Release mechanism of high mobility group nucleosome binding domain 1 from lipopolysaccharide-stimulated macrophages

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Abstract. Alarmins are identified as endogenous mediators that have potent immune-activating abilities. High mobility group nucleosome binding domain 1 (HMGN1), a highly conserved, non-histone chromosomal protein, which binds to the inner side of the nucleosomal DNA, regulates chromatin dynamics and transcription in cells. Furthermore, HMGN1 acts as a cytokine in the extracellular milieu by inducing the recruitment and maturation of antigen-presenting cells (dendritic cells) to enhance Th1-type antigen-specific immune responses. Thus, HMGN1 is expected to act as an alarmin, when released into the extracellular milieu. The present study investigated the release mechanism of HMGN1 from macrophages using mouse macrophage-like RAW264.7 cells. The results indicated that HMGN1 was released from lipopolysaccharide (LPS)-stimulated RAW264.7 cells, accompanied by cell death as assessed by the release of lactate dehydrogenase (LDH). Subsequently, the patterns of cell death involved in HMGN1 release from LPS-stimulated RAW264.7 cells were determined using a caspase-1 inhibitor, YVAD, and a necroptosis inhibitor, Nec-1. YVAD and Nec-1 did not alter LPS-induced HMGN1 and LDH release, suggesting that pyroptosis (caspase-1-activated cell death) and necroptosis are not involved in the release of HMGN1 from LPS-stimulated RAW264.7 cells. In addition, flow cytometric analysis indicated that LPS stimulation did not induce apoptosis but substantially augmented necrosis, as evidenced by staining with annexin V/propidium iodide. Together these findings suggest that HMGN1 is extracellularly released from LPS-stimulated RAW264.7 macrophage-like cells, accompanied by unprogrammed necrotic cell death but not pyroptosis, necroptosis or apoptosis.

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Abbreviations: HMGN1, high mobility group nucleosome binding domain 1; LPS, lipopolysaccharide; HMGB1, high mobility group box 1; HMG, high mobility group; NF-κB, nuclear factor κB; MAPK, mitogen activated protein kinase

Key words: alarmin, endotoxin shock, sepsis, high mobility group nucleosome binding domain 1, macrophage

Introduction

Sepsis is caused by polymicrobial infections associated with severe systemic inflammatory response syndrome that leads to multiple organ failure, including acute lung injury and renal and hepatic failure, as well as septic shock (1-3). Bacterial endotoxin lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria and is important for stimulating mononuclear phagocytes (macrophages and monocytes) to secrete various inflammatory mediators (e.g. cytokines, reactive oxygen species, prostanoids/leukotrienes, proteases and nitric oxide), including alarmins (1,3,4).

Alarmins are identified as endogenous mediators that have potent immune-activating abilities. To date, distinct molecules of alarmins have been identified, including antibacterial peptide (defensin and cathelicidin), cytokines (IL-1β and IL-33), heat shock proteins, adenosine triphosphate, uric acid and nuclear components (HMG proteins, nucleosome and histone) (4-9). Generally, alarmins are extracellularly released by the following mechanisms: i) Passive release by necrotic (unprogrammed) cell death, which is caused by infective or noninfective tissue damage or ii) active release by certain immune cells without cell death by a specialized secretion pathway, in which alarmins are transported from the nucleus to the cytoplasm, then into the lysosome and eventually released into the extracellular space through exocytosis (9,10). Additionally, previous studies have revealed that pyroptosis, a form of programmed necrotic cell death accompanied by the formation of inflammasomes, caspase-1 activation and IL-1β production, induces the secretion of certain alarmins (11,12). Alarmins augment the innate and adaptive immune responses by distinct mechanisms and are capable of promoting the recruitment and activation of immune cells, including antigen-presenting cells (13,14). Under septic conditions, excessive release of several alarmins, including HMGB1 and IL-1β, leads to uncontrolled inflammation in the host (15,16). By contrast, alarmins also exhibit beneficial effects on the host by promoting the mobilization and activation of immune cells to eliminate

potential pathogens (13,14). Thus, alarmins are crucial in the pathological process of sepsis/septic shock.

High mobility group nucleosomal binding protein-1 (HMGN1), a highly conserved, non-histone chromosomal protein, which binds to the inner side of the nucleosomal DNA, regulates chromatin dynamics and transcription in cells (17,18). By contrast, HMGN1 acts as a cytokine in the extracellular milieu. HMGN1 induces the recruitment and maturation of antigen-presenting cells (dendritic cells) to enhance Th1-type antigen-specific immune responses (13). In addition, HMGN1 promotes the secretion of inflammatory cytokines from dendritic cells via direct interaction with Toll-like receptor 4 (TLR4), followed by the activation of myeloid differentiation primary response gene 88/nuclear factor κB (NF-κB) and the mitogen activated protein kinase (MAPK) pathway (14). Thus, HMGN1 is expected to act as an alarmin, when released into the extracellular milieu, and to be important in the pathogenesis of sepsis/septic shock. However, the mechanism of HMGN1 release from immune cells remains to be elucidated. The present study therefore investigated the release mechanism of HMGN1 from macrophages, which is important in sepsis/septic shock by producing inflammatory mediators in response to bacterial pathogen-associated molecular patterns (PAMPs), using mouse macrophage-like RAW264.7 cells.

Materials and methods

Reagents. LPS (from *E. coli* serotype O111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ac-Tyr-Val-Ala-Asp-H (Ac-YVAD-CHO), a specific caspase-1 inhibitor was purchased from Peptide Institute, Inc. (Osaka, Japan). Necrostatin-1 (Nec-1), a selective inhibitor of necroptosis and its inactive analog necrostatin-1 (Nec-1i) were purchased from Merck Millipore (Darmstadt, Germany).

Quantification of HMGN1 released from LPS-stimulated RAW264.7 cells. A murine macrophage cell line RAW264.7 was purchased from the European Collection of Cell Cultures (Wiltshire, UK) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal calf serum (endotoxin level <10 EU/ml; Cell Culture Technologies, Herndon, VA, USA) and 100 U/ml penicillin/100 μg/ml streptomycin (Nacalai Tesque, Inc., Kyoto, Japan) at 37˚C in a 5% CO_2 incubator. RAW264.7 cells ($2x10^5$ cells/well) were seeded into a 12-well culture plate and incubated overnight at 37˚C in 5% CO_2. Subsequently, cells were washed with fresh media and incubated with or without LPS (100 ng/ml) in the absence or presence of Ac-YVAD-CHO, Nec-1 or Nec-1i at a final concentration of 10 μM (each) in the media. Culture supernatants were recovered at 20 h after the incubation and HMGN1 in the culture supernatants was quantitated by western blotting. Prior to electrophoresis, supernatants were concentrated by trichloroacetic acid (TCA) precipitation. Briefly, 900 μl of supernatants were added with ice-cold 1/9 volume of 100% TCA (100 μl), vortexed and incubated on ice for 30 min. The mixtures were centrifuged at 8,000 x g at 4˚C for 15 min and the supernatants were aspirated. Pellets were washed with 1 ml of 100% ice-cold acetone and centrifuged at 8,000 x g at 4˚C for 5 min, and dissolved in 1X Laemmli sample buffer. Dissolved samples were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Nacalai Tesque, Inc.), and the resolved proteins were electrophoretically transferred onto polyvinylidine difluoride nitrocellulose membranes (Immobilon-P; Merck Millipore). The membranes were blocked in Blocking One (Nacalai Tesque, Inc.) and sequentially probed with rabbit anti-HMGN1 polyclonal antibody (2 μg/ml; cat. no. ab5212; Abcam, Cambridge, MA, USA), and horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (1:2,000 dilution; cat. no. AP187P; Chemicon International, Temecula, CA, USA). HMGN1 was finally visualized using Super Signal West Dura Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, USA) and the detected bands were analyzed using Multi Gauge software (version 3.0) and an LAS-3000 Image Analyzer (Fujifilm, Tokyo, Japan).

Quantification of lactate dehydrogenase (LDH) activities. LDH activity in the supernatants of LPS-stimulated RAW264.7 cells was determined for evaluating cell death. LDH activity in the supernatants and 1% Triton X-100-lysed cells (as a total activity of 100%) were measured using a commercially available LDH assay kit (Takara Bio, Inc., Shiga, Japan), according to the manufacturer's instructions.

Quantification of IL-1β. The quantity of IL-1β in the culture supernatants of LPS-stimulated RAW264.7 cells was measured using a commercially available mouse IL-1β ELISA kit (detection limits of 8 pg/ml; eBioscience, San Diego, CA, USA), according to the manufacturer's instructions.

Quantification of apoptosis. Apoptotic and necrotic cell death was assessed by staining cells with annexin V-fluorescein isothiocyanate and propidium iodide (PI). RAW264.7 cells ($2x10^5$ cells/well) were seeded into a 12-well culture plate and incubated overnight at 37˚C in 5% CO_2. Subsequently, cells were washed with fresh media and incubated with or without LPS (100 ng/ml) in the absence or presence of Ac-YVAD-CHO, Nec-1 or Nec-1i (10 μM each). After 8 h, cells were washed with 0.05% ethylenediaminetetraacetic acid (EDTA)/phosphate-buffered saline (PBS) and detached with 0.25% trypsin-EDTA (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, annexin V/PI staining was performed using a MEBCYTO Apoptosis kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), according to the manufacturer's instructions. Briefly, cells were centrifuged in a 1.5 ml tube (200 x g, 5 min) and supernatants were discarded. Cells were sequentially rinsed with DMEM and ice-cold PBS, and resuspended in 85 ml of binding buffer. A total of 10 μl of annexin V stock solution and 5 μl of PI stock solution was then added to the cells, and incubated for 15 min in the dark at room temperature. Thereafter, cells were immediately analyzed by flow cytometry (FACSCalibur cell analyzer; Becton-Dickinson, San Jose, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical significance was determined by Student's t-test or one-way analysis of variance (GraphPad

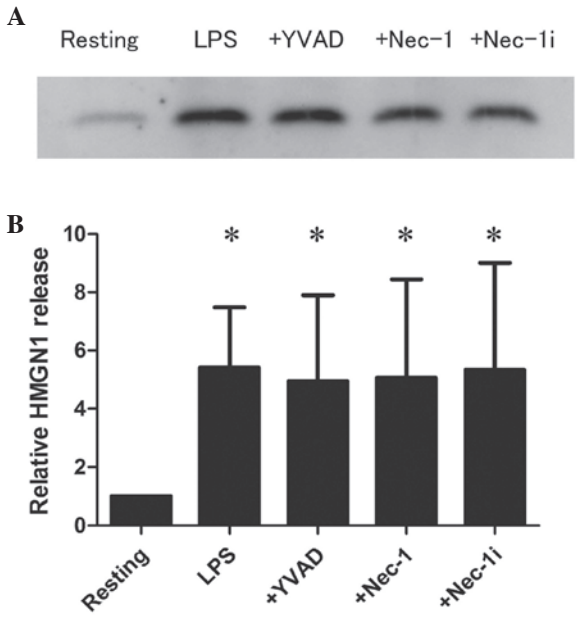

Figure 1. Release of HMGN1 from LPS-stimulated RAW264.7 cells. (A) RAW264.7 cells were incubated with LPS (100 ng/ml) or without (resting) in the absence or presence of YVAD, Nec-1 or Nec-1i (10 μM each) at 37˚C for 20 h. HMGN1 was detected by western blotting. Images are representative of eight separate experiments. (B) HMGN1 release is expressed as a fold increase relative to resting. Data are presented as the mean ± standard deviation of eight independent experiments. Values are compared between resting and LPS, +YVAD, +Nec-1 or +Nec-1i. *P<0.05. HMGN1, high mobility group nucleosome binding domain 1; LPS, lipopolysaccharide; Nec-1, Necrostatin-1.

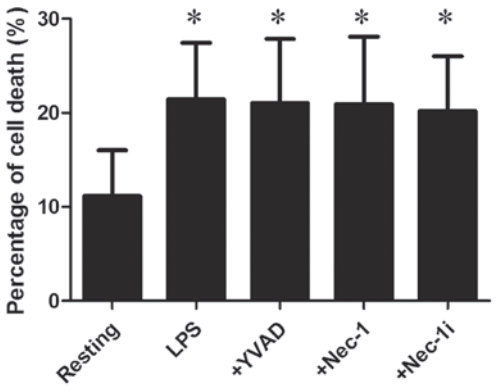

Figure 2. Release of LDH from LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with LPS (100 ng/ml) or without (resting) in the absence or presence of YVAD, Nec-1 or Nec-1i (10 μM each) at 37˚C for 20 h. LDH was quantitated using a commercially available LDH assay kit. Data are presented as the mean ± standard deviation of 10 independent experiments. Values are compared between resting and LPS, +YVAD, +Nec-1 or +Nec-1i. *P<0.05. LPS, lipopolysaccharide; LDH, lactate dehydrogenase; Nec-1, Necrostatin-1.

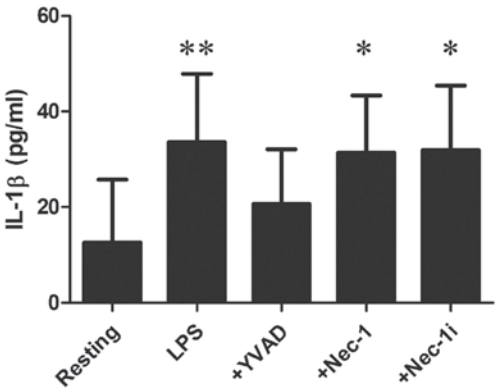

Figure 3. Release of IL-1β from LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with LPS (100 ng/ml) or without (resting) in the absence or presence of YVAD, Nec-1 or Nec-1i (10 μM each) at 37˚C for 20 h, and IL-1β was quantitated by enzyme-linked immunosorbent assay. Data are presented as the mean ± standard deviation of 10 independent experiments. Values are compared between resting and LPS, +YVAD, +Nec-1 or +Nec-1i. *P<0.05, **P<0.01. LPS, lipopolysaccharide; IL-1β, interleukin-1β; Nec-1, Necrostatin-1.

Prism Software Inc., La Jolla, CA, USA) with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Release of HMGN1 from LPS-stimulated RAW264.7 cells. Initially, the present study aimed to determine whether LPS stimulation induces the extracellular release of HMGN1 from RAW264.7 cells. As shown in Fig. 1, the level of HMGN1 in the supernatants increased by 5.4-fold following LPS (100 ng/ml) stimulation compared with resting (P<0.05). The percentage of HMGN1 release was calculated from the total quantity of HMGN1 in the cell lysates. HMGN1 release was <1% in resting cells; however, increased up to 2.7-5.9% in LPS-stimulated cells (data not shown).

Furthermore, LDH assay indicated that the LDH activity in the supernatants increased from 11.2±4.9% in resting cells to 21.5±6.0% in LPS-stimulated cells (P<0.05; Fig. 2), suggesting that HMGN1 release is accompanied by cell death.

Effects of Ac-YVAD-CHO and Nec-1 on HMGN1 release from LPS-stimulated RAW264.7 cells. Subsequently, the patterns of cell death involved in HMGN1 release from LPS-stimulated RAW264.7 cells were determined using Ac-YVAD-CHO (YVAD) and Nec-1. YVAD, a selective inhibitor of caspase 1, suppresses pyroptosis, whereas Nec-1, an allosteric inhibitor of the death domain receptor-associated adaptor kinase RIP1, inhibits necroptosis (19). YVAD did not affect the release of HMGN1 or LDH from LPS-stimulated RAW264.7 cells (Figs. 1 and 2), although it inhibited the release of IL-1β as a marker of caspase-1 activation (Fig. 3). Similarly, Nec-1 did not affect HMGN1 release or LDH release from LPS-stimulated RAW264.7 cells (Figs. 1 and 2). A previous study confirmed that Nec-1 suppressed the release of HMGN1 from RAW264.7 cells treated with LPS plus zVAD (a pan-caspase inhibitor), which induces necroptosis of macrophages (20,21).

These observations suggest that HMGN1 release from LPS-stimulated RAW264.7 cells is not associated with pyroptosis (caspase-1-acivated cell death) or necroptosis (RIP1-dependent programmed cell death).

Involvement of necrosis in HMGN1 release from LPS-stimulated RAW264.7 cells. Finally, the involvement

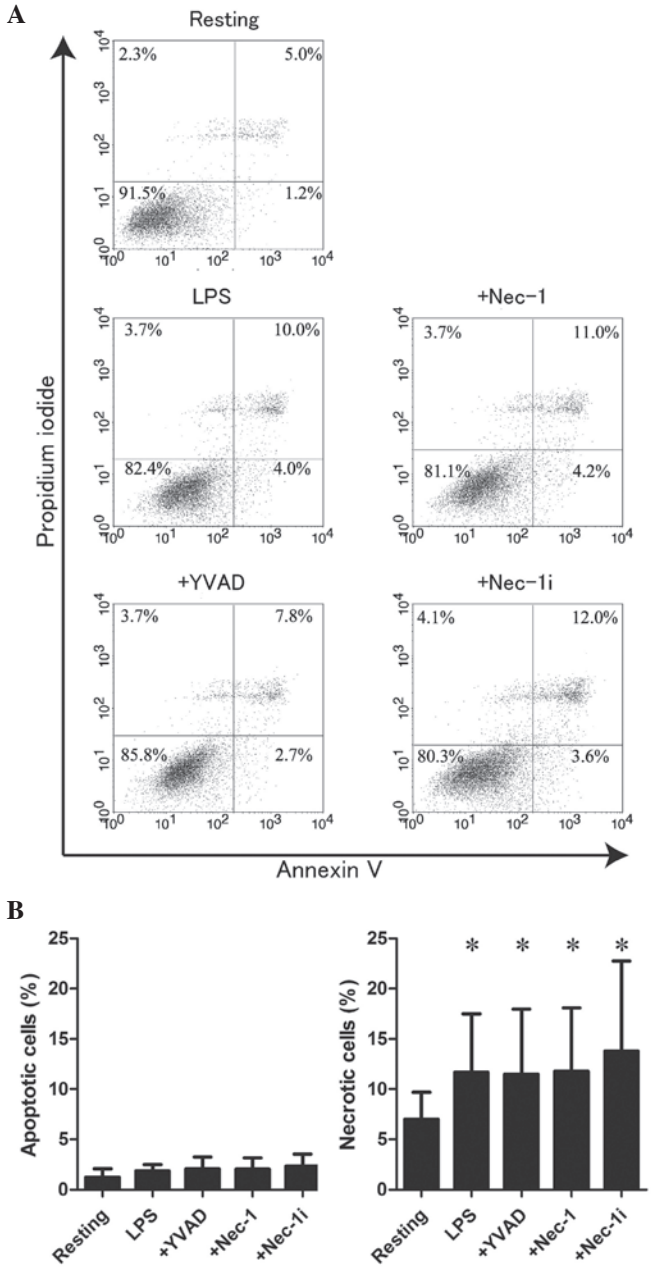

Figure 4. Assay for apoptosis and necrosis of LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with LPS (100 ng/ml) or without (resting) in the absence or presence of YVAD, Nec-1 or Nec-1i (10 μM each) at 37˚C for 8 h, and then stained with fluorescein isothiocyanate-labeled annexin V and PI. (A) Representative plots showing the distribution of annexin V and PI staining for resting and LPS-stimulated cells incubated with or without YVAD, Nec-1 or Nec-1i. The percentage of the cells delimited by each quadrant is also shown. Images are representative of 10 separate experiments. (B) Quantitative analysis of the percentage of apoptotic and necrotic cells. Apoptotic and necrotic cells were defined as annexin V^+/PI^- (lower right) and annexin V^+/PI^+ (upper left + right), respectively. Data are presented as the mean ± standard deviation of 10 independent experiments. Values are compared between resting and LPS, +YVAD, +Nec-1 or +Nec-1i. $^*P<0.05$. LPS, lipopolysaccharide; PI, propidium iodide; Nec-1, Necrostatin-1.

of necrotic cell death in HMGN1 release was determined by staining LPS-stimulated RAW264.7 cells with annexin V and PI. As shown in Fig. 4A and B, LPS stimulation did not significantly increase apoptotic cells (lower right division) but significantly increased necrotic cells ($P<0.05$, upper right and left divisions) compared with resting. Of note, it has been reported that PI-positive cells are increased by pyroptosis and necroptosis (22,23). However, YVAD and Nec-1 did not affect these patterns of cell death. Together these findings suggest that LPS stimulation is not likely to induce apoptosis, pyroptosis or necroptosis of RAW264.7 cells, and HMGN1 is released from PI-positive necrotic RAW264.7 cells stimulated with LPS.

Discussion

Endotoxin/septic shock is a severe and abnormal condition that is induced during infections with gram-negative bacteria. It is characterized by systemic inflammatory responses of the host to invading microorganisms, PAMPs and damage-associated molecular patterns, including endogenous danger signal molecules termed alarmins (4). A previous study revealed that HMGN1, an alarmin, acts as a cytokine in the extracellular milieu, which enhances Th1-type antigen-specific immune responses (13). In addition, HMGN1 stimulates immune cells, including monocytes and dendritic cells, to secrete inflammatory cytokines via the TLR4/NF-κB and MAPK pathways (14). Since the activation of NF-κB and excessive production of inflammatory cytokines from monocytes/macrophages are key events in sepsis, the extracellular release of HMGN1 from monocytes/macrophages is expected to be important in the pathogenesis of sepsis/septic shock (14). However, the release mechanism of HMGN1 from monocytes/macrophages remains to be elucidated. The present study revealed that HMGN1 is extracellularly released from LPS-stimulated RAW264.7 macrophage-like cells, accompanied by unprogrammed necrotic cell death but not pyroptosis, necroptosis or apoptosis.

Cell death has been discussed dichotomously as either apoptosis or necrosis (24). Apoptosis is typically described as a programmed process of self-destruction that avoids release of inflammatory cellular contents (25). The execution of apoptosis minimizes the leakage of cellular constituents by forming an apoptotic body, a result of non-lytic cellular shrinkage (26). By contrast, necrosis is the most common form of cell death, which is characterized by membrane rupture and leakage of prophlogistic cellular constituents (24). Thus, necrosis is considered to be the potent source of immunoactivating danger signal molecules, including alarmins. In addition, a previous study expanded categorization of the two types of novel cell deaths i.e. pyroptosis and necroptosis (26). Pyroptosis is identified as caspase-1-dependent cell death of macrophages and dendritic cells found in bacterial infection (27). Pyroptosis is induced by the formation of the inflammasome that facilitates the activation of caspase-1, and the generation of cytokines, including IL-1β, based on the cleavage of their precursors by activated caspase-1 (12). During pyroptosis, cellular contents are rapidly released into the extracellular space by pore formation and plasma membrane loss (28). Another form of cell death, necroptosis was originally defined as a potent immunogenic programmed cell death induced by the presence of zVAD-fmk (a pan-caspase inhibitor) and tumor necrosis factor receptor signaling that involves the activation of RIP1 and mitochondrial instability (20,29). During necroptosis, swelling of cellular organelles and plasma membrane disruption are induced, and consequently inflammatory cellular contents are extracellularly released (25).

In the present study, it was revealed that HMGN1 is released from LPS-stimulated RAW264.7 cells, accompanied by cell death as assessed by the release of LDH. Subsequently, the patterns of cell death involved in HMGN1 release from LPS-stimulated RAW264.7 cells were determined using YVAD (a caspase-1 inhibitor) and Nec-1 (a RIP1 inhibitor). YVAD and Nec-1 did not alter LPS-induced HMGN1 and LDH release, although YVAD and Nec-1 inhibited LPS-stimulated IL-1β release and LPS/zVAD-stimulated HMGN1 release, respectively. These observations suggest that pyroptosis (caspase-1-activated cell death) and necroptosis (RIP1-dependent programmed cell death) are not involved in the release of HMGN1 from LPS-stimulated RAW264.7 cells. In addition, flow cytometric analysis indicated that LPS stimulation did not induce apoptosis but substantially augmented necrosis, as evidenced by annexin V/PI-staining. Together these findings suggest that HMGN1 is released from necrotic (unprogrammed cell death) but not apoptotic, pyroptotic or necroptotic (a programmed form of necrosis) RAW264.7 cells following stimulation with LPS. Supporting this theory, pyroptosis and necroptosis were not induced in RAW264.7 cells by LPS stimulation under our experimental condition, since YVAD (a pyroptosis inhibitor) and Nec-1 (a necroptosis inhibitor) did not inhibit LPS-induced LDH release and PI staining, although these inhibitors were capable of suppressing LDH release and PI staining associated with pyroptosis and necroptosis (27,30-32). In addition, IL-1β was extracellularly released from RAW264.7 cells by LPS stimulation, but its maximum level was 30 pg/ml (Fig. 3). Notably, pyroptosis-associated IL-1β release is reported to reach 1-2 ng/ml (11,33,34). These observations suggest that in our experiment, caspase-1 is activated by LPS stimulation to process and release IL-1β; however, it is unlikely that the activation is sufficient to induce pyroptosis.

In conclusion, the release mechanism of HMGN1 from macrophages was evaluated using RAW264.7 cells and it was revealed that its extracellular release from LPS-stimulated RAW264.7 cells is predominantly dependent on necrosis (unprogrammed cell death) but not apoptosis, pyroptosis or necroptosis. Under conditions of sepsis/septic shock, alarmins are hypothesized to be released from immune cells due to cell death or cell activation at the site of infection and inflammation, and have a fundamental role in the regulation of host defense and tissue repair (35,36). Therefore, HMGN1 released from macrophages and other immune cells by stimulation with PAMPs, including LPS, is likely to be important in sepsis/septic shock as an alarmin. Anti-cytokine based therapies for sepsis-associated systemic inflammation have not been successful (37). Therefore, the modulation of cell death/cell activation and subsequent release of alarmins, including HMGN1 is expected to be a promising target for a novel therapeutic strategy against sepsis/septic shock.

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