

Invasive *Escherichia coli* are a feature of Crohn's disease

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Crohn's disease (CD) and ulcerative colitis (UC) are idiopathic inflammatory conditions of the gut. Our goal was to investigate if invasive *Escherichia coli* strains were present in patients with inflammatory bowel disease (IBD). Bacterial strains were isolated from biopsy material obtained from normal controls, and patients with a clinical diagnosis of CD and UC. Invasive bacteria were characterized by gentamicin protection assay and biochemical profiling (Api-20E). Strains were characterized by induction of cytokine expression in epithelial and macrophage cell cultures, measurement of epithelial barrier function, and confocal microscopy. Of all invasive bacterial strains in CD 98.9% were identified as *E. coli* as opposed to 42.1% in UC and 2.1% in normal controls. Epithelial invasion *in vitro* was significantly higher for CD-associated *E. coli* (8.4%, ± 5.5 of initial inoculum (I/O)) in comparison to UC (2.5%, ± 0.4 I/O), but highest for strains from inflamed CD tissue (11.3%, ± 4.3 I/O). Both, CD and UC *E. coli* strains induced high mean TNF- α expression in macrophage cell lines (2604.8 pg/10⁵ cells, ± 447.4 ; 2,402.6 pg/10⁵ cells, ± 476.3 , respectively), but concentrations were significantly higher for isolates from inflamed CD tissue (3071.3 pg/10⁵ cells, ± 226.0). Invasive *E. coli* from IBD tissue induced similar concentrations of interleukin (IL)-8 in epithelial cell cultures, but strains from inflamed CD tissue induced significantly less epithelial IL-8 (674.1 pg/10⁵ cells, ± 58.0 vs 920.5 pg/10⁵ cells, ± 94.6). IBD-associated *E. coli* strains significantly decreased transepithelial resistance, induced disorganization of F-actin and displacement of ZO-1, and E-cadherin from the apical junctional complex (AJC). In comparison to normal controls and UC, *E. coli* are more prevalent in CD, are highly invasive, and do not encode for known effector proteins. *E. coli* strains from IBD patients regulate cytokine expression and epithelial barrier function, two pathological features of IBD.

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Inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD), and ulcerative colitis (UC) are chronically remitting intestinal inflammatory processes of unknown etiology.¹ IBD predominantly affects young adults, and patients suffer from bloody diarrhea, abdominal pain, and weight loss. In a significant number of patients, inflammation is not limited to the intestine but affects extraintestinal sites, involving liver, eyes, and joints.² Immunologically, IBD is characterized by a markedly increased cytokine expression from T lymphocytes, neutrophils, macrophages, and epithelial cells.³ Among others, significant cytokine induction has been observed for TNF- α ⁴ and interleukin (IL)-8,⁵ two cytokines central to the pathogenesis of CD and UC. Increased colonic IL-8 concentration has been correlated with neutrophil infiltration of IBD tissue,⁶ whereas TNF- α is implicated in systemic manifestations of disease, like

anorexia, weight loss, and depletion of whole-body protein and lipids.⁷ Additional pathophysiological change observed in IBD is elevated intestinal permeability that correlates with disease activity,⁸ and it has been utilized experimentally as a marker for relapsing disease.⁹ It has been speculated that the two essential components for the development of gastrointestinal inflammation in CD and UC are genetic predisposition of the host and environmental factors, although neither one alone is sufficient to induce chronic inflammation.

There is mounting evidence that the environmental factors implicated in the pathogenesis of IBD are bacteria and their components.¹⁰ Both, CD and UC primarily affect intestinal areas with high bacterial counts, and in CD, surgical diversion of fecal stream or treatment with bowel rest and total parental nutrition have been used therapeutically to control

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enteric inflammation.¹¹ Further evidence implicating a role for enteric flora is that treatment with antibiotics and probiotics was found to be beneficial, and is now utilized for induction and maintenance of remission, respectively.¹² In addition, immunological studies have shown that the majority of patients with IBD develop serological and T-cell response to their own enteric flora.¹³

Recently, a novel class of *E. coli*, termed adherent-invasive *E. coli* (AIEC), has been associated with CD.¹⁴ The representative lactose-fermenting strain LF82 efficiently invaded epithelial cell cultures *in vitro* and was able to survive in the intracellular compartment for prolonged period of time.¹⁵ The goal of the current study was to characterize invasive *E. coli* strains in an American population with CD, UC, or normal controls without IBD. Further, we sought to answer if invasive *E. coli* displays pathogenicity traits that have been identified in human IBD, namely invasion of epithelial cells, regulation of barrier function, and cytokine production.

MATERIALS AND METHODS

Biopsy Material

All studies were approved by the Emory Institutional Review Board. Patients with clinically confirmed IBD were recruited between July 2002 and August 2005 at a single center. With the exception of a single CD patient, research subjects did not have exposure to antibiotics 3 months before colonoscopy. Medications allowed during the study included azathioprine, prednisone, and mesalamine preparations. Patients receiving other medications for the treatment of IBD were excluded. Control patients constituted asymptomatic subjects with a normal colonoscopic examination and without evidence of inflammation or colonic polyps. After written consent, usually four biopsies were obtained during outpatient colonoscopy from 15 patients with CD (mean age 42.6 years, 60% women), 12 with UC (mean age 37.5 years, 75% women), and 12 normal control subjects (mean age 54 years, 54.3% women). For patients with a diagnosis of IBD, an attempt was made to gather tissue from macroscopically inflamed and non-inflamed appearing segments. Biopsies were immediately incubated in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 100 µg/ml gentamicin (Invitrogen) for 1 h, washed three times in PBS and lysed in 1% Triton-X-100/PBS. Aliquots were cultured on MacConkey agar plates at 37°C overnight and lactose-fermenting colonies were enumerated and propagated in LB broth for 4 h at 37°C under aerobic conditions. Individual clones were stored in 50% glycerol at -80°C until further use.

Cell Culture

Caco-2 epithelial cells and J774A.1 macrophages (ATCC, Manassas, Virginia) were grown in DMEM (Invitrogen), gentamicin 50 µg/ml (Invitrogen), 10% FCS (Gemini, Calabasas, CA, USA) in a 5% CO₂, 37°C atmosphere, and

were used between passages 45–75 and 10–15, respectively. For invasion assays, epithelial cells and macrophages were seeded on collagen-coated 96-well plates. Caco-2 cells were allowed to differentiate for 5–7 days before infection.

Invasion Assay

Bacterial invasion was measured as described previously.¹⁶ Briefly, individual bacterial strains were grown aerobically in 2 ml LB broth at 37°C overnight. To minimize basolateral bacterial invasion, only differentiated Caco-2 epithelial cells were infected in triplicate at multiplicity of infection (MOI) 10:1 with an overnight culture. Following centrifugation at 500g, 15 min, 37°C, infections were allowed to proceed for 3 h without antibiotics. Monolayers were washed in PBS four times and incubated for an additional hour in DMEM supplemented with 100 µg/ml gentamicin. Mean bactericidal concentration (MBC) for gentamicin was determined at 1 µg/ml and was used at 100-fold the MBC. Cell cultures were rinsed again in PBS after gentamicin killing and monolayer integrity was confirmed microscopically after the final wash. Epithelial cells were lysed in 1% Triton-X-100/PBS and serial dilutions were plated on LB plates. Colonies were counted the following day after 37°C incubation overnight. To determine the percentage of intracellular bacteria in relation to the initial inoculum (I/O), aliquots of individual overnight LB cultures were serially diluted, plated on LB agar plates, and enumerated. Significant epithelial invasion was determined by comparing pathogenic *E. coli* strains enteroinvasive *E. coli* strain E12860/0 (EIEC, kindly provided by MS Sonnenberg), CD reference strain LF82 (kindly provided by A. Darfeuille-Michaud), to nonpathogenic, commensal strains EFC-1, and FN414. Invasion was considered significant if a minimum of ≥1% of I/O (*t*-test) could be recovered from the intracellular compartment. All bacterial strains used for this study are listed in Table 1.

Identification of Invasive Bacterial Strains

All bacterial strains considered to be invasive were grown on LB agar plates and a single colony was diluted in water. Aliquots of bacterial solutions were cultured in Api-20E test strips (Biomérieux, Durham, NC, USA) under aerobic and anaerobic conditions overnight according to the manufacturer's specification. Invasive bacteria classified as *E. coli* by the Api-20E system with likelihood values of ≥92.4% were considered for further analysis.

Multiplex PCR for Pathogenicity Genes in *E. Coli* Strains

E. coli strains determined to be invasive were subjected to multiplex PCR to screen for genes associated with pathogenicity as described previously. Briefly, invasive *E. coli* from our study and positive control strains, were boiled in PBS and amplified with primer combinations listed in Table 2. Pathogenic *E. coli* strains functioning as positive controls included Enterohemorrhagic *E. coli* O157:H7 (EHEC), EIEC E50851/0 (EIEC), Enteropathogenic *E. coli* 2348/69 (EPEC),

Table 1 *E. coli* strains used in this study

Strain	Characteristics	Source	Reference
FN414	Commensal from healthy volunteer	R. Hull	17
EFC-1	Commensal from healthy volunteer	UMB	17
E12860/0	Enteroinvasive <i>E. coli</i> O124:H-	B. Rowe	18
E50851/0	Enteroinvasive <i>E. coli</i> O164:H-	B. Rowe	18
EDL933	Enterohemorrhagic <i>E. coli</i> O157:H7, outbreak in Michigan	CDC	19
H10407	Enterotoxigenic <i>E. coli</i> O78:H11	C. Deneke	20
JM221	Enterotoxigenic <i>E. coli</i>	J. Nataro	21
17-1	Enterotoxigenic <i>E. coli</i>	J. Nataro	21
CFT073	Uropathogenic <i>E. coli</i>	UMAB	17
BF1072	Uropathogenic <i>E. coli</i>	UMAB	17
E2348/69	Enteropathogenic <i>E. coli</i> O127:H6 from infant diarrhea	B. Rowe	20
LF82	Invasive <i>E. coli</i> strain from inflamed appearing CD tissue	Darfeuille-Michaud	14
4F	Invasive <i>E. coli</i> isolate from normal appearing CD tissue	This study	
13I	Invasive <i>E. coli</i> isolate from inflamed appearing CD tissue	This study	
30A	Invasive <i>E. coli</i> isolate from normal appearing UC tissue	This study	
150F	Invasive <i>E. coli</i> isolate from normal control subject	This study	

Enterotoxigenic *E. coli* H10407, Enterotoxigenic *E. coli* JM221 and 17-2, and Uropathogenic *E. coli* CFT073 and BF1072 (Table 1). PCR was performed with primer combinations for bacterial toxins (*VT1*, *VT2*, *VT2e*, *ST1*, *ST2*, *LT-1*, *cnf1*, *cnf2*, *astA*, *cdt*), adhesion molecules (*fimH*, *eaeA*, *espB α* , *papC*, *afaB-afaC*, *sfaD-sfaE*, *bmaE/afaE8*, pCVD432, *bfp*), *EAF*, and *ipaH* (Table 2). Bacterial DNA was amplified in 35 cycles with denaturing DNA at 94°C, annealing at 55°C, and extension at 68°C for 1 min each. PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Pulsed Field Gel Electrophoresis of Invasive *E. Coli*

Bacteria were grown on Trypticase soy agar plates with 5% sheep blood (TSA-SB; Becton Dickinson and Company, Sparks, MD, USA) at 37°C for 14–16 h. Pulsed field gel electrophoresis (PFGE) was performed using the PulseNet standardized protocol for *E. coli* O157:H7 as described by Ribot *et al.*²² Briefly, bacterial cells were suspended in cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0), and cell concentration adjusted to a turbidity reading of 0.48–0.52 (Dade Behring Microscan turbidity meter; Dade Behring Inc., Deerfield, IL, USA). A 400 μ l aliquot of cell suspension was transferred to a sterile microcentrifuge tube containing 20 μ l of proteinase K (20 mg/ml stock; Invitrogen, Carlsbad, CA, USA) followed by gentle mixing. Four hundred microliters of melted 1% SeaKem Gold agarose (FMC, Rockland, ME, USA) containing 1% SDS, made in standard 0.01 M Tris-EDTA buffer, were added to each cell suspension and mixed gently by pipetting up and down two to three

times, immediately dispensing into the wells of PFGE plug molds (Bio-Rad, Hercules, CA, USA). The plugs were allowed to solidify for 5 to 10 min at room temperature. The solidified agarose plugs were transferred to a tube containing 5 ml of lysis buffer (50 mM Tris, 50 mM EDTA, 1% Sarkosyl (pH 8.0)) and 25 μ l of proteinase K (20 mg/ml). Lysis was allowed to proceed at 54°C in a shaking water bath for 2 h. Plugs were washed two times with sterile water for 15 min each time and four times with sterile Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8.0) for 15 min each time in a shaking water bath. Agarose-embedded DNA was cut (2.0 mm in thickness) and restricted with 50 U of *XbaI* and *BlnI* (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 2 h at 37°C. The digested DNA plugs were loaded on the PFGE gel consisting of 1% SeaKem Gold agarose prepared in 0.5 \times Tris-Borate-EDTA (TBE) buffer (Sigma, St Louis, MO, USA). The electrophoresis was carried out on a CHEF Mapper (Bio-Rad) with switch times of 2.16–54.17 s at 6 V/cm for 19 h and TBE buffer temperature at 14°C. Gels were stained using ethidium bromide (1 mg/ml) and destained with two deionized water washes. Gel images were obtained using a Gel Doc EQ imager (Bio-Rad) under UV transillumination. Analysis of PFGE patterns was performed using BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium).

Cytokine Analysis

Caco-2 epithelial cells and J774A.1 macrophages were prepared as described above. Cell culture media were infected in triplicate with an aliquot of an overnight culture of control *E. coli* EFC-1, EPEC, and CD isolate LF82. Induction of

Table 2 Primer combinations for multiplex PCR and expected product size in base pair (bp)

Primer	Bacterial gene	Size (bp)
Fp: ACGTTACAGCGTGTTCRGGGATC	<i>VT1</i>	121
Rp: TTGCCACAGACTGCGTCAGTRAGG	<i>VT1</i>	
Fp: TGTGGCTGGTTCGTTAATACGGC	<i>VT2</i>	102
Rp: TCCGTTGTCATGGAACCGTTGTC	<i>VT2</i>	
Fp: CCAGAATGTCAGATAACTGGCGAC	<i>VT2e</i>	322
Rp: GCTGAGCACTTTGTAACAATGGCTG	<i>VT2e</i>	
Fp: TTTCCCTCTTTTAGTCAGTCAACTG	<i>ST1</i>	160
Rp: GGCAGGATTACAACAAAGTTCACAG	<i>ST1</i>	
Fp: CCCCTCTCTTTTGCACCTCTTTCC	<i>ST2</i>	423
Rp: TGCTCCAGCAGTACCATCTCTAACCC	<i>ST2</i>	
Fp: TGGATTCATCATGCCACACAAGG	<i>LT-1</i>	360
Rp: CCATTCTCTTTTGCCTGCCATC	<i>LT-1</i>	
Fp: GGCGACAAATGCAGTATTGCTTGG	<i>cnf₁</i>	552
Rp: GACGTTGGTTCGGTAATTTTGGG	<i>cnf₁</i>	
Fp: GTGAGGCTCAACGAGATTATGCACTG	<i>cnf₂</i>	839
Rp: CCACGCTTCTTTCAGTTGTTCTC	<i>cnf₂</i>	
Fp: CCATCAACACAGTATATCCGA	<i>astA</i>	111
Rp: GGTCGCGAGTGACGGCTTTGT	<i>astA</i>	
Fp: CCAACAACACTGAGTTTCTCG	<i>cdt</i>	860
Rp: CAGTCAACGTTGCAGAAGCTG	<i>cdt</i>	
Fp: TGCAGAACGGATAAGCCGTGG	<i>fimH</i>	508
Rp: GCAGTCACCTGCCCTCCGGTA	<i>fimH</i>	
Fp: TGAGCGGCTGGCATGAGTCATAC	<i>eaeA</i>	241
Rp: TCGATCCCCATCGTACCAGAGG	<i>eaeA</i>	
Fp: TATCGATAATAACAATGCGG	<i>espBα</i>	930
Rp: CATGCGATTAATAAGGTCAG	<i>espBα</i>	
Fp: GACGGCTGTACTGCAGGGTGTGGCG	<i>PapC</i>	328
Rp: ATATCCTTTCTGCAGGGATGCAATA	<i>PapC</i>	
Fp: GCTGGGCAGCAAAGTATAACTCTC	<i>afaB-afaC</i>	750
Rp: CATCAAGCTGTTTGTCTCCGCCG	<i>afaB-afaC</i>	
Fp: CGGAGGAGTAATTACAACCTGGCA	<i>sfaD-sfaE</i>	410
Rp: CTCCGGAGAAGTGGGTGCATCTTAC	<i>sfaD-sfaE</i>	
Fp: CTAACCTGCCATGCTGTGACAGTA	<i>BmaE/afaE8</i>	302
Rp: TTATCCCCTGCGTAGTTGTGAATC	<i>BmaE/afaE8</i>	
Fp: AGACTCTGGCGAAAGACTGTATC	<i>PCVD432</i>	194
Rp: ATGGCTGTCTGTAATAGATGAGAAC	<i>PCVD432</i>	
Fp: AATGGTGCTTGCCTTGCTGC	<i>Bfp</i>	326
Rp: GCCGCTTTATCCAACCTGGTA	<i>Bfp</i>	
Fp: CAGGGTAAAAGAAAGATGATAA	<i>EAF</i>	397
Rp: TATGGGGACCATGTATTATCA	<i>EAF</i>	
Fp: TGGAAAACTCAGTGCTCTGCGG	<i>lpaH</i>	140
Rp: TTCTGATGCCTGATGGACCAGGAG	<i>lpaH</i>	

cytokine expression was compared to strain 4F (CD, inflamed tissue), 13I (CD, normal tissue), and 30A (UC, normal tissue). Cells were infected at MOI 10:1 for 3 h, washed three times with PBS, and the growth medium was changed to DMEM supplemented with gentamicin 100 μ g/ml, penicillin, and streptomycin. Cell supernatant was harvested after a total of 24 h and stored at -20°C until further use. Conditioned media were analyzed by ELISA (Invitrogen) in duplicate in three independent experiments for IL-8 and TNF- α from Caco-2 and J774A.1 cells, respectively, according to the manufacturers specification. Data were expressed as mean with standard error (s.e.m.) per 1×10^5 cells.

Examination of Epithelial Barrier Function *In Vitro*

Well-differentiated Caco-2 cells were grown on polycarbonate membrane supports, pore size 0.4 μ m (Costar, Corning, NY, USA). Subtracting filter resistance, epithelial monolayers were grown to an average transepithelial resistance (TER) of 327.9 Ωcm^2 , ± 20.6 and infected with commensal *E. coli* strain EFC-1, pathogenic E2348/69 and representatives from patients with CD (LF82, 4F, 13I) and UC (30A) at MOI 10:1. *E. coli* isolates 4F, 13I, and 30A from our population were chosen for their high TNF- α -inducing capability. Changes in TER over time were determined hourly with an equilibrated Epithelial Voltometer (World Precision Instruments, Berlin, Germany). Triplicate TER readings from three independent experiments were normalized, averaged, and expressed as percent change with standard error. The effect of bacterial isolates on adherens junctions (AJ) and tight junctions (TJ) was analyzed by confocal microscopy. Following infection for 4 h, cells were fixed in ice-cold 100% ethanol, and blocked with 5% goat serum in Hank's Balanced Salt Saline⁺ (HBSS) overnight. Epithelial monolayers were incubated with monoclonal mouse anti-ZO-1 (Zymed, San Francisco, CA, USA; dilution 1:1000) and mouse anti-E-cadherin (Becton Dickinson, Franklin Lakes, NJ, USA; dilution 1:300). Secondary antibodies used in our laboratory were goat anti-mouse Alexa546 and goat anti-rabbit Alexa647 (Molecular Probes, Carlsbad, CA, USA; dilution 1:1000). All monolayers were labeled with Alexa488-phalloidin to stain for F-actin (dilution 1:1000), nuclear stain Hoechst (dilution 1:10 000), and mounted in ProLong Gold (Invitrogen). Labeled slides were visualized using $\times 63$ objective magnification in vertical (x/z) and horizontal plane (x/y) on an LSM510 confocal microscope (Zeiss, Jena, Germany).

Western Blot Analysis of Tight and Adherens Junctional Proteins

Polarized Caco-2 monolayers were propagated on permeable transwell polycarbonate membrane supports (Corning, NY, USA) and serum starved for 16 h before infection. Cells were infected with EFC-1, EPEC, LF82, 4F, 13I, or 30A at MOI of 10:1 for 4 h at 37°C with 5% CO_2 . Cells were lysed and scraped in 50 μ l cell extraction buffer (Invitrogen) supplemented with complete protease inhibitor cocktail

(Roche Applied Science, Indianapolis, IN, USA) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturers' specifications. Protein concentration was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). A 20–30 μ g portion of cell extract was subjected to gel electrophoresis and transferred onto nitrocellulose membrane (Bio-rad). Membranes were blocked in 5% milk for 1 h at room temperature and labeled with primary antibodies at 4°C overnight. Primary antibodies for human TJ protein ZO-1 (Invitrogen), AJ protein E-cadherin (kindly provided by Dr Charles Parkos), and actin (Calbiochem, San Diego, CA, USA) were used at dilution 1:1000, 1:1000, and 1:500, respectively. Following labeling with primary antibodies, membranes were washed in TTBS and blotted with 1:20 000 HRP-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Membranes were developed with ECL substrate (Pierce, Rockford, IL, USA) and exposed to Kodak BioMax MR film (Eastman Kodak Company, Rochester, NY, USA). Film exposures from three independent experiments were subjected to UN-SCAN-IT densitometry analysis and data expressed as mean pixel with bars for s.e.m.

Statistical Analysis

For Figures 1–3 and 5 data were calculated as mean \pm s.e.m. The Student's *t*-test was used to determine statistical significance for invasion assays, ELISA, and measurements of TER. *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Identification of Invasive Bacteria in CD, UC, and Normal Controls

Bacterial growth on MacConkey agar plates was detected in the form of individual lactose-fermenting and nonfermenting colonies. Colony density in biopsy material varied from patient to patient from a single to several hundred colonies. Bacterial growth following overnight incubation was detected in tissue from 12 of 15 patients with CD, 3 of 12 with UC, and 10 of 12 normal controls. Culture of IBD tissue samples resulted in bacterial growth regardless of whether biopsies were taken from macroscopically inflamed or normal appearing tissue. Following propagation under aerobic condition overnight, a total of 830 bacterial isolates were further analyzed in triplicate by an *in vitro* invasion assay with differentiated Caco-2 epithelial cells: 378 strains from CD, 111 from UC, and 341 from normal tissue (Table 3a). Significant invasion ($\geq 1\%$ I/O) was observed in 159 strains from all patients that underwent colonoscopy: 93 of 378 strains from patients with CD (24.6%), 19 of 111 from UC (17.1%), and 47 of 341 from normal control subjects (13.8%).

For identification purposes, 159 strains considered invasive were further subjected to biochemical testing by the Api-20E system. The majority of isolates were identified as *E. coli* (90.0%, Table 3b), followed by *Enterobacter asburiae* (4.5%), *Klebsiella pneumoniae* (1.8%), *Hafnia alvei* (0.9%),

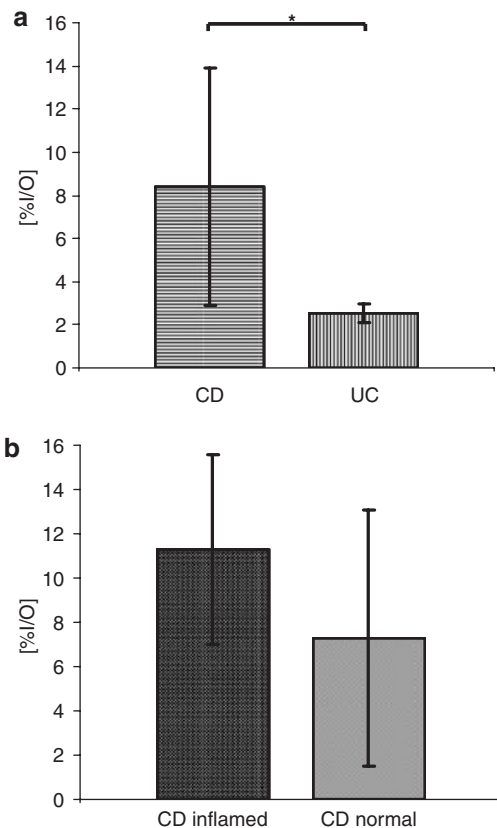


Figure 1 (a) Comparison of mean invasion indices with \pm s.e.m. for *E. coli* isolates from patients with IBD. Differentiated Caco-2 cells in 96 wells were infected in triplicate for 3 h, culture medium changed, and supplemented with gentamicin. Aliquots of cell lysates and bacterial cultures were incubated on LB agar plates and bacterial invasion expressed as percentage of I/O. Mean invasion indices for *E. coli* from patients with CD were 8.4%, ± 5.5 and UC 2.5%, ± 0.4 . Differences in mean invasion indices (Student's *t*-test) were considered significant comparing CD and UC ($*P = 1.22 \times 10^{-7}$). (b) Mean invasion indices with \pm s.e.m. for *E. coli* isolates from macroscopically normal and inflamed CD tissue. Mean invasion indices for *E. coli* isolated from inflamed tissue were higher (11.3%, ± 4.3) in comparison with strains from normal appearing mucosa (7.3%, ± 5.8). This did not reach statistical significance ($P = 5.34 \times 10^{-2}$).

Buttiauxella agrestis (0.9%), and *Escherichia hermannii* (0.9%). Half of all patients with CD harbored invasive *E. coli* strains, whereas only two out of 15 subjects with UC were positive for invasive *E. coli*. Interestingly, invasive *E. coli* were only propagated from normal appearing UC tissue, but not from tissue samples taken from macroscopically inflamed areas.

Applying our experimental conditions, almost all invasive strains in CD were identified as belonging to the species *E. coli* (92 out of 93 strains, 98.9%; Table 3c). Invasive *E. coli* were less common in UC (42.1%). Out of 47 bacterial strains considered invasive, only a single *E. coli* (strain 150F, 2.1%) was identified in normal controls.

E. Coli Invasion Assay

We next determined invasion indices for all *E. coli* strains from patients with CD and UC, regardless of whether isolated

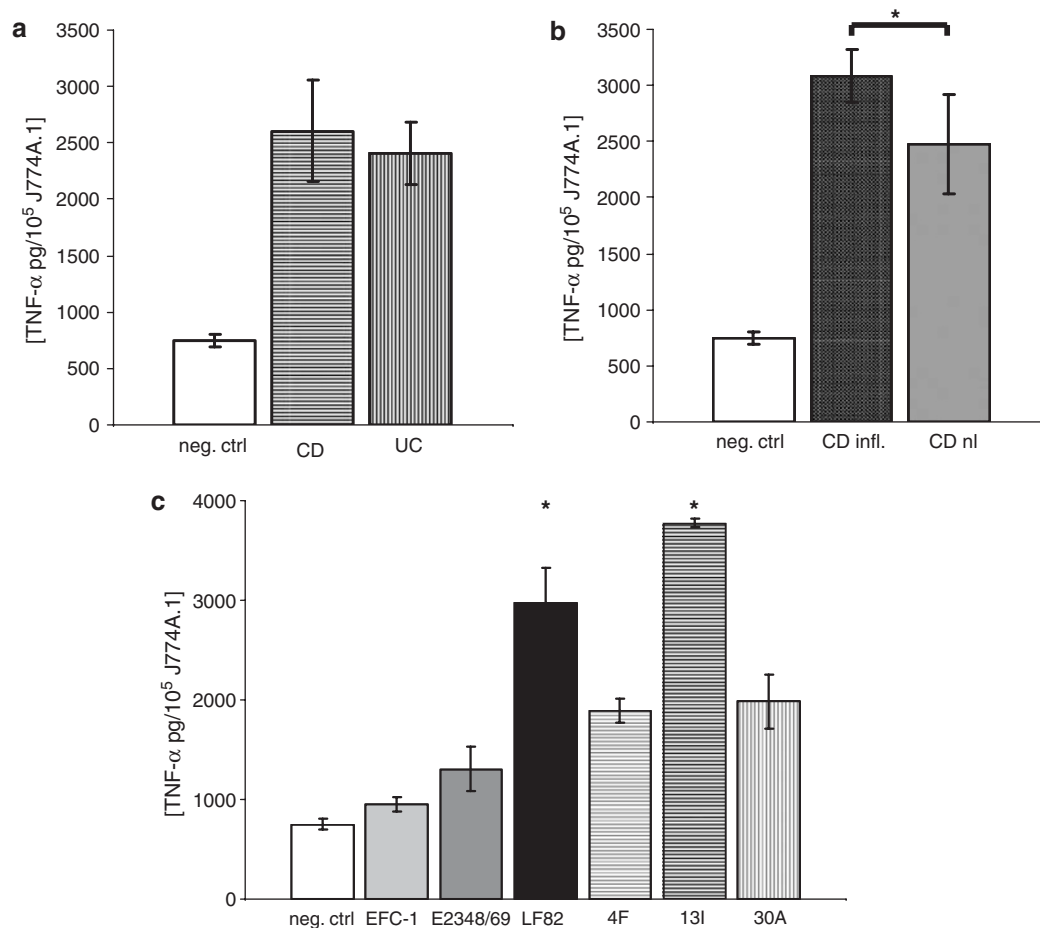


Figure 2 (a) *E. coli* strains from patients with CD and UC induce TNF- α expression in macrophage cultures *in vitro*. Individual invasive *E. coli* strains from patients with CD and UC were incubated with J774A.1 macrophages in duplicate for 3 h with an OD-adjusted aliquot of an overnight culture. Following 3 h infection, growth media were exchanged, now containing antibiotics, and TNF- α concentration in the supernatant was measured after 24 h. Data were expressed as mean TNF- α concentration per 1×10^5 cells with \pm s.e.m. from three independent experiments ($n = 6$) for isolates from CD (2604.8 pg/10⁵ cells, ± 447.4), UC (2402.6 pg/10⁵ cells, ± 476.3), and negative control (752.3 pg/10⁵ cells, ± 55.2). Differences in *E. coli* from CD and UC (Student's *t*-test) were not considered statistically significant ($P = 0.291$). (b) *E. coli* from inflamed CD tissue induce high concentration of TNF- α *in vitro*. Separate analysis for induction of TNF- α expression in J774A.1 was performed for invasive *E. coli* strains propagated from inflamed and macroscopically normal appearing CD mucosa. *E. coli* from inflamed areas (3071.3 pg/10⁵ cells, ± 226.0) induced significantly more TNF- α in comparison to strains from normal mucosa (2476.5 pg/10⁵ cells, ± 447.9 ; $*P = 5.16 \times 10^{-6}$). (c) *E. coli* strains from patients with IBD induce high TNF- α concentrations in macrophage cell cultures *in vitro*. J774A.1 macrophage cultures were infected at MOI 10:1 with *E. coli* control strains EFC-1, E2348/69, and LF82 and isolates 4F, 13I, and 30A from our IBD population for 3 h. TNF- α concentrations were determined after a total of 24 h by ELISA. Data are expressed as mean concentration per 10⁵ cells with \pm s.e.m. ($n = 6$). Isolates LF82 (2978.3 pg/10⁵ cells, ± 346.3) and 13I (3771.9 pg/10⁵ cells, ± 41.9) from inflamed CD tissues induced significantly more TNF- α in comparison to *E. coli* EFC-1, E2348/69, 4F, and 30A.

from inflamed or normal appearing tissue. Mean invasion indices after 3 h of infection were significantly higher for *E. coli* isolates from CD patients (8.4%, ± 5.5 ; Figure 1a) in comparison with strains from patients with UC (2.5%, ± 0.4 , $p 1.22 \times 10^{-7}$). Mean invasion index for the single invasive *E. coli* strain isolated from a normal control subject was 1.8%, ± 1.4 (data not shown), which was significantly lower in comparison with *E. coli* isolates from patients with CD ($P = 0.026$), but not UC ($P = 0.9$).

In CD, invasive *E. coli* was cultured from both macroscopically normal and inflamed appearing tissue. In contrast to CD, we were only able to culture invasive *E. coli* from

normal appearing UC tissues. Therefore, only invasion indices for *E. coli* strains from macroscopically inflamed and normal appearing CD tissue were further analyzed. Invasion indices for *E. coli* isolates from inflamed appearing mucosa were higher (11.3%, ± 4.3 ; Figure 1b) in comparison to strains from non-inflamed areas (7.3%, ± 5.8). However, this did not reach statistical significance ($P = 5.34 \times 10^{-2}$).

Invasive *E. Coli* from IBD Tissue Induce Cytokine Expression *In Vitro*

LF82 is an AIEC representative strain isolated from a chronic CD lesion in the terminal ileum. In comparison to LPS, LF82

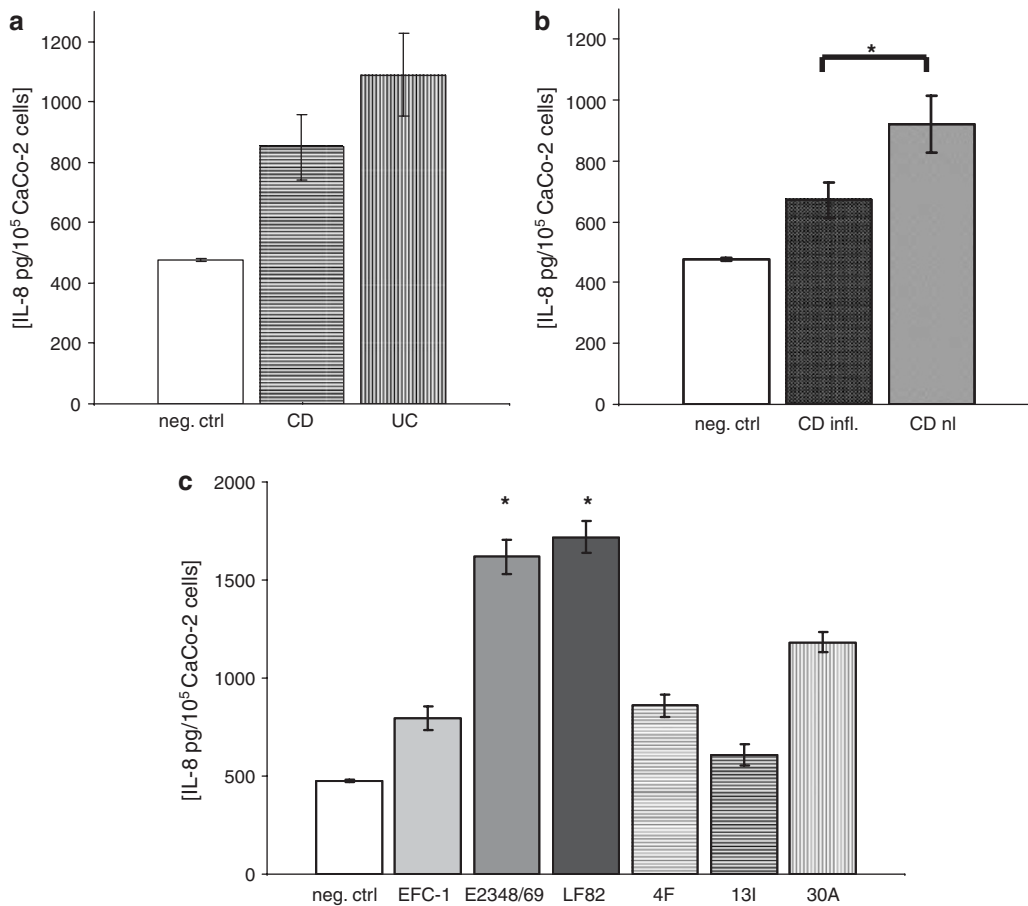


Figure 3 (a) *E. coli* strains from patients with CD and UC induce IL-8 expression in Caco-2 cells *in vitro*. Individual *E. coli* strains from CD and UC patients were incubated in duplicate with Caco-2 monolayers. Epithelial IL-8 production was determined by ELISA after a total of 8 h from three independent experiments ($n = 6$). Mean IL-8 concentrations were 850.8 pg/10⁵ cells, ± 107.3 for CD isolates and 1089.3 pg/10⁵ cells, ± 136.3 for UC *E. coli*. IL-8 induction was not statistically different for *E. coli* from CD and UC ($P = 0.0747$). (b) Invasive *E. coli* from inflamed CD tissue induces less IL-8 expression *in vitro*. Mean IL-8 concentration in the supernatant of Caco-2 epithelial cells infected for 3 h with *E. coli* strains isolated from inflamed CD tissue was lower (674.1 pg/10⁵ cells, ± 58.0) in comparison with strains cultured from macroscopically normal appearing mucosa (920.5 pg/10⁵ cells, ± 94.6). This difference was statistically significant ($*P = 6.16 \times 10^{-13}$). (c) Epithelial IL-8 expression in response to infection with individual *E. coli* strains. Supernatant of three independent experiments of Caco-2 cells infected with EFC-1, E2348/69, LF82, 4F, 13I, and 30A was analyzed by ELISA and data expressed as mean IL-8 concentration with \pm s.e.m. ($n = 6$). Control strains E2348/69 (1619.0 pg/10⁵ cells, ± 87.7) and LF82 (1719.4 pg/10⁵ cells ± 80.1) induced significantly higher IL-8 protein concentrations in comparison with nonpathogenic EFC-1 and isolates from our IBD population 4F, 13I, and 30A.

was previously found to induce 2.7-fold higher TNF- α concentrations in J774A.1 macrophage cultures after 24 h. We therefore sought to determine if Gram-negative isolates from our IBD population displayed a similar phenotype. *E. coli* cultures were incubated with J774A.1 macrophages at MOI 10:1 for 3 h, media supplemented with gentamicin, and TNF- α was measured in the cell supernatant after a total of 24 h. Mean TNF- α concentration for CD *E. coli* strains was 2604.8 pg/10⁵ cells, ± 447.4 (Figure 2a), which was similar for *E. coli* from patients with UC (2402.6 pg/10⁵ cells, ± 447.4 , $P = 2.5 \times 10^{-2}$), and was significantly increased from uninfected baseline (753.3 pg/10⁵ cells, ± 55.2). Analogous to invasion indices for CD *E. coli* from normal and inflamed tissue, we compared their ability to induce TNF- α expression in our macrophage culture system. *E. coli* from inflamed CD tissue induced significantly higher TNF- α secretion in the

supernatant of J774A.1 cells compared with strains from normal appearing mucosa (Figure 2b; 3071.3 pg/10⁵ cells, ± 226.0 vs 2476.5 pg/10⁵ cells, ± 447.9 ; $P = 5.16 \times 10^{-6}$).

Next, we focused on individual invasive bacterial isolates from our IBD population that induced very high TNF- α production in J774A.1 cultures. Isolates 30A from normal UC biopsy material, 4F and 13I from normal and inflamed CD tissue, respectively, were compared to the reference strain LF82, non-pathogenic EFC-1, and pathogenic EPEC strain E2348/69 in their ability to induce TNF- α . TNF- α expression in J774A.1 macrophage cultures was highest after infection with LF82 (Figure 2c; 2978.3 pg/10⁵, ± 346.3) and 13I (3991.9 pg/10⁵, ± 41.9 , $P = 0.14$). In comparison, infection with EFC-1 (955.0 pg/10⁵ cells, ± 73.7), E2348/69 (1302.8 pg/10⁵, ± 222.6), 4F (1890.8 pg/10⁵ cells, ± 119.9), 30A (1982.2 pg/10⁵ cells, ± 271.1), and invasive strain 150F from

Table 3a Characterization of invasive bacterial strains isolated from biopsy material from normal controls and patients with IBD by Gentamicin protection assays

Diagnosis	No. of strains analyzed	No. of invasive strains	% invasive strains
CD	378	93	24.6
UC	111	19	17.1
Normal controls	341	47	13.8
Total no. of strains tested: 830		Total no. of invasive strains 159	

Intracellular CFU \geq 1% I/O were considered significant for bacterial invasion.

Table 3b Api-20E system identification and frequency in percent of all invasive bacteria from patients with IBD and normal control populations

Bacterial species	% all invasive strains
<i>Escherichia coli</i>	90.0
<i>Enterobacter asburiae</i>	4.5
<i>Klebsiella pneumoniae</i>	1.8
<i>Hafnia alvei</i>	0.9
<i>Buttiauxella agrestis</i>	0.9
<i>Escherichia hermannii</i>	0.9

Table 3c Distribution of invasive *E. coli* in biopsy material according to clinical diagnosis

Diagnosis	No. of invasive strains	No. of invasive <i>E. coli</i>	% invasive <i>E. coli</i>
CD	93	92	98.9
UC	19	8	42.1
Normal controls	47	1	2.1
Total no. of invasive strains: 159		Total no. of invasive <i>E. coli</i> : 101	

normal control tissue (1464.6 pg/10⁵ cells, \pm 197.1, data not shown) led to significantly less TNF- α expression after 24 h in J774A.1 cultures. These results show that mucosal *E. coli* isolates from IBD patients induce significantly increased TNF- α expression, which is highest for isolates from the inflamed tissue.

Non-invasive and in particular invasive bacteria induce high IL-8 expression in epithelial cell cultures.²³ We next subjected Caco-2 monolayer cultures to *E. coli* strains isolated

from our IBD population and analyzed IL-8 expression in the supernatant of infected cell cultures. Monolayers were infected at MOI 10:1 in triplicate for 3 h without antibiotics, and changed to DMEM with antibiotics for the remainder of the experiment. Induction of IL-8 expression in epithelial cells increased from the baseline 477.3 pg/10⁵ cells, \pm 4.4 (Figure 3a) to 850.8 pg/10⁵ cells, \pm 107.3 for all CD strains and 1089.3 pg/10⁵ cells, \pm 136.3 for isolates from UC tissue which did not reach statistical significance over CD strains ($P=0.055$). However, in contrast to increased TNF- α production in J774A.1 macrophage cultures, *E. coli* from inflamed as opposed to normal CD tissue induced significantly less IL-8 protein expression (Figure 3b; 672.2 pg/10⁵ cells, \pm 58.5 vs 922.2 pg/10⁵ cells, \pm 93.9). This difference in IL-8 expression for inflamed vs non-inflamed isolates was significant ($P=3.95 \times 10^{-13}$).

Analogous to experiments performed in macrophage cultures, we also compared IL-8 induction for individual IBD isolates from our population (4F, 13I, 30A) to *E. coli* reference strains EFC-1, E2348/69, and LF82. Highest concentrations of IL-8 expression in epithelial monolayer cultures were observed for E2348/69 (1619.0 pg/pg/10⁵ cells, \pm 87.7) and LF82 (1,719.4 pg/10⁵ cells \pm 80.1). None of our invasive IBD isolates (4F: 860.8 pg/10⁵ cells, \pm 57.6; 13I: 606.4 pg/10⁵ cells, \pm 53.5; 30A: 1183.6 pg/10⁵ cells, \pm 52.4) or *E. coli* strain 150F from a control subject (389.3pg/10⁵ cells, \pm 70.7; data not shown) was capable of inducing comparable level of IL-8 in the supernatant of infected Caco-2 monolayer cultures. These results indicate that invasive *E. coli* isolates from our population are either capable of weakly inducing or suppressing IL-8 expression in Caco-2 monolayer cultures.

Examination of Genetic Similarity among Invasive *E. Coli* Strains from IBD Patients

Following phenotypic characterization by invasion assay and cytokine expression, we determined genetic relatedness of IBD *E. coli* isolates by PFGE (Figure 4). To increase accuracy of genetic analysis, whole bacterial DNA isolated from EFC-1, LF82, 4F, 13I, and 30A was subjected to digestion with restriction enzymes *Xba*I and *Bln*I and gel electrophoresis. The highest degree of similarity was determined for isolates 4F and 13I (64.71%) from our CD patient population. However, this did not reach statistical significance. Genetic similarities were even lower for all other *E. coli* strains tested, including LF82 and EFC-1. Therefore, *E. coli* from CD and UC patients appear to share certain phenotypic characteristics like epithelial invasion and cytokine induction, but are not genetically related.

Analysis for Effector Proteins in Invasive *E. Coli* from IBD Tissue

Amplification of bacterial DNA with all primer combinations for bacterial toxins and adhesion molecules resulted in a PCR product of correct size only in positive control strains. Unlike control strains, none of the *E. coli* isolates from our IBD

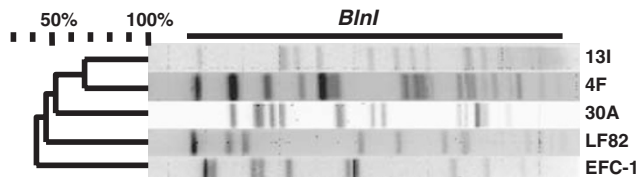


Figure 4 *E. coli* PFGE. Agarose-embedded bacterial DNA from strains EFC-1, LF82, 4F, 13I, and 30A was digested with *Xba*I and *Bln*I, and electrophoresis carried out with switch times of 2.16 to 54.17 s at 6 V/cm. Gels were stained using ethidium bromide and images obtained with a Gel Doc EQ imager under UV transillumination. Analysis of PFGE patterns was performed using BioNumerics software package. No significant similarity was determined for IBD isolates from patients with either CD or UC.

population yielded a positive result for any of the tested primer combinations, including *afaB-afaC* and *fimH* (data not shown). These findings indicate that our *E. coli* strains might harbor novel genes associated with bacterial adhesion and invasion.

Regulation of Barrier Function in Response to Infection with IBD *E. Coli*

Increased intestinal permeability is a feature of patients with IBD and their relatives, in particular CD.²⁴ We therefore sought to determine if our IBD bacterial isolates regulate protein members of the apical junctional complex (AJC) in differentiated epithelial cell culture *in vitro*. Epithelial monolayer was grown to an average TER of 327.9 Ωcm^2 , ± 20.6 and infected with commensal *E. coli* strain EFC-1, E2348/69, and representatives from patients with CD (LF82, 4F, 13I) and UC (30A) at MOI 10:1. In comparison to uninfected negative control and EFC-1 infected monolayers, E2348/69 and all IBD strains tested (LF82, 4F, 13I, 30A) induced comparable and significant decrease in TER over time regardless of whether they were isolated from CD or UC tissue samples (Figure 5). These differences in TER became apparent at 4 h, were significant at 5 h, and progressed until the end of the experiment at 11 h. Our results indicate that *E. coli* strains from patients with IBD regulate barrier function.

For confocal microscopy, Caco-2 monolayers grown on membrane supports were infected at MOI 10:1 for 4 h and analyzed by confocal microscopy. In comparison to uninfected control slides, nonpathogenic commensal strain EFC-1 induced minimal reorganization of F-actin, but not displacement of TJ protein ZO-1 or AJ protein E-cadherin (Figure 6). However, E2348/69 (Figure 6, inset) and LF82, isolated from inflamed CD tissue, reorganized F-actin, and displaced ZO-1 and E-cadherin from the AJC. Similar changes were observed for strain 13I and 30A from our study population, isolated from inflamed CD and normal UC tissue, respectively. Displacement of ZO-1 and E-cadherin, and the disorganization of F-actin were less severe after infection with an *E. coli* isolates from non-inflamed CD (4F). Regardless of the Gram-negative strains used to infect Caco-2

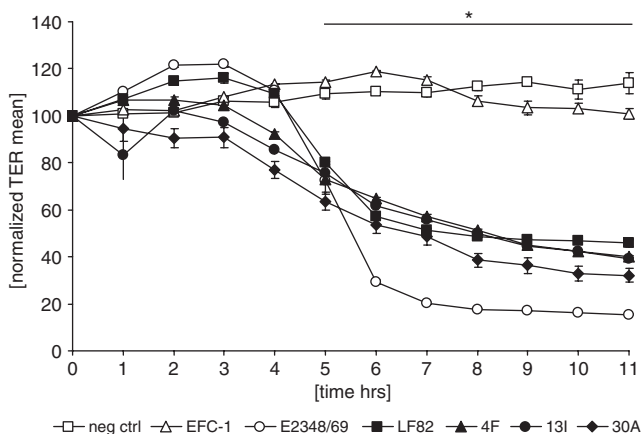


Figure 5 Invasive *E. coli* from CD and UC reduce epithelial barrier function. Epithelial monolayers cultures were grown to an average TER of 327.9 Ωcm^2 , ± 20.6 and were infected in triplicate with commensal, nonpathogenic EFC-1, E2348/69, and representative *E. coli* strains from patients with CD (LF82, 4F, 13I) and UC (30A) at MOI 10. TER was measured hourly and followed up to 11 h, data from three independent experiments ($n=9$) normalized and expressed as percent change over baseline with standard error. As opposed to untreated negative control monolayer and infection with EFC-1, treatment with E2348/69, LF82, 4F, 13I, and 30A led to reduction of TER evident at 4 h and significant (*) at 5 h which progressed until the end of the experiment at 11 h.

cells, all induced significant apical aggregation of F-actin in comparison to negative control cell cultures (Figure 6, *x/z*). Despite disorganization of F-actin and displacement of ZO-1, and E-cadherin in cell cultures infected with *E. coli*, columnar cell shape was maintained as determined by nuclear stain and F-actin organization (data not shown). Additionally, infection with any *E. coli* strains used in this study did not result in changes of total cellular concentrations for ZO-1, E-cadherin, or β -actin as determined by Western blot analysis and densitometry (Supplementary data). Invasive IBD *E. coli* strains induce displacement of AJC family of proteins which results in decreased epithelial barrier function *in vitro*.

DISCUSSION

This study investigated whether invasive *E. coli* are present in an American population of normal controls and patients with IBD. Our findings show that invasive *E. coli* are predominantly associated with CD and to a lesser extent with UC, but not normal control subjects. Interestingly, almost all invasive bacterial strains isolated from CD patients were identified as *E. coli* regardless of tissue disease activity. Applying our methods of bacterial isolation, we were unable to isolate invasive *E. coli* strains from inflamed UC tissue. Invasion indices for CD *E. coli* were significantly higher in comparison to those from UC and even higher for strains from inflamed CD tissue. We found that *E. coli* from both CD and UC induced significant TNF- α expression in macrophage supernatant comparable to the reference strain LF82 from a patient with CD. Strains from both IBD populations induced

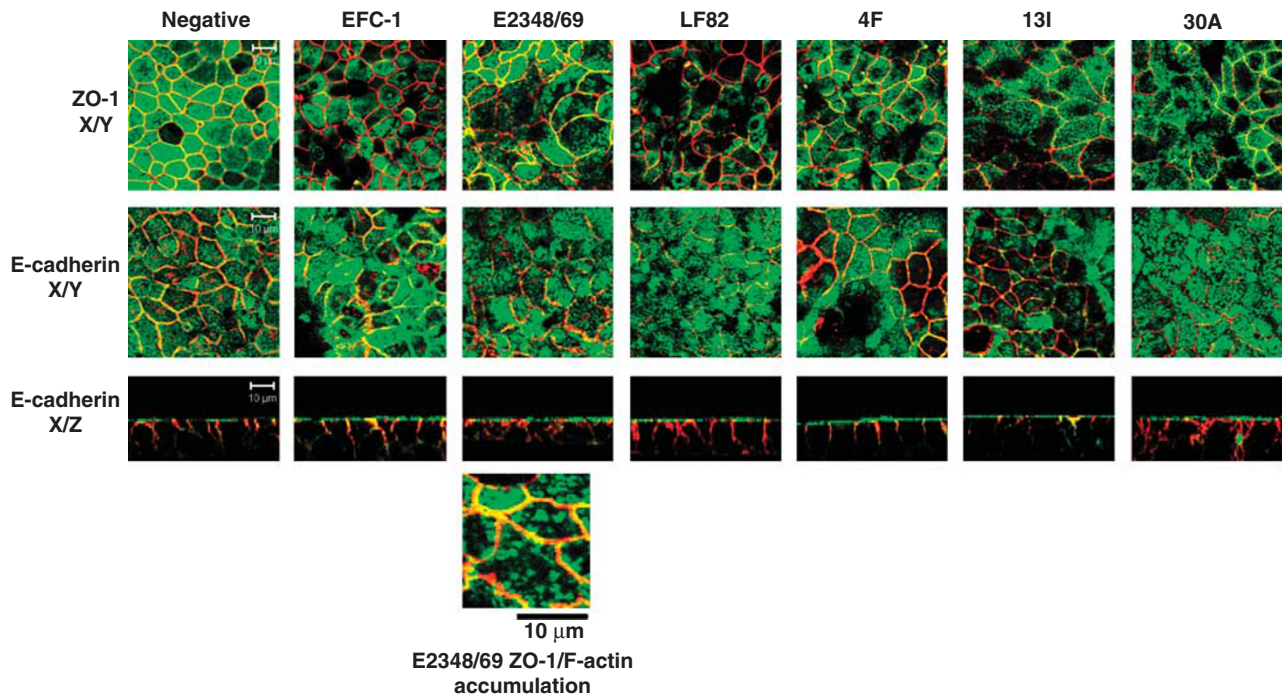


Figure 6 Examination of Caco-2 monolayer cultures infected with *E. coli* by confocal microscopy. Polarized Caco-2 cells were grown on membrane supports and infected for 4 h with nonpathogenic commensal strain EFC-1, positive control E2348/69, reference strain LF82, and invasive *E. coli* from our population: 4F (inactive CD), 13I (inflamed CD), 30A (normal UC). Monolayers were fixed, stained, and examined by confocal microscopy. Infection with LF82, 13I, and 30A resulted in significant displacement of ZO-1 (red), E-cadherin (red), and F-actin (green). Displacement of AJC proteins was less severe for strain 4F isolated from normal CD tissue and minimal for commensal EFC-1. Infection with all *E. coli* isolates resulted in apical accumulation of F-actin (x/z; red: E-cadherin, green: F-actin). Monolayer integrity was not affected in negative controls or experimental samples infected with *E. coli* (data not shown).

IL-8 in epithelial cells, but *E. coli* from inflamed CD tissue induced significantly less. In addition, invasive *E. coli* strains from our IBD population decreased epithelial barrier function through displacement of the key AJC proteins ZO-1 and E-cadherin. Induction of IL-8 and TNF- α expression⁵ and decrease in barrier function^{25,26} have been described as pathophysiological features of IBD.

***E. coli* in IBD**

E. coli is the predominant facultative aerobic inhabitant of human intestine and part of normal enteric flora. There is mounting evidence that *E. coli* is more prevalent in patients with IBD in comparison to normal control subjects, particularly in CD tissue.²⁷ Initial studies, determining bacterial colonization in resected ileal and colonic surgical specimens, identified significantly increased *E. coli* colony counts in CD, but not tissue from patients with UC or normal controls.²⁸ Recently, higher numbers of *E. coli* were also identified in neoterminal ileum of CD, especially in early recurrent disease when compared with normal control tissue.²⁹ Alternatively, PCR-based methods detected *E. coli* 16S rRNA and DNA more frequently in CD biopsy material than control tissue^{30,31} In comparison to tissue infected with other enteric pathogens, immunohistochemistry identified *E. coli* antigens in biopsy material from 10 out of 16 CD patients, especially

in ulcerations, lamina propria, and along fissures.³² However, several studies found IBD-associated *E. coli* with both CD and UC. Culture-independent analysis of bacterial ribosomal RNA revealed significantly higher prevalence of *E. coli* in IBD in comparison with normal control subjects.^{33,34} This was confirmed with a culture-dependent method, as epithelium-associated *E. coli* was propagated from CD and UC tissue, but not from normal controls.³⁵

An increasing number of pathogenicity features are being identified in *E. coli* from patients with CD^{36–38} and in animal models, one of them being bacterial invasion. LF82 is an invasive *E. coli* isolated from an ileal biopsy that efficiently adheres to and invades epithelial cells *in vitro*,¹⁴ and has been used in this and other studies as a reference strain. The association of CD with invasive *E. coli* was confirmed by Martin *et al*³⁹ as 29% of CD patients were infected with intramucosal *E. coli* in comparison with 9% of control subjects. Bacterial invasion was verified by infecting epithelial cell cultures *in vitro*. Similar results were described for granulomatous colitis, an IBD equivalent in boxer dogs, identifying adherent, invasive, and replicating *E. coli* within cultured epithelial cells.⁴⁰ A role of invasive bacteria in CD is indirectly supported by the fact that *E. coli* were more frequently identified in serosa and mesenteric lymph nodes from CD surgical specimen than healthy control tissue^{41,42} Our findings

reported here support these studies and support a role for invasive *E. coli* in the pathogenesis of CD.

Our findings are in contrast to other studies investigating quantitative changes of the intestinal flora in IBD.⁴³ Swidzinski et al⁴⁴ described *E. coli* adherent to, but not invading the epithelial layer, even in inflamed mucosa. In that particular study, invasive bacteria were rare and identified predominantly as *Bacteroides fragilis* and *Eubacterium rectale* populations. These differences in results might be explained by alternative experimental approaches utilized. We speculate that *in vivo*, *E. coli* strains tightly adhere to epithelial cells and are prevented from invading tissue by host factors, including the mucosal immune system, peristalsis, and mucus production. However, propagation *ex vivo* unmasks bacterial pathogenicity traits and *E. coli* become invasive in a system devoid of additional host defense mechanisms.

Invasive *E. Coli* Induce TNF- α Expression

In comparison with nonpathogenic commensal strains and LPS, we show that *E. coli* isolates from our IBD population induced significantly higher TNF- α expression in macrophage cell cultures *in vitro*. Maximum expression of TNF- α in J774.A1 cultures in response to our isolates was comparable to concentrations induced by reference *E. coli* strain LF82 propagated from a chronic small bowel CD lesion.¹⁵ Higher TNF- α stimulatory activity of isolates from CD and UC patients might be due to quantitative and qualitative differences in LPS, flagella, or due to a specific bacterial effector gene or gene products. LPS and flagellin mediate activation of the innate immune response through Toll-like receptor-4 and -5 (TLR), respectively, leading to marked upregulation of TNF- α .⁴⁵ TLR-mediated induction of cytokine expression in IBD appears to be a strong candidate pathway as stimulation of macrophages collected from IBD patients with TLR-2 agonists zymosan and lipoteichoic acid induces TNF- α *in vitro*.⁴⁶ This study also identified an increased expression of TLR-2 on macrophages isolated from patients with active CD in comparison to cells from patients in remission or control subjects. An additional candidate receptor possibly mediating increased TNF- α expression is TLR-4, which was upregulated on macrophages from inflamed CD and UC tissue.⁴⁷ This requires further clarification, considering the central role of TNF- α in the pathogenesis and therapy of patients with IBD.^{48–51}

Invasive *E. Coli* Regulate IL-8 Activation

Pathogenic bacteria, and in particular strains that penetrate the epithelial barrier, induce high expression of pro-inflammatory cytokines, including TNF- α and IL-8.²³ Similar to the reference strain LF82, invasive *E. coli* strains from our patient population with CD and UC induced high expression of epithelial IL-8. Maximum IL-8 expression was not different among the disease groups, but significantly decreased in epithelial cultures infected with *E. coli* from inflamed tissue. This novel finding was unexpected, considering a stimulatory

effect of IBD-associated *E. coli* from our population on TNF- α expression in macrophage cultures as outlined above.

Suppression of IL-8 is a common feature among Gram-negative pathogenic and nonpathogenic bacteria, and has been described for *Salmonella* spp. and *Yersinia* spp. Following epithelial cell invasion, *Salmonella enterica* serovar *typhimurium* was found to inhibit NF- κ B-dependent gene expression, including IL-8.⁵² The effector proteins responsible for this effect were identified as SspH1 and SptP. Similarly, nonpathogenic *Salmonella typhimurium* Pho1Pc and *S. pullorum* express AvrA, a bacterial protein that prevents TNF- α -induced IL-8 transcription.⁵³ YopJ, a bacterial effector similar to AvrA, blocked transcription of IL-8 in bronchial epithelial cells.⁵⁴ In addition to IL-8, YopJ also reduced expression of RANTES and ICAM-1 in the same *in vitro* system. Even though IL-8 is increased in CD tissue,⁵⁵ we speculate that our invasive, activated *E. coli* isolated from inflamed areas express a single or multiple effector molecules that prevent maximal expression of IL-8 by epithelial cells.

Regulation of Barrier Function

Our study clearly showed for the first time that *E. coli* isolated from patients with IBD decrease TER and induce disorganization of F-actin, and displacement of ZO-1, and E-cadherin. Previous studies have identified a twofold increase of intestinal permeability in patients with CD and their relatives.⁵⁶ This has been attributed to a decrease in mRNA and protein concentrations of E-cadherin²⁵ and ZO-1⁵⁷ among other molecules. Besides, cytokines such as TNF- α ,⁵⁸ α -hemolysin from CD *E. coli*⁵⁹ has been implicated in the regulation of barrier function. It is therefore conceivable that our invasive *E. coli* elicit, at least in part, a direct and indirect effect on barrier function through upregulation of TNF- α .

In conclusion, our present findings associate invasive *E. coli* with IBD, in particular CD, and describe novel pathogenicity features of these bacteria including regulation of cytokine expression and barrier function. Disturbance of immune balance and barrier function by invasive bacteria might account for some of the pathophysiological changes and intestinal inflammation observed in IBD. Future studies will include serological and genetic analyses of our invasive *E. coli* and dissection of TNF- α regulatory pathways through TLR.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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