Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages

Susan L. Fink¹ and Brad T. Cookson²*
¹Molecular and Cellular Biology Program and ²Departments of Laboratory Medicine and Microbiology, University of Washington, Seattle, WA, USA.

Summary

Salmonella enterica serovar Typhimurium invades host macrophages and induces a unique caspase-1-dependent pathway of cell death termed pyroptosis, which is activated during bacterial infection in vivo. We demonstrate DNA cleavage during pyroptosis results from caspase-1-stimulated nuclease activity. Although poly(ADP-ribose) polymerase (PARP) activation by fragmented DNA depletes cellular ATP to cause lysis during oncosis, the rapid lysis characteristic of Salmonella-infected macrophages does not require PARP activity or DNA fragmentation. Membrane pores between 1.1 and 2.4 nm in diameter form during pyroptosis of host cells and cause swelling and osmotic lysis. Pore formation requires host cell actin cytoskeleton rearrangements and caspase-1 activity, as well as the bacterial type III secretion system (TTSS); however, insertion of functional TTSS translocons into the host membrane is not sufficient to directly evoke pore formation. Concurrent with pore formation, inflammatory cytokines are released from infected macrophages. This mechanism of caspase-1-mediated cell death provides additional experimental evidence supporting pyroptosis as a novel pathway of inflammatory programmed cell death.

Introduction

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a facultative intracellular pathogen that invades macrophages and induces caspase-1-mediated host cell death in vitro (Hersh et al., 1999) and in vivo (Monack et al., 2000). Caspase-1-deficient mice have a 1000-fold higher oral LD₅₀ than wild-type animals and even with high oral doses are remarkably resistant to systemic bacterial dissemination, demonstrating that activation of caspase-1 is a crucial component of bacterial pathogenesis in vivo (Monack et al., 2000; 2001a). Caspase-1-mediated cell death, termed pyroptosis, is a newly identified pathway of programmed cell death that inherently produces an inflammatory outcome, but the molecular mechanism of cell death is poorly understood (Cookson and Brennan, 2001; Fink and Cookson, 2005).

The ability of S. Typhimurium to induce rapid macrophage pyroptosis requires the Salmonella pathogenicity island 1 (SPI-1) type III secretion system (TTSS) (Chen et al., 1996a) and the translocated effector, SipB, has been shown to interact with caspase-1 (Hersh et al., 1999). The host adaptor protein ASC and the NOD-LRR protein Ipaf are also required for caspase-1 activation in response to Salmonella infection, suggesting that formation of a multiprotein complex termed the inflammasome triggers pyroptosis (Mariathasan et al., 2004). The human pathogen S. Typhi also stimulates pyroptosis in human-derived macrophages (Monack et al., 2001b). Pyroptosis is not limited to macrophages, as caspase-1-dependent death of Salmonella-infected dendritic cells has also been described (van der Velden et al., 2003).

Pyroptosis is distinguished from other forms of cell death in part by its unique morphological and biochemical characteristics. S. Typhimurium-infected macrophages contain fragmented DNA diffusely present within the nucleus, which contrasts with the typical condensed nuclear morphology observed during apoptosis (Brennan and Cookson, 2000). In addition, the cleaved DNA does not display the oligonucleosomal pattern characteristic of apoptosis (Watson et al., 2000). During pyroptosis, activation of apoptotic caspases, including caspase-3, -6, -7 or -8, does not occur, and mitochondrial integrity remains intact (Brennan and Cookson, 2000; Jesenberger et al., 2000). S. Typhimurium-induced cell death results in loss of membrane integrity and release of cytoplasmic contents (Brennan and Cookson, 2000). However, the molecular mechanisms mediating DNA fragmentation and cell lysis during pyroptosis have not been identified.

Caspase-1, also known as interleukin (IL)-1-beta-converting enzyme, is a cysteine protease that cleaves and activates the pro-forms of host inflammatory cytokines, IL-1β and IL-18 (Fantuzzi and Dinarello, 1999). Both cytokines are processed and released by S. Typhimurium-infected macrophages (Monack et al., 2001b; Obregon...
et al., 2003), but neither is required for macrophage death (Monack et al., 2001b). Active IL-1β and IL-18 are inflammatory mediators that potentiate cytokine induction, recruitment of immune cells, enhancement of T and NK cell function, and production of diverse secondary cytokines (Dinarello, 1998; Delaleu and Bickel, 2004). Thus, macrophage pyroptosis during Salmonella infection may contribute to the inflammation and tissue destruction observed in vivo (Everest et al., 2001; Kaufmann et al., 2001) via caspase-1-dependent production of active IL-1β and IL-18 and release of inflammatory intracellular contents during lysis (Scaffidi et al., 2002; Shi et al., 2003).

Pyroptosis occurs in response to a number of bacterial and viral pathogens in addition to Salmonella (Chen et al., 1996; Hilbi et al., 1997; 1998; Sansonetti et al., 2000; 2005; Kelk et al., 2003; 2005; Sun et al., 2005; Thumbikat et al., 2005). Recent studies have demonstrated inflammasome-mediated caspase-1 activation not only in response to exogenous foreign stimuli, but also to endogenous ‘danger signals’ (Kanneganti et al., 2006; Mariahasan et al., 2006; Martinon et al., 2006). Caspase-1 has been implicated in non-infectious inflammatory diseases (Cookson and Brennan, 2001), suggesting that the caspase-1-mediated events occurring during pyroptosis may have broad biological significance. The findings we describe here illuminate the molecular mechanism leading to the features of cell death and cytokine secretion during pyroptosis of S. Typhimurium-infected macrophages.

Results

Nuclease- and caspase-1-dependent DNA cleavage in Salmonella-infected macrophages

Degradation of chromosomal DNA is a terminal event in the life of a cell and a hallmark of cell death (Zhang and Xu, 2002). Additionally, DNA cleavage can be mechanistically coupled to cell lysis. To investigate the molecular mechanism of DNA fragmentation during pyroptosis, DNA cleavage in S. Typhimurium-infected J774A.1 macrophages was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) reaction (Fig. 1A, B, and F). Macrophages infected for 4 h with wild type (Fig. 1B), but not non-cytotoxic SPI-1-deficient prgH (Fig. 1C) or sipB (Fig. 1D) mutant S. Typhimurium, demonstrate DNA damage. Control macrophages induced to undergo apoptosis by treatment with gliotoxin (Waring et al., 1988; Brennan and Cookson, 2000) demonstrate similar levels of DNA cleavage (Fig. 1E). The nuclease primarily responsible for DNA fragmentation during apoptosis, caspase-activated DNase (CAD), is present in living cells bound to the inhibitor ICAD.
(Enari et al., 1998). Caspase-mediated cleavage of ICAD results in release and activation of CAD (Sakahira et al., 1998), and recombinant caspase-1 cleaves ICAD in vitro (Zhou et al., 2000). We hypothesized that ICAD proteolysis may lead to DNA fragmentation during pyroptosis, and therefore examined ICAD degradation by immunoblot. Although the abundance of full-length ICAD protein decreased in apoptotic macrophages treated with gliotoxin (Fig. 1G), detectable ICAD degradation did not occur in TUNEL positive macrophages infected with wild-type S. Typhimurium (Fig. 1G). These results indicate DNA fragmentation during pyroptosis is unlikely to involve the apoptotic mechanism regulated by ICAD proteolysis.

DNA fragmentation during cell death can result from nuclease activity or reactive oxygen species (Halliwell and Aruoma, 1991; Salgo et al., 1995; Muratori et al., 2003; Nagata et al., 2003). The specific caspase-1 inhibitor, YVAD (Garcia-Calvo et al., 1998), prevented DNA fragmentation in infected macrophages (Fig. 2), confirming that capsase-1 activity is required for DNA fragmentation (Monack et al., 2001b). Furthermore, the nuclease inhibitor aurantricarboxylic acid (ATA) (Escargueil-Blanc et al., 1997) inhibited DNA cleavage in infected macrophages (Fig. 2) indicating nuclease-mediated DNA fragmentation; DNase I-mediated DNA cleavage in control macrophages was also inhibited by ATA (Fig. 2). In contrast, reactive chemical species generated by hydrogen peroxide and S-nitroso-N-acetyl-penicillamine (SNAP) (Wang et al., 2003) caused DNA damage that was not nuclease-dependent and was not inhibited by ATA (Fig. 2). In S. Typhimurium-infected primary bone marrow-derived macrophages (BMDM), DNA fragmentation was also prevented by both YVAD and ATA (data not shown). Prevention of DNA cleavage by nuclease inhibition excludes the possibility that DNA damage during Salmonella infection results from reactive chemical species. Together these results demonstrate DNA fragmentation during pyroptosis results from the activity of a caspase-1-activated nuclease.

**Lysis of Salmonella-infected macrophages is independent of DNA cleavage and PARP activity**

Fragmented DNA activates the enzyme poly(ADP-ribose) polymerase-1 (PARP) to catalyse ADP-ribosylation of nuclear proteins using NAD+ as a substrate (Herceg and Wang, 2001). Mild DNA damage stimulates PARP and promotes DNA repair (Herceg and Wang, 2001). However, excessive PARP activity depletes cellular NAD+ and, consequently ATP, which leads to cell lysis (Ha and Snyder, 1999). PARP is cleaved and inactivated during apoptosis (Herceg and Wang, 2001), but remains in its active, uncleaved state in S. Typhimurium-infected macrophages (Brennan and Cookson, 2000). This suggested PARP activity, stimulated by caspase-1-dependent DNA damage (Figs 1 and 2), could lead to cell lysis during pyroptosis. We tested this hypothesis by examining release of cytosolic lactate dehydrogenase (LDH) from S. Typhimurium-infected J774A.1 macrophages after 6 h (Fig. 3A). LDH release was not inhibited by the PARP inhibitors 3-amino benzamide (3AB) (Schraufstatter et al., 1986; Filipovic et al., 1999) or 4-hydroxyquinazoline (Lee and Shacter, 1999; Veres et al., 2004), nor did they block LDH release from infected BMDM (data not shown). In contrast, macrophage exposure to hydrogen peroxide stimulates PARP-dependent LDH release and cell lysis (Schraufstatter et al., 1986; Filipovic et al., 1999), which was inhibited by 3AB (Fig. 3A). Together these data indicate lysis during pyroptosis is independent of PARP activity.
DNA damage can also lead to cell lysis via mechanisms distinct from PARP activation (Ueda et al., 1995), and we therefore examined the possibility that PARP-independent mechanisms could couple DNA fragmentation to lysis during pyroptosis. ATA prevented DNA fragmentation during pyroptosis (Fig. 3B), but not LDH release from infected J774A.1 macrophages (Fig. 3C) or infected BMDM (data not shown). These results indicate the rapid loss of membrane integrity characteristic of Salmonella-infected macrophages occurs independently of both PARP activity and DNA fragmentation.

Ethidium bromide uptake by Salmonella-infected macrophages requires caspase-1 and actin-mediated processes

Localizing active caspase-1 to the plasma membrane has led to speculation that membrane pores form and release the leaderless cytokines, IL-1β and IL-18 (Singer et al., 1995). In addition, cytolytic pores are observed concurrently with caspase-1 activation in response to extracellular ATP and potassium efflux by lipopolysaccharide-activated macrophages (Steinberg et al., 1987; Solle et al., 2001; Kahlenberg and Dubyak, 2004). Using uptake of small membrane impermeant dyes as our experimental approach to examine pore formation (Schilling et al., 1999; Lajdova et al., 2004; Chen et al., 2005), we stained S. Typhimurium-infected macrophages with the red membrane impermeant dyes, ethidium bromide (EtBr; MW 394 Da) or ethidium homodimer-2 (EthD2; MW 1293 Da), and counterstained with the membrane permeable dye SYTO 62. Mock-infected cells excluded both impermeant dyes (Fig. 4A, E and I) while cells treated with the detergent, triton X-100, uniformly allowed influx of both EtBr and EthD2 (Fig. 4D and H). After 2.5 h of infection by S. Typhimurium, macrophages became permeable to EtBr (Fig. 4B) while excluding the larger EthD2 (Fig. 4F). These results are consistent with the formation of discrete membrane lesions allowing the passage of small molecules such as EtBr, but excluding larger molecules like EthD2. In contrast, gliotoxin-treated macrophages formed apoptotic bodies but excluded both EtBr and EthD2 (Fig. 4C and G) consistent with maintenance of an intact plasma membrane throughout apoptosis (Fink and Cookson, 2005).

EtBr uptake by S. Typhimurium-infected macrophages was completely prevented by inhibition of caspase-1 with YVAD-CMK (Fig. 4J and O), while AAF-CMK, a structurally similar peptide inhibitor with no activity towards caspase-1, failed to prevent EtBr uptake by infected cells (Fig. 4K). YVAD did not inhibit EtBr uptake by cells treated with triton X-100 (data not shown). Cytochalasin D (CD), which prevents bacterial uptake and S. Typhimurium-induced macrophage death by inhibiting actin cytoskeleton rearrangements (Guilloteau et al., 1996; Monack et al., 1996), also prevented EtBr uptake by infected cells (Fig. 4L). These findings indicate processes mediated by activated caspase-1 and downstream of actin polymerization, are required for EtBr uptake during pyroptosis.

The bacterial SPI-1 TTSS is required for Salmonella to stimulate caspase-1 activation and pyroptosis in infected macrophages (Hersh et al., 1999; Mariathasan et al., 2004), and we consistently observed that macrophages infected with non-cytotoxic SPI-1-deficient prgH (Fig. 4M) or sipB (Fig. 4N) mutant S. Typhimurium did not become permeable to EtBr (Fig. 4K). Insertion of the TTSS translocon in host cells has been suggested to directly create defects that permeabilize host cell membranes to small molecules in the extracellular media (Neyt and Cornelis, 1999; Dacheux et al., 2001; Roy et al., 2004). In contrast, it is also thought that the TTSS constitutes a continuous channel, directly connecting the bacteria and host cell,
while excluding any molecules from the environment (Gauthier and Finlay, 2001). Our results suggest EtBr uptake by infected macrophages is mediated by host cell processes as it was completely inhibited by CD (Fig. 4L), which does not prevent formation of the SPI-1 TTSS or translocation of SPI-1 effectors (Collazo and Galan, 1997). We demonstrated that SPI-1 translocation in infected macrophages was not altered by YVAD (Fig. 5), which inhibited both EtBr uptake (Fig. 4J) and LDH release (Hersh et al., 1999; Brennan and Cookson, 2000; Monack et al., 2001b). To detect TTSS-mediated effector translocation, we used S. Typhimurium expressing the SPI-1 effector SspH1 fused to CyaA (Miao and Miller, 2000), which catalyses the formation of cAMP in the presence of the cofactor calmodulin present in the host cytosol. Successful translocation of the SspH1–CyaA fusion protein into the host cell, in the presence or absence of YVAD, resulted in increased cAMP (Fig. 5). These findings demonstrate that although the SPI-1 TTSS is required to induce caspase-1 activation and pyroptosis, formation of the TTSS translocon is not sufficient to cause EtBr uptake by macrophages, as effector translocation occurs in the presence of either CD (Collazo and Galan, 1997) or YVAD (Fig. 5), which both inhibited influx of EtBr (Fig. 4L and J). During infection at a low multiplicity of infection (moi), our results exclude the possibility that EtBr uptake occurs directly through the bacterial translocation apparatus inserted in the host cell membrane.

**Caspase-1-dependent pore formation leads to swelling and osmotic lysis of Salmonella-infected macrophages**

Plasma membrane defects cause osmotic lysis by dissipating cellular ionic gradients; retention of large cytoplasmic constituents increases intracellular colloid osmotic pressure and leads to water influx and cell swelling (Clinkenbeard and Thiessen, 1991; Lobo and Welch, 1994). The formation of caspase-1-dependent membrane defects indicated by EtBr uptake suggested the possibility that S. Typhimurium-infected macrophages may undergo cell swelling prior to lysis. We tested this hypothesis using flow cytometry, as forward scatter measurements provide

---

**Fig. 4.** Caspase-1-dependent pore formation occurs in S. Typhimurium-infected macrophages. J774A.1 macrophages adherent to glass coverslips were treated with PBS, 1% triton or 5 µM gliotoxin or uniformly infected with GFP expressing S. Typhimurium at a moi of 10:1 for 2.5 h. Cells were stained with the membrane permeable dye SYTO 62 (blue) and the membrane impermeant dyes (red), EtBr (MW 394) or EthD2 (MW 1293). Adherent cells were visualized by fluorescence microscopy (40x objective). The arrow in (C) points to visible apoptotic bodies produced by gliotoxin-treated cells. Macrophages were infected in the presence of the specific caspase-1 inhibitor YVAD in (J). As a negative control, a similarly sized peptide inhibitor AAF, with no activity towards caspase-1, fails to prevent EtBr uptake by infected cells (K). Macrophages were infected in the presence of CD which prevents bacterial uptake by inhibiting actin cytoskeleton rearrangements in (L). Infection with non-cytotoxic prgH (M) or sipB (N) mutant S. Typhimurium does not cause EtBr uptake. A minimum of 500 cells were examined for each experimental condition; means and standard deviations are shown (I and O). Results are representative of five independent experiments.
Pore formation during pyroptosis

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd, Cellular Microbiology, 8, 1812–1825

A quantitative assessment of cell size and have been used to examine apoptotic cell shrinkage (Stephenson et al., 1998; Bortner and Cidlowski, 2003; Elliott and Higgins, 2003; Patel et al., 2003). A proportion of macrophages infected with cytotoxic wild-type S. Typhimurium demonstrated an increase in forward scatter after 3 h of infection and by 4 h the majority of the cells within the population demonstrated increased forward scatter (Fig. 6) consistent with an increase in cell size. This was not observed with the non-cytotoxic prgH (Fig. 6) or sipB (data not shown) mutant bacteria, demonstrating that increased cell size is not a consequence of infection per se. Parallel analysis of microsphere size standards confirmed the linear relationship of forward scatter values with particle size (Fig. 6, inset) (Haynes, 1988) and indicated after 4 h the diameter of S. Typhimurium-infected macrophages increased 45% compared with mock infected cells. In contrast, cells treated with gliotoxin demonstrated a decrease in forward scatter, consistent with apoptotic cell shrinkage (data not shown).

Swelling of S. Typhimurium-infected macrophages is dependent on caspase-1 activity, as indicated by elimination of increased forward scatter by treatment with YVAD (Fig. 6), but not AAF (data not shown). Cell swelling and water uptake due to membrane defects are a function of loss of ionic equilibrium (Lobo and Welch, 1994) where pathological ion fluxes can be non-specifically inhibited by the cytoprotective agent glycine (Frank et al., 2000). Glycine prevented swelling of S. Typhimurium-infected macrophages as measured by flow cytometry (Fig. 7A) and inhibited lysis (Fig. 7B), without affecting EtBr uptake (Fig. 7C). These findings indicate caspase-1-dependent membrane defects, and the resultant ion fluxes that can be blocked by glycine, lead to swelling and lysis during pyroptosis of Salmonella-infected macrophages.

Osmotic lysis resulting from membrane pores is prevented by osmoprotectant molecules with molecular diameters greater than the functional diameter of the pores (Lobo and Welch, 1994; Noronha et al., 1996; Kirby et al., 1998; Dacheux et al., 2001). These molecules are retained in the extracellular medium, where they counterbalance the increased intracellular osmotic pressure thereby preventing water influx, swelling and lysis. We sought to determine if discretely sized pores contribute to lysis during pyroptosis. Osmoprotectants of 2.4 nm in diameter or larger (Scherrer and Gerhardt, 1971) protected infected J774A.1 macrophages (Fig. 8) and BMDM (data not shown) from lysis, whereas those smaller than 1.1 nm had no effect. None of the osmoprotectants prevented lysis of gliotoxin-treated controls (Fig. 8). 2.4 nm osmoprotectants also prevented swelling of S. Typhimurium-infected cells measured by flow cytometry, without inhibiting EtBr uptake (data not shown). Importantly, osmotic lysis resulting from membrane pores is prevented by osmoprotectant molecules with molecular diameters greater than the functional diameter of the pores (Lobo and Welch, 1994; Noronha et al., 1996; Kirby et al., 1998; Dacheux et al., 2001). These molecules are retained in the extracellular medium, where they counterbalance the increased intracellular osmotic pressure thereby preventing water influx, swelling and lysis. We sought to determine if discretely sized pores contribute to lysis during pyroptosis. Osmoprotectants of 2.4 nm in diameter or larger (Scherrer and Gerhardt, 1971) protected infected J774A.1 macrophages (Fig. 8) and BMDM (data not shown) from lysis, whereas those smaller than 1.1 nm had no effect. None of the osmoprotectants prevented lysis of gliotoxin-treated controls (Fig. 8). 2.4 nm osmoprotectants also prevented swelling of S. Typhimurium-infected cells measured by flow cytometry, without inhibiting EtBr uptake (data not shown). Importantly,
osmoprotection is not observed when irregular membrane damage occurs (Viboud and Bliska, 2002). The resolution of the size of membrane defects, indicated by osmoprotectants to be within a narrow size range, supports the hypothesis that discrete transmembrane pores between 1.1 and 2.4 nm in diameter form during pyroptosis. These data are supported by our observation that infected macrophages allowed influx of EtBr (MW 394), while simultaneously excluding EthD2 (MW 1293). Together, our data indicate formation of caspase-1-dependent plasma membrane pores leads to dissipation of ionic gradients, water influx, cell swelling and ultimately cell lysis during pyroptosis.

Release of caspase-1-activated cytokines from Salmonella-infected macrophages occurs in the absence of lysis and is temporally associated with pore formation

Caspase-1 cleaves and activates the pro-forms of IL-18 and IL-1β and both cytokines are released by S. Typhimurium-infected macrophages undergoing pyroptosis (Monack et al., 2001b; Obregon et al., 2003). Unlike other cytokines, IL-1β and IL-18 lack secretion signals and several mechanisms have been proposed to account for their release (Rubartelli et al., 1990; Hamon et al., 1997; Andrei et al., 1999; 2004; MacKenzie et al., 2001; Wewers, 2004; Marty et al., 2005). Infection with wild type, but not non-cytotoxic prgH or sipB mutant bacteria, stimulated macrophages to release processed IL-18 (Fig. 9A) and IL-1β (Fig. 9B). This required caspase-1 activity, as it was prevented by treatment with YVAD (Fig. 9A and B). It has been hypothesized that membrane rupture and cell lysis

Fig. 7. Glycine prevents swelling and lysis of S. Typhimurium-infected macrophages without inhibiting EtBr uptake. J774A.1 macrophages were treated with PBS, 1% triton or uniformly infected with S. Typhimurium at a moi of 10:1 in the presence of glycine, which non-specifically inhibits ion fluxes. After staining with 7-AAD, the forward scatter (FSC) of intact cells after 4 h of infection was evaluated by flow cytometry (A). LDH released into the supernatant by dying macrophages after 6 h was quantified and the means and standard deviations from triplicate samples are shown (B). Adherent macrophages infected with GFP expressing bacteria (green) for 2.5 h were stained with the membrane permeable dye SYTO 62 (blue) and the membrane impermeant dye EtBr (red) and visualized by fluorescence microscopy (40x) (C). Results are representative of at least three independent experiments for each (A, B and C).

Fig. 8. Osmotic lysis via pore formation contributes to LDH release in S. Typhimurium-infected macrophages. J774A.1 macrophages were uniformly infected with S. Typhimurium at a moi of 10:1 for 6 h or treated with 5 μM gliotoxin for 8 h in the presence of osmoprotectants of varying sizes. LDH released into the supernatant by dying macrophages was quantified. The means and standard deviations from triplicate samples are shown. Results are representative of seven independent experiments.

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd, Cellular Microbiology, 8, 1812–1825
Pore formation during pyroptosis

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd, Cellular Microbiology, 8, 1812–1825

May be required for release of caspase-1-activated cytokines (Monack et al., 2001a; Hogquist et al., 1991a,b). However, glycine completely protected S. Typhimurium-infected macrophages from lysis (Fig. 7B), yet did not prevent release of processed IL-18 (Fig. 9A) or IL-1β (Fig. 9B). Thus, lysis is not required for cytokine release. Pore formation may provide a mechanism for release of processed cytokines, and we considered this hypothesis by concurrently assessing pore formation, cell lysis and accumulation of processed IL-18 in the supernatant of S. Typhimurium-infected macrophages. Between 2 and 3 h after infection EtBr permeability increased maximally, and by 4 h most cells were permeable to EtBr (Fig. 9C). Correspondingly, appearance of processed IL-18 increased in the supernatant between 2 and 3 h after infection, and reached a maximum at 4 h (Fig. 9D) when fewer than 50% of the cells in the population had proceeded to lysis (Fig. 9C). Together, these results suggest release of activated cytokines during pyroptosis can occur in the absence of lysis and is temporally associated with pore formation.

Discussion

Our findings describe events in the molecular pathway of pyroptosis, in which caspase-1 is responsible not only for the features of cell death but also for the activation and release of inflammatory cytokines (Fig. 10). Actin cytoskeleton rearrangements are required for host cell death (Guilloteau et al., 1996; Monack et al., 1996) and mediate uptake of Salmonella, which secretes effectors via the SPI-1 TTSS leading to activation of caspase-1 (Fig. 10A) (Hersh et al., 1999; Mariathasan et al., 2004). Fragmentation of infected macrophage DNA results from caspase-1-dependent nuclease activity, and in contrast to apoptosis, is unlikely to involve ICAD degradation. PARP activity and DNA fragmentation are not responsible for lysis during pyroptosis. Instead, actin polymerization and caspase-1 activity lead to the formation of plasma membrane pores between 1.1 and 2.4 nm in diameter (Fig. 10B). Concurrently, inflammatory cytokines activated by caspase-1 are released in a manner that does not require cell lysis (Fig. 10C). The kinetics of cytokine release indicate this process may be facilitated by pore formation. In addition, caspase-1-dependent membrane pores dissipate ionic gradients (Fig. 10D) leading to water uptake and cell swelling, ultimately culminating in osmotic lysis with release of inflammatory intracellular contents (Fig. 10E).

The enzyme directly responsible for DNA fragmentation during pyroptosis is not known. Endonuclease G, which is normally sequestered in mitochondria, but is released during mitochondrial breakdown (Zhang and Xu, 2002), is not likely to mediate DNA fragmentation during pyroptosis, as mitochondrial integrity remains intact in S. Typhimu- rium-infected macrophages (Jesenberger et al., 2000). DNase II resides in the lysosomes of phagocytes and degrades exogenous DNA after uptake (McIlroy et al., 2000), but its role in cell-autonomous DNA fragmentation is unknown. DNase I is normally bound and inhibited by monomeric actin, which is an in vitro substrate for caspase-1 proteolysis (Kayalar et al., 1996), suggesting...
that caspase-1 cleavage of actin may activate DNase I to fragment DNA during pyroptosis.

Extensive DNA fragmentation causes the enzyme PARP to deplete its substrate NAD leading to energy depletion and oncosis (Ha and Snyder, 1999). Lysis of S. Typhimurium-infected macrophages does not require PARP activity or DNA fragmentation, distinguishing pyroptosis from oncosis. We have also observed that inhibition of lysis by either glycine or PEG 1450 does not prevent DNA fragmentation (data not shown), indicating DNA cleavage is independent of membrane breakdown. Therefore, DNA fragmentation and cell lysis are both activated by caspase-1, but proceed via independent pathways.

Our findings demonstrate that membrane defects form during pyroptosis and contribute to cell lysis. S. Typhimurium-infected macrophages become permeable to EtBr (MW 394 Da) while simultaneously excluding the slightly larger EthD2 (MW 1293 Da). These results are consistent with the pore size suggested by our osmoprotection experiments, in which PEG 1450 (MW 1450 Da), similar in size to EthD2, prevents swelling and lysis while smaller molecules do not. Together, these findings demonstrate that discretely sized membrane pores with a functional diameter of 1.1–2.4 nm form during pyroptosis. Prevention of EtBr uptake by YVAD indicates pore formation is caspase-1-dependent and, as YVAD does not affect translocation of type III effectors, these data exclude the possibility that EtBr uptake results directly from insertion of the bacterial type III translocation apparatus in the host cell membrane (Viboud and Bliska, 2002; Roy et al., 2004). In addition, CD blocks EtBr uptake, without inhibiting translocation of SPI-1 effectors (Collazo and Galan, 1997), further demonstrating that the S. Typhimurium type III translocon by itself does not directly form the EtBr permeable pore we observe in the infected macrophage plasma membrane.

Pore formation is also induced by other stimuli that activate caspase-1. Ligation of the P2X7 purinergic receptor by extracellular ATP and treatment with maitotoxin, a potent marine toxin that causes ciguatera seafood poisoning, both trigger potassium efflux leading to inflammasome-mediated caspase-1 activation and the formation of membrane pores with similar characteristics to those we have observed in S. Typhimurium-infected cells (Steinberg et al., 1987; Schilling et al., 1999; Solle et al., 2001; Estacion et al., 2003; Kahlenberg and Dubyak, 2004; Lundy et al., 2004; Verhoef et al., 2004; Mariathasan et al., 2006). The mechanism of P2X7 receptor-stimulated pore formation is controversial (Liang and Schwiebert, 2005), but recent investigations have demonstrated the involvement of caspases (Donnelly Roberts et al., 2004). This suggests the possibility that common caspase-1-dependent pores are formed in response to P2X7 receptor ligation, maitotoxin exposure and Salmonella infection. Further investigation is needed to determine if caspase-1-dependent pore formation results from insertion of pore forming molecules into the plasma membrane, opening of pre-existing membrane channels or another mechanism.

Pyroptosis is unique not only because of the distinct molecular mechanism we have outlined here, but also because the enzyme responsible for cellular suicide is primarily known for its cytokine processing function. IL-1β and IL-18 are multifunctional cytokines that play central roles in acute and chronic inflammation (Braddock et al., 2006).
and their activation during pyroptosis contributes to the inflammatory outcome predicted for this pathway. In addition, rapid lysis of cells undergoing pyroptosis releases intracellular contents, which additionally incites an inflammatory outcome (Scalfi et al., 2002; Shi et al., 2003). Therefore, pyroptosis is a program of cellular self-destruction that is intrinsically inflammatory (Fink and Cookson, 2005).

In addition to Salmonella, other infectious agents may activate caspase-1-mediated pyroptosis. Macrophage infection by Shigella flexneri stimulates caspase-1-dependent cell death and activation of cytokines, which contributes to inflammation during infection in vivo (Chen et al., 1996b; Hilbi et al., 1997; 1998; Sansonetti et al., 2000). Actinobacillus actinomycetemcomitans and Mannheimia haemolytica both produce leukotoxins that activate caspase-1-dependent cell death (Kelk et al., 2003; 2005; Thumbikat et al., 2005) and viral infections also may induce pyroptosis (Stasakova et al., 2005). Caspase-1 has additionally been implicated in the pathogenesis of non-infectious diseases that share the phenotype of inflammation accompanying cellular demise. These include septic shock, inflammatory bowel disease, myocardial infarction, cerebral ischemia, multiple sclerosis, and Huntington’s disease (Li et al., 1997; Schielke et al., 1998; Ona et al., 1999; Ming et al., 2002; Siegmund, 2002; Frantz et al., 2003). The molecular events that we have described during pyroptosis of S. Typhimurium-infected macrophages may be a common pathway of cell death occurring in these diverse disease states that couples the outcomes of cellular demise and inflammation.

Experimental procedures

Bacterial strains, macrophages and growth conditions

S. Typhimurium strain SL1344 and its prgH1::TnphoA (Behlau and Miller, 1993) and sipB::aphT (Kaniga et al., 1995) derivatives were used for all experiments. Plasmid DNA was purified from S. Typhimurium expressing GFP under the Trc promoter (Cummings et al., 2005) or an SspH1–CyaA fusion (a gift of Dr Samuel Miller) (Miao and Miller, 2000) using the QIAGEN (Valencia, CA) Miniprep system and electroporated into wild-type SL1344 and its prgH1::TnphoA and sipB::aphT derivatives. Bacteria were grown for infection experiments as described previously (Brennan and Cookson, 2000). 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 cold sterile PBS, and kept on ice before macrophage infections. The macrophage-like cell line J774A.1 was obtained from the American Type Culture Collection. BMDM were isolated from the femur exudates of C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and cultured with 30% L-cell-conditioned medium as described (Swanson and Isberg, 1995; Molofsky and Swanson, 2003). After 6–7 days of incubation, BMDM were collected by gentle washing with ice-cold PBS containing 1 mM EDTA. Macrophages were cultured at 37°C in 5% CO2 in Dulbecco’s minimal essential medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 5 mM Hepes, 0.2 mg ml⁻¹ L-glutamine, 0.05 mM β-mercaptoethanol, 50 mg ml⁻¹ gentamicin sulphate and 10 000 U ml⁻¹ penicillin and streptomycin.

Reagents

Gliotoxin (Sigma-Aldrich, St Louis, MO) was used at 5 μM to induce macrophage apoptosis (Waring et al., 1988; Monack et al., 1996; Brennan and Cookson, 2000) and 1 mM H2O2 was used to induce oncosis (Schaufflatter et al., 1986; Filipovic et al., 1999). Macrophages were treated with 0.5 mM ATA (Sigma-Aldrich, St Louis, MO), 3AB (Sigma-Aldrich, St Louis, MO), 5 μM CD (Sigma-Aldrich, St Louis, MO), or 5 mM glycine (Sigma-Aldrich, St Louis, MO), for 1 h before infection or induction of cell death. Caspase-1 was inhibited with 250 μM acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD; Calbiochem, San Diego, CA) for 1 h before infection, or cells were treated with 250 μM H-Ala-Ala-Phe-chloromethylketone (AAF; Calbiochem, San Diego, CA) for 1 h before infection. In osmoprotection experiments, infections were preformed in 30 mM solutions of sucrose, raffinose, PEG 1450, or PEG 3350 (Sigma-Aldrich, St Louis, MO) made up in supplemented antibiotic free DMEM containing 5% FCS and filter sterilized prior to use.

Invasion protocol

Macrophages in supplemented antibiotic free DMEM containing 5% FCS were allowed to adhere for 18–24 h before infection, and fresh media were replaced 1 h before infection. Using a previously described invasion protocol (Chen et al., 1996a; Brennan and Cookson, 2000), bacteria were added to macrophages at a moi of 10:1 and allowed to invade for 2 h. Media containing 15 μg ml⁻¹ gentamicin were then added to kill extracellular bacteria and the invasion was continued for specified lengths of time. Macrophage infection for each experiment was confirmed by microscopy or determining the number of gentamicin-protected intracellular bacteria. Gentamicin protection assays demonstrated that ATA, 3AB, YVAD, AAF, glycine and osmoprotectants do not inhibit bacterial uptake or intracellular survival. CD reduced the number of intracellular bacterial by over 90% as measured by recovery of gentamicin-protected bacteria, consistent with previous observations (Collazo and Galan, 1997).

TUNEL staining

DNA fragmentation was detected using TUNEL as described previously (Brennan and Cookson, 2000). Macrophages grown on glass coverslips were uniformly infected with S. Typhimurium at a moi of 10:1 for 4 h, treated with gliotoxin for 6 h or treated with a combination of 5 mM H2O2 and 1 mM SNAP (Calbiochem, San Diego, CA) for 30 min. Adherent cells were washed with PBS, fixed, and permeabilized. Cells were treated with 1000 U ml⁻¹ DNase I (Invitrogen, Carlsbad, CA) for 10 min as a positive control for DNA fragmentation, and washed three times with PBS. DNA strand breaks were labelled using the In Situ Cell Death Detection Kit as directed by the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN). Coverslips were mounted using ProLong antifade (Molecular Probes, Eugene, OR) and analysed using a Bio-Rad MRC-600 or Leica SL con-
focal microscope in the W. M. Keck Center for Advanced Studies in Neural Signaling. Images were reduced in size for publication using Adobe Photoshop. The means and standard deviations were derived from counting a minimum of four fields from two different coverslips per sample.

**Immunoblotting**

Macrophages grown in 75 cm² flasks were uniformly infected with *S. Typhimurium* at a moi of 10:1: For ICAD immunoblotting, cells were removed after 4 h of infection or 6 h of glitoxin treatment using ice-cold PBS containing 1 mM EDTA and pooled with non-adherent cells recovered by centrifugation. Total protein from 4 × 10⁶ cells was separated by 16.5% SDS-PAGE and transferred to nitrocellulose membranes. ICAD cleavage was assessed by Western blotting using an anti-ICAD antibody (BD Pharmingen, San Diego, CA) and an anti-p44/p42 MAP kinase antibody (Cell Signaling Technology, Beverly, MA) to confirm equal loading. For IL-1β and IL-18 immunoblotting, supernatant from macrophages infected in serum free media was filtered using 0.2 µm syringe filters (Pall Corporation, Ann Arbor, MI), and concentrated using Centricron Plus-20 centrifugal filter devices (Millipore, Billerica, MA). Concentrated supernatant from 2 × 10⁶ cells was separated by 15% SDS-PAGE, transferred to nitrocellulose membranes, and cytokine processing was assessed by Western blotting using anti-IL-18 antibody M-19 (Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-IL-1β antibody (R and D Systems, Minneapolis, MN). Membranes were incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and detected with an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, England, UK). Detection of processed cytokines in supernatant samples was not saturated under the immunoblot conditions used as determined by loading of recombinant protein. Quantification of immunoblots was performed by densitometry using NIH Image, version 1.63.

**Lactate dehydrogenase release**

Macrophages grown in 96 well plates were uniformly infected with *S. Typhimurium* at a moi of 10:1. Supernatants were evaluated for the presence of the cytoplasmic enzyme LDH using the Cytotox 96 kit as directed by the manufacturer’s instructions (Promega, Madison, WI) after 4 h of infection or at the indicated time point. Percentage cytotoxicity was calculated as 100 × (experimental LDH – spontaneous LDH)/(maximum LDH release – spontaneous LDH).

**Ethidium bromide and EthD2 staining**

Macrophages grown on glass coverslips were uniformly infected with *S. Typhimurium* at a moi of 10:1 or treated with 1% triton X-100 or 5 µM glitoxin. After 2.5 h of infection or at the indicated time point, adherent cells were washed once with HBSS, and stained with SYTO 62 (Molecular Probes, Eugene, OR) and either EtBr at 25 µg ml⁻¹ (Sigma-Aldrich, St Louis, MO) or EthD2 as directed by the manufacturer’s instructions (Molecular Probes, Eugene, OR). Coverslips were analysed using a Bio-Rad MRC-600 or Leica SL confocal microscope in the W. M. Keck Center for Advanced Studies in Neural Signaling. Images were reduced in size for publication using Adobe Photoshop. The means and standard deviations were derived from counting a minimum of four fields from two different coverslips per sample.

**CyaA-based translocation assay**

Secretion of the SspH1–CyaA fusion was assessed as previously described (Miao and Miller, 2000). Macrophages grown in 24 well plates were uniformly infected with *S. Typhimurium* at a moi of 10:1 for 4 h. Cells were removed using ice-cold PBS containing 1 mM EDTA and inhibitors as indicated and pooled with non-adherent cells recovered by centrifugation. Cells were stained with 7-AAD (Molecular Probes, Eugene, OR) as described previously (Brennan and Cookson, 2000) and immediately analysed on a BD FACSscan flow cytometer. FlowJo software (Tree Star, Ashland, OR) was used to analyse the data and plot forward scatter for intact, 7-AAD negative cells. Microsphere size standards in the flow cytometry size calibration kit (Molecular Probes, Eugene, OR) were analysed in parallel with cell samples and used to confirm the linear relationship of forward scatter values with particle size.

**Acknowledgements**

We thank Dr Samuel Miller for providing the SspH1–CyaA fusion and sipB mutant bacteria; Matthew Johnson and Laura Mayeda for assistance with tissue culture; Sara Barrett for help with flow cytometry; Molly Bergman and Tessa Bergsbaken for experimental assistance; members of the Cookson laboratory for critical review of the manuscript; and the W. M. Keck Center for Advanced Studies in Neural Signaling for confocal microscopy support. This work was supported by AI47242 and P50 HG02360. S.L.F. was supported by a Poncin Fellowship and ARCS Fellowship.

**References**

Andrei, C., Dazzi, C., Lotti, L., Torrisi, M.R., Chimini, G., and Rubartelli, A. (1999) The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles. *Mol Biol Cell* 10: 1463–1475.

Andrei, C., Margiocco, P., Poggi, A., Lotti, L.V., Torrisi, M.R., and Rubartelli, A. (2004) Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: implications for inflammatory processes. *Proc Natl Acad Sci USA* 101: 9745–9750.

Behlau, I., and Miller, S.I. (1993) A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. *J Bacteriol* 175: 4475–4484.

Bortner, C.D., and Cidlowski, J.A. (2003) Uncoupling cell shrinkage from apoptosis reveals that Na⁺ influx is required for advanced studies in neural signaling. *Cellular Microbiology*, 8, 1812–1825
for volume loss during programmed cell death. J Biol Chem 278: 39176–39184.

Braddock, M., Quinn, A., and Canvin, J. (2004) Therapeutic potential of targeting IL-1 and IL-18 in inflammation. Expert Opin Biol Ther 4: 847–860.

Brennan, M.A., and Cookson, B.T. (2000) Salmonella induces macrophage death by caspase-1-dependent necrosis. Mol Microbiol 38: 31–40.

Chen, Y., Smith, M.R., Thirumalai, K., and Zychlinsky, A. (1997) The interleukin-1beta-converting enzyme, caspase-1, is required for cell shrinkage, phosphatidylserine translocation and death in T lymphocyte apoptosis. EMBO Rep 4: 189–194.

Everest, P., Wain, J., Roberts, M., Rook, G., and Dougan, G. (2001) The molecular mechanisms of severe typhoid fever. Trends Microbiol 9: 316–320.

Fautuzzi, G., and Dinarello, C.A. (1999) Interleukin-18 and interleukin-1 beta: two cytokine substrates for ICE (caspase-1). J Clin Immunol 19: 1–11.

Filipovic, D.M., Meng, X., and Reeves, W.B. (1999) Inhibition of PARP prevents oxidant-induced necrosis but not apoptosis in LLC-PK1 cells. Am J Physiol 277: F428–F436.

Fink, S.L., and Cookson, B.T. (2005) Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect Immun 73: 1907–1916.

Frank, A., Rauen, U., and de Groot, H. (2000) Protection by glycine against hypoxic injury of rat hepatocytes: inhibition of ion fluxes through nonspecific leaks. J Hepatol 32: 58–66.

Frantz, S., Ducharme, A., Sawyer, D., Rohde, L.E., Kobzik, L., Fukazawa, R., et al. (2003) Targeted deletion of caspase-1 reduces early mortality and left ventricular dilatation following myocardial infarction. J Mol Cell Cardiol 35: 685–694.

Garcia-Calvo, M., Peterson, E.P., Leiting, B., Ruel, R., Nicholson, D.W., and Thornberry, N.A. (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem 273: 32608–32613.

Gauthier, A., and Finlay, B.B. (2001) Bacterial pathogenesis: the answer to virulence is in the pore. Curr Biol 11: R264–R267.

Gilloteau, L.A., Wallis, T.S., Gautier, A.V., MacIntyre, S., Platt, D.J., and Lax, A.J. (1996) The Salmonella virulence plasmid enhances Salmonella-induced lysis of macrophages and influences inflammatory responses. Infect Immun 64: 3385–3393.

Halliwell, B., and Aruoma, O.I. (1991) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci USA 96: 13978–13982.

Hamon, Y., Luciani, M.F., Becc, F., Verrier, B., Rubartelli, A., and Chimini, G. (1997) Interleukin-1beta secretion is impaired by inhibitors of the Atp binding cassette transporter, ABC1. Blood 90: 2911–2915.

Haynes, J.L. (1988) Principles of flow cytometry. Cytometry Suppl 3: 7–17.

Herceg, Z., and Wang, Z.Q. (2001) Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. Mutat Res 477: 97–110.

Hersh, D., Monack, D.M., Smith, M.R., Ghori, N., Falkow, S., and Zychlinsky, A. (1999) The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc Natl Acad Sci USA 96: 2396–2401.

Hibili, H., Chen, Y., Thirumalai, K., and Zychlinsky, A. (1997) The interleukin 1beta-converting enzyme, caspase 1, is activated during Shigella flexneri-induced apoptosis in human monocyte-derived macrophages. Infect Immun 65: 5165–5170.

Hibili, H., Moss, J.E., Hersh, D., Chen, Y., Arondel, J., Banerjee, S., et al. (1998) Shigella-induced apoptosis is dependent on caspase-1 which binds to IpaB. J Biol Chem 273: 32895–32900.

Hogquist, K.A., Nett, M.A., Unanue, E.R., and Chaplin, D.D. (1991a) Interleukin 1 is processed and released during apoptosis. Proc Natl Acad Sci USA 88: 8485–8489.
infected by virulent bacteria expressing SipB are a major source of active interleukin-18. "Infect Immun" 71: 4382–4388.

Ona, V.O., Li, M., Vonsattel, J.P., Andrews, L.J., Khan, S.Q., Chung, W.M., et al. (1999) Inhibition of caspase-1 slows disease progression in a mouse model of Huntington’s disease. "Nature" 399: 263–267.

Patei, K., Harding, P., Haney, L.B., and Glass, W.F., 2nd (2003) Regulation of the mesangial cell myofibroblast phenotype by actin polymerization. "J Cell Physiol" 195: 435–445.

Roy, D., Liston, D.R., Di Ione, V.J.A., Nelson, D.J., Pujol, C., et al. (2004) A process for controlling intracellular bacterial infections induced by membrane injury. "Science" 304: 1515–1518.

Rubartelli, A., Cozzolino, F., Talio, M., and Sita, R. (1990) A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. "EMBO J" 9: 1503–1510.

Sakahira, H., Enari, M., and Nagata, S. (1998) Cleavage of IL-1beta and IL-18 are essential for bacterial lipopolysaccharide-induced inflammation. "Nature" 391: 96–99.

Salgo, M.G., Bermudez, E., Squadrito, G.L., and Pryor, W.A. (1995) Peroxynitrite causes DNA damage and oxidation of thiols in rat thymocytes [corrected]. "Arch Biochem Biophys" 322: 500–505.

Sansonetti, P.J., Phalipon, A., Arondel, J., Thirumalai, K., Sansen, O., and Prat, F. (1998) Reduced ischemic brain injury in interleukin-1 beta null mice lacking P2X(7) receptors. "Science" 282: 1447–1459.

van der Velden, A.W., Velasquez, M., and Starnbach, M.N. (2003) Salmonella rapidly kill dendritic cells via a caspase-1-dependent mechanism. "J Immunol" 171: 6742–6749.

Veres, B., Radnai, B., Gallyas, F., Jr, Varbiro, G., Berente, Z., Osz, E., and Sumegi, B. (2004) Regulation of kinase cascades and transcription factors by a poly(ADP-ribose) polymerase-1 inhibitor, 4-hydroxyquinazoline, in lipopolysaccharide-induced inflammation in mice. "J Pharmacol Exp Ther" 310: 247–255.

Wang, J.Y., Shum, A.Y., and Ho, Y.J. (2003) Oxidative neurotoxicity in rat cerebral cortex neurons: synergistic effects of H2O2 and NO on apoptosis involving activation of p38 mitogen-activated protein kinase and caspase-3. "J Neurosci Res" 72: 506–519.

Waring, P., Eichner, R.D., Mullbacher, A., and Sjaarda, A. (1988) Gliotoxin induces apoptosis in macrophages unrelated to its antiphagocytic properties. "J Biol Chem" 263: 18493–18499.

Watson, P.R., Gautier, A.V., Paulin, S.M., Bland, A.P., Jones, P.W., and Wallis, T.S. (2000) Salmonella enterica serovars Typhimurium and Dublin can lyse macrophages by a mechanism distinct from apoptosis. "Infect Immun" 68: 3744–3747.

Wexers, M.D. (2004) IL-1beta: an endosomal exit. "Proc Natl Acad Sci USA" 101: 10241–10242.

Zhang, J., and Xu, M. (2002) Apoptotic DNA fragmentation and tissue homeostasis. "Trends Cell Biol" 12: 84–89.

Zhou, X., Gordon, S.A., Kim, Y.M., Hoffman, R.A., Chen, Y., Zhang, X.R., et al. (2000) Nitric oxide induces thymocyte apoptosis via a caspase-1-dependent mechanism. "J Immunol" 165: 1252–1258.