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# Bacterial Endotoxin Induces the Release of High Mobility Group Box 1 via the IFN- $\beta$ Signaling Pathway<sup>1</sup>

Ju-Hyun Kim,\*<sup>†</sup> Seon-Ju Kim,\*<sup>†</sup> Im-Soon Lee,<sup>‡</sup> Myung-Shik Lee,<sup>§</sup> Satoshi Uematsu,<sup>¶</sup> Shizuo Akira,<sup>¶</sup> and Kwon Ik Oh<sup>2\*†</sup>

Sepsis is a devastating condition characterized by a systemic inflammatory response. Recently, high mobility group box 1 (HMGB1) was identified as a necessary and sufficient mediator of the lethal systemic inflammation caused by sepsis. However, despite its clinical importance, the mechanism of HMGB1 release has remained to be elusive. In this study, we demonstrate that the IFN- $\beta$ -mediated JAK/STAT pathway is essential for LPS or *Escherichia coli*-induced HMGB1 release, which is dependent on Toll/IL-1R domain-containing adaptor-inducing IFN- $\beta$  adaptor. Additionally, we show that NO acts as a downstream molecule of the IFN- $\beta$  signaling. Furthermore, the JAK inhibitor treatment as well as the STAT-1 or IFN- $\beta$  receptor deficiency reduced HMGB1 release in a murine model of endotoxemia. Our results suggest that HMGB1 release in sepsis is dependent on the IFN- $\beta$  signaling axis; thus, therapeutic agents that selectively inhibit IFN- $\beta$  signaling could be beneficial in the treatment of sepsis. *The Journal of Immunology*, 2009, 182: 2458–2466.

Sepsis is a major cause of death in intensive care units, accounting for over 200,000 deaths per year in the U.S. alone. Despite the recent advances in intensive care treatment and the discovery of antibiotics, sepsis remains associated with a high mortality rate (1, 2). Sepsis is commonly initiated by an infection, but its pathogenesis is characterized by an overwhelming systemic inflammatory response and subsequent immune dysfunction, which can lead to lethal multiple organ failure (3). Endotoxin and other pathogen components stimulate macrophages to produce proinflammatory cytokines, including TNF, which play roles as mediators in inflammatory responses and organ injuries. However, although TNF has been known as a main cause of the lethal systemic inflammation, as shown in the TNF neutralization experiment that prevented the development of septic shock in animal models, TNF-blocking therapy demonstrated only partial clinical effects in seriously ill patients, implying other mediators involved in the pathogenesis of sepsis (4).

Recently, it has been reported that another late proinflammatory mediator, high mobility group box 1 (HMGB1),<sup>3</sup> may contribute to the progression of sepsis (5). HMGB1 was initially described as a nuclear nonhistone DNA-binding protein that functions as a struc-

tural cofactor critical for proper transcriptional regulation (6). It also facilitates numerous nuclear events, including replication, recombination, and DNA transposition (7). Recently, it has been reported that HMGB1 is secreted into the extracellular milieu by activated macrophages, and identified as a late mediator of lethal systemic inflammation in sepsis (8). Exposure to HMGB1 leads to various cellular responses, including chemotaxis (9, 10), increased permeability of cell monolayer (11), and the release of various proinflammatory cytokines such as TNF, IL-1, IL-6, and MIP-1 (12).

In contrast to the clarity of the functions of HMGB1, the mechanisms by which macrophages release HMGB1 in the presence of LPS have been controversial. In the stimulated macrophages with LPS, the release of HMGB1 is an active, but delayed response, involving the hyperacetylation, the phosphorylation, the translocation from the nucleus to secretory lysosomes, and eventually the extracellular release of the molecule (13, 14). Some proinflammatory cytokines induced by LPS, such as TNF and IFN- $\gamma$ , can activate macrophages to secrete HMGB1 even in the absence of LPS (8, 15). In addition, the inhibition of HMGB1 secretion by various methods is usually accompanied with down-regulation of TNF or IFN- $\gamma$  (16–19). Based on the above findings, it could be speculated that the LPS-induced early inflammatory cytokines may function as intermediating molecules in HMGB1 secretion through enhancing the hyperacetylation and phosphorylation of HMGB1. In contrast, several other reports showed that the inhibition of the early inflammatory cytokines or LPS signaling molecules did not significantly affect HMGB1 release (19, 20). Thus, for the complete understanding of the HMGB1 release, the identification of additional molecules that are closely related to the event, but to date elusive, is necessary.

In this study, we have investigated crucial signal pathways related to the HMGB1 release induced by LPS or live bacteria, and demonstrated that LPS exploited TLR4/IFN- $\beta$ /STAT-1 signaling axis to induce HMGB1 release. Furthermore, NO plays a role as a downstream molecule of the IFN- $\beta$  signaling. Our study suggests that various agents targeting IFN- $\beta$  signaling could be potential candidates for the treatment of lethal systemic inflammation caused by sepsis.

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<sup>3</sup> Abbreviations used in this paper: HMGB1, high mobility group box 1; CaMK, calcium/calmodulin-dependent protein kinase; poly(I:C), polyinosinic-polycytidylic acid; TRIF, Toll/IL-1R domain-containing adaptor-inducing IFN- $\beta$ .

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## Materials and Methods

### Cells and reagents

A mouse macrophage cell line, RAW 264.7 (American Type Culture Collection), was maintained in DMEM supplemented with 10% FBS and 0.1% penicillin/streptomycin (Invitrogen Korea). LPS (O55:B5) and polyinosinic-polycytidylic acid (poly(I:C)) were purchased from Sigma-Aldrich. JAK inhibitor, pyridone 6 (21), was obtained from Calbiochem. IFN- $\beta$  was purchased from PBL IFN Source. The following Abs were used in this study: anti-HMGB1 (Abcam or R&D Systems), anti-IFN- $\beta$  (clone 7F-D3; Abcam), anti-TNF (clone G281-2626; BD Biosciences), anti-phospho-STAT1, anti-phospho-JNK (Cell Signaling Technology), and anti-STAT1 (Santa Cruz Biotechnology).

### Mice

IFN- $\beta$  receptor<sup>-/-</sup> (IFNAR<sup>-/-</sup>, 129 background) and 129Sv/Ev control mice were obtained from B&K Universal. MyD88<sup>-/-</sup> mice were purchased from Oriental BioService. MyD88<sup>-/-</sup>, Toll/IL-1R domain-containing adaptor-inducing IFN- $\beta$  (TRIF)<sup>-/-</sup>, and STAT1<sup>-/-</sup> (22) mice were C57BL/6 (B6) background. All mice were housed under specific pathogen-free conditions at the animal facility of Hallym University College of Medicine. Experiments were performed after the approval of the Animal Experimentation Committee at Hallym University.

### Macrophage isolation

Macrophages were obtained from the peritoneal cavity under thioglycolate-induced inflammatory conditions by peritoneal lavage. The cells were rested for attachment on the plate, and then stimulated for the indicated periods.

### Measurement of cytokines

Concentrations of TNF, IL-6, and IFN- $\beta$  were measured using ELISA kits (BD Biosciences or PBL IFN Source), according to the manufacturer's instructions. The level of HMGB1 was determined by Western blotting using anti-HMGB1 Abs, and quantified by densitometric scanning of the exposed x-ray film. Relative densitometric value of HMGB1 of each sample shown as arbitrary units in the figures was calculated by arbitrarily assigning that of control (a LPS (or *Escherichia coli*)-treated sample) as 1 (16).

### Western blotting

Samples of peritoneal lavage fluid or culture supernatant were concentrated with centricon (Milipore) and then separated on 12% SDS-PAGE gels, transferred to nitrocellulose membranes. The membranes were probed with anti-HMGB1 Abs, and subsequently incubated with a HRP-conjugated secondary Ab. Bands were detected using SuperSignal West Femto kit (Pierce).

### Preparation of pervanadate

Pervanadate stock solution (1 mM) was prepared by adding 10  $\mu$ l of 100 mM vanadate and 50  $\mu$ l of 100 mM hydrogen peroxide to 940  $\mu$ l of water. Excess hydrogen peroxide was removed by adding catalase 5 min after mixing the vanadate and hydrogen peroxide. The pervanadate solutions were used within 5 min to minimize decomposition of the vanadate-hydrogen peroxide complex (23, 24).

### NO assay

NO concentrations were measured in cell culture supernatants using Griess system (Promega), according to the manufacturer's instructions.

### Statistical analysis

All values in the figures and the text are expressed as mean  $\pm$  SEM, and  $p < 0.05$  was considered to be statistically significant. Survival data were analyzed by the log-rank test, and all other data were evaluated by unpaired Student's  $t$  test (nonparametric, two-tailed).

## Results

### TLR4 and JAK molecules are indispensable to HMGB1 secretion

To evaluate a potential role of TLR4 in the LPS-induced HMGB1 release, peritoneal macrophages from wild-type and *Tlr4* mutant C3H/HeJ mice were stimulated with LPS, and the levels of HMGB1 in the culture supernatants were compared. In contrast to the poly(I:C) stimulation ( $p = 0.3649$ ), HMGB1 was barely de-

tected in the *Tlr4* mutant macrophages treated by LPS (Fig. 1A), which suggests that LPS induces HMGB1 release via TLR4. To further investigate signaling pathways associated with this event, macrophage-like RAW 264.7 cells were treated with various protein inhibitors at 30 min before LPS stimulation, and the changes in the levels of HMGB1 release were examined. The inhibition of MAPKs (PD98059, SB202190, and U0126) or NF- $\kappa$ B (MG132) did not affect the HMGB1 release. Interestingly, however, the inhibition of JAK (pyridone 6) and PI3K (LY294002) significantly attenuated the HMGB1 release induced by LPS (Fig. 1B), clearly indicating positive roles of JAK and PI3K activities in this process.

TLR4 uses two different adaptors, the MyD88 and the TRIF, and cytokines can be induced in MyD88- or TRIF-deficient mice in the presence of LPS albeit at low levels, depending on activated pathways. Thus, by stimulating macrophages from MyD88- or TRIF-deficient mice with LPS, we tested which one of the two adaptors, MyD88 and TRIF, is involved in the HMGB1 release. Interestingly, the amounts of extracellular HMGB1 in the TRIF<sup>-/-</sup> macrophages were significantly reduced (Fig. 1C), whereas those in the MyD88<sup>-/-</sup> macrophages were increased (Fig. 1D). As expected, the level of TNF that is known to be stimulated by LPS through the MyD88 adaptor was greatly lower in the MyD88<sup>-/-</sup> macrophages (Fig. 1E).

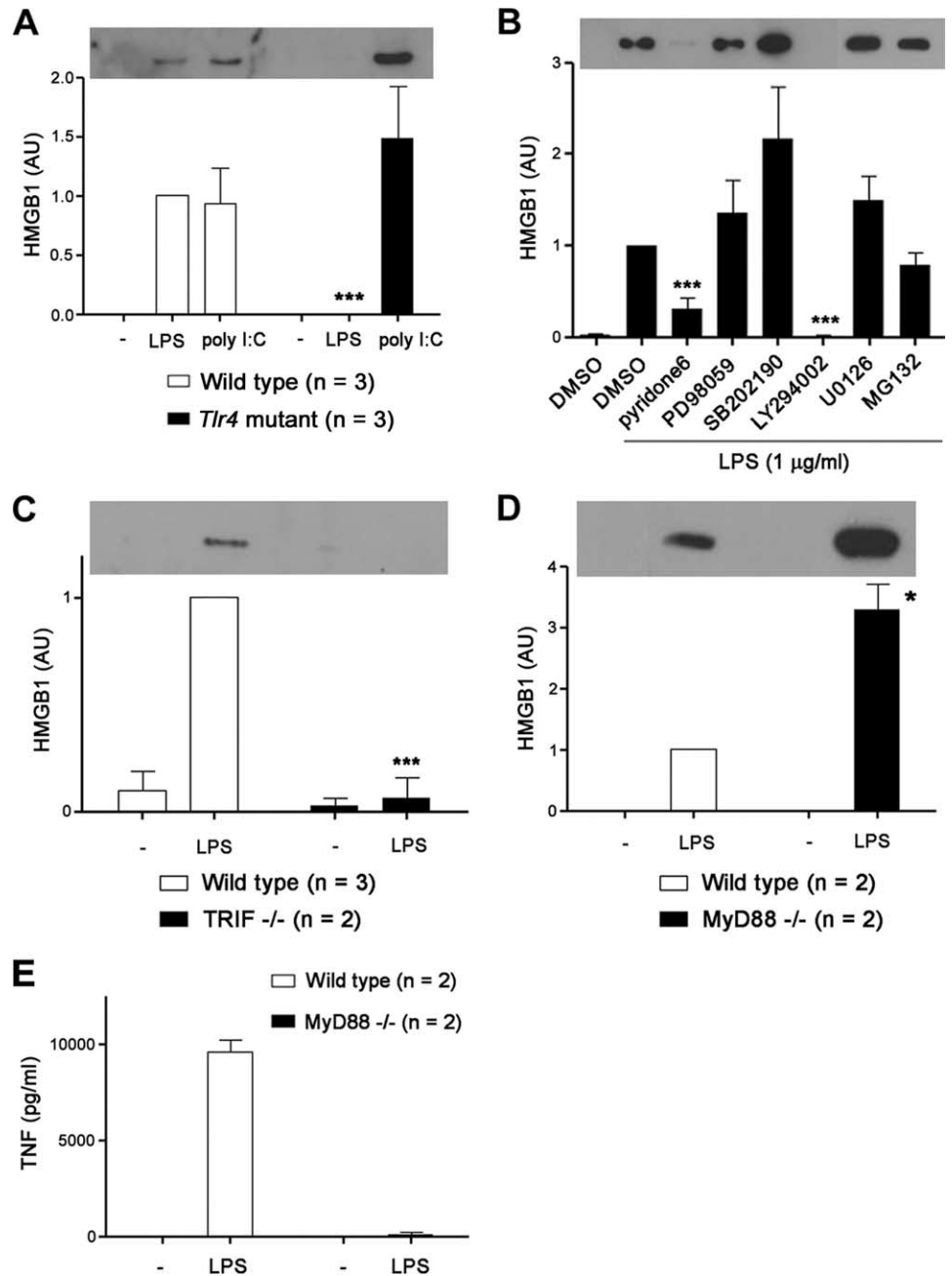
### IFN- $\beta$ signaling is essential for HMGB1 secretion

To dissect a role of JAK in the HMGB1 release, we treated both primary macrophages and RAW cells with various doses of pyridone 6, and examined the changes in the HMGB1 secretion. As demonstrated in the above data, the treatment of pyridone 6 inhibited the HMGB1 release in a dose-dependent manner in both cells (Fig. 2, A and B). Because TLR4 signaling by LPS results in activation of the JAK-STAT signaling pathway, which is independent of MyD88 and secondary to the action of IFN- $\beta$  (25), we tested whether IFN- $\beta$  is essential to the LPS-induced HMGB1 secretion. In both of the macrophages and the RAW cells treated with LPS, anti-IFN- $\beta$  Ab reduced extracellular HMGB1 release in a dose-dependent manner (Fig. 2, C and D). Because it has been controversial whether TNF is involved in LPS-induced HMGB1 release (20), we also investigated the role of TNF using a neutralizing Ab. In contrast to IFN- $\beta$ , any significant changes in the HMGB1 levels by addition of anti-TNF Ab were not observed (Fig. 2C).

Although the results of the above experiments strongly imply the importance of IFN- $\beta$  signaling pathway in the HMGB1 release in the LPS-treated cells, other bacterial molecules may affect HMGB1 release through pattern recognition receptors different from TLR4 in clinical settings. In addition, it was reported that TLR4<sup>-/-</sup> mice compared with wild-type mice did not show any difference of mortality in the polymicrobial sepsis model (26). Therefore, we examined whether IFN- $\beta$  signaling molecules are also essential in *E. coli*-induced HMGB1 release. We blocked IFN- $\beta$  signals using anti-IFN- $\beta$  Ab or a chemical inhibitor, pyridone 6, and measured HMGB1 after overnight incubation. Surprisingly, the levels of HMGB1 were significantly reduced in all conditions (Fig. 2E). To compare the signaling mechanisms associated with the HMGB1 release induced by *E. coli* with LPS, we treated primary macrophages with *E. coli* in the presence of several chemicals previously reported to inhibit the LPS-induced HMGB1 release (16, 19, 27) and found similar signaling pathways can be exploited by both LPS and *E. coli* (K. Oh and J. Kim, unpublished observation).

Next, we examined the role of STAT-1 in the HMGB1 release using peritoneal macrophages from STAT-1<sup>-/-</sup> mice. Upon treatment of LPS or *E. coli*, the HMGB1 release was

**FIGURE 1.** TLR4 and JAK molecules are indispensable to HMGB1 secretion. *A*, Peritoneal macrophages from wild-type and *Tlr4* mutant mice are incubated with LPS (1  $\mu\text{g/ml}$ ) or poly(I:C) (50  $\mu\text{g/ml}$ ) overnight, and the levels of HMGB1 in supernatants were measured by Western blotting. The relative densitometric values of HMGB1 bands are shown as arbitrary units (AU). Densitometric value from the LPS-treated wild-type macrophages was taken as 1. *B*, RAW cells were preincubated with the indicated inhibitor for 30 min and then treated with LPS (1  $\mu\text{g/ml}$ ) for overnight. The supernatants were concentrated with centricon, and the HMGB1 levels were measured by Western blotting ( $n = 3$ ). *C* and *D*, Peritoneal macrophages isolated from B6 wild-type and *TRIF*<sup>-/-</sup> (*C*) or *MyD88*<sup>-/-</sup> (*D*) mice were exposed to LPS (1  $\mu\text{g/ml}$ ) overnight. Supernatants were concentrated and analyzed for HMGB1. Graphs show the relative changes in the levels of HMGB1, as determined by densitometric analysis. The relative densities of the HMGB1 bands were analyzed the same as in *A*, and the mean values  $\pm$  SEM fold changes relative to the control (the LPS-treated sample) are shown. *E*, For the control, the levels of TNF of wild-type B6 and *MyD88*<sup>-/-</sup> macrophages were measured by ELISA. AU, arbitrary unit. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  compared with LPS-treated control samples (LPS-treated wild-type macrophages (*A*, *C*, and *D*) or RAW cells (*B*)).



completely blocked in the *STAT-1*<sup>-/-</sup> macrophages compared with the macrophages from wild-type mice (Fig. 3A). In addition, when we treated the wild-type macrophages with sodium pervanadate to enhance tyrosine phosphorylation of *STAT-1*, the amount of extracellular HMGB1 was dramatically increased (Fig. 3B). In contrast, the levels of TNF were not dependent on *STAT-1* (Fig. 3C).

The findings that the PI3K inhibitor (LY294002) as well as pyridone 6 inhibited LPS-induced HMGB1 release (Fig. 1B) and that IFN- $\beta$  was a principal mediator in the HMGB1 release event (Fig. 2) led us to examine whether LY294002 reduced HMGB1 level via IFN- $\beta$  signaling. First, we treated RAW cells with LY294002 plus LPS and measured the levels of IFN- $\beta$ . Interestingly, LY294002 inhibited IFN- $\beta$  production (Fig. 4A), and subsequently reduced the tyrosine phosphorylation of *STAT-1* (Fig. 4B) in a dose-dependent manner. To examine whether LY294002 also attenuates the downstream signaling of IFN- $\beta$ , we added IFN- $\beta$  pro-

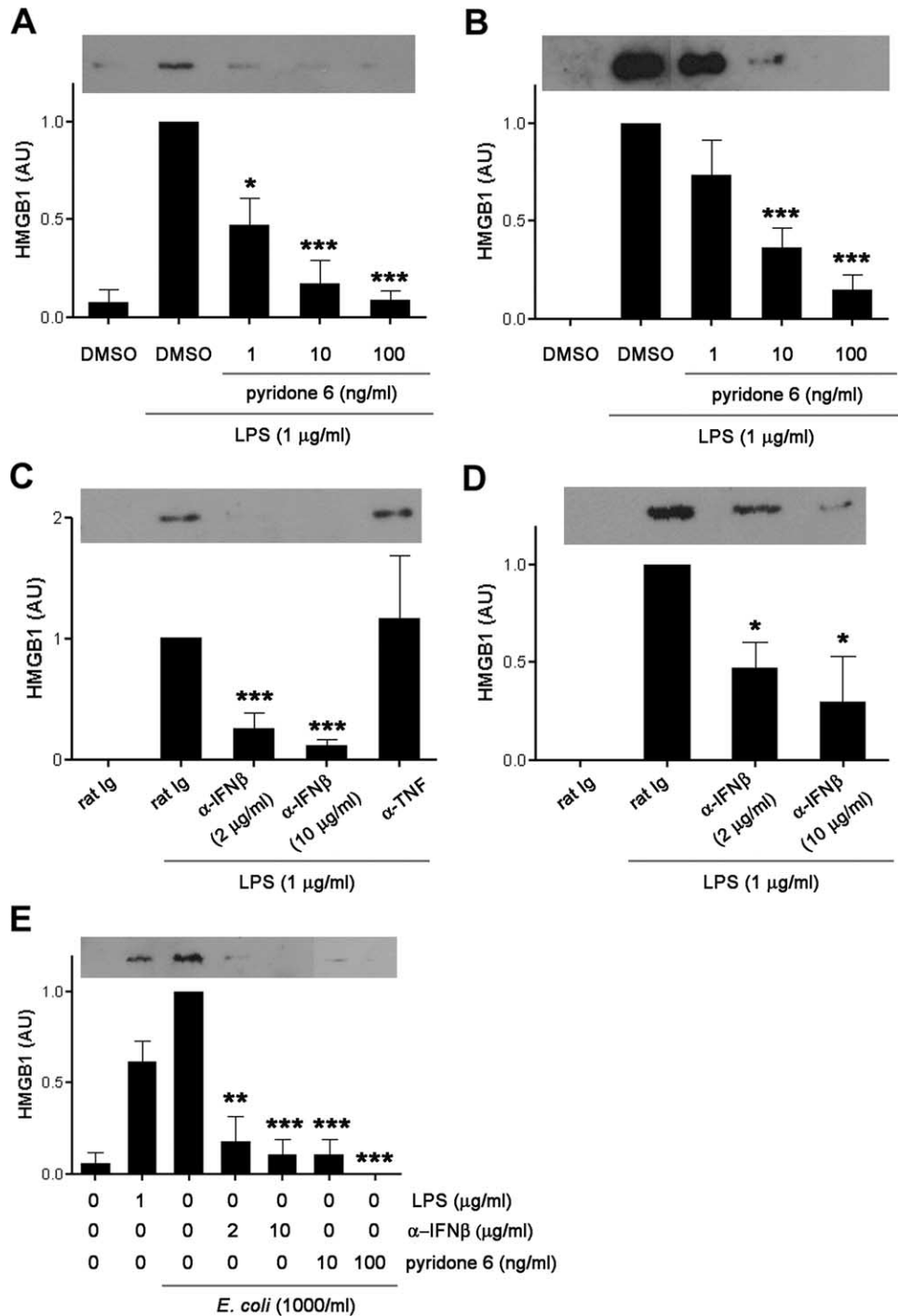
tein into the medium, and measured the levels of tyrosine phosphorylation of *STAT-1* in RAW cells in the presence of LY294002. Even in the increased concentration of LY294002 up to 50  $\mu\text{M}$ , the level of the *STAT-1* phosphorylation induced by the addition of IFN- $\beta$  was not changed (Fig. 4C). Therefore, the results indicate that LY294002 inhibits LPS-induced HMGB1 release by blocking IFN- $\beta$  synthesis.

#### *NO* is a downstream molecule of IFN- $\beta$ signaling related to the HMGB1 release

We treated RAW cells and primary macrophages with IFN- $\beta$  protein to examine whether IFN- $\beta$  stimulation is sufficient for HMGB1 release. Despite the treatment of IFN- $\beta$  (500 U/ml) or LPS, up to 6 h, no HMGB1 was detectable in the RAW cells (Fig. 5A). Interestingly, however, after 18 h, both treatments induced the secretion of HMGB1. In the primary macrophages, the induction patterns of HMGB1 in the two treatments were different. Although



**FIGURE 2.** Inhibition of IFN- $\beta$  signals blocks HMGB1 secretion. *A* and *B*, JAK inhibitor pyridone 6 inhibits the HMGB1 release in LPS-treated macrophages (*A*,  $n = 7$ ) and RAW cells (*B*,  $n = 5$ ) in a dose-dependent manner. Peritoneal macrophages (*A*) or RAW cells (*B*) were stimulated with LPS (1  $\mu\text{g/ml}$ ) in the presence of vehicle (DMSO) or various doses of pyridone 6 overnight. The supernatants were concentrated, subjected to Western blot assay, and quantified by densitometry for HMGB1. The value of the LPS-treated cells was arbitrarily assigned a value of 1, and the relative densitometric values of HMGB1 bands are shown. *C* and *D*, Effects of anti-IFN- $\beta$  Ab on the HMGB1 release in LPS-treated macrophages (*C*,  $n = 5$ ) and RAW cells (*D*,  $n = 3$ ). Peritoneal macrophages (*C*) or RAW cells (*D*) were stimulated with LPS (1  $\mu\text{g/ml}$ ) in the presence of control Ab (rat Ig) or various doses of anti-IFN- $\beta$  Ab overnight. Levels of HMGB1 in each supernatant were measured and quantified as described in *A* and *B*. *E*, Both anti-IFN- $\beta$  Ab and pyridone 6 inhibit HMGB1 release in macrophages treated with *E. coli* ( $n = 3$ ). Peritoneal macrophages were preincubated with various doses of pyridone 6 or anti-IFN- $\beta$  Ab, followed by *E. coli* ( $10^3/\text{ml}$ ) stimulation overnight. The densitometric value of the *E. coli*-treated sample was arbitrarily assigned a value of 1, and the relative densitometric values of HMGB1 bands are shown. The HMGB1 data were normalized to LPS- or *E. coli*-treated sample that was arbitrarily assigned a value of 1, and expressed as mean  $\pm$  SEM. AU, arbitrary unit. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  compared with LPS (*A–D*) or *E. coli* alone (*E*).

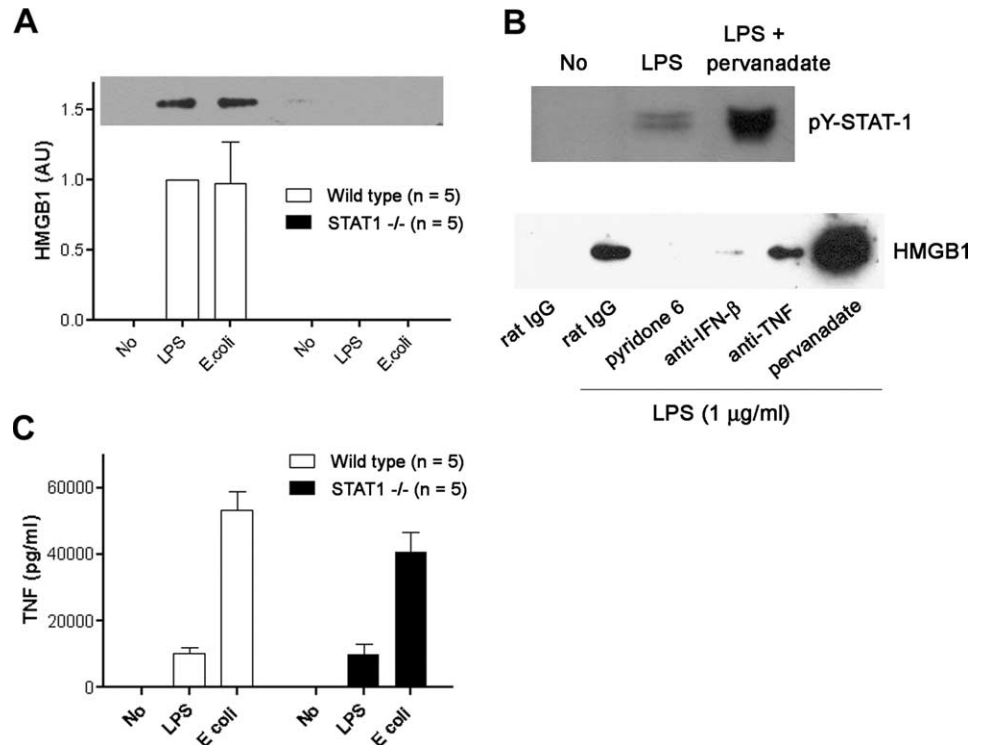


HMGB1 was induced by LPS after 18 h, the treatment of 3 h was enough for IFN- $\beta$  to induce HMGB1, but the fold induction was almost 7-fold lower to the full stimulation by LPS. However, we observed a supportive role of IFN- $\beta$  in LPS-induced HMGB1 release in primary macrophages. When we treated primary macrophages with IFN- $\beta$  in the presence of LPS, IFN- $\beta$  increased HMGB1 release more than 2.5-fold, compared with LPS treatment alone (Fig. 5C). The above data suggest that IFN- $\beta$  is a necessary, but not sufficient mediator in LPS-induced HMGB1 release and the presence of another factor is essential in case of primary macrophages, unlike RAW cells. To identify another molecule, we treated primary macrophages with various doses of TNF plus IFN- $\beta$  and measured the HMGB1 level. However, the addition of

TNF did not change the HMGB1 levels (data not shown). These results indicate that IFN- $\beta$  as well as other unknown molecule, but not TNF, are needed in LPS-induced HMGB1 release in primary macrophages.

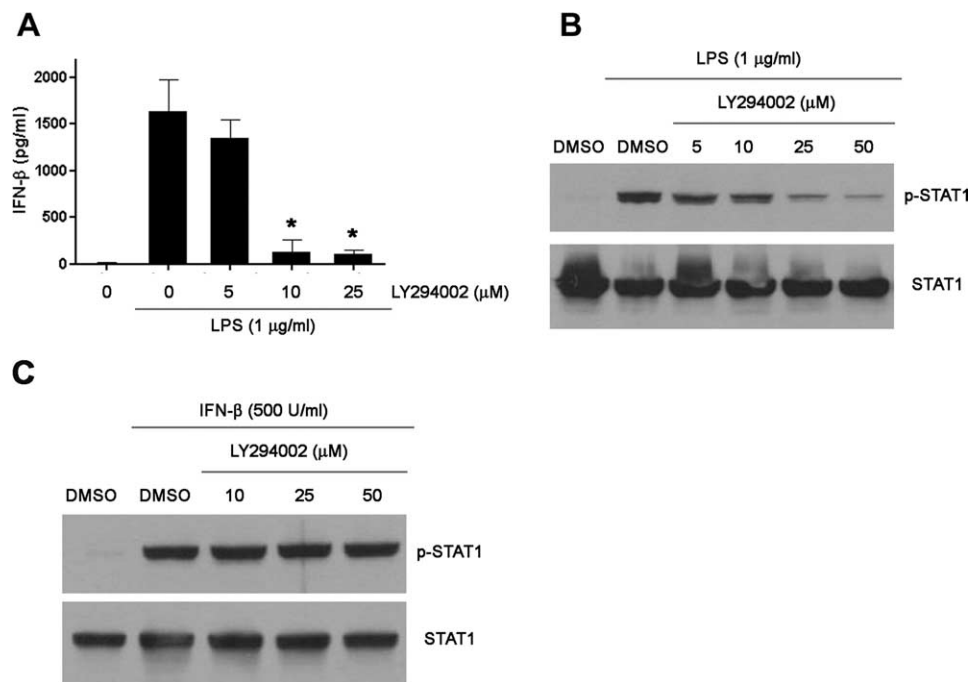
Because several signaling molecules have been previously shown to be involved in HMGB1 secretion (16, 19, 27) (K. Oh, unpublished observation), we tested NO among them as a downstream molecule of the IFN- $\beta$  activity related to the HMGB1 release. Upon the treatment with LPS, NO expression was induced, and the LPS-induced NO production was greatly diminished by the treatment of pyridone 6, an IFN- $\beta$  signaling inhibitor (Fig. 6A). Moreover, NO production was induced by IFN- $\beta$  (Fig. 6B), suggesting a role of NO as a molecule directly

**FIGURE 3.** STAT-1 molecule regulates LPS-induced HMGB1 release. *A*, The HMGB1 release was inhibited in STAT-1 deficient macrophages ( $n = 5$ ). Macrophages isolated from wild-type B6 or STAT1<sup>-/-</sup> mice were treated with either LPS (1  $\mu\text{g/ml}$ ) or *E. coli* ( $10^3/\text{ml}$ ). The levels of HMGB1 in culture supernatants were concentrated and measured by Western blotting. *B*, Pervanadate treatment enhanced STAT-1 phosphorylation and HMGB1 release. *Upper panel*, Peritoneal macrophages were treated with LPS in the presence of pervanadate (20  $\mu\text{M}$ , LPS + pervanadate) for 2 h, lysed, and then subjected to tyrosine-phosphorylated STAT-1 (pY-STAT-1) Western blotting. In control, the cultures were left untreated (No), or treated with LPS only (LPS). *Lower panel*, Peritoneal macrophages were treated as indicated overnight, and the levels of HMGB1 in culture supernatants were measured by Western blotting ( $n = 2$ ). *C*, The levels of TNF of wild-type B6 and STAT-1<sup>-/-</sup> macrophages were measured by ELISA ( $n = 5$ ).

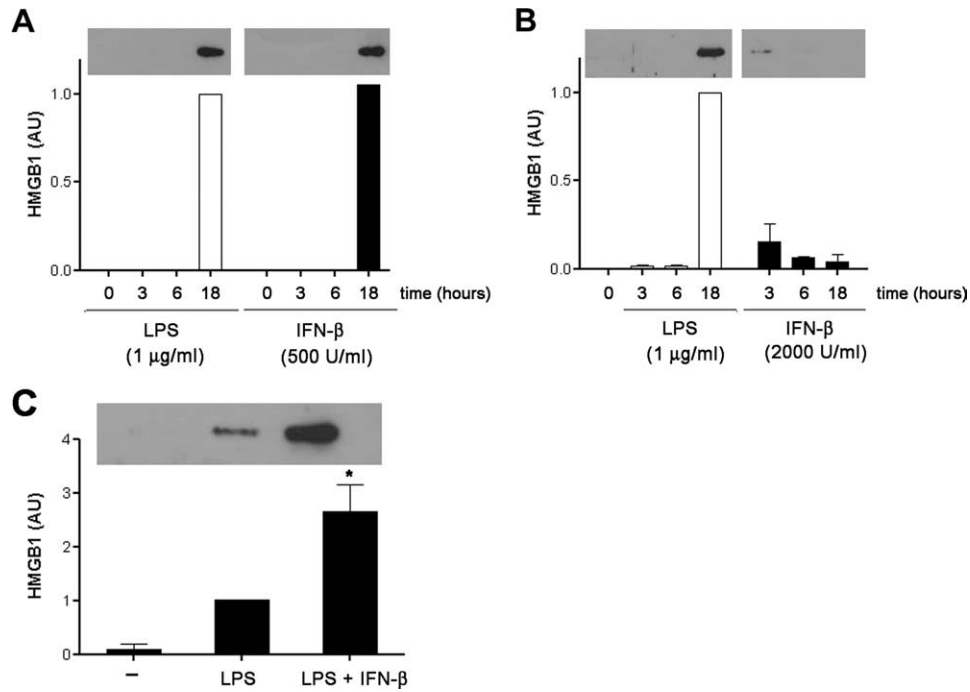


associated with the IFN- $\beta$  signaling. To examine the role of NO in IFN- $\beta$ -induced HMGB1 release, we treated RAW cells with 1400W (inducible NO synthase inhibitor) plus IFN- $\beta$ , and measured the changes in the levels of HMGB1. Interestingly, the

addition of 1400W in RAW cell cultures inhibited HMGB1 release induced by IFN- $\beta$  significantly (Fig. 6C). Thus, IFN- $\beta$  seems to induce HMGB1 release, at least in part, through NO signaling pathway.



**FIGURE 4.** LY294002 prevents LPS-induced HMGB1 release through inhibition of IFN- $\beta$  production. *A*, PI3K inhibitor LY294002 inhibits LPS-induced IFN- $\beta$  production in a dose-dependent manner ( $n = 3$ ). RAW cells were preincubated with various doses of LY294003 for 30 min and subsequently treated with LPS (1  $\mu\text{g/ml}$ ) overnight. The levels of IFN- $\beta$  were measured by ELISA. *B*, LY294002 inhibits LPS-induced tyrosine phosphorylation of STAT-1 in a dose-dependent manner ( $n = 3$ ). RAW cells were stimulated with LPS plus LY294003 for 2 h. Total cell lysates were harvested, subjected to Western blotting, and probed with anti-tyrosine-phosphorylated (*upper*) or total STAT-1 (*lower*) Abs. The representative blot of three independent experiments is shown. *C*, LY294002 does not affect the tyrosine phosphorylation of STAT-1 induced by the treatment of IFN- $\beta$  ( $n = 2$ ). RAW cells were stimulated with IFN- $\beta$  (500 U/ml) plus LY294003, and the levels of tyrosine phosphorylation of STAT-1 were measured by Western blotting. The representative blot of two independent experiments is shown. AU, arbitrary unit. \*,  $p < 0.05$  compared with LPS alone.



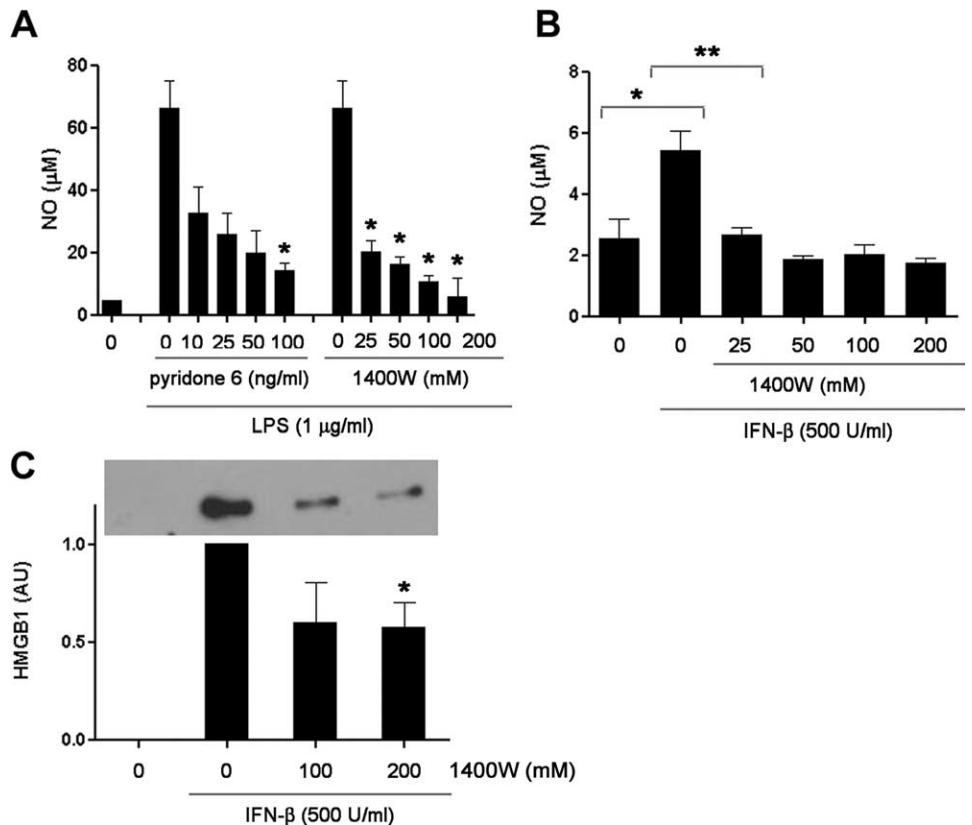
**FIGURE 5.** IFN- $\beta$  treatment induces HMGB1 in RAW cells. *A*, IFN- $\beta$  increases HMGB1 secretion as efficiently as LPS dose in RAW cells ( $n = 3$ ). Cells were treated with either LPS or IFN- $\beta$  for 3, 6, and 18 h, and at each time point the culture supernatant was collected and subjected to Western blotting to measure HMGB1 levels. *B*, Primary macrophages resist releasing HMGB1 in the response to IFN- $\beta$  ( $n = 3$ ). Macrophages were treated with LPS or IFN- $\beta$  for 3, 6, and 18 h, and the levels of HMGB1 in culture supernatants were measured and quantified. *C*, IFN- $\beta$  enhances LPS-induced HMGB1 release in primary macrophages. The supernatants from macrophages treated with LPS (1  $\mu$ g/ml) alone or LPS (1  $\mu$ g/ml) plus IFN- $\beta$  (500 U/ml) overnight were concentrated and subjected to Western blotting to measure HMGB1. The HMGB1 data were normalized to the LPS-treated sample that was arbitrarily assigned as a value of 1. AU, arbitrary unit. \*,  $p < 0.05$  compared with LPS alone.

*IFN- $\beta$  signals are important to HMGB1 release in vivo*

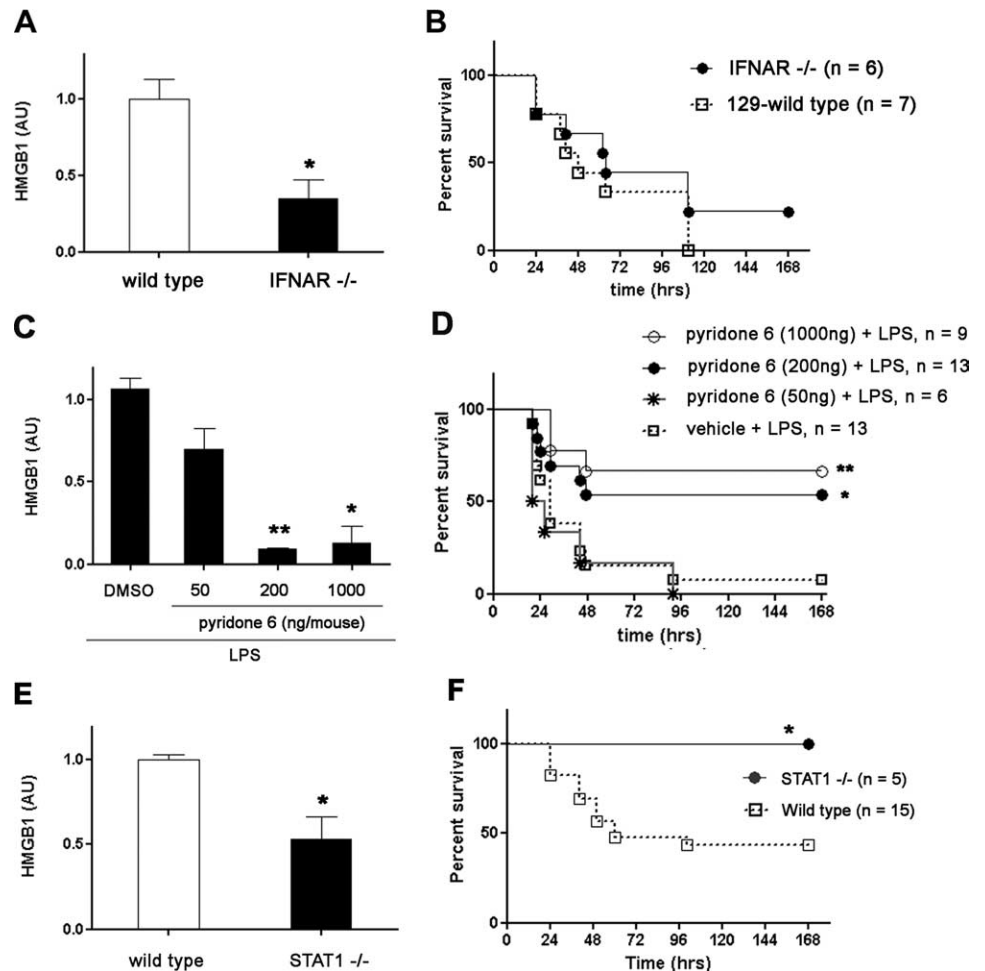
To evaluate the role of IFN- $\beta$  signaling in HMGB1 release in vivo, we used the murine endotoxemia model. Because IFNAR<sup>-/-</sup> mice

were 129 background and 129 mice were more resistant to LPS than B6, we administered a higher dose of LPS (65 mg/kg) i.p. to the control wild-type or IFNAR<sup>-/-</sup> mice and measured their

**FIGURE 6.** NO is a downstream molecule of IFN- $\beta$ . *A*, JAK inhibitor pyridone 6 inhibits LPS-induced NO production in a dose-dependent manner ( $n = 2$ ). RAW cells were preincubated with various doses of pyridone 6 or 1400W (positive control) for 30 min and subsequently treated with LPS. NO levels were determined by Griess assay. *B*, IFN- $\beta$  enhanced NO production, which is blocked by 1400W ( $n = 4$ ). RAW cells were stimulated by IFN- $\beta$  in the absence or presence of 1400W, and NO levels were determined by Griess assay. *C*, 1400W attenuates HMGB1 release in IFN- $\beta$ -treated RAW cells ( $n = 4$ ). RAW cells were stimulated by IFN- $\beta$  in the presence of 1400W, and HMGB1 levels were determined. The HMGB1 data were normalized to the IFN- $\beta$ -treated sample that was arbitrarily assigned a value of 1, and expressed as mean  $\pm$  SEM. AU, arbitrary unit. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with LPS (*A*), medium control (*B*), or IFN- $\beta$  alone (*B* and *C*).



**FIGURE 7.** LPS-induced HMGB1 release partially involves IFN- $\beta$  signaling pathway in vivo. **A**, LPS (65 mg/kg) was administered i.p. to 129 wild-type and IFNAR<sup>-/-</sup> mice (male, 22–25 g). HMGB1 levels were measured at 18 h after LPS (65 mg/kg) injection by Western blot analysis. Data are presented as mean  $\pm$  SEM ( $n = 3$ –5). **B**, IFNAR<sup>-/-</sup> and wild-type mice were treated by LPS (65 mg/kg), and their survival was monitored for 1 wk. **C**, HMGB1 levels in B6 mice treated with LPS (40 mg/kg) plus various doses of pyridone 6 or vehicle (DMSO) were measured by Western blotting ( $n = 3$ ). **D**, B6 mice were injected with various doses of pyridone 6, at 1 h before LPS (40 mg/kg) infusion. Injections of pyridone 6 were then given twice per day for 3 days. Mice were observed for 1 wk after LPS administration. **E**, HMGB1 levels in wild-type B6 and STAT1<sup>-/-</sup> mice treated by LPS (20 mg/kg) were measured by Western blotting ( $n = 3$ –5). **F**, Wild-type B6 and STAT1<sup>-/-</sup> mice were injected with 20 mg/kg LPS, and survival was monitored for 1 wk. HMGB1 data are presented as mean  $\pm$  SEM. AU, arbitrary unit. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with wild-type mice (**A** and **E**) or LPS plus vehicle-treated mice (**C** and **D**).



HMGB1 levels. The HMGB1 levels of the IFNAR<sup>-/-</sup> mice were lower than those of the control mice (Fig. 7A). However, the difference in mortality between the control and the IFNAR<sup>-/-</sup> mice was not statistically significant (Fig. 7B). We also performed the same experiments using pyridone 6-treated B6 mice. B6 mice were subjected to vehicle or various doses of pyridone 6, and the lethal dose of LPS (40 mg/kg) administration. Pyridone 6 was treated in four different concentrations (0, 50, 200, and 1000 ng/mouse), and the treatment reduced both HMGB1 release (Fig. 7C) and the sepsis mortality (Fig. 7D) in a dose-dependent manner. To confirm the role of STAT-1 in HMGB1 release in vivo, the level of HMGB1 was examined in STAT1<sup>-/-</sup> mice compared with the B6 wild-type mice, and we found that STAT-1 deficiency also reduced the levels of HMGB1 (Fig. 7E). In the case of survival, as previously reported (28, 29), the survival rates of the STAT1<sup>-/-</sup> mice compared with those of the B6 wild-type mice were increased only in low-dose LPS-treated group (20 mg/kg; Fig. 7F). When we injected with high dose (40 mg/kg) of LPS, we could not observe any significant difference of mortality between B6 wild-type and STAT1<sup>-/-</sup> mice (data not shown).

## Discussion

In this study, we have shown that IFN- $\beta$  functions as a common and essential molecule in the HMGB1 release induced by LPS as well as by *E. coli*. We observed that the LPS-induced HMGB1 release is associated with TLR4 (Fig. 1A), TRIF (Fig. 1C), IFN- $\beta$ , and JAK-STAT signaling (Fig. 2). Inhibition of any of these signals dramatically reduced the HMGB1 release. In addition, the activation of PI3K was also involved in LPS-induced IFN- $\beta$  pro-

duction (Fig. 4A), and NO played as a downstream molecule of the IFN- $\beta$  signaling (Fig. 6). Together, these data strongly reveal that IFN- $\beta$  plays a pivotal role in HMGB1 release, suggesting it as a potential target molecule in sepsis treatment (30) (Fig. 7).

It has been reported that NO is involved in LPS-induced HMGB1 release (16), and in this study we showed that IFN- $\beta$  is critical in the same process. These data suggest that NO may play roles as an IFN- $\beta$  signaling molecule in the HMGB1 release. Interestingly, LPS-induced NO production was inhibited by pyridone 6 (Fig. 6A), and IFN- $\beta$  treatment directly induced NO production (Fig. 6B). In addition, 1400W (inducible NO synthase inhibitor) treatment abolished IFN- $\beta$ -induced HMGB1 secretion (Fig. 6C). These data suggest that NO plays as a downstream molecule of IFN- $\beta$  in LPS-induced HMGB1 release, at least partially.

Recently, it was reported that the apoptosis and apoptosis-related enzymes, caspases, are important for HMGB1 release (19, 27). We also observed the data showing that benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone treatment effectively attenuates *E. coli*-induced HMGB1 release in macrophages (K. Oh, unpublished observation) and investigated the possible relation between IFN- $\beta$  and apoptosis like the above NO study. However, IFN- $\beta$  treatment did not induce apoptosis, and the effect of LPS on apoptosis in macrophages is also still controversial (19, 22). Recently, LPS was reported to induce apoptosis often in a cell type-specific manner and sometimes to show an antiapoptotic effect in monocytes, neutrophils, and macrophages (22, 31–34). We also observed the similar results (LPS did not increase the apoptotic cell populations) in macrophages using flow cytometric analysis (data not shown). Interestingly, a group of caspases was reported to be



essential for the processing and maturation of the inflammatory cytokines IL-1 $\beta$  and IL-18 (35). Furthermore, caspase-8 has been shown to have additional functions unrelated to cell death, such as T cell homeostasis, proliferation, and activation (36, 37). Therefore, the effect of benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone on HMGB1 release can be attributed to the inhibition of not only apoptosis, but also other events, such as the modulation of inflammation.

We also investigated the effects of apoptosis on HMGB1 release in vivo. Sepsis may induce massive apoptosis of lymphocytes, especially thymocytes (38), and the administration of caspase inhibitors reduced thymocyte apoptosis (39) as well as HMGB1 release (27). To determine to what extent the apoptosis of thymocytes contributes to HMGB1 release in vivo, we administered LPS into athymic nude mice, and measured HMGB1 levels. Interestingly, the HMGB1 levels of the nude mice were as high as those of the control mice (K. Oh, unpublished data). This result suggests that HMGB1 release should be induced by apoptosis of other types of cells in addition to thymocytes, as previously described in an experiment with B lymphocytes (40). Another possibility is that the administration of caspase inhibitors could affect HMGB1 release indirectly through the modulation of the systemic inflammatory responses.

Recently, it was published that calcium/calmodulin-dependent protein kinase (CaMK) mediated HMGB1 release from macrophages treated with LPS (41). By using various methods to prevent CaMK activation, such as chemical inhibitors (STO609 and KN93), RNA interference, as well as gene knockout mice, the authors showed that CaMK promotes the extracellular release of HMGB1. We tested whether CaMK activation is also involved in IFN- $\beta$  synthesis, and interestingly found that STO609 (a CaMKIV inhibitor) reduced significantly the IFN- $\beta$  production in a dose-dependent manner (S. Kim and K. Oh, unpublished data), indicating that IFN- $\beta$  is indeed involved in CaMK-related HMGB1 release. The effect of STO609 seems almost the same as that of LY294002 (Fig. 4). Both inhibitors reduce LPS-induced HMGB1 release via blocking IFN- $\beta$  synthesis, supporting that IFN- $\beta$  signaling is critical to HMGB1 release. Further study regarding how the CaMK activation and the IFN- $\beta$  production are related will provide insights into the mechanism of HMGB1 release.

To investigate the role of IFN- $\beta$  in LPS-induced HMGB1 release in vivo, we used a neutralizing anti-IFN- $\beta$  Ab, knockout mice (IFNAR $^{-/-}$ , STAT1 $^{-/-}$ ), and a chemical inhibitor (pyridine 6). First, we monitored the HMGB1 levels and mortality in B6 mice treated with anti-IFN- $\beta$  Ab plus lethal dose of LPS. Unexpectedly, treatment with anti-IFN- $\beta$  Ab changed neither the HMGB1 levels nor the survival rates (data not shown). Because it has been reported that IFN- $\beta$  $^{-/-}$  mice were less sensitive to LPS-induced mortality (27), the above results were most likely caused by incomplete blocking of all the functional IFN- $\beta$  molecules. Then, we substituted IFN- $\beta$  receptor gene-deficient (IFNAR $^{-/-}$ ) mice. Although the levels of HMGB1 in IFNAR $^{-/-}$  mice were significantly lower than 129 control mice (Fig. 7A), the survival rates were comparable (Fig. 7B). These data were also surprising, because IFNAR $^{-/-}$  mice were reported to be less succumbed to polymicrobial sepsis (42). The reason for this discrepancy is currently not clear. However, because the early proinflammatory cytokines such as TNF play a dominant role in LPS-induced mortality and the TNF level in IFNAR $^{-/-}$  mice is comparable to wild-type mice, the protective effect of IFN- $\beta$  receptor deficiency on HMGB1 levels could be neglected. In addition, a significant amount ( $\sim$ 1/3) of HMGB1 molecules still remained in LPS-treated IFNAR $^{-/-}$  mice (Fig. 7A), and the amount could be enough for the functions of HMGB1 (12, 43, 44) in systemic

inflammatory conditions. This assumption implicates that the complete abrogation of HMGB1 release is needed to improve the survival rates. Consistently, pyridone 6 reduced HMGB1 levels more efficiently (Fig. 7C) and subsequently improved survival rates (Fig. 7D).

We showed that LPS-induced HMGB1 release was almost totally abrogated in STAT-1 $^{-/-}$  macrophages (Fig. 3A). However, the effects of the STAT-1 deficiency on HMGB1 release (Fig. 7E) were not obvious in vivo, compared with pyridone 6 treatment. This discrepancy could be due to their different environmental settings, because although LPS induces HMGB1 release almost entirely through STAT-1 signaling in the cultured macrophages, other unknown processes such as cell necrosis (45) could also contribute to HMGB1 release in a JAK-dependent, but STAT-1-independent manner in in vivo settings. The evidence supporting the importance of JAK in LPS-induced lethality in vivo has been previously described in several reports, in which STAT-1 $^{-/-}$  mice are more susceptible to sepsis, compared with JAK protein tyrosine kinase family member-deficient Tyk2 $^{-/-}$  mice (28, 29).

Although we demonstrated that STAT-1 is critical in HMGB1 release, the detailed molecular mechanism of HMGB1 release via STAT-1 remains to be further characterized. One of the interesting questions is whether STAT-1 is directly involved in the posttranslational modulation of HMGB1, such as phosphorylation and acetylation, which is a critical step in its nuclear transport toward secretion. To test this hypothesis, we performed immunoprecipitation and Western blotting assay, but could not find the changes of the acetylations or phosphorylations in STAT-1 $^{-/-}$  macrophages due to the strong backgrounds. We are currently investigating the alterations of HMGB1 in STAT-1 $^{-/-}$  macrophages using the two-dimensional electrophoresis. These studies will provide a detailed mechanism of HMGB1 release and an insight into the understanding of protein transport from nucleus to cytoplasm and the posttranslational modification process.

## Disclosures

The authors have no financial conflict of interest.

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