Salmonella Rapidly Kill Dendritic Cells via a Caspase-1-Dependent Mechanism[1]

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Dendritic cells provide a critical link between innate and acquired immunity. In this study, we demonstrate that the bacterial pathogen *Salmonella enterica* serovar Typhimurium can efficiently kill these professional phagocytes via a mechanism that is dependent on *sipB* and the *Salmonella* pathogenicity island 1-encoded type III protein secretion system. Rapid phosphatidylserine redistribution, caspase activation, and loss of plasma membrane integrity were characteristic of dendritic cells infected with wild-type *Salmonella*, but not *sipB* mutant bacteria. Caspase-1 was particularly important in this process because *Salmonella*-induced dendritic cell death was dramatically reduced in the presence of a caspase-1-specific inhibitor. Furthermore, dendritic cells obtained from caspase-1-deficient mice, but not heterozygous littermate control mice, were resistant to *Salmonella*-induced cytotoxicity. We hypothesize that *Salmonella* have evolved the ability to selectively kill professional APCs to combat, exploit, or evade immune defense mechanisms. ***The Journal of Immunology*****, 2003, 171: 6742–6749.**

Immune surveillance mediated by dendritic cell is of central importance in innate and acquired immunity. To engage T lymphocytes during infection, dendritic cells internalize and destroy invading microbes and display pathogen-derived peptides on their surface in the context of MHC molecules (1–4). Recognition of Ag by T cells is critically important in acquired resistance against a variety of microbes, including the facultative intracellular bacterial pathogen *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). For example, selective depletion of $CD4^+$ T lymphocytes, and to a lesser extent $CD8^+$ T cells, significantly impairs the ability of adoptively transferred immune splenocytes to protect against challenge with virulent *Salmonella* (5). Furthermore, mice that are defective in MHC-dependent signaling to $CD4^+$ and $CD8^+$ T cells are highly susceptible to *Salmonella* infection (6, 7). Given the critical role of dendritic cells in stimulating the cellular arm of the adaptive immune system, it is important to better understand interactions between these professional phagocytes and *Salmonella*.

Ingestion of *S. typhimurium* results in a lethal, typhoid-like systemic infection in mice that is characterized by bacterial invasion of the distal ileum, followed by systemic dissemination and colonization of the liver and spleen. Following extensive replication within splenic and hepatic phagocytes, *Salmonella* re-enter the bloodstream, causing infected animals to succumb to septic shock and multiple organ failure (8). Numerous studies indicate that within professional APCs, *Salmonella* occupy an altered vacuolar compartment (9), suggesting that *Salmonella* have evolved strategies to resist and overcome innate immune defenses. Indeed, long-term residence and replication within phagocytic cells appear to be essential for *Salmonella* virulence because mutant bacteria that cannot survive or multiply inside macrophages are attenuated for systemic infection in mice (10). The complex nature of this host-pathogen interaction is further illustrated by recent evidence that *Salmonella* can kill macrophages via at least two distinct mechanisms, resulting in the rapid activation of host caspase-1 and caspase-2 (11–21). Caspase-1 is a member of a family of cysteine-containing aspartate-specific proteases that play an important role in apoptosis (22, 23). Intriguingly, caspase-1-deficient mice are resistant to intragastric, but not i.p. infection (24), which suggests that *Salmonella* exploit caspase-1 to disseminate systemically. Furthermore, caspase-1-dependent bacterial colonization of ileal Peyer's patches is characterized by the presence of numerous dying cells, as determined by TUNEL (24). Because the phenotype of these cells is not known, it is important to identify other Peyer's patch cell types that are susceptible to caspase-1-dependent *Salmonella*-induced cytotoxicity. For example, significant numbers of dendritic cells, which are among the first immune cells to encounter invading *Salmonella* that traverse the intestinal barrier, are found in the interfollicular regions and follicle-associated epithelium in the subepithelial dome of ileal Peyer's patches.

We report in this work that *Salmonella* can rapidly kill dendritic cells, and demonstrate that mutant bacteria that are unable to express either SipB or a functional *Salmonella* pathogenicity island (SPI[3])-1-encoded type III secretion system (TTSS) cannot induce dendritic cell death. Host caspase-1 contributes to *Salmonella*-induced cytotoxicity because dendritic cells obtained from caspase-1-deficient mice are significantly more resistant to *Salmonella*-induced death than are dendritic cells obtained from wild-type littermate control mice. These findings have important implications for understanding both *Salmonella* pathogenesis and host response to infection.

Materials and Methods

Dendritic cell preparations and culture conditions

Dendritic cells were cultured from the bone marrow of 8- to 10-wk-old female C57BL/6J, BALB/cByJ, or C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME). Caspase-1-deficient mice (25) and caspase-$1^{+/-}$

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[3] Abbreviations used in this paper: SPI, *Salmonella* pathogenicity island; LDH, lactate dehydrogenase; MOI, multiplicity of infection; TTSS, type III secretion system.

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littermate controls were a generous gift from Abbott Bioresearch Center (Worcester, MA). Bone marrow cultures were maintained for 5–7 days at 37°C and 7% CO_2 in DMEM (Invitrogen, Grand Island, NY), supplemented with 10% FBS, IL-4 (1 ng/ml; Roche Diagnostics, Indianapolis, IN), 10 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ), 25 mM HEPES buffer, and 1% penicillin/streptomycin. To prepare cells for infection, bone marrow cultures were incubated overnight in medium without antibiotics, after which $CD11c^+$ dendritic cells were purified using anti-CD11c-conjugated MACS microbeads and magnetic separation columns (Miltenyi Biotec, Auburn, CA). Where indicated, dendritic cells were matured or activated using antibiotic-free medium supplemented with 5 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) for the last 20–24 h of culture. LPS-stimulated dendritic cells expressed high levels of MHC-II on their cell surface and were poorly phagocytic (data not shown).

Flow cytometry

In preparation for flow cytometric analysis, dendritic cell populations were stained with allophycocyanin-conjugated anti-CD11b, PE-conjugated anti-CD11c, and FITC-conjugated anti-MHC-II mAb. To characterize the mechanism of *Salmonella*-induced cytotoxicity, purified dendritic cells were stained with FITC-conjugated annexin V and propidium iodide. All reagents were purchased from BD Biosciences (San Diego, CA). Cells were analyzed on a BD Biosciences FACSCalibur flow cytometer (Mountain View, CA).

Bacterial strains and growth conditions

Bacterial cultures were grown to stationary phase under aerobic conditions at 37°C in 3 ml Luria-Bertani broth (Difco Laboratories, Detroit, MI). When required, chloramphenicol and kanamycin were added to the medium at final concentrations of 30 and 60 μg/ml, respectively. Bacterial culture supernatants were collected following centrifugation of cultures grown to a cell density that would have killed dendritic cells. Heat-killed bacteria were obtained by incubating bacterial suspensions at 65°C for 10 min. Wild-type *S. typhimurium* SR-11 χ3041 and a set of isogenic *invA*, *sipB*, and *spiB* mutant strains have been described elsewhere (14).

Cytotoxicity assays

Immature dendritic cells (5×10^4 in a total of 50 μl/well) were infected with bacterial cultures grown to late-log phase, as described previously (14), at an input multiplicity of infection (MOI) of ~15 in a final volume of 100 μl, unless indicated otherwise. Three hours postinfection (except where noted differently), leakage of lactate dehydrogenase (LDH) from the dendritic cell cytoplasm was quantified colorimetrically using the Cytotox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI). The absorbance (A_{490}) was measured using a microtiterplate reader (SPECTRAmax; Molecular Devices, Sunnyvale, CA), after which the percentage of cytotoxicity was calculated using the following formula: 100% × ((experimental release − spontaneous release)/(maximum release − spontaneous release)). Spontaneous release is the amount of LDH released from the cytoplasm of uninfected cells, whereas the maximum release is the amount of LDH released from uninfected cells after treatment with lysis solution.

Inhibitor studies

Actin polymerization was blocked by pretreating dendritic cells for 15 min with 10-fold serial dilutions of cytochalasin D (ranging from 0.1 to 100 μg/ml; ICN Biomedicals, Aurora, OH). Caspase activity was chemically inhibited by pretreatment of dendritic cells for 1 h with 100 μM Z-VAD-fmk, a general caspase inhibitor (Bachem Bioscience, King of Prussia, PA). To specifically inhibit caspase-1, dendritic cells were incubated for 1 h with 2-fold serial dilutions of Ac-YVAD-cmk (ranging from 3.13 to 100 μM; ICN Biomedicals). To block nonspecific ion fluxes across the plasma membrane, dendritic cells were pretreated for 1 h with medium containing 5 mM glycine, as described previously (15). One-half of the antibiotic-free medium (50 μl) containing cytochalasin D, Z-VAD-fmk, Ac-YVAD-cmk, or glycine was removed following centrifugation, after which dendritic cells were resuspended and infected with *Salmonella*, as described above.

Dendritic cell invasion assays

Bacterial entry into immature dendritic cells was determined using a gentamicin protection assay that has been described previously (10). Bacteria that were not cell associated were removed 30 min after infection by washing cells three times with PBS. Infected dendritic cells were incubated for additional 1 h in the presence of medium containing gentamicin (25 μg/ml), an antibiotic that kills extracellular, but not intracellular, bacteria. Bacterial invasion was determined by plating for CFUs on Luria-Bertani-agar plates after washing and lysing infected dendritic cells with Triton X-100 (Sigma-Aldrich).

Results

Salmonella *efficiently kill* $CD11c^+$ *dendritic cells*

Microscopic studies aimed at improving our understanding of dendritic cell interactions with *Salmonella* revealed that *Salmonella* were cytotoxic to these professional APCs. *Salmonella*-induced death of C57BL/6J-derived $CD11c^+$ dendritic cells (Fig. 1*A*) was morphologically apparent within several hours of infection (data not shown) and was quantified by measuring leakage of intracellular LDH into the culture supernatant. Release of cytoplasmic LDH from *Salmonella*-infected dendritic cells was dependent on the MOI with near maximum release 3 h postinfection at an MOI of ~16 (Fig. 1*B*). Neither heat-killed bacteria nor concentrated bacterial culture supernatants induced significant release of LDH (Fig. 1*C*), indicating that a physical interaction between dendritic cells and live bacteria is required for *Salmonella*-induced cytotoxicity. The ability of *Salmonella* to kill dendritic cells does not appear to be regulated by growth phase because bacteria grown to late-log phase and stationary phase were equally cytotoxic (data not shown). Furthermore, splenic dendritic cells and human monocyte-derived dendritic cells were similarly sensitive to *Salmonella*-induced cytotoxicity (data not shown).

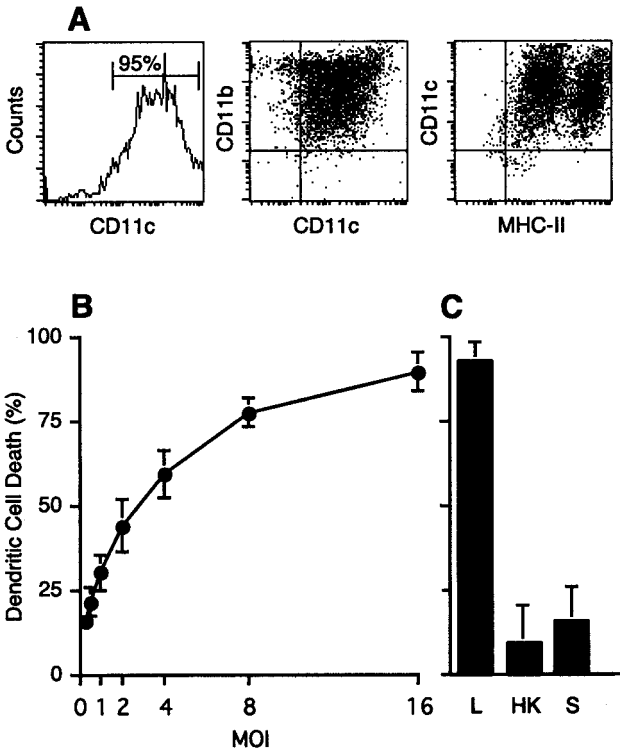

FIGURE 1. *Salmonella* efficiently kill $CD11c^+$ dendritic cells. Bone marrow-derived $CD11c^+$ dendritic cells obtained from C57BL/6J mice were purified and characterized by flow cytometry before infection. As expected, cell surface expression of CD11b, CD11c, and class II MHC varied among purified dendritic cells, indicating ongoing development and maturation (*A*). *Salmonella*-induced dendritic cell death, as quantified by measuring leakage of LDH from the dendritic cell cytoplasm 3 h postinfection, was dependent on the multiplicity of infection (*B*) and required live bacteria (*C*). Bacterial culture supernatant (S) and heat-killed (HK) *Salmonella* were noncytotoxic (*C*). Data are representative (*A*) or arithmetic means (*B* and *C*) of at least three independent experiments. Error bars indicate the SD of the mean.

Salmonella *rapidly kill dendritic cells via a* sipB- *and SPI-1-dependent mechanism*

Immature dendritic cells are highly phagocytic and readily ingest foreign Ags, such as microbes, via macropinocytosis (1, 3). To determine whether bacterial internalization is required for *Salmonella*-induced cytotoxicity, dendritic cells were treated with cytochalasin D before infection. As shown in Fig. 2*A*, the presence of cytochalasin D, which prevents cytoskeletal rearrangements by blocking actin polymerization, diminished *Salmonella*-induced killing of both immature and LPS-stimulated dendritic cells in a dose-dependent manner, suggesting that bacterial internalization is required for optimal cytotoxicity.

Because mature dendritic cells, which are highly efficient at stimulating T lymphocytes, do not readily ingest exogenous Ags (1, 3), these results suggest that *Salmonella* may actively induce their own uptake into these cells. Bacterial entry into nonphagocytic cells is dependent on the SPI1-encoded TTSS (26, 27). *Salmonella* use this highly specialized protein export apparatus to inject proteins that manipulate the actin-based cytoskeleton into the host cell cytoplasm (28). To determine whether the SPI1 invasion machinery is required for *Salmonella*-induced dendritic cell death, we tested a *Salmonella* strain deficient in *invA*, which encodes an essential structural component of this TTSS (26, 27). As shown in Fig. 2*B*, *Salmonella*-induced cytotoxicity was abrogated in immature dendritic cells infected with *invA*-mutant bacteria. *Salmonella*-induced dendritic cell death was also dependent on a functional *sipB* gene (Fig. 2*B*), which encodes a SPI1-secreted type III effector protein that is both necessary and sufficient to kill macrophages (20). In contrast to *invA* and *sipB* mutant bacteria, *spiB*-deficient *Salmonella* were fully cytotoxic (Fig. 2*B*), indicating that the SPI2-encoded TTSS, which is required for delayed induction of programmed macrophage cell death (14), is not required for dendritic cell killing. Notably, similar results were obtained when LPS-stimulated dendritic cells (Fig. 2*C*) and stationary phase bacterial cultures were used (data not shown), suggesting that macrophages (14) and dendritic cells respond differently to infection with *Salmonella*.

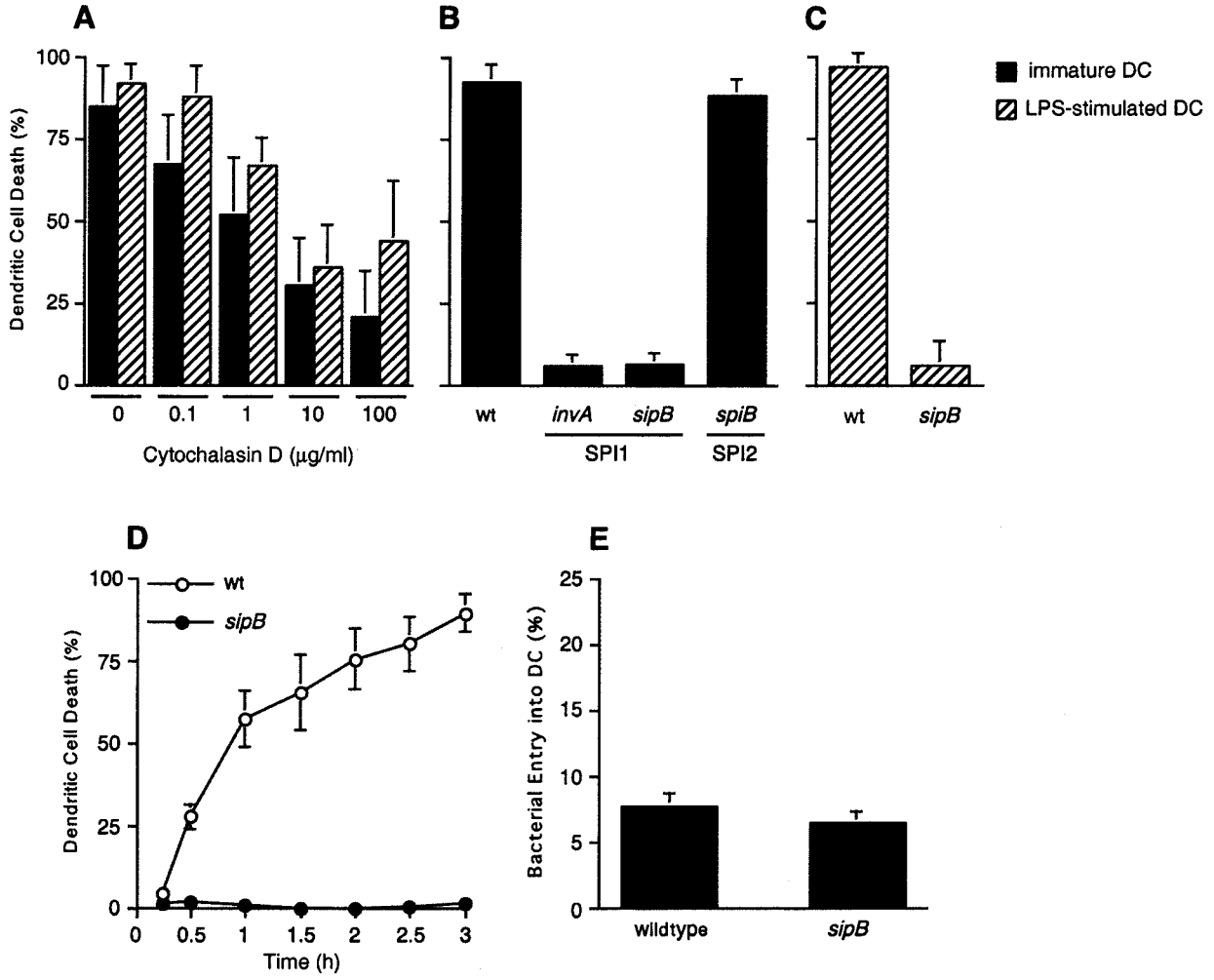

FIGURE 2. *Salmonella*-induced dendritic cell death is dependent on *sipB* and the SPI1-encoded TTSS. Pretreatment of immature and LPS-stimulated dendritic cells with cytochalasin D, which blocks bacterial internalization, reduced *Salmonella*-induced cytotoxicity in a dose-dependent manner (*A*). Bacterial induction of immature (*B*) and mature (*C*) dendritic cell death was dependent on the SPI1-encoded TTSS and *sipB*, encoding a SPI1-secreted effector molecule, but did not require the SPI2-encoded type III protein export apparatus. *Salmonella*-induced dendritic cell death was monitored over a 3-h period and quantified at 30-min intervals by measuring the release of intracellular LDH from dendritic cells infected with either wild-type or *sipB*-deficient bacteria (*D*). Comparable numbers of wild-type and *sipB*-deficient *Salmonella* were internalized by immature dendritic cells, as was determined using a gentamicin protection assay (*E*). Data from all graphs are arithmetic means of at least three independent experiments. Error bars indicate the SD of the mean.

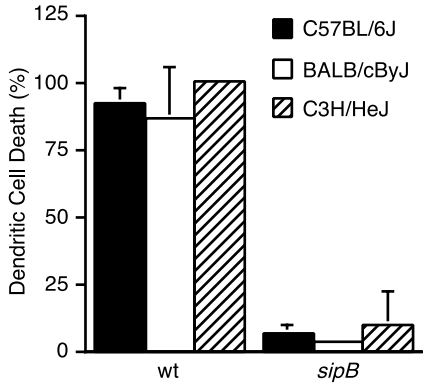

FIGURE 3. *Salmonella* efficiently kill dendritic cells obtained from mice with distinct genetic backgrounds. Dendritic cells obtained from susceptible C57BL/6J and BALB/cByJ mice and resistant C3H/HeJ mice were infected with either wild-type or *sipB* mutant bacteria at an MOI of ~15. *Salmonella*-induced cytotoxicity was quantified 3 h postinfection by measuring the release of cytoplasmic LDH. Data are arithmetic means of at least three independent experiments. Error bars indicate the SD of the mean.

When *Salmonella*-induced cytotoxicity was measured over time, it was revealed that dendritic cells infected with wild-type *Salmonella*, but not *sipB* mutant bacteria, released significant quantities of cytoplasmic LDH as early as 30 min postinfection (Fig. 2*D*). At this time, infected dendritic cells had internalized comparable numbers of wild-type and mutant bacteria as determined in a gentamicin protection assay (Fig. 2*E*). Collectively, these data indicate that *Salmonella* rapidly kill dendritic cells via a *sipB* and SPI1-dependent, SPI2-independent mechanism (Fig. 2, *B* and *C*). Furthermore, these results are evidence that long-term intracellular survival and replication are not required for *Salmonella*-induced dendritic cell death (Fig. 2*D*).

Dendritic cells from mice with naturally resistant and susceptible genetic backgrounds are similarly sensitive to Salmonella*-induced killing*

To demonstrate that *sipB*- and SPI-1-dependent *Salmonella*-induced cytotoxicity was not specific to C57BL/6J-derived dendritic cells, we confirmed that *Salmonella* are able to kill dendritic cells obtained from BALB/cByJ and C3H/HeJ mice (Fig. 3). Importantly, C3H/HeJ mice, but not C57BL/6J or BALB/cByJ mice, express functional Slc11a1 (Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1, also called Nramp1) and are resistant to infection with *Salmonella* (29, 30). These results therefore demonstrate that dendritic cells from mice with differentially susceptible genetic backgrounds are equally sensitive to *Salmonella*-induced cytotoxicity. Furthermore, C3H/HeJ mice are endotoxin resistant due to a spontaneous mutation in the *Tlr4* gene (31), which suggests that LPS-mediated signaling via Toll-like receptor 4 is not required for *Salmonella*-induced dendritic cell death.

Salmonella *rapidly induce phosphatidylserine redistribution, caspase activation, and membrane damage in infected dendritic cells*

To determine the nature of *Salmonella*-induced cytotoxicity, dendritic cells were stained with FITC-conjugated annexin V and analyzed by flow cytometry. Annexin V specifically binds to phosphatidylserine, a plasma membrane lipid that rapidly relocalizes from the inner leaflet to the outer leaflet in cells that are undergoing programmed cell death. As shown in Fig. 4*A*, a significant

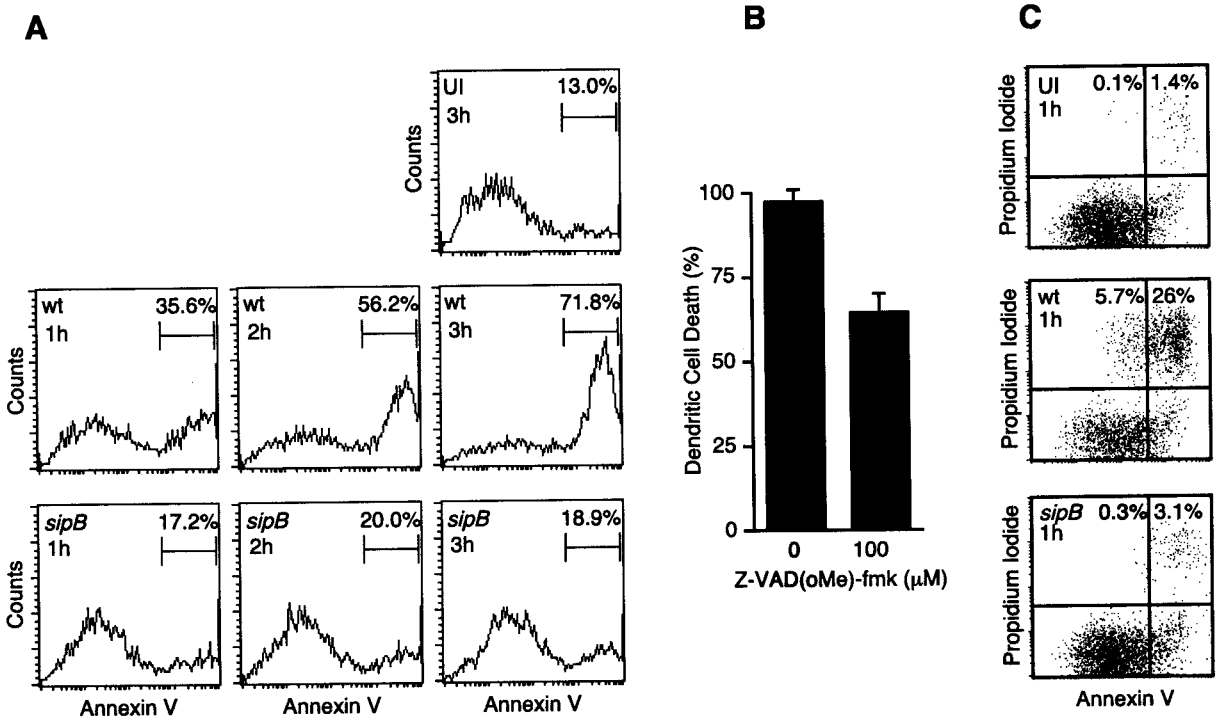

FIGURE 4. *Salmonella*-infected dendritic cells rapidly undergo programmed cell death. When compared with uninfected cells (UI), dendritic cells infected with wild-type *Salmonella* (wt), but not *sipB*-deficient bacteria (*sipB*), rapidly redistributed phosphatidylserine from the inner to the outer leaflet of the plasma membrane over time, as was determined by annexin V binding (*A*). In the presence of Z-VAD(oMe)-fmk, a general caspase inhibitor, *Salmonella*-induced dendritic cell death was partially blocked during a 3-h infection (*B*). Rapid loss of plasma membrane integrity as measured by propidium iodide staining was detected in dendritic cells infected for 1 h with wild-type *Salmonella*, but not *sipB* mutant bacteria, even in the absence of annexin V binding (*C*). Data are arithmetic means (*B*) or representative (*A* and *C*) of three independent experiments. Error bars indicate the SD of the mean.

number of wild-type *Salmonella*-infected dendritic cells stained positive for annexin V within 1 h, while the total number of annexin V-positive cells increased dramatically over time. In contrast, the degree of annexin V staining of dendritic cells infected with *sipB*-deficient *Salmonella* was comparable to that of uninfected control cells, even at 3 h postinfection (Fig. 4*A*). These results suggest that *Salmonella*, via a *sipB*- and SPI-1-dependent mechanism, induce a pathway(s) of programmed cell death in infected dendritic cells.

The major executors of programmed cell death are caspases, a family of cysteine-containing aspartate-specific proteases that facilitate systematic disassembly and disintegration of cellular structures (22, 23). To determine whether caspases were activated upon infection with *Salmonella*, dendritic cells were pretreated with Z-VAD(oMe)-fmk, an irreversible pan-caspase inhibitor. Although *Salmonella*-induced cytotoxicity was reduced in the presence of this chemical inhibitor during a 3-h infection, substantial amounts of cytoplasmic LDH continued to be released into the extracellular environment (Fig. 4*B*). One possible explanation for these results is that caspase activity was only partially blocked in Z-VAD(oMe)-fmk-treated dendritic cells. Alternatively, these observations may point to a rapid loss of plasma membrane integrity and subsequent leakage of cytoplasmic contents into the extracellular environment.

To distinguish between these two possibilities, dendritic cells were stained with FITC-conjugated annexin V and propidium iodide, a DNA intercalator that is excluded from cells with an intact plasma membrane. After only 1 h of infection, a significant proportion of dendritic cells infected with wild-type *Salmonella*, but not *sipB* mutant bacteria, stained positive for both annexin V and propidium iodide (Fig. 4*C*). In contrast, dendritic cells treated for several hours with gliotoxin, an apoptosis-inducing fungal toxin, stained positive for annexin V only (data not shown). Importantly, a small, but significant number of wild-type *Salmonella*-infected dendritic cells stained positive for propidium iodide only (Fig. 4*C*), suggesting that phosphatidylserine redistribution is not a prerequisite for *Salmonella*-induced loss of plasma membrane integrity. In summary, our results indicate that wild-type *Salmonella*, via a *sipB*- and SPI-1-dependent mechanism, induce phosphatidylserine translocation (Fig. 4*A*), caspase activation (Fig. 4*B*), and rapid loss of plasma membrane integrity (Figs. 2*D* and 4*C*) in infected dendritic cells.

Caspase-1 contributes to Salmonella*-induced dendritic cell death*

Recent evidence suggests that SipB is both necessary and sufficient to activate macrophage caspase-1 (20), although the precise mechanism of caspase-1 activation is not yet known (32). To determine whether caspase-1 plays a role in *Salmonella*-induced dendritic cell death, cells were pretreated with Ac-YVAD-cmk, an irreversible caspase-1 inhibitor. As shown in Fig. 5*A*, dendritic cells pretreated with Ac-YVAD-cmk were protected from *Salmonella*-induced cytotoxicity in a dose-dependent manner, although substantial amounts of LDH continued to be released into the extracellular environment, even at high concentrations of this caspase-1-specific inhibitor (Fig. 5*A*). Release of cytoplasmic LDH was not due to nonspecific leakage or drug-related cytotoxicity because LDH release from Ac-YVAD-cmk-treated cells infected with *sipB* mutant bacteria was negligible (Fig. 5*A*).

To determine whether caspase-1 is required for *Salmonella*-induced cytotoxicity, dendritic cells from caspase-1-deficient (25) and heterozygous littermate control mice were infected with either wild-type *Salmonella* or *sipB* mutant bacteria. As expected, caspase-$1^{+/-}$ dendritic cells rapidly released cytosolic contents

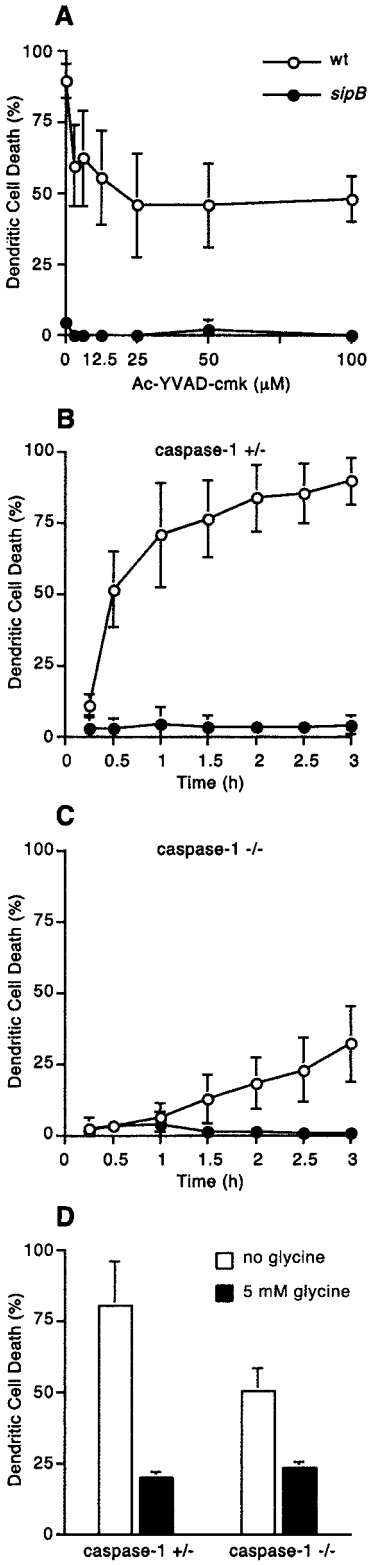

FIGURE 5. *Salmonella* rapidly kill dendritic cells via a caspase-1-dependent mechanism. Pretreatment of dendritic cells with the caspase-1-specific inhibitor Ac-YVAD-cmk reduced *Salmonella*-induced cytotoxicity in a dose-dependent manner (*A*), whereas dendritic cells from caspase-1-deficient mice (*C*), but not heterozygous littermate control mice (*B*), were resistant to *Salmonella*-induced killing. Dendritic cells obtained from caspase-1-deficient mice and heterozygous littermate control mice were protected from *Salmonella*-induced cytotoxicity in the presence of exogenous glycine (*D*), which inhibits necrotic cell death by blocking nonspecific ion fluxes across the plasma membrane. Data are arithmetic means of three independent experiments. Error bars indicate the SD of the mean.

into the extracellular environment following infection with wild-type, but not *sipB* mutant bacteria (Fig. 5*B*). In contrast, however, dendritic cells from caspase-1-deficient mice were significantly more resistant to *Salmonella*-induced cytotoxicity (Fig. 5*C*). Over time, some *sipB*-specific cytoplasmic leakage was detected in caspase-1-deficient dendritic cells (Fig. 5*C*), the linear increase of which suggests that *Salmonella* may inflict constant but permanent damage to the dendritic cell plasma membrane. To test this hypothesis, dendritic cells were pretreated with glycine, which inhibits necrotic cell death by blocking nonspecific ion fluxes across the plasma membrane (33–36). In agreement with recent evidence that glycine can inhibit bacterially induced host cell death (15, 37), *Salmonella*-induced cytotoxicity to both caspase-$1^{+/-}$ and caspase-$1^{-/-}$ dendritic cells was dramatically reduced in the presence of exogenous glycine (Fig. 5*D*). Cumulatively, these data are evidence that caspase-1 plays an important role in *Salmonella*-induced dendritic cell death. Some *sipB*- and SPI1-dependent toxicity was observed in caspase-1-deficient dendritic cells, which suggests that membrane damage-related toxicity is accelerated by caspase-1.

Discussion

Essential virulence strategies of the enteric pathogen *S. typhimurium* include survival and replication within macrophages (10). Recent evidence, however, suggests that during the initial gastrointestinal phase of infection, *Salmonella* may be internalized by dendritic cells (38–40). Although several studies suggest that internalized bacteria survive inside these professional APCs (41, 42), we demonstrate in this work that *Salmonella* can rapidly kill $CD11c^+$ dendritic cells. Mutant bacteria that do not assemble a functional SPI1-encoded type III protein export apparatus or strains that are unable to express the SPI1-secreted effector molecule SipB were noncytotoxic. Dendritic cells infected with wild-type, but not *sipB*-deficient *Salmonella*-redistributed phosphatidylserine, rapidly lost plasma membrane integrity, and were partially protected from *Salmonella*-induced cytotoxicity when pretreated with either a pan-caspase- or caspase-1-specific inhibitor. Furthermore, dendritic cells obtained from caspase-1-deficient mice, but not heterozygous littermate control mice, were significantly more resistant to *sipB*-dependent *Salmonella*-induced cytotoxicity, demonstrating that caspase-1 plays an important role in *Salmonella*-induced dendritic cell death. Some *sipB*- and SPI1-dependent toxicity was observed in the absence of caspase-1, however, suggesting that caspase-1 accelerates membrane damage-related toxicity. We have found only minor differences in the kinetics of SPI1-mediated, caspase-1-dependent cell death of macrophage vs dendritic cells. Most significantly, we have found that unlike *Salmonella*-induced macrophage cell death, the ability of *Salmonella* to kill dendritic cells does not appear to be regulated by bacterial growth phase. We hypothesize that macrophages and dendritic cells respond differently to infection with *Salmonella*.

Our results, which resemble caspase-1-dependent necrosis (15), have important implications for understanding *Salmonella*-induced macrophage and dendritic cell death, even though the molecular mechanisms of SipB translocation and caspase-1 activation are not yet fully understood (20, 43–46). Recent evidence indicates that certain extracellular stimuli, including ATP and nigericin, trigger caspase-1-dependent proteolytic processing of pro-IL-1β and externalization of mature IL-1β by inducing a net efflux of intracellular potassium (47–49). ATP-induced, but not nigericin-mediated release of active IL-1β is dependent on the $P2X_7$ receptor (50). Macrophages that express this nonspecific ATP-gated ion channel initially appear to selectively release mature IL-1β in response to ATP treatment, although leakage of other cytoplasmic contents, including LDH, is detected over time (47, 50). Thus, stimulation of the $P2X_7$ receptor channel causes cytoplasmic leakage and ultimately cell death. We hypothesize that a host cell receptor channel such as the $P2X_7$ receptor or perhaps the pore-forming components of the SPI1-encoded TTSS may play a role in *Salmonella*-inflicted damage to the dendritic cell plasma membrane.

The results of this study are likely to have important implications for understanding *Salmonella* pathogenesis and host response to infection. *Salmonella*, unlike viruses, are facultative intracellular pathogens that do not solely rely on host cells for survival and replication. Therefore, dendritic cell death does not prematurely terminate the bacterial life cycle per se. In fact, *Salmonella* appear to have evolved successfully to exploit programmed host cell death. Mouse virulence studies indicate that *Salmonella* critically depend on host caspase-1 for systemic dissemination. Following oral infection of caspase-1-deficient mice, virulent *Salmonella* are less efficient in colonizing ileal Peyer's patches and are unable to spread via mesenteric lymph nodes to the liver and spleen (24). As a result, these mice are 1000-fold more resistant to oral infection than wild-type mice. Caspase-$1^{-/-}$ mice are fully susceptible to i.p. challenge (24), however, which suggests that *Salmonella* exploit caspase-1 during the gastrointestinal phase of infection. In support of this hypothesis, bacterial colonization of ileal Peyer's patches is characterized by the presence of numerous dying cells (24), the identity of which remains unknown. Previously published evidence that *Salmonella* kill infected macrophages via a caspase-1-dependent mechanism (11, 13, 20) has led to the hypothesis that *Salmonella* exploit macrophages to spread to the liver and spleen (14, 51). This hypothesis is supported by mouse virulence studies demonstrating that SPI1-deficient *Salmonella*, which are unable to induce rapid, caspase-1-dependent macrophage cell death, are attenuated following intragastric, but not i.p. infection (52). Our results, however, suggest that *Salmonella*, to disseminate systemically, may also take advantage of resident dendritic cells in the gut-associated lymphoid tissue.

A role for dendritic cells in systemic dissemination of *Salmonella* is consistent with the presence of many TUNEL-positive cells in Peyer's patches obtained from *Salmonella*-infected wild-type, but not caspase-1-deficient mice (24). In addition, ex vivo ileal loop assays demonstrate that *Salmonella* infect Peyer's patch dendritic cells (38). It was also shown recently that *Salmonella* can cross the intestinal barrier within CD18-expressing phagocytes (39, 40), which include dendritic cells. There most likely will be differences in how *Salmonella* interact with and exploit macrophages vs dendritic cells in vivo. It is therefore important to better understand the effects of *Salmonella* infection on both of these cell types.

Tissue destruction, inflammation, and infiltration of polymorphonuclear lymphocytes and monocytes are hallmarks of gastrointestinal infection with *Salmonella*. Järveläinen et al. (32) proposed recently that nonspecific caspase-1-dependent inflammation-induced pathology may allow *Salmonella* to spread systemically following secondary invasion of a compromised intestinal barrier. Alternatively, caspase-1-mediated release of proinflammatory cytokines may be required for the recruitment of immune cells to the site of infection, potentially providing *Salmonella* with a new intracellular niche and a mode of transport to the liver and spleen (14, 51). Indeed, activation of caspase-1 results in the release of potent stimulators of cell migration, including IL-1β and IL-18 (42, 53–56). The hypothesis that dendritic cells may be the only way for bacteria to disseminate systemically (32) may be correct, but should be reviewed in light of the evidence presented in this study that dendritic cells and macrophages are similarly susceptible to *Salmonella*-induced cytotoxicity. Because the outcome of host-pathogen interactions during the natural course of

infection is profoundly influenced by the temporal and spatial expression of bacterial virulence factors and host proteins alike, these hypotheses may not be mutually exclusive and warrant further investigation. It is unlikely, although not impossible, that ineffective systemic dissemination of *Salmonella* in caspase-1-deficient mice is due to increased or altered innate phagocyte effector functions because bacterial survival within dendritic cells (unpublished observations) and macrophages (24) in vitro was unaffected by the absence of caspase-1. We do find that in the presence of gentamicin (an antibiotic that kills extracellular, but not intracellular, bacteria), the total number of wild-type bacteria recovered per well gradually diminishes over time when compared with *sipB*-deficient *Salmonella*. However, because *Salmonella*-infected dendritic cells rapidly lose plasma membrane integrity (Figs. 2*D*, 4*C*, and 5, *B–D*), it is difficult to dissect whether bacterial replication within dendritic cells is affected or whether gentamicin is getting into the cell, killing the bacteria.

It has become increasingly clear that dendritic cells play a central role in the generation of acquired immunity against microbial pathogens. The ability of an infected host to generate a protective immune response may therefore be affected more greatly by *Salmonella*-induced dendritic cell death than if we only understood macrophages to be killed. Dendritic cells, because of their ability to degrade invading microbes and efficiently present microbial peptides to T lymphocytes, form an important link between the innate and adaptive immune system. However, despite the pivotal role of T lymphocytes in acquired resistance to *Salmonella*, it is not known when and where *Salmonella*-specific T cell responses are initiated and what population(s) of professional APCs participates in the priming of these responses. An important role for dendritic cells in engaging T lymphocytes during *Salmonella* infection is suggested by in vitro evidence that these professional phagocytes efficiently present *Salmonella*-derived Ags to both $CD4^+$ and $CD8^+$ T cells either directly or following ingestion of infected macrophages that have undergone *Salmonella*-induced death (57, 58). Ex vivo data showing that *Salmonella* reside within Peyer's patch dendritic cells (38) and in vivo evidence demonstrating that flagellin-specific $CD4^+$ T cells, in response to *Salmonella* ingestion, rapidly migrate to and expand within the gut-associated lymphoid tissue (59) further suggest that resident dendritic cells may stimulate T lymphocyes during natural infection. To better understand these intricate interactions, we are currently investigating how *Salmonella*-induced macrophage and dendritic cell death affects the ability of an infected host to mount a protective immune response against this frequently lethal pathogen.

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