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Molecular Medicine

HMGB1 Mediates Anemia of Inflammation in Murine Sepsis Survivors

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Patients surviving sepsis develop anemia, but the molecular mechanism is unknown. Here we observed that mice surviving polymicrobial gram-negative sepsis develop hypochromic, microcytic anemia with reticulocytosis. The bone marrow of sepsis survivors accumulates polychromatophilic and orthochromatic erythroblasts. Compensatory extramedullary erythropoiesis in the spleen is defective during terminal differentiation. Circulating tumor necrosis factor (TNF) and interleukin (IL)-6 are elevated for 5 d after the onset of sepsis, and serum high-mobility group box 1 (HMGB1) levels are increased from d 7 until at least d 28. Administration of recombinant HMGB1 to healthy mice mediates anemia with extramedullary erythropoiesis and significantly elevated reticulocyte counts. Moreover, administration of anti-HMGB1 monoclonal antibodies after sepsis significantly ameliorates the development of anemia (hematocrit 48.5 ± 9.0% versus 37.4 ± 6.1%, $p < 0.01$; hemoglobin 14.0 ± 1.7 versus 11.7 ± 1.2 g/dL, $p < 0.01$). Together, these results indicate that HMGB1 mediates anemia by interfering with erythropoiesis, suggesting a potential therapeutic strategy for anemia in sepsis.

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INTRODUCTION

Severe sepsis, the clinical syndrome that occurs in response to infection or injury (1), occurs in more than 700,000 cases annually in the United States. The mortality rate is >20%; survivors of severe sepsis have a cumulative 5-year mortality of 74% (2,3). Anemia, defined as a decrease in the hematocrit and hemoglobin, is a frequent complication of severe sepsis, with >60% of septic patients developing anemia, and 90% of these patients will remain anemic for several weeks (4).

Anemia in the critically ill patient is a predictor of poor outcome (5). This "anemia of inflammation" affects patients with chronic infections, autoimmune disorders, and malignancies, as well as sepsis (6). Hemoglobin levels of 7–9 g/dL are typical in septic patients (7) and characterized by microcytic and hypochromic erythrocytes (4,8). Despite its high prevalence and significant adverse impact on quality of life, the mechanisms of anemia of inflammation remain unclear (7).

Patients with sepsis develop significant long-term elevations in circulating HMGB1 levels that correlate with in-hospital mortality (9). HMGB1 is a

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proinflammatory cytokine that exists in three redox isoforms, termed disulfide, all-thiol and sulfonyl, each possessing differential signaling activities (10,11). Disulfide HMGB1 binds to the cell surface receptor complex MD2-TLR4 (12) to enhance release of TNF and IL-6, cytokines that have been implicated in the onset of anemia of inflammation in sepsis and negatively regulate erythropoiesis (13,14). We recently identified HMGB1 as a mediator of persistent morbidity and mortality in sepsis survivors (15,16). Here we show that HMGB1 is both necessary and sufficient to induce anemia in murine sepsis survivors and that HMGB1 is a therapeutic target.

MATERIALS AND METHODS

Mice

All experiments were performed in accordance with the National Institutes of Health guidelines, under protocols approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research. Male BALB/c mice were purchased from Charles River. Mice were 3–4 months old at the moment of surgery and weighed between 25 and 28 g. Mice were housed in groups of five in a pathogen-free facility in an enriched environment. Animals were on a 12-h daylight cycle with ad libitum access to water and normal chow. After cecal ligation and puncture (CLP), investigators and technicians from the Feinstein animal facility ascertained animal welfare twice a day.

Induction of Sepsis

Severe polymicrobial abdominal sepsis was induced in BALB/c mice by CLP as previously described (15). Briefly, after isolation of the cecum, we ligated below the ileo-cecal valve and punctured it once with a 22-G needle. Stool (~1 mm) was mechanically extruded, the cecum returned to the abdominal cavity and the wound closed with surgical clips. Resuscitation immediately after CLP included 1 mL saline solution, as well as a dose of Imipenem-Cilastatin (0.5 mg/kg diluted in a 0.9% saline solution), both administered subcutaneously. Survival rates in the CLP model are typically between 50 and 70%.

Recombinant HMGB1 Administration to Healthy BALB/c Mice

Recombinant rat HMGB1 was expressed in *Escherichia coli* and purified as previously described (17). Recombinant disulfide HMGB1 was prepared as described elsewhere (18,19), 500 μg diluted in 650 μL phosphate-buffered saline (PBS) and administered intraperitoneally to healthy BALB/c mice daily for 21 d. Control mice received PBS injections. This dose of HMGB1 induces an inflammatory response that lasts ~24 h. One day after the last injection, blood, bone marrow and spleen were harvested.

Administration of Anti-HMGB1 Neutralizing Monoclonal Antibody

The anti-HMGB1 monoclonal antibody (clone 2G7) was generated as previously described (20). This is a neutralizing antibody that detects all redox isoforms of HMGB1. It does not need complement activation or Fc-receptor interactions to perform as an HMGB1 antagonist. It does not react with HMGB2. The monoclonal antibody detects an epitope in sequence 46–63 of the box A domain, more specifically around glycine in position 58, since this is the single discriminating residue between HMGB1 and HMGB2 in this sequence. On d 9–11 after surgery, CLP survivors received intraperitoneal injections of either anti-HMGB1 monoclonal antibody (50 μg/day in 200 μL PBS) or mouse IgG2b (ESMD Chemicals) as isotype control (15). Specificity of 2G7 has been previously demonstrated (15,16).

Complete Blood Counts

Blood was collected by cardiac puncture and transferred to ethylenediaminetetraacetic acid (EDTA)-coated tubes. Complete blood counts were obtained using the automated hematology analyzer AcT diff (Beckman Coulter). Reticulocytes were determined by flow cytometry using Thiazole orange (Retic Count®; BD Biosciences) according to the manufacturer's instructions.

Characterization of Murine Erythropoiesis

To evaluate stress erythropoiesis, spleens were flushed with 1 mL ice-cold PBS, and cells were expelled using gentle compression. Bone marrow cells were obtained after dislocating two limbs and two long bones. Bones were cleaned and the epiphysis cut. Each diaphysis was then flushed with PBS repeatedly until no obvious macroscopic hematopoietic tissue remained. Flushed cells (splenic and bone marrow) were homogenized into single cell solution, filtered using a 40 μmol/L sterile cell strainer (Fisherbrand®; Fisher Scientific).

To quantitate the degree of erythropoiesis in the bone marrow and the spleen of animals, we used positive magnetic sorting with anti-CD45 beads (Milteny Biotec). Erythropoiesis was monitored as described previously (21). Briefly, we gated on $CD45^{neg}$, $CD11b^{neg}$ and $GR\text{-}1^{neg}$ cells within the $7\text{-}AAD^{neg}$ gate. Those cells were then gated on $Ter119^{+}$ cells, followed by CD44 versus forward scatter. This revealed six different cell populations corresponding to the six stages of terminal erythroid differentiation. All flow data were subsequently analyzed using FlowJo v9.4 (Tree Star).

Cytokine Determination

Erythropoietin (EPO) and ferritin were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions: EPO (R&D Systems, cat: MEP00B) and ferritin (Abcam, cat. no. ab157713). The levels of TNF and IL-6 were determined using a mouse a multiplex kit (Meso Scale Discovery), according to the manufacturer's instructions. HMGB1 levels were measured by immunoblotting analysis as described previously (19). Briefly, plasma samples were loaded on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred to a poly(vinylidene difluoride) (PVDF)

immunoblot membrane (Bio-Rad). After blocking, membranes were probed with either specific anti-HMGB1 antiserum or purified IgG from anti-HMGB1 antiserum for Western blot analysis. The levels of HMGB1 were determined by reference to standard curves generated with purified HMGB1, and the relative band intensity was quantified by ImageJ (National Institutes of Health).

Spleen Histopathology

Whole spleens were harvested and fixed using 4% (vol/vol) paraformaldehyde-PBS. The samples were then sent to AML Laboratories for processing, slicing and hematoxylin and eosin (H&E) staining. Photomicrographs were obtained on a Zeiss Axio Imager Z.1 with an Axiocam 506 color camera and further processed using Zen software to match exposure and color balance across specimens.

Statistics

Data are expressed as mean ± standard deviation (SD). Differences between means were determined by using a two-tailed *t* test. *p* values <0.05 were considered significant.

RESULTS

HMGB1 Is a Mediator of Anemia in Sepsis Survivors

To study the development of anemia of inflammation during sepsis, we induced severe sepsis in BALB/c mice by CLP, using an established protocol with survival rates of 50–70% (15). Complete blood counts, obtained for up to 28 d after CLP, revealed a significant decrease in hematocrit (Figure 1A), hemoglobin (Figure 1B) and red blood cells (Figure 1C) as early as 3 d after the onset of sepsis that persisted for at least 28 d in survivors, except for the number of red blood cells. The anemia was normocytic and normochromic for at least 7 d, thereafter becoming microcytic and hypochromic, as evidenced by progressive decreases in the mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) (Figures 1D, E). During the first week after CLP, the reticulocyte count was significantly decreased in CLP survivors compared with sham animals (Figure 1F). Circulating EPO levels increased from d 5–7 (not shown), followed by a transient increase in the reticulocyte count on d 15 (Figure 1F). These results indicate that a microcytic, hypochromic anemia persists for at least 28 d in murine severe sepsis survivors. The ferritin levels were elevated during the first 2 wks after CLP compared with the sham group (not shown), precluding a possible iron deficiency in the CLP model.

Prolonged experimental administration of recombinant TNF induces anemia and reduces red blood cell survival (14), and IL-6 negatively regulates erythropoiesis, in part by inducing hepcidin (13,22). However, TNF and IL-6 increase only transiently after sepsis onset in patients, returning to normal levels as anemia persists (23). CLP survivors had a rapid and transient increase in IL-6 and TNF, but circulating levels of those cytokines returned to levels similar to the sham group after d 5 (Figures 1G, H). Hepcidin, a master regulator of iron bioavailability, is reduced in response to acute blood loss, resulting in increased intestinal absorption of iron (7). During inflammation, IL-6 induces hepcidin, leading to hypoferrinemia and anemia (13). Circulating hepcidin was elevated only transiently 7 d after CLP (data not shown). In contrast to other inflammatory mediators, circulating HMGB1 increased after d 7 and remained elevated for at least 28 d (Figure 1I), a pattern consistent with previous observations that HMGB1 is a late-occurring mediator of sepsis. This result indicates a role for HMGB1 in sustaining the anemic phenotype in CLP survivors.

To study the mechanism of anemia in mice surviving sepsis, spleen and bone marrow were collected from animals 10 d after the onset of sepsis. We recently demonstrated the expansion of the leukocyte compartment in the spleen of CLP survivors (15). In agreement with these results, we observed that the spleens are macroscopically enlarged (Figure 2A). Analysis of spleen sections revealed disruption of the normal architecture, with expansion of the red pulp that is characteristic of stress erythropoiesis (24) (Figure 2B). After depletion of the $CD45^{pos}$ compartment (that is, lymphoid populations), splenocytes were analyzed by flow cytometry on the basis of the expression of the erythroid marker Ter119. $Ter119^{+}$ cells were then analyzed by CD44 expression and forward scatter as previously described (21). CD44, the hyaluronate receptor, presents an expression pattern that decreases over 30-fold during terminal erythroid differentiation (25). The different stages of terminal erythroid differentiation, from pro- to orthochromatic erythroblasts, were significantly expanded in the CLP survivors (Figures 2C, D). Bone marrow cell mass was reduced, both before and after depletion of the lymphoid compartment, but was not statistically significant (Figure 2E). Flow cytometry analyses revealed significant increases in the erythroid precursor population with accumulation of orthochromatic erythroblasts (Figures 2F, G). During erythropoiesis, there is progressive expansion and differentiation with one proerythroblast (P) producing two basophilic (B), four polychromatophilic (Po) and eight orthochromatic (O) erythroblasts (21). In the CLP animals, we observed that the progression toward orthochromatic erythroblasts in the bone marrow is altered, with the ratio becoming 1 (P), 1.8 (B), 5 (Po) and 11.6 (O). This accumulation of late-stage cells indicates that defective erythropoiesis contributes to the anemia phenotype and that there is an increased production of red cell precursors in both cellular compartments.

HMGB1 Induces Anemia and Stress Erythropoiesis in Mice

We then administered rHMGB1 to BALB/c mice for 21 consecutive days and observed the onset of anemia within 7 d, characterized by a significant increase in the reticulocyte count

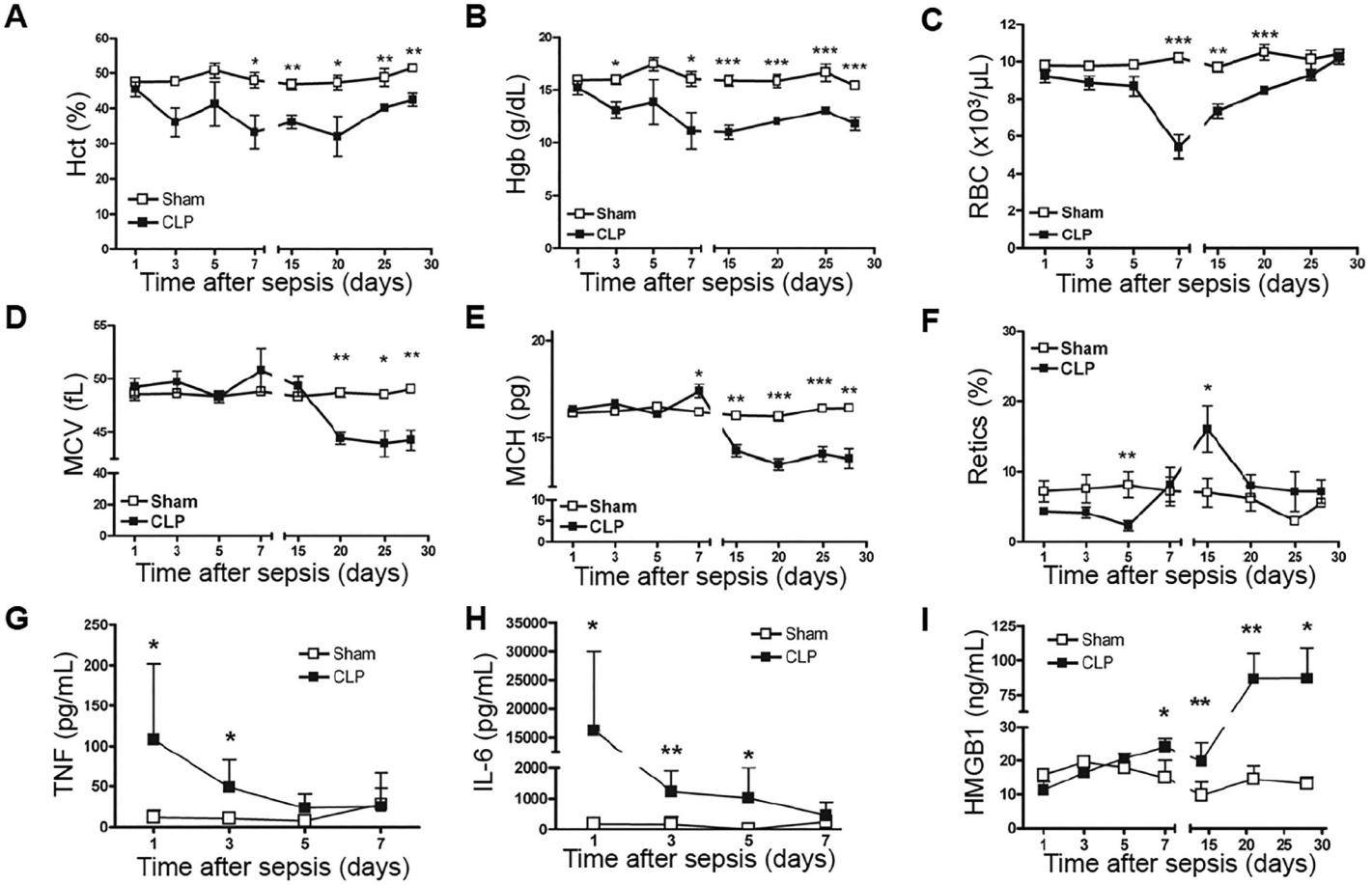

Figure 1. Sepsis survivors develop sustained anemia, as well as a delayed elevation in HMGB1. Mice were subject to CLP to induce severe sepsis, or sham surgery as control. Compete blood counts were performed at prespecified time points (A–F). TNF (G) and IL-6 (H) were measured by multiplex ELISA. HMGB1 was determined by semiquantitative immunoblotting (I). Data are expressed as mean ± SD (n = 4–5 mice/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus sham.

(Figure 3A). Total cell counts in the bone marrow did not differ significantly between groups ($1.1 \pm 0.3 \times 10^8$ cells versus $0.79 \pm 0.3 \times 10^8$cells, respectively; $p = 0.2$). We found, however, a significant depletion of the erythroid compartment in animals exposed to HMGB1 ($0.25 \pm 0.05 \times 10^8$ cells versus $0.1 \pm 0.02 \times 10^8$ cells, respectively; $p < 0.001$) (Figure 3B). We also observed an expansion of erythropoiesis in the bone marrow in response to HMGB1, with an accumulation of polychromatophilic and orthochromatic erythroblasts (Figures 3C, D) and a ratio of 1 (P), 1.5 (B), 3.9 (Po) and 9.1 (O). The degree of abnormality was clearly less in the rHMGB1-treated animals than in CLP [1 (P), 1.8 (B), 5 (Po) and 11.6 (O)]. Macroscopic examination of the spleens revealed that injecting HMGB1 induced splenomegaly (Figure 3E). Analysis of the spleen showed increased total cellularity ($1.9 \pm 0.05 \times 10^8$ cells in the vehicle group versus $3.8 \pm 1.1 \times 10^8$ cells in the HMGB1-treated group; $p = 0.035$) and specific expansion of the erythroid compartment ($0.86 \pm 0.2 \times 10^8$ versus $1.7 \pm 0.2 \times 10^8$, respectively; $p = 0.002$) (Figure 3F). Flow cytometry analyses revealed that exposure to HMGB1 led to increased erythropoiesis (Figure 3G), with significant increases in the four populations of precursors from pro- to orthrochromatic erythroblast (Figure 3H) when compared with the untreated group.

Administration of Anti-HMGB1 Monoclonal Antibodies Ameliorates Anemia in the CLP Model

To evaluate the role of HMGB1 in the anemia of sepsis, mice received neutralizing monoclonal anti-HMGB1 antibody (clone 2G7) or isotype control (IgG-2b) on d 9–11 after CLP. The rationale for treating the mice at this point was to evaluate the effect of neutralizing HMGB1 after anemia onset. Sepsis survivors receiving isotype control developed persistent anemia, whereas survivors receiving the anti-HMGB1 had similar levels of hemoglobin and hematocrit as the sham group (Figures 4A, B). Although hemoglobin and hematocrit normalized within days after the administration of anti-HMGB1, MCH

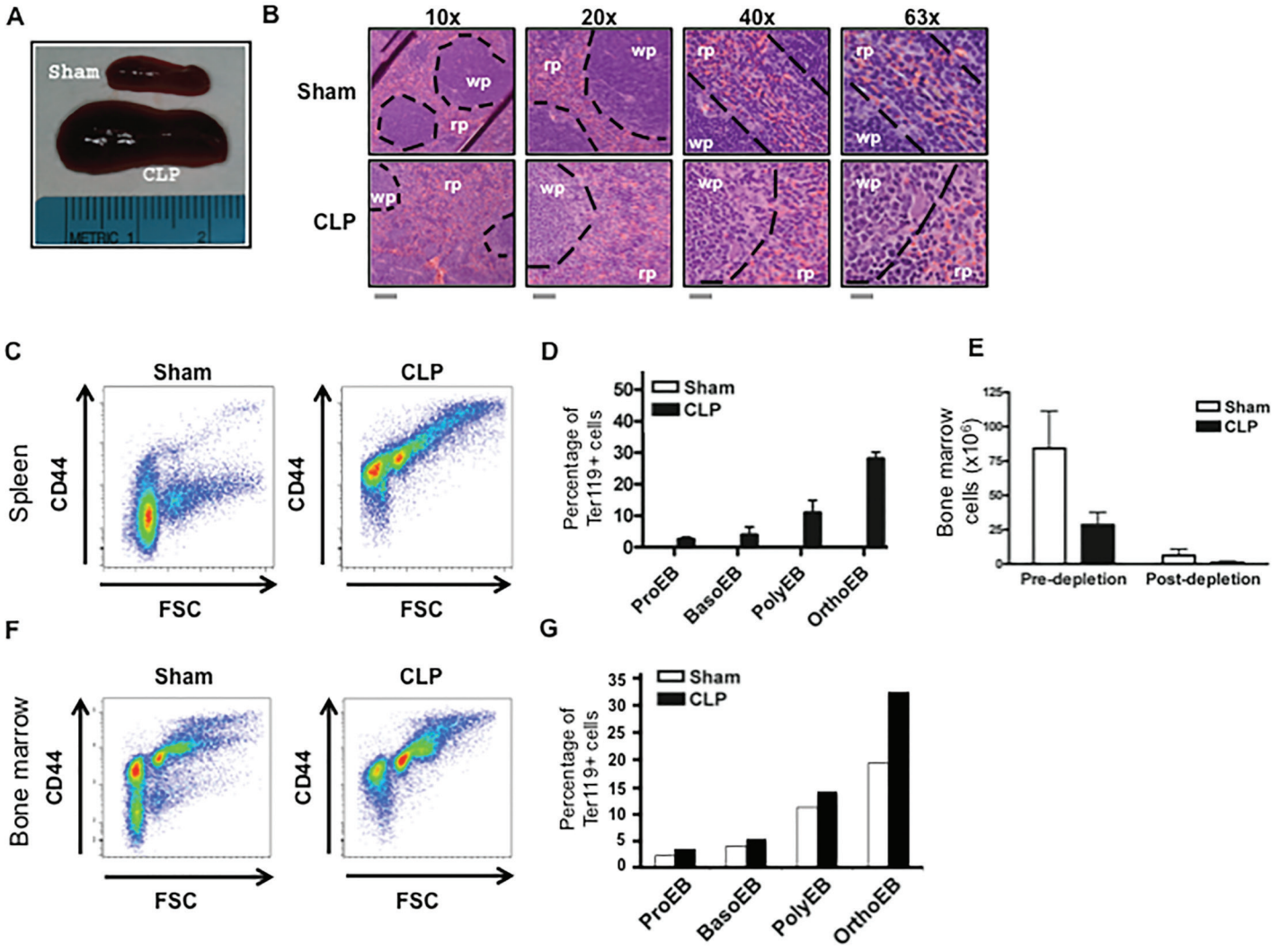

Figure 2. Sepsis survivors have defective terminal erythroid differentiation. Spleen and bone marrow were obtained from sepsis survivors 10 d after mice were subjected to CLP or sham surgery. CLP survivors developed splenomegaly (A) and an expansion of the red pulp (B), suggesting stress erythropoiesis. Dash borders denote the separation between the red pulp (rp) and the white pulp (wp). Numerical apertures for the different magnifications: 10× = 0.45; 20× = 0.80; 40× = 0.75; 63× oil = 1.4. Scale bar: 125 μm (10×), 62 μm (20×), 31 μm (40×) and 20 μm (63×). Analysis of the erythroid compartment by flow cytometry shows an expansion of immature erythroid cells in the spleen of CLP animals (C, D). Bone marrow total cellular content was not different between groups, either before or after depletion (E). Flow analysis shows an increase in orthochromatic erythroblasts (F, G). Cell counts of total and post-depletion bone marrow represents the number of cells from femur and tibia obtained from both hind limbs. Data shown are mean ± SD (n = 3–4 mice/group).

remained lower in CLP survivors than in the sham group 3 wks after CLP, independent of treatment (data not shown). MCV showed a trend to normalizing in the treated group (data not shown).

DISCUSSION

HMGB1 is a mediator in the immunepathogenesis of severe sepsis (18,20,26) and also induces renal (27) and cognitive dysfunctions (16). Our present study reveals a novel role for HMGB1 in the pathogenesis of anemia of inflammation in sepsis survivors.

Anemia can result from increased red cell destruction or defective red cell production. Although we did not measure red cell survival in our murine model of sepsis survivors, we did observe that erythropoiesis is defective in the bone marrow of the CLP animals. Indeed, during normal terminal erythroid differentiation, three mitoses lead to the production of eight orthochromatic erythroblasts from one proerythroblast. Therefore, a ratio of 1:2:4:8 is normally observed under steady-state conditions. In the CLP model, however, we observed a ratio of 1:1.8:5:11.6, suggesting that the defect occurs between basophilic

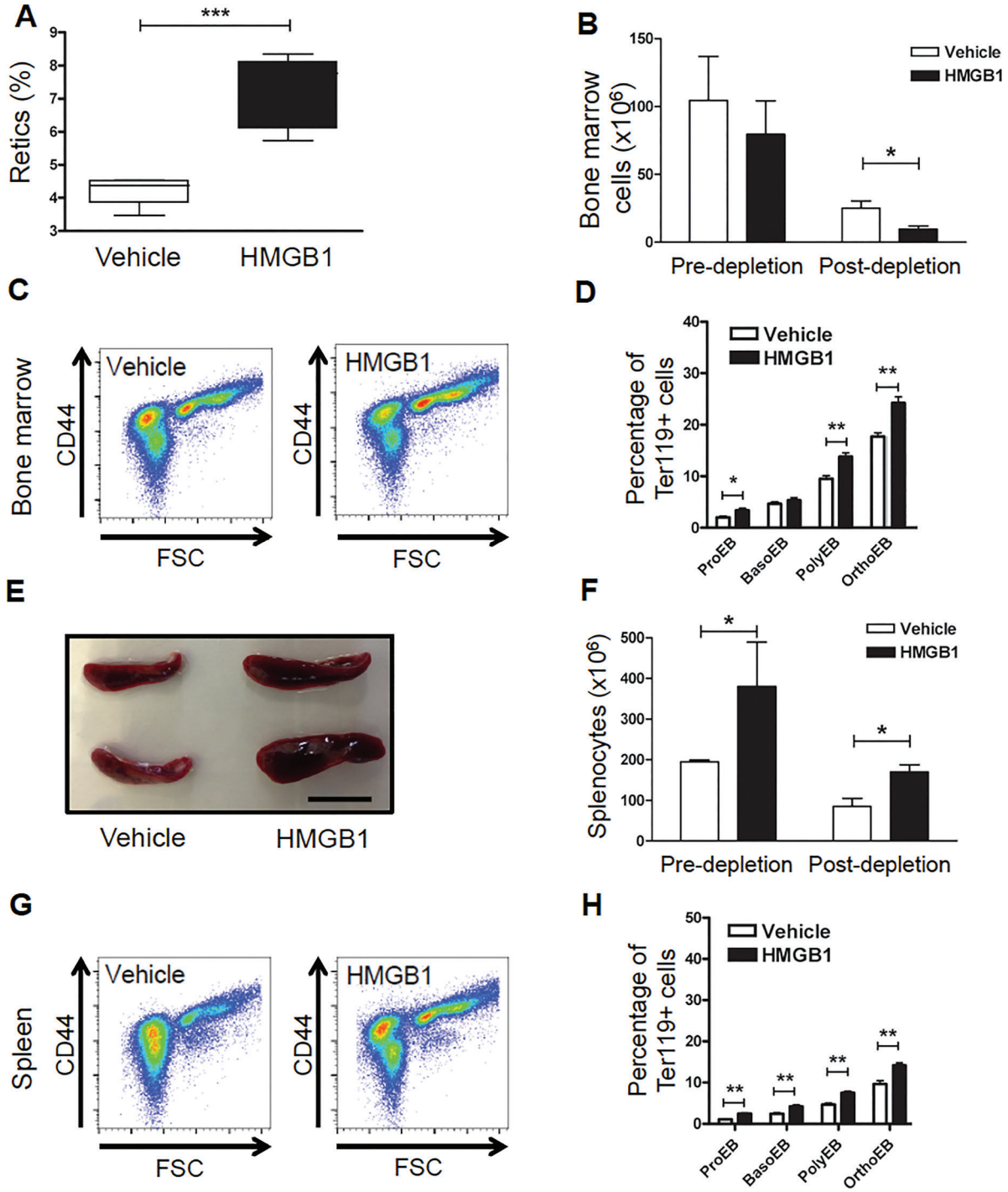

Figure 3. HMGB1 induces anemia and impairs late erythropoiesis. BALB/c mice were injected with rHMGB1 (500 μg/mouse/d, intraperitoneally) or vehicle for 7 d; blood, spleen and bone marrow were collected 24 h after the last injection. (A) HMGB1-induced anemia with an elevated reticulocyte count. (B) Bone marrow total cellular content was not different between groups, but the erythroid compartment was significantly reduced. (C, D) Analysis of the erythroid compartment by flow cytometry shows an expansion of terminal erythroid precursors. (E) Administration of HMGB1 also induced splenomegaly (scale bar: 1 cm). (F) Analysis of the splenic cell compartment showed that the total cell count, as well as the erythroid compartment (post–CD45 depletion), were significantly increased. (G, H) Flow cytometry analyses indicated an increase in the different erythroid populations. Data are expressed as box and whisker plots (A) or mean ± SD (B, D, F, H) (n = 3–4 mice/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus vehicle.

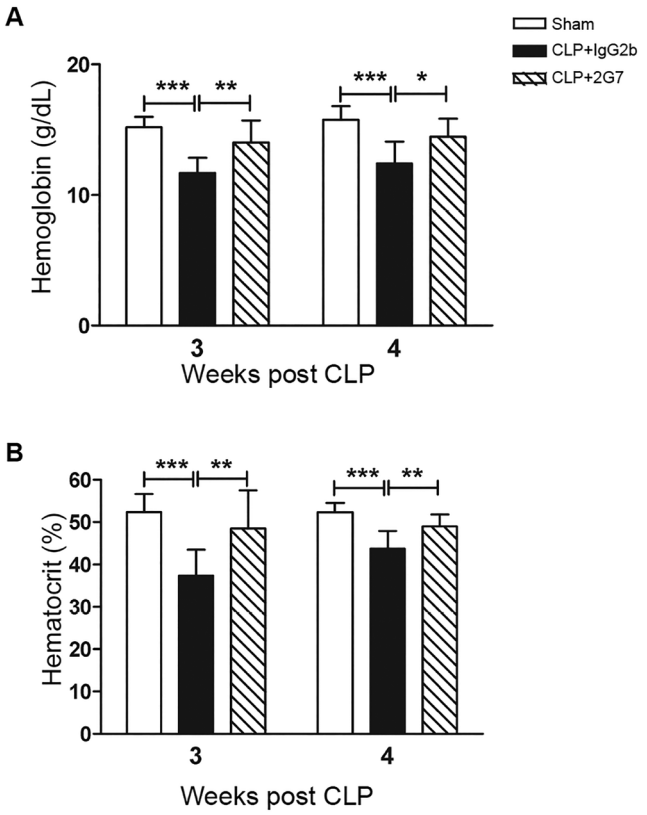

Figure 4. Neutralizing HMGB1 reverses anemia in sepsis survivors. CLP survivors were randomly assigned to receive 50 μg/mouse/day of either neutralizing anti-HMGB1 mAb (2G7) or isotype control on d 9–11 after CLP. Whole blood was collected on d 21 or 28 after CLP (4 and 10 d after the last dose of 2G7). Administration of 2G7 rescued septic mice from anemia, as determined by evaluation of the hemoglobin (A) and hematocrit (B) parameters. Data are mean ± SD (n = 4–5 mice/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and orthochromatic stages, with increased numbers of mitotic events. Data collected 3 wks after CLP revealed that hematologic recovery is occurring because red blood cell numbers return to normal levels. This result is consistent with increased production, although resulting in smaller red cells containing less hemoglobin. This step produces a microcytic and hypochromic phenotype that persists in sepsis survivors.

Inflammatory cytokines have been implicated in the anemia of inflammation. Prolonged administration of recombinant TNF induces anemia and reduces red blood cell survival (14,28). IL-6 negatively regulates erythropoiesis, in part by inducing hepcidin (13,22,29). In the CLP model, IL-6 peaked 24 h after CLP, with plasma concentrations decreasing rapidly before manifestation of anemia, suggesting a role for IL-6 only in the early stages preceding the onset of anemia. Hepcidin is another important mediator in anemia of inflammation and, during systemic inflammation, hepcidin production leads to a reduction in iron availability for erythropoiesis (30). In the CLP model, we found that the hepcidin levels were only transiently increased, going back to normal within 7 d after surgery. Moreover, the administration of rHMGB1 failed to induce a significant increase in circulating hepcidin, together suggesting that hepcidin does not play a significant role in the prolonged phase of anemia after sepsis.

Plasma levels of HMGB1 were not significantly increased for the first 7 d after CLP, but they remained persistently elevated thereafter, suggesting a role for HMGB1 in the chronic phase. To evaluate this possibility, we administered recombinant HMGB1 for 21 d and observed an increase in the reticulocyte count as well as splenomegaly and stress erythropoiesis. We also observed a reduction in total hemoglobin as well as MCH, a similar pattern to the one observed in CLP survivors, where hypochromic anemia developed before microcytosis. This result suggests that HMGB1 may induce anemia by interfering with hemoglobin homeostasis. In the bone marrow compartment of animals receiving HMGB1, we observed increased populations of all terminal erythroid stages, and the ratios from pro- to orthochromatic were closer to normal [1 (P), 1.5 (B) 3.9 (Po), 9.1 (O)], indicating that rHMGB1 induces an increase in the number of mitoses.

Monoclonal anti-HMGB1 antibodies have been studied in animal models of sepsis (20), endotoxin (31), ischemia reperfusion (32), chronic infectious arthritis (33), pain and transplantation (34). Here, the administration of a monoclonal anti-HMGB1 antibody (2G7) conferred significant protection against anemia, indicating that HMGB1 is necessary for pathogenesis of anemia in sepsis survivors.

CONCLUSION

Our results suggest a novel role for HMGB1 in the pathogenesis of anemia of inflammation in severe sepsis survivors and that HMGB1 may be a therapeutic target in the treatment of anemia of inflammation. Together, these results define a new model of anemia similar to the clinical scenario (4), which can be targeted with a monoclonal antibody toward therapeutic advantage in sepsis survivors.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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