

Hemoadsorption Reprograms Inflammation in Experimental Gram-negative Septic Peritonitis: Insights from *In Vivo* and *In Silico* Studies

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Improper compartmentalization of the inflammatory response leads to systemic inflammation in sepsis. Hemoadsorption (HA) is an emerging approach to modulate sepsis-induced inflammation. We sought to define the effects of HA on inflammatory compartmentalization in *Escherichia coli*-induced fibrin peritonitis in rats. Hypothesis: HA both reprograms and re-compartmentalizes inflammation in sepsis. Sprague Dawley male rats were subjected to *E. coli* peritonitis and, after 24 h, were randomized to HA or sham treatment (sepsis alone). Venous blood samples collected at 0, 1, 3 and 6 h (that is, 24–30 h of total experimental sepsis), and peritoneal samples collected at 0 and 6 h, were assayed for 14 cytokines along with $\text{NO}_2^-/\text{NO}_3^-$. Bacterial counts were assessed in the peritoneal fluid at 0 and 6 h. Plasma tumor necrosis factor (TNF)- α , interleukin (IL)-6, CXCL-1, and CCL2 were significantly reduced in HA versus sham. Principal component analysis (PCA) suggested that inflammation in sham was driven by IL-6 and TNF- α , whereas HA-associated inflammation was driven primarily by TNF- α , CXCL-1, IL-10 and CCL2. Whereas peritoneal bacterial counts, plasma aspartate transaminase levels and peritoneal IL-5, IL-6, IL-18, interferon (IFN)- γ and $\text{NO}_2^-/\text{NO}_3^-$ were significantly lower, both CXCL-1 and CCL2 as well as the peritoneal-to-plasma ratios of TNF- α , CXCL-1 and CCL2 were significantly higher in HA versus sham, suggesting that HA-induced inflammatory re-compartmentalization leads to the different inflammatory drivers discerned in part by PCA. In conclusion, this study demonstrates the utility of combined *in vivo/in silico* methods and suggests that HA exerts differential effects on mediator gradients between local and systemic compartments that ultimately benefit the host.

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INTRODUCTION

Sepsis is a complex systemic inflammatory clinical syndrome that occurs because of an overwhelming inflammatory/immune response to infection and is associated with the so-called systemic inflammatory response syndrome (SIRS) (1).

This multifaceted inflammatory activation occurs via stimulation of the host immune effector cells, leading to the release of chemokines, cytokines and reactive oxygen/nitrogen species. This process functions properly when these mediators are restricted to specific tissues, wherein local

injury or infection induces a well-regulated inflammatory response. However, once the levels of these cytokines rise sufficiently so that they begin to appear in the bloodstream at levels capable of inducing a systemic response, inflammation takes on many of its harmful characteristics. Sustained elevations of both pro- and anti-inflammatory cytokines, and presumably their complex interactions with inflammatory, endothelial and parenchymal cells, identify those sepsis patients who subsequently develop multiple organ dysfunction syndrome (MODS), shock and death (2).

This complex immunobiology may help explain the failure of therapeutic at-

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tempts to control inflammation via blocking a single inflammatory mediator (3). An alternative strategy involves the removal of excessive inflammatory mediators from the circulation. Blood purification therapy techniques that use an extracorporeal device can remove a wide array of inflammatory mediators from circulating blood nonspecifically (4–6). This multimediator targeting may promote the downregulation of systemic inflammation and assist the body in regaining homeostasis (7).

We previously described an extracorporeal hemoadsorption (HA) device that can nonspecifically remove low-molecular weight proteins (including cytokines and chemokines) from circulating blood by using biocompatible sorbent beads (CytoSorb; CytoSorbents, Princeton, NJ, USA). CytoSorb HA beads are polystyrene-divinylbenzene porous particles (450 μm average particle diameter, 0.8–5 nm pore diameter, 850 m^2g^{-1} surface area) with a biocompatible polyvinyl-pyrrolidone coating. Adsorption to the internal pore surface is accomplished by a putative combination of nonspecific hydrophobic interactions, as well as size exclusion of large-molecular weight solutes >70 kDa, such as albumin and immunoglobulins. This device has been effective in removing tumor necrosis factor (TNF- α), interleukin (IL)-6 and IL-10 and other middle-molecular weight proteins in both *in vitro* and *ex vivo* studies and at improving survival in experimental models of endotoxemia and sepsis (8,9). In the present study, we tested HA in a rat model of *E. coli*-induced fibrin clot peritonitis and sought to determine the principal components responsible for the effects of HA on acute inflammation accompanying experimental sepsis.

MATERIALS AND METHODS

Surgical Preparation and Fibrin Clot Implantation

The study was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conforms

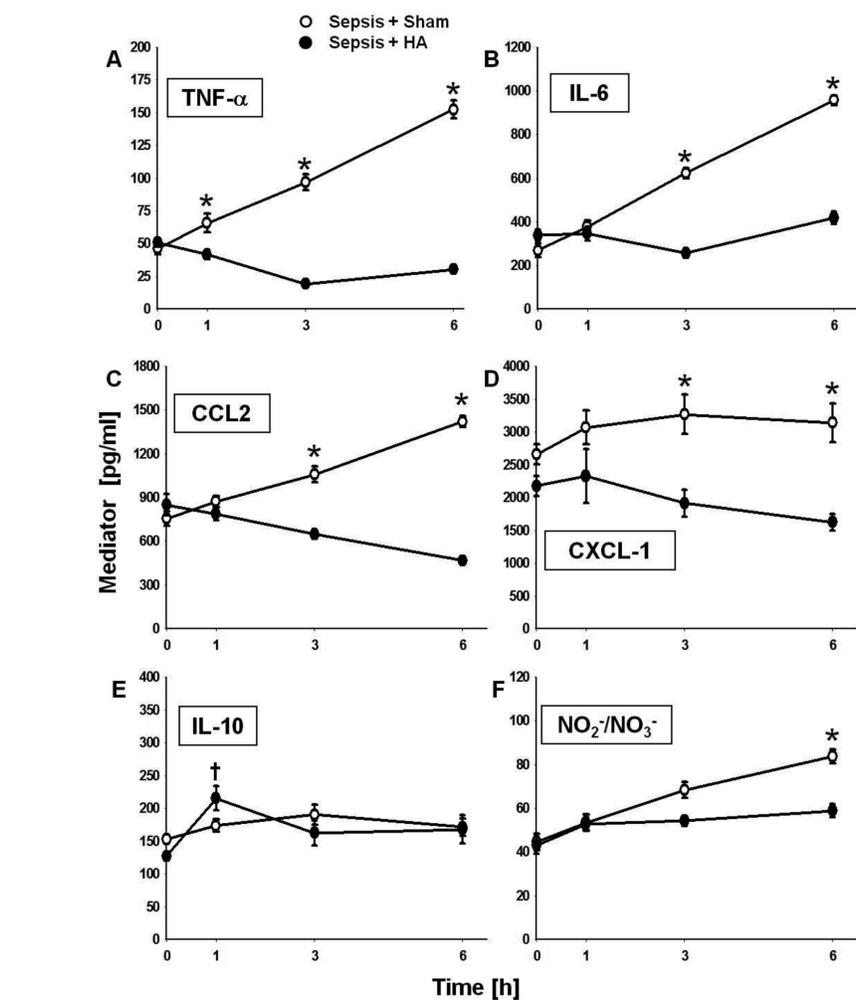


Figure 1. Effects of HA on circulating levels of inflammatory mediators. Postcannulation baseline values (that is, 24 h after implantation of an *E. coli*-impregnated fibrin clot and hence established sepsis) for all cytokines were not significantly different between the two experimental groups. Circulating TNF- α (A), IL-6 (B), CCL2 (C) and CXCL-1 (D) were significantly lower in the HA group versus the sham group (* $p < 0.05$ versus sham group; [†] $p < 0.05$ versus HA at 0 h). Circulating NO₂⁻/NO₃⁻ after 6 h of treatment was significantly lower (F) (* $p < 0.001$ versus sham; [†] $p < 0.05$ versus HA at 0 h). Note: Some error bars are not visible, since they are overlapped by the black symbol.

to U.S. National Institutes of Health guidelines for the care and use of laboratory animals. After acclimatization for 5 d, adult male Sprague Dawley rats ($n = 21$, 24–28 wks old, 430–480 g body weight, from Harlan Laboratories) were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneally). The animals were then subjected to an *Escherichia coli* (strain ATCC 25922; American Type Culture Collection, Manassas, VA, USA) inoculum in a fibrin clot (1.5×10^8

to 2×10^8 colony-forming units [CFUs]/clot; see details below) introduced into the peritoneum via laparotomy (10). Twenty-four hours later, the rats were re-anesthetized and the femoral and the internal jugular veins were isolated by dissection and cannulated with 0.97-mm polyethylene 50 tubing, for use in the extracorporeal circuit.

The rats were then treated with HA or a sham circuit ($n = 8$ rats each) for 6 h, for a total of 30 h of experimental sepsis,

as follows. All the tubing in the extracorporeal circulation was flushed with heparinized saline (5 units/mL). The extracorporeal circuit was started from the femoral vein and returned back to the ipsilateral internal jugular vein by action of a mechanic minipump (Fisher Scientific). For HA, a 1.5-g CytoSorb cartridge (Cytosorbents) with a dead space of 0.3–0.4 mL was placed in the circuit of animals randomized to the HA group, whereas no cartridge was used in the sham group. Blood flow was maintained by the minipump at a rate of 0.8–1.0 mL/min and regulated by a stopcock at the precartridge side. Both the size of the device and the blood flow rate were scaled to those used for humans (approximately a 300-g device on the basis of body weight with a blood flow of 100–150 mL/min on the basis of comparable blood volume). Blood samples (0.4 mL) were collected from the internal jugular vein at 0, 1, 3 and 6 h (that is, 24–30 h of total experimental sepsis). After blood withdrawal, the catheters were flushed with an equal volume of heparinized saline (5 U/mL). After 6 h, the treatment was stopped and an intraperitoneal fluid sample was collected by injecting 10 mL sterile isotonic saline into the peritoneum via an 18-gauge needle. The abdomen was gently manipulated and the peritoneal fluid was aspirated for cytokine assay (n = 6, HA or sham) and total bacterial count (n = 8, HA or sham). The rats were then euthanized by administering an overdose of pentobarbital sodium followed by cervical dislocation.

For the control animals, a separate group of animals (n = 9) were subjected to a laparotomy incision followed by implantation of a fibrin clot that lacks *E. coli*. After 24 h after implantation of the fibrin clot, the animals were euthanized and blood samples were collected via a cardiac puncture.

Preparation and Dose Estimation of *E. Coli*-Impregnated Fibrin Clot

Bacteria were quantified using a spectrophotometer (DU 530 UV/VIS; Beckman Coulter, Brea, CA, USA) on the day

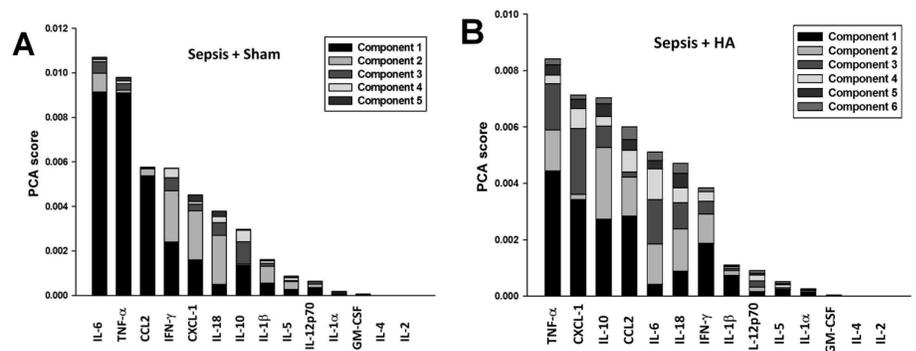


Figure 2. Rats were subjected to sepsis by the implantation of a fibrin clot impregnated with *E. coli* as described in the Materials and Methods. PCA suggests different drivers of inflammation in HA versus sham. Principal component analysis suggested that the circulating inflammatory response in the sham group was primarily driven by IL-6 and TNF-α (A), whereas the response to HA was primarily driven by TNF-α, CXCL-1, IL-10 and CCL2 (B).

of bacterial fibrin clot implantation. A portion of this bacterial culture was added to a Lysogeny broth agar plate and incubated overnight at 37°C to obtain a count of implanted *E. coli* in a given rat. We determined that rats that received a fibrin clot containing 0.8×10^8 to 1.2×10^8 CFUs/clot did not show signs of septicemia 24 h after implantation, but rats that received an inoculum of 1×10^8 to 2.0×10^8 CFUs/clot had a mortality rate of ~40–45% after 48 h of clot implantation (data not shown). An *E. coli* inoculum of 1.5×10^8 to 2.0×10^8 induced lethargy, hypothermia, reluctance to feed, tachycardia and tachypnea in rats, with a mortality rate of ~20–25% at 24 h (data not shown). Rats that received inocula higher than 2.0×10^8 CFUs/clot had a mortality rate of 80% during the first 24 h of implantation (data not shown). Thus, we chose an *E. coli* inoculum ranging from 1.5×10^8 to 2.0×10^8 CFUs/clot for the present study.

Blood Collection and Cytokine, $\text{NO}_2^-/\text{NO}_3^-$ and Aspartate Transaminase (AST)/Alanine Aminotransferase (ALT) Analysis

Blood (0.4 mL) was withdrawn from HA-treated and sham rats from the internal jugular vein line at time 0 (24 h after implantation of the *E. coli*-impregnated fibrin clot) and 1, 3 and 6 h later. Plasma samples (n = 8 per group) were prepared and stored at –80°C until analysis.

Plasma cytokines were measured with a multiplex bead immunoassay system (Luminex; Millipore, Billerica, MA, USA). The cytokine assays included granulocyte/macrophage colony-stimulating factor (GM-CSF), keratinocyte-derived chemokine (GRO/KC, CINC-1, CXCL-1), interferon (IFN)-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p70, IL-18, monocyte chemoattractant protein-1 (MCP-1, CCL2) and TNF-α. Plasma $\text{NO}_2^-/\text{NO}_3^-$ was measured by the nitrate reductase method using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA). AST and ALT were measured using a commercially available kit (Fujifilm, Asaka-shi, Satama, Japan; distributed by Heska Corporation, Loveland, CO, USA) according to the manufacturer's instructions.

Peritoneal Fluid Collection and Analyses

To determine baseline levels of peritoneal cytokines and bacterial count at 0 h, an *E. coli*-impregnated fibrin clot was implanted in 12 separate rats that were not included in the total number of rats used for the two major experimental groups (HA and sham). After 30 h of sepsis (immediately after euthanasia), 10 mL sterile isotonic saline was injected into the peritoneum. The peritoneal irrigation fluid was recovered after a laparotomy for cytokine assay (n = 6 per group) and

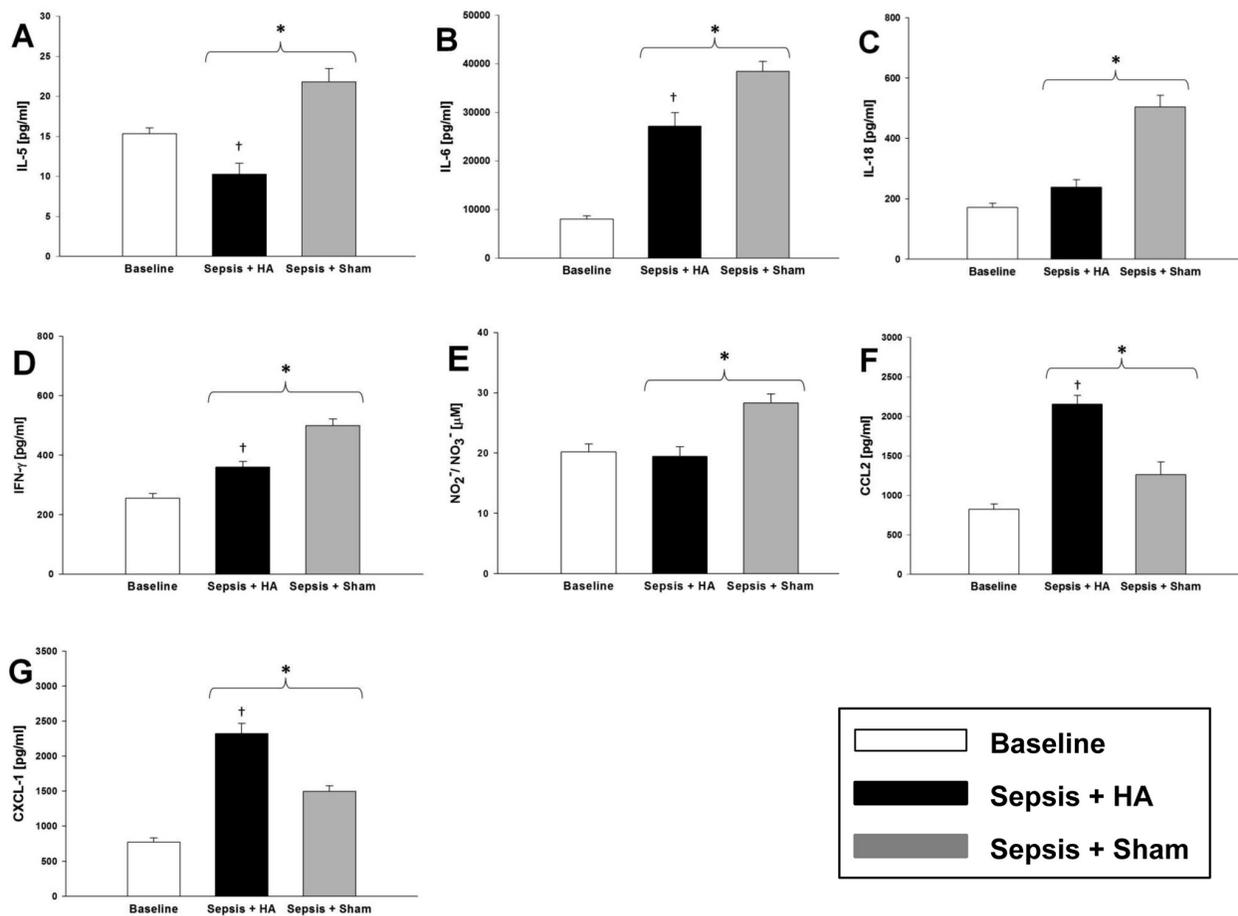


Figure 3. Effects of HA on peritoneal inflammatory mediators and bacteria. Rats were subjected to sepsis by the implantation of a fibrin clot impregnated with *E. coli* followed by sham or HA treatment, and peritoneal fluid was obtained as described in the Materials and Methods. Peritoneal IL-5 (A), IL-6 (B), IL-18 (C), IFN- γ (D) and NO₂⁻/NO₃⁻ (E) were significantly lower (n = 6 rats; *p < 0.05 versus sham group; †p < 0.05 versus baseline). CCL2 (F) and CXCL-1 (G) concentrations were significantly higher (n = 6 rats; *p < 0.05 versus sham group; †p < 0.05 versus baseline).

bacterial count (n = 8 per group) (11). A portion of the peritoneal fluid was streaked on an agar plate and incubated overnight at 37°C. After 24 h incubation, bacterial colonies were quantified as described above. The remaining peritoneal fluid was then stored at -80°C for cytokine assay (see above).

Statistical Analyses

All data are expressed as mean \pm standard error of the mean. Statistical analysis was performed by one-way analysis of variance on ranks followed by the Holm-Sidak test, using SigmaPlot 11 software (Systat Software, San Jose, CA, USA), with p < 0.05 considered significant. A power calculation was performed

and suggested that n = 8 rats was a cohort size sufficient to observe statistically significant differences in inflammatory mediators between treatment groups. Principal component analysis (PCA) was carried out essentially as described recently (12) using MATLAB software 7.6.0 (MathWorks, Natick, MA, USA).

All supplementary materials are available online at www.molmed.org.

RESULTS

Overview

In total, 21 animals were implanted with *E. coli*-impregnated fibrin clots; of these, 5 animals died after 24 h of the fib-

rin clot implantation (23.8% mortality), but before randomization to HA or sham circuit groups. Accordingly, these rats were excluded from further analysis. The remaining 16 animals were randomized to HA (n = 8) or sham (n = 8). The levels of circulating inflammatory mediators in the control group 24 h after implantation of a fibrin clot lacking *E. coli* compared with circulating inflammatory mediators of the sham group are shown in Supplementary Figure S1.

Effects of HA on Circulating Levels of Inflammatory Mediators and Markers of Liver Damage

We initially sought to determine if HA altered the balance of inflammatory me-

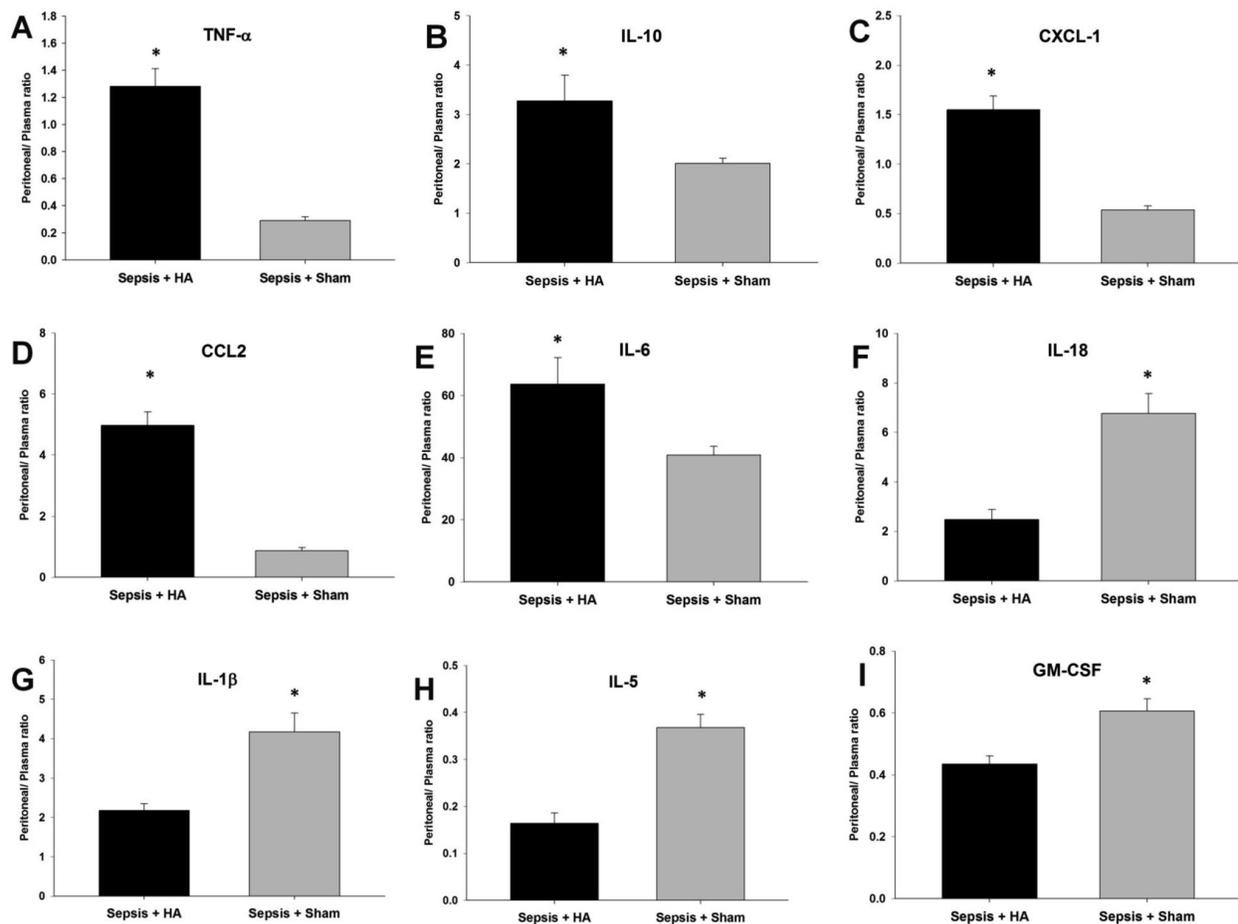


Figure 4. Ratios of local (peritoneal) mediators to systemic concentrations at 6 h for all the mediators that were measured in both sites. Rats were subjected to sepsis by the implantation of a fibrin clot impregnated with *E. coli* followed by sham or HA treatment, and plasma as well as peritoneal fluid was obtained as described in the Materials and Methods. Animals in the HA group had significantly higher localized TNF- α (* $p = 0.0010$ versus sham) (A), IL-10 (* $p = 0.040$ versus sham) (B), CXCL-1 (* $p = 0.001$ versus sham) (C), CCL2 (* $p = 0.001$ versus sham) (D) and IL-6 (* $p = 0.029$ versus sham) (E). The sham group had significantly higher ratios of IL-18 (* $p = 0.001$ versus HA) (F), IL-1 β (* $p = 0.003$ versus HA) (G), IL-5 (* $p = 0.001$ versus HA) (H) and GM-CSF (* $p = 0.005$ versus HA) (I). There was no significant difference in the peritoneal/plasma ratios of IL-1 α and IFN- γ .

diators in the setting of septic peritonitis; accordingly, we examined the effect of HA on circulating cytokines. The differences in circulating plasma cytokines for animals treated with HA or sham ($n = 8$ per group) are shown in Figure 1. Postcannulation baseline values (that is, 24 h after implantation of an *E. coli*-impregnated fibrin clot and hence established sepsis) for all cytokines were not significantly different between the two experimental groups. However, the concentrations of TNF- α (Figure 1A), IL-6 (Figure 1B), CCL2 (Figure 1C) and CXCL-1 (Figure 1D) were significantly lower in the HA group after

treatment compared with the sham group. Moreover, after only 1 h of the treatment, the concentrations of TNF- α (see Figure 1A), IL-6 (see Figure 1B) and CCL2 (see Figure 1C) were significantly lower in the HA group. At 3 and 6 h, TNF- α (see Figure 1A), IL-6 (see Figure 1B), CCL2 (see Figure 1C) and CXCL-1 (see Figure 1D) were still lower in the HA group compared with sham. Interestingly, circulating IL-10 levels were significantly higher ($p = 0.002$) after 1 h of treatment in the HA group compared with baseline, whereas the levels of IL-10 in sham animals were not (Fig-

ure 1E). However, there was no statistically significant difference in IL-10 levels between HA and sham at any time point. The concentration of circulating NO₂⁻/NO₃⁻ at 6 h of treatment was significantly lower in the HA group when compared with the sham group (Figure 1F). The levels of circulating IFN- γ , IL-1 β , IL-12 p70, IL-5, IL-18, GM/CSF and IL-1 α were unchanged over time in both sham and HA animals, whereas levels of circulating IL-4 and IL-2 were undetectable in both groups (Supplementary Figure S2). Circulating levels of AST were significantly lower ($p = 0.041$) in the HA

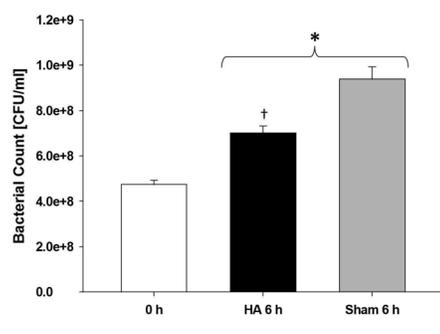


Figure 5. Peritoneal bacterial count in HA and sham groups at 6 h versus the baseline at 0 h. Rats were subjected to sepsis by the implantation of a fibrin clot impregnated with *E. coli* followed by sham or HA treatment, and peritoneal fluid was obtained as described in the Materials and Methods. Peritoneal bacterial counts were significantly lower (n = 8 rats; **P* < 0.001 versus sham) in the HA group ($7 \times 10^8 \pm 3.0 \times 10^7$ CFUs/mL) when compared with the sham group ($9.4 \times 10^8 \pm 5.3 \times 10^7$ CFUs/mL).

group (186.25 ± 18.98 units/liter [U/L]) versus the sham group (252 ± 22.42 U/L) at 6 h after treatment. However, there was no statistically significant difference (*p* = 0.075) in plasma ALT between treatment groups (117.5 ± 3.65 U/L in HA versus 138.7 ± 10.42 U/L in sham group).

PCA Suggests Different Drivers of Inflammation in HA Versus Sham

We next used PCA in an attempt to identify the subsets of circulating mediators that are most strongly indicative of membership in the HA or sham treatment group and that thereby might be considered principal drivers of the inflammatory response pattern seen in each group. PCA suggested that the circulating inflammatory response in the sham group was primarily driven by IL-6 and TNF- α (Figure 2A), whereas the sepsis response in the HA group was primarily driven by TNF- α , CXCL-1, IL-10 and CCL2 (Figure 2B). This analysis suggests that, in this animal model (and in the time range studied), TNF- α and IL-6 are principal drivers of sepsis and that HA modifies this process via CXCL-1,

IL-10 and CCL2 (and with a concomitantly reduced role for IL-6).

Effects of HA on Peritoneal Inflammatory Mediators and Bacteria

We next hypothesized that removal of mediators from the plasma by HA would not lead to reduced local inflammatory mediator concentrations and that, by increasing the ratio of local to systemic concentrations, we would observe improved bacterial clearance (13–17). We tested these hypotheses by examining the peritoneal fluid levels of inflammatory mediators (n = 6 per group) as well as changes in peritoneal bacterial counts (n = 8 per group). Figure 3 shows the difference in concentrations of peritoneal cytokines in both HA and sham groups at 6 h (that is, 30 h after implantation of an *E. coli*-impregnated fibrin clot) versus the baseline at 0 h (24 h after clot implantation). The peritoneal fluid concentrations of IL-5 (Figure 3A), IL-6 (Figure 3B), IL-18 (Figure 3C) and IFN- γ (Figure 3D) and the stable NO reaction products NO₂⁻/NO₃⁻ (Figure 3E) were significantly lower in the HA group when compared with the sham group. Interestingly, CCL2 (Figure 3F) and CXCL-1 (Figure 3G) concentrations in the peritoneal fluid were significantly higher in the HA group when compared with the sham group. Peritoneal IL-2 (Supplementary Figure S3A) and GM-CSF (Supplementary Figure S3B) were elevated in the HA animals at 6 versus 0 h, but no statistically significant differences were observed between HA and sham.

The PCA of plasma inflammatory mediators suggested that HA was associated with effects on TNF- α , CXCL-1, IL-10 and CCL2. Because a healthy inflammatory response remains localized, whereas a hallmark of detrimental inflammation in sepsis manifests in the systemic spillover of inflammatory mediators (18), we hypothesized that HA may act in some fashion to maintain localized levels of these principal mediators and reduce their systemic spillover. To test this hypothesis, we calculated the ratios of local (peritoneal) mediators to sys-

temic concentrations at 6 h for the all mediators that were detectable in both sites. This analysis demonstrated that animals in the HA group had significantly higher localized TNF- α (*p* = 0.0010; Figure 4A), IL-10 (*p* = 0.040; Figure 4B), CXCL-1 (*p* = 0.001; Figure 4C), CCL2 (*p* = 0.001; Figure 4D) and IL-6 (*p* = 0.029; Figure 4E). In contrast, the sham group had significantly higher ratios of IL-18 (*p* = 0.001; Figure 4F), IL-1 β (*p* = 0.003; Figure 4G), IL-5 (*p* = 0.001; Figure 4H) and GM-CSF (*p* = 0.005; Figure 4I). There was no significant difference in the peritoneal/plasma ratios of IL-1 α and IFN- γ (Supplementary Figure S4).

We next hypothesized that the altered local inflammatory response after HA treatment would result in reduced local bacterial burden. Figure 5 shows the peritoneal bacterial count in both HA and sham groups at 6 h versus the baseline at 0 h. As can be seen in Figure 5, peritoneal bacterial counts were significantly lower in the HA group when compared with the sham group.

DISCUSSION

The inflammatory response is compartmentalized both structurally and across multiple scales of organization (19–22). We recently hypothesized that acute inflammation proceeds at a given “nested” level or scale until positive feedback exceeds a “tipping point.” Below this tipping point, inflammation is contained and manageable; when this threshold is crossed, inflammation becomes disordered and dysfunction propagates systemically (23,24). Thus, circulating inflammatory mediators, including cytokines and free radical reaction products such as NO₂⁻/NO₃⁻, play an important role in the pathophysiology of sepsis (1). In patients with established sepsis, both proinflammatory and antiinflammatory mediators coexist in the circulation in markedly increased amounts (25–30), whereas paradoxically, the capacity to combat infection or to mount appropriate inflammatory responses is blunted (31). On the basis of these observations, we and others hypothesized that re-

removal of inflammatory mediators would be beneficial in sepsis. Indeed, HA has demonstrated efficacy in animal models (6,8) and is currently in clinical trials for the treatment of sepsis (clinicaltrials.gov, NCT00559130).

The primary goal of the present study was to begin to test the hypothesis that some of the benefit of HA could be ascribed to reprogramming miscompartmentalized and dysregulated inflammation in sepsis, by defining the effects of HA on the nature, intensity and compartmentalization of inflammatory mediators. This experimental model is associated with a clinically realistic mortality of 25–30%. Our results show that HA with the CytoSorb polymer can effectively remove proinflammatory cytokines (TNF- α and IL-6) as well as chemokines (CCL2 and CXCL-1) from the circulation, while also resulting in lower IL-5, IL-6, IL-18, IFN- γ and NO₂⁻/NO₃⁻ (along with higher levels of CXCL-1 and CCL2) in the peritoneal fluid after 6 h of HA.

Except for circulating IL-10 and IL-1 β , which did not show any statistical significance in HA versus sham treatment, the present results are consistent with other studies on the effects of HA on cytokine removal, both in *ex vivo* and rat cecal ligation and perforation models. The likely explanation is that, since molecular capture with the HA device depends on concentration, molecules with lower concentrations will be captured with lower efficacy compared with those with higher concentrations. Various experimental models of sepsis have differences in cytokine expression, and this particular model is associated with a relatively low expression of IL-10. Similar to the rat cecal ligation and perforation model, our septic peritonitis model is one of true infection, thereby approximating human sepsis. We also note that HA treatment did affect the ratio of plasma to peritoneal IL-10, suggesting that compartmental effects of this cytokine may be more important than systemic effects. Importantly, the mortality rate in our animal model that was seen at 20–24 h after

introducing the *E. coli*-impregnated fibrin clot and immediately before establishing the extracorporeal circuit (approximately 25–30%) is similar to mortality rates seen in the human intensive care unit (ICU) patients with sepsis (32). However, it is important to note that these patients receive antibiotic and fluid therapy, unlike in our animal model. Moreover, the timing of mortality is different between our animal model and the clinical setting. We also note that HA treatment was initiated 24 h after the implantation of an *E. coli*-impregnated fibrin clot and continued until 30 h postimplantation, reflecting a clinically realistic timing of therapy. One advantage of the animal model used in the present study over cecal ligation and perforation is the ability to quantify both input and final numbers of bacteria, and we were thus able to demonstrate a significant reduction in peritoneal bacteria in HA versus sham.

To better understand the effects of HA on inflammation in our rat model, we sought to leverage the insights gained from data-driven analyses into quasi-mechanistic insights regarding the dynamics of inflammation after sepsis. We therefore used PCA to identify the subsets of mediators that are most strongly indicative of HA or sham and that thereby might be considered principal drivers of the inflammatory response pattern seen in each group. Importantly, PCA is on the basis of time-dependent changes in variance (33); therefore, we hypothesized that this analysis would yield insights into the dynamic responses of the various inflammatory mediators. We recently demonstrated the utility of this method, along with other data-driven analyses, in suggesting the principal drivers of trauma/hemorrhagic shock in mice (12). In the present study, PCA suggested that IL-6 and TNF- α are the principal circulating drivers of sepsis (as seen in the sepsis + sham experimental group) and that HA modifies this process via CXCL-1, IL-10 and CCL2 (with a reduced contribution of IL-6). We confirmed this computationally derived hy-

pothesis, in part, in that the peritoneal-to-plasma ratios of TNF- α , IL-10, CXCL-1 and CCL2 were higher in the HA group. However, the peritoneal-to-plasma ratio for IL-6 was also higher in the HA group compared with sham; the reason and potential role for elevated local IL-6 is at this point unclear, but these findings suggest that HA does more than simply remove cytokines. Rather, this treatment appears to lead to inflammatory reprogramming and re-compartmentalization. This effect is also consistent with the results of a recent study from our group, where we demonstrated reduced organ injury and improved survival with HA in CLP sepsis, even when we scaled the intervention back to a level at which typical circulating inflammatory mediators (for example, TNF- α and IL-6) were not affected (34).

Although at first glance it would appear that the simplest explanation for reduced inflammatory mediators in the plasma is linked to the reduced bacterial counts in the peritoneum, it is not obvious how this would have happened when the HA therapy (which to our knowledge has no direct antibiotic effect) was administered to the systemic circulation rather than to the peritoneal space. We hypothesize that HA helps to maintain relevant inflammatory mediators in the peritoneal space and thereby reduces the number of bacteria. Presumably, as bacterial numbers decrease in the peritoneum, the forward-feedback proinflammatory loop driven by peritoneal bacteria results in further decreases in circulating inflammatory mediators. Although this hypothesis is consistent with our data, alternative explanations such as sequestration of inflammatory mediators and bacteria to other organs/compartments are also consistent with these data. In addition, it is possible that, whereas liver damage was ameliorated by HA, that other measures of organ pathophysiology (for example, hemodynamic parameters) were not improved. Further studies will be required to examine these issues.

Finally, and perhaps most importantly, we have shown results compatible with

the hypothesis that reprogramming and re-compartmentalization of sepsis-induced inflammation resulted in improved bacterial clearance and reduced liver injury. We interpret these results as being consistent, since better leukocyte homing to the nidus of infection would have decreased bacterial counts and reduced remote liver damage caused by activated leukocytes. Moreover, tissue samples taken from the rats' kidney, liver and lung from either the HA or sham groups for hematoxylin and eosin staining suggested worse pathology in the sham- versus HA-treated rats. However, these trends did not reach statistical significance in semiquantitative analysis (data not shown). Thus, our findings further the hypothesis that immune modulation induced by blood purification results in better localization of the inflammatory response in severe sepsis and provide proof of concept for a new paradigm in the treatment of sepsis (35). This hypothesis is not limited to HA but may apply to other forms of blood purification including high-volume hemofiltration and plasma exchange. For example, Yekebas *et al.* (36) demonstrated that a combination of hemofiltration and adsorption (accomplished with frequent filter changes) resulted in marked improvement of immune function in animals with experimental pancreatitis. However, we note that most studies of blood purification have failed to examine immune responses.

CONCLUSION

We recognize that there are several limitations in our model. For example, the animals did not receive supplemental oxygen, fluid resuscitation, vasopressors or other therapies. Antibiotics were not administered, since they may alter the inflammatory response and may well have obscured any "signal." We would also point out that many patients suffering from sepsis in the ICU acquire infections with organisms that are resistant to antibiotics or inadequate source control (37). Because the animals were euthanized 30 h after the initiation of septic

peritonitis, we cannot comment on the effects of HA on short- or long-term survival. The animal model involved sepsis induced by a single strain of *E. coli*, and our results may have been different in the setting of polymicrobial infection (although prior studies in the setting of cecal ligation and perforation [8,34] suggest that HA is effective in the setting of realistic, polymicrobial infections). Another limitation of our study is that, while we observed reduced bacterial counts in the peritoneal fluid of HA rats, the possibility remains that these bacteria migrated to distal organs rather than being killed. We believe that this is an unlikely possibility, since the reduced peritoneal bacterial counts were observed in tandem with reduced plasma inflammatory mediators and markers of liver damage, as well as with reduced histological damage in HA rats compared with sham animals.

Another important limitation to the interpretation of our data is the fact that it is likely that many noncytokine, low-molecular-weight proteins adsorb to the HA beads, proteins that may affect inflammation and/or liver dysfunction via alternative mechanisms. A further limitation of our study is that the number of mediators, particularly antiinflammatory mediators, was restricted by the necessary use of rats for these experiments, an experimental consideration that limited the number of analytes we could measure using commercially available, rat-specific Luminex™ beadsets. Similarly, we note that pathogen-associated (for example, LPS) and damage-associated (for example, HMGB-1) pattern molecules are also important in the pathophysiology of sepsis and were not evaluated in this study. Finally, we note that *in silico* methods alone cannot validate associations and that more interventions (as well as true mechanistic computational modeling) are needed to demonstrate specific causes and effects. We note that tools such as PCA serves only to suggest the primary drivers in a certain experimental condition by ranking the top inflammatory mediators that contributed most in

that specific experimental condition. PCA does this by measuring the variability among multiple inflammatory mediators with respect to evolution of these mediators over time (12,24,33,38). Thus, by suggesting the components that contributed most, we hypothesized that PCA would raise specific hypotheses and direct our future work to focus on demonstrating these associations.

In conclusion, we suggest that HA that uses a large surface-area polymer appears to reduce, relocalize and reprogram sepsis-induced acute inflammation, while simultaneously reducing infectious burden and liver damage. Future studies are needed to fully define the effects of this emerging sepsis therapy.

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DISCLOSURE

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