Interaction of *Yersinia enterocolitica* with Macrophages Leads to Macrophage Cell Death through Apoptosis

KLAUS RUCKDESCHEL,^{1*} ANDREAS ROGGENKAMP,² VIRGINIE LAFONT,¹ PAUL MANGEAT,³
JÜRGEN HEESEMANN,² AND BRUNO ROUOT¹

INSERM U431,¹ and CNRS UMR 5539,³ Université Montpellier II, 34095 Montpellier Cedex 05, France,
and Max von Pettenkofer-Institut für Hygiene und Mikrobiologie, 80336 München, Germany²

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Suppression of the host defense is one of the hallmarks of *Yersinia enterocolitica* infection. This enteric pathogen resists phagocytosis and interferes with macrophage functions from an extracellular localization (oxidative-burst generation and tumor necrosis factor alpha production). In this study, we investigated the fate of the *Y. enterocolitica*-infected macrophage. We found that murine J774A.1 macrophages and macrophages derived from human monocytes were killed by infection with *Y. enterocolitica*. Analysis of cellular morphology and DNA fragmentation revealed that macrophage cell death occurs through the induction of apoptosis. A total of $92\% \pm 5\%$ (mean $\pm$ standard deviation) of murine J774A.1 macrophages and $74\% \pm 6\%$ of human monocyte-derived macrophages underwent apoptosis upon *Yersinia* infection after 4 and 20 h, respectively. The broad-spectrum caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone blocked completion of the *Yersinia*-induced apoptotic program but not the surface exposure of phosphatidylserine as an early-stage apoptotic event. Analysis of different *Yersinia* mutants showed that macrophage apoptosis depends on a functional *Y. enterocolitica* type III protein secretion system. Apoptotic cell death of macrophages was not related to the YopE-mediated cytotoxic effect of *Yersinia*, since disruption of actin microfilaments by a *Y. enterocolitica* strain expressing a restricted repertoire of yop genes, including YopE, did not result in macrophage apoptosis. Furthermore, *Yersinia*-induced cytotoxic alterations in epithelial HeLa cells, which are conferred by YopE, did not lead to apoptosis. Our data demonstrate for the first time that *Y. enterocolitica* promotes the apoptosis of macrophages, an effect which is clearly distinct from the morphological alterations mediated by *Yersinia* on epithelial HeLa cells.

Yersinia enterocolitica is an enteric pathogen which has developed strategies to resist the immune defense of the host. Following ingestion, the bacteria transit to the ileum, where they invade the intestinal mucosa and proliferate within the lymphoid follicles (Peyer's patches) (21). Replication of *Y. enterocolitica* in the host lymphoid tissue depends on the presence of a 70-kb virulence plasmid (pYV) that encodes a set of secreted proteins, termed Yops (*Yersinia* outer proteins) (25, 44). The Yop proteins mediate resistance to host defense mechanisms. YopH is a protein tyrosine phosphatase (20) which dephosphorylates host cell proteins (3, 8). It is assumed that YopH subverts normal signal transduction processes of the eukaryotic cell (3, 8). YopE causes disruption of the actin microfilament structure (38), which induces rounding and detachment of mammalian cells from the extracellular matrix (18, 37–39). Together, these two proteins are largely responsible for the inhibition of phagocytosis and the suppression of the oxidative burst of professional phagocytes (7, 15, 23, 36, 37, 41). In addition, yersiniae inhibit tumor necrosis factor alpha production by macrophages, a feature which is distinct from the effects mediated by YopH and YopE (40). At least four Yops, YopH, YopE, YopO, and YopM, are translocated inside the eukaryotic cell by surface-bound bacteria upon host cell contact (13). Export of the Yop proteins is mediated by a virulence-plasmid-encoded type III protein secretion system. Yop translocation is controlled by YopN and requires the translocator proteins YopD and YopB as well as LcrV (for a recent review on the *Yersinia* Yop virulon, see reference 13).

The *Yersinia* Yop virulon constitutes a sophisticated bacterial system for subverting eukaryotic cells which enables survival and extracellular multiplication of the bacteria in the host lymphoid tissue (13, 22). Virulence-plasmid-cured yersiniae are nonpathogenic and rapidly eliminated in the intestinal lymphoid follicles by the activity of phagocytic cells (22). In vivo studies in the murine infection model provide evidence for a central role of phagocytes in *Yersinia* pathogenesis (5, 22) and imply that evasion of the microbicidal action of phagocytes is a critical step in the outcome of *Y. enterocolitica* infection.

Despite severe interference with macrophage antibacterial activities, the fate of the *Yersinia*-infected macrophage is far from clear. Two other enteropathogenic bacteria, *Shigella flexneri* and *Salmonella typhimurium*, have recently been shown to kill macrophages by activation of their intrinsic cell death program, leading to apoptosis (10, 29, 31, 48). Apoptosis is an innate cell suicide mechanism that is essential for normal development of the multicellular organism. It is involved in the ablation of unwanted, damaged, or potentially neoplastic cells. Initiation of apoptosis activates a core apoptotic program present in all cells (2). Interleukin 1 β -converting enzyme (ICE)-related proteases, designated caspases (1), are the major executors of this apoptotic program, causing the morphological changes on apoptotic cells (30, 32). Main characteristics are shrinkage of the cytoplasm, condensation of the nuclear chromatin, and fragmentation of the chromosomal DNA into multimers of 200 bp (2). Now there is increasing evidence that this form of cell death also plays a role in the pathogenesis of infectious diseases.

In this study, we show that the interaction of *Y. enterocolitica* with macrophages leads to macrophage cell death through apoptosis. *Yersinia*-induced apoptosis of macrophages must be distinguished from the morphological changes seen on HeLa

* Corresponding author. Mailing address: INSERM U431, Université Montpellier II, Place E. Bataillon, CC100, F-34095 Montpellier Cedex 05, France. Phone: (33) 4-67-14-42-44. Fax: (33) 4-67-14-33-38.

TABLE 1. *Y. enterocolitica* strains used in this study

Strain	Relevant characteristic(s)	Reference
WA-314	Serogroup O8; clinical isolate harboring virulence plasmid pYVO8	24
WA-C	Plasmidless derivative of strain WA-314	24
WA-C(pYV-7146)	Mutant strain, deficient in YopH secretion; insertional inactivation of <i>sydH</i> , the gene for the YopH-specific chaperone SydH	41
WA-C(pYV-515)	Mutant strain, deficient in secretion of Yops; insertional inactivation of <i>lcrD</i> , the gene encoding LcrD, which is essential for Yop secretion	41
WA-C(pLCR)	Strain harboring plasmid pLCR encoding the secretion apparatus of <i>Y. enterocolitica</i> , including the genes for YopD, YopB, YopN, and LcrV	35
WA-C(pLCR, pB8-23)	Strain WA-C(pLCR) harboring an additional plasmid encoding the genes for YopH, YopE, and YadA	35

cells upon *Yersinia* infection and may reflect an additional strategy of this bacterial pathogen to overcome the host defense.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Overnight cultures grown at 26°C were diluted 1:20 in fresh Luria-Bertani broth and grown for 2 h at 37°C as described previously (40, 41). Then the bacteria were washed once and resuspended in phosphate-buffered saline (PBS) at a concentration of 10^8 bacteria/ml. The desired bacterial concentration was adjusted by measuring the optical density at 600 nm and checked by plating serial dilutions of the samples on agar and counting CFU after incubation at 26°C for 20 h.

Cell culture, isolation of blood monocytes, and infection conditions. The murine macrophage-like cell line J774.A1 and the human epithelial HeLa cell line were routinely grown in cell growth medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 5 mM L-glutamine) (Life Technologies, Cergy, Pontoise, France). Peripheral blood monocytes were isolated from healthy donors by standard Ficoll-Hypaque (Sigma, St. Louis, Mo.) gradient centrifugation as previously described (9). They were resuspended in cell growth medium and incubated overnight in plastic culture plates. Thereafter, monocytes were thoroughly washed to remove nonadherent cells. The purity of monocytes obtained by this technique was more than 90%, as determined by binding of monoclonal antibodies to CD14 (9). Differentiation to macrophages was performed by incubation with 500 U of granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Levallois-Perret, France) per ml for 5 days. Cells were always incubated at 37°C and with 5% CO₂ in a humidified atmosphere. In some experiments, the ICE-related protease inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (ZVAD.fmk) (Bachem, Voisins le Bretonneux, France) was included. In these cases, cells were pretreated with ZVAD.fmk (100 µM) for 2 h before infection. Infections were performed at a multiplicity of infection (MOI) of 50 bacteria per cell for 90 min unless stated otherwise. After infection, HeLa cells and GM-CSF-differentiated monocytes were centrifuged for 5 min at $400 \times g$ to facilitate contact between bacteria and cells. After 90 min of infection, the medium was replaced by fresh culture medium supplemented with 100 µg of gentamicin per ml. When the ICE-related protease inhibitor ZVAD.fmk was included, the medium was not changed, but bacteria were killed by the addition of gentamicin (100 µg/ml). Incubation of the cells was continued for the time periods indicated in the experiments.

Analysis of cell viability. Cell viability was determined by trypan blue exclusion. At the indicated time points after infection, cells grown in culture plates were overlaid with trypan blue (0.1%) and scored visually with an inverted microscope. A minimum of 100 cells per sample was analyzed. Each experiment was done twice.

Analysis of DNA fragmentation. Fragmentation of DNA was analyzed as described previously (26). A total of 5×10^6 cells per sample were lysed with lysis

buffer (0.5% Triton X-100–20 mM EDTA–5 mM Tris [pH 7.4]). After centrifugation, the supernatant containing low-molecular-weight DNA was treated with proteinase K (150 µg/ml) for 1 h at 60°C and, thereafter, overnight at 37°C. Lysates were extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) and once with an equal volume of chloroform-isoamyl alcohol (24:1 [vol/vol]). The samples were left for more than 1 h, with continuous shaking after the addition of phenol-chloroform-isoamyl alcohol. The DNA in the aqueous phase was precipitated with ethanol–0.3 M sodium acetate containing 40 µg of glycogen (Boehringer, Mannheim, Germany)/ml, washed with 70% ethanol, and resuspended in 10 mM Tris (pH 8.0)–1 mM EDTA–2 µg of RNase/ml. Samples were incubated overnight at 37°C and subjected to electrophoresis on a 1% agarose gel. DNA fragments were visualized by ethidium bromide staining.

Assessment of apoptosis by fluorescence microscopy. Apoptotic cells were detected and quantified by an assay based on the detection of phosphatidylserine exposed on the outer leaflet of apoptotic cells. Unfixed cells were stained with fluorescein-conjugated annexin V, which has high affinity to membrane-exposed phosphatidylserine according to the manufacturer's instructions (Annexin-V-Fluos; Boehringer). The simultaneous application of the DNA stain propidium iodide (Sigma) allowed the discrimination of apoptotic from necrotic cells, since the nuclei of necrotic cells retain their shapes but the nuclei of apoptotic cells appear to be strongly condensed (2). The percentages of apoptotic cells were determined by counting a minimum of 100 cells per sample in a fluorescence microscope (Leica DM IRB). Experiments were repeated three times.

DNA fragmentation in individual apoptotic cells was also detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL reaction). The MEBSTAIN apoptosis kit (Immunotech; Marseille, France) was used to label free 3' OH ends of DNA fragments with fluorescein as recommended by the manufacturer.

Immunofluorescent labeling of F-actin. F-actin was stained with BODIPY FL phalloidin in accordance with the manufacturer's instructions (Molecular Probes, Leiden, The Netherlands). Briefly, infected cells were fixed in PBS–3.7% paraformaldehyde for 20 min, permeabilized with PBS–0.2% Triton X-100 for 5 min, and labeled with BODIPY FL phalloidin for 20 min. Thereafter, bacteria were stained with a rabbit polyclonal anti-*Y. enterocolitica* O8 (41) and a second rhodamine-conjugated anti-rabbit immunoglobulin G antibody (Sigma). Labeled preparations were observed with a fluorescence microscope.

RESULTS

Morphological changes and viability of J774A.1 macrophages upon *Y. enterocolitica* infection. The murine macrophage cell line J774A.1 was infected with the virulent wild-type (WA-314) and a nonpathogenic, virulence-plasmid-cured (WA-C) *Y. enterocolitica* strain at a ratio of 50 bacteria per cell. After 90 min, extracellular bacteria were killed with gentamicin. Morphological changes of J774A.1 macrophages were analyzed by phase-contrast microscopy following 4 h of incubation. Compared to untreated cells (Fig. 1a), macrophages infected with the non-virulent strain WA-C appeared to be strongly swollen (Fig. 1b), originating from the high rate of phagocytosis of this strain (40). In contrast, the virulent wild-type strain WA-314, which resists phagocytosis by J774A.1 cells and remains extracellularly located at the macrophage surface (40), induced a heterogeneous picture of morphological changes in J774A.1 cells (Fig. 1c). Some cells were remarkably shrunken and condensed, while other cells acquired an intense granular appearance and a ruffled plasma membrane. These processes strongly resembled membrane blebbing and cytoplasmic vacuolization, which are ultrastructural characteristics of cells undergoing apoptosis (2).

Trypan blue exclusion revealed that more than 30% of J774A.1 macrophages infected with the virulent strain WA-314 were already dead after 4 h of incubation. The percentage of dead cells increased to more than 80% after 8 h, whereas more than 90% of the macrophages which were not treated or infected with the nonvirulent strain WA-C remained viable at that time point.

Virulence-plasmid-bearing *Y. enterocolitica* induces DNA fragmentation of J774A.1 macrophages. To find out whether J774A.1 cells killed by virulent *Y. enterocolitica* undergo apoptotic cell death, we investigated DNA fragmentation of the dying cells, which is one of the main characteristics of apopto-

FIG. 1. Morphology of J774A.1 macrophages infected with *Y. enterocolitica*. J774A.1 cells were either untreated (a) or infected with the virulence-plasmid-cured strain WA-C (b) or the virulence-plasmid-harboring strain WA-314 (c) (MOI, 50:1; 90 min). Cells were analyzed by phase-contrast microscopy following 4 h of incubation. Magnification, $\times 1,000$.

sis. DNA fragmentation was analyzed over a period of 270 min, starting 90 min after the onset of infection (Fig. 2a). After 90 min, no DNA fragmentation was observed, whether cells were treated with virulent (Fig. 2a, lane 3) or nonvirulent *Y. entero-*

colitica (Fig. 2a, lane 2). However, after 150 min, the virulent strain WA-314 induced DNA degradation, characterized by a typical ladder pattern in the agarose gel (Fig. 2a, lane 6). The extent of DNA fragmentation increased at 210 min (Fig. 2a, lane 9) and was already slightly reduced at 270 min (Fig. 2a, lane 12). The proof of internucleosomal DNA fragmentation suggests that the killing of macrophages by *Y. enterocolitica* occurs, at least in part, through the induction of apoptosis.

Cell death of J774A.1 macrophages in response to *Y. enterocolitica* infection occurs through apoptosis. To determine the proportion of apoptotic cells in *Y. enterocolitica*-infected J774A.1 macrophages, we labeled apoptotic cells with fluorescein-conjugated annexin V, which binds with high affinity to phosphatidylserine exposed on the outer membranes of cells undergoing apoptosis (14, 46). Phosphatidylserine exposure occurs in the early stages of apoptosis, prior to the loss of membrane integrity (46). In cells which had already lost their membrane integrity, the simultaneous application of the membrane-impermeable DNA stain propidium iodide allowed the analysis of chromatin condensation as a late-stage apoptotic event (46). The kinetics of *Y. enterocolitica*-induced apoptosis (Fig. 2b) was followed over the same time course as the DNA fragmentation (Fig. 2a). After 150 min (Fig. 2, lane 6), $31\% \pm 6\%$ (mean $\pm$ standard deviation [SD]) of the J774A.1 cells infected with the virulent strain WA-314 were already annexin V positive, i.e., had started the apoptotic process. This result correlated with the appearance of DNA fragmentation at the same time point. The percentage of apoptotic cells increased to $95\% \pm 4\%$ after 270 min (Fig. 2, lane 12). In contrast to the virulent *Y. enterocolitica* strain WA-314, the virulence-plasmid-cured strain WA-C failed to induce apoptosis of J774A.1 macrophages (Fig. 2a and b and Fig. 3a). Cells undergoing apoptosis exhibited a heterogeneous picture of apoptotic features

FIG. 2. *Y. enterocolitica* induces apoptosis in J774A.1 cells. (a) Time course of J774A.1 cell DNA fragmentation in response to *Y. enterocolitica* infection. (b) Quantification of apoptotic J774A.1 cells. Cells were untreated (lanes 1, 4, 7, and 10), or infected with the virulence-plasmid-cured strain WA-C (lanes 2, 5, 8, and 11), or the virulence-plasmid-harboring strain WA-314 (lanes 3, 6, 9, and 12) (MOI, 50:1; 90 min). Isolation of low-molecular-weight DNA (a) and quantification of apoptotic cells (b) were performed following 90 min (lanes 1, 2, and 3), 150 min (lanes 4, 5, and 6), 210 min (lanes 7, 8, and 9), and 270 min (lanes 10, 11, and 12) of incubation. Apoptosis was quantified by counting apoptotic cells after staining with fluorescein-conjugated annexin V and propidium iodide. The percentages of apoptotic cells are mean values from three different experiments (100 cells were analyzed in each experiment). Lane M in panel a displays the molecular size marker (lambda DNA/EcoRI plus HindIII).

FIG. 3. Surface phosphatidylserine exposure and nuclear morphology of J774A.1 cells infected with *Y. enterocolitica*. J774A.1 cells were infected with the virulence-plasmid-cured strain WA-C (a) or the virulence-plasmid-harboring strain WA-314 in the absence (b) or presence (c) of 100 μ M caspase inhibitor ZVAD.fmk (MOI, 50:1; 90 min). After 4 h of incubation, unfixed cells were stained simultaneously with propidium iodide and fluorescein-conjugated annexin V and analyzed by fluorescence microscopy. Magnification, $\times 1,000$. Cells in panels a and c exhibit weak background staining with propidium iodide.

in the fluorescence microscope (Fig. 3b); cells in earlier stages of apoptosis had an almost normal appearance but were annexin V positive (green fluorescence), while apoptotic cells in later stages shrank remarkably and lost membrane integrity, and the condensed chromatin was strongly stained with propidium iodide (red fluorescence). The proportion of propidium iodide-positive cells closely corresponded to the proportion of cells which could not exclude trypan blue.

In situ DNA fragmentation could also be demonstrated by the TUNEL reaction, an assay based on the labeling of free DNA ends with fluorescein (data not shown). Forty to fifty percent of WA-314-infected J774A.1 cells exhibited TUNEL-positive chromatin after 4 h. However, the percentages of TUNEL-positive cells appearing at later time points were difficult to determine, since many cells were already detached from the matrix.

Apoptosis of J774A.1 cells induced by the virulent *Y. enterocolitica* strain depended on MOI and time of infection. At an MOI of 50 bacteria per cell and an infection time of 90 min, $92\% \pm 5\%$ of the macrophages underwent apoptosis within 4 h. At an MOI of 10, $78\% \pm 8\%$ of the cells were apoptotic. Reduction of the infection time to 60 and 30 min at an MOI of 50 decreased the proportion of apoptotic cells to $88\% \pm 5\%$ and $75\% \pm 4\%$, respectively. Nevertheless, these data clearly show that promotion of J774A.1 cell apoptosis by *Y. enterocolitica* is very efficient, indicating that killing of J774A.1 cells due to *Yersinia* infection does occur through the induction of programmed cell death.

The caspase inhibitor ZVAD.fmk prevents completion of the apoptotic process induced by *Y. enterocolitica* in J774A.1 cells.

To determine whether caspases are involved in *Y. enterocolitica*-induced apoptosis, we treated J774A.1 macrophages with the cell-permeable, broad-spectrum caspase inhibitor ZVAD.fmk at a concentration of 100 μ M (30). ZVAD.fmk treatment inhibited shrinkage of J774A.1 cells infected with the virulent strain WA-314, and cells retained their normal shapes as analyzed by phase-contrast microscopy (data not shown). Furthermore, membrane integrity was not affected, as more than 95 and 70% of the cells showed no trypan blue dye at 8 and 20 h after infection, respectively. Similar results were obtained with ZVAD.fmk concentrations of 25 and 50 μ M. However, after 4 h of incubation, $87\% \pm 11\%$ of the cells exhibited fluorescence after labeling with fluorescein-conjugated annexin V (Fig. 3c), indicating that the external display of phosphatidylserine was not prevented by ZVAD.fmk. Analyzing the effect of ZVAD.fmk on DNA fragmentation at 4 and 20 h after infection (Fig. 4), we found that nucleosome laddering induced by the virulent strain WA-314 (Fig. 4, lane 3) was completely inhibited (Fig. 4, lanes 4 and 8). Figure 4 also reveals that DNA fragmentation due to infection with virulent *Yersinia* was completed after 20 h, since no more fragments were detected (Fig. 4, lane 7). On the other hand, J774A.1 cells infected with the nonvirulent strain WA-C exhibited weak DNA degradation after 20 h (Fig. 4, lane 6), a phenomenon which can be attributed to a low rate of apoptosis occurring in macrophages after phagocytosis of extracellular bacteria (6).

***Y. enterocolitica*-induced apoptosis of J774A.1 macrophages depends on a functional type III protein secretion system but is not a consequence of *Yersinia*-mediated disruption of actin microfilaments.** We investigated different *Y. enterocolitica* mutants and strains to restrict the number of potential virulence factors involved in the promotion of apoptosis. Figure 5 displays the results obtained with the different *Y. enterocolitica* strains with respect to DNA fragmentation (Fig. 5a) and phosphatidylserine exposure (Fig. 5b). In these experiments, staurosporine (5 μ M) was included as a positive control, inducing

FIG. 4. The caspase inhibitor ZVAD.fmk prevents *Y. enterocolitica*-induced DNA fragmentation of J774A.1 cells. Cells were untreated (lanes 1 and 5) or infected with the virulence-plasmid-cured strain WA-C (lanes 2 and 6) or the virulence-plasmid-harboring strain WA-314 in the absence (lanes 3 and 7) or the presence (lanes 4 and 8) of ZVAD.fmk (100 μ M) (MOI, 50:1; 90 min). Low-molecular-weight DNA of J774A.1 cells was isolated after 4 h (lanes 1 to 4) or after 20 h (lanes 5 to 8) of incubation. Lane M displays the molecular size marker (lambda DNA/EcoRI plus *Hind*III).

apoptosis of $95\% \pm 2\%$ of the cells within 4 h (data not shown). Strain WA-C(pYV-7146) is selectively impaired in the secretion of YopH by insertional inactivation of the YopH-specific chaperone gene *ycpH* (41). This strain (Fig. 5, lane 4) was as efficient as the virulent wild-type strain WA-314 (Fig. 5, lane 3) in the induction of apoptosis. Similar results were obtained with a mutant directly affected in *yopH* expression (data not shown), indicating that apoptosis is not mediated by YopH. The protein LcrD is an essential element of the *Yersinia* type III secretion apparatus (34), and thus, inactivation of *lcrD* in strain WA-C(pYV-515) prevents the secretion of all of the Yops (41). The *lcrD* mutant WA-C(pYV-515) (Fig. 5, lane 5) failed to induce apoptosis of J774A.1 macrophages almost to the same extent as the virulence-plasmid-cured strain WA-C (Fig. 5, lane 2). This result proves that macrophage-programmed cell death triggered by *Y. enterocolitica* is mediated by secreted virulence factors depending on a functional Yop secretion system. In this context, we tested two other *Y. enterocolitica* strains expressing a restricted repertoire of yop genes. Strain WA-C(pLCR) harbors the fragment of the *Y. enterocolitica* virulence plasmid encoding the Yop secretion machinery and the genes for the translocator and/or regulator proteins YopD, YopB, LcrV, and YopN. The second strain, referred to as WA-C(pLCR, pB8-23), carries an additional plasmid encoding the genes for YopH and YopE and for the adhesin YadA. In contrast to strain WA-C(pLCR), strain WA-C(pLCR, pB8-23) is highly resistant to phagocytosis and suppresses the macrophage oxidative burst (40), virulence properties which are known to be conferred by YopH and YopE (7, 15, 23, 36, 37, 41). However, neither strain was efficient in inducing apoptosis of J774A.1 cells (Fig. 5, lanes 6 and 7). These findings imply that the proteins secreted by strain WA-C(pLCR, pB8-23) (YopD, YopB, LcrV, YopN, YopH, and YopE) are not (at least not solely) responsible for the induction of macrophage apoptosis.

The actin-depolymerizing activity of YopE is known to confer morphological changes to mammalian cells (37, 38). To

examine the possibility that *Yersinia*-induced disruption of the actin cytoskeleton causes macrophage apoptosis, we infected J774A.1 cells with different *Yersinia* strains and analyzed the behavior of the actin cytoskeleton. Immunofluorescent labeling of F-actin with phalloidin demonstrated rapid disruption of the actin filaments upon infection with the virulent strain WA-314 (Fig. 6b) and the YopH/YopE-translocating strain WA-C(pLCR, pB8-23) (Fig. 6c). Both strains induced a change in the microfilament structure from an ordered appearance to a disordered granular pattern (38), which was obviously mediated by extracellularly bound bacteria. Most likely, this was the effect of YopE (38). In contrast, the virulence-plasmid-cured strain WA-C appeared to be efficiently phagocytosed, but the actin microfilament structure of the macrophages was not affected (Fig. 6a). However, since strain WA-314, but not strain WA-C(pLCR, pB8-23), was able to induce apoptosis of J774A.1 cells (Fig. 5), these results show that *Y. enterocolitica*-mediated disruption of the actin filaments is not responsible for triggering macrophage apoptosis.

***Y. enterocolitica* triggers apoptosis of human macrophages but not of epithelial HeLa cells.** We wondered whether *Y. enterocolitica*-induced apoptosis is unique to J774A.1 murine macrophages or whether it also occurs in macrophages derived from human monocytes and in epithelial HeLa cells. GM-CSF-differentiated human macrophages were infected either with virulence-plasmid-cured (WA-C) or virulence-plasmid-bearing yersiniae (WA-314) or with strain WA-C(pLCR, pB8-23). Af-

FIG. 5. Apoptosis of J774A.1 cells infected with different *Y. enterocolitica* mutants and strains. (a) J774A.1 cell DNA fragmentation. (b) Quantification of apoptotic J774A.1 cells. Cells were either untreated (lane 1) or infected with the virulence-plasmid-cured strain WA-C (lane 2), the virulence-plasmid-harboring strain WA-314 (lane 3), the *ycpH* mutant WA-C(pYV-7146) (lane 4), the *lcrD* mutant WA-C(pYV-515) (lane 5), strain WA-C(pLCR) secreting YopD, YopB, YopN, and LcrV (lane 6), and strain WA-C(pLCR, pB8-23) secreting YopD, YopB, YopN, LcrV, YopH, and YopE (lane 7) (MOI, 50:1; 90 min). Isolation of low-molecular-weight DNA (a) and staining of cells with fluorescein-conjugated annexin V and propidium iodide (b) were performed after 4 h of incubation. The percentages of apoptotic cells are mean values from three different experiments (100 cells were analyzed in each experiment). Lane M in panel a displays the molecular size marker (lambda DNA/EcoRI plus *Hind*III).

FIG. 6. Immunofluorescent labeling of the actin microfilament structure of *Y. enterocolitica*-infected J774A.1 macrophages. J774A.1 cells were either infected with the virulence-plasmid-cured strain WA-C (a), the virulence-plasmid-harboring strain WA-314 (b), or strain WA-C(pLCR, pB8-23) secreting YopD, YopB, YopN, LcrV, YopH, and YopE (c). After 60 min, cells were fixed, permeabilized, and labeled with BODIPY FL phalloidin to detect F-actin (green fluorescence). Bacteria were stained with a rabbit anti-*Y. enterocolitica* O8 antibody followed by a rhodamine-conjugated antirabbit antibody (red fluorescence). Cells were analyzed by fluorescence microscopy. Magnification, $\times 2,500$.

TABLE 2. *Y. enterocolitica* induces apoptosis of human monocyte-derived macrophages but not of HeLa cells

Treatment	Mean % of apoptotic cells $\pm$ SD ^a	
	Human macrophages	HeLa cells
Untreated	3 $\pm$ 2	6 $\pm$ 1
Staurosporine ^b	97 $\pm$ 2	94 $\pm$ 4
WA-C	2 $\pm$ 1	4 $\pm$ 1
WA-314	74 $\pm$ 6	6 $\pm$ 5
WA-C(pLCR, pB8-23)	7 $\pm$ 2	9 $\pm$ 1

^a Apoptosis was assayed 20 h after infection (MOI, 50:1; 90 min) by staining the cells with fluorescein-conjugated annexin V and propidium iodide. Results are expressed as mean percentages of apoptotic cells $\pm$ SDs from three independent experiments. One hundred cells per sample were analyzed in each experiment.

^b Staurosporine was included as a positive control at a concentration of 5 μ M.

ter incubation for 4 h, selective macrophages treated with strain WA-314 exhibited characteristic membrane blebbing (data not shown) and underwent apoptosis after a final incubation of 20 h (74% $\pm$ 6% apoptotic cells [Table 2]). Macrophages infected with strain WA-C(pLCR, pB8-23) were strongly affected after 4 h. Cells rounded up and shrunk remarkably, an effect probably mediated by YopE (data not shown). After 20 h, cells recovered almost completely (Table 2). Thus, although this process seems to be delayed, *Y. enterocolitica* triggers apoptosis in human macrophages in the same fashion as in J774A.1 cells.

Human epithelial HeLa cells treated with the strains WA-314 and WA-C(pLCR, pB8-23) exhibited the classical morphological alterations attributed to the YopE actin-depolymerizing effect (37, 38) after only 90 min of incubation; cells rounded up and tended to detach from the culture dish (Fig. 7c and e). These morphological changes were still visible after 8 h, but no significant cell apoptosis occurred within that period, as determined by detection of surface-exposed phosphatidylserine (Fig. 7d and f). A single apoptotic cell is included in every image in Fig. 7. The majority of the HeLa cells had not undergone apoptosis up to 20 h after infection (Table 2), whether they were treated with virulence-plasmid-cured yersiniae, wild-type yersiniae, or strain WA-C(pLCR, pB8-23). Similar results were obtained by the trypan blue exclusion assay. Only 10 to 20% of the infected HeLa cells were found to be dead after 20 h. Thus, HeLa cells do not remarkably lose viability and clearly respond differently to *Y. enterocolitica* infection than macrophages.

DISCUSSION

In this study, we have shown that *Y. enterocolitica* kills murine J774A.1 macrophages and macrophages derived from human monocytes through the induction of apoptosis. Macrophages infected with *Y. enterocolitica* exhibited characteristic features of cells undergoing programmed cell death, such as DNA fragmentation, external exposure of phosphatidylserine, membrane blebbing, and chromatin condensation. These effects are mediated by surface-bound bacteria, since yersiniae resist phagocytosis and are located extracellularly at the macrophage membrane (15, 36, 37, 40, 43). We used the cell-permeable caspase inhibitor ZVAD.fmk to reveal a crucial role for caspases in *Y. enterocolitica*-induced apoptosis, since ZVAD.fmk is an effective inhibitor of all tested caspases (4, 17, 30). In J774A.1 cells, ZVAD.fmk prevented DNA fragmentation and membrane permeabilization induced by *Y. enterocolitica* but did not prevent the external display of phosphatidyl-

FIG. 7. Morphology and assessment of apoptosis of *Y. enterocolitica*-infected HeLa cells. HeLa cells were infected with the virulence-plasmid-cured strain WA-C (a and b), the virulence-plasmid-harboring strain WA-314 (c and d), or strain WA-C(pLCR, pB8-23) secreting YopD, YopB, YopN, LcrV, YopH, and YopE (e and f) (MOI, 50:1; 90 min). After 8 h of incubation, uninfected cells were stained with fluorescein-conjugated annexin V and propidium iodide and analyzed by phase-contrast (a, c, and e) and fluorescence microscopy (b, d, and f) to identify apoptotic cells. Magnification, $\times 500$.

serine as an early-stage apoptotic event. This implies that caspases are involved not in the onset of J774A.1 cell apoptosis, as characterized by surface phosphatidylserine exposure, but in the completion of the program, once it is initiated. This feature does not seem to be unique to infection with *Y. enterocolitica*, since under our experimental conditions, treatment of J774A.1 cells with the apoptosis-inducing agent staurosporine also did not prevent phosphatidylserine exposure in the presence of ZVAD.fmk (data not shown).

We compared the effects observed on macrophages with the events triggered on HeLa cells by *Y. enterocolitica* infection. The human epithelial HeLa cell line is a very well-established model of infection for the study of *Yersinia*-host cell interactions (37–39). The events occurring on HeLa cells were in sharp contrast to the apoptotic processes taking place in macrophages. HeLa cells exhibited the classical alterations mediated by YopE, i.e., they rounded up and tended to detach from the matrix (37, 38), but they did not undergo apoptosis, and cell viability was not affected. Indications for different behaviors of macrophages and HeLa cells in response to *Yersinia* infection have already been provided in the literature. Goguen et al. (18) found that macrophage cell viability was reduced, as tested in a ^{51}Cr release assay, whereas Rosqvist and Wolf-Watz (39) revealed no impaired viability of HeLa cells upon treatment with yersiniae. However, until now, the literature has not discriminated between the morphological alterations occurring on HeLa cells and macrophages; both were designated “cytotoxic” effects (18, 39). Our results now clearly demonstrate that HeLa cells and macrophages respond differently to *Yersinia* infection. Actin disruption mediated by YopE, which causes the global morphological changes on HeLa cells (38), does not seem to be the driving force of macrophage apoptosis. Accordingly, a *Y. enterocolitica* strain secreting only a restricted rep-

ertoire of Yops, including YopE, induced strong actin depolymerization of J774A.1 macrophages and severe morphological alterations in human macrophages but did not trigger apoptosis. *Yersinia* mutants with impaired *yopE* expression were previously reported to induce the same morphological changes on macrophages as wild-type yersiniae after 3 h of infection (23) and to be cytotoxic (37). In fact, these effects may be due to modifications resulting from macrophage apoptosis. In our study, a *yopE* mutant of *Yersinia pseudotuberculosis* [strain YPIII(pIB522), kindly provided by H. Wolf-Watz] was able to trigger apoptosis in J774A.1 cells (data not shown). Taken together, these data provide strong evidence that YopE is not responsible for the induction of macrophage apoptosis. The results obtained with the *lcrD* mutant, a strain with impaired secretion of all Yops, showed that apoptosis triggered by *Y. enterocolitica* depends on a functional type III protein secretion system. This finding implies that one or more secreted *Yersinia* virulence factors do specifically induce the macrophage cell death program. Previous studies suggested that macrophage cytotoxicity observed with the *yopE* mutant may be an effect of YopH (37). However, analysis of a *Y. enterocolitica* mutant whose YopH secretion was affected excluded the possibility that apoptosis is mediated by YopH.

A type III protein secretion system similar to that of *Yersinia* spp. has been shown to be required for the apoptotic effects induced by *S. typhimurium* and *S. flexneri* on macrophages (10, 29, 31, 48). Nevertheless, the mechanism and the strategy by which *Y. enterocolitica* triggers apoptosis appears to differ from those of *Salmonella*- and *Shigella*-mediated apoptosis. Cell death induced by these facultative intracellular pathogens shows a close correlation with the capability of these bacteria to invade infected cells, implying that the virulence factors mediating cell invasion are also responsible for apoptosis (10,

31, 48). In contrast, *Y. enterocolitica* avoids the internalization of macrophages and appears to trigger macrophage cell death from an extracellular localization. Furthermore, recent studies demonstrated that *S. flexneri* kills human monocyte-derived macrophages by the induction of necrosis, whereas murine J774A.1 cells undergo apoptotic cell death upon *Shigella* infection (16). Our work shows that *Yersinia* infection of both cell types results in cell death by apoptosis. This suggests that the mechanism used by *Yersinia* to induce apoptosis is more general than that used by *Shigella*. In *S. flexneri*, the invasion plasmid antigen B (IpaB) was found to induce apoptosis by binding directly to caspase-1/interleukin 1 β -converting enzyme (11). IpaB binding appears to upregulate caspase-1 activity, and specific inhibition of caspase-1 with peptide Ac-Tyr-Val-Ala-aspartic acid aldehyde (Ac-YVAD-CHO) prevents *Shigella*-induced apoptosis (11). In our study, the caspase-1 inhibitor did not block apoptosis induced by *Y. enterocolitica* (data not shown). Together with the finding that the broad-spectrum caspase inhibitor ZVAD.fmk blocked the completion, but not the onset, of *Yersinia*-induced apoptosis, these results strongly indicate that *Yersinia*, unlike *Shigella*, initiates apoptosis at a level upstream of caspase activation, independently of caspase-1. Several reports claim a role of mitogen-activated protein kinase (MAPK) modules in the regulation of apoptosis (12, 19, 27, 28, 33, 42, 45, 47), and there is good evidence that parallel cascades of MAPK families serve to regulate cell proliferation and cell death (2). A differential activation state of one or several groups of MAPKs may also be involved in *Yersinia*-induced programmed cell death, since macrophage ERK1/2, p38, and JNK MAPKs are initially stimulated and subsequently deactivated by *Y. enterocolitica* infection (40). However, the mechanism of *Yersinia*-induced apoptosis and the virulence factor(s) involved remain to be elucidated.

In conclusion, our study emphasizes the complexity of the strategy used by *Y. enterocolitica* to neutralize host defenses. Interaction of *Y. enterocolitica* with macrophages, characterized by interference with macrophage phagocytosis, oxidative-burst generation, and tumor necrosis factor α production, culminates in the induction of the intrinsic macrophage cell death program. Macrophage apoptosis has not been recognized for many years because it was masked by the "cytotoxic" YopE effect, which, in fact, appears not to be responsible for macrophage cell death. Apoptosis of macrophages may be viewed as another outstanding hallmark of *Yersinia*-mediated subversion of the eukaryotic immune defense. Our study indicates the existence of a novel strategy used by a bacterial pathogen to promote macrophage suicide.

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REFERENCES

- Alnemri, E. S., D. J. Livingston, D. W. Nicholson, G. Salvesen, N. A. Thornberry, W. W. Wong, and J. Yuan. 1996. Human ICE/CED-3 protease nomenclature. *Cell* 87:171.
- Anderson, P. 1997. Kinase cascades regulating entry into apoptosis. *Microbiol. Mol. Biol. Rev.* 61:33-46.
- Andersson, K., N. Carballera, K.-E. Magnusson, C. Persson, O. Stendahl, H. Wolf-Watz, and M. Fällman. 1996. YopH of *Yersinia pseudotuberculosis* interrupts early phosphotyrosine signalling associated with phagocytosis. *Mol. Microbiol.* 20:1057-1069.
- Armstrong, R. C., T. Aja, J. Xiang, S. Gaur, J. F. Krebs, K. Hoang, X. Bai, S. J. Korsmeyer, D. S. Karanewsky, L. C. Fritz, and K. J. Tomaselli. 1996. Fas-induced activation of the cell death-related protease CPP32 is inhibited by Bcl-2 and by ICE family protease inhibitors. *J. Biol. Chem.* 271:16850-16855.
- Autenrieth, I. B., V. Kempf, T. Sprinz, S. Preger, and A. Schnell. 1996. Defense mechanisms in Peyer's patches and mesenteric lymph nodes against *Yersinia enterocolitica* involve integrins and cytokines. *Infect. Immun.* 64:1357-1368.
- Baran, J., K. Guzik, W. Hryniewicz, M. Ernst, H.-D. Flad, and J. Pryjma. 1996. Apoptosis of monocytes and prolonged survival of granulocytes as a result of phagocytosis of bacteria. *Infect. Immun.* 64:4242-4248.
- Bliska, J. B., and D. S. Black. 1995. Inhibition of the Fc receptor-mediated oxidative burst in macrophages by the *Yersinia pseudotuberculosis* tyrosine phosphatase. *Infect. Immun.* 63:681-685.
- Bliska, J. B., J. C. Clemens, J. E. Dixon, and S. Falkow. 1992. The *Yersinia* tyrosine phosphatase: specificity of a bacterial virulence determinant for phosphoproteins in the J774A.1 macrophage. *J. Exp. Med.* 176:1625-1630.
- Caron, E., A. Gross, J.-P. Liautard, and J. Dornand. 1996. *Brucella* species release a specific, protease-sensitive, inhibitor of TNF- α expression, active on human macrophage-like cells. *J. Immunol.* 156:2885-2893.
- Chen, L. M., K. Kaniga, and J. E. Galán. 1996. *Salmonella* spp. are cytotoxic for cultured macrophages. *Mol. Microbiol.* 21:1101-1115.
- Chen, Y., M. R. Smith, K. Thirumalai, and A. Zychlinsky. 1996. A bacterial invasin induces macrophage apoptosis by binding directly to ICE. *EMBO J.* 15:3853-3860.
- Chen, Y.-R., Y. Wang, D. Templeton, R. J. Davis, and T.-H. Tan. 1996. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and γ radiation: duration of JNK activation may determine cell death and proliferation. *J. Biol. Chem.* 271:31929-31936.
- Cornelis, G. R., and H. Wolf-Watz. 1997. The *Yersinia* Yop virulon: a bacterial system for subverting eukaryotic cells. *Mol. Microbiol.* 23:861-867.
- Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148:2207-2216.
- Fällman, M., K. Andersson, S. Hakansson, K.-E. Magnusson, O. Stendahl, and H. Wolf-Watz. 1995. *Yersinia pseudotuberculosis* inhibits Fc receptor-mediated phagocytosis in J774 cells. *Infect. Immun.* 63:3117-3124.
- Fernandez-Prada, C. M., D. L. Hoover, B. D. Tall, and M. M. Venkatesan. 1997. Human monocyte-derived macrophages infected with virulent *Shigella flexneri* in vitro undergo a rapid cytolytic event similar to oncosis but not apoptosis. *Infect. Immun.* 65:1486-1496.
- Fraser, A., and G. Evan. 1996. A license to kill. *Cell* 85:781-784.
- Goguen, J. D., W. S. Walker, T. P. Hatch, and J. Yother. 1986. Plasmid-determined cytotoxicity in *Yersinia pestis* and *Yersinia pseudotuberculosis*. *Infect. Immun.* 51:788-794.
- Goillot, E., J. Raingeaud, A. Ranger, R. I. Tepper, R. J. Davis, E. Harlow, and I. Sanchez. 1997. Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. *Proc. Natl. Acad. Sci. USA* 94:3302-3307.
- Guan, K., and J. E. Dixon. 1990. Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*. *Science* 249:553-556.
- Hanski, C., U. Kutschka, H. P. Schmoranz, M. Naumann, A. Stallmach, H. Hahn, H. Menge, and E. O. Riecken. 1989. Immunohistochemical and electron microscopic study of interaction of *Yersinia enterocolitica* serotype O8 with intestinal mucosa during experimental enteritis. *Infect. Immun.* 57:673-678.
- Hanski, C., M. Naumann, A. Grützkau, G. Pluschke, B. Friedrich, H. Hahn, and E. O. Riecken. 1991. Humoral and cellular defense against intestinal murine infection with *Yersinia enterocolitica*. *Infect. Immun.* 59:1106-1111.
- Hartland, E. L., S. P. Green, W. A. Phillips, and R. M. Robins-Browne. 1994. Essential role of YopD in inhibition of the respiratory burst of macrophages by *Yersinia enterocolitica*. *Infect. Immun.* 62:4445-4453.
- Heesemann, J. 1987. Chromosomal-encoded siderophores are required for mouse virulence of enteropathogenic *Yersinia* species. *FEMS Microbiol. Lett.* 48:229-233.
- Heesemann, J., U. Gross, N. Schmidt, and R. Laufs. 1986. Immunochemical analysis of plasmid-encoded proteins released by enteropathogenic *Yersinia* sp. grown in calcium-deficient media. *Infect. Immun.* 54:561-567.
- Hockenbery, D., G. Nunez, C. Millman, R. D. Schreiber, and S. J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334-336.
- Ichijo, H., E. Nishida, K. Irie, P. ten Dijke, M. Saitoh, T. Moriguchi, M. Takagi, K. Matsumoto, K. Miyazono, and Y. Gotoh. 1997. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 275:90-94.
- Johnson, N. L., A. M. Gardner, K. M. Diener, C. A. Lange-Carter, J. Gleavy, M. B. Jarpe, A. Minden, M. Karin, L. I. Zon, and G. L. Johnson. 1996. Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death. *J. Biol. Chem.* 271:3229-3237.
- Lindgren, S. W., I. Stojiljkovic, and F. Heffron. 1996. Macrophage killing is

- an essential virulence mechanism of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **93**:4197–4201.
30. McCarthy, N. J., M. K. B. Whyte, C. S. Gilbert, and G. I. Evan. 1997. Inhibition of ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. J. Cell Biol. **136**:215–227.
 31. Monack, D. M., B. Raupach, A. E. Hromockyj, and S. Falkow. 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. USA **93**:9833–9838.
 32. Nagata, S. 1997. Apoptosis by death factor. Cell **88**:355–365.
 33. Nishina, H., K. D. Fischer, L. Radvanyi, A. Shahinian, R. Hakem, E. A. Rubie, A. Bernstein, T. W. Mak, J. R. Woodgett, and J. M. Penninger. 1997. Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3. Nature **385**:350–353.
 34. Plano, G. V., and S. C. Straley. 1993. Multiple effects of *lcrD* mutations in *Yersinia pestis*. J. Bacteriol. **175**:3536–3545.
 35. Roggenkamp, A., S. Schubert, C. A. Jacobi, and J. Heesemann. 1996. Dissection of the *Yersinia enterocolitica* virulence plasmid pYVO8 into an operating unit and virulence genes modules. FEMS Microbiol. Lett. **134**:69–73.
 36. Rosqvist, R., I. Bölin, and H. Wolf-Watz. 1988. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. Infect. Immun. **56**:2139–2143.
 37. Rosqvist, R., A. Forsberg, M. Rimpiläinen, T. Bergman, and H. Wolf-Watz. 1990. The cytotoxic protein YopE of *Yersinia* obstructs the primary host defence. Mol. Microbiol. **4**:657–667.
 38. Rosqvist, R., A. Forsberg, and H. Wolf-Watz. 1991. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect. Immun. **59**:4562–4569.
 39. Rosqvist, R., and H. Wolf-Watz. 1986. Virulence plasmid-associated HeLa cell induced cytotoxicity of *Yersinia pseudotuberculosis*. Microb. Pathog. **1**: 229–240.
 40. Ruckdeschel, K., J. Machold, A. Roggenkamp, S. Schubert, J. Pierre, R. Zumbihl, J.-P. Liautard, J. Heesemann, and B. Rouot. 1997. *Yersinia enterocolitica* promotes deactivation of macrophage mitogen-activated protein kinases extracellular signal-regulated kinase-1/2, p38, and c-Jun NH₂-terminal kinase: correlation with its inhibitory effect on tumor necrosis factor alpha production. J. Biol. Chem. **272**:15920–15927.
 41. Ruckdeschel, K., A. Roggenkamp, S. Schubert, and J. Heesemann. 1996. Differential contribution of *Yersinia enterocolitica* virulence factors to evasion of microbicidal action of neutrophils. Infect. Immun. **64**:724–733.
 42. Schwenger, P., P. Bellosta, I. Vietor, C. Basilico, E. Y. Skolnik, and J. Vilcek. 1997. Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated kinase activation. Proc. Natl. Acad. Sci. USA **94**:2869–2873.
 43. Simonet, M., S. Richard, and P. Berche. 1990. Electron microscopic evidence for in vivo extracellular localization of *Yersinia pseudotuberculosis* harboring the pYV plasmid. Infect. Immun. **58**:841–845.
 44. Straley, S. C., G. V. Plano, E. Skrzypek, and J. B. Bliska. 1993. Yops of *Yersinia* spp. pathogenic for humans. Infect. Immun. **61**:3105–3110.
 45. Verheij, M., R. Bose, X. H. Lin, B. Yao, W. D. Jarvis, S. Grant, M. J. Birrer, E. Szabo, L. I. Zon, J. M. Kyriakis, A. Haimovitz-Friedman, Z. Fuks, and R. N. Kolesnick. 1996. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature **380**:75–79.
 46. Verhoven, B., R. A. Schlegel, and P. Williamson. 1995. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J. Exp. Med. **182**:1597–1601.
 47. Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270**:1326–1331.
 48. Zychlinsky, A., M. C. Prevost, and P. J. Sansonetti. 1992. *Shigella flexneri* induces apoptosis in infected macrophages. Nature **358**:167–169.

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