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Shigella-induced Apoptosis Is Dependent on Caspase-1 Which Binds to IpaB*

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We report here that the Shigella invasion plasmid antigen (Ipa)B, which is sufficient to induce apoptosis in macrophages, binds to caspase (Casp)-1, but not to Casp-2 or Casp-3. Casp-1 is activated and its specific substrate interleukin-1β is cleaved shortly after Shigella infection. Macrophages isolated from Casp-1 knock-out mice are not susceptible to Shigella-induced apoptosis, although they respond normally to other apoptotic stimuli. Shigella kills macrophages from *casp-3*, *casp-11*, and *p53* knock-out mice as well as macrophages overexpressing Bcl-2. We propose that Shigella induces apoptosis by directly activating Casp-1 through IpaB, bypassing signal transduction events and caspases upstream of Casp-1. Taken together these data indicate that Shigella-induced apoptosis is distinct from other forms of apoptosis and seems uniquely dependent on Casp-1.

Shigellae are the etiological agents of bacillary dysentery, an acute form of diarrhea accompanied by blood and mucus. Dysentery is an epidemiologically important disease which is often fatal in children. Shigellae are invasive bacteria that penetrate the colonic tissue and initiate an acute inflammation which is predominantly responsible for the ensuing symptoms (1, 2). *In vitro*, Shigellae invade eukaryotic cells and escape the phagosome to reach the cytoplasm (3). Both invasion and phagosome escape require the secretion of the three invasion plasmid antigens (Ipa)[1] proteins: IpaB, IpaC, and IpaD (4, 5).

Shigella induces apoptosis in macrophages, but not other cell types (6). *In vivo,* induction of apoptosis in Shigellosis was demonstrated in an animal model (7) and dysenteric patients (8). IpaB is sufficient to induce apoptosis in macrophages (9). This invasin is secreted by intracellular bacteria and distributed in the cell cytoplasm (10) where it binds to a caspase, and activates apoptosis (9).

Caspases are cysteine proteases that cleave after aspartate residues and activate apoptosis in eukaryotic cells. Eleven different caspases have been identified in mice and humans, each with a distinct substrate specificity. These enzymes are translated as zymogens which autocatalyze their cleavage into mature enzymes. The amino-terminal domain of caspase precursors does not contribute to the mature enzyme and may be involved in the regulation of maturation. Mature caspases are heterodimers formed by two subunits derived from the carboxyl-terminal domain of the precursor (11).

Based on sequence comparison and substrate specificity, caspases can be classified into three subfamilies. Caspase-1 (Casp-1, interleukin-1β converting enzyme), Casp-2 (Nedd-2, Ich-1), and Casp-3 (CPP32/Yama/Apopain) are prototypic examples of each subfamily (11, 12). Casp-1 was originally purified based on its ability to convert the proinflammatory cytokine interleukin-1β (IL-1β), from a 30-kDa precursor to a biologically active 17-kDa protein (13, 14). Casp-1 also activates IL-18, a cytokine that induces interferon-γ (15, 16). Caspases are homologues of the *Caenorhabditis elegans* cell death gene *ced-3*. Casp-1, as well as most other caspases, activates apoptosis when overexpressed in culture cell lines (17). Surprisingly, mice with a targeted deletion in *casp-1* do not have an overt apoptosis-related phenotype. Yet, they are relatively resistant to endotoxin-induced shock, presumably because they cannot process IL-1 (18, 19) and IL-18 (15, 16). ATP induced apoptosis is as efficient in macrophages explanted from *casp-1* −/− as in macrophages from wild type mice (18). Thus, Casp-1 is not believed to be necessary for apoptosis during development or in homeostasis.

Casp-11 (Ich-3) is closely related to Casp-1 (20). As opposed to Casp-1 which is constitutively expressed, Casp-11 is induced in macrophages only after LPS stimulation. Overexpression of Casp-11 results in IL-1β cleavage, even though Casp-11 does not directly cleave this cytokine. Moreover, overexpression of Casp-1 in cells from *casp-11* −/− mice does not induce apoptosis. Similar to *casp-1* −/− mice, *casp-11* knock-outs develop normally and are resistant to endotoxin-induced shock (21). Taken together, these data suggest that Casp-11 acts upstream of Casp-1.

Casp-3, the mammalian caspase most highly related to Ced-3, cleaves a number of homeostatic, repair, and structural proteins (22–24). Casp-3 is activated in many different systems. In contrast to *casp*-1- and *casp-11*-deficient mice, *casp-3* knockouts die prematurely and have a profound defect in brain development (25). Thymocytes from these mice are susceptible,

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[1] The abbreviations used are: Ipa, invasion plasmid antigen; Casp, caspase; IL, interleukin; LPS, lipopolysaccharide.

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however, to some apoptotic stimuli. Casp-3 is therefore required in many, but not all pathways that lead to programmed cell death. Casp-2 is alternatively spliced and generates a pro- and an anti-apoptotic molecule (26). In *casp-2* −/− mice apoptosis mediated by perforin and granzyme B was defective but developmental cell death of motor neurons was accelerated (27). The position of Casp-2 in a "caspase cascade" is still unclear (28, 29).

Here, we show that IpaB binds to Casp-1, but not Casp-2 or Casp-3. As expected, Casp-1 is activated and IL-1β is cleaved during a Shigella infection. Using macrophages elicited from mice with different targeted deletions, we demonstrate that Shigella requires Casp-1, but not Casp-3 or Casp-11 to induce apoptosis. Shigella-induced apoptosis proceeds in the absence of the apoptosis promoter p53 and in the presence of the anti-apoptotic protein Bcl-2. We propose a model where Shigella bypasses upstream apoptotic regulators to directly activate Casp-1.

EXPERIMENTAL PROCEDURES

Ligand Blot of His-tagged Caspase Fusion Proteins—His-tagged Casp-1 was constructed by releasing the cDNA from pJ.348 (17) by a *Bam*HI, *Sal*I digestion, cloning into pGSTag (30), and subsequently subcloning into pRSETA (Invitrogen, Carlsbad, CA). *casp-2* (31) and *casp-3* (22) were amplified by polymerase chain reaction from pMSN2.4 and pSK-CPP32α, respectively (kindly provided by Dr. S. Kumar; Hanson Center for Cancer Research, Adelaide, Australia, and Dr. E. S. Alnemri; Jefferson Cancer Institute, Philadelphia, PA). The polymerase chain reaction products were cloned into the *Bam*HI and *Pst*I sites of pRSETA and sequenced.

The constructs (*His-casp-1*, *-2,* and*-3*) were transformed into *Escherichia coli* BL21(DE3)/pLysS and induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside at an OD_{595} of 0.8–1.0 (1 h at 37 °C). Bacterial pellets were resuspended in binding buffer containing 1 mM phenylmethylsulfonyl fluoride, lysed by sonication, and the His-tagged caspases were purified by nickel chelate chromatography according to the manufacturer's instructions (Novagen, Madison, WI). His-Casp-3 was soluble and stable under the above conditions, while His-Casp-2 had to be solubilized from inclusion bodies by addition of 1.5% Sarcosyl. His-Casp-1 and -2 were stabilized by addition of either 100 μM acetyl-Tyr-Val-Ala-Asp-CHO (YVAD-CHO, Biomol, Plymouth Metting, PA) or 100 μM YVAD-CHO and acetyl-Asp-Glu-Val-Asp-CHO (DEVD-CHO), respectively. The purified His-tagged caspases were quantified by Western blot using a monoclonal anti-T7-Tag antibody (Novagen).

The probes for the ligand blot experiment, GSTag-IpaB and GSTag, were purified and labeled with protein kinase A/[γ-^{32}P]ATP as described (9). Nitrocellulose membranes containing similar amounts of His-Casp-1, -2, and 3 were blocked for 2 h at room temperature in 20 mM Tris, pH 7.5, 20 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, 5% milk. The blocked membranes were probed with ^{32}P-labeled GSTag-IpaB ([^{32}P]GSTag-IpaB) or [^{32}P]GSTag, diluted in blocking buffer containing 50 mM NaCl but no milk (1.5 h, room temperature), and washed twice for 30 min with blocking buffer without milk. The radiolabeled membranes were exposed to a PhosphorImager screen.

Bacteria and Growth Conditions—M90T, an invasive isolate of *Shigella flexneri* serotype 5, is our virulent strain of reference (32). An IpaB deletion mutant of M90T, referred to as Δ*ipaB* (4), or a plasmid-less non-virulent derivative, BS176, were used as negative controls. Bacteria were grown in tryptic soy broth at 37 °C with aeration. The bacteria were washed and resuspended in RPMI 1640 medium before infection of macrophages.

Collection of Peritoneal Macrophages—casp-1 (18), *casp-3* (25), and *casp-11* (21) knock-out mice between 6 and 7 weeks old were obtained from the Animal Resource Facility at BASF Bioresearch Corp., Yale and Harvard Universities, respectively. *p53* knock-out and C57BL/6 control mice were obtained from Jackson Laboratory (Bar Harbor, ME). Four days after an intraperitoneal injection of 1 ml of thioglycolate, macrophages were collected by peritoneal lavage with cold phosphate-buffered saline and washed. Macrophages were isolated by adherence at 37 °C for 90 min and cultured in RPMI-1640 glutamine medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), penicillin (10,000 units/ml), and streptomycin (10,000 μg/ml).

Macrophage Infection and Cytotoxicity Assays—Macrophages were infected at a multiplicity of infection of 100 bacteria (colony forming units) per cell as described previously (33). Cytotoxicity was measured using the CytoTox 96 assay, according to the manufacturer's instructions (Promega, Madison, WI). LDH release is a measure of cell death, not specifically of apoptosis. Nevertheless, we have previously established that Shigella kills by apoptosis (6). Furthermore, in order to detect apoptotic cells, similar preparations were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, stained with propidium iodide at a concentration of 0.01 mg/ml (Molecular Probes, Inc., Eugene, OR), and observed with a fluorescence microscope. Macrophage like RAW cells transfected with *bcl-2* were kindly provided by Dr. Brune (34). For Etoposide (Sigma) experiments, 10^6 cells were treated with 50 μM for 24 h.

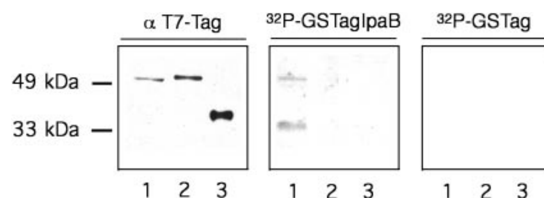

FIG. 1. **IpaB binds to Casp-1 but not to Casp-2 or Casp-3.** Purified His-tagged Casp-1 (*lane 1*), Casp-2 (*lane 2*), and Casp-3 (*lane 3*) were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an anti-T7 Tag antibody to assess the amount of protein loaded. Identical membranes were probed with either [^{32}P]GSTagIpaB or [^{32}P]GSTag as a control. [^{32}P]GSTagIpaB bound to Casp-1 but not to the other two caspases. The control [^{32}P]GSTag did not bind to any of the caspases.

Maturation of ICE and IL-1β in Peritoneal Macrophages Infected with Shigella—Peritoneal macrophages were elicited from Swiss Webster mice, selected and infected as described above. 0.5 × 10^6 macrophages were activated overnight with 1 μg/ml LPS (Shigella serotype 1A; Sigma), when indicated. Prior to infection, the cells were washed with serum-free RPMI 1640, and, when indicated, the cells were incubated for 1 h with 50 μM YVAD-CMK. After infection at a multiplicity of infection of 50 the macrophages were lysed *in situ* at given time points as described previously (35). Samples containing equal protein amounts were separated on 15% SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed by Western blot using either a rabbit anti-Casp-1 (kindly provided by Dr. D. Miller; Merck, Rahway, NJ), or a goat anti-IL-1β (R&D Systems, Minneapolis, MN) antibody.

RESULTS

IpaB Binds to Casp-1, But Not to Casp-2 or Casp–3—The *S. flexneri* invasin IpaB was shown to bind to a caspase in macrophage lysates both *in vitro* and *in vivo* (9). The IpaB-binding caspase was tentatively identified as Casp-1 using a polyclonal antiserum (9). To confirm that Casp-1 binds IpaB, and test whether other caspases also bind this bacterial protein, we performed a ligand blot assay. We chose to test Casp-1, Casp-2, and Casp–3 as representative members of the three caspase subfamilies (11).

Purified His-tagged Casp-1, Casp-2, and Casp–3 were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose. An anti-T7 monoclonal antibody which recognizes an epitope within the His-tag was used to verify the amount of protein loaded into each lane (Fig. 1). We used protein-kinase A/[γ-^{32}P]ATP to radiolabel purified GSTag and GSTagIpaB (30). Identical membranes containing the different caspases were probed with either [^{32}P]GSTagIpaB or [^{32}P]GSTag as a control. As shown in Fig. 1, [^{32}P]GSTagIpaB binds to Casp-1 but not to Casp-2 or Casp–3, while [^{32}P]GSTag did not bind to any of these caspases. IpaB bound both to the Casp-1 zymogen of 45 kDa as well as to a degradation product of around 30 kDa. This indicates that IpaB specifically binds to Casp-1.

Casp-1 Is Activated during Shigella Infections—Since IpaB binds to Casp-1 we tested whether Casp-1 matures from its zymogen to its active form during infection. In these experiments we infected macrophages activated with LPS, to be able to detect IL-1β. Lysates were made at the indicated time points and analyzed by immunoblots for the maturation of Casp-1 and

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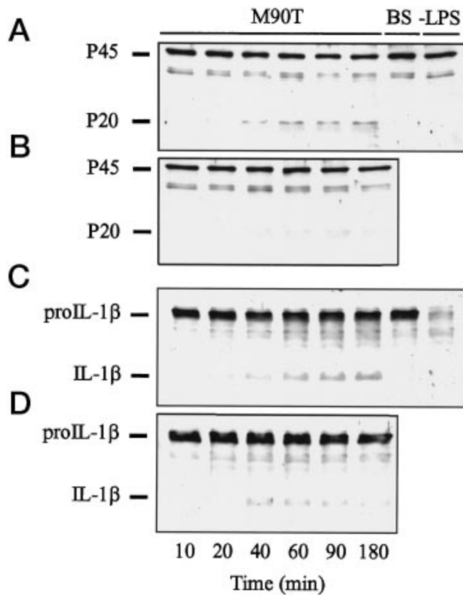

FIG. 2. **Casp-1 matures during *S. flexneri* infection.** Naive or LPS-activated peritoneal macrophages from wild type mice were infected with virulent (*M90T*) or avirulent (*BS176*) *S. flexneri*. Lysates collected at the indicated post-infection time points were analyzed by Western blot. *A,* Western probed with an anti-Casp-1 antibody. Casp-1 is expressed at comparable levels in naive and LPS-activated macrophages and matures shortly after infection with M90T as indicated by the appearance of the 20-kDa band. *B,* Western probed with an anti-Casp-1 antibody of macrophages treated with the Casp-1 inhibitor YVAD-cmk before infection. Casp-1 maturation is blocked by YVAD-cmk. *C,* Western probed with an anti-IL-1β antibody. IL-1β is expressed only in LPS-activated macrophages and matures after infection with M90T as indicated by the appearance of the 17-kDa band. *D,* Western probed with an anti-IL-1β antibody of macrophages treated with the Casp-1 inhibitor YVAD-cmk before infection. As expected, IL-1β maturation is blocked by YVAD-cmk.

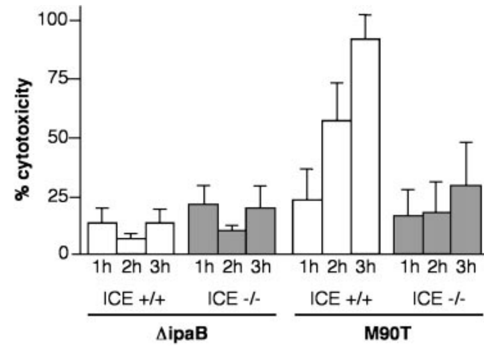

FIG. 3. **Macrophages from *casp-1* −/− mice are not susceptible to *S. flexneri* induced apoptosis.** Peritoneal macrophages were isolated from wild type or *casp-1* −/− mice and infected with either M90T or a deletion mutant in *ipaB* (Δ*ipaB*). At the indicated time points, cytotoxicity was determined by measuring the release of cytoplasmic lactate dehydrogenase (*LDH*). Only macrophages from wild type mice are susceptible to Shigella-induced apoptosis.

IL-1β. We detected Casp-1 maturation from the 45-kDa precursor to the 20-kDa subunit as early as 20 min after infection (Fig. 2*A*). This anti-Casp-1 antibody recognizes the 20 kDa, but not the 10-kDa subunit of the mature enzyme. As expected, expression of Casp-1 was not up-regulated after activation with LPS and Casp-1 did not mature in macrophages infected with the non-virulent strain BS176. We also tested whether the caspase inhibitor YVAD could block caspase maturation. YVAD inhibited Casp-1 maturation (Fig. 2*B*), as well as Shigella-induced apoptosis (9).

IL-1β, a substrate of Casp-1, was cleaved with similar kinetics to Casp-1. Not surprisingly, expression of IL-1β was only detected in LPS-activated macrophages. IL-1β was not cleaved in macrophages infected with BS176 (Fig. 2*C*). IL-1β maturation was significantly retarded in macrophages preincubated with YVAD (Fig. 2*D*). These data show that Casp-1 is activated in Shigella-infected macrophages.

Casp-1 −/− Macrophages Are Resistant to Shigella-induced Apoptosis in Vitro—To further investigate the role of Casp-1 in Shigella-induced apoptosis, we infected peritoneal macrophages from wild type and *casp-1* −/− mice (18), with either the *S. flexneri* wild type strain M90T or an isogenic strain with a deletion in *ipaB* (Δ*ipaB*, Ref. 4). M90T killed more than 90% of a culture of macrophages from wild type mice within 3 h of infection (Fig. 3). In contrast, macrophages from *casp-1* −/− mice were resistant to Shigella-induced cell death. Macrophages infected with Δ*ipaB* were not killed. Infected macrophages were also stained with propidium iodide and scored for apoptotic morphology. These experiments confirmed that *S. flexneri* induces apoptosis in wild type but not in *casp-1* −/− macrophages (data not shown). Thus, unlike apoptosis induced by other stimuli (18), Shigella-mediated macrophage death requires *casp-1*.

Casp-3 and Casp-11 Are Not Necessary for Shigella-induced Apoptosis—We tested whether macrophages from *casp-3* knock-out mice (25) were susceptible to Shigella-induced cytotoxicity. Peritoneal macrophages isolated from wild type and *casp-3* −/− mice were killed with equal efficiency by Shigella (Fig. 4A). As expected, BS176 was not cytotoxic.

Similar results were obtained with macrophages from *casp-11* knock-out mice (21). Casp-11 appears to be an upstream regulator of Casp-1 activation (21). As shown in Fig. 4*B*, virulent Shigella killed macrophages isolated from control or *casp 11* −/− mice to the same extent. BS176 was not cytotoxic to macrophages from either mice lineage. Since Casp-11 is induced after LPS activation, we also tested LPS-activated macrophages from control and *casp 11* −/− mice. M90T induced cell death with equal efficiency in both types of activated macrophages (data not shown).

Shigella-induced Apoptosis Is Independent of p53 and Cannot be Inhibited by Bcl-2—To further investigate the nature of the apoptotic cascade engaged in macrophages infected by Shigella, we tested the role of the tumor suppressor, *p53,* as well as the apoptosis inhibitor oncogene *bcl-2*. p53 is necessary for apoptosis induced by numerous stimuli (36). Conversely, apoptosis is often inhibited by overexpression of Bcl-2 (37, 38). Shigella killed *p53* −/− as efficiently as wild type macrophages (Fig. 5*A*), demonstrating that Shigella-induced apoptosis is independent of p53. BS176 was unable to kill either wild type or *p53* −/− macrophages.

In order to test whether overexpression of Bcl-2 affects Shigella-induced apoptosis, the macrophage-like cell line RAW stably transfected with either the vector alone, or the vector expressing Bcl-2 (34) was infected with M90T or BS176. As shown in Fig. 5*B*, overexpression of Bcl-2 did not protect macrophages from Shigella killing. BS176 is not cytotoxic to either *bcl-2* or vector-transfected cells. Similar results were obtained with differentiated U937 cells overexpressing Bcl-x_L, another anti-apoptotic protein of the Bcl-2 family (Ref. 39, data not shown).

Expression of Bcl-2 in the transfected macrophages was confirmed by Western blot (data not shown). Furthermore, in order to determine whether the Bcl-2 overexpressed in RAW cells was functional, we treated these macrophages with the topoisomerase inhibitor Etoposide, an inducer of apoptosis that is known to be inhibited by Bcl-2. Twenty-four hours after treatment almost 50% of the vector control cells yet only 5% of the Bcl-2 expressing cells died (Fig. 5*C*), indicating that Bcl-2 expressed in RAW cells is biologically active.

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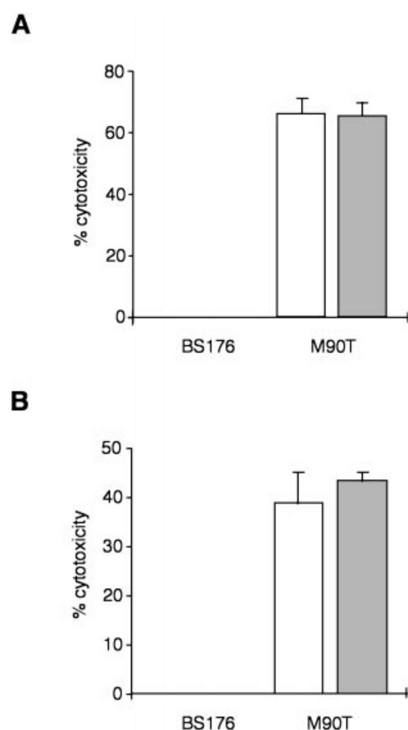

FIG. 4. ***S. flexneri* is cytotoxic to macrophages from both *casp-3* and *casp-11* knock-out mice.** *A,* peritoneal macrophages were isolated from wild-type (□) or *casp-3* −/− (▨) mice or *B,* from *casp-11* −/− (▨) mice. Macrophages were infected either with M90T or BS176. Cytotoxicity was determined by measuring the release of LDH after 3 h. Shigella is cytotoxic to macrophages from both *casp-3* −/− and *casp-11* −/− mice.

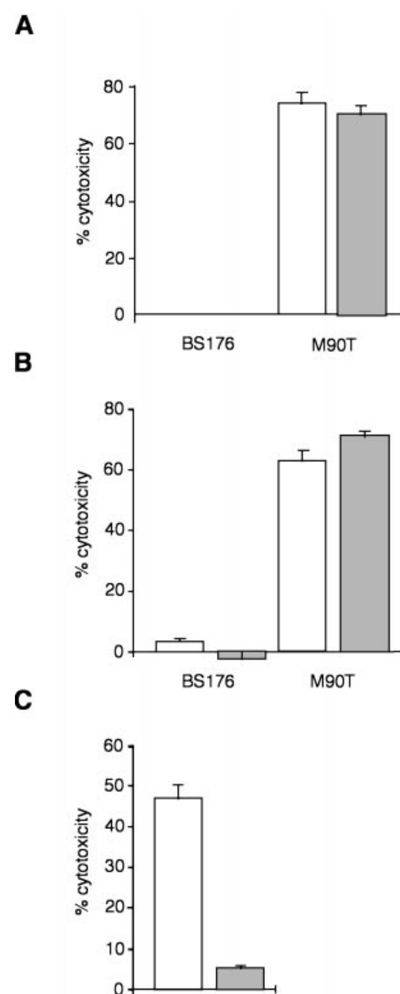

FIG. 5. ***S. flexneri* is cytotoxic to macrophages from *p53* knock-out mice and to RAW cells overexpressing Bcl-2.** *A,* peritoneal macrophages isolated from wild-type (□) or *p53* −/− (▨) mice and *B,* RAW cells transfected with the vector control (□) or with the vector encoding *bcl-2* (▨) were infected with either M90T or BS176. *C,* RAW cells transfected with the vector control (□) or with the vector encoding *bcl-2* (▨) were treated with Etoposide for 24 h. Cytotoxicity was determined by measuring the release of LDH. M90T is cytotoxic to macrophages from both *p53* knock-out mice and to RAW cells overexpressing Bcl-2. However, Bcl-2 could protect RAW cells from Etoposide-induced apoptosis.

DISCUSSION

Among the caspases, Casp-1 appears to be unique since it induces apoptosis and activates two potent proinflammatory cytokines, IL-1β and IL-18 (15, 16). Interestingly, these cytokines do not encode a signal sequence and it is still unknown how they reach the extracellular space after cleavage by Casp-1 (40, 41). The link between apoptosis and inflammation by Casp-1 is surprising, since apoptosis is classically considered to be a immunologically silent cell death (42). Indeed, during development and homeostasis, numerous apoptotic events occur without eliciting inflammation. The role of macrophage cell death in the release of IL-1β and IL-18 is still not understood.

Although Casp-1 induces apoptosis when overexpressed in tissue culture cells (17) the role of this enzyme in apoptosis has remained enigmatic. *casp-1* −/− mice develop normally and macrophages from these mice undergo apoptosis induced by several stimuli (18, 19). These results suggest either that Casp-1 function is redundant or is not required in the pathways leading to apoptosis in development or in response to certain stimuli.

The Shigella invasin IpaB binds specifically to Casp-1 but not Casp-2 or Casp-3 (Fig. 1). Based on sequence homology, Casp-1, Casp-2, and Casp-3 are representatives of the three caspase subfamilies (11). It seems unlikely that IpaB binds to caspases closely related to Casp-1, since Shigella kills macrophages derived from *casp-11* −/− mice. Casp-11 is closely related to *casp-1* and is classified within the same caspase family (Fig. 4). IpaB bound both the zymogen of Casp-1 as well as a protein of 30 kDa that could be an intermediate maturation product.

Casp-1 is processed shortly after Shigella infection suggesting that IpaB can promote this process (Fig. 2*A*). Cleavage of pro-IL-1β indicates that the mature Casp-1 is active during infection (Fig. 2*C*). The irreversible inhibitor YVAD-cmk has higher affinity for Casp-1 than for other caspases. This inhibitor blocks Shigella-induced apoptosis (9) and also Casp-1 activity as shown by its inhibition of IL-1β cleavage (Fig. 2*D*). Surprisingly, YVAD also inhibits Casp-1 maturation (Fig. 2*B*). Although in cell lysates, YVAD has a significantly higher affinity for mature Casp-1 than for its zymogen (43), the affinity of the Casp-1 zymogen for YVAD *in vivo* is not known. Our results could indicate that in Shigella infections: 1) YVAD inhibits the autocatalytic activity of the zymogen, 2) that mature Casp-1 activity is necessary to activate the Casp-1 precursor, or 3) that a different caspase with high affinity for YVAD is necessary for Casp-1 activation.

Casp-1 is necessary for Shigella-induced apoptosis since these bacteria do not kill macrophages from mice with a targeted deletion in this protease (Fig. 3). These results are surprising, as macrophages isolated from *casp-1* −/− are susceptible to other apoptotic stimuli (18). Although Casp-3 is implicated in many different apoptotic processes and is thought to be a downstream effector protease in a caspase cascade (11), it is not necessary for Shigella killing (Fig. 4).

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Recently, Casp-11 was shown to regulate Casp-1 activation (21). Data presented here suggest that Shigella directly activates apoptosis through Casp-1, since macrophages from *casp-11* −/− mice are insensitive to Shigella cytotoxicity (Fig. 4). The independence of Shigella-induced apoptosis of Casp-11 is not surprising since both naive and activated wild type or *casp-11* macrophages are susceptible to Shigella-induced cell death (Figs. 2 and 4, Ref. 33), although Casp-11 is up-regulated only in activated macrophages. Thus, Shigella induces a unique form of apoptosis which is dependent on Casp-1, does not require Casp-3, and bypasses the requirement for Casp-11 activation.

The pro-apoptotic function of Casp-1 in Shigella-induced apoptosis appears to be independent of mature IL-1β. Pretreatment of macrophages with IL-1 receptor antagonist, a natural competitor of IL-1, does not prevent Shigella-induced apoptosis.[2] Furthermore, filtered, IL-1 rich, supernatants of macrophages infected with Shigella are not cytotoxic to naive macrophages.[3] These results strongly suggest that Casp-1-induced apoptosis does not function through IL-1β.

Consistent with the model that Shigella induces apoptosis by directly activating Casp-1 is the observation that this process does not require p53 (Fig. 5). p53 is a transcriptional activator that is necessary for apoptosis initiated by diverse stimuli after G_1 arrest in the cell cycle (36). The susceptibility of *p53* −/− macrophages to Shigella is not surprising, since *S. flexneri*-induced apoptosis does not require transcription or translation (6) and can occur at any point during the cell cycle.[3] Bcl-2, and other members of the same family, are thought to be pore forming proteins that regulate traffic across mitochondrial and probably other membranes (38). Bcl-2 (Fig. 5*B*) and Bcl-x_L (data not shown), two anti-apoptotic members of the Bcl-2 family, do not inhibit Shigella-induced cell death. These data suggest that Bcl-2 acts upstream of Casp-1 activation or that these anti-apoptotic proteins cannot inhibit Casp-1-dependent cell death in this system.

We propose the following model for apoptosis during Shigellosis. Shigella secretes IpaB into the cytoplasm (10) where it binds to Casp-1. Casp-1 is then activated and cleaves pro-IL-1β as well as apoptosis effector proteins. Direct binding of IpaB to Casp-1 bypasses the need for a signal transduction pathway and caspases upstream of Casp-1, such as Casp-11. Although we have ruled out the possibility of Casp-3 being a necessary substrate for Casp-1 in Shigella-induced apoptosis, it is still unclear whether other downstream caspases are needed. Whether this pathogenic mechanism is specific for Shigella infections remains to be determined. Salmonella, however, encodes a protein homologous to IpaB, the Salmonella invasion protein (Sip)B which also binds to Casp-1.[4]

Macrophages infected with Shigella release large amounts of IL-1 (33) and treatment of animals with the IL-1 receptor antagonist prevents inflammation after infection with Shigella (44). Here we demonstrate that the Shigella invasin IpaB specifically binds to Casp-1 and that this caspase is absolutely required for Shigella-induced apoptosis. These data indicate that in contrast to developmental or homeostatic apoptosis, Shigella-induced apoptosis requires Casp-1. In view of the dual role of Casp-1 as a pro-apoptotic and pro-inflammatory enzyme, we propose that Casp-1-dependent cell death results in inflammation. Since IL-1β does not encode a signal sequence, apoptosis might allow for the release of the mature cytokine into the extracellular milieu. This model of proinflammatory apoptosis is particularly attractive in Shigellosis, in which the bacteria activate a Casp-1-dependent apoptosis and IL-1 is the triggering cytokine of the inflammatory response. Furthermore, using dominant negative Casp-1 transgenic mice, it was recently shown that this enzyme is required for ischemic brain injury (45), suggesting a role for Casp-1 in other acute pathologies.

Dysentery is self-limiting if patients do not succumb to dehydration. Thus, although the inflammatory response initially allows the disease to progress, it eventually controls the infection. It is interesting to speculate that Casp-1, with its dual pro-apoptotic and pro-inflammatory function, evolved together with IL-1 and IL-18 as triggers of an acute inflammatory response.

[2] H. Hilbi, J. E. Moss, D. Hersh, Y. Chen, J. Arondel, S. Banerjee, R. A. Flavell, J. Yuan, P. J. Sansonetti, and A. Zychlinsky, unpublished results.

[3] H. Hilbi, J. E. Moss, D. Hersh, Y. Chen, J. Arondel, S. Banerjee, R. A. Flavell, J. Yuan, P. J. Sansonetti, and A. Zychlinsky, unpublished observation.

[4] D. Hersh, D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky, manuscript in preparation.

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