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Coordinated Host Responses during Pyroptosis: Caspase-1–Dependent Lysosome Exocytosis and Inflammatory Cytokine Maturation

Tessa Bergsbaken,* Susan L. Fink,† Andreas B. den Hartigh,‡ Wendy P. Loomis,‡ and Brad T. Cookson*‡

Activation of caspase-1 leads to pyroptosis, a program of cell death characterized by cell lysis and inflammatory cytokine release. Caspase-1 activation triggered by multiple nucleotide-binding oligomerization domain-like receptors (NLRs) (1). NLR proteins trigger formation of a multi-protein inflammasome complex, which includes the cysteine protease caspase-1 (2). Association of these proteins facilitates the processing and activation of caspase-1 (2), leading to a conserved program of inflammatory cell death termed pyroptosis (3). The features of pyroptosis include cellular DNA damage and rapid formation of plasma membrane pores, resulting in cell lysis and release of inflammatory intracellular contents. Pyroptosis is accompanied by caspase-1–dependent processing and activation of the inflammatory cytokines IL-1β and IL-18 (4).

IL-1β and IL-18 lack classical secretion signals, and several methods of cytokine secretion have been proposed. Evidence suggests IL-1β processing in macrophages occurs in the cytosol (5), and membrane pores formed during pyroptosis may allow cytokine release (4). Budding of mature IL-1β–containing microvesicles from the cell surface has also been observed (6–8), which is consistent with cytosolic processing of IL-1β. Other groups have suggested active caspase-1 and cytokines reside in lysosomes, with lysosome exocytosis, or fusion of lysosomes with the cell surface, mediating cytokine release (9–12). Thus, a unifying mechanism for cytokine secretion during pyroptosis has yet to be identified.

In addition to its proposed role in cytokine secretion, lysosome exocytosis is involved in myriad cellular processes ranging from immune function to skin pigmentation (13, 14). In addition to the conventional lysosomal hydrolases that mediate intracellular protein degradation, specialized secretory lysosomes contain a unique set of cell type-specific proteins destined for secretion (14). Examples of secretory lysosomes include lytic granules of cytotoxic T cells, MHC class II compartments of APCs, and melanin-containing granules of melanocytes (13, 14). The importance of this exocytic process in host defense is illustrated by the immunodeficiencies that arise in humans with mutations in genes regulating lysosome fusion events (13). Conventional lysosomes have also been demonstrated to fuse with the cell surface after plasma membrane damage (15–18), facilitating membrane repair and rescue cells from lysis (16, 17).

Host activation of caspase-1 controls replication of pathogens in vivo, and contributes to the pathophysiology of several inflammatory disorders (3). Importantly, the protective functions of caspase-1 during infection are not solely due to processing and activation of IL-1β and IL-18 (19, 20), suggesting additional caspase-1–dependent processes are providing protection against infection and contributing to pathological inflammation in vivo. Therefore, defining the mechanistic features of pyroptosis will provide insight into how this form of cell death contributes to inflammatory processes and control of microbial infection. This study identifies lysosome exocytosis as a conserved caspase-1–dependent feature of pyroptosis. We show that caspase-1 activation leads to increased membrane permeability and an influx of calcium, which results in fusion of lysosomes with the cell surface and release of lysosomal contents. Secretion of processed IL-1β and IL-18 in macrophages undergoing pyroptosis occurs independently of lysosome exocytosis. We have demonstrated that multiple stimuli, acting through a diverse set of NLR proteins, lead to two conserved caspase-1–dependent secretion events: the release of processed inflammatory cytokines and lysosome-mediated release of antimicrobial host factors and degraded microbial products.
Materials and Methods

Macrophages
Bone marrow-derived macrophages were isolated from the femur exudates of C57BL/6 (The Jackson Laboratory) mice and cultured at 37°C in 5% CO2 in DMEM (Invitrogen) supplemented with 10% FCS, 5 mM HEPES, 0.2 mg/ml l-glutamine, 0.05 mM 2-ME, 50 mg/ml gentamicin sulfate, and 10,000 U/ml penicillin and streptomycin with 30% L cell-conditioned medium (21). After 6–7 d of incubation, macrophages were collected by washing with ice-cold PBS containing 1 mM EDTA, resuspended in supplemented antibiotic-free DMEM containing 5% FCS, and allowed to adhere for 18–24 h before infection. Macrophages were primed with 100 ng/ml LPS ~18 h before Salmonella infection when assaying IL-18 secretion. Bone marrow-derived macrophages from lethal toxin (LT)-susceptible BALB/c mice (The Jackson Laboratory) were used to examine LT-induced pyroptosis.

Infections were done in supplemented antibiotic-free DMEM containing 5% FCS and 5 mM glycine, unless otherwise indicated. Ac-YV AD-CMK was added to 200 μM h before infection, Calcium-free DMEM lacking CaCl2 and containing 1 mM EDTA was added to cells 1 h before infection. All infections were done in the presence of 5 mM glycine, which prevented cell lysis in response to all stimuli used to activate caspase-1 (4, 22). All infections were done in the presence or absence of glycine and showed identical results.

Induction of macrophage cell death
Macrophages were pretreated with 50 ng/ml ultrapure LPS from Salmonella minnesota (List Biologicals) prior to treatment with 20 μM nigericin or 5 mM ATP (Sigma-Aldrich) in the presence of 50 ng/ml LPS. For LT treatment, both toxin subunits, protective Ag and lethal factor, were added at 1 μg/ml (List Biologicals, Salmonella strain SL1344 and a sipB::aphT derivative (23) (type III secretion system [T3SS]-null) were used for all experiments except calcium flux and lysosomal release assays, which were done using Salmonella strain LT2. Salmonella expressing GFP contained plasmid pDW5 (24). Bacteria were grown for infection experiments, as described previously (22). Briefly, overnight cultures back-diluted 1:15 into L-broth containing 0.3 M sodium chloride were grown at 37°C with shaking for 3 h, washed, and resuspended in sterile PBS, and used to infect macrophages at a multiplicity of infection of 10:1.

Fluorescence staining
Macrophages grown on glass coverslips were incubated with 0.5 mg/ml TAM dextran for 1 h, followed by a 3-h chase with fresh media. Macrophages were then infected with Salmonella in the presence of 5 μM carboxyfluorescein-YVAD-fluoromethyl ketone (FAM-YVAD; Immunochromy Technology). Macrophages were washed thoroughly to remove unbound FAM-YVAD and fixed, and DNA was stained with To-Pro (Molecular Probes). To identify surface LAMP1 exposure, intact macrophages were incubated with anti-LAMP1 Abs (BD Pharmingen), then fixed and stained with PE-conjugated secondary Abs and the nuclear stain, To-Pro3. To confirm that binding of anti-LAMP1 Abs is restricted to the cell surface, FAM-YVAD-treated macrophages were infected with Salmonella for 20 min and then stained with Abs specific for the cytoplasmic protein, ASC, a component of the multliprotein inffammasome complex. ASC- and LAMP1-specific staining was compared in the presence and absence of permeabilization with Cytofix/Cytoperme (BD Biosciences). Coverslips were mounted using ProLong antifade reagent (Molecular Probes) and analyzed using a Leica SL confocal microscope in the W. M. Keck Center for Advanced Studies in Neural Signaling.

Immunoblotting
Macrophages were infected in serum-free DMEM, and at the indicated time points the supernatant was removed, sterilized using a 0.22-μm filter, and concentrated using a 10,000 MWCO Centricron Plus 20 centrifugal filter device (Millipore). Supernatants were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes, and protein processing and release were analyzed by Western blot using anti–IL-18 M19 (Santa Cruz Biotechnology), anti–IL-1β AF-401-NA (R&D Systems), or anti-cathepsin D C-20 (Santa Cruz Biotechnology), and peroxidase-conjugated secondary Abs. Immunoblots were developed with an ECL system (Amersham Biosciences).

Calcium flux
Macrophages were incubated with PBS containing 2.5 μM fluo-4 and 0.01% Pluronic F127 (Molecular Probes) at room temperature for 20 min.

Cells were washed once and resuspended in serum-free media containing 2.5 mM probenecid and 5 mM glycine (Sigma-Aldrich) and incubated for 30 min at 37°C in 5% CO2 before infection or treatment. Images were taken of multiple fields prior to treatment (time = 0) and every 15 s for 20–25 min following treatment using a DeltaVision Live Cell microscope (Applied Precision) in the W. M. Keck Center for Advanced Studies in Neural Signaling. Temperature was maintained at 37°C without CO2 throughout the experiment. Time-lapse videos were generated using DelaVision SoftWorx.

Release of yeast particles from lysosomes
Macrophages were incubated with 0.5 mg/ml Alexa-488 dextran for 1 h, followed by a 3-h chase with fresh media containing Alexa-594 zymosan. Extracellular zymosan was washed away and replaced with media containing 5 mM glycine, which protects cells from caspase-1–dependent cell lysis (25). Macrophages were infected with Salmonella, and images were taken of multiple fields every 20 s for 20–25 min following treatment using a DeltaVision Live Cell microscope (Applied Precision). During this time period, caspase-1–dependent cell lysis is negligible in the presence of 5 mM glycine (Supplemental Fig. 1A) (25). Temperature was maintained at 37°C without CO2 throughout the experiment. Prior to taking the final image, the membrane-impermeant dye trypan blue (0.2% trypan blue in 0.2 M sodium citrate buffer [pH 4.4]) was added to quench extracellular fluorescence.

Isolation of supernatant proteins and antimicrobial activity assay
Wild-type macrophages were incubated in phenol red-free, serum-free media containing 5 mM glycine with or without 1.8 mM CaCl2 and infected with Salmonella for 30 min. Wild-type and C57BL/6 macrophages were stimulated with 50 ng/ml LPS for 3 h, followed by incubation in phenol red-free, serum-free media containing 5 mM glycine and 5 mM ATP with or without 1.8 mM CaCl2. Supernatants were harvested and filter sterilized, CaCl2 was added back to those lacking calcium, and then supernatants were acidified to pH 2–3 using trifluoroacetic acid (TFA). An Oasis HLB Plus cartridge (Waters) was wetted with acetonitrile and washed once in 0.1% TFA before application of acidified supernatant. The cartridge was washed thoroughly with 0.1% TFA, and proteins were eluted in 60% acetonitrile/0.1% TFA. The samples were lyophilized and stored at −80°C. Lyophilized samples were reconstituted in 0.1% TFA to original magnification ×500, the starting concentration. Approximately 2 × 106 Salmonella/ml were incubated in Luria–Bertani broth containing ×40 concentrated supernatant and incubated at 37°C for 1 h and then diluted and plated on Luria–Bertani agar to determine the number of CFUs.

Results
Pyroptosis is accompanied by lysosome exocytosis
Lysosome exocytosis has been proposed to mediate release of caspase-1–processed cytokines in response to ATP (9, 10); therefore, the fate of lysosomal compartments was examined in Salmonella-infected macrophages undergoing pyroptosis. Macrophages were incubated with TAM dextran to identify lysosomes (Fig. 1A) and then infected with Salmonella in the presence of the fluorescent probe (FAM-YVAD), which specifically binds to active caspase-1 (26). Wild-type Salmonella infection triggered activation of caspase-1, and many caspase-1–positive macrophages were devoid of lysosomes (Fig. 1A, Supplemental Fig. 1B). Loss of lysosomes required injection of proteins into the host cell cytosol via the bacterial T3SS, as T3SS-null Salmonella failed to stimulate caspase-1 activation and remained lysosome positive (Fig. 1A). The reduced number of lysosomes during pyroptosis could be explained by their fusion with the cell surface as a result of lysosome exocytosis, and this was quantified by examining surface exposure of the lysosomal transmembrane protein LAMP1. Salmonella infection resulted in surface localization of LAMP1 on ~35% of macrophages, compared with <5% of uninfected or T3SS-null (SipB−) Salmonella-infected macrophages (Fig. 1B, 1C). These data indicate activation of caspase-1 is accompanied by loss of lysosomal compartments and surface exposure of LAMP1 via lysosome exocytosis.
Active caspase-1 mediates membrane pore formation, calcium influx, and lysosome exocytosis

Fusion of both conventional and secretory lysosomes with the cell surface requires an influx of calcium ions from the extracellular media (16). Caspase-1 activation leads to formation of plasma membrane pores between 1.1 and 2.4 nm in diameter, large enough to allow the influx of extracellular calcium ions required for lysosome exocytosis (4). We examined the kinetics of pore formation during pyroptosis by monitoring uptake of ethidium bromide (EtBr) during infection with GFP-expressing *Salmonella*. At 5 min postinfection, the majority of wild-type macrophages were infected, but had not yet taken up EtBr, and by 20 min ~50% of macrophages were EtBr positive (Fig. 2A). *Casp1−/−* macrophages failed to take up EtBr at any time point in response to *Salmonella* infection (Fig. 2A), indicating pore formation during pyroptosis is a caspase-1–dependent process.

Despite formation of caspase-1–dependent membrane pores, plasma membranes remain intact and retain cytoplasmic lactate dehydrogenase early in pyroptosis (Supplemental Fig. 1A) (4). Membrane integrity during LAMP-1 staining was confirmed using Abs specific for the cytoplasmic protein ASC, a component of the multiprotein inflammasome complex. Abs-mediated detection of ASC foci was only possible when the macrophages were first permeabilized with Cytolix/Cytoperm (Supplemental Fig. 1C), demonstrating that *Salmonella*-induced LAMP-1 staining (Fig. 1B) is specific for molecules exposed on the cell surface during lysosome exocytosis of otherwise intact cells.

The influx of calcium into *Salmonella*-infected macrophages was addressed using the calcium indicator fluo-4, which has increased fluorescence intensity in response to calcium binding. Increased intracellular calcium levels were observed in individual macrophages ~8 min after *Salmonella* infection (Fig. 2B, Supplemental Video 1). In contrast, infection of *Casp1−/−* macrophages did not result in increased intracellular calcium (Fig. 2B, Supplemental Video 2). The change in mean fluorescence intensity of individual wild-type and *Casp1−/−* macrophages was quantified, and representative traces are shown (Fig. 2C). Fifty percent of wild-type *Salmonella*-infected macrophages underwent a calcium influx during 20 min of infection, compared with <2% of *Salmonella*-infected *Casp1−/−* macrophages during the same period (Fig. 2D).Removing calcium from the extracellular media prevented increased intracellular calcium, confirming the influx of the calcium was from the extracellular milieu and not intracellular calcium stores (T. Bergsbaken, unpublished data). These data are consistent with caspase-1–induced membrane pores allowing a calcium influx from the extracellular media.

To directly examine the role of caspase-1 activation and extracellular calcium in lysosome exocytosis, macrophages were infected with *Salmonella* in the presence of the caspase-1 inhibitor YVAD-CMK or in calcium-free media, and surface LAMP-1–positive cells were quantified. Surface LAMP-1 was detected on 44% of *Salmonella*-infected macrophages, and this was reduced to 7% in the presence of YVAD-CMK (Fig. 3A, 3B). In the absence of extracellular calcium, only 4% of *Salmonella*-infected macrophages became surface LAMP1 positive (Fig. 3A, 3B). Calcium-free media had no effect on the uptake of *Salmonella* into macrophages (Supplemental Fig. 1D), nor does the absence of extracellular calcium alter *Salmonella*-induced pyroptosis (25). Lysosome exocytosis also results in secretion of lysosomal protein (14), and we confirmed release of lysosomal cathepsin D into the supernatant during *Salmonella* infection (Fig. 3C). Consistent with the pattern of surface LAMP1 exposure, cathepsin D secretion during infection was inhibited in the presence of YVAD-CMK or calcium-free media (Fig. 3C). These data suggest that during *Salmonella* infection caspase-1 activation leads to an increase in membrane permeability and an influx of calcium from the extracellular media. Elevated intracellular calcium facilitates lysosome exocytosis, resulting in surface LAMP1 exposure and lysosomal protein secretion.

Caspase-1–dependent lysosome exocytosis is a conserved host response

Previous research has indicated caspase-1–dependent membrane pore formation occurs in response to multiple stimuli (25), suggesting events downstream of pore formation, like lysosome exocytosis, may also be conserved. *Salmonella* activates caspase-1 via NLRC4. Additional stimuli that signal through distinct NLRs were assayed for their ability to trigger caspase-1–dependent lysosome exocytosis. Treatment of wild-type macrophages with *Bacillus anthracis* LT or the H+/K+ antiporter nigericin [which activate caspase-1 via NLRP1b and NLRP3, respectively (27, 28)] resulted in the surface exposure of LAMP1 (Fig. 4A, 4B). This process required active caspase-1, as *Casp1−/−* or YVAD-treated macrophages did not become surface LAMP1 positive under the same conditions (Fig. 4A, 4B). These data confirmed caspase-1–dependent lysosome exocytosis is a conserved response to a diverse group of stimuli.

In macrophages and monocytes treated with the P2X7 receptor ligand ATP, caspase-1 is activated, but lysosome exocytosis is
caspase-1 independent (10–12). One possible explanation for this distinct mechanism of lysosome exocytosis is the ability of ATP treatment to initiate a rapid calcium influx prior to detectable caspase-1 activation. Increased intracellular calcium was detected within 30 s of ATP stimulation of both wild-type and Casp1<sup>−/−</sup> macrophages (Fig. 4D), in contrast to the delayed calcium influx observed during Salmonella infection (Fig. 2D). Surface LAMP1 was apparent in both wild-type and Casp1<sup>−/−</sup> macrophages after ATP treatment and was inhibited by removing calcium (Fig. 4D), unlike Salmonella infection in which surface LAMP1 exposure was both caspase-1 and calcium dependent (Fig. 3). Thus, stimuli capable of mediating calcium influxes independently of caspase-1 activation (10, 17) can initiate lysosome exocytosis without requiring caspase-1 activation; however, caspase-1 activation via the NLRs NLRP1b, NLRP3, and NLRC4 leads to the conserved processes of pyroptosis and lysosome exocytosis.

Caspase-1–dependent cytokine secretion occurs independently of lysosome exocytosis

During pyroptosis, the inflammatory cytokines IL-1β and IL-18 are cleaved and released from the cell. Membrane pore-mediated secretion (4), lysosome exocytosis (9, 10), and multivesicular body release (11, 12) have all been proposed as mechanisms of secretion. During Salmonella infection, mature IL-18 and IL-1β were released from macrophages, and secretion was inhibited by YVAD-CMK (Fig. 5A). Inhibition of lysosome exocytosis by removal of calcium from the extracellular media (Figs. 3, 4D) did not affect cytokine processing and release (Fig. 5A), indicating lysosome exocytosis is not required for cytokine release during Salmonella infection. The same pattern of mature cytokine secretion was observed after ATP treatment, in which IL-1β cleavage and secretion required caspase-1 activity, but was not altered by inhibition of lysosome exocytosis (Fig. 5B). Therefore,
mature cytokine secretion during pyroptosis occurs by a caspase-1-dependent process distinct from lysosome exocytosis.

**Lysosome exocytosis mediates release of antimicrobial factors and microbial products during pyroptosis**

Phagocytic uptake of microbes often leads to their transport into acidic phagolysosomal compartments for destruction. Our data suggest that intact or degraded microbes residing in lysosomes may be released by lysosome exocytosis during pyroptosis. We examined the ability of macrophages undergoing pyroptosis to release phagocytosed yeast particles via lysosome exocytosis. Macrophages were loaded with Alexa-488 dextran (to identify lysosomes) and Alexa-594–labeled yeast particles, and the majority of yeast particles were located within lysosomes in both wild-type and Casp1−/− macrophages (Fig. 6A–C). Macrophages were infected with *Salmonella* and monitored by live cell microscopy to observe whether lysosomal compartments (containing both Alexa-488 dextran and Alexa-594 yeast) underwent exocytosis and fused with the cell surface. Release of Alexa-594 yeast into the extracellular medium was confirmed using the membrane-impermeant dye trypan blue, which quenches extracellular fluorescence (Fig. 6A, Supplemental Fig. 1E). Thirty-six percent of wild-type macrophages released one or more yeast particles during the course of *Salmonella* infection (Fig. 6B, 6D). In contrast, <10% of Casp1−/− macrophages released yeast particles during *Salmonella* infection (Fig. 6C, 6D), indicating caspase-1 can mediate release of microbial products within lysosomes. These studies were performed in the presence of glycine and at time points prior to cell lysis (Supplemental Fig. 1A). Therefore, release of lysosomal contents during the early stages of pyroptosis is mediated by exocytosis.

Recent studies have indicated host lysosomal proteins retain their antimicrobial activity when released into the extracellular space (17, 29), suggesting lysosome exocytosis could also function to control replication of extracellular bacteria in the vicinity of cells undergoing pyroptosis. To examine this possibility, wild-type macrophages were infected with *Salmonella*, and secreted factors were collected under conditions in which lysosome exocytosis occurred (S+LE) or lysosome exocytosis was inhibited (S−LE). Exocytosed factors were concentrated and incubated with exponentially growing *Salmonella*, and the surviving bacteria were quantified. Only 1.4% of the initial number of *Salmonella* were recovered after incubation with S+LE; however, when incubated with S−LE, bacterial survival increased to >22% (Fig. 6E). The increased antimicrobial activity of supernatants containing lysosomal components was also observed using macrophages treated with ATP (T. Bergsbaken, unpublished data). These data indicate lysosome exocytosis enhances the antimicrobial nature of pyroptosis by releasing molecules that have direct antimicrobial activity against extracellular bacteria.

**Discussion**

We demonstrate that caspase-1–dependent lysosome exocytosis is a conserved feature of pyroptosis. Activation of caspase-1 stimulates membrane perturbations and an influx of calcium from the extracellular milieu. Increased intracellular calcium levels...
Several pathogens use specialized secretion systems to inject proteins into the host cell cytosol, and this requires formation of a membrane-spanning translocation pore in the host cell membrane. It has been suggested that the pore formed by the T3SS stimulates a calcium influx and fusion of lysosomes with the cell surface (30); however, we have demonstrated that pores formed in the macrophage membrane during Salmonella and Yersinia infection are dependent on host caspase-1 (4, 31) (Fig. 2A). Casp1−/− macrophages, or macrophages treated with caspase-1 inhibitor, fail to undergo a calcium influx or lysosome exocytosis in response to Salmonella infection (Figs. 2B, 3A), and infection of macrophages with Yersinia also stimulates lysosome exocytosis in a caspase-1–dependent manner (T. Bergsbaken, unpublished data). This indicates that during infection, host caspase-1, and not the bacterial secretion system per se, is critical for allowing the calcium influx required for lysosome exocytosis. We have shown caspase-1 activation mediates lysosome exocytosis, and others have demonstrated caspase-1 plays a role in limiting intracellular bacterial survival via phagosome maturation (32–34). An overlapping set of host cell processes regulate lysosome exocytosis and phagosome maturation (30, 35), and our studies and others implicate caspase-1 as a regulator of both lysosome exocytosis and phagosome maturation during bacterial infection (32–34). Macrophages that have active caspase-1 restrict bacterial replication by enhancing transit to lysosomes, and cells destined to undergo pyroptosis continue to exert antimicrobial activity by releasing host antimicrobial factors.

Fusion of conventional lysosomes with the cell surface is able to mediate membrane repair in multiple cell types (16). However, the stimuli used in our studies led to robust caspase-1 activation and ultimately resulted in cell lysis, indicating lysosome exocytosis was not sufficient to completely rescue cells from lysis under these conditions. Caspase-1 activation is clearly not an all or nothing phenomenon, as macrophages and other cell types can activate caspase-1 without immediately undergoing cell death (32, 36–39). It is possible that intermediate levels of caspase-1 activation result in membrane alterations and cytokine secretion, with subsequent lysosome exocytosis repairing membrane defects, rescuing cells from lysis, and simultaneously releasing antimicrobial factors. Once threshold levels of active caspase-1 are reached, membrane defects outstrip membrane repair and cell death occurs. Further experiments will be required to address the ability of lysosome exocytosis to rescue cells from pyroptosis.

Lysosomes contain factors with known antimicrobial activity in the context of the acidified lysosome; however, some lysosomal contents retain antimicrobial activity in the extracellular milieu (29, 40) (Fig. 6E). Several proteins with adjuvant activity have also been localized to lysosomes (14, 41, 42). For intracellular pathogens such as Salmonella, the lysosomes of infected macrophages may also contain degraded bacteria; their fusion with the cell surface could mediate the release of Ags for presentation by neighboring cells, and, consistent with this, bacterial lipids are released from Mycobacterium tuberculosis-infected macrophages (43). We have demonstrated caspase-1–dependent release of microbial products during pyroptosis. Together these findings suggest inflammatory host cell suicide by pyroptosis is not only an innate defense mechanism, but also drives concurrent release of inflammatory cytokines, molecules with direct antimicrobial activity, and microbial products and other inflammatory contents that could function to amplify the inflammatory response by sensitizing nearby host cells to pyroptosis (31). Additionally, release of Ags in the presence of caspase-1–dependent inflammation would facilitate robust adaptive immune responses.
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microvesicle shedding from macrophages restricts Salmonella infection of mouse macrophages. 


A process for controlling intracellular bacterial virulence that involves induced membrane injury. 

Phospholipases C and A2 control lysosome-mediated IL-1β release in murine macrophages. 

Interleukin-1β is cytosolic and precedes cell death. 

Secretion of interleukin-1β by microvesicle shedding. 


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Cryopyrin activates the inflammasome in response to toxins and ATP. 


Phospholipases C and A2 control lysosome-mediated IL-1β secretion: implications for inflammatory processes. 


SUPPLEMENTAL MATERIAL

Figure S1:

A) Exogenous glycine protects cells from caspase-1-dependent cell lysis. Bone marrow-derived macrophages were infected with *Salmonella* in the presence (dashed line) or absence (solid line) of 5mM glycine. At the indicated times, supernatants were harvested and placed on ice. Triplicate 50µl aliquots of supernatant were transferred to a 96-well plate and the presence of the cytoplasmic enzyme LDH was measured using the Cytox96 kit (Promega, Madison, WI). Representative graph from 3 independent experiments. * P<0.005

B) *Salmonella* infection of macrophages results in the loss of lysosomal compartments via lysosome exocytosis. Bone marrow-derived macrophages were incubated with TMR-dextran to label lysosomes (red) and infected for 20 minutes with *Salmonella* in the presence of FAM-YVAD-FMK to label active caspase-1 (green). DNA was stained using TO-PRO3 (blue). During infection macrophages contain active caspase-1 (green) and a few contain both active caspase-1 and lysosomal staining (red), see cell a. The majority of macrophages with active caspase-1 are devoid of lysosomal compartments, see cell b. A macrophage without active caspase-1 (c) is shown in the same field for comparison.

C) Early in pyroptosis, lysosome exocytosis occurs in intact cells containing active caspase-1. Bone marrow-derived macrophages were infected for 20 minutes with *Salmonella* in the presence of FAM-YVAD-FMK to label active caspase-1 (green). Cells were washed to remove unbound FAM-YVAD prior to further treatment. Half of the samples (Non-permeabilized; top row) were stained with primary rat anti-LAMP1 and rabbit anti-ASC antibodies (AL177; Enzo Life Sciences) for 30 minutes on ice prior to fixation with 2%
paraformaldehyde. Remaining samples (Permeabilized; bottom row) were treated with Cytofix/Cytoperm (BD Biosciences) for 30 minutes, washed and then stained with primary rat anti-LAMP1 and rabbit anti-ASC antibodies. All samples were incubated with the same secondary antibodies: Goat anti-Rat-Alexa 555 (LAMP1, red) and Goat anti-Rabbit-Alexa 405 (ASC, orange). DNA was stained using TO-PRO3 (blue). Arrows indicate punctate staining of inflammasome complexes, containing both ASC and active caspase-1, that form during pyroptosis (ref. 2). Foci of active caspase-1 and ASC co-localize in permeabilized cells, resulting in yellow puncta in the merged image. ASC foci are not visible in non-permeabilized cells (puncta are green in merged image), demonstrating the plasma membrane is intact and anti-LAMP1 staining is surface-restricted. Representative of 3 experiments.

D) *Salmonella* infection is calcium-independent. The number of internalized bacteria per macrophage was determined using sequential labeling of bacteria before and after permeabilization. Wild-type macrophages were incubated in serum-free media containing 5mM glycine with or without 1.8mM CaCl₂ and infected with *Salmonella* for 30 minutes. To label extracellular bacteria, cells were incubated with a *Salmonella* group B LPS-specific antibody, washed, fixed, and incubated with a secondary antibody conjugated to Alexa-555. The macrophages were then permeabilized with Cytofix/Cytoperm, incubated once more with the anti-LPS antibody to label both intracellular and extracellular bacteria, and incubated with a secondary antibody conjugated to Alexa-405. Intracellular bacteria were enumerated in 5 randomly selected fields. Graph represents the average of 3 independent experiments. P= 0.14

E) Pyroptosis triggers release of lysosome-bound zymosan into the extracellular media. Wild-type macrophages were loaded with Alexa-488 dextran to label lysosomes (green) and Alexa-594 yeast particles (red), infected with *Salmonella*, and imaged every 20s for 20 minutes.
Representative images highlight the dextran-positive compartments described in Figure 6 A. Dextran-positive compartments containing (a) or lacking (b) zymosan particles were released sequentially over the 20 min infection period. Extracellular Alexa-594 yeast particles are red. Released dextran molecules readily diffuse away from the macrophage, while the larger zymosan particles remain visible after exocytosis.

**Figure S2: Mechanism of lysosome exocytosis during pyroptosis.** (A) Caspase-1-dependent lysosome exocytosis and cytokine secretion. (1) During *Salmonella* infection, *B. anthracis* lethal toxin, or nigericin treatment, the NLRs NLRC4, NLRP3, and NLRP1b trigger activation of caspase-1 (2). Active caspase-1 leads to plasma membrane damage, resulting in an influx of extracellular calcium (3). Increased intracellular calcium leads to fusion of lysosomes with the cell surface and secretion of lysosomal contents, or lysosome exocytosis (4). Caspase-1 activation also mediates processing of IL-1β and IL-18 (5) and secretion by an unknown mechanism that is independent of calcium influx and lysosome exocytosis (6). (B) Caspase-1-independent lysosome exocytosis. Stimuli like ATP (via the P2X7 receptor) and membrane pore forming toxins act directly on the cell surface (1), leading to membrane perturbations and a calcium influx from the extracellular media (2). Increased intracellular calcium leads to lysosome exocytosis, which does not require caspase-1 activity (3). These stimuli also trigger potassium efflux, which facilitates caspase-1 activation via NLRP3 (4). Similar to (A), caspase-1 activation leads to cytokine processing (5) and release (6). Microvesicle shedding and mutivesicular body release have been suggested to mediate processed cytokine release during ATP treatment of macrophages (refs. 11, 12); however, it is not yet known whether these
findings represent a universal mechanism of cytokine release that can be extended to all caspase-1 activating stimuli.

**Videos S1 & S2:** The *Salmonella*-induced Ca2+ flux is caspase-1-dependent. Wild-type (Video S1) and *Casp1/-* (Video S2) macrophages were loaded with the calcium indicator fluo-4 and infected with *Salmonella*. Images were taken every 15s for 25 minutes. Representative of multiple fields from 3 experiments.