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Interaction of *Yersinia pestis* with Macrophages: Limitations in YopJ-Dependent Apoptosis

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The enteropathogenic *Yersinia* strains are known to downregulate signaling pathways in macrophages by effectors of the type III secretion system, in which YopJ/YopP plays a crucial role. The adverse effects of *Yersinia pestis*, the causative agent of plague, were examined by infecting J774A.1 cells, RAW264.7 cells, and primary murine macrophages with the EV76 strain and with the fully virulent Kimberley53 strain. *Y. pestis* exerts YopJ-dependent suppression of tumor necrosis factor alpha secretion and phosphorylation of mitogen-activated protein kinases and thus resembles enteropathogenic *Yersinia*. However, *Y. pestis* is less able to activate caspases, to suppress NF-κB activation, and to induce apoptosis in macrophages than the high-virulence *Y. enterocolitica* WA O:8 strain. These differences appear to be related to lower efficiency of YopJ effector translocation by *Y. pestis*. The efficiencies of effector translocation and of apoptosis induction can be enhanced either by using a high bacterial load in a synchronized infection or by overexpressing exogenous YopJ in *Y. pestis*. Replacing YopJ with the homologous *Y. enterocolitica* effector YopP can further enhance these effects. Overexpression of YopP in a *yopJ*-deleted *Y. pestis* background leads to rapid and effective translocation into target cells, providing *Y. pestis* with the high cytotoxic potential of *Y. enterocolitica* WA O:8. We suggest that the relative inferiority of *Y. pestis* in triggering cell death in macrophages may be advantageous for its in vivo propagation in the early stages of infection.

The genus *Yersinia* includes three pathogenic species, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*. These species are closely related genetically, yet they differ significantly in pathogenesis (route of infection, symptoms and severity of disease). Infection by *Y. pestis*, the etiological agent of plague, occurs by intradermal fleabites or inhalation and leads to fatal bubonic or pneumonic plague. *Y. enterocolitica* and *Y. pseudotuberculosis*, on the other hand, cause mild and self-limiting gastrointestinal syndromes after infection by the oral route (4, 37). In spite of the differences in pathogenesis, the three species have similar mechanisms for evading host innate immunity. This ability is attributed mainly to genes encoded by ~70-kb plasmids present in the three pathogenic *Yersinia* species (designated pCD1 in *Y. pestis* and pYV in *Y. enterocolitica* and *Y. pseudotuberculosis*), which exhibit extensive sequence similarity (7, 19, 25, 36, 38, 49). These plasmids, which are essential for virulence (1, 16, 21, 39), encode a type III secretion system (TTSS) comprised of a secretion apparatus, chaperones, and several effectors (Yops) (9). *Yersinia* TTSS expression is induced by temperature (a shift to 37°C) and contact with a host cell, both of which lead to transfer of Yops from the bacterium into the host cell cytosol (9, 28, 51). Among the most pronounced effects of the Yop translocon are disruption of the target cell signaling network and cytoskeleton rearrangement, which are necessary for phagocytosis by host macrophages and polymorphonuclear neutrophils. These effects involve the action of several Yops, including YopE, YopH, YopO/YpkA, and YopT (32).

An additional effector, designated YopP in *Y. enterocolitica* and YopJ in *Y. pseudotuberculosis*, has been shown to have a variety of adverse effects on interactions with host cells. These effects include inhibition of the mitogen-activated protein kinase (MAPK) and NF-κB pathways (3, 33, 35, 42, 46, 58), suppression of cytokine production (3, 35, 46), and induction of apoptosis of infected macrophages (29, 30, 43). Induction of programmed cell death involves activation of an early step in the caspase pathway (12), as well as inhibition of NF-κB activation, which prevents upregulation of antiapoptotic protein expression (42, 58). Recently, the involvement of MAPK signaling pathways in the apoptotic response has also been demonstrated (58).

The actual role of YopJ/YopP functions in *Yersinia* pathogenesis has not been resolved. Some studies have reported that deletion of *yopP*/*yopJ* by mutagenesis does not affect the 50% lethal doses for mice infected by *Y. pseudotuberculosis* (20), *Y. enterocolitica* (13), and *Y. pestis* (50; unpublished data for the Kimberley53 strain in a bubonic plague model). However, other studies have revealed involvement of YopP/YopJ in the virulence of *Y. enterocolitica* (53) and *Y. pseudotuberculosis*, as well as actual induction of macrophage apoptosis in vivo (31).

It has been suggested that YopJ belongs to a family of cysteine proteases related to the ubiquitin-like protein proteases (34). Sequence comparisons and mutagenesis studies have underlined the role of the presumptive cysteine protease catalytic triad His109-Glu128-Cys172 in YopJ function (12, 34, 58). Recently, YopJ was shown to be a promiscuous deubiquitinating enzyme capable of removing ubiquitin moieties from IκBα, thus inhibiting its proteasomal degradation and

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TABLE 1. Bacterial Strains and plasmids used

Strain or plasmid	Relevant characteristic(s)	Reference or source
Y. pestis strains		
Kimberley53	Virulent strain	18
Kimberley53Δp70/Δp10	Spontaneously pPCP1- and pCD1-cured Kimberley53 strain	18
EV76	*pgm* (Girard's strain)	1
EV76Δ*yopJ*	EV76 with knockout in *yopJ* gene; Kan^r	This study
EV76Δ*yopJ*+*yopP*	EV76Δ*yopJ* carrying p*yopP*	This study
EV76Δ*yopJ*+*yopJ*	EV76Δ*yopJ* carrying p*yopJ*	This study
Y. enterocolitica strains		
Y. entero.	Virulent strain; WA O:8	ATCC 27729[a]
*Y. entero*Δp70	Spontaneously pYV-cured WA O:8	27
Plasmids		
pGFPuv	*lac*-controlled *gfp* gene on pUC vector; Amp^r	CLONTECH, USA
p*yopP*	pGFPuv in which the *gfp* gene is replaced by the *Y. enterocolitica* O:8 *yopP* gene	This study
p*yopJ*	pGFPuv in which the *gfp* gene is replaced by the *Y. pestis* Kimberley53 *yopJ* gene	This study
pKOBEG:*sacB*	Carries the λ phage *red* operon and *sacB* gene; Cm^r	14

[a] See reference 27.

leading to downregulation of NF-κB functions (60). In addition, analysis of high- and low-virulence *Y. enterocolitica* strains belonging to two different serotypes revealed the role of Arg143 in YopP-related effects (44). In this context, it is interesting that these *Y. enterocolitica* serotypes exhibit variations in YopP translocation efficiency (13).

The functional studies with YopJ/YopP were conducted with the enteropathogenic organisms *Y. enterocolitica* and *Y. pseudotuberculosis* (32, 41, 57). TTSS-mediated apoptosis of macrophages by *Y. pestis* was demonstrated under specific conditions that included tight interaction (impact of a high bacterial load and prolonged incubation [54]) between J774A.1 cells and an avirulent CO92 strain (*pgm*).

In this study, we focused on the YopJ-related functions of *Y. pestis* in murine macrophages. We found that *Y. pestis* harbors machinery for impairment of MAPK pathway activation and secretion of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α). We also showed, using various infection conditions, that two *Y. pestis* strains, the *pgm* attenuated EV76 strain and the fully virulent Kimberley53 strain, are both less able to induce macrophage apoptosis, to impair NF-κB activation, and to activate caspase pathways than the virulent *Y. enterocolitica* O:8 strain. These limitations correlate with inefficient translocation of YopJ by *Y. pestis*, which leads to slow accumulation of the effector in the target cells. In addition, we found that *Y. enterocolitica*-derived YopP can provide effective apoptotic potential to *Y. pestis*, and we suggest that this can be attributed to the fact that *Y. enterocolitica* O:8 YopP is better adapted to translocation by the *Yersinia* TTSS than YopJ.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Yersinia* strains and plasmids used in this study are listed in Table 1. *Yersinia* strains were isolated from stocks on selective BIN medium (2). Routine propagation was performed on brain heart infusion agar (BHIA) (Difco). For cell infection experiments, bacteria were grown at 28°C in heart infusion broth (HIB) for 18 h in a gyratory shaker at 150 rpm. Bacteria were then diluted in HIB to obtain an optical density at 660 nm of 0.05 and grown for additional 3 h at 37°C (100 rpm). The bacteria were washed and then resuspended in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, and the density was adjusted to the desired concentration for infection of eukaryotic cells.

Cell culture. Murine macrophage-like cell lines J774A.1 and RAW264.7 were obtained from ATCC. Bone marrow macrophages were isolated from ICR female mice (Charles River Margate UK Ltd.). Cells collected from two femurs were suspended in 20 ml culture medium (see below) supplemented with 50 ng/ml macrophage colony-stimulating factor (R&D Systems) and were seeded directly into 96-well plates. After 5 days, nonadherent cells were removed, and the medium was replaced. At this stage, >95% of the cells stained positive for the macrophage marker CD11b.

All cells were grown at 37°C in the presence of 5% CO_2 in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin, and streptomycin. One day prior to infection, cells were transferred to an antibiotic-free medium.

YopJ knockout mutagenesis of *Y. pestis*. *yopJ* deletion mutagenesis of the *Y. pestis* EV76 strain was performed by replacing the 519-bp central region of this gene (between two BamHI sites) with a kanamycin resistance cassette (Pharmacia) by homologous recombination. The protocol used was based on previously established methodologies (11, 14). A linear PCR fragment in which kanamycin sequences were flanked by *yopJ* sequences was electroporated into EV76 bacteria expressing the λ phage *red* system from pKOBEG::*sacB* (a generous gift from E. Carniel). Electroporation was performed in 10% glycerol and 10% polyethylene glycol 8000 (Sigma), and the bacteria were incubated in HIB for 2 h at 28°C. Transformants were selected on BHIA containing 50 μg/ml kanamycin, after which the pKOBEG::*sacB* plasmid was cured by growing the bacteria on BHIA supplemented with 10% sucrose. The expected knockout phenotype (EV76Δ*yopJ*) was verified by PCR, Southern blot, and Western blot analyses.

Sequence analysis and construction of *yopP* and *yopJ* expression vectors. The coding sequence of YopP from the virulent *Y. enterocolitica* WA O:8 strain and the coding sequence of YopJ from the virulent *Y. pestis* Kimberley53 strain were cloned by PCR using the Expand high-fidelity PCR system (Roche Applied Science). The primers used for both *yopP* and *yopJ* cloning were 5′CCCCATT ACCCCAAGCTTGATGATCGGACCAATATCACAAATAAA3′ (sense primer) and 5′AACAAAGGAGTCCGGAATTCGTATTCCCATACTGGAGCA AG3′ (antisense primer).

All the synthetic DNA oligodeoxynucleotides were prepared using an automatic Applied Biosystems DNA synthesizer. The resulting PCR products were inserted downstream of the *lac* promoter in pGFPuv (Clontech Lab) (replacing the *gfp* gene between the HindIII and EcoRI sites). Plasmids were introduced into the EV76Δ*yopJ* knockout strain by electroporation, which resulted in generation of the EV76Δ*yopJ*+*yopJ* and EV76Δ*yopJ*+*yopP* strains. The cloned gene sequences were verified with an ABI310 genetic analyzer (Applied Biosystems), using an ABI PRISM BigDye terminator reaction kit. The deduced amino acid sequence of the *yopP* gene of the *Y. enterocolitica* WA O:8 strain used by us was found to be identical to the sequence published previously for the A127/90 O:8 strain (gi:23630581). The *Y. pestis* Kimberley53 *yopJ* gene sequence was found to be identical to the sequence of *Y. pestis* CO92 (gi:16082759).

Cell cytotoxicity assay. Cells were seeded at a concentration of 2×10^4 cells/well in 96-well tissue culture plates 15 h prior to infection. Bacterial suspensions were applied to the monolayers at a multiplicity of infection (MOI) of

50 or at another MOI, as indicated below, and incubated for 1 h. Immediately after infection, cultures were washed three times with phosphate-buffered saline (PBS) to remove extracellular bacteria. Medium containing 50 μg/ml gentamicin was added in order to kill the remaining extracellular bacteria. This procedure was designated the "overlay procedure." In some of the experiments an alternative protocol was used; bacteria were forced onto the cells by centrifugation at 130 × *g* for 5 min. Infected cultures were incubated for 1 h before addition of gentamicin to a final concentration of 50 μg/ml without removal of the extracellular bacteria. This procedure was designated the "impact procedure." For both protocols, plates were incubated for an additional 6 h. The amounts of released lactate dehydrogenase (LDH) in supernatants of infected macrophages were determined colorimetrically using the Cytotox 96 nonradioactive cytotoxicity assay (Promega) according to the manufacturer's instructions. The A_{490} was determined using a microplate reader (Sunrise; Tecan, Austria). Cytotoxicity caused by bacteria was expressed as the percentage of cell death. The percent cytotoxicity was calculated using the following formula: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. The spontaneous release reflected the amount of LDH released from the cytoplasm of uninfected macrophages, whereas the maximum release was the amount of LDH released from detergent-lysed uninfected macrophages. Experiments were performed at least three times in triplicate. In parallel, the morphology of cells was monitored for up to 24 h by light microscopy.

Assessment of apoptosis by fluorescence microscopy. Apoptotic cells were detected and quantified by annexin-V staining for phosphatidylserine exposed on the outer leaflet of the cells. A total of 1×10^5 cells were cultured in four-well chamber slides (Nunc) 15 h prior to infection. Cells were infected by adding bacteria to cell monolayers and incubated for 30 min as described above (overlay procedure). Cell staining was performed 2 to 3 h postinfection with Alexa-conjugated annexin-V (Boehringer, Mannheim, Germany). Simultaneous DNA staining with 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma), which cannot penetrate apoptotic cells, allowed discrimination between apoptotic and necrotic cells. The percentage of apoptotic cells was determined by counting 100 cells per sample. Experiments were performed at least three times. In addition, nuclear morphology was examined by DAPI staining of cells after methanol fixation (which allowed stain penetration) in order to determine nuclear fragmentation of apoptotic cells.

Assay for MAPK phosphorylation. RAW264.7 cells (5×10^6) were seeded into 25-cm^2 flasks and infected at an MOI of 100 (by the overlay procedure) for different times. Cell lysates were prepared as described previously (56) and were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). After transfer to nitrocellulose membranes, duplicate membranes were developed with phospho-specific anti-p38 antibodies (New England Biolabs, Inc.), followed by horseradish peroxidase-conjugated second antibody, and were visualized by ECL. Stripped membranes were reprobed with standard anti-extracellular signal-regulated kinase 1/2 antibodies (Sigma) as a control for loading. Experiments were repeated at least three times.

TNF-α secretion. RAW264.7 macrophages (2×10^5 cells) were seeded into 24-well plates and infected at an MOI of 100 (by the overlay or impact procedure) for 2 h. The concentration of TNF-α in the cell medium was determined by an enzyme-linked immunosorbent assay, using the DuoSet mouse TNF-α immunoassay system (R&D Systems).

EMSA: assessment of NF-κB nuclear translocation. Cells (5×10^6) were plated in 75-cm^2 flasks (TPP, Switzerland) 15 h prior to infection. Monolayers were overlaid with bacteria for 90 min at an MOI of 50. Following infection, nuclear cell extracts were prepared, and an electrophoretic mobility shift assay (EMSA) was performed as described previously (55), with minor modifications. The nuclear protein extracts (8 μg) were preincubated for 15 min on ice with 2 μg poly(dI-dC) (Amersham Pharmacia Biotech) in a binding buffer (25 mM HEPES [pH 7.9], 0.5 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol). The radiolabeled NF-κB binding oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (1.75 ng) was then added to the DNA-protein complexes, which were incubated for an additional 30 min. The DNA-protein complexes were separated on 5% neutral PAGE gels using Tris-borate-EDTA running buffer. The gels were developed by X-ray radiography. For supershift analysis, rabbit polyclonal anti-p65 NF-κB antibodies (sc-109X; Santa Cruz Biotechnology, California) were added for 15 min prior to the incubation with labeled oligonucleotide probe. Competition studies were carried out with a 100-fold molar excess of unlabeled oligonucleotides that was added to the reaction mixture before the radiolabeled oligonucleotides were added. Experiments were repeated at least three times.

Caspase 3/caspase 7 assay. Cells were grown and infected as described above for the cell cytotoxicity assay. Three hours after infection, caspase 3 and caspase 7 activities were measured with an Apo-ONE homogeneous caspase 3/7 assay kit (Promega) used according to the manufacturer's instructions.

Western blot analysis of *Yersinia* proteins YopJ and YopP. Bacteria were lysed with Laemmli sample buffer (Bio-Rad), and proteins were subjected to SDS-PAGE prior to transfer to nitrocellulose membranes. To monitor expression of the YopP and YopJ, the membranes were developed with polyclonal rabbit anti-peptide YopP/YopJ antibodies, which reacted with both YopP and YopJ, followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. The antipeptide antibodies were raised by immunizing rabbits with maleimide-activated keyhole limpet hemocyanin (Pierce) conjugated to the synthetic peptide CHDKLDPYLPVTFYKHTQGKKR (amino acids 211 to 231 of YopJ/YopP). Antibodies were affinity purified with a Sulfolink peptide coupling gel column (Pierce) used according to the manufacturer's instructions.

Monitoring YopP/YopJ translocation. RAW264.7 macrophages were infected with *Y. enterocolitica* or *Y. pestis* at an MOI of 100, either by overlaying the bacterial suspension or by centrifugation at 130 × *g* for 5 min. The infected cell cultures were incubated for different times. Cells were then washed with cold PBS, resuspended in PBS containing 0.1% Triton X-100 and a complete protease inhibitor cocktail (Roche), and incubated for 20 min on ice. Cell membranes and debris were removed by centrifugation (15,000 × *g*, 15 min, 4°C). Subsequently, 50 μg of cytosolic protein extract (equivalent to ~1×10^6 cells) was subjected to 10% SDS-PAGE. After electrophoresis, the gels were blotted onto nitrocellulose membranes, which were then probed with a rabbit polyclonal anti-YopP/YopJ antiserum (see above) at 4°C overnight. In some of the experiments, we used antipeptide antibodies which were further purified by affinity binding. Primary antibody binding was detected with a horseradish peroxidase-conjugated second antibody, followed by ECL. YopH translocation was monitored by the same approach using antibodies raised against the synthetic peptide CRQTAVRADLNANYIQY FRQS (amino acids 284 to 305).

RESULTS

Defining the *Y. pestis* cell infection model. The prototype *Y. pestis* EV76 strain was used in most of the macrophage infection experiments. This strain is an attenuated *pgm*-deficient strain; its virulence for mice can be restored by coinjection of inorganic iron (4). In selected cases, macrophage infection was also performed with the highly virulent *Y. pestis* Kimberley53 strain (18, 24).

Macrophage infection conditions were designed to reflect early events of natural infection, in which bacteria propagating in the flea gut at a low temperature are exposed to a temperature shift upon infection of a mammalian host. Accordingly, we used bacteria that were pregrown at 28°C and shifted to 37°C for 3 h. Under these conditions Yops are expressed and primed for translocation into phagocytic cells, yet bacteria do not carry the F1 capsule (data not shown), which is known to inhibit phagocytosis of *Y. pestis* by macrophages (6, 15).

The efficient cytotoxicity conferred by *Y. enterocolitica* WA O:8 infection of macrophages is not manifested in *Y. pestis* EV76 infection. Macrophage-like J774A.1 or RAW264.7 cells were infected by overlaying cell monolayers with bacterial suspensions of *Y. enterocolitica* WA O:8 or *Y. pestis* EV76. Cytotoxicity was monitored by measuring LDH release; annexin-V and DAPI staining were used to confirm apoptotic death. No LDH release was observed 6 h after infection of J774A.1 or RAW264.7 cells with either *Y. pestis* EV76 or its pCD1-cured derivative, EV76Δp70 (Fig. 1A). Moreover, microscopic observations indicated that the *Y. pestis*-infected cells remained intact even at 20 h postinfection (Fig. 1B). In contrast, as expected from previous studies (45), macrophages infected with *Y. enterocolitica* O:8 using the same infection protocol did release LDH very effectively within 6 h (Fig. 1A), and cells detached from the plates as early as 4 h postinfection. As expected, the cytotoxic effect of *Y. enterocolitica* O:8 on mac-

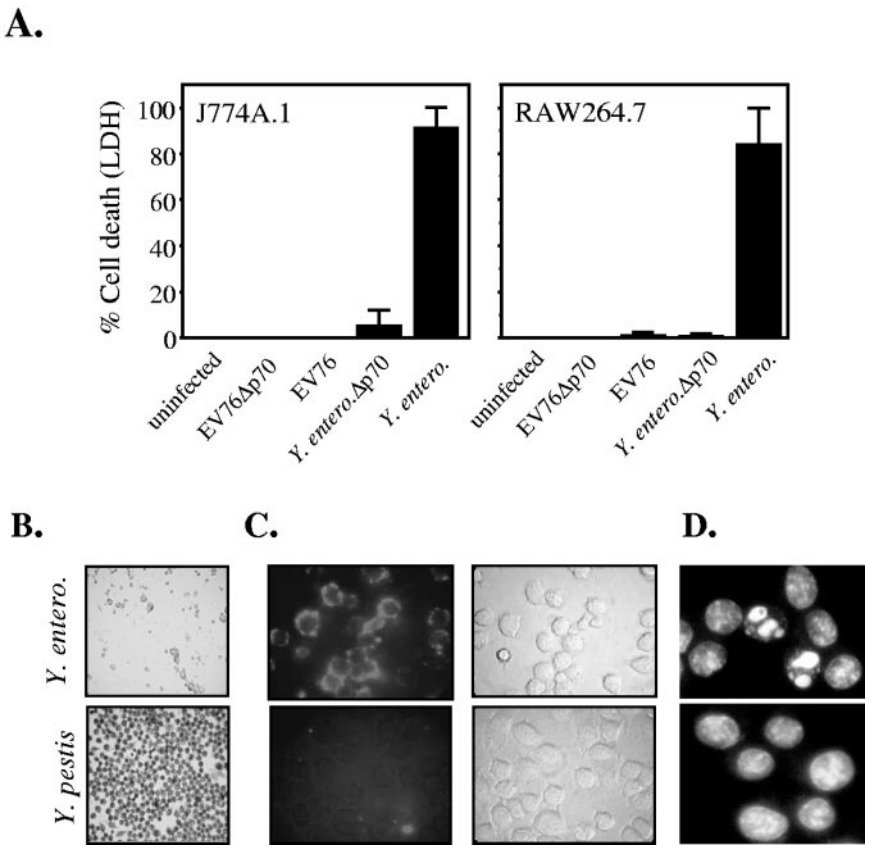

FIG. 1. Induction of apoptotic cell death by *Y. pestis* EV76 and *Y. enterocolitica* WA O:8 and their derivatives. Cell death and apoptosis were monitored upon infection by overlay of macrophages with various *Yersinia* strains (bacterial suspensions were added to the cell monolayers and washed off after incubation as described in Materials and Methods). The death of J774A.1 cells, as well as RAW264.7 cells, infected with the *Yersinia* strains was determined by LDH release 6 h postinfection (A), and detachment of cells (J774A.1) from culture wells was examined 20 h postinfection by light microscopy of Gimenez-stained monolayers (B). (C) The onset of J774A.1 cell apoptosis was evaluated 3 h postinfection by annexin-V staining (left panels). In the right panels the same fields visualized by phase-contrast microscopy are shown. (D) Nuclear morphology was determined by DAPI staining (magnification, ×1,000) at 4 h postinfection. *Y. entero*., *Y. enterocolitica*.

rophages was found to be dependent on the presence of the pYV plasmid (Fig. 1A) and exhibited characteristic features of an apoptotic process, as manifested by (i) positive staining by annexin-V, which binds to phosphatidylserine exposed on the outer membrane of cells at the early stages of apoptosis (63% of the cells were positive [Fig. 1C]), and (ii) condensed chromatin formations (Fig. 1D). These markers of apoptosis were practically absent in macrophages infected with *Y. pestis* EV76 under the same experimental conditions. The percentage of annexin-V-stained cells was less than 2.5%, and condensed chromatin formations were not detected in the *Y. pestis*-infected macrophages.

The levels of apoptosis conferred by *Y. pestis* and *Y. enterocolitica* correlate with the efficiencies of suppression of NF-κB activation and caspase 3 and 7 induction. The well-documented relationship between induction of macrophage apoptosis by enteropathogenic *Yersinia* and suppression of NF-κB activation (42–44, 46) led us to examine the effects of *Y. pestis* infection on nuclear translocation of NF-κB.

J774A.1 cells were infected with either *Y. pestis* EV76 or *Y. enterocolitica* O:8, as well as their derivatives cured of the 70-kb virulence plasmids, and nuclear translocation of NF-κB was analyzed by EMSAs (Fig. 2A). As expected, infection with the plasmid-cured *Y. enterocolitica* strain led to an increase in specific binding of NF-κB to the NF-κB consensus DNA probe, whereas infection with wild-type *Y. enterocolitica* led to suppression of NF-κB activation (Fig. 2A, lanes 5 and 6).

Infection with the plasmid-cured *Y. pestis* EV76 strain also resulted in NF-κB activation, suggesting that the pathogen-associated molecular patterns of *Y. pestis* can activate NF-κB nuclear translocation to the same extent as *Y. enterocolitica* O:8 (Fig. 2A, compare lanes 3 and 5). Nevertheless, in contrast to the results obtained with *Y. enterocolitica*, suppression of such activation was not observed when the virulence plasmid harboring *Y. pestis* was used (Fig. 2A, lane 4). It thus appears that the Yop translocon of *Y. pestis* EV76, in contrast to the Yop translocon of *Y. enterocolitica* O:8, is not capable of suppressing NF-κB activation under the same infection conditions.

An additional pathway involved in apoptosis regulation by *Yersinia* is the activation of procaspases (12). This led us to examine caspase 3 and 7 activity in macrophages infected with *Y. enterocolitica* and *Y. pestis*. In the case of *Y. enterocolitica* O:8, we found that there was a ~100-fold increase in the level of caspase 3/caspase 7 activation compared to the activation in untreated cells. In contrast, no detectable activation was observed upon infection with *Y. pestis* EV76 (Fig. 3).

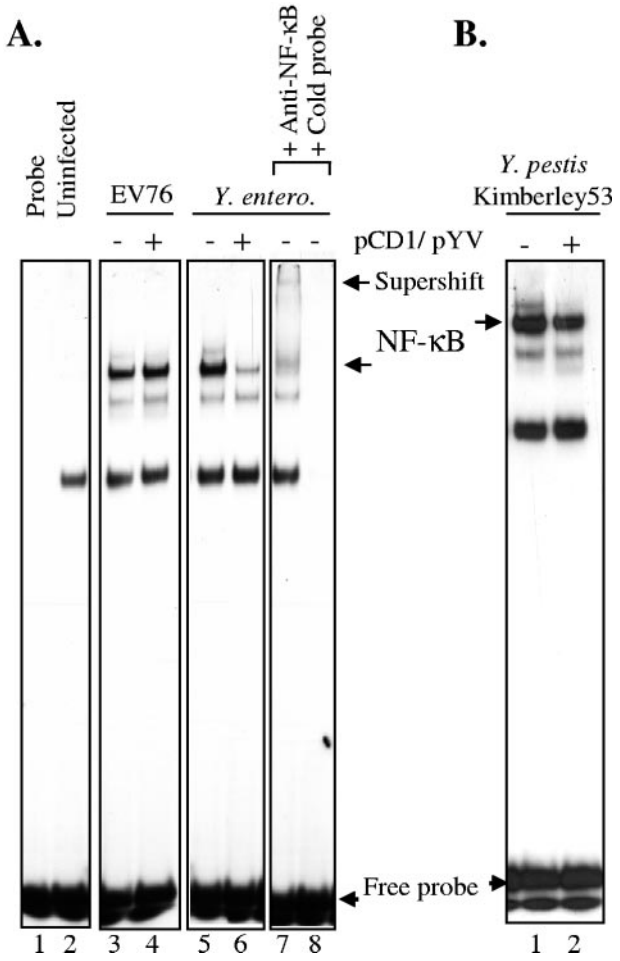

FIG. 2. NF-κB DNA-binding activity in J774A.1 macrophages infected with *Y. pestis* or *Y. enterocolitica*. J774A.1 cells were exposed by using the overlay procedure to different *Yersinia* strains and their plasmid-cured derivatives (in panel A, *Y. enterocolitica* O:8 and *Y. pestis* EV76 and their pCD1/pYV-cured derivatives, and in panel B, *Y. pestis* Kimberley53 and its pCD1- and pPCP1-cured derivative). Nuclear lysates were tested for interaction with an NF-κB-specific DNA probe by EMSAs. The specificity of binding was demonstrated by a supershift assay using antibodies directed against the p65 NF-κB subunit (panel A, lane 7) and by competition with a 100-fold molar excess of the unlabeled oligonucleotide (panel A, lane 8). Nuclear lysate of uninfected cells was used as a control (panel A, lane 2). *Y. entero*., *Y. enterocolitica*.

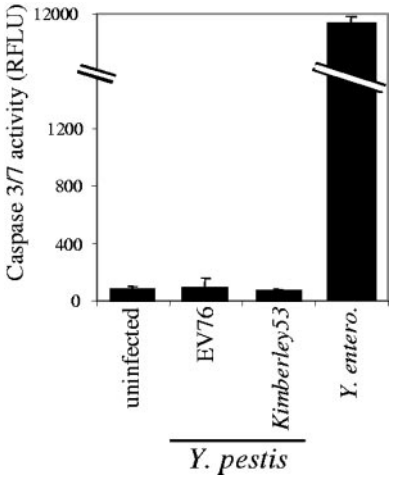

FIG. 3. Induction of caspase 3/caspase 7 activity in macrophages infected with *Y. pestis* or *Y. enterocolitica*. J774A.1 cells infected by using the overlay procedure with *Y. pestis* strains EV76 and Kimberley53 were compared to cells infected with *Y. enterocolitica* WA O:8. Caspase 3/caspase 7 activity was measured 3 h after infection as described in Materials and Methods and is expressed in relative fluorescence units (RFLU). *Y. entero*., *Y. enterocolitica*.

Thus, the difference between the abilities of *Y. pestis* and *Y. enterocolitica* O:8 to confer programmed cell death of host cells correlates well with the difference between the abilities of the organisms to affect two pathways implicated in the apoptotic response.

Weak apoptotic effect of *Y. pestis* on macrophages is also exhibited by a highly virulent strain. To check that the effects observed for the interaction of *Y. pestis* EV76 with macrophages are not unique to this attenuated strain, we infected J774A.1 cells with the highly virulent *Y. pestis* strain Kimberley53. Macrophages infected with this strain, like macrophages infected with EV76, remained viable even 20 h following infection. Likewise, the Kimberley53 strain, in contrast to *Y. enterocolitica*, did not activate caspase 3/caspase 7 in J774A.1 cells (Fig. 3), and no inhibitory effect on NF-κB activation was observed (Fig. 2B). These observations indicate that the *Y. pestis* features described above are not restricted to the EV76 attenuated strain but could also be attributed to a wild-type, fully virulent strain which is able to kill mice at a 50% lethal dose of ~1 CFU (18, 24).

Together, these results suggest that highly virulent *Y. pestis* is less able to induce apoptotic cell death of macrophages than the high-virulence *Y. enterocolitica* O:8 strain. Interestingly, in this respect, *Y. pestis* resembles the low-virulence *Y. enterocolitica* O:9 strain (44).

Correlation between *Yersinia*-induced cytotoxicity and the efficiency of YopJ/YopP translocation into host cells. The experiments described above indicate that *Y. pestis* does not have the high cytotoxic potential of *Y. enterocolitica* O:8. Nevertheless, *Y. pestis*-induced apoptosis can occur under specific experimental conditions. Forcing a high dose of bacteria onto cells by centrifugation, combined with maintaining the contact between the gentamicin-treated bacteria and the cells for a long time, was shown previously to promote cell death (54). Side-by-side comparison of such infection conditions with conditions in which contact of the bacteria with cells is less extensive (overlay of bacterial suspension on cells without centrifugation, followed by washing the bacteria away), again revealed the difference between *Y. pestis* and *Y. enterocolitica* O:8. While both assay conditions were effective for promoting cell death by *Y. enterocolitica*, extensive contact between the macrophages and *Y. pestis* was required in order to observe measurable, YopJ-dependent cell death (Fig. 4A). Nevertheless, even under these favorable conditions, the cytotoxicity induced by *Y. pestis* was less than that induced by *Y. enterocolitica* in RAW264.7 cells, as well as in bone marrow-derived murine macrophages (Fig. 4A). As expected, *Y. pestis*-induced cell death due to increased interaction with cells was also reflected

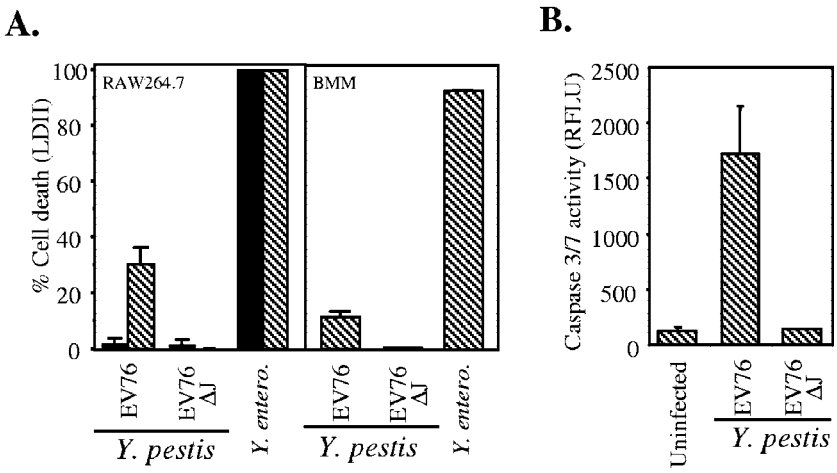

FIG. 4. Enhancing the cytotoxic effect of *Y. pestis* EV76 by forcing bacteria onto cells. (A) RAW264.7 cells were infected with *Y. enterocolitica*, *Y. pestis*, and *Y. pestis* with a knockout in *yopJ* (Δ*yopJ* [ΔJ]), using two different infection techniques, forcing bacterial contact by centrifugation (impact procedure) (striped bars) and overlay of a bacterial suspension (solid bars), and examined for LDH release 6 h postinfection; bone marrow macrophages (BMM) were infected only by the impact procedure and were evaluated for LDH release 5 h postinfection. (B) Caspase 3/caspase 7 activity was determined 3 h postinfection by the impact procedure. RFLU, relative fluorescence units; *Y. entero*., *Y. enterocolitica*.

in more effective induction of caspase 3/caspase 7 activation (compare Fig. 3 and 4B).

The consistent inequality in the abilities of *Y. pestis* and *Y. enterocolitica* O:8 to induce apoptosis led us to compare the levels of expression of *Y. enterocolitica* YopP to the levels of expression of the homologue YopJ in *Y. pestis* and the translocation of these molecules into macrophage cells. We found that the two bacteria expressed comparable amounts of the YopJ/YopP effectors 3 h after a temperature shift to 37°C (Fig. 5A). In contrast, the amount of translocated YopP in RAW264.7 cells was much larger than the amount of YopJ. This was most apparent when infection was carried out by the less stringent overlay method (Fig. 5B). Furthermore, even with extensive cell contact, a marked difference between the strains was observed, as shown by differences in the rate of translocation, following "synchronized" initiation of infection (Fig. 5C). In the case of *Y. enterocolitica* infection, YopP could be detected within 15 min after the initiation of infection, and the level reached a plateau within 45 to 60 min. However, in *Y. pestis*-infected cells, only trace levels of YopJ were observed after 30 min, and after 90 min the levels were comparable to those observed for YopP translocated by *Y. enterocolitica* at 15 min after infection. Even though differences in the stabilities of the effectors within the cells cannot be ruled out, the kinetic profile of intracellular effector accumulation and the lack of detectable degradation products as determined by Western blotting suggest that this is not a major contributor to the observed difference in the accumulation of YopJ and YopP in the same target cells.

Together, these observations suggest that the fact that *Y. pestis* is less able to trigger host cell death than *Y. enterocolitica* is a consequence of the reduced efficiency of YopJ translocation into macrophage cells. This could result from differences in the effectiveness of the surface interactions between macrophages and the two *Yersinia* strains or from intrinsic differences in the translocation of YopJ and YopP. Enumeration of cell-associated bacteria (by viable counting following 1 h of interaction and extensive washes) revealed that the number of associated *Y. enterocolitica* cells was higher than the number of *Y. pestis* cells by a factor of less than 3, independent of the mode of infection (overlay or impact). Such a difference does not

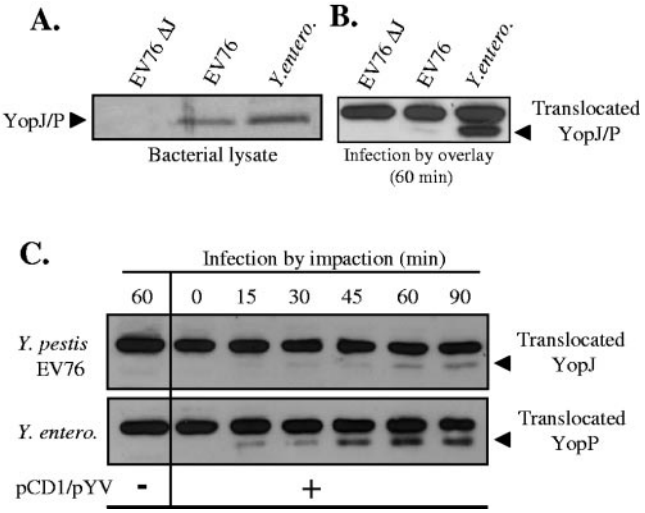

FIG. 5. Levels of expression of YopJ/YopP in *Y. pestis* EV76 and *Y. enterocolitica* O:8 and amounts of effector translocated into infected macrophages. (A) For evaluation of bacterial YopJ/YopP expression, *Yersinia* strains were grown for 3 h at 37°C. Then 3×10^7 bacteria were subjected to SDS-PAGE and developed with polyclonal rabbit anti-peptide YopP/YopJ antibodies as described in Materials and Methods. (B and C) For evaluation of effector translocation following infection by the overlay procedure (B) or the impact procedure (C), infection was arrested at the times indicated, and cytosolic fractions of infected RAW264.7 cells were obtained by lysis with 0.1% Triton X-100. Aliquots equivalent to 1×10^6 cells (normalized by protein measurement) were subjected to SDS-PAGE. Gels were blotted and developed with rabbit polyclonal anti-peptide YopP/YopJ antibodies. Bands were visualized by ECL. Note that the blot in panel B is overexposed compared to the blot in panel C. *Y. entero*., *Y. enterocolitica*.

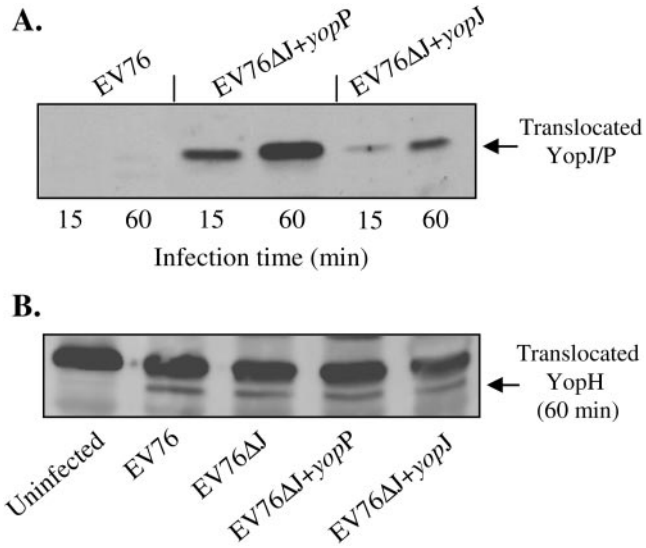

FIG. 6. Overexpression of YopJ and YopP in *Y. pestis* background: effect on translocation. (A) Translocation of effectors YopJ and YopP was evaluated by infecting RAW264.7 cells by the impact procedure and monitoring for the presence of the effectors in cell lysates by SDS-PAGE at different times after infection. Blots were developed as described in the legend to Fig. 5, except that the anti-peptide YopP/YopJ antibodies were purified by antigen affinity chromatography to reduce the nonspecific background. (B) In parallel, translocation of YopH was examined 60 min postinfection. YopH was visualized as described in Materials and Methods.

appear to fully account for the marked difference observed in YopJ/YopP translocation (Fig. 5B), suggesting that additional mechanisms are involved in determining the limited apoptotic potential of *Y. pestis*. To further address this issue, YopJ derived from *Y. pestis* and YopP derived from *Y. enterocolitica* were expressed in the same genetic background of a *Y. pestis* strain.

YopJ is less adapted to translocation than YopP. To evaluate the contribution of the effector dose, as well as the nature of the effector, to *Yersinia*-induced cytotoxicity, a *yopJ*-deleted *Y. pestis* strain (EV76Δ*yopJ*) was engineered to express high levels of either YopJ or YopP. To do this, the corresponding genes from the virulent *Y. enterocolitica* WA O:8 and *Y. pestis* Kimberley53 strains were cloned by PCR. The deduced amino acid sequences were found to be identical to those published previously for the *Y. enterocolitica* A127/90 O:8 and *Y. pestis* CO92 strains, respectively. Each of the two YopJ/YopP coding sequences (which differ by 17 of 288 amino acids) was inserted downstream of the *lac* promoter. The YopJ and YopP expression plasmids were introduced into the *Y. pestis* EV76Δ*yopJ* strain, yielding two new strains, EV76Δ*yopJ*+*yopP* and EV76Δ*yopJ*+*yopJ*, which express the two effectors at comparable high levels (more than 1 order of magnitude higher than the endogenous YopJ levels in *Y. pestis*).

The efficiencies of YopP/YopJ translocation following infection with the EV76Δ*yopJ*+*yopJ* and EV76Δ*yopJ*+*yopP* strains were determined and compared to the efficiency of YopJ translocation by the nonmanipulated *Y. pestis* EV76 strain. Figure 6A shows that overexpression of both effectors by the bacterium led to more effective translocation of the effectors into infected cells. Nevertheless, the rate of accumulation of YopP in the cells was clearly higher than the rate of accumulation of YopJ; at 15 min, as well as 60 min, after the initiation of infection, the YopP levels in the target cells were substantially higher than the levels of YopJ. These results are not a reflection of differences in cell interactions, since all the *Y. pestis* derivatives associated with RAW264.7 cells at comparable efficiencies (data not shown). Moreover, the two complemented strains, EV76Δ*yopJ*+*yopJ* and EV76Δ*yopJ*+*yopP*, as well as the nonmanipulated EV76 strain, injected equal amounts of the YopH effector (Fig. 6B), suggesting that overexpression of YopJ or YopP does not differentially affect the TTSS machinery. It thus appears that there are intrinsic differences in the abilities of YopJ and YopP to translocate into macrophages following interactions with *Yersinia*.

These results imply that the extent of *Yersinia*-induced apoptosis is controlled by the rate of YopJ/YopP accumulation. To further study the quantitative aspects of induction of cell death, we infected RAW264.7 cells with different doses of bacteria expressing variable amounts of either YopJ or YopP under conditions which are assumed to be more favorable (impact procedure) or less favorable (overlay procedure) for effector translocation. When cells were infected by the impact procedure, the levels of cytotoxicity (6 h postinfection) conferred by *Y. enterocolitica* O:8 were ~60% at an MOI as low as 10 and more than 90% at an MOI of 25 (Fig. 7A). In contrast, in *Y. pestis*-infected cells, no cytotoxicity was observed at low MOIs, while an MOI of 100 was required to obtain 30% cytotoxicity. It should be noted that the greater cytotoxicity of *Y. enterocolitica* was manifested not only by the extent of cell killing but also by rapid onset of the effect (3 h after infection no effect was observed in *Y. pestis*-infected macrophages, while complete lysis was observed with *Y. enterocolitica* at an MOI of 50 [data not shown]).

Overexpression of YopJ in *Y. pestis* (EV76Δ*yopJ*+*yopJ*) significantly increased *Y. pestis*-mediated cytotoxicity. In this case, ~20% cell death was observed at an MOI of 25, and 80% cell death was observed at an MOI of 50. A more pronounced increase in cytotoxicity was observed, however, when the heterologous effector YopP was overexpressed in the *Y. pestis* background (EV76Δ*yopJ*+*yopP*). In this case, 100% cytotoxicity was observed at an MOI as low as 10, a bacterial load that was 10 times lower than that required to obtain full cell destruction with the isogenic EV76Δ*yopJ*+*yopJ* strain (Fig. 7A). The difference between the bacteria overexpressing YopP and the bacteria overexpressing YopJ was even more striking when infection was performed using the overlay procedure, which could be more relevant for simulation of in vivo conditions (Fig. 7B). Again, only 50% cell killing was observed at an MOI of 100 with EV76Δ*yopJ*+*yopJ*, while nearly 100% cell death was induced at a 10-fold-lower MOI (MOI of 10) with EV76Δ*yopJ*+*yopP*. As noted above, under these conditions native *Y. pestis* failed to trigger cell death, while native *Y. enterocolitica* O:8 was very effective (Fig. 7B).

In conclusion, these results indicate that similar levels of expression of the two orthologous effectors (YopJ and YopP) in a *Y. pestis* background lead to a pronounced difference in induction of cell death upon infection of macrophages. Thus, YopP expressed either in a *Y. enterocolitica* O:8 background or in a *Y. pestis* background appears to be more amenable than

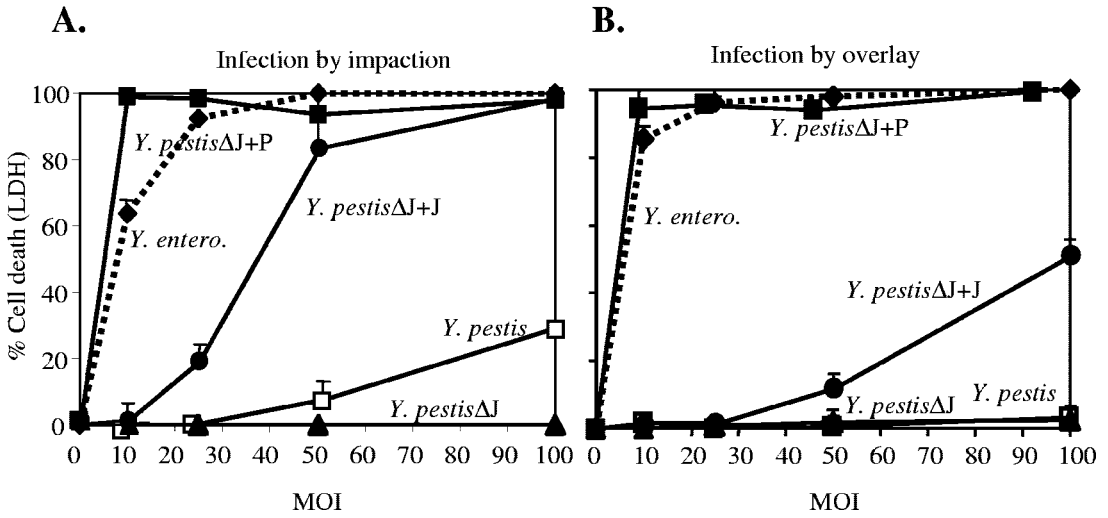

FIG. 7. Overexpression of YopJ and YopP in *Y. pestis* background: effects on cytotoxicity to macrophages. Cytotoxicity was evaluated by monitoring LDH release 6 h following infection of RAW264.7 cells by the impact procedure (A) and by the overlay procedure (B) with different amounts of bacterial strains (MOIs, 10 to 100). □, infection by *Y. pestis* (EV76); ▲, infection by *Y. pestis* Δ*yopJ* (*Y. pestis*ΔJ); ●, infection by *Y. pestis* Δ*yopJ*+*yopJ* (*Y. pestis*ΔJ+J); ■, infection by *Y. pestis* Δ*yopJ*+*yopP* (*Y. pestis*ΔJ+P); ◆ and dashed line, infection by *Y. enterocolitica* (*Y. entero*.).

YopJ for translocation from the bacterium into the host cell, and it can readily reach cellular concentrations which are sufficient to trigger cell death.

***Y. pestis* effectively suppresses proinflammatory functions of infected cells.** The marked limitation of *Y. pestis* for inducing the YopJ-dependent apoptotic response led us to examine the ability of this organism to counteract TNF-α production, yet another adverse function attributed to YopJ/YopP in *Yersinia* species (3, 35, 42, 58). Infection of RAW264.7 cells by EV76, EV76Δp70, and EV76ΔJ (Fig. 8A) revealed that *Y. pestis* can suppress secretion of TNF-α in a YopJ-dependent manner and thus resembles *Y. enterocolitica* WA O:8 in this respect (Fig. 8A).

p38, Jun N-terminal protein kinase, and extracellular signal-regulated kinase 1/2 MAPK pathways are important regulators of TNF-α production in response to bacterial infection (41). Therefore, we examined the kinetics of activation and suppression of these kinases upon infection with *Y. pestis* EV76 using Western blot immunostaining with antibodies against the phosphorylated forms of the MAPKs. The results for p38 are shown

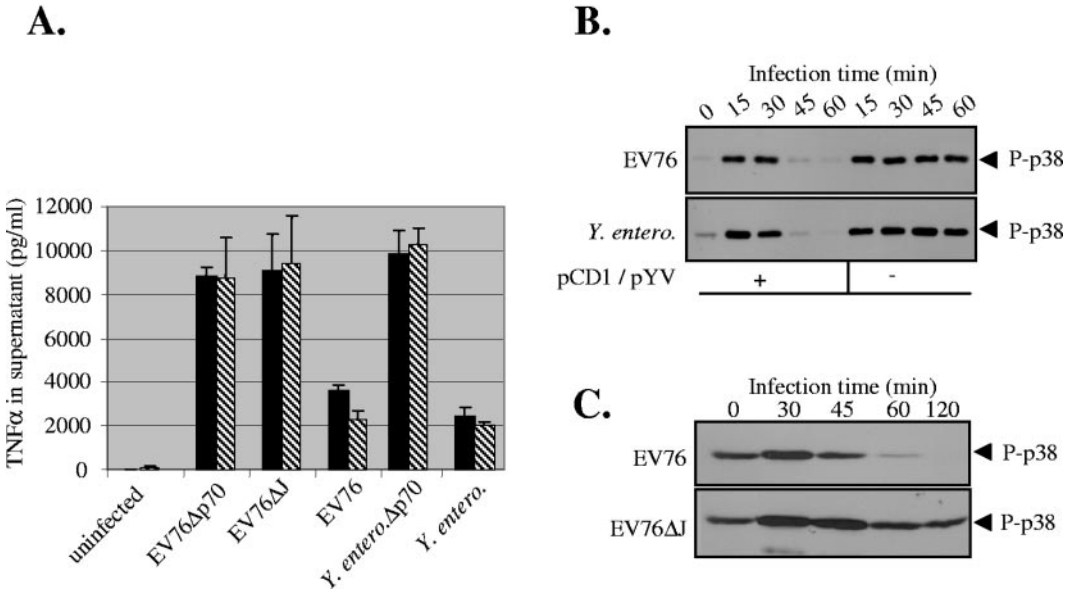

FIG. 8. Suppression of TNF-α secretion and p38 phosphorylation in *Yersinia*-infected macrophages. (A) To assay TNF-α secretion, J774A.1 cells were infected by the overlay procedure (solid bars) or by the impact procedure (striped bars) with different *Yersinia* strains. The secretion of TNF-α into cell medium was monitored 2 h after initiation of infection by an enzyme-linked immunosorbent assay. (B and C) Activation and suppression of p38 phosphorylation were examined in cells infected by the overlay procedure for different times. Macrophage lysates were analyzed by immunoblotting with phospho-specific p38 antibodies (P-p38). *Y. entero*., *Y. enterocolitica*.

in Fig. 8; similar results were obtained when Jun N-terminal protein kinase or extracellular signal-regulated kinase 1/2 phosphorylation was monitored (data not shown). Exposure of cells to *Y. pestis* for short periods of time (15 to 30 min) resulted in an increase in MAPK phosphorylation, independent of the presence of pCD1 (Fig. 8B). Prolonged incubation of *Y. pestis* with macrophages (for 45 min or longer) reduced p38 phosphorylation to basal levels and even lower levels in a *yopJ*-dependent manner (Fig. 8C). A side-by-side comparison of *Y. pestis* and *Y. enterocolitica* revealed similarities in the patterns and time courses of their effects on the p38 phosphorylation profile (Fig. 8B).

The fact that *Y. pestis* and *Y. enterocolitica* exhibit comparable YopJ/YopP-dependent suppressive effects on MAPK activation and TNF-α secretion is puzzling in view of the marked difference in effector translocation by these two organisms. This could be explained by the need for a low YopJ threshold concentration in the cell for induction of these suppressive effects. Indeed, effective suppression of TNF-α secretion by *Y. pestis* is observed even when infection is carried out by the overlay method (Fig. 8A), which is characterized by translocation of small amounts of YopJ (Fig. 5B).

Thus, examination of the YopJ-dependent functions of *Y. pestis* revealed a distinctive phenotype which readily allowed suppression of the proinflammatory responses but was much more demanding when it came to induction of cell death.

DISCUSSION

In comparative functional analyses of *Y. pestis* and enteropathogenic *Yersinia* workers previously focused on genes carried on the two unique plasmids of *Y. pestis*, pMT1 and pPCP1 (37). The recent sequencing of *Y. pestis* genomes (two strains virulent for humans and an avirulent strain), as well as of *Y. pseudotuberculosis* and *Y. enterocolitica* genomes, shifted attention toward the dynamic nature of the *Y. pestis* genome and the degeneration of many genes, as well as the acquisition of other genes (7, 40, 59). Nevertheless comparative functional studies of intact genes common to all pathogenic yersiniae, such as the genes for pCD1/pYV virulence plasmid-encoded Yop effectors, should not be overlooked.

In this study we attempted to characterize the YopJ-related functions of *Y. pestis* in infected macrophages and to compare these functions to those of the homologue effector YopP of the high-virulence *Y. enterocolitica* O:8 strain. The study was conducted with bacteria that were precultured at 28°C and then shifted to 37°C for 3 h to allow induction of expression of the various Yops. These experimental conditions were supposed to simulate early events of a fleabite-caused *Y. pestis* infection, in which a shift from the ambient temperature to body temperature occurs, and enough time was allowed for recruitment of macrophages to the site of infection.

However, simulation of the actual in vivo interaction between bacteria and macrophages could be more complicated. Issues such as the number of bacteria interacting with a single cell, the duration of the interaction, the nature of cell-bacterium contact, and the effect of the immediate environment should be considered, even though their relevance to in vivo infection is not straightforward. For instance, the high bacterial dose delivered during a fleabite (5) implies that a high multiplicity of infection should be used in in vitro studies. Nevertheless, the extremely low infective dose of *Y. pestis* in mouse models (4, 18) and the high infectivity via aerosols (10) argue for the use of low MOIs. In our studies we resorted to experimental protocols in which the MOIs ranged from 5 to 100 and the contact between the bacteria and the cells was either mild (overlay of bacterial suspensions on monolayers) or intense (bacterial impact due to centrifugation which led to a fourfold increase in the number of cell-associated bacteria). Some of these protocols were used previously to study *Y. pestis* pCD1-dependent cytotoxicity (22) and TTSS-dependent caspase induction (54).

Systematic comparison of macrophage infection under various experimental conditions allowed us to detect a substantial limitation in the YopJ-dependent apoptosis induced by *Y. pestis* compared to the YopP-dependent apoptosis induced by *Y. enterocolitica* O:8 (Fig. 1). In the case of *Y. enterocolitica* O:8, cytotoxicity was induced at low MOIs under all infection conditions tested, whereas partial cell death was observed with *Y. pestis* at elevated MOIs and with an efficacious cell-bacterium interaction. Moreover, compared to *Y. enterocolitica*, *Y. pestis* was less effective in suppressing NF-κB nuclear translocation and in inducing caspase 3/caspase 7 activation (Fig. 2 and 3), two well-documented pathways involved in *Yersinia*-induced apoptosis (41, 57). It is important to emphasize that we were able to demonstrate that the limitations in suppression of NF-κB activation and apoptosis induction can be identified not only in the attenuated *Y. pestis* EV76 strain but also in the fully virulent *Y. pestis* Kimberley53 strain.

Comparative analysis of YopJ and YopP translocation into macrophages by *Y. pestis* and *Y. enterocolitica* O:8 indicated that the observed species-specific differences in apoptosis induction could result from differences in effector accumulation within the infected cells. The YopJ of *Y. pestis* was translocated less efficiently into macrophages, as reflected by its retarded accumulation in host cells compared to the accumulation of *Y. enterocolitica* YopP (Fig. 5).

To distinguish the contribution of YopJ and YopP from other factors in determining the disparity between apoptosis induced by *Y. pestis* and apoptosis induced by *Y. enterocolitica*, we compared YopJ functionality and YopP functionality by overexpressing these effectors in the same *Y. pestis* genetic background. This revealed that YopP is indeed translocated more efficiently than YopJ. Actually, the rapid accumulation of YopP in cells infected by the recombinant *Y. pestis* strain resembled the accumulation observed for the parental wild-type *Y. enterocolitica* strain (Fig. 5C and 6A). The differences in the cytosolic levels of YopJ/YopP upon infection by *Y. pestis* and its engineered derivative strains appear to be correlated to the extent of the cytotoxic effects induced by the various strains (Fig. 7). All these observations suggest that at least part of the low cytotoxic potential of *Y. pestis* originates from the low level of compatibility of the YopJ effector with the translocation machinery. Actually, YopJ of *Y. pestis* appears to be less adapted for translocation by its parent injectisome than the heterologous effector YopP. Nevertheless, one cannot overlook the contribution of other factors to the limited apoptotic potential of *Y. pestis*. These factors could include differences in the architecture of the injection machinery (32), as well as differences in the extent of interaction of the various *Yersinia*

bacteria with the target cells resulting from known differences in their bacterial surface compositions (15, 17, 23, 37, 48). Indeed, we observed a certain advantage in the binding of *Y. enterocolitica* O:8 compared with the binding of of *Y. pestis* under the various infection conditions which we used (up to a threefold difference). However, results presented here, as well as in previous studies of the different *Y. enterocolitica* serotypes (13), suggest that the nature of the effector plays a major, perhaps overriding, role in determining the amount of YopJ/YopP translocated into the cell. The 17-amino-acid difference between the *Y. pestis* Kimberley53-derived YopJ and *Y. enterocolitica* WA O:8-derived YopP could allow functional differences governing translocation, stability, and catalytic activity. It should be noted, however, that amino acids previously implicated in YopJ/YopP function, like the protease-related catalytic triad His109-Glu128-Cys172 (12, 34, 58), are conserved in YopJ and YopP of the bacterial strains used here. Furthermore, the amino acid at position 143 in both YopJ of *Y. pestis* and YopP of *Y. enterocolitica* O:8 is arginine. In *Y. enterocolitica* O:9, this position is occupied by serine, which was shown to be responsible for the limited YopP functionality of this strain (44). It should be noted, however, that among the 17 amino acids by which the *Y. enterocolitica* WA O:8 strain differs from the sequenced *Y. pestis* strains (strains Kimberley53, CO92 [gi:16082759], KIM [gi:3822046], and 91001 [gi:45478515]), 5 also discriminate between *Y. enterocolitica* O:8 (10 strains) and *Y. enterocolitica* O:9 (9 strains) serotypes (13). Thus, Glu55, Ile62, Gly106, Thr144, and Ser189 are shared by *Y. pestis* and *Y. enterocolitica* O:9, both of which appear to translocate YopP/J rather poorly (Fig. 5) (13), while *Y. enterocolitica* O:8, which is an "effective translocator," has Lys, Thr, Asp Ala, and Asn, respectively, at these positions. Whether these five residues (three of which represent nonconservative changes) are indeed involved in regulating YopJ/YopP translocation efficiency remains to be resolved by site-directed mutagenesis.

Another observation made in this study is related to the differential effects of intracellular YopJ/YopP levels on the various adverse functions of this effector in macrophages. While, as discussed above, induction of apoptosis occurs when high intracellular YopJ/YopP threshold levels are reached, YopJ-dependent suppression of MAPK phosphorylation and TNF-α secretion appears to occur under less stringent conditions. The latter effects can be also observed when small amounts of YopJ are detected in the cells (infection with *Y. pestis* by the overlay procedure) (Fig. 8). This suggests that different thresholds of cytosolic effector concentrations are required for initiating the various YopJ-related cellular events. Interestingly, different thresholds were observed for *Y. pestis* TTSS-dependent induced phagocytosis and cytotoxicity (54).

Recent observations with *Y. pseudotuberculosis* (60) indicate that IκBα, (which sequesters NFκ-B in the cytoplasm and prevents its translocation into the nucleus) is phosphorylated, ubiquitinated, and degraded as early as 5 min after initiation of infection, which allows immediate translocation of NF-κB into the nucleus. Thus, effective concentrations of YopJ in the cell should be reached within a short time for exerting the deubiquitinase activity on IκB and sequestering NF-κB in the cytoplasm. It appears that this can be achieved upon infection with *Y. enterocolitica* O:8 but not upon infection with *Y. pestis* (Fig. 2 and 5B). On the other hand, suppression of activation of the cytoplasmic MAPK kinases appears to be a more prolonged process (33) and could be less sensitive to the slow accumulation of YopJ. Thus, it appears that the rates of accumulation of YopJ and YopP in *Yersinia*-infected macrophages are determinative for the differential modulation of the two adverse effects, induction of suppression of the proinflammatory response and induction of apoptosis.

The low efficiency of *Y. pestis* for conferring YopJ-mediated death upon macrophages (Fig. 3 and 4) may be a consequence of the evolution of the organism into a highly pathogenic species. The intradermal injection of *Y. pestis* bacteria through fleabites is believed to involve two seemingly contradictory processes. On the one hand, bacteria can be ingested by resident professional phagocytes, which allows replication in a shielded environment and eventually transport, as viable pathogens, to target organs (6, 8, 26). On the other hand, *Yersinia* can exert a variety of adverse effects (blocking phagocytosis, suppressing proinflammatory responses, and promoting apoptosis) on such phagocytic cells in order to downregulate activation of the innate immune response (4, 47, 52, 54, 57). The coexistence of these two processes could be facilitated by a variety of temperature-regulated processes, providing temporal separation between effective cellular uptake of newly injected bacteria and the host cell inactivation events. Yet, if productive *Y. pestis* infection is indeed dependent on uptake by macrophages, a very effective mechanism of cell killing at the site of invasion would be disadvantageous. Thus, the relative impairment of YopJ translocation into resident phagocytic cells, which on the one hand allows downregulation of the proinflammatory pathways but on the other hand does not allow induction of cell death, may contribute to the extremely low number of *Y. pestis* bacteria needed to cause a fatal infection (4, 18). One could therefore envision that the evolution of *Y. pestis* included partial incapacitation of the YopJ translocation potential which contributes to the relatively inefficient macrophage killing. This could be an additional example of the presumed reductive evolution (7, 59) of this pathogen.

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