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Delayed Neutralization of IL-6 Reduces Organ Injury, Selectively Suppresses Inflammatory Mediator and Partially Normalizes Immune Dysfunction following Trauma and Hemorrhagic Shock

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Abstract

An excessive and uncontrolled systemic inflammatory response is associated with organ failure, immunodepression, and increased susceptibility to nosocomial infection following trauma. Interleukin-6 (IL-6) plays a particularly prominent role in the host immune response after trauma with hemorrhage. However, as a result of its pleiotropic functions, the effect of IL-6 in trauma and hemorrhage is still controversial. It remains unclear whether suppression of IL-6 after hemorrhagic shock and trauma will attenuate organ injury and immunosuppression. In this study, C57BL/6 mice were treated with anti-mouse-IL-6 monoclonal antibody (anti-IL-6 mAb) immediately prior to resuscitation in an experimental model combining hemorrhagic shock and lower extremity injury (HS+T). Interleukin-6 levels and signaling were transiently suppressed following administrations of anti-IL-6mAb following HS+T. This resulted in reduced lung and liver injury, as well as suppression in the levels of key inflammatory mediators including IL-10, KC, MCP-1, and MIP-1 α at both 6 and 24h. Furthermore, the shift to Th2 cytokine production and suppressed lymphocyte response were partly prevented. These results demonstrate that IL-6 is not only a biomarker but also an important driver of injury-induced inflammation and immune suppression in mice. Rapid measurement of IL-6 levels in the early phase of post-injury care could be used to guide IL-6 based interventions.

Keywords

MCP-1; KC; IP10

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AUTHORSHIP

Y.Z. – designed and conducted the experiments, analyzed data, and wrote the paper

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T.B. – designed experiments, oversaw the project, analyzed the data and wrote the paper

Conflict of Interest Disclosure

The authors declare no conflict of interest.

INTRODUCTION

Traumatic injury accompanied by hemorrhage is characterized by marked early inflammatory response and concomitant depression of some immune functions. An excessive and uncontrolled inflammatory response and associated immunodepression increases susceptibility to organ failure and nosocomial infection (1). Many inflammatory mediators and pathways are elevated following trauma/hemorrhage, among the most consistent of which is interleukin-6 (IL-6)(2). Studies have shown that IL-6 mRNA and protein are produced in the lungs (3), liver (4), cardiac tissue (5), and spleen (6) of mice subject to trauma/hemorrhage. The magnitude of IL-6 elevation has been correlated with the extent of injury severity and organ dysfunction in both experimental and clinical studies (7, 8) Furthermore, IL-6 is a prognostic indicator of outcome in patients with multiple injuries; sustained elevation in circulating IL-6 levels correlates with poor outcome after trauma/hemorrhage (8, 22).

Interleukin-6 targets multiple cells types and induces a variety of biologic responses, including the regulation of the acute phase response (APR), stimulation of immunoglobulin production and lymphocytic differentiation, chemokine production, and recruitment of leukocytes. As a result of these pleiotropic functions, IL-6 plays a dual role in the inflammatory response to injury, often classified as pro-inflammatory locally and anti-inflammatory systemically. A previous collaborative study involving our group has demonstrated that the local pro-inflammatory effects dominate the liver of IL-6-deficient mice subjected to hemorrhagic shock (9). Apart from its inflammatory action, IL-6 also functions as an immunoregulatory cytokine. For instance, the IL-6-triggered acute phase response induces macrophages to release prostaglandin E2 (PGE2) (10), which is a powerful endogenous immune suppressant.

Trauma/hemorrhage causes suppression of cell-mediated immunity characterized by a shift in the Th1/Th2 balance toward Th2 responses (11). Th1 cells promote cellular immunity to kill intracellular pathogens, whereas Th2 cells promote humoral immunity to provide a defense against extracellular parasites. A depression in Th1 immunity has been reported to increase the risk of infectious complications. Studies have suggested that IL-6 plays a key role in the Th1 to Th2 differentiation (12). However, the shift of Th1/Th2 balance in response to trauma/hemorrhage is complex and poorly understood.

The overall impact of excessive IL-6 production in trauma/hemorrhage is still controversial. Studies have shown beneficial effects of IL-6 deficiency in experimental paradigms of thermal injury, sepsis, and trauma/hemorrhage (13, 14, 15). In contrast, other studies demonstrate that IL-6 administration prevents epithelial cell and cardiomyocyte apoptosis induced by trauma/hemorrhage (16, 17). Systemic infusion of IL-6 following hemorrhagic shock reduces inflammation and injury in the liver and lung (18). Some authors have concluded that the effect of IL-6 may be model-specific. Others have concluded that the effects of IL-6 are dependent on the levels and duration of production (5). In this scenario, the anti-inflammatory response to IL-6 might result in immune suppression with an enhanced susceptibility to infectious complications. Therefore, it remains unclear whether

blockade of IL-6 will attenuate immunosuppression induced in the setting of trauma/hemorrhage.

In the present study, C57BL/6 mice were treated with anti-mouse-IL-6 monoclonal antibody (anti-IL-6 mAb) after injury and establishment of shock and immediately prior to resuscitation, in an experimental model combining hemorrhagic shock and lower extremity injury (HS+T). Because the duration of hemorrhagic shock was 2h, IL-6 produced during that time was allowed to exert its adaptive actions. Interleukin-6 levels and signaling were suppressed transiently following administrations of anti-IL-6mAb following HS+T. This resulted in reduced lung and liver injury, as well as a selective suppression in circulating inflammatory mediators. Furthermore, the shift to Th2 cytokine production and suppressed lymphocyte responses were partly prevented by anti-IL-6mAb. These results demonstrate that IL-6 is not only a biomarker but also an important driver of injury-induced inflammation and immune suppression.

MATERIALS AND METHODS

Reagents

Monoclonal anti mouse STAT3 antibody(MAB1799), neutralizing rat anti-IL-6 mAb (MAB406), and isotype control immunoglobulin G1 (IgG1) mAb (MAB005), mouse interleukin 6 (IL-6), IL-10, IFN- γ , TNF- α , KC, MCP-1, and IP10 enzyme-linked immunosorbent assay (ELISA) kits were purchased from R & D Systems (Minneapolis, MN); rabbit polyclonal anti-SOCS3 antibody (ab 16030) and anti-GAPDH were from AbCam (AbCam, Cambridge, MA); RPMI 1640 and L-glutamine were from Lonza BioWhittaker (Walkersville, Md); heat-inactivated fetal bovine serum, nonessential amino acids, and sodium pyruvate were from Hyclone Lab, Fisher Scientific (Logan, Utah); penicillin-streptomycin and 2-mercaptoethanol were from Gibco Life Technologies Corp (Grand Island, NY); concanavalin A (ConA) was from GE Healthcare Corp (Piscataway, NJ)

Animal Preparation

C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME), 8–12 weeks old and weighing 20–30g, were used in this study. All mice were maintained in the animal research center to acclimatize to the animal facility for 1 week before the experiments. These experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Mice used in the experimental protocols were housed in compliance with National Institutes of Health (NIH) animal care guidelines. The animals were maintained in the animal facility with a 12:12h light-dark cycle and free access to standard laboratory food and water.

Pseudo-fracture Combined with Hemorrhage Model

Animals were anesthetized with intraperitoneal pentobarbital (70 mg/kg); repeated doses of Pentobarbital Sodium (10 mg/kg, i.p.) were given as necessary throughout the course of the experiment. Bilateral Pseudofracture (PF) and hemorrhagic shock (HS) were performed as previously described by our group (19, 20). Briefly, donor mice were euthanized via

overdose of pentobarbital and the femurs and tibias were removed from both hind limbs and crushed using a sterile mortar and pestle in the biohazard hood. The crushed bone fragments were suspended in 2ml phosphate-buffer solution (PBS), crushed again, and kept in a sterile tube. Recipient animals were anesthetized and both thighs were crushed with a hemostat for 30s to induce a soft tissue lesion. A bone mixture (0.15 ml) suspended in PBS was injected in the area of the soft tissue injury of each thigh using a 1cc syringe and a 20-gauge needle. A unilateral groin incision (1cm) was performed and the femoral artery was dissected and cannulated with a sterile PE-10 catheter. Blood was withdrawn via one femoral artery cannula until MAP reached 25 mmHg. The contralateral cannula was connected to a blood pressure monitor and the mean arterial pressure (MAP) was recorded. After 2h of HS+T, animals were resuscitated with three times the volume of shed blood with Ringer lactate. Mice were sacrificed at 4h, 22h or 46h following the initiate of the resuscitation.

Experimental Groups

Animals were randomized into four groups, each assessed at three different time points: control+IgG; control+anti-IL-6 mAb; HS+T+IgG; HS+T+anti-IL-6 mAb. Either rat anti-mouse-IL-6mAb (3mg/kg) or isotype control rat IgG (3mg/kg) was administered intraperitoneally after hemorrhagic shock and just prior to the onset of resuscitation. Control mice were used to obtain physiologic parameters. The control mice that received anti-IL-6 mAb or IgG were killed under inhalational anesthesia with no other experimental manipulation performed.

Alanine Aminotransferase Measurement

Alanine aminotransferase (ALT) was measured by using the Dry-Chem Veterinary Chemistry Analyzer (HESKA, Loveland, CO; slides from Fujifilm Corporation, Asaka-shi Saitama, Japan).

Histopathological Examination

Briefly, the liver and lung were removed and fixed in 2% paraformaldehyde for 4h and then transferred through a series of alcohol processing steps (70 to 100%), followed by tissue incubation in xylene. Samples were embedded in paraffin, sectioned at 5µm, and stained with hematoxylin and eosin (H&E). Images were taken on an Olympus Provis light microscope (Malvern, NY) with 100× magnification.

Analyses of Lung Injury

To analyze lung injury, 8–10 randomly selected fields (magnification 20X) of left upper lung lobes were assessed in each mouse by two pathologists independently who were blinded to the identity of the specimens. Quantitative morphometric assessment of injury was performed by grading four histological findings: congestion, edema, inflammation, and hemorrhage. The lung injury degree was scored on a scale of 0 to 4 (0=normal, 1=mild, 2=moderate, 3=severe, and 4=very severe) for each feature with a cumulative maximum score of 16.

Detection of SOCS3 and Phosphorylated STAT3 by Western-blot

Total proteins were extracted by lysis buffer (Cell Signaling Technologies) containing 20mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM β -glycerophosphate, 1mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin, and 1mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by three cycles of freeze and thaw, and cytosolic fractions were prepared by centrifugation at 13,000g for 15min at 4 °C. Protein concentration was determined by the BCA method (Pierce, Rockford, IL.). Proteins (40 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding was blocked with TBS-Tween (0.01%) containing 5% nonfat milk for 1h at room temperature. The membranes were hybridized with primary antibodies for rabbit anti-STAT3 (Abcam, Cambridge, MA), p-STAT3, SOCS3 and GAPDH (Santa Cruz Biotech, CA) in TBS-T for overnight. Membranes were washed three times with TBS-T and Horseradish peroxidase-conjugated secondary antibodies were then used in a standard enhanced chemiluminescence reaction according to manufacturer's instructions (Pierce).

Serum Cytokines Assays

Mice were anesthetized with isoflurane and blood was harvested by cardiac puncture. Serum was collected by centrifuging the blood at 300 $\times$ g for 10 min at 4 °C. Serum was divided into aliquots and stored at -80°C for analysis. Serum IL-6, IL-10, KC, MCP-1, IP10 and MIP-1 α were assessed by ELISA according to manufacturer's instructions.

Preparation of Splenocytes and Stimulation

The spleens were removed aseptically and placed in ice-cold, 4°C PBS, and digested with collagenase D (1mg/mL; Roche Diagnostics Corp, Indianapolis, Ind) followed by gently passing through a 70- μm mesh strainer to produce a single-cell suspension. The suspension was centrifuged at 300 $\times$ g for 15 min at 4 °C, The pellet was suspended in PBS, erythrocytes were lysed with red blood cell lysing buffer (Sigma-Aldrich Co), and the remaining cells were washed with PBS by centrifugation at 300 $\times$ g for 15 min. The splenocytes were resuspended to a concentration of 1×10^6 cells/ml in RPMI medium 1640 containing 10% fetal bovine serum, L-glutamine, penicillin-streptomycin, nonessential amino acids, sodium pyruvate, and 2-mercaptoethanol. Cell viability of > 90% was confirmed with trypan blue stain. The ability of the splenocyte cultures to produce cytokines in response to a mitogenic stimulation was assessed by stimulation with ConA (2.5 $\mu\text{g}/\text{mL}$) and subsequent culture for 48h at 37°C. After that period, the splenocyte culture supernatants were collected and centrifuged at 300 $\times$ g for 10 min. The supernatants were harvested, aliquoted, and frozen for cytokine analysis. Th1 (IFN- γ and TNF- α) and Th2 (IL-10) cytokines were measured by ELISA according to manufacturer's instructions.

Splenocyte Proliferation

Splenocyte proliferation capability in response to mitogenic stimulation with Concanavalin A was measured by [^3H]thymidine incorporation technique. Spleen cell cultures (1×10^6 cells/ml) were carried out in 96-well round-bottom tissue-culture plates (BD Falcon; BD Biosciences). The splenocytes were cultured with 2.5 $\mu\text{g}/\text{mL}$ ConA for 48h, then [^3H]thymidine (1 $\mu\text{Ci}/\text{well}$; Perkin Elmer Inc, Waltham, Mass) was added and incubation

was continued for an additional 18h. Radioactivity was then measured using a Topcount scintillation counter (Perkin Elmer Inc) and expressed as counts per minute (cpm). Each experiment was repeated three times.

Statistical Analysis

The results presented in the study are expressed as the mean $\pm$ SEM. Comparisons between groups were performed using one-way analysis of variance (ANOVA) using SigmaPlot 11.0 software (Systat Software Inc, San Jose, Calif).

RESULTS

Neutralizing Antibody to IL-6 Suppresses Circulating IL-6 Levels and Signaling Following Trauma and Hemorrhage

Neutralizing antibody against IL-6 (IL-6 mAb) was administered following 2h of hemorrhagic shock and immediately prior to resuscitation in a mouse model of hemorrhagic shock plus trauma (HS+T). Thus, anti-IL-6 was administered in a clinically relevant fashion rather than prophylactically. Levels of IL-6 as well as markers of downstream IL-6 signaling were measured at 6, 24, and 48h following the induction of HS+T. As shown in figure 1(A–C), HS+T led to a dramatic up regulation in circulating levels of IL-6 in mice treated with control antibody by 6 h. These levels remained elevated at 24h and declined by 48h (though remaining slightly elevated). The administration of anti-IL-6 mAb immediately prior to resuscitation suppressed levels of IL-6 to nearly undetectable levels 4h later (6h of HS+T total). By 24h, IL-6 levels were still significantly suppressed; however, by 48h IL-6 levels were equivalent between the control antibody treated mice and the anti-IL-6 mAb treated mice following HS+T.

Interleukin-6 activates cell signaling that leads to the phosphorylation of STAT3 (p-STAT) (4, 16). There is also a simultaneous up regulation of suppressor of cytokine signaling 3 (SOCS3) (26). To determine if the neutralization of IL-6 also suppressed these IL-6 signaling pathways, levels of p-STAT3 and SOCS3 were measured in liver lysates. By 6h following HS+T, there was a marked increase in both p-STAT3 and SOCS3 (Figure 1, D–J) in the liver of mice treated with control antibody. This increase persisted to 24h and to a lesser degree at 48h. The administration of anti-IL-6 mAb blocked the upregulation in both of these parameters nearly completely at 6 h, and this suppression of HS+T induced p-STAT3 and SOCS3 upregulation persisted partially until 24h. However, by 48h the levels of p-STAT3 and SOCS3 in the liver were essentially equal in the animals treated with control or neutralizing antibody, respectively. Taken together, these data indicate that a single dose of neutralizing anti-IL-6 antibody given immediately prior to resuscitation from HS+T results in a marked suppression in IL-6 levels as well as in IL-6 signaling at the peak time point of 6h, with partial suppression persisting to 24h. However, by 48h both IL-6 levels and IL-6 signaling diminish, and there is no obvious impact of anti-IL-6 mAb.

The Effects of Anti-IL-6 Antibody on Lung and Liver Injury Following Trauma and Hemorrhage

We next sought to determine if a single dose of neutralizing anti-IL-6 antibody given after HS+T and immediately prior to resuscitation would reduce the organ injury characteristic of this experimental model. Consistent with our previous reports (20), we found that both lung injury (Figure 2, A–L) and liver injury (Figure 2, M–X) were maximal between 6 and 24h. Lung injury was assessed by histology and lung injury score (Figure 3, A–C) while liver damage was assessed histologically and by circulating levels of ALT (Figure 3, D–F). Administration of the anti-IL-6 mAb remarkably suppressed lung injury at all three time points. Liver damage was suppressed at the 6 and 24h. By 48h, there was minimal evidence of liver damage in any of the groups. These data show that neutralizing IL-6 just prior to resuscitation reduces the marked end-organ damage observed in this model of hemorrhagic shock plus tissue trauma.

Neutralizing Anti IL-6 Antibody Suppresses Increases in Circulating Cytokine and Chemokine Levels Following Trauma and Hemorrhage

HS+T leads to elevations in several cytokines and chemokines that are thought to drive the organ injury and immune suppression following trauma. Therefore, we measured the impact of anti-IL-6 mAb on the levels of the cytokine IL-10 and the chemokines KC, MCP-1, IP10, and MIP-1 α . There was no difference in the baseline levels of these mediators between the control IgG-treated and anti-IL-6 mAb-treated control mice (Fig 4). HS+T led to significant elevations in all five inflammatory mediators. Levels for all were highest at the 6h time point and then declined by 24h, but were still elevated over controls. By 48h, the levels of all five mediators had returned to near baseline. Anti-IL-6 mAb given immediately prior to resuscitation significantly reduced the levels of IL-10, KC, MCP-1, and MIP-1 α at both 6h and 24h following HS+T. Interestingly, IP10 levels were not impacted by the administration of IL-6mAb. These data show that IL-6 contributes to the elevation in several chemokines as well as IL-10. However, IP10 elevations measured after resuscitation appear to be independent of IL-6.

Neutralization of IL-6 Suppresses the Conversion from a Th1 to Th2 Phenotype Following Trauma and Hemorrhage

Trauma is associated with immune dysfunction that is thought to render victims susceptible to nosocomial infections. This has been associated with a suppression of adaptive immune responses which is characterized by a conversion from a Th1 to a Th2 T-cell phenotype. In trauma models, this phenomenon can be assessed by the capacity of splenocytes isolated from injured animals to produce either Th1 (TNF- α and IFN- γ) or Th2 (IL-10) cytokines in response to stimulation with mitogens such as ConA. To assess the impact of neutralizing IL-6 on Th1 to Th2 conversion, we isolated splenocytes at 6, 24, and 48h from control mice or injured mice treated with either control antibody or IL-6mAb. As shown in Figure 5, HS +T led to a marked reduction in splenocyte TNF- α and IFN- γ release in response to ConA *in vitro*. This reduction in Th1 cytokines was observed at all three time points studied. Splenocytes isolated from mice that had received anti-IL-6 mAb following HS+T produced significantly higher levels of both TNF- α and IFN- γ in response to ConA at all three time

points. The splenocyte production of IL-10 in response to ConA was increased following injury. Treatment with IL-6 mAb antibody significantly suppressed IL-10 production by splenocytes *in vitro* at the 24 and 48h time points. These data suggest that neutralizing IL-6 following hemorrhagic shock and immediately prior to resuscitation not only suppresses the systemic inflammatory response but also reduces the conversion of T-cells from a Th1 to the Th2 phenotype.

Neutralizing IL-6 Partially Prevents the Suppression in Splenocyte Proliferation Following Trauma and Hemorrhage

Another marker of immune dysfunction in trauma models is the suppression of proliferation of splenocytes in response to mitogens *in vitro*. Consistent with previous studies (21), we found that splenocytes isolated from animals subjected to HS+T exhibit a marked suppression in proliferative capacity in response to ConA. The inhibition was most significant at the 24h time point following HS+T (Figure 6). The *in vivo* administration of IL-6 mAb prior to resuscitation had no impact on the *in vitro* suppression of splenocyte proliferation measured at 6h after injury. However, at 24h and 48h anti-IL-6 mAb treatment partially prevented the suppression in splenocyte proliferation induced by HS+T. These data suggest that mediators other than IL-6 drive the suppression of proliferation of splenocytes at 6h following injury but that at later time points IL-6 contributes to this parameter of immune dysfunction.

DISCUSSION

This study was undertaken to determine if inhibition of IL-6 at the time of resuscitation from hemorrhagic shock and trauma (HS+T) would alter the subsequent inflammatory response, end-organ injury, and immune suppression induced by injury in mice. We chose this time point for intervention to be consistent with a therapeutic time frame. We also chose this time point with the idea that IL-6 exerts both positive and negative effects following trauma/hemorrhage, and that complete inhibition from the onset of trauma may not be desirable. We have shown that administration of anti-IL-6 monoclonal antibody suppresses the subsequent end-organ injury, selectively suppresses circulating cytokine and chemokine levels, and partially normalizes the responses of splenocyte to ConA. These results support the notion that transient and delayed suppression of IL-6 signaling could be a beneficial therapeutic goal in blunt trauma associated with hemorrhagic shock when IL-6 is overproduced.

It is well known that circulating IL-6 levels correlate with the magnitude of the injury and subsequent organ failure in human trauma (7, 22). Experimental studies have shown both detrimental and beneficial effects of IL-6 in models of trauma. For example, IL-6 pre-treatment limits organ damage in hemorrhagic shock models (18). Conversely, IL-6 knock-out mice reduced liver damage following hemorrhagic shock (9). These paradoxical observations have led to a view that IL-6 may be a good biomarker of the inflammatory response following injury, but not a viable therapeutic target. However, another concept is that organ damage and immune dysfunction result from a sustained and excessive production of inflammatory mediators such as IL-6 (23). In this scenario, it would be reasonable to hypothesize that transient suppression of key drivers of the inflammatory

response could be of benefit. Here, we delayed IL-6 inhibition until 2h following injury. This had a dramatic effect on the end-organ damage measured only 4h later. The protection persisted for the 48h duration of the study and was associated with a partial suppression of other inflammatory mediators. These observations suggest that partial, transient, or even delayed inhibition of IL-6 could be of therapeutic value, especially in situations in which IL-6 levels are found to be above the thresholds associated with adverse outcomes (24).

We provide confirmation that IL-6 and its downstream signaling pathways, STAT3 and SOCS3, are markedly up-regulated by HS+T. Many of the detrimental effects attributed to IL-6 are mediated through activation of the JAK-STAT pathways. For example, STAT3 phosphorylation can lead to neutrophil infiltration through the up-regulation of adhesion molecules such as ICAM-1 (3) while over-expression of STAT3 in pulmonary epithelial cells leads to inflammation in the lung (25). Although SOCS3 is associated with counter-inflammatory actions, this signaling molecule can also contribute to the pro-inflammatory effects of IL-6 (26). We show here that the neutralization of IL-6 nearly completely blocks STAT3 phosphorylation and SOCS3 up-regulation in the liver following HS+T. These results not only show that the antibody was effective in blocking IL-6, but also that IL-6 is the dominant driver of these pathways in the liver in the setting of experimental HS+T. Our results do not directly link STAT3 or SOCS3 inhibition mechanistically to the protective effects observed with IL-6 inhibition, however.

Many of the complications of severe injury are thought to be due to the combined effects of over-production of multiple cytokine and chemokine mediators. Whereas the initial up-regulation of these inflammatory pathways is likely to be the result of the activation of pattern recognition receptors such as TLR4, TLR9, and TLR2 (27, 28) the role of these early inflammatory mediators in driving the subsequent production of other mediators is unknown. Here, we show that delayed neutralization of IL-6 suppresses circulating IL-10, KC, MCP-1, and MIP1 α (but not IP-10). Interleukin-10 (29), KC (30), MCP-1 (31), and MIP1 α (32) have all been linked to inflammation-associated organ injury in trauma models. Therefore, the organ injury associated with IL-6 over-production and could be driven through these downstream mediators. Conversely, the lower production of some mediators could be the consequence of reduced tissue damage. It is interesting that neutralization of IL-6 did not impact IP10 levels at 6 or 24h following HS+T. This finding suggests that IP-10 alone does not mediate lung or liver damage. Furthermore, IL-6 does not appear to be a driver of IP-10 production in HS+T in mice. Alternatively, IP-10 may be triggered by mechanisms involving IL-6 that are activated prior to 2h (2).

We also sought to determine if delayed and transient IL-6 inhibition would impact on some of the parameters associated with immune dysfunction in our trauma model. In both human (33) and animal models (2, 21) there is a shift from Th1 to a Th2 lymphocyte phenotype. This has been associated with an increased susceptibility to infection and elevated mortality. It has also been reported that IL-6 inhibits Th1 differentiation by a negative feedback mechanism (34). Interleukin-6 may also be involved in the Th1 to Th2 balance through indirect pathways such as those mediated via SOCS3. These data demonstrate that SOCS3 has an important role in regulating the Th1/Th2 balance.

Interleukin-10 might also contribute to the depressed splenocyte Th1 cytokine release and shift towards a Th1 response after injury. Anti-IL-10 treatment prevented T-cell immunosuppression and improved the survival rate after injury (35, 36). Therefore, the decreased circulating IL-10 levels observed following treatment with anti-IL-6 antibody in our study might also contribute to the preserved Th1/Th2 balance observed subsequently. MCP1 also acts as a potent factor in polarization of Th cells toward a Th2 phenotype (37). Previous studies in mice have shown maximal immune suppression between 24 and 48h post-trauma (21). In the present study, we show that the suppressed production of Th1 cytokines and increased production of IL-10, a Th2 cytokine, by splenocytes was partially reversed at 24 and 48h following IL-6 neutralization.

Several animal and clinical studies have demonstrated that mitogen-induced lymphocyte proliferation is suppressed following trauma (11, 13, 21, 35). Our results confirm this phenomenon, and further demonstrate that the suppression in splenocyte proliferation measured at 48h after HS+T could be restored partially by early administration of anti-IL-6 antibody. Similar results have been obtained in a model of burn injury plus ethanol (13, 38) however, our's is the first to show that IL6 is a proximal driver of trauma-induced lymphocyte alterations in a model of blunt polytrauma. We postulate that the magnitude of the early systemic inflammatory response is one determinant of the extent and duration of the T-cell changes that follow severe injury.

In summary, our results confirm that IL-6 is not simply a biomarker of the injury response, but rather is a key driver. Delayed and transient IL-6 neutralization, or perhaps suppression of IL-6 signaling, could be a viable strategy to limit the consequences of sustained inflammation following severe injury. Rapid measurement of IL-6 levels in the early phase of post-injury care could be used as a guide to such IL-6 based interventions.

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ABBREVIATIONS

STAT3	Signal transducer and activator of transcription 3
SOCS3	Suppressor of Cytokine signaling 3
IL6	Interleukin 6
TNF-α	Tumor necrosis factor alpha
IFN γ	Interferon gamma
HS + T	Hemorrhagic Shock plus Trauma
ALT	<i>alanine-aminotransferase</i>
KC	Keratinocyte-derived chemokine
IP10	Interferon induced protein 10
TH	T-helper cell

MIP-1alpha Macrophage inhibitory protein 1 alpha

References

1. Chaudry IH, Ayala A. Mechanism of increased susceptibility to infection following hemorrhage. *Am J Surg*. 1993; 165(2A Suppl):59S–67S. [PubMed: 8439001]
2. Mi Q, Constantine G, Ziraldo C, Solovyev A, Torres A, Namas R, Bentley T, Billiar TR, Zamora R, Puyana JC, Vodovotz Y. A dynamic view of trauma/hemorrhage-induced inflammation in mice: principal drivers and networks. *PLoS One*. 2011; 6(5):e19424. [PubMed: 21573002]
3. Hierholzer C, Kalff JC, Omert L, Tsukada K, Loeffert JE, Watkins SC, Billiar TR, Tweardy DJ. Interleukin-6 production in hemorrhagic shock is accompanied by neutrophil recruitment and lung injury. *Am J Physiol*. 1998; 275(3 Pt 1):L611–L621. [PubMed: 9728057]
4. Hierholzer C, Kalff JC, Bednarski B, Memarzadeh F, Kim YM, Billiar TR, Tweardy DJ. Rapid and simultaneous activation of Stat3 and production of interleukin 6 in resuscitated hemorrhagic shock. *Arch Orthop Trauma Surg*. 1999; 119(5–6):332–336. [PubMed: 10447634]
5. Yang S, Zheng R, Hu S, Ma Y, Choudhry MA, Messina JL, Rue LW 3rd, Bland KI, Chaudry IH. Mechanism of cardiac depression after trauma-hemorrhage: increased cardiomyocyte IL-6 and effect of sex steroids on IL-6 regulation and cardiac function. *Am J Physiol Heart Circ Physiol*. 2004; 287(5):H2183–H2191. [PubMed: 15475534]
6. Zhu XL, Zellweger R, Zhu XH, Ayala A, Chaudry IH. Cytokine gene expression in splenic macrophages and Kupffer cells following hemorrhage. *Cytokine*. 1995; 7(1):8–14. [PubMed: 7749070]
7. Gebhard F, Pfeisch H, Steinbach G, Strecker W, Kinzl L, Bruckner UB. Is interleukin 6 an early marker of injury severity following major trauma in humans? *Arch Surg*. 2000; 135(3):291–295. [PubMed: 10722030]
8. Sperry JL, Friese RS, Frankel HL, West MA, Cuschieri J, Moore EE, Harbrecht BG, Peitzman AB, Billiar TR, Maier RV, Remick DG, Minei JP. Inflammation and the Host Response to Injury Investigators: Male gender is associated with excessive IL-6 expression following severe injury. *J Trauma*. 2008; 64(3):572–578. discussion 578–9. [PubMed: 18332795]
9. Meng ZH, Dyer K, Billiar TR, Tweardy DJ. Essential role for IL-6 in postresuscitation inflammation in hemorrhagic shock. *Am J Physiol Cell Physiol*. 2001; 280(2):C343–C351. [PubMed: 11208530]
10. Stapleton PP, Strong VE, Freeman TA, Winter J, Yan Z, Daly JM. Gender affects macrophage cytokine and prostaglandin E2 production and PGE2 receptor expression after trauma. *J Surg Res*. 2004; 122(1):1–7. [PubMed: 15522307]
11. Kimura F, Shimizu H, Yoshidome H, Ohtsuka M, Miyazaki M. Immunosuppression following surgical and traumatic injury. *Surg Today*. 2010; 40(9):793–808. [PubMed: 20740341]
12. Diehl S, Rincon M. The two faces of IL-6 on Th1/Th2 differentiation. *Mol Immunol*. 2002; 39(9):531–536. [PubMed: 12431386]
13. Fontanilla CV, Faunce DE, Gregory MS, Messingham KA, Durbin EA, Duffner LA, Kovacs EJ. Anti-interleukin-6 antibody treatment restores cell-mediated immune function in mice with acute ethanol exposure before burn trauma. *Alcohol Clin Exp Res*. 2000; 24(9):1392–1399. [PubMed: 11003205]
14. Yang S, Hu S, Choudhry MA, Rue LW 3rd, Bland KL, Chaudry IH. Anti-rat soluble IL-6 receptor antibody down-regulates cardiac IL-6 and improves cardiac function following trauma-hemorrhage. *J Mol Cell Cardiol*. 2007; 42(3):620–630. [PubMed: 17313958]
15. Mommsen P, Barkhausen T, Frink M, Zeckey C, Probst C, Krettek C, Hildebrand F. Productive capacity of alveolar macrophages and pulmonary organ damage after femoral fracture and hemorrhage in IL-6 knockout mice. *Cytokine*. 2011; 53(1):60–65. [PubMed: 20934884]
16. Moran A, Tsimelzon AI, Mastrangelo MA, Wu Y, Yu B, Hilsenbeck SG, Poli V, Tweardy DJ. Prevention of trauma/hemorrhagic shock-induced lung apoptosis by IL-6-mediated activation of Stat3. *Clin Transl Sci*. 2009; 2(1):41–49. [PubMed: 20443866]

17. Alten JA, Moran A, Tsimelzon AI, Mastrangelo MA, Hilsenbeck SG, Poli V, Tweardy DJ. Prevention of hypovolemic circulatory collapse by IL-6 activated Stat3. *PLoS One*. 2008; 3(2):e1605. [PubMed: 18270592]
18. Meng ZH, Dyer K, Billar TR, Tweardy DJ. Distinct effects of systemic infusion of G-CSF vs. IL-6 on lung and liver inflammation and injury in hemorrhagic shock. *Shock*. 2000; 14(1):41–48. [PubMed: 10909892]
19. Kobbe P, Kaczorowski DJ, Vodovotz Y, Tzioupi CH, Mollen KP, Billiar TR, Pape HC. Local exposure of bone components to injured soft tissue induces Toll-like receptor 4-dependent systemic inflammation with acute lung injury. *Shock*. 2008; 30(6):686–691. [PubMed: 18461022]
20. Cai C, Cao Z, Loughran PA, Kim S, Darwiche S, Korff S, Billiar TR. Mast cells play a critical role in the systemic inflammatory response and end-organ injury resulting from trauma. *J Am Coll Surg*. 2011; 213(5):604–615. [PubMed: 21920785]
21. Darwiche SS, Pfeifer R, Menzel C, Ruan X, Hoffman M, Cai C, Chanthaphavong RS, Loughran P, Pitt BR, Hoffman R, Pape HC, Billiar TR. Inducible nitric oxide synthase contributes to immune dysfunction following trauma. *Shock*. 2012; 38(5):499–507. [PubMed: 23042189]
22. Cuschieri J, Bulger E, Schaeffer V, Sakr S, Nathens AB, Hennessy L, Minei J, Moore EE, O'Keefe G, Sperry J, et al. Early elevation in random plasma IL-6 after severe injury is associated with development of organ failure. *Shock*. 2010; 34(4):346–351. [PubMed: 20844410]
23. Xiao W, Mindrinos MN, Seok J, Cuschieri J, Cuenca AG, Gao H, Hayden DL, Hennessy L, Moore EE, Minei JP, et al. A genomic storm in critically injured humans. *J Exp Med*. 2011; 208(13):2581–2590. [PubMed: 22110166]
24. Roumen RM, Hendriks T, van der Ven-Jongekrijg J, Nieuwenhuijzen GA, Sauerwein RW, van der Meer JW, Goris RJ. Cytokine patterns in patients after major vascular surgery, hemorrhagic shock, and severe blunt trauma. Relation with subsequent adult respiratory distress syndrome and multiple organ failure. *Ann Surg*. 1993; 218(6):769–776. [PubMed: 8257227]
25. Li Y, Du H, Qin Y, Roberts J, Cummings OW, Yan C. Activation of the signal transducers and activators of the transcription 3 pathway in alveolar epithelial cells induces inflammation and adenocarcinomas in mouse lung. *Cancer Res*. 2007; 67(18):8494–8503. [PubMed: 17875688]
26. Yasukawa H, Ohishi M, Mori H, Murakami M, Chinen T, Aki D, Hanada T, Takeda K, Akira S, Hoshinima M, et al. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat Immunol*. 2003; 4(6):551–556. [PubMed: 12754507]
27. Gill R, Ruan X, Menzel CL, Namkoong S, Loughran P, Hackam DJ, Billiar TR. Systemic inflammation and liver injury following hemorrhagic shock and peripheral tissue trauma involve functional TLR9 signaling on bone marrow-derived cells and parenchymal cells. *Shock*. 2011; 35(2):164–170. [PubMed: 20577143]
28. Gill R, Tsung A, Billiar TR. Linking oxidative stress to inflammation: Toll-like receptors. *Free Radic Biol Med*. 2010; 48(9):1121–1132. [PubMed: 20083193]
29. Schneider CP, Schwacha MG, Chaudry IH. The role of interleukin-10 in the regulation of the systemic inflammatory response following trauma-hemorrhage. *Biochim Biophys Acta*. 2004; 1689(1):22–32. [PubMed: 15158910]
30. Frink M, Hsieh YC, Hsieh CH, Pape HC, Choudhry MA, Schwacha MG, Chaudry IH. Keratinocyte-derived chemokine plays a critical role in the induction of systemic inflammation and tissue damage after trauma-hemorrhage. *Shock*. 2007; 28(5):576–581. [PubMed: 18084824]
31. Hildebrand F, Hubbard W, Choudhry MA, Frink M, Pape HC, Kunkel SL, Chaudry IH. Kupffer cells and their mediators: the culprits in producing distant organ damage after trauma-hemorrhage. *Am J Pathol*. 2006; 169(3):784–794. [PubMed: 16936255]
32. Hsieh CH, Frink M, Hsieh YC, Kan WH, Hsu JT, Schwacha MG, Choudhry MA, Chaudry IH. The role of MIP-1 alpha in the development of systemic inflammatory response and organ injury following trauma hemorrhage. *J Immunol*. 2008; 181(4):2806–2812. [PubMed: 18684972]
33. Mack VE, McCarter MD, Maama HA, Calvano SE, Daly JM. Dominance of T-helper 2-type cytokines after severe injury. *Arch Surg*. 1996; 131(12):1303–1308. discussion 1308–9. [PubMed: 8956772]

34. Diehl S, Anguita J, Hoffmeyer A, Zapton T, Ihle JN, Fikrig E, Roncon M. Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity*. 2000; 13(6):805–815. [PubMed: 11163196]
35. Ayala A, Lehman DL, Herdon CD, Chaudry IH. Mechanism of enhanced susceptibility to sepsis following hemorrhage. Interleukin-10 suppression of T-cell response is mediated by eicosanoid-induced interleukin-4 release. *Arch Surg*. 1994; 129(11):1172–1178. [PubMed: 7979949]
36. Lyons A, Goebel A, Mannick JA, Lederer JA. Protective effects of early interleukin 10 antagonism on injury-induced immune dysfunction. *Arch Surg*. 1999; 134(12):1317–1323. discussion 1324. [PubMed: 10593329]
37. Huang DR, Wang J, Kivisakk P, Rollins BJ, Ransohoff RM. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J Exp Med*. 2001; 193(6):713–726. [PubMed: 11257138]
38. Faunce DE, Gregory MS, Kovacs EJ. Acute ethanol exposure prior to thermal injury results in decreased t-cell responses mediated in part by increased production of IL-6. *Shock*. 1998; 10(2): 135–140. [PubMed: 9721981]

Fig. 1.

Effects of anti-IL-6 mAb on serum IL-6 levels and expression of phosphorylated STAT3 and SOCS3 following HS+T. Serum samples and livers were collected at 6 h, 24 h, or 48 h following HS+T or control treated with Isotype control Ab or anti-IL-6 mAb. Serum concentrations of IL-6 (A, B, C) were determined by ELISA as described in Methods. The expression of STAT3, phosphorylated STAT3 and SOCS3 was assessed by Western blotting analysis (D). Relative intensity of paired bands was compared after normalization with the density ratio of GAPDH expressions (E~J). Data are expressed as mean $\pm$ SE from 4 to 6

mice for each group. * $P < 0.05$ HS+PF vs. control and # $P < 0.05$ Isotype control Ab vs. Anti-IL-6 mAb by ANVOA

Fig. 2.

Histology of liver and lung injury following HS+T. Mice were sacrificed at 6 h, 24 h and 48 h time points following HS+T or control treated with Isotype control Ab or anti-IL-6 mAb, hepatic and pulmonary histopathological analysis was performed using Hematoxylin and eosin (H&E) staining as described in Materials and Methods (scale bar=50 μ m).

Representative results from 4 mice are shown for each group

Fig. 3.

Effect of IL-6 neutralization on lung injury scores (A, B, C) and serum ALT levels (D, E, F) at 6, 24, and 48 h following HS+T or control treated with Isotype control Ab or anti-IL-6 mAb. Data are expressed as mean $\pm$ SE from 5 to 6 mice for each group. * $P < 0.05$ HS+PF vs. control and # $P < 0.05$ Isotype control Ab vs. Anti-IL-6 mAb by ANOVA

Fig. 4. Effect of IL-6 neutralization on serum cytokines/chemokines Levels following HS+T. Serum samples were collected via cardiac puncture at 6, 24, and 48 h following HS+T or control treated with Isotype control Ab or anti-IL-6 mAb. Serum concentrations of IL-10 (A, B, C) and KC (D, E, F), MCP-1 (G, H, I), IP-10 (J, K, L) and MIP-1a (M, N, O) were determined by ELISA as described in Methods. Data are expressed as mean $\pm$ SE from 5 to 6 mice for each group. * $P < 0.05$ HS+T vs. control and # $P < 0.05$ Isotype control Ab vs. Anti-IL-6 mAb by ANOVA

Fig. 5.

Effect of IL-6 neutralization on splenocyte cytokine production with ConA stimulation following HS+T. Mice were sacrificed at 6 h (A, D, G), 24 h (B, E, H) and 48 h (C, F, I) time points following HS+T separately; spleens were aseptically removed and processed for single-cell suspension. Splenocytes were cultured with ConA (2.5 μg/ml) for 48 h. Supernatants were harvested for measurement of cytokines TNF-α (A, B, C), IFN-γ (D, E, F) and IL-10 (H, I, J). Data are mean ± SE from 5 to 6 mice for each group. * $P < 0.05$ HS+T vs. control and # $P < 0.05$ Isotype control Ab vs. Anti-IL-6 mAb by ANOVA

Fig. 6.

Neutralizing IL-6 Partially Prevents the Suppression in Splenocyte Proliferation Following HS+T Splenocytes were isolated from control or HS+T mice at 6 h(A), 24 h(B) and 48 h(C) following HS+T separately; spleens were aseptically removed and processed for single-cell suspension. Splenocyte were cultured with ConA (2.5 μ g/ml) for 48h, and proliferation was measured by the [3 H] thymidine incorporation technique. cpm, Counts per min. Data are mean $\pm$ SE from 5 to 6 mice for each group. * P <0.05 HS+PF vs. control and # P <0.05 Isotype control Ab vs. Anti-IL-6 mAb