Macrophage Apoptosis Triggered by IpaD from Shigella flexneri

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Shigellosis, a potentially severe bacillary dysentery, is an infectious gastrointestinal disease caused by Shigella spp. Shigella invades the human colonic epithelium and avoids clearance by promoting apoptosis of resident immune cells in the gut. This process is dependent on the Shigella type III secretion system (T3SS), which injects effector proteins into target cells to alter their normal cellular functions. Invasion plasmid antigen D (IpaD) is a structural component that forms a complex at the tip of the T3SS apparatus needle. Recently, IpaD has also been shown to indirectly induce apoptosis in B lymphocytes. In this study, we explored the cytotoxicity profile during macrophage infection by Shigella and discovered that the pathogen induces macrophage cell death independent of caspase-1. Our results demonstrate that IpaD triggers apoptosis in macrophages through activation of host caspases accompanied by mitochondrial disruption. Additionally, we found that the IpaD N-terminal domain is necessary for macrophage killing and SipD, a structural homologue from Salmonella, was found to promote similar cytotoxicity. Together, these findings indicate that IpaD is a contributing factor to macrophage cell death during Shigella infection.

Shigellosis is a gastrointestinal human disease caused by infectious pathogens of the genus Shigella. The global burden of shigellosis has been estimated at 165 million cases with 600,000 deaths per year (1). Children under 5 years of age are considered most at risk for shigellosis, which has previously been associated with malnutrition, stunted growth with cognitive impairment, and death in this population (2). In the developing world, endemic shigellosis is a prominent childhood disease with most cases caused by Shigella flexneri (3). As these nations become more developed, there is a transition to Shigella sonnei as the predominant cause of shigellosis (4).

S. flexneri is a Gram-negative bacillus that infects the colonic epithelium and is transmitted by the oral-fecal route. A 200 kb virulence plasmid is at the core of S. flexneri invasiveness and survival in the host. Loss of this plasmid renders the bacterium avirulent (5). The virulence plasmid encodes invasion plasmid antigens IpaB, C and D that contribute to formation of a type III secretion system apparatus (T3SA) tip complex and translocon that form at the T3SS needle tip to complete a conduit to the host cell for delivering effector proteins into the host cell cytoplasm. T3SSs allow bacteria to modulate host-cell processes as an integral part of their infection cycles (6). Shigella uses its T3SS-dependent mechanism to cause the death of immune cells in mucosa-associated lymphoid tissues of the gut. In vivo apoptosis of macrophages, T and B cells has been suggested to occur during Shigella infection (7). Similarly, mucosal biopsy specimens of patients with shigellosis have shown apoptotic cell death of macrophages in the lamina propria is a hallmark of the disease (8).

Several reports have described necrosis and oncosis as mechanisms of S. flexneri-induced macrophage cell death (9–11). Early studies focused on the potential of IpaB to cause an inflammatory cell death known as pyroptosis (12–14). This type of cell death is dependent on caspase-1 activation and its cleavage of pro-interleukin-1β (IL-1β) (13, 15, 16). Furthermore, pyroptosis caused by caspase-1 canonical and caspase-11 noncanonical inflammasomes in Shigella-mediated macrophage death has been studied in recent years (17). It has also been shown that death of monocyteic cells is not entirely dependent on caspase-1 since a specific inhibitor is unable to completely block cytotoxicity in dendritic cells, whereas treatment with the pan-caspase inhibitor z-VAD was found to completely abrogate cell death in these cells (18).

It was recently shown that IpaD can induce apoptosis in B lymphocytes but only in conjunction with an additional unknown factor (19). Here we present evidence that IpaD is also able to elicit macrophage cell death. Our results suggest that Shigella-induced apoptosis in macrophages occurs by an IpaD-dependent mechanism independent of caspase-1 and -11 activation. During this process, IpaD appears to activate other caspases and trigger loss of mitochondrial membrane potential (∆Ψm). Based on the findings presented here, a model is proposed for a cascade of events in which IpaD elicits mitochondrial damage as it promotes macrophage death, which occurs in parallel with pyroptosis.

MATERIALS AND METHODS

Materials. CyToTox 96 nonradioactive cytotoxicity assay kit and caspase luminescent substrates are from Promega, Madison, WI. Caspase inhibitors are from R&D Systems, Minneapolis, MN. A mouse T1/2/T2 9-Plex Ultrafecrit assay kit was purchased from and scanned with a MESO QuickPlex SQ 120 plate reader from Meso Scale Discovery, Rockville, MD. N,N-Dimethyl odendecylamine N-oxide (LDAO) solution was obtained from Sigma-Aldrich, St. Louis, MO. Murine macrophage cell line J774A.1 (TIIB-67) was from the American Type Culture Collection, Manassas, VA. All media and supplements were from Mediatech, Manassas, VA. Glass-bottom plates were from MatTek, Ashland, MA. The FITC-Annexin V/Dead Cell apoptosis kit, Hoechst stain, SlowFade gold reagent, Alexa Fluor 568, and live cell imaging solution were from Life Technologies, Grand Island, NY. Accutase cell detachment solution and cytometer setup
and tracking beads were from BD Biosciences, San Jose, CA. NucView 488 Caspase-3 assay kit, staurosporine, and JC-1 mitochondrial membrane potential detection kit were from Biotium, Hayward, CA. Cleaved Caspase-3 (Asp 175) antibody was from Cell Signaling Technology, Danvers, MA. TOM20 antibody was from Santa Cruz Biotechnology, Dallas, TX. IRDye 800 CW Goat anti-mouse IgG and Odyssey blocking buffer (TBS) were from LI-COR, Lincoln, NE. The Trans-Blot Turbo RTA Mini nitrocellulose transfer kit was from Bio-Rad, Hercules, CA.

**Protein purification.** npGpET15b/Thun(T33E), sipppET15b//Tun(T33E), or krrpET15b/Thun(T33E) were used to overexpress His tag-labeled proteins, which were then purified by using a method previously reported (20). ipApDipET9a/Thun(T33E) was used to overexpress IpaD, which was then purified by anion-exchange chromatography. Proteins in phosphate-buffered saline (PBS) containing 0.1% LDAO were stored at −80°C until use. Concentrations were determined by measuring the A_{280} (21).

**Cell culture.** Cell lines J774 and RAW264.7 were cultured in Dulbecco modified Eagle medium or RPMI supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Bone marrow-derived macrophages (BMDMs) were obtained from bone marrow samples processed and incubated with granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce differentiation of monocytes to M1 macrophages (22). Macrophages were seeded at a density of 2 × 10^4 cells per well in a 96-well plate and cultured at 37°C in supplemented media overnight. Cells were primed for 3 or 5 h with lipopolysaccharide (LPS) from Salmonella enterica serumov serovar Typhimurium SL1344 (2 μg/ml) and infected with S. flexneri strains at a multiplicity of infection (MOI) of 100 or stimulated with recombinant proteins for 2 h (unless otherwise noted) in serum-free, antibiotic-free media.

**Strains.** S. flexneri 2457T (wild-type) and SF622 (IpaD^-), ipApDipWPS4 (IpaD^+), and ipApDipPS4 (IpaD^-) strains were grown in tryptic soy broth or on tryptic soy agar containing Congo red and appropriate antibiotics.

**Chloroquine resistance assay.** A modified gentamicin protection assay was performed as described elsewhere (23–25). Briefly, J774 cells were seeded at 2.5 × 10^5 per well in 24-well plates 24 h prior to infection. Cells were infected at an MOI of 10:1, and infection allowed to progress for 30 min. The cells were then washed and incubated for 2 h in media with 50 μg of gentamicin/ml, with or without 400 μM chloroquine. Cells were washed and lysed in 0.9% NaCl–0.2% Triton X-100. Lysates were serially diluted and plated. CFUs were counted after 16 h of incubation. The percent bacteria in the cytosol was determined as follows: ([CFU in gentamicin plus chloroquine/CFU in gentamicin]) × 100.

**Cytotoxicity.** J774 macrophages were treated as described above. The release of lactate dehydrogenase (LDH) was measured as an indicator of cell death. LDH measurements were performed with a CytoTox 96 non-radioactive cytotoxicity assay kit as described in the manufacturer’s protocol. The percentage of cytotoxicity was calculated as: 100 × [(experimental release − background release)/(total release − spontaneous release)]. In this formula, the background release represents the amount of LDH present in the supernatant of a vehicle control (cells exposed to 0.25% dimethyl sulfoxide (DMSO) in caspase-inhibitor assays or 0.001% LDAO for purified protein experiments), the spontaneous release represents the amount of LDH present in the supernatant of cells exposed to PBS, and the total release is the amount of LDH by cell lysis with a Triton X-100 solution.

**Caspase activity.** J774 macrophages were stimulated with purified IpaD for 30 min. The activity of caspase-2, caspase-3, caspase-8, and caspase-9 was measured by using caspase luminescent substrates. Assays were performed as per the manufacturer’s protocol for Caspase-Glo systems. The luminescence was measured 30 min after addition of the substrates.

**Cell death inhibition.** J774 macrophages were incubated with the inhibitors Z-VAD-FMK (pan-caspase, 50 μM), Z-WEHD-FMK (caspase-1, 50 μM), Z-YVAD-FMK (caspase-1, 50 μM), and Z-LEHD-FMK (caspase-9 or -11, 50 μM) for 1 h prior to exposure to S. flexneri strains. Cell death elicited by mutant strains was normalized to the complemented strain ipApDDipWPS4. Similarly, the effect of inhibitors on cell death was measured in relation to cytotoxicity elicited by purified protein. The inhibitors Z-VDVAD-FMK (caspase-2, 50 μM), Z-DEVAD-FMK (caspase-3, 50 μM), and Z-IEFD-FMK (caspase-8, 50 μM) were also tested in these assays. Inhibition was calculated as 100 − the percent cytotoxicity of treated versus untreated samples.

**Cytokine release.** J774 macrophages or BMDMs were stimulated for 48 h. Secreted cytokine levels were detected in supernatants with an MSD T2/L2 9-plex cytokine kit and scanned with a Meso QuickPlex SQ 120 plate reader from Meso Scale Discovery.

**Flow cytometry.** J774 macrophages were exposed to purified IpaD at varied time intervals and recovered as a single cell suspension with Accucut assay detachment solution. Samples were then processed with NucView 488 Caspase-3 assay kit according to the manufacturer’s instructions.

Cells exposed to staurosporine (1 μM) were used as a positive control for caspase-3 activation. J774 macrophages exposed to purified IpaD for 1 h were processed with a FITC Annexin V/Dead Cell apoptosis kit. J774 macrophages exposed to purified IpaD for 30 min were processed with a JC-1 mitochondrial membrane potential detection kit. Samples were analyzed by flow cytometry with blue (488 nm) and yellow-green (561 nm) lasers and appropriate filters on a BD FACSAria Fusion cell sorter.

**Live cell imaging.** J774 macrophages in sterile glass-bottom 96-well plates were incubated for 15 min with Alexa-labeled recombinant proteins. Cells were washed extensively. Live cell imaging was performed, and images collected 1 h after infection with an inverted fluorescence microscope (Olympus IX-83 motorized microscope) equipped with a 20× objective lens and differential interference contrast, TRITC (tetramethyl rhodamine isothiocyanate) filters. All images were obtained and analyzed identically on cellSens software.

**Immunoblots.** J774 macrophages exposed to IpaD for 1 h were collected, washed in PBS, and sonicated to prepare lysates. The protein concentration was measured by using a bicinchoninic acid assay, and 25 μg of total protein was separated on a standard sodium dodecyl sulfate–containing 15% polyacrylamide gel. The proteins were then transferred onto nitrocellulose for immunoblot analysis using primary antibodies against cleaved caspase-3 and TOM20 with appropriate secondary antibodies. Blots were developed with an Odyssey CLX infrared image system from LI-COR.

**Statistical analysis.** All graphs and statistical analyses were performed with GraphPad Prism 5.04. A P value of <0.05 (P < 0.05) in analysis of variance (ANOVA) or multiple t tests was considered significant in all cases. Data values are expressed as the means ± the standard deviations. All experiments were performed at least twice with triplicate samples. Representative data sets are shown.

**RESULTS**

**Shigella cytotoxicity to macrophages is dependent on factors in addition to caspase-1 activation.** To determine whether events in addition to caspase-1 activation contribute to cytotoxicity during macrophage invasion by Shigella, we investigated the toxicity profile during wild-type S. flexneri infection of J774 macrophages. As expected, S. flexneri induced significant levels of cell death compared to controls (Fig. 1). When cells were incubated with the pan-caspase inhibitor Z-VAD-FMK prior to bacterial infection, cell death was reduced by 40% under the conditions used, which is similar to the levels observed by others (9). This is in contrast with specific caspase-1 inhibitors that only reduced cell death by 20%. No difference was found between the effects of the two caspase-1 inhibitors Z-WHED-FMK and Z-YVAD-FMK. These results suggest that the observed level of macrophage cell death elicited by S. flexneri invasion is not solely dependent on caspase-1 activation as observed by the effect of the pan-caspase inhibitor. This finding...
raises the possibility that other caspases are involved in Shigella-mediated macrophage killing.

**Shigella cytotoxicity can be partially attributed to IpaD.** It has been largely accepted that Shigella causes pyroptosis in macrophages via caspase-1 activation accompanied by subsequent IL-1β release (14, 15, 26). Based on the data above, however, it appears that there is a caspase-1-independent mechanism that also contributes to macrophage killing. Because the amount of IpaD produced by Shigella exceeds what is likely needed for its role in controlling type III secretion as a T3SA needle tip component (27), we propose this protein works as an effector in certain host cells, with caspase-1 being a downstream effector.

To determine whether IpaD is a functional effector in Shigella infection, we explored the possibility that Shigella infection could be due to IpaD as an effector in certain host cells. We found that IpaD-mediated cell death could be due to IpaD as an effector in certain host cells.

An *ipaD*-null Shigella mutant (strain SF622) complemented with the gene for full-length *ipaD* (*IpaD*+ ) was found to behave similarly to wild-type *S. flexneri* for macrophage cytotoxicity (data not shown). The *IpaD*+ strain was then compared to the SF622 strain complemented with a mutant *ipaD* gene (IpaDΔ41−80 mutant), which encodes IpaD with a large deletion within the IpaD N terminus. This strain was previously identified as virulent and this deletion did not eliminate IpaD-mediated macrophage killing. This strain was previously identified as virulent and this deletion did not eliminate IpaD-mediated macrophage killing.

We refined the experiment to include inhibitors of specific caspases in addition to caspase-1. Activation of caspase-11 has been reported upon *S. flexneri* infection (28) and inflammasome activation by caspase-11 can occur independently (29) or as a mechanism upstream of caspase-1 (30). The caspase-11 noncanonical inflammasome pathway appears to be triggered by the large amount of LPS presented by the cytoplasmic localization of these Gram-negative bacteria (28). Thus, we included the inhibitor W-LEHD-FMK to block LPS-mediated cell death by caspase-11 (31, 32) when examining the ability for the IpaDΔ41−80 strain to cause macrophage death.

**The inflammatory cytokine IL-1β does not play a role in IpaD-mediated cell death.** Studies centered on the role of inflammasome pathways in macrophage cell death by *Shigella* have shown that cell death mediated by caspase-1 or caspase-11 lead to secretion of cytokines IL-1β and IL-18 (17, 34, 35). Both cytokines had previously been shown to mediate *Shigella* induced inflammasome (36). To determine whether either of these inflammatory cytokines are involved in IpaD-induced cell death, the levels of secreted IL-1β were monitored after macrophage infection by *Shigella* IpaD+ or IpaDΔ41−80 strains (Fig. 3). No differences in IL-1β secretion were observed in cells infected with either IpaD+ or IpaDΔ41−80 strains. Treatment of macrophages with a pan-caspase
inhibitor or combined caspase-1 and -11 inhibitors greatly reduced the levels of secreted IL-1β. No differences were found between the strains in their reduced ability to elicit IL-1β secretion. Similar results were observed in cultured macrophages (J774 cells, Fig. 3A) and primary cells (BMDMs, Fig. 3B). This further suggests that mechanisms beyond IL-1β induction are involved in macrophage killing.

IpaD-induced cytotoxicity can be demonstrated using purified protein. Because our experiments with mutant Shigella indicate that IpaD could be involved in macrophage cell death, we tested an in vitro model of cell death using purified recombinant IpaD. Because IpaD is a highly polar protein (37) that is not readily internalized by host cells, we used a method for transduction of proteins in solution in the presence of mild detergent (38). We mixed IpaD with LDAO, a zwitterionic detergent used to maintain IpaB in a soluble state, for use in similar assays to promote its uptake. To determine whether IpaD was indeed carried into cultured macrophages using this method, we exposed macrophages to Alexa-568-labeled IpaD in the presence or absence of LDAO and analyzed the cellular distribution and cell morphology by microscopy. Cells were incubated with treatment for 15 min and then washed extensively. Live cells were imaged 1 h after treatment. IpaD delivered in PBS was unable to enter cells as shown by a low intracellular signal (see Fig. S2 in the supplemental material), and thus no change in cell morphology was observed compared to control cells. Meanwhile, cells exposed to IpaD with 0.001% LDAO showed rapid internalization and intracellular localization of IpaD. The number of cells with internalized IpaD was 97.9% ± 2.3% (five slides were quantified). Cells treated with IpaD-LDAO exhibited an apoptotic morphology, including changes in refractive index, cell shrinkage, and the appearance of apoptotic bodies compared to the typical morphology observed in a vehicle (0.001% LDAO) control (see Fig. S2 in the supplemental material).

Upon exposed macrophages to different concentrations of IpaD (0.2 to 2.4 μM), we established that purified IpaD elicits dose-dependent cell death in J774 cells (Fig. 4A). We observed the same effect in another cell line of cultured macrophages (RAW264.7 cells; Fig. 4B). IpaD-driven cytotoxicity was also identified in primary cells (BMDMs), although the levels of cell death triggered were somewhat reduced (Fig. 4C). IpgC, a T3SS chaperone protein with no known effector activity, was used as a negative control and exhibited no cytotoxicity (see Fig. S3 in the supplemental material). Thus, the model developed with recombinant purified protein should allow identification of the events that lead to IpaD-mediated cell death.

An N-terminal IpaD domain that spans amino acids 1 to 120 has been shown to fold independently of the rest of the protein (33). When IpaD possessing a deletion of the region corresponding to amino acids 41 to 80 (to yield the IpaD341–80 strain) is incubated with the cells, the recombinant protein causes negligible levels of cytotoxicity (2.23% ± 0.26% [data not shown]). The N-terminal IpaD domain was previously described as having a self-chaperoning domain and is present in a family of IpaD structural homologues (SipD from Salmonella and BipD from Burkholderia pseudomallei). This domain is not present in other, more distant homologues in an alternative family of T3SA needle tip proteins (LcrV from Yersinia spp. and PcrV from Pseudomonas aeruginosa) (39, 40). To determine whether the presence of related N-terminal domains between IpaD and SipD imparted cytotoxic potential to the latter, we examined the effect of purified SipD (Salmonella) in macrophages. Indeed, we found that purified SipD also triggers cell death in macrophages in a dose-dependent manner (Fig. 4D). In contrast, the homologue LcrV (Yersinia), which lacks a discrete N-terminal domain, exhibited negligible levels of cytotoxicity (Fig. 4E). These data imply that macrophage killing by T3SA needle tip proteins is limited to the family of tip proteins possessing an N-terminal domain like that of IpaD and SipD (41).

IpaD increases caspase activity in macrophages. Because of the possibility that multiple caspases are involved in Shigella-mediated cell death and the cytotoxic effect of IpaD, we performed caspase activity assays after exposure of J744 macrophages to purified IpaD for 30 min. Compared to vehicle controls lacking IpaD, significant increases in the activities of initiator caspase-2, caspase-8, and caspase-9 were observed (Fig. 5A). We also found a minor, though significant, increase in the activity of the effector caspase-3 (Fig. 5A). These caspases have been reported to be activated as part of classical apoptosis pathways (42).

FIG 3 The deletion mutant IpaD341–80 and the complemented IpaD+ strain elicit similar levels of IL-1β secretion. Cytokine IL-1β levels were measured in supernatants of cells exposed to complemented S. flexneri SF622 (ipaD-null) strains. Cells exposed to a Shigella strain complemented with a full-length ipaD gene are indicated by black bars and to a complemented strain (the IpaD341–80 mutant) are indicated by gray bars. (A) IL-1β levels in J774 cells exposed to each strain show no significant difference. IL-1β levels were also assessed under conditions of caspase inhibition with a pan-caspase inhibitor and by the combined caspase-1 and -11 inhibitors. The amounts of IL-1β secreted by the ipaD-null SF622 strain were minimal. (B) A similar experiment was performed with BMDMs. Significance was calculated with a one-way ANOVA (*P < 0.05). *, versus the IpaD+ strain.
that exposure of IpaD to macrophages for 30 min resulted in elevated initiator caspase activities, but inhibition of effector caspase-3 also disrupted IpaD-induced cell death (Fig. 5B). To study the event timeline that results in macrophage cell death by IpaD, we analyzed caspase-3 activation through a time course from 5 to 120 min. Substrate (NucView 488) fluorescence was monitored as an indicator of caspase-3 activity. By 1 h, IpaD had caused caspase-3 activation in a major portion (40%) of the cell population (Fig. 6). As cell death progressed, caspase-3 activity was reduced as seen for the 2-h time point (Fig. 6). This is likely reflecting the progression of the macrophage population to apoptotic bodies and dead cells. Staurosporine was used as a positive control of apoptosis and exhibited caspase-3 activity at 2 h postexposure (Fig. 6, bottom panel) as reported elsewhere (43).

Characterization of IpaD-induced apoptosis. To determine whether intracellular localization of IpaD in macrophages is related to activation of an intrinsic apoptotic pathway, we exposed macrophages to IpaD for 30 min. A single cell suspension was probed with JC-1, a cell-permeable fluorophore that forms aggregates in mitochondria and monomers in the cytoplasm which fluoresce in the red and green channels, respectively. Loss of mitochondrial membrane potential (∆Ψm) was observed upon exposure to IpaD, as reflected in decreased red fluorescence in the cell population (Fig. 7, right panel). The vehicle (LDAO) control did not have any observed effect on ∆Ψm (Fig. 7, middle panel) compared to a PBS control (Fig. 7, left panel). Also, we evaluated the ability of IpaD⁺ or IpaD³⁴¹–⁸⁰ Shigella strains to disrupt mitochondria (see Fig. S4 in the supplemental material). IpaD⁺ Shigella strains are able to elicit mitochondrial disruption in 17.8% of the cell population, whereas the IpaD³⁴¹–⁸⁰ mutant showed a reduced ability to do so (9.2%).

**FIG 4** Purified IpaD elicits cytotoxicity in macrophages. (A) J774 cells were exposed to purified IpaD at concentrations ranging from 2.4 to 0.2 μM for 2 h. Cytotoxicity was measured with an LDH release assay in which the percent LDH release is directly proportional to the amount of cell death. (B) RAW264.7 cells exposed to purified IpaD. (C) BMDM cell death caused by IpaD. The amount of cell death is reduced from that observed in the macrophage cell lines. (D) J774 cells exposed to purified SipD, an IpaD homologue from Salmonella. (E) J774 cells exposed to purified LcrV, an IpaD homologue from Yersinia.

**FIG 5** IpaD promotes caspase activation. (A) Cells were incubated with a vehicle control or 2.4 μM IpaD for 30 min. Caspase activity was measured with luminemt substrates specific for caspase-2, caspase-3, caspase-8, and caspase-9. The luminescence measured is directly proportional to the amount of caspase activity. The fold increase in caspase activity relative to the vehicle control is shown. The activity of all caspases assayed was found to be significantly different from the vehicle control using a multiple Student t test analysis (P < 0.05). (B) Cells were pretreated with appropriate caspase inhibitors (see Materials and Methods) for 1 h and subsequently exposed to IpaD for 2 h. Cell death inhibition relative to a control exposed to IpaD alone (determined as 0% inhibition of cell death) was assessed. Significance was calculated with a one-way ANOVA (P < 0.05).
The IpaD^- null strain does not induce any substantial mitochondrial disruption.

Additionally, we incubated J774 macrophages with a vehicle control (LDAO), 2.4 μM IpaD, 2.4 μM IpgC, or 1 μM staurosporine for 1 h and stained with annexin V and propidium iodide (PI). Annexin V staining indicates cell populations that are in midstage apoptosis. Populations that are progressing toward cell death further lose the integrity of their membrane and also exhibit PI staining. Cells that are dead only exhibit PI staining. We found that IpaD is able to push cells toward mid-stage apoptosis at 1 h, whereas IpgC, which we had shown causes no cytotoxicity, does not (see Fig. S5 in the supplemental material).

**DISCUSSION**

Macrophage cell death is important during *S. flexneri* pathogenesis as a survival method that allows the pathogen to avoid rapid infection. Cells that are dead only exhibit PI staining. We found that IpaD is able to push cells toward mid-stage apoptosis at 1 h, whereas IpgC, which we had shown causes no cytotoxicity, does not (see Fig. S5 in the supplemental material).

**FIG 6** IpaD exposure results in time-dependent caspase-3 activation. (A) Cells were exposed to 2.4 μM IpaD or to a vehicle control as a function of time. The cells were then washed and NucView 488 added. Green fluorescence of NucView 488 increases if caspase-3 is in its active form. IpaD exposure for 1 h shows a population with active caspase-3. Staurosporine (Stp) was used as a positive control for caspase-3 activation (bottom panel) using the 2-h time point. (B) Lysates from cells exposed to IpaD for 1 h or a vehicle control were probed for cleaved caspase-3.

**FIG 7** IpaD causes loss of mitochondrial membrane potential (ΔΨm). Cells were exposed to PBS, a vehicle (LDAO) control, or 2.4 μM IpaD in LDAO for 30 min. After detachment as a single cell suspension, JC-1 was added, and the fluorescence measured using a cell sorter. Red fluorescence indicated JC-1 aggregates accumulated in the mitochondria. Green fluorescence indicated cytoplasmic localization of JC-1 monomers. A shifted population to lower fluorescence in the red channel shows the mitochondrial membrane potential is compromised.
caspases (caspase-2, -8, and -9) leads to activation of an effector triggered by IpaD. An increased activity in multiple initiator caspase-8 (51), and caspase-9 is required for activation of caspase-3, able to create a feedback loop by cleaving caspases and their responses would commit a cell to apoptosis (10, 44, 45).

IpAD is the tip protein of the Shigella T3SS apparatus (46, 47) and, like the translocators IpA and IpAC, it is secreted when the proper stimulus is applied (46). By blocking inflammatory cell death, we were able to reduce the cytotoxic profiles of infection and identify IpAD as an additional effector of macrophage killing. Furthermore, a Shigella strain expressing IpAD with a mutation within the N-terminal domain is unable to induce this arm of cytotoxicity. In vitro, these events were mimicked by exposing macrophages to recombinant purified IpAD in the presence of a mild detergent vehicle (LDAO). Purified IpAD elicited dose-dependent cell death in the micromolar range possibly through its N-terminal domain. A similar observation was found for SipD, the IpAD structural homologue from Salmonella, which also triggers cell death in macrophages. Conversely, the Yersinia counterpart LcrV, which lacks the additional N-terminal structures present in IpAD and SipD (40, 48), is unable to cause macrophage cell death (Fig. 4).

The cytotoxicity observed is an effect of an apoptotic pathway triggered by IpAD. An increased activity in multiple initiator caspases (caspase-2, -8, and -9) leads to activation of an effector caspase (caspase-3) (49), and studies show that temporality of caspase activation is necessary for correct assembly of the apoptosome (50). Moreover, apoptosis caused by release of cytochrome c into the cytoplasm allows activation of caspase-9, which in turn activates caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, and caspase-10 (49). Feedback loops and shared networks of caspase activation have been widely described. For example, caspase-3 is able to create a feedback loop by cleaving caspase-8 (51), and caspase-9 is required for activation of caspase-8 in a mitochondrion-mediated positive-feedback loop (52). However, activation of a single caspase is usually not the sole source of apoptotic events, and instead a combination of activated caspases and their responses would commit a cell to apoptosis (53–55).

The mechanism of apoptosis mediated by IpAD seems to involve the mitochondria since IpAD was found to cause activation of caspase-9, which is part of a mitochondrial apoptotic pathway (56). Moreover, we observed that purified IpAD or the IpAD+ Shigella strain trigger a loss of mitochondrial membrane potential (∆Ψm, Fig. 7 and see also Fig. S4 in the supplemental material), which is a decisive step for the activation of caspase-2 and caspase-9 (57). Thus, IpAD is likely causing mitochondrial disruption as an initial step in its mechanism of macrophage cell death. Identification of the definitive molecular mechanism for IpAD-mediated apoptosis in macrophages will require further study. Caspase-2 and caspase-8 have been identified as processors of Bid, a protein that activates intrinsic apoptosis (58, 59). Our findings with these caspases merit assessment of this pathway in future studies.

It is likely that Shigella benefits from killing resident macrophages through an integrated response of apoptosis and pyroptosis, avoiding clearance, and gaining access to the basolateral side of the epithelium. Apoptosis would not result in pathogen release since Shigella spp. would presumably be enclosed in apoptotic bodies and therefore unable to invade the intestinal epithelium. This measure, however, would reduce the overall inflammation and neutrophil infiltration (60, 61). Meanwhile, parallel pyroptosis in other macrophage populations ensures that enough bacteria escape to the extracellular environment to allow epithelial cell invasion, at the cost of producing inflammation.

It is also possible, however, that macrophage apoptosis could have an effect similar to apoptosis in dendritic cells, which can lead to a tolerogenic switch (62). Thus, resident macrophages may be switched to a regulatory phenotype that would be beneficial to the pathogen by allowing it to continue colonizing the region with reduced immune recognition. Furthermore, it is known that macrophages in the lamina propria that are hyporesponsive to Toll-like receptor signaling suppress inflammatory dendritic cells (63). An apoptotic response could also be a response mounted by the host. Resident macrophages might be signaling for rapid cell death to limit replication of Shigella once it escapes the phagosome (64).

Based on the evidence presented here, we conclude that IpAD is a contributing factor to macrophage cell death seen during Shigella infection. Furthermore, we have developed an in vitro model that looks at the events elicited only by IpAD, eliminating other known pathogen factors that contribute to macrophage cell death. This in vitro model will allow further studies directed at understanding the role of secreted IpAD in Shigella pathogenesis. As seen in our results, these studies could be relevant to other pathogens with T3SSs, such as Salmonella. One caveat of using the mouse immortal and primary cells used in these experiments is the fact that Shigella is a host restricted pathogen whose only natural hosts are humans and, to a lesser extent, nonhuman primates. Nevertheless, mouse and guinea pig models have been successfully used to assess Shigella pathogenesis, as well as the innate and adaptive immune responses elicited during shigellosis. It will thus be important to next consider the implications of IpAD as an effector protein during human infection as this work progresses.

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