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Crohn disease–associated adherent-invasive *E. coli* bacteria target mouse and human Peyer's patches via long polar fimbriae

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Crohn disease (CD) is a multifactorial disease in which an abnormal immune response in the gastrointestinal (GI) tract leads to chronic inflammation. The small intestine, particularly the ileum, of patients with CD is colonized by adherent-invasive *E. coli* (AIEC) — a pathogenic group of *E. coli* able to adhere to and invade intestinal epithelial cells. As the earliest inflammatory lesions are microscopic erosions of the epithelium lining the Peyer's patches (PPs), we investigated the ability of AIEC bacteria to interact with PPs and the virulence factors involved. We found that AIEC bacteria could interact with mouse and human PPs via long polar fimbriae (LPF). An LPF-negative AIEC mutant was highly impaired in its ability to interact with mouse and human PPs and to translocate across monolayers of M cells, specialized epithelial cells at the surface of PPs. The prevalence of AIEC strains harboring the *lpf* operon was markedly higher in CD patients compared with controls. In addition, increased numbers of AIEC, but not LPF-deficient AIEC, bacteria were found interacting with PPs from *Nod2*$^{-/-}$ mice compared with WT mice. In conclusion, we have identified LPF as a key factor for AIEC to target PPs. This could be the missing link between AIEC colonization and the presence of early lesions in the PPs of CD patients.

Introduction

Crohn disease (CD) and ulcerative colitis (UC) are multifactorial diseases, occurring in individuals with genetic predisposition in whom an environmental or infectious trigger causes an abnormal immune response (1–3). Several lines of evidence suggest that bacteria play a role in the onset and perpetuation of IBD (4). *E. coli* bacteria have been assigned a putative role in CD. They are abnormally predominant in early and chronic ileal lesions of CD, and most *E. coli* strains isolated from the ileal mucosa of CD patients adhere to intestinal epithelial cells (IECs) (5–7). In addition to their ability to adhere, these *E. coli* bacteria are able to invade IECs and belong to a new pathogenic group of *E. coli*, designated adherent-invasive *E. coli* (AIEC) (8). Several independent studies have reported the abnormal presence of AIEC associated with ileal mucosa of CD patients (7, 9–13) owing to increased ileal expression of CEACAM6, which acts as a receptor for AIEC binding via type 1 pili to the intestinal mucosa (14, 15). The adhesion and invasion process of reference AIEC strain LF82 involves, in addition to type 1 pili, flagella, outer membrane proteins, and outer membrane vesicles (OMVs) (16–18). In particular, the LF82 invasion process occurs via the interaction between the ER-localized stress response chaperone Gp96 and the outer membrane protein OmpA expressed at the surface of OMVs, allowing OMVs to fuse with IECs (19). In addition, analysis of the genome sequence of AIEC strain LF82 revealed the presence of several known virulence genes and 4 putative pathogenic islands carrying virulence-related genes (20).

Clinical observations suggest that the sites of initial inflammation in ileal CD are the lymphoid follicles (21), since microscopic erosions are observable at the specialized follicle-associated epithelium (FAE), which lines Peyer's patches (PPs) (22). The most prominent feature of FAE is the presence of specialized membranous/microfold cells (M cells), which are optimized for antigen adherence and transport and for immunological sampling of microorganisms (23). Several microorganisms, particularly invasive bacteria, take advantage of the transcytotic characteristics of M cells to use them to cross the intestinal barrier. For example, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* cross the intestinal epithelial barrier through M cells of the FAE (24), as does rabbit enteroadherent *E. coli* (25). The mechanism responsible for *Y. pseudotuberculosis* targeting M cells is essentially mediated by invasion (26). *Salmonella* Typhimurium bacteria selectively interact ex vivo with PPs (27), and a recent study reported that glycoprotein 2 (GP2), specifically expressed on the apical plasma membrane of M cells among enterocytes (28), is recognized by FimH, the adhesin of type 1 pili on bacteria (29). These observations demonstrate that the entry of type 1 piliated bacteria, such as *E. coli* and *Salmonella* Typhimurium, into M cells could occur by means of the GP2-FimH recognition. In addition, the long polar fimbriae (LPF), encoded by the *lpf* operon, were equally responsible for *Salmonella* Typhimurium specific adherence to M cells of the murine FAE (30). In the case of *Shigella flexneri*, bacteria entry occurs essentially through the M cell monolayer of the FAE, as shown in a rabbit ligated intestinal loop assay of infection, with the involvement of IpaB and IpaC effectors (31).

As the earliest observable lesions of CD are microscopic erosions of the FAE lining the PPs and as AIEC bacteria colonize the ileal mucosa of CD patients, we aimed in the present study to

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search for the presence of virulence factors in AIEC that could be involved in the targeting of PPs by these invasive bacteria. We report here, in addition of the involvement of type 1 pili, the presence of a functional *lpf* operon, encoding LPF in AIEC, that allows AIEC bacteria to interact with murine and human PPs and to translocate across M cells.

Results

Analysis of the AIEC bacteria interaction with PPs. With the aim of identifying AIEC bacterial factors putatively involved in the targeting of PPs, we studied the interaction between AIEC LF82 bacteria and isolated PPs in Ussing chambers. As *E. coli* bacteria were previously reported to interact with PPs via the recognition of GP2 specifically expressed on apical plasma membrane of M cells by type 1 pili (28, 29), we studied the interaction of LF82 bacteria with murine PPs in the presence of antibodies raised against GP2. The addition of anti-GP2 antibodies during AIEC LF82 infection statistically decreased the number of LF82 bacteria interacting with PPs, from $31.89 \times 10^6 \pm 8.73 \times 10^6$ CFU in the absence of antibodies to $6.75 \times 10^6 \pm 2.38 \times 10^6$ CFU in the presence of anti-GP2 (Figure 1A). Immunostaining experiments using anti-GP2 antibodies to visualize M cells and anti-O83 antibodies to visualize LF82 bacteria confirmed that numerous LF82 wild-type bacteria colocalized with GP2 (Figure 1B). We also observed decreased numbers of AIEC bacteria interacting with murine PPs for the type 1 pili–negative mutant LF82-Δ*fimA*. However, we still observed type 1 pili–negative LF82 mutant associated with GP2-positive cells. In addition, we also still observed LF82 bacteria interacting with PPs when 0.5% methyl α-D-mannopyranoside was added in order to block the binding of type 1 pili to GP2. Together these results indicated that LF82 should express additional factor(s) targeting PPs in a mannose-independent manner.

Identification of lpf operon in CD-associated E.coli strains. We analyzed the genome sequence of AIEC strain LF82 (Genoscope, ColiScope Project, NC_011993 890) with the aim of identifying AIEC putative virulence factors involved in PP targeting other than type 1 pili expressed in the entire family of Enterobacteriaceae and by about 60% of fecal isolates (32). This screening indicated the presence of a putative functional *lpf* operon encoding LPF (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI44632DS1).

The AIEC LF82-ΔlpfA mutant was impaired in its ability to interact with murine and human isolated PPs. To identify the involvement of LPF in the ability of AIEC strain LF82 to target PPs, we generated the LF82-Δ*lpfA* isogenic mutant. After 3 hours infection of murine PPs in the presence of 0.5% methyl α-D-mannopyranoside to better assess the role of LPF, a significant ($P < 0.01$), 6.7-fold decrease in the number of associated AIEC LF82 bacteria was observed for the LF82-Δ*lpfA* isogenic mutant compared with wild-type AIEC strain LF82 (Figure 1C). This was not due to differences in bacterial growth, since growth curves for the wild-type strain LF82 and the LF82-Δ*lpfA* mutant were similar (Supplemental Figure 2). Nor was this due to lack of expression of type 1 pili, since the LPF-negative mutant still expressed these pili at a level similar to that in LF82 wild-type bacteria (Supplemental Figure 3). The transcomplementation of the mutant with the cloned *lpf* operon restored the interaction with murine PPs. FISH experiments using probe EUB338 to visualize bacteria indicated numerous bacteria inside PPs for the wild-type strain LF82 (Figure 1D), and very few PP-associated bacteria for the LF82-Δ*lpfA* mutant. The number of PP-associated bacteria was fully restored when the LF82-Δ*lpfA* mutant was transcomplemented with cloned *lpf* operon, indicating that LPF are essential for AIEC LF82 bacteria to interact with murine PPs. To confirm these ex vivo data with murine intestinal tissues, we performed in vivo experiments using mouse ileal loop containing one PP. We observed a significant ($P < 0.01$), 6.5-fold decrease in the number of PP-associated bacteria for the LF82-Δ*lpfA* isogenic mutant compared with wild-type AIEC strain LF82, and the wild-type phenotype was fully restored for the LPF-negative mutant expressing cloned LPF (Figure 1E). To identify whether LPF also mediate AIEC adhesion to IECs, we performed additional experiments in Ussing chambers using small intestine without PPs. Results interestingly showed that the ability of the LF82-Δ*lpfA* isogenic mutant to adhere to intestinal epithelium was similar to that of the wild-type strain LF82 (Figure 1F). Thus, this indicated that the difference in the ability of LF82 bacteria and LPF-negative mutant to bind PPs was related to a specific adhesion of LPF to FAE. In addition, the interaction of bacteria with PPs was studied in competition experiments between LF82 and nonpathogenic *E. coli* MG1655 expressing or not expressing LPF. A slight, 1.6-fold-decreased adhesion of LF82 to PPs was observed during coinfection with the nonpathogenic MG1655 *E. coli* strain, probably due to competition of type 1 pili binding to the glycoprotein GP2; and a 4.5-fold-decreased LF82 adhesion to PPs was observed during coinfection with MG1655 strain expressing LPF, confirming that the adhesion of LF82 bacteria to PPs is related to the presence of LPF (Figure 1G).

To analyze the involvement of LPF expressed by AIEC bacteria to target PPs in humans, we performed similar experiments using human isolated PPs. Confocal microscopy analyzing the uptake of bacteria across the FAE and into the subepithelial dome (SED) of human PPs using Ussing chamber indicated that, compared with wild-type strain LF82, LF82-Δ*lpfA* mutant showed 4.9-fold-decreased transepithelial uptake ($P < 0.01$ vs. LF82) (Figure 2, A and B). Transcomplementation of the mutant with the cloned *lpf* operon restored its ability to interact with human PPs, indicating that LPF are also essential for AIEC LF82 bacteria to interact with human PPs.

The AIEC LF82-ΔlpfA isogenic mutant was impaired in its ability to translocate across M-like cell monolayer and to interact with murine M cells in vivo. We used the in vitro model of the specialized M cells, in which polarized enterocyte-like Caco-2-cl1 cells were cocultured with the Raji B cell line (33). To analyze whether these M-like cells express only a few developed microvilli as previously described (34), we performed F-actin phalloidin-TRITC labeling, and results were consistent with the presence of cells displaying morphological characteristics of M cells (Supplemental Figure 4A).

We investigated the interaction of AIEC strain LF82 with Caco-2-cl1 monocultures compared with M-like cells in the presence of 0.5% methyl α-D-mannopyranoside. Confocal analysis showed numerous AIEC LF82 bacteria interacting with the monolayer of M-like cells compared with the Caco-2-cl1 monolayer (Figure 3, A and B). This was also observed in the absence of methyl α-D-mannopyranoside and related to an absence of CEACAM6 expression by Caco-2-cl1 monolayers (Supplemental Figure 4B). Analysis of translocation across these monolayers indicated that only low numbers of LF82 bacteria translocated across Caco-2-cl1 monolayers even after 4 hours of infection, whereas high numbers of LF82 bacteria translocated across M cell monolayers (Figure 3D). As transepithelial electrical resistance (TEER) stayed constant during the 4 hours of infection (data not shown), this indicated that the large increase in the number of translocated bacteria was not the result of a loss of monolayer integrity.

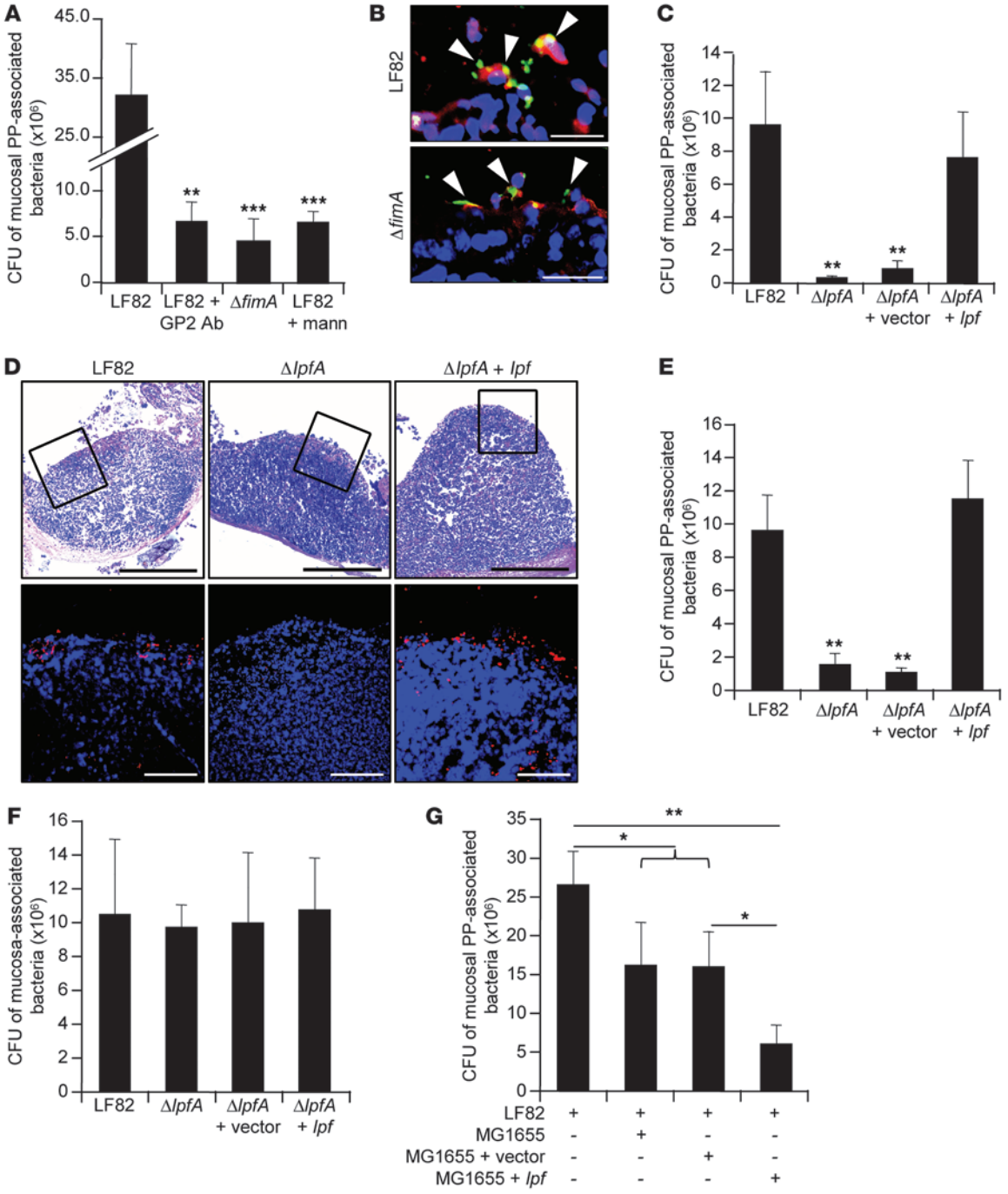

Figure 1

Interaction of AIEC bacteria with PPs. (**A**) Interaction of bacteria with murine PPs placed in Ussing chambers after a 4-hour infection period, with or without anti-GP2 antibody (1 μg/ml) and with or without 0.5% methyl α-D-mannopyranoside (mann). When needed, PPs were preincubated with anti-GP2 antibody. (**B**) Confocal analysis of PP sections after labeling of AIEC LF82 with LPS O83 antibody (green), of M cells with anti-GP2 antibody (red), and DNA with Hoechst (blue). Scale bars: 20 μm. Arrowheads show clear colocalization of bacteria and M cells. (**C**) Interaction of wild-type LF82 and *lpf* mutant with murine PPs placed in Ussing chambers after a 4-hour infection period. (**D**) HES staining and confocal analysis of indicated areas after Cy3-EUB228 FISH staining to detect bacteria (red) and Hoechst to identify DNA (blue). Scale bars: 100 μm for HES staining and 20 μm for confocal analysis. Images in the bottom row correspond to the boxed regions in the top row. (**E**) In vivo interaction of wild-type LF82 and *lpf* mutant with murine PPs using ileal loop assay after a 4-hour infection period in the presence of 0.5% methyl α-D-mannopyranoside. (**F**) Interaction of wild-type LF82 and *lpf* mutant with murine small intestine mucosa without PPs after a 4-hour infection period in Ussing chambers. All results are expressed as numbers of mucosa-associated bacteria; each value is the mean ± SEM of at least 5 separate experiments. (**G**) Interaction of wild-type LF82 bacteria with murine PPs placed in Ussing chambers and coincubated with nonpathogenic MG1655 *E. coli* K-12 strain expressing or not expressing LPF_{LF82}. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

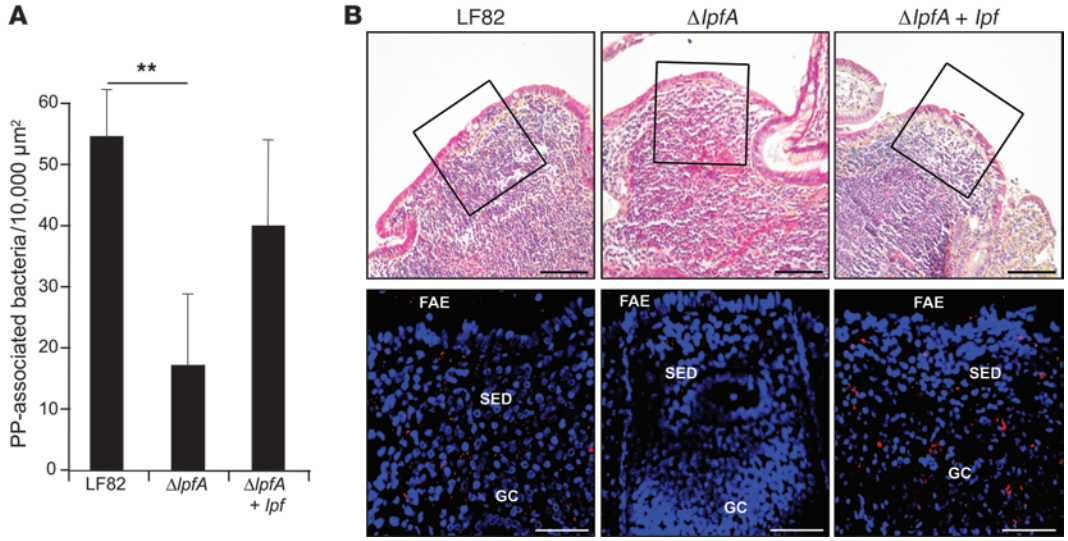

Figure 2
Involvement of LPF to promote interaction between AIEC LF82 bacteria and PPs. (**A**) Quantification of intra-human PP-associated bacteria by confocal microscopy. Each value is the mean ± SEM of 2–4 separate experiments, with 3–5 sections studied for each experiment. $^{**}P < 0.01$. (**B**) Representative confocal photomicrographs of uptake of bacteria across human FAE. Scale bars: 100 μm for HES staining and 50 μm for confocal analysis. Images in the bottom row correspond to the boxed regions in the top row. See the Figure 1D legend for staining.

Decreased interaction of the LF82-Δ*lpfA* mutant compared with the wild-type strain was observed with M-like cells (Figure 3, B and C), and after 4 hours of infection, significantly decreased translocation was observed for the LF82-Δ*lpfA* mutant compared with the wild-type strain (Figure 3E). Transcomplementation of LF82-Δ*lpfA* mutant with cloned *lpf* fully restored the phenotype, indicating that LPF play a key role in targeting M cells. The observation that LPF expression is crucial for AIEC interaction with M cells was confirmed ex vivo using PPs isolated from mice and confocal analysis to search for colocalization of bacteria with M cells stained with TRITC-labeled *Ulex europaeus* agglutinin 1 at the surface of whole PPs. Results showed that the number of bacteria associated with M cells was significantly higher ($P < 0.001$) for wild-type strain LF82 than for the LF82-Δ*lpfA* mutant (Figure 3, F and G).

Prevalence of lpf-positive adherent-invasive E. coli strains in CD patients and controls. Various *lpf* operons are observed among bacteria belonging to the Enterobacteriaceae family, including *Shigella* spp., *Salmonella* spp., *Citrobacter rodentium*, enterohemorrhagic *E. coli* (EHEC), and rabbit enteropathogenic *E. coli* (REPEC). BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that LpfA protein from AIEC strain LF82 (CU651637) was most closely similar to that of *Salmonella* Typhimurium strain LT2 (NC_003197), with 86% similarity, and to that of EHEC strain EDL-933 encoded by *lpf1* (NC_002655), with 82% similarity (Supplemental Figure 1 and Supplemental Figure 5A). DNA sequencing of the LpfA-encoding genes of all the CD-associated *E. coli* strains positive in hybridization showed that, among ileal-associated *E. coli* strains isolated from 55 CD patients, none harbored the *lpfA* gene of *Salmonella* Typhimurium. $lpfA_{LF82}$-positive *E. coli* strains were found in 8 (14.5%) patients, and $lpfA_{Shigella}$-positive *E. coli* strains were found in 23 (41.8%) patients (Supplemental Figure 5). We only observed *E. coli* strains positive for $lpfA1_{EHEC}$ or $lpfA2_{EHEC}$ in 4 patients, in 2 of whom the *E. coli* strains associated with the ileal mucosa also harbored the $lpfA_{Shigella}$ gene.

As a high prevalence of putatively functional $lpfA_{Shigella}$-positive AIEC strains was observed in CD patients, we compared $LPF_{Shigella}$ and LPF_{LF82} in their ability to mediate bacterial interaction with PPs. Thus, experiments were performed with CD-associated AIEC strain LF110 harboring an *lpfA* gene similar to that of *S. flexneri* 2a strain 301 (with 99% similarity, Supplemental Figure 5B) and the corresponding *lpfA*-negative mutant. The comparison of the ability of AIEC strain LF110 and strain LF82 to interact with murine PPs revealed similar numbers of PP-associated bacteria for the two AIEC strains (Figure 4). Like the LF82-Δ*lpfA* mutant, the LF110-Δ*lpfA* mutant showed a significantly decreased ability to interact with murine PPs compared with the wild-type strain. Therefore, the analysis of the presence of any *lpf* operon in ileal-associated *E. coli* strains indicated a statistically significant difference ($P = 0.006$) in the prevalence of *lpfA*-positive *E. coli* strains associated with ileal specimens between CD patients and controls (Table 1). Of the 55 CD patients, 26 (47.3%) harbored *lpfA*-positive *E. coli* strains, in contrast to only 5 (17.2%) of the 29 controls without IBD. When we analyzed the presence of the *lpfA* gene in AIEC and non-AIEC strains, we observed that 12 CD patients harbored *lpfA*-positive AIEC strains. Fourteen CD patients harbored *lpfA*-positive *E. coli* strains that were not initially classified as AIEC on the basis of the expression of AIEC-specific virulence properties. Even without considering a possible underestimation of the AIEC phenotype, the prevalence of subjects harboring *lpf*-positive AIEC strains was significantly ($P = 0.027$) higher in CD patients than in controls (21.8% versus 3.5%). Of note, most of the *E. coli* strains associated with the ileal mucosa of controls were non-AIEC and did not harbor the *lpfA* gene, which was highly significant compared with CD patients ($P = 0.00006$).

LPF-dependent increased interaction of AIEC LF82 bacteria with Nod2$^{-/-}$ PPs. Confocal quantification of M cells on PPs using TRITC-labeled UEA1 indicated a 2.9-fold-higher number in *Nod2*$^{-/-}$ mice

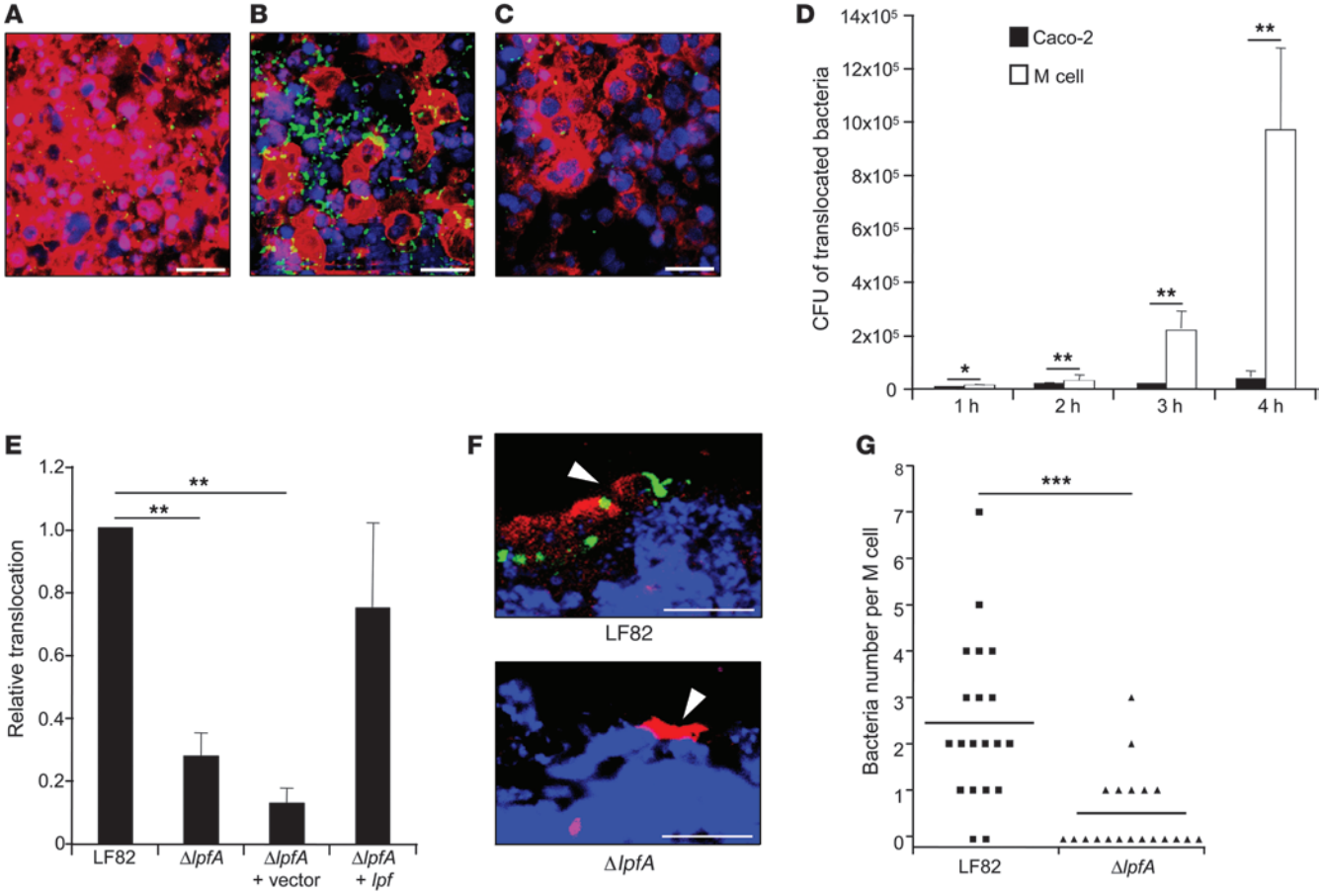

Figure 3

LPF are involved in the ability of AIEC strain LF82 to target M cells. Interaction of AIEC bacteria LF82 (**A** and **B**) and LF82-Δ*lpfA* isogenic mutant (**C**) with Caco-2-cl1 (**A**) or M-like cell (**B** and **C**) monolayers in the presence of 0.5% methyl α-D-mannopyranoside. Phalloidin-TRITC labeling of F-actin (red), anti-O83 antibody labeling of LF82 bacteria (green), and Hoechst labeling of DNA (blue) were used. Confocal photomicrographs of interaction of bacteria with in vitro M cells are representative of 3 separate experiments. Scale bars: 25 μm. (**D**) Translocation across Caco-2-cl1 or M-like cell monolayers. Results are expressed as CFU of translocated bacteria. Each value is the mean ± SEM of at least 5 separate experiments. (**E**) Translocation of LF82 bacteria and LF82-Δ*lpfA* isogenic mutant across M-like cell monolayers after 4 hours infection. Results are expressed as translocated bacteria relative to those obtained for strain LF82, taken as 1. (**F**) Confocal analysis of murine PP sections after labeling of AIEC LF82 with LPS O83 antibody (green), of M cell with UEA-1 TRITC (red), and DNA with Hoechst (blue). Scale bars: 20 μm. Arrowheads indicate UEA-1–positive cells. (**G**) Quantification of murine M cell–associated bacteria by confocal microscopy analysis. Bars represent the mean. $^{**}P < 0.01$, $^{***}P < 0.001$.

compared with wild-type mice (Figure 5, A–C), confirming previous findings by Barreau et al. (35). In addition, quantification of GP2-positive cells using anti-GP2 antibody indicated a 2.8-fold-higher number of GP2-positive cells in *Nod2*$^{-/-}$ mice compared with wild-type mice (Figure 5, D–F). As GP2 expression is restricted to M cells in FAE (29), this confirmed an increased number of M cells in PPs of *Nod2*$^{-/-}$ mice compared with wild-type mice.

We investigated the ability of AIEC LF82 bacteria and LF82-Δ*lpfA* mutant to interact with murine PPs isolated from wild-type and *Nod2*$^{-/-}$ mice. Results presented in Figure 5G show that the number of PP-associated LF82 bacteria was significantly higher for PPs from *Nod2*$^{-/-}$ mice than from wild-type mice ($P < 0.05$). In contrast, the number of LPF-negative LF82 mutant interacting with PPs was similar regardless of the *Nod2* genotype, indicating that the increased number of LF82 bacteria observed with PPs from *Nod2*$^{-/-}$ mice was linked to the expression of LPF.

Discussion

Clinical observations suggest that the sites of initial inflammation in ileal CD are the lymphoid follicles (21, 22). A correlation exists between peak of CD case frequency (between 15 and 25 years old) and peak representing the number of PPs according to age, suggesting that CD develops as an inflammatory process specifically targeting these important lymphoid structures (36). Moreover, recent studies reported a defect in the intestinal barrier to control bacterial translocation across PPs of CD (37, 38). It is well documented that several enteroinvasive pathogens, such as *Yersinia*, *Shigella*, and *Salmonella*, invade the intestinal mucosa via the M cells of the FAE (24, 27, 31). The molecular mechanism of the specific interaction between the invasive pathogens and M cells involve the Inv invasin for *Yersinia* (26), the IpaB and IpaC effectors for *Shigella* (31), and LPF for *Salmonella* (30). It is also known that enteropathogenic *E. coli* (EPEC)

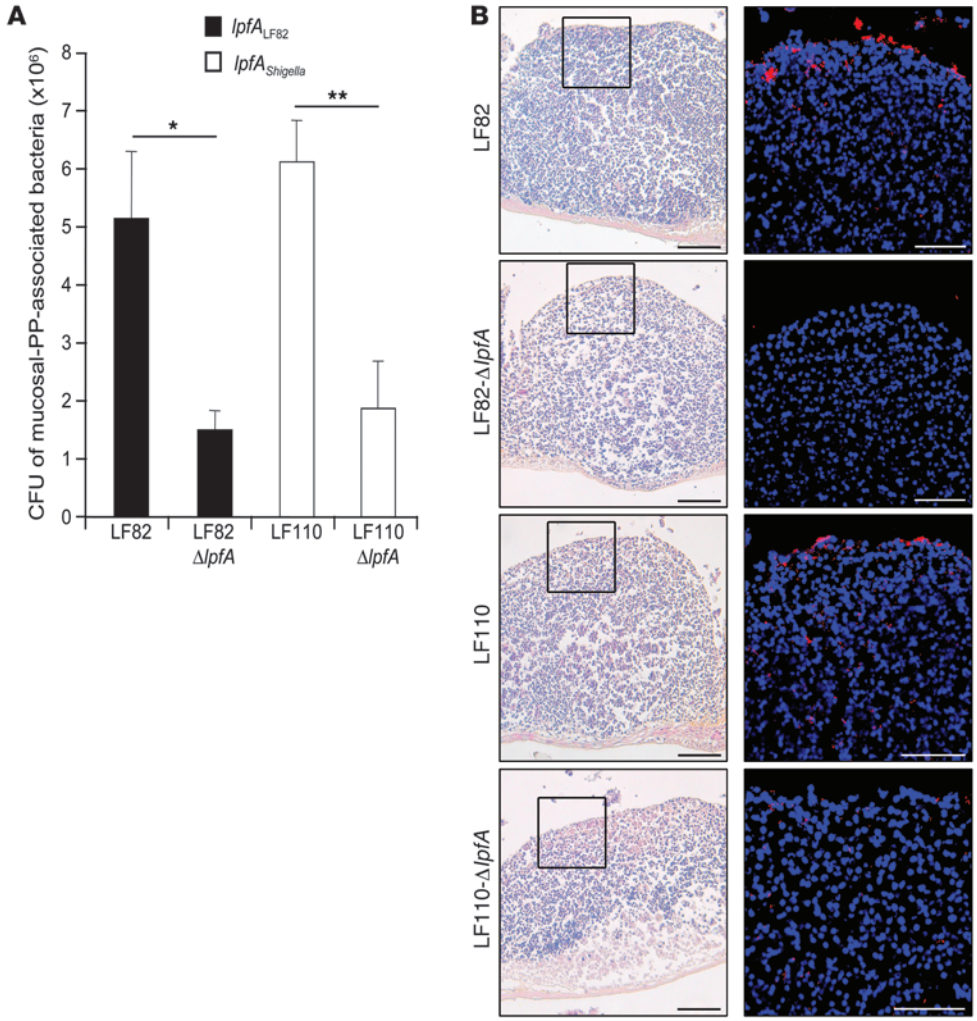

Figure 4
Comparison of ability of $LPF_{Shigella}$ and LPF_{LF82} bacteria to interact with murine PPs. (**A**) Quantification of murine PP-associated bacteria for AIEC strains LF82 and LF110 and corresponding Δ*lpfA* mutants. See the legend to Figure 1C. Each value is the mean ± SEM of at least 5 separate experiments. (**B**) Visualization of bacteria interacting with murine isolated PPs. Scale bars: 100 μm for HES staining and 50 μm for confocal analysis. Images in the right column correspond to the boxed regions in the left row. See the legend to Figure 1D. $^{*}P < 0.05$, $^{**}P < 0.01$.

are able to translocate across an in vitro M cell model and that this translocation is regulated by the type III secretion system, but the bacterial effector involved is not yet known (34).

Here we show that AIEC bacteria are able to interact with PPs involving at least one virulence factor in addition to type 1 pili. The analysis of the genome sequence of AIEC reference strain LF82 indicates the presence of an *lpf* operon encoding LPF. Such *lpf* operons were reported in *Salmonella* Typhimurium, *Shigella boydii*, and *S. flexneri* and in EHEC EDL933 (30, 39), but this is the first report to our knowledge of the presence of an *lpf* operon in CD-associated AIEC bacteria. In *Salmonella* Typhimurium, LPF promote bacterial interaction to murine PPs (30), and ex vivo assays performed with a murine intestinal organ culture model showed that their interaction with PPs leads to full expression of LPF (40). No role has been yet reported for LPF in *Shigella*. For EHEC, experiments in pigs and sheep with O157:H7 strain 86-24 indicated that LPF contribute to intestinal colonization (41). To study the involvement of LPF in AIEC LF82 interaction with PPs, we performed in vitro experiments with M cells, and ex vivo assays with murine and human PPs. We observed that LPF have a crucial and type 1 pili–independent role in mediating the interaction between AIEC LF82 bacteria and PPs. Numerous AIEC LF82 bacteria were observed within PPs, as shown by ex vivo assays using murine and human PPs for wild-type strain, but not for the LPF-negative LF82 mutant.

Table 1
Prevalence of adherent-invasive *E. coli* positive for *lpfA* gene in ileal specimens from CD patients and controls

Origin of the strains	Total no. of subjects	Total no. of subjects harboring *lpfA*-positive *E. coli* (%)	No. of subjects (%)			
			AIEC⁺ *lpfA*⁺	AIEC⁺ *lpfA*⁻	Non-AIEC *lpfA*⁺	Non-AIEC *lpfA*⁻
Ileal CD patients	55	26 (47.3)	12 (21.8)	6 (10.9)	14 (25.4)	22 (40.0)
Ileal controls	29	5 (17.2)[A]	1 (3.5)[B]	0 (0.0)[C]	4 (13.8)[C]	24 (82.7)[D]

P values for controls versus CD patients: [A]$P = 0.006$; [B]$P = 0.027$; [C]$P > 0.05$; [D]$P = 0.0002$.

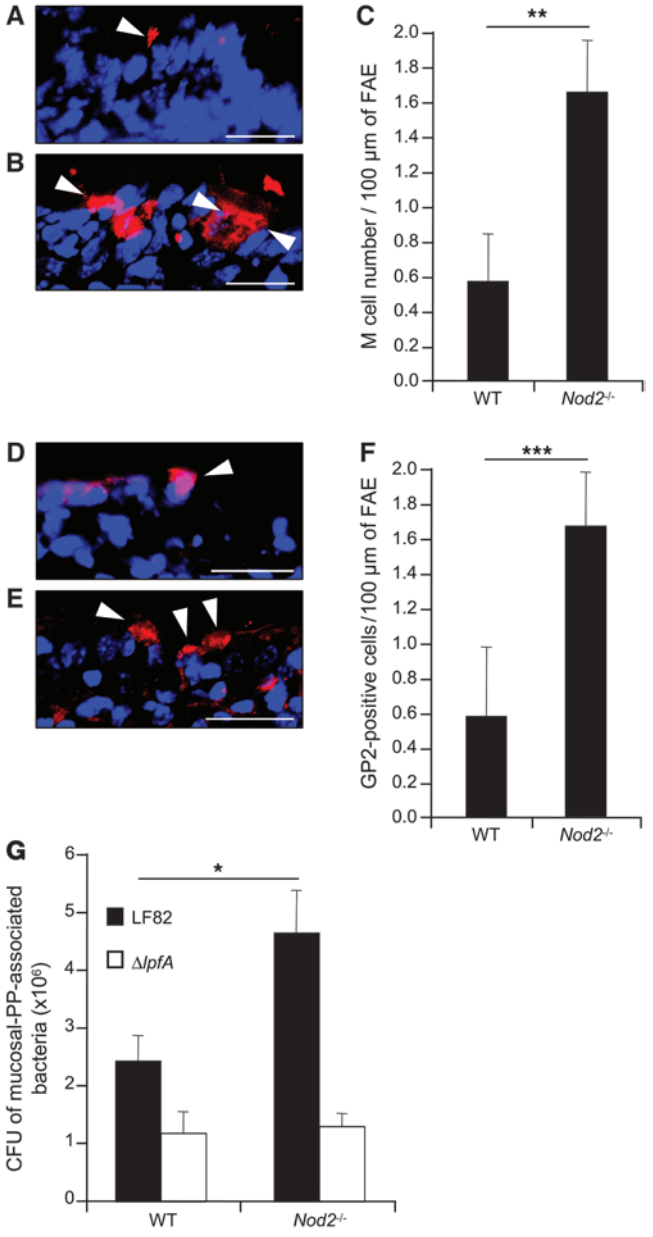

Figure 5
LPF-dependent increased interaction of AIEC LF82 bacteria with *Nod2*$^{-/-}$ PPs. Visualization (**A**, **B**, **D**, and **E**) and quantification (**C** and **F**) of UEA-1 TRITC–stained M cells (red) (**A**–**C**) and GP2-positive cells (red) (**D**–**F**) present in FAE of wild-type (**A** and **D**) and *Nod2*$^{-/-}$ (**B** and **E**) mice after cryostat section. Scale bars: 20 μm. Arrowheads indicate UEA-1– or GP2-positive cells. In **C** and **F**, results are expressed as number of M cells present in 100 μm of FAE. Each value is the mean ± SEM of 6 separate experiments, with 5–6 sections studied for each experiment. (**G**) Ability of wild-type LF82 bacteria and LPF-negative mutant to interact with murine PPs from wild-type and *Nod2*$^{-/-}$ mice. See the legend to Figure 1C. Each value is the mean ± SEM of at least 5 separate experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

M cells can serve as an entry portal for many enteroinvasive pathogens. We observed here that AIEC LF82 bacteria translocated at a very high level through M cell monolayers, but not as the result of the loss of monolayer integrity, since we observed that the TEER of M cell monolayers was not modified after infection with strain LF82. A decreased ability to translocate was observed for the LPF-negative mutant, and confocal analysis showed that AIEC LF82 bacteria interacted with M cells at the surface of murine PPs only when LPF were expressed. Together, these experiments demonstrate a functional role for the LPF of AIEC strain LF82 in targeting M cells on the surface of PPs. Of note, the enteric pathogens crossing the FAE need to survive phagocytosis and bacterial killing by resident and recruited macrophages in the dome of the lymphoid follicle. This is not really a problem for AIEC bacteria, since one of the characteristics of AIEC is their ability to survive and replicate at a high rate in phagolysosomes within macrophage (42, 43). Thus, the expression of LPF in AIEC bacteria is not deleterious for these invasive bacteria crossing the FAE.

Mutations in the *CARD15/NOD2* gene are associated with abnormal development and function of PPs, since *Nod2*-knockout mice exhibit an increased number of PPs also characterized by an excess of M cells and CD4$^+$ T cells (35). In the present study, probably owing to the increased numbers of M cells, we observed LPF-dependent increases in numbers of AIEC LF82 bacteria interacting with PPs from *Nod2*$^{-/-}$ compared with WT mice. Interestingly, AIEC bacteria are more frequently isolated during early recurrence of ileal CD (9), and *Nod2* mutations are mostly associated with ileal involvement, structuring complications, and a slightly earlier age of onset (44). In this context, the analysis of a large cohort of CD patients is necessary to search for a possible correlation between *NOD2* polymorphisms and the presence of adherent-invasive *E. coli* expressing LPF. Of note, NOD2-deficient mice do not develop macroscopically or clinically evident colitis in response to their own microbiota or when challenged with AIEC bacteria (N. Barnich, unpublished observations), indicating that some other genetic or environmental factors are required to create the necessary conditions for increasing host susceptibility to invasion by LPF-expressing bacteria. Perhaps the generation of humanized transgenic mice expressing CEACAM6 in the small bowel using a villin promoter and knockout for *Nod2* could indicate whether ileal bacterial colonization involving abnormal CEACAM6 expression is a missing host susceptibility factor.

Two major *lpf* operons were identified in CD-associated *E. coli* strains, similar to those found in strain LF82 and *Shigella*. Interestingly, we show here that the roles of the $LPF_{Shigella}$ and LPF_{LF82} in their ability to interact with PPs are similar, and that there was a statistically significant increase in the prevalence of *lpfA*-positive *E. coli* strains, whatever the *lpfA* origin, in ileal specimens from CD patients compared with controls. We therefore suggest that *lpfA* probes are very useful and sensitive molecular tools for investigating the presence of fully pathogenic AIEC in CD patients, since we observed some *lpfA*-positive CD-associated *E. coli* strains that were not initially classified as AIEC on the basis of all AIEC-specific virulence properties.

In conclusion, our findings, in addition to the suspected major role of PPs in CD as observed by histological and functional examination of the gut mucosa, show that CD-associated AIEC bacteria by expressing LPF can use PPs as an open gate to induce early stages of the disease. This observation could be very relevant, since there is a potential role of gastrointestinal infections in the onset of IBD as reported by two studies, including a recent

Danish population-based cohort long-term follow-up study that showed that gastroenteritis caused by nontyphoid *Salmonella* or thermophilic *Campylobacter* increases the risk of developing IBD (45, 46). LPF are expressed by *Salmonella*, giving weight to the idea that PPs are major players in IBD. Further analyses are therefore needed to investigate the presence of LPF-expressing pathogens in CD tissues. In particular, such studies should address whether the Gram-negative intramucosal bacteria observed in 73% of CD patients express LPF, since 58% of these intramucosal bacteria were not identified as *E. coli* (7). Taken together, our data reinforce the idea that underlying genetic components in hosts affected by IBD might create the necessary conditions for increasing host susceptibility to invasion by LPF-expressing bacteria, which aggravates the disease. This is another example of co-evolution between pathogens and the host, as we previously reported that AIEC bacteria express type 1 pili variant to better bind to abnormally expressed CEACAM6 by ileal enterocytes of CD patients (14, 15). From the data presented here, the co-evolution could involve, on the host side, increased numbers of PPs and M cells in patients and, on the pathogen side, the presence of virulence genes encoding LPF. We previously reported that CEACAM6 expression by IECs is positively regulated by AIEC adhesion and also by proinflammatory cytokine stimulation, and so we can speculate that more bacteria will reach the FAE underlying macrophages, and that more proinflammatory cytokines will be synthesized, leading to an amplification loop of colonization and inflammation.

Methods

Reference bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Supplemental Table 1. Bacteria were grown routinely in LB broth or in cell culture medium (EMEM medium supplemented with 10% fetal bovine serum [PAA]), with 2% wt/vol bile salts (sodium cholate, Sigma-Aldrich), overnight at 37°C and without shaking.

Construction of isogenic mutants and transcomplementation assays. Isogenic mutants were generated with a PCR product using the method described by Datsenko et al. (47). Primers used are listed in Supplemental Table 2. For transcomplementation assays, a PCR product containing the entire 5,454-bp *lpf* operon was cloned into the pBAD24 vector (48) (Supplemental Tables 1 and 2).

Interaction of bacteria with murine PPs using Ussing chamber and mouse ileal loop experiments. Biopsy samples from ileum containing PPs or without PPs of 6-week-old FVB wild-type or *Nod2*$^{-/-}$ male mice were placed in a 0.196-cm^2 chamber with 1.6 ml of circulating oxygenated Ringer solution at 37°C with or without 0.5% methyl α-D-mannopyranoside. Bacterial interactions were studied using viable bacteria at a final concentration of 8×10^7 CFU/ml in the mucosal reservoir. Four hours after infection, biopsy samples were washed and crushed with an Ultra-Turrax in the presence of 0.1% Triton X-100, and bacteria numbers were determined by plating. When needed, anti-GP2 antibody (Santa Cruz Biotechnology Inc.) was added at a final concentration of 1 μg/ml in the circulating mucosal Ringer solution during 30 minutes of preinfection and during the 4 hours of infection. For coincubation experiments, 8×10^7 CFU/ml of LF82 strain were mixed with 8×10^7 CFU/ml of nonpathogenic *E. coli* strain MG1655, MG1655 + pBAD24, or MG1655 + pBAD24-*lpf*. After 4 hours of infection, the number of LF82 bacteria was determined by plating on erythromycin-containing agar medium to select for AIEC LF82.

For visualization of the interaction, 3-μm sections of paraffin-embedded PPs were stained with hematoxylin/eosin/safranin (HES) or subjected to FISH using Cy3-labeled probe EUB338 (49). To search for a colocalization between bacteria and M cells, TRITC-labeled *U. europaeus* agglutinin 1 (Sigma-Aldrich) or anti-GP2 antibody was used to label M cells and antibodies raised against *E. coli* LPS O83 were used to label LF82 bacteria. The slides were examined with a Zeiss LSM 510 Meta confocal microscope.

Interactions of bacteria with PPs were also studied using mouse ileal loops, as previously described (50). Briefly, mice were starved for 24 hours before operation, with water available ad libitum. Mice were anesthetized, and their intestines exteriorized through a midline incision. Two or 3 intestinal segments (about 1 cm) containing 1 PP were ligated, and 5×10^7 CFU were injected into the loop. Four hours after injection, the animals were killed, the loops were excised, and the number of PP-associated bacteria was determined as previously described. Animal protocols were approved by the Committee for Ethical Issues, CEMEA Auvergne (Clermont-Ferrand, France).

Interaction of bacteria with human isolated PPs. PPs were isolated from human ileum using dissection microscopy as previously described in detail (38). PPs from CD patients were obtained from macroscopically normal ileum of 10 patients undergoing surgery for recurrence at a previous ileocolonic anastomosis (6 men, 4 women; median age, 30 years, range, 21–59; 5 on maintenance with mesalamine, 3 on azathioprine, 2 no medication). Routine histology showed no inflammation to mild inflammation in the sampled part of the ileum. PPs from controls were obtained from macro- and microscopically normal ileal specimens of patients who had undergone surgery for right-sided colon cancer. The control patients had no signs of generalized disease, and none had received preoperative chemo- or radiotherapy. The study was approved by the Regional Human Ethics Committee in Linköping, Sweden, and all subjects had given informed consent. Bacterial uptake across FAE was performed in Ussing chambers, as previously described (38). Experiments were performed in the presence of 0.5% methyl α-D-mannopyranoside.

Translocation across M cell monolayers. The coculture conditions were as follows: 5×10^5 Raji B cells were resuspended in complete DMEM (PAA) and added to the basolateral chamber of 14-day-old Caco-2-cl1 cell monolayers grown on 12-mm Transwell cell culture inserts (Millipore), and the cocultures were maintained for 4–6 days (33, 51, 52). The corresponding monocultures of Caco-2-cl1 cells on matched filter supports were used as controls. The integrity of cell monolayers was measured by monitoring TEER with an Millicell-ERS (Millipore).

For translocation assay, the apical surface of M cell monolayers was infected in the presence of 0.5% methyl α-D-mannopyranoside with 10 μl bacterial suspension containing 1×10^7 bacteria. Basolateral medium was removed 4 hours after infection, and samples were diluted and plated onto Müller-Hinton agar plates to determine the number of CFU of translocated bacteria.

For visualization of the monolayers, Caco-2 cells cultured alone or with Raji B cells, with or without infection, were washed thoroughly in PBS and fixed in 2% paraformaldehyde at 4°C for at least 45 minutes. After permeabilization of cells with Triton X-100 (0.3% in PBS for 20 minutes), Transwells were incubated with rabbit anti-O83 antibody (1:200 in PBS) for 45 minutes at room temperature and, after washing with PBS, with FITC-conjugated anti-rabbit antibodies (1:100 in PBS). F-actin was simultaneously localized by inclusion of phalloidin-TRITC with the secondary antibody. Transwells were then washed thoroughly in PBS and mounted in Vectashield containing DAPI (Vector Laboratories). Transwells were examined with a Zeiss LSM 510 Meta confocal microscope.

E. coli strains isolated from IBD patients and controls, and DNA dot blot hybridization and sequencing. A total of 249 *E. coli* strains were isolated from ileal specimens from 55 of 63 CD patients tested who had undergone ileocolectomy with end-to-end ileocolonic anastomosis and from ileal specimens from 29 of 36 controls with right-sided colon cancer who had undergone right hemicolectomy. None of the patients received antibiotics in the 4 weeks before sampling. All patients and controls gave informed consent, and approval from the local ethics committee (CCPPRB, Lille) was obtained. None of the controls developed IBD at follow-up evaluation.

Hybridizations were performed on extracted DNA from *E. coli* strains with PCR fragments as DNA probes corresponding to *lpfA* from AIEC strain LF82, *Salmonella* Typhimurium, *Shigella boydii*, and EHEC O157:H7 $lpfA_1$ and $lpfA_2$ (Supplemental Table 2). Single-stranded DNA was sequenced, translated, and analyzed using ExPASy (http://expasy.org/tools/dna.html) and EBI-ClustalW software (http://www.ebi.ac.uk/ clustalw/) using the default options.

Statistics. Numerical values are expressed as means with SEM. Statistical comparisons were performed using 2-tailed Student's *t* test or Mann-Whitney *U* test when appropriate, unless the variables required a 2-tailed Fisher exact test. For experiments with multiple treatment groups, ANOVA was performed, followed by pairwise comparisons using Bonferroni's multiple comparison tests. All tests were 2 sided. A *P* value less than 0.05 was considered statistically significant.

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