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The Role of IFN-α and Nitric Oxide in the Release of HMGB1 by RAW 264.7 Cells Stimulated with Polyinosinic-Polycytidylic Acid or Lipopolysaccharide[1]

Weiwen Jiang* and David S. Pisetsky[2]*†

High mobility group protein 1 (HMGB1) is a nonhistone nuclear protein with a dual function. Inside the cell, HMGB1 binds to DNA and modulates a variety of processes, including transcription. Outside the cell, HMGB1 displays cytokine activity and can promote inflammation, serving as a mediator in models of shock and arthritis. In in vitro studies, proinflammatory molecules such as LPS, lipoteichoic acid, dsRNA, TNF-α, and IFN-γ can induce HMGB1 release from macrophages. To define further the release process, we investigated the role of the downstream mediators, NO and IFN-α, in the release of HMGB1 from RAW 264.7 macrophage cells stimulated with LPS or polyinosinic-polycytidylic acid (poly(I:C)). In these experiments, 1400W, an inhibitor of NO production by the inducible NO synthase, reduced HMGB1 release stimulated by LPS, but not poly(I:C), whereas neutralizing IFN-α prevented HMGB1 release induced by poly(I:C), but not LPS. The addition of an NO donor and rIFN-α to RAW 264.7 cells caused HMGB1 release. Furthermore, inhibition of JNK activation attenuated HMGB1 release induced by either LPS or poly(I:C). Analysis of bone marrow-derived macrophages stimulated by LPS or poly(I:C) showed patterns of HMGB1 release similar to those of RAW 264.7 cells. Together, these experiments indicate that, although both LPS and poly(I:C) induce HMGB1 release from RAW 264.7 cells and murine macrophages, the response is differentially dependent on NO and IFN-α. ***The Journal of Immunology,*** **2006, 177: 3337–3343.**

High mobility group protein 1 (HMGB1)[3] is a nonhistone nuclear protein with both intracellular and extracellular functions. Inside the cell, HMGB1 binds to DNA and promotes its interaction with transcriptional factors as well as other proteins (1, 2). HMGB1 is a highly mobile protein, and can shuttle between the nucleus and cytoplasm as well as exit from the cell (3). Outside the cell, HMGB1 can serve as a cytokine and induce an array of proinflammatory responses that resemble those of LPS (4, 5). Indeed, HMGB1 can mediate the late effects of LPS and plays an important role in sepsis (6).

HMGB1 can exit the cell by both an active and passive process. With monocytes or macrophages stimulated with LPS, the release of HMGB1 is active, involving protein acetylation, translocation from the nucleus, and secretion in vesicles (7). When cells die by necrosis, HMGB1, because of its mobility and weak binding to chromatin, can float away from the nucleus. Among cytokines, TNF-α and IFN-γ can also induce the active translocation of HMGB1 into the extracellular milieu (8, 9).

In its extracellular form, HMGB1 can induce a variety of cellular responses, including the expression of proinflammatory mediators such as TNF-α, IL-1, and NO, and induction of dendritic cell maturation (10–13). In its role as a cytokine, HMGB1 has a complex structure, with different regions manifesting opposing actions. Thus, while the HMGB1 B box displays cytokine activity, the A box may antagonize the inflammatory response of HMGB1 (14, 15). An important role of HMGB1 in immunopathogenesis is suggested by the demonstration of elevated extracellular levels in disease settings and the effects of anti-HMGB1 in animal models of endotoxin-induced shock and arthritis (4, 16).

The release of HMGB1 from cells was first demonstrated with LPS and in the setting of sepsis (4, 17). Among immune stimulants, LPS induces responses by interacting with TLR4 (18), a member of the family of pattern recognition receptors. These pattern recognition receptors recognize bacterial or viral products and can trigger innate immunity. The release of HMGB1 is not unique to LPS. In a previous study, we demonstrated that, in addition to LPS, lipoteichoic acid, a ligand of TLR2, and the synthetic dsRNA, polyinosinic-polycytidylic acid (poly(I:C)), a TLR3 ligand, can induce the release of HMGB1 by RAW 264.7 cells (19). Of note, CpG DNA, the ligand of TLR9, did not induce this release, suggesting specificity in the process.

In this study, we have investigated further the role of other inflammatory mediators and signal transduction pathways in HMGB1 release induced by LPS and poly(I:C) in RAW 264.7 cells. In results presented in this work, we show that NO and IFN-α can both induce HMGB1 release, although their role varies depending on the stimulus. As such, NO mediates HMGB1 release induced by LPS, whereas IFN-α mediates the release induced by poly(I:C). Nevertheless, for both LPS and poly(I:C), HMGB1 release is dependent on JNK activation, as inhibitors of this pathway can prevent the extracellular release of this molecule. Together, these studies clarify the mechanisms by which HMGB1 release

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[3] Abbreviations used in this paper: HMGB1, high mobility group protein 1; BMMC, bone marrow-derived macrophage; iNOS, inducible NO synthase; L-NIL, L-N^6-iminoethyl-lysine; MCMV, mouse CMV; pDC, plasmacytoid dendritic cell; poly(I:C), polyinosinic-polycytidylic acid; siRNA, small inhibitory RNA; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-β.

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can be triggered in innate immunity and suggest strategies to interdict this process.

Materials and Methods

Cells and reagents

RAW 264.7 cells were purchased from American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% FBS and 200 μg/ml gentamicin. To obtain bone marrow-derived macrophages (BMMCs), femurs from C57BL/6 mice (The Jackson Laboratory) were dissected free of connective tissue and flushed with RPMI 1640 medium. Bone marrow cells were seeded at 1×10^6 cells/ml in the presence of 30% conditioned medium from L929 cells. On day 7 of culture, nonadherent cells were removed by vigorous washing with RPMI 1640 medium. The adherent BMMCs were cultured with fresh RPMI 1640 medium until additional experiments. All protocols for animal use were approved by the Institutional Animal Care and Use Committee of the Durham Veterans Affairs Medical Center.

LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich, and poly(I:C) was purchased from InvivoGen. Abs against MAPKs (p38, phospho-p38, phospho-ERK1/2 (ERK1/2), and phospho-JNK) were purchased from Cell Signaling Technology. MAPK inhibitors (SB203580, PD90580, and JNK inhibitor) were purchased from Calbiochem. DETA NONOate (NOC-18), 1400W, and L-N^6-iminoethyl-lysine (L-NIL) were purchased from Alexis. rIFN-αA and rat anti-IFN-α Ab were purchased from PBL Biomedical Laboratories. Goat anti-TNF-α-neutralizing Ab and goat IgG were purchased from R&D Systems.

Cell culture

RAW 264.7 cells were plated in 6-well culture plates for 2–3 h and washed twice with Opti-MEM (Invitrogen Life Technologies). Cells were then stimulated with LPS, poly(I:C), NOC-18, or rIFN-αA for 20–24 h, and supernatants were collected for NO, cytokine, and HMGB1 assay. For inhibition studies, 1400W, anti-IFN-α Ab, or MAPK inhibitors were preincubated with cells for 30 min and subsequently stimulated with LPS or poly(I:C) for 20 h. Similar experimental conditions were used in experiments with BMMCs. For MAPK assays, RAW 264.7 cells were stimulated with LPS or poly(I:C) for 15, 60, or 90 min. Cells were then washed twice with ice-cold PBS and lysed by M-PER (Pierce). Protein concentration was determined by the Bradford method. For MAPK inhibition assays, cells were preincubated with various MAPK inhibitors for 30 min, followed by the addition of LPS or poly(I:C) for 60 min. Cellular proteins were isolated, and MAPK activities were measured by Western blotting.

Biochemical and immunochemical assays

The levels of TNF-α and IFN-α in supernatants were measured by ELISA. TNF-α capture and detection Ab pairs were purchased from R&D Systems, and ELISA were performed as described previously (20). An IFN-α ELISA kit was purchased from PBL Biomedical Laboratories, and assays were performed in accordance with manufacturer's instructions. Nitrite levels in culture supernatants were measured based on the Griess method, as published previously (20).

For Western blotting of HMGB1, supernatants were collected after 20–24 h of culture and concentrated by Centricon YM-10 (Millipore). The volume of the concentrated supernatants was adjusted to 70 μl for equal loading, and samples were resolved on 4–12% NuPAGE Tris-Bis SDS polyacrylamide gel (Invitrogen Life Technologies). Protein was transferred to polyvinylidene difluoride membranes (Invitrogen Life Technologies), blocked with 5% dry milk in TBS-Tween, and blotted with rabbit anti-HMGB1 polyclonal Ab (gift from K. J. Tracey, North Shore University Hospital, New York University School of Medicine, Manhasset, NY). The membrane was then incubated with HRP-conjugated anti-rabbit IgG, followed by SuperSignal West Femto substrate (Pierce). Images were captured by exposing the membrane to a charge-coupled device camera (FluorChem8900; Alpha Innotech).

The relative levels of HMGB1 in culture supernatants were quantified using AlphaEasyFC version 3.1.2 after capturing Western blot images by a charge-coupled device camera. The density of HMGB1 was expressed as fold increase over that of the medium control or fold decrease over that of LPS- or poly(I:C)-stimulated samples in inhibition studies. To quantify the relative amount of HMGB1 released by RAW 264.7 cells stimulated with either LPS or poly(I:C), a standard curve was generated on the same blot during electrophoresis by serial dilutions of rHMGB1 (Sigma-Aldrich). The density of the HMGB1 in culture supernatants was recorded and plotted against the standard curve to obtain the concentration of HMGB1. Results are expressed as ng/ml.

Confocal imaging

RAW 264.7 cells or BMMCs were cultured in 8-well chamber slides and stimulated with LPS (0.5 μg/ml) or poly(I:C) (0.25 μg/ml) for 20–24 h. Cells were then washed with ice-cold PBS, fixed, and permeabilized using the Cytofix/Permeable kit (BD Biosciences). The rabbit anti-HMGB1 Ab was added, followed by Alexa anti-rabbit IgG 488 (Molecular Probes). Cell-permeable DRAQ5 (Alexis) was used for nuclear staining. Images were captured by a confocal laser-scanning microscope (Zeiss LSM510; Zeiss).

Results

The effects of NO on HMGB1 release

In this study, we compared the induction of HMGB1 release by two molecules that differ in their pattern of TLR stimulation. Thus, poly(I:C), a model for dsRNA, depends on TLR3, whereas LPS depends on TLR4. In RAW 264.7 cells, both poly(I:C) and LPS induce HMGB1 translocation and extracellular release, as shown by Western blotting (Fig. 1*A*) and confocal imaging (Fig. 1*B*). In these experiments, the amount of HMGB1 in the culture supernatants was determined by densitometry, using purified HMGB1 as a standard. In culture supernatants from cells stimulated with LPS, 64.5 ± 3.0 ng/ml HMGB1 was observed, whereas the amount of HMGB1 induced by poly(I:C) was 342.5 ± 28.8 ng/ml. In general, the quantity of HMGB1 in medium-treated RAW 264.7 cell cultures was <5 ng/ml.

A comparison of the relative intensities of HMGB1 bands also reflected stronger HMGB1 release by cells induced by poly(I:C) than that induced by LPS, with 15.4 ± 6.9-fold vs 3.8 ± 1.7-fold increases over the medium control, respectively (Fig. 1*A*). In addition to stimulating HMGB1 release, both poly(I:C) and LPS induced TNF-α and NO production in RAW 264.7 cells (Fig. 1*C*).

As shown previously, TNF-α promotes the induction of HMGB1 release induced by LPS in RAW 264.7 cells (21). Confirming previous studies, we showed that neutralizing TNF-α by Abs reduced HMGB1 release induced by LPS, but not by poly(I:C) (Fig. 2*A*). Control goat IgG did not affect HMGB1 release induced by LPS or poly(I:C) (Fig. 2*B*). We therefore assessed the effect of other mediators.

To evaluate the role of NO in HMGB1 release, the specific inducible NO synthase (iNOS) inhibitor 1400W was used. As shown in Fig. 3*A*, the addition of 1400W in RAW 264.7 cell cultures inhibited NO production induced by either LPS or poly(I:C) in a dose-dependent manner. The addition of 1400W also reduced HMGB1 release induced by LPS, whereas HMGB1 release induced by poly(I:C) was not affected (Fig. 3*B*). 1400W alone did not result in HMGB1 release in RAW 264.7 cells (data not shown). Another iNOS inhibitor, L-NIL, also caused a dose-dependent inhibition of HMGB1 release by LPS, although the dose of L-NIL required for such effects was much higher (2 mM) than that used for 1400W; the extent of inhibition was also less (data not shown).

To evaluate further the effects of NO on HMGB1 release, we added an NO donor, NOC-18, to RAW 264.7 cultures and measured HMGB1 release in the medium by Western blotting. As shown in Fig. 3*C*, NOC18 dose dependently induced the release of HMGB1 by RAW 264.7 cells. These results are consistent with a role of NO in HMGB1 release, although its extent may depend on the stimulus for cell activation.

The effects of IFN-α on HMGB1 release

In in vitro studies, IFN-γ, a type II IFN, induced HMGB1 release by RAW 264.7 cells (8). The role of IFN-α, a type I IFN, on HMGB1 release was therefore studied. In RAW 264.7 cells, poly(I:C) induced the production of IFN-α, whereas LPS did not (Fig. 4*A*). To evaluate the role of IFN-α in HMGB1 release, we assessed the effects of a neutralizing Ab to IFN-α in RAW 264.7

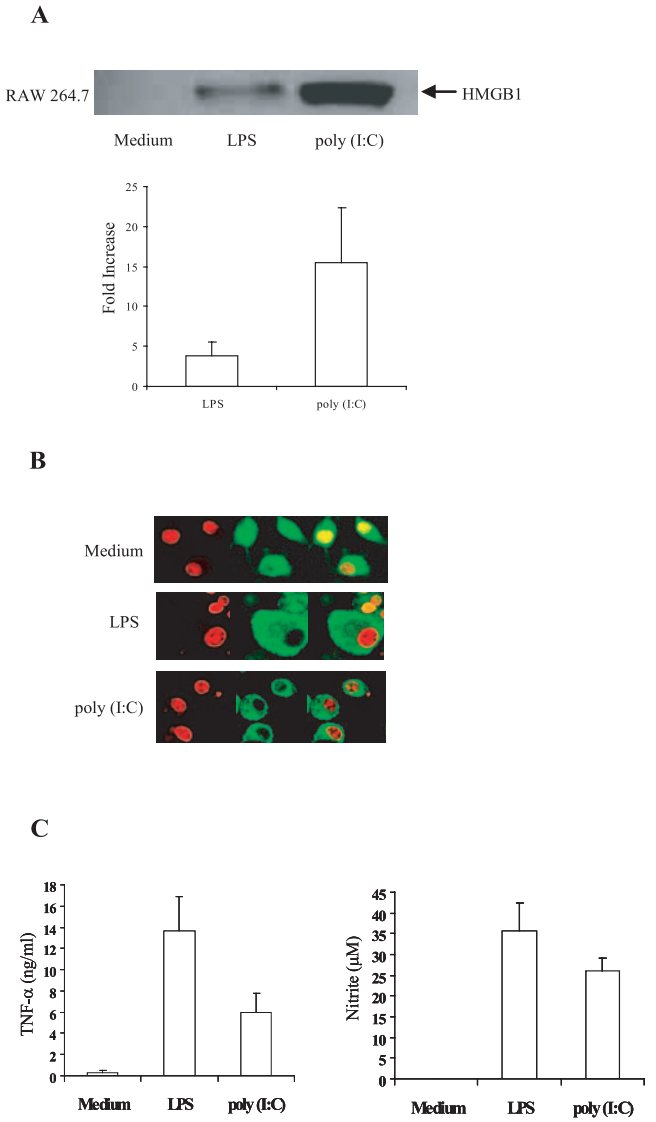

FIGURE 1. The release of HMGB1 by poly(I:C) or LPS. RAW 264.7 cells were stimulated with poly(I:C) (0.25 μg/ml) or LPS (0.5 μg/ml) for 20–24 h. HMGB1 in culture supernatants was detected by Western blotting, and the density of the HMGB1 band was expressed as fold increase over medium control (*A*). HMGB1 translocation was visualized by confocal imaging (*B*) using anti-HMGB1 Ab (green); nuclei were counterstained with DRAQ5 (red). TNF-α and NO levels in RAW 264.7 cell culture supernatant were also determined (*C*).

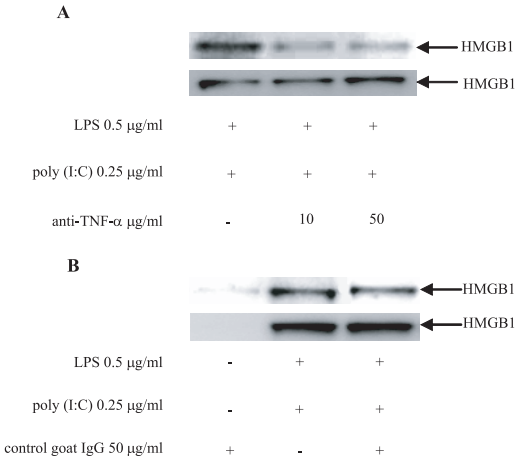

FIGURE 2. The role of TNF-α in HMGB1 release induced by poly(I:C) or LPS. RAW 264.7 cells were stimulated with LPS or poly(I:C) with or without anti-TNF-α Ab for 20 h, and supernatant HMGB1 was measured by Western blotting (*A*). Goat IgG served as a control with or without LPS or poly(I:C) stimulation (*B*).

cell cultures stimulated with LPS or poly(I:C). As shown in Fig. 4*B*, the neutralizing Ab to IFN-α dose dependently inhibited HMGB1 release by RAW 264.7 cells stimulated by poly(I:C), but not LPS. Control rat IgG did not affect HMGB1 release induced by poly(I:C).

To evaluate further the role of IFN-α in HMGB1 release, mouse rIFN-αA was added into RAW 264.7 cell cultures and HMGB1 in the medium was measured. As shown in Fig. 4*C*, rIFN-αA dose dependently induced the release of HMGB1 in RAW 264.7 cell cultures. The increased release of HMGB1 induced by rIFN-αA was not due to endotoxin as the addition of polymixin B did not inhibit the release of HMGB1 (Fig. 3*C*). These findings indicate a direct role of IFN-α in HMGB1 release.

The effects of inhibiting JNK on HMGB1 release

Although TLR ligands can lead to different patterns of responses, they nevertheless induce similar downstream signaling pathways that involve MyD88, IL-1R-associated kinase, and further activation of MAPKs and NF-κB (22). To assess the signaling pathways induced by LPS and poly(I:C), the effects of MAPK activation was investigated. As results shown in Fig. 5*A* indicate, both LPS and poly(I:C) induced the activation of all three MAPKs (p38, ERK, and JNK).

Because MAPK activation is required for the production of mediators such as TNF-α, IL-6, IFN-γ, and NO (23–26), we therefore investigated the role of MAPKs in the release of HMGB1. For this purpose, we used specific MAPK inhibitors and measured HMGB1 release by Western blotting. As shown in Fig. 5*B*, the inhibition of p38 and MEK activation did not affect HMGB1 release induced by either LPS or poly(I:C). In contrast, inhibition of JNK activation blocked HMGB1 release induced by either LPS or poly(I:C).

To assess inhibition of MAPKs under these conditions, cells were preincubated with various MAPKs inhibitors, followed by stimulation with LPS or poly(I:C). Cellular proteins were isolated and blotted against Abs specific to activated MAPKs. As shown in Fig. 5*C*, specific MAPK inhibitors inhibited MAPK activation induced by either LPS or poly(I:C). JNK inhibition also reduced IFN-α and NO levels in RAW 264.7 cells stimulated with poly(I:C) or LPS (Fig. 6). Together, these findings point to a predominant role of JNK activation in the release of HMGB1 induced by LPS and poly(I:C).

To assess whether primary macrophages function in a similar manner as do the RAW 264.7 cells, BMMCs were stimulated with LPS or poly(I:C) and supernatant levels of HMGB1 were determined from the density of HMGB1 on Western blots. Similar to results with RAW 264.7 cells, poly(I:C) induced more HMGB1 release from BMMCs than did LPS, with an 8.4 $\pm$ 2.5- vs 1.6 $\pm$ 0.7-fold increase over medium control, respectively (Fig. 7*A*).

Confocal imaging also confirmed the translocation of HMGB1 from the nucleus (Fig. 7*B*). It is of note that, following culture, the macrophages (both RAW 264.7 cells and BMMCs) stimulated by poly(I:C) appeared to be smaller than those stimulated by LPS. The differences in macrophage morphology may relate to the pattern of cytokines and other mediators induced as well as downstream signaling pathways that are operative. In these experiments, the staining of HMGB1 in BMMCs was primarily localized in the nucleus, although the translocation of HMGB1 did not appear as

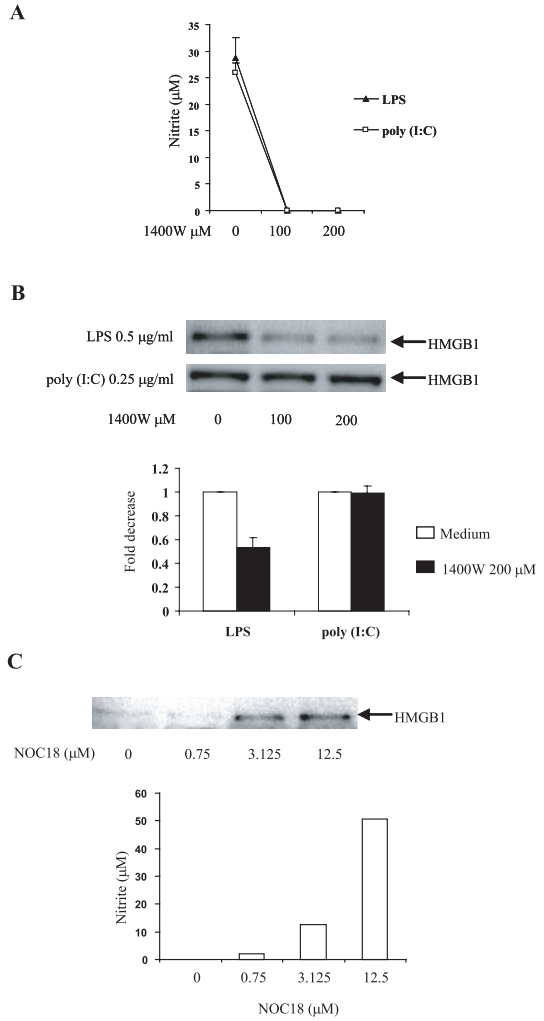

FIGURE 3. The role of NO in HMGB1 release induced by poly(I:C) or LPS. For iNOS inhibition, RAW 264.7 cells were preincubated with 1400W for 30 min and subsequently stimulated with either LPS or poly(I:C) for 20 h. Nitrite and HMGB1 levels in supernatants were assayed, and fold decrease in HMGB1 levels was determined by densitometry (*A* and *B*). The NO donor, NOC18, was added to RAW 264.7 cells and, after 24 h, supernatant HMGB1 levels were assayed (*C*).

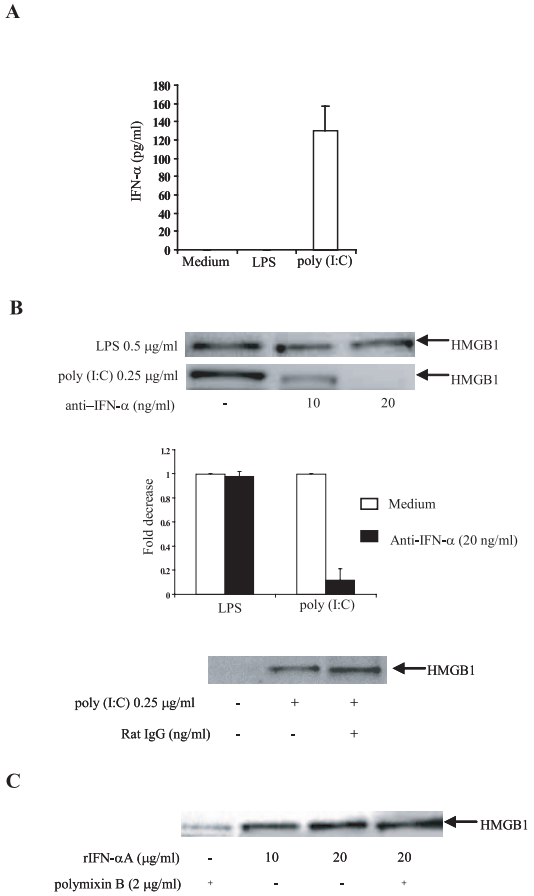

FIGURE 4. The role of IFN-α in HMGB1 release induced by poly(I:C) or LPS. RAW 264.7 cells were stimulated with LPS (0.5 μg/ml) or poly(I:C) (0.25 μg/ml) for 24 h and supernatants were collected for measurement of IFN-α by ELISA (*A*). RAW 264.7 cells were preincubated with anti-IFN-α Ab, followed by stimulation with either LPS or poly(I:C) for 20 h. A rat IgG control was included with or without poly(I:C) stimulation. Supernatant HMGB1 levels were measured by Western blotting and quantified by densitometry (*B*). rIFN-αA was added to RAW 264.7 cells, with or without polymixin B; supernatant was collected and assayed for HMGB1 (*C*).

complete as that observed in RAW 264.7 cells. In addition, HMGB1 release by BMMCs was delayed when compared with that of RAW 264.7 cells.

For inhibition assays, BMMCs were stimulated with LPS or poly(I:C) in the presence of 1400W, anti-IFN-α, or the JNK inhibitor, and HMGB1 levels in the supernatants were measured. As results in Fig. 7*C* show, HMGB1 release even in the absence of stimulation was observed at levels that exceeded those of RAW 264.7 cells; this release may be related to the culture conditions and the background rate of cell death. Nevertheless, both LPS and poly(I:C) increased these levels. With LPS, HMGB1 levels were reduced by 1400W, but not by anti-IFN-α. In contrast, with cells stimulated by poly(I:C), anti-IFN-α, but not 1400W, inhibited HMGB1 release. For both stimuli, JNK inhibition reduced HMGB1 levels. These results thus indicate that the response of primary macrophages is similar to those of RAW 264.7 cells, pointing to a common mechanism for HMGB1 release.

Discussion

Results of this study clarify the mechanisms by which TLR ligands induce HMGB1 release from macrophages and indicate that inflammatory mediators can differentially regulate this process. Thus, we have shown that IFN-α and NO play distinct roles in the release of HMGB1 from RAW 264.7 cells as well as BMMCs stimulated by either poly(I:C) or LPS, ligands of TLR3 or 4, respectively. Nevertheless, for both stimuli, inhibition of JNK activation reduced HMGB1 release. Together, these results suggest distinct pathways for HMGB1 release and the role of various mediators in the key processes by which HMGB1 translocates from the nucleus for secretion into the extracellular milieu.

Of small molecule mediators of inflammation, NO, a free radical gas, is critical to innate immunity, with stimulation of macrophages by TLR ligands leading to up-regulation of iNOS expression and production of NO. NO displays a host of activities in inflammation as well as processes such as apoptosis and the regulation of vascular tone. In sepsis, NO production is highly induced under conditions in which serum levels of HMGB1 are also elevated (27). Although these correlations, as well as the kinetics of NO production and HMGB1 release in vitro (i.e., late appearance of both), could suggest a role of NO in HMGB1 release, this step of the inflammatory cascade had not previously been investigated.

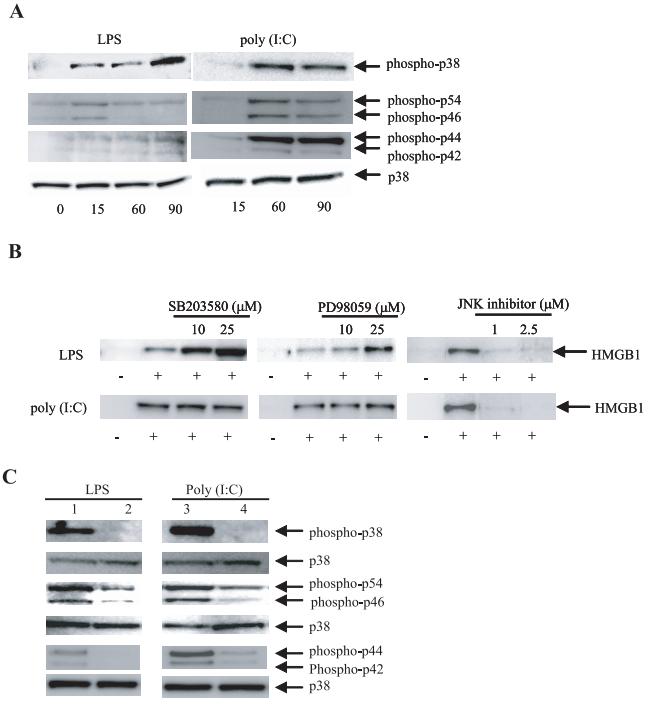

FIGURE 5. The role of JNK activation in HMGB1 release induced by poly(I:C) or LPS. RAW 264.7 cells were stimulated with either LPS (0.5 μg/ml) or poly(I:C) (0.25 μg/ml) for 0, 15, 60, and 90 min. Cellular proteins were extracted and assayed for MAPK activation (*A*). Total p38 levels served as control for protein equal loading. For inhibition assays, RAW 264.7 cells were preincubated with different MAPK inhibitors, followed by stimulation with LPS or poly(I:C) for 20 h. HMGB1 level in supernatants were determined by Western blotting (*B*). To demonstrate inhibition of MAPK activation, RAW 264.7 cells were pretreated with various specific MAPK inhibitors for 30 min, followed by 60-min stimulation with either LPS or poly(I:C). Cellular proteins were then extracted and assayed for MAPK inhibition (*C*: *lane 1*, 0.5 μg/ml LPS; *lane 2*, LPS with various MAPK inhibitors; *lane 3*, 0.25 μg/ml poly(I:C); and *lane 4*, poly(I:C) with various MAPK inhibitors). Cellular p38 levels were used as protein loading control.

The results presented in this work show that NO induces the release of HMGB1 from RAW 264.7 cells and that inhibiting iNOS attenuates HMGB1 release induced by LPS. With respect to

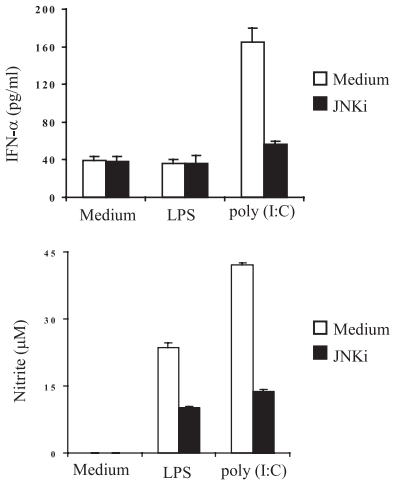

FIGURE 6. The effects of JNK inhibition on IFN-α and NO production induced by poly(I:C) or LPS. RAW 264.7 cells were pretreated with JNK inhibitor and subsequently stimulated with poly(I:C) or LPS for 20 h. Supernatants were collected and assayed for IFN-α and NO.

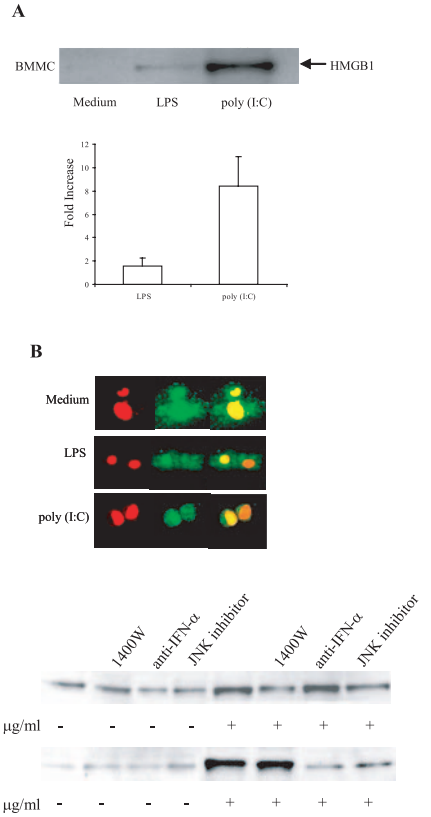

FIGURE 7. The effects of 1400W, anti-IFN-α, and JNK inhibition on HMGB1 release induced by LPS or poly(I:C) in murine BMMCs. BMMCs were stimulated with poly(I:C) (10 μg/ml) or LPS (1 μg/ml) for 24 h. HMGB1 levels in culture supernatants were detected by Western blotting, and the density of the HMGB1 band was expressed as fold increase over medium control (*A*). HMGB1 translocation was visualized by confocal imaging (*B*) using anti-HMGB1 Ab (green); nuclei were counterstained with DRAQ5 (red). BMMCs were preincubated with either 1400W, anti-IFN-α, or JNK inhibitor for 30 min and then stimulated with LPS or poly(I:C) for 24 h. Supernatants were collected and HMGB1 levels were measured by Western blotting (*C*). Results are representative of three experiments performed at separate times.

immune changes induced in sepsis, it is possible that HMGB1 acts as a downstream effector of NO, with selective inhibition of iNOS attenuating the HMGB1 release. In addition to NO, other mediators induced by LPS such as TNF-α may induce HMGB1 release. In our study, in agreement with a previous report, neutralizing TNF-α inhibited HMGB1 release (21).

Interestingly, despite the effects of the 1400W on HMGB1 release induced by LPS, the inhibition of iNOS did not affect the release of HMGB1 induced by poly(I:C). These findings suggest that other mediators or pathways induced by poly(I:C) modulate the effects of NO. Although LPS and poly(I:C) use similar signaling pathways, significant differences in the outcome of stimulation exist. Among these, the robust production of IFN-α, a type I IFN, is a prominent feature of activation by dsRNA and other viral products. IFN-α, mainly produced by plasmacytoid dendritic cells (pDCs) in humans (28), can prime the immune system for subsequent stimulation, with IFN-α augmenting responses to IFN-γ and IL-6 (29, 30). IFN-α also exerts a positive feedback autocrine loop (31) and can induce the maturation of dendritic cells (32, 33).

In this study, we showed that IFN-α can also induce the active release of HMGB1 in vitro. The effect of IFN-α may explain the differential effects of CpG DNA on HMGB1 release by macrophages and pDCs. In a previous study, we reported that CpG DNA did not induce HMGB1 release by RAW 264.7 cells (19). In

contrast, other investigators have shown that CpG DNA can induce human pDCs to release HMGB1 (13). The differences in the effects of CpG DNA may be related to its ability to induce IFN-α in the two cell types, with the IFN-α promoting HMGB1 release in the pDCs. The inhibition of HMGB1 release induced by poly(I:C) by neutralizing IFN-α further supports a role of this cytokine in active HMGB1 release.

Similar to the results of other investigators (34), we have found that poly(I:C), but not LPS, induces IFN-α production in RAW 264.7 macrophages. Furthermore, we have observed that, although both LPS and poly(I:C) induce the production of IFN-β, the amount induced by poly(I:C) is significantly greater. Interestingly, in these experiments, an Ab to IFN-β could reduce HMGB1 release with either stimulant (W. Jiang and D. S. Pisetsky, unpublished observations). The effects on HGMB1 release induced by LPS may result from the inhibition of NO production, however, because, as other investigators have shown, in murine macrophages, NO production is dependent on IFN-β (35). In this regard, CpG DNA, which does not cause HMGB1 release, induces very little IFN-β production by RAW 264.7 cells (W. Jiang, unpublished observations). Together, these observations suggest that differences in the stimulation of HMGB1 release by TLR ligands may relate to the pattern and amount of type I IFN production induced. These studies are ongoing.

In addition to the mediators induced, TLR ligands may differ in their stimulation of downstream signaling pathways that can affect cell function and activation. Thus, CpG DNA stimulation depends on MyD88, whereas LPS has both MyD88 and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathways (36–38). Poly(I:C) also depends on TRIF, although it may induce other downstream pathways (39). It is of interest that TLR ligands (i.e., LPS and poly(I:C)) that induce HMGB1 release activate TRIF, whereas CpG DNA, which does not have TRIF-dependent activation, fails to induce this release. These observations suggest the ability of TLR ligands to induce HMGB1 release in macrophages may depend on TRIF activation.

Among TLR ligands, both LPS and dsRNA activate signaling pathways, including MAPKs. MAPKs are important regulators of inflammation (23, 24, 26, 40–42), with inhibitors of these pathways modulating the pathogenesis of inflammatory diseases in experimental models (43). Among its effects, JNK activation plays an important role in iNOS expression induced by LPS, IFN-γ, and TNF-α (24, 40, 44). In our study, both LPS and poly(I:C) activated JNK and induced the production of NO in RAW 264.7 cells. Although JNK inhibition blocked HMGB1 release induced by LPS and poly(I:C), blocking p38 and ERK activation did not inhibit this process. These results suggest that p38 and ERK1/2 may not be involved in HMGB1 release induced by LPS or poly(I:C); this finding is in agreement with a previous study on HMGB1 release induced by LPS (21).

It is of note that, in our experiments, inhibition of p38 and ERK1/2 led to higher levels of HMGB1 release in cells stimulated by LPS. The basis of this effect is not known, although an increase in HMGB1 release at the higher doses of the inhibitors used might result from cytotoxicity and the release of HMGB1 during cell death. In addition, cross-talk between signaling pathways may also contribute to the increased levels of HMGB1 seen after inhibiting p38 and ERK activation. Although the interplay of these pathways requires further investigation, these results indicate the operation, in a stimulus-dependent manner, of NO and IFN-α in HMGB1 release.

In these experiments, poly(I:C) has been studied as a model for a viral molecule that could elicit responses during infection. To determine whether such release in fact occurs during infection, we investigated in preliminary experiments the presence of extracellular HMGB1 in cultures of RAW 264.7 cells infected with mouse CMV (MCMV). MCMV is a DNA virus, although, similar to the situation with other viruses, dsRNA may play a role in its life cycle; indeed, both TLR3 and TLR9 mediate the host response to MCMV (45). Results of these experiments indicated that during infection with MCMV, HMGB1 release from cells occurs depending on the multiplicity of infection (W. Jiang and D. S. Pisetsky, unpublished observations). Similar results have been obtained by other investigators with West Nile virus and salmon anemia virus infection (46, 47). Although virus infection is a complicated event and can involve both apoptotic and necrotic death, our results nevertheless indicate that HMGB1 release may occur during viral exposure, supporting the use of dsRNA as a model.

Our studies may also have relevance to immune responses occurring with the use of small inhibitory RNA (siRNA). siRNAs are small duplex RNA molecules that can block target gene expression at the level of mRNA and, as such, represent novel agents for modifying gene expression both in vitro and in vivo. Although siRNA are designed for high specificity, several reports have demonstrated cell activation by these compounds (48–50). If siRNA, like dsRNA, causes HMGB1 release, its use may be complicated by the effects of HMGB1 and its downstream mediators. Future experiments will define further the range of conditions in which HMGB1 release occurs and the optimal approaches for blocking this mediator in the setting of inflammatory disease.

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Disclosures

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