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Leptospira interrogans Induces Apoptosis in Macrophages via Caspase-8- and Caspase-3-Dependent Pathways▽

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Apoptosis of host cells plays an important role in modulating the pathogenesis of many infectious diseases. It has been reported that *Leptospira interrogans*, the causal agent of leptospirosis, induces apoptosis in macrophages and hepatocytes. However, the molecular mechanisms responsible for host cell death remained largely unknown. Here we demonstrate that *L. interrogans* induced apoptosis in a macrophage-like cell line, J774A.1, and primary murine macrophages in a time- and dose-dependent manner. Apoptosis was associated with the activation of cysteine aspartic acid-specific proteases (caspase-3, caspase-6, and caspase-8), the increased expression of Fas-associated death domain (FADD), and the cleavage of the caspase substrates poly(ADP-ribose) polymerase (PARP) and nuclear lamina protein (lamin A and lamin C). Caspase-9 was activated to a lesser extent, whereas no release of cytochrome *c* from mitochondria was detectable. Inhibition of caspase-8 impaired *L. interrogans*-induced caspase-3 and -6 activation, as well as PARP and lamin A/C cleavage and apoptosis, suggesting that apoptosis is initiated via caspase-8 activation. Furthermore, caspase-3 was required for the activation of caspase-6 and seemed to be involved in caspase-9 activation through a feedback amplification loop. These data indicate that *L. interrogans*-induced apoptosis in macrophages is mediated by caspase-3 and -6 activation through a FADD–caspase-8-dependent pathway, independently of mitochondrial cytochrome *c*–caspase-9-dependent signaling.

Apoptosis is a genetic and biochemical process that plays an essential role in morphogenesis, host defense, and homeostasis of all tissues (29, 46). Caspases are a family of cysteine proteases that mediate apoptosis induced by a variety of stimuli. Based on their structures and order in cell death pathways, caspases can be divided into initiators (such as caspase-2, -8, -9, -10, and -12) and effectors (such as caspase-3, -6, and -7) (13). Two pathways, the intrinsic and extrinsic death pathways, have been identified in most cases of caspase-dependent apoptosis (9). The intrinsic death pathway involves mitochondrial release of cytochrome *c*, which interacts with Apaf-1 and dATP to promote procaspase-9 autoactivation, which in turn activates downstream effectors such as caspase-3, -6, and -7 (19). The extrinsic death pathway is initiated by the engagement of cell surface death receptors (CD95/Fas/APO-1 and tumor necrosis factor receptor). The complex of death receptors and ligands leads to the recruitment of the adapter molecule FADD and the activation of caspase-8. In some cells, active caspase-8 is sufficient to activate caspase-3 directly. In other cells, however, caspase-8 indirectly mediates caspase-3 activation by cleaving the proapoptotic Bcl-2 family member Bid, which induces mitochondrial cytochrome *c* release and thereby links the two pathways (37). After activation, caspases cleave various cellular substrates (such as PARP and lamin A/C), resulting in membrane blebbing, chromatin condensation, and the formation of apoptotic bodies.

There is increasing evidence that apoptosis can be triggered by a wide range of microbial pathogens (12). To grow inside the host and cause disease, microbial pathogens have evolved different survival strategies. Pathogen-induced host cell apoptosis might be advantageous for pathogens in helping them evade host defenses, allowing bacterial replication, and facilitating the release of intracellular bacteria after the termination of intracellular replication. From the point of view of the host, death of pathogen-infected cells may be required in order to kill the intracellular pathogens and reduce or eliminate the production of viable pathogenic organisms (15). The role of host cell apoptosis and the underlying molecular processes differ among pathogens, and this reflects the diversity of the pathogenic mechanisms involved in a given type of infection (16).

Leptospirosis is a worldwide zooanthroponotic disease caused by spirochetes of the genus *Leptospira* (4, 28). The clinical manifestations of human leptospirosis range from mild febrile illness to severe multiorgan failure, characterized by jaundice, pulmonary hemorrhage, and renal failure (23). Leptospires can be divided into pathogenic (e.g., *Leptospira interrogans*, *L. borgpetersenii*, *L. weilii*, and *L. kirschneri*) and nonpathogenic (e.g., *L. biflexa*) species. Pathogenic leptospires are highly motile and invasive spirochetes that have the capacity to survive and grow in tissues by escaping natural defense mechanisms. In recent years, many leptospiral adhesins and lipoproteins have been reported to play roles in invasion and colonization during pathogenesis (3, 8, 21, 32). In contrast to our knowledge of these processes in the pathogen, much less is

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known about the molecular and functional responses of host cells. *Leptospira* infection induces NF-κB activation, p38 phosphorylation, and inflammatory chemokine production in microglia (5). Studies by others have shown that *L. interrogans* induces apoptosis in macrophages in vitro and in hepatocytes in vivo (30, 31). However, the underlying mechanisms responsible for *L. interrogans*-induced apoptosis remain uncharacterized. The present study was therefore designed to investigate the molecular requirements for apoptosis induction by *L. interrogans*. We propose a model in which *L. interrogans*-induced murine macrophage apoptosis is mediated through an extrinsic pathway of apoptosis, namely, through caspase-3 and -6 activation downstream of a FADD–caspase-8-dependent pathway, in the absence of cytochrome *c*–caspase-9-dependent signaling.

MATERIALS AND METHODS

Abbreviations. Caspase, cysteine aspartic acid-specific protease; Apaf-1, apoptosis proteinase-activating factor-1; FADD, Fas-associated death domain; PARP, poly(ADP-ribose) polymerase; lamin A/C, nuclear lamina protein (lamin A and lamin C); EMJH, Ellinghausen-McCullough-Johnson-Harris; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; MOI, multiplicity of infection; LDH, lactate dehydrogenase; PI, propidium iodide; TEM, transmission electron microscopy; AFC, 7-amino-4-trifluoromethyl coumarin; Ac-DEVD-AFC, acetyl-Asp-Glu-Val-Asp-AFC; Ac-VEID-AFC, acetyl-Val-Glu-Ile-Asp-AFC; Ac-IETD-AFC, acetyl-Ile-Glu(OMe)-Thr-Asp(OMe)-AFC; Ac-LEHD-AFC, acetyl-Leu-Glu-His-Asp-AFC; fmk, fluoromethyl ketone; Z-DEVD-fmk, benzyloxycarbonyl-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-fmk; Z-VEID-fmk, benzyloxycarbonyl-Val-Glu-Ile-Asp(OMe)-fmk; Z-IETD-fmk, benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fmk; Z-LEHD-fmk, benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fmk; OMe and OCH_3, hydroxymethyl; SDS, sodium dodecyl sulfate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SD, standard deviations.

Bacterial strains and culture conditions. *L. interrogans* serovar Lai strain Lai 56601 (39) (serogroup Icterohaemorrhagiae) and *L. biflexa* serovar Patoc strain Patoc I (serogroup Samaranga) were used in this study. They were purchased from the National Institute for the Control of Pharmaceutical and Biological Products in Beijing, China. Both leptospiral strains were cultivated at 28°C in EMJH (14) liquid medium supplemented with 8% heat-inactivated rabbit serum. To maintain virulence, strain Lai was preserved by deep freezing and propagated by iterative passage in specific-pathogen-free Dunkin-Hartley ICO:DH (Poc) guinea pigs (10 to 12 days old; each weighing less than 150 g). Guinea pigs were infected intraperitoneally with low-passage-number leptospires in EMJH medium. Control animals were injected with sterile EMJH medium alone by the same route. When prostration and anorexia appeared after inoculation, leptospires were isolated from the blood of infected guinea pigs and stored at −70°C. For infection experiments, frozen *L. interrogans* was thawed and passaged <2 times in liquid medium. Animal protocols were approved by the Animal Ethics Review Committee of Zhejiang University.

Cell lines, cultivation, and infection. The murine monocyte/macrophage-like cell line J774A.1, the human alveolar epithelial cell line A549, and the HUVEC lines EVC304 and HUV-EC-C were purchased from the American Type Culture Collection (Manassas, VA). J774A.1 and EVC304 cells were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY). A549 and HUV-EC-C cells were maintained in F-12K medium (ATCC, Manassas, VA). Both media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO). For HUV-EC-C cells, F-12K medium was further supplemented with 0.1 mg/ml heparin (Sigma) and 0.03 mg/ml endothelial cell growth supplement (Sigma). Cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2. For infection, J774A.1 (1×10^5 cells/well), EVC304 (5×10^4 cells/well), HUV-EC-C (5×10^4 cells/well), and A549 (5×10^4 cells/well) cells were seeded in 12-well tissue culture plates. *Leptospira* was harvested by centrifugation (12,000 × *g*, 15 min, 15°C), washed twice with PBS (0.01 M; pH 7.2), and resuspended in warm (28°C) RPMI 1640 or F-12K medium without antibiotics. *Leptospira* organisms were counted by dark-field microscopy with a Petroff-Hausser counting chamber (Fisher Scientific, Pittsburgh, PA). Cell monolayers were washed three times with PBS to remove antibiotics and loosely adherent cells and were then infected at different MOIs for the indicated times. As a negative control, uninfected cells were incubated in RPMI 1640 medium without antibiotics.

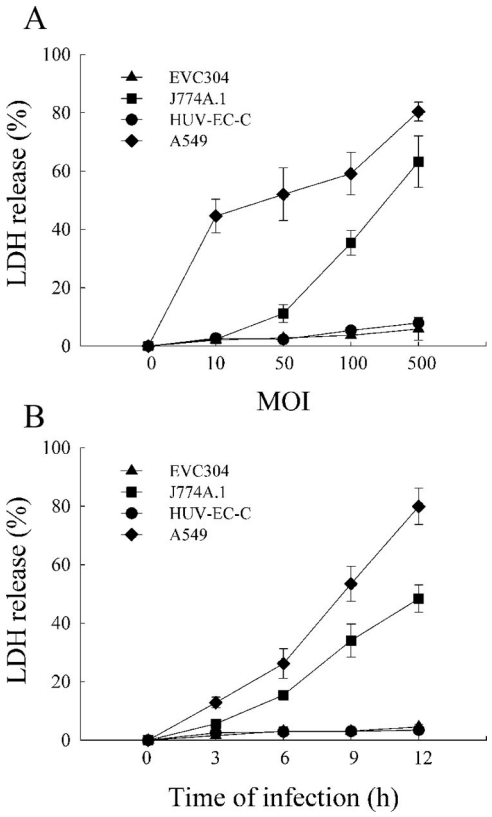

FIG. 1. Effects of *L. interrogans* infection on the viability of host cells. (A) Cells were incubated with *L. interrogans* at different MOIs for 8 h. (B) J774A.1, EVC304, HUV-EC-C, and A549 cells were incubated with or without *L. interrogans* (at MOIs of 100, 100, 100, and 10, respectively) for different periods. Cell viability was measured by LDH release assays. Results are means ± SD for triplicate wells from three separate experiments.

Murine macrophage isolation and infection. Murine peritoneal macrophages were isolated from male BALB/c mice (6 to 8 weeks old; Zhejiang Experimental Animal Center) by washing the peritoneal cavities with cold RPMI 1640 medium (40). Macrophages were seeded in 12-well tissue culture plates (2×10^5 cells/well) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and were incubated for 2 h at 37°C under a humidified atmosphere containing 5% CO_2. After the incubation, nonadherent cells were removed by gentle washing, and the remaining macrophages were infected with *Leptospira* by following the protocol described above. Animal protocols were approved by the Animal Ethics Review Committee of Zhejiang University.

Cytotoxicity assays. LDH release assays were used to quantify cell death. Briefly, cells were grown in multiwell plates and infected as described above except that serum-free RPMI 1640 medium was used to avoid the LDH activity present in serum. The supernatant was then collected, and LDH activity was measured colorimetrically using a CytoTox 96 cytotoxicity assay kit (Promega) and was read on a Bio-Rad model 550 microplate reader at 490 nm. Spontaneous LDH release was determined by assaying supernatants from uninfected cells, and total LDH was determined by using uninfected cells that had been lysed by 0.5% Triton X-100. The percentage of LDH release (cytotoxicity) was calculated as $(LDH_{infected} - LDH_{spontaneous})/(LDH_{total} - LDH_{spontaneous}) \times 100$.

Flow cytometry assay of apoptosis. After treatment and incubation, adherent and floating cells were collected by trypsinization and centrifuged at 1,000 × *g* for 5 min. Cell pellets were washed three times with PBS, resuspended in annexin V binding buffer with fluorescein isothiocyanate-conjugated annexin V, and incubated for 15 min in the dark according to the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). After PI was added, the cell suspension was immediately analyzed by FACSCalibur flow cytometry and CellQuest Pro soft-

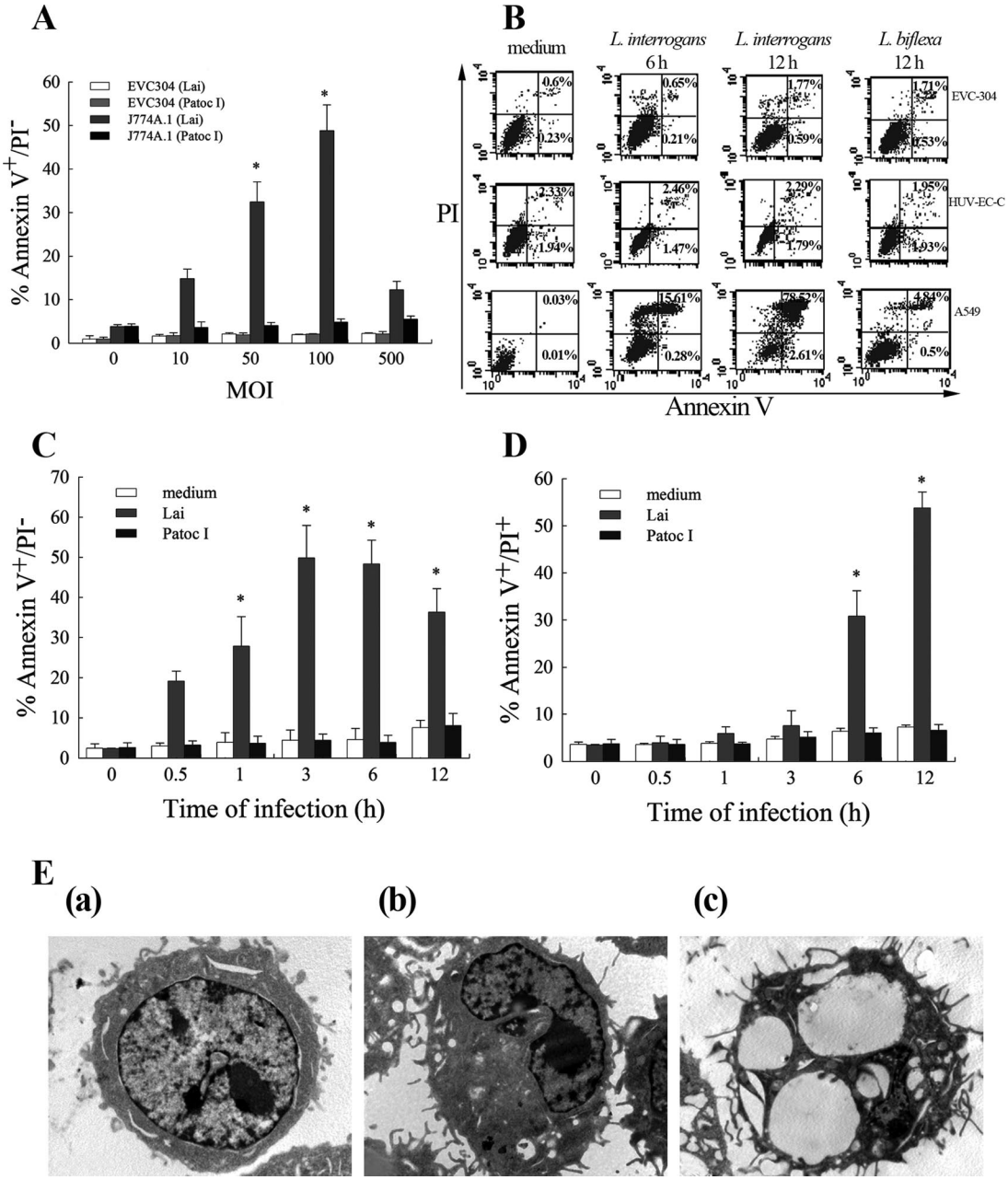

FIG. 2. Cell-specific cell death induced by *L. interrogans*. (A) J774A.1 and EVC304 cells were incubated with *L. interrogans* or *L. biflexa* at the indicated MOIs for 5 h. (B) EVC304, HUV-EC-C, and A549 cells were incubated with or without leptospires at MOIs of 100, 100, and 10, respectively. Representative scatter plots for three independent experiments are shown. (C and D) J774A.1 cells were incubated with or without *L. interrogans* and *L. biflexa* at an MOI of 100 for different periods. The proportions of annexin V^+ PI^- and annexin V^+ PI^+ cells were measured by flow cytometry. Data are means ± SD for three independent experiments (∗, $P < 0.01$ for comparison with uninfected cells). (E) Transmission electron micrographs of uninfected and *L. interrogans*-infected J774A.1 cells. (a) Control cells showed normal cellular morphology. (b and c) J774A.1 cells infected by *L. interrogans* for 2 h (b) or 6 h (c) demonstrated characteristic features of apoptosis, including cytoplasmic vacuolation and condensed nuclear chromatin.

ware (Beckman Coulter, Fullerton, CA). Cells in early phases of apoptosis bind annexin V but exclude PI, while those in the late process or necrotic cells stain simultaneously with annexin V and PI.

TEM assay of apoptosis. Cells were washed three times with PBS and collected by centrifugation at 1,000 × *g* for 10 min. The cell pellets were fixed for 2 h at 4°C in a solution containing 2.5% formaldehyde and were washed three times with PBS. Pellets were then postfixed with osmium tetroxide, rinsed, dehydrated, and embedded in epoxy resin (Sigma). Ultrathin sections were collected on 100- to 150-mesh nickel grids (Plano, Wetzlar, Germany), stained with lead citrate and uranyl acetate, and then examined by TEM (Tecnai-10; Philips, Eindhoven, The Netherlands).

Fluorometric analysis of caspase activity. The enzymatic activities of caspase-3, -6, -8, and -9 were measured according to the manufacturer's instructions (caspase fluorometric assay kit; BioVision, Inc., Mountain View, CA). Briefly, control and treated cells were collected, washed with ice-cold PBS, and resuspended in 50 μl of chilled cell lysis buffer for 10 min on ice. Lysates were clarified by centrifugation (10,000 × *g*, 10 min, 4°C), aliquoted, and stored at −80°C until assay. Total-protein concentrations in lysates were determined using

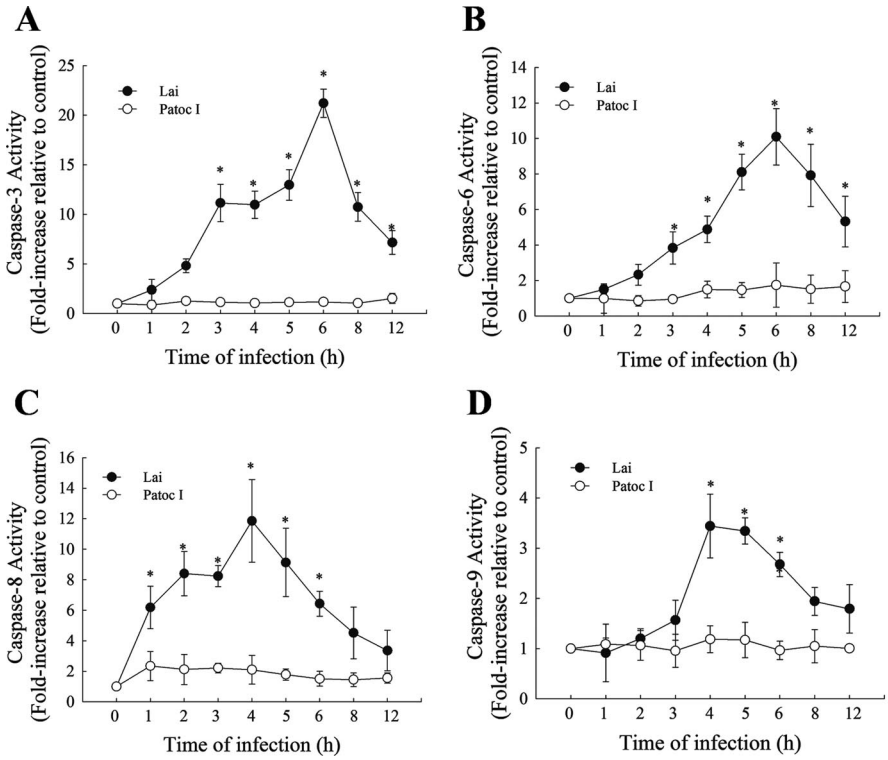

FIG. 3. Caspase activation during *L. interrogans* infection. J774A.1 cells were incubated with or without *L. interrogans* or *L. biflexa* at an MOI of 100 for the indicated times. Cell lysates were prepared, and caspase activities were determined with the fluorogenic substrates Ac-DEVD-AFC (for caspase-3), Ac-VEID-AFC (for caspase-6), Ac-IETD-AFC (for caspase-8), and Ac-LEHD-AFC (for caspase-9). Data are means for three independent experiments ± SD (*, $P < 0.01$ for comparison with the uninfected control). The value for the uninfected control was set at onefold).

the bicinchoninic acid assay (Beyotime Institute of Biotechnology, China). For assays, equal amounts of lysate were incubated with 50 μl reaction buffer containing 10 mM dithiothreitol and 50 μM caspase fluorometric substrates (Ac-DEVD-AFC for caspase-3, Ac-VEID-AFC for caspase-6, Ac-IETD-AFC for caspase-8, and Ac-LEHD-AFC for caspase-9) at 37°C for 1 h. The fluorescence of the released AFC was measured with a fluorometer (Synergy HT; Bio-Tek Instruments, Inc.) using excitation and emission wavelengths of 400 and 505 nm, respectively. The increase in caspase activity was then determined relative to the activity of the uninfected control.

Use of caspase inhibitors. The caspase-3 inhibitor Z-DEVD-fmk, the caspase-6 inhibitor Z-VEID-fmk, the caspase-8 inhibitor Z-IETD-fmk, and the caspase-9 inhibitor Z-LEHD-fmk were purchased from BioVision, Inc. All inhibitors were used at 100 μM, a concentration known to optimally inhibit specific caspase activity. Cells were pretreated with the inhibitors for 1 h at 37°C. After treatment, cells were infected with *Leptospira* at an MOI of 100 for the indicated times in the presence of corresponding inhibitors.

Western blot analysis. Infected and uninfected cells were harvested at the times indicated, washed three times in PBS, and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) with 10 mg/ml protease inhibitor mixture (Calbiochem, Germany) for 15 min on ice. Lysates were centrifuged (10,000 × *g*, 10 min, 4°C), and supernatants were collected. Protein concentrations were estimated by the bicinchoninic acid protein assay. Equivalent amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) using a Trans-Blot semidry transfer cell (Bio-Rad Laboratories, Inc., Hercules, CA). The membranes were blocked in 20 mM Tris (pH 7.6)–137 mM NaCl–0.1% Tween 20 containing 5% nonfat milk and were probed with the following antibodies separately overnight at 4°C: rabbit polyclonal antibodies against FADD (BioVision, Inc., Mountain View, CA), lamin A/C, and PARP (both from Cell Signaling Technology, Beverly, MA). Membranes were then incubated with peroxidase-conjugated anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories Inc., PA) for 2 h at room temperature and were visualized on X-ray film using enhanced chemiluminescence reagents (Millipore, Billerica, MA). A horseradish peroxidase-conjugated monoclonal antibody against GAPDH (KangChen Bio-tech, China) was used as an internal control. The intensities of the bands were estimated by densitometric scanning using the GelDoc 2000 system (Bio-Rad) and Quantity One software (Bio-Rad). Data shown are from at least three independent experiments.

Statistical analysis. Data from a minimum of three experiments were averaged and presented as means ± SD. One-way analysis of variance followed by Dunnett's multiple-comparison test was used to determine significant differences. Statistical significance was defined as a P value of <0.01.

RESULTS

***L. interrogans* reduced cell viability and induced apoptosis in J774A.1 cells.** In our initial experiments, the cytotoxic effects of *Leptospira* infection on J774A.1 cells, A549 cells, and HUVEC were determined by LDH release assays. *L. interrogans* reduced the viability of J774A.1 cells in a dose-dependent manner, with significant cytotoxicity at an MOI of 50 (Fig. 1A). This cytotoxicity was also seen in the alveolar epithelial cell line (A549 cells), for which a significant reduction in viability was observed at an MOI as low as 10. Cell death during *L. interrogans* infection was also time dependent. At an MOI of 100, a loss in J774A.1 cell viability was detected 3 h after infection and reached a significant level in comparison with the uninfected control after 6 to 12 h (Fig. 1B). A549 cells showed similar kinetics of cell death, with an 80% decrease in viability by 12 h. HUVEC (the EVC 304 and HUV-EC-C cell lines) infected by *L. interrogans* exhibited morphological alterations after a long incubation time (>12 h after infection). Cells

rounded up and tended to detach from the culture dish (data not shown). However, the cell viability of HUVEC was always >90%, indicating that these cells responded differently to *L. interrogans* infection than J774A.1 and A549 cells. *L. biflexa*, used as a nonpathogenic control bacterium, did not reduce the viability of J774A.1 cells, HUVEC, or A549 cells (data not shown).

We subsequently investigated whether *L. interrogans*-induced cell death was due to induction of apoptosis. When measured by flow cytometry using annexin V and PI staining, *L. interrogans*-infected J774A.1 cells exhibited a typical early-apoptotic profile (annexin V^+ PI^-) at MOIs from 10 to 100. The maximum level of apoptosis reached 53.85% at an MOI of 100. In contrast to the cell viability measurements, there was no further increase in early apoptosis at the higher MOI of 500 (Fig. 2A). Under the same conditions, there was no measurable apoptosis in either HUVEC or A549 cells (data not shown). Similar results were obtained when HUVEC were infected at an MOI of 100 for different times. Interestingly, a low MOI of 10 is sufficient to induce time-dependent necrotic cell death in A549 cells (Fig. 2B). Time course analysis showed rapid apoptosis of J774A.1 cells infected at an MOI of 100 in comparison with HUVEC and A549 cells. Control cells generally exhibited a relatively low level of background apoptosis (<5%). Apoptotic cells began to appear as early as 0.5 h after infection. The maximal percentage of early-apoptotic cells was reached at 3 to 6 h (Fig. 2C). In later stages of infection (>6 h after infection), *L. interrogans* triggered either primary necrosis or postapoptotic necrosis (annexin V^+ PI^+), as assessed by flow cytometry of the same cell population (Fig. 2D). The morphological changes in *L. interrogans*-infected J774A.1 cells observed by TEM further confirmed the apoptotic response. Infected J774A.1 cells manifested the characteristic features of apoptosis, including cytoplasmic vacuolation, chromatin condensation, and margination (Fig. 2Eb and c). No morphological changes were observed in uninfected cells (Fig.2Ea). Taken together, these findings show that *L. interrogans* induces apoptosis in a cell-specific manner.

***L. interrogans*-induced caspase activation in J774A.1 cells.** To determine whether caspases are involved in *L. interrogans*-induced apoptosis of murine macrophage-like cells, we examined the kinetics of caspase activation over 12 h of *Leptospira* stimulation. The results showed that *L. interrogans* but not *L. biflexa* increased the activities of caspase-3,-6, -8, and -9 within 4 to 6 h; however, these activities declined afterwards. Compared with those in uninfected cells, the activities of caspase-3 and -6 had increased significantly 3 h after infection ($P < 0.01$) and peaked at 6 h (Fig. 3A and B). The activation of caspase-8 was already significant 1 h after infection, and caspase-8 attained maximal levels at 4 h after infection (Fig. 3C). Caspase-9 was activated to a much lower extent, and the significant increase in caspase-9 activity occurred later than those of caspase-3, -6, and -8 (Fig. 3D). In EVC304 cells, however, we found no caspase activation during *L. interrogans* infection (data not shown).

L. interrogans-induced caspase-3 and -6 activation in J774A.1 cells was further confirmed by measuring the cleavage of two known caspase substrates. PARP, an enzyme involved in DNA repair, is cleaved during apoptosis and serves as a substrate for caspase-3. Immunoblot analysis showed that infection with *L. interrogans* caused the cleavage of PARP in a time-dependent manner to yield a characteristic 89-kDa fragment. PARP cleavage products were evident at 4 h, and their levels increased further at 6 and 8 h (Fig. 4A). Lamin A/C, a substrate for caspase-6, was cleaved into a large (40- to 45-kDa) and a small (28-kDa) fragment in cells infected by *L. interrogans*. The cleavage kinetics of lamin A/C was similar to that of PARP, but the degree of cleavage was lower (Fig. 4B and C).

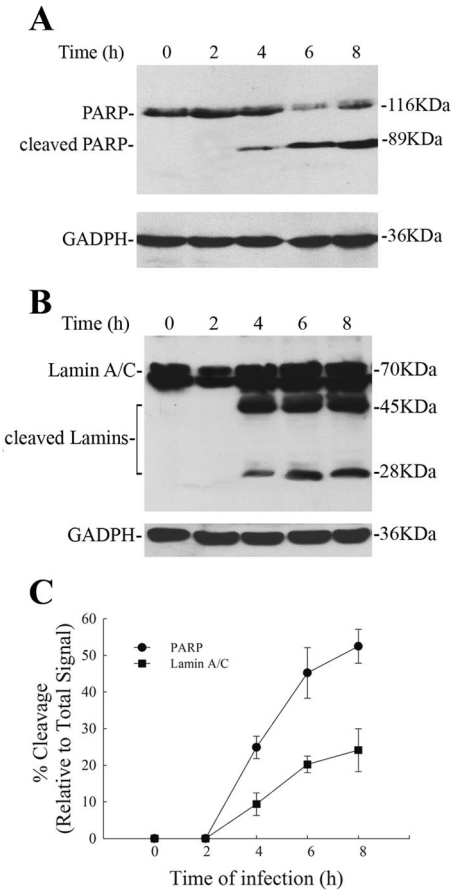

FIG. 4. *L. interrogans* infection induced the cleavage of PARP and lamin A/C. (A and B) Western blot analysis of PARP and lamin A/C cleavage. Cells were incubated with *L. interrogans* at an MOI of 100. At the indicated times, total-protein extracts were prepared, and proteins were separated by SDS-polyacrylamide gel electrophoresis followed by Western blotting with antibodies to PARP, lamin A/C, or GAPDH. GAPDH was used as a loading control. Data are representative of three independent experiments. (C) The intensities of the PARP or lamin A/C bands were quantified by densitometry. Data are expressed as the percentage of cleaved fragments relative to the total expression level in each sample.

Caspase-8 but not caspase-9 is responsible for *L. interrogans*-induced J774A.1 cell apoptosis. Taking into account the fact that caspase-8 and -9 are initial caspases in many cases of apoptotic cell death, we set out to determine which of them may be required for *L. interrogans*-induced J774A.1 cell apoptosis. In contrast to a caspase-9 inhibitor (Z-LEHD-fmk), a specific inhibitor for caspase-8 (Z-IETD-fmk) reduced the proportion of annexin V^+ PI^- cells induced by *L. interrogans* (18.29% ± 4.63% versus 47.37% ± 8.64% in untreated cells; $P < 0.01$), implying a major role for caspase-8 in apoptosis

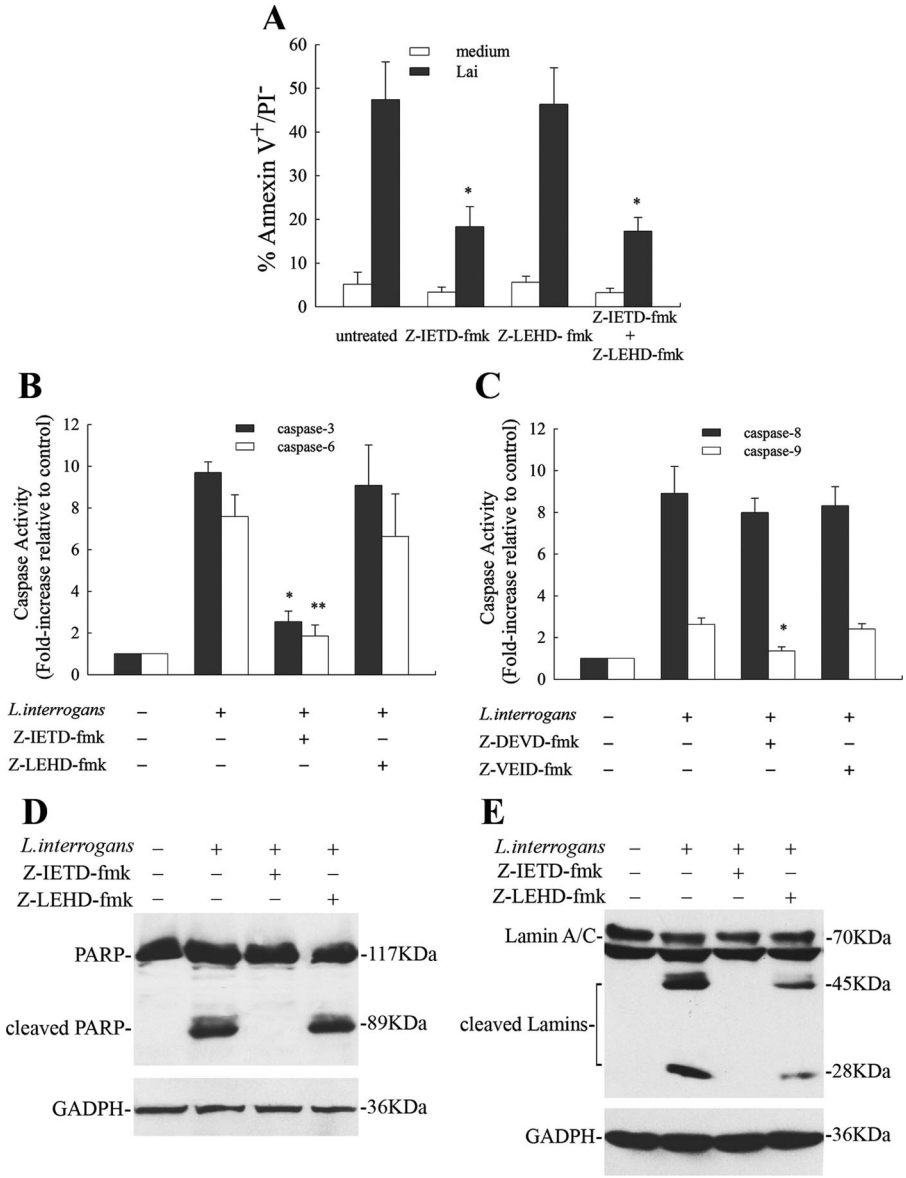

FIG. 5. Caspase-8 is required for *L. interrogans*-induced apoptosis. J774A.1 cells were either left untreated or treated with Z-IETD-fmk (100 μM) or Z-LEHD-fmk (100 μM) for 1 h prior to incubation with or without *L. interrogans* at an MOI of 100 for 5 h. (A) The percentage of annexin V^+ PI^- cells was measured by flow cytometry. Values are means ± SD for three independent experiments. (*, $P < 0.01$ for comparison with untreated, *L. interrogans*-infected cells). (B and C) Fluorometric analyses of caspase activity. Data are means for three independent experiments ± SD. * and **, significant differences ($P < 0.01$) between treatment groups and corresponding untreated, *L. interrogans*-infected cells. (D and E) Western blot analysis of the cleavage of PARP and lamin A/C. GAPDH was used as a loading control. Results are representative of three independent experiments.

(Fig. 5A). This conclusion was confirmed by evaluating the impact of the caspase-8 and -9 inhibitors on the activation of downstream caspases. Interestingly, Z-IETD-fmk but not Z-LEHD-fmk markedly decreased *L. interrogans*-induced activation of caspase-3 and -6 (Fig. 5B). Consistent with this result, the cleavage of PARP or lamin A/C was also blocked by Z-IETD-fmk, while the caspase-9 inhibitor had a much smaller effect (Fig. 5D and E). In addition, we found that caspase-9 activation was reduced by caspase-3 inhibitors, whereas caspase-8 activation was not affected (Fig. 5C). Taken together, these data suggest that caspase-8 is involved in macrophage apoptosis during infection and is upstream from caspase-3 and -6.

The effects of caspase-8 and -9 inhibitors on apoptosis and the activation of downstream executor caspases suggest the involvement of a FADD-dependent apoptotic pathway. In order to determine whether FADD plays a role in our infection model, we analyzed the expression of FADD. Western blotting and densitometric scanning results showed that the FADD protein level increased following *L. interrogans* infection, suggesting that infection could sensitize host cells to death recep-

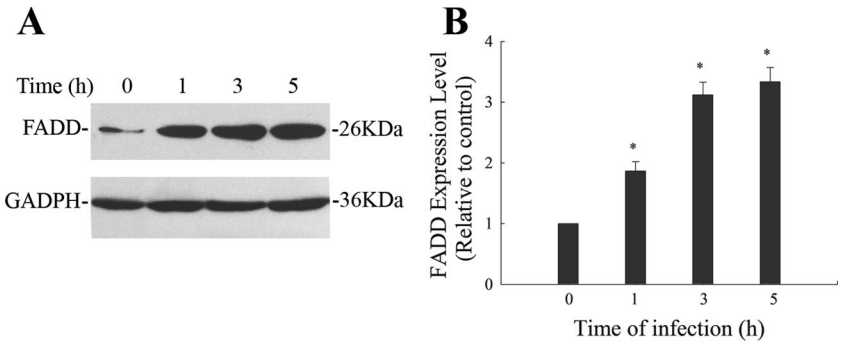

FIG. 6. FADD protein levels increased in J774A.1 cells after *L. interrogans* infection. (A) Western blot analysis of the expression levels of FADD. GAPDH was used as a loading control. Results are representative of three independent experiments. (B) The intensities of FADD were quantified by densitometry and normalized to those of GAPDH signals in the same samples. Results are expressed as the increase (*n*-fold) in the expression level over that for the uninfected control. Data are means from three independent experiments ± SD (*, $P < 0.01$).

tor signaling pathways by increasing FADD translation or decreasing its degradation (Fig. 6A and B). In parallel, we did not detect any release of cytochrome *c* from mitochondria (data not shown). These data further reinforce the notion that apoptosis is strongly associated with the FADD- and caspase-8-related pathways.

Caspase-3 and caspase-6 are both required for *L. interrogans*-induced apoptosis in J774A.1 cells. To characterize the roles of caspase-3 and caspase-6 in apoptosis induced by *L. interrogans*, J774A.1 cells were pretreated with a specific inhibitor of caspase-3 (Z-DEVD-fmk) or caspase-6 (Z-VEID-fmk) before infection. Z-DEVD-fmk treatment reduced apoptosis by approximately 60% (20.95% ± 5.13% versus 47.37% ± 8.64% in untreated cells; $P < 0.01$ [Fig. 7A]). Inhibition of apoptosis was also seen in cells pretreated with Z-VEID-fmk, although to a lesser extent than with the caspase-3 inhibitor. However, apoptosis was further decreased in cells pretreated with both the caspase-3 and caspase-6 inhibitors (Fig. 7A).

We further investigated the relationship between the activation of caspase-3 and that of caspase-6. Western blotting showed that Z-VEID-fmk completely inhibited lamin A/C cleavage but failed to inhibit PARP degradation. In contrast, Z-DEVD-fmk largely abrogated the proteolysis of both PARP and lamin A/C (Fig. 7B and C). This result can be explained by the facts that caspase-3 is required for activation of caspase-6 during *L. interrogans*-induced J774A.1 cell apoptosis and that lamins are primarily substrates for caspase-6. As further confirmation of this explanation, caspase-6 activation was also completely inhibited by Z-DEVD-fmk, whereas caspase-3 activity was unaffected by Z-VEID-fmk treatment under the same conditions (Fig. 7D and E). These data suggest that caspase-3 plays a predominant role in mediating *L. interrogans*-induced macrophage-like cell apoptosis, although caspase-6 also contributes.

***L. interrogans* triggers caspase-dependent apoptosis in primary macrophages.** Since the experiments described above were performed with an immortalized macrophage-like cell line, further experiments were performed to investigate whether the results accurately reflect interactions between *L. interrogans* and primary macrophages. In fact, flow cytometric analysis showed that macrophage apoptosis was induced efficiently by *L. interrogans* at a low MOI of 10 (Fig. 8A and B). During a high-MOI infection, however, necrotic cell death was prominent, a finding that was confirmed further by an LDH release assay (data not shown). At an MOI of 10, macrophage apoptosis occurred significantly as early as 0.5 h and rapidly reached a plateau at 3 h after infection (Fig. 8C). These results suggest that primary murine macrophages are more susceptible to induction of apoptosis by *L. interrogans* than J774A.1 cells. The higher sensitivity of primary cells was further confirmed by the observation that the activities of caspase-3, -6 and -8 increase faster than in J774A.1 cells. The increase in caspase-9 activity was delayed and did not begin to appear until 3 h after infection (Fig. 9A). By use of specific caspase inhibitors, the relationships between the activation of individual caspases and their roles in *L. interrogans*-induced macrophage apoptosis were also investigated. In results similar to those for J774A.1 cells, apoptosis in primary murine macrophages required caspase-3 and -6 activation, which is mediated by the caspase-8-dependent pathway (Fig. 9B, C, and D).

DISCUSSION

Macrophage-pathogen interactions play an important role in the pathogenesis of bacterial infections (35). Previous studies reported active interactions between *L. interrogans* and murine macrophage-like cells, resulting in *L. interrogans* invasion and macrophage apoptosis. However, the underlying molecular processes responsible for macrophage death remained unknown. In this study, we attempted to dissect the molecular basis for *L. interrogans*-induced cell death in J774A.1 cells and primary macrophages. The results demonstrated that J774A.1 cells infected with *L. interrogans* show significant reductions in cell viability compared with uninfected cells. This effect was partly due to cells undergoing apoptosis, which was characterized by morphological changes and surface exposure of phosphatidylserine. Apoptosis in J774A.1 cells was observed at MOIs from 10 to 100, with no further increase at higher MOIs. This finding allows us to speculate that at high MOIs, cells undergo rapid and extensive damage of the plasma membrane, resulting in necrosis rather than apoptosis. Certain bacteria are known to exert this type of effect; for example, *Legionella pneumophila* induces necrosis at high MOIs, with no signs of

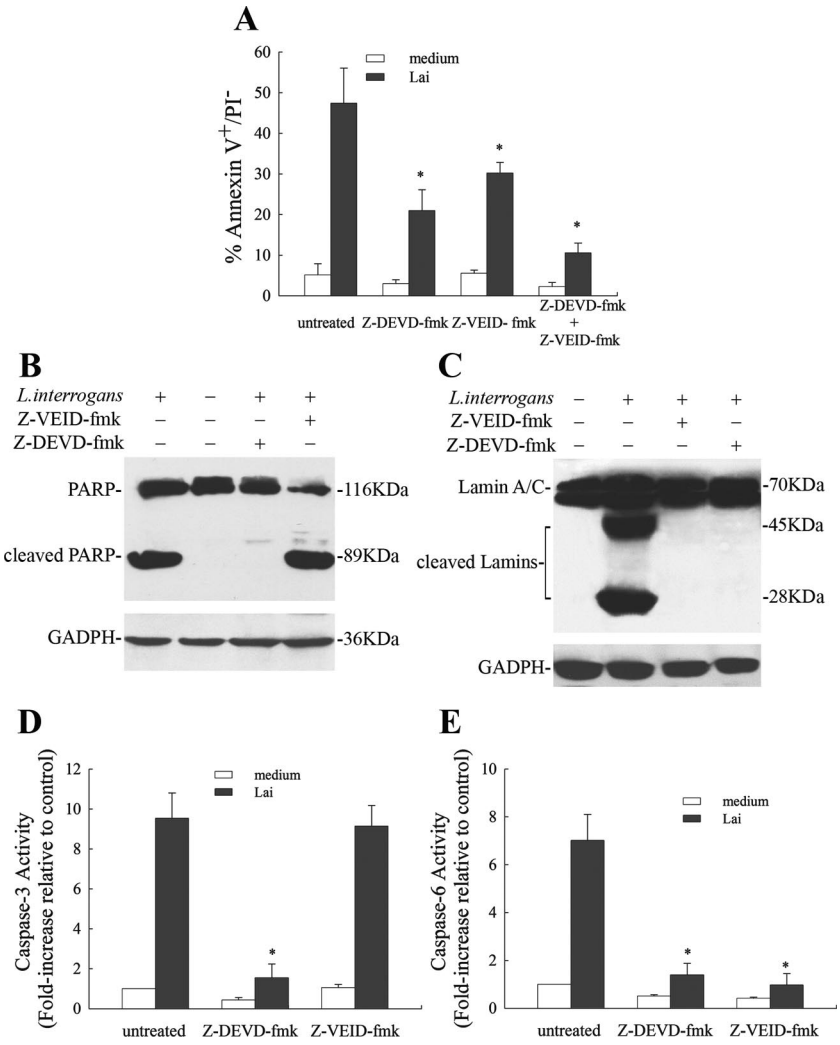

FIG. 7. Effects of caspase-3 and -6 inhibitors on *L. interrogans*-induced apoptosis. J774A.1 cells were either left untreated or treated with Z-DEVD-fmk (100 μM) or Z-VEID-fmk (100 μM) for 1 h prior to incubation with or without *L. interrogans* at an MOI of 100 for 5 h. (A) The percentage of annexin V^+ PI^- cells was measured by flow cytometry. Values are means ± SD for three independent experiments (∗, $P < 0.01$ for comparison with untreated, *L. interrogans*-infected cells). (B and C) Western blot analysis of the cleavage of PARP and lamin A/C. GAPDH was used as a loading control. Results are representative of three independent experiments. (D and E) Fluorometric analyses of caspase-3 and caspase-6 activities. Results are means for three independent experiments ± SD (∗, $P < 0.01$ for comparison with untreated, *L. interrogans*-infected cells).

apoptosis (20). An apoptotic response was also confirmed in murine peritoneal macrophages. Significant apoptosis was observed at a low MOI of 10, whereas necrosis instead of apoptosis was observed at an MOI of 100. Thus, primary murine macrophages are more sensitive to apoptosis due to *L. interrogans* infection than J774A.1 cells, a finding that was further confirmed by caspase activation assays.

Our data revealed that *L. interrogans* induces rapid apoptosis in both immortalized J774A.1 cells and primary macrophages, since apoptosis was measurable within 30 min after infection. Previous studies have shown that *L. interrogans* is internalized quickly, within 20 min after infection (30). Thus, it appears that *L. interrogans*-induced macrophage apoptosis occurs almost immediately after internalization and is independent of *L. interrogans* intracellular replication. But it remains unclear whether the host or *L. interrogans* benefits the most from this rapid apoptotic outcome. The induction of macrophage apoptosis may play a role in aiding *L. interrogans* to evade the immune response, or, alternatively, apoptosis of the infected macrophage may be part of a defense strategy against infection by depriving *L. interrogans* of a sanctuary and limiting the spread of infection. Further studies to address this problem are currently under way.

Because pulmonary capillary hemorrhage and hemoptysis can be the most prominent manifestations of severe leptospirosis, we extended our observations to investigate the effects of *L. interrogans* on capillary vessels and lung tissue. The HUVEC 304 cell line is a well-established model for the study of vascular endothelial injury (11, 18, 27). Apoptosis was not observed in EVC304 cells during the time studied, and this result was confirmed with HUV-EC-C cells. However, after prolonged infection, the morphology of HUVEC

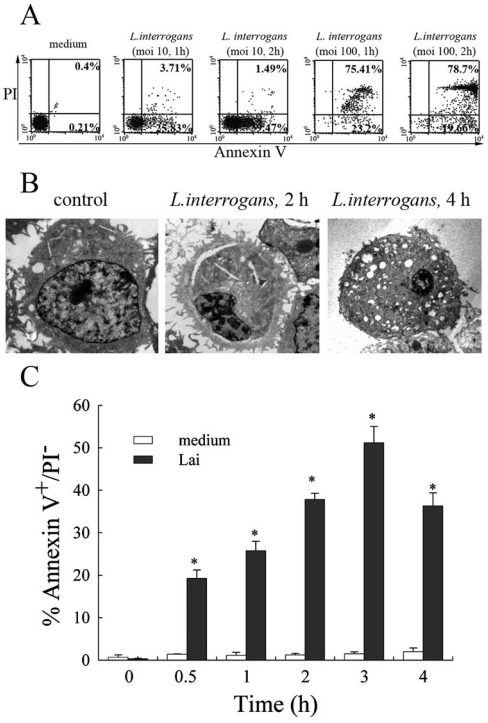

FIG. 8. *L. interrogans* induced apoptosis in primary macrophages. (A) Murine peritoneal macrophages were incubated with *L. interrogans* at MOIs of 10 and 100. Scatter plots representative of three independent experiments are shown. (B) Transmission electron micrographs of uninfected and *L. interrogans*-infected murine peritoneal macrophages. (C) Macrophages were incubated with or without *L. interrogans* at an MOI of 10 for different periods. The percentage of annexin V^+ PI^- cells was measured by flow cytometry. Data are means ± SD for three independent experiments (*, $P < 0.01$ for comparison with uninfected cells).

changed. These changes could expand the intercellular space and lead to increased vascular permeability, which agrees with a previous proposal that primary alterations of capillary permeability could be a salient event in leptospirosis (10). Previous examinations of lung tissues have revealed that pulmonary edema was the principal consequence of leptospiral infection (25). Our results show that *L. interrogans* infection induced time-dependent necrosis in the human lung epithelial cell line A549. Contrary to the existing viewpoint that leptospirosis pathogenesis is mediated by leptospiral toxin or an immune response of the host (4, 34), our results suggest that pneumopathy can also be directly triggered by intact leptospires (36). These findings also highlight the fact that the cellular effects of *L. interrogans* infection occur in organ- and cell-type-specific contexts. Several pathogens have been reported to induce cell death in this cell-specific manner. For example, *Shigella flexneri* and *Salmonella enterica* serovar Typhimurium induce apoptosis in macrophages but not in epithelial cells (6, 26).

Caspase-3, serving as an executor caspase, plays a pivotal role in the apoptosis induced by various pathogens (38). Our data clearly show that activation of caspase-3 is not the only mechanism leading to *L. interrogans*-induced J774A.1 cell and murine macrophage apoptosis, because complete inhibition of caspase-3 with Z-DEVD-fmk only partially inhibited apoptosis. Other executor caspases may also contribute to apoptosis induced by *L. interrogans*. In line with this notion, we found that caspase-6 was also activated, and apoptosis was partially inhibited by the caspase-6 inhibitor.

The sequential activation of caspase-3 and -6 in apoptotic pathways is a subject of intense interest. Studies have demonstrated that caspase-6 activation may depend on caspase-3 activation (43, 45), occurs upstream of caspase-3 activation (1), or occurs independently of caspase-3 activation (33). Our study showed that Z-DEVD-fmk reduced caspase-6 activity and blocked the cleavage of lamin A/C, whereas the activation of caspase-3 was not affected by Z-VEID-fmk. Based on these results, it appears that caspase-3 functions as an upstream activator of caspase-6 in *L. interrogans*-induced murine macrophage apoptosis. In addition, the specific role played by caspase-6 during apoptosis is complex. Caspase-6 is a lamin protease and is required for chromatin condensation and nuclear disassembly during the execution phase of apoptosis (41). However, some studies reported that caspase-6 may also be involved in the direct activation of caspase-8 (7, 22), suggesting that caspase-6 plays a more upstream role as a regulator of caspase activation rather than as a destructive caspase in the demolition phase of apoptosis. In the present study, we found that caspase-6 did not activate caspase-8. Thus, and in view of the lamin A/C cleavage results, it remains possible that caspase-6 plays a role as an effector caspase in *L. interrogans*-induced apoptosis in macrophages.

Two caspase-dependent pathways are well recognized to lead to effector caspase activation: the death receptor caspase-8-dependent pathway and the mitochondrial caspase-9-dependent pathway. We demonstrated that both caspase-8 and caspase-9 were activated by *L. interrogans* infection but that caspase-9 was activated at a much lower level than caspase-8. *L. interrogans*-induced apoptosis, effector caspase activation, and cleavage of caspase substrates (lamin A/C and PARP) were effectively inhibited by the caspase-8 inhibitor, revealing an important role for caspase-8 in this form of apoptosis.

Caspase-8 activation is usually involved in the extrinsic, death receptor pathway of apoptosis. Accordingly, we examined the expression of FADD, a death receptor adapter protein that promotes caspase-8 activation. Our results indicate that *L. interrogans* infection increased the expression level of FADD in the macrophage-like cell line. Therefore, in agreement with findings for other bacteria, including *Pseudomonas aeruginosa* (17), *Helicobacter pylori* (42), and *Staphylococcus aureus* (2), which trigger apoptosis via death receptor pathways, these results collectively indicate that apoptosis induced by *L. interrogans* is strongly associated with FADD-dependent caspase-8 activation upstream of caspase-3 and -6. Further studies to investigate the death receptors and signal transduction pathways involved are currently under way.

An interesting observation was that treatment of infected macrophages with the caspase-9 inhibitor did not reduce apoptosis. Moreover, *L. interrogans* infection failed to induce the release of cytochrome *c* from mitochondria. Since caspase-9 was activated at a low level, one can propose two possibilities:

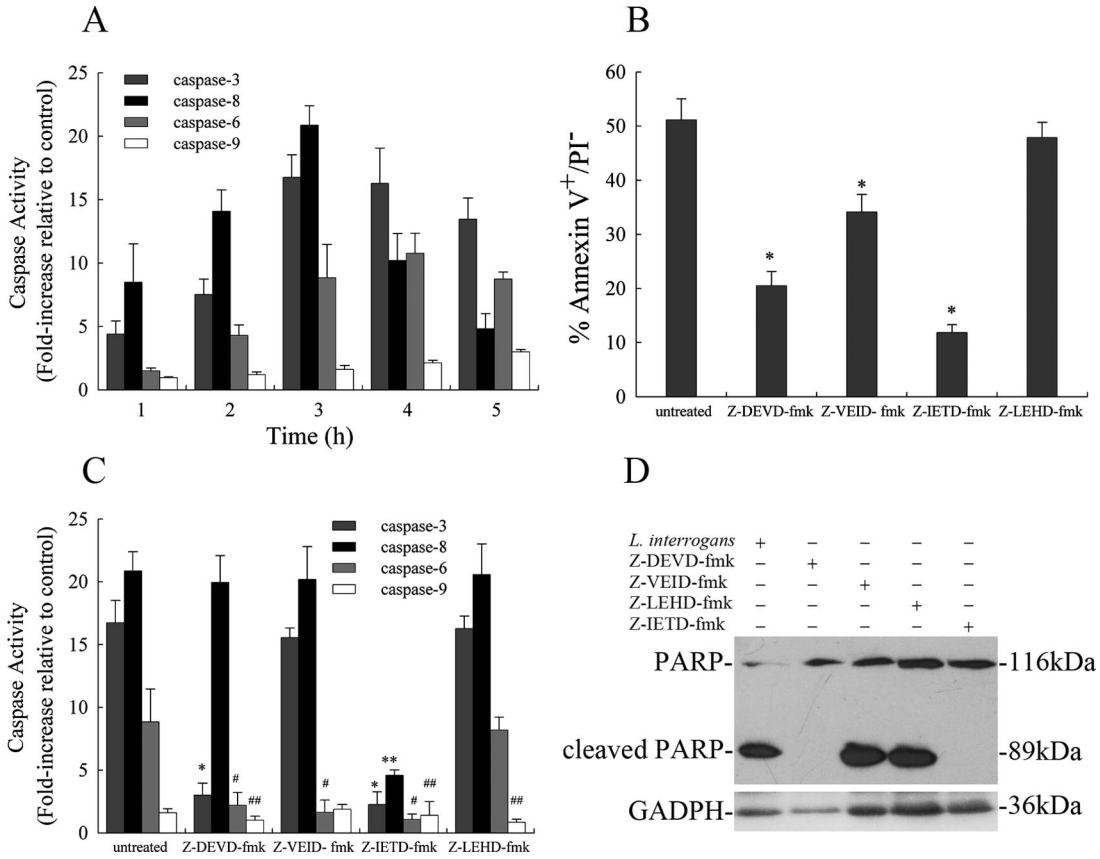

FIG. 9. Roles of individual caspases in *L. interrogans*-induced apoptosis of primary macrophages. (A) Murine peritoneal macrophages were incubated with *L. interrogans* at an MOI of 10. Caspase activity was measured and expressed relative to the activity of the uninfected control at every time point. Data are means for three independent experiments ± SD. (B and C) Macrophages were either left untreated or treated with caspase inhibitors for 1 h prior to incubation with *L. interrogans* at an MOI of 10 for 3 h. (B) The percentage of annexin V^+ PI^- cells was measured by flow cytometry. Data are means ± SD for three independent experiments (*, $P < 0.01$ for comparison with untreated, *L. interrogans*-infected cells). (C) Caspase activity was measured and expressed relative to that of uninfected, untreated cells. Data are means for three independent experiments ± SD. *, **, #, and ## indicate significant differences ($P < 0.01$) within each treatment group relative to the activity of untreated, *L. interrogans*-infected cells. (D) Western blot analysis of the cleavage of PARP. GAPDH was used as a loading control. Results are representative of three independent experiments.

either a small amount of caspase-9 was activated by undetectable amounts of released cytochrome *c*, or caspase-9 was processed at an alternative cleavage site, since caspase-9 can be activated not only by Apaf-1/cytochrome *c*-related autoprocessing but also by activated caspase-3 via an apoptosis amplification feedback loop (44, 47). The latter possibility is plausible, since our data revealed that the activation of caspase-9 was downstream of caspase-3 activation and was blocked by the caspase-3 inhibitor. From these results, we conclude that the Apaf-1/cytochrome *c* apoptotic pathway does not play a significant role in *L. interrogans*-induced macrophage apoptosis. However, given that we previously observed that *L. interrogans* infection triggers mitochondrial damage such as swelling (24), we cannot rule out the possibility that mitochondria are involved in the caspase-independent component of cell death induced by *L. interrogans*.

In conclusion, we show that *L. interrogans* induces apoptosis and caspase activation in macrophages through a FADD–caspase-8-dependent pathway but independently of mitochondrial cytochrome *c* release. Our findings provide promising insights into the host cell response in leptospirosis and may provide a molecular basis for more detailed understanding of leptospiral pathogenesis.

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