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Role of Macrophages in Mobilization of Hematopoietic Progenitor Cells from Bone Marrow after Hemorrhagic Shock

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Abstract

The release of hematopoietic progenitor cells (HPC) from bone marrow (BM) is under tight homeostatic control. Under stress conditions, HPC migrate from BM and egress into circulation to participate in immune response, wound repair, or tissue regeneration. Hemorrhagic shock with resuscitation (HS/R), resulting from severe trauma and major surgery, promotes HPC mobilization from BM, which in turn affects post-HS immune responses. In this study, we investigated the mechanism of HS/R regulation of HPC mobilization from BM. Using a mouse HS/R model we demonstrate that the endogenous alarmin molecule high-mobility group box 1 (HMGB1) mediates HS/R-induced G-CSF secretion from macrophages (Mφ) in a RAGE signaling-dependent manner. Secreted G-CSF, in turn, induces HPC egress from BM. We also show that activation of β-adrenergic receptors on Mφ by catecholamine mediates the HS/R-induced release of HMGB1. These data indicate that HS/R, a global ischemia/reperfusion stimulus, regulates HPC mobilization through a series of interacting pathways that include neuro-endocrine and innate immune systems, in which Mφ play a central role.

Keywords

G-CSF; HMGB1; RAGE; innate immune; β-adrenergic receptor

INTRODUCTION

Hematopoietic stem and progenitor cells (HPC) reside in the bone marrow (BM) and produce large amounts of mature blood cells daily to replenish ones that are destroyed. Under physiological homeostatic conditions, there are small numbers of circulating HPC in the blood, which traffic between the BM and peripheral organs. The release of HPC from BM is normally regulated by circadian oscillations (1). However, under stress conditions,

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robust HPC migration from BM can occur with egress into the circulation, and it has been proposed that BM HPC migration into sites of injury is a mechanism by which damaged tissues are repaired (2, 3). Studies have shown that trauma and hemorrhagic shock (HS) cause prolonged and exaggerated mobilization of HPC to the peripheral blood, and these cells can be seeded at injury sites, such as injured lungs (4). These findings suggest that HPC from BM may play an important role in the functional recovery of peripheral organs in response to HS. Alternatively, HS-induced HPC mobilization has been postulated to be a cause of post-HS BM suppression, which clinically manifests as a persistent anemia and altered wound healing (5). Taken together, HPC mobilization from BM following trauma and HS is an important mechanism that modulates patient prognosis although the mechanism underlying HS-induced mobilization of HPC from BM is not fully understood.

High-mobility group box 1 (HMGB1) is a DNA-binding nuclear protein that is passively released from necrotic cells, or actively secreted by innate immune cells in response to microbial products or other inflammatory stimuli. Increasing evidence demonstrates that HMGB1 is the prototypic damage-associated molecular pattern (DAMP) molecule and acts as potent mediator of injury in animal models of sepsis, ischemia, trauma, and HS (6). Extracellular HMGB1 interacts with several receptors, including Toll-like receptors (TLR) and receptor for advanced glycation end products (RAGE) (7). Recent evidence has shown that HMGB1 can also act as a cytokine to induce immune cell proliferation and migration, for example, HMGB1 plays a chemoattractant role in inducing the recruitment of immature dendritic cells, neutrophils, and mesoangioblasts (8, 9). HMGB1 can also stimulate the migration of endothelial progenitor cells after ischemic injury in a RAGE-dependent manner (10).

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that selectively stimulates the proliferation of neutrophilic precursor cells and augments their activation and release from BM stores (11). In addition to the mobilization of neutrophil, G-CSF is known to effectively promote BM-derived stem cell mobilization into the peripheral circulation (12) and HPC egress from BM in normal and traumatic conditions, and therefore increasing the number of colony forming cells in peripheral blood (13, 14).

In the current study, we investigated the mechanism underlying HS-induced HPC mobilization from BM. We demonstrate that HMGB1 mediates HS-induced G-CSF secretion from macrophages (M ϕ) in a RAGE signaling-dependent manner. Secreted G-CSF, in turn, induces HPC egress from BM. We also show that activation of β -adrenergic receptors on M ϕ by catecholamine mediates HS-induced release of HMGB1. This study sheds light on the central role of M ϕ in mediating HPC mobilization following HS.

MATERIAL AND METHODS

Mouse Strain and Reagents

Male wild-type (WT) C57BL/6 mice, TLR4 knockout (TLR4^{-/-}) mice and receptor for advanced glycation endproducts (RAGE) knockout (RAGE^{-/-}) mice, both have a C57BL/6 background, were bred in Dr. Timothy Billiar's lab at the University of Pittsburgh. All experimental protocols involving animals were approved by Institutional Animal Care and Use Committees of Veterans Affairs Pittsburgh Healthcare System and University of Pittsburgh. Mice were 8–12 wk of age at the time of experiments and were maintained on standard rodent chow and water ad libitum. The mice were not fasted before experiments.

Polyclonal neutralizing antibody against HMGB1, prepared as described previously, was kindly provided by Dr. K. J. Tracey (Feinstein Institute for Medical Research, Manhasset, NY). Polyclonal anti-HMGB1 antibody for Western blotting was purchased from Cell

Signaling Technologies (Danvers, MA). ELISA for mouse G-CSF was from R&D Systems (Minneapolis, MN). Nonspecific rabbit IgG (Cat. no. I5006), diphenyleneiodonium, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), except where noted.

Mouse Model of Hemorrhagic Shock and Resuscitation (HS/R)

The HS/R mouse model was performed as previously described (15). Briefly, animals were anesthetized with 50 mg/kg of ketamine and 5 mg/kg of xylazine via intraperitoneal (i.p.) administration. Femoral arteries were cannulated for monitoring of mean arterial pressure, blood withdrawal and resuscitation. HS was initiated by blood withdrawal and reduction of the mean arterial pressure to 30 mm Hg within 20 min. After a hypotensive period of 2 h, animals were resuscitated with three times shed-blood volume of Ringer's lactate solution over a period of 60 min. Sham animals underwent the same surgical procedure without HS/R. In some experiments, mice were injected i.p. with neutralizing antibodies against HMGB1 (600 µg per mouse) or G-CSF (100 µg per mouse; Abcam, Cambridge, MA) 10 min before hemorrhage. At 2 or 4 h after resuscitation, peripheral blood was collected from the right ventricle. To block catecholamine signaling, mice were injected i.p. with propranolol (2 mg/kg; Sigma-Aldrich, St. Louis, MO), a β -adrenergic receptor antagonist, 10 min before HS/R (PBS for control group).

Colony Forming Unit in Culture (CFU-C) Assay

HPC levels in the blood after HS/R were evaluated using CFU-C assay. Blood was collected from the right ventricle after the mice were euthanized. The mononuclear cells were isolated from 0.5 ml peripheral blood by Ficoll™ density gradient separation by following the manufacturer's instructions (Ficoll-Paque Premium 1.084, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After washing with PBS, cells were mixed with 1.0 ml GF M3434 methylcellulose medium that contained fetal bovine serum and recombinant mouse stem cell factor (Stem Cell Technologies, Wancue, Canada), then seeded into two wells of a 24-well plate. Cultures were incubated at 37°C in 5% CO₂. The total numbers of CFU-C colonies were counted at day 7 by microscope. Pellets from the Ficoll™ density gradient separation were also washed with PBS and cultured for CFU-C in order to test the recovery of the colony forming cells. No CFU-C was observed from the pellet cells.

In Vivo M ϕ Depletion and Repletion

M ϕ depletion was induced using clodronate encapsulated liposome (16). At 24 h before HS, liposome containing clodronate (100 µl per mouse, Encapsula Nano Sciences, Nashville, TN) or liposome only (control) was injected i.p. into WT and RAGE^{-/-} mice. M ϕ (1×10^7 cells/mouse) isolated from BM of WT or RAGE^{-/-} mice were then given back to clodronate liposome-treated mice by tail vein injection during resuscitation phase following HS in order to achieve M ϕ repletion.

M ϕ Isolation and Treatment

Immunomagnetic separation (BD Biosciences Pharmingen, San Diego, CA) was used to isolate BM M ϕ . Magnetic nanoparticle-conjugated antibodies (anti-mouse Gr-1, anti-CD4, anti-CD8, and anti-CD45R/B220 antibodies; BD Biosciences Pharmingen, San Diego, CA) were used to label and remove PMN and lymphocytes. The resulting cells consisted of >98% M ϕ , and the cell viability was >95% as assessed by Wright-Giemsa staining and trypan blue exclusion, respectively. Isolated M ϕ were incubated in DMEM containing 10% FCS at a concentration of 1×10^6 cell/ml of medium. The cultures were treated with HMGB1 (0.5 µg/ml) for 30 min. At various time points after stimulation with HMGB1, cell culture medium was harvested for G-CSF measurement by ELISA.

Western blot analysis

Serum was harvested from collected blood after centrifugation and diluted with 3 volumes of PBS. Aliquots of diluted serum from each sample (~30 μ l) were loaded for SDS-PAGE and subjected to Western blot using rabbit polyclonal antibody against HMGB1 (Cell Signaling).

ELISA

G-CSF levels in cell culture medium and serum were evaluated by ELISA (R&D Systems) according to the manufacturer's instructions.

Data Presentation and Statistics Analysis

The data are presented as mean $\pm$ SEM of the number of determinations indicated in each experiment. Data were analyzed by one-way ANOVA. When individual studies are demonstrated, these are representative of at least three independent studies.

RESULTS

HPC mobilization from BM following HS/R

Colony formation in culture is one of the characteristics of HPC (17). To study the changes in HPC mobilization following HS/R, we used an established murine HS/R model and examined changes of HPC number in the blood after HS/R by counting the colony forming unit in culture (CFU-C). As shown in Fig. 1A, there were small numbers of CFU-C in the circulation of sham or naïve mice. However, HS/R resulted in a nearly 4-fold increase in peripheral HPC CFU-C by 4 h after HS/R ($p < 0.01$). CFU-C remained statistically increased compared to sham-operated groups for at least 8 h (Fig. 1A). In addition, HPC colonies in the blood of HS/R mice were significantly larger than those in sham control animals (Fig. 1B). Based on the results shown in Fig. 1A, blood samples for CFU-C assay were collected at 4 h after HS/R in subsequent experiments.

Catecholamine and HMGB1 mediate HS/R induced HPC mobilization

It is well established that major trauma induces a rapid and sustained release of catecholamines (18). To test whether HS/R-induced rapid HPC egress from BM is mediated through a catecholamine pathway, mice were injected i.p. with nonselective β -adrenergic blocker, propranolol (2 mg/kg), 10 min before HS/R, and circulating HPC growth at 4 h after HS/R was then examined. As shown in Fig 2A, propranolol prevented the HS/R-induced rapid increase in CFU-C. To further confirm the role of catecholamines in HPC mobilization from BM, we injected epinephrine (2 mg/kg by weight) directly into mice that were not subjected to HS/R. Epinephrine alone induced a significant increase in circulating HPC, similar to levels seen after HS/R (Fig. 2A). These results indicate that the catecholamine pathway contributes to HPC egress after HS/R.

Studies have shown that HMGB1 release can also be regulated by the autonomic nervous system and catecholamine release (19), and we have previously shown that HMGB1 released through stimulation by catecholamines is responsible for PMN mobilization from BM after HS/R (15). We next addressed whether HMGB1 is also involved in catecholamine-induced HPC mobilization after HS/R. Neutralizing antibody to HMGB1 (600 μ g/mouse) was administered to mice 10 min before HS/R or epinephrine administration. As shown in Fig. 2A, treatment with anti-HMGB1 antibody significantly prevented HS/R-induced and epinephrine-induced increases in circulating HPC. These results suggest that both HMGB1 and epinephrine are important mediators of HS/R-induced HPC mobilization.

We further investigated the release of HMGB1 into the blood after HS/R and also the effect of epinephrine on this release after HS/R in WT mice. As shown in Figure 2B, HS/R caused an increase of serum HMGB1 level at as early as 2 h after HS/R, and this was prevented by propranolol treatment. Similarly, treatment of sham mice with epinephrine i.p. resulted in an increase in circulating HMGB1 to similar levels. These results are consistent with our previous observation that catecholamine is a major mediator that mediates HS/R induced HMGB1 release (15).

In order to define a role of M ϕ in releasing HMGB1 in response to catecholamine, BM M ϕ were treated with epinephrine (2 μ g/ml) and/or propranolol (2 μ g/ml) for 2 h, then HMGB1 in the supernatant was assessed by immunoblotting. Epinephrine induced HMGB1 release from BM M ϕ and this was blocked by propranolol (Fig. 2C). Taken together these results indicate that both catecholamine and HMGB1 are required for HS/R-induced HPC mobilization from BM to peripheral blood, and catecholamine serves as a key mediator for HS/R-induced HMGB1 secretion from BM M ϕ .

HMGB1 mainly acts through RAGE rather than TLR4 to induce HPC mobilization

HMGB1 has been shown to bind to at least five membrane receptors, including RAGE (20), TLR2, TLR4 (21), triggering receptor expressed on myeloid cells-1 and CD24 (22). Studies have shown that in HS and ischemia/reperfusion injury, TLR4 and RAGE are two major receptors mediating HMGB1-induced inflammation (15, 23). To address whether TLR4 and RAGE are required for HMGB1-mediated HPC mobilization, TLR4^{-/-} mice and RAGE^{-/-} mice were subjected to HS/R followed by measurement of peripheral HPC numbers. TLR4 deficiency did not block HS/R-induced HPC mobilization, whereas HS/R-induced circulating HPC numbers were significantly reduced in RAGE-deficient mice (Fig. 3A). These data therefore suggest that HMGB1 signaling via RAGE and not TLR4 regulates HPC mobilization from BM after HS/R.

G-CSF is a known important mediator promoting HPC mobilization from BM (14). We therefore wanted to determine whether G-CSF mediates HMGB1/RAGE-induced HPC mobilization. To do this we first measured serum G-CSF levels at 2 h after HS/R in WT and RAGE^{-/-} mice. As shown in Fig. 3B, RAGE deficiency significantly decreased serum G-CSF levels compared to levels in WT HS/R mice. Similarly, WT mice treated with neutralizing antibody to HMGB1 also showed significantly decreased serum G-CSF levels (Fig. 3B). These findings indicate that both HMGB1 and RAGE are required for HS/R-induced G-CSF release.

RAGE signaling in macrophage is required for G-CSF release and HPC mobilization

We next wanted to address the role of G-CSF in HS/R-induced HPC mobilization. To do this we administered neutralizing antibody against G-CSF to WT and RAGE^{-/-} mice 10 min before HS/R and then measured HPC mobilization into the circulation as before. Fig. 4A shows that treatment of WT mice with anti-G-CSF antibody prevented the HS/R-induced increase in peripheral CFU-C.

Recent studies have shown that M ϕ in the BM are sufficient to elicit HPC mobilization (24). We therefore wanted to understand the role of RAGE signaling on M ϕ during HS/R in HPC mobilization. We first prepared chimeric mice, in which M ϕ in WT mice were replaced with RAGE^{-/-} M ϕ , and M ϕ in RAGE^{-/-} mice were replaced with WT M ϕ . We did this using M ϕ depletion and repletion techniques as described in Methods, and used WT/WT and RAGE^{-/-}/RAGE^{-/-} depletions and repletions as controls. Global RAGE^{-/-} had significantly decreased HS/R-induced HPC egress into blood (Fig. 4A) and significantly decreased levels of serum G-CSF (Fig. 4B) compared with WT after HS/R. Interestingly,

replenishing RAGE^{-/-} mice with WT Mφ restored the HS/R-induced increase in peripheral HPC and serum G-CSF (Fig. 4A and 4B). However replenishing WT mice with RAGE^{-/-} Mφ prevented the increases in HS/R-induced HPC egress and serum G-CSF (Fig. 4A and 4B). These results indicate that RAGE signaling on Mφ is an important determinant of HS/R regulation of HPC mobilization.

The role of Mφ RAGE signaling in mediating G-CSF release was further recapitulated in *in vitro* experiments. HMGB1 caused an increase of G-CSF release in WT Mφ starting at 2 h after HMGB1 stimulation and reached a 35-fold increase by 5 h. However, HMGB1 failed to induce a significant increase in G-CSF release in RAGE^{-/-} Mφ (Fig. 4C). These *in vitro* experiments further suggest a role for HMGB1 signaling via RAGE on Mφ to promote G-CSF release.

DISCUSSION

HPC mobilization from BM following trauma and HS is a common phenomenon and plays an important role in regulating immune responses and tissue repair (2, 17). In this study we sought to determine how the global ischemia/reperfusion injury initiated during resuscitated HS regulates HPC mobilization from BM. We show that HMGB1 mediates HS/R-induced G-CSF secretion from Mφ in a RAGE signaling-dependent manner. Secreted G-CSF, in turn, induces HPC egress from BM after HS/R. We also show that activation of β-adrenergic receptor of Mφ by catecholamine mediates the HS/R-induced release of HMGB1. The data support a central role of Mφ in HS-induced HPC mobilization.

Trauma and HS result in substantial and sustained release of catecholamines that lead to a wide range of hemodynamic, metabolic, behavioral, and immune changes (18). It has been reported that norepinephrine signaling via the sympathetic nervous system regulates HPC egress from BM through a mechanism that involves G-CSF-induced suppression of bone-lining osteoblasts and downregulation of the chemokine SDF-1 (19). A recent study demonstrated that administration of propranolol significantly reduced post-shock BM suppression (25). These data suggested an important role of catecholamine in promoting HPC migration. In the current study, we found that in HSβ-adrenergic receptor activation by catecholamine promotes HPC mobilization by inducing

HMGB1 release and subsequent G-CSF secretion. We show here thatβ-adrenergic receptor antagonist propranolol significantly decreased HS-induced HMGB1 levels and HPC in circulation. In addition, administration of epinephrine directly induced HMGB1 release and increased HPC in circulation. These findings indicate that catecholamines are responsible for HS-induced HMGB1 release. Since TLR4 knockout did not prevent HS/R-induced increase in peripheral CFU-C, LPS, which may be derived from a post-HS infection or translocation from the gut, should not a key mediator for HS/R-induced HMGB1 release. HMGB1 is becoming recognized as the prototypic alarmin (26). Increasing evidence now indicates that HMGB1 can act as an early inflammatory mediator in ischemia (27, 28), hemorrhagic shock (29, 30), and non-infectious hepatitis (31). Recently, chemoattractant roles for HMGB1 in inducing migration of immature dendritic cell (26), smooth muscle cells (32), and mesoangioblasts (9) have also been reported. HMGB1 acts through RAGE to induce the migration of these cells directly. However, no role had been ascribed to HMGB1 in the regulation of HPC mobilization. In this study, we identified an important role for HMGB1 in inducing HPC egress from BM following HS/R. This effect of HMGB1 is mediated via RAGE signaling to induce G-CSF release from Mφ and subsequent HPC egress into the circulation. We observed that HS/R leads to increased HMGB1 levels in serum, which is consistent with previous studies that demonstrated elevated HMGB1 in human HS/R (6), mouse ischemia/reperfusion injury (27), and in acute lung injury (33). We

further found that neutralizing antibody to HMGB1 blocks HS/R-induced release of G-CSF and blocks the increase in circulating HPC in a RAGE-dependent, but TLR4-independent, manner. These data support an important but indirect role of HMGB1 in inducing HPC migration.

G-CSF secretion can be induced by a wide variety of exogenous agents, including LPS, lipoteichoic acid, and phytohaemagglutinin (34). Mφ from different organs have been shown to be a major source of G-CSF in the presence of bacteria and LPS (35). In the current study we also observed a central role of Mφ responding to upstream signaling to release HMGB1 and G-CSF, to promote subsequent HPC mobilization. Using chimeric mice, in which WT Mφ were replaced by those from RAGE^{-/-} mice, we show that deficiency of RAGE in Mφ, rather than other cell populations, significantly prevented HS/R-induced HPC egress from BM, and this effect resulted decreased release of G-CSF in response to HMGB1. We have previously reported that HMGB1 acting through TLR4 mediates HS/R-induced IL-23 secretion and subsequent IL-17 release, which, in turn, promoted neutrophil mobilization from bone marrow through G-CSF (15). We observed that TLR4-mutant mice decreased serum IL-23 and IL-17 following HS/R, and caused a partial decrease in serum G-CSF. The observation suggested the existence of other pathways by which HS induces G-CSF release. The current study, however, supports an alternative pathway, in which HMGB1 through Mφ RAGE bypasses the TLR4-IL-23-IL-17 pathway and induces G-CSF release and subsequent HPC mobilization. HS/R-induced myeloid cell mobilization from bone marrow is a complicated process and the mechanisms for neutrophil and HPC egress may not be the same. It is plausible that some unknown factors are involved in the different mechanisms that underlie TLR4- and RAGE-dependent regulation of PMN and HPC mobilization, respectively.

In summary, the present study demonstrates a novel mechanism connecting the insult of HS/R and HPC mobilization from BM. Catecholamine mediates the HS/R-induced release of HMGB1, which in turn, mediates HS/R-induced G-CSF secretion from macrophages in a RAGE signaling-dependent manner. Secreted G-CSF then induces HPC egress from BM. These data indicate that HS/R, a global ischemia/reperfusion stimulus, regulates HPC mobilization through a series of interacting pathways that include neuro-endocrine and innate immune systems.

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Figure 1. HS/R induces robust mobilization of HPC

A. HS/R increases CFU-C numbers in the blood. WT (C57BL/6) mice were subjected to HS/R (HS) or sham operation (SM). Blood samples were collected 4 and 8 h after HS/R and CFU-C assay was performed. The graph shows the mean $\pm$ SEM (n=6; *p < 0.01 compared with the groups labeled with no *asterisk*). **B.** Morphology of colonies. Colonies in HS group (4 h after HS) are much larger than that in sham mice (4 h after sham). Images are representative of six independent experiments with similar results.

Figure 2. Catecholamine-induced HMGB1 release mediates HPC mobilization

A. Catecholamine blockade and neutralizing antibody against HMGB1 prevent HS-induced HPC mobilization. Mice were injected i.p. with neutralizing antibody against HMGB1 (Ab; 600 μ g per mouse) or propranolol (Prop; 2 mg/kg) 10min before HS/R or injection of epinephrine (Ep; 2 mg/kg i.p.). Peripheral blood samples were collected 4 h after HS/R (HS) or injection of epinephrine from the right ventricle after the mice were euthanized for CFU-C assay. The graph shows the mean $\pm$ SEM (n=6; *p < 0.01 compared with the groups labeled with no *asterisk*). **B.** β -adrenergic receptor antagonist prevents HS/R-induced increase in serum HMGB1. Propranolol (Prop; 2 mg/kg, i.p.) or epinephrine (Ep; 2 mg/kg, i.p.) were given to WT mice 10 min before HS/R (HS) or sham operation (SM), respectively, and serum was collected after 2 h and HMGB1 detected by Western blotting. The blots are representative of 5 independent studies. The graph depicts the mean $\pm$ SEM of the fold changes in HMGB1 serum levels, as compared to sham/saline (SM/SAL) group. n= 5 mice. **C.** Epinephrine activation of β -adrenergic receptor induces HMGB1 release from M ϕ . M ϕ collected from WT mice BM were stimulated with epinephrine (Ep; 2 μ g/ml) and/or propranolol (Prop; 2 μ g/ml) for 2 h, HMGB1 in the supernatant was analyzed using Western blotting. Cells with no treatment are control. The blots shown are representative of three independent experiments with similar results. The graph depicts the mean $\pm$ SEM of the fold changes in HMGB1 in the supernatants as compared to the group with no treatment.

Figure 3. RAGE mediates HS/R-induced HPC mobilization

A. HMGB1 mainly acts through RAGE rather than TLR4 to induce HPC mobilization. WT (C57BL/6) mice, TLR4^{-/-} mice and RAGE^{-/-} mice were subjected to HS/R (HS) or sham operation (SM). Blood samples were collected at 4 h after HS/R and CFU-C assay was performed. The graph shows the mean $\pm$ SEM (n=4; *p < 0.01 compared with the groups labeled with no *asterisk*). **B.** Neutralizing antibody against HMGB1 or RAGE^{-/-} mice attenuate HS/R-induced release of G-CSF in circulation. WT and RAGE^{-/-} mice were injected i.p. with neutralizing antibody against HMGB1 (Ab; 600 μ g per mouse) or non-specific IgG (IgG; 100 μ g per mouse) 10min before HS/R. Peripheral blood samples were collected 2 h after HS/R (HS) or sham (SM) and serum G-CSF levels were determined by ELISA. Data represent mean $\pm$ SEM (n = 5 mice; *p < 0.01 compared with the groups labeled with no *asterisk*).

Figure 4. RAGE on Mφ mediates HMGB1-induced G-CSF release and HPC mobilization
A. Changes in HPC mobilization in chimeric mice. Mφ in WT and RAGE^{-/-} mice were depleted using chlodronate liposome 2 days prior to HS/R, and the mice were replenished with BM Mφ isolated from WT or RAGE^{-/-} mice and injected via tail vein (1×10^7 cells per mouse) 15 min prior to HS/R. In some groups mice were also given with neutralizing antibody against G-CSF (Ab; 100 μg/mouse) 10 min before HS/R. Blood CFU-C was measured at 4 h after HS/R (HS) or sham operation (SM). Mice received empty liposome injection were used as a control. Data represent mean $\pm$ SEM (n = 4; * p < 0.01 as compared to the groups with no asterisk). **B.** Changes in serum G-CSF level in chimeric mice. Mφ in WT and RAGE^{-/-} mice were depleted and repleted as described in “A”. Serum G-CSF level was measured by ELISA. Mice received empty liposome injection were used as a control. Data represent mean $\pm$ SEM (n = 4 mice; * p < 0.01 compared with the groups with no asterisk). **C.** *In vitro* Mφ release of G-CSF in response to HMGB1 stimulation. BM Mφ were isolated from either WT or RAGE^{-/-} mice and stimulated with HMGB1 (50 μg/ml) for up to 5h. G-CSF in the cell culture medium was measured by ELISA. Data represent mean $\pm$ SEM. (n = 5 for each time point; *p < 0.01 compared to WT group at that time point).