

# Inhibition of Neutrophil Migration by Hemopexin Leads to Increased Mortality Due to Sepsis in Mice

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**Rationale:** The reduction of neutrophil migration to the bacterial focus is associated with poor outcome in sepsis.

**Objectives:** The objective of this study was to identify soluble substances in the blood of septic mice that inhibit neutrophil migration.

**Methods:** A pool of serum obtained from mice 2 hours after the induction of severe sepsis by cecal ligation and puncture inhibited the neutrophil migration. The proteins with inhibitory activity on neutrophil migration were isolated by Blue-Sepharose chromatography, high-performance liquid chromatography, and electrophoresis, and identified by mass spectrometry.

**Measurements and Main Results:** Hemopexin was identified as the serum component responsible for the inhibition of neutrophil migration. In sepsis, the pretreatment of wild-type mice with hemopexin inhibited neutrophil migration to the focus of infection and decreased the survival rate from 87.5 to 50.0%. Hemopexin-null mice subjected to severe sepsis presented normal neutrophil migration, low bacteremia, and an improvement of 40% in survival rate. Moreover, hemopexin inhibited the neutrophil chemotaxis response evoked by C5a or macrophage inflammatory protein-2 and induced a reduction of CXCR2 and L-selectin as well as the up-regulation of CD11b expression in neutrophil membranes. The inhibitory effect of hemopexin on neutrophil chemotaxis was prevented by serine protease inhibitors or ATP. In addition, serum levels of ATP were decreased 2 hours after severe sepsis.

**Conclusions:** These data demonstrate for the first time the inhibitory role of hemopexin in neutrophil migration during sepsis and suggest that the therapeutic inhibition of hemopexin or its protease activity could improve neutrophil migration to the focus of infection and survival in sepsis.

**Keywords:** neutrophil migration; sepsis; hemopexin; survival; cecal ligation and puncture

Activation of endothelial cells by chemokines (1) during a localized infection process stimulates the rolling and tight adhesion of neutrophils to the endothelium and provides directional migration cues (2). Once at the site of infection, neutrophils internalize pathogens and produce toxic substances that kill the

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## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Reduction of neutrophil migration to the bacterial focus is associated with poor outcome in sepsis. The mechanism involved in this process is not completely understood; however, serum factors may be responsible for the inhibition of neutrophil migration in severe sepsis.

### What This Study Adds to the Field

Abrogation of the acute-phase protein hemopexin improves neutrophil migration to the focus of infection and survival in sepsis.

invading microorganisms (3, 4). However, when the host fails to contain the pathogens, they or their products spread to the circulation, resulting in overproduction of inflammatory mediators and impaired immune function (5). We have shown that the severity of sepsis induced by cecal ligation and puncture (CLP) (6), by gram-positive (7) or gram-negative (8) intraperitoneal bacterial inoculation is associated with inhibition of neutrophil recruitment to the sites of infection. The inability of neutrophils to migrate results in an increased number of bacteria in the peritoneal cavity and blood, lung leukocyte sequestration, and high mortality. Furthermore, compared with neutrophils from healthy subjects, neutrophils obtained from severely septic patients have greatly reduced chemotactic responses (9).

The mechanism involved in the reduction of neutrophil migration observed in severe sepsis is not completely understood. However, bacteria and/or their products spreading and inducing activation of the systemic Toll-like receptors (TLRs) result in excessive circulating cytokines/chemokines (8, 10), which could inhibit neutrophil migration. In fact, intravenous administration of LPS (6), tumor necrosis factor (TNF)- $\alpha$ , or IL-8 inhibits neutrophil migration to the mouse peritoneal cavity. The monoclonal anti-TNF- $\alpha$  antibody partially prevented the inhibition of neutrophil migration in an endotoxemia model (11). Moreover, TNF receptor type 1 and type 2 (TNFR1/R2) double-knockout mice are more resistant to severe sepsis (12).

Despite the evidence that cytokines mediate the failure of neutrophil migration, clinical trials using neutralizing antibodies against cytokines or soluble receptors did not show significant beneficial effects on septic patients (13). However, other serum factors may also be responsible for the inhibition of neutrophil migration because massive fresh plasma transfusions restore neutrophil function in newborns suffering from sepsis (14). In addition, we showed that the acute-phase protein  $\alpha_1$ -acid glycoprotein (AGP) is a serum factor that inhibits neutrophil migration in severe human sepsis (15).

In experimental mouse sepsis, the serum AGP concentration was significantly increased only 6 hours after the initiation of severe sepsis (F. Spiller and F. Q. Cunha, unpublished data). However, 2 hours after the induction of severe sepsis in mice, there is a decrease in rolling and adhesion of leukocytes to the endothelium as well as internalization and desensitization of chemokine receptor CXCR2 on the neutrophil membrane (16), which are essential for neutrophil migration. Therefore, other serum factors may be involved in the failure of neutrophil migration during the early phase of severe sepsis and knowledge of these mediators should help in the management of severe sepsis. Thus, the objective of the present study was to identify soluble substances other than AGP in the blood of septic mice (15) that inhibit neutrophil migration during early phases of sepsis.

## METHODS

Experimental details are provided in the online supplement.

### Neutrophil Migration

Neutrophil migration was determined 6 hours after CLP or thioglycolate administration (17).

### Sepsis Models

Polymicrobial sepsis was induced by CLP model (18). A single puncture was made with a 21-gauge needle to induce mild sepsis (MS) or four punctures were made with an 18-gauge needle to induce severe sepsis (SS). Gram-negative sepsis was induced by *Escherichia coli* (25922; American Type Culture Collection, Bethesda, MD) inoculation.

### Neutrophil Isolation and Chemotaxis Assay

Bone marrow neutrophils were isolated by Percoll gradient (10) and treated (1 h) with 1  $\mu$ M hemopexin-hemin (Hx-Hem), bovine serum albumin (BSA)-Hem, or hemopexin (0.5, 1, and 5  $\mu$ M). One group was pretreated (30 min) with zinc deuteroporphyrin 2,4-bis glycol (30  $\mu$ M) before hemopexin. In another set of experiments, hemopexin (5  $\mu$ M) was incubated (30 min, 37°) with phenylmethylsulfonyl fluoride (PMSF, 50–200  $\mu$ M), aprotinin (AP, 2.5–10  $\mu$ M), or ATP (2.5–10  $\mu$ M), and then these complexes were incubated with neutrophils for 1 hour. After that, neutrophil chemotaxis was determined in response to RPMI (random migration) or to macrophage-inflammatory protein (MIP)-2 (20 ng/ml) or C5a (50 or 100 nM).

### Flow Cytometric Analysis

Isolated mouse bone marrow neutrophils were incubated for 1 hour with hemopexin (1, 5, and 10  $\mu$ M) and stained with antibodies against CD62 ligand, CD11b, or CXCR2. The cells were then analyzed by flow cytometry (FACSsort; BD Biosciences, Mountain View, CA).

### Blue-Sephadex Chromatography

A pool of 30 ml of serum from severely septic mice was chromatographed on a Blue Sepharose 6 Fast Flow column to remove most of the albumin (19). The material with low albumin content was denoted fraction B<sup>-</sup>.

### HPLC

Fraction B<sup>-</sup>, which contained most of the migration-inhibitory activity, was subjected to HPLC on a Vydac C<sub>18</sub> column (218TP510; Grace Vydac, Hesperia, CA), using a Shimadzu chromatography system (Shimadzu, Tokyo, Japan).

### Native Electrophoresis

The HPLC effluent containing neutrophil migration-inhibitory activity, F1 (Figure 2), was subjected to non-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Eight micrograms of protein of the sample per lane was applied and the protein was localized by Coomassie blue and eluted by diffusion (20).

## Mass Spectrometry

Proteins purified by PAGE and a commercial hemopexin sample were heat denatured and hydrolyzed with modified trypsin (Promega, Madison, WI) (15). Peptide sequences were obtained by collision-induced dissociation-tandem mass spectrometry (CID-MS/MS). The amino acid sequences of four tryptic peptides, indicated by an asterisk (\*) in Table E1 in the online supplement, were deduced from the series of *b* and *y* fragment ions produced by CID. The masses of tryptic peptides and fragment ions from CID were used to search GenBank at the National Center for Biotechnology Information, using ProteinProspector MS-Fit and MS-Tag software (<http://prospector.ucsf.edu/>).

## Statistical Analysis

Data are reported as means  $\pm$  SEM (except for survival curves) and are representative of two or three independent experiments with five mice in each group. Differences between two unpaired groups were compared by Student *t* test. The mean differences between three or more groups were compared by analysis of variance. When significant differences were identified, individual comparisons were subsequently tested with Bonferroni's *t* test. Statistical significance was set at *P* < 0.05. Survival rates (*n* = 10–12) were expressed as percentages, and a log-rank test (chi-square test) was used to examine differences between survival curves.

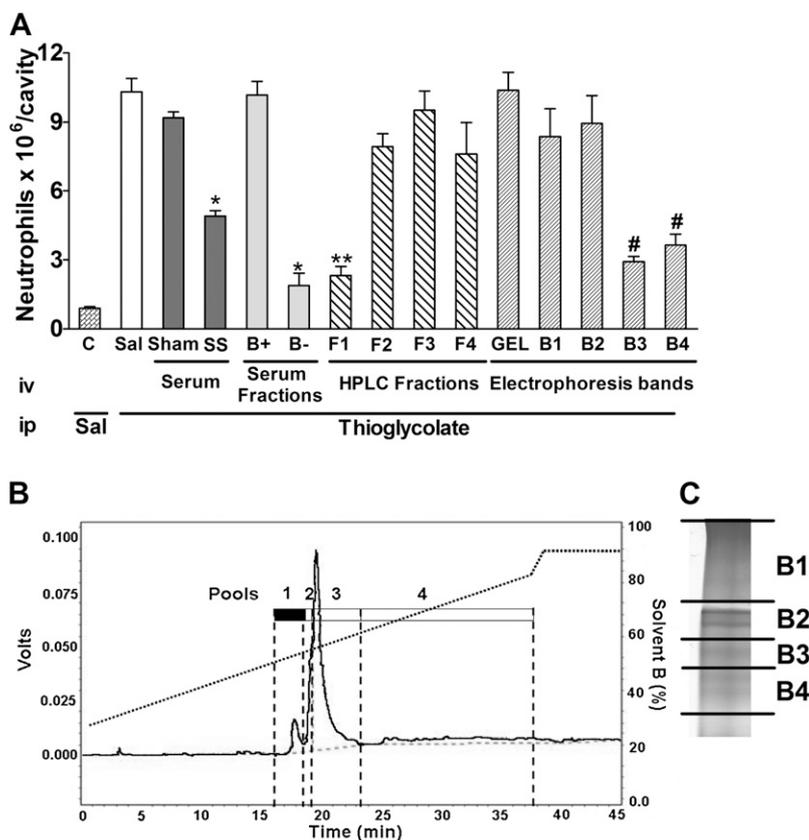
## RESULTS

### Serum and Serum Fractions from Severe Septic Mice Inhibit Neutrophil Migration

Mice subjected to severe sepsis (SS) presented 100% mortality (48 h after surgery) and failure of neutrophil migration to the focus of infection (6 h after surgery), as previously demonstrated (6). However, mice subjected to mild sepsis (MS) presented approximately 10% mortality and efficient neutrophil migration to the focus of infection (data not shown). The intravenous pretreatment of naive mice with serum collected from SS mice 2 hours after CLP significantly reduced thioglycolate-induced neutrophil migration compared with mice pretreated with saline or serum from sham-operated (sham) mice (Figure 1A). The intravenous pretreatment of naive mice with serum from MS mice did not significantly inhibit neutrophil migration (data not shown). The next experiments were designed to identify the soluble substances in the serum from SS mice that inhibit neutrophil migration.

Serum from SS mice was filtered through an affinity column to remove albumin (Blue Sepharose column). Figure 1A shows that most of the neutrophil migration-inhibitory activity was recovered in the fraction that was not adsorbed to the resin (B<sup>-</sup>). This fraction with low albumin content (B<sup>-</sup>) was then filtered through an HPLC C<sub>18</sub> column. A typical ultraviolet absorbance profile of fraction B<sup>-</sup> is shown in Figure 1B. Neutrophil migration-inhibitory activity was detected only in fraction 1. When a portion of fraction 1 was subjected to PAGE under native nondenaturing conditions (Figure 1C), the neutrophil migration-inhibitory activity was demonstrable only in the diffusates of band 3 (B3) and band 4 (B4) (Figure 1A). In contrast, the part of the gel that did not stain for protein (GEL) did not inhibit neutrophil migration.

Mass spectrometric analysis of the tryptic peptides identified hemopexin as the protein in fractions B3 and B4. The peptide mass fingerprint of fractions B3 and B4 contained nine peptides with *m/z* values that were less than 0.3 atomic mass unit ( $\mu$ ) different from the values expected on the basis of the amino acid sequence of hemopexin (Figure E1 and Table E1). Four peptides were subjected to CID-MS/MS and their amino acid sequences were deduced from the ion fragment pattern (Table E1 and Figure E2). Moreover, the tryptic mass fingerprint



**Figure 1.** Serum from severe septic mice and their fractions inhibit neutrophil migration. (A) Neutrophil migration to the peritoneal cavity of mice. Six hours after injection of thioglycolate (3%, 500  $\mu$ l/cavity, intraperitoneal), cells in the peritoneal cavity were collected and neutrophils were counted (see METHODS). Test substances were administered intravenously 30 minutes before thioglycolate. Data are reported as mean ( $\pm$  SEM)  $\times 10^6$  neutrophils per cavity ( $n = 5$ ) and are the result of three independent experiments. Data were analyzed by analysis of variance followed by Bonferroni's *t* test. Serum from sham-operated mice (Sham) or mice with severe sepsis (SS) was injected at 9.6 mg of protein per mouse. Protein from the serum of septic mice that adsorbed to the Blue Sepharose column (B+) and protein that did not adsorb (B-) were injected intravenously at 7.40 and 2.05 mg of protein per mouse, respectively. \**P* < 0.001 compared with the saline (Sal) plus thioglycolate group or the B+ plus thioglycolate group. The effluent fractions from HPLC (F1–F4) were injected intravenously at 60  $\mu$ g of protein per mouse. \*\**P* < 0.01 versus the Sal plus Thioglycolate group. Diffusates of gel bands 1 to 4 were injected intravenously at 0.41  $\mu$ g of protein per mouse. The gel control (GEL) was a diffusate from a portion of the gel that was free of protein. #*P* < 0.05 compared with Sal or GEL. (B) HPLC profile of serum fraction B-. The fraction of the septic serum pool with low albumin content (B-) was applied to a C<sub>18</sub> column and eluted with a linear gradient of acetonitrile in trifluoroacetic acid as described in METHODS. The effluent was combined in four pools as indicated on the horizontal line and the neutrophil migration-inhibiting activity of each pool is shown in (A). (C) Native gel electrophoresis diffusates from HPLC fraction 1 (F1). ip = intraperitoneal; iv = intravenous.

obtained from a commercial sample of hemopexin did not differ significantly from that obtained from fraction B3 or B4 (data not shown). The apparent molecular masses measured by SDS-PAGE of bands B3 and B4 were approximately 57 and 69 kD (Figure E1C), respectively, as calculated with program ImageJ version 1.37 (National Institutes of Health, Bethesda, MD). The molecular mass of hemopexin is reported to be approximately 60 kD (20). A monoclonal antibody against hemopexin detected this protein in fractions B3 and B4 by Western analysis (Figure E1D).

### Hemopexin Inhibits Neutrophil Migration and Increases Mortality in Sepsis

Mass spectrometry indicated that hemopexin was the migration-inhibitory protein in SS serum. We used commercial hemopexin to confirm the inhibitory action of hemopexin on neutrophil migration. Figure 2A shows that intravenous pretreatment of mice with various amounts of hemopexin significantly inhibited the neutrophil migration stimulated by thioglycolate. BSA used as a negative control, as expected, did not inhibit neutrophil migration. In addition, serum collected from hemopexin-null mice 2 hours after severe sepsis did not significantly inhibit neutrophil migration, in contrast to serum collected from wild-type mice under the same conditions (Figure 2A).

To determine the role of hemopexin in sepsis, mice were treated with commercial hemopexin (10, 30, or 150  $\mu$ g/mouse, intravenous) 30 minutes before the induction of mild sepsis. As shown in Figure 2B, hemopexin treatment resulted in significantly reduced neutrophil migration to the focus of infection compared with wild-type mice treated with saline and subjected to the same septic stimulus (Figure 2B). The inhibition of neutrophil migration induced by hemopexin was accompanied

by an increased mortality rate among MS mice. Figure 2C shows that the survival rate of mice subjected to MS and pretreated with saline was 100% 3 days after surgery and 90% thereafter. The intravenous pretreatment of MS mice with hemopexin (10 or 30  $\mu$ g/mouse) reduced the survival rate to approximately 65% 3 days after sepsis. The reduction of the survival rate was more evident when the MS mice were pretreated with hemopexin at 150  $\mu$ g/mouse. Under these experimental conditions the survival rate was approximately 50% 3 days after sepsis. After treatment (6 h after CLP) with hemopexin (150  $\mu$ g/mouse), the survival rate among MS mice was also decreased (Figure E3).

Moreover, pretreatment of MS hemopexin-null mice with hemopexin (150  $\mu$ g/mouse, intravenous) also inhibited neutrophil migration to the focus of infection (Figure E4A). We also evaluated the levels of hemopexin after administration of this protein in hemopexin-null mice by ELISA. As shown in Figure E4B, hemopexin was not detected in the serum from hemopexin-null mice pretreated with saline. However, 6 hours after intravenous administration of hemopexin (15–300  $\mu$ g/mouse) we were able to detect circulating levels of this protein in naive and MS hemopexin-null mice.

### Hemopexin-null Mice Are More Resistant to Sepsis

In the previous section we demonstrated the detrimental role of hemopexin in sepsis. To further investigate this issue, CLP was performed in hemopexin-null mice. The data in Figure 2D show that hemopexin-null mice subjected to SS had a significant increase in neutrophil number in the peritoneal cavity 6 hours after surgery when compared with wild-type mice subjected to the same SS stimulus. The pretreatment (30 min) of hemopexin-null mice subjected to SS with hemopexin (150  $\mu$ g/mouse, intravenous) abrogated this improvement of neutrophil migra-

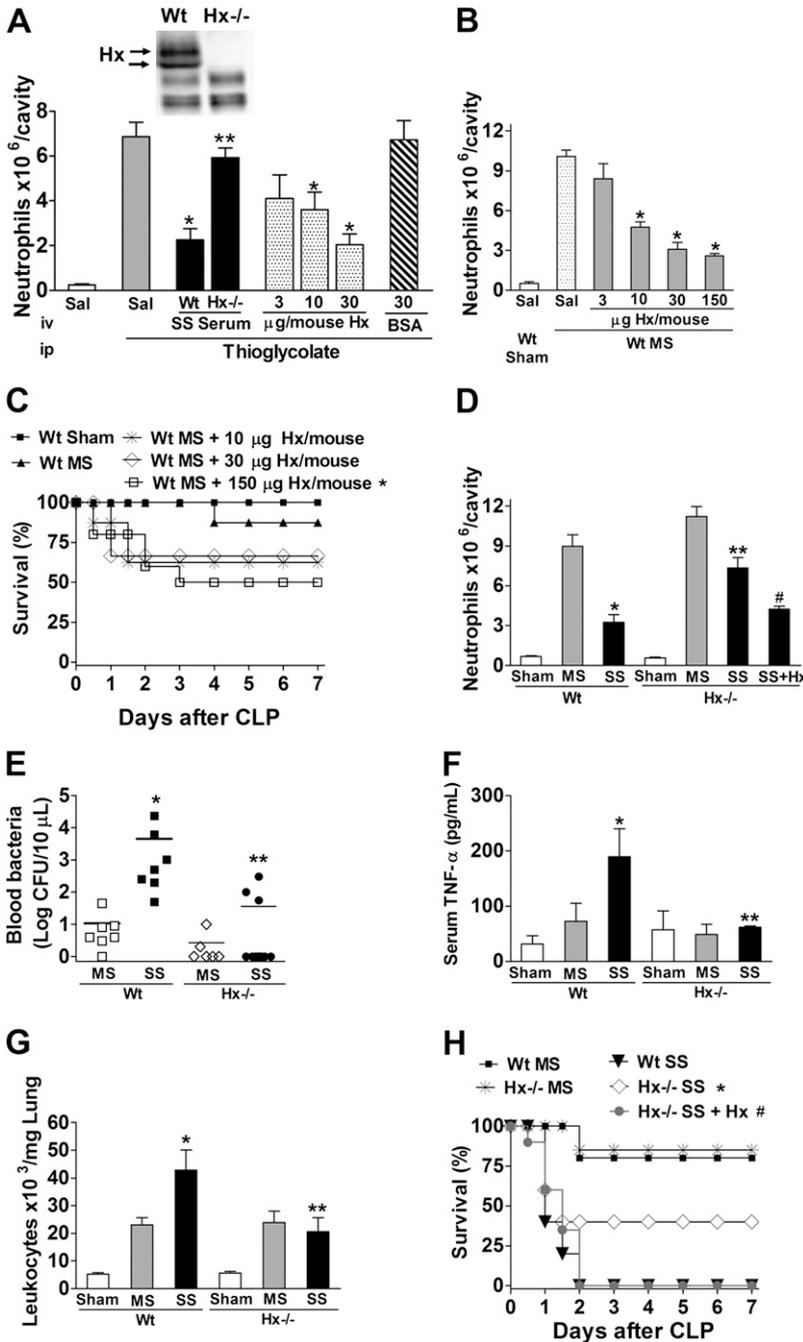
tion to the focus of infection. During MS, both strains presented similar effective neutrophil recruitment to the peritoneal cavity 6 hours after surgery. However, when neutrophil migration was evaluated 4 hours after MS induction, we observed that hemopexin-null mice had a significantly higher amount ( $P < 0.05$ ,  $n = 5$ ) of neutrophils in the peritoneal cavity [ $7.86 (\pm 0.97) \times 10^6$  neutrophils per cavity] compared with wild-type mice [ $3.92 (\pm 0.82) \times 10^6$  neutrophils per cavity]. Levels of the cytokines IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and IL-10 in the peritoneal cavity 6 hours after the induction of sepsis were similar in both mouse strains (Figure E5).

We determined the bacterial load in the peritoneal cavity and blood 6 hours after CLP. As shown in Figure 2E, SS hemopexin-null mice exhibited low levels of bacteria in the blood when compared with SS wild-type mice. The infectious focus of hemopexin-null mice also contained low levels of

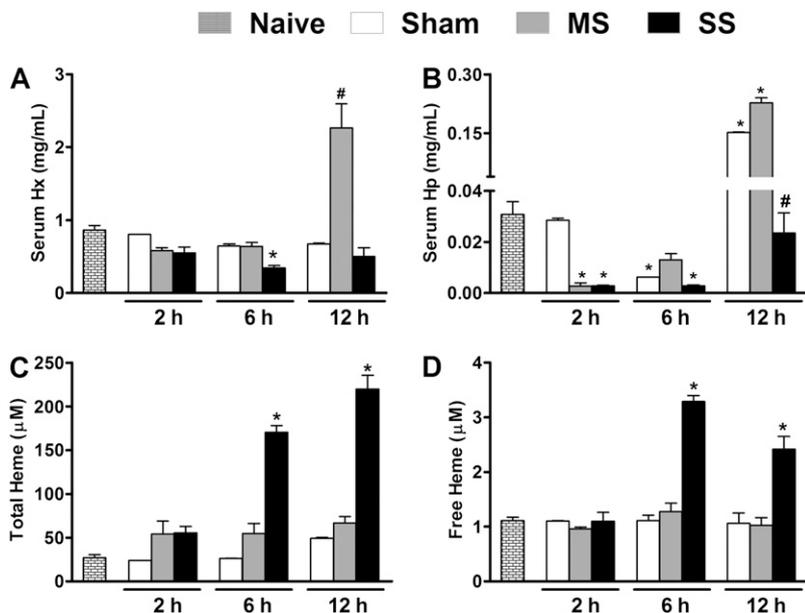
bacteria (data not shown). In MS both mouse strains showed similar bacterial contents.

We also evaluated the systemic inflammatory response of hemopexin-null and wild-type mice to CLP. We first determined the serum levels of TNF- $\alpha$  6 hours after sepsis induction. We confirmed that the levels of TNF- $\alpha$  were increased in wild-type mice during severe sepsis when compared with nonsevere sepsis. However, in hemopexin-null mice with SS, the increase in TNF- $\alpha$  was not observed (Figure 2F). Furthermore, leukocyte sequestration (measured by myeloperoxidase activity) in lung tissue 3 hours after sepsis was significantly higher in wild-type SS mice than in sham or MS mice (Figure 2G). This increase in lung neutrophil sequestration was not observed in hemopexin-null mice with SS.

Last, we evaluated the mortality of wild-type and hemopexin-null mice undergoing MS and SS induced by CLP. As shown in



**Figure 2.** A commercial sample of plasma hemopexin inhibits neutrophil migration and hemopexin-null mice are more resistant to severe sepsis. (A) Serum collected 2 hours after the induction of severe sepsis from wild-type mice (Wt, 9.50 mg of protein per mouse) or from hemopexin-null mice ( $Hx^{-/-}$ , 9.50 mg of protein per mouse) and bovine serum albumin (BSA, 30  $\mu$ g/mouse) or commercial hemopexin (Hx, 3–30  $\mu$ g/mouse) was injected intravenously 30 minutes before thioglycolate (3%, 500  $\mu$ l/cavity, intraperitoneal). Six hours after thioglycolate administration, neutrophils in the peritoneal cavity were counted (see METHODS). Data are reported as mean ( $\pm$  SEM)  $\times 10^6$  neutrophils per cavity and are the result of three independent experiments. \* $P < 0.01$  compared with the saline (Sal) plus thioglycolate group; \*\* $P < 0.05$  compared with the serum Wt plus thioglycolate group. The inset in (A) is a Western blot analysis of serum samples from Wt and  $Hx^{-/-}$  mice with severe sepsis (SS); a monoclonal antibody against Hx was used for detection. We used 10–12 mice per group for survival analysis and 5 mice per group for the other data. A log-rank test (chi-square test) was used to examine differences between survival curves and the other data were analyzed by analysis of variance followed by Bonferroni's *t* test. Commercial Hx (3–150  $\mu$ g/mouse, intravenous) was injected 30 minutes before the induction of mild sepsis (MS) by cecal ligation and puncture (CLP). Six hours later, (B) neutrophil migration to the focus of infection was determined and (C) survival rates were determined up to 7 days after surgery. \* $P < 0.05$  compared with the Sal plus MS group. (D–F) MS and severe sepsis (SS) were induced in  $Hx^{-/-}$  and Wt mice. Commercial Hx (150  $\mu$ g/mouse, intravenous) was injected 30 minutes before the induction of SS in  $Hx^{-/-}$  mice. Six hours after surgery (D) neutrophils and (E) the bacterial content in blood and (F) serum TNF- $\alpha$  levels were determined. (G) Leukocyte sequestration in lung was determined 3 hours after surgery with a myeloperoxidase assay and (H) the survival rates were also determined; \* $P < 0.05$  compared with the MS Wt group; \*\* $P < 0.05$  compared with the SS Wt group. CFU = colony-forming units; ip = intraperitoneal; iv = intravenous; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .



**Figure 3.** Serum levels of hemopexin haptoglobin and total and free heme during sepsis. Serum samples were collected from naive, sham-operated (Sham), subjected to mild sepsis (MS) or severe sepsis (SS) mice 2, 6, and 12 hours after cecum ligation and puncture (CLP) surgery. (A) Serum levels of hemopexin (Hx); \* $P < 0.05$  compared with MS and Sham groups; # $P < 0.01$  compared with all groups. (B) Serum levels of haptoglobin (Hp); \* $P < 0.05$  compared with naive group; # $P < 0.05$  compared with the MS 12-hour group. (C) Serum total heme and (D) free heme levels; \* $P < 0.05$  compared with Sham and MS groups. These experiments were repeated three times ( $n = 5$ ).

Figure 2H, both mouse strains subjected to MS presented survival rates of approximately 80% throughout the 7-day observation period. In contrast, 100% of wild-type mice subjected to SS died within 2 days, whereas about 40% of hemopexin-null mice subjected to SS survived throughout the 7-day observation period. Interestingly, when hemopexin-null mice were pretreated with hemopexin (150  $\mu\text{g}/\text{mouse}$ , intravenous) all mice died within 2 days after CLP. In another model of sepsis induced by intraperitoneal inoculation with *E. coli* ( $2 \times 10^8$  cfu/mouse), the survival rate of wild-type mice was 48% over a 7-day observation period, as opposed to 86% for hemopexin-null mice. When wild-type mice were pretreated with hemopexin all mice died within 1 day after *E. coli* inoculation (Figure E6A).

Confirming the antiinflammatory role of hemopexin, Figure E6 shows that hemopexin (150  $\mu\text{g}/\text{mouse}$ , intravenous) pretreatment inhibited LPS-induced leukocyte infiltration in lungs and mortality (from  $\sim 100$  to  $\sim 30\%$ ) in mice. This dose of LPS (5 mg/kg, intraperitoneal) induces a systemic inflammatory response that is responsible for the low survival of the animals.

#### Serum Hemopexin Concentration during Sepsis

It has been described that serum levels of hemopexin are up-regulated during the infection process (21); however, it was not fully addressed in sepsis. As shown in Figure 3A, serum levels of hemopexin were significantly down-regulated 6 hours after severe CLP; however, 12 hours after sepsis induction, there was a significant increase in the concentration of hemopexin only in mice subjected to MS. In contrast, the mRNA expression of hemopexin was increased in MS and SS sepsis 6 hours after surgery, and this up-regulation was sustained at 12 hours in SS mice (Figure E7A). Although the serum level of hemopexin was not increased during SS, the level of this protein was significantly higher in the liver of severely septic mice when compared with sham-operated mice (Figure E7B). The better characterized function of hemopexin is to scavenge free heme, following by its transport to the liver (22). Thus, the absence of the correspondent protein in the serum could be explained by its high turnover due to the transport of heme to the liver. To confirm this hypothesis, we measured the haptoglobin levels (a known marker of hemolysis) and the levels of total and free heme. Figure 3B shows that the levels of haptoglobin were down-regulated, indicating a scenario of hemolysis during severe sepsis. Moreover,

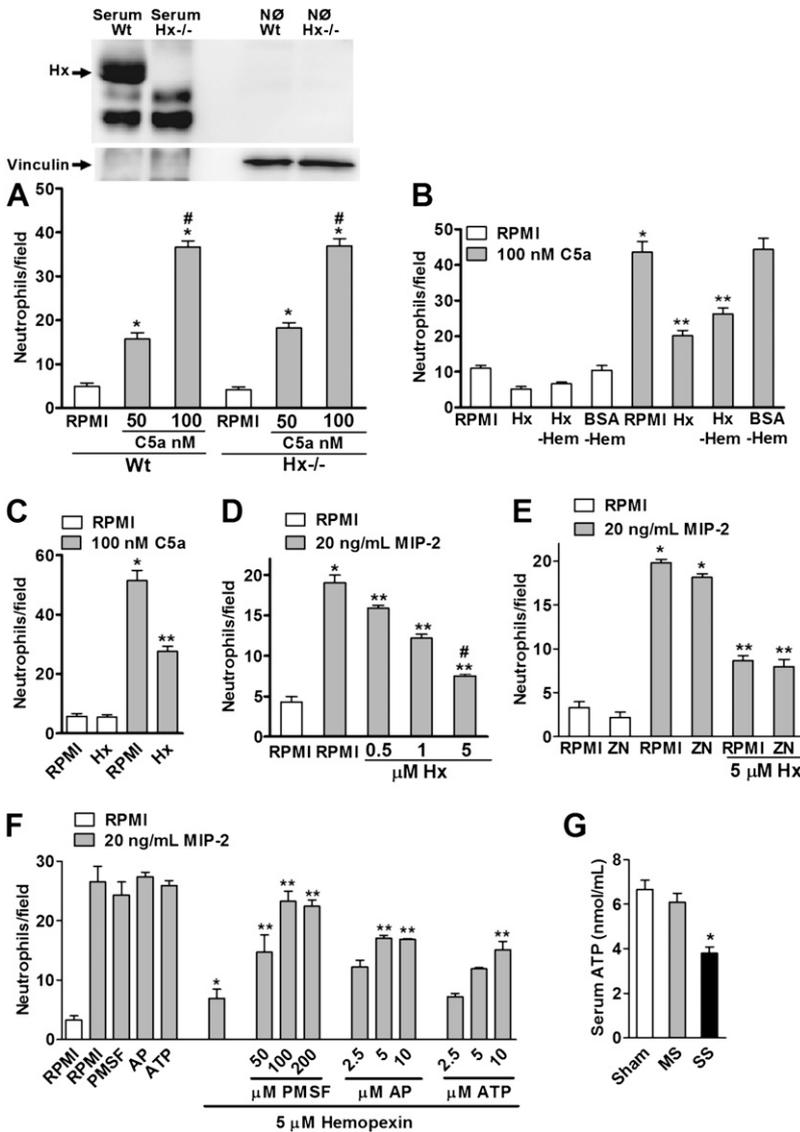
the serum levels of total (Figure 3C) and free heme (Figure 3D) were significantly increased 6 and 12 hours after surgery only in SS.

The production of hemopexin is known to be regulated by proinflammatory cytokines, such as IL-6 and TNF- $\alpha$  (20), and therefore we evaluated the serum levels of hemopexin after CLP in TNFR1/R2 double-knockout mice (TNFR1/R2 $^{-/-}$ ) and in IL-6-deficient mice (IL-6 $^{-/-}$ ). The hemopexin serum levels 2 and 6 hours after MS and SS sepsis induction were similar in both strains of mice when compared with wild-type mice (data not shown). However, 12 hours after MS induction, the serum levels of hemopexin was significantly decreased in IL-6 $^{-/-}$  mice when compared with TNFR1/R2 $^{-/-}$  or wild-type mice (Figure E7C).

#### Hemopexin Inhibits the Neutrophil Chemotactic Response *in Vitro*

Bone marrow cells were collected from wild-type and hemopexin-null mice and neutrophils were isolated for testing the *in vitro* chemotactic response in a Boyden chamber. The chemotactic responses of neutrophils from both strains were similar. As shown in Figure 4A, C5a (50 nM) induced a significant chemotactic response in neutrophils from both wild-type and hemopexin-null mice. There was a significant difference between the responses to 50 and 100 nM C5a, indicating that the response was dose dependent. The inset in Figure 4A is a Western blot analysis of serum and of a bone marrow neutrophil extract from wild-type and hemopexin-null naive mice. A monoclonal antibody against hemopexin detected hemopexin only in serum but not in neutrophils from wild-type mice or in serum from hemopexin-null mice.

The effect of hemopexin on the chemotaxis of neutrophils from the bone marrow of wild-type mice was then evaluated. As heme content in serum is increased during severe sepsis, the effect of the heme-hemopexin complex on neutrophil chemotaxis was also evaluated. Neutrophils were incubated at 37°C for 1 hour in the absence (control, RPMI) or presence of hemopexin (1  $\mu\text{M}$ ), heme-hemopexin (Hx-Hem, 1  $\mu\text{M}$ ), or heme-albumin (BSA-Hem, 1  $\mu\text{M}$ ) as a negative control and then the cells (95% viable by trypan blue exclusion) were allowed to migrate toward 100 nM C5a. As shown in Figure 4B, hemopexin and Hx-Hem pretreatment resulted in a marked inhibition of C5a-induced chemotaxis. Pretreatment with BSA-Hem had no effect on chemotaxis at the concentration and time studied.



Similarly, hemopexin also inhibited the chemotaxis of neutrophils from hemopexin-null mice to C5a (Figure 4C). Pretreatment of wild-type neutrophils with hemopexin also induced a marked dose-dependent inhibitory effect on chemotactic response to macrophage-inflammatory protein (MIP)-2 (Figure 4D). Moreover, pretreatment (30 min) of neutrophils with the nonspecific inhibitor of heme oxygenase (HO), zinc deuteroporphyrin 2,4-bis glycol, was not able to block the inhibitory effect of hemopexin on neutrophil chemotaxis (Figure 4E).

Several studies have provided data demonstrating a protease activity for plasma hemopexin. Hemopexin purified from human plasma or the recombinant protein was able to induce transient protein leakage after contact with rat kidney *ex vivo* and showed protease activity that could be inhibited with various serine protease inhibitors or ATP *in vitro* (23, 24). To test the role of hemopexin protease activity in the inhibition of neutrophil chemotaxis, we preincubated (30 min, 37°C) hemopexin with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 50–200 μM) or aprotinin (AP, 2.5–10 μM) or with ATP (2.5–10 μM), and then these complexes were added

**Figure 4.** Hemopexin inhibits the neutrophil (NØ) chemotactic response induced by C5a or macrophage-inflammatory protein (MIP)-2. (A) Neutrophils purified from the bone marrow of wild-type (Wt) and hemopexin-null (Hx<sup>-/-</sup>) mice were subjected to chemotaxis stimulated by C5a (50 or 100 nM) or RPMI (control) in a Boyden chamber. Data are reported as the mean (± SEM) number of neutrophils per field and are the results of three independent experiments performed in triplicate. \**P* < 0.001 compared with RPMI; #*P* < 0.001 compared with 50 nM C5a (analysis of variance followed by Bonferroni's test). At the top of (A) is a Western blot analysis of serum and bone marrow neutrophil extracts from Wt and Hx<sup>-/-</sup> naive mice. A monoclonal antibody against hemopexin (Hx) or vinculin was used for detection. (B) Neutrophils purified from the bone marrow of Wt mice were incubated with RPMI medium, 1 μM Hx, 1 μM Hx-hemin (Hx-Hem) complex, or 1 μM bovine serum albumin-Hem complex (BSA-Hem) for 1 hour at 37°C before chemotaxis stimulated by 100 nM C5a or RPMI (control) in a Boyden chamber. \**P* < 0.01 compared with RPMI plus RPMI; \*\**P* < 0.01 compared with RPMI plus C5a. (C) Neutrophils purified from the bone marrow of hemopexin-null mice were incubated with RPMI medium or 1 μM Hx for 1 hour at 37°C before chemotaxis stimulated by 100 nM C5a or RPMI (control) in a Boyden chamber. \**P* < 0.01 compared with RPMI plus RPMI; \*\**P* < 0.01 compared with RPMI plus C5a. (D) Hx (0.5–5 μM) also inhibited neutrophil chemotaxis stimulated by MIP-2 (20 ng/ml) in a dose-dependent manner. \**P* < 0.01 compared with RPMI plus RPMI; \*\**P* < 0.01 compared with RPMI plus MIP-2; #*P* < 0.01 compared with 0.5 μM Hx plus MIP-2. (E) The inhibitor of heme oxygenase, zinc deuteroporphyrin 2,4-bis glycol (ZN, 30 μM), did not block the effect of Hx on neutrophil chemotaxis. \**P* < 0.001 compared with RPMI plus RPMI; \*\**P* < 0.01 compared with RPMI plus MIP-2. (F) Hemopexin (5 μM) was incubated (30 min, 37°C) with phenylmethylsulfonyl fluoride (PMSF, 50–200 μM), aprotinin (AP, 2.5–10 μM), or ATP (2.5–10 μM). These complexes were added to neutrophils for 1 hour and the chemotaxis response to 100 nM C5a or RPMI (control) in a Boyden chamber was evaluated. \**P* < 0.01 compared with RPMI plus MIP-2; \*\**P* < 0.01 compared with hemopexin plus C5a. (G) Levels of ATP were measured in serum collected from mice 2 hours after mild sepsis (MS) or severe sepsis (SS) induction or from sham-operated mice.

to neutrophils for 1 hour. After that, the chemotactic response of neutrophils to MIP-2 was determined. As shown in Figure 4F, PMSF, AP, or ATP significantly prevented the inhibitory effect of hemopexin in neutrophil chemotaxis. In addition, Figure 4G shows that, when compared with sham-operated or MS mice, serum from severely septic mice had a significant decrease in the levels of ATP 2 hours after surgery; a condition that could induce the protease activity of hemopexin *in vivo*.

We isolated neutrophils from TLR4- and MyD88-null mice to determine whether the inhibitory effect of hemopexin on neutrophil chemotaxis was mediated by contamination with LPS in the sample. Hemopexin (1 or 5 μM) also inhibited the MIP-2-induced chemotaxis of neutrophils from TLR4- or MyD88-null mice. As expected, LPS inhibited MIP-2-induced chemotaxis of neutrophils from wild-type mice, but was ineffective on neutrophils from TLR4<sup>-/-</sup> mice (Figure E8).

The leakage of intracellular lactate dehydrogenase (LDH) into the extravascular medium is an indicator of cell death due to cell membrane damage (25). Therefore we measured the LDH concentration in the supernatant of neutrophils treated

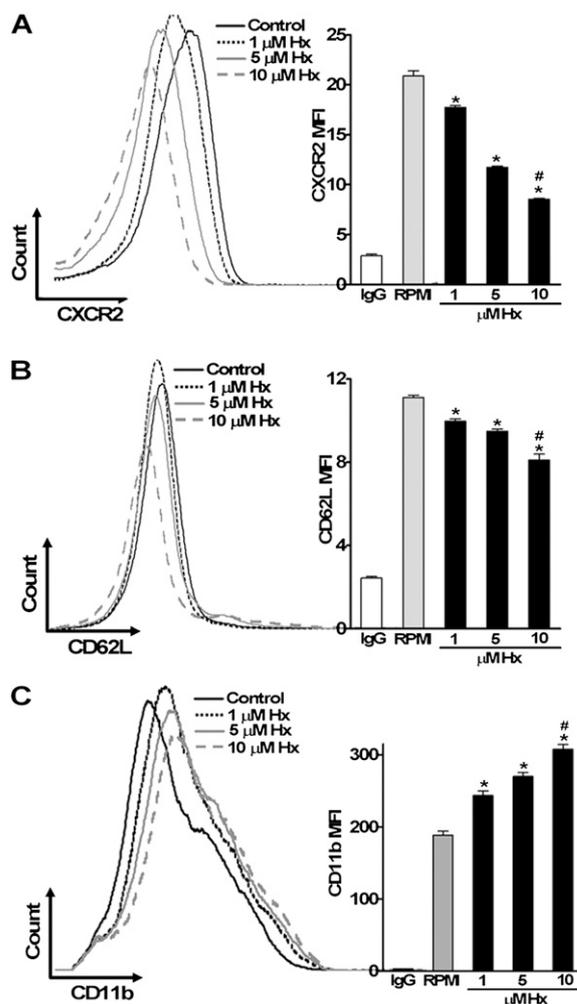
with hemopexin. Neutrophils ( $1 \times 10^6/\text{ml}$ ) incubated (4 h,  $37^\circ\text{C}$ ) with all doses of hemopexin showed similar levels of LDH in supernatants compared with neutrophils incubated with RPMI. However, neutrophils incubated with RPMI and subjected to liquid nitrogen lysis showed an approximately 85% increase in LDH in the supernatant when compared with cells incubated with RPMI without nitrogen lysis (data not shown). The incubation of hemopexin (5 and  $10 \mu\text{M}$  for 4 h at  $37^\circ\text{C}$ ) with neutrophils also did not induce the membrane expression of annexin V or propidium iodide accumulation in the cells when compared with control neutrophils (Figure E9).

#### Hemopexin Down-modulates CXCR2 and L-Selectin and Up-modulates CD11b Expression in Neutrophils

*In vitro* studies have demonstrated that the down-regulation of CXCR2 is responsible for the reduced neutrophil response to chemokines that bind to CXCR2 (26). Moreover, down-regulation of CXCR2 on the neutrophil surface is an important event for the failure of neutrophil migration to the focus of infection during severe sepsis (15). Therefore, we tested whether *in vitro* hemopexin neutrophil stimulation leads to CXCR2 down-modulation. Flow cytometric analysis showed that pretreatment with hemopexin (1, 5, and  $10 \mu\text{M}$ ) significantly decreased the expression of CXCR2 on neutrophils (Figure 5A). Moreover, hemopexin also induced neutrophil L-selectin shedding (Figure 5B) and up-modulation of CD11b (Figure 5C). All three effects were dose dependent. L-selectin shedding and increased expression of CD11b on circulating neutrophils have been used as predictors of severity for septic patients (27, 28).

#### DISCUSSION

The failure of neutrophil migration to the focus of infection may contribute to the high mortality rate of severe sepsis (6–8). Several lines of evidence have shown that serum factors induced in the host during the systemic inflammatory response syndrome are responsible for the inhibition of neutrophil functions such as migration and oxidative burst (13, 14, 28). In a search for serum mediators of the inhibition of neutrophil migration during sepsis, we demonstrated here that the acute-phase protein hemopexin participates in this process. First, we showed that serum collected from wild-type mice 2 hours after severe sepsis inhibited thioglycolate-induced neutrophil migration to the mouse peritoneal cavity. Hemopexin was isolated from this serum and the purified protein inhibited thioglycolate-induced neutrophil migration to the peritoneal cavity, an activity also demonstrable for commercial hemopexin. In a CLP model of mild sepsis, hemopexin inhibited neutrophil migration to the focus of infection and increased the mortality rate. As expected, hemopexin-null mice subjected to severe sepsis did not present failure of neutrophil migration to the focus of infection. Consequently, these mice presented less bacteremia and an improved survival rate when compared with wild-type mice. When hemopexin-null mice were treated with hemopexin before severe sepsis, their survival rate, as well as recruitment of neutrophils to the peritoneal cavity, were comparable to what was observed in severely septic wild-type animals. Moreover, hemopexin inhibited neutrophil chemotaxis *in vitro* and induced down-modulation of CXCR2 and CD62 ligand, and up-modulation of CD11b expression in neutrophils. In addition, we demonstrated that the inhibitory effect of hemopexin on neutrophil chemotaxis depends on its protease activity, because serine protease inhibitors or ATP inhibited this process. Thus, the present study has demonstrated for the first time a negative role for hemopexin in sepsis.



**Figure 5.** Hemopexin decreased the expression of CD62 ligand (CD62L) and CXCR2 and increased CD11b expression on neutrophils. Flow cytometry of (A) CXCR2, (B) CD62L, and (C) CD11b expression on bone marrow neutrophils incubated with RPMI medium or hemopexin (Hx; 1, 5, and  $10 \mu\text{M}$ ) for 1 hour at  $37^\circ\text{C}$ . \* $P < 0.05$  compared with RPMI; # $P < 0.01$  compared with  $1 \mu\text{M}$  Hx. Data are reported as the mean ( $\pm$  SEM) fluorescence intensity (MFI) and are the result of the three independent experiments performed in triplicate.

The methods used to isolate hemopexin, that is, Blue Sepharose,  $\text{C}_{18}$  HPLC, and PAGE, were standard. However, the difficulty in quantifying the biological response to hemopexin made it impossible to monitor the recovery of activity of the active substance at each purification step or to improve the recovery at any step. We recovered approximately  $300 \mu\text{g}$  of hemopexin from 30 ml of serum, which is low, considering that the blood of septic mice contains approximately  $0.50 \text{ mg}$  of hemopexin per milliliter.

As is the case for many acute-phase proteins, the serum concentration of hemopexin could increase during infection (21, 29). It is a 60-kD plasma glycoprotein with a high binding affinity for heme, with which it forms a 1:1 molar complex. After heme binding, hemopexin undergoes a conformational change permitting interaction with a specific receptor, expressed mainly on the hepatocyte membrane, and then is internalized (20, 30). Hemopexin is the main protein responsible for the transport of intravascular heme, thus preventing both free heme-catalyzed oxidative damage and heme-bound iron loss (20). Here, for the first time, we demonstrated the course of serum hemopexin

levels during sepsis. Hemopexin mRNA and protein levels in the liver strongly increased after severe sepsis whereas the serum protein level decreased, suggesting a rapid turnover caused by intravascular hemolysis. This is in agreement with what occurs in many other pathologic conditions associated with hemolysis including trauma, hemoglobinopathies, hemorrhages, and malaria (22). In addition, our data suggested that IL-6 is the main regulator of hemopexin production in sepsis, because IL-6<sup>-/-</sup> mice showed lower levels of hemopexin than did wild-type mice.

We showed here the inhibitory effect of hemopexin on neutrophil migration. When hemopexin was administered by the intravenous route to mice subjected to mild sepsis, neutrophil migration to the focus of infection was inhibited. Consequently, control of infection was impaired, with increased mortality of the mice. In another model of a local sterile inflammation, hemopexin also inhibited thioglycolate-induced neutrophil migration to the peritoneal cavity. Moreover, hemopexin-null mice subjected to severe sepsis presented a significant increase in neutrophil content at the focus of infection when compared with wild-type mice and were more resistant to sepsis. Of note, in apparent contradiction to these findings, hemopexin-null mice are more susceptible to heme overload or intravascular hemolysis (29–31). Moreover, in the present study, hemopexin protected animals from LPS-induced mortality. These models have similar physiopathological events. Oxidative stress, and endothelial cell and leukocyte activation, are the main factors responsible for the tissue injury (31–33). These alterations are triggered mainly by free heme in heme overload and intravascular hemolysis models and by LPS in LPS-induced shock, respectively. In the first two models hemopexin, by sequestering heme, prevents endothelial and tissue damage. In LPS-induced shock the ability of hemopexin to inhibit neutrophil migration prevents neutrophil accumulation in vital organs, such as the lungs. In contrast, the efficient neutrophil migration observed in hemopexin-null mice after the induction of severe sepsis improved the control of infection, as indicated by the reduction in colony-forming unit content at the focus of infection and in blood. Bacteria and their products in the blood stream could induce the activation of TLRs and, therefore, increase the serum levels of cytokines and trigger leukocyte activation, which accumulated in the lungs and other specific organs (8, 34). Low serum TNF- $\alpha$  levels and the reduction of lung leukocyte sequestration probably account for the improvement of survival observed in hemopexin-null mice after sepsis.

The higher amounts of neutrophil in the infection focus observed in hemopexin-null mice undergoing sepsis, when compared with wild-type mice, were not due to a higher concentration of cytokines at the site of infection, because both mouse strains presented similar levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 in the exudate. Nevertheless, neutrophil chemotaxis was inhibited when hemopexin was incubated with neutrophils from wild-type or from hemopexin-null mice. Of note, we observed a similar chemotactic response of neutrophils from the bone marrow of wild-type and hemopexin-null mice. Together, these results suggest that the main effect of hemopexin is on neutrophil locomotion. In addition, Suzuki and colleagues (35) demonstrated that hemopexin inhibits phorbol myristate acetate-induced neutrophil adhesion to fibrinogen- and serum-coated surfaces (36). There is also evidence that hemopexin inhibits the production of cytokines by LPS-stimulated macrophages (37). However, it seems that it does not happen in the context of sepsis, because the level of cytokine production in the focus of infection in hemopexin-null mice was similar to that in wild-type mice.

Inhibition of chemotaxis was observed with both hemopexin and the Hx-heme complex, suggesting that this effect is in-

dependent of the low-density receptor-related protein (LRP/CD91) present in these cells, because hemopexin alone has low affinity for this receptor. LRP/CD91 has been reported to internalize Hx-heme complexes (30). Heme is rapidly catabolized by HO that opens the porphyrin ring, producing biliverdin and carbon monoxide (CO) and releasing iron that can then be bound by ferritin (38). It has been shown that the activation of HO by LPS or heme with consequent CO production inhibits the neutrophil chemotactic response and that HO inhibitors prevent this inhibition of the chemotactic response (A. Freitas and F. Q. Cunha, unpublished data). However, the nonspecific inhibitor of HO did not block the hemopexin effect on neutrophil chemotaxis, suggesting that the inhibitory effect of hemopexin is independent of the CD91/HO/CO pathway.

As mentioned previously, hemopexin had protease activity that could be inhibited by serine protease inhibitors or ATP (24). Here we demonstrated that coincubation of hemopexin with serine protease inhibitors or ATP prevented the reduction of neutrophil chemotaxis induced by hemopexin. Moreover, the serum level of ATP was decreased in mice with severe sepsis. These data suggest that severe sepsis triggers an inhibitory activity of hemopexin, for example, enhancements of its protease activity and/or decrease the efficiency of their endogenous inhibitor. This hypothesis may explain why only septic serum inhibited neutrophil migration. Moreover, an intriguing finding of the present study was that the inhibitory effect of human hemopexin on neutrophil migration is achieved with a low dose ( $\sim$ 8–10 times less than the physiological concentration in mice). As human hemopexin behaves like the hemopexin found in septic mice, it is possible that the human protein is more efficient in inhibiting migration and/or that the purification procedure from human serum unmasks the inhibitory effect.

Chemokines coordinate the migration and chemotaxis of leukocytes by binding to specific chemokine receptors. CXCR2 plays a central role in the recruitment of neutrophils from the circulation to the site of inflammation (39). Impaired neutrophil migration during sepsis is correlated with early down-regulation of CXCR2 expression on circulating neutrophils (15). We have identified hemopexin as a mediator of this phenomenon, because hemopexin induced the down-regulation of CXCR2 on neutrophils. Interestingly, this decreased CXCR2 expression was accompanied by reduced chemotaxis in response to MIP-2. Moreover, hemopexin induced shedding of L-selectin and up-regulation of CD11b on neutrophils. These findings suggest that the circulating hemopexin during sepsis could promote the activation of circulating neutrophils and therefore induce the down-regulation of CXCR2 and L-selectin and the up-regulation of CD11b and, as a consequence, the inhibition of neutrophil migration to the focus of infection. In accordance, the protease activity of hemopexin is able to down-regulate angiotensin II receptor-1 in human monocytes, endothelial cells, and rat aortic rings (40).

The present study provides evidence for another example of an acute-phase protein with antiinflammatory effects. It has been shown that C-reactive protein (41), serum amyloid A (42), AGP (14), pentraxin-3 (43), and now hemopexin inhibit neutrophil migration/chemotaxis. The antiinflammatory action of acute-phase proteins could be beneficial by limiting tissue neutrophil infiltration in pathological conditions with systemic inflammatory response syndrome without a focus of infection. However, in sepsis, the overwhelming antiinflammatory response in the early phase of sepsis inhibits neutrophil migration and, consequently, impairs bacterial killing. Thus, therapeutic inhibition of acute-phase proteins such as hemopexin, AGP, C-reactive protein, and serum amyloid A in

the early phase of sepsis could improve neutrophil migration to the focus of infection and, as a consequence, improve the survival rate.

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