Yersinia enterocolitica Induces Apoptosis and Inhibits Surface Molecule Expression and Cytokine Production in Murine Dendritic Cells

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Yersinia enterocolitica **evades innate immunity by expression of a variety of pathogenicity factors. Therefore, adaptive immunity including CD4$^+$ T cells plays an important role in defense against *Y. enterocolitica*. We investigated whether *Y. enterocolitica* might target dendritic cells (DC) involved in adaptive T-cell responses. For this purpose, murine DC were infected with *Y. enterocolitica* wild-type and mutant strains prior to incubation with ovalbumin (OVA) as antigen and 5-(6)-carboxyfluorescein diacetate *N*-succinimidyl ester-labeled OVA-specific T cells from DO11.10 mice. While T-cell proliferation was partially affected by infection of DC with plasmid-cured and YopP-deficient *Yersinia* mutant strains, no T-cell proliferation occurred after infection of DC with wild-type *Y. enterocolitica*. Infection of DC with *Y. enterocolitica* wild type resulted in decreased up-regulation of major histocompatibility complex class II, CD54 (intercellular adhesion molecule 1), CD 80, and CD86 expression. Experiments with plasmid-cured *Y. enterocolitica* or a YopP-deficient mutant strain revealed that YopP accounts for inhibition of surface molecule expression. Wild-type *Y. enterocolitica* suppressed the release of KC, tumor necrosis factor alpha, interleukin-10 (IL-10), and IL-12 by DC, while infection of DC with plasmid-cured *Y. enterocolitica* or with the YopP-deficient mutant resulted in the production of these cytokines. Moreover, infection with wild-type *Y. enterocolitica* induced apoptosis in DC mediated by YopP. Apoptosis occurred despite translocation of NF-κB to the nucleus, as demonstrated by electromobility shift assays. Together, these data demonstrate that *Y. enterocolitica* targets functions of murine DC that are required for T-cell activation. This might contribute to evasion of adaptive immune responses by *Y. enterocolitica*.**

Yersinia enterocolitica is a gram-negative bacterial pathogen in humans and rodents which causes diarrhea and systemic infections, including abscesses in the spleen and liver, as well as immunopathological sequelae, such as reactive arthritis. The action of several pathogenicity factors of *Y. enterocolitica*, encoded by the chromosome and the pYV plasmid, are required. The pYV plasmid, including the *yop* virulon, encodes a type III secretion system by which *Yersinia* outer proteins (Yops) are secreted and translocated into the cytosol of host cells. YopB and YopD are part of a needle by which effector Yops (YopE, YopH, YopM, YopO, YopP, and YopT) are injected into the cytoplasm of mammalian target cells (9). YopE, YopT, and YopO cause disruption of the actin microfilament structures, and YopH dephosphorylates host cell proteins, such as focal adhesion kinase, paxillin, and p130Cas (5, 22). By this means, *Y. enterocolitica* prevents uptake into host cells. In fact, electron microscopy studies demonstrated that *Y. enterocolitica* is predominantly extracellularly located in host tissues (16). YopP inhibits both NF-κB signaling pathways by targeting IκB kinase-β and mitogen-activated protein kinase cascades by interacting with various members of the mitogen-activated protein kinase kinase superfamily, resulting in inhibition of cytokine production and apoptosis in macrophages (20, 26).

There is substantial evidence that elimination of *Y. enterocolitica* from infected tissue is accomplished by T-cell-activated macrophages. Therefore, T-cell responses, in particular those of CD4$^+$ T cells, as well as the production of gamma interferon (IFN-γ), are essential to limit yersiniosis (1, 6, 7). In fact, T-cell-deficient nude mice or mice deficient in interleukin-12 (IL-12), IL-18, TNFRp55, or IFN-γ receptor are highly susceptible to *Y. enterocolitica* and cannot eliminate the pathogen from infected tissues. Consequently, immunity against a lethal *Y. enterocolitica* infection can be adoptively transferred by *Yersinia*-specific T-cell clones into naïve mice.

T cells need to be stimulated by antigen-presenting cells (APC) in order to become activated. Dendritic cells (DC) are the most important APC, as indicated by observations that DC are able to stimulate T cells even in the presence of minute quantities of antigen and by their capacity to activate naïve T cells (12).

Previous studies demonstrated that *Y. enterocolitica* might also target DC (27). Those authors demonstrated that although infection of human DC with *Y. enterocolitica* did not result in DC death, a transient down-regulation of major histocompatibility complex (MHC) class II and CD80 molecules was observed and accompanied by a decreased T-cell activation capacity as indicated by mixed lymphoid reaction analyses (20). A detailed analysis of these events and the effect of *Y. enterocolitica* on murine DC, however, has not yet been examined. In addition to DC, macrophages may also serve as APC. The effect of *Yersinia* infection on murine macrophages was

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analyzed, and induction of apoptosis was found to be one of the most important features of impairment of macrophage function by *Y. enterocolitica* (10, 25).

Therefore, we analyzed whether *Y. enterocolitica* O:8 impedes the CD4 T-cell activation capacity of bone marrow-derived murine DC by means of 5-(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE)-labeled ovalbumin (OVA)-specific T cells from DO11.10 mice. Various apoptosis assays were performed to examine whether induction of host cell death might be involved in decreased T-cell activation. Furthermore, expression patterns of molecules contributing to immunological synapse formation (MHC class II, CD80, CD86, and intercellular adhesion molecule 1 [ICAM-1]) and also secretion of cytokines (IL-12, IL-10, KC, and tumor necrosis factor alpha [TNF-α]) were determined.

MATERIALS AND METHODS

Mice. BALB/c mice were purchased from Harlan Winkelmann (Borchen, Germany). DO11.10 mice (19) were maintained under specific-pathogen-free conditions. The majority (>95%) of T cells in the DO11.10 mice are CD4$^+$ T cells which bear a T-cell receptor that recognizes an OVA-derived peptide, OVA$_{323-339}$, which is presented by I-A^d. The experiments were performed with 6- to 12-week-old female mice.

Bacterial strains and growth conditions. The following *Y. enterocolitica* strains were used in this study: *Y. enterocolitica* WA-314 of serotype O:8 wild type carrying the virulence plasmid pYV (designated as WA-pYV$^+$), a YopP-deficient mutant (WA-pYV$^+$YopP$^-$; kindly provided by K. Ruckdeschel, Munich, Germany), and a plasmid-cured *Y. enterocolitica* strain (WA-pYV$^-$). All strains of *Y. enterocolitica* were grown overnight in Luria-Bertani broth at 27°C supplemented with chloramphenicol (20 μg/ml), nalidixic acid (10 μg/ml; ICN Biomedicals, Irvine, Calif.), spectinomycin (100 μg/ml), or kanamycin (25 μg/ml; Sigma, Deisenhofen, Germany). A 1:20 dilution of the overnight *Yersinia* culture was incubated for an additional 1.5 h at 37°C. The bacteria were washed once in phosphate-buffered saline (PBS; Invitrogen, Karlsruhe, Germany), and the optical density at 600 nm was determined. Heat-killed WA-pYV$^+$ cells were generated by heating the bacteria at 60°C for 4 h. The bacteria were added at a multiplicity of infection (MOI) of 10 to the bone marrow-derived DC.

Cell cultures and infection. DC and T cells were grown in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine (Invitrogen), 100 U of penicillin/ml, 100 μg of streptomycin (Biochrom)/ml, 50 μM β-mercaptoethanol (Sigma), 1% (vol/vol) nonessential amino acids (Biochrom), and 1 mM sodium pyruvate (Biochrom). DC were prepared according to the protocol of Lutz et al. (17). Briefly, 2 × 10^6 bone marrow cells, flushed from the femurs and tibias of BALB/c mice, were seeded in 100-mm dishes in 10 ml of medium containing 200 U of granulocyte-macrophage colony-stimulating factor (GM-CSF; produced by mouse myeloma strain P3X63)/ml. After 3 days, an additional 10 ml of fresh medium containing 200 U of GM-CSF/ml was added to the cultures. After 6 and 8 days, half of the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in 10 ml of fresh medium containing GM-CSF and given back to the original plate. At days 8 and 9, the slightly attached cells were used for the experiments described herein. More than 90% of these cultures expressed CD11c as assessed by flow cytometric analysis, and characteristic clustering of DC was observed by light microscopy.

For infection of DC, 4 × 10^5 cells (apoptosis assays), 10^6 cells (proliferation assays and enzyme-linked immunosorbent assay [ELISA]), or 5 × 10^6 cells (adhesion molecule expression analyses, electrophoretic mobility shift assay, and Western blotting) were seeded in 24- or 6-well plates in medium without antibiotics and infected with an MOI of 10 or stimulated with 5 μg of lipopolysaccharide (LPS; from *Salmonella enterica* serovar Typhimurium; Sigma)/ml or 1 μM staurosporine (Sigma). The bacteria were sedimented onto the cells at 400 × *g* for 5 min. After 1 h of infection, 0.1 mg of gentamicin (Sigma)/ml was added to kill the extracellular bacteria. Where indicated, the cells were cultured further for various experiments.

Flow cytometry analysis of adhesion molecules. To examine the regulation of adhesion molecules of *Yersinia*-infected DC, 5 × 10^6 cells were infected as described above. At 3 h postinfection, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-I-A/I-E (2G9), anti-CD80 (16-10A1), anti-CD86 (GL1), or anti-CD54 (3E2) (BD PharMingen, Heidelberg, Germany)

antibodies and propidium iodide (Calbiochem, La Jolla, Calif.). Samples were analyzed on a FACSCalibur flow cytometer (BD Immunocytometry Systems) with gating on the propidium iodide-negative cells. Data were analyzed with WINMDI version 2.3 software (J. Trotter, Scripps Institute, La Jolla, Calif.).

Determination of cytokine production by ELISA. To study the effects of different *Y. enterocolitica* strains on cytokine release, murine DC (10^6) were infected as described above and the supernatants were collected after 6 h for KC ELISA (Duo set; R&D Systems, Wiesbaden, Germany) and after 24 h for IL-10, IL-12 (p70), and TNF-α ELISA (OptEIA ELISA set; BD PharMingen). Cytokine levels were measured according to the manufacturers' instructions.

Assessment of apoptosis. (i) Assessment of mitochondrial transmembrane potential. DC (4 × 10^5) were infected with different *Yersinia* strains as described above. The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analyzed using the $\Delta\Psi_m$-specific stain TMRE (Molecular Probes, Eugene, Oreg.). Briefly, TMRE (40 nM) was added at 3.75 h postinfection and incubated for a further 15 min at 37°C. Thereafter, the cells were additionally stained with propidium iodide (40 ng/ml) and analyzed by flow cytometry.

(ii) Assessment of DNA fragmentation: Nicoletti assay. At 24 h after infection, the cells were transferred into fluorescence-activated cell sorter tubes and centrifuged at 400 × *g* for 5 min. The pellet was resuspended in 400 μl of Nicoletti reagent (50 μg of propidium iodide/ml, 0.1% trisodium citrate-2-hydrate, and 0.1% Triton X-100 dissolved in H$_2$O), incubated on ice for 10 min, and analyzed by flow cytometry.

(iii) TUNEL assay. Twenty-four hours after infection, cells were fixed and processed to detect fragmented genomic DNA in a terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assay (Roche, Mannheim, Germany) according to the manufacturer's instructions. Cells were analyzed by flow cytometry.

(iv) Assessment of phosphatidylserine residue translocation. Translocation of phosphatidylserine residues from the inner to the outer leaflet of the cell membrane and the loss of cell membrane integrity were measured at 4 h postinfection by incubating the cells with Annexin-V–fluorescein and propidium iodide according to the manufacturer's instructions (Annexin-V-FLUOS staining kit; Roche). Cells were analyzed by flow cytometry.

Transmission electron microscopy. Cell pellets were fixed for 24 h in 4% glutaraldehyde in a 0.05 M phosphate-buffered solution containing 0.15 M NaCl at pH 7.3 at room temperature. Postfixation was based on 1% osmium tetroxide containing 1% potassium dichromate in 0.85% NaCl at pH 7.3 for 45 min. After embedding in glycide ether, the blocks containing cells were cut using an ultramicrotome (Ultracut; Reichert, Vienna, Austria). Ultra-thin sections (80 nm) were stained with 0.5% uranyl acetate for 10 min at 30°C and with 2.7% lead citrate for 5 min (Ultrastainer; LKB, Uppsala, Sweden) at 20°C. Grids were examined using a Zeiss EM 902 transmission electron microscope (Zeiss, Oberkochen, Germany) operating at 80 kV and at magnifications between ×2,000 and ×50,000.

Purification of DO11.10 CD4$^+$ T cells. CD4$^+$ T cells were purified from spleens of DO11.10 mice by preparing a single-cell suspension, followed by lysis of red blood cells (80 mM NH$_4$Cl, 5 mM KHCO$_3$, 5 mM Na$_2$EDTA) and passage through a nylon wool column. Using the CD4-negative T-cell isolation kit and a magnetic-activated cell sorter system (Miltenyi Biotech, Bergisch-Gladbach, Germany), MHC class II$^+$, B220$^+$, and CD8$^+$ cells were depleted. The resulting T-cell preparations, containing 97 to 99% CD4$^+$ T cells, were used without further purification.

T-cell proliferation assay. At 1 h postinfection, 0.1 mg of gentamicin/ml and 0.1 mg of OVA (Sigma)/ml were added and the cells were incubated at 37°C for a further 2 h. Freshly isolated DO11.10 T cells were labeled with CFSE (Molecular Probes) as previously described (15, 18) with minor modifications. Briefly, 5 × 10^7 T cells/ml were washed twice with ice-cold PBS and incubated with CFSE (5 μM) in PBS for 3 min at 37°C. The cells were then washed twice with ice-cold heat-inactivated fetal calf serum, resuspended in cell culture medium, and used for the experiments. CFSE-labeled T cells (4 × 10^5) were coincubated with the OVA-loaded DC (10^5) in 48-well plates. Following 4 days of coincubation, the proliferation rate was analyzed by flow cytometry with excitation at 488 nm and the FL1 detection channel.

Electrophoretic mobility shift assay. DC (5 × 10^6) were infected in six-well plates as described above. At various intervals postinfection, nuclear extracts were prepared as previously described (28). Aliquots of the supernatants containing nuclear proteins were stored at −80°C. Protein concentrations were determined using the Bradford assay. Oligonucleotide probes were labeled with [γ-^{32}P]ATP (Amersham Biosciences, Freiburg, Germany) using T4 polynucleotide kinase (New England Biolabs, Frankfurt, Germany) and purified on a NucTrap probe purification column (Stratagene, Amsterdam, The Netherlands). The following oligonucleotides were used: NF-κB wild type (NF-κBc; 5′-ATGT
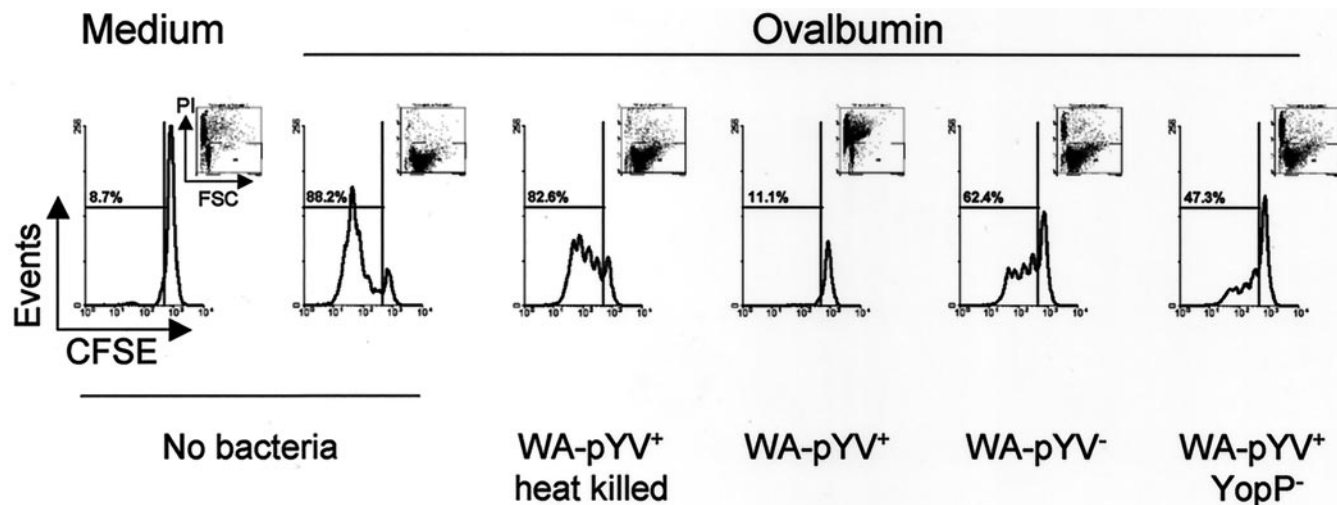
FIG. 1. Proliferation of T cells after coculture with DC infected with *Y. enterocolitica*. DC were incubated with various strains of *Y. enterocolitica*, wild type (WA-pYV$^+$), plasmid cured (WA-pYV$^-$), YopP deficient (pYV$^+$ YopP$^-$), and heat-killed wild type (WA-pYV$^+$ heat killed), at an MOI of 10. After 1 h, gentamicin (100 μg/ml) and OVA (100 μg/ml) were added. DC were cocultured with CFSE-labeled OVA-specific T-cell receptor transgenic T cells from DO11.10 mice at a ratio of 4 (one DC to four T cells). T-cell proliferation was analyzed 4 days later by flow cytometry by gating on propidium iodide-negative cells (inserts). Results are representative of seven experiments.

GAGGGGACTTTCCCAGGC-3′), NF-κB mutant (NF-κBm; 5′-ATGTGAGG cGACTTTCCCAGGC-3′ [the lowercase letter represents the mutation]; Santa Cruz, Heidelberg, Germany). Nuclear extracts (3 to 6 μg) were incubated with 30,000 cpm of the ^{32}P-labeled oligonucleotide probe for 45 min on ice in a buffer containing 5% glycerol, 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 7.2), and 1 μg of dI-dC. Antibodies against p50 (sc-1190X) and p65 (sc-372X) (both from Santa Cruz) were included in the binding reaction mixture for supershift analysis. Samples were resolved on a 5% nondenaturing polyacrylamide gel using 0.5× TBE (25 mM Tris-HCl, 25 mM boric acid, 0.5 mM EDTA) as running buffer. Gels were transferred to Whatman 3M paper (Schleicher & Schüll, München, Germany) and dried under vacuum. Protein binding was assessed by autoradiography.

Statistics. Data were analyzed by the unpaired two-tailed Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

T-cell activation after infection of DC with *Y. enterocolitica*. T-cell responses are required for elimination of *Y. enterocolitica* from infected tissues (2, 3). T-cell proliferation assays were performed to analyze whether *Yersinia* infection alters the T-cell activation capacity of DC. For this purpose, DC were infected with *Y. enterocolitica* wild-type and mutant strains. Then, OVA was added as an antigen as well as OVA-specific CFSE-labeled T cells from DO11.10 mice. After 4 days of incubation, cultures were analyzed by flow cytometry to assess T-cell proliferation.

Strong T-cell proliferation was observed in uninfected control cultures. OVA-induced T-cell proliferation was completely inhibited after infection of DC with wild-type *Yersinia* (WA-pYV$^+$), while T-cell proliferation was observed when DC were infected with heat-killed wild-type *Yersinia* or with viable plasmid-cured *Y. enterocolitica* (Fig. 1). Lower T-cell proliferation was observed upon exposure of DC to a YopP-deficient *Yersinia* mutant strain compared with plasmid-cured *Y. enterocolitica*. Together, these data suggest that gene products encoded by the virulence plasmid affect DC functions required to promote T-cell proliferation.

***Yersinia* induces apoptosis in DC.** Previous studies demonstrated that *Y. enterocolitica* induces apoptosis in murine macrophages (10, 25) but not in human DC (27). To assess whether *Y. enterocolitica* induces death of murine DC and whether this might be involved in the ability of DC to stimulate T cells, apoptosis assays were performed with *Yersinia*-infected murine DC. Wild-type *Y. enterocolitica* (WA-pYV$^+$) induced death of murine DC, as illustrated in Fig. 2. Four hours after *Y. enterocolitica* infection, DC were TMRE negative and propidium iodide positive, indicating the loss of mitochondrial potential and damage of the cellular membrane, respectively. In agreement with this finding, Annexin-V staining was also positive. Nuclear and DNA fragmentation were both obvious 24 h after infection as indicated by positive staining in Nicoletti and TUNEL assays (data not shown). Cellular fragmentation and nuclear condensation in apoptotic DC was confirmed by electron microscopy (Fig. 2). These data suggest that *Y. enterocolitica*-mediated cell death of murine DC is predominantly an apoptotic process.

In contrast to infection with viable wild-type *Y. enterocolitica* (WA-pYV$^+$), apoptosis was not induced by plasmid-cured (WA-pYV$^-$), YopP deficient (WA-pYV$^+$ YopP$^-$), or heat-killed wild-type (WA-pYV$^+$) *Yersinia* (Fig. 3). Therefore, YopP appears to be the most important factor involved in cell death caused by *Yersinia*.

Surface molecule expression in DC after infection with *Y. enterocolitica*. Maturation of DC is accompanied by changes in the expression of surface molecules. To examine whether *Y. enterocolitica* modulates maturation of DC, the expression of molecules which contribute to immunological synapse formation, including the expression of MHC class II and costimulatory molecules (ICAM-1, CD80, and CD86), was analyzed by flow cytometry.

Three hours after incubation with either LPS, *Y. enterocolitica* WA-pYV$^-$, or *Y. enterocolitica* WA-pYV$^+$ YopP$^-$, ex-
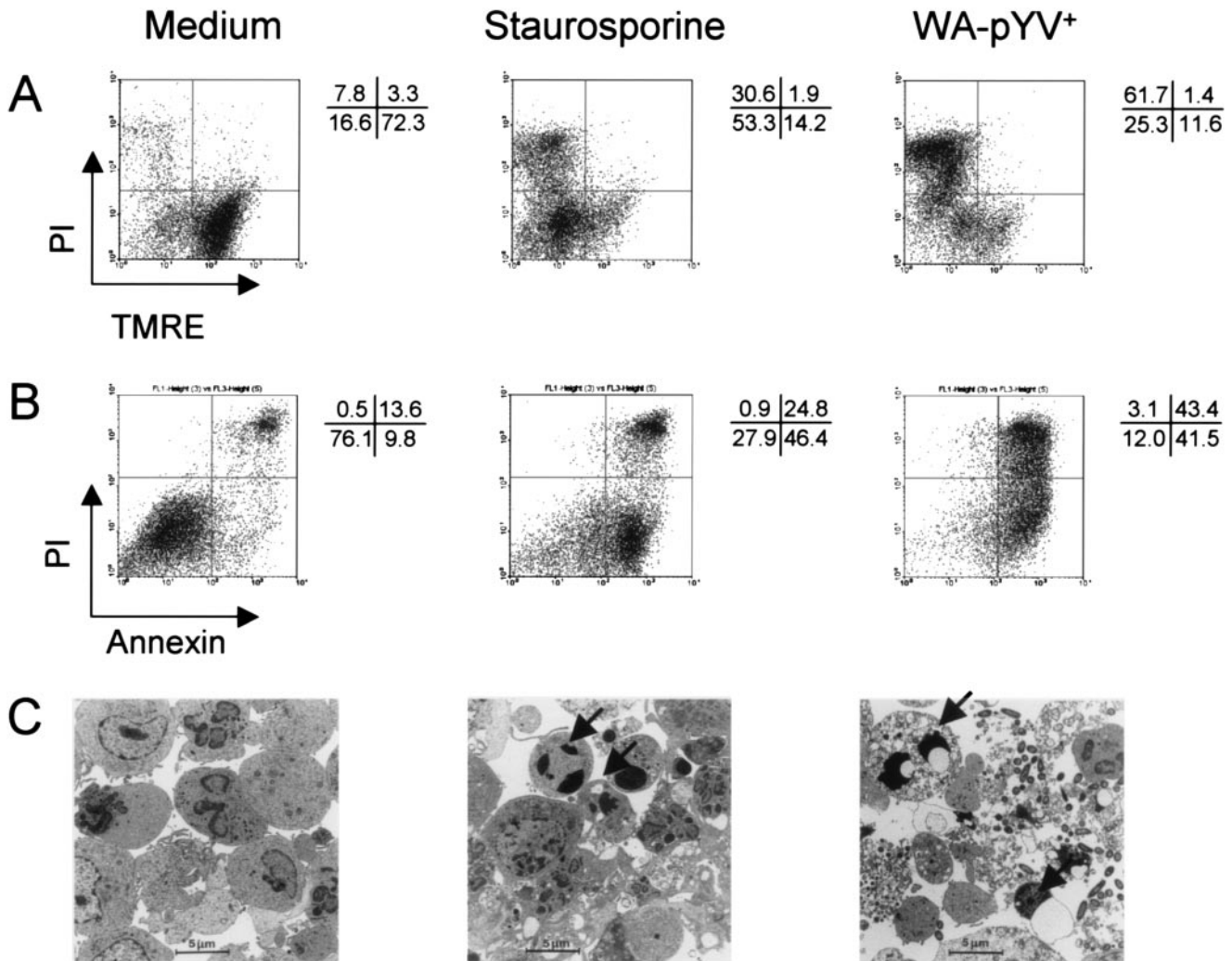
FIG. 2. Death of DC after infection with *Y. enterocolitica*. (A and B) DC were left untreated or incubated with staurosporine or wild-type *Y. enterocolitica* (WA-pYV$^+$; MOI, 10). Four hours postinfection, DC were stained with TMRE and propidium iodide (PI) (A) or with FITC-conjugated Annexin-V and PI (B). Numbers indicate percentages of cells in each quadrant. Results are representative of 16 (A) and 9 (B) experiments. (C) Electron microscopy analysis of DC incubated with medium demonstrated normal morphology of the cells, while treatment with staurosporine or infection with wild-type *Y. enterocolitica* (4 h) induced numerous apoptotic bodies containing cytoplasmic and chromatin remnants (arrows).

pression of MHC class II was markedly increased compared to that in untreated DC (Fig. 4). Increased MHC class II expression was also observed after infection with the *Y. enterocolitica* wild-type strain (WA-pYV$^+$), although expression was less than after infection with *Yersinia* mutant strains deficient for YopP (WA-pYV$^+$YopP$^-$). The costimulatory molecules CD86, CD54 (ICAM-1), and CD80 were strongly expressed after infection of DC with *Y. enterocolitica* WA-pYV$^-$ or *Y. enterocolitica* WA-pYV$^+$YopP$^-$, whereas no increase in expression was observed after infection with wild-type (WA-pYV$^+$) *Y. enterocolitica*. Together these results suggest that wild-type (WA-pYV$^+$) *Y. enterocolitica* inhibits maturation of DC and that YopP accounts for this effect.

Cytokine production after infection of DC with *Y. enterocolitica*. To analyze whether cytokine release by murine DC is altered after infection with *Y. enterocolitica*, DC were exposed

to *Y. enterocolitica* strains and mutants as described above and the cytokines IL-12, IL-10, KC, and TNF-α were determined in culture supernatants by ELISA. Infection with wild-type (WA-pYV$^+$) *Yersinia* did not result in IL-12 secretion. In contrast, IL-12 secretion was induced by LPS and by plasmid-cured (WA-pYV$^-$) or YopP-deficient (WA-pYV$^+$ YopP$^-$) *Y. enterocolitica* mutants (Fig. 5). While maximum IL-12 concentrations were observed after treatment of DC with LPS, the highest concentrations of IL-10 were detected after infection with plasmid-cured *Yersinia*, and these were significantly higher compared to incubation with LPS or heat-killed *Yersinia* (Fig. 5).

IL-8 is known to attract neutrophil granulocytes and also to contribute to T-lymphocyte attraction (31). We analyzed whether *Y. enterocolitica* may also affect the ability of murine DC to secrete KC, the murine equivalent of IL-8. As shown in Fig. 5, incubation of DC with LPS or *Yersinia* mutant strains
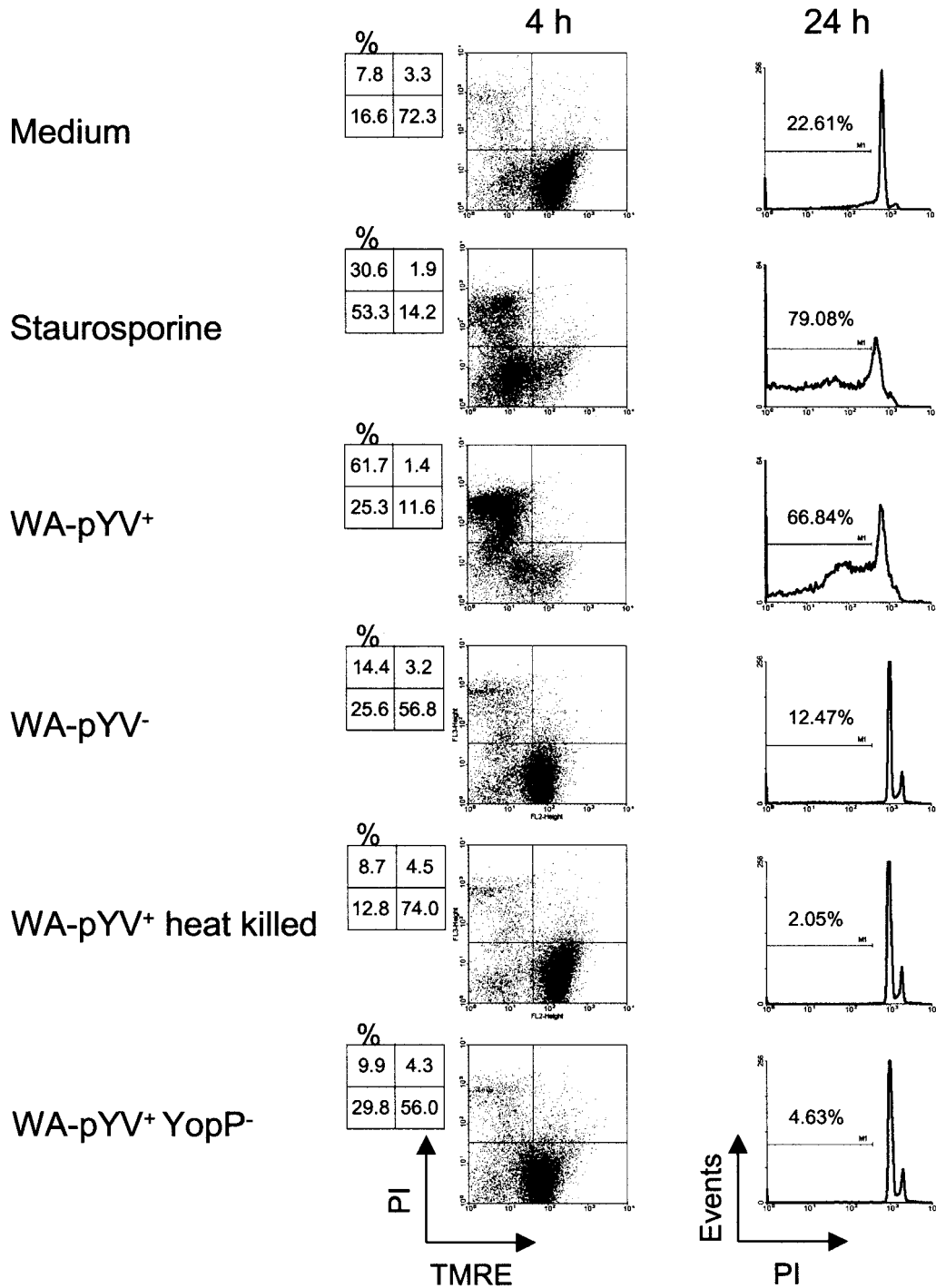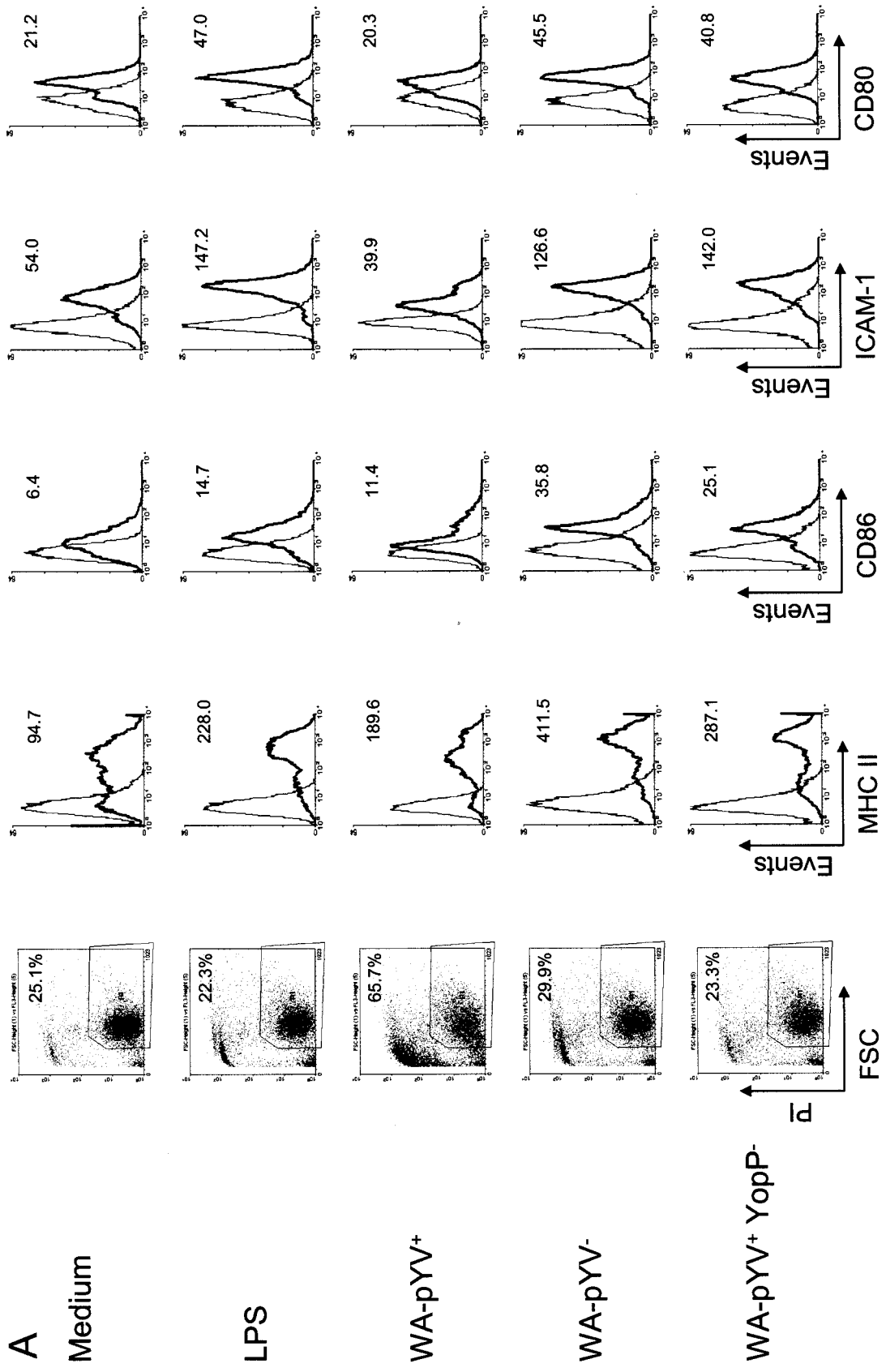
FIG. 3. Death of DC after infection with *Y. enterocolitica*. DC were left untreated or incubated with staurosporine or different strains of *Y. enterocolitica*: wild type (WA-pYV$^+$), plasmid cured (WA-pYV$^-$), YopP deficient (pYV$^+$ YopP$^-$), and heat-killed wild type (WA-pYV$^+$ heat killed). Four hours postinfection DC were stained with TMRE and propidium iodide (PI), and 24 h postinfection the Nicoletti procedure was performed. Results are representative of three experiments.

resulted in increased secretion of KC. In contrast, secretion of KC was not increased following infection with wild-type *Y. enterocolitica* (WA-pYV$^+$). Similar results were observed regarding the release of TNF-α after *Yersinia* infection. Interestingly, maximum TNF-α was observed in DC treated with the

YopP-deficient mutant strain (WA-pYV$^+$ YopP$^-$) (Fig. 5). These results suggest that *Y. enterocolitica* suppresses cytokine production by DC predominantly via the action of YopP, and the results are consistent with previously published data for macrophages and epithelial cells (8, 21, 26, 29).

A

Medium

LPS

WA-pYV+

WA-pYV-

WA-pYV+ YopP-

	CD80	ICAM-1	CD86	MHC II	PI / FSC
Medium	21.2	54.0	6.4	94.7	25.1%
LPS	47.0	147.2	14.7	228.0	22.3%
WA-pYV+	20.3	39.9	11.4	189.6	65.7%
WA-pYV-	45.5	126.6	35.8	411.5	29.9%
WA-pYV+ YopP-	40.8	142.0	25.1	287.1	23.3%

CD80 — Events

ICAM-1 — Events

CD86 — Events
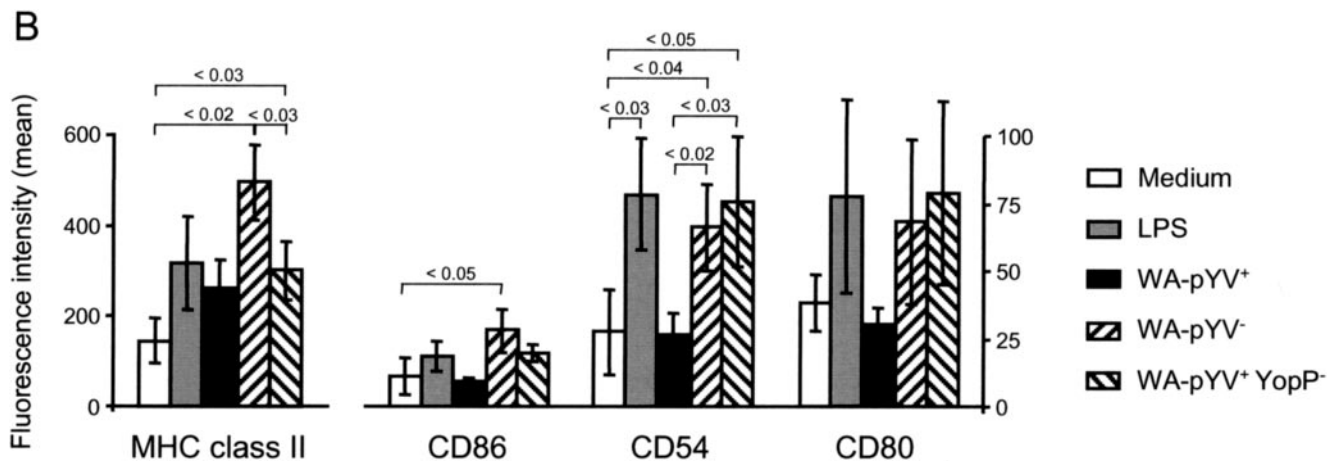
MHC II — Events

FSC — PI

FIG. 4. Expression of MHC class II and costimulatory molecules by DC after infection with *Y. enterocolitica*. (A) DC were incubated with various strains of *Y. enterocolitica:* wild type (WA-pYV$^+$), plasmid cured (WA-pYV$^-$), YopP deficient (pYV$^+$ YopP$^-$), and heat-killed wild type (WA-pYV$^+$ heat killed). Three hours after starting infection DC were stained (black lines) for MHC class II, CD86, CD54 (ICAM-1), and CD80 and analyzed by flow cytometry. By gating out propidium iodide-positive cells, 10,000 live cells were examined in each measurement. Gray lines, isotype-matched immunoglobulin G controls. The differences between the means of monoclonal antibodies to isotype controls are indicated in the top corner of each events graph. (B) Means of four independent experiments are shown. Numbers above graphs indicate *P* values.

NF-κB activation after infection of DC with *Y. enterocolitica*. The transcription factor NF-κB promotes transcription of various immune response genes, including those encoding adhesion molecules (e.g., ICAM-1) and cytokines (e.g., TNF-α). While *Y. enterocolitica* invasin protein was found to activate NF-κB in epithelial cells, YopP inhibits the activation of NF-κB, and this, in concert with other effects, causes apoptosis of murine macrophages (24). Nuclear extracts showed low NF-κB binding activity in the control samples but increased binding activity in DC treated with LPS or plasmid-cured *Y. enterocolitica* WA-pYV$^-$. Infection with YopP-deficient *Y. enterocolitica* (WA-pYV$^+$ YopP$^-$) resulted in a moderate increase in the concentration of nuclear NF-κB. A moderate increase in the binding activity of NF-κB was also observed 30 and 60 min after infection with wild-type *Y. enterocolitica* (WA-pYV$^+$); however, this binding activity decreased markedly at 90 min postinfection (Fig. 6).
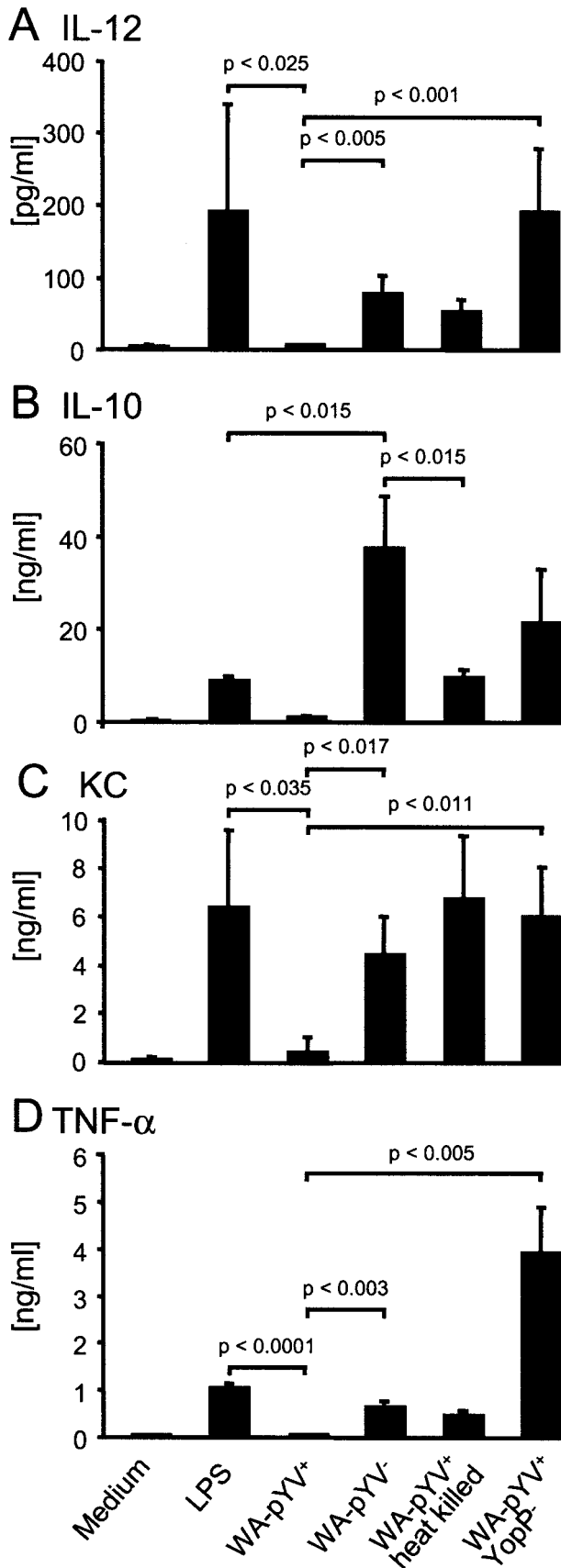
DISCUSSION

The present study demonstrates that *Y. enterocolitica* may affect several important functions of murine DC, including maturation as well as cytokine production. In addition, *Y. enterocolitica* induces apoptosis in DC. These alterations of DC by *Y. enterocolitica* obviously contribute to decreased antigen-specific CD4 T-cell activation. Although these observations have been obtained in in vitro experiments, it is tempting to speculate that by targeting the aforementioned functions of DC *Y. enterocolitica* might at least partially subvert the adaptive immune defense mechanisms of the host which are required for elimination of this pathogen from infected tissue. However, further studies are now required in order to investigate whether the *Yersinia*-induced changes of DC observed herein can also be observed in vivo, e.g., upon infection of mice with pathogenic *Y. enterocolitica* strains.

The most prominent effect of *Y. enterocolitica* on DC was the

induction of cell death. This observation is in contrast to a recent report that provided some evidence that human DC do not undergo cell death after infection with *Y. enterocolitica* (27). However, cell death is also observed in *Yersinia*-infected murine macrophages, and this effect is caused by YopP (10, 25). Consistent with these results we found that in contrast to wild-type *Y. enterocolitica* (WA-pYV$^+$), a YopP-deficient (WA-pYV$^+$ YopP$^-$) mutant strain did not induce cell death in DC. Moreover, it was found that LPS-tolerant macrophages that had been pretreated with LPS were much less susceptible to *Yersinia*-induced cell death by apoptosis (25). Likewise, we found that mature DC that had been prestimulated with LPS were less susceptible to *Yersinia* YopP-induced cell death than immature DC (data not shown). These data suggest that besides macrophages, DC may also undergo cell death upon interaction with pathogenic yersiniae, and YopP appears to be the most important factor for induction of DC death.

Exposure of DC to *Y. enterocolitica* caused a decrease in the mitochondrial potential, DNA fragmentation, and translocation of phosphatidylserine residues from the inner to the outer cellular membrane leaflet, strongly suggesting that *Yersinia*-mediated DC death is predominantly caused by apoptosis. In the present study the frequency of apoptotic DC 4 h after infection with *Yersinia* was similar to that observed in experiments with murine macrophages (25). While macrophages were infected with *Yersinia* at an MOI of 50 for 90 min (25), the DC used in this study were only infected at an MOI of 10 for 60 min. In our experiments higher MOIs caused a rapid and total disruption of DC, suggesting that murine DC are more susceptible to *Yersinia*-induced apoptosis than macrophages (data not shown). In fact, we infected macrophages (J774 murine macrophages) and DC with *Y. enterocolitica* in parallel, and 4 h later survival of macrophages was markedly higher (61%) than survival of DC (35%) (data not shown).

In macrophages, YopP-mediated inhibition of NF-κB activation appears to be involved in *Yersinia*-induced apoptosis

FIG. 5. Production of IL-12 (A), IL-10 (B), KC (C), and TNF-α (D) by DC after infection with *Y. enterocolitica*. DC from BALB/c mice were incubated with LPS or with various strains of *Y. enterocolitica*: wild type (WA-pYV$^+$), plasmid cured (WA-pYV$^-$), YopP deficient (pYV$^+$ YopP$^-$), and heat-killed wild type (WA-pYV$^+$ heat killed). Gentamicin (100 μg/ml) was added 1 h after infection. Three hours (KC) or 23 h (IL-12, IL-10, and TNF-α) later, supernatants were harvested and analyzed by ELISA. Data represent the means of results from three independent experiments.
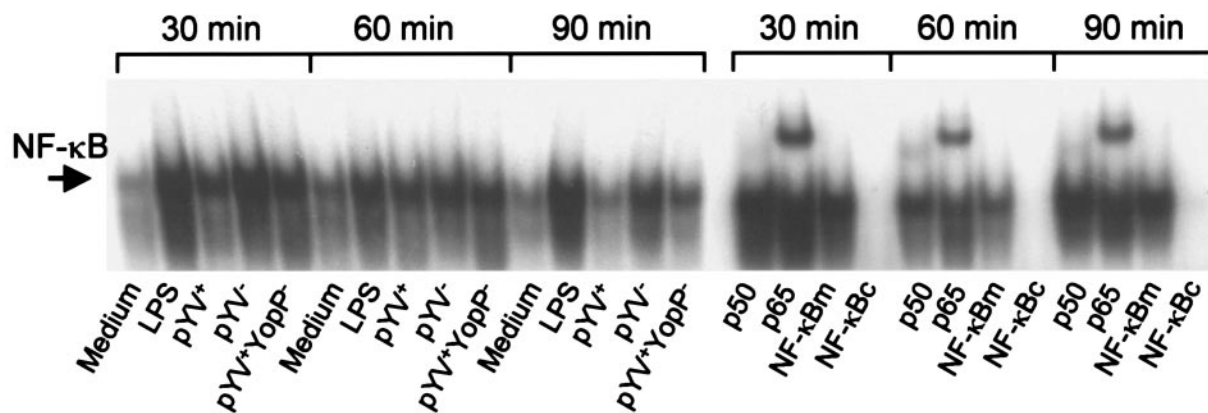
(24). According to this finding, a biphasic NF-κB activation course was found in *Yersinia*-infected macrophages. NF-κB binding activity was observed after 30 min but was absent 90 min after *Yersinia* infection. In DC we observed a slight activation of NF-κB at 30 and 60 min, even after infection with wild-type *Y. enterocolitica* (WA-pYV$^+$); however, this signal declined significantly after 90 min. The faster prevention of NF-κB signaling in macrophages might result from the higher *Yersinia* MOI used for infection in the experiments carried out by Ruckdeschel et al. (24). Despite the fact that nuclear NF-κB activity was obtained for longer periods in *Yersinia*-infected DC than in macrophages, DC turned out to be more susceptible to killing by *Yersinia* than macrophages. Therefore, at present it is not clear whether and to what extent prevention of NF-κB activation in DC triggers apoptosis or if other mechanisms contribute to the induction of apoptosis.

The temporary partial activation of DC observed after infection with wild-type *Yersinia* (WA-pYV$^+$) might explain the difference between the up-regulation of MHC class II molecules and the lack of cytokine release. In contrast to cytokines, preformed MHC class II molecules are abundant in intracellular compartments of nonactivated DC (13). While limited DC activation might be sufficient to transmit preformed MHC class II molecules to the DC cell surface, de novo cytokine synthesis requires a prolonged period of cell activation.

Together, these results indicate that YopP is the most potent pathogenicity factor of *Y. enterocolitica* and prevents DC-mediated activation of CD4$^+$ T cells, predominantly by induction of apoptosis. On the other hand, YopP-deficient (WA-pYV$^+$ YopP$^-$) or plasmid-cured (WA-pYV$^-$) *Yersinia* mutants were also able to decrease DC-mediated T-cell activation to some degree.

Exposure of DC to plasmid-cured (WA-pYV$^-$) *Yersinia* induced moderate IL-12 levels but very high IL-10 levels. This cytokine production profile might contribute to the reduction of T-cell proliferation observed after infection with plasmid-cured *Yersinia* in vitro. In fact, IL-10 may cause a reduction of T-cell proliferation by at least two mechanisms. Firstly, the formation of regulatory T cells might be induced by IL-10, as reported for *Bordetella pertussis* infections (11). Secondly, IL-10 might induce genes of suppressor proteins, as discussed recently (4, 14, 30).

As the mechanisms of IL-10 and IL-12 production on T-cell proliferation in yersiniosis are actually unclear, further studies are required. Although it is unclear which compound of plasmid-cured (WA-pYV$^-$) *Yersinia* contributes to stimulation of IL-10 production, we speculate that *Yersinia* LPS might be the responsible compound. In fact, although smooth LPS from *Escherichia coli* preferentially induces secretion of IL-12, LPS from *Porphyromonas gingivalis* induces IL-10 secretion (23).

FIG. 6. *Y. enterocolitica*-induced activation of NF-κB in DC. DC were incubated with LPS or with various strains of *Y. enterocolitica:* wild type (WA-pYV⁺), plasmid cured (WA-pYV⁻), YopP deficient (pYV⁺ YopP⁻), and heat-killed wild type (WA-pYV⁺ heat killed). After 30, 60, and 90 min, nuclear extracts were prepared and analyzed in gel shift experiments with ³²P-labeled probes for NF-κB. (A) Time course of *Yersinia*-induced NF-κB activation. (B) Supershift analyses were performed by incubating nuclear extracts with antibodies against NF-κB subunits p50 or p65 and for competition experiments with a 100-fold excess of unlabeled NF-κB probe. NF-κBc, consensus sequence; NF-κBm, mutated sequences. Results are representative of three experiments.

The semirough LPS from *Yersinia* might cause host responses similar to those from *P. gingivalis*. Based on these in vitro observations, it might be possible that plasmid-cured (WA-pYV⁻) *Yersinia* also mediates antiinflammatory properties in mice.

Although YopP was found to exert the most pronounced inhibitory effects on DC and T-cell activation, the use of a YopP-deficient (WA-pYV⁺ YopP⁻) strain and a plasmid-cured (WA-pYV⁻) *Yersinia* mutant strain strongly suggests that in addition to YopP other factors exert inhibitory effects on DC-triggered T-cell proliferation. In keeping with this assumption, YopH was reported to suppress activation of T and B lymphocytes by inhibition of early phosphorylation events of the antigen receptor signaling complex of T cells (32). However, this was a direct effect of Yops on the T cells, an effect that can be excluded in the experimental system described herein. However, similar to our observations in DC, it was also found that in B cells *Yersinia pseudotuberculosis* inhibits up-regulation of surface expression of costimulatory molecules such as B7.2 (32).

In conclusion, our study demonstrates that *Y. enterocolitica* is able to impair the T-cell activation capacity of murine DC by various means. The most important factor might be YopP, which causes rapid apoptosis in DC. On the other hand, other yet-undefined Yops also impair T-cell activation.

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