

Microreview

***Salmonella*-induced macrophage death: multiple mechanisms, different outcomes**

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Summary

The facultative intracellular pathogen *Salmonella enterica* triggers programmed cell death in macrophages. The close examination of this phenomenon has revealed an unusually complex picture involving diverse mechanisms that lead to different types of programmed cell death. It appears that the outcome of the interaction of salmonella with macrophages depends on the relative contribution of two type III protein secretion systems, in conjunction with the stimulation of innate immunity outputs through conserved determinants collectively known as ‘pathogen-associated molecular patterns’ (PAMPs). These interactions result in a breakdown of the balance between survival and pro-apoptotic cellular pathways, which eventually leads to macrophage cell death. The relative significance for the infection process of the different types of macrophage cell death triggered by salmonella remains to be established

Introduction

Many microbial pathogens that have coevolved with their hosts often display a remarkable degree of adaptation, which allows them to modulate or even exploit a variety of host cellular processes (Galán, 2002). These pathogen adaptations often lead to the manipulation of the very systems that the host utilizes to detect and eliminate them. For example, many microbial pathogens have evolved mechanisms to modulate macrophage cell functions such as vesicular trafficking, antigen presentation, or antimicrobial responses (Rosenberger and Finlay, 2003). Some pathogens have even evolved the ability to trigger programmed cell death in these special-

ized cells (Navarre and Zychlinsky, 2000). Examples of the latter are all the serovars of *Salmonella enterica*, which have evolved multiple mechanisms to induce macrophage cell death (Chen *et al.*, 1996a; Guilloteau *et al.*, 1996; Monack *et al.*, 1996). Since first reported in 1996 (Chen *et al.*, 1996a; Guilloteau *et al.*, 1996; Lindgren *et al.*, 1996; Monack *et al.*, 1996), the mechanisms of salmonella-induced macrophage cell death have been the subject of some confusion and controversy (Boise and Collins, 2001; Cookson and Brennan, 2001; Knodler and Finlay, 2001). While some groups reported rapid macrophage death upon infection with salmonella (Chen *et al.*, 1996a; Monack *et al.*, 1996; Brennan and Cookson, 2000), others reported the need for prolonged infection (24 h) for cell death to occur (Guilloteau *et al.*, 1996; Lindgren *et al.*, 1996). Furthermore, some groups observed that macrophages killed by salmonella exhibited the typical signs of apoptosis such a chromatin fragmentation (Chen *et al.*, 1996a; Monack *et al.*, 1996), caspase-3 activation (Jesenberger *et al.*, 2000), and the presence of nucleosomes in the cytoplasm (Chen *et al.*, 1996a), while others reported features more consistent with necrosis, such as lack of caspase-3 activation and loss of membrane integrity (Chen *et al.*, 1996a; Brennan and Cookson, 2000; Watson *et al.*, 2000). These seemingly contradictory observations, which as it will be discussed later are most likely the consequence of different experimental conditions, underscore the fact that salmonella-induced macrophage cell death is much more complex than initially thought. More recently, the use of different bacterial mutants as well as mouse strains deficient in relevant pathways, has brought some clarification to a death process that does not seem to easily fit any conventional categorization. In this article, without attempting to be comprehensive, we will discuss what is known about the different mechanisms of salmonella-induced cell death and will attempt to put in context seemingly contradictory observations. For more discussion of this topic readers are referred to other excellent articles (Navarre and Zychlinsky, 2000; Boise and Collins, 2001; Knodler and Finlay, 2001; Monack *et al.*, 2001b; Jarvelainen *et al.*, 2003).

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Salmonella pathogenicity island 1 (SPI-1)-dependent macrophage cell death

S. enterica encodes two type III protein secretion systems (TTSS), which through the delivery of specific effector proteins into host cells, operate at different steps of the infection cycle (Galán, 2001). One of the systems, encoded within the SPI-1, is involved in the intestinal phase of infection (Galán, 1999). The other, encoded within the pathogenicity island 2 (SPI-2), is essential for systemic infection (Shea *et al.*, 1999; Hensel, 2000; Waterman and Holden, 2003). Consequently, the expression of these systems is differentially regulated: the expression of SPI-1 TTSS is stimulated by environmental cues (e.g. high osmolarity, low oxygen tension) present in the intestinal tract, while the expression of the SPI-2 TTSS is stimulated by conditions present within host cells (e.g. low Mg⁺⁺ concentrations, acidic pH) (Galán, 2001). Remarkably, both TTSSs have the capacity to induce macrophage cell death, although by apparently different mechanisms and at different times after infection.

A caspase-1 dependent pathway leads to rapid macrophage death

In vitro, salmonella-mediated macrophage cell death occurs rapidly (i.e. within ~45 min of infection) if bacteria have been grown under conditions that allow optimal expression of the SPI-1 TTSS (Chen *et al.*, 1996a; Monack *et al.*, 1996). The fact that some growth conditions do not allow optimal expression of the SPI-1 TTSS has led to some confusion not only in terms of the requirement of this system to induce cell death but also in terms of the kinetics of salmonella-induced macrophage cell death, or even the predominant features that underlie the death process. As discussed above, macrophages undergoing cell death induced by salmonella exhibits features that are not easily categorized within any of the well-characterized forms of programmed cell death (Boise and Collins, 2001). Furthermore, as it was specifically noted in one of the original reports (Chen *et al.*, 1996a), it is clear that macrophages undergoing salmonella-induced death are not homogeneous and exhibit sometimes significantly different features (Fig. 1). Some macrophages undergoing death can be seen exhibiting features that would be consistent with apoptosis, such as chromatin condensation and the appearance of nucleosomes in the cytoplasm (Chen *et al.*, 1996a; Monack *et al.*, 1996). Others, however, exhibit features that are more consistent with a necrotic-type of cell death, such as the disruption of the plasma membrane and internal organelles (Chen *et al.*, 1996a; Watson *et al.*, 2000), and the absence of chromatin condensation or poly-ADP-ribose polymerase (PARP) cleavage (Brennan and Cookson, 2000), an indi-

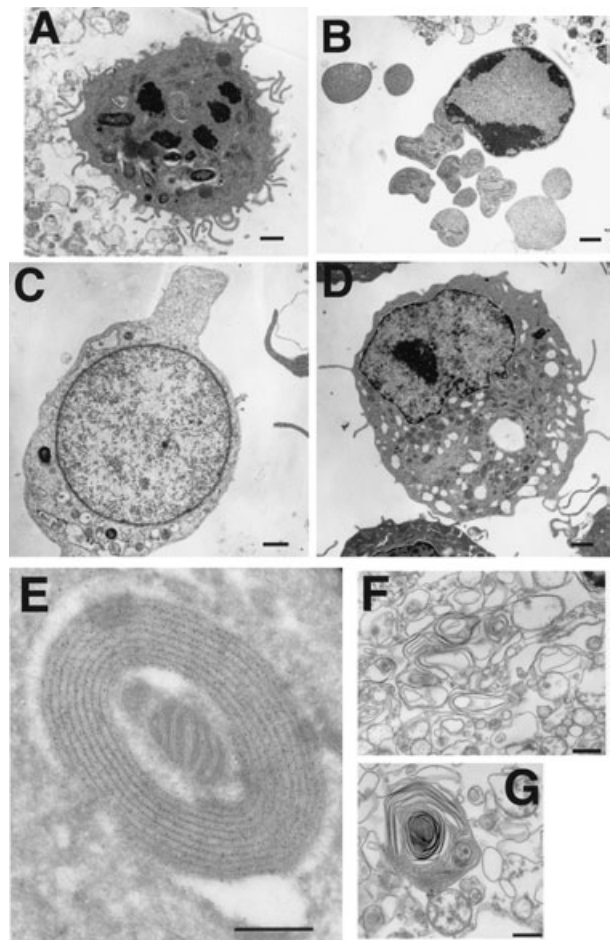


Fig. 1. Salmonella infection results in different types of macrophage cell death. A–D show electron micrographs of J774A.1 macrophages infected with *Salmonella typhimurium* (taken from Chen *et al.*, 1996a; scale bar = 1 μ m).

A and B. Typical features of 'apoptosis' such as membrane blebbing, fragmentation and condensation of chromatin, and the presence of apoptotic bodies.

C. Macrophages exhibiting features of necrosis.

D. Uninfected macrophages.

E. An immuno-electron micrograph of SipB-transfected COS-2 cells showing the formation of multilamellar structures (labelled by an antibody directed to SipB), which contains an apparently damaged mitochondrion (scale bar = 0.250 μ m).

F and G. Macrophages undergoing autophagy as a consequence of *Salmonella typhimurium* infection (scale bars = 0.5 and 0.250 μ m for F and G, respectively). E–G are taken from Hernandez *et al.* (2003).

cation of caspase-3 activation and a hallmark of apoptosis. Indeed, the proportion of one or the other morphological type is highly dependent not only on the levels of expression of the SPI-1 TTSS but also on the multiplicity of infection (Chen *et al.*, 1996a). At low multiplicity of infection or suboptimal expression of the SPI-1 TTSS, the proportion of 'apoptotic-looking' macrophages increases (L. Hernandez and J. E. Galán, unpubl. obs.). In contrast, maximum expression of the SPI-1 TTSS in conjunction with medium or high multiplicity of infection favours the

appearance of 'necrotic-looking' macrophages (L. Hernandez and J. E. Galán, unpubl. obs.). It is clear, however, that regardless of the growth conditions and/or multiplicity of infection, this rapid form of cell death does not occur when macrophages are infected with an SPI-TTSS-deficient salmonella strain (Chen *et al.*, 1996a; Monack *et al.*, 1996; Lundberg *et al.*, 1999; Brennan and Cookson, 2000; Jesenberger *et al.*, 2000; van der Velden *et al.*, 2000; Forsberg *et al.*, 2003). Because the SPI-1 TTSS can deliver effector proteins from the outside of the cell [in fact, this system is responsible for mediating bacterial uptake into non-phagocytic cells (Galán and Zhou, 2000)], addition of agents that block bacterial uptake by disrupting the actin cytoskeleton cannot prevent salmonella-induced macrophage cell death (Chen *et al.*, 1996a).

The different morphological features observed in macrophages undergoing salmonella SPI-1-dependent cell death not only underscore the difficulty of classifying this type of cell death following conventional paradigms (i.e. 'apoptosis' or 'necrosis'), but also indicate that more than one mechanism is involved in this process. In fact, 'necrosis' or 'apoptosis' are increasingly seen as extreme manifestations of related processes that encompass a variety of mechanisms that can all be placed under the umbrella of 'programmed cell death' (Lockshin and Zakeri, 2002; Yuan *et al.*, 2003; Gozuacik and Kimchi, 2004). It is now established that the very rapid (~45 min after infection) form of macrophage cell death induced by salmonella requires caspase-1 (Hersh *et al.*, 1999; Brennan and Cookson, 2000; Jarvelainen *et al.*, 2003). Thus, caspase-1-defective macrophages are clearly more resistant to salmonella-induced programmed cell death *in vitro*, although these macrophages eventually (~3–4 h after infection) succumb to bacterial infection (Hernandez *et al.*, 2003). The involvement of caspase-1 in programmed cell death is unusual in that this caspase has not been implicated in the 'classical' apoptotic mechanisms involved in either the so-called 'extrinsic' (caspase-9 dependent) or 'intrinsic' (caspase-8 dependent) apoptotic pathways that result in the activation of the caspase-3. Consistent with the lack of involvement of these caspases in this form of programmed cell death, caspase-3 is not activated in salmonella-infected macrophages infected by SPI-1-TTSS-competent salmonella that are undergoing rapid cell death (Brennan and Cookson, 2000). In addition, caspase-1 deficiency in mice does not lead to overt defects in physiological cell death pathways (Kuida *et al.*, 1995). The lack of involvement of caspase-1 in 'classical' apoptosis is consistent with its involvement in the less conventional form of rapid programmed cell death observed in macrophages after salmonella infection, whose features (lack of chromatin fragmentation, loss of membrane integrity, etc.) do not resemble 'classical' apoptosis and has therefore been

described as 'programmed necrosis' (Brennan and Cookson, 2000).

Studies conducted by Zychlinsky and colleagues, first in *Shigella* spp. (Chen *et al.*, 1996b) and subsequently in *S. enterica* (Hersh *et al.*, 1999), determined that the related proteins IpaB and SipB, from *Shigella* and salmonella, respectively, are involved in triggering programmed cell death in a caspase-1 dependent manner. Supporting a direct role for these proteins in the induction of this process, mutant strains of *Shigella* spp. or *S. enterica* lacking IpaB or SipB, respectively, were shown to be defective in the induction of rapid macrophage cell death (Chen *et al.*, 1996a,b; Hersh *et al.*, 1999; Santos *et al.*, 2001). However, although translocated into host cells by type III secretion systems, both proteins are essential for the function of these systems because they mediate the transfer of effector proteins through the host cell membrane (Collazo and Galán, 1997). Because the absence of these proteins results in the inability of the bacteria to translocate all type III secretion effector proteins (Collazo and Galán, 1997), the interpretation of these results is inconclusive. In support of the direct involvement of IpaB/SipB in the induction of caspase-1 dependent macrophage cell death, Zychlinsky and collaborators showed that microinjection of either SipB or IpaB results in macrophage cell death (Chen *et al.*, 1996b; Hersh *et al.*, 1999). Furthermore, immunoprecipitation experiments suggested that IpaB/SipB may form a complex with caspase-1 (Chen *et al.*, 1996b; Hersh *et al.*, 1999), although a direct interaction between these proteins was not demonstrated. In addition to caspase-1, several other cellular proteins have also been reported to bind IpaB or SipB (Chen *et al.*, 1996b; Watarai *et al.*, 1996; De Geyter *et al.*, 2000; Skoudy *et al.*, 2000). Although this may suggest multiple functions for this protein family, it is also possible that the detection of some of these interactions may be the consequence of the intrinsic 'sticky' nature of IpaB and SipB, which display exposed hydrophobic domains (Hayward *et al.* 2000; McGhie *et al.*, 2002; Hume *et al.*, 2003). Therefore, more studies will be necessary to unambiguously establish whether IpaB/SipB directly interact with and activate caspase-1 to trigger programmed cell death. As it will be discussed below, recent studies have uncovered innate immune pathways, undoubtedly activated by *Shigella* spp. and *S. enterica* through their conserved 'pathogen-associated molecular patterns' (PAMPs), which by themselves are able to stimulate caspase-1-dependent programmed cell death (Yoo *et al.*, 2002b; Stehlik *et al.*, 2003; Zhang *et al.*, 2003; Hsu *et al.*, 2004). Whether SipB and IpaB contribute to the stimulation of this pathway in a manner different from that involving their TTSS translocase activity remains to be established.

Autophagy at the centre of a SipB-dependent, caspase-1-independent pathway of macrophage cell death. Bone-

marrow-derived mouse macrophages from caspase-1-deficient mice remain susceptible to salmonella-induced cytotoxicity although cell death occurs with delayed kinetics (Hernandez *et al.*, 2003). Although wild-type macrophages succumb to salmonella infection within 45 min of infection, it takes up to 3 h for caspase-1-deficient macrophages to show signs of cytotoxicity. This caspase-1 independent death pathway is also dependent on the SPI-1 TTSS and more specifically, on the SipB protein (Hernandez *et al.*, 2003). A mutant salmonella lacking all known effectors of the SPI-1 type III secretion system but having a functional type III secretion and translocation system retained the same ability as wild type to kill caspase-1 deficient macrophages. Furthermore, transient expression of SipB (but not the other translocases SipC or SipD) in caspase-1 deficient macrophages led to cell death, further supporting the role of this protein in this caspase-1-independent death pathway (Hernandez *et al.*, 2003).

How does SipB mediate caspase-1 independent macrophage cell death? Transient expression of SipB in cells that are relatively resistant to salmonella-induced death led to the formation of multilamellar structures resembling autophagosomes (Hernandez *et al.*, 2003) (Fig. 1). Under the electron microscope, these structures were frequently observed in close association to mitochondria or even containing what appear to be degrading mitochondria. Intriguingly, on occasions mitochondria appeared to be fusing to the SipB-containing multilamellar structures. Although it has not been formally demonstrated that these structures are the product of mitochondria fusion, some observations suggest this possibility: (i) purified SipB has the ability to induce the fusion of liposomes and this activity was enhanced if cardiolipin, an abundant lipid in mitochondrial membranes, was included in the liposomes (Hayward *et al.*, 2000); (ii) a SipB domain shown to be essential for its membrane fusion activity was shown to be also essential for the induction of the multilamellar structures when expressed in mammalian cells (Hernandez *et al.*, 2003); (iii) the SipB-induced multilamellar structure closely resemble other structures such as drosophila *nebenkern*, which are formed as a consequence of mitochondrial fusion (Hales and Fuller, 1997; Hernandez *et al.*, 2003); (iv) the modular organization of SipB is similar to that of the product of the drosophila *fuzzy onion* gene, which is responsible for the formation of *nebenkern* (McGhie *et al.*, 2002; Rojo *et al.*, 2002); (v) mitochondrial markers were present in the SipB-induced multilamellar structures (Hernandez *et al.*, 2003); and (vi) SipB localized to mitochondria during salmonella infection (Hernandez *et al.*, 2003). Similar multilamellar structures to those observed after transient SipB expression were observed in salmonella-infected caspase-1-defective macrophages (Her-

nandez *et al.*, 2003) (Fig. 1). These structures were readily stained by the autophagosomal marker monodansylcadaverine (MDC), further supporting their autophagic origin (Hernandez *et al.*, 2003). Taken together, these results indicate that the SipB-mediated macrophage cell death may be the consequence of its ability to induce autophagy by either damaging mitochondria or by altering the balance between mitochondria fusion and fission. Autophagy has been linked to the induction of a less-well understood form of programmed cell death known as 'type II programmed cell death' (Schwartz *et al.*, 1993; Lemasters, 1999; Xue *et al.*, 1999; Bursch *et al.*, 2000; Cohen *et al.*, 2002). In this type of death, caspases are often not required for the execution of the death pathway. Addition of pancaspase inhibitors did not prevent the salmonella-induced cytotoxicity of caspase-1-deficient macrophages (Hernandez *et al.*, 2003), which is consistent with the hypothesis that this is a type II programmed cell death.

Salmonella SPI-2-dependent macrophage cell death

Salmonella enterica mutant strains that are deficient in the SPI-1 TTSS or that have been grown under conditions that are not optimal for the expression of this system, can still kill macrophages (Guilloteau *et al.*, 1996; Libby *et al.*, 1997; Lindgren and Heffron, 1997; van der Velden *et al.*, 2000; Santos *et al.*, 2001; Browne *et al.*, 2002). However, killing requires prolonged incubation (up to 24 h). Although the mechanisms of this delayed killing are not well understood, it appears that it is dependent on the activity of the SPI-2 TTSS. *S. enterica* carrying mutations in essential components of this system or in genes that regulate their expression, were shown to be defective in their ability to induce this delayed programmed cell death (van der Velden *et al.*, 2000; Monack *et al.*, 2001a). Although the SPI-2 TTSS has been shown to be required for intracellular growth and survival (Waterman and Holden, 2003), it appears that this activity may not be the main contribution of this system to the induction of macrophage cell death because some *Salmonella typhimurium* mutants, which are deficient in intracellular growth and survival, are still able to kill macrophages (van der Velden *et al.*, 2000). It is therefore possible that some effector protein(s) delivered into cells by the SPI-2 TTSS may specifically trigger this programmed cell death. Although the identity of such a factor is unknown, a candidate protein may be the virulence plasmid encoded protein SpvB. A deletion mutation in *spvB* abolished the ability of *S. typhimurium* to trigger this delayed form of programmed cell death (Browne *et al.*, 2002) and expression of SpvB in mammalian cells induced apoptosis (Kurita *et al.*, 2003). It is possible that SpvB may be delivered into cells by the SPI-2 TTSS, although evidence to

the contrary has been recently reported (Gotoh *et al.*, 2003). SpvB is an ADP ribosylating toxin and actin has been proposed as the main target for this activity (Lesnick *et al.*, 2001). However, it is unknown whether disruption of the actin cytoskeleton by SpvB is responsible for the induction of macrophage programmed cell death.

A recent report suggests that the delayed form of salmonella-induced macrophage cell death may require the stimulation of TLR4 and the dsRNA responsive kinase PKR (Hsu *et al.*, 2004). It is becoming increasingly clear that stimulation of the innate immune system by PAMPs such as LPS lead to both anti-apoptotic as well as pro-apoptotic pathways. In many instances, pathogen specific mechanisms appear to modulate these responses so that the pro-apoptotic pathway prevails resulting in macrophage cell death. It is therefore possible that salmonella, through the activity of some SPI-2 TTSS effector protein in conjunction with SpvB may favour the pro-apoptotic responses that follow TLR activation, thereby triggering programmed cell death. Intriguingly, this delayed form of apoptosis also appears to require caspase-1. Caspase-1 defective macrophages were shown to be resistant to the SPI-2-dependent programmed cell death (Monack *et al.*, 2001a). Furthermore, the delayed macrophage cell death was accompanied by a delayed release of IL1 β , a process dependent on the activity of caspase-1 (Monack *et al.*, 2001a). The mechanisms of caspase-1 activation in this pathway are not clear. However, increasing evidence indicates that activation of the innate immune system leads to the activation of caspase-1. For example, Nod1 stimulation leads to the activation of caspase-1 through its direct association with caspase-1 or RIP2 (Yoo *et al.*, 2002a; Zhang *et al.*, 2003). Nod1 is a member of a growing family of proteins that contain nucleotide-binding and a leucine-rich repeat domains and that are thought to work as intracellular receptors of microbial products to stimulate innate immunity outputs (Inohara and Nunez, 2003). RIP2 is a serine/threonine kinase that is downstream from signalling pathways emanating from both TLR and Nod-family receptor members (Kobayashi *et al.*, 2002). It is therefore possible that a common thread to many of the pathways leading to both, the rapid as well as the delayed form of programmed cell death may be salmonella's ability to potently stimulate innate immunity outputs leading to caspase-1 activation as well as interfering with survival pathways so as to tilt the balance towards cell death (Fig. 2). Consistent with this hypothesis, infection of macrophages with salmonella has been reported to lead to the degradation of Raf-1, a kinase that is central to many survival pathways (Jesenberger *et al.*, 2001). Furthermore, inhibition of Cdc42 and Rac1, two Rho-family GTPases that are activated by salmonella SPI-1 TTSS effector proteins, reduced the cytotoxic effects of salmonella on macrophages (Forsberg *et al.*, 2003), suggesting

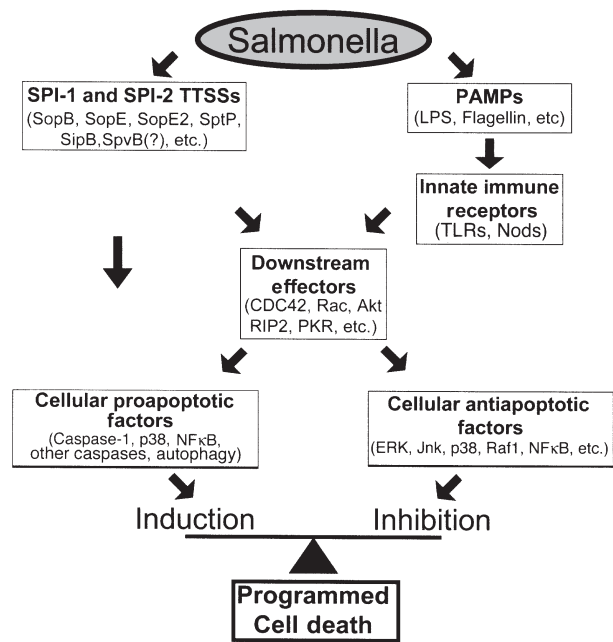


Fig. 2. Model for salmonella-induced macrophage cell death (see text for details).

that the specific activation of these GTPases by salmonella effector may provide additional signals favouring cell death.

Biological significance of salmonella-mediated macrophage cell death

Very little is known about the significance of the different types of macrophage cell death triggered by salmonella as well as their relative contribution to pathogenesis or host defence. It is not at all clear whether macrophage cell death is triggered by salmonella to counteract a host defence mechanisms or whether it constitutes a host response to halt bacterial replication. It is even less clear whether the different types of macrophage cell death represent evolutionary adaptations of the pathogen or the host. Are all types of macrophage cell death equally 'pro-inflammatory' or is the caspase-1-independent cell death less pro-inflammatory because it does not lead to the processing of inactive pro-IL1 β into its bioactive form? More importantly, when, where and in what type of cells do the different types of salmonella-induced cell death become predominant? It has been reported that caspase-1-deficient mice are more resistant to salmonella infection (Monack *et al.*, 2000) and it has been proposed that this resistance is the consequence of the inability of salmonella to cross the intestinal epithelial barrier in the absence of profuse inflammation. However, it is not clear whether the reduced inflammatory response in the gut of salmonella-infected caspase-1-deficient mice is the con-

sequence of reduced apoptosis or reduced levels of IL-1 β because of the lack of the converting enzyme. More studies undoubtedly to come will help to understand the biological and immunological significance of these different types of cell death and their relative contribution to the pathogenesis of and defense against salmonella infections.

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