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Yersinia Outer Protein P Inhibits CD8 T Cell Priming in the Mouse Infection Model

Konrad Trulzsch,‡ Gernot Geginat,† Thorsten Sporleder,* Klaus Ruckdeschel,* Reinhardt Hoffmann,* Jürgen Heesemann,* and Holger Rüssmann*

Pathogenic yersiniae translocate a mixture of effector proteins called Yersinia outer proteins (Yops) into the cytosol of eukaryotic cells by their type III secretion system. YopP is one of the best characterized of these effector proteins and known to inhibit the proinflammatory response of the host by interfering with NF-κB signal transduction and inducing apoptosis of macrophages. The effects of YopP on the immune response were studied by using Yersinia Ag-independent approach using bacteria that translocate the well-characterized model Ag listeriolysin O of Listeria monocytogenes via their type III secretion system. In this study we demonstrate a novel function for YopP in vivo. It is shown for the first time that YopP not only counteracts the innate immune defense but also inhibits the adaptive immune system by suppressing the development of an effective CD8 T cell response in a mouse model. A possible mechanism for this could be the inhibition of Ag presentation by dendritic cells (DC). In vitro this is shown to be due to the rapid induction of programmed DC death and to inhibition of DC maturation. Using this approach we could further show that the listeriolysin O-specific CD8 T cells generated in vivo by the yopP mutant are functional and are able to protect mice against a lethal challenge with wild type Listeria. The Journal of Immunology, 2005, 174: 4244–4251.

Pathogenic yersiniae cause a wide range of diseases in humans ranging from bubonic plague caused by Yersinia pestis to self-limiting gastroenteritis and lymphadenitis caused by the enteric pathogens Yersinia pseudotuberculosis and Yersinia enterocolitica. In the mouse infection model enteropathogenic yersiniae invade Peyer’s patches by entering through specialized epithelial cells called M cells (1, 2). Subsequently yersiniae disseminate to lymph nodes, spleen, and liver where they form microcolonies/microabscesses (3, 4). In contrast to other enteric pathogens such as Salmonella and Shigella, Yersinia is predominantly an extracellular pathogen (1, 5–7). Initially innate host defenses such as polymorphonuclear leukocytes (PMNs), macrophages, and NK cells are involved in controlling Yersinia infection (7–10). But subsequently a robust adaptive immune response is required to overcome Yersinia infection. Specific Abs (11, 12) as well as IFN-γ-producing CD4 and CD8 T cells (4, 12–17) play an essential role in clearing Yersinia infection and have been shown to mediate protection in adoptive transfer experiments (4, 13, 15). It has been demonstrated that infection of rats with Y. pseudotuberculosis leads to the induction of a class I-restricted cytotoxic CD8 T cell response but the Ags that are presented to CD8 T cells in a class I context are unknown (16). However, cells infected with Yersinia have been shown to present an epitope of YopH to MHC class I-restricted CD8 T cells (18).

To overcome host defense mechanisms, pathogenic yersiniae have acquired a complex arsenal of effector proteins that inhibit the innate and adaptive immune system of the host. These major antihost determinants are located on a 70-kb virulence plasmid pYV, which encodes a protein microinjection apparatus called the type III secretion system (TTSS) and at least six effector proteins, Yersinia outer proteins (Yops; YopH, YopO/YpkA, YopP/YopJ, YopE, YopM, and YopT) that are translocated into the cytosol of eukaryotic cells (reviewed in Ref. 19). The main function of these Yops is to inhibit the immune response of the host. At least four Yops (YopH, YopE, YopT, and YopO/YpkA) are involved in inhibiting phagocytosis of yersiniae by disrupting the cytoskeleton of PMNs and macrophages (20–23). YopH is a phosphorytrosine phosphatase (24) that dephosphorylates focal adhesion kinase, paxillin, Fyn-binding protein, p130cas, and SKAP-HOM (Src kinase-associated phosphoprotein of 55 kDa homologue) thereby disrupting focal adhesions (25–31), and that suppresses the production of reactive oxygen intermediates by macrophages and PMNs (20, 29). YopH has been shown to not only contribute to evasion of the innate but also the adaptive immune response by impairing T cell and B cell activation in vitro (32, 33). YopE is a GTPase-activating protein that acts preferentially on Rac GTPases, which may explain its inhibitory effect on phagocytosis and reactive oxygen intermediate production (34–36). YopT is a cysteine protease that preferentially inactivates RhoA GTPases by cleavage of the C-terminal geranylglycerol-cysteine residue (37, 38). YopO is an autophosphorylating serine/threonine kinase that interacts with RhoA, Rac, and actin (37, 39, 40). YopO is an autophosphorylating serine/threonine kinase that interacts with RhoA, Rac, and actin (37, 39, 40). YopO is a leucine-rich repeat protein that traffics to the nucleus of infected cells (41) and forms a protein complex with two cellular kinases protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1) (42). Recently it was shown that YopM might interfere with innate immunity by causing a depletion of NK cells (10).

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Besides paralyzing phagocytic cell, Yops also inhibit the proinflammatory response elicited by infected cells. This has been most extensively studied for YopP (YopJ in Y. pseudotuberculosis), which has been shown to inhibit TNF-α release by macrophages and IL-8 release by epithelial and endothelial cells (43–47). It has also been shown to reduce the expression of adhesion molecules, such as ICAM-1 and E-selectin on endothelial cells, which might inhibit recruitment of PMN to the infection site (44). All these effects have been attributed to the inhibition of the activation of the transcription factor NF-κB (43, 47, 48). YopP inhibits the NF-κB-activating IκB kinase β resulting in inhibition of NF-κB dissociation from IκB and NF-κB translocation to the nucleus (49). Besides inhibiting the NF-κB pathway, YopP also disrupts at least three MAPK pathways (ERK1/2, JNK1, and p38) that are involved in the immune response (43, 46, 49–51). These inhibitory effects of YopP also contribute to the ability of Yersinia to induce apoptosis in macrophages but not other cell types (52, 53). Despite the extensive study of YopP effects in vitro, there is little evidence for the precise role of YopP in vivo. Monack et al. (54) have shown that Y. pseudotuberculosis induces apoptosis of Mac-1+ cells in mice and that this is probably responsible for the attenuated phenotype of a yopJ mutant.

In this communication we demonstrate a novel function for YopP which is to suppress the adaptive immune response of the host. For this purpose we constructed Yersinia mutants that translocate the model Ag listeriolysin O (LLO) of Listeria monocytogenes by their TTSS. This enables the study of specific CD8 T cell induction and protection independent of Yersinia by their TTSS. This enables the study of specific CD8 T cell genes and harbors all Yops (59). The sodA mutant is deficient in manganese-cofactored superoxide dismutase which has been shown to inhibit TNF-β (43, 47, 48). YopP inhibits the NF-κB pathway, YopP also disrupts at least three MAPK pathways (ERK1/2, JNK1, and p38) that are involved in the immune response (43, 46, 49–51). These inhibitory effects of YopP also contribute to the ability of Yersinia to induce apoptosis in macrophages but not other cell types (52, 53). Despite the extensive study of YopP effects in vitro, there is little evidence for the precise role of YopP in vivo. Monack et al. (54) have shown that Y. pseudotuberculosis induces apoptosis of Mac-1+ cells in mice and that this is probably responsible for the attenuated phenotype of a yopJ mutant.

### Materials and Methods

#### Bacterial strains and plasmids

Y. enterocolitica WA-314 is a clinical isolate of serotype O:8 (56). WA-C is a plasmid-less derivative of WA-314 (56). WA-C(pYYV) (57) harbors a chloramphenicol cassette in a noncoding region of the pYV plasmid and is as virulent as WA-314 (57). WA-C(pYYVΔP) and WA-C(pYYVΔE) are isogenic yopP and yopE deletion mutants, respectively, that were previously described (57). WA-C(pYYV5-151) harbors a TNF insertion in low calcium response gene lcrD and is deficient in translocation of all Yops (58). The sodA mutant is deficient in manganese-cofactored superoxide dismutase and harbors all Yops (59). The sodA(yopP) double mutant was constructed by transforming plasmid pYYVopP::TnMax25 (60) into WA-314ΔsodA. Plasmid pHR430 (61) bears the genetic information for the specific Yop chaperone SycE as well as a fusion protein consisting of the first 138 amino acids of YopE and amino acids 51–363 of LLO of L. monocytogenes. The fusion protein was C-terminal tagged with an M45 epitope. Genes yopE and sycE are under the control of their wild-type promoter. This plasmid was transformed into the above named strains by electroproporation (62). Bacteria were cultured aerobically in Luria-Bertani (LB) broth or on LB agar plates (Difco) at 27°C (Yersinia) or 37°C (Escherichia coli). Antibiotics were used at the following concentrations; kanamycin, 25 µg/ml; nalidixic acid, 60 µg/ml; chloramphenicol, 20 µg/ml; spectinomycin, 50 µg/ml.

#### Oral infection and in vivo protection assay

Six- to 8-wk-old female CB6F1 mice (Füllinsdorf) were kept under specific pathogen-free conditions (positive-pressure cabinet) and were provided with food and water ad libitum. Mice were infected orally with 1 × 10⁶ yersiniae from frozen stock suspensions. Stock suspensions were prepared by growing bacteria to stationary phase in LB medium at 27°C, and freezing in 15% glycerol. After appropriate dilutions bacteria were washed twice with PBS and mice were fed 50 µl using a microliter pipette. Mice were subjected to fasting 16 h before oral infection. The actually administered dose was determined by plating serial dilutions on Mueller-Hinton agar for 36 h at 27°C. Mice were sacrificed by CO₂ asphyxiation and spleens were aseptically removed. Control mice were immunized i.p. with 5 × 10⁶ CFU of L. monocytogenes.

For the challenge experiments, groups of 10 mice were injected with 10⁶ CFU of L. monocytogenes in 100 µl of PBS into the lateral tail vein. Three days after the challenge, CFU/organ were determined by plating serial dilutions of homogenates on PALCAM Listeria selective agar (Merck). Colonies were counted after 48 h incubation. All experiments were repeated twice.

#### Bone marrow cultures

DCs were obtained from bone marrow of BALB/c mice as previously described (63) and grown in DMEM supplemented with 10% FBS, 10 ng of GM-CSF (R&D Systems), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 50 µM 2-ME. DCs were seeded at a density of 2 × 10⁵ cells per 10 ml of medium in bacteriologic petri dishes and were fed with 100 µl of GM-CSF-supplemented medium every 3 days. DCs were purified by magnetic cell sorting using MACS CD11c MicroBeads (Miltenyi Biotec) according to manufacturer’s recommendations. DCs were used after 5 days of culture.

#### Ag presentation assay

CD8 T cells against H-2k-restricted epitope LLO91–99 were derived from spleens of L. monocytogenes-infected BALB/c mice and were propagated by repeated restimulation in the presence of 10⁻⁹ M peptide as previously described (64). T cell recognition of infected bone marrow-derived DCs was measured by detection of IFN-γ in culture supernatants as previously described (64). Briefly, DCs were infected with Y. enterocolitica in 96-well flat-bottom microwell plates at a multiplicity of infection (MOI) of 1, 10, and 100 by centrifugation for 10 min at 200 × g. After 1, 2, and 4 h at 37°C, infected DCs were washed twice and the culture medium was supplemented with 100 µg of gentamicin/ml and 3 × 10⁵ T cells were added to each well containing 3 × 10⁴ DCs (E:T ratio 1:1). After 12–18 h at 37°C, supernatants were harvested and the IFN-γ concentration was measured using an IFN-γ-specific ELISA.

#### ELISPOT assay

The frequency of LLO91–99-specific CD8 T lymphocytes in mice immunized with the Y. enterocolitica mutants was determined by an IFN-γ-specific ELISPOT assay as previously described (61). Assays were performed with nitrocellulose-backed 96-well microtiter plates (Nunc) that were coated with rat anti-mouse IFN-γ mAb (BioSource International). Unseparated splenocytes (5 × 10⁶) from each mouse were set up in three different wells for triple determination of the T cell frequency. Splenocytes were stimulated for 6 h in round-bottom microtiter plates in the presence of 10⁵ M CD8 T cell epitope LLO₉₁₋₉₉. Subsequently activated cells (5 × 10⁴) were transferred to ELISPOT plates and incubated overnight. ELISPOT plates were developed with biotin-labeled rat anti-mouse IFN-γ mAb, HRP-Streptavidin conjugate (BD Biosciences), and aminoethylcarbazole dye solution. The frequency of Ag-specific cells was calculated as the number of spots per 10⁶ splenocytes seeded. The specificity and sensitivity of the ELISPOT assay were controlled with T cell lines specific for LLO₉₁₋₉₉. Recovery of seeded T cells was higher than 90%.

#### Assessment of programmed DC death by fluorescent microscopy

Assessment of programmed cell death was performed essentially as previously described (65). DCs were infected with the Y. enterocolitica mutants was determined by an IFN-γ-specific ELISPOT assay as previously described (61). Assays were performed with nitrocellulose-backed 96-well microtiter plates (Nunc) that were coated with rat anti-mouse IFN-γ mAb (BioSource International). Unseparated splenocytes (5 × 10⁶) from each mouse were set up in three different wells for triple determination of the T cell frequency. Splenocytes were stimulated for 6 h in round-bottom microtiter plates in the presence of 10⁵ M CD8 T cell epitope LLO₉₁₋₉₉. Subsequently activated cells (5 × 10⁴) were transferred to ELISPOT plates and incubated overnight. ELISPOT plates were developed with biotin-labeled rat anti-mouse IFN-γ mAb, HRP-Streptavidin conjugate (BD Biosciences), and aminoethylcarbazole dye solution. The frequency of Ag-specific cells was calculated as the number of spots per 10⁶ splenocytes seeded. The specificity and sensitivity of the ELISPOT assay were controlled with T cell lines specific for LLO₉₁₋₉₉. Recovery of seeded T cells was higher than 90%.

#### FACS analysis

DCs were infected at an MOI of 10 for 2 h and were either treated 2 h with 10 µg/ml LPS (Sigma-Aldrich) or remained untreated. After 2 h, cells were washed twice and medium was supplemented with 100 µg/ml gentamicin.
After 16 h CD11c− cells were recovered by immunomagnetic separation and for cytofluorometry analysis, cells were stained with FITC-labeled hamster anti-rat CD11c, PE-labeled hamster anti-mouse CD80–PE (IgG2, clone 16-10A1; BD Biosciences), PE-labeled rat anti-mouse CD86–PE (IgG2a mAb, clone GL1; BD Biosciences), PE-labeled rat anti-mouse CD8a (IgG2a mAb, clone 53-6-7; BD Biosciences) or isotope-matched control mAb (BD Biosciences).

Double immunofluorescence staining

Bone marrow-derived DCs were cultured as described earlier and centrifuged onto glass coverslips. One hour before the addition of bacteria, DMEM was replaced by 500 µl of HBSS. Yersinia were grown overnight in LB medium, diluted 1/40 in fresh medium and grown to exponential phase (OD600 0.6) at 37°C. DCS were infected with yersiniae at an MOI of 10 for 2 h at 37°C, 5% CO2. Cells were washed three times with HBSS to remove non-cell-associated bacteria and fixed in 3.7% formaldehyde. To differentiate cell-associated extracellular bacteria from intracellularly located yersiniae, the double immunofluorescence technique was used (66).

Bacteria were stained with a mouse anti-Y. enterocolitica YadA (Yersinia adhesin is a pYY-encoded surface exposed outer membrane protein) mAb and a secondary anti-mouse tetramethylrhodamine isothiocyanate (TRITC) conjugate (1/500 in 3% BSA/PBS; Sigma-Aldrich). Subsequently DCS were permeabilized with 0.1% Triton X-100 in PBS for 3 min. Intracellular and extracellular bacteria were stained with the YadA mAb and a secondary anti-mouse FITC conjugate (1/100 in 3% BSA/PBS; Sigma-Aldrich). Coverslips were mounted on glass slides and analyzed by fluorescence microscopy.

Statistical analysis

Statistical analysis of the in vitro experiments were performed using the Newman-Keuls multiple comparison test at the 0.05 significance level. All tests were performed with WINKS statistical analysis software (TeksAsoft).

Results

YopP inhibits CD8 T cell response in vivo

To analyze the effects of Yops on the development of a CD8 T cell response during murine yersiniosis, we chose to use the well-characterized Ag LLO of L. monocytogenes for intracutaneous delivery by the Yersinia TTSS. We used the previously described construct pHR430 (61), which harbors the genetic information for secretion and translocation of YopE (amino acids 1–138) fused to amino acids 51–363 of LLO. This fragment of LLO harbors the immunodominant H-2Kd-restricted CD8 T cell epitope LLO91–99. To facilitate immunodetection, the fusion protein was labeled at its C terminus with an M45 epitope tag. Plasmid pH4R30 also harbors the sycE gene. Transcription of the gene fusion and sycE is mediated by the wild-type yopE/yopE, as well as the previously described yopP and yopE mutants WA-C(pYVYΔP) and WA-C(pYVΔE), respectively (57).

To determine the role of Yops in the development of a CD8 T cell response in vivo, we orally infected C57BL/6 mice with 1 × 108 CFU of the yopP, yopE, sodA as well as a sodA/yopP double mutant translocating YopE/LLO/M45. Control mice were infected i.p. with a sublethal dose (5 × 104 CFU) of L. monocytogenes. Six weeks after immunization, IFN-γ ELISPOT assays were performed to determine the number of LLO-specific CD8 T cells in spleens of mice. The frequency of LLO91–99–specific CD8 T cells was determined by counting the number of IFN-γ spots generated per 105 spleen cells in the presence of synthetic LLO91–99 nonamer-peptide. Mice immunized with the yopP mutant translocating YopE/LLO/M45 revealed similar numbers of IFN-γ-producing cells reactive with LLO91–99 as did mice infected with L. monocytogenes (Fig. 1). In contrast mice immunized with the yopE mutant, which we have shown to colonize mouse tissues to a comparable extent (57), did not show a significant response. Because we cannot use the parental strain of the yop mutants for these experiments (because it is lethal for mice), we also performed immunizations using the highly attenuated sodA mutant background. Again these experiments show that only a significant CD8 T cell response can be induced using the sodA/yopP double mutant but not the sodA mutant expressing YopP. The number of IFN-γ spots generated by the sodA/yopP double mutant were however significantly lower than for the yopP mutant indicating that colonization of the spleen is beneficial for inducing a CD8 T cell response. Besides the yopE mutant we also analyzed the CD8 T cell response using highly attenuated yopH, yopM, and yopQ mutants translocating YopE/LLO/M45, but none of these strains were able to induce significant numbers of LLO91–99–specific CD8 T cells (data not shown). This indicates that the CD8 T cell response is dependent on the absence of YopP.

To determine whether the CD8 T cells generated in vivo were functional and could protect mice against a lethal challenge with L. monocytogenes, mice were orally immunized with the yopP, yopE, and sodA mutants translocating YopE/LLO/M45. As controls, mice were immunized with the yopP mutant not harboring YopE/LLO/M45 and with a sublethal i.p. dose of L. monocytogenes. Eight weeks after immunization with the different strains, mice were challenged with 5 × 104 CFU of L. monocytogenes i.v. Three days after the challenge, spleens were removed and the number of surviving listeriae were determined (Fig. 2). Mice immunized with the yopP mutant not harboring YopE/LLO/M45 as well as mice immunized with the yopP or sodA mutants translocating YopE/LLO/M45 showed high colony counts of Listeria in spleens. In

![FIGURE 1. Frequency of LLO-specific CD8 T cells in spleens of mice orally infected with 1 × 108 CFU of the indicated Y. enterocolitica mutants translocating YopE/LLO/M45. As a control, spleens from mice infected with L. monocytogenes were used. The frequency of LLO-specific T cells in spleens was determined by ELISPOT assay. The number of cells reactive with LLO91–99 per 105 splenocytes is shown ± SD from 15 mice. * Significant difference from the negative control group.](http://www.jimmunol.org/content/182/5/4246.F1)

![FIGURE 2. Protective immunity against listeriosis in mice orally immunized with the indicated Y. enterocolitica yop mutants translocating YopE/LLO/M45. As a negative control mice were immunized with the yopP mutant not harboring YopE/LLO/M45. Positive control mice received a sublethal i.p. dose of 5 × 104 CFU of L. monocytogenes. Eight weeks after immunization, mice were i.v. challenged with 106 CFU of L. monocytogenes serovar 1/2a EGD. The bacterial load of L. monocytogenes in spleen of mice was determined 72 h postinfection. Results are shown as the mean log10 CFU ± SD from 20 mice per group. * Significant difference from the negative control group.](http://www.jimmunol.org/content/182/5/4246.F2)
was completely protected and showed five log 10 monocytogenes M45. The control group immunized with a sublethal dose of YopE/LLO/M45. Presentation of the MHC class I-restricted epitope LLO91–99 by DCs was measured in an in vitro Ag presentation assay with epitope-specific CD8 T cells. Activation of T cells was detected by the amount of IFN-γ secreted into the culture supernatant. The IFN-γ concentration is shown in picograms per milliliter and the SD of triplicate determinations. The detection limit of the IFN-γ ELISA was 0.05 ng/ml. Similar results were obtained in two independent experiments.

contrast, mice immunized with the yopP mutant translocating YopE/LLO/M45 showed two to three log_{10} less CFUs in spleens than mice immunized with the yopE or sodA mutants translocating YopE/LLO/M45 or the yopP mutant not harboring YopE/LLO/M45. The control group immunized with a sublethal dose of L. monocytogenes was completely protected and showed five log_{10} less CFU than the other strains. This shows that immunization with the yopP mutant translocating YopE/LLO/M45 protects against a challenge with wild-type L. monocytogenes. This protection is presumably mediated by LLO_{91–99}-specific CD8 T cells.

YopP inhibits MHC class I-restricted Ag presentation by DCs in vitro

The finding that we can only induce a CD8 T cell response against a translocated heterologous Ag using yopP-deficient Y. enterocolitica strains prompted us to perform in vitro Ag presentation assays to determine whether this effect might be due to inhibition of Ag display by DCs. To test this assumption we directly compared the ability of the yopP mutant and WA-C(pYV) translocating YopE/LLO/M45 to deliver LLO_{91–99} with the MHC class I-restricted Ag presentation pathway. For this purpose we infected 5-day-old murine bone marrow-derived DCs with the yopP mutant and WA-C(pYV) either translocating or lacking YopE/LLO/M45.

DCs were infected for 1, 2, or 4 h at an MOI of 1, 10, and 100. The ability of infected DCs to present LLO_{91–99} to specific CD8 T cells derived from spleens of mice infected with L. monocytogenes was assessed by measuring IFN-γ secretion into the culture supernatant (Fig. 3). DCs infected with the yopP mutant translocating YopE/LLO/M45 were efficiently recognized by LLO_{91–99}-specific T cells, whereas DCs infected with WA-C(pYV) translocating YopE/LLO/M45 stimulated a much weaker LLO-specific T cell response. As expected DCs infected with yersiniae not expressing YopE/LLO/M45 did not induce a significant IFN-γ response. The highest IFN-γ concentrations in the supernatant were detected after 1 h of infection with IFN-γ concentrations continually falling after longer infection periods. Generally Ag display by DCs was also lower at very high (100) or low (1) MOI. This is probably due to nonspecific toxic effects of the other translocated Yops after long infection periods, artificially high numbers of infecting bacteria, or insufficient translocation of heterologous Ag at very low MOI.

YopP induces programmed cell death and prevents maturation of DCs

Because YopP is known to induce apoptosis of macrophages (but not other cell types), we wondered whether this was also the case for DCs and whether this could explain the inhibitory effect of YopP on Ag presentation. For this purpose we infected 5-day-old bone marrow-derived DCs with the yopP mutant or WA-C(pYV), DCs were infected at an MOI of 10:1. Two hours after infection, gentamicin was added to kill yersiniae and programmed DC death was quantified 3 and 5 h postinfection by counting cells that were stained with fluorescein-conjugated annexin V and propidium iodide. Results shown are the means from three experiments (100 cells analyzed for each experiment), * , Significant difference from the negative control group.

According to the established concept, the uptake of Ags by DCs is followed by their maturation with subsequent down-regulation of phagocytic activity and concomitant up-regulation of costimulatory molecules. A failure to trigger the expression of costimulatory molecules could explain the effect of YopP-mediated CD8 T cell priming by DCs.
cell suppression in vivo. Remarkably, cytofluorometric analysis of DCs after infection with WA-C(pYY) and the yopP mutant revealed a failure to up-regulate CD80 and CD86 on DCs after infection with WA-C(pYY) (Fig. 5). This, however, is not necessarily a direct effect of YopP on the expression of costimulatory molecules because most DCs infected with WA-C(pYY) are undergoing programmed cell death at the same time that an up-regulation of costimulatory molecules is taking place.

Yersinia resists phagocytosis by DCs

The TTSS microinjection apparatus of yersiniae is activated after bacteria are closely attached to the host-cell surface but is downregulated after internalization (67, 68). Therefore we determined whether Yersinia could also inhibit its internalization by DCs. We therefore performed double immunofluorescence staining to determine the number of intracellular and extracellular bacteria. For this purpose we infected DCs with WA-C(pYY), yopP, and yopE mutants for 2 h. As a control we used an lcrD mutant, which is deficient in translocation of all Yops and should be unable to resist phagocytosis by Yop translocation. Adherence of all tested Yersinia strains to DCs was in a comparable range (on average 10 bacteria/cell). Extracellularly located bacteria were detected with an anti-YadA mAb and a TRITC marked secondary Ab. After permeabilization with Triton X-100, intracellular bacteria were stained with the anti-YadA mAb and a FITC-marked secondary Ab. The percentage of phagocytosed bacteria was determined by the ratio of intracellular bacteria (Fig. 6, FITC signal, green) to the total number of cell-associated bacteria (Fig. 6, FITC and TRITC signals, red and green, respectively). As can be seen in Fig. 6, only 6.4 ± 3% of WA-C(pYY) yersiniae were located intracellularly in DCs. As expected, the yopP mutant showed a comparable ratio of intracellular yersiniae as WA-C(pYY), whereas 44.8 ± 5% and 25 ± 5% of cell-associated lcrD and yopE mutants, respectively, were internalized by DCs. This indicates that wild type yersiniae are located predominately extracellularly and that Yops enable yersiniae to resist phagocytosis by DCs. The phagocytosis rate of yersiniae was however significantly lower than previously reported for macrophages or neutrophils (23).

Discussion

Previously we demonstrated that a yopP knockout mutant of Y. enterocolitica was attenuated in an oral mouse infection model (57). In contrast to mice infected with the WA-C(pYY) strain, mice infected with the yopP mutant were able to control infection, eliminating bacteria from spleens and livers by day 12 postinfection. We wondered whether this effect might be due to the development of a stronger adaptive immune response after infection with the yopP mutant vs the wild type. This prompted us to study a possible immnosuppressive action of the effector protein YopP on the adaptive immune response using a Yersinia-Ag independent approach. We decided to use Yersinia mutants that translocate the well-characterized listerial Ag LLO by their TTSS to study the CD8 T cell response. Using this approach we could demonstrate for the first time that the Yersinia effector protein YopP 1) inhibits the adaptive immune response of the host by suppressing the development of a CD8 T cell response in vivo; 2) inhibits MHC class I-restricted Ag presentation by DCs; 3) rapidly induces DC cell death; and 4) prevents maturation of DCs.

YopP is one of the best characterized effector proteins of Yersinia that has been previously shown to exert multiple effects in vitro, such as suppression of cytokine release by macrophages and epithelial cell, and to induce apoptosis in macrophages. YopP/ YopJ has been shown to inhibit the adaptive immune response in vivo by induction of apoptosis of macrophages and this inhibition was made responsible for the attenuated phenotype of a yopJ mutant in the mouse infection model (54).

In this study we show in vivo that strains lacking yopP are able to induce a strong CD8 T cell response to a translocated heterologous Ag. In contrast attenuated yopH, yopE, yopM, and yopQ mutants translocating YopE/LLO/M45 were not able to induce a significant CD8 T cell response against LLO in vivo. The immune response to bacteria depends to a large extent on the degree of colonization of the host (colonization of Peyer’s patches only vs colonization of liver/spleen). This might account for the poor CD8 T cell response after infection of mice with the yopH, yopM, and yopQ mutants, which are highly attenuated and able to colonize only the gut and Peyer’s patches (57). However, because the yopE

![FIGURE 5](http://www.jimmunol.org/)  
**FIGURE 5.** DC maturation. Expression of CD80 and CD86 was monitored on DCs in the presence of different stimuli. DCs were infected with WA-C(pYY) or the yopP mutant, or were treated with LPS or remained untreated. DCs were isolated by immunomagnetic separation, stained, and were subjected to cytofluorometric analysis. Similar results were obtained in two independent experiments.

![FIGURE 6](http://www.jimmunol.org/)  
**FIGURE 6.** Phagocytosis of *Yersinia* by DCs. DCs were infected by WA-C(pYY), yopP, yopE, and lcrD mutants for 2 h. Extracellular yersiniae were detected by an anti-YadA primary and TRITC-marked secondary Ab. After permeabilization intracellular bacteria were detected by an anti-YadA primary and FITC-marked secondary Ab. Phagocytosis rates are shown as a percentage of intracellular bacteria (green) relative to total number of cell-associated bacteria. Values are the mean ± SD from three experiments. *, Significant difference from WA-C(pYY).
mutant is comparable in virulence to the yopP mutant (57) differences in colonization are not likely to have influenced this result.

To have more comparable strains, however, we also performed these experiments using a highly attenuated sodA mutant (harboring all yop) translocating YopE/LLO/M45 and compared the immune response with a sodA/yopP double mutant translocating YopE/LLO/M45. These experiments confirmed our previous results with only the sodA/yopP double mutant inducing a CD8 T cell response compared with the sodA mutant.

The development of an immune response depends on the amount of Ag that is translocated into the cytosol of APCs and it has been shown that hypertranslocation of heterologous Ag by a Y. pseudotuberculosis yopK knockout mutant results in a superior immune response (61). To exclude the possibility that the CD8 T cell response induced by yopP-deficient mutants could be the result of compensatory hypertranslocation of YopE/LLO/M45, we demonstrated by Western blot analysis that the deletion of yopP does not influence the amount of YopE/LLO/M45 that is translocated into DCs (data not shown). Furthermore WA-C(pYV), yopP, and yopE mutants resist phagocytosis by DCs and remain predominantly extracellular. These results demonstrate for the first time that YopP functions to subvert the CD8 T cell response of the host in vivo. To show that CD8 T cells generated after infecting mice with yopP lacking Yersinia strains are functional, we challenged mice that had been immunized orally with different yersiniae translocating YopE/LLO/M45 with wild type L. monocytogenes. These experiments demonstrate that protection against murine listeriosis can only be achieved by immunizing mice with the yopP mutant translocating YopE/LLO/M45. Presumably this protection is mediated by CD8 T cells. This shows for the first time that Y. enterocolitica mutants can be used as oral live vaccine carriers for immunization against an intracellular pathogen.

To find a correlate for the in vivo CD8 T cell suppression in a cell culture model, we performed Ag presentation assays with DCs using WA-C(pYV) and the yopP knockout mutant. Previously, we could demonstrate efficient MHC class I-restricted Ag presentation by macrophages using wild-type Y. enterocolitica translocating YopE/p60 (55). Repeating these assays with the yopP mutant however showed no difference in the ability of these macrophages to present heterologous Ag to specific CD8 T cells (data not shown). This led us to examine the role of YopP on Ag presentation by DCs, which are known to be the most potent stimulators of naive T cells. These experiments showed that MHC class I-restricted Ag presentation and CD8 T cell stimulation by DCs after infection with the yopP knockout mutant was several orders of magnitude higher than for WA-C(pYV) and shows that YopP inhibits Ag presentation by DCs in vitro.

The most obvious reason for the inhibitory effect of YopP on Ag presentation by DCs would be the induction of programmed cell death. Up to now apoptosis induction by YopP has been demonstrated into DCs. One group showed a decrease in immunostimulatory capacity of DCs after infection with Y. enterocolitica of serotype O:3 (69, 70). The proliferative response of T cells was analyzed and an MLR was performed that showed an inhibitory effect on T cell proliferation. These effects were transient in nature and live yersiniae were not required suggesting other mechanisms than those described in our study. The authors could not detect any apoptosis or necrosis induction by yersiniae over the course of several days. These differences might be due to the different Yersinia strains used. Our study was performed with Y. enterocolitica serotype O:8, which is known to be a substantially more effective inducer of apoptosis than serotype O:3 (71).

Because neither primary T cell lines nor T cell hybridomas have been found to be susceptible to YopP/YopJ-mediated apoptosis induction (32), it is likely that DCs are a major target of YopP/YopJ and that this mechanism is responsible for the YopP-mediated CD8 T cell suppression in vivo. In this study, we would also like to emphasize that the yopP mutant still translocates the protein tyrosine phosphatase YopH, which is believed to suppress the T cell and B cell response (32, 33). Therefore, YopP is the first effector protein shown to inhibit the adaptive immune response in vivo and the second effector protein besides YopH shown to inhibit the adaptive immune response in a cell culture model.

Our results are consistent with the model in which yersiniae kill APC by programmed cell death to induce a “silent” nonimmunogenic death and to prevent the development of a T cell response. This is in contrast to studies of apoptosis induction by Mycobacterium tuberculosis (72) and Salmonella typhimurium (73). These facultative intracellular bacteria induce apoptosis of APC. In contrast to Yersinia, apoptosis has been shown to aid in the development of a CD8 T cell response by cross-presentation of apoptotic vesicles by bystander DCs. So, in contrast to the beneficial function of apoptotic cell death during infection with intracellular bacteria, apoptosis induction by extracellular yersiniae is detrimental to the host by inhibiting both innate and adaptive immune responses. Possibly bystander DCs are paralyzed by antiphagocytic Yops during Yersinia infection, which prevents cross-presentation of apoptotic blebs. In addition to programmed cell death as a possible mechanism for the in vivo CD8 T cell suppression, we found that Yersinia prevents maturation of DCs as evidenced by reduced costimulator (CD80 and CD86) expression by WA-C(pYV) but not by the yopP mutant.

One major question that arises is why yersiniae, which are considered to be predominately extracellular pathogens, would strive to inhibit the development of a CD8 T cell response in the host. CD8 T cells function to lyse infected target cells to release intracellular bacteria into the extracellular environment where they can be more effectively killed by activated macrophages. However, T cells are also known to produce large quantities of IFN-γ, which increases the bactericidal capacity of macrophages and this is known to be essential for overcoming Yersinia infection. CD8 T cells could have direct bactericidal activity against yersiniae. Such an effect has been shown for granulysin from CD8 T cells, which kills extracellular M. tuberculosis (74). Another possibility is that Y. enterocolitica survives intracellularly at some stage of infection, which has been suggested for Y. pestis (75) and Y. pseudotuberculosis (76). Possibly it is advantageous for Yersinia to prevent cytolysis of infected cells that have been reprogrammed by the injection of Yops to function in a favorable fashion for Yersinia. Finally the discovery that YopP inhibits the development of a CD8 T cell response will be useful for designing novel Y. enterocolitica based vaccine carrier strains translocating heterologous Ags for CD8 T cell induction.

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Disclosures

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References


