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Integrin-Mediated First Signal for Inflammasome Activation in Intestinal Epithelial Cells

Josephine Thinwa,* Jesus A. Segovia,⁎† Santanu Bose,⁎†1 and Peter H. Dube⁎†

How intestinal epithelial cells (IECs) recognize pathogens and activate inflammasomes at intestinal surfaces is poorly understood. We hypothesized that IECs use integrin receptors to recognize pathogens and initiate inflammation within the intestinal tract. We find that IECs infected with Yersinia enterocolitica, an enteric pathogen, use β1 integrins as pathogen recognition receptors detecting the bacterial adhesin invasin (Inv). The Inv–integrin interaction provides the first signal for NLRP3 inflammasome activation with the type three secretion system translocon providing the second signal for inflammasome activation, resulting in release of IL-18. During infection, Yersinia employs two virulence factors, YopE and YopH, to counteract Inv-mediated integrin-dependent inflammasome activation. Furthermore, NLRP3 inflammasome activation in epithelial cells requires components of the focal adhesion complex signaling pathway, focal adhesion kinase, and rac1. The binding of Inv to β1 integrins rapidly induces IL-18 mRNA expression, suggesting integrins provide a first signal for NLRP3 inflammasome activation. These data suggest integrins function as pathogen recognition receptors on IECs to rapidly induce inflammasome-derived IL-18–mediated responses. The Journal of Immunology, 2014, 193: 000–000.

Mucosal surfaces are exclusively sensitive to inflammation-mediated immune pathologies, necessitating strict regulation of inflammatory responses (1, 2). To minimize unwanted inflammation, epithelial cells in environments with high microbial burdens, such as the intestine, selectively express pathogen-associated molecular pattern (PAMP) receptors (PAMPR) on the basolateral surface or in endosomal compartments (3). Additionally, production of inflammatory cytokines IL-1β and IL-18 is tightly regulated transcriptionally by PAMPRs and posttranslationally by the inflammasome (4–7).

Inflammasomes are macromolecular machines that promote activation of caspase-1 in response to PAMP molecules, cellular stress, or cellular damage (8). Ultimately, inflammasome activation leads to the initiation of inflammatory signaling through cleavage of pro–IL-1β and IL-18 to their active forms and secretion of these cytokines from the cell. Inflammasomes are usually composed of a sensor protein from the nucleotide-binding domain and leucine-rich repeat protein family (NLR), an adaptor protein such as apoptosis-associated speck-like protein containing a CARD, and caspase-1. The activation of inflammasomes is thought to involve two signals; the first signal results from PAMP recognition and leads to the increased expression of the inflammasome components and substrate cytokines; various cellular insults such as pore formation can provide the second signal for the NLRP3 inflammasome, resulting in caspase-1 cleavage and cytokine release. Inflammasome activation is best studied in macrophages; however, several cell types can produce IL-1β and IL-18. Intestinal epithelial cells (IECs) do not express IL-1β, but they do express pro–IL-18, and IL-18 derived from IECs and hematopoietic cells is known to protect against colitis and colorectal cancer in mouse models (9–11).

The intestinal epithelium resides in a unique immunological environment, where it is potentially in contact with microorganisms constituting the normal flora. Additionally, the intestine is a major portal for infectious diseases, suggesting IECs evolved mechanisms to distinguish between innocuous flora and dangerous pathogens (1). The mechanisms underlying inflammasome activation in response to infection of IECs are not understood.

However, a critical step in the pathogenesis of food-borne bacterial pathogens is attachment or attachment and invasion of IECs (12). Some of the best-characterized invasive pathogens of the intestine are Yersinia enterocolitica and the closely related Yersinia pseudotuberculosis.

Y. enterocolitica is a zoonotic bacterial food-borne pathogen of humans causing terminal ileitis, enterocolitis, and mesenteric lymphadenitis (13, 14). To attach to and invade intestinal tissues, Yersinia expresses an adhesin called invasin (Inv) (15, 16). Inv binds to β1 integrins predominantly expressed on microfold epithelial cells overlying Peyer’s patches (PP) (17). Inv-mediated integrin binding facilitates invasion of the intestinal epithelium and PP colonization. Once Y. enterocolitica establishes infection of the PP, it is an extracellular pathogen that utilizes numerous virulence factors to modulate host responses to infection (18, 19).

Y. enterocolitica encodes virulence factors on both the chromosome and the 70-kb virulence plasmid, pYY (14, 19). pYY encodes several effector toxins and a type three secretion system (TTSS) that provides a conduit to secrete these effectors from the bacterial cytosol directly into the host cell cytoplasm (19, 20). The TTSS translocon proteins form a pore in the host cell membrane...
serving as a signal for NLRP3 inflammasome activation and inflammatory cytokine secretion from macrophages (21–24). The effectors known as *Yersinia* outer proteins (Yops) are mostly enzymes; for example, YopH is a protein tyrosine phosphatase that dephosphorylates focal adhesion kinase (FAK), p130 cas, and other components of the focal adhesion complex to disrupt the actin cytoskeleton in epithelial cells (23–30). YopE, a GTPase-activating protein, targets signaling of the small G proteins Rac, Rho, and Cdc42 (31). Both YopP and YopE act together to inhibit β1 integrin signaling. YopP/J1, a protease and protein acetylase, has been implicated in modulation of the inflammasome in macrophages through inhibition of NF-κB. Yops also inhibit cytokine expression as a means of immune evasion (23, 24, 32–35). The molecular mechanisms used by other Yops, such as YopM, have yet to be completely defined. Interestingly, YopM in the closely related pathogens *Y. pseudotuberculosis* and *Yersinia pestis* does not appear to be an enzyme but is a protein that binds and sequesters host-signaling proteins, including caspase-1. In activated macrophages, YopM can be a potent inhibitor of the inflammasome through its interaction with caspase-1 (24). However, mechanisms of *Y. enterocolitica*-derived inflammasome signaling in IECs and the role of the Yops in this process are completely unknown.

In the current study, we identify the mechanism of NLRP3 inflammasome activation in IECs. We also describe a novel first-signal signaling pathway for NLRP3 inflammasome activation in IECs requiring signaling through the focal adhesion-signaling complex. We determine Yops E and H as the virulence factors acting on intestinal epithelial cells requiring signaling through the focal adhesion-signaling complexes. We find that YopM inhibits the first signal for inflammasome activation in nonmyeloid cells.

**Materials and Methods**

**In-frame Yop deletions of *Y. enterocolitica***

All *Y. enterocolitica* strains used in this project were derived from JB80V (Supplemental Table I). In-frame deletions of YopE, YopH, and YopQ were generated through homologous recombination by using the 500-bp upstream and downstream sequences of each gene. The up- and downstream sequences were first amplified by PCR from the virulence plasmid pYEL0801 with predesigned primers (Supplemental Table II). These DNA fragments were cloned into pCR2.1-TOPO vector by TOPO TA cloning (K4500) from Invitrogen, according to the manufacturer’s instructions. Primers were made with 5' overhangs of SalI and NcoI restriction site sequences (underlined), which allowed for the 500-bp up- and downstream fragments to be cloned into a single pCR2.1-TOPO vector. Subsequently, the ~1-kb DNA fragment was cloned into the SalI and NcoI sites of the suicide vector pSR47s and introduced into *Escherichia coli* s17pir mating strain. Mating between JB80V and s17pir conjugated the suicide plasmid into JB80V. Kanamycin and nalidixic acid resistance selected for recombination of the suicide plasmid into the virulence plasmid YPY. Loss of the integrated pSR47s from the kanamycin and nalidixic acid-resistant JB80V780V clones was achieved by selecting for sucrose resistance. PCR and SDS-PAGE Coomassie blue staining of all secreted Yops verified the successful deletion of each Yop.

**Inv single amino acid substitutions**

Single amino acid substitutions to generate *E. coli* expressing Inv D760A and Inv D809A were performed through the QuikChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer’s instructions. The mutagenic oligonucleotide primers used to generate the substitution were listed in Supplemental Table II.

**RT-PCR**

Caco-2 cells were treated for 1 h with 5 μM PF-431396 and 100 μM NSC 23766 to inhibit FAK and rac1, respectively. Control cells were treated with DMSO (solvent). Cells were infected with the indicated strain of *Y. enterocolitica* for 30 min, and then total RNA was extracted using the TRIzol method. Two micrograms of total RNA was reverse transcribed to cDNA. Following cDNA synthesis, RT-PCR was performed for caspase-1, nlrp3, and GAPDH, with the resulting product fractionated by agarose gel electrophoresis.

**Cells and infection**

Human enterocyte cell line Caco-2 was cultured at 37°C with 5% CO2 in 20% FBS MEM. For infections, cells were grown to confluence (4 d) in 12- or 6-well dishes. *Yersinia* (Supplemental Table II) were grown overnight in Luria–Bertani (LB) medium containing 20 μg/ml nalidixic acid at 28°C. The cultures were then diluted 1:100 into fresh media and grown at 37°C for 2 h. *Salmonella* were grown overnight in LB medium 37°C, diluted into fresh LB medium, and grown for 3.5 h at 37°C. All bacteria were washed with PBS and diluted into OPTI-MEM at an OD of 0.2 before infecting Caco-2 cells at a multiplicity of infection (MOI) of 10 or as otherwise specified. After 1 h of infection, 20 μg/ml gentamicin was added to kill extracellular bacteria and maintained throughout the course of the experiment. *E. coli* HB101 strains (Supplemental Table II) were also grown at 37°C overnight in LB medium under 100 μg/ml ampicillin selection and then diluted and grown at 37°C for an additional 1 h. Caco-2 cells were infected with *E. coli* strains for 2 h and subsequently treated with 25 μM nigericin (Sigma-Aldrich) and 20 μg/ml gentamicin for the reminder of the infection time course.

**Chemical inhibition studies**

Caco-2 cells cultured on 12- or 6-well plates were pretreated for 1 h with 100 μM irreversible caspase-1 inhibitor Ac-YVAD-cmk from Bachem. Potassium efflux was blocked with either 50 μM ion-channel blocker glyburide or 130 mM KCl, both from Sigma Aldrich. For Rac1 and FAK inhibition, 50 or 100 μM Rac1 inhibitor NSC23766 (EMD Millipore) and the indicated concentrations of the FAK inhibitor PF-431396 (Sigma-Aldrich) were used. Solvents used were water for NSC23766 and DMSO for the remaining inhibitors; solvents also served as the negative (vehicle) controls. After pretreatment with the inhibitors, cells were infected as indicated for 8 h with inhibitors maintained in culture throughout the infection.

IL-18 ELISA assay

We assessed the production of IFN-γ from KG-1 cells, a human myelomonocytic cell line, stimulated by conditioned media from Caco-2 cells. KG-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS in 96-well plates seeded at 2 × 10⁵ cells/well. Cell culture supernatants from Caco-2 cells infected with *Y. enterocolitica* strains or *Salmonella typhimurium* were also added on the KG-1 cells. Mature rIL-18 (1 ng/ml) was also added as a positive control. After 24-h incubation, samples were analyzed for the concentration of IFN-γ using the human IFN-γ ELISA from BD Biosciences. Immunoblots of cell lysates or supernatants were used to monitor protein expression and the cleavage products of IL-18 and caspase-1. Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris [pH 8], 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) supplemented with protease inhibitor mixture (RPI) and 2 μM PMSF. Supernatant proteins were precipitated by the methanol–chloroform extraction method, as described (36). Protein concentration in the cell lysate was determined using bicinchoninic acid assay kit. Equal concentrations of protein were separated through 10–15% denaturing SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with primary Abs diluted (1:1000) against human integrin αβ1, integrin, FAK, and phospho-FAK, all from Cell Signaling Technology. Other Abs used were against human IL-18 from Santa Cruz Biotechnology and against Rac1 from Epitomics, a loading control Ab against heat shock protein 70 from Santa Cruz Biotechnology. Blots were then washed as before, and protein bands were visualized by chemiluminescence. Relative caspase-1 protein levels were determined by densitometric analysis of Western blot bands using a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA).

**β1 integrin blocking**

Confluent Caco-2 cells were treated with 0.5 mM inhibitory peptide cyclo-GRGDSP or a nonblocking control peptide cyclo-GRGESP, from AnaSpec. Integrins were blocked with the indicated concentrations of anti-human integrin αβ1 mAb, JB85, from EMD Millipore, and a control Ab

Inflammasome activation in intestinal epithelium
against integrin α5 chain clone V5 (BD Biosciences) or mIgG from Sigma-Aldrich. Prior to infection, Caco-2 cells were pretreated with Abs for 30 min at room temperature and then infected and incubated at 37°C for the remaining time course.

**RNA interference**

Caco-2 cells were cultured to 50% confluency and then transfected with small interfering RNAs (siRNAs) using Lipofectamine RNAiMAX (Invitrogen), according to manufacturer’s instructions. siRNAs included 50 nM cryopyrin NLRP3 (sc-45469; Santa Cruz Biotechnology); 100 nM FAK siRNA and control siRNA (Cell Signaling Technology); and ON-TARGETplus SMARTpool siRNA (100 nM) against β1 integrin (L-004506-00-0005) and control siRNA (D-001810-10-05; Dharmacon, Thermo Scientific). Rac1 knockdown was achieved by transfecting 0.5 μg/well Rac1 short hairpin RNA (shRNA) vector (pRNAT.mCherry shRac1) or control (pRNAT.mCherry shScr) for 48–72 h before infection with bacteria. The knockdown of transcription was verified by quantitative RT-PCR (qRT-PCR) or immunoblot analysis.

**Statistical analysis**

ANOVA was performed using the Prism 4 software package (GraphPad, La Jolla, CA). Data are presented as means ± SD, with p values <0.05 considered significant.

**Results**

Y. enterocolitica YopE and YopH inhibit secretion of active IL-18 from IECs

In mouse models of yersiniosis, IL-18 is critical in the host response to Y. enterocolitica infection (37). To investigate whether IL-18 secretion is modulated during Y. enterocolitica infection of IECs, we infected the human enterocyte cell line Caco-2 with Y. enterocolitica or S. typhimurium as a positive control. Wild-type bacteria failed to induce the secretion of IL-18 (Fig. 1A), but we hypothesized that Y. enterocolitica employs one or more Yop(s) to disrupt the production and secretion of active IL-18. IECs were infected with Yop mutants, and ELISA determined the concentrations of IL-18 secreted into the culture supernatants. IL-18 secretion was detected from cells infected with the yopE mutant. In contrast to the yopE mutant, infection of cells with the yopH or other Yop mutants did not result in detectable IL-18 secretion. However, a yopEH mutant induced robust secretion of IL-18 (Fig. 1A, 1B). These findings indicate YopH and YopE act synergistically to inhibit secretion of IL-18 from IECs. In the closely related pathogen, Y. pseudotuberculosis, the YopK protein was reported to inhibit activation of caspase-1 and IL-1β secretion from bone marrow–derived macrophages (23). We found the Y. enterocolitica yopQ mutant (homologous to YopK) did not alter secretion of IL-18, suggesting differences in inflammasome modulation between IECs and macrophages (Fig. 1). Notably, Y. enterocolitica JB580v, a strain lacking the pVY virulence plasmid encoding the Yops and TTSS machinery, is unable to stimulate IL-18 (Fig. 1). This finding suggests that other properties of the TTSS such as the translocon are required for IL-18 secretion during Y. enterocolitica infection of IECs (22).

IL-18 bioactivity was tested by assaying the ability of conditioned media from cells infected with the Y. enterocolitica mutants to induce the production of IFN-γ from KG-1 cells (38). As shown in Fig. 1C, IFN-γ production was induced by the supernatants of cells infected with the yopEH mutant but not with supernatants from the other Yersinia strains tested. These results indicate that, in the absence of Yops E and H, Y. enterocolitica induces the secretion of mature IL-18.

Activation of the inflammasome in macrophages leads to the maturation of IL-1β. However, literature suggests that IECs produce IL-18 but do not produce IL-1β (39). To test the ability of IECs to produce IL-1β in response to Yersinia infection, we infected Caco-2 cells with the various strains of Yersinia or Salmonella as a positive control. As shown in Fig. 1D, Caco-2 cells do not secrete appreciable levels of IL-1β under our infection conditions, with all samples having IL-1β concentrations below the limit of detection for the assay.

**qRT-PCR**

qRT-PCRs were performed with TaqMan gene expression assays, according to the manufacturer’s instructions, and the reaction was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). Probes from Life Technologies were used, as follows: NLRP3, Hs00198082 m1; IL-18, Hs01038788 m1; and GAPDH, Hs99999905-m1. Expression levels were dependent on caspase-1, we treated cells with an irreversible caspase-1 inhibitor (Ac-YVAD-cmk) prior to infection and then monitored maturation of IL-18. IL-18 maturation was dramatically reduced in the presence of inhibitor, indicating caspase-1 activity was required for IL-18 maturation (Fig. 2A, 2B). The reduction in mature IL-18 corresponded with a reduction in the active form of caspase-1 p20 from IECs infected with Yersinia strains or Salmonella. Corresponding to reductions in cleaved IL-18 in the supernatant following caspase-1 inhibitor treatment, inhibitor treatment reduced caspase-1 band intensity from 0.19, 0.40, and 0.48 arbitrary units (au) in the solvent-treated cells to 0.12, 0.02, and 0.17 au in the inhibitor-treated cells for JB580v, yopEH, and Salmonella, respectively (Fig. 2B). Altogether, these data suggest that production of mature IL-18 from IECs requires active caspase-1.

The NLRP3 inflammasome is activated in macrophages infected with Yersinia (23, 24, 35). To investigate NLRP3 inflammasome activation in IECs, we used siRNA-targeting nlrp3 and verified significant siRNA-mediated knockdown of nlrp3 transcript (Fig. 2C). When Caco-2 cells were transfected with NLRP3–specific siRNA, followed by infection with the yopEH mutant, the secretion of IL-18 was markedly decreased relative to cells transfected with nontargeting siRNA (Fig. 2D).

**Bacterial pore-forming toxins can trigger NLRP3 inflammasome activation (40) through efflux of intracellular potassium. To examine K+ efflux in Y. enterocolitica-induced inflammasome activation, we infected Caco-2 cells in the presence of 130 mM extracellular KCl, which led to a 50% reduction in secretion of IL-18 from cells infected with the yopEH mutant (Supplemental Fig. 1). To further test the role of K+, we pretreated cells with glibenclamide to block potassium efflux. In agreement with the KCl results, infection of Caco-2 cells with the yopEH mutant, the secretion of IL-18 was markedly decreased relative to cells transfected with nontargeting siRNA (Fig. 2D).

**Inv binding to αβ3 integrins promotes IL-18 secretion**

The major adhesin of Yersinia, Inv, binds β1 integrins, aiding in contact-dependent TTSS, and facilitates phagocytosis (41). The injection of Yops E and H by the TTSS disrupts signaling downstream of the β1 integrin, inhibiting phagocytosis (19). We hypothesized that Inv-activated β1 integrin signaling acts as a primary trigger for inflammasome activation in IECs. Therefore, we infected Caco-2 cells with a Y. enterocolitica yopEH inv triple mutant and found that the absence of Inv resulted in 40-fold
expression of masome-signaling pathways in epithelial cells. We silenced receptors are a component of the pathogen detection and inflam-
cell transfected with integrins stimulate caspase-1 cleavage and IL-18 secretion. Caco-2
its Inv expression.

Importantly, using nonpathogenic

Because our data suggest that Inv provides the first signal for

We hypothesized that if pore formation were present as a sec-

... E. coli Inv expressed on

Inflammasome activation (Fig. 3D). Furthermore, RGD-containing peptide (cyclo-

Although Inv binds to several β-integrins, Inv preferentially

Because our data suggest that Inv provides the first signal for

Because our data suggest that Inv provides the first signal for

we tested whether β-integrin receptors are a component of the pathogen detection and inflam-

Because our data suggest that Inv provides the first signal for

Importantly, using nonpathogenic E. coli expressing Inv allowed
supernatants were detected by immunoblotting. (strains of ELISA 8 h postinfection. (of two to three independent experiments. *inhibitor prior to infection with the indicated Y. enterocolitica strains or S. typhimurium (Stm). Cell supernatants were evaluated for IL-18 secretion by ELISA 8 h postinfection. (B) Levels of procaspase-1, IL-18, and heat shock protein 70 in the cell lysates and mature IL-18 and caspase-1 p20 in cell supernatants were detected by immunoblotting. (C and D) Caco-2 cells were transfected with NLRP3 siRNA or control nontargeting siRNA for 72 h. (C) The knockdown of the Nlrp3 transcript was confirmed by qRT-PCR. (D) siRNA-transfected cells were infected with wild-type (JB580V) and yopEH mutant strains of Y. enterocolitica, and supernatants were evaluated for IL-18 secretion by ELISA. See also Supplemental Fig. 1. Data are presented as mean ± SD of two to three independent experiments. *p < 0.05, ***p < 0.0001.

us to test the ability of Caco-2 cells to respond to common PAMPs present on other common Gram-negative bacteria that are part of the normal flora.

Caco-2 cells were infected with E. coli-expressing Y. enterocolitica inv (E. coli plus Inv), E. coli strain carrying an empty vector (E. coli vector), and JB580c, in the presence or absence of nigericin. E. coli plus Inv and JB580c induced IL-18 secretion 25- and 4-fold, respectively, when compared with uninfected cells and controls (Fig. 5A). IL-18 secretion was MOI dependent when cells were infected with E. coli plus Inv and JB580c, but not with E. coli plus vector (Fig. 4A, 4B, respectively). IL-18 secretion during E. coli plus Inv infection was abrogated by the α5β1 integrin-neutralizing Ab and reduced by >2-fold by cyclo-GRGDSP (Fig. 5B, SD). These data suggest that Inv, when coupled with pore formation, is sufficient to stimulate IL-18 secretion in IECs. Importantly, these data also suggest that IECs are not responding to PAMPs other than Inv as a means to detect the presence of pathogenic bacteria.

Inv from Y. pseudotuberculosis contains an aspartic acid at position 911 that is essential for integrin binding (44, 45). This critical amino acid is at position 760 in Y. enterocolitica Inv. We tested whether D760 was necessary for IL-18 production and secretion by infecting cells with an Inv-D760A point mutant. We also infected IECs with a control Inv mutant, D809A, an aspartic acid predicted to be on the same face of Inv as D760, but not involved in integrin binding (45). In comparison with IECs infected with E. coli expressing wild-type Inv, IL-18 secretion decreased by >2-fold when cells were infected with E. coli Inv D760A (Fig. 5C). The Inv D809A mutant was still capable of inducing IL-18 to similar levels as E. coli expressing wild-type Inv. Altogether, these data suggest that Inv binding to integrins through the critical amino acid D760 is necessary for the induction of IL-18 secretion.

FAK and Rac1 are involved in inflammasome signaling and IL-18 production in IECs

Our data suggest Y. enterocolitica utilizes Yops H and E to disrupt integrin signaling to attenuate caspase-1 activity and IL-18 secretion from IECs. β1 integrin binding by Inv causes receptor clustering, triggering the assembly of focal adhesion complexes and tyrosine kinase activity (41). FAK is a major target of YopH (27, 29). We hypothesized FAK contributes to the integrin signal activating the inflammasome in IECs. To test FAK, we assessed IL-18 secretion from IECs treated with increasing concentrations of PF-431396, an inhibitor that blocks FAK phosphorylation. Following infection with the yopEH mutant, we found that phosphorylation of FAK was decreased with inhibitor concentrations starting at 1 μM, corresponding to a dose-dependent decrease in IL-18 secretion (Supplemental Fig. 2). Cells were pretreated with increasing concentrations of PF-431396 and infected with wild-type Y. enterocolitica, the yopEH mutant, and as a positive control S. typhimurium. Each incremental increase in inhibitor concentration decreased IL-18 secretion by ∼2-fold from cells infected with the yopEH mutant, whereas IL-18 secretion from cells infected with S. typhimurium or wild-type Yersinia was not impacted (Fig. 6A). To further test FAK, we used RNA interference to deplete FAK prior to infection. Caco-2 cells were transfected with siRNA targeting FAK as well as control siRNA. Following FAK depletion, cells were infected with wild-type Y. enterocolitica, the yopEH mutant, or S. typhimurium. Analysis of...
the culture media indicated that knockdown of FAK decreased IL-18 concentrations by 3-fold in comparison with control siRNA-transfected cells, but had no significant effect on IL-18 secreted by cells infected with \textit{S. typhimurium}. These data suggest FAK signaling is involved in inducing the secretion of IL-18 during \textit{Y. enterocolitica} infection with the \textit{yopEH} mutant.

Inv-mediated integrin signaling activates \textit{Rho} GTPases such as Rac1 to regulate actin cytoskeleton rearrangements. YopE is an inhibitor of Rac1, Cdc42, and RhoA activity (31, 33). To test Rac1 in inflammasome activation, we used the Rac1 inhibitor NSC23766, which does not affect the activity of Cdc42 and RhoA (46). Caco-2 cells were treated with increasing concentrations of NSC23766 prior to infection. Concentrations of secreted IL-18 following infection with the \textit{yopEH} mutant decreased in a dose-dependent manner, resulting in a 2-fold decrease at the highest inhibitor concentration (Fig. 6C). Additionally, Caco-2 cells were transfected with shRNA plasmid targeting rac1. As shown in Fig. 6D, \textit{yopEH} mutant infection of cells after rac1 knockdown reduced IL-18 by 2-fold relative to controls. Rac1 inhibitor or shRNA, our data do not rule out other YopE targets in IL-18 secretion, including RhoA (47).

\textbf{Yops E and H synergistically inhibit inflammasome activity.} We tested whether this synergism could be chemically complemented by inhibiting FAK and Rac1 activity prior to \textit{yopEH} mutant infection. Caco-2 cells were pretreated with either FAK inhibitor (5 \(\mu\)M) or Rac1 inhibitor (100 \(\mu\)M) or a combination of the two inhibitors. In comparison with vehicle-treated cells, FAK inhibitor alone dramatically reduced secreted IL-18 4-fold relative to the rac1 inhibitor, which reduced IL-18 concentrations 2-fold (Fig. 6E). The combination of the two inhibitors reduced IL-18 secretion to levels just above the limit of detection, corresponding to a 50-fold decrease in IL-18 concentrations relative to controls (Fig. 6E). These data suggest FAK and Rac1 act synergistically to transduce first signals from integrins to the inflammasome.

\textbf{IL-18 expression is regulated by integrin signaling in IECs}

We investigated whether integrin signaling provides a first signal for inflammasome activation in IECs. Caco-2 cells infected with wild-type or the \textit{yopEH} mutant were evaluated for changes in expression of casp1, \textit{nlrp3}, and IL-18 mRNA. Expression of casp1 mRNA was not modulated at the early time points tested and...
remained similar to uninfected cells (data not shown). Expression of \textit{nlrp3} was inhibited by FAK and rac1 inhibitors in mock-treated IECs, wild-type \textit{Yersinia}-infected cells, and \textit{yopEH} mutant-infected IECs relative to untreated cells demonstrating decreased basal \textit{nlrp3} mRNA expression, but mRNA levels were similarly decreased in all treatment groups (data not shown). These data suggest that integrin binding to matrix most likely leads to basal \textit{nlrp3} expression, but that infection does not modulate \textit{nlrp3} expression at the early time points tested. However, there was an early induction (0.5 hours post infection) in \textit{IL-18} mRNA in cells infected with the \textit{yopEH} mutant. This induction resulted in a 2-fold increase in \textit{IL-18} mRNA relative to cells infected with wild-type \textit{Yersinia} and uninfected cells. Wild-type \textit{Y. enterocolitica} failed to augment the expression of \textit{IL-18} mRNA, resulting in \textit{IL-18} transcript levels similar to uninfected cells (Fig. 7A). To test whether integrin signaling was responsible for upregulating \textit{IL-18} mRNA expression, we pretreated cells with either JBS5 \textit{α5β1} neutralizing Ab or \textit{α5β1} nonneutralizing Ab. Caco-2 cells pretreated with JBS5 Ab and infected with the \textit{yopEH} mutant showed a 2-fold decrease in \textit{IL-18} expression relative to the nonneutralizing Ab. In the presence of JBS5 Ab, \textit{IL-18} mRNA levels from infected cells remained the same as from uninfected cells (Fig. 7B). We tested FAK and rac1 in the expression of \textit{IL-18} mRNA by pretreating cells with the FAK and rac1 inhibitors, and then the levels of \textit{IL-18} mRNA were evaluated after 30 min of infection. \textit{IL-18} transcript levels during infection with the \textit{yopEH} mutant decreased to basal levels similar to uninfected cells and cells infected with wild-type \textit{Y. enterocolitica} (Fig. 7C). These data suggest that integrin signaling involving the activities of both FAK and Rac1 can influence inflammasome activity by upregulating \textit{IL-18} mRNA. Altogether, these data suggest that integrins can provide the first signal for inflammasome activation in epithelial cells (Fig. 8).

\textbf{Discussion}

IECs serve as central regulators of gut homeostasis responding to insults by secreting cytokines (IL-18) to initiate both innate immunity and epithelial repair. In this study, to our knowledge, we demonstrate for the first time that \textit{α5β1} integrins on IECs when bound to \textit{Y. enterocolitica} Inv trigger IL-18 secretion through activation of the NLRP3 inflammasome. As part of integrin-mediated signaling to the inflammasome, we report that the activities of FAK and Rac1 are involved in signal transduction (Fig. 8).
However, the full inflammasome signal requires both Inv-dependent integrin binding and pore formation. We further show *Y. enterocolitica* utilizes at least two TTSS effectors, YopE and YopH, to block the integrin-mediated inflammasome signal (Fig. 8).

In the intestine, the response of IECs to microbes requires an acute ability to discriminate pathogenic bacteria from commensal flora. Because both normal flora and pathogenic microbes share conserved PAMPs sensed by host PAMPRs, we hypothesized that intestinal IECs must sense and respond to other PAMPs on enteric pathogens. Adhesins such as Inv are common among invasive bacteria and often bind their receptors with affinities significantly higher than natural ligands (48). A recent report demonstrated that *Treponema denticola* surface protein Td92 binds to α5β1 integrins, leading to the full activation of the NLRP3 inflammasome in THP-1 monocytes (49). Although our findings similarly show that Inv-mediated integrin signaling is important for inflammasome activation in response to *Y. enterocolitica* infection, we found that, in IECs, unlike macrophages that express other PAMPRs, integrins provide the inflammasome-priming signal by upregulating IL-18 transcription. These data strongly suggest the Inv–integrin inter-
suggesting that IECs are sensing Inv as a PAMP through inter- 
active site binding (44, 45), abolished IL-18 secretion, strongly 
IL-18 secretion. A point mutation in Inv, D760A, which disrupts 
that block the receptor–ligand interaction dramatically reduced 
a PAMP required the Inv–integrin interaction as Abs or peptides
presence of nigericin. Furthermore, the IEC response to Inv as
vector failed to stimulate significant amounts of IL-18 in the
genic
rectly tested this hypothesis in experiments utilizing nonpatho-
further implies that IECs use other mechanisms to rapidly detect
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ings, our data found no demonstrable role for Y. enterocolitica YopQ, the homolog of YopK, in counteracting the activation of the
inflammasome in IECs. In fact, the yopEH mutant is YopQ and
YopM sufficient and induces significant IL-18, suggesting that these Yops are not major inhibitors of inflammasome activity in
IECs. The reason for this discrepancy might be due to Yersinia species-specific differences in YopQ/YopK activity. However, it is
reasonable to speculate that the differences observed in Yops-
regulating inflammasome activity are due to cell-type differences.
In support of this hypothesis, recent work investigating the targets
of YopH in neutrophils isolated from infected animals clearly
showed cell-type–specific targets of YopH in vivo (30).

Collectively, these data highlight the importance of integrin
signaling for inflammasome-mediated inflammatory responses in
IECs and show that Y. enterocolitica escapes these responses by
utilizing YopE and YopH to interfere with integrin signaling. These data illustrate how pathogens can control inflammasome-
mediated inflammatory signaling by blocking signaling pathways
required for the first signal. Elucidating the potential of integrin
signaling as a common inflammasome activation mechanism in
epithelial cells will provide important insight into host–pathogen
interactions dictating early inflammatory responses at mucosal

FIGURE 8. A model depicting the proposed mechanism for invasin-
mediated first signals and induction of inflammasome activity. Inv binding
to β1 integrins on epithelial cells triggers a signaling cascade involving
focal adhesion proteins, including FAK, followed by the activation of the
Rho GTPase Rac1. Activation of the focal adhesion complex ultimately
leads to IL-18 gene expression. This integrin signaling pathway coupled
with TTSS translocon-mediated pore formation leads to the activation of
the NLRP3 inflammasome and the secretion of IL-18. This signaling
cascade is potently inhibited by the activity of the virulence factors YopH
and YopE during infection with wild-type Yersinia enterocolitica.

action provides the first signal in a two-signal model of inflam-
masome activation. Full activation of the inflammasome requires
an additional signal triggered by Yersinia TTSS or in some of our
experimental systems by treating cells with the pore-forming toxin
nigericin (50).

Pore formation by the Yersinia TTSS translocon in LPS-primed macrophages activates the NLRP3 inflammasome (21, 23, 34, 35,
51). However, to our knowledge, our work is the first to demon-
strate that Inv binding to αβ1 integrins can facilitate the priming
step, often called the first signal. This is of particular importance
during intestinal innate immunity because IECs have muted
responses to LPS due to low expression of TLR4, MD-2, and
CD14 (3, 52). The relatively low expression of TLRs is critical for
immune tolerance to commensal bacteria during homeostasis and
further implies that IECs use other mechanisms to rapidly detect
pathogenic bacteria.

A tenet of our hypothesis is that IECs discriminate between
normal flora and pathogens by utilizing novel PAMPRs. We di-
rectly tested this hypothesis in experiments utilizing nonpatho-
genic E. coli, a component of the normal flora. E. coli ectopically
expressing Inv could induce IL-18 expression in the presence of
nigericin. However, even at high MOIs, E. coli with an empty
vector failed to stimulate significant amounts of IL-18 in the
presence of nigericin. Furthermore, the IEC response to Inv as
a PAMP required the Inv–integrin interaction as Abs or peptides
that block the receptor–ligand interaction dramatically reduced
IL-18 secretion. A point mutation in Inv, D760A, which disrupts
active site binding (44, 45), abolished IL-18 secretion, strongly
suggesting that IECs are sensing Inv as a PAMP through inter-
actions with integrins. These data also demonstrate that IECs
did not respond to common PAMPs present on both E. coli and
Yersinia. Altogether, these data strongly support the hypothesis
that integrins expressed on IECs can function as PAMPRs dis-
criminating between pathogens and normal flora. Pathogens are
capable of exploiting integrins for attachment and uptake because
they often bind with much higher affinity than natural ligands; for
example, Inv binds with a 100-fold higher affinity than fibronectin
(48). This property of pathogen-associated adhesins could poten-
tially provide a mechanism for how IECs accomplish integrin-
dependent pathogen detection.

Many pathogens gain access to host cells and tissues by bind-
ing to integrins. Interestingly, vaccinia virus uptake is mediated
by β1 integrins, and, like Yersinia, vaccinia has numerous virulence
factors targeting inflammasome signaling (53). Considering the
large number of pathogens exploiting integrins for invasion, our
findings potentially highlight a common mechanism evolved to
facilitate rapid recognition and inflammasome responses to inva-
sive pathogens at mucosal surfaces.

Analysis of the impact of Yops on inflammasome activity in
macrophages identified an inhibitory activity for YopK/YopQ,
YopE, and YopM (23, 24, 33). However, the inhibitory activity
of YopE is not yet clear because overexpression studies found
that Y. enterocolitica YopE disrupted inflammasome complex
assembly, whereas Y. pseudotuberculosis YopE did not exhibit
inflammasome inhibitory activity in mouse macrophages (23, 33).
Our data demonstrate that YopE alone does not fully account for
the potent inhibitory capability of Y. enterocolitica on inflam-
masome activity but acts in concert with YopH. However, we did not
test overexpression of YopE in the IEC model, and it is possible
that it would be sufficient to disrupt inflammasome assembly.
Prior to our work, YopH had not been identified as an inflam-
masome inhibitor. Previous work showed that Y. pseudotubercu-
losis YopK masks the Yersinia TTSS translocon from detection by the
inflammasome in macrophages (23). Contrary to these find-
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Disclosures
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