

Yersinia enterocolitica Adhesin A Induces Production of Interleukin-8 in Epithelial Cells

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The major invasive factor of *Yersinia enterocolitica*, the invasin (Inv) protein, induces proinflammatory host cell responses, including interleukin-8 (IL-8) secretion from human epithelial cells, by engagement of $\beta 1$ integrins. The Inv-triggered $\beta 1$ integrin signaling involves the small GTPase Rac; the activation of MAP kinases, such as p38, MEK1, and JNK; and the activation of the transcription factor NF- κ B. In the present study, we demonstrate that *Y. enterocolitica* YadA, which is a major adhesin of *Y. enterocolitica* with pleiotropic virulence effects, induces IL-8 secretion in epithelial cells. The abilities of YadA and Inv to promote adhesion to and invasion of HeLa cells and to induce IL-8 production by the cells were investigated by expression of YadA and Inv in *Escherichia coli*. While YadA mediates efficacious adhesion to HeLa cells, it mediates marginal invasion compared with Inv. Both YadA and Inv trigger comparable levels of IL-8 production. Conformational changes of the YadA head domain by mutation of NSVAIG-S motifs, which abolish collagen binding, also abolish adhesion of *Yersinia* to HeLa cells and YadA-mediated IL-8 secretion. Furthermore, experiments in which blocking antibodies against $\beta 1$ integrins were used demonstrate that $\beta 1$ integrins are crucial for YadA-mediated IL-8 secretion. Inhibitor studies demonstrate the involvement of small GTPases and MAP kinases, such as p38, MEK1, and JNK, indicating that $\beta 1$ integrin-dependent signaling mediated by Inv or YadA involves similar signaling pathways. These data present YadA, in addition to Inv, YopB, and *Yersinia* lipopolysaccharide, as a further inducer of proinflammatory molecules by which *Y. enterocolitica* might promote inflammatory tissue reactions.

Yersinia pseudotuberculosis and *Yersinia enterocolitica* are enteropathogenic to humans and cause gastrointestinal disorders, such as enterocolitis and mesenteric lymphadenitis, as well as reactive arthritis, erythema nodosum, and septicemia (5, 12). The pathogenicity of *Yersinia* is determined by a number of virulence factors that are encoded either by genes of the bacterial chromosome or by genes present in the pYV (*Yersinia* virulence) plasmid (8, 11). The factors encoded by the virulence genes comprise adhesins and invasins, such as invasin (Inv) and *Yersinia* adhesin A (YadA), as well as the secreted *Yersinia* outer proteins (Yops) (9, 10).

Previous studies demonstrated that Inv is important in the early phase of intestinal infection (39, 40), most likely for efficient translocation of the bacteria through the M cells and colonization of the Peyer's patches (47). The 192 and 195 carboxy-terminal amino acids of *Y. pseudotuberculosis* and *Y. enterocolitica* Inv proteins, respectively, are required for binding to $\beta 1$ integrins and induction of invasion (28, 31, 46, 58). Beads coated with the C-terminal 195 amino acids of *Y. enterocolitica* Inv may induce interleukin-8 (IL-8) synthesis by triggering the degradation of I κ B α and subsequent translocation of NF- κ B p50/p65 and p65/p65 dimers to the nucleus (46). Adhesion of the bacteria to the host cell without internaliza-

tion is adequate for the induced IL-8 expression (49), suggesting that Inv acts as a ligand for a receptor-mediated signaling pathway. Moreover, it was demonstrated that Rac1 and mitogen-activated protein (MAP) kinases, such as p38 and JNK, are involved in Inv-triggered IL-8 production (21).

Upon infection with *Yersinia* or other bacteria, such as *Salmonella*, *Shigella*, or *Escherichia coli* (enteropathogenic *E. coli*), intestinal epithelial cells produce a variety of proinflammatory cytokines, including IL-8 (29, 43). IL-8 is an important chemoattractant and activator of polymorphonuclear leukocytes, and epithelial cells (43) were considered "watchdogs" for the innate and adaptive immune system (15). The production of chemokines may play an ambiguous role in infections, as, e.g., *Yersinia* is resistant to phagocytosis by polymorphonuclear leukocytes. Therefore, recruitment of these cells may cause tissue disruption, dissemination of the bacteria, and disease rather than contribute to infection control (1, 20, 33).

The *inv* gene is expressed predominantly at 27°C (38). However, recent work has demonstrated that Inv is also expressed at 37°C at low pH (38). In vivo studies with Inv-deficient mutants revealed that the *inv* gene is not essential for establishing an infection. Thus, although the initial infection process in the Peyer's patches is delayed upon infection with *Y. enterocolitica* *inv* mutant strains, the 50% lethal dose of an *inv* mutant strain is unaltered (39).

In contrast, YadA appears to be essential for establishing an infection, as *yadA*-deficient *Y. enterocolitica* mutants are avirulent in mice (14, 37). YadA mediates adhesion to host cells and confers resistance against complement and defensin bac-

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description	Reference or source
<i>Y. enterocolitica</i> pYV ⁻	Serotype O:8; virulence plasmid-cured derivative of <i>Y. enterocolitica</i> WA-314	24
<i>Y. enterocolitica</i> pYV ⁻ Δ <i>inv</i>	Serotype O:8; virulence plasmid-cured derivative of <i>Y. enterocolitica</i> WA-314; <i>inv</i> gene is knocked out by a kanamycin resistance cassette	41
<i>E. coli</i> HB101	F ⁻ <i>D(gpt-proA)62 leuB6 glnV44 ara-14 galK2 lacY1 D(mcrC-mrr) rpsL20(Str^r) xyl-5 mtl-1 recA13</i>	42
pEEV1	pTM100 derivative with a SacI restriction site engineered in the <i>cat</i> gene	34
pYMS4505	<i>yadA</i> _{Y_eO3} cloned as a 1,753-bp PCR fragment into the BamHI site of pTM100; Cm ^r ; <i>yadA</i> under control of the interrupted tetracycline resistance gene of the vector	52
pYMS4505-M6	pYMS4505 expressing YadA-M6 (V156D, A157D, I158E); Cm ^r ; <i>yadA</i> under control of the interrupted tetracycline resistance gene of the vector	52
pYL8	<i>yadA</i> _{Δ83-104} and the <i>lcrF</i> genes of <i>Y. enterocolitica</i> O:3 cloned into pTM100; Cm ^r ; under control of the natural <i>yadA</i> promoter and <i>lcrF</i>	53
pNIV 136	Derivative of pIN-III-ompA2; artificial DNA cloning vector (gi392964, AJ223122)	19, 49
pInv1914	Derivative of pNIV136, expressing Inv of <i>Y. enterocolitica</i> O:9 under control of an IPTG-inducible <i>lpp</i> promoter containing a <i>lacUV5</i> promoter-operator region	45
pYadA ^{O:8}	Derivative of pNIV136 expressing YadA ^{O:8} under control of an IPTG-inducible <i>lpp</i> promoter containing a <i>lacUV5</i> promoter-operator region	This study

tericidal activity (56). Structural analysis of YadA revealed lollipop-shaped surface projections composed of trimeric YadA proteins (25, 26, 36). Although there is evidence that YadA may bind to host cell β1 integrins, its role in host-pathogen interaction is still unclear. Recent data has suggested that under certain growth conditions (low Ca²⁺ and high Mg²⁺ concentrations) or by overexpression of YadA in *E. coli*, YadA of *Y. pseudotuberculosis* mediates uptake into host cells, possibly via extracellular-matrix-dependent bridging between YadA and host cell β1 integrins (16). From these data, it was concluded that YadA mediates internalization into host cells under environmental conditions in which Inv is repressed. Although inhibitor studies revealed that Ser/Thr kinases, as well as phosphatidylinositol-3-kinase, are involved in the uptake process, the host cell signaling processes activated by YadA are not clear.

The aim of the study presented here was to investigate whether YadA, like Inv, might induce cytokine production in host cells. Moreover, we wanted to know whether YadA also exploits β1 integrin signaling pathways to accomplish this. Finally, we addressed whether the signaling checkpoints, small GTPases and MAP kinase, are involved in YadA-triggered cytokine production. Our results indicate that YadA in fact induces activation of a signaling pathway leading to NF-κB activation and IL-8 production, suggesting that the induction of inflammatory host cell responses should be added to the list of pleiotropic virulence functions of YadA.

MATERIALS AND METHODS

DNA constructs. The plasmids pYMS4505 (52), expressing *Y. enterocolitica* YadA from serotype O:3; pYMS4505-M6 (52); pYL8 (53), expressing *Y. enterocolitica* YadA^{O:3} mutants; and pEEV1 (with an additional restriction site compared to pTM100), used as a control, have been described previously and are derived from the expression vector pTM100 (34) (Table 1).

The vector pYadA^{O:8} was constructed by amplifying the *yadA* gene from pYV plasmid *Y. enterocolitica* serotype O:8 by PCR using the forward primer 5'-CC ATGGGAAGCTAAAGATTTTAAGATCAGTG-3' (the NcoI restriction site is underlined) and the reverse primer 5'-AGATCTATTAGGATTAATACAGGC GCAg-3' (the BglII restriction site is underlined) according to the published sequence (51). The amplified DNA fragments (digested with NcoI and BglII) were cloned into the pNIV136 expression vector, resulting in pYadA^{O:8}.

Bacterial strains and growth conditions. All of the bacterial strains used are listed in Table 1. Plasmid-cured *Y. enterocolitica* WA-314 pYV⁻ (24), a *Y. enterocolitica* WA-314 pYV⁻ Δ*inv* mutant (41), noninvasive *E. coli* HB101 pNIV136 (49), and *E. coli* HB101 pInv1914 expressing *Y. enterocolitica* Inv under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter (49) and *E. coli* HB101 pYadA^{O:8} expressing *Y. enterocolitica* YadA from serotype O:8 under the control of an IPTG-inducible promoter (this work) were grown in Luria-Bertani broth (LB). The same medium was used for the cultivation of *E. coli* and *Y. enterocolitica* WA-314 pYV⁻ Δ*inv* pYMS4505 expressing *Y. enterocolitica* YadA from serotype O:3 and *E. coli* and *Y. enterocolitica* WA314 pYV⁻ Δ*inv* pYMS4505-M6 and pYL8 (52), both expressing collagen-binding mutants of YadA, and *E. coli* and *Y. enterocolitica* WA314 pYV⁻ Δ*inv* pEEV1 (used as a control).

Cell culture and infection. Human HeLa cervical epithelial cells (ATCC CCL-2.1; American Type Culture Collection, Manassas, Va.) were maintained in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 2 mM glutamine (Invitrogen, Karlsruhe, Germany) and 10% fetal calf serum (Sigma, Taufkirchen, Germany). The fibroblast-like GD25 and GD25-β1A cell lines were a kind gift from R. Faessler (MPI, Munich, Germany). The murine GD25 cells were derived from the embryonic stem cell clone G201, which is deficient in the β1 integrin subunit (18). The stably transformed cell line GD25β-1A was obtained by electroporating wild-type integrin β-1A cDNA into GD25 cells (57). GD25 cells which did not express β1 integrins were cultivated in DMEM (Gibco, Karlsruhe, Germany) supplemented with 2 mM glutamine (Invitrogen) and 10% FCS (Sigma), and the GD25-β1A cell line expressing β1 integrins was maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10 μg of puromycin (Sigma)/ml. For infection, cells were grown overnight in 24-well plates and infected with either *Y. enterocolitica* or *E. coli* strains expressing Inv, YadA, or YadA mutants. Before infection, the cells were washed three times with prewarmed phosphate-buffered saline (PBS), and fresh RPMI 1640 medium was added. The bacteria were grown overnight in LB at either 27°C for *Y. enterocolitica* WA-314 pYV⁻ and *Y. enterocolitica* WA-314 pYV⁻ Δ*inv* or 37°C for *Y. enterocolitica* WA-314 pYV⁺ and *E. coli* strains. The bacteria were diluted 1:10 in LB and grown for another 2 to 3 h at 27 or 37°C. If necessary, IPTG was added to a final concentration of 0.1 mM 30 min before the bacteria were harvested. The bacteria were collected by centrifugation and washed twice with PBS. After determination of the optical density at 600 nm, appropriate dilutions of the bacteria in PBS were performed to infect the cells with a multiplicity of infection (MOI) of 100 bacteria/cell. An MOI of 100 was used in all infection experiments. Monolayers of cells and bacteria were incubated for 1 h at 37°C. After removal of the medium, the cells were washed three times with PBS to remove extracellularly located bacteria, and fresh medium with 100 μg of gentamicin/ml to kill remaining extracellular bacteria was added. After a further 3 h, the supernatants of the cells were collected and the levels of cytokines were determined by enzyme-linked immunosorbent assay (ELISA). TNF-α was a kind gift from G. Adolf (Bender, Vienna, Austria).

Cell adhesion and invasion assays. For preparation of the cell adhesion and uptake assay, 10⁵ HeLa or GD25 cells per well were seeded and grown overnight

in 24-well cell culture plates (Nunc Life Technologies, Wiesbaden, Germany) to a confluent cell layer to avoid adherence of the bacteria to the plastic. Cell monolayers were washed once with phosphate-buffered saline and incubated for 2 h with fresh RPMI 1640 or DMEM without antibiotics before the addition of bacteria (MOI, 100). For blocking studies, increasing concentrations (from 0 to 10 $\mu\text{g/ml}$) of the anti-human monoclonal $\beta 1$ integrin (CD29) antibody Lial/2 (Immunotech, Marseille, France) were added to the medium 0.5 h before infection. For adhesion assays, the cells were washed three times with phosphate-buffered saline 15 min postinfection. The total number of adherent bacteria was determined by cell lysis with 1% Triton X-100 and plating on selective LB agar plates. Bacterial invasion was assessed 15, 30, and 60 min after infection. After this time, the cells were washed three times with phosphate-buffered saline, new medium with 100 μg of gentamicin/ml was added, and the cells were incubated for another 2 h at 37°C and then lysed with 1% Triton X-100 and plated on selective LB agar plates in appropriate dilutions. All adhesion and invasion experiments for each condition were performed in duplicate, and at least three independent experiments were done.

Inhibitors. *Clostridium difficile* toxin TcdB10463 was purified as previously described (6, 35). The MAP kinase inhibitors SB202190, PD98059, and SP600125 were purchased from Calbiochem (Schwalbach, Germany).

IL-8 ELISA. The amount of IL-8 secreted by HeLa cells into the supernatant was determined as previously described (49) using an ELISA with optimal concentrations of a mouse anti-human IL-8 monoclonal antibody (G265-5; BD Biosciences Pharmingen, San Diego, Calif.) and a biotinylated mouse anti-human IL-8 monoclonal antibody (G265-8; BD Biosciences Pharmingen) as the detecting antibody. IL-8 concentrations were calculated from the straight-line portion of a standard curve derived using recombinant human IL-8 (BD Biosciences Pharmingen).

Electrophoretic mobility shift assay (EMSA). HeLa cells (5×10^6) were infected as described above. At various intervals postinfection, nuclear extracts were prepared as previously described (44). Aliquots of the supernatant containing nuclear proteins were stored at -80°C . Protein concentrations were determined by the Bradford assay. The oligonucleotide probes described below were labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Amersham Biosciences, Uppsala, Sweden) using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) and then purified on a NucTrap probe purification column (Stratagene, Amsterdam, The Netherlands). The following oligonucleotides were used: IL-8- κB wild type (5'-ATCG TGGAATTCCTCTGA-3'; Metabion, Munich, Germany) and IL-8- κB mutant (5'-ATCCTGCAATGTCGTCTGA-3'; Metabion). 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 poly(dI-dC) for NF- κB shifts. Samples were resolved on a 5% nondenaturing polyacrylamide gel using $0.5 \times \text{TBE}$ (25 mM Tris-HCl, 25 mM boric acid, 0.5 mM EDTA) as a running buffer. The gels were transferred to Whatman 3M paper and dried under vacuum. Protein binding was detected by autoradiography.

Immunofluorescence microscopy. HeLa cells were seeded on coverslips and grown overnight at 37°C. Cells were infected with *E. coli* pYadA^{O:8} or *E. coli* pInv1914 for 2 h. After 3% paraformaldehyde (Sigma) fixation of the monolayers, extracellular *E. coli* pYadA^{O:8} was stained with a polyclonal rabbit anti-P1.8 antibody (diluted 1:100) and *E. coli* pInv1914 was stained with a polyclonal rabbit anti-Inv 3.1 antibody (diluted 1:100), followed by Cy-5-conjugated goat anti-rabbit antibodies (Dianova, Hamburg, Germany). After three washings with phosphate-buffered saline, the cells were permeabilized by 2% Triton X-100 in phosphate-buffered saline and washed, and intracellular bacteria were stained by polyclonal rabbit anti-P1.8 antibody or polyclonal rabbit anti-Inv 3.1 antibody, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (Dianova). F-actin was stained by tetramethyl rhodamine isothiocyanate-conjugated phalloidin (Sigma). The fluorescence images were obtained with a DM IRE2 confocal laser scan microscope (Leica, Wetzlar, Germany).

SDS-PAGE and Western blot analysis. Bacterial strains were lysed in sodium dodecyl sulfate (SDS) sample buffer ($\sim 5 \times 10^7$ *E. coli* bacteria were loaded per lane). Separation of the proteins was performed using SDS-polyacrylamide gel electrophoresis (PAGE). For immunoblotting, the proteins were transferred electrophoretically onto polyvinylidene difluoride membranes (1.2 mA/cm² for 60 min). The membranes were blocked overnight with PBS-5% milk at 4°C. For detection of Inv, a polyclonal rabbit anti-Inv 3.1 antibody (diluted 1:1,000) and a peroxidase-conjugated secondary anti-rabbit antibody (diluted 1:5,000; Amersham Biosciences) were added for 1 h each. For detection of YadA, a polyclonal rabbit anti-P1.8 antibody (diluted 1:1,000) and a peroxidase-conjugated secondary anti-rabbit antibody (diluted 1:5,000; Amersham Biosciences) were added for 1 h each. The detection was carried out using the ECL detection kit from Amersham Biosciences.

Statistics. The data shown in the figures are from representative experiments. Comparable results were obtained in at least two additional experiments. Differences between mean values were analyzed using Student's *t* test. A *P* value of <0.05 was considered statistically significant.

RESULTS

YadA mediates adhesion to and upregulation of IL-8 in HeLa cells. As previously shown (45, 46, 48, 49), Inv is able to induce proinflammatory cytokines, such as IL-8, and a high number of other gene products (4) in a $\beta 1$ integrin-dependent manner. Based on reports demonstrating that YadA adheres to and promotes uptake into host cells via $\beta 1$ integrins (16, 50, 54), we speculated that YadA may also trigger IL-8 secretion.

To address this question, YadA and Inv were cloned into the IPTG-inducible vector pNIV136 and transformed into *E. coli* HB101. Overexpression of YadA and Inv to comparable degrees by *E. coli* HB101 was confirmed by Western blot analysis and SDS-PAGE, as indicated in Fig. 1A. Since adhesion is a prerequisite for Inv-induced IL-8 secretion, we next compared the adhesion of Inv- and YadA-expressing *E. coli* to HeLa cells. For this purpose, HeLa cells were infected with *E. coli* HB101 harboring the vector pNIV136 (vector control), pInv1914 (*inv*⁺), or pYadA^{O:8} (*yadA*⁺), and the number of bacteria adhering to epithelial cells was determined 15 min postinfection (Fig. 1B). We found that upon infection of HeLa cells with either *E. coli* pInv1914 or *E. coli* pYadA^{O:8}, adherence to HeLa cells was significantly ($P < 0.05$) increased compared to that with the control strain *E. coli* HB101 pNIV136 (Fig. 1B). Moreover, YadA overexpressed in *E. coli* mediated a twofold-higher adhesion to HeLa cells than Inv overexpressed in *E. coli*.

To investigate the role of $\beta 1$ integrins in adhesion, infection experiments with the fibroblast cell line GD25, which lacks $\beta 1$ integrin expression, and the cell line GD25- $\beta 1\text{A}$, in which $\beta 1$ integrins are overexpressed, were performed. In general, adhesion to GD25- $\beta 1\text{A}$ fibroblasts mediated by YadA was slightly but not significantly higher than adhesion mediated by Inv. Adhesion of *E. coli* pInv1914 or *E. coli* pYadA^{O:8} to GD25 fibroblasts was significantly reduced (75 and 76%) compared to adhesion to GD25- $\beta 1\text{A}$ cells (Fig. 1C). These data show that $\beta 1$ integrins are required for YadA- and Inv-mediated adhesion.

To determine internalization of *E. coli* pInv1914 or *E. coli* pYadA^{O:8}, gentamicin killing assays (Fig. 1D) and immunostaining (Fig. 1E) were performed. Inv- or YadA-expressing *E. coli* strains showed increased internalization compared to *E. coli* pNIV136 in a time-dependent manner. However, YadA O:8 was much less efficient in mediating internalization than Inv. Immunostaining of *E. coli* pInv1914- or *E. coli* pYadA^{O:8}-infected HeLa cells confirmed these results (Fig. 1E). Thus, upon infection with *E. coli* pYadA^{O:8}, most bacteria were extracellularly located and only a few were internalized.

To determine whether YadA triggers IL-8 secretion, HeLa cells were infected with *E. coli* pNIV136, *E. coli* pInv1914, or *E. coli* pYadA^{O:8}, and IL-8 secretion was detected at different time points postinfection. YadA- and Inv-expressing *E. coli* strains induced comparable amounts of IL-8 6 h postinfection (Fig. 2A). However, the onset of IL-8 secretion induced by

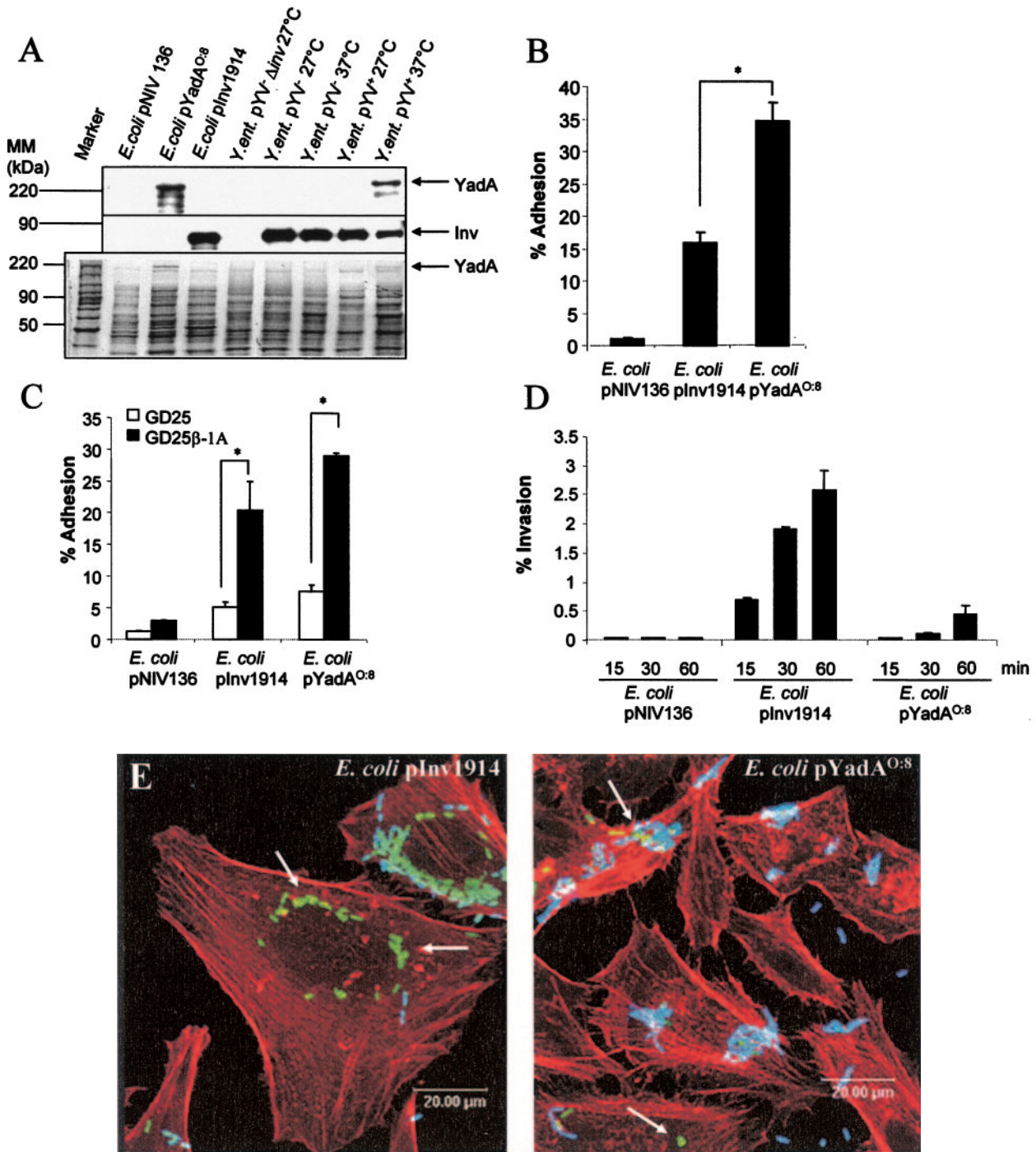


FIG. 1. YadA- and Inv-mediated adhesion and invasion. (A) Expression of YadA and Inv of *E. coli* pYadA^{O:8}, *E. coli* pInv1914, *Y. enterocolitica* pYV⁺, and *Y. enterocolitica* pYV⁻. *Yersinia* and *E. coli* strains were grown overnight in LB at 27 and 37°C and subcultured for a further 3 h at the indicated temperatures. Subsequently, Western blot analysis using polyclonal antibodies against Inv (middle) and YadA (top) was performed. In parallel, SDS-PAGE (bottom) was performed as a loading control. Inv and YadA expression is indicated by arrows. (B) HeLa cells were infected with Inv- or YadA-expressing bacteria (MOI, 100). Adhesion of bacteria to HeLa cells was determined 15 min postinfection, as described in Materials and Methods. The asterisk indicates a significant difference between adhesion of *E. coli* pYadA^{O:8} and that of *E. coli* pInv1914 to HeLa cells ($P < 0.05$). The error bars indicate standard deviations. (C) GD25 cells were infected with Inv- or YadA-expressing bacteria (MOI, 100). Adhesion of bacteria to GD25-β1A and GD25 cells was determined 15 min postinfection. The asterisks indicate a significant difference between the adhesion of *E. coli* pInv1914 and that of *E. coli* pYadA^{O:8} to GD25 cells versus GD25-β1A cells ($P < 0.05$). (D) Uptake of bacteria into HeLa cells was analyzed by determining the number of intracellularly viable bacteria using gentamicin killing at the indicated time points. (E) Internalization of *E. coli* pInv1914 or *E. coli* pYadA^{O:8} into HeLa cells was visualized by immunofluorescence using polyclonal antibodies against Inv or YadA, respectively, as indicated in Material and Methods. Overlays of multicolor staining are shown. The cytoskeleton was stained with phalloidin-tetramethyl rhodamine isothiocyanate (red). Extracellularly localized bacteria, blue and green staining; internalized bacteria, exclusively green staining. The arrows indicate examples of intracellularly located bacteria.

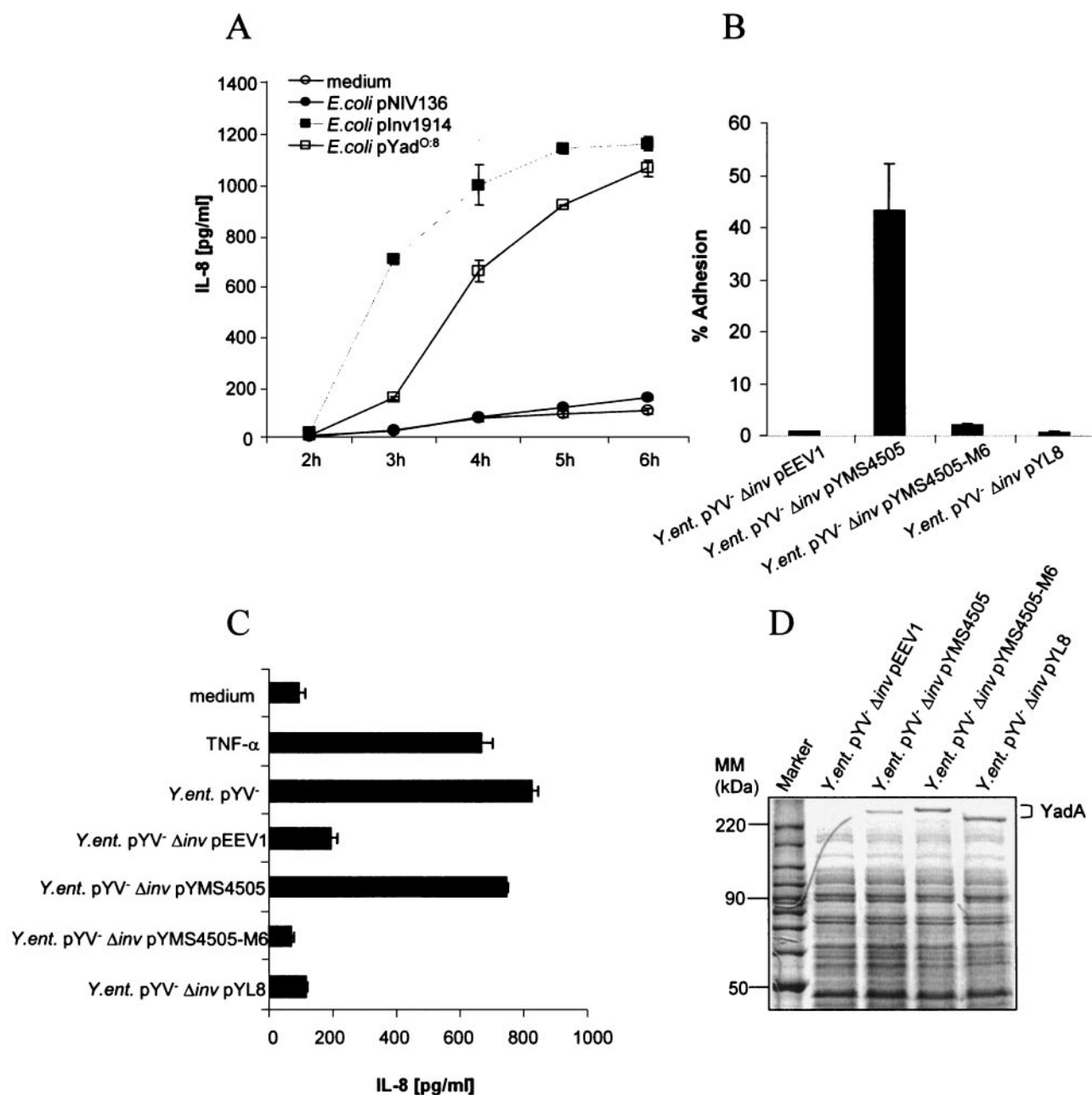


FIG. 2. IL-8 secretion by and adhesion to HeLa cells upon infection with *E. coli* or *Y. enterocolitica* expressing YadA and/or Inv (MOI, 100). HeLa cells were infected (A) with *E. coli* strains or stimulated with TNF- α for the indicated times or (B) with *Y. enterocolitica* strains for 4 h. IL-8 levels in culture supernatants were determined by ELISA. The error bars indicate standard deviations. (C) HeLa cells were infected with the indicated *Yersinia* strains for 15 min, and subsequently, adhesion assays were performed as described in Materials and Methods. The experiments were representative of three further independent experiments. (D) SDS-PAGE was performed to show YadA protein expression of *Yersinia* strains used in panels B and C. *E. coli* strains were grown in LB at 37°C, and *Yersinia* strains were grown overnight at 27°C and subcultured for 3 h the next day at 27 or 37°C as indicated. MM, molecular mass.

YadA was time delayed compared to Inv-induced IL-8 secretion.

As described previously, mutation of NSVAIG-S motifs in the head domain of YadA abrogates collagen binding due to conformational change of the structure of the YadA head domain (36, 52). To address whether these structural changes of YadA affect *Y. enterocolitica* YadA-mediated adhesion and

IL-8 induction, HeLa cells were infected with *Y. enterocolitica* pYV⁻ Δ inv transformed with control vector pEEV1, vector pYMS4505 expressing YadA O:3, or vector pYMS4505-M6 or pYL8, which carry YadA O:3 mutants deficient in collagen binding. HeLa cells were infected with different *Y. enterocolitica* strains, and adhesion, as well as IL-8 secretion, was detected (Fig. 2B and C). Furthermore, SDS-PAGE was

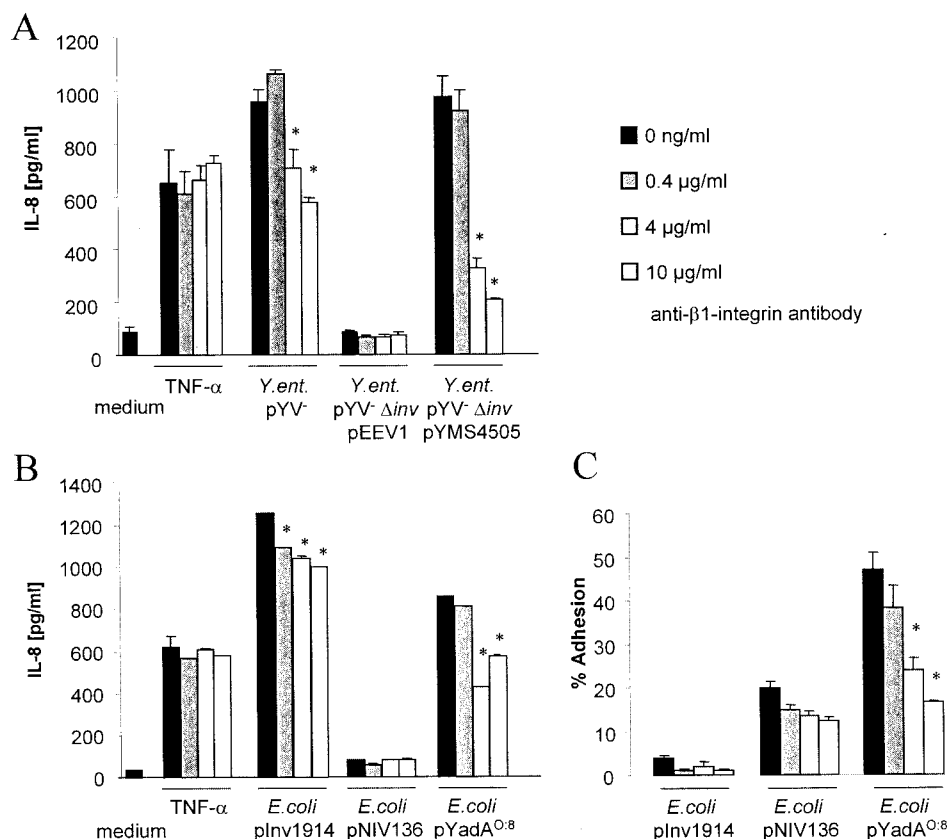


FIG. 3. *YadA*-mediated IL-8 secretion and adhesion by HeLa cells is β 1 integrin dependent. Prior to infection of HeLa cells with *Yersinia* strains (A) or *E. coli* strains (B and C), the cells were incubated with the indicated amounts of blocking anti- β 1 integrin antibody Lia1/2. IL-8 levels in culture supernatants were determined by ELISA 4 h postinfection (A and B) or adhesion experiments were performed as indicated in Materials and Methods (C). The experiments are representative of three further independent experiments. The error bars indicate standard deviations.

performed to show *YadA* expression of the *Yersinia* strains used in these experiments (Fig. 2D). Adhesion assays revealed that in contrast to *Y. enterocolitica* pYV⁻ Δ inv pYMS4505 expressing *YadA* O:3, the strains *Y. enterocolitica* pYV⁻ Δ inv pYMS4505-M6 and *Y. enterocolitica* pYV⁻ Δ inv pYL8, which carry mutations of either the *YadA* head-forming domain M2/3 or M6, and *Y. enterocolitica* pYV⁻ Δ inv pEEV1, which carries a control vector, were not able to adhere to HeLa cells (Fig. 2B). In a similar manner, IL-8 secretion was significantly increased compared to basal IL-8 levels only upon infection with *Y. enterocolitica* pYV⁻ Δ inv pYMS4505 but not upon infection with *Y. enterocolitica* pYV⁻ Δ inv pYMS4505-M6, *Y. enterocolitica* pYV⁻ Δ inv pYL8, or *Y. enterocolitica* pYV⁻ Δ inv pEEV1 (Fig. 2C). Taken together, these data demonstrate that *YadA*, like *Inv*, can induce IL-8 secretion in epithelial cells. Moreover, conformational changes in the head domain of *YadA* affect collagen binding (36, 52), adhesion, and induction of IL-8 secretion.

To investigate, whether IL-8 secretion mediated by *YadA* involves β 1 integrins, the β 1 integrin-specific blocking antibody Lia1/2 was added prior to infection of HeLa cells with *Inv*- or *YadA*-expressing *Yersinia* strains (Fig. 3A). *Inv*- and *YadA*-induced IL-8 secretion levels were significantly reduced (40 and 79%) by the addition of neutralizing anti- β 1 integrin antibodies. The impact of anti- β 1 integrin antibodies on IL-8

secretion mediated by *YadA* was even higher than that on *Inv*-induced IL-8 secretion. To exclude the possibility that the higher impact of anti- β 1 integrin antibodies on IL-8 secretion mediated by *YadA* compared to that on IL-8 secretion mediated by *Inv* might be due to different expression levels of *YadA* and *Inv*, neutralization experiments were performed using *E. coli* strains expressing *Inv* and *YadA* in the same vector system under the control of the same promoter (IPTG-inducible *lpp* promoter) (Fig. 3B). *Inv*- and *YadA*-induced IL-8 secretion levels were significantly reduced (21 and 49%). To address whether reduced IL-8 secretion corresponds to reduced adhesion, adhesion assays were performed (Fig. 3C). Pretreatment with anti- β 1 integrin antibodies significantly reduced adhesion of *E. coli* pInv1914 and *E. coli* pYadA^{O:8} (64 and 49%), indicating that reduced adhesion to β 1 integrins corresponds to reduced IL-8 secretion.

***YadA* induces IL-8 expression by engagement of Rho GTPases, MAP kinase cascade, and NF- κ B activation.** To investigate whether signaling events activated by *YadA* involve the same pathways as that elicited by *Inv*, we studied whether *YadA* leads to NF- κ B activation. HeLa cells were infected with *E. coli* expressing either *YadA* or *Inv*, and subsequently, nuclear extracts were tested by EMSA for binding activity to an NF- κ B consensus sequence (Fig. 4). Indeed, like *Inv* but to a lesser extent, *YadA* induced binding of NF- κ B to the NF- κ B

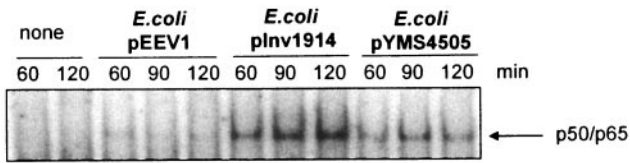


FIG. 4. Determination by EMSA of NF-κB activation in nuclear extracts of HeLa cells after stimulation with Inv- or YadA-expressing *E. coli*. Sixty, 90, or 120 min postinfection, nuclear extracts were prepared and analyzed in gel shift experiments with ³²P-labeled NF-κB consensus probes. The experiments are representative of three further independent experiments.

consensus sequence. The involvement of Rho GTPases was tested by treating the cells with *C. difficile* toxin TcdB10463, which inhibits the Rho GTPases Rac, Rho, and Cdc42, prior to infection with YadA- or Inv-expressing *E. coli* (Fig. 5A). TcdB10463 inhibits Inv- and YadA-mediated IL-8 secretion by >60%, indicating the involvement of one or more of these Rho GTPases in IL-8 secretion. The involvement of MAP kinases was tested by using a specific inhibitor of p38 (SB202190), MEK1 (PD98059), or JNK (SP600125). The addition of SB202190, PD98059, or SP600125 reduced YadA-induced IL-8 secretion up to 70, 67, or 69%, respectively (Fig. 5B to D). All three MAP kinase inhibitors, SB202190, PD98059, and

SP600125, reduced YadA- and Inv-mediated IL-8 secretion in a dose-dependent manner. Taken together, these data show that the signaling cascade leading to IL-8 secretion mediated by YadA involves β1 integrins; Rho GTPases; the MAP kinases MEK1, JNK, and p38; and NF-κB activation.

DISCUSSION

Infection of epithelial cells with *Y. enterocolitica* triggers the expression of a high number of genes, including genes encoding proinflammatory molecules, such as IL-8 (4). Inv was shown to be a major inducer of IL-8 expression and secretion. Plasmid-encoded factors, such as YopP, are able to counteract this response (4). The induction of a proinflammatory response by Inv is known to be dependent on adhesion to β1 integrins. Taking into account that β1 integrins are also involved in adhesion via YadA, we hypothesized that YadA may also be involved in triggering a proinflammatory host response. Experiments in this study confirmed this hypothesis and demonstrated that YadA can mediate IL-8 secretion. Moreover, we showed that, like Inv, β1 integrins are involved in this process. Furthermore, the downstream signaling events triggered by YadA involve the same components as in Inv-mediated signaling, namely, Rho GTPases and the MAP kinases MEK1, JNK, and p38.

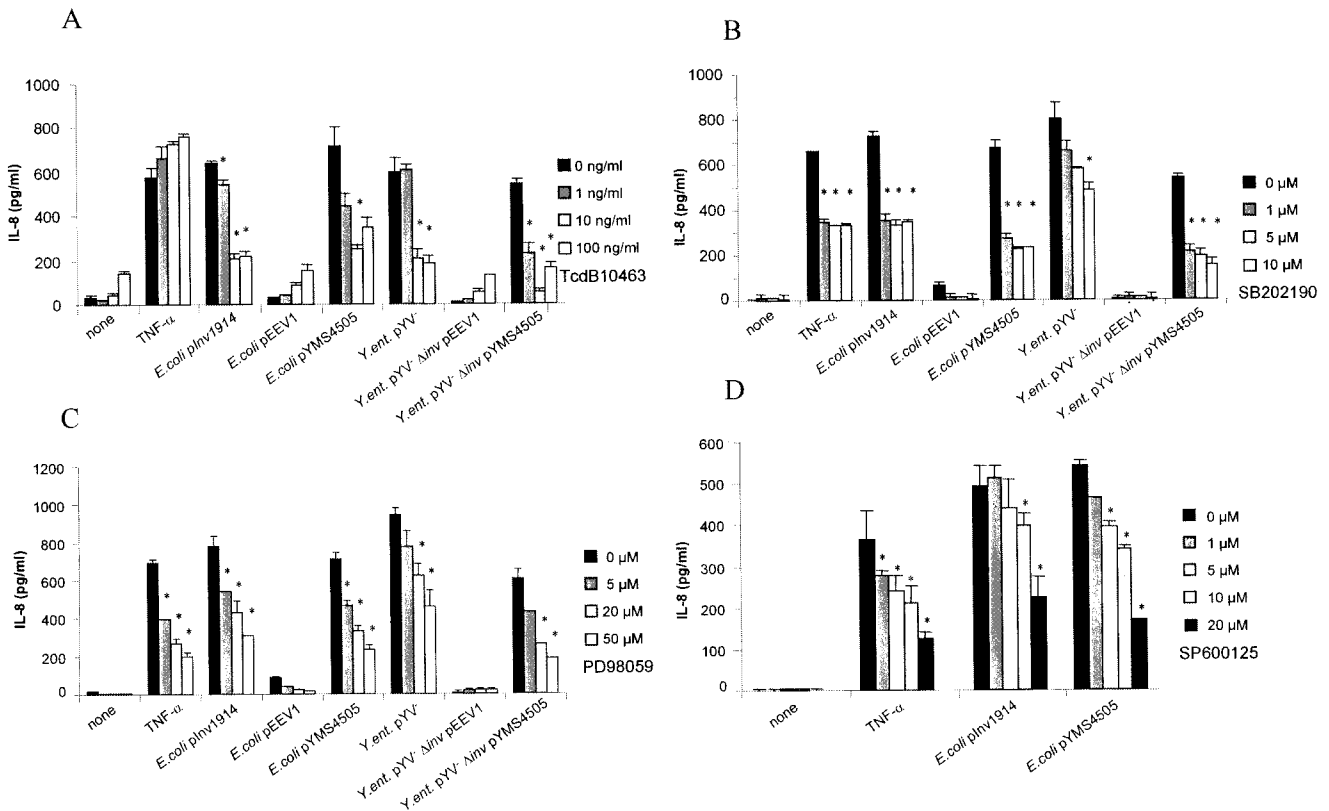


FIG. 5. Modulation of YadA- and Inv-induced IL-8 secretion by inhibitory drugs. HeLa cells were treated with (A) *C. difficile* toxin TcdB10463 (an inhibitor of Rac, Rho, and Cdc42) 24 h prior to infection or (B) SB202190 (a p38 inhibitor), (C) PD98059 (a MEK1 inhibitor), or (D) SP600125 (a JNK inhibitor) 30 min prior to infection with Inv- or YadA-expressing bacteria or stimulation with tumor necrosis factor alpha (TNF-α) (50 ng/ml). IL-8 production was determined by ELISA in supernatants collected 4 h after infection. The experiments are representative of three further independent experiments. The asterisks indicate a significant difference between IL-8 secretion of infected or stimulated cells treated with inhibitors compared with that of untreated cells ($P < 0.05$). The error bars indicate standard deviations.

YadA was expressed either in *E. coli* strains or in plasmid-cured *Yersinia* strains. These strains do not express any of the virulence factors (e.g., YopP) that are known to counteract a proinflammatory response induced by *Y. enterocolitica* in vitro. In this setting, YadA and Inv are similarly effective in mediating adhesion to GD25- β 1A fibroblasts and even more effective in mediating adhesion to HeLa cells, as proven with *E. coli* overexpressing YadA and Inv by the same vector system. The different efficacies of YadA and Inv in adhering to HeLa and GD25- β 1A cells may be due to cell-type-specific differences.

By using the fibroblast cell line GD25 (18), which lacks β 1 integrin expression in comparison with the fibroblast cell line GD25- β 1A overexpressing β 1 integrins (57), we demonstrated that β 1 integrins are crucial for YadA-mediated adhesion of *Yersinia* to cells. The data presented here are in line with previous reports (3, 16) that β 1 integrins are required for YadA-mediated binding to cells. Eitel and Dersch (16) showed that YadA of *Y. pseudotuberculosis* can mediate highly efficient uptake into epithelial cells when overexpressed in *E. coli* or when *Y. pseudotuberculosis* is grown under specific growth conditions. This process can be blocked using neutralizing antibodies against fibronectin and β 1 integrins, which led to the suggestion that internalization mediated by YadA occurs via extracellular-matrix-dependent bridging between YadA and the host cell β 1 integrin receptors (16).

In line with previous data (23) and in contrast to YadA of *Y. pseudotuberculosis*, even overexpression of *Y. enterocolitica* YadA can only weakly mediate uptake of *Y. enterocolitica* into epithelial cells. Thus, YadA of *Y. enterocolitica* is much less efficient (18-fold) in mediating uptake than Inv of *Y. enterocolitica*. The reason why YadA of *Y. enterocolitica* may be less efficient in mediating uptake than *Y. pseudotuberculosis* is unclear. Although the amino acid sequence of YadA is highly homologous between the two species, there are two additional amino acid sequences in the head domain of *Y. pseudotuberculosis* and one additional amino acid sequence in the stalk domain of *Y. enterocolitica*. One can speculate that these sequences could be responsible for the different ability to invade cells mediated by YadA. Further studies will be necessary to address this question.

The interaction between the *Yersinia* Inv protein and the β 1 integrin receptor permits bacterial attachment to the host cell and triggers internalization (27). Previous studies uncovered a role for Inv-mediated adhesion in the production of IL-8 in epithelial cells infected with *Y. enterocolitica* (30, 45, 49). In this study, we demonstrate that YadA expressed in *E. coli* in the same vector system as Inv also induces IL-8 secretion. Since both YadA and Inv mediate adhesion but only Inv efficiently mediates uptake, these data support previous studies showing that adhesion, but not internalization, of *Yersinia* is essential for triggering IL-8 secretion via β 1 integrins (4, 49).

Tahir et al. (52) reported that NSVAIG-S motifs in the N-terminal half of YadA are required for collagen binding. A recent study revealed that mutations of these putative collagen binding domains are located in the center of the YadA trimers and therefore cannot be directly involved in collagen binding. Mutation of these domains led to conformational changes of the head domain of YadA, which abolishes collagen binding. Interestingly, it was also shown that binding of *Y. enterocolitica* to fibronectin and laminin is not affected by these mutations

(52). Using *Y. enterocolitica* pYV⁻ Δ inv-expressing YadA mutants in which different NSVAIG-S motifs were mutated, we demonstrated that these mutations were sufficient to abolish YadA-mediated adhesion and subsequently IL-8 secretion. This is in keeping with the idea that collagen binding could be involved in adhesion and thus in subsequent IL-8 secretion.

Adhesion mediated by YadA and Inv is a prerequisite for IL-8 secretion. At least for HeLa cells, YadA mediates stronger adhesion than Inv. In contrast, the time delay of IL-8 secretion mediated by YadA compared to that mediated by Inv indicates that YadA is less effective in mediating IL-8 secretion than Inv. Moreover, YadA-mediated adhesion to and IL-8 secretion by HeLa cells can be blocked more efficiently by anti- β 1 integrin antibodies than Inv-mediated adhesion and IL-8 secretion. Previously obtained results showed high-affinity binding of Inv to integrins (13), which may explain why blocking of adhesion and IL-8 secretion with antibodies is not effective. In contrast, there is evidence that the binding of YadA to β 1 integrins may be an indirect effect via bridging to extracellular matrix proteins, such as fibronectin (16). Low-affinity binding to β 1 integrins via extracellular matrix proteins may explain the more efficient adhesion and the delayed onset of IL-8 secretion, as well as the higher blocking capacities of anti- β 1 integrin antibodies.

The involvement of β 1 integrins in Inv- and YadA-triggered IL-8 secretion also indicated a common signaling pathway for YadA- and Inv-triggered proinflammatory responses. By cross-linking β 1 integrins with antibodies, it was clearly shown that β 1 integrins are directly involved in a signal cascade leading to IL-8 secretion via Rac1 and MAP kinase-p38 signaling pathways (32). Similarly, for Inv-mediated IL-8 secretion, it was shown that Rac1 is required for activation of NF- κ B (20, 46) and that Inv activates the MAP kinases p38, MEK1, and JNK (20). The comparison of Inv- and YadA-mediated signaling presented here confirmed that the factors involved in Inv-mediated signaling leading to IL-8 secretion are also required for YadA-mediated signaling.

Taking these data together, we can conclude that YadA, in addition to a number of other biological properties, such as serum resistance (2, 7, 17), arthrogenicity (22), and epithelial-cell adhesion (23), contributes to the proinflammatory response of the host to infection with *Y. enterocolitica*. Our results and recent observations suggest that factors of *Yersinia* which can induce a proinflammatory response involving NF- κ B activation seem to be very redundant. Thus, Viboud et al. (55) demonstrated for *Y. pseudotuberculosis* that YopB can also induce IL-8 by activation of the small GTPase Ras and NF- κ B. However, the actual contributions of these various factors, like Inv, YadA, and YopB, as well as lipopolysaccharide, to triggering a proinflammatory response in vivo in different cell types in concert with the mechanisms of *Yersinia* which counteract the proinflammatory response in vivo are still unclear and will have to be addressed in future studies.

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