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*J Immunol* 2008; 181:4240-4246; doi: 10.4049/jimmunol.181.6.4240

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High Mobility Group Protein-1 Inhibits Phagocytosis of Apoptotic Neutrophils through Binding to Phosphatidylserine

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Phagocytosis of apoptotic cells, also known as efferocytosis, is an essential feature of immune responses and critical to resolution of inflammation. Impaired efferocytosis is associated with an unfavorable outcome from inflammatory diseases, including acute lung injury and pulmonary manifestations of cystic fibrosis. High mobility group protein-1 (HMGB1), a nuclear nonhistone DNA-binding protein, has recently been found to be secreted by immune cells upon stimulation with LPS and cytokines. Plasma and tissue levels of HMGB1 are elevated for prolonged periods in chronic and acute inflammatory conditions, including sepsis, rheumatoid arthritis, acute lung injury, burns, and hemorrhage. In this study, we found that HMGB1 inhibits phagocytosis of apoptotic neutrophils by macrophages in vivo and in vitro. Phosphatidylserine (PS) is directly involved in the inhibition of phagocytosis by HMGB1, as blockade of HMGB1 by PS eliminates the effects of HMGB1 on efferocytosis. Confocal and fluorescence resonance energy transfer demonstrate that HMGB1 interacts with PS on the neutrophil surface. However, HMGB1 does not inhibit PS-independent phagocytosis of viable neutrophils. Bronchoalveolar lavage fluid from Scnn+ mice, a murine model of cystic fibrosis lung disease which contains elevated concentrations of HMGB1, inhibits neutrophil efferocytosis. Anti-HMGB1 Abs reverse the inhibitory effect of Scnn+ bronchoalveolar lavage on efferocytosis, showing that this effect is due to HMGB1. These findings demonstrate that HMGB1 can modulate phagocytosis of apoptotic neutrophils and suggest an alternative mechanism by which HMGB1 is involved in enhancing inflammatory responses. The Journal of Immunology, 2008, 181: 4240–4246.

Phagocytosis of apoptotic cells, also known as efferocytosis, is an essential feature of the immune response. Rapid clearance of apoptotic cells by professional phagocytes is critical in protecting the surrounding tissue from harmful exposure to the inflammatory or immunogenic contents of dying cells (1). In addition, ingestion of apoptotic cells by macrophages results in the release of anti-inflammatory mediators, including TGF-β1 and PGE2, and suppresses the production of proinflammatory cytokines, such as IL-8 and TNF-α, as well as other proinflammatory mediators, including TXA2, by macrophages (2, 3). Defects in the clearance of dying cells have been shown in patients with acute lung injury (4, 5), cystic fibrosis (6, 7), as well as those with chronic autoimmune diseases (8, 9).

The initial event in efferocytosis is the recognition of an “eat me” signal on the target cell by a broad set of receptors localized on the surface of the phagocyte. Phosphatidylserine (PS) is the best-explored eat me signal on apoptotic cells (10). In the early stages of apoptosis, PS redistributes from the inner to the outer membrane leaflet in a process involving inhibition of aminophospholipid translocase and activation of lipid scramblase (10). Interaction of PS with annexin V results in shielding of the PS eat me signal and inhibition of apoptotic and necrotic cell uptake by macrophages (11, 12).

High mobility group protein-1 (HMGB1), originally described as a nuclear nonhistone DNA-binding protein, has more recently been shown to act as an extracellular mediator of inflammation (13, 14). Experiments in mice found that HMGB1 levels in serum are increased at late time points after endotoxin exposure and also that delayed administration of Abs to HMGB1 attenuates endotoxin lethality (13, 15). However, recent studies have demonstrated that HMGB1 itself has weak proinflammatory activity, and only develops the ability to induce cytokine production from macrophages and other cell populations after binding to DNA or bacterially derived materials (16, 17). Because there is evidence that HMGB1 can bind to PS in vitro (16, 18), we hypothesized that HMGB1 may inhibit recognition of apoptotic cells by phagocytes through covering PS on the apoptotic cell’s surface. Thus, in this study, we not only confirmed that HMGB1 binds to PS on apoptotic neutrophils, but additionally demonstrated that HMGB1 has an inhibitory effect on efferocytosis.

Materials and Methods

Reagents

Custom mixture Abs and negative selection columns for neutrophil isolation were from Stem Cell Technologies. Penicillin-streptomycin and Brewer thiglycollate were from Sigma-Aldrich. Annexin V-FITC and propidium iodide (PI) were from R&D Systems, PS, phosphatidylcholine, and 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-PS were from Avanti Polar Lipids. Rabbit anti-HMGB1 polyclonal Abs were from Abcam. Mouse anti-CD47 mAbs were from BD Biosciences. Chromo 546 and Chromo 642 fluorescent labeling kits were from Active Motif. Purified recombinant annexin V was from BD Biosciences.
Preparations, was washed. Neutrophil purity, as determined by HEMA 3 stained cytospin to pass through by negative selection. The cells were then pelleted and the entire cell suspension was then placed into a column, surrounded by a magnet. The T cells, B cells, RBC, monocytes, and macrophages were captured in the column, allowing the neutrophils to pass through by negative selection. The cells were then pelleted and washed. Neutrophil purity, as determined by HEMA 3 stained cytospin preparations, was >97%. Cell viability, as determined by trypan blue exclusion, was consistently >98%.

Apoptosis was induced by heating at 42°C for 60 min and followed by incubation at 37°C in 5% CO₂ for 3 h. To monitor apoptosis, 10⁶ cells were stained with annexin V-FITC and PI, according to the manufacturer’s protocol. Cells were analyzed without fixation by flow cytometry within 30 min of staining.

Culture of mouse peritoneal macrophages
Peritoneal macrophages were elicited in 8–10-wk-old mice by intraperitoneal injection of 1 ml of 3% Brewer thioglycollate. Cells were harvested 5 days later by peritoneal lavage. Cells were plated on 96-well plates at a concentration of 2 × 10⁴ cells/well. After 2 h at 37°C, nonadherent cells were removed by washing with medium. Fresh medium was added to the cells and changed approximately every 3 days. One hour before the phagocytosis assay, the medium was replaced by Opti-MEM medium with 5% mouse serum.

In vitro phagocytosis assays
Phagocytosis was assayed by adding 10⁶ preincubated apoptotic neutrophils suspended in 100 μl Opti-MEM medium to each well of the 96-well plate containing adherent macrophage monolayers at 37°C for 90 min. For studies investigating inhibition of phagocytosis, apoptotic neutrophils were preincubated with HMGB1, lipid vesicles, anti-HMGB1 Abs, annexin V (supplemented with 2 mM CaCl₂), or bronchoalveolar lavage (BAL) fluid from WT or Scnn mice. PMNs were heated at 42°C for 60 min and then incubated for 3 h at 37°C. Apoptosis was analyzed by flow cytometry after annexin V and PI staining. Neutrophils were heated at 42°C for 60 min and then incubated for 15 min at 4°C. Anti-biotin tetrameric Ab complexes (100 μl) were then added, and the cells were incubated for 15 min at 4°C. Following this, 60 μl of colloidal magnetic dextran iron particles were added and incubated for 15 min at 4°C. This entire cell suspension was then placed into a column, surrounded by a magnet. The T cells, B cells, RBC, monocytes, and macrophages were captured in the column, allowing the neutrophils to pass through by negative selection. The cells were then pelleted and washed. Neutrophil purity, as determined by HEMA 3 stained cytospin preparations, was >97%. Cell viability, as determined by trypan blue exclusion, was consistently >98%.

Isolation and induction of apoptosis in neutrophils
All of the animal protocols have been reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of University of Alabama at Birmingham. Mouse neutrophils were purified from bone marrow cell suspensions as described previously (20). In brief, bone marrow cells were incubated with 20 μl of primary Abs specific to the cell surface markers F4/80, CD4, CD45R, CD5, and TER119 for 15 min at 4°C. Anti-biotin tetrameric Ab complexes (100 μl) were then added, and the cells were incubated for 15 min at 4°C. Following this, 60 μl of colloidal magnetic dextran iron particles were added and incubated for 15 min at 4°C. The entire cell suspension was then placed into a column, surrounded by a magnet. The T cells, B cells, RBC, monocytes, and macrophages were captured in the column, allowing the neutrophils to pass through by negative selection. The cells were then pelleted and washed. Neutrophil purity, as determined by HEMA 3 stained cytospin preparations, was >97%. Cell viability, as determined by trypan blue exclusion, was consistently >98%.

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Confocal microscopy and fluorescence resonance energy transfer (FRET) analysis
Following apoptosis induction, NBD-PS and Chromeo546 labeled HMGB1 were added to the apoptotic neutrophils at a final concentration of 1 μg/ml and incubated at 37°C for 3 h. The cells were washed and then fixed with 4% paraformaldehyde in PBS for 10 min and cytospin slides were prepared.

FRET analysis was performed as previously described (22). In brief, proximity of cell surface proteins was investigated for FRET measurements by the acceptor photobleaching technique. An excitation wavelength of 450 nm and an emission wavelength of 525–550 nm were used for donor probes (NBD), and an excitation wavelength of 530 nm and an emission wavelength of 570–625 nm were used for the acceptor (Chromeo546). FRET-positive pixels and efficiencies were calculated by directly subtracting images obtained before acceptor photobleaching from images acquired immediately after acceptor photobleaching to reveal the increased fluorescence of the donor. After an initial confocal image was acquired, a portion of a single cell was masked, and the acceptor fluorescence (Chromeo546) in the masked area was bleached with a pumped dye laser to <30%. The remaining area of the same cell was left unbleached and used as a control. After pixel alignment between the pre- and postbleach images was ensured, individual pixel FRET efficiencies were calculated by expressing the increased donor fluorescence as a fraction of the postbleach intensity. The noise level in unbleached areas was eliminated to identify FRET-positive areas and calculate image statistics.
Statistical analysis

For each experimental condition, the entire group of animals was prepared and studied at the same time. Data are presented as mean ± SD (in vitro experiments) or mean ± SEM (in vivo experiments) for each experimental group. One-way ANOVA followed by the Tukey-Kramer analysis was performed for comparisons among multiple groups and Student’s t test was used for comparisons between two groups. A value of p < 0.05 was considered significant.
Results

**PS is necessary for phagocytosis of apoptotic neutrophils**

To confirm apoptosis of the neutrophils after heating and incubation at 37°C for 3 h, the cells were stained with annexin V-FITC and PI for flow cytometry. As shown in Fig. 1A, after heating for 60 min, constantly >40% of the neutrophils were apoptotic (i.e., annexin V+). Phagocytosis of apoptotic neutrophils obtained after 60 min heating was ~3-fold higher than that found with viable, nonapoptotic neutrophils (Fig. 1B).

Previous studies have demonstrated that PS expressed on the surface of apoptotic lymphocytes is required for phagocytosis by macrophages (23). To test whether the appearance of PS on the surface of apoptotic neutrophils was also associated with the development of the ability to efficiently phagocyte apoptotic cells, phagocytosis assays were performed using apoptotic bone marrow neutrophils and peritoneal macrophages. PS vesicles were used to compete with the PS presented on apoptotic neutrophils (24). The lipid PC, which is present on both the viable and apoptotic cell surface and is not involved in recognition of apoptotic cells, was used as a negative control for PS. Due to its ability to bind to PS with high affinity, annexin V was also used to examine the role of neutrophil PS expression on phagocytosis (25). As shown in Fig. 1C, both PS vesicles and annexin V, but not PC vesicles, significantly decreased efferocytosis of apoptotic neutrophils. These results indicate that exposed PS on the apoptotic neutrophils serves as a signal for phagocytosis.

**HMGB1 inhibits phagocytosis of apoptotic neutrophils**

To determine whether extracellular HMGB1 can modulate efferocytosis, apoptotic neutrophils were incubated with HMGB1 for 30 min before being exposed to macrophages for phagocytosis assays. As shown in Fig. 2A, HMGB1 dose-dependently inhibits phagocytosis of apoptotic neutrophils with diminished phagocytosis apparent at 0.1 μg/ml HMGB1 and maximal effects present at 1 μg/ml HMGB1. As a positive control, annexin V also was able to inhibit phagocytosis, whereas BSA had no effects. Fig. 2B showed representative images of phagocytosis of neutrophils treated with control BSA or HMGB1.

To demonstrate that the inhibitory effect of HMGB1 on phagocytosis of apoptotic neutrophils is not due to contaminants, such as LPS, presented in the purified protein preparation, the HMGB1 protein was boiled for 15 min before being incubated with neutrophils. The boiled protein no longer inhibited phagocytosis, indicating that the active form of HMGB1 is required to modulate phagocytosis (Fig. 2C).

To determine whether the inhibition of phagocytosis by HMGB1 is a specific effect, we added anti-HMGB1 Abs to the phagocytosis assays. As shown in Fig. 2D, anti-HMGB1 Abs abolished the inhibitory effects of HMGB1 on engulfment of apoptotic neutrophils by macrophages. In contrast, control rabbit IgG had no effect on HMGB1 associated decreases in efferocytosis (Fig. 2D).

To rule out the possibility that abrogation of the inhibitory effect of HMGB1 by anti-HMGB1 Abs is due to Ab-associated secondary stimulation of FcR on the macrophages, macrophages were pre-treated with anti-FcR Abs before the addition of neutrophils to the cultures. As shown in Fig. 2E, even after FcR blockade, anti-HMGB1 Abs still efficiently eliminated the inhibitory activity of HMGB1 on macrophage phagocytosis of neutrophils.

To demonstrate that HMGB1 inhibits phagocytosis in vivo, apoptotic neutrophils incubated with mouse albumin or recombinant HMGB1 were injected intratracheally into mice and BAL fluid harvested 90 min later for determination of alveolar uptake of neutrophils. As shown in Fig. 2F, incubation of apoptotic neutrophils to HMGB1, but not mouse albumin, before administration into the lungs resulted in diminished phagocytosis by alveolar macrophages in vivo. To determine whether HMGB1 binds to the surface of apoptotic neutrophils, HMGB1 was labeled with Chromo 642 fluorescent dye and then incubated with the apoptotic neutrophils that subsequently were injected intratracheally. Chromo 642 labeled HMGB1, but not Chromo 642 labeled mouse albumin, bound to the apoptotic cell surface (Fig. 2, G and H).

**PS is involved in the inhibition of phagocytosis by HMGB1**

PS exposure on the cell surface is essential for efferocytosis of apoptotic cells (7, 11, 12, 23). Because HMGB1 can bind to PS in vitro (16), we therefore hypothesized that the inhibitory effect of HMGB1 on neutrophil phagocytosis involves PS. Thus, we examined whether PS can abolish the inhibitory effect of HMGB1 or vice versa. As shown in Fig. 3, preincubation of HMGB1 with PS abrogated the inhibitory effect of either HMGB1 or PS on efferocytosis. In contrast, PC was unable to block the inhibitory effects of HMGB1 neutrophil phagocytosis.

**HMGB1 binds to PS on apoptotic neutrophils**

It has been shown that HMGB1 can bind to the PS in vitro (16, 18). Additionally, as shown in Fig. 3, we found that PS can abolish the inhibitory effect of HMGB1. Thus, we investigated whether HMGB1 can interact with PS on intact cells. For these experiments, apoptotic neutrophils were incubated with PS (NBD-PS) and Chromo 546-labeled HMGB1. The distribution of PS and HMGB1 on the cell surface was then determined by confocal imaging, and the molecular interaction of HMGB1 and NBD-PS was measured by FRET.

As shown in Fig. 4A, both PS and HMGB1 were localized to the surface of apoptotic neutrophils. FRET spots, demonstrating interaction between HMGB1 and PS, appeared on the cell surface with the Acceptor (Chromo 546-HMGB1, red fluorescence) being photo-bleached. In contrast, fewer FRET spots were observed on the cell surface of the same neutrophils that were not photo-bleached (Fig. 4A). The FRET efficiency between PS and HMGB1
was significantly greater in the bleached area of the cell membrane as compared with the unbleached area (Fig. 4B).

**HMGB1 does not inhibit PS-independent phagocytosis**

CD47 acts as a “don’t eat me” signal on the surface of viable cells, blocking phagocytosis by binding to signal-regulatory protein (SIRPα), a heavily glycosylated transmembrane protein with an ITIM (26). To test whether HMGB1 inhibits phagocytosis specifically through interactions with PS or whether CD47 might also be involved, we used anti-CD47 Abs to block CD47 function, which induced the engulfment of functional, nonapoptotic neutrophils by macrophages (Fig. 5). There was no effect of HMGB1 on the phagocytosis of viable neutrophils following treatment with CD47 Abs, indicating the specificity of the interaction between PS and HMGB1 in inhibiting efferocytosis.

**Contribution of increased pulmonary HMGB1 to alterations in neutrophil efferocytosis**

Cystic fibrosis is associated with the accumulation of large numbers of neutrophils into the lungs, as well as a higher percentage of apoptotic neutrophils among the population of pulmonary neutrophils (7, 27). Previous studies have indicated that the increased percentage of apoptotic neutrophils in the Airways of patients with cystic fibrosis reflects decreased efferocytosis (7). In recent studies, we found elevated levels of HMGB1 in BAL from Scnn−/− mice, a murine model for cystic fibrosis, and also in sputum from patients with this disorder (28). Given the findings of the present experiments, which showed that HMGB1 inhibits phagocytosis of apoptotic neutrophils, we hypothesized that the elevated pulmonary HMGB1 contributes to alterations in neutrophil efferocytosis. A, BAL fluid of Scnn−/− mice significantly inhibits phagocytosis of apoptotic neutrophils by macrophages. Phagocytosis assays were performed in the presence of 12 μl of PBS, wild-type mouse BAL fluids, Scnn−/− BAL fluids, Scnn−/− BAL fluids preincubated with 1 μg mouse anti-HMGB1 mAbs for 30 min, or Scnn−/− BAL fluids preincubated with 1 μg mouse IgG was added to cultures of macrophages and apoptotic neutrophils. Phagocytosis assays were performed and the phagocytic index determined. **, p < 0.01 compared with wild-type; #, p < 0.05 compared with Scnn−/−.

**FIGURE 6.** Increased BAL HMGB1 contributes to alterations in neutrophil efferocytosis. A, BAL fluid of Scnn−/− mice significantly inhibits phagocytosis of apoptotic neutrophils by macrophages. Phagocytosis assays were performed in the presence of 12 μl of PBS, BAL fluids from WT mice (n = 4), or BAL fluids from Scnn−/− mice (n = 5). Data are presented as mean ± SD. **, p < 0.01 compared with PBS and WT groups. B, Anti-HMGB1 Ab abolishes increased phagocytosis of neutrophils induced by exposure to Scnn−/− BAL fluids. Twelve microliters of PBS, wild-type mouse BAL fluids, Scnn−/− BAL fluids, Scnn−/− BAL fluids preincubated with 1 μg mouse anti-HMGB1 mAbs for 30 min, or Scnn−/− BAL fluids preincubated with 1 μg mouse IgG was added to cultures of macrophages and apoptotic neutrophils. Phagocytosis assays were performed and the phagocytic index determined. ***, p < 0.001 compared with wild-type; #, p < 0.05 compared with Scnn−/−.
concentrations of HMGB1 associated with cystic fibrosis contribute to the decreased efferocytosis present in this condition. To examine this issue, we measured phagocytosis of apoptotic neutrophils exposed to BAL samples from control and Scnn−/− mice.

As shown in Fig. 6A, phagocytosis of apoptotic neutrophils was significantly reduced after incubation with bronchoalveolar lavage from Scnn−/− mice, but not after exposure to BAL from control mice. Measured concentrations by quantitative Western blotting of HMGB1 in Scnn−/− BAL were 1.36 ± 0.23 µg/ml, and were 0.76 ± 0.30 µg/ml in BAL from control mice. Incubation of BAL from Scnn−/− mice with anti-HMGB1 Abs restored the phagocytic index to control levels (Fig. 6B), indicating that HMGB1 contained in these BAL was responsible for their inhibitory effects on efferocytosis.

Discussion

Extracellular release of HMGB1 has potent proinflammatory effects and contributes to acute lung injury as well as other organ failures after pathophysiologic events such as endotoxemia, bacterial peritonitis, hemorrhage, ischemia/reflow injury, and burns (13, 29–36). In these settings, blockade of the actions of HMGB1 through administration of anti-HMGB1 Abs or through the use of inhibitory fragments of HMGB1, such as the A box, decreased organ injury and improved survival. However, because recent studies (16, 37) have found that highly purified HMGB1 loses much of its ability to activate cells to produce cytokines and other proinflammatory mediators, determination of the mechanisms by which HMGB1 contributes to organ injury remains an important question.

The present experiments demonstrate that HMGB1 can diminish the phagocytosis of apoptotic neutrophils by macrophages both in vitro and in vivo. The mechanism of such inhibitory actions of HMGB1 appears to be through interaction with PS on the neutrophil membrane, thereby blocking an important ligand involved in phagocytosis. The ability of HMGB1 to bind to PS has previously been demonstrated under in vitro conditions (16), and the present experiments extend those findings to the intact cell. Of note, because HMGB1 was unable to affect the ability of anti-CD47 Abs to increase phagocytosis of viable neutrophils, its role in efferocytosis appears to be specific and directly linked to interaction with PS. Although previous studies (22, 38–40) have found that HMGB1 is capable of interactions with multiple intracellular and extracellular molecules, the present results, showing direct association between HMGB1 and PS by FRET as well as blockade of HMGB1’s inhibitory effects on neutrophil phagocytosis by exogenous PS, indicate that its role in efferocytosis is specifically due to interactions with PS.

Acute lung injury is characterized by the accumulation of activated neutrophils in the lungs (41). These pulmonary neutrophils produce proinflammatory cytokines, such as IL-1β and TNF-α, and release reactive oxygen species as well as other mediators capable of damaging adjacent cell populations and contributing to tissue injury. Pulmonary levels of HMGB1 are increased in the setting of acute lung injury, and HMGB1 itself can produce pulmonary injury (30, 36). Therefore, interaction of elevated tissue concentrations of HMGB1 with PS exposed on the surfaces of neutrophils that have entered into early apoptosis, but remain activated to produce proinflammatory mediators, may diminish engulfment and removal of such neutrophils, allowing them to continue to contribute to and exacerbate the proinflammatory milieu associated with acute lung injury. In addition, inhibition by HMGB1 of phagocytosis of neutrophils in late apoptosis may exacerbate pulmonary inflammation by permitting these cells to release their intracellular contents directly into the lungs, rather than being neutralized within macrophages. Therefore, even if HMGB1 itself is unable to directly activate neutrophils, macrophages, and other cellular populations involved in acute lung injury, its ability to diminish phagocytosis and clearance of neutrophils from the lungs would provide an important mechanism enhancing the severity of acute lung injury. Such inhibitory actions of HMGB1 on efferocytosis would also explain the beneficial effects of anti-HMGB1 therapies in acute lung injury.

Recent studies have shown that elevated circulating and tissue concentrations of HMGB1 are present in chronic inflammatory conditions, such as rheumatoid arthritis and cystic fibrosis (15, 28, 42–46). Although the role of activated neutrophils is not well defined in chronic autoimmune disorders, neutrophils have been demonstrated to play a central role in contributing to lung injury in cystic fibrosis (6, 27). Neutrophils are also involved in other pulmonary inflammatory conditions, including chronic obstructive pulmonary disease, pneumonia, and chronic bronchiectasis (47, 48). Recent data have found elevated HMGB1 levels in cystic fibrosis and in patients with severe pneumonia (28, 49). In the present studies, HMGB1 in BAL from Scnn−/− mice, a murine model for cystic fibrosis, was shown to be directly involved in inhibiting phagocytosis of apoptotic neutrophils. The ability of HMGB1 to inhibit neutrophil clearance in cystic fibrosis and other pulmonary conditions associated with ongoing neutrophil dependent inflammation would be expected to contribute to ongoing pulmonary injury, suggesting a role for anti-HMGB1 therapies in these settings.

Acknowledgments

We thank Albert Tousson of the UAB imaging microscopy core facility for preparation of the FRET images. We thank Drs. Steven Rowe and Jaroslaw Zujewski for insightful suggestions. We also thank Youhong Zhang for general assistance.

Disclosures

The authors have no financial conflict of interest.

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