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# IFN- $\gamma$ Induces High Mobility Group Box 1 Protein Release Partly Through a TNF-Dependent Mechanism<sup>1</sup>

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We recently discovered that a ubiquitous protein, high mobility group box 1 protein (HMGB1), is released by activated macrophages, and functions as a late mediator of lethal systemic inflammation. To elucidate mechanisms underlying the regulation of HMGB1 release, we examined the roles of other cytokines in induction of HMGB1 release in macrophage cell cultures. Macrophage migration inhibitory factor, macrophage-inflammatory protein 1 $\beta$ , and IL-6 each failed to significantly induce the release of HMGB1 even at supraphysiological levels (up to 200 ng/ml). IFN- $\gamma$ , an immunoregulatory cytokine known to mediate the innate immune response, dose-dependently induced the release of HMGB1, TNF, and NO, but not other cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , or IL-6. Pharmacological suppression of TNF activity with neutralizing Abs, or genetic disruption of TNF expression (TNF knockout) partially (50–60%) inhibited IFN- $\gamma$ -mediated HMGB1 release. AG490, a specific inhibitor for Janus kinase 2 of the IFN- $\gamma$  signaling pathway, dose-dependently attenuated IFN- $\gamma$ -induced HMGB1 release. These data suggest that IFN- $\gamma$  plays an important role in the regulation of HMGB1 release through a TNF- and Janus kinase 2-dependent mechanism. *The Journal of Immunology*, 2003, 170: 3890–3897.

**M**ediators produced by macrophages and other immune cells contribute to the damaging sequelae of lethal systemic inflammation (e.g., endotoxemia and sepsis). The interaction of these mediators influences the development of a beneficial or pathological response (1, 2). Recent evidence reveals a role for high mobility group box 1 protein (HMGB1)<sup>3</sup> as a late mediator of delayed endotoxin lethality (3, 4). HMGB1 was first purified from nuclei ~30 years ago, and named high mobility group for its rapid mobility on electrophoresis gels (5). Nuclear HMGB1 has been implicated in diverse cellular functions, including determination of nucleosomal structure and stability, and binding of transcription factors to cognate DNA sequences (6, 7). In addition to the nucleus and cytoplasm, HMGB1 has also been localized to the cell membrane of neurites and tumor cells (8, 9), where it colocalizes and interacts with tissue plasminogen activator (8) and the receptor for advanced glycation end products (RAGE) (9, 10). Engagement of RAGE with HMGB1 activates mitogen-activated protein kinase (MAPK) pathways (11), and the blockade of HMGB1-RAGE interaction suppresses the activation of MAPKs and the growth and metastases of tumors in mice (9).

We discovered that HMGB1 is actively released by activated macrophages/monocytes in vitro and that serum HMGB1 levels are elevated in endotoxemic animals (3) and in patients with surgical sepsis (3) or hemorrhagic shock (12). Recently, Scaffidi et al. (13) demonstrated that HMGB1 is passively released by necrotic or damaged cells, and triggers an inflammatory response, supporting a role for extracellular HMGB1 in inflammation mediated by tissue injury and trauma. Interestingly, HMGB1 is not released by apoptotic cells even after subsequent secondary necrosis, indicating that apoptotic cells are programmed to die without broadcasting an inflammatory signal to neighboring cells (13). Exposure to HMGB1 leads to various cellular responses, including chemotactic cell movement (11), increase in permeability of Caco-2 enterocytic monolayers (14), and the release of various proinflammatory mediators such as TNF, IL-1 (15, 16), and NO (14). Administration of exogenous HMGB1 to animals causes derangements in intestinal barrier function (14), anorexia (17), tissue injury (15), and lethality (3). Administration of anti-HMGB1 Abs confers protection against LPS-induced acute lung injury (15) and lethality (3), even when the first dose of Ab is administered after the early TNF response. Suppression of HMGB1 release with anti-inflammatory compounds (such as ethyl pyruvate) also protects animals in a model of lethal systemic inflammation (18), suggesting that therapeutic agents targeting HMGB1 may prove to be useful in the treatment of lethal systemic inflammation diseases.

Inflammatory stimuli (e.g., bacterial endotoxin) activate MAPKs (e.g., p38, extracellular signal-regulated kinase (ERK)1/2, and c-Jun N-terminal kinase) (19, 20), and stimulate the sequential release of early proinflammatory cytokines (e.g., TNF, IL-1, IL-6, and IFN- $\gamma$ ) (21–24) and late proinflammatory mediators such as macrophage migration-inhibitory factor (MIF) (25–27) and HMGB1 (3). IFN- $\gamma$  plays important roles in the innate immune response by enhancing the production of proinflammatory cytokines such as TNF and IL-1 $\beta$  (28–31). Consistently, administration of IFN- $\gamma$  increases lethality in animal models of sepsis (e.g.,

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<sup>3</sup> Abbreviations used in this paper: HMGB1, high mobility group box 1 protein; RAGE, receptor for advanced glycation end products; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ERK, extracellular signal-regulated kinase; MIF, macrophage migration-inhibitory factor; JAK, Janus kinase; HuPBMC, human PBMC.

cecal ligation and puncture) (32), whereas inhibition of IFN- $\gamma$  activity or expression protects against lethality in animal models of endotoxemia (33–37). Because multiple proinflammatory mediators interact and contribute to the damaging sequelae of lethal systemic inflammation (1, 4), it is important to examine the potential roles of IFN- $\gamma$  in the regulation of HMGB1 release. In the present study, we have demonstrated IFN- $\gamma$  induces HMGB1 release through TNF- and Janus kinase (JAK)2-dependent mechanisms.

## Materials and Methods

### Cell culture

Murine macrophage-like RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS and 2 mM glutamine. At 80–90% confluency, RAW 264.7 cells were washed twice with, and subsequently cultured in, serum-free OPTI-MEM I medium (Life Technologies). After preincubation for 2 h, cell cultures were stimulated with various stimuli (IL-6 (catalog no. 1 444 581); IFN- $\gamma$  (catalog no. 1 276 905); Roche, Nutley, NJ).

Thioglycolate-elicited peritoneal macrophages were isolated from young mice (male, 7–8 wk) (C3H/HeJ (*Tlr4<sup>Lps-d</sup>*) (stock no. 000659); C57BL/6  $\times$  129S6 (TNF<sup>-/-</sup>) (stock no. 003008); and C57BL/6J (stock no. 000664); The Jackson Laboratory, Bar Harbor, ME) as previously described (3), and cultured in RPMI 1640/10% FBS/2 mM glutamine on six-well tissue culture plates (4  $\times$  10<sup>6</sup> cells/2 ml/well). After preculture for 12 h, the culture medium was replaced with serum-free OPTI-MEM-I medium, and cell cultures were stimulated with LPS or IFN- $\gamma$ .

Human PBMC (HuPBMC) were isolated by density gradient centrifugation through Ficoll (Ficoll-Paque PLUS; Pharmacia, Piscataway, NJ) as previously described (38), and cultured in RPMI 1640/10% heat-inactivated human serum/2 mM L-glutamine overnight. Nonadherent cells were subsequently removed, and adherent monocyte-enriched cultures were stimulated with IFN- $\gamma$  or LPS.

### Immunoassays for HMGB1 and cytokines

The levels of HMGB1 in the culture medium or macrophage cells were assayed by Western blotting analysis using rabbit polyclonal Abs as previously described (3). Western blots were scanned with a silver image scanner (SilverScanner II; Lacie, Beaverton, OR), and the relative band intensity was quantified by using the NIH Image 1.59 software. The levels of HMGB1 were calculated with reference to standard curves generated with purified rHMGB1.

Cellular HMGB1 levels were also assessed by immunostaining of macrophage cell cultures using Ag affinity-purified anti-HMGB1 polyclonal Abs following a previously described protocol (39). Briefly, macrophage cell cultures were transferred onto adhesion slides (Lab-Tek chamber slides; catalog no. 62407335; VWR, Bristol, CT), and incubated at 37°C for 1 h to allow adherence. The adherent cells were fixed with phosphate-buffered formaldehyde (4%; pH 7.4; 15 min), and permeabilized with Triton X-100 (0.3%; pH 7.4; 10 min). After blocking the slides with 10% BSA (37°C; 1 h), cells were sequentially incubated with anti-HMGB1 Abs and FITC-conjugated anti-rabbit IgG (catalog no. F9887; Sigma-Aldrich, St. Louis, MO). Following several extensive washings, the slides were mounted immediately on a Vectashield (catalog no. H-1000; Vector Laboratories, Burlingame, CA) to acquire images using a confocal microscope (1  $\times$  70, fluoroview; Olympus, Melville, NY).

The levels of cytokines (TNF, IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6) in the culture medium were determined using commercially available ELISA kits (catalog nos. MTA00, MLA00, MLB00, and M6000, respectively; R&D Systems, Minneapolis, MN) as previously described (38, 40). The levels of cytokines were calculated with reference to standard curves of purified recombinant cytokines at various dilutions. The levels of NO in the culture medium were determined indirectly by measuring the NO<sub>2</sub><sup>-</sup> production with a colorimetric assay based on the Griess reaction as previously described (41). Briefly, 100  $\mu$ l of culture medium was mixed with 900  $\mu$ l of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine, and 2.5% H<sub>3</sub>PO<sub>4</sub>) and incubated at room temperature for 10 min, and the OD was read at 560 nm. NO<sub>2</sub><sup>-</sup> concentrations were determined with reference to a standard curve generated with sodium nitrite at various dilutions.

### RNase protection assay

Total RNA was extracted from cells using TRIzol reagent (catalog no. 15596-026; Life Technologies), and the levels of HMGB1 and  $\beta$ -actin mRNA were measured using an RNase protection assay kit following the

manufacturer's instructions (catalog no. 1414; Ambion, Austin, TX). Briefly, an HMGB1 cDNA fragment (201 nt) was generated by PCR using two primers: a sense primer (5'-CCG AAT TCG CTT CTG TCA ACT TCT CAG AGT TTT CC-3') and an antisense primer (5'-GCG TAA TAC GAC TCA CTA TAG GGC GAG GAT CCC GAA GGA GGC CTC TTG GGT GCA TTG-3') containing a T7 promoter site. The [ $\alpha$ -<sup>32</sup>P]UTP-radiolabeled antisense RNA probe was generated with T7 RNA polymerase using an *in vitro* transcription kit (Maxiscript; catalog no. 1308-1326; Ambion), and hybridized with sample RNA. After removing free probe by ribonuclease digestion, the hybrid of antisense RNA was analyzed by electrophoresis on 5% TB-urea polyacrylamide gel (catalog no. 161-1187; Bio-Rad, Hercules, CA). The relative mRNA levels were quantified with a molecular imager-imaging densitometer (GS-700; Bio-Rad) after exposure to x-ray film.

### Assay of phosphorylation state of MAPKs and STAT1

Macrophage cells were lysed in SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue), and the cellular concentrations of phospho-MAPK and phospho-STAT1 were determined by Western blot analysis using commercially available Ab kits following the manufacturer's instructions (p38 (Thr<sup>180</sup>/Tyr<sup>182</sup>) MAPK Ab kit (catalog no. 9210); MEK1/2 (Ser<sup>217/221</sup>) MAPK Ab kit (catalog no. 9120); p44/42 MAPK Ab kit (catalog no. 9100); phospho-STAT1 (Tyr<sup>701</sup>) Ab (catalog no. 9171); Cell Signaling Technology, Beverly, MA). To verify equal loading for different samples, the samples were reprobbed with a different Ab specific to total MAPKs or STAT1 (STAT1 Ab (catalog no. 9172)). The roles of MAPKs and JAKs in IFN- $\gamma$ -induced HMGB1 release were assessed by using specific inhibitors (p38 MAPK inhibitor, SB203580 (catalog no. 559389); MEK1/2 inhibitor, U0126 (catalog no. 662005); JAK2 inhibitor, AG490 (catalog no. 658401); Calbiochem, La Jolla, CA).

### Statistical analysis

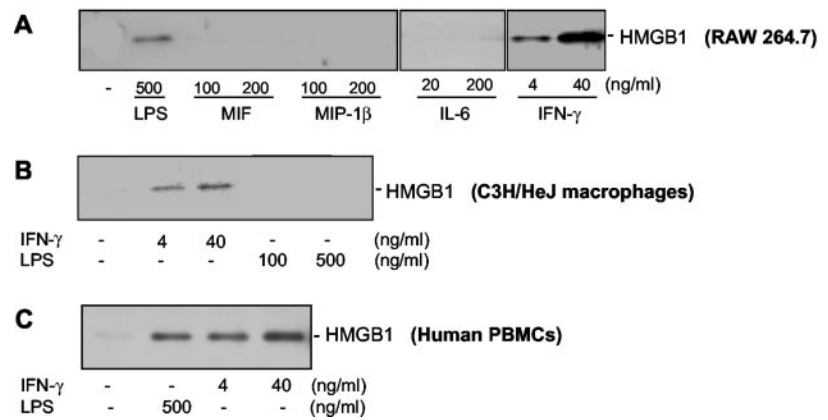
Values in the figures were expressed as mean  $\pm$  SEM of two to three independent experiments either in duplicates or triplicates ( $n = 6-9$ ). Student's two-tailed *t* test was used to compare means between groups. A value of  $p < 0.05$  was considered to be statistically significant.

## Results

### Effect of various proinflammatory cytokines on HMGB1 release

To evaluate the potential role of proinflammatory cytokines in stimulating HMGB1 release, cultures of macrophage-like RAW 264.7 cells were stimulated by the addition of cytokines, and levels of HMGB1 in the culture medium were subsequently measured by immunoblotting analysis. HMGB1 was barely detected in the culture medium in the absence of inflammatory stimuli (–), but was detected after stimulation of cells with bacterial endotoxin (Fig. 1A). MIF, macrophage-inflammatory protein 1 $\beta$ , and IL-6 each failed to significantly induce the release of HMGB1, even at supraphysiological concentrations (up to 200 ng/ml) (Fig. 1A). However, IFN- $\gamma$ , at concentrations as low as 4 ng/ml (20 U/ml), triggered a significant increase of HMGB1 levels in the culture medium (Fig. 1A). To eliminate the possibility that the IFN- $\gamma$ -induced HMGB1 release was due to LPS contamination (<1  $\times$  10<sup>-7</sup> endotoxin U/ng), primary peritoneal macrophages of LPS-resistant mice (C3H/HeJ) were isolated and stimulated with IFN- $\gamma$ . Consistent with our previous report (3), LPS, even at concentrations up to 500 ng/ml, failed to induce HMGB1 release in these LPS-resistant macrophages (Fig. 1B). However, IFN- $\gamma$ , even at a concentration as low as 4 ng/ml, induced a marked HMGB1 release (Fig. 1B), confirming that the IFN- $\gamma$ -induced HMGB1 release was not dependent upon LPS signal transduction pathways. The inducible nature of HMGB1 release was further confirmed in primary HuPBMC. HMGB1 was not detectable in the absence of inflammatory stimuli (–), but extracellular levels were markedly increased after stimulation with IFN- $\gamma$  or LPS (Fig. 1C). Thus, IFN- $\gamma$  effectively induced HMGB1 release in both macrophage and monocyte cell cultures.

**FIGURE 1.** Effect of IFN- $\gamma$  on HMGB1 release in macrophage/monocyte cell cultures. Murine macrophage-like RAW 264.7 cells (A), primary peritoneal macrophages of LPS-resistant C3H/HeJ mice (B), or HuPBMC (C) were stimulated with indicated inflammatory stimuli for 16 h, and assayed for HMGB1 accumulation in the culture medium by Western blotting analysis.



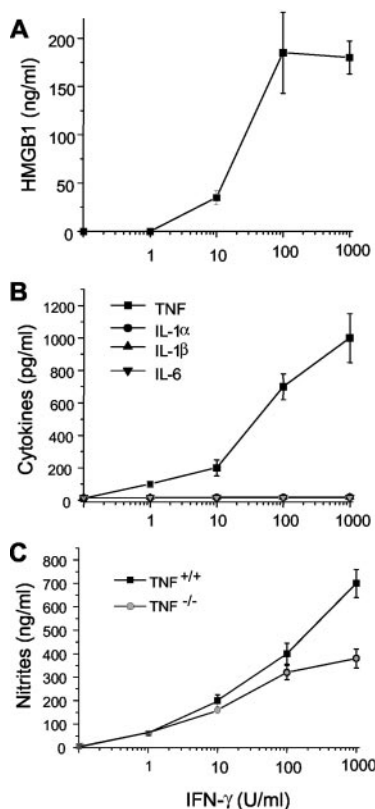
#### Effect of IFN- $\gamma$ stimulation on the release of other proinflammatory cytokines

To address the specificity of IFN- $\gamma$ -mediated cytokine release, macrophage cultures were stimulated with IFN- $\gamma$  at various concentrations. At pathophysiological concentrations (0–40 ng/ml), IFN- $\gamma$  induced HMGB1 release in a dose-dependent manner, with a significant amount of HMGB1 release after stimulation with IFN- $\gamma$  at concentrations as low as 20 U/ml (4 ng/ml; Fig. 2A). Cell viability, as assessed by trypan blue exclusion, was unaffected by IFN- $\gamma$  at concentrations that induced effective HMGB1 release (control cell viability = 95–97%; vs IFN- $\gamma$ -treated cells (100 U/ml), cell viability = 95–98%, 16 h after stimulation), confirming

that the IFN- $\gamma$ -induced HMGB1 release was specific and not due to cell death.

IFN- $\gamma$  also dose-dependently induced the release of TNF and NO, but not other cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6 (Fig. 2, B and C), indicating that IFN- $\gamma$  specifically induces the release of TNF, NO, and HMGB1 in macrophage cultures. Because TNF itself induces HMGB1 release in macrophage cultures (3), it was possible that IFN- $\gamma$  induced HMGB1 release partially via induction of TNF production. To test this possibility, we first examined the effect of TNF-neutralizing Abs on IFN- $\gamma$ -mediated HMGB1 release in RAW 264.7 cell cultures. TNF-neutralizing Abs dose-dependently abrogated IFN- $\gamma$ -induced HMGB1 release, with a significant, maximal, suppression of HMGB1 release by ~50% ( $p < 0.05$ ; Fig. 3A). Irrelevant IgGs or IL-1 $\beta$ -specific neutralizing Abs (R&D Systems; data not shown) did not affect IFN- $\gamma$ -induced HMGB1 release at these concentrations (5, 10, 20  $\mu$ g/ml; Fig. 3A), indicating that IFN- $\gamma$ -induced TNF partly contributes to IFN- $\gamma$ -mediated HMGB1 release.

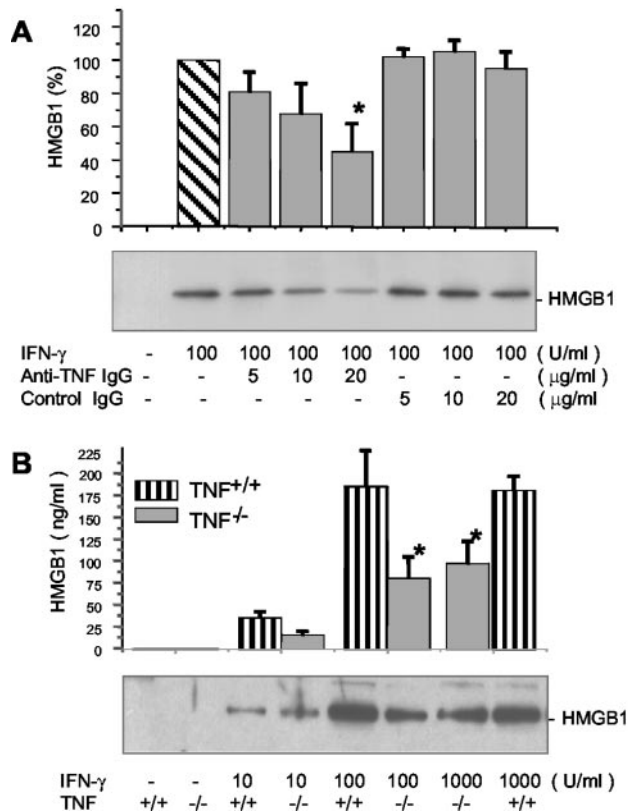
To further confirm the role of TNF in IFN- $\gamma$ -induced HMGB1 release, primary peritoneal macrophages were isolated from TNF-deficient C57BL/6 mice or control littermates, and stimulated with IFN- $\gamma$  at various concentrations. As shown in Fig. 3B, IFN- $\gamma$  stimulation triggered dose-dependent HMGB1 release in primary peritoneal macrophages from both TNF-deficient (TNF<sup>-/-</sup>) and control C57BL/6 (TNF<sup>+/+</sup>) mice. A wide concentration range of IFN- $\gamma$  (10, 100, and 1000 U/ml) induced significantly less HMGB1 release in cultures of TNF-deficient (TNF<sup>-/-</sup>) macrophages as compared with normal (TNF<sup>+/+</sup>) macrophages ( $p < 0.01$ ; Fig. 3B), indicating an important role for TNF in IFN- $\gamma$ -induced HMGB1 release. Similarly, a wide concentration range of IFN- $\gamma$  (10, 100, and 1000 U/ml) also caused significantly less release of NO in cultures of TNF-deficient (TNF<sup>-/-</sup>) macrophages as compared with normal (TNF<sup>+/+</sup>) macrophages ( $p < 0.01$ ; Fig. 2C), implicating a possible role for NO in the regulation of IFN- $\gamma$ -induced HMGB1 release. Cell viability, as assessed by trypan blue exclusion, was unaffected by IFN- $\gamma$  even at concentrations up to 1000 U/ml (control cell viability = 94–96%; vs IFN- $\gamma$ -treated cells (1000 U/ml), cell viability = 95–97%, 16 h after stimulation), indicating that the IFN- $\gamma$ -induced HMGB1 release was not due to cell death.



**FIGURE 2.** IFN- $\gamma$  induces the release of HMGB1, TNF, and NO in a dose-dependent manner. Primary peritoneal macrophages were isolated from C57BL/6J mice, and stimulated with IFN- $\gamma$  at various concentrations for 16 h. The levels of HMGB1 (A), other cytokines (i.e., TNF, IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6) (B), and NO (C) in the culture medium were expressed as mean  $\pm$  SEM of two independent experiments.

#### Effects of IFN- $\gamma$ stimulation on phosphorylation of MAPKs and STAT1

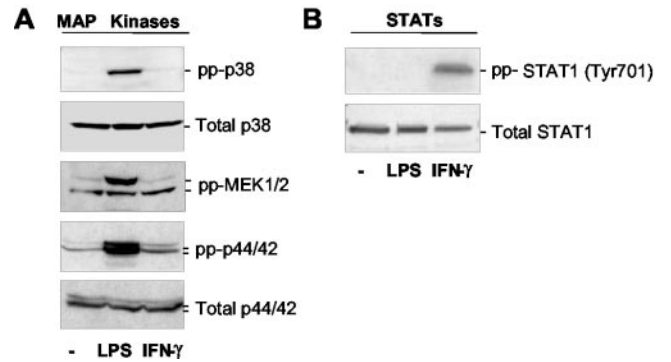
It has been well demonstrated that IFN- $\gamma$  and bacterial endotoxin (LPS) use different signal transduction pathways to activate macrophages. To evaluate the potential roles of different signaling pathways in the regulation of HMGB1 release, we examined the



**FIGURE 3.** Roles of TNF in IFN- $\gamma$ -induced HMGB1 release. *A*, Anti-TNF Abs partially abrogated IFN- $\gamma$ -induced HMGB1 release. RAW 264.7 cells were stimulated with IFN- $\gamma$  (100 U/ml) either alone or in the presence of anti-TNF Abs (anti-TNF IgG) or irrelevant (control IgG) Abs for 16 h, and the levels of HMGB1 in the culture medium were expressed as percentage (%) of maximal stimulation (i.e., + IFN- $\gamma$  alone). Shown in the upper panel are mean  $\pm$  SEM of two independent experiments; shown in the lower panel is a representative Western blot. *B*, TNF-deficient (TNF<sup>-/-</sup>) macrophages released significantly less HMGB1 in response to IFN- $\gamma$  stimulation. Primary peritoneal macrophages were isolated from normal C57BL/6J (TNF<sup>+/+</sup>) or TNF-deficient (TNF<sup>-/-</sup>) C57BL/6 mice, stimulated with IFN- $\gamma$  for 16 h at indicated concentrations, and assayed for HMGB1 levels by Western blotting analysis. Shown in the upper panel are means  $\pm$  SEM of two independent experiments, and shown in the lower panel is a representative Western blot.

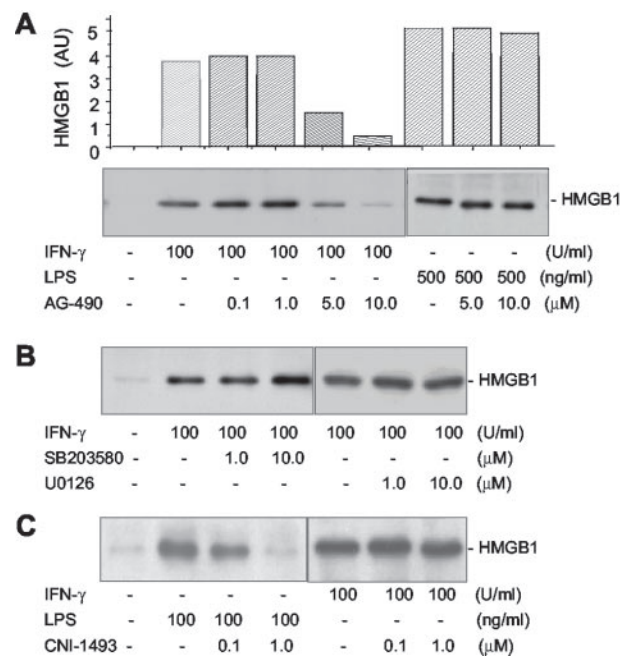
effects of IFN- $\gamma$  stimulation on phosphorylation of various MAPKs and STAT1 in macrophage cultures. As expected, LPS, but not IFN- $\gamma$ , induced a significant phosphorylation of p38, MEK1/2, and p44/42 (ERK1/2) (Fig. 4A), indicating that IFN- $\gamma$ -induced HMGB1 release was not dependent on MAPK activation. CNI-1493, a tetravalent guanlylhydrazone inhibitor of p38 and c-Jun N-terminal kinase MAPK (38, 42), dose-dependently inhibited HMGB1 release induced by LPS, but not by IFN- $\gamma$  (Fig. 5C). Other specific inhibitors for p38 (SB203580) or MEK1/2 (U0126) failed to inhibit IFN- $\gamma$ -induced HMGB1 release even at high concentrations (up to 10  $\mu$ M) (Fig. 5B), indicating that IFN- $\gamma$  mediates HMGB1 release through MAPK-independent mechanisms. Notably, CNI-1493, even at concentrations up to 1  $\mu$ M, failed to suppress IFN- $\gamma$ -induced release of NO (data not shown), further implicating a possible role for NO in the regulation of IFN- $\gamma$ -induced HMGB1 release.

In contrast, IFN- $\gamma$ , but not LPS, triggered specific phosphorylation of STAT1 (Tyr<sup>701</sup>) at concentrations that were also effective in inducing HMGB1 release (Fig. 4B). JAK2 kinase is critical in transmitting signal from IFN- $\gamma$  to downstream molecules (e.g.,

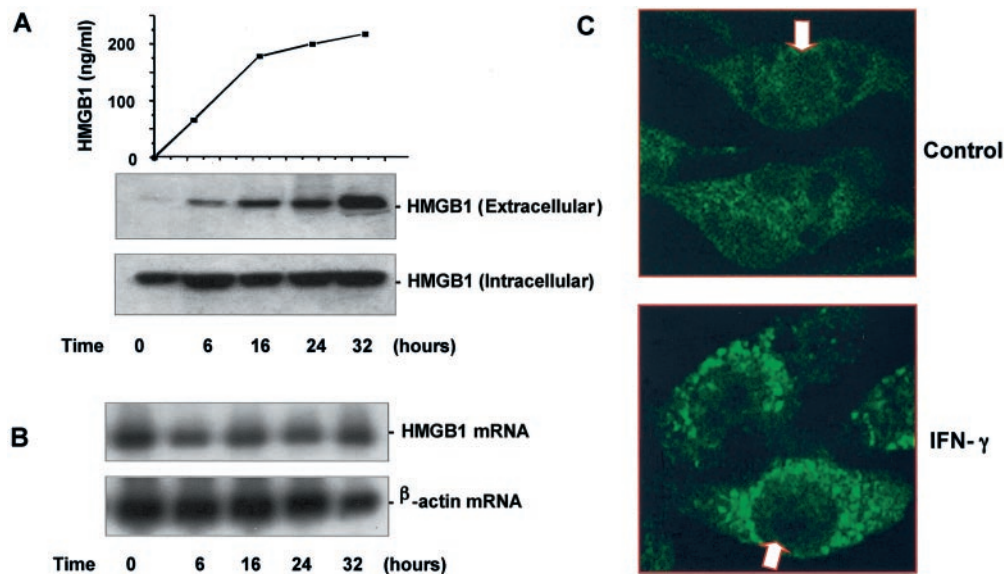


**FIGURE 4.** Effect of IFN- $\gamma$  on phosphorylation of STAT1 and MAPKs (p38, MEK1/2, and ERK1/2). RAW 264.7 cell cultures were stimulated with LPS or IFN- $\gamma$  for 30 min, and the concentrations of phospho-MAPKs (*A*) and phospho-STAT1 (*B*) were determined by Western blotting analysis using specific Abs.

STAT1), because JAK2-deficient cells fail to respond to IFN- $\gamma$  (43). A specific JAK2 inhibitor, AG490, dose-dependently abrogated IFN- $\gamma$ -induced HMGB1 release (Fig. 5A). The suppressive effect of AG490 on IFN- $\gamma$ -induced HMGB1 release was specific, because AG490 did not affect LPS-induced HMGB1 release (Fig. 5A), confirming that a JAK2-independent response is not affected by AG490. Furthermore, the suppression of AG490 on IFN- $\gamma$ -induced HMGB1 release was not due to cell toxicity, because, at low concentrations (from 0 to 10  $\mu$ M) effective for inhibiting HMGB1 release, AG490 did not affect the cell viability of macrophage cultures (control cell viability = 95–98%; vs AG490-treated cells (10  $\mu$ M), cell viability = 95–97%). This is consistent with a previous report demonstrating that AG490 is nontoxic, and effective in animal models of allergic encephalomyelitis (44). However, at higher concentrations (from 50 to 250  $\mu$ M), AG490 did exhibit a dose-dependent cytotoxicity to macrophage cultures (AG490-treated cells (50  $\mu$ M),



**FIGURE 5.** Effect of inhibitors of JAK2 and MAPKs on HMGB1 release. Murine macrophage-like RAW 264.7 cells were stimulated for 16 h with IFN- $\gamma$  either alone, or in the presence of various inhibitors for JAK2 (*A*) or MAPKs (*B* and *C*), and assayed for HMGB1 levels by Western blotting analysis.



**FIGURE 6.** Effect of IFN- $\gamma$  stimulation on HMGB1 release and localization in macrophage cultures. Murine macrophages cells were stimulated with IFN- $\gamma$  (100 U/ml) for various time periods, and cell cultures and cell-conditioned culture medium were separately assayed for levels of HMGB1 protein by Western blotting analysis (A), or HMGB1 mRNA by RNase protection assay (B). A, Effect of IFN- $\gamma$  stimulation on levels of intracellular and extracellular HMGB1 in macrophage cultures. The extracellular HMGB1 levels were time-dependently increased in the culture medium, whereas the intracellular HMGB1 levels remained unchanged after IFN- $\gamma$  stimulation. B, Effect of IFN- $\gamma$  stimulation on levels of HMGB1 mRNA in macrophage cultures. The levels of HMGB1 mRNA, as normalized by the levels of a housekeeping gene ( $\beta$ -actin) mRNA, were not significantly changed by IFN- $\gamma$  stimulation. C, Effect of IFN- $\gamma$  stimulation on localization of cellular HMGB1 in macrophage cultures. HMGB1 staining appeared to be diffusely distributed in the cytoplasm and nuclear region of unstimulated (control) macrophage cultures, but was observed predominantly in the cytoplasm as aggregated granules after IFN- $\gamma$  stimulation, indicating a possibility that IFN- $\gamma$ -stimulated macrophages actively translocate nuclear HMGB1 to the cytoplasm before releasing it into the extracellular milieu. The arrows point to central, nuclear regions of representative cells.

cell viability = 80–90%), and consequently increased (rather than decreased) IFN- $\gamma$ -stimulated HMGB1 release (data not shown). This is expected, because recent studies by Scaffidi et al. (13) suggested that necrotic cells passively release HMGB1. Notably, AG490 also dose-dependently inhibited IFN- $\gamma$ -induced release of TNF (TNF<sub>IFN- $\gamma$  alone</sub> = 885  $\pm$  75 pg/ml; TNF<sub>IFN- $\gamma$  + 1  $\mu$ M AG490</sub> = 757  $\pm$  87 pg/ml; TNF<sub>IFN- $\gamma$  + 5  $\mu$ M AG490</sub> = 647  $\pm$  65 pg/ml; and TNF<sub>IFN- $\gamma$  + 10  $\mu$ M AG490</sub> = 355  $\pm$  56 pg/ml). Thus, it will be important to further investigate the potential roles of JAK2-STAT1 signaling pathway in the regulation of IFN- $\gamma$ -mediated release of TNF and HMGB1 release in future studies.

#### Effect of IFN- $\gamma$ stimulation on HMGB1 cellular localization

IFN- $\gamma$  stimulated a time-dependent release of HMGB1, beginning at  $\sim$ 6 h and peaking between 16 and 24 h after IFN- $\gamma$  stimulation (Fig. 6A). To determine whether the increase of extracellular HMGB1 levels was associated with a decline of intracellular levels, culture medium and cells were assayed for HMGB1. Intracellular HMGB1 levels were not decreased after IFN- $\gamma$  stimulation, even though extracellular HMGB1 levels were significantly increased after IFN- $\gamma$  stimulation (Fig. 6A), suggesting that nascent HMGB1 may be continuously synthesized to replenish the intracellular pool of HMGB1 following IFN- $\gamma$  stimulation. The effect of IFN- $\gamma$  stimulation on cellular HMGB1 mRNA levels was evaluated by RNase protection assay. Consistent with our earlier observations (3), RAW 264.7 cell cultures constitutively expressed HMGB1 mRNA, and maintained a basal level even in the absence of inflammatory stimuli (Fig. 6B). IFN- $\gamma$  stimulation did not significantly change its cellular mRNA levels in macrophage cultures (Fig. 6B), although the total (i.e., intracellular plus extracellular) levels of HMGB1 protein were markedly increased after IFN- $\gamma$  stimulation.

To determine whether IFN- $\gamma$  stimulation affects HMGB1 cellular localization, macrophage cultures were immunostained with Ag affinity-purified anti-HMGB1 Abs. Quiescent macrophages constitutively expressed HMGB1 and maintained an intracellular pool of HMGB1 in the cytoplasm and nuclear regions (Fig. 6C). The levels of HMGB1, as revealed by the intensity of fluorescent immunostaining, did not change substantially in macrophage cultures after IFN- $\gamma$  stimulation (Fig. 6C). However, the pattern and localization of HMGB1 staining were noticeably altered as early as 6 h after IFN- $\gamma$  stimulation. HMGB1 staining appeared to be diffusely distributed in both the cytoplasm and nucleus regions of unstimulated macrophage cultures, but was observed predominantly in the cytoplasm of IFN- $\gamma$ -stimulated macrophages as numerous aggregated granules (Fig. 6C). Similar changes of HMGB1 localization have been observed by others for monocytes in response to LPS stimulation (45). This suggests that IFN- $\gamma$ -stimulated macrophages actively translocate nuclear HMGB1 to the cytoplasm before releasing it into the extracellular milieu.

#### Discussion

IFN- $\gamma$  is produced by activated immune cells (e.g., T cells, NK cells, and macrophages) (46, 47), and plays important roles in the innate immune response by enhancing the production of proinflammatory cytokines (e.g., TNF and IL-1 $\beta$ ) (28–31). Administration of IFN- $\gamma$  increases lethality in animal models of sepsis (e.g., cecal ligation and puncture) (32). Pharmacological inhibition of IFN- $\gamma$  with neutralizing Abs (33, 34), or genetic disruption of IFN- $\gamma$  expression (35–37), protects against lethality in animal models of endotoxemia. In this study, we have demonstrated that IFN- $\gamma$  specifically induces the release of TNF, NO, and HMGB1,

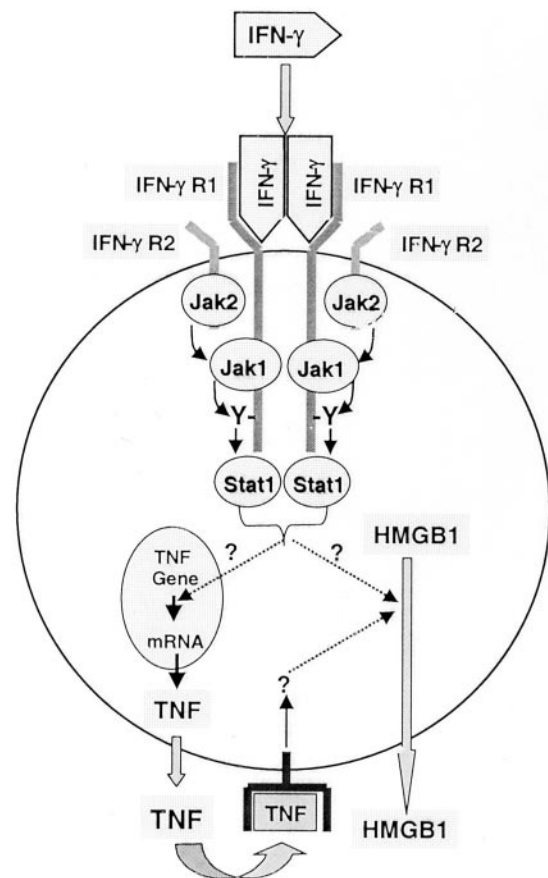
but not other cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6. IFN- $\gamma$ -induced HMGB1 release was not due to LPS contamination, because IFN- $\gamma$  effectively induced HMGB1 release even in peritoneal macrophages isolated from LPS-resistant C3H/HeJ mice, but failed to activate MAPKs (e.g., p38, MEK1/2, and p44/42) at concentrations that effectively induced HMGB1 release. It was also not dependent on cell death, because cell viability was not affected by IFN- $\gamma$  at concentrations that effectively induced HMGB1 release. The IFN- $\gamma$ -induced HMGB1 release was only partially dependent on the induction of TNF, because pharmacological inhibition of TNF activity with neutralizing Abs, or genetic disruption of TNF expression (TNF knockout), consistently led to a partial (40–50%) inhibition of IFN- $\gamma$ -mediated HMGB1 release. Previous studies demonstrate that IFN- $\gamma$  can effectively stimulate macrophages to release NO (48, 49), and *S*-nitrosylation can regulate functions of proteins in specific subcellular compartments (50). It is plausible that NO may occupy an important role in the regulation of HMGB1 translocation from one cellular compartment to another (potentially through *S*-nitrosylation), and thereby regulate its subsequent release into the extracellular milieu. This hypothesis has been supported by interconnected, parallel changes of NO and HMGB1 release under several experimental conditions. Genetic disruption of TNF expression led to a parallel inhibition of IFN- $\gamma$ -induced release of both NO (51) and HMGB1 in macrophage cultures. Furthermore, a macrophage-deactivating agent, CNI-1493, simultaneously attenuated LPS-mediated release of NO (41) and HMGB1, but failed to suppress IFN- $\gamma$ -induced release of NO and HMGB1. Thus, it is important to test this hypothesis using specific inducible NO synthase/NO synthase inhibitors and/or inducible NO synthase-deficient cell/cell line (e.g., p388D1) in future studies. Nevertheless, it is now reasonable to consider the possibility that IFN- $\gamma$  may exert its immune-stimulating function partly through induction of early (i.e., TNF) and late (i.e., HMGB1, NO) proinflammatory mediators.

Although various inflammatory stimuli (e.g., LPS) induce the sequential release of early (e.g., TNF (1–2 h), IL-1 (4–6 h), and IFN- $\gamma$  (4–6 h)) (22, 23), and late (e.g., MIF and HMGB1) proinflammatory cytokines (3, 4, 25–27), the mechanisms underlying the regulation of these early and late proinflammatory mediators are quite different. For instance, TNF is produced in vanishingly small amounts (if any at all) in quiescent macrophage cells, but its transcription and translation are rapidly up-regulated upon stimulation by a variety of inflammatory stimuli, which enables the synthesis and release of large quantities of TNF within a short time period (52). In contrast, HMGB1 is constitutively expressed in quiescent macrophage cells to maintain a large preformed pool of HMGB1 in both the nucleus and cytoplasm regions. For instance, upon stimulation with IFN- $\gamma$ , the intracellular levels of HMGB1 mRNA and protein remain unchanged, but HMGB1 is found predominantly in the cytoplasm as numerous aggregated granules. This raises the possibility that IFN- $\gamma$ -stimulated macrophages actively translocate nuclear HMGB1 to the cytoplasm before releasing it into the extracellular milieu. Indeed, using cell fractionation and immunostaining techniques, Gardella et al. (45) demonstrated that activated monocytes also translocated HMGB1 from the nucleus to cytoplasmic organelles for subsequent active secretion. Similarly, Kokkola et al. (53) observed that activated macrophages/monocytes in inflammatory synovial tissue of both experimentally induced arthritis and clinical rheumatoid arthritis appeared to translocate nuclear HMGB1 to the cytoplasm.

The regulation of early and late proinflammatory cytokines may use different signal transduction pathways. For instance, MAPKs (e.g., p38 and ERK1/2) play important roles in the regulation of TNF and IL-1 $\beta$  production in macrophage cultures (19, 52), and therapeutic agents targeting p38 MAPK (e.g., SB203580, SB239063, and

RWJ-67657) (54) exhibited certain efficacy in animal models of endotoxemia (55), polymicrobial sepsis (56), arthritis (57), chronic airway disease (58), and cerebral ischemia (59). In contrast, MAPKs are not important in the regulation of IFN- $\gamma$ -induced HMGB1 release, because 1) MAPKs (such as p38, ERK1/2, and MEK1/2) were not effectively activated by IFN- $\gamma$ , and 2) MAPK inhibitors did not effectively inhibit IFN- $\gamma$ -induced HMGB1 release in macrophage cultures. Thus, IFN- $\gamma$  induced HMGB1 release through a MAPK-independent mechanism.

IFN- $\gamma$  activates several groups of signaling molecules, including the receptor-associated Janus tyrosine kinases (e.g., JAK1 and JAK2) (60, 61), tyrosine kinase (62), the STATs (e.g., STAT1) (63), and IFN regulatory factors (64, 65) (Fig. 7). The critical roles of JAK-STAT signaling pathways in IFN- $\gamma$ - or IL-6-mediated cellular response have been well demonstrated using transgenic mice deficient in STAT1, JAK1, or JAK2 (66–68). For instance, JAK2-deficient cells are still responsive to IL-6 (67), but fail completely to respond to IFN- $\gamma$ , indicating a critical, nonredundant role of JAK2 in the IFN- $\gamma$ -signaling pathway. In light of our observations that IL-6 failed to induce HMGB1 release and that a specific inhibitor of JAK2, AG490 (69, 70), abrogated IFN- $\gamma$ -induced



**FIGURE 7.** Conceptual relationships between IFN- $\gamma$ , JAK2, TNF, and HMGB1 release in macrophage cultures. Engagement of IFN- $\gamma$  with its receptor leads to aggregation of receptor complex (with two molecules of IFN- $\gamma$ , JAK1, JAK2, IFN- $\gamma$ R1, and IFN- $\gamma$ R2 subunits), sequential phosphorylation of JAK2, JAK1, and IFN- $\gamma$ R1 (at a tyrosine residue near the C terminus), and subsequent recruitment of STAT1. Once bound to the receptor complex, STAT1 is phosphorylated by activated JAK2 kinase and translocated to the nucleus, where it participates in the regulation of expression of various genes. In this study, we demonstrated that IFN- $\gamma$  induced the release of TNF and HMGB1 potentially via a JAK2 signaling pathway, although the intricate molecular cascade underlying regulation of IFN- $\gamma$ -induced HMGB1 release remains to be further investigated.

HMGB1 release, it is now reasonable to consider an important role of JAK2 in the regulation of IFN- $\gamma$ -induced HMGB1 release. However, our present data cannot eliminate the potential involvement of other IFN- $\gamma$  signaling molecules including tyrosine kinase and IFN regulatory factor in regulation of the release of HMGB1. Thus, it will be important to investigate the potential roles of these IFN- $\gamma$  signaling transduction pathways in the regulation of TNF production, NO release, or HMGB1 secretion in future studies. Although early (e.g., TNF, IL-1 $\beta$ , and IFN- $\gamma$ ) and late (e.g., HMGB1) proinflammatory mediators may interact and contribute to the damaging sequelae of lethal systemic inflammation, therapeutic agents targeting JAK2 and other signaling pathways may prove to be useful in the treatment of lethal systemic inflammation diseases.

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