that IL-10 may play an important role in an early stage of T-H.

**IL-10 levels in the isolated liver perfusate.** The liver was isolated and perfused with Krebs-Henseleit-bicarbonate buffer to measure the release of IL-10 from the livers following T-H. The perfusate was collected at 0, 30, and 60 min. The levels of IL-10 in the perfusate were significantly higher in the T-H group compared with sham at every time point (Fig. 2). These results indicate that the production of IL-10 in the liver is significantly upregulated following T-H.

**IL-10 release by the isolated Kupffer cells.** Because macrophages could be a major source of anti-inflammatory cytokine release, Kupffer cells were isolated at 2 h following T-H or sham operation and were incubated for 16 h to evaluate their production of IL-10. The average production of IL-10 from the isolated Kupffer cells was almost 20 times higher in the T-H group compared with sham (Fig. 3). These results suggest that Kupffer cells could be one of the major sources of IL-10.

**The effects of anti-IL-10 Abs on proinflammatory cytokine release.** To determine whether or not an inhibition of IL-10 activity alters the production of proinflammatory cytokine release, the isolated Kupffer cells were incubated with different concentrations of either anti-IL-10 or control MAb (0, 0.1, 1, and 10 μg/ml) for 16 h and the levels of IL-6 and TNF-α in the supernatant were measured. At 10 μg/ml of anti-IL-10 Ab, the production of IL-6 was significantly enhanced compared with other concentrations in both sham and T-H groups (Fig. 4A). However, these changes were not observed when the cells were incubated with control IgG. Anti-IL-10 MAb showed similar effects on the production of TNF-α, with the effects being greater in the T-H group (Fig. 4B). There was a significant difference between the sham and T-H groups in the production of TNF-α when the cells were incubated with 1 and 10 μg/ml of anti-IL-10 MAb. These results indicate that IL-10 produced by Kupffer cells early following T-H inhibits the release of proinflammatory cytokines, possibly in an autocrine or paracrine manner. Furthermore, the results suggest that IL-10 contributes more toward attenuating Kupffer cell production of TNF-α rather than IL-6 in the T-H group.

**The effects of IL-6 and TNF-α on IL-10 production.** The isolated Kupffer cells were incubated with different concentrations of recombinant rat-IL-6 or TNF-α to evaluate whether these cytokines stimulated the production of IL-10. Kupffer cell cultures were treated with neutralizing MAbs for TNF-α or IL-6. Neither MAb had any effect on IL-10 production. Likewise, recombinant TNF-α or IL-6 had no effect on IL-10 production by Kupffer cells from sham and T-H animals (Fig. 5).

**DISCUSSION**

The production of IL-10 is triggered by several stresses (31). Endotoxin administration is the most extensively studied stress model to induce IL-10 release from the monocytes and macrophages (6). Other than endotoxin, Le Moine et al. (14) reported that reactive oxygen intermediates have been shown to induce IL-10 release after reperfusion of cold-preserved livers. In human studies, peripheral blood mononuclear cells respond to burns or severe trauma with the production of IL-10 (18). Our results lead us to suggest that a combination of severe trauma and hemorrhagic shock could likewise be a strong inducer of IL-10 production. IL-10 is an important immunoregulatory cytokine produced by B and T lymphocytes (5, 21) and monocytes and macrophages (4, 6). These cells rapidly

![Fig. 2. IL-10 levels in the perfusate during the isolated liver perfusion. At 2 h following T-H and resuscitation or sham operation, animals were anesthetized and the livers were isolated and perfused in situ. Perfusate was collected before the recirculating perfusion began (0 min) and after 30 and 60 min of recirculating perfusion. Data are means ± SE. *P < 0.05 vs. sham.](image)

![Fig. 3. IL-10 levels in the supernatant from the isolated Kupffer cell cultures.](image)

![Fig. 4. Effects of anti-IL-10 antibodies (Abs) on IL-6 (A) and TNF-α (B) production. Kupffer cells were isolated at 2 h following T-H or sham operation and were incubated for 16 h with different concentrations of anti-IL-10 Ab. Same concentrations of IgG were used as control. *P < 0.05 vs. other concentrations of anti-IL-10 Ab; #P < 0.05 vs. sham; $P < 0.05 vs. same concentration of IgG.](image)
secretes IL-10 in response to stress, suggesting an important counterregulatory role for this anti-inflammatory cytokine. Our results, in concordance with other reports, showed a marked elevation of plasma IL-10 levels at 2 and 5 h following T-H, which declines after 24 h. Therefore, we have focused on the role of IL-10 at an early stage of T-H (2 h).

Our previous studies have shown increased production of Kupffer cell proinflammatory cytokine following T-H (1), which appeared to contribute to the elevated plasma proinflammatory cytokine levels (22) and hepatic damage (13, 33). Kupffer cells also have been shown to produce IL-10 in response to endotoxin challenge (10). However, it remains unclear whether Kupffer cell IL-10 production is enhanced following T-H in a rat model. Therefore, in this study, we sought to determine the role of Kupffer cells in producing IL-10 and in regulating inflammatory responses following T-H.

The results from isolated liver perfusion showed significantly higher production of IL-10 from the liver at 2 h after T-H compared with sham. Furthermore, the levels of IL-10 released from the Kupffer cells isolated from the T-H group were markedly higher than in the sham group. Therefore, we speculate that Kupffer cells might be a major contributor to the elevated plasma IL-10 levels following T-H.

Our working hypothesis is that Kupffer cell IL-10 acts early to regulate inflammatory cytokine responses in T-H, possibly acting in a paracrine/autocrine fashion. This assertion is supported by numerous studies (28, 32) that report that IL-10 inhibits the production of IL-6 and TNF-α by macrophages and monocytes under conditions of stress. In concordance with previous reports using different stress models or different cell types, the production of IL-6 and TNF-α was significantly increased by inhibiting the activity of IL-10 by using neutralizing anti-IL-10 MAb. Interestingly, these effects were more apparent in the production of TNF-α than IL-6 following T-H. However, our data did not elucidate the precise reason for this difference.

It should be noted that the methodology used in this study contains potential limitations. The isolated liver perfusion does not completely simulate the in vivo conditions. Therefore, the actual in vivo kinetics and extent of IL-10 release from the liver remain unknown. The condition for isolating Kupffer cells is also not physiological. In the liver, cytokines are not only produced by Kupffer cells but also by other cells such as hepatocytes (23) and sinusoidal endothelial cells (11). These cells closely interact in regulating the production of proinflammatory and anti-inflammatory cytokines. Additionally, a recent study has shown that hepatic stellate cells activated by LPS or bile duct ligation overexpress IL-10 mRNA and release IL-10 protein (36). Because our study used only isolated Kupffer cells, an interaction between Kupffer cells and other types of hepatic cells with regard to the production and regulation of pro- and anti-inflammatory cytokines remains to be elucidated. These relationships should be revealed in future studies.

We measured the effects of proinflammatory cytokines on the production of IL-10 by the Kupffer cells. IL-10 release is significantly higher in T-H groups compared with sham, yet neither group demonstrated any effects on IL-10 levels from treatment with either IL-6 or TNF-α. Furthermore, neutralization of proinflammatory cytokines with anti-IL-6 or anti-TNF-α MAbs also did not produce any change in the release of IL-10. These results suggest that the production of IL-10 by Kupffer cells is not likely to be triggered by proinflammatory cytokines but rather by some other mechanism. We speculate that one of the possible candidate mechanisms is hypoxic stress on the liver induced by severe hypotension during hemorrhagic shock (12). Furthermore, the results did not support the notion of a feedback-downregulatory mechanism of IL-6 and TNF-α on Kupffer cell IL-10 release.

On the basis of these results, it could be postulated that IL-10 would be a useful therapeutic agent in severe trauma and hemorrhagic patients. This adjunct might ameliorate the inflammatory response in the liver by decreasing the production of proinflammatory cytokines and subsequently decreasing hepatic injury in an acute phase of T-H (27). However, previous reports using in vivo administration of recombinant IL-10 or anti-IL-10 Ab in different models have yielded conflicting results. Treating the animals with recombinant IL-10 30 min before LPS challenge not only decreased serum TNF-α levels but also decreased serum alanine aminotransferase concentrations and the degree of severe hepatic necrosis (16). The authors in this study concluded that IL-10 is involved in the hepatoprotective mechanism under septic conditions. In a similar study, pretreatment with IL-10 reduced the LPS/galactosamine-induced liver neutrophil margination and up-regulation of adhesion molecules both on liver specimens and circulating neutrophils (26). However, other studies have shown deleterious rather than salutary effects for IL-10. Treating mice with anti-IL-10 Ab 1 day after burn injury significantly improved survival rate from the subsequent septic stress induced by cecal ligation and puncture (17). Additionally, anti-IL-10 Ab treatment 1 day after burn injury restored T cell function and cytokine production. Therefore, the authors concluded that in vivo inhibition of IL-10 at an early stage of burn injury might be a useful approach in preventing the development of immune dysfunction. In studies of hemorrhagic shock, the results are more controversial. Pretreatment of animals with recombinant IL-10 in severe hemorrhagic shock did not improve survival rates, although serum TNF-α levels were significantly attenuated (9). Administration of recombinant IL-10 during resuscitation following hemorrhage produces salutary effects on the depressed immune responses only in males but did not further enhance immune function in females under those conditions (7, 8). Therefore, we speculate that the
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prevention of organ damage or immune suppression induced by severe trauma and hemorrhagic shock would not be achieved by only controlling the activity of IL-10. Furthermore, the role of IL-10 might be more complicated when gender is taken into account.

In summary, our results indicate that the IL-10 released by the Kupffer cells early after T-H potentially plays a pivotal role in attenuating the proinflammatory cytokine release in an autocrine/paracrine manner in male rats. Although the release of IL-6 or TNF-α from the Kupffer cells is regulated by IL-10, neither IL-6 nor TNF-α showed an effect on the release of IL-10 from the Kupffer cells.

GRANTS

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