A Novel Caspase-1/Toll-like Receptor 4-independent Pathway of Cell Death Induced by Cytosolic Shigella in Infected Macrophages

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Shigella-induced macrophage cell death is an important step in the induction of acute inflammatory responses that ultimately lead to bacillary dysentery. Cell death was previously reported to be dependent upon the activation of caspase-1 via interaction with IpaB secreted by intracellular Shigella, but in this study, we show that Shigella infection of macrophages can also induce cell death independent of caspase-1 or IpaB activity. Time-lapse imaging and electron microscopic analyses indicated that caspase-1-dependent and -independent cell death is morphologically indistinguishable and that both resemble necrosis. Analyses of Shigella mutants or Escherichia coli using co-infection with Listeria suggested that a component common to Gram-negative bacteria is involved in inducing caspase-1-independent cell death. Further studies revealed that translocation of bacterial lipid A into the cytosol of macrophages potentially mediates cell death. Notably, cell death induced by cytosolic bacteria was TLR4-independent. These results identify a novel cell death pathway induced by intracellular Gram-negative bacteria that may play a role in microbial-host interactions and inflammatory responses.

Shigella are highly adapted human pathogens that cause bacillary dysentery (also referred to shigellosis). The prominent pathogenic feature of Shigella is the ability to invade a variety of host cells, including macrophages, dendritic cells, and epithelial cells, which leads to severe inflammatory responses in intestinal tissue. When Shigella reach the colon and rectum, the bacteria translocate through the epithelial barrier via M cells that overlie the solitary lymphoid nodules (1). Once they reach the underlying M cells, Shigella invade the resident macrophages, and the infecting bacteria escape from the phagosome into the cytosol. Shigella multiply in the cytoplasm and induce cell death through activation of caspase-1 mediated by IpaB protein secreted via the type III secretion system (TTSS), in turn leading to the maturation and release of IL-1β (2, 3). Shigella released from the killed macrophages enter the surrounding enterocytes through their basolateral surface by inducing membrane ruffling and macro-pinocytosis (4). Once the bacterium is surrounded by a membrane vacuole, it immediately disrupts the vacuole membrane and escapes into the cytoplasm. Shigella multiply in the cytoplasm and move about by inducing actin polymerization at one pole of the bacterium, allowing intracellular bacteria to spread within the cytoplasm as well as into the adjacent epithelial cells (5, 6). During bacterial multiplication within the epithelial cells, peptidoglycan (PGN) is released from the cytoplasmic bacterium, and the murpeptidase composed of a diaminopimelate-containing N-acetylmuramyl-L-alanyl-D-isoglutamine dipeptide is recognized by Nod1/CARD4, which engages the formation of IκB kinase (IKK)-RICK complex required for the activation of NF-κB, thus leading to the production of interleukin (IL)-8 (7–11). As a result, the proinflammatory chemokines and cytokines produced by the macrophages, and epithelial cells infected with Shigella elicit strong inflammation in the intestinal tissue.

Although it has been reported that macrophages infected by Shigella undergo apoptosis as a result of IpaB-mediated caspase-1 activation (12–14), the notion that Shigella induces cell death through apoptosis is controversial. For example, some studies showed that macrophage cell death induced by Shigella causes necrotic cell death (15, 16). In addition, Shigella infection of human mononuclear cell line U937 cells differentiated to macrophages elicits apoptosis, whereas infection differentiated toward the myeloid lineage cells caused necrotic cell death (17, 18). Moreover, other studies have indicated that cell death of macrophages by Shigella infection is not totally suppressed by caspase-1-deficiency (19, 20). Therefore, it is likely that multiple cell death pathways are engaged in the macrophage cell death response to Shigella infection.

A similar situation has also been noted in Salmonella-induced macrophage cell death (21, 22), and the morphological features of infected macrophages have been shown to be both

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1 The abbreviations used are: TTSS, type III secretion system; BM, bone marrow; BMDCs, BM-derived dendritic cells; BMMs, BM-derived macrophages; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; PGN, peptidoglycan; PI, propidium iodide; FACS, flow cytometry; EM, electron microscopy; TNF-α, tumor necrosis factor-α; TRITC, tetramethylrhodamine isothiocyanate; IL, interleukin; N-WASP, neural-Wiskott Aldrich syndrome protein.
necrotic and apoptotic; cell death occurred in Salmonella infection (21, 23, 24). Salmonella infection of macrophages mediated by Salmonella pathogenicity island 1 (SPI-1) TTSS is capable of inducing both caspase-1-dependent and -independent cell death (25, 26), whereas the cell death mediated by SPI-2 TTSS is partly dependent on caspase-1 (27). The rapid caspase-1-dependent cell death caused by Salmonella infection is also referred to as “programmed necrotic death,” implying that the cell death is somewhat different from the well-characterized apoptotic cell death (23). A recent study showed that Salmonella infection leads to caspase-1-independent cell death, in which autophagosome formation in mitochondria is induced by SipB protein secreted by Salmonella SPI-1 TTSS (28). Thus, the results of these studies have strongly suggested that the cell death responses of macrophage by Salmonella infection are variable, perhaps reflecting the activation of different host cell death pathways by pathogenic bacteria.

In this context, we wished to evaluate the impact of caspase-1-independent macrophage cell death on Shigella infection by using caspase-1-deficient macrophages and dendritic cells and to identify the bacterial component(s) involved in inducing cell death. The results showed that caspase-1-independent cell death occurs in Shigella infection independent of IpaB secreted by Shigella TTSS. Analysis of the morphological features of the caspase-1-dependent and -independent macrophage cell death induced by Shigella indicated that the cells undergo a necrotic type of cell death. Co-infection experiments with a series of Shigellosis mutants or Escherichia coli K-12 with Listeria monocytogenes have indicated that bacterial component(s) common to Gram-negative bacteria in the macrophage cytosol can induce cell death regardless of the presence or absence of caspase-1 activation. The analysis designed to identify bacterial surface components that stimulate cell death identified lipA as a potent inducer of cell death in the macrophage cytosol. Importantly, the putative lipA-mediated death pathway is distinct from the pathway mediated by Toll-like receptor 4 (TLR4), and we herein propose that at least lipA released by Shigella into the macrophage cytosol stimulates a cell death pathway through a mechanism that is independent of caspase-1 and TLR4.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The wild-type Shigella flexneri 2a YSH6000 and L. monocytogenes 12/1b derived from previously (29). Shigella mutants, S25 (mxiA::Tn5) (30), ipoC gene null mutant (31), and large plasmid-cured strain YSH6200 (32) were used in this study. The ipoB and ipoD null mutants were constructed by allele replacement strategies according to the procedures described previously (33). Staphylococcus aureus (H9262) and Bacillus subtilis (H11005) were obtained from the Laboratory of Culture collection in our department. To visualize living bacteria in infected cells, a GFP gene was cloned into pUC19-Tp (34) and transformed into Shigella. The Shigella and Listeria strains were grown routinely in brain-heart infusion broth (BD Biosciences). The Staphylococcus and Bacillus strains were grown in tryptic soy broth (BD Biosciences).

Mouse—C57BL/6 mice were purchased from Japan Clea. The caspase-1-deficient mice (C57BL/6 background) and TLR4-deficient mice (C57BL/6 background) have been described previously (35, 36). Mouse handling conformed to the requirements of the Animal Care and Use Committee in Institute of Medical Science, University of Tokyo.

Reagents—Lipopolysaccharide (LPS) and alkaline-detoxified LPS derived from E. coli B5505 and purified lipA preparations of E. coli F583 were purchased from Sigma. PGN from S. aureus was from Fluka-Chemie. PGN from Shigella S225 mutant was purified by the methods described previously (10). The purified Shigella PGN was further treated with polymyxin B-immobilized beads (Detoxi-Gel; Pierce) to remove contaminating LPS. The following antibodies were obtained commercially: rabbit anti-mouse caspase-1 (Santa Cruz Bio-technology), rabbit anti-mouse caspase-9 and -3 (Cell Signaling), and anti-mouse Fas (Jo2; Pharmingen).

Bone Marrow (BM) Culture—Bone marrow-derived dendritic cells (BMDCs) were isolated by plating BM cells from femurs and tibias at 1 × 10^7 cells/ml in 6-well plates with 10% fetal calf serum-RPMI 1640 supplemented with 10 ng/ml murine granulocyte-macrophage colony-stimulating factor (PeproTech), as described previously (15). At day 5, loosely adherent cells were harvested by gentle pipetting and enriched for the DC11c-expressing population by using a magnetic cell sorter and N418 magnetic beads (Miltenyi Biotec). Approximately 90% of the enriched population stained positive for CD11c with antibody HL3 (BD Biosciences) according to the flow cytometry analysis. Bone marrow-derived macrophages (BMMs) were isolated by plating BM cells in 10% fetal calf serum-RPMI 1640 supplemented with 30% supernatant from L929 cells. At day 5, strongly adherent macrophages were harvested by incubating in PBS on ice for 10 min.

Bacterial Infection—DCs or macrophages were seeded at 5 × 10^5 cells/ml in 24-well plates containing 10% fetal calf serum-RPMI 1640. The cells were infected with wild-type Shigella or Listeria at a multiplicity of infection of ~10/cell. In the case of non-invasive strains, cells were infected with ~40 bacteria/cell because preliminary data indicated that ~4 times fewer macrophages phagocytosed non-invasive bacteria than invasive strains. The cells were centrifuged at 600 × g for 10 min to synchronize the stage of infection, and 30 min later, gentamicin (100 μg/ml) and kanamycin (60 μg/ml) were added to kill the extracellular bacteria. For DC maturation, the wells were extensively washed with RPMI 1640 to remove residual extracellular bacteria before adding antibiotics and then incubated for additional 24 h.

Lactate Dehydrogenase (LDH) Assay, Flow Cytometry, and Enzyme-Linked Immunosorbent Assay—At the time indicated after infection, the LDH activity of the culture supernatants from infected cells was measured by using the CytoTox 96 assay kit (Promega) according to the manufacturer’s protocol. For DC maturation, at 24 h after infection, surviving DCs were incubated with anti-CD16/32 (2.4G2; Pharmingen) to minimize nonspecific staining, and the cells were then stained with biotinylated anti-CD40 (3/23; Pharmingen) and developed with streptavidin-phycerothyrin (Pharmingen). For detection of apoptosis/necrosis, at the indicated time after infection, the macrophages were stained with annexin-V-FITC (Roche Applied Science) for 20 min on ice and stained with propidium iodide (Sigma). Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences) with CellQuest software. Cytokine production in the culture supernatants was measured with an enzyme-linked immunosorbent assay kit (R&D Systems).

Murine Pulmonary Infection and TUNEL Assay—Anesthetized mice were intranasally inoculated with 2 × 10^5 bacteria in 20 μl as described previously (38). The mice were sacrificed at the indicated time, and their lungs were removed and fixed in 4% paraformaldehyde in PBS. The tissue was embedded in paraffin, sectioned, and stained with a Leica cryostat (model CM1900). A TUNEL assay was performed using the DeadEnd fluorometric TUNEL system (Promega). The sections were also immunostained with TRITC-anti-Shigella LPS antibody and counterstained with hematoxylin. The sections were analyzed under a confocal microscope (LSM510, Carl Zeiss), and the cells that contained a nucleus with an intensity higher than an arbitrary threshold of 50 in non-infected lung area were defined as TUNEL-positive.

Time-lapse Imaging and Transmission Electron Microscopy—Static or moving images of infected cells were collected with a cooled CCD camera and a time-lapse imaging system (Roper Scientific), and they were analyzed using IPLab Spectrum software (Signal Analytics Corp.). For electron microscopy, infected cells were fixed with a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in PBS, and after post-fixing with osmium tetroxide, the samples were dehydrated in ethanol and embedded in Epon. Thin sections were examined with a Hitachi (H-7100) electron microscope.

Adorption on the Latex Beads—Exponentially growing bacteria (OD_{600} = 1.0) were collected by centrifugation and resuspended in one-fifth volume of PBS. The bacterial cells were disrupted by sonication and clarified by filtration. When indicated, bacterial lysates were boiled for 1 h at 100 °C or treated with proteinase K (20 μg/ml) for 2 h at 37 °C. Two microliters of latex beads (carboxylated microparticles, 2 μm in diameter; Polyscience) were mixed with 100 μl of bacterial lysates or the bacterial components (1 mg/ml) for 16 h at 4 °C. The beads were collected by centrifugation at 13,000 × g for 5 min, washed in PBS, and then added to the macrophages at a density of 10–20 beads/cell, with or without Listeria.

RESULTS

Shigella Infection of Macrophages and Dendritic Cells Induces Caspase-1-independent Cell Death—To investigate the Shigella-induced macrophage death in the absence of
caspase-1 BMDCs expanded with granulocyte-macrophage colony-stimulating factor or BMMs isolated from wild-type or caspase-1-deficient mice were infected with YSH6000 (wild-type *Shigella*). Although no release of LDH by caspase-1-deficient BMDCs was detected until 2 h after infection, LDH release began 2 h after infection and increased greatly to a level similar to that in wild-type BMDCs by 5 h (Fig. 1A). A similar cell response was seen in BMMs infected with YSH6000 (Fig. 1A), suggesting that caspase-1-independent cell death occurs upon infection of DCs or macrophages with *Shigella*. By contrast, the *ipaB* mutant (Δ*ipaB*), which cannot escape into the cytoplasm, did not cause a significant level of LDH release (Fig. 1A). We then assessed the maturation of caspase-1-deficient BMDCs to confirm that caspase-1-independent cell death was caused by *Shigella* infection. Wild-type and caspase-1-deficient BMDCs infected with *Shigella* for 24 h were examined for IL-12 production and cell surface expression of CD40. IL-12 production and CD40 up-regulation were observed upon infection of wild-type and caspase-1-deficient BMDCs with mutant Δ*ipaB* and S225 (TTSS-deficient mutant), but not YSH6000 (Fig. 1, B and C), confirming that all infected DCs had been destroyed by *Shigella* infection before maturation. A previous study revealed reduced apoptotic cells in the lung of caspase-1-deficient mice when compared with wild-type mice at 6 h after infection with *Shigella* (38). To assess apoptosis in the lung, we infected caspase-1-deficient and wild-type mice with *Shigella* via nasal route and determined the number of apoptotic cells by the TUNEL assay. After 6 h of infection, TUNEL-positive macrophage-like cells were detected in both the wild-type and the mutant mice, although there were fewer apoptotic cells in the lung of caspase-1 mutant mice (Fig. 1, D and E), consistent with the previous study (38). By 12 h after infection, however, the number of apoptotic cells was similar in the lung of wild-type and mutant mice (Fig. 1, D and E). Thus, caspase-1 contributes to the early phase of cell death, but it is not required for the induction of cell death in macrophages and DCs upon infection with *Shigella*.

**Characterization of Shigella-induced Cell Death**—To characterize the cell death induced by *Shigella* in wild-type and caspase-1-deficient BMMs, we collected time-lapse images of infected BMMs. When wild-type BMMs were infected with YSH6000, extensive membrane blebbing and nuclear shrinkage were observed within 60 min after infection (Fig. 2A and supplemental movie Fig. 2Amovie1.mov). Similar blebbing was observed 150 min after infection of caspase-1-deficient BMMs (Fig. 2A and supplemental movie Fig. 2Amovie2.mov), and these delayed cellular responses were comparative to that of the LDH release by infected caspase-1-deficient cells (Fig. 1A). Anti-Fas antibody-induced apoptosis in wild-type BMMs did not cause such extensive membrane blebbing, and small blebs protruded along the cell periphery instead (Fig. 2A). Moreover, transmission electron microscopy revealed that YSH6000 caused the necrotic type of cell death by both wild-type (1 h after infection) and caspase-1-deficient BMMs (3 h after infection). Indeed, both types of infected cells showed cell membrane disappearance and loss of cytoplasm, findings that were strikingly different from typical apoptotic cellular events (Fig. 2B). Thus, although the onset of cell death in caspase-1-deficient macrophages occurred later than wild-type macrophages, the cellular responses of the wild-type and caspase-1-deficient cells to *Shigella* infection were similar and resembled necrosis rather than apoptosis.

**Bacterial Components Common to Gram-negative Bacteria Have Cell Death-inducing Activity**—*IpaB* secreted by *Shigella* has been reported to be the sole bacterial agent capable of triggering macrophage programmed cell death because the protein interacts with and activates caspase-1 (12, 13, 20). *IpaB* and *IpaC* are essential for TTSS function because they mediate the transfer of effector proteins through the host cell membrane by forming membrane pores at the points of contact.

**FIG. 1.** Wild-type *Shigella* induce caspase-1-independent cell death of infected antigen-presenting cells. **A,** LDH release from infected wild-type and caspase-1-deficient BMDCs and BMMs. **B,** IL-12 production in infected wild-type and caspase-1-deficient BMDCs 24 h after infection. **C,** surface expression of CD40 in infected wild-type and caspase-1-deficient BMDCs 24 h after infection. **D,** TUNEL staining of mouse lung cells after intranasal infection by wild-type *Shigella*. Lung sections from wild-type or caspase-1-deficient mice 6 or 12 h after infection were stained with fluorescent TUNEL and TRITC-labeled anti-LPS serum and counterstained with hematoxylin. Arrows indicate TUNEL-positive infected cells with macrophage morphology in wild-type mice 6 h after infection. **E,** quantitation of TUNEL-positive cells in the infected lung sections. The data are mean values of five digitized images (field area 0.45 mm²) from at least three sections.
between the TTSS needles and the host membrane. The activities are also involved in the ability of *Shigella* to disrupt the phagocytic vacuoles and escape into the macrophage cytoplasm. Thereby the *ipaB* and *ipaC* mutants are unable to escape from phagocytic vacuole into the cytoplasm (12, 39). To investigate whether *IpaB* and *IpaC* (and *IpaD*) are involved in inducing macrophage cell death, we created a co-infection system of *Shigella* mutants and *L. monocytogenes* in which secretion of listeriolysin O by *Listeria* helps the non-invasive *Shigella* mutants to escape into the cytoplasm. Infection of macrophages with *Listeria* alone did not induce appreciable cell death by 5 h after infection (data not shown). To validate this system, RAW264.7 cells expressing N-WASP (N-WASP is required for *Shigella* VirG-mediated actin polymerization) (40) were infected with S325 (a TTSS-deficient *Shigella* mutant that expresses VirG, which is required to mediate actin polymerization) together with *Listeria*, and the ability of S325 to induce actin assembly was investigated by immunofluorescence staining with anti-Listeria LPS antibody and phalloidin (40). With this system, the bacterium residing within the cytosol could be assembled by polymerized actin. As seen in Fig. 3A, YSH6000 formed actin assemblies by 30 min after infection of RAW264.7 expressing N-WASP. Under the same condition, the number of actin-positive S325 co-infected with *Listeria* also gradually increased (Fig. 3A), thus demonstrating that S325 resided within the macrophage cytoplasm. By exploiting a similar system using ΔJ774A.1 macrophages, the ability of Δ*ipaB* to cause cell death was examined when co-infected with *Listeria*. At 2 h after infection, Δ*ipaB* was able to induce cell death as judged by LDH release, and the induced cell death resembled necrotic cell death with extensive blebbing and was indistinguishable from that induced by YSH6000 (Fig. 3B and supplemental movies Fig. 3Bmovie1.mov and Fig. 3Bmovie2.mov). In a similar manner, Δ*ipaC*, Δ*ipaD*, S325, and YSH6200 (large plasmid-cured *Shigella* mutant) were all still capable of inducing cell death when co-infected with *Listeria* (Fig. 3C). Furthermore, an *E. coli* strain, DH5α, also induced cell death when co-infected with *Listeria* (Fig. 3C). Intriguingly, no LDH release was observed when Gram-positive bacteria, such as *B. subtilis* or *S. aureus*, were co-infected with *Listeria*, strongly suggesting that bacterial component(s) common to Gram-negative bacteria have potent activity to induce necrotic cell death.

To further characterize the cell death induced by the Gram-negative bacteria internalized into macrophage cytoplasm, surface exposure of phosphatidylserine (PS) and incorporation of propidium iodide (PI) into cell nucleus were determined by annexin-V labeling and incorporation of PI, respectively, in the macrophages infected with *Shigella* since PS exposure occurs prior to nuclear PI incorporation during classical apoptosis (41). Although a small population of PS exposure alone was detected, PS exposure and PI incorporation occurred concomitantly in macrophages infected with YSH6000 (Fig. 3D) (18). Although the rates of increase in both PS exposure and PI incorporation were slightly slower than during YSH6000 infection, all of the *Shigella* mutants, including DH5α, increased both PS exposure and PI incorporation when co-infected with *Listeria* (Fig. 3D), further indicating that the bacterial component(s) common to Gram-negative bacteria possess activity to stimulate necrotic cell death.

To further investigate whether Gram-negative bacteria residing in macrophage cytoplasm are capable of inducing cell death regardless of the presence of caspase-1, wild-type and caspase-1-deficient BMMs were infected with S325 and *Listeria*, and the levels of LDH release were measured. As shown in Fig. 3E, although LDH release by wild-type cells had begun 2 h after infection, it gradually increased in the caspase-1-deficient cells after 3 h and increased further by 7 h. Thus, these results indicate that in addition to *Shigella*, Gram-negative bacteria that are delivered to the cytosol also stimulate caspase-1-independent cell death pathway.

**Activation of the Caspases Is Involved in Triggering the Rapid Cell Death Event**—When Gram-negative bacteria, such as S325 (TTSS-deficient *Shigella*), YSH6200 (plasmid-cured *Shigella*), or DH5α, were translocated into the cytoplasm of wild-type BMMs, rapid cell death was induced. We therefore investigated whether activation of caspase-1 is involved in inducing cell death by immunoblotting with anti-caspase-1 antibody. As seen in Fig. 4A, cleavage of caspase-1 was detected when J774 A.1 cells were infected with Δ*ipaB*, Δ*ipaC*, Δ*ipaD*, S325, and DH5α together with *Listeria*; the same was true of caspase-3 and -9 activation. Since infection of each of the strains without *Listeria*, or with *Listeria* alone, did not result in caspase activation, the results suggested that the bacterial component(s) are capable of stimulating the caspase-mediated...
cell death cascade. To determine whether it does, we investigated LDH release in wild-type or caspase-1-deficient BMMs infected with YSH6000 in the presence of Z-VAD, a broad caspase inhibitor. The addition of Z-VAD suppressed LDH release in BMMs infected with YSH6000 by 2 h after infection, as reported previously (13, 14, 39); however, no further effect was observed after 3–5 h of infection (Fig. 4B). By contrast, no inhibitory effect of the addition of Z-VAD on LDH release was observed in caspase-1-deficient BMMs at all during this period. Since the cell death responses in Z-VAD-treated wild-type BMMs were similar to that observed in caspase-1-deficient BMMs (Figs. 1A and 3E), these findings suggested that activation of caspases is involved in triggering the rapid cell death event.

Lipid A Is a Potent Inducer of Macrophage Death—To identify the bacterial component(s) that are capable of stimulating macrophage cell death, we first tested whether the bacterial lysates can trigger cell death by using a co-infection system with Listeria. The addition of lysates from wild-type Shigella with Listeria slightly increased LDH release from infected J774A.1 cells at 5 h after infection (Fig. 5B). To facilitate the access of bacterial components to the cytosol of macrophages, bacterial lysates were immobilized on carboxylated latex microbeads to increase their uptake through phagocytosis in the presence of Listeria. The immobilization on the beads was confirmed by immunostaining with FITC-labeled anti-LPS antibody (Fig. 5A). LDH release from the macrophages was evaluated after adding the lysate-adsorbed beads together with Listeria. As shown in Fig. 5B, LDH release by J774A.1 cells was increased significantly, and the cells protruded extensive membrane blebbing similar to that observed in bacterial infection. Similar cellular responses were also observed when beads coated with bacterial lysates from E. coli DH5α were added (data not shown). Intriguingly, the cytotoxic activity present in the bacterial lysates was not diminished by boiling or exposure to proteinase K (Fig. 5C), suggesting that some non-protein bacterial component(s), such as LPS or PGN, is involved in stimulating cell death. We therefore examined purified LPS, alkaline-detoxified LPS (inactivated lipid A, aLPS), lipid A, and PGN from Shigella and S. aureus for their ability to stimulate macrophage cell death by the same methods as for the bacterial lysates. The quality of each of the components was checked by using NF-κB reporter gene assay in 293T cells harboring expression plasmids encoding TLR4/MD-2 (for LPS) (42), Nod1 (for diaminopimelate-type PGN) (7), and Nod2 (for diaminopimelate- and Lys-type PGN) (43) (data not shown). These components were adsorbed on the beads and added to J774A.1 macrophages with Listeria. As seen in Fig. 5D, LPS and lipid A, but not aLPS, Gram-negative, or -positive PGN, induced LDH release with extensive membrane blebbing only when co-infected with Listeria, strongly indicating that LPS, including lipid A, is a potent inducer for stimulating macrophage cell death.

**Fig. 3.** Components common to Gram-negative bacteria induce both caspase-1-dependent and -independent necrotic cell death. **A**, quantitative analysis of intracytoplasmic bacteria. RAW 264.7 macrophages expressing N-WASP were infected with green fluorescent protein-expressing wild-type Shigella or the green fluorescent protein-expressing S325 mutant with or without Listeria. The number of actin-associated intracytoplasmic Shigella in infected cell was counted. The immunofluorescence images were shown at 2 h after infection. F-actin and intracellular Listeria were visualized with rhodamine-phalloidin and Cy5-labeled anti-Listeria antibody, respectively. Actin-associated green fluorescent protein-expressing Shigella are visualized as yellow. Bar, 10 μm. B, time-lapse images of infected J774A.1 macrophages with wild-type Shigella and IpaB mutants with Listeria. Arrows indicate the large extensive membrane blebbing from the cells. C, LDH release from J774A.1 macrophages infected with the bacteria indicated with or without Listeria. D, flow cytometric analysis of infected macrophages. Infected cells were stained with annexin-V-FITC and PI. The data represent the results of three individual experiments. E, LDH release from wild-type or caspase-1-deficient BMMs co-infected with the S325 mutant and Listeria.
death. To confirm the cytotoxic activity of LPS and exclude some effects of the co-infected Listeria, each component was microinjected directly into the cytosol of BMMs. The macrophages microinjected with LPS and lipid A, but not with aLPS or PBS, caused PS exposure, determined by staining with annexin-V (Fig. 5E), whereas the addition of the components to

Fig. 4. Caspase activation in infected J774A.1 macrophages. A, full-length and cleaved fragments of caspase-1, -9, or -3 were detected by Western blotting with their specific antibodies. B, effect of Z-VAD on LDH release from Shigella-infected wild-type or caspase-1-deficient BMMs. Me2SO, used for solubilizing Z-VAD, was also added to the control cells and had a slight inhibitory effect on the LDH release.

Fig. 5. Lipid A is a potent inducer of cell death in the cytosol of J774A.1 macrophages. A, LPS-adsorbed latex beads. Beads incubated with or without Shigella lysates were immunostained with FITC-labeled anti-LPS antibody. Bar, 10 μm. B, enhancement of LDH release from macrophages by bacterial lysates-adsorbed latex beads. The beads were added to macrophages with and without Listeria. C, the effect of boiling and proteinase K treatment on lysates that induced LDH release. D, LDH release from macrophages by purified component-adsorbed latex beads. E, microinjection of purified components into the cytosol of BMMs. The components (at a needle concentration of 0.5 mg/ml) were injected with Cy5-labeled anti-mouse goat IgG. At 30 min after injection, the cells were stained with annexin-V-Alexa 568 to detect PS exposed on the cell surface. F, LDH release from infected wild-type and caspase-1-deficient BMMs with YSH6000 or isogenic msbB1/2 mutant. Student’s t tests were performed to measure statistical significance (*, significantly different at p < 0.05; **, significantly different at p < 0.01).
Macrophage invasion (data not shown). Decreases the biological activity of lipid A (44, 45) but not lipid A (44, 45). The absence of the secondary myristate residue acyl-oxy-acylation by myristic acid at the C3 of glucosamine of lipid A (44, 45). The absence of the secondary myristate residue decreases the biological activity of lipid A (44, 45) but not macrophage invasion (data not shown). \( \Delta \text{msbB1/2} \)-infected BMMs were compared with YSH6000-infected BMMs for their ability to release LDH. As shown in Fig. 5F, the rate of LDH release by wild-type BMMs infected with the mutant was significantly less than the rate of release by YSH6000. Thus, a Shigella mutant expressing lipid A with reduced biological activity exhibited attenuated the ability to induce cell death in macrophages. Indeed, the extensive membrane blebbing by wild-type BMMs infected with \( \Delta \text{msbB1/2} \) was delayed when compared with the cells infected with YSH6000 in 1–2 h after infection (data not shown). The effect of the \( \text{msbB1/2} \) mutation was also observed in LDH release from caspase-1-deficient BMMs (Fig. 5F), suggesting that the lipid A activity is involved in the induction of caspase-1-independent cell death.

Shigella-induced Macrophage Cell Death Is Distinct from the Cell Death Mediated by TLR4—Since lipid A is well known to be the major bacterial component able to stimulate the TLR4-mediated signaling pathway (46), we used BMMs derived from TLR4-deficient mice (36) to investigate whether the pathway is involved in the lipid A-mediated cell death events by Shigella infection. As shown in Fig. 6A, TLR4-deficiency had no effect on the cell death pathway induced by Shigella or Gram-negative bacteria delivered to the cytosol. By contrast, the extracellular lipid A stimulation induced IL-1\( \beta \) and IL-6 production in wild-type BMMs but not in TLR4-deficient BMMs (Fig. 6B), indicating that the cytosolic bacteria-mediated cell death pathway is independent of TLR4 signaling.

### DISCUSSION

In the present study, we used caspase-1-deficient BMMs and BMDCs to investigate the impact of caspase-1-independent cell death in Shigella infection and obtained the following findings. (i) In the absence of caspase-1, Shigella infection of BMMs and BMDCs is capable of eliciting cell death 2 h later than in the presence of caspase-1; (ii) the infected cells undergo necrotic rather than apoptotic cell death; (iii) the cell death and caspase-1 activation induced by Shigella are not dependent on IpaB activity but on the bacterial internalization into the cytoplasm; (iv) translocation of non-invasive Shigella mutants and \( E. \) coli K-12, but not Gram-positive bacteria, into the macrophage cytoplasm is capable of stimulating cell death regardless of the caspase-1 activity; (v) lipid A translocated into macrophage cytoplasm is capable of inducing cell death; and (vi) the cell death induced by the cytoplasmic lipid A is independent of that mediated by TLR4. Based on these findings, we propose that the observed caspase-1- and IpaB-independent cell death events induced by Shigella (or Gram-negative bacteria) internalized into the macrophage cytoplasm is an important mechanism for eliciting inflammatory responses, which seem to be mediated by unidentified pathway(s).

Shigella Induce Macrophage Cell Death Independent of Caspase-1 Activity—Shigella infection of macrophages has been indicated to induce rapid cell death, and the ability of IpaB to interact with and activate caspase-1 is the most crucial issue since the \( \text{ipaB} \) mutant showed no activity to induce cell death and wild-type Shigella infection of caspase-1-deficient macrophages did not result in induction of cell death until 2 h after infection (Fig. 1). Because the \( \text{ipaB} \) mutant is unable to be translocated into the cytoplasm of macrophages and the IpaB activity is essential for the TTSS function, the direct role of IpaB in inducing apoptotic death by Shigella infection of macrophages remains unclear. In this study, we found that

**FIG. 6. Intracytosolic bacteria-mediated cell death pathway is distinctive from that mediated by TLR4.** A, LDH release from wild-type and TLR4-deficient BMMs after infection with the bacteria indicated. B, extracellular lipid A stimulation induced IL-1\( \beta \) and IL-6 production in wild-type BMMs but not in TLR4-deficient BMMs. After adding lipid A (1 \( \mu \)g/ml), expression of IL-1\( \beta \) and \( \beta \)-actin (as a control) was analyzed by Western blotting with specific antibodies. The amounts of IL-6 in the cell culture supernatants 24 h after stimulation were measured by enzyme-linked immunosorbent assay.
abrupt cell death by *Shigella* assessed on the basis of LDH release by caspase-1-deficient cells occurred after 2 h later than in the presence of caspase-1, and the kinetics for the LDH release by caspase-1-deficient cells were substantially comparable with that in the wild-type cells. Furthermore, translocation of TTSS-deficient mutant unable to secrete IpaB into cytoplasm caused cell death of both wild-type and caspase-1-deficient macrophages. The ability of *Shigella* to induce cell death in the absence of caspase-1 was also demonstrated in a murine pulmonary infection model. Although the number of TUNEL-positive cells in the sections of the lungs of caspase-1-deficient mice in the initial stage of infection, such as at 6 h after infection, was still one-third that of the wild-type, by 12 h after infection, it had dramatically increased to a level similar to that in the wild-type mice. Thus, these data clearly showed that although *Shigella* infection promotes rapid cell death in the presence of caspase-1, the infection of caspase-1-deficient cells induces extensive cell death via a different pathway from that triggered by caspase-1 activity.

*Shigella Induce Necrotic Macrophage Cell Death—* As mentioned in the Introduction, the type of macrophage cell death caused by *Shigella* infection is a matter of controversy (14, 15, 17). In this study, time-lapse imaging and electron microscopic analysis clearly indicated that both caspase-1-dependent and -independent cell death as a result of *Shigella* infection resembled necrotic cell death rather than typical apoptotic cell death. Indeed, the morphological features of infected wild-type or the caspase-1-deficient macrophages are indistinguishable with extensive membrane blebbing and membrane integrity. The same was true of BMDCs and peritoneal macrophages from wild-type and caspase-1-deficient mice, and necrotic cell death was also observed in J774A.1 macrophages (Fig. 3), mouse RAW264.7 macrophages, or human monocyte-derived macrophages infected with *Shigella*. LDH assay has been widely used to detect the cytotoxicity of infected macrophages. Cyttoplasmic enzymes such as LDH are thought to be released only by necrotic cells as a result of loss of cell membrane integrity, however, under certain conditions, apoptotic cells undergo secondary necrosis accompanied by the release of LDH. When wild-type or caspase-1-deficient macrophages were infected with *Shigella*, LDH release was detected concomitant with the formation of large membrane blebbing, suggesting that LDH release by the cells killed by *Shigella* infection does not result from the secondary necrotic event. This notion was confirmed by double staining with annexin-V and PI of infected J774A.1 cells, in which the double-positive population, but not annexin-V single-positive cell population (apoptosis), had significantly increased during infection (Fig. 3D). Taken together, our results strongly suggest that infection of macrophages by *Shigella* stimulates the necrotic cell death pathway and that when caspase-1 is intact, its activity together with that of other caspases, such as caspase-3 and -9, may also play an important role in stimulating rapid cell death (Fig. 4).

*Shigella Can Induce IpaB-independent Cell Death in Macrophages—* Since the *ipaB* mutant, S325 (the TTSS-deficient mutant), or *E. coli* K-12, when translocated into the macrophage cytoplasm by using the activity of *Listeria* listerialysin O to disrupt phagocytic vacuole, was demonstrated to elicit cell death, we concluded that some bacterial component(s) common to Gram-negative bacteria is involved in stimulating cell death and that the components induce activation of caspase-1, -3, and -9 in the absence of IpaB. These results were strikingly different from those of a previous study in which *ipa* mutants expressing *E. coli* hemolysin allowed the mutant to escape from phagocytic vacuoles and *ipaB* mutant, but not *ipaC* or *ipaD* mutant, failed to induce cell death (12). Analysis of various microbead-immobilized bacterial components for the ability to induce cell death indicated that the lipid A moiety of LPS is a potent inducer of the death pathway when translocated into the macrophage cytoplasm via the *Listeria* co-infection system. To confirm the participation of lipid A activity in the bacteria-induced cell death event, we created the *msbB12* double mutant from *Shigella* YSH6000 (wild-type S. flexneri), which lacks the secondary myristate residue of lipid A, and investigated it for an effect on macrophage cell death. The results showed that infection of BMMs derived from wild-type or the caspase-1-deficient mice with the *msbB12* mutant significantly decreased the capacity to induce cell death as judged by LDH release. However, since *msbB12* mutation does not completely inhibit LDH release from infected macrophages, the other bacterial factor rather than lipid A may also be involved in the cell death pathway. Moreover, when lipid A or LPS was added to the external medium of macrophages, it was unable to induce cell death. TLR4 is well known to recognize LPS (lipid A) (47), which is localized on the cell surface and also recruited into the phagosomal compartments. LPS has been indicated to have both proapoptotic and anti-apoptotic activities in various cells, such as endothelial cells and macrophages, in which the activities have been indicated to depend on the TLR4-mediated pathways (2, 48). We therefore used TLR4-deficient BMMs to investigate whether bacterial translocation into macrophage cytoplasm to induce cell death also depends on the TLR4 activity, and the results strongly suggested that bacterial LPS or the other components released into the cytosol of macrophages is likely to be recognized by a yet unidentified host factor other than TLR4. In this regard, it is worth noting the negative results obtained with the involvement of Nod1 and Nod2 in mediating cell death since growing evidence has indicated that cytosolic bacterial components recognized as pathogen-associated molecular patterns are involved in the activation of caspase-1 and in innate immune systems. The members of Nod family proteins that contain nucleotide-binding and leucine-rich repeat domains, Nod1 and Nod2, can recognize bacterial PGN and mediate the induction of caspase-1 and NF-κB activation (8). Indeed, Nod1 senses PGN derived from intracellular bacteria in *Shigella*-infected epithelial cells and activates NF-κB and IL-8 production (9, 10). However, when Nod1- or Nod2-deficient BMMs derived from the knock-out mice were infected with *Shigella*, they were still able to induce cell death the same as in wild-type BMMs. Since no host molecule has yet been identified as responsible for the recognition of cytoplasmic bacterial LPS (lipid A), we are currently striving to identify the putative factor(s) that mediate the cell death pathway. Since LPS including lipid A released by bacteria has been shown to enter subcellular organelles such as Golgi apparatus (49, 50), we believe that this putative death pathway is also important as a mechanism of stimulating the host inflammatory responses by recognizing the pathogen-associated molecular patterns in the macrophage cytosol.

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