Monocytes regulate systemic coagulation and inflammation in abdominal sepsis

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ABDOMINAL SEPSIS is a leading cause of hospital-related deaths (3, 16). Sepsis-induced mortality ranges between 18% and 30%, and the presence of disseminated intravascular coagulation increases the mortality rate to 35% (8, 13, 31). The high mortality rate in septic patients is in part related to an incomplete understanding of the underlying pathophysiology. Intestinal contamination of the abdominal cavity with toxins and microbes causes local formation of proinflammatory substances, such as cytokines and chemokines (18). Translocation of microbes, toxins, and proinflammatory substances into the circulation triggers a systemic inflammatory response characterised by widespread activation of innate immune cells, such as monocytes and neutrophils, leading to organ injury (5, 28). Several studies have shown that pulmonary recruitment of neutrophils is a rate-limiting step in septic lung injury (9, 11). Neutrophils are particularly sensitive to C-X-C motif (CXC) chemokines, including chemokine CXC ligand (CXCL)1, CXCL2, and CXCL5 (20). Although it is widely held that monocytes play an important role in systemic inflammation, the literature on the role of monocytes in controlling pulmonary neutrophilia is complex and partly contradictory (1, 35). Thus, we decided to examine the role of blood monocytes for pulmonary accumulation of neutrophils as well as cytokine and chemokine formation in abdominal sepsis.

Hemostatic dysfunction is one of the most prominent features in sepsis. Hemostatic alterations in sepsis are typified by an early hypercoagulable phase concomitant with impaired anticoagulation and fibrinolysis (33). This is followed by a hypocoagulable phase due to consumption of platelets and coagulation factors (41). Hemostatic cascades are complex processes consisting of a dynamic interplay between several discrete elements (23, 30). Individual quantification of these elements by classical assays, including the ones used to measure coagulation factors and inhibitors, do not capture the global effect of all these elements in hemostasis (34). Instead, global hemostasis assays, including thrombin generation tests and thromboelastometry, have emerged as effective tools to obtain more comprehensive evaluations of hemostasis (10, 32). Thrombin generation has been shown to be useful in the evaluation of diseases with complex changes in hemostasis, such as chronic liver disease (37), trauma-induced coagulopathy (12), and liver transplantation (15). Recent clinical studies have shown that thrombin generation is greatly diminished in patients with sepsis (4, 26). In the present study, we hypothesized that monocytes might play a role in regulating thrombin generation and coagulation factor consumption in sepsis.

Based on the considerations above, the aim of the present study was to define the role of monocytes in regulating systemic inflammation and coagulation in abdominal sepsis. For this purpose, we used a sepsis model based on ligation and puncture of the cecum.

MATERIALS AND METHODS

Animals. All experiments were conducted in accordance with legislation on the protection of animals and were approved by the Regional Ethical Committee for Animal Experimentation of Lund University. Male C57Bl/6 mice (20–25 g) were housed in an animal facility with a 12:12-h light-dark cycle at 22°C and fed a laboratory diet and water ad libitum. Mice were anesthetized with 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kilogram of body weight.

Experimental protocol of sepsis. Polymicrobial sepsis in mice was induced by a cecal ligation and puncture (CLP) procedure as previ-
ously described in detail (40). Briefly, animals were anesthetized, and a midline incision was made to expose the cecum, which was filled with feces by milking stool backward from the ascending colon. The cecum was then ligated with a 5-0 silk suture. The cecum was soaked with PBS (pH 7.4) and punctured twice with a 21-gauge needle on the antimesenteric border. The cecum was returned into the peritoneal cavity, and the abdominal incision was sutured. Sham mice underwent identical laparotomy and resuscitation procedures, but the cecum was neither ligated nor punctured. Animals were reanesthetized 6 h after CLP to collect samples for further analysis.

Monocyte and neutrophil depletion. Mice were administered intraperitoneally with 20 mg/kg clodronate liposomes (Encapsula Nano Sciences) 24 h before CLP, and animals treated with PBS liposomes served as controls. An antibody directed against lymphocyte antigen (Ly)6G (20 mg/kg, clone 1A8, Bioxcell, West Lebanon, NH) was given intraperitoneally 24 h before CLP to deplete animals of neutrophils. Control animals received a control antibody (20 mg/kg, Bioxcell).

Flow cytometry. Blood was collected into syringes containing 1:10 acid citrate dextrose 6 h after CLP induction. Blood samples were incubated with an anti-CD16/CD32 antibody (10 min at room temperature), which blocked FcγIII/II receptors, to reduce nonspecific labeling and then incubated with FITC-conjugated anti-Mac-1 (clone M1/70, BD Pharmingen, San Jose, CA), phycoerythrin-conjugated anti-Ly6G (clone 1A8, BD Pharmingen), and phycoerythrin-Cy-conjugated anti-Ly6C (clone AL-21, BD Pharmingen) antibodies. Leukocytes were recovered after centrifugation. Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and a viable gate was used to exclude dead and fragmented cells. Monocytes were defined as Ly6C+Ly6G− cells.

Myeloperoxidase activity. Myeloperoxidase (MPO) levels in lung tissue were assayed according to a standard protocol (38). Briefly, frozen tissue was thawed and homogenized in 0.02 M phosphate buffer (pH 7.4). The supernatant was discarded after centrifugation for 10 min at 14,000 rpm at room temperature, and the pellet was then dissolved by adding 1 ml of 0.5% hexadecyltrimethylammonium bromide. Samples were stored at −20°C overnight, thawed, and then kept at 60°C in a water bath for 2 h followed by 90-s sonication. The supernatant was collected after 5 min of centrifugation at 14,000 rpm, and MPO activity in the supernatant was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H2O2 (450 nm, with a reference filter of 540 nm, 25°C). Values are expressed as MPO units per gram of tissue.

Systemic leukocyte and platelet counts. Blood was collected from the tail vein and mixed with Turk's solution [0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% (vol/vol)] in a dilution of 1:20 for the identification of platelets in a Burker chamber.

ELISA. Plasma levels of prothrombin, factor V, factor VII, factor X, activated protein C, and thrombin-antithrombin (TAT) complexes were quantified using commercially available ELISA kits (Molecular Innovations, Peary Ct, Novi; USCN Life Science, Wuhan, Hubei, China; and Siemens, Marburg, Germany). Both plasma and lung tissue levels of IL-6 as well as CXCL1, CXCL2, and CXCL5 were determined by commercial ELISA kits (R&D Systems, Abingdon, Oxon, UK). Lungs were harvested 6 h after the induction of CLP and immediately frozen at liquid nitrogen. Samples were collected 6 h after the induction of CLP from the vena cava (1:10 acid citrate-dextrose), centrifuged at 14,000 rpm for 10 min at 4°C, and stored at −20°C until use. Linearity was assessed and confirmed by samples containing recombinant mouse prothrombin factor V, factor VII, factor X, activated protein C, IL-6, CXCL1, CXCL2, CXCL5, and TAT standard plasma serially diluted with a calibrator diluent.

Thrombin generation assay. For analysis of thrombin generation, mouse blood was collected from the inferior vena cava (1:10 acid citrate-dextrose). As previously described (6), platelet-poor plasma was obtained by a series of centrifugations to remove platelets and cell fragments. Samples were first centrifuged at 19,000 g for 15 min and then at 20,500 g for 40 min to obtain platelet-poor plasma. Phospholipid vesicles containing 10% phosphatidyl serine, 20% phosphatidylethanolamine, and 70% phosphatidylcholine were prepared by extrusion as previously described (22). Recombinant mouse tissue factor was obtained from R&D Systems. The fluorogenic substrate Z-Gly-Gly-Arg-AMC was obtained from Bachem (Bubendorf, Switzerland). A working solution of recombinant mouse tissue factor (1 pm) was prepared in HEPES-buffered saline solution containing 25 mM HEPES, 140 mM NaCl, and 5 mg/ml BSA, and a mixture of fluorescent substrate (0.3 mM) and CaCl2 (16 mM) was freshly prepared in HEPES-buffered saline. To start thrombin generation, 10 μl phospholipid vesicles (0.5 mM), 40 μl platelet-poor plasma, 10 μl tissue factor, and 20 μl fluorogenic substrate with CaCl2 were mixed. The formation of thrombin was monitored with accumulated fluorescence in a Tecan Infinite 200 microplate reader (Männedorf, Switzerland) at an excitation wavelength of 360 nm and emission wavelength of 460 nm. All experiments were carried out in duplicate at 37°C.

Statistics. Data are presented as means ± SE; n represents the number of animals. Statistical evaluations were performed using Kruskal-Wallis one-way ANOVA on ranks followed by multiple comparisons versus the sham group (Dunnnett’s method). P values of <0.05 were considered statistically significant.

RESULTS

Monocyte depletion and activation. Clodronate liposomes were used to deplete mice of monocytes as previously described (22). Intraperitoneal administration of clodronate liposomes decreased the circulating number of monocytes by 96% (Fig. 1, A and B). Treatment with clodronate liposomes had no other effect on any leukocyte subtype (data not shown). To study whether monocytes are activated in the CLP model, we examined CD11b levels on circulating monocytes and found a clear-cut increase in monocyte expression of CD11b in septic animals (Fig. 1, C and D).

Monocytes regulate thrombin generation. CLP significantly increased time to peak formation by 134%, reduced peak formation by 71%, and decreased total thrombin generation by 57% (Fig. 2, A–D). Monocyte depletion significantly reduced time to peak thrombin formation and restored peak and total thrombin generation back to levels similar to those in sham animals (Fig. 2, A–D). TAT complexes formed after neutralization of thrombin by antithrombin III have been used as a surrogate marker for thrombin generation (39). In the present study, we observed that CLP markedly increased plasma levels of TAT complexes and that the administration of clodronate significantly reduced CLP-induced TAT complex levels in plasma by 54% (Fig. 3).

Monocytes regulate consumption of coagulation factors. CLP caused a significant decrease in plasma levels of prothrombin (Fig. 4A), factor V (Fig. 4B), factor X (Fig. 4C), and protein C (Fig. 4D). We found that plasma levels of prothrombin, factor V, factor X, and protein C were markedly higher in septic mice depleted of monocytes compared with CLP animals treated with vehicle (Fig. 4, A–D).

Neutrophil infiltration in the lung. Systemic inflammation in abdominal sepsis is characterized by the accumulation of neutrophils in the lung. Indeed, we found that CLP triggered a 5.4-fold increase in pulmonary activity of MPO, a marker of...
neutrophils (Fig. 4). Depletion of monocytes reduced CLP-induced MPO activity by 44% in the lung (Fig. 4). Administration of the anti-Ly6G antibody decreased circulating numbers of neutrophils by 90% (Table 1 and Fig. 5B). Depletion of neutrophils decreased CLP-induced MPO levels in the lung by 82% (Fig. 5A).

Systemic levels of IL-6 and CXC chemokines. CLP increased plasma levels of IL-6 by 1,214-fold and pulmonary levels of IL-6 by 15-fold (Fig. 6A). Monocyte depletion attenuated levels of IL-6 by 66% and 39% in the plasma and lung, respectively, in septic animals (Fig. 6A). In addition, CLP markedly enhanced CXCL1, CXCL2, and CXCL5 levels in the plasma and lung (Fig. 6, B–D). We found that depletion of monocytes significantly reduced plasma and lung levels of CXCL1, CXCL2, and CXCL5 in CLP mice (Fig. 6, B–D).
DISCUSSION

Systemic changes in inflammation and coagulation are key components in sepsis. This study documents an important role of peripheral blood monocytes for sepsis-induced thrombin generation and consumption of coagulation factors. Moreover, our findings show that monocytes are critical for the formation of proinflammatory compounds and neutrophil accumulation in the lung in abdominal sepsis. These results increase our understanding of the important role of monocytes in the pathophysiology of sepsis.

Convincing evidence has demonstrated that neutrophil recruitment is a rate-limiting step in the pathophysiology of septic lung injury (5, 28). It is generally held that monocytes/macrophages play a central role in the host response against bacterial infections, although the published data on the role of monocytes/macrophages for pulmonary neutrophilia are complex and contradictory. For example, on the one hand, one study (35) reported that alveolar macrophages are a positive regulator endotoxin-induced neutrophil accumulation in the lung. On the other hand, another study (2) has shown that depletion of alveolar macrophages has no effect or even increases pulmonary recruitment of neutrophils in response to endotoxin (2). Similarly, in a similar model of ischemia-reperfusion-induced lung injury, one report (22) showed that depletion of alveolar macrophages decreased neutrophil infiltration, whereas another study (1) demonstrated that depletion of alveolar macrophages increased neutrophil accumulation in the lung. In the present study, we observed that CLP increased surface expression of Mac-1 on circulating monocytes, indicating that monocytes are activated in this model of abdominal sepsis.

Moreover, we found that depletion of peripheral blood monocytes decreased CLP-induced pulmonary activity of MPO by 44%, suggesting that blood monocytes are important regulators of neutrophil recruitment in septic lung injury. This notion is in line with two previous studies (7, 19) on endotoxin-induced neutrophil accumulation in the lung showing that blood monocytes rather than alveolar macrophages control neutrophil infiltration in the inflamed lung. In addition, our findings are corroborated by a previous study (36) showing that clodronate protects against hepatic accumulation of neutrophils and tissue damage in a model of septic peritonitis. Moreover, our findings show that blood monocytes are important regulators of plasma and lung levels of CXCL1, CXCL2, and CXCL5 in abdominal sepsis. Knowing that these CXC chemokines are potent activators and chemoattractants of neutrophils (42, 43), it may be forwarded that blood monocyte-dependent formation of CXC chemokines is one mechanism by which blood monocytes control neutrophil activation and accumulation in septic lung injury. In this context, it should be mentioned that systemic administration of clodronate might also deplete other cells, such as Kupffer cells and alveolar macrophages, that could contribute to the effects of clodronate.

Patients with sepsis are characterized by progressive dysfunction of hemostasis typified by consumption of plate-
lets and coagulation factors causing a state of hypocoagu-
lation. Previous studies on the hemostatic response in sepsis
have mainly been based on analyses of individual elements
of the coagulation system, which may not capture the net
effect of sepsis on hemostasis. Instead, more recent efforts
have been focused on global tests of hemostasis, such as
thromboelastometry and thrombin generation (10, 32). Of
these tests, thrombin generation has been most extensively
used to characterize complex coagulopathies (29). In the
present study, we found that abdominal sepsis markedly
attenuated thrombin generation, which is in line with four
recent clinical reports on patients with sepsis (4, 17, 26, 27).
Knowing that tissue factor is a potent stimulator of thrombin
formation in vitro and that monocyte-derived tissue factor is
the dominating source of intravascular tissue factor in sepsis
(24), it was of great interest to examine the role of blood
monocytes for thrombin generation in abdominal sepsis. In
the present study, we found that depletion of blood mono-
cytes decreased time to peak generation of thrombin as well
as increased peak and total thrombin formation in CLP
animals, showing, for the first time, that blood monocytes
regulate thrombin generation in abdominal sepsis. This
conclusion is also supported by our findings showing that
clodronate markedly reduce the enhanced plasma levels of
TAT in septic animals. Interestingly, a previous study (25)
showed that endotoxin-induced activation of the coagulation
system is dependent on myeloid (granulocytes and mono-
cytes) cell-derived tissue factor. Whether the monocyte-
dependent formation of thrombin in abdominal sepsis ob-
served here is mediated by tissue factor is a topic for further
studies. A common observation in patients with sepsis is a
progressive loss of coagulation factors in plasma. We next
examined the role of monocytes in sepsis-induced consump-
tion of coagulation factors. We observed that CLP induction
reduced plasma levels of prothrombin, factor V, and factor
X as well as protein C by \( \frac{57}{1022} \). Notably, depletion of

Table 1. Systemic platelet and leukocyte differential counts

<table>
<thead>
<tr>
<th></th>
<th>Platelets</th>
<th>Monomorphonuclear Leukocytes</th>
<th>Polymorphonuclear Leukocytes</th>
<th>Total Leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1224 ± 23</td>
<td>6.2 ± 0.4</td>
<td>1.9 ± 0.1</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td>PBS + CLP</td>
<td>1149 ± 21</td>
<td>6.2 ± 0.2</td>
<td>0.6 ± 0.1*</td>
<td>6.7 ± 0.2*</td>
</tr>
<tr>
<td>Clodronate + CLP</td>
<td>856 ± 26†</td>
<td>3.0 ± 0.1†</td>
<td>1.4 ± 0.1†</td>
<td>4.4 ± 0.1†</td>
</tr>
<tr>
<td>Control antibody + CLP</td>
<td>1127 ± 30</td>
<td>6.1 ± 0.1</td>
<td>0.5 ± 0.1*</td>
<td>6.6 ± 0.1*</td>
</tr>
<tr>
<td>Anti-Ly6G antibody + CLP</td>
<td>939 ± 48‡</td>
<td>4.3 ± 0.3‡</td>
<td>0.2 ± 0.0*‡</td>
<td>4.5 ± 0.4‡</td>
</tr>
</tbody>
</table>

Data are means ± SE of \( 10^6 \) cells/ml; \( n = 5 \). Blood was collected from PBS, clodronate (20 mg/kg), control antibody-, or anti-lymphocyte antigen 6G antibody (20 mg/kg)-treated animals exposed to cecal ligation and puncture (CLP) for 6 h as well as from sham-operated (sham) mice. Leukocytes were identified as monomorphonuclear leukocytes and polymorphonuclear leukocytes. *\( P < 0.05 \) vs. sham; †\( P < 0.05 \) vs. PBS + CLP; ‡\( P < 0.05 \) vs. control antibody + CLP.
Monocytes restored plasma levels of these factors in septic animals, suggesting that monocytes play a central role in the consumption of coagulation factors in abdominal sepsis. In this context, it is interesting to note that among its many activities, IL-6 has been shown to exert a pivotal role in hemostasis. For example, IL-6 can induce a prothrombotic state by increasing platelet production and reactivity as well as promoting expression of tissue factor and coagulation factors (14). Indeed, one recent study (21) has demonstrated that IL-6 can increase thrombin generation. In the present study, we found that the induction of CLP triggered increased plasma levels of IL-6 and that monocyte depletion abolished the sepsis-evoked increase in IL-6 production. These findings suggest that monocytes are important for increased formation of IL-6 in sepsis and could help to explain, at least in part, the monocyte-dependent activation of systemic coagulation as observed in the present study.

Taken together, our novel results demonstrate that blood monocytes regulate both systemic coagulation and inflammation in abdominal sepsis. These findings help elucidate the role of monocytes in the pathophysiology of sepsis and encourage further attempts to target monocytes to ameliorate hemostatic dysfunction and pathological inflammation in abdominal sepsis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
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