Pyroptotic cells externalize eat-me and release find-me signals and are efficiently engulfed by macrophages

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Abstract

Pathogenic intracellular bacteria often hijack macrophages for their propagation. The infected macrophages release IL-1β and IL-18 and simultaneously commit suicide, which is called pyroptosis; both responses require caspase-1. Here, we found that pyroptotic cells induced by microbial infection were efficiently engulfed by human monocytic THP-1-cell-derived macrophages or mouse peritoneal macrophages. This engulfment was inhibited by the D89E mutant of milk fat globule (MFG) epidermal growth factor (EGF) factor 8 (MFG-E8; a phosphatidylserine-binding protein) that has been shown previously to inhibit phosphatidylserine-dependent engulfment of apoptotic cells by macrophages, suggesting that the engulfment of pyroptotic cells by macrophages was also phosphatidylserine dependent. Using a pair of cell lines that respectively exhibited pyroptosis or apoptosis after muramyl dipeptide treatment, we showed that both pyroptotic and apoptotic cells bound to a T-cell immunoglobulin and mucin domain-containing 4 (Tim4; another phosphatidylserine-binding protein)-coated plate, whereas heat-killed necrotic cells did not, indicating that phosphatidylserine was externalized in pyroptosis and apoptosis but not in accidental necrosis. Macrophages engulfed apoptotic cells most efficiently, followed by pyroptotic and then heat-killed necrotic cells. Pyroptotic cells also released a macrophage attractant(s), ‘find-me’ signal, whose activity was diminished by apyrase that degrades nucleoside triphosphate to nucleoside monophosphate. Heat-killed necrotic cells and pyroptotic cells released ATP much more efficiently than apoptotic cells. These results suggest that pyroptotic cells, like apoptotic cells, actively induce phagocytosis by macrophages using ‘eat-me’ and find-me signals. Based on these results, a possible role of coordinated induction of pyroptosis and inflammatory cytokine production is discussed.

Keywords: cell death, eat-me signal, find-me signal, microbial infection, phagocytosis

Introduction

Intracellular infection by pathogenic microbes often induces cell death with necrotic morphology. This mode of cell death was previously considered to be passive cell death that does not involve cellular signaling. However, recent studies have revealed that the necrotic cell death of murine macrophages that follows infection by a variety of pathogenic microbes is actually induced by a host-cell mechanism involving caspase-1 (1). Because caspase-1 simultaneously induces the proteolytic maturation and release of pro-inflammatory cytokines such as IL-1β and IL-18, this programmed cell death mode is inherently pro-inflammatory, and thus it was named pyroptosis (2). Although cell death of macrophages induced by bacterial infection has been most extensively studied as a prototype of pyroptosis, caspase-1-dependent cell death has been observed in other tissues, including central nervous and cardiovascular systems, under pathological conditions, suggesting that pyroptosis plays a role in a variety of biological systems (2).

In the course of apoptosis, the cell body and nucleus shrink and become fragmented, but the barrier function of the plasma membrane is maintained until the very end of the process. Meanwhile, phosphatidylserine (PS),
which is normally present in the inner leaflet of the plasma membrane, is expressed on the cell surface. The exposed PS functions as an ‘eat-me’ signal that facilitates engulfment of the cell corpse by macrophages. PS is recognized by macrophages' PS-binding proteins, such as milk fat globule (MFG) epidermal growth factor (EGF) factor 8 (MFG-E8) and T-cell immunoglobulin and mucin domain-containing 4 (Tim4) (3). In addition, apoptotic cells release ‘find-me’ signals, such as fractalkine, lysophosphatidylcholines, sphingosine-1 phosphate and the nucleotides ATP and uridine triphosphate (UTP), which recruit macrophages to the corpse (4). By these mechanisms, apoptotic cells are engulfed in vivo by macrophages without the spillage of intracellular contents that would otherwise cause inflammation and autoimmune disease (3). In contrast to apoptosis, in the course of necrosis, the plasma membrane ruptures quickly, and the leaked intracellular contents cause inflammation. Nevertheless, recent studies demonstrated that necrotic cells are also engulfed by macrophages in a PS-dependent manner (5, 6). Whether pyroptotic cells are efficiently engulfed by macrophages and whether they externalize eat-me and release find-me signals have not been reported.

In this study, we investigated the efficiency of and mechanisms underlying the phagocytic clearance of pyroptotic cells by macrophages and found that pyroptotic cells, like apoptotic cells, use find-me and eat-me signals to attract macrophages and to induce phagocytosis, respectively.

Methods

Reagents

Phorbol myristate acetate (PMA), staurosporine (Wako Pure Chemical Industries, Tokyo, Japan), muramyl dipeptide (MDP), PKH26, PKH67 (Sigma-Aldrich) and apyrase (New England Biolabs Japan, Tokyo, Japan) were purchased. Fas ligand was prepared as described previously (7). PKH26 and PKH67 are general membrane-labeling fluorescent dyes. However, we have observed punctate intracellular staining of cells with these dyes after culture of stained cells, probably due to redistribution of the dyes.

Human cell lines

The THP-1 human monocytic leukemia cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The COLO205 human colon adenocarcinoma cell line was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Miyagi, Japan). The CLC12N2-Pyr (previously termed CLC12N2-Nec) and CLC12N2-Apo cell lines were COLO205-derived stable transfectants expressing an NLRC4-NOD2 (C12N2) chimeric protein (8). CLC12N2-Pyr and CLC12N2-Apo cells exhibit pyroptotic or apoptotic cell death, respectively, in response to MDP stimulation. THP1C12N2 and SKC12N2 cell lines are THP-1-derived and SK-MEL-28 human melanoma cell line-derived stable transfectants expressing C12N2 (8). THP1C12N2 and SKC12N2 cells exhibit pyroptotic cell death in response to MDP stimulation (8). All cells were cultured in 10% FCS–RPMI 1640 medium.

Preparation of mouse peritoneal macrophages

Thioglycollate medium (3%, 1.5ml; Difco, Detroit, MI, USA) was injected intra-peritoneally into C57BL/6 mice. Four days after thioglycollate injection, peritoneal exudate cells were recovered by washing the peritoneal cavity with 4ml PBS twice (total 8ml PBS), washed three times and suspended in 10% FCS–RPMI 1640. To further isolate peritoneal macrophages, 2×10⁶ peritoneal exudate cells per well were cultured in 24-well plates for 2h, and non-adherent cells were removed by aspiration. All animal experiments were performed according to the guidelines for the use of experimental animals of the Animal Research Committee of Kanazawa University.

Bacterial infection

To generate bacterium-induced pyroptotic cells, THP-1 cells or mouse peritoneal exudate cells were infected with Staphylococcus (S.) aureus (strain Smith, kindly provided by Dr Nakanishi, Kanazawa University, Kanazawa, Ishikawa, Japan), Pseudomonas (P.) aeruginosa (JCM14847, Japan collection of micro-organisms, Riken Bioresource Center, Tsukuba, Ibaraki, Japan) or Salmonella (S.) typhimurium (JCM1652, Japan collection of micro-organisms) as follows. S. aureus, P. aeruginosa and S. typhimurium in the log phase were used for infection. THP-1 cells were infected with S. aureus or P. aeruginosa in antibiotic-free medium in 24-well plates. The plates were briefly centrifuged (240 × g, for 1 min) to improve the interaction between the cells and bacteria. One and a half hours after the infection, gentamicin (50 µg ml⁻¹) was added to kill the extracellular bacteria. Cells were further cultured for 1h for total 2.5-h infection period. In some experiments, S. aureus was first treated with gentamicin (50 µg ml⁻¹) in RPMI 1640 medium at room temperature for 1h. THP-1 cells were then co-cultured with the killed bacteria for 2.5h. Mouse peritoneal exudate cells were infected with S. typhimurium in antibiotic-free medium in a 35-mm suspension culture dish (430588, Corning, Inc. Corning, NY, USA). One and a half hours after the infection, gentamicin (50 µg ml⁻¹) was added to kill the extracellular bacteria. Cells were further cultured for 1h for total 2.5-h infection period.

In vitro phagocytosis assay

To generate PKH67-labeled phagocytes, THP-1 cells or mouse peritoneal macrophages were first stained with PHK67 according to the manufacturer’s protocol. PKH67-labeled THP-1 cells were then cultured with 160nM PMA for 72h to induce a macrophage-like phenotype. The PMA was removed by replacing the culture medium before addition of target cells. To label target cells, cells were stained with PHK26 according to the manufacturer's protocol before cell death induction. To specifically stain membrane-permeabilized target cells, cells were stained with propidium iodide (PI, 2.5 µg ml⁻¹) after cell death induction. Target cells were lifted from culture plates by gentle pipetting and then added to phagocytes and incubated for 2h. Phagocytosis was observed under a fluorescence inverted microscope (BIOREVO BZ-9000, KEYENCE, Osaka, Japan) or a confocal fluorescence microscope (Axiovert...
100M confocal laser scanning microscopy, Carl Zeiss, Jena, Germany). To analyze phagocytosis using flow cytometry (FACS Canto II, BD Biosciences), target cells remaining unphagocytosed were removed by washing culture wells with PBS, and then macrophages were detached from plates using 0.25% Trypsin–1 mM EDTA–PBS, followed by a wash with 10% FCS–RPMI 1640. Percent phagocytosis was calculated as percentage of PKH26–PKH67+ cells among total PKH67+ cells. In some experiments, dead cells were pre-incubated with the culture supernatant from either HEK293T cells expressing the MFG-E8-D89E mutant (9) or mock transfectants for 30 min at room temperature and then used in the phagocytosis assays.

**Tim4-binding assay**

Mouse Tim4-Fc (10) (2 μg ml−1) was bound to flat-bottomed 96-well plates (MaxiSorp, Thermo Fisher Scientific, Waltham, MA, USA) with coating buffer (0.1 M sodium carbonate buffer, pH 9.5) for 1 to 2 h at 37°C. Cells (1 × 105) stained with PKH26 were added to each well and incubated at room temperature for 30 min. Unbound cells were gently washed by immersing the plates in 0.05% Tween–PBS and reversing the plate a couple of times in the buffer. The PKH26 fluorescence of each well was measured by Fluoroskan Ascent (Thermo Fisher Scientific) before and after the wash, and the proportion of bound cells was calculated as a percentage of the input cells.

**Lactate dehydrogenase release assay**

Lactate dehydrogenase (LDH) release assays were performed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol.

**WST-1 assay**

To determine the cell viability, WST-1 assays were performed as described previously (11). To determine the proportion of migrated cells in the Transwell assays, the WST-1 assay was adapted to a 24-well-plate format. Aliquots (100 μl) of culture supernatants collected from cultures after their incubation with assay reagents and fresh medium (similarly incubated with assay reagents, as a background control) were transferred to flat-bottomed 96-well plates. The net absorbance at 450 nm was determined using a microplate reader. The net absorbance of control wells containing the same number of cells in the input chamber was defined as 100%.

**Transwell migration assay and ATP measurement**

THP-1-derived cells (6 × 105), COLO205 or COLO205-derived cells (3 × 105) or SKC12N2 cells (2 × 105) were stimulated with MDP (1 μg ml−1), staurosporine (2 mM) or Fas ligand (1000 units ml−1) for 1–5 h or infected with S. aureus for 2.5 h in RPMI 1640 medium containing 1% BSA and 10 mM HEPES (pH 7.2). Otherwise, cells were heated at 90°C for 3 min using Block Incubator (ASTEC, Fukuoka, Japan) or irradiated with UVC (100 mJ cm−2) using Stratalinker 2400 (Stratagene, La Jolla, CA, USA) and then cultured for 4 h. The supernatants were then collected. For the Transwell migration assays, THP-1 cells (2 × 105) in 200 μl fresh culture medium were added to an 8-μm pore-size cell culture insert (upper chamber, BD Falcon), and 700 μl of one of the above culture supernatants was placed in the companion plate (lower chamber). After a 90-min incubation at 37°C, the proportion of cells that migrated into the lower chamber as the percentage of cells added to the upper chamber was determined by the WST-1 assay as described herein. To measure ATP, culture supernatants were boiled for 2 min immediately after collection to inactivate any phosphatases. The ATP concentration was then determined using an ATP determination kit (Wako, Tokyo, Japan) according to the manufacturer’s protocol.

**Statistics**

All experiments were done in duplicate and repeated at least three times. Graphs were generated using cumulative data from two representative experiments. Statistical significance of difference between two experimental groups was assessed by the two-tailed Student’s t-test.

**Results**

**Pyroptotic cells are engulfed by macrophages in a PS-dependent manner**

Human monocytic cell lines such as THP-1 and NOMO-1 infected with S. aureus or P. aeruginosa exhibit a necrotic morphology when killed (8) (Supplementary Figure S1A and C and Supplementary Movie S1, available at International Immunology Online). This necrotic cell death is pyroptosis, because it is mediated by caspase-1 and accompanied by IL-1β release (8) (Supplementary Figure S2, available at International Immunology Online). To investigate the phagocytosis of pyroptotic cells, THP-1 cells were treated with PMA to induce their differentiation into macrophagic cells (hereafter THP-1 macrophages) and labeled with PKH67 (green fluorescent dye). As the target, uninfected or S. aureus- or P. aeruginosainfected THP-1 cells were labeled with PKH26 (red fluorescent dye). THP-1 macrophages and target THP-1 cells were then co-cultured for 2 h. Green THP-1 macrophages that had engulfed red pyroptotic cells were observed under a confocal fluorescence microscope (Fig. 1A). Flow cytometric analyses revealed that about 15–25% of the phagocytes engulfed target cells under these conditions (Fig. 1B and C). Phagocytosis efficiency of target cells treated with killed bacteria was not significantly different from that of untreated target cells, excluding the possibility that bacterial components induced phagocytosis of live target cells (Fig. 1D).

To confirm that pyroptotic THP-1 cells were engulfed by THP-1 macrophages, S. aureus-infected THP-1 cells were stained with PI (red fluorescent dye) that stains only membrane-permeabilized cells. THP-1 macrophages and target THP-1 cells were then co-cultured for 2 h. Efficient phagocytosis of pyroptotic THP-1 cells (red) by THP-1 macrophages (green) was observed under a fluorescence microscope (Fig. 1E).

COLO205 colon adenocarcinoma-derived CLC12N2-Apo and CLC12N2-Pyr cells are a pair of cell lines that exhibit apoptotic and pyroptotic cell death, respectively, in response to MDP stimulation (8) (as described in Methods; see Supplementary Figure S3A, available at International Immunology Online).
After MDP stimulation, both CLC12N2-Apo cells (labeled with PKH26) and CLC12N2-Pyr cells (labeled with PI) were engulfed by THP-1 macrophages (Fig. 1F). To compare the phagocytosis efficiency of apoptotic and pyroptotic cells by THP-1 macrophages under the same conditions, CLC12N2-Apo and CLC12N2-Pyr cells were stained with PKH26 and treated with MDP. COLO205 cells not killed by MDP treatment were used as a control. These
target cells were then co-cultured with PKH67-labeled THP-1 macrophages for 2h. CLC12N2-Pyr cells were engulfed more efficiently than COLO205 cells but less efficiently than CLC12N2-Apo cells (Fig. 1G and H). Phagocytosis of CLC12N2-Apo and CLC12N2-Pyr cells by THP-1 macrophages was confirmed using a confocal fluorescence microscope (Fig. 1I). We also compared the phagocytosis efficiency of heat-killed necrotic COLO205 cells with that of other target cells (Fig. 2A). Heat-killed COLO205 cells were engulfed slightly more efficiently than untreated COLO205 cells but less efficiently than MDP-treated CLC12N2-Apo and CLC12N2-Pyr cells.

MFG-E8 is a PS-binding protein that carries an RGD integrin-binding motif and it links apoptotic cells to phagocytes (9). The D89E mutant of MFG-E8 lacks the RGD motif and can inhibit the PS-dependent phagocytosis of apoptotic cells (9). As expected, the D89E mutant efficiently inhibited the phagocytosis of MDP-treated CLC12N2-Apo cells by THP-1 macrophages (Fig. 2A). Similarly, D89E inhibited the phagocytosis of MDP-treated CLC12N2-Pyr cells (Fig. 2A). Importantly, the phagocytosis of S. aureus- and P. aeruginosa-killed THP-1 cells was also strongly diminished by D89E (Fig. 2B). Similar results were obtained using PMA-activated NOMO-1 cells as phagocytes and S. aureus- or P. aeruginosa-infected NOMO-1 cells as target cells (Supplementary Fig. S4, available at International Immunology Online). The intracellular bacterium S. typhimurium induces pyroptosis in mouse peritoneal macrophages (1,2,8). Similar results were also obtained using thioglycollate-induced peritoneal macrophages as phagocytes and pyroptotic mouse peritoneal macrophages induced by S. typhimurium infection as target cells (Fig. 2C).

Taken together, these results indicate that pyroptotic cells are engulfed by macrophages in a PS-dependent manner, albeit less efficiently than apoptotic cells.

Pyroptotic cells expose PS

When dead cells are stained with soluble fluorescein-conjugated protein probes for PS, such as Annexin V or Tim4-Fc (a soluble fusion protein of the extracellular region of Tim4 and the Fc portion of human IgG1), not only apoptotic cells but also pyroptotic and heat-killed necrotic cells are labeled (Supplementary Figures S1D, S3D and S5, available at International Immunology Online). However, the plasma membrane of pyroptotic and heat-killed necrotic cells rapidly ruptures, so soluble PS probes can enter the dead cells. Therefore, it is not clear whether the PS-binding probe labels PS that is inside the cells or that is exposed on the plasma membrane surface.

To investigate PS exposure on the cell surface, we first examined whether dead cells would bind to Annexin V-coated plates. However, significant adhesion of dead cells, including apoptotic cells, to Annexin V-coated plates was not observed under our experimental conditions. Then we examined whether dead cells would bind to a Tim4-Fc-coated plate (Fig. 3). Because live THP-1 cells bound to Tim4-Fc-coated plates (probably through their Fcγ receptor), COLO205-derived cell lines were used as the source of dead cells. As expected, apoptotic (MDP-treated CLC12N2-Apo) cells bound to Tim4-Fc-coated but not uncoated plates, whereas live (MDP-treated COLO205) cells bound to neither plate. Like the apoptotic cells, pyroptotic (MDP-treated CLC12N2-Pyr) cells bound to Tim4-Fc-coated but not uncoated plates. Heat-killed necrotic COLO205 cells bound to Tim4-Fc-coated
Fig. 3. Pyroptotic and apoptotic cells expose PS. COLO205, CLC12N2-Apo and CLC12N2-Pyr cells were labeled with PKH26. COLO205 cells were left untreated (COLO205) or heat-killed by incubation at 90°C for 3 min (COLO205-Heat) and then incubated at 37°C for 3 h. CLC12N2-Apo and CLC12N2-Pyr were treated with MDP (1 µg ml⁻¹) for 3 or 5 h, respectively. In (C), CLC12N2-Pyr cells were pre-cultured with or without 10 or 20 µM Ac-YVAD-CMK for 1 h before MDP stimulation. Cells were then transferred to untreated [Tim4-Fc(-)] or Tim4-Fc-coated [Tim4-Fc(+)] plates and incubated for 30 min at room temperature. Unbound cells were removed by gentle washing as described in Methods. Before and after the wash, photographs were taken (A) and fluorescence intensity was measured (B and C). Binding (%) = fluorescence intensity after wash/fluorescence intensity before wash. Error bars and asterisks indicate standard deviation and statistical significance, respectively. *P < 0.05; ns, not significant.

ATP released from pyroptotic cells induces THP-1 cell migration

The chemotactic activity released from S. aureus-infected THP-1 cells was not attenuated by heat treatment (90°C, 3 min; Fig. 5A), suggesting that the major chemotactic activity was not a protein. Because ATP and UTP have been found to be major find-me signals of apoptotic cells (14), we investigated whether ATP was released from pyroptotic cells. THP-1 cells killed by S. aureus or P. aeruginosa indeed released ATP into the culture medium (Fig. 5B). Treatment of THP-1 cells with gentamicin-killed bacteria did not induce ATP release, indicating that stimulation by bacterial components without intracellular infection did not induce ATP release (Fig. 5C). Furthermore, treatment of the culture supernatants from microbially infected THP-1 cells with apyrase, which degrades nucleoside triphosphates into nucleoside monophosphates, abolished the chemotactic activity (Fig. 5B). Similarly, MDP-stimulated CLC12N2, SKC12N2 and THP1C12N2 cells released ATP into the culture medium, and apyrase treatment eliminated ATP and the chemotactic activity (Fig. 5D). Intracellular bacterium S. typhimurium infection also induced ATP release in mouse peritoneal macrophages (Fig. 5E). These results indicate that nucleotides, including ATP, are the major macrophage chemoattractants released by pyroptotic cells.

Next, we sought to compare the amounts of ATP and chemotactic activity released from cells killed by the different
cell death modalities. To this end, THP-1 cells were infected with *S. aureus* to induce pyroptosis or treated with (i) UVC or staurosporine to induce apoptosis or (ii) heat to induce accidental necrosis (Supplementary Fig. S1C and D, available at International Immunology Online). Strong macrophage chemotactic activity was detected in the supernatants from pyroptotic and heat-killed necrotic cells, whereas no and weak chemotactic activity was detected in the supernatants from UVC- and staurosporine-induced apoptotic cells, respectively (Fig. 6A).

Consistent with the chemotactic activity, heat-induced necrosis led to the greatest ATP release, and pyroptotic THP-1 cells released more ATP than apoptotic THP-1 cells. The calculated amount of ATP released per cell for the different cell death modalities was $1161.1 \pm 92.1 \mu$mol for heat-induced necrosis, $52.5 \pm 2.5 \mu$mol for pyroptosis and $7.9 \pm 0.1 \mu$mol for staurosporine-induced apoptosis. No significant ATP release was detected from UVC-killed apoptotic THP-1 cells. Similar results were obtained when we used NOMO-1 human monocytic cells instead of THP-1 cells (Supplementary Figure S6, available at International Immunology Online). We also found that both MDP-induced pyroptosis and heat-induced necrosis of CLC12N2-pyr cells resulted in much more efficient ATP release than did the UVC- or Fas ligand-induced apoptosis of this cell line (Fig. 6B).

**Discussion**

In this study, by using mainly two models of pyroptosis (bacterium-infected THP-1 cells and MDP-treated CLC12N2-Pyr cells), we have demonstrated that pyroptotic cells release ATP that attracts monocytes and thereafter were efficiently engulfed by THP-1 macrophages. The engulfment was inhibited by a PS-binding protein MFG-E8-D89E, indicating that the phagocytosis was PS dependent. Consistent results were obtained using another human monocytic cell line (NOMO-1 cells) and mouse peritoneal macrophages. Thus, PS-dependent phagocytosis of pyroptotic cells was commonly observed using at least two independent human monocytic cell lines and primary mouse macrophages. As physical evidence for the PS exposure of pyroptotic cells, we demonstrated that MDP-treated CLC12N2-Pyr cells bound to plates coated with Tim4-Fc, another PS-binding protein. We failed to demonstrate PS exposure of pyroptotic THP-1 cells by the Tim4-Fc-binding assay, because of the limitation of the assay system that cannot exclude Fc-mediated binding of THP-1 cells to Tim4-Fc. Although the inhibition of engulfment of pyroptotic THP-1 cells by MFG-E8-D89E can be considered as biological evidence for PS exposure of pyroptotic THP-1 cells, further study is required to confirm PS exposure on pyroptotic monocytes.

Previously, Hirt and Leist (5) and Brouckaert et al. (6) have demonstrated that phagocytosis of cells killed by programmed necrosis occurs through a PS-dependent mechanism. Hirt and Leist demonstrated that necrotic human Jurkat T lymphoid cells killed with staurosporine under ATP-depleted conditions were engulfed by human monocyte-derived macrophages more preferentially than apoptotic Jurkat cells. On the other hand, Brouckaert et al. showed that necrotic mouse L929 fibroblastic cells killed by tumor necrosis factor were engulfed by mouse M4/4 macrophages less efficiently than apoptotic L929 cells. Thus, the efficiency of necrotic cell engulfment by macrophages may vary depending on the experimental model.
Although PS exposure of apoptotic cells is caspase-dependent, we found that a caspase-1 inhibitor did not block PS exposure in MDP-treated CLC12N2-Pyr cells, consistent with our previous finding that pyroptotic cell death requires caspase-1 protein but not its catalytic activity. These results may indicate that the signal pathway involved in PS exposure of pyroptotic cells is quite different from that in apoptotic cells. Our future study will aim to identify molecules involved in the pyroptosis signal transduction downstream of caspase-1.

Although ATP is a major find-me signal of apoptotic cells (14), pyroptotic and necrotic cells are also expected to release ATP because their plasma membranes rupture relatively quickly. In fact, we found that pyroptosis and heat-induced necrosis induced release of greater amounts of ATP than did apoptosis (which resulted in release of small amounts of ATP in our experimental conditions). Thus, ATP may play a more important role in macrophage recruitment to necrotic and pyroptotic cells than in macrophage recruitment to apoptotic cells.

Extracellular ATP plays important roles in inflammation by activating purinergic receptors such as the P2Y<sub>2</sub> and P2X<sub>7</sub> receptors (15). ATP's macrophage-attracting activity is mediated by the P2Y<sub>2</sub> receptor, expressed on macrophages. This function of ATP can be achieved at 10–100 nM (14). On the other hand, ATP in the millimolar range activates the NLRP3 inflammasome, which induces the maturation and secretion of pro-inflammatory cytokines, such as IL-1β and IL-18 (16, 17). This function of ATP is mediated by the P2X<sub>7</sub> receptor. Because the concentration of cytoplasmic ATP is in the millimolar range, the latter function of ATP would be exhibited...
only in close proximity to pyroptotic or necrotic cells. Thus, pyroptotic and necrotic cells might attract macrophages and induce their production of pro-inflammatory cytokines. In contrast, ATP released from apoptotic cells might be sufficient to attract macrophages but not to induce cytokine production. Thus, the difference between the amount of ATP released from pyroptotic/necrotic cells and that from apoptotic ones could result in pyroptosis/necrosis being more inflammatory than apoptosis.

Some important remaining questions are whether phagocytosis of pyroptotic cells by macrophages benefits the host animal and why the activation of the inflammasome simultaneously causes the production of the pro-inflammatory cytokines IL-1β and IL-18 and subsequent pyroptotic cell death. Pathogenic intracellular bacteria hijack macrophages for their propagation in the host animal. The pyroptosis of infected macrophages could help the host by plundering the reproductive niche used by the intracellular bacteria. However, pyroptosis would also cause the spillage of live bacteria, and some bacteria would remain in the macrophage corpses. Therefore, when a macrophage engulfs pyroptotic cells, it also internalizes bacteria. If the macrophage has been activated, it should be able to kill the bacteria, which would benefit the host. If not, the engulfing macrophage too might be hijacked for bacterial reproduction. The role of cytokine production could be explained in this context. That is, the secretion of IL-18 could play an important role in macrophage activation by inducing T and NK cells to produce IFNγ (18), which in turn would activate the bacterial activity of macrophages (19). Meanwhile, IL-1β would induce endothelial cells to produce chemokines such as IL-8 (20), which would in turn recruit neutrophils to engulf the spilled bacteria (21). Therefore, the coordinated regulation of cytokine production and cell death by the inflammasome could be very important for managing intracellular bacterial infections.

Supplementary data

Supplementary data are available at International Immunology Online.

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References

Phagocytosis of pyroptotic cells


