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Identification of Hemopexin as an Anti-Inflammatory Factor That Inhibits Synergy of Hemoglobin with HMGB1 in Sterile and Infectious Inflammation

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Hemoglobin is released from lysed RBCs in numerous clinical settings. High mobility group box 1 (HMGB1) is a nuclear and cytosolic DNA-binding protein released from injured cells that has been shown to play an important role in inducing inflammation. Because both of these endogenous molecules are frequently present in sites of necrosis and inflammation, we studied their interaction on the activation of macrophages. We report in this article that hemoglobin and HMGB1 synergize to activate mouse macrophages to release significantly increased proinflammatory cytokines. Addition of microbial ligands that activate through TLR2 or TLR4 resulted in further significant increases, in a “three-way” synergy between endogenous and microbial ligands. The synergy was strongly suppressed by hemopexin (Hx), an endogenous heme-binding plasma protein. The findings suggest that hemoglobin may play an important role in sterile and infectious inflammation, and that endogenous Hx can modulate this response. Administration of Hx may be beneficial in clinical settings characterized by elevated extracellular hemoglobin and HMGB1. The Journal of Immunology, 2012, 189: 2017–2022.

Materials and Methods

Materials

The following TLR agonists were purchased: smooth LPS from E. coli O55: B5 (List Biologicals) and Pam3Cys (EMC Microcollections). All these TLR agonists were dissolved in pyrogen-free H2O and saved as aliquots at

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; DPI, diphenyldielenidoiodonium; HMGB1, high mobility group box 1; Hx, hemopexin; KO, knockout; NAC, N-acetyl-i-cysteine; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; Syk, spleen tyrosine kinase; TRIF, TIR1/IL-1R domain-containing adapter-inducing IFN-β.

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−80°C. Diphenylethionium (DPI), N-acetyl-L-cysteine (NAC), allo-
arginine, l-NAME, and picatennol were obtained from Sigma. C57BL/6, TL2 knockout (KO), C3H/HeN, and C3H/HeJ mice were obtained from Charle
River Labs. MyD88 KO mice were a kind gift from Dr. Joseph El-Khoury (Department of Medicine, Massachusetts General Hospital, Boston, MA). Toll/IL-1R domain-containing adapter-inducing IFN-β (TRIF) KO mice were obtained from Jackson Labs. Receptor for ad-
vanced glycation end products (RAGE) KO mice were obtained from Hein
rna Erlandsson-Harris (Department of Medicine, Karolinska Institute, Stockholm, Sweden). The Institutional Animal Care and Use Committee at Massachusetts General Hospital approved the animal protocols used in this
study. Recombinant rat HMGB1 was expressed in E. coli and purified to
homogeneity as previously described (13, 14).

Purification of mouse and human hemoglobin

Hemoglobin was purified as previously described with modifications (15),
using pyrogen-free conditions. In brief, mouse blood was collected from
C57BL/6 mice by cardiac puncture. Human blood was collected asepti-
cally from healthy human volunteers. The blood was washed with an equal
weight of isotonic saline solution (0.9% NaCl, w/v) three times by centri-
fugation at 1000 × g to remove serum proteins. Equal volumes of saline
were added to the pellet containing RBCs, and this solution was sonicated
5 × 10 s at amplitude 40% with 1-min laps between pulses in Branson 450
sonicator from Bronson Ultrasone Corporation (Danbury, CT). The hem-
oglobin solution was dialuted with an equal volume of saline and sub-
jected to a second centrifugation at 2000 × g for 1 h. The resulting
hemoglobin solution, removed from the center layer, was filtered through
0.22-µm Millipore membranes and saved at −20°C in the dark. The
concentration of purified hemoglobin was measured by Micro-BCA. The
purity of the hemoglobin was confirmed to be >99% by non-denaturing
PAGE and high-pressure liquid chromatography.

Purification of Hx from mouse or human serum

Mouse or human serum Hx was purified by using hem affinity chroma-
tography essentially as we have described previously (16). In brief, serum
was filtered through 0.22-µm Millipore membranes; then albumin was
precipitated and removed by adding cold 1.68% rivanol solution (pH 8.0).
The resulting postrivanol precipitation sample was dialyzed against
pyrogen-free PBS. Protease inhibitors (0.5 mM AEBSF, 10 µM E-64, 2
µg/ml aprotinin, and 1 µM pepstatin A) were added to the dialyzed
postrivanol precipitation with gentle agitation for 15 min at 4°C. The
mixture was then applied to a 6-ml hemin-agarose column (Sigma) three
times, followed by extensive washing with 1200 ml PBS containing 0.5 M
NaCl overnight at 4°C to remove unbound proteins. Hx bound to the
column was eluted using 0.2 M citric acid (pH 2.0) and then immediately
neutralized with 10 M NaOH. Proteins in the buffer were exchanged,
consolidated in PBS at 4°C using Centriprep YM-30 (Millipore, MA), and
saved in aliquots at −80°C.

Limulus amebocyte lysate assay

The Limulus amebocyte lysate assay was performed as previously described
(17) to verify the endotoxin level to be <0.01 EU/mg in HMGB1, purified
hemoglobin, and Hx before use in cell cultures.

Preparation of macrophages

Bone marrow-derived macrophages (BMDMs) were prepared from mice, as
described previously (18) with minor modifications (16, 19). BMDMs were
seeded in wells of 96-well culture plates at a density of 4.0 × 10³ cells/cm²
(1.28 × 10⁴/well) and were incubated at 37°C under humidified 5% CO₂ to
allow cells to adhere before use in assays.

Macrophage culture and cytokine assays

BMDMs were washed three times in serum-free medium, followed by
incubation overnight with HMGB1, with or without hemoglobin, or with
different TLR agonists as desired at indicated concentrations. Purified mouse
or human hemopexin was added to the culture in some experiments as noted.
Concentrations of TNF and IL-6 in the supernatants were quantitated by
ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s
instructions.

Statistics

Except where indicated, representative data from at least three experiments
are presented in the figures. Data are expressed as means, and error bars
represent SE. The data were analyzed by GraphPad Prism 5 (GraphPad
Software, La Jolla, CA). We used t tests to compare conditions with and
without hemoglobin or with and without Hx. One-way ANOVA was used
to analyze the data for the dose-dependent synergy between hemoglobin
and HMGB1, the dose-dependent effect of Hx, and the dose-dependent
effect of picatennol. The p values <0.05 were considered to be statisti-
cally significant.

Results

Hemoglobin strongly synergizes with HMGB1 to induce TNF
and IL-6 from macrophages

Different concentrations of HMGB1 were incubated with a pre-
determined optimized concentration of mouse hemoglobin in cell
culture with BMDMs, and concentrations of TNF and IL-6 were
measured in the culture supernatants. As expected, treatment with
HMGB1 significantly induced TNF and IL-6 release by macro-
phages (Fig. 1). Hemoglobin alone did not induce detectable TNF
at concentrations up to 1000 µg/ml (8), but significantly enhanced
TNF and IL-6 production by BMDMs induced by different con-
centrations of HMGB1 (Fig. 1A, 1B). The effect of hemoglobin
on TNF and IL-6 production was dose dependent (Fig. 1C, 1D).
Human hemoglobin also synergized with HMGB1 to induce high
concentrations of TNF and IL-6 (data not shown). Together, these
results indicate that hemoglobin synergistically increases HMGB1-
dependent macrophage activation. We also found that free heme at
low concentrations (<1 µM) synergizes with HMGB1 to induce
increased TNF and IL-6 production from macrophages (data not shown).

FIGURE 1. Hemoglobin (Hb) synergizes with
HMGB1. BMDMs from C57BL/6 mice were
washed three times with serum-free medium and
then cultured overnight with HMGB1 at different
dilutions alone or with mouse Hb (30 µg/ml) (A, B),
or HMGB1 at 4 µg/ml with mouse Hb
at different concentrations (C, D). Concentrations
of TNF (A, C) or IL-6 (B, D) in the supernatants
were determined by ELISA. Results represent
mean ± SE and are representative of more than
three independent experiments. *p < 0.05, **p <
0.01 compared between cells treated with and
without Hb.
Synergistic induction of proinflammatory cytokines by HMGB1 and hemoglobin are partially dependent on TLR2, TLR4, RAGE, MyD88, and TRIF

It has been proposed that the cytokine release induced by HMGB1 may be mediated by several receptors including TLR2, TLR4, and RAGE (12). Thus, we next assessed the role of TLR2, TLR4, and RAGE signaling pathways in the synergy between hemoglobin with HMGB1. We compared cytokine responses of BMDMs from wild-type and KO mice incubated with HMGB1 in the absence or presence of hemoglobin. Responses of BMDMs from TLR2 and RAGE KO mice were compared with those of BMDMs from control wild type C57BL/6 mice, and responses of BMDMs from TLR4-deficient (C3H/HeJ) mice were compared with those of BMDMs from control (C3H/HeN) mice (Fig. 2). TNF induced by HMGB1 alone was variably decreased in the TLR2 KO cells, the C3H/HeJ cells, and the RAGE KO cells compared with the control wild-type cells (data not shown). However, the synergy between HMGB1 and hemoglobin was still detected, despite the diminished responses in mice deficient in TLR2 (Fig. 2A) or TLR4 (Fig. 2B) or RAGE (Fig. 2C); although in some cases, there was a small percentage decrease in the synergy. These findings suggest that TLR2, TLR4, or RAGE each could be partially involved but are not required for the synergy. We next studied cells obtained from MyD88 KO mice and TRIF KO mice to investigate whether the adaptor proteins MyD88 and TRIF were involved in the synergy. Although, as expected, BMDMs from TRIF and MyD88 KO mice produced much less TNF (only ∼5%) as compared with the wild type cells, there was still detectable synergy between HMGB1 and hemoglobin in cytokine production by both MyD88 and TRIF KO BMDMs (Fig. 2D). Together, these data suggest that TLR signaling may be involved but is not essential for the synergy to be present.

Involvement of induction of reactive oxygen species and activation of spleen tyrosine kinase in the mechanisms of synergy

We used pharmacological inhibitors to investigate the role of reactive oxygen species (ROS) in the synergy between hemoglobin and HMGB1. As shown in Fig. 3A, the antioxidant NAC significantly decreased and the flavoprotein inhibitor DPI partially decreased TNF that was synergistically induced by hemoglobin and HMGB1, whereas the xanthine oxidase inhibitor allopurinol and NO synthase inhibitor l-NAME did not decrease the synergy (Fig. 3A). NAC also decreased TNF induced by HMGB1 in the absence of hemoglobin. It has been reported that the synergy of heme with LPS is mediated through spleen tyrosine kinase (Syk)-dependent ROS generation (7). To evaluate the role of Syk in the hemoglobin-HMGB1 synergy, we used the selective Syk inhibitor piceatannol to pretreat the macrophages. As shown in Fig. 3B, the synergy was significantly reduced by piceatannol in a dose-dependent manner. Piceatannol also decreased the signal in cells that received HMGB1 alone. These results suggest that Syk-dependent ROS generation plays an important role in the induction of TNF by HMGB1, as well as in the synergy of HMGB1 with hemoglobin.

Hx blocks the synergistic induction of proinflammatory cytokines from macrophages by HMGB1 and hemoglobin

We have shown that Hx, the major heme scavenger in the plasma (10, 20), significantly blocks the synergy of hemoglobin with LPS (8). In addition, Hx has some immunomodulatory activities in that it modestly downregulates proinflammatory cytokines from macrophages (16) and functions as an anti-inflammatory component of serum high-density lipoprotein in atherosclerosis (21). It was therefore of interest to assess whether Hx would also affect the synergistic induction of proinflammatory cytokines that were induced by hemoglobin with HMGB1. We observed that addition of Hx significantly suppressed the synergistic induction of TNF in a dose-dependent manner (Fig. 4A). Similar results were obtained...
with IL-6 (Fig. 4B). Addition of equal concentrations of endotoxin-free albumin (Sigma) under the same conditions did not suppress the response (data not shown).

TLR2 and TLR4 agonists, HMGB1, and hemoglobin synergize to induce macrophages to release high concentrations of TNF and IL-6 that are suppressed by Hx

Because it is common that tissue damage, necrosis, and bleeding coexist in some settings of infection, we next assessed the synergy from three types of stimuli including HMGB1, hemoglobin, and the microbial-derived TLR agonist, LPS. Very low concentrations of LPS (250 pg/ml), HMGB1 (2 μg/ml), and hemoglobin (10 μg/ml) were used to stimulate BMDMs. Significantly higher concentrations of TNF and IL-6 (Fig. 5A, 5B) were observed in the culture after overnight incubation, compared with the cultures with the stimulus alone or any combinations of two stimuli. Similar results were observed using the TLR2 agonist Pam3Cys (1 ng/ml) instead of LPS (Fig. 5C, 5D). The addition of Hx to the culture resulted in remarkable suppression in synergy between the three types of stimuli (Fig. 6A, 6B). These data suggest that synergy between HMGB1, hemoglobin, and microbial products influences the magnitude of the innate immune response to injury and invasion, and that Hx may play a role in limiting this response.

Discussion

Damage to blood vessel walls with release of blood into tissues occurs frequently in diseases and after medical procedures. RBCs are either reabsorbed without rupture or undergo secondary degradation of cellular membranes with the release of hemoglobin into the extracellular space. Often there is an associated primary or secondary local inflammation, with generation and release of

![FIGURE 4. Hx suppresses hemoglobin (Hb) synergy with HMGB1 on BMDMs. BMDMs from C57BL/6 mice were washed three times with serum-free medium and then cultured overnight with 4 μg/ml HMGB1 in the absence or presence of 30 μg/ml mouse Hb and indicated concentrations of Hx. Concentrations of TNF (A) and IL-6 (B) in the supernatants were determined by ELISA. Results denote the mean ± SE and are representative of more than four independent experiments. **p < 0.01 compared between cells treated with and without Hx.](http://www.jimmunol.org/)

![FIGURE 5. Synergistic inflammation induced by hemoglobin (Hb), HMGB1, and TLR agonist LPS or Pam3Cys (P3C). BMDMs from C57BL/6 mice were washed three times with serum-free medium and then cultured overnight with Hb (10 μg/ml), HMGB1 (2 μg/ml), LPS (250 pg/ml) (A, B), or P3C (1 ng/ml) (C, D) alone or combinations of two or three of the stimuli. Concentrations of TNF (A, C) and IL-6 (B, D) in the supernatants were determined by ELISA. Results represent mean ± SE and are representative of three independent experiments. **p < 0.01 compared between cells treated with three stimuli and with one or two stimuli.](http://www.jimmunol.org/)
HMGB1, an intracellular protein that is integral in causing and increasing inflammation through active secretion after activation of NF-κB, or when released by permeabilized cell membranes during necrosis. We describe in this article that very low concentrations of hemoglobin synergize with HMGB1 to increase proinflammatory cytokines from macrophages. The results suggest that breakdown products of RBCs that are released into tissues likely play an important role in amplifying sterile inflammation that is present when blood is released into tissues.

This finding extends an observation made many years ago that hemoglobin synergizes with LPS in vitro and in vivo to increase release of TNF (1–3). There is extensive literature on the role of HMGB1 in promoting inflammation in tissues in which degrading blood cells would be present (11). Although difficult to obtain a numerical value for the concentration of hemoglobin in this complex tissue microenvironment, synergy with HMGB1 was observed at a hemoglobin concentration of 3 μg/ml, which is 1/50,000th of the total hemoglobin concentration in whole blood. Hemoglobin concentrations in areas of necrosis or other inflamed fluids almost certainly exceed this.

Several earlier studies indicate that LPS and hemoglobin physically interact (3, 22, 23). However, the finding that hemoglobin leads to synergistic increases in cytokine production with multiple other ligands, including HMGB1, suggests that the synergy may occur at the cellular level. Synergy of hemoglobin with LPS is due to the heme moiety (6), and synergy of heme with LPS appears to be mediated through ROS generation that is dependent on Syk (7). The mechanisms for the synergy of hemoglobin with HMGB1 may involve similar pathways. Our results using cells from several types of KO mice suggest that TLR2, TLR4, and RAGE signaling might contribute, but is not essential, to the synergy. Our studies on ROS and Syk using inhibitors are consistent with the concept that Syk-dependent ROS generation is important, but not specific, to the synergy. In our studies, the stimulation of macrophages after removal of several of the receptors reported to be involved in HMGB1 signaling, or after removal of adaptor signaling pathways, or with pharmacological inhibition of ROS or Syk resulted in suppressed responses to different extents with HMGB1 alone, confounding interpretation of the specificity of these mechanisms on the synergy. Further studies on additional receptors including CD163 for hemoglobin-bound haptoglobin and CD91 for heme-bound Hx may provide more understanding of the synergistic mechanisms.

We found that the induction of the proinflammatory cytokines TNF and IL-6 as a result of synergy between hemoglobin and HMGB1 was further increased by the presence of low concentrations of microbial ligands such as LPS or Pam3Cys that activate TLR4 or TLR2, respectively. This increase was not only additive but synergistic, leading to a “three-way synergy” in which three compounds were much more potent than any two of the other compounds. Countless studies have used single TLR agonists such as LPS alone in model systems as reagents to probe immune interactions. However, isolated activation by any single TLR agonist ligand is unlikely to occur in nature (24), and symmetry between hemoglobin, HMGB1, and microbial ligands is likely important in sepsis syndrome (7, 14, 25). Our finding confirms and extends prior studies reporting synergistic activation of signaling pathways, and subsequent induction of proinflammatory cytokines and chemokines triggered by different TLR agonists (26–32).

Although our findings seem of particular importance to extracellular hemoglobin in tissues outside of the vasculature, in some situations, a similar synergy between extracellular hemoglobin and HMGB1 may occur in the bloodstream itself, such as in sickle cell crisis, hemolysis caused by infection including hemorrhagic fevers and malaria, ischemic-perfusion syndrome, cardiopulmonary bypass, severe sepsis, and after transfusions in which there is liberation of hemoglobin into blood (33–37).

Hx strongly suppressed the synergistic inflammation of HMGB1 with hemoglobin. Hx has been considered primarily as a protein that binds heme rather than hemoglobin (10). Our findings in this study are consistent with the concept that Hx may transfer or “steal” heme from degrading hemoglobin, as has been described by Hrkal et al. (9). Sequestration of a low amount of heme would lead to a potent effect because low concentrations of hemoglobin greatly amplify cytokine release. Transfer of heme out of hemoglobin would also generate heme-free globin, which itself has an anti-inflammatory effect on macrophages (6). Other mechanisms for the suppression are also possible, including downstream induction of heme-oxigenase 1 with secondary anti-inflammatory effects (38).

Hx concentrations in blood are decreased in some clinical situations, including during intravascular hemolysis (39), in premature infants (40), and during severe sepsis (25). It is possible that low concentrations could reflect a decrease in heme buffering capacity, in which case measurement of the concentrations of Hx might be helpful as a biomarker, alone or perhaps in combination with other inflammatory markers including HMGB1. It was recently reported that infused Hx decreases organ failure and mortality in a cecal ligation and puncture model in mice (25), a finding that raises the possibility of administering Hx as a therapy to treat sepsis, as well as other infections in which hemoglobin is liberated such as hemorrhagic fevers or malaria. The findings in this study raise the possibility that infusion of Hx might likewise be helpful in settings where hemoglobin and HMGB1 are both present in the absence of infection, such as in sterile inflammation or necrosis.

Disclosures

H.S.W. has declared Hx to Massachusetts General Hospital as a potential candidate molecule to help decrease inflammation, and the institution has filed for provisional patent protection. The other authors have no financial conflicts of interest.

References


