

Salmonella Exploits Caspase-1 to Colonize Peyer's Patches in a Murine Typhoid Model

By Denise M. Monack,* David Hersh,[§] Nafisa Ghori,*
Donna Bouley,[‡] Arturo Zychlinsky,[§] and Stanley Falkow*

From the *Department of Microbiology and Immunology and the [‡]Department of Comparative Medicine, Stanford School of Medicine, Stanford University, Stanford, California 94305; and the [§]Skirball Institute, Department of Microbiology and Kaplan Cancer Center, New York University School of Medicine, New York, New York 10016

Abstract

Salmonella typhimurium invades host macrophages and induces apoptosis and the release of mature proinflammatory cytokines. SipB, a protein translocated by *Salmonella* into the cytoplasm of macrophages, is required for activation of Caspase-1 (Casp-1, an interleukin [IL]-1 β -converting enzyme), which is a member of a family of cysteine proteases that induce apoptosis in mammalian cells. Casp-1 is unique among caspases because it also directly cleaves the proinflammatory cytokines IL-1 β and IL-18 to produce bioactive cytokines. We show here that mice lacking Casp-1 (*casp-1*^{-/-} mice) had an oral *S. typhimurium* 50% lethal dose (LD₅₀) that was 1,000-fold higher than that of wild-type mice. *Salmonella* breached the M cell barrier of *casp-1*^{-/-} mice efficiently; however, there was a decrease in the number of apoptotic cells, intracellular bacteria, and the recruitment of polymorphonuclear lymphocytes in the Peyer's patches (PP) as compared with wild-type mice. Furthermore, *Salmonella* did not disseminate systemically in the majority of *casp-1*^{-/-} mice, as demonstrated by significantly less colonization in the PP, mesenteric lymph nodes, and spleens of *casp-1*^{-/-} mice after an oral dose of *S. typhimurium* that was 100-fold higher than the LD₅₀. The increased resistance in *casp-1*^{-/-} animals appears specific for *Salmonella* infection since these mice were susceptible to colonization by another enteric pathogen, *Yersinia pseudotuberculosis*, which normally invades the PP. These results show that Casp-1, which is both proapoptotic and proinflammatory, is essential for *S. typhimurium* to efficiently colonize the cecum and PP and subsequently cause systemic typhoid-like disease in mice.

Key words: apoptosis • pathogenesis • intestine • inflammation • macrophages

Introduction

Salmonellae cause a variety of disease syndromes, ranging from gastroenteritis to systemic infections like typhoid fever. After ingestion, *Salmonella* reaches the small intestine where it invades the mucosa by crossing the epithelial barrier through M cells overlying the lymphoid follicles of Peyer's patches (PP).¹ *Salmonella* invasion results in characteristic tissue changes, including infiltration of polymor-

phonuclear lymphocytes (PMNs) and monocytes, which contribute to the erosion of the intestinal mucosa (1). After *Salmonella* traverses the epithelial barrier, it encounters tissue macrophages and other host cells in the lamina propria. After replication in the PP, the bacteria enter the lymphatics and bloodstream and spread to deeper tissues (2). *Salmonella* invasion of host cells depends on bacterial proteins encoded in the chromosomal locus *Salmonella* pathogenicity island 1 (SPI1). SPI1 genes encode components of a type III secretion apparatus, regulatory proteins, and secreted effector proteins and their chaperones (1). In an oral inoculation, SPI1-defective mutants are attenuated in their ability to cause systemic infections, yet they are fully virulent if the bacteria are injected intraperitoneally (3–6). Thus, SPI1 appears to be necessary for the initial phase of the disease process.

Address correspondence to Denise M. Monack, Dept. of Microbiology and Immunology, Stanford School of Medicine, 299 Campus Dr., Fairchild Bldg. D 037, Stanford, CA 94305. Phone: 650-498-6953; Fax: 650-723-1837; E-mail: dmonack@leland.stanford.edu

¹Abbreviations used in this paper: Casp-1, Caspase-1; LB, Luria broth; MLNs, mesenteric lymph nodes; PMNs, polymorphonuclear lymphocytes; PP, Peyer's patches; SPI1, *Salmonella* pathogenicity island 1; TUNEL, TdT dUTP-biotin nick-end labeling.

The SPI1-encoded SipB protein (*Salmonella* invasion protein) is required to promote inflammation in infected ileum (7), to cause diarrhea in a calf model (1, 8), and to promote host cell invasion through aiding in the delivery of additional effector molecules (9–12). SipB interacts with Casp-1, which we believe leads to its activation within the macrophage cytoplasm, resulting in apoptosis (13). Unlike other mice with targeted deletions in other caspase genes, the *casp-1*^{-/-} mice do not display any defects in apoptotic pathways involved in development, homeostasis, or T cell selection (14). Macrophages from *casp-1*^{-/-} mice are resistant to *Salmonella typhimurium*-induced apoptosis (13), although they still undergo apoptosis in response to several physiological stimuli (15, 16); macrophages from *casp-3*^{-/-} and *casp-11*^{-/-} mice remain susceptible to *Salmonella*-induced apoptosis (Hersh, D., unpublished observation). In addition to playing a role in the apoptotic pathway in macrophages (17, 18), Casp-1 cleaves the proinflammatory cytokines IL-1 β and IL-18 to their mature forms (16). The biologically inactive forms of these two cytokines are stored

in the cytoplasm of macrophages (19–22). Indeed, *Salmonella* infection of cultured macrophages and dendritic cells induces secretion of active IL-1 β and IL-18 (13, 23, 24), suggesting that *Salmonella*-induced apoptosis is proinflammatory. Here we report on the role of the *Salmonella*-induced apoptotic and inflammatory response in the outcome of this host–pathogen interaction in vivo.

Materials and Methods

Bacterial Strains. Wild-type *S. typhimurium* SL1344 (25) and the isogenic *orgA* mutant strain BJ66 (3) were grown overnight with aeration in Luria broth (LB) before oral inoculation of mice. Bacteria were grown standing overnight in high-salt LB before inoculation of ligated loops. For gentamicin protection assays performed in peritoneal macrophages, SL1344 was grown overnight with aeration, followed by a 15-min incubation at 37°C in normal mouse serum to opsonize bacteria.

Mice. 8–10-wk-old B10.RIII-H2^bH2-T18^b(17NS)/Sn *casp-1*^{+/+} *nramp*^{s/s} (The Jackson Laboratory), C57BL/6 H-2^b *casp-1*^{+/+} *nramp*^{s/s} (The Jackson Laboratory), and B10.RIII-H2^b H2-

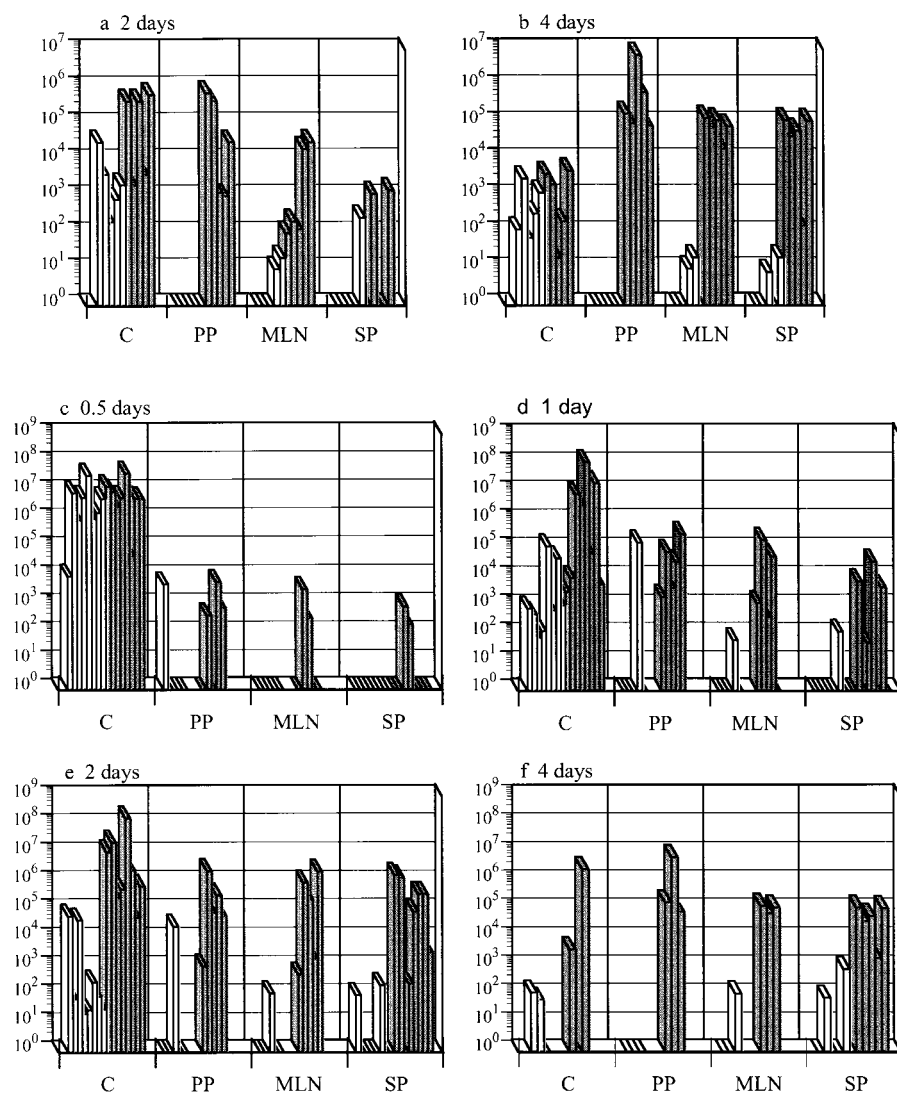


Figure 1. *Casp-1*^{-/-} mice are resistant to *S. typhimurium* colonization after an oral infection. Wild-type mice (gray bars) and *casp-1*^{-/-} mice (white bars) were inoculated intragastrically with wild-type *S. typhimurium*. The tissue colonization of mice inoculated with 2×10^7 CFU of *S. typhimurium* SL1344 was determined for (a) 2 and (b) 4 d after inoculation. C, cecum; PP, Peyer's patches; MLN, mesenteric lymph nodes; SP, spleen. $n = 5$ for both days. Tissue colonization after intragastric inoculation with 10^{10} CFU of *S. typhimurium* SL1344 for (c) 0.5, (d) 1, (e) 2, and (f) 4 d after inoculation. Cecum and spleen, $n = 8$ for days 0.5, 1, and 2; $n = 3$ for day 4. PP and MLNs, $n = 5$ for days 0.5, 1, and 2; $n = 3$ for day 4. Day 1 for PP and MLNs for wild-type compared with *casp-1*^{-/-} mice: $P = 0.0236$ and $P = 0.0343$, respectively. Day 2 for PP, MLNs, spleens, and livers: $P = 0.0132$, $P = 0.0343$, $P = 0.0025$, and $P = .0283$, respectively. Day 4 for PP, MLNs, spleens, and livers: $P = 0.0369$, $P = 0.0038$, $P = 0.0207$, and $P = 0.0278$, respectively. Rectangles on the x axis represent zero bacterial colonies present in undiluted tissue. These data represent results from one experiment, performed on age-matched *casp-1*^{-/-} and wild-type mice, which is representative of three experiments. The variability in mouse colonization at early times is reproducible and typical of what is seen after an oral inoculation.

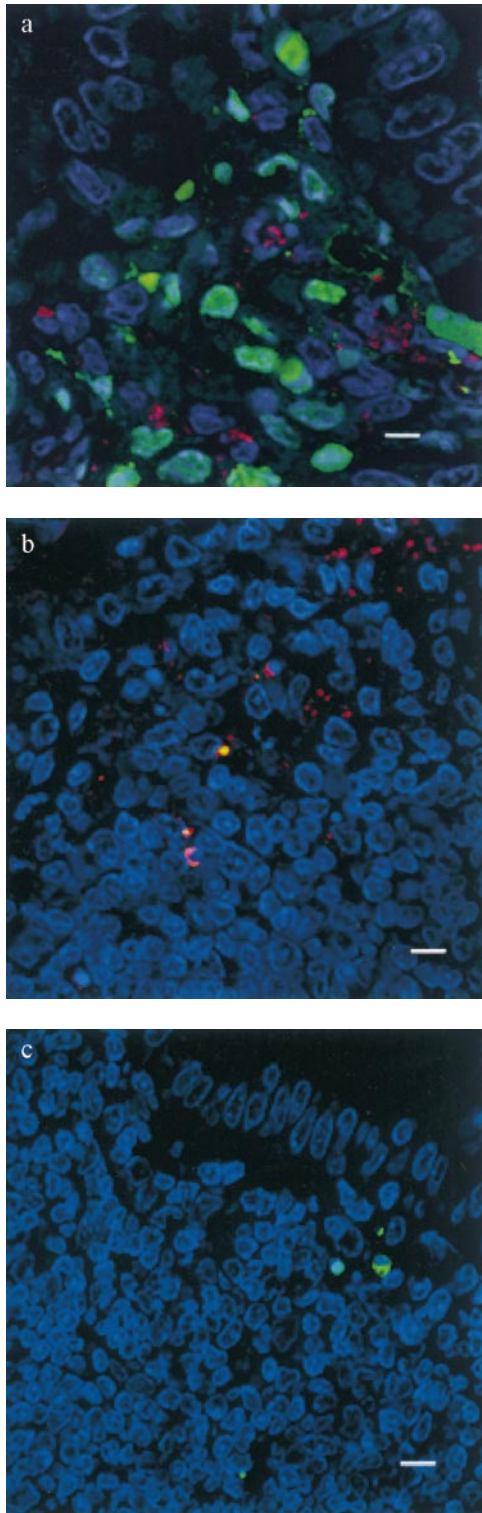


Figure 2. TUNEL⁺ cells colocalize with *S. typhimurium* in the dome area of PP from *casp-1*^{+/+} mice. Cross sections of PP from ligated intestinal loops performed in (a) wild-type or (b) *casp-1*^{-/-} mice infected with wild-type *Salmonella* or (c) wild-type mice inoculated with LB for 1 h were fixed and stained for apoptotic nuclei in green, *Salmonella* in red, and host cell nuclei in blue. The scalebar represents 8 μ m in a and 10 μ m in b and c. Images are oriented such that the intestinal lumen is at the top and the lymphoid follicle (not shown) is toward the bottom.

T18^b(17NS)/Sn *casp-1*^{-/-} *nramp*^{+/s} (Merck Research Laboratories) mice were kept under specific pathogen-free conditions in filter-top cages. Interbreeding of mice lacking *casp-1*, which were backcrossed eight generations onto the B10.RIII background, was performed to obtain the *casp-1*^{-/-} mice. Mice were provided with sterile water and food ad libitum.

Oral Inoculation of Mice. Wild-type and *casp-1*^{-/-} mice were deprived of food 14 h before orogastric inoculation. For LD₅₀ calculations, 35 *casp-1*^{+/+} and 35 *casp-1*^{-/-} mice were inoculated orogastrically through a gastric tube, with serial 10-fold dilutions ($n = 5$ mice/inoculum) ranging from 1.38×10^4 to 1.38×10^{10} CFU. The health of the animals was followed for 30 d after inoculation, and deaths were recorded. For colonization experiments, mice were inoculated orogastrically with the indicated dose of wild-type *S. typhimurium* SL1344. Mice were killed on days 0.5, 1, 2, and 4 followed by dissection of cecum, PP, mesenteric lymph nodes (MLNs), and spleens. Tissues were homogenized and weighed, and dilutions were plated on Luria agar plates containing Streptomycin (200 μ g/ml) to obtain CFU per gram tissue.

Ligated Intestinal Loop Model and Measurement of Gentamicin-protected Bacteria. Mice were starved for 15 h before they were anesthetized by i.p. inoculation of 1.5 mg pentobarbital sodium as previously described (26). A 10–15-cm intestinal loop was injected with 10^8 SL1344. At 0.5, 1, 2, and 3 h after infection, PP were dissected away from the small intestine and processed for transmission electron microscopy (26). Separate mice were used for the measurement of gentamicin-protected bacteria. At 0.5 and 3 h, the intestinal loops were flushed three times with 10 ml of PBS containing gentamicin (100 μ g/ml), followed by dissection of individual PP, which were then incubated separately with gentle agitation in 1 ml of DMEM supplemented with 10% fetal bovine serum and 100 μ g/ml gentamicin for 100 min at 37°C. This was followed by two 10-min washes in 1 ml of PBS to clear the gentamicin. Each PP was homogenized and serial dilutions were plated as described above.

Histology and TUNEL Reactions on Fixed PP. For histological examinations and TUNEL (TdT dUTP-biotin nick-end labeling) reactions on tissue sections, PP were fixed in 10% buffered neutral formalin, embedded in paraffin, and sectioned. Some sections were then stained with hematoxylin and eosin. Sections for TUNEL reactions were processed as described for the In Situ Cell Death Detection Kit, Fluorescein (Boehringer). The sections were labeled for 1 h at 37°C, rinsed with PBS, incubated in anti-*Salmonella* polyclonal rabbit antiserum, and finally incubated with anti-rabbit-TRITC antibody (Sigma-Aldrich) and ToTo-3 (Molecular Probes) to stain host cell nuclei. Coverslips were mounted over anti-quench and sealed, and tissue sections were analyzed on a confocal microscope (MRC1024; Bio-Rad Laboratories). Tissues for electron microscopy were prepared exactly as previously described (26).

Peritoneal Macrophages and Gentamicin Protection Assay. Macrophages were elicited to the peritoneum of mice and isolated 4 d after injection of 3% thioglycolate broth. After 2 h, nonadherent cells were removed and the remaining macrophages (2.5×10^5 /well) were incubated in DMEM supplemented with 10% heat-inactivated FCS, 50 μ g/ml penicillin, 50 μ g/ml streptomycin, 50 IU/ml IFN- γ (Sigma-Aldrich), and 50 ng/ml LPS from *Escherichia coli* (Sigma-Aldrich) at 37°C, 5% CO₂ overnight. The monolayers were then washed with fresh tissue culture media before infections. Infections were performed with a multiplicity of infection of 10 to 1. Bacteria were spun onto the cells at 1,000 rpm for 5 min and incubated for 30 min, followed by incubation

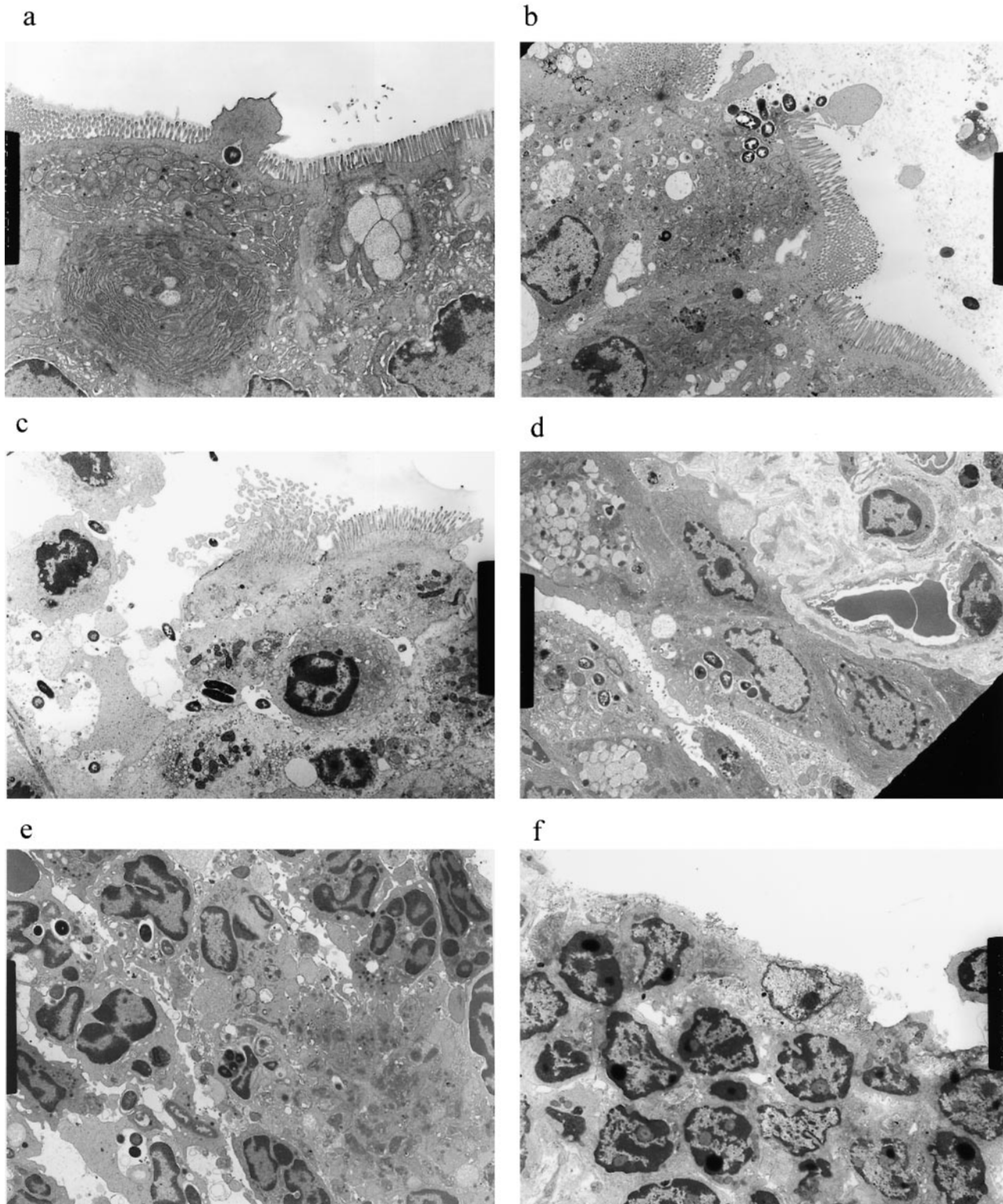


Figure 3. (continues on facing page). *S. typhimurium* induces inflammation in wild-type PP but not in *casp-1*^{-/-} PP. Transmission electron micrographs of infected PP revealed bacterial invasion of M cells at 0.5 h in wild-type (a) and *casp-1*^{-/-} (b) PP, and at 1 h in (c) extracellular *Salmonella* in wild-type and (d) intracellular bacteria in *casp-1*^{-/-} PP. At 3 h, wild-type PP dome revealed an infiltration of PMNs and intracellular *Salmonella* (e), whereas *casp-1*^{-/-} PP revealed an absence of PMNs and bacteria (f). Hematoxylin and eosin stains of PP from *casp-1*^{+/+} (g) mice infected for 3 h revealed clusters of PMNs (black arrows) and pyknotic cells (arrowheads), whereas *casp-1*^{-/-} PP did not (h). Uninfected, wild-type PP are shown for comparison (i). Yellow arrows point to the PP dome area. Serial tissue sections were stained with anti-*Salmonella* antibody to confirm that the PP shown was infected. All sections are oriented with intestinal lumen at the top and lymphoid follicle at the bottom.

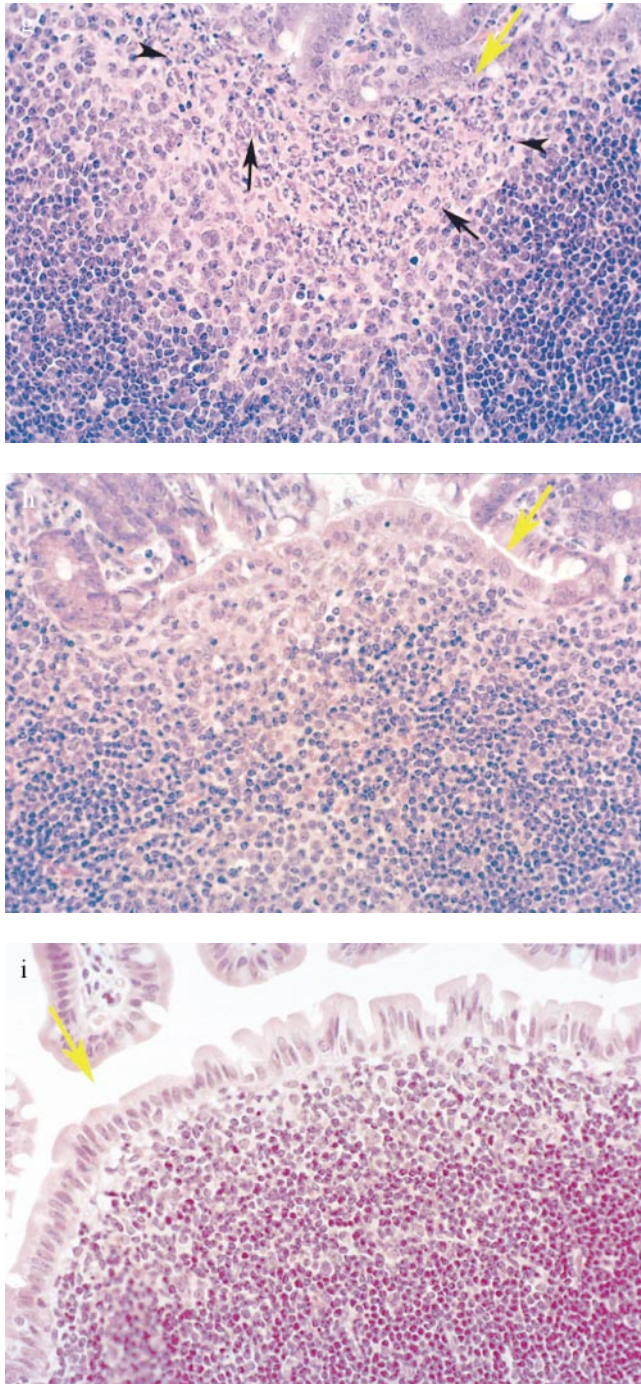


Figure 3. (continued).

in 100 $\mu\text{g/ml}$ gentamicin for 90 min. The tissue culture media was then replaced with a minimum inhibitory concentration of gentamicin (10 $\mu\text{g/ml}$) for the remainder of the assay. At 2, 4, 8, and 24 h the cells were lysed with 1% Triton X-100 for 10 min, diluted with LB, and plated for CFU per well.

Statistical Analysis. The colonizations of tissue from wild-type and *casp-1*^{-/-} mice were compared by the Mann-Whitney U test. F-test for analysis of variance revealed $P < 0.0001$ for all tissues on days 1, 2, and 4. The mean gentamicin-protected CFU were compared by Student's *t* test.

Results

Oral LD₅₀ for *S. typhimurium* is 1,000-fold Higher in *casp-1*^{-/-} Mice. To assess the role of the host protein, Casp-1, during *Salmonella* infection, we determined the LD₅₀ for mice lacking this enzyme. Mice were inoculated intragastrically with 10-fold serial dilutions of the wild-type *S. typhimurium* strain SL1344. The infected animals were followed for 30 d and the estimated value for the 50% death point was calculated by the Reed-Muench method (27). The oral LD₅₀ in *casp-1*^{-/-} mice was 1.67×10^8 , which was 1,000-fold higher than the 1.56×10^5 LD₅₀ value obtained for wild-type mice. To allow for comparisons with published reports using the susceptible mouse strain C57BL/6, the oral LD₅₀ in C57BL/6 was determined to be 2.4×10^5 .

***casp-1*^{-/-} PP Colonization Is Significantly Lower after Oral Inoculation with *S. typhimurium*.** We orally infected *casp-1*^{-/-} mice with wild-type *S. typhimurium* to investigate at what stage of a systemic infection Casp-1 plays a role. Wild-type and *casp-1* null mice were inoculated orogastrically with 2×10^7 wild-type *S. typhimurium*, a dose that is ~ 100 -fold above the LD₅₀ in wild-type mice. *casp-1*^{-/-} mice were colonized in the cecum but not in the PP at 2 and 4 d after inoculation (Fig. 1, a and b). Two out of five *casp-1*^{-/-} mice were colonized in the MLNs at both 2 and 4 d, whereas all of the wild-type mice were colonized ($P = 0.032$ for 2 d after inoculation; $P = 0.0083$ for 4 d after inoculation). By 4 d, all wild-type mice were heavily colonized in the spleens, whereas three of the *casp-1*^{-/-} mice were not colonized, with two mice containing fewer than 20 CFU ($P = 0.0207$). Thus, a dose of wild-type *S. typhimurium* that is sufficient to colonize and eventually kill all wild-type mice poorly colonized the *casp-1*^{-/-} mice.

We next inoculated mice with 10^{10} CFU of wild-type *S. typhimurium*, which is ~ 100 -fold higher than the LD₅₀ for *casp-1* null mice. In this experiment, the *casp-1*^{-/-} mice were colonized at early time points in the cecum but not in the PP, whereas wild-type mice were colonized at both sites (Fig. 1, c and d). 1 d after infection with this high dose, the PP and MLNs of wild-type mice had significantly more bacteria than these same tissues in *casp-1*^{-/-} animals (Fig. 1 d). By day 2 of infection, *Salmonella* were found in large numbers in the spleens of wild-type mice, whereas the *casp-1*^{-/-} mice still were not significantly colonized in any of the tissues tested except the cecum (Fig. 1 e). By day 4 of infection, wild-type but not *casp-1*^{-/-} animals were noticeably ill and bacteria were present in all tissues tested. The number of bacteria on day 4 was significantly higher in all tissues of *casp-1*^{+/+} than in *casp-1*^{-/-} mice, except in the cecum (Fig. 1 f). Eventually the *casp-1*^{-/-} mice died when inoculated with such a high dose, but the time to death was very different from that of wild-type mice. The mean time to death was 5.4 d for wild-type mice, whereas the mean time to death for *casp-1*^{-/-} mice was 9.6 d ($P = 0.0106$).

Increased Apoptosis in Wild-type PP Correlates with Colonization. To test whether *Salmonella* induces apoptosis in PP and whether this apoptosis is Casp-1 dependent, apop-

otic cells within PP sections of infected mice were detected by the TUNEL reaction (25). The PP were studied in the ligated intestinal loop system, which allows for the analysis of early time points. At 1 h after infection, wild-type PP contained far more TUNEL⁺ cells than did *casp-1*^{-/-} or uninfected PP from either mouse background. Furthermore, many apoptotic cells colocalized with bacteria in the dome area of wild-type PP (Fig. 2). Thus, in wild-type mice *Salmonella* induced many cells in the PP to undergo apoptosis either directly or indirectly, whereas in *casp-1*^{-/-} mice *Salmonella* induced relatively few cells to undergo apoptosis. Furthermore, *Salmonella* failed to survive in *casp-1*^{-/-} mice.

The Early Colonization in Wild-type PP Is Associated with an Increase in Inflammatory Cells and an Increase in Intracellular *Salmonella* by 3 h in the PP. Bacterial colony counts and tissue samples for microscopic examination were obtained from the ligated loops of wild-type and *casp-1*^{-/-} mice infected for 0.5, 1, and 3 h. At 0.5 h after infection in both wild-type and *casp-1*^{-/-} mice, *Salmonella* invaded M cells and produced the characteristic membrane ruffles followed by the destruction of M cells (28; Fig. 3, a and b). At 1 h, the macrophages in the dome area of wild-type PP were killed and extruded into the intestinal lumen, and bacteria were extracellular (Fig. 3 c). By 3 h, the epithelium overlying the PP in wild-type mice was virtually destroyed by invading *Salmonella* and there were many PMNs containing bacteria that were localized to the dome area (Fig. 3, e and g). In contrast, in PP from *casp-1*^{-/-} mice infected for 1 h, the majority of bacteria were inside monocytes (Fig. 3 d). By 3 h, very few bacteria were observed and the *casp-1*^{-/-} PP, which showed little inflammation, were devoid of PMNs (Fig. 3, f and h). Thus, *Salmonella* initiates an inflammatory response in *casp-1*^{+/+} mice, presumably due in part to Casp-1-mediated IL-1 β and IL-18 activation.

We quantitated the observed decline in bacteria during the 3-h ligated loop experiment. The number of intracellular *S. typhimurium* within PP after infection was measured using a gentamicin protection assay to enrich for intracellular bacteria (29). Consistent with the histopathological findings, 10-fold more gentamicin-resistant (intracellular) bacteria were enumerated in the *casp-1*^{-/-} mice than in the wild-type mice at this 0.5-h time point (Fig. 4). In contrast to wild-type mice at 3 h, there was a fivefold decrease in bacteria in the *casp-1*^{-/-} animals, consistent with the dwindling numbers seen on microscopic analysis. Thus, wild-type *Salmonella* can efficiently invade *casp-1*^{-/-} PP, but is unable to survive within them.

***casp-1*^{-/-} and Wild-type Mice Are Equally Susceptible to Intraperitoneally Injected *Salmonella*.** The oral colonization data suggested that Casp-1 plays a role in the initial intestinal colonization phase of *Salmonella* infections, but we could not determine what role, if any, Casp-1 might play in the colonization of other tissues. To determine whether Casp-1 is required for *Salmonella* to colonize extraintestinal organs, the gastrointestinal tract was bypassed by inoculating mice intraperitoneally. Under this protocol, *casp-1*^{-/-} and wild-type mice had similar infection progressions ($1.92 \times$

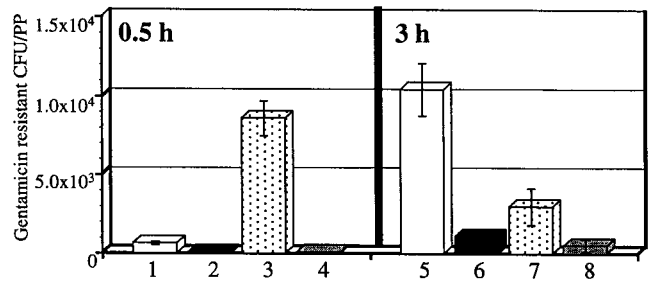


Figure 4. *S. typhimurium* CFU decrease in *casp-1*^{-/-} PP. Ligated intestinal loops were performed for 0.5 and 3 h and gentamicin-protected CFU per PP was determined. Lanes 1 ($n = 9$) and 5 ($n = 10$), *casp-1*^{+/+} mice infected with SL1344. Lanes 2 ($n = 9$) and 6 ($n = 11$), *casp-1*^{+/+} mice infected with noninvasive mutant BJ66 (3). Lanes 3 ($n = 10$) and 7 ($n = 13$), *casp-1*^{-/-} mice infected with SL1344. Lanes 4 ($n = 12$) and 8 ($n = 12$), *casp-1*^{-/-} mice infected with BJ66. Unpaired *t* test revealed $P < 0.0001$ and $P = 0.0013$ for SL1344 in *casp-1*^{-/-} compared with *casp-1*^{+/+} at 0.5 and 3 h, respectively; $P = 0.0001$ for SL1344 in *casp-1*^{+/+} mice for 0.5 versus 3 h; $P = 0.0028$ for SL1344 in *casp-1*^{-/-} mice for 0.5 versus 3 h. The noninvasive mutant produced numbers that were not statistically different between the two mouse strains. The mean and standard deviations are shown from two separate experiments.

$10^5 \pm 4.06 \times 10^4$ and $2.77 \times 10^5 \pm 3.29 \times 10^4$ CFU/gram per wild-type and *casp-1*^{-/-} spleen, respectively, 2 d after inoculation). Furthermore, these two strains of mice showed equal mortality after i.p. inoculation with 100 wild-type *S. typhimurium* (Fig. 5). These data show that Casp-1 activity is not needed for successful infection by the i.p. route, but appears to be essential in order for *Salmonella* to establish a successful gastrointestinal infection by the mucosal route.

Intracellular Survival of *Salmonella* Is the Same in Macrophages from Wild-type and *casp-1*^{-/-} Mice in an In Vitro Assay. Many reports correlate the ability of *S. typhimurium* to cause mouse typhoid fever with in vitro bacterial survival within cultured macrophages (30–33). To determine whether the failure of *Salmonella* to disseminate in *casp-1*^{-/-} mice was due to an increased killing of *Salmonella* by macrophages from *casp-1*^{-/-} mice, *Salmonella* interactions with peritoneal macrophages derived from wild-type and *casp-1*^{-/-} mice were compared in vitro. We tested the killing of bacteria under conditions where apoptosis of the macrophages was eliminated either by growing wild-type *S. typhimurium* to late stationary phase or by using a mutant *S. typhimurium* that cannot deliver SipB (25). No difference in survival over the course of the 24-h assay was found in the killing of *Salmonella* (Fig. 6). The initial recovery of intracellular bacteria at 2 h was the same for both wild-type and *casp-1*^{-/-} macrophages. This was followed by similar levels of bacteria killing, nearly 1.5 logs, over the 24-h assay. Therefore, macrophages from *casp-1*^{-/-} mice are not inherently different in their capacity to kill *Salmonella* compared with wild-type-derived macrophages.

The PP, MLNs, and Spleens of *casp-1*^{-/-} and Wild-type Mice Are Equally Colonized by *Yersinia pseudotuberculosis*. Could another enteric pathogen, which has been shown to induce apoptosis in macrophages in vitro by a Casp-1-independent mechanism (34), colonize the *casp-1*^{-/-} mice?

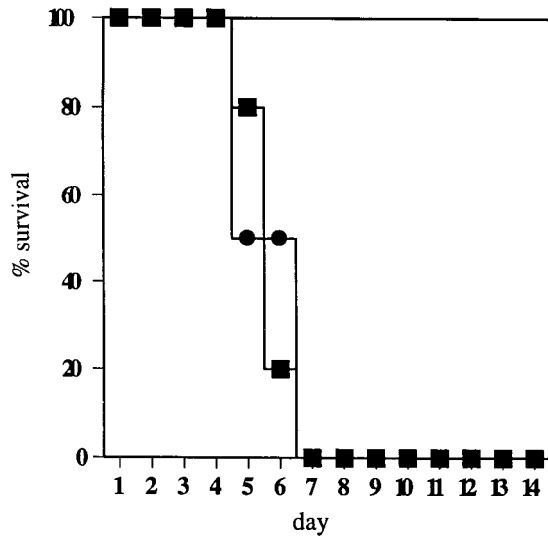


Figure 5. *casp-1^{-/-}* mice are susceptible to an i.p. route of inoculation with *S. typhimurium*. 100 SL1344 bacteria were injected into the peritoneal cavity of *casp-1* null (■) and wild-type (●) mice. The percentage of survival was recorded for 14 d. All *casp-1*-deficient mice died by day 7.

To test this idea, we inoculated wild-type and *casp-1^{-/-}* mice with 10⁹ CFU wild-type *Y. pseudotuberculosis*, a dose that is fivefold higher than the *Y. pseudotuberculosis* LD₅₀ for wild-type mice. In contrast to *Salmonella*, *Y. pseudotuberculosis* colonized the cecum and PP of both mouse strains (Fig. 7, a and b). The bacteria then spread to the MLNs and

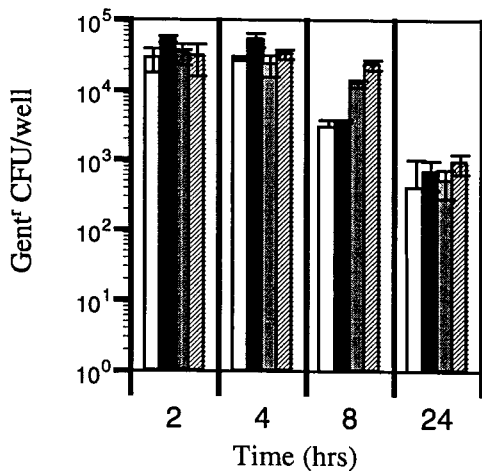


Figure 6. Activated peritoneal macrophages from wild-type and *casp-1^{-/-}* mice kill *S. typhimurium* in the absence of apoptosis. Peritoneal macrophages from wild-type mice were infected with opsonized, stationary phase SL1344 (white bars) or the noninvasive *S. typhimurium* mutant, BJ66 (black bars). Peritoneal macrophages from *casp-1^{-/-}* mice were infected with opsonized, stationary phase SL1344 (gray bars) or the noninvasive *S. typhimurium* mutant, BJ66 (hatched bars). The multiplicity of infection was 10 bacteria per macrophage. Under these conditions, there was no macrophage death as measured by assaying the culture supernatants for the presence of lactate dehydrogenase with a Cytotox96 Cell Death Kit (Promega).

spleens of both wild-type and *casp-1^{-/-}* mice in equal numbers (Fig. 7, a and b). Thus, we conclude that the relative inability of *Salmonella* to colonize the PP of *casp-1^{-/-}* mice was not due to a general antimicrobial compensatory mechanism specific to the genetic deficiency in the Casp-1 knockout mice.

Discussion

Previously we and others have shown that *Salmonella* induces apoptosis in macrophages and that during in vitro infection of macrophages this induction is dependent on the SPI1-encoded type III secretion protein SipB (13, 25, 35). We have also shown that *Salmonella*-induced apoptosis is dependent on the host protein Casp-1 (13). We find in this study that mice deficient for the host cysteine protease, Casp-1, are markedly resistant to colonization by wild-type *S. typhimurium*. Indeed, it requires 1,000-fold more bacteria by oral challenge to kill 50% of the *casp-1^{-/-}* mice as compared with wild-type mice. Yet, when *casp-1^{-/-}* mice are challenged intraperitoneally with *Salmonella*, there is no difference in susceptibility compared with wild-type mice as measured by spleen colonization or mortality. Thus, Casp-1 plays an essential role, that of allowing for bacterial colonization, in the innate immunity of the gastrointestinal tract to *Salmonella* infection.

We further addressed this role of Casp-1 in innate immunity by analyzing PP infected in a ligated intestinal loop model by confocal and electron microscopy as well as by a gentamicin protection assay. *Salmonella* was able to invade and induce membrane ruffling on the surface of M cells in the absence of Casp-1. At 1 h after infection, PP from wild-type but not from *casp-1^{-/-}* mice contained a significant increase in the number of cells undergoing apoptosis, both by electron microscopy and by an apoptotic-specific DNA stain (TUNEL reaction). Although some of the TUNEL⁺ cells in the dome area of the PP colocalized with *Salmonella*, we cannot rule out the possibility that after initial bacterium-dependent apoptosis there is rapid cytokine-dependent apoptosis of neighboring cells. Nevertheless, the apoptosis observed within 1 h of PP infection was Casp-1 dependent. The increase in apoptosis in the infected wild-type PP correlated with an increase in intracellular *Salmonella* and inflammation. Taken together, these data highlight the consequences of the *Salmonella*-Casp-1 interaction. Early in infection of the PP, the bacteria escape the macrophages by inducing apoptosis. The simultaneous recruitment of PMNs and other cellular elements of the immune system to the site of the infection appears to provide a new intracellular niche for the bacteria. In the *casp-1^{-/-}* mice, the lack of apoptosis and failure to recruit new immune cells results in the bacteria remaining within the host immune cells underlying the follicle-associated epithelium, which are likely to include resident macrophages and dendritic cells. Presumably the intracellular bacteria in the *casp-1^{-/-}* mice are eliminated by the resident immune cells. *Salmonella* survive within an intracellular site of wild-type PP, and whether or not PMNs are a safe haven at early times in the PP for in-

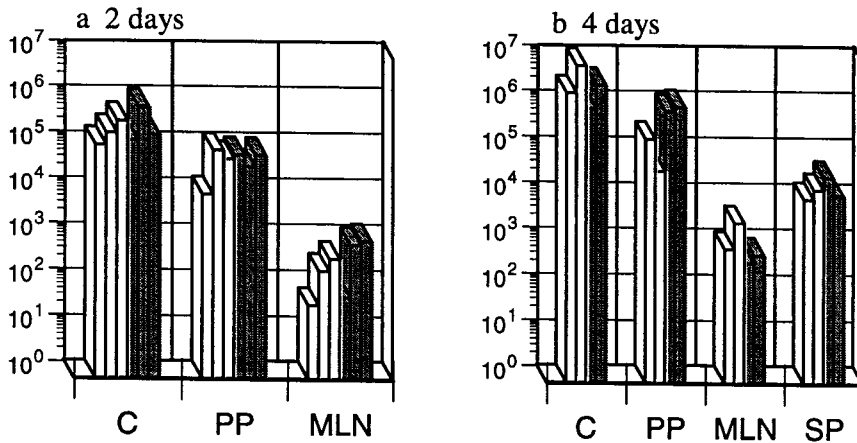


Figure 7. *casp-1*^{-/-} mice are colonized with *Y. pseudotuberculosis*. Wild-type mice (gray bars) and *casp-1*^{-/-} mice (white bars) were inoculated intragastrically with a wild-type *Y. pseudotuberculosis* strain, YPIIIpYV. C, cecum; PP, Peyer's patches; MLN, mesenteric lymph nodes; SP, spleen. *n* = 3 for day 2; *n* = 2 for day 4.

tracellular *Salmonella* as suggested in the spleen remains to be determined (36, 37).

Furthermore, *Y. pseudotuberculosis*, an enteric pathogen that induces Casp-1-independent macrophage apoptosis, infects both wild-type and *casp-1* null mice normally. Thus, *Y. pseudotuberculosis*, which also targets M cells to invade PP (38, 39) and induces apoptosis of macrophages in vitro and of Mac-1⁺ cells in vivo (40–42) when administered orally, was able to colonize the cecum, PP, MLNs, and spleens of both wild-type and *casp-1* null mice. [Q7] Therefore, the inability of *S. typhimurium* to disseminate from the intestine to the spleens of *casp-1*^{-/-} mice is not due to a mechanism that is nonspecifically inhibiting the dissemination of all enteric pathogens, and instead specifically requires Casp-1.

The data presented here, together with the identification of SipB as necessary for the activation of Casp-1 (13), indicate that SPI1-encoded proteins are important for the invasion of intestinal epithelium, which then allows for the subsequent dissemination of bacteria after oral infection (4–6). Recently, an SPI1-independent route for bacterial dissemination outside the gastrointestinal tract was described (43). This additional route is dependent on CD-18 cell types, which include macrophages and dendritic cells. It is noteworthy that CD-18-deficient mice are resistant to infection by this pathway. We believe that the five *casp-1*^{-/-} mice that contained low levels of *Salmonella* in the spleen but were not colonized in the PP (Fig. 1) were colonized via the CD-18-dependent route.

Richter-Dahlfors et al. have shown that *S. typhimurium* can induce apoptosis of macrophages in the liver after an intravenous route of inoculation (44). The roles of Casp-1 and SipB were not investigated. Our i.p. inoculation data indicate that, similar to SipB, Casp-1 is not necessary for *Salmonella* virulence once the bacteria have breached the gastrointestinal barrier. Thus, the apoptosis found in the liver (44) is either Casp-1 independent or not critical for *Salmonella* survival and replication in the spleen and liver.

The induction of Casp-1-dependent apoptosis immediately following *Salmonella* entry into PP coincided with the recruitment of PMNs and other immune cells and is associated with tissue destruction. Our data do not indicate

whether both the proapoptotic and the proinflammatory activities of Casp-1 are necessary for *Salmonella* to successfully colonize the PP. We are currently conducting experiments to address the relative contributions of these proinflammatory cytokines to PP colonization and systemic spread. *Shigella*, the causative agent of bacterial dysentery, which induces Casp-1-dependent apoptosis in macrophages through IpaB, a homologue of SipB, also initiates inflammation by a Casp-1-dependent mechanism (29, 45). Thus, *Salmonella* and *Shigella* appear to share the initial steps in setting up a gastrointestinal infection even though they produce quite different diseases.

The mechanisms of immunity to *Salmonella* require the action of many cytokines and soluble factors including IL-18, TNF- α , IL-12, IFN- γ , and nitric oxide (9–12, 23, 46–56). Thus, it is likely that once the bacteria breach the mucosal surface to enter the PP, there are multiple host defense and bacterial virulence factors that come into play. However, we have shown that the absence of a single host cell enzyme, Casp-1, renders mice resistant to *Salmonella* gastrointestinal infection. Thus, in wild-type mice, Casp-1 activation is a critical step in the establishment of a systemic disease.

Since Casp-1 can trigger a proinflammatory response, which is considered an antimicrobial host response, it is perhaps initially counterintuitive to learn that a host enzyme that initiates a proinflammatory response is required by *Salmonella* to produce a successful infection. One interpretation of this result is that *Salmonella* does not require the proinflammatory response at all and that the proapoptotic Casp-1 activity is all that is necessary for PP colonization. Even so, it seems remarkable that in *casp-1*^{-/-} mice the invading *Salmonella* do not simply avoid phagocytosis by entering the more numerous cell populations within the PP, such as B and T lymphocytes or the adjacent epithelium. Furthermore, the findings that some *Salmonella* mutants that are unable to induce apoptosis in vitro are not attenuated for virulence (57) indicate that this interpretation is too simplistic. The alternative explanation, which we favor, is that it is not only the capacity of *Salmonella* to induce apoptosis of PP macrophages that is required, but the

inflammatory cascade induced by *Salmonella* is also required for bacteria to cross cellular barriers and allow for their eventual replication and persistence in the reticuloendothelial system. The precise timing with which this pathogen triggers this cascade is probably important to the establishment of infection. Our results underscore the idea that bacterial pathogens have evolved to not only circumvent or subvert a host defense mechanism, but indeed to directly manipulate the host innate immune system.

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