Published in final edited form as:

J Trauma. 2004 April ; 56(4): 742–748.

Putative Mechanism of Hemorrhage-Induced Leukocyte

Hyporesponsiveness:

Induction of Suppressor of Cytokine Signaling-3

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Abstract

Background—After hemorrhagic shock, macrophages are less responsive to lipopolysaccharide (LPS) regarding cytokine production and receptor expression. However, mechanisms responsible for this are poorly understood. Suppressors of cytokine signaling (SOCS) proteins have been found to play a prominent role in LPS tolerance and cytokine desensitization in macrophages. Therefore, the purpose of this study was to determine whether hemorrhagic shock induced SOCS expression.

Methods—Male C3H/HeN mice were subjected to hemorrhage or sham hemorrhage. Twenty-four hours after each procedure, tissues were harvested, the cells were processed for protein, and SOCS expression was examined.

Results—Our data show that SOCS-1 expression does not change after hemorrhage, but SOCS-3 is up-regulated in a tissue and cell population (e.g., macrophage)-specific manner.

Conclusion—These data suggest that cytokines or other inflammatory mediators present during the first 24 hours after the induction of shock have the ability to induce tolerance to LPS or cytokines and suppress the function of immune cells by up-regulating SOCS-3.

Keywords

Immunosuppression; Shock; Mouse

Hemorrhage that arises from soft tissue trauma or bone injury can lead to life-threatening loss of blood and is a common complication in most trauma patients.¹ The impact of trauma/hemorrhage on the immune system has been an active area of study for almost three decades.¹ Several studies have shown that the immune system is “primed” by traumatic insults, such as hemorrhage, resulting in increased cytokine production and tissue injury mediated primarily by neutrophil infiltration and subsequent activity.^{2–4} However, hemorrhage alone, without a coinciding insult such as tissue trauma or bone injury, has also been shown to induce immunosuppression by reducing antigen presentation,⁵ reducing clearance and killing of microorganisms,⁶ altering cytokine expression,^{7–11} suppressing mitogen-induced lymphocyte function,^{10–12} and enhancing susceptibility to sepsis.¹³

Many of the functions listed above are regulated by a variety of signal transduction pathways, both positively and negatively. The signal transduction mechanisms used in response to

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Presented at the 62nd Annual Meeting of the American Association for the Surgery of Trauma, September 11–13, 2003, Minneapolis, Minnesota.

hemorrhage are less well studied, but nuclear factor- κ B, Janus kinase 1 (JAK1), and members of the signal transducers and activators of transcription (STAT) family have been found to be activated after hemorrhage.¹⁴⁻¹⁶ As immunosuppression is observed within 24 hours and can last several days, unresponsiveness to further insults may be a result of a blunting mechanism induced by these pathways. Recently, a family of proteins that has the potential to negatively regulate responses after hemorrhage has emerged and is collectively known as the suppressors of cytokine signaling (SOCS) proteins (reviewed in Alexander,¹⁷ Greenhalgh and Hilton,¹⁸ and Cooney¹⁹). Although initially identified as inhibitors of the JAK-STAT pathway, the SOCS proteins have also been shown to interfere with cell signaling by promoting the degradation of signaling proteins^{20,21} as well as binding to a variety of signal transducers.^{17,22-24}

SOCS proteins are induced by a wide variety of stimuli, many of whose actions are inhibited by SOCS, thereby representing a negative feedback control mechanism. Again, these proteins were first identified as being induced by cytokines that use the JAK-STAT pathway,^{18,20} but they can also be induced in a JAK-STAT-independent manner.^{25,26} Regarding immunosuppression, SOCS-1 and SOCS-3 are the only members of this family that appear to have roles in the immune system,¹⁷ and have been shown to be induced by numerous cytokines and inflammatory factors.²⁵⁻²⁷ Indeed, SOCS-3 has been found to be up-regulated in the liver of septic rats²⁸ and rats subjected to burn trauma.^{29,30} Similarly, SOCS-1 has also been found to be up-regulated in the spleen in response to burn trauma.²⁹ Although the exact roles of the SOCS proteins in these models have not been fully elucidated, their expression has been linked to organ dysfunction, and in one case coincides with decreased STAT3.³⁰

A putative role for the SOCS proteins in the immunosuppression observed after hemorrhage has emerged by means of a variety of studies. As mentioned above, one classic measure of immunosuppression is a reduced function in response to exogenous stimulation, which may be blocked by SOCS. In addition, the SOCS proteins have been shown to be crucial mediators of lipopolysaccharide (LPS) tolerance both in vitro³¹⁻³³ and in vivo,³² and therefore may be an important factor in the increased mortality that has been documented for hemorrhaged animals hit with a septic insult.¹³ From these data, we proposed the hypothesis that SOCS protein expression, SOCS-1 and SOCS-3, is pathologically up-regulated in the immune cells of hemorrhaged mice and contributes to the immunosuppression seen in these animals. To begin to examine their roles in the immune response, we profiled the expression patterns of SOCS-1 and SOCS-3 over 24 hours after hemorrhage.

MATERIALS AND METHODS

Buffers and reagents used included Hank's balanced salt solution and phosphate-buffered saline (PBS), obtained from Invitrogen (Grand Island, NY). SOCS-3 (M-20) and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotech, Inc. (Santa Cruz, CA). SOCS-1 antibody (Abcam ab9870) was obtained from Novus Biologicals, Inc. (Littleton, CO).

Animal Use

Male mice were used in all studies. C3H/HeN (HeN) mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were used at 6 to 8 weeks of age and weighed approximately 20 to 25 g. The studies performed here were all carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Rhode Island Hospital Committee on Animal Use and Care.

In Vitro Induction of SOCS Protein

The macrophage cell line RAW264.7 was used as a positive control for SOCS expression. RAW264.7 were incubated in RPMI-1640 containing 10% fetal calf serum (Hyclone, Logan, UT) with or without 10 µg/mL of LPS (Sigma, St. Louis, MO) for 4 hours. The dose of LPS used is a standard dose used in our laboratory for in vitro stimulation.^{34,35} The cells were lysed in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, 150 mmol/L sodium chloride, 10 mmol/L sodium phosphate, 10 mmol/L sodium fluorescein, 1 mmol/L sodium orthovanadate, 0.5% Triton X100, and Sigma's protease inhibitor cocktail) for 30 minutes on ice, and protein was quantitated using the Bio-Rad Protein Assay (Hercules, CA) and stored at -80°C until use.

Hemorrhage Model of Shock

Shock was induced in the mouse using a hemorrhage model previously established in our laboratory.^{4,13} In brief, under anesthesia with Metofane (Schering-Plough Animal Health Corp., Union, NJ), polyethylene No. 10 catheters were placed into both femoral arteries. The animals were allowed to awaken and the blood pressure was constantly monitored through one catheter using a model P23 ID strain gauge transducer (Gould, Inc., Oxnard, CA). The mouse was bled through the other catheter to maintain a mean arterial blood pressure of 30 ± 5 mm Hg, which was maintained for 90 minutes. The amount of shed blood was recorded, and at the end of the 90-minute period, the mouse was resuscitated with lactated Ringer's solution at four times the volume of shed blood. The catheters were then removed, the vessels were ligated, and the incisions were closed. Sham-operated controls (Sham) had their femoral arteries ligated and were restrained for periods of time equal to their hemorrhaged counterparts, but no blood was shed. Animals were allowed food and water ad libitum.

Sample Preparation

Twenty-four hours postsurgery, the mice were killed, and the blood, thymus, lung, liver, peritoneal leukocytes, and spleen were collected. Blood leukocytes were obtained by dextran sedimentation of whole blood. Thymocytes and splenocytes were obtained by gently grinding the organs between ground-glass slides. Contaminating red blood cells in all populations were lysed with hypotonic lysis. Splenocytes were further separated with antimouse B220-coated Dynabeads (Dyna, Great Neck, NY) to enrich B cells or adherence to plastic plates to enrich macrophages. The nonadherent cells consisted of T cells, neutrophils, dendritic cells, and other cells. Cells were then lysed in lysis buffer for 30 minutes on ice. Liver and lung samples were homogenized in lysis buffer using a PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA). Protein content in the lysates was determined using the Bio-Rad protein assay.

Western Blot Analysis

Thirty to 50 µg of protein was separated on 16% poly-acrylamide gels and transferred to PVDF according to the manufacturer's instructions (Novex, San Diego, CA). Membranes were then blocked with 5% milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 2 hours at room temperature. SOCS-1 (0.5 µg/mL) or SOCS-3 (1:500) antibody was diluted in 2% milk in TBST, added to blots, and incubated overnight at 4°C. Membranes were washed three times with TBST and incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. After washing three times with TBST and once with Tris-buffered saline, proteins were visualized by ECL (Amersham Biosciences, Piscataway, NJ). Protein bands were quantitated by densitometry using SigmaGel software.

Statistical Analysis

The Student's *t* test was used to determine significant differences between the sham and hemorrhage groups. Statistical analysis was performed using SigmaStat software. Data were considered significant at a value of $p < 0.05$.

RESULTS

To determine which populations exhibited an up-regulation of SOCS-1 or SOCS-3 protein in response to hemorrhagic shock, mice were subjected to sham or hemorrhage operations. Twenty-four hours postsurgery, the lung, thymocytes, blood leukocytes, peritoneal leukocytes, splenocytes, and liver were examined for SOCS expression by Western blot analysis. In all tissues tested, SOCS-1 protein was readily detectable and did not appear to change after hemorrhagic shock (Fig. 1). Similarly, although SOCS-1 mRNA is induced in RAW264.7 cells on LPS stimulation,^{25,33} the protein levels did not change dramatically (Fig. 1).

Consistent with previously published reports,²⁵ SOCS-3 protein was low to undetectable in unstimulated RAW264.7 cells (data not shown) and was significantly up-regulated on stimulation with LPS (RAW+) (Fig. 2A). Similarly, SOCS-3 was low to undetectable in all tissues tested from the sham control animals (Fig. 2A), suggesting that the trauma of the procedure itself (i.e., extended restraint, small incision, and vessel ligation) does not induce SOCS-3 expression. Just as we previously observed in sepsis,³⁶ SOCS-3 expression in response to hemorrhagic shock was tissue-specific. SOCS-3 was undetectable in the liver, thymus, and peripheral blood leukocytes 24 hours posthemorrhage (Fig. 2A). SOCS-3 protein was up-regulated in the peritoneal leukocytes, spleen, and lung tissue 24 hours posthemorrhage (Fig. 2A). However, in contrast to what was seen in sepsis, where the most prominent increase in SOCS-3 occurred in the peritoneal leukocytes,³⁶ the largest increase in SOCS-3 expression occurred in the spleen (Fig. 3). Therefore, in an effort to identify the cell population contributing to this increase in SOCS-3 signal, splenocytes were further separated to determine the cellular source. Similar to our previous observation in sepsis, we found that splenic macrophages are the major cell population that expresses SOCS-3 in response to hypotension (Fig. 2B).

DISCUSSION

The SOCS proteins are an emerging family of regulatory proteins that inhibit cell signaling and cellular functions by means of a variety of mechanisms. Therefore, the aim of this study was to profile the expression pattern of SOCS-1 and SOCS-3 after hemorrhage to begin to ascertain its putative role in the immunosuppression observed after hemorrhagic shock. The results presented here both support (SOCS-3) and contradict (SOCS-1) our hypothesis that these proteins play a role in immunosuppression.

The observation that SOCS-1 protein is readily detectable in all tissues and did not vary was not expected (Fig. 1) and does not support our hypothesis of SOCS-1 being an immunosuppressive agent. However, these data are in agreement with similar studies using a sepsis model, in which SOCS-1 is readily detectable in all tissues and only changes in the spleen and thymus (C.S. Chung, unpublished data). This brought the accuracy of the antibody into question, but mRNA analysis by reverse-transcriptase polymerase chain reaction confirmed our results. The consistent expression of SOCS-1 may also be attributed to its hypothesized role as a hematologic regulator, as SOCS-1 knockout mice die within weeks of birth because of uncontrolled infiltration into organs (liver, lungs, pancreas, and heart) and a depletion of B and T lymphocytes.³⁷ This latter scenario is in agreement with our data, as hemorrhage does not induce changes in immune cell populations or induce deleterious levels of lymphocyte infiltration into organs.

In contrast, SOCS-3 expression is tissue- and stimulus (hemorrhage)-dependent. Similar to our sepsis model,³⁶ SOCS-3 was undetectable in the blood leukocytes and thymus under all conditions (Fig. 2). However, in contrast to sepsis^{28,36} and burn trauma,^{29,30} SOCS-3 was also undetectable in the liver (Fig. 2). This suggests that SOCS-3 induction in this tissue is dependent on the type of trauma received and not a general response to a traumatic insult or infection. The absence of SOCS-3 in the liver is also consistent with the studies by Hierholzer et al. in which STAT3 activity is increased in the liver after hemorrhage,¹⁴ which is also in contrast to that seen in the liver after burn trauma.³⁰

SOCS-3 expression is induced by hemorrhagic shock in the lung, spleen, and peritoneal leukocytes (Fig. 2), with the most prominent up-regulation being in the spleen (Fig. 3). The importance of SOCS-3 expression and its exact role in immunosuppression is difficult to ascertain. First, the low level of induction in the lung may be a result of a dilution effect of the affected populations (i.e., SOCS-3 might be dramatically up-regulated in alveolar macrophages), but this is masked by the noninduction of SOCS-3 in lung tissue itself. Therefore, further investigation is needed to determine whether SOCS-3 is involved in immunosuppression in lung macrophages. Second, peritoneal macrophages have altered functions after hemorrhage; however, the low level of SOCS-3 (Fig. 3), versus a 10-fold induction observed after sepsis,³⁶ may or may not be enough to induce the immunosuppression. This too will take further investigation. In contrast, the up-regulation of SOCS-3 in the spleen fully supports the hypothesis that it plays a significant role in the immunosuppression observed in this tissue. Previous reports have indicated that SOCS-3 is induced in T cells, B cells, neutrophils, and macrophages by a variety of agents both in vitro and in vivo.^{17,26,38,39} Consistent with our previous report in sepsis,³⁶ splenic macrophages are the primary cells that express SOCS-3 protein 24 hours posthemorrhage.

The SOCS family proteins have been recently identified as important feedback negative regulatory factors in cytokine signaling through the JAK-STAT pathway. Gene knockout experiments suggest a crucial role for SOCS-1 (i.e., regulation of T-cell development and interferon- γ signaling) and SOCS-3 (i.e., fetal liver erythropoiesis and lymphocyte proliferation; reviewed in Greenhalgh and Hilton,¹⁸ Chen et al.,⁴⁰ and O'Shea et al.⁴¹). Although both SOCS-1 and SOCS-3 inhibit JAK-STAT signaling, the specificity of biological function is dependent on the nature of the stimuli as well as the target organs/tissues/cells. Ram and Waxman⁴² reported that inhibition of JAK-STAT signaling by the various SOCS proteins use different mechanisms/sites of interaction with distinct targets on the cytokine receptor-JAK complex. The different expression patterns observed for SOCS-1 and SOCS-3 within the hemorrhagic mouse as well as those seen in different models, such as burn or endotoxemia,²⁹ suggest that depending on the nature of stimuli, cell/tissue sites, or time course, various SOCS family proteins are induced differentially. It will be important to understand how these different SOCS proteins contribute to the regulation of the cytokine network, which plays a crucial role in the mortality and morbidity in trauma/shock patients. In this regard, our data may provide the groundwork for the initiation of future studies delineating the role of SOCS proteins in shock.

In summary, SOCS-3 has been found to be up-regulated in the lungs, spleens, and peritoneal leukocytes in response to hemorrhagic shock and may contribute to the immunosuppression observed. The specific roles SOCS-3 play in this immunosuppression remain the primary focus of ongoing research. We have obtained SOCS-1 and SOCS-3 knockout mice in which we will be able to more closely examine the effect each of these proteins has in the ability of the animal to recover from hemorrhagic shock or respond to further insults such as sepsis. From these studies, we hope to gain a further understanding of the pathologic aspects of hemorrhagic shock and possibly provide information that will help in the discovery of new therapies to help treat patients.

Acknowledgements

Supported by National Institutes of Health grant RO1-GM46354 (to A.A.).

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Fig. 1.

SOCS-1 expression is not altered by hemorrhage. Blood, spleen, lung, liver, thymus, and peritoneal leukocyte samples were examined for SOCS-1 expression 24 hours after sham hemorrhage or hemorrhage by Western blot analysis. RAW264.7 cells incubated for 4 hours, either unstimulated (Raw-) or stimulated with LPS (Raw+), were used as controls. Each lane represents samples taken from individual animals. Blots shown are representative of three individual experiments (three different groups of mice, two sham and two hemorrhaged mice analyzed per group).

Fig. 2.

Tissue-specific expression of SOCS-3 after hemorrhage. Blood, spleen, lung, liver, thymus, and peritoneal leukocyte samples were examined for SOCS-3 expression 24 hours after sham hemorrhage or hemorrhage by Western Blot analysis (A). RAW264.7 cells stimulated with LPS (Raw+) for 4 hours were used as controls. Each lane represents samples taken from individual animals. Splenic cellular source of SOCS-3 expression in B cells, macrophages (Mφ), or nonadherent cells (NAdh) from hemorrhaged mice was also assessed (B). Blots shown are representative of three individual experiments (three different groups of mice, two sham and two hemorrhaged mice analyzed per group).

Fig. 3.

SOCS-3 expression in lung, spleen, and peritoneal leukocytes. The relative up-regulation of SOCS-3 expression in the lung, spleen, and peritoneal leukocytes represented in Figure 2 was determined. Protein bands were quantitated by means of densitometry and expressed as optical density per centimeter squared (arbitrary units). Results presented as mean $\pm$ SEM (n = 6).

*SOCS-3 expression after hemorrhage that was significantly greater than that measured in sham controls (p < 0.05).