

# ***Salmonella*-induced Caspase-2 Activation in Macrophages: A Novel Mechanism in Pathogen-mediated Apoptosis**

By Veronika Jesenberger,\* Katarzyna J. Procyk,\* Junying Yuan,<sup>§</sup>  
Siegfried Reipert,<sup>‡</sup> and Manuela Baccarini\*

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From the \*Department of Cell- and Microbiology, Institute of Microbiology and Genetics, and the  
<sup>‡</sup>Department of Molecular Cell Biology, Institute of Biochemistry and Molecular Cell Biology, Vienna  
Biocenter, 1030 Vienna, Austria; and the <sup>§</sup>Department of Cell Biology, Harvard Medical School,  
Boston, Massachusetts 02115

## **Abstract**

The enterobacterial pathogen *Salmonella* induces phagocyte apoptosis in vitro and in vivo. These bacteria use a specialized type III secretion system to export a virulence factor, SipB, which directly activates the host's apoptotic machinery by targeting caspase-1. Caspase-1 is not involved in most apoptotic processes but plays a major role in cytokine maturation. We show that caspase-1-deficient macrophages undergo apoptosis within 4–6 h of infection with invasive bacteria. This process requires SipB, implying that this protein can initiate the apoptotic machinery by regulating components distinct from caspase-1. Invasive *Salmonella typhimurium* targets caspase-2 simultaneously with, but independently of, caspase-1. Besides caspase-2, the caspase-1-independent pathway involves the activation of caspase-3, -6, and -8 and the release of cytochrome *c* from mitochondria, none of which occurs during caspase-1-dependent apoptosis. By using caspase-2 knockout macrophages and chemical inhibition, we establish a role for caspase-2 in both caspase-1-dependent and -independent apoptosis. Particularly, activation of caspase-1 during fast *Salmonella*-induced apoptosis partially relies on caspase-2. The ability of *Salmonella* to induce caspase-1-independent macrophage apoptosis may play a role in situations in which activation of this protease is either prevented or uncoupled from the induction of apoptosis.

Key words: monocytes/macrophages • cell death • proteases • natural immunity • bacteria

## **Introduction**

*Salmonella* species cause a variety of enteric diseases ranging from self-limiting gastroenteritis (mainly due to *S. typhimurium*) to the more severe systemic typhoid fever (caused by *S. typhi*). Infections are generally food borne and pose a particularly serious health hazard in regions where the hygienic conditions are inappropriate. After consumption of contaminated food or water, *Salmonellae* reach the intestine, where they cross the epithelial barrier by invading the specialized M cells of the ileac Peyer's patches. By activating the host signal transduction cascades controlling the actin cytoskeleton, *Salmonella* induces the formation of membrane ruffles localized at the contact point between bacterium and host cell and is ultimately taken up in large vacu-

oles (1). By destroying infected M cells, the bacteria gain access to the mesenteric lymph follicles, where they face the host's macrophages. For *Salmonella*, as well as for many other facultative intracellular pathogens, surviving this encounter is the key to a successful infection. Invasive *Salmonella* is capable of persisting within the macrophages in spacious vacuoles uncoupled from the normal endocytic route. These vacuoles do not acquire lysosomal markers like cathepsin D or L and may therefore represent a relatively safe intracellular site in which the bacteria can survive and multiply (2).

Besides its ability to survive in infected macrophages, invasive *Salmonella* induces phagocyte apoptosis in vitro (3–6). Apoptosis is mediated by a cell-intrinsic suicide program, the activation of which is regulated by different signals originating from both the intracellular and the extracellular milieu. The situation during an in vivo infection is certainly different from experimental setups in vitro in which all of the bacteria are uniformly invasive, and the inoculum is rather overwhelming. A certain amount of

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K.J. Procyk's present address is Protein Phosphorylation Lab, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.

Address correspondence to Manuela Baccarini, Dept. of Cell- and Microbiology, Institute of Microbiology and Genetics, Vienna Biocenter, Dr-Bohrgasse 9, 1030 Vienna, Austria. Phone: 431-4277-54607; Fax: 431-4277-9546; E-mail: manuela@gem.univie.ac.at

phagocyte apoptosis can be detected after infection of mice with *Salmonella* in vivo (7), but the extent to which apoptosis contributes to the pathogenesis of *Salmonella* infections is at present unknown. *Salmonella* species (spp.) share the ability of inducing macrophage apoptosis with *Yersinia* spp. (8–10) and *Shigella* spp. (11–15), suggesting that this may represent a hallmark of, and perhaps a selective advantage for, the establishment of enterobacterial infections. Both epithelial cell invasion and induction of macrophage apoptosis depend on a functional type III secretion system. Type III protein secretion systems are specialized protein secretion apparatuses capable of translocating bacterial proteins into host cells, and they play a pivotal role in the interaction between a variety of mammalian and plant pathogenic bacteria with their hosts (16). In *Salmonella*, the type III secretion genes essential for epithelial cell invasion and apoptosis induction are clustered in a region denoted *Salmonella* pathogenicity island 1 (SPI-1)<sup>1</sup> at centisome 63 of the chromosome (17, 18). SipB, a protein encoded by SPI-1, is essential for *Salmonella*-mediated macrophage apoptosis (3).

Caspases (cystein/aspartic acid proteases) are the effector molecules of the apoptotic program. All enzymes in the family are produced as zymogens and are activated by proteolysis. The so-called “initiator caspases” can activate other family members (the “effector caspases”), establishing a proteolytic cascade. Initiator caspases generally feature a long prodomain containing protein interaction motifs. These motifs mediate the direct or adaptor-mediated oligomerization and activation of the initiator caspases. Effector caspases possess short prodomains, which are cleaved by initiator caspases. Thus activated, the effector caspases process the so-called death substrates, whose cleavage irreversibly commits the cell to apoptosis (19, 20).

Despite its evolutionary conservation, the cell death pathway in mammals is complex, and it includes multiple mammalian caspases with apparently similar apoptotic function. A number of caspase genes have been inactivated in the mouse, but the study of caspase-deficient mice has not yielded the expected clues, possibly because of caspase redundancy in most apoptotic processes. In the case of caspase-1, the general conclusion of the study of the mutant mice is that apoptosis is essentially normal in these animals but that they have a severe defect in cytokine production (21–29). It was therefore somewhat surprising that both *Salmonella*- and *Shigella*-induced macrophage apoptosis require caspase-1. Apoptosis is induced by two structurally similar proteins exported by type III secretion systems, SipB of *Salmonella typhimurium* and IpaB of *Shigella flexneri*, by binding to and activating caspase-1 in the host cells (11, 30, 31). In this study, we show that invasive *Salmonella* rapidly activates caspase-2 in a caspase-1-independent manner and that caspase-1 is not absolutely required for macrophage apoptosis. Caspase-1-deficient macrophages are killed by invasive *Salmonella* in a comparatively slow process (4–6 h). SipB is essential for caspase-1-independent macrophage

apoptosis, demonstrating that this protein can activate the apoptotic machinery by regulating components distinct from caspase-1. Apoptosis involves the activation of caspase-2, -3, -6, and -8 and the release of cytochrome *c* from mitochondria. With the exception of caspase-2 activation, these phenomena are not observed during the fast, caspase-1-dependent apoptosis. Caspase-2 plays a role in caspase-1-independent apoptosis and, by contributing to caspase-1 activation, in rapid apoptosis.

## Materials and Methods

**Bacteria.** *Salmonella typhimurium* strains SR11 (wild type [wt]), SB111 (*invA*<sup>-</sup>; unable to secrete proteins via the type III pathway), and SB169 (*sipB*<sup>-</sup>) were grown in 5 ml of Luria-Bertani broth (1% Bacto Tryptone, 0.5% yeast extract, 1% sodium chloride) at 37°C overnight (16–20 h) under agitation. To obtain highly invasive bacteria, overnight cultures were diluted to an OD<sub>600</sub> of 0.02 in 50 ml of TYP broth (1.6% Bacto Tryptone, 1.6% yeast extract, 0.5% sodium chloride, 0.25% dipotassium phosphate) and incubated for 5 h under agitation (5).

**Cell Culture and Infection.** Bone marrow-derived macrophages from caspase-1- (28) or caspase-2-deficient (32) mice and wt controls were cultured in DMEM supplemented with 10% FCS and 20% L-conditioned medium as a source of CSF-1. Confluent cells (~5 × 10<sup>6</sup> cells per 100-mm-diameter tissue culture dish) were cultured for 16–20 h in medium without CSF-1 and then infected with bacterial cultures. In selected experiments, the cells were treated with a caspase-2-specific inhibitor (Z-VDVAD-fmk, 100 μM [33]; R&D Systems) for 90 min before infection with *Salmonella*. A multiplicity of infection (m.o.i.; bacteria per macrophage) of 25 was used. After 30 min of infection, gentamycin was added to the medium (Sigma-Aldrich; 50 μg/ml for 1 h and then 10 μg/ml) to kill extracellular bacteria. All experiments were repeated three to five times.

**Cell Lysis and Western Blotting.** Cells from one 100-mm-diameter cell culture dish were washed twice with PBS and lysed in 300 μl of solubilization buffer (10 mM Tris-HCl, pH 7.0; 50 mM sodium chloride; 30 mM sodium pyrophosphate; 1% Triton X-100). Insoluble material was removed by centrifugation (20,000 g, 30 min). For immunoblotting, 30–50 μg of lysates was separated by 10 or 15% SDS-PAGE and transferred onto nitrocellulose membranes. After 1 h blocking in TTBS (10 mM Tris-HCl, pH 8.0; 150 mM sodium chloride; 0.1% Tween 20) supplemented with 5% milk powder, the membranes were probed with the appropriate primary antibodies (actin, caspase-1, caspase-2, and caspase-3 from Santa Cruz Biotechnology, Inc.; caspase-6 and caspase-8 from Chemicon; cytochrome *c* from BD PharMingen; and cytochrome *c* oxidase subunit IV [cox-IV] from Molecular Probes) diluted in 1% BSA (fraction V; Sigma-Aldrich) in TTBS before incubation with peroxidase-conjugated secondary antibodies and detection by an enhanced chemiluminescence system (Pierce Chemical Co.).

**Subcellular Fractionation.** Cells were scraped into Mito buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 μM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail [Boehringer Mannheim]). After incubation on ice for 30 min, cells were disrupted at 4°C in a 1-ml syringe fitted with a 25-gauge hypodermic needle (15 strokes). Nuclei were removed by centrifugation at 700 g for 5 min at 4°C. Supernatants were then further centrifuged at 13,000 g for 20 min at 4°C. The resulting su-

<sup>1</sup>Abbreviations used in this paper: SPI-1, *Salmonella* pathogenicity island 1; wt, wild type.

pernatants were considered as cytosolic and the pellets as mitochondrial fractions.

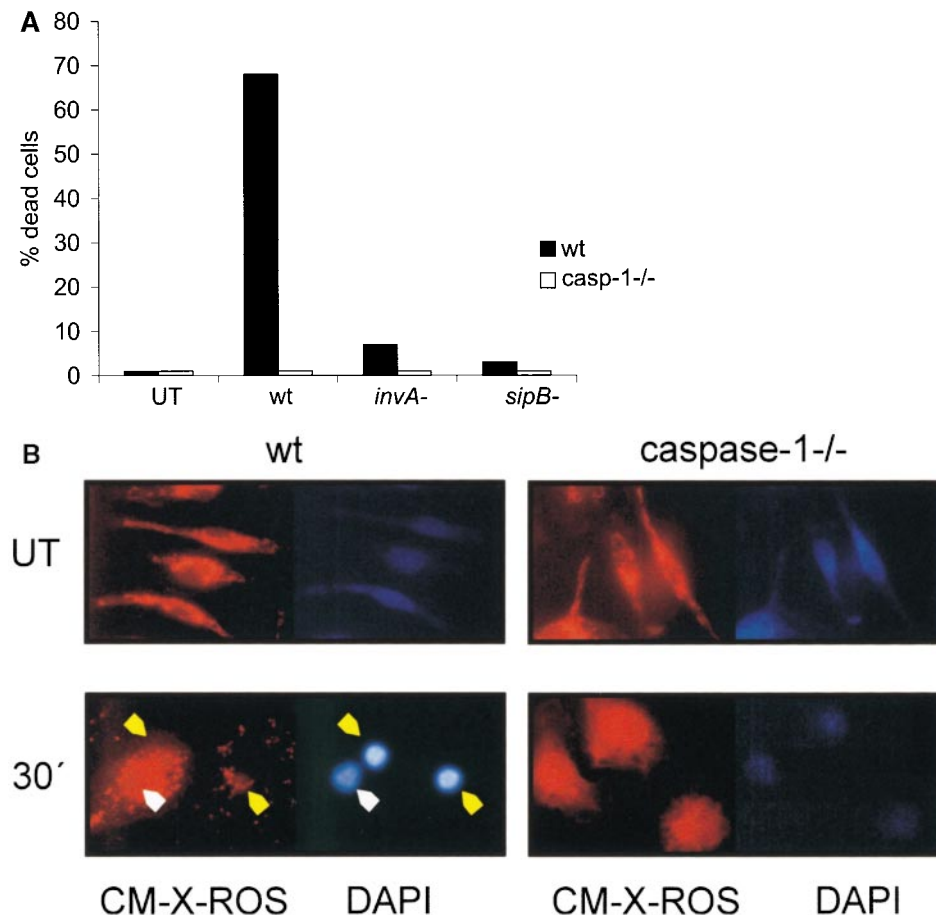
**Nuclear and Mitochondrial Staining.**  $3 \times 10^5$  macrophages were seeded on a coverslip in a well of a six-well cell culture dish. Mitochondria were stained with Chloromethyl-X-Rosamine (CM-X-ROS, MitoTracker™ Red; Molecular Probes), a potential-sensitive fluorochrome that withstands fixation and permeabilization of cells (34). Before infection, CM-X-ROS was added to the medium (final concentration 50 nM), and macrophages were incubated for 10 min at 37°C before infection with *Salmonella*. Chromatin condensation in infected macrophages was determined by staining with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). In brief, medium was removed and 70  $\mu$ l of DAPI (0.5  $\mu$ g/ml; Sigma-Aldrich) was placed on the coverslip. Cells were incubated for 1 min, washed twice with PBS, and fixed with 3% formaldehyde in PBS for 10 min at room temperature. After washing with PBS twice, coverslips were mounted in 20% Mowiol (Sigma-Aldrich) in PBS. Chromatin condensation was assessed in randomly chosen areas of the sample by independent experimenters (300–500 cells per sample).

**Electron Microscopy.** Cells were grown on glass coverslips and washed three times before fixation in glutaraldehyde (3% 0.15 M Sorensen's buffer, pH 7.4, for 1 h). Cells were then washed thrice with the same buffer and postfixed in 1% OsO<sub>4</sub> in Sorensen's buffer for 1 h. The cells were subsequently dehydrated in ethanol and flat-embedded in epoxy resin (Agar 100). Thin sections (60–80 nm) were mounted on copper grids and contrasted by uranyl acetate and lead citrate. Sections were viewed at 80 kV in a JEM-1210 electron microscope (Jeol Ltd.).

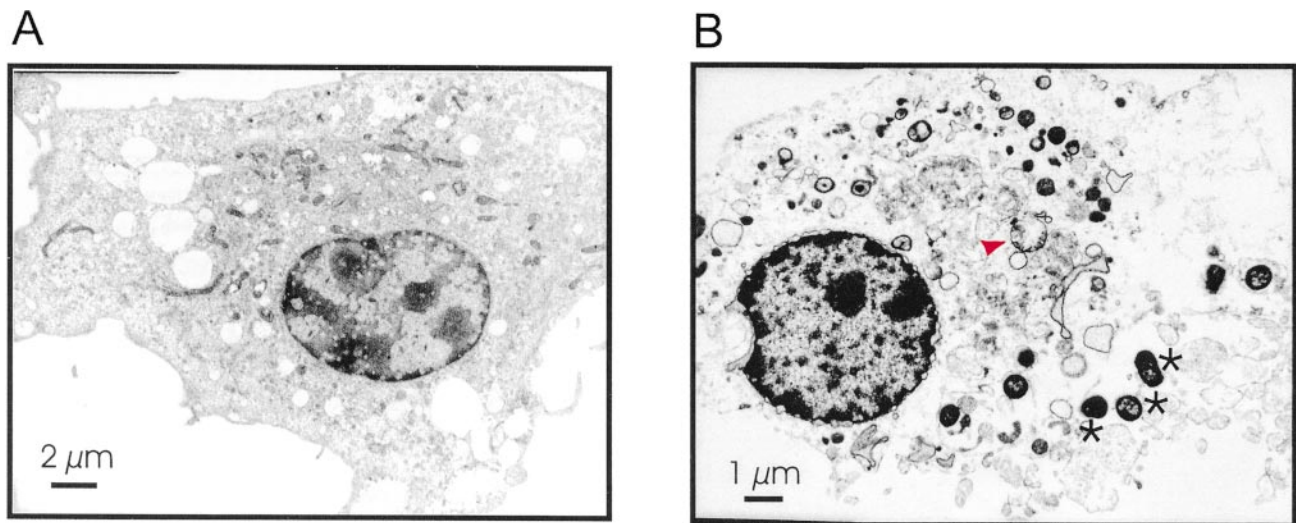
## Results

**Caspase-1 Is Required for Rapid *Salmonella*-induced Apoptosis.** *Salmonella* grown to the transition between the logarithmic and the stationary phase induces macrophage apoptosis with the fastest kinetics reported to date (5). At a m.o.i. of 25, 85% of the cells die within 30 min of infection, showing the morphological hallmarks of apoptosis (shrinkage, chromatin condensation, membrane blebbing; see Fig. 2 B). A functional type III secretion system is essential for the ability of *Salmonella* to induce apoptosis, and both an *invA*<sup>-</sup> and a *sipB*<sup>-</sup> mutant are incapable of doing so (Fig. 1 A). SipB of *Salmonella* (30) and the related protein IpaB of *Shigella* (15, 31) have been reported to bind to caspase-1 and activate it, thereby causing apoptosis. In agreement with this, primary bone marrow-derived macrophages from caspase-1-deficient mice failed to undergo apoptosis within the first 30 min of infection with wt *Salmonella* (Fig. 1, A and B).

To gain more insight in the mechanism of rapid *Salmonella*-induced apoptosis, we monitored simultaneously loss of mitochondrial transmembrane potential and chromatin changes during apoptosis. Cells labeled with the potential-sensitive dye CM-X-ROS were infected with invasive *Salmonella* and stained with DAPI 30 min after infection. The infected cells showed a change in shape, and the CM-X-ROS staining appeared to be more diffused than in the un-



**Figure 1.** Caspase-1 is required for rapid macrophage apoptosis. Primary bone marrow-derived macrophages were isolated from wt or caspase-1-deficient mice (casp-1<sup>-/-</sup>). Cells were stained with CM-X-ROS before infection with *Salmonella* strains (m.o.i. 25). 25 min after infection, cells were stained with DAPI to reveal chromatin condensation and observed under a fluorescent microscope. (A) wt (closed bars) or casp-1<sup>-/-</sup> macrophages (open bars) were infected with wt *Salmonella* or with the invasion-defective *invA*<sup>-</sup> and *sipB*<sup>-</sup> strains. UT, untreated cells. The percentage of cells containing condensed chromatin was determined by microscopical examination of triplicate samples. The SD was <5% in all cases, and it has been omitted. (B) photomicrographs of wt or caspase-1-deficient (caspase-1<sup>-/-</sup>) macrophages infected with wt *Salmonella*.



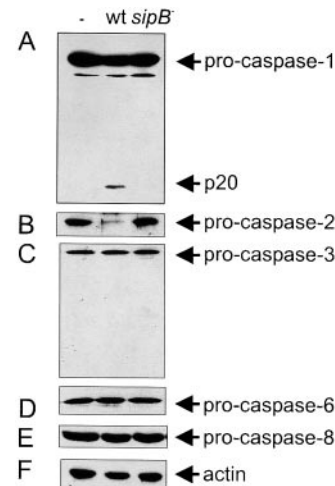
**Figure 2.** Ultrastructural features of rapid *Salmonella*-induced macrophage apoptosis. (A) Uninfected macrophage. (B) wt macrophage after a 20-min infection with invasive *Salmonella*; both nucleus and cytoplasm are significantly smaller as in uninfected macrophages (compare the bar in A = 2  $\mu\text{m}$  with the bar in B = 1  $\mu\text{m}$ ). The nucleus displays electron-dense material and a blistered nuclear envelope. The cytoplasm is in a progressed stage of lysis. It contains structurally intact mitochondria as well as swollen mitochondria with reduced matrix density (red arrowhead). Note also numerous *Salmonella* (marked by asterisks) associated with the macrophage.

infected controls (Fig. 1 B). These changes could also be observed in caspase-1-deficient macrophages, which at this time were not undergoing *Salmonella*-induced cell death (Fig. 1 A) and were therefore not related to apoptosis. DAPI staining of wt macrophages showed cells with different degrees of chromatin condensation (Fig. 1 B, wt 30 min). In cells with moderate chromatin condensation, the mitochondria retained CM-X-ROS, showing that their inner transmembrane potential was maintained (Fig. 1 B, white arrowhead). Upon completion of apoptosis, the nuclear staining appeared characteristically bright. The mitochondria of these cells released CM-X-ROS, indicating a loss of inner transmembrane potential (Fig. 1 B, yellow arrowheads). This is consistent with the dramatic swelling of some of these organelles observed in transmission electron microscopy of cells in the terminal stage of apoptosis (Fig. 2 B, red arrowhead). Other ultrastructural features of rapid *Salmonella*-induced macrophage apoptosis were the extreme shrinkage of both nucleus and cytoplasm (compare the bar in Fig. 2 A = 2  $\mu\text{m}$  with that in Fig. 2 B = 1  $\mu\text{m}$ ), the blebbing of the cytoplasmic membrane, and the blistered nuclear envelope.

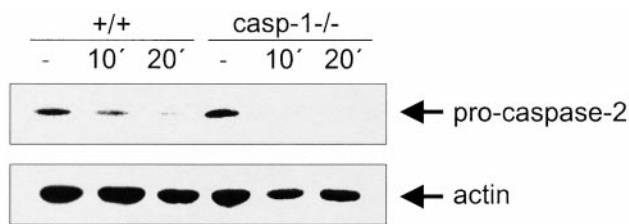
**Caspase Activation in the Course of Rapid *Salmonella*-induced Apoptosis.** To investigate the molecular mechanisms underlying rapid *Salmonella*-induced apoptosis, quiescent primary bone marrow-derived macrophages were either left untreated or infected with wt or *sipB*<sup>-</sup> *Salmonella* strains. The caspase profile of the cells and the activation state of these enzymes 15 min after infection was assessed in whole cell lysates by immunoblotting with specific antisera. Primary bone marrow-derived macrophages express caspase-1, -2, -3, -6, and -8 (Fig. 3). We monitored the activation of caspase-1 by immunoblotting with an antiserum that recognizes both the zymogen (procaspase-1) and the long

subunit of the active enzyme (p20). We confirmed that infection with a wt, but not with a *sipB*<sup>-</sup> *Salmonella* strain, activates this protease (Fig. 3 A). In addition, however, we observed the activation of caspase-2, measured as a decrease in the caspase-2 zymogen. At this early time point, little of the zymogen was cleaved, and intermediate forms (see Fig. 8) could not be detected. Again, the *sipB*<sup>-</sup> strain failed to activate caspase-2 (Fig. 3 B). Caspase-3, -6, and -8 were not activated in the course of this rapid apoptosis (Fig. 3, C, D, and E, respectively). The specific release of cytochrome *c* from mitochondria was not observed during rapid *Salmonella*-induced apoptosis (not shown).

**Caspase-2 Activation by Invasive *Salmonella* Occurs Independently of Caspase-1.** To assess whether activation of caspase-2 was part of the response initiated by caspase-1, we monitored zymogen cleavage in the early phases of infection



**Figure 3.** Caspase-1 and -2 are rapidly activated by invasive *Salmonella*. Primary bone marrow-derived macrophages isolated from wt mice were infected with wt *Salmonella* or with the invasion-defective *sipB*<sup>-</sup> strain. After 15 min of infection, cells were lysed and the activation state of the caspases was analyzed by immunoblotting. (A) caspase-1; (B) caspase-2; (C) caspase-3; (D) caspase-6; (E) caspase-8; (F) actin, used as a loading control.



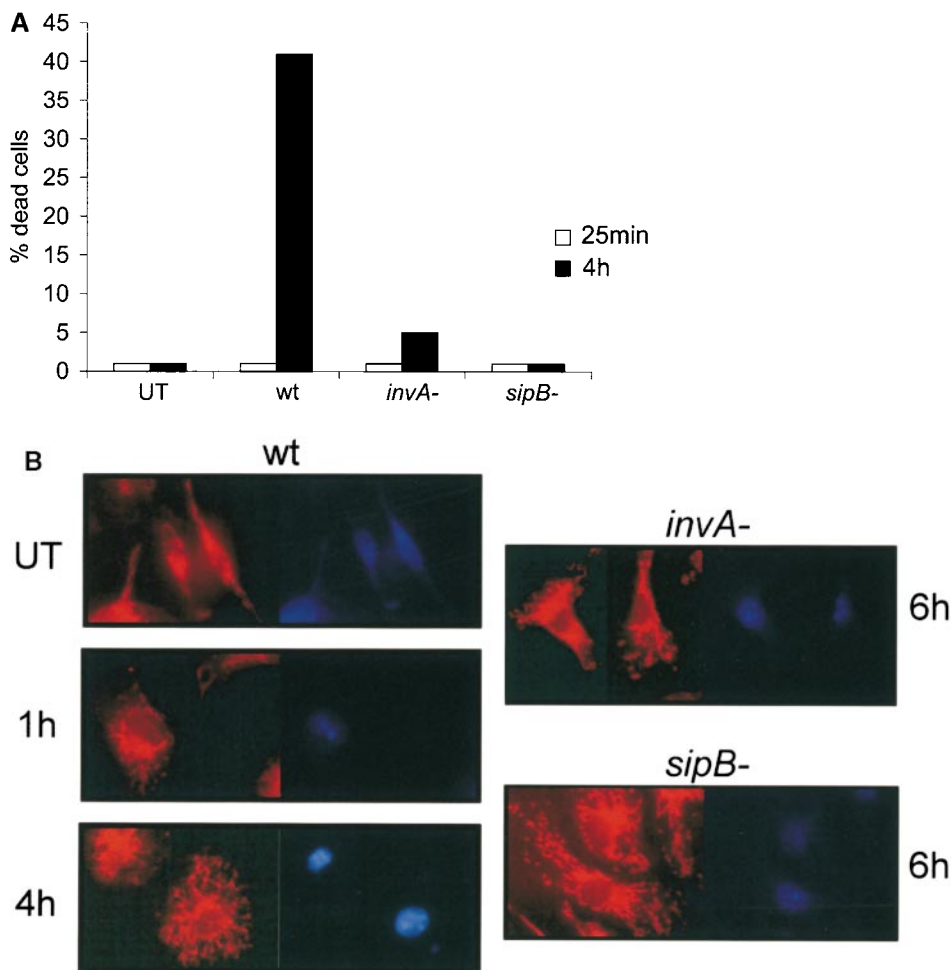
**Figure 4.** Invasive *Salmonella* activates caspase-2 in a caspase-1-independent manner. Primary bone marrow-derived macrophages from wt or caspase-1-deficient mice (*casp-1*<sup>-/-</sup>) were infected with invasive *Salmonella* (m.o.i 25). 10 and 20 min after infection, cells were lysed and the activation state of caspase-2 was analyzed by immunoblotting. An actin immunoblot is shown as a control for equal loading.

in primary bone marrow-derived macrophages from wt and caspase-1-deficient mice. Caspase-2 was rapidly activated in both cases (Fig. 4). As discussed above, only a small fraction of zymogen was cleaved, and intermediate forms were not detected at these early time points. Thus, *Salmonella* targeted caspase-2 directly, simultaneously with caspase-1.

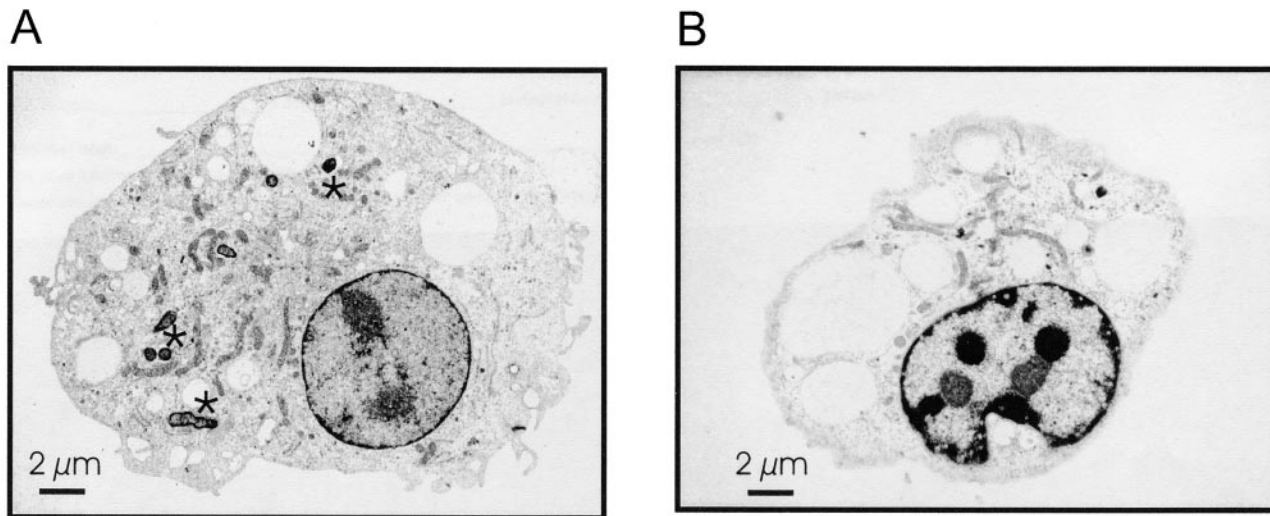
*Caspase-1-deficient Macrophages Undergo SipB-dependent, Salmonella-induced Apoptosis.* We next tested whether caspase-1-deficient macrophages underwent apoptosis upon

longer infection with the bacteria. Bone marrow-derived, caspase-1-deficient macrophages were either left untreated or were infected with wt or *sipB*<sup>-</sup> *Salmonella* strains. These macrophages succumbed to *Salmonella*-induced apoptosis after a 4-h infection. As in the case of the rapid apoptosis observed in wt macrophages, induction of cell death requires the SPI-1-encoded type III secretion apparatus, and the *invA*<sup>-</sup> and *sipB*<sup>-</sup> strains were not cytotoxic (Fig. 5 A). Caspase-1-deficient macrophages showed chromatin condensation after a 4-h infection with *Salmonella*, but nuclear staining never attained the characteristic compactness and brightness observed in the case of the wt macrophages (compare Fig. 1 B and Fig. 5 B). The mitochondria of these cells, although reduced in number (Fig. 5 B), retained the CM-X-ROS staining, indicating that their transmembrane potential did not dissipate. In infected cells, the mitochondria were organized in a more punctuate pattern. This reorganization was induced by the wt bacteria as well as by the *invA*<sup>-</sup> and the *sipB*<sup>-</sup> mutant strains and therefore was a consequence of infection rather than of cell death (Fig. 5 B).

To characterize the *Salmonella*-induced death of caspase-1-deficient macrophages ultrastructurally, we performed transmission electron microscopy of cells after a 4-h infection either with wt bacteria or with a *sipB*<sup>-</sup> mutant (Fig. 6).



**Figure 5.** Caspase-1-deficient macrophages are not resistant to apoptosis induced by invasive *Salmonella*. Primary bone marrow-derived macrophages isolated from wt caspase-1-deficient mice were stained with CM-X-ROS and DAPI and infected with wt *Salmonella* or with the invasion-defective *invA*<sup>-</sup> and *sipB*<sup>-</sup> strains (m.o.i 25) as described in the legend to Fig. 1. (A) Caspase-1-deficient macrophages were infected with wt *Salmonella* or with the invasion-defective *invA*<sup>-</sup> and *sipB*<sup>-</sup> strains. The percentage of cells containing condensed chromatin was determined as described in the legend to Fig. 1, 25 min (open bars) and 4 h (closed bars) after infection. UT, untreated cells. The SD was <5% in all cases, and it has been omitted. (B) Photomicrographs of caspase-1-deficient macrophages infected with *Salmonella*.



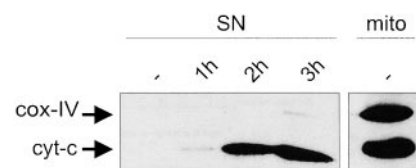
**Figure 6.** Electron microscopy of wt and caspase-1-deficient macrophages infected with invasive or invasion-deficient *Salmonella*. (A) Caspase-1-deficient macrophage after a 4-h infection with the *sipB*<sup>-</sup> strain; the size ratio between nucleus and cytoplasm shows no significant differences in comparison with uninfected macrophages. Moreover, both the nucleus and the cytoplasm do not show any morphological differences in comparison to control cells (A), and mitochondria do not display any signs of morphological alterations. A number of *Salmonella* (marked by asterisks) within the section indicate the infection of the cell. (B) Caspase-1-deficient macrophage after a 4-h infection with invasive *Salmonella*; the nucleus is slightly reduced in size and displays electron-dense material as a result of strong nucleolar segregation. The ratio in size between nucleus and cytoplasm is significantly reduced. The cytoplasm contains giant vacuoles. Absence of vacuoles and a dramatic reduction of cytoplasmic content in a late stage of cell death suggest a release of the vacuolar content into the culture medium (data not shown). Similar to control cells (A), mitochondria appear to be elongated and structurally intact.

The cell infected with wt strain showed a morphology compatible with apoptosis, with cell and nuclear shrinkage and chromatin condensation (Fig. 6 B). Most of the cell was occupied by large vacuoles, and the mitochondria were reduced in number. In contrast to the situation observed in wt macrophages infected with invasive *Salmonella* (Fig. 2 B), however, these organelles did not show any gross anomalies. Caspase-1-deficient macrophages infected with a *sipB*<sup>-</sup> mutant strain were viable, contained fewer vacuoles and some bacteria, and showed no signs of cell damage (Fig. 6 A). Thus, *Salmonella* is capable to induce caspase-1-independent apoptosis in macrophages, and this requires a functional *sipB* gene.

*Cytochrome c Is Released during Caspase-1-independent, Salmonella-induced Apoptosis.* The mitochondria of infected cells retained their membrane potential even during the late stages of apoptosis, and their morphology was not dramatically altered (Fig. 5 B and Fig. 6 B). However, invasive *Salmonella* induced progressive release of cytochrome *c* from the mitochondria of caspase-1-deficient macrophages. The appearance of cytochrome *c* in the mitochondrial supernatant preceded apoptosis, starting 1 h after infection and accumulating in the later phases (2 and 3 h). In contrast, cox-IV, an integral mitochondrial enzyme, was not present in the mitochondrial supernatant (Fig. 7). This served as a control for the purity of our mitochondrial supernatant and for the specificity of cytochrome *c* release.

*Caspase Activation during Caspase-1-independent, Salmonella-induced Apoptosis.* Caspase activation was monitored in the early phases of caspase-1-independent apoptosis (1 h; at this time chromatin condensation has not started and the cells are morphologically indistinguishable from cells in-

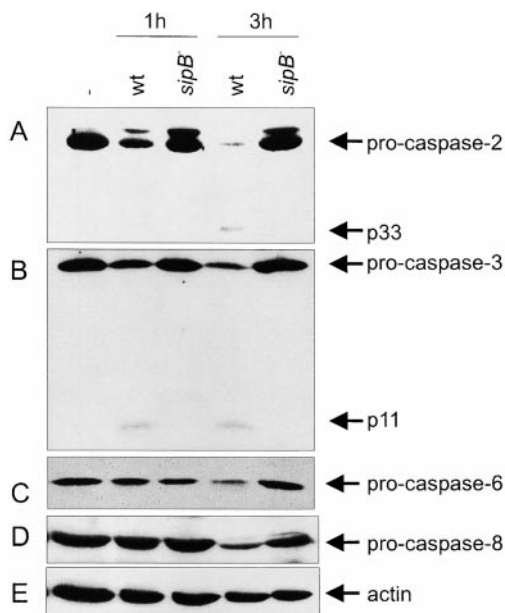
fectured with the noninvasive strains) as well as in the later phases of the process (3 h; at this time chromatin condensation is evident and the cells are clearly distinguishable from cells infected with noninvasive strains). Progressive caspase-2 activation was readily observed in these cells, as shown by the disappearance of the zymogen (procaspase-2). At later time points, when most of the zymogen was cleaved, the antiserum revealed a 33-kD intermediate form (p33). The apparent discrepancy with the kinetics of procaspase-2 cleavage shown in Fig. 4 stems from different exposure times used. The immunoblot shown in Fig. 4 was exposed for a short time, to visualize the relatively small changes in the amount of procaspase-2 at these early time points. Conversely, the immunoblot in Fig. 8 was exposed longer, to visualize the more dramatic cleavage of procaspase-2 and the appearance of p33 occurring at these later time points. Caspase-2 processing was not observed in cells infected



**Figure 7.** Cytochrome *c* (cyt-*c*) release during caspase-1-independent macrophage apoptosis. Primary bone marrow-derived macrophages isolated from caspase-1-deficient mice were infected with wt *Salmonella*. The presence of cytochrome *c* in the mitochondria (mito) or in the mitochondrial supernatant (SN) was assessed by immunoblotting at different times after infection. The blots were reprobed with antisera against cox-IV to control for the purity of our mitochondrial supernatant and for the specificity of cytochrome *c* release.

with a *sipB*<sup>-</sup> mutant strain (Fig. 8 A). We further investigated whether the other caspases expressed in primary macrophages were activated during caspase-1-independent apoptosis. Caspase-3 activation was assessed using an antiserum that recognizes both the zymogen (procaspase-3) and the short subunit of the active enzyme (p11). Caspase-3 was activated within 1 h of infection and remained active in the late phases of apoptosis (3 h; Fig. 8 B). The activation of caspase-6 and -8 was assessed by immunoblotting with antisera that recognize the zymogens only (procaspase-6 and -8). Processing of caspase-6 and -8 was only evident in the late phases of apoptosis, 3 h after infection with invasive *Salmonella*. The decrease in the procaspase-6 and -8 bands was specific, as shown by re-probing the blot with an antiserum against actin as a normalization control (Fig. 8, C–E). As in the case of caspase-2, caspase-3, -6, and -8 were not activated during infection with the *sipB*<sup>-</sup> mutant strain.

**Caspase-2 Inhibition Delays Apoptosis in Both wt and Caspase-1-deficient Macrophages.** To gain more insight into the role of caspase-2 in *Salmonella*-induced apoptosis, we treated primary bone marrow-derived macrophages with a caspase-2 inhibitor before infection with invasive bacteria. The inhibitor-treated cells showed markedly delayed kinetics of apoptosis, although they succumbed to *Salmonella* 60 min after infection (Fig. 9 B). This result was confirmed by comparing the kinetics of *Salmonella*-induced apoptosis of primary macrophages from wt or caspase-2-deficient mice

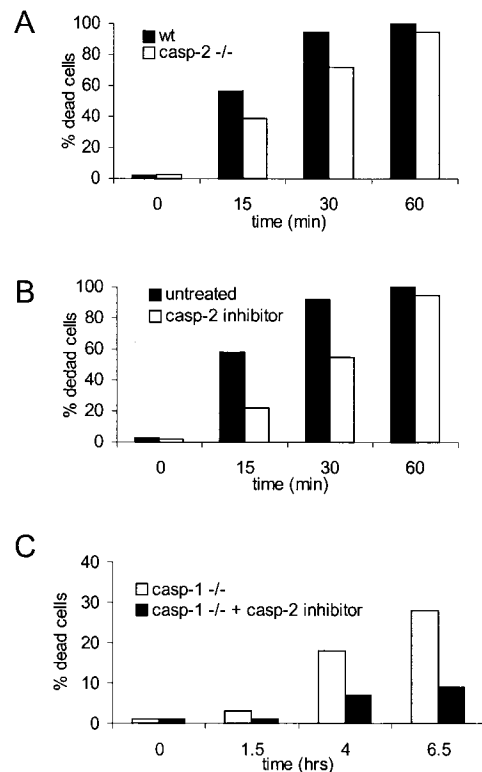


**Figure 8.** Caspase activation during caspase-1-independent macrophage apoptosis. Primary bone marrow-derived macrophages isolated from caspase-1-deficient mice were infected with wt *Salmonella* or with the invasion-defective *sipB*<sup>-</sup> strain. After 1 and 3 h of infection, cells were lysed, and the activation state of the caspases was analyzed by immunoblotting. (A) caspase-2; (B) caspase-3; (C) caspase-6; (D) caspase-8; (E) actin, used as a loading control. The caspase-2 blot was exposed longer than the immunoblots in Fig. 3 B and Fig. 4 to reveal the progressive cleavage of the zymogen and the appearance of the 33-kD intermediate band.

(Fig. 9 A). The effect of the inhibitor, however, was more marked than that of gene ablation (compare A and B in Fig. 9).

We next investigated the effect of the caspase-2 inhibitor on caspase-1-independent apoptosis. Treatment of caspase-1-deficient macrophages with the inhibitor resulted in a clear delay in the kinetics of *Salmonella*-induced apoptosis (Fig. 9 C). As noted above, however, the macrophages did succumb to the infection at later time points (20 h; data not shown).

**Caspase-1 Activation by Invasive *Salmonella* Is Partially Dependent on Caspase-2.** To investigate the role of caspase-2 in caspase-1-dependent apoptosis, we monitored zymogen cleavage in the early phases of infection in primary bone marrow-derived macrophages from wt mice treated with a caspase-2-specific inhibitor. Interestingly, caspase-1 cleavage was clearly impaired in the inhibitor-treated cells (Fig. 10 A). Similar, albeit less pronounced, effects were observed by monitoring caspase-1 cleavage in caspase-2-defi-



**Figure 9.** Chemical inhibition or genetic ablation of caspase-2 delays apoptosis induced by invasive *Salmonella*. Primary bone marrow-derived macrophages isolated from wt, caspase-2-deficient (*casp-2*<sup>-/-</sup>), or caspase-1-deficient (*casp-1*<sup>-/-</sup>) mice were stained with DAPI and infected with wt *Salmonella* as described in the legend to Fig. 1. (A) wt (closed bars) and caspase-2-deficient (open bars) macrophages were infected with wt *Salmonella*. (B) wt macrophages were either left untreated (closed bars) or were treated with a caspase-2 inhibitor (Z-VDVAD-fmk, 100  $\mu$ M; open bars) for 90 min before infection. (C) Caspase-1-deficient macrophages were either left untreated (open bars) or were treated with a caspase-2 inhibitor as described above. The percentage of cells containing condensed chromatin was determined as described in the legend to Fig. 1, at different points after infection. The SD was <5% in all cases, and it has been omitted.

cient macrophage (Fig. 10 B). Thus, while *Salmonella* targeted caspase-2 independently of caspase-1, caspase-2 contributed to caspase-1 activation.

## Discussion

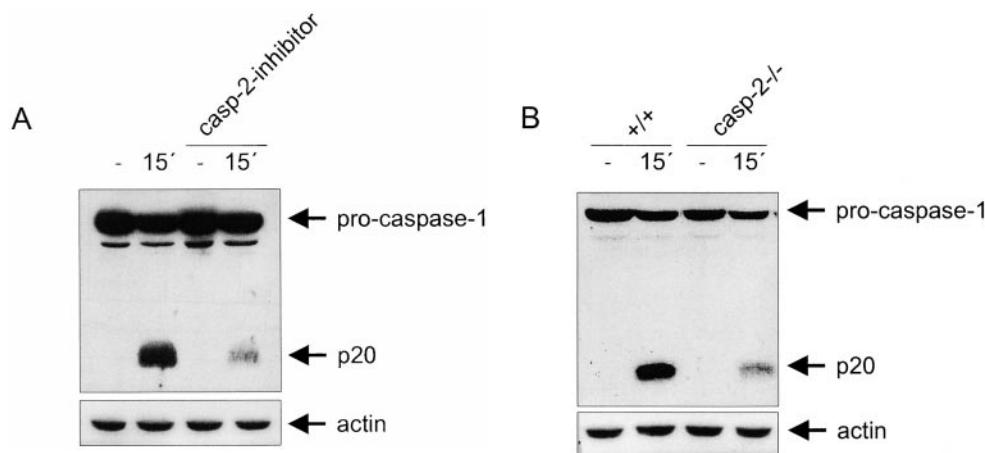
The discovery of the induction of macrophage apoptosis by gram-negative pathogens is one of the most exciting developments in the field of host-parasite relationships. The apoptotic event is one of the fastest reported and is dependent on caspase-1. This finding is particularly intriguing, as caspase-1 is commonly regarded as an enzyme mainly involved in cytokine maturation and release. Here we report a previously unrecognized, caspase-1-independent form of apoptosis induced by invasive *Salmonella* in macrophages. This apoptotic process is slower than the caspase-1-dependent cell death. It involves both the activation of a caspase cascade comprising caspase-2, -3, -6, and -8 and the release of the apoptogenic cytochrome *c* from the mitochondria of the infected cells. Caspase-2 possesses a long prodomain, which identifies it as an initiator caspase (35). This protease is activated already 10 min after infection and therefore appears to be at the top of the caspase cascade induced by *Salmonella*. The second caspase to be activated is caspase-3, followed in the late phase by caspase-6 and -8 (processed 3 h after infection). This order has been derived by comparing the kinetics of caspase degradation using immunoblotting with the respective antibodies and is therefore tentative. Nevertheless, the data are compatible with earlier observations indicating that activation of caspase-2 by various apoptotic stimuli precedes caspase-3 cleavage (36, 37) as well as with the ability of caspase-3 to sequentially activate caspase-6 and -8 in vitro (38). In further support of the role of caspase-2 in caspase-1-independent apoptosis, chemical inhibition of caspase-2 significantly delays this process, although it does not prevent it completely. It is therefore still possible that *Salmonella* may directly target other caspases in addition to caspase-1 and -2.

Specific release of cytochrome *c* from the mitochondria is a further feature of caspase-1-independent apoptosis. Judging by its kinetics, this event is likely to be a conse-

quence of rather than the reason for the activation of the caspase cascade (Fig. 7). Caspase-3, -6, -7, and -8 can reportedly stimulate cytochrome *c* release (39). Caspase-3 and -8 may do so by cleaving Bid, a member of the Bcl-2 family, and generating a proapoptotic fragment that induces cytochrome *c* release (40, 41). In *Salmonella*-infected macrophages, cytochrome *c* is set free in the absence of loss of mitochondrial membrane potential (Fig. 5 B) or of detectable mitochondrial swelling (Fig. 6 B), as has been reported in the case of Bid-induced release (41, 42). It is conceivable that cytochrome *c* escaping from the mitochondria may feed back on the caspase cascade and accelerate the apoptotic process.

Caspase-2 plays a role in both caspase-1-dependent and -independent apoptosis. Genetic ablation of caspase-2 or its chemical inhibition results in a significant delay in the kinetics of rapid, caspase-1-dependent apoptosis; surprisingly for us, this correlates with a reduced activation of the only other caspase activated at this point, caspase-1. Chemical inhibition of caspase-2 seems to be more effective than genetic ablation. This might be because the inhibitor has unspecific effects other than caspase-2 inhibition. As an alternative explanation, it should be considered that the caspase-2 mice lack both the proapoptotic (caspase-2<sub>L</sub>) and antiapoptotic (caspase-2<sub>S</sub>; references 43 and 44) forms of the enzyme. As a result of this, accelerated apoptosis has been described in some cell types derived from these animals (32). The lack of both the pro- and antiapoptotic form of caspase-2 in the knockout mice might be responsible for the different efficacy of the chemical inhibitor and of the genetic modification in inhibiting *Salmonella*-induced apoptosis and caspase-1 activation. Be that as it may, it is clear that invasive *Salmonella* targets caspase-1 not only directly via direct SipB binding but also indirectly, via caspase-2 activation.

It is intriguing that both caspase-1-dependent and -independent apoptosis require the function of the type III secretion system encoded for by SPI-1 and, more specifically, the presence of the SipB protein (reference 30 and this study). SipB and its close relative, IpaB from *Shigella flexneri*, bind caspase-1 directly, but the exact mode of caspase



**Figure 10.** Caspase-1 activation by invasive *Salmonella* is partially dependent on caspase-2. (A) Primary bone marrow-derived macrophages from wt mice were treated with a caspase-2 inhibitor (Z-VDVAD-fmk, 100  $\mu$ M) for 90 min before infection with invasive *Salmonella* (m.o.i 25). (B) Macrophages from wt or caspase-2-deficient mice (casp-2<sup>-/-</sup>) were infected with invasive *Salmonella* (m.o.i 25). 15 min after infection, cells were lysed and the activation state of caspase-1 was analyzed by immunoblotting. Actin immunoblots are shown as loading controls.



activation has not yet been elucidated. It is tempting to speculate that binding of SipB or IpaB to the protease will cause aggregation and autoactivation of the enzyme (induced proximity model). In this context, it should be noted that caspase-1 and -2, although rather different in sequence, both contain long prodomains featuring highly homologous CARDs (caspase recruitment domains). These domains mediate homophilic binding of caspases to adaptor proteins, leading to caspase aggregation and activation (35). IpaB does not bind to caspase-2 directly (31), and given the similarity between the two bacterial proteins, it is reasonable to predict the same for SipB. This leaves open two main possibilities: (a) SipB may form a complex with a CARD-containing adaptor which in turn will bind to, and cause the activation of, caspase-2; and (b) SipB may function as a translocase for other virulence factors, one of which may be responsible for caspase-2 activation. Clarification of this issue must await further work.

In vitro, caspase-1-dependent apoptosis is a rapid and complete process that kills all exposed macrophages in a remarkably short time. Why, then, does *Salmonella* initiate a distinct apoptotic process, targeting a different host of apoptogenic proteins? When would this “fallback” apoptosis become relevant? First, there is evidence that, in vivo, caspase activation does not always correlate with apoptosis. Processing of caspases has been observed in nonapoptotic T lymphocytes, and it is in fact required for a number of physiological processes, including cell proliferation (45, 46). Interestingly, only a subset of the caspases activated during T cell apoptosis is processed during proliferation. Similarly, active caspase-1 can be found in nonapoptotic, activated mucosal macrophages from patients with inflammatory bowel disease, a relevant example in our context (47). It is conceivable that, in vivo, both caspase-1 and caspase-2 must cooperate to induce efficient apoptosis. Second, it should be taken into account that during infection, the activation state of the macrophages encountered by *Salmonella* changes dramatically and that activated macrophages are reportedly more resistant than control cells against *Salmonella*-dependent apoptosis (6). The cells at the site of the infection will be confronted with *Salmonella*-derived LPS, which has been recently shown to upregulate a novel, endogenous caspase-1-specific inhibitor termed ICEBERG (Dixit, V., personal communication). These cells would then become resistant against caspase-1-dependent apoptosis but would still be killed by the alternative pathway described in this paper.

In addition, macrophages at the site of infection will produce nitric oxide, which will, in turn, decrease the activity of several caspases (48, 49), including caspase-1 (50). The transcription factors nuclear factor (NF)- $\kappa$ B and activator protein (AP)-1 will be also be activated in these macrophages. Both factors play a major role in the expression of inflammatory cytokines. At the same time, NF- $\kappa$ B (51–53) and AP-1 (52, 53) have been reported to antagonize macrophage apoptosis. If caspase activity in general is lowered by nitric oxide or if the cells become more resistant to apoptosis due to the stimulation of antiapoptotic mechanisms

linked to macrophage activation, *Salmonella* might increase its chances to induce apoptosis by simultaneously targeting caspase-1 and -2.

The occurrence of apoptosis during the early phases of *Salmonella* infection has been demonstrated experimentally (7). In vivo, the induction of macrophage apoptosis would disable the very cell type that efficiently reduces the bacterial load (54). In addition, the macrophages encountering invasive bacteria would die before being able to produce inflammatory cytokines. Of further advantage for the microbe, apoptosis, in contrast to necrosis, does not lead to massive release of cellular components and therefore does not trigger inflammation. The rapid induction of macrophage apoptosis may be instrumental in establishing/maintaining systemic infection, and if so, it may represent an attractive therapeutic target. However, general caspase inhibitors may interfere with T cell function (45, 46), and caspase-1-specific inhibitors might prevent the production of cytokines, which play an important role in the host resistance to infection (55). Understanding the alignment of the apoptotic pathways initiated by *Salmonella* might prove important for the design of therapeutic protocols that reduce macrophage apoptosis without altering the inflammatory response of the host.

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