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Inflammasome-Dependent Release of the Alarmin HMGB1 in Endotoxemia

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Endotoxin administration recapitulates many of the host responses to sepsis. Inhibitors of the cysteine protease caspase 1 have long been sought as a therapeutic because mice lacking caspase 1 are resistant to LPS-induced endotoxic shock. According to current thinking, caspase 1-mediated shock requires the proinflammatory caspase 1 substrates IL-1β and IL-18. We show, however, that mice lacking both IL-1β and IL-18 are normally susceptible to LPS-induced splenocyte apoptosis and endotoxic shock. This finding indicates the existence of another caspase 1-dependent mediator of endotoxemia. Reduced serum high mobility group box 1 (HMGB1) levels in caspase 1-deficient mice correlated with their resistance to LPS. A critical role for HMGB1 in endotoxemia was confirmed when mice deficient for IL-1β and IL-18 were protected from a lethal dose of LPS by pretreatment with HMGB1-neutralizing Abs. We found that HMGB1 secretion from LPS-primed macrophages required the inflammasome components apoptotic speck protein containing a caspase activation and recruitment domain (ASC), caspase 1 and Ipaf, whereas HMGB1 secretion from macrophages infected in vitro with Salmonella typhimurium was dependent on caspase 1 and Ipaf. Thus, HMGB1 secretion, which is critical for endotoxemia, occurs downstream of inflammasome assembly and caspase 1 activation. The Journal of Immunology, 2010, 185: 4385–4392.

Lethal endotoxemia in mice models mimics many features of septic shock in patients, including elevated cytokine production and extensive leukocyte apoptosis (1). The molecular mechanisms underlying lethal endotoxemia are not understood completely, but one important mediator is high mobility group box 1 (HMGB1) (2–4), which was identified originally as a highly conserved DNA-binding factor in the nucleus with roles in DNA transcription, replication, and repair (5). Subsequent studies showed HMGB1 at high levels in the serum of septic humans and animals (2, 6). A role for HMGB1 in shock-associated lethality was indicated when passive immunization with HMGB1-neutralizing Abs prevented organ damage in animal sepsis models (3).

Macrophage cell lines have been shown to release HMGB1 in response to LPS and during necrosis, but not when exposed to apoptotic stimuli (7). These observations fueled the concept that HMGB1 is a prototypical endogenous danger signal or alarmin, which is released from activated macrophages and necrotic cells. Secreted HMGB1 binds immune receptors, including TLRs and the receptor for advanced glycation end products (RAGE), to elicit proinflammatory responses (8). HMGB1 lacks a classic secretion signal, so the mechanism by which HMGB1 is released from cells remains unclear. Proinflammatory cytokines IL-1β and IL-18 also lack a signal peptide for the classic endoplasmic reticulum–Golgi exocytosis pathway. These cytokines are both produced as inactive precursors in the cytosol and are released in an ill-defined manner following their maturation by the cytosolic protease caspase 1. The cytosolic zymogen form of caspase 1 consists of an N-terminal procaspase domain, followed by two subunits that together form the protease (9). Procaspase 1 is processed and activated within large protein complexes termed inflammasomes, which are assembled on nucleotide-binding oligomerization domain-like receptor (NLR) protein scaffolds (10).

NLR family members are believed to recognize conserved microbial and viral components called pathogen-associated molecular patterns (PAMPs), as well as endogenous, intracellular danger-associated molecular patterns (10). The N terminus of an NLR generally contains a homotypic interaction motif, such as the caspase activation and recruitment domain (CARD) or pyrin domain. A central nucleotide-binding oligomerization domain is thought to be involved in self-oligomerization, whereas the C-terminal leucine-rich repeats sense specific PAMPs and autoregulate NLR activity. Adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) bridges the interaction between NLR proteins and caspase 1, having an N-terminal pyrin domain and C-terminal CARD. Whereas ASC is always essential for activation of caspase 1 (11), the NLR protein Ipaf is only required for caspase 1 activation in macrophages infected with bacterial pathogens Salmonella (11–13), Pseudomonas (14–16), Legionella (17), and Shigella (18). The related NLR family member Nalp3 mediates caspase 1 activation in response to bacterial PAMPs such as LPS when combined with...
a second stimulus, such as the P2X7 receptor ligand ATP or the cation ionophore nigericin (19–23). Nalp3 also activates caspase 1 in LPS-primed macrophages exposed to uric acid, silica, and asbestos crystals (24–26) or when macrophages are infected with *Staphylococcus aureus* (19, 27) or *Klebsiella pneumonia* (28).

Caspase 1-deficient mice are highly resistant to LPS-induced endotoxemia (29), but the contributions of caspase 1 substrates IL-1β and IL-18 to endotoxic shock are less clear. Mice lacking IL-1β are normally susceptible to LPS-induced endotoxemia (30). We compared the response of caspase 1-deficient mice to that of animals lacking both IL-1β and IL-18. Unlike mice lacking caspase 1, animals lacking both IL-1β and IL-18 were normally susceptible to LPS-induced splenocyte apoptosis and endotoxic shock. We show that caspase 1 and its upstream activators are required for secretion of a third proinflammatory mediator called HMGB1, and when this is neutralized in vivo, IL-1β/IL-18 doubly deficient mice are protected from endotoxemia.

**Materials and Methods**

**Mice**

*Nalp3*^−/−*, Ipaf*^−/−*, *ASC*^−/−*, caspase 1^−/−*, caspase 7^−/−*, and *IL-1β*^−/−*/*IL-18*^−/−* mice have been described (11, 19, 31–33). All strains were generated with C57BL/6 embryonic stem cells or backcrossed to C57BL/6 mice for at least 10 generations. Studies complied with the National Institutes of Health Guide for the Care and Use of Laboratory animals and were approved by the Genentech Institutional Animal Care and Use Committee (South San Francisco, CA).

**Macrophages**

Bone marrow-derived macrophages (BMDMs) were prepared as described (33). Briefly, bone marrow from femurs of 6–12-wk-old mice were cultured in IMDM containing 10% heat-inactivated FBS, 20% L cell-conditioned medium, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2. Adherent cells after 5–7 d of culture were replated in 6-well or 24-well dishes in IMDM containing 10% heat-inactivated FBS and antibiotics.

**Endotoxemia**

Male mice (6–8 wk old) were maintained in microisolator cages and received food and water ad libitum according to American Association of Laboratory Animal Care guidelines. Lethal endotoxemia was induced by i.p. injection of 40 mg/kg LPS (Escherichia coli serotype 0111:B4; Sigma-Aldrich, St. Louis, MO). The spleen was harvested after 24 h, and formalin-fixed, paraffin-embedded sections were stained with H&E. IL-1β^−/−/*IL-18* doubly deficient mice were injected i.p. with 100 μg/kg rabbit polyclonal HMGB1-neutralizing Abs (34) or control rabbit IgGs (15006; Sigma-Aldrich) 1 h prior to i.p. injection of 20 mg/kg LPS. Group survival was analyzed with the Kaplan-Meier test in Prism5 (GraphPad, San Diego, CA). A p value < 0.05 was considered statistically significant.

**Bacterial infection and stimulation with microbial ligands**

*Salmonella enterica* serovar *typhimurium* strain SL1344 and the isogenic orgA mutant strain B66 (SipB−) were kindly provided by D. Monack.
Glutamine at 37˚C in a humidified atmosphere containing 5% CO₂. Cells were transfected with lipofectamine (Invitrogen, Carlsbad, CA) for 12 h, and then lysates were prepared in 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.4), 200 mM NaCl, 5 mM EDTA, and 10% glycerol.

**Plasmids and transfection**

pCMV-Sport6–pro-IL-1β and pCMV-Sport6-HMGB1 plasmids encoding mouse HMGB1 and pro-IL-1β were purchased from American Type Culture Collection (Manassas, VA), and cDNA inserts were verified by DNA sequencing. 293T cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM t-glutamine at 37˚C in a humidified atmosphere containing 5% CO₂. Cells were transfected with lipofectamine (Invitrogen, Carlsbad, CA) for 12 h, and then lysates were prepared in 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.4), 200 mM NaCl, 5 mM EDTA, and 10% glycerol.

**In vitro caspase 1 cleavage assay**

pCMV-Sport6–pro-IL-1β and pCMV-Sport6-HMGB1 (250 ng each) were transcribed and translated in vitro with a SP6-promoter-driven wheat germ-extract protein expression system (Promega, Madison, WI) and [35S]methionine. Translation products (2 μl) were incubated with 1 U recombinant caspase 1 (BioVision, Mountain View, CA) in 23 μl cell-free system buffer [10 mM HEPES (pH 7.4), 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM sodium pyruvate, and 1 mM DTT] for up to 1 h at 37˚C. Where indicated, recombinant caspase 1 was pretreated with 1 μM YVAD-cmk (Calbiochem, San Diego, CA) for 10 min.

**Fluorescence microscopy**

A total of 100,000 BMDMs were plated on glass cover slips (BD Biosciences, San Jose, CA), and 24 h later, cells were stimulated, washed in PBS, and fixed in 4% paraformaldehyde for 10 min. Cells were permeabilized with 0.1% Triton X-100 for 5 min. Blocking was with Image-iT FX Signal enhancer (Invitrogen) and then blocking buffer (10% goat serum in PBS, 0.1% Triton X-100, 0.1% saponin) for 60 min. Cells were stained overnight with 1 μg/ml HMGB1 Ab (Abcam, Cambridge, MA) in blocking buffer. After washing, bound Ab was revealed with 1 μg/ml Texas Red-conjugated goat anti-rabbit Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Slides were mounted with Prolong gold-DAPI (Invitrogen) and analyzed at room temperature under a Zeiss LD Plan-NEOFLUAR 20×0.4 PCor2 korr objective on a Zeiss Axiosvert 200M microscope equipped with a Zeiss AxioCam CCD digital camera and Axiovision Rel.4.6 software (Zeiss, Oberkochen, Germany). Fluorescence signals in digital black-and-white fluorographs were artificially colored with Adobe Photoshop (Adobe Systems, San Jose, CA).

**Caspase 1 Ab** was a generous gift of Dr. P. Vandenabeele (Ghent University, Ghent, Belgium). The HMGB1 Ab was purchased from Abcam (catalog number ab18256).

**HMGB1 and cytokine release**

HMGB1 in serum and culture supernatants was quantified with an HMGB1 ELISA (Shino test). Mouse cytokines were measured by Luminex assay (Bio-Rad, Hercules, CA). Data were analyzed with the Student t test. A p value < 0.05 was considered statistically significant.

**Results**

Caspase 1-dependent release of HMGB1 is essential for LPS-induced endotoxemia

Although many of the downstream proinflammatory effects of caspase 1 activation are mediated by caspase 1 substrates IL-1β and IL-18 (35), it still is not clear why mice lacking caspase 1 are...
resistant to LPS-induced splenocyte apoptosis and lethality (29). IL-1β-deficient mice, unlike caspase 1-deficient mice, are susceptible to LPS-induced endotoxemia (30), so we determined whether this is because they still produce the proinflammatory cytokine IL-18. Wild-type (WT), caspase 1−/−, and il-1β−/−/il-18−/− mice were given a lethal dose of LPS. All WT and il-1β−/−/il-18−/− mice had succumbed by 72 h (Fig. 1A). By contrast, the caspase 1−/− mice remained viable after 7 d. Consistent with these data, significant endotoxin-induced lymphocyte apoptosis (1, 36) was evident at 24 h in the spleens of WT and il-1β−/−/il-18−/− mice, but this was markedly reduced in caspase 1−/− spleens (Fig. 1B). Splenocyte apoptosis in WT and il-1β−/−/il-18−/− mice was due to the LPS treatment, because apoptosis was not seen in mice receiving saline alone. We counted apoptotic cells in the splenic white pulp of 4 mice of each genotype using five randomly chosen high-power fields (×400), and this confirmed that caspase 1−/− mice contained significantly fewer apoptotic lymphocytes than WT or il-1β−/−/il-18−/− mice (p = 0.0006 and p = 0.00002, respectively) (Fig. 1C). These results indicate that neither IL-1β nor IL-18 is essential for LPS-induced splenocyte apoptosis and lethal endotoxic shock. Given that HMGB1 is an important mediator of shock (2, 3, 6), we measured its levels in the serum of LPS-treated WT and caspase 1−/− mice. In agreement with published data (2), LPS treatment increased serum HMGB1 levels in WT mice 5-fold (Fig. 1D). In stark contrast, serum HMGB1 levels in caspase 1−/−/il-1β−/−/il-18−/− mice differed little from vehicle-treated WT or caspase 1−/− mice. This caspase 1-dependent HMGB1 release was crucial for endotoxemia because HMGB1-neutralizing Abs, but not control IgGs, protected against LPS-induced lethality in il-1β−/−/il-18−/− mice (Fig. 1E).

**HMGB1 release from LPS-primed macrophages requires the NALP3 inflammasome**

Next we sought direct evidence that inflammasome components are required for HMGB1 release from primary mouse macrophages. Although monocytes and macrophage-like cell lines such as RAW264.7 release HMGB1 (2) and IL-1β (37) in response to LPS stimulation alone, it is well established that mouse BMDMs activate caspase 1 and release IL-1β only when LPS priming is accompanied by a second stimulus such as ATP (10, 11, 33, 37). We observed a similar phenomenon for caspase 1-dependent HMGB1 release: BMDMs cultured in 10 μg/ml LPS alone for up to 24 h did not release HMGB1 (Fig. 2A). Only BMDMs that were primed with LPS and then exposed to 5 mM ATP for 30 min secreted significant amounts of HMGB1. ATP stimulation of BMDMs in the absence of LPS-priming did not cause HMGB1 release (Fig. 2A). As a control for LPS functionality, proinflammatory TNF-α was released from BMDMs in response to LPS alone (Fig. 2B). Release of HMGB1 from BMDMs treated with LPS plus ATP was dependent on Nalp3 (Fig. 2C, 2D), ASC (Fig. 2E, 2F), and caspase 1 (Fig. 2E, 2F), further extending the similarities between HMGB1 and IL-1β secretion from BMDMs. The ionophore nigericin can substitute for ATP in Nalp3-dependent IL-1β secretion from BMDMs (19, 33). We found that BMDMs primed with 10 μg/ml LPS for 3 h and then treated with 20 μM nigericin for 30 min also released significant amounts of HMGB1 in a Nalp3− (Fig. 3A, 3B), ASC− (Fig. 3C, 3D), and caspase 1-dependent manner (Fig. 3C, 3D). In agreement, the caspase 1 inhibitor Ac-YVAD-cmk prevented the release of HMGB1 from LPS plus ATP and LPS plus nigericin-treated BMDMs (Supplemental Fig. 1). In contrast, HMGB1 release was not appreciably affected by the cathepsin B inhibitor CA-074-OMe (Supplemental Fig. 2).

**Salmonella-induced HMGB1 release requires the Ipaf inflammasome**

Whereas LPS/ATP and LPS/nigericin induce caspase 1 activation via the Nalp3 inflammasome, macrophages infected with the Gram-negative bacterial pathogen *Salmonella typhimurium* (*Salmonella*) respond by activating caspase 1 through the Ipaf inflammasome (11–13). Ipaf, in a manner yet to be determined, senses bacterial flagellin, which appears to access the cytosol using the bacterial type III secretion system (11–13). We used *Salmonella* infection of BMDMs to determine whether HMGB1 secretion was linked to the Nalp3 inflammasome specifically, or was a general outcome of caspase 1 activation. BMDMs infected with WT *Salmonella*, but not flagellin-mutant *fljB*/*fljC* or type III secretion system mutant *sipB*−/−, activated caspase 1 (Supplemental Fig. 3) and released significant amounts of HMGB1 into the culture medium, as determined by HMGB1 immunoblotting (Fig. 4A) and HMGB1-specific ELISA (Fig. 4B). TNF-α released in response to the two mutant bacteria, however, was comparable to that induced by WT *Salmonella* (Fig. 4C), demonstrating that impaired HMGB1 release was not due to a general lack of macrophage stimulation. The correlation between caspase 1 activation and HMGB1 secretion in macrophages infected with WT *Salmonella* suggested that Ipaf inflammasome activation was upstream of HMGB1 secretion. Indeed, BMDMs from ipaf−/− and caspase 1−/− mice failed to secrete HMGB1 in response to WT *Salmonella* (Fig. 4D–G), although...
they released normal amounts of TNF-α (11) (Supplemental Fig. 4). In agreement, the caspase 1 inhibitor Ac-YVAD-cmk prevented
the release of HMGB1 from
Salmonella-infected BMDMs (Supplemental Fig. 1), whereas the cathepsin B inhibitor CA-074-OMe had no affect (Supplemental Fig. 2). These results demonstrate that
the Ipaf inflammasome is essential for HMGB1 release from
Salmonella-infected macrophages and that HMGB1 release is a
general consequence of caspase 1 activation.

Inflammasome-mediated translocation and release of full-
length HMGB1 is independent of IL-1β, IL-18, and caspase-7

HMGB1 exists in the nucleus of resting cells, where it is linked to
DNA transcription, replication, and repair (5). We made use of
immunofluorescence microscopy to determine the role of the
inflammasome in the subcellular localization of HMGB1 in LPS-
primed BMDMs. WT and
nalp3−/− BMDMs contained nuclear
HMGB1 both before (Fig. 5A, leftmost panels) and after LPS
priming (Fig. 5A, second to left panels), the latter observation
consistent with a second stimulus being required for HMGB1
release (Fig. 2A). Subsequent exposure to either ATP or nigericin
resulted in almost complete depletion of the nuclear pool of
HMGB1 in WT BMDMs (Fig. 5A, second to right and right upper
panels). By contrast, nuclear HMGB1 levels were not reduced in
LPS/ATP- and LPS/nigericin-treated
nalp3−/− BMDMs (Fig. 5A, second to right and right lower panels). These results demonstrate
that LPS priming is not sufficient for Nalp3-dependent trans-
location of nuclear HMGB1 out of macrophages.

Secreted IL-1β and IL-18 undergo caspase 1-dependent matu-
rature (35), but we found no evidence of HMGB1 cleavage upon
release from BMDMs. HMGB1 recovered from the culture medium
of BMDMs exposed to either LPS/ATP or
Salmonella showed
comparable migration by SDS-PAGE to full-length HMGB1 over-
expressed in 293T cells (Fig. 5B). In addition, recombinant caspase
1 converted pro-IL-1β to mature IL-1β within 30 min, but under the
same conditions, recombinant caspase 1 did not cleave in vitro-
translated HMGB1, even after 60 min (Fig. 5C). Caspase 7 also
is activated downstream of caspase 1 (38). However, caspase 7
does not mediate caspase 1-dependent release of HMGB1 because
caspase 7−/− BMDMs stimulated with LPS/ATP or infected with
Salmonella secreted normal levels of HMGB1 (Fig. 5D). As
expected from our in vivo studies (Fig. 1A), release of HMGB1 was not compromised in IL-1β−/−/IL-18−/− BMDMs (Fig. 5E). Therefore, HMGB1 does not undergo proteolytic processing and is released independently of the known caspase 1 substrates IL-1β, IL-18, and caspase 7.

Discussion

The evolutionarily conserved cysteine protease caspase 1 is the
effector protein of large (700 kDa) cytosolic protein complexes
called inflammasomes (10). The stimulus-dependent oligomeri-
cation of certain NLR family members triggers inflammasome

FIGURE 4. HMGB1 release from Salmonella-infected macrophages requires bacterial flagellin, a functional type III secretion and the Ipaf inflammasome. A–C, BMDMs were left untreated (CTRL), infected with WT Salmonella (MOI 10), or with the type III secretion system-deficient (SipB−) or flagellin-deficient (fljB−/fliC−) mutants for 1 h. Culture supernatants were collected and analyzed for secreted HMGB1 by Western blotting (A) and ELISA (B) and for secreted TNF-α by Luminex assay (C). D and E, BMDMs from WT and Ipaf−/− mice were left untreated (CTRL) or infected with WT Salmonella (MOI 10) for 1 h. Culture supernatants were collected and analyzed for secreted HMGB1 by Western blotting (D) and ELISA (E). F and G, BMDMs from WT and caspase 1−/− mice were left untreated (CTRL) or infected with WT Salmonella (MOI 10) for 1 h. Culture supernatants were analyzed for secreted HMGB1 by Western blotting (F) and ELISA (G). Black arrowhead on Western blots marks HMGB1 (29 kDa). ELISA and Luminex data represent the mean ± SD of triplicate samples from a single experiment, and all results are representative of three independent experiments.
assembly and the proximity-induced autoproteolytic activation of caspase 1 (10, 39–41). Activated caspase 1 subsequently processes its substrates, which include caspase 7 (38) and the proinflammatory cytokines IL-1β and IL-18 (29, 42, 43). Caspase 1 also mediates a specialized form of programmed cell death in myeloid cells that is often referred to as pyroptosis, as it differs from apoptosis and programmed necrosis (44). The molecular mechanisms of pyroptosis remain elusive.

Mice lacking inflammasome components Nalp3 and caspase 1 are resistant to LPS-induced endotoxemia (19, 21, 29, 43), but the roles of the caspase 1 substrates IL-1β and IL-18 in endotoxic shock are less clear. Whereas il-1β−/− mice are susceptible to LPS-induced endotoxemia (30), the response of il-18−/− mice has not been reported. Our finding that il-1β−/−/il-18−/− mice are normally susceptible to LPS-induced endotoxic shock indicates that caspase 1 elicits endotoxemia through other effectors. il-1β−/−/ il-18−/− mice also succumbed to Escherichia coli-induced sepsis, whereas caspase 1-deficient mice cleared the infection (32). HMGB1 is a nuclear protein that is believed to cause inflammation when released into the extracellular space (2, 7). rHMGB1 produced in E. coli is a potent inducer of proinflammatory cytokines when administered to cells or injected in mice (2, 45–49), but studies using HMGB1 purified from eukaryotic sources such as rat brain (49) or calf thymus (50) suggested a requirement for endogenous or microbial cofactors for HMGB1 to potently activate RAGE and induce cytokine production. Thus, HMGB1 that is released in the bloodstream during infection might bind microbial components or substances released from injured tissue to induce or augment the production of inflammatory mediators. The assembly of such immunostimulatory complexes has been demonstrated for RAGE receptor activation by an HMGB1–CpG DNA complex (50). In addition to RAGE, HMGB1 was suggested to induce NF-κB signaling through TLR2 and TLR4 activation (51, 52), but this has been contested by others (50).

We showed in this study that serum HMGB1 levels are markedly reduced in LPS-challenged caspase 1−/− mice. The pan-caspase inhibitor zVAD-fmk was shown previously to both reduce serum HMGB1 and suppress apoptosis in the spleen and thymus of septic mice, but the critical caspase associated with HMGB1 release was not evident (53). HMGB1-neutralizing Abs protected il-1β−/− / il-18−/− mice from a normally lethal dose of LPS, underscoring the critical role of HMGB1 release in endotoxemia. Because HMGB1
neutralization is being evaluated in clinical trials as a treatment for sepsis, bacteremia, and ARDS (4, 54), our finding that HMGB1 release occurs in splenocytes during endotoxemia and in isolated macrophages exposed to LPS/ATP or Salmonella, the caspase 1 substrate that promotes HMGB1 release remains to be identified. Caspase 1 has been suggested to allow secretion of leaderless cytokines, such as IL-1β and IL-18, demonstrating that inflammasomes engage various independent effector mechanisms to promote inflammation.

In summary, our results show that HMGB1 release is a new mechanism by which caspase 1 contributes to inflammation and endotoxemia. Caspase 1-mediated HMGB1 release occurred in the absence of IL-1β and IL-18, demonstrating that inflammasomes engage various independent effector mechanisms to promote inflammation.

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Disclosures
M.L., A.C.V., and V.M.D. are/were employees and/or shareholders of Genentech.

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