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Escherichia coli isolated from a Crohn's disease patient adheres, invades, and induces inflammatory responses in polarized intestinal epithelial cells

Tonyia Eaves-Pyles[a,*], Christopher A. Allen[a], Joanna Taormina[a], Alexander Swidsinski[c], Christopher B. Tutt[a], G. Eric Jezek[a], Martha Islas-Islas[d], Alfredo G. Torres[a,b]

[a]*Department of Microbiology and Immunology, University of Texas Medical Branch, 301 University Blvd., Galveston, Texas 77555-1070, USA*
[b]*Department of Pathology, University of Texas Medical Branch, 301 University Blvd., Galveston, Texas 77555-1070, USA*
[c]*Innere Klinik, Gastroenterologie, Charite Humboldt Universitat, 10098 Berlin, Germany*
[d]*Centro de Investigaciones en Ciencias Microbiológicas, B. Universidad Autónoma de Puebla, Puebla 72570, México*

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Abstract

Inflammatory diseases of the intestinal tract are a major health concern both in the United States and around the world. Evidence now suggests that a new category of *Escherichia coli*, designated Adherent Invasive *E. coli* (AIEC) is highly prevalent in Crohn's Disease (CD) patients. AIEC strains have been shown to colonize and adhere to intestinal epithelial cells (IEC). However, the role AIEC strains play in the induction of an inflammatory response is not known. Therefore, we examined several *E. coli* strains (designated LF82, O83:H1, 6604 and 6655) that were isolated from CD patients for their ability to induce inflammation in two IEC, Caco-2BBe and T-84 cells. Results showed that each strain had varying abilities to adhere to and invade IEC as well as induced cytokine secretion from polarized IEC. However, *E. coli* O83:H1 displayed the best characteristics of AIEC strains as compared to the prototype AIEC strain LF82, inducing cytokine secretion from IEC and promoting immune cell migration through IEC. Upon further analysis, *E. coli* O83:H1 did not harbor virulence genes present in known pathogenic intestinal organisms. Further characterization of *E. coli* O83:H1 virulence determinants showed that a non-flagellated O83:H1 strain significantly decreased the organism's ability to adhere to and invade both IEC and elicit IEC cytokine secretion compared to the wild type and complemented strains. These findings demonstrate that *E. coli* O83:H1 possesses the characteristics of the AIEC LF82 strain that may contribute to the low-grade, chronic inflammation observed in Crohn's disease.

Keywords: Inflammation; Intestine; Bacteria; Crohn's disease; Immune cells

Introduction

The cause of inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is still unknown. However, IBD is thought to result from inappropriate and continual activation of the intestinal mucosal immune system due to a complex interaction of genetic, environmental and microbial factors. Clinical and experimental data implicate luminal bacteria and/or bacterial products in both the initiation and perpetuation of chronic intestinal inflammation (Liu et al., 1995; Schultsz et al., 1997; Burke and Axon, 1988; Lamps et al., 2003). Some of the

*Corresponding author. Tel.: +409 772 9429; fax: +409 747 6869.
E-mail address: tdeavesp@utmb.edu (T. Eaves-Pyles).

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pathological characteristics seen in CD, including ulcers of the mucosa, abscesses, and macrophage recruitment and activation, also occur in other intestinal diseases, such as shigellosis, salmonellosis and *Yersinia* enterocolitis, where invasiveness of the bacteria is a vital virulence process (Liu et al., 1995; Lamps et al., 2003). It has been shown that early and chronic ileal lesions of CD patients are abnormally colonized (up to 50%) with high numbers of *E. coli* strains, and it has been suggested that these strains participate in the pathogenesis (Martin et al., 2004; Darfeuille-Michaud, 2002; Darfeuille-Michaud et al., 2004; Boudeau et al., 1999; Schultsz et al., 1999). This new category of *E. coli* has been designated Adherent Invasive *Escherichia coli* (AIEC) and is found in CD patients (Burke and Axon, 1988; Martin et al., 2004; Darfeuille-Michaud, 2002; Darfeuille-Michaud et al., 2004; Boudeau et al., 1999). AIEC strains colonize the intestinal mucosa by adhering to intestinal epithelial cells (IEC; Martin et al., 2004; Darfeuille-Michaud, 2002; Boudeau et al., 1999). These AIEC strains are also true invasive pathogens, able to invade IEC via a macropinocytosis-like process, and to survive and replicate intracellularly after lysis of the endocytic vacuole (Darfeuille-Michaud, 2002; Boudeau et al., 1999). All these virulence properties designate AIEC as a potential pathogen, with the ability to induce persistent intestinal inflammation, by crossing and breaching the intestinal barrier, moving to deep tissues, and continuously activating macrophages (Darfeuille-Michaud, 2002).

Bacterial adhesion to IEC is the first step in the pathogenicity of many bacteria involved in infectious diseases of the gut. Adhesion enables the bacteria to colonize the gut, thus limiting clearance from the intestine. The adherence of AIEC strains likely require flagella that have a direct role in the adherence process via active motility and also play an undefined role in the invasive process (Barnich et al., 2003). Flagella are the bacterial appendages that are found on the surface of Gram-negative and some Gram-positive organisms, serving essentially as helical propellers. A flagellum filament is made of polymerized flagellin subunits attached by a hook structure to the basal body (Yonekura et al., 2003). The number and distribution of flagella on bacteria vary depending on the surrounding environmental conditions. Flagella are known to move the bacteria through their aqueous environment; however, they are more than organelles of motility. Flagella play a crucial role in bacterial pathogenesis by contributing to the adhesion of the bacterium to host cells and enhancing bacterial invasion (Giron et al., 2002; Dibb-fuller et al., 1999; Schmitt et al., 2001).

Upon the adhesion and/or invasion of IEC by certain pathogens, the IκBα/NF-κB signal transduction pathway is activated, resulting in the induction of inflammatory responses. Upon cell stimulation by various factors, including bacteria and their products, IκBα is degraded, freeing NF-κB to move to the nucleus and begin gene transcription of inflammatory mediators such as IL-6, IL-8 and nitric oxide (Xiao and Ghosh, 2005; Baldwin, 1996; Tak and Firestein, 2001). It has been shown that *E. coli* strains isolated from inflammatory bowel disease patients were able to activate NF-κB (La Ferla et al., 2004). The production of cytokines is important for recruiting certain immune cells, such as dendritic cells (DC) and neutrophils, to the site of infection (Liu et al., 2004; Kucharzik and Williams, 2003; Stagg et al., 2003). These immune cells are the first line of defense and are crucial against invading pathogens (Liu et al., 2004; Kucharzik and Williams, 2003; Stagg et al., 2003).

DC are important mediators between the innate and adaptive immune system. Intestinal DC are located in gut-associated lymphoid tissue, such as Peyer's patches, and are spread throughout the lamina propria (Stagg et al., 2003). Upon secretion of CCL20 (also known as MIP-3α) from IEC, DC are activated and recruited to the site of infection (Stagg et al., 2003). These cells play a key role in presenting antigen to T cells and can skew the adaptive immune response to Th1 vs. Th2 pathways. Additionally, DC can also destroy the pathogens that have been taken up via phagocytosis (Stagg et al., 2003). Similarly, neutrophils play a central role in innate immunity following a pathogenic bacterial infection. These short-lived cells contain host-defense molecules in their cytoplasm for rapid mobilization following bacterial infection (Liu et al., 2004; Segal, 2005; Pilsczek et al., 2005; Allen et al., 2005). Upon secretion of IL-8 by IEC, neutrophils are recruited out of the blood through the endothelium and then through the tight junctions of IEC to reach the infection site (Kucharzik et al., 2005; Hurley et al., 2004). Immune cell infiltration into the intestine has been shown to be a hallmark of IBD (Eksteen et al., 2004; Murakami et al., 2002). However, it is not known whether AIEC strains induce an inflammatory response in IEC and/or immune cell migration.

We have obtained several *E. coli* strains that were isolated from CD patients and each were tested for adhesion and invasion. We found that one of the *E. coli* strains, belonging to the serotype O83:H1 (designated *E. coli* O83:H1 throughout this study), possessed the characteristics of an AIEC strain more so than the other *E. coli* strains and as compared to the prototype AIEC LF82. Therefore, the current study was designed to further characterize the interaction between *E. coli* O83:H1 and two human IEC, Caco-2BBe and T-84. We utilized polarized IEC to determine whether *E. coli* O83:H1 interaction with the apical surface would elicit an inflammatory response. Our results showed that *E. coli* O83:H1 could adhere to, invade and induce inflammatory responses in IEC resulting in immune cell

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migration through IEC. Importantly, we confirmed that flagella are required for the adhesive and invasive phenotype observed with *E. coli* O83:H1, compared to non-flagellated *E. coli* O83:H1 that was unable to even elicit an inflammatory response from IEC. Additionally, upon further analysis, *E. coli* O83:H1 did not harbor virulence genes present in known pathogenic intestinal organisms.

Materials and methods

Intestinal epithelial cell culture models

Caco-2BBe, a human adenocarcinoma intestinal epithelial cell line, and the colonic epithelial cell line T-84 (American Type Culture Collection, Bethesda, MA) were used between passages 5 and 15. Cells were seeded on 24-well tissue culture plates or on 0.4 or 3.0 μm-filter membrane inserts (Costar®, Corning, NY) until confluent. The Caco-2BBe cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 110 mg/l of sodium pyruvate and antibiotics (Gibco BRL, Grand Island, NY) at 37 °C in 5% CO_2. The T-84 cells were cultured in DMEM/Ham's F-12 medium (1:1) supplemented with 5% FBS. The cells were grown for 5–6 days, which allowed them to develop into a confluent, differentiated, polarized monolayer establishing microvilli, brush borders and tight junctions. Using polarized IEC grown on transwell filters is important, because this allows us to mimic the physiological positioning of IEC *in vivo*, thereby, permitting cultured IEC to form distinct apical and basolateral surfaces that express surface-specific proteins, including receptors (Haller et al., 2004). Following verification that the cells on the membrane insert achieved a transepithelial electrical resistance (TEER) of 400–500 $\Omega\,cm^2$ with a voltohmeter (EVOM, World Precision Instruments, Inc., Sarasota, FL), then after subtraction of resistance across a cell-free filter they were employed for these studies.

All experiments in these studies were performed using serum- and antibiotic-free tissue culture medium to ensure no potential interference by serum-containing elements regarding bacteria interaction with the host cell.

Bacterial cultures

E. coli strains O83:H1, 6604, 6655 (provided by Dr. Alexander Swidsinski, Charite Hospital, Germany) were taken from biopsies of the ileum, ascending and descending colon of Crohn's disease patients. The biopsies were washed 4 times in saline then lysed. Bacteria were isolated from the saline wash fluid and hypotonic lysis. *E. coli* LF82 was provided by Ed Boedeker (University of Maryland School of Medicine) who obtained this strain from Dr. Darfeuille-Michaud (Universite d'Auvergne, Clermont-Ferrand, France), and *E. coli* HS strain is a human commensal isolate from a healthy adult (ATCC; Tarr et al., 2000). All organisms were inoculated in 10 mL of LB broth overnight at 37 °C in a shaking incubator. Cultures were centrifuged at 10,000 rpm for 10 min and washed twice with sterile phosphate buffered saline (PBS). Bacterial pellets were resuspended in 10 mL of PBS, and bacteria were counted using a Petroff Hausser counting chamber (Hausser Scientific, Horsham, PA) to determine colony-forming units (CFU) and confirmed bacterial CFU by plating 10-fold dilutions. Then bacterial concentrations used in the experiments were adjusted to the desired concentration using standard dilutions.

Construction of *E. coli* O83:H1 *fliC* mutant and complementation of the *fliC* mutation

The *E. coli* O83:H1 isogenic mutant defective in flagella production, designated CBTO1 (O83:H1 *fliC::cat*), was constructed by inactivation of the *fliC* gene by marker exchange as follows. The suicide vector containing the *fliC* gene disrupted with the *cat* gene (from pACYC184) was used to perform marker exchange as previously described (Giron et al., 2002; Torres et al., 2001). Briefly, the suicide plasmid was introduced into *E. coli* O83:H1 by conjugation using the donor SM10 ($\lambda^{\circ}pir$) strain. The resulting mutants were screened for the appropriate resistance patterns, loss of the suicide plasmid, and PCR analysis for the presence of the interrupted gene in the O83:H1 genome, as well as using motility plates (non-motile phenotype).

To complement the CBT01 strain, the region within the *fli*ZACD operon from *E. coli* O83:H1 containing the *fliC* gene (region encoding genes *fliA* and *fliC*) was amplified by PCR with primer pair 5PZFLID (5′-CACAGCTGACCCGACTCCCAGC-3′) and 3PZFLID (5′-TCCAGCTGTGTTTAAAGTCTTTAAG-3′) and cloned into plasmid pGEMTEasy, to produce pGEMT: $fliC_{O83}$. The resulting plasmid was introduced by electroporation into CBT01 then the ability to restore swimming phenotype was evaluated using motility agar plates.

To ensure that there was no disruption in type I fimbrae expression by the non-flagellated CBTO1 mutant, CBTO1 and O83:H1 organisms were fixed on sterile microscope slide covers in 24-well plates with 4% paraformaldehyde for 20 min followed by two washes with sterile PBS. Fixed cells were then incubated with rabbit serum raised against type I fimbrae (1:1000),

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kindly provided by Matthew Duncan (Duke University Medical Center), for 1 h followed by two PBS washes. Next, fixed cells were labeled with goat anti-rabbit IgG Alexa 488 (1:3000) (Molecular Probes, Eugene, OR) for 1 h at room temperature. Cells were washed twice with sterile PBS and air-dried. Mounting solution-containing DAPI to label the cell nucleus (VECTASHIELD®, Vector Laboratories, Burlingame, CA) was added in a drop-wise manner to microscope slides then covered with inverted cover slips containing the labeled bacteria. Fluorescent images of fixed CBTO1 and O83:H1 was acquired using an Olympus BX51 Research Microscope System (Olympus America, Inc., Center Valley, PA) at a magnification of 100 × (oil immersion).

Bacterial adhesion and invasion assays

The ability of the *E. coli* strains to adhere to Caco-2BBe and T-84 cells was assessed as previously described (Tarr et al., 2000; Torres and Kaper, 2003). The cells were grown on 24-well plates to confluence at 37 °C in 5% CO_2. Before use, the cells were washed with sterile PBS (pH 7.4) and replenished with DMEM. Cells were then infected with the individual *E. coli* strains (multiplicity of infection [MOI] of approximately 100:1) for 3 h at 37 °C. For qualitative analysis, the cells were washed, fixed, and stained with Giemsa solution and evaluated by light microscopy. To quantify adherence of the *E. coli* strains, the infected cells were washed twice with PBS and lysed for 10 min with 200 µl of 0.1% Triton X-100 in PBS buffer. The adherent bacteria were recovered and plated on Luria-Bertani (LB) agar plates. Plates were incubated at 37 °C overnight and then colonies were counted for statistical analysis. To obtain accurate counts of the bacteria that adhered to IEC, the experiments examining bacterial invasion were performed in parallel to the bacterial adhesion experiments. Then the number of bacteria that invaded the cells was subtracted from the number of bacteria that adhered to the IEC.

To determine the invasive capabilities of the *E. coli* strains, Caco-2BBe and T-84 cells were infected as above. Following 3 h incubation at 37 °C and 5% CO_2, cells were washed twice with sterile PBS and then incubated with DMEM containing 50 µg/mL gentamicin for 1 h to kill extracellular bacteria. Cells were washed twice with sterile PBS, lysed with 0.1% Triton X-100 in PBS buffer, plated on LB agar plates and incubated at 37 °C. Colonies were counted the following day as above.

Purification and transmigration of DC and neutrophils

DC were isolated from monocytes purified from PBMC (peripheral blood mononuclear cells) by negative selection using the magnetic column separation system (StemCell Technologies, Inc.) as previously described (Elkord et al., 2005). Briefly, monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, HEPES, sodium pyruvate, antibiotics, GM-CSF (100 ng/mL) and IL-4 (50 ng/mL) and set up in 24-well tissue culture plates at 5×10^5/mL. Cytokines were replenished every 3 days. Non-adherent, immature DC were obtained at 7 days of culture, and only homogeneous immature DC populations, characterized by high levels of CD11a and no CD83 expression, were used in these experiments.

Neutrophils were purified as previously described (Kucharzik and Williams, 2003; Soler-Rodriguez et al., 2000). Briefly, anti-coagulated venous blood was collected from normal volunteers. Neutrophils were isolated using dextran sedimentation and Hypaque-Ficoll (Amersham Biosciences, Piscataway, NJ) density-gradient separation. Purified neutrophils were resuspended in RPMI 1640 before use in migration experiments. Additionally, apical and basolateral supernatants from DC and neutrophils migration were analyzed to determine cytokine secretion.

Immune cell transmigration assay

Caco-2BBe and T-84 cells, grown on 3-µm filters and maintained as described above, were infected on the apical surface with 1×10^6 CFU of O83:H1, LF82, or HS for 2 h at 37 °C and 5% CO_2. Then freshly isolated DC, or neutrophils (1×10^5) were added to the basolateral surface and their migration assessed at 4 h via collection of the apical and basolateral supernatants followed by immediate DC or neutrophil counts using a hemocytometer. Un-stimulated IEC with DC or neutrophils added to the apical surface served as a negative control.

Detection of cytokines

Where applicable, Caco-2BBe and T-84 cell supernatants were collected at 6 h following bacterial stimulation of cells. Human IL-8 (Pierce/Endogen, Woburn, MA) and MIP-3α (CCL20; R&D Systems, Minneapolis, MN) levels in the supernatants were quantified by ELISA.

Multiplex PCR to detect pathogenic *E. coli* and *Shigella* virulence factors

The multiplex PCR assay was performed as previously described (Aranda et al., 2007). Briefly, The bacterial strains were subjected to multiplex PCR with specific primers for the detection of the following virulence markers: *eae* (intimin gene of EPEC and

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EHEC), *bfpA* (bundle-forming pili gene of EPEC), *aggR* (transcriptional activator of EAEC), *elt* (heat-labile enterotoxin of ETEC), *ipaH* (invasion plasmid antigen H of *Shigella*), and *stx* (Shiga toxin gene of EHEC). Primer sequences and concentrations used in the reaction are described by (Aranda et al., 2007). The PCR samples were subjected to the following cycling conditions: 50 °C (2 min, 1 cycle), 95 °C (5 min, 1 cycle), 40 cycles of 95 °C (1 min), 50 °C (1 min), 72 °C (1 min) and 72 °C (7 min, 1 cycle) and the PCR products were separated in 2% agarose gel.

Statistical analyses

Where applicable, numerical results were expressed as the mean ± SEM of three or four independent experiments. Statistical differences were considered if the p value was <0.05 as determined by ANOVA followed by Student's t-test.

Immune cell migration was calculated by collecting the apical and basolateral supernatants and then counting immune cells in both supernatants to determine cell migration. Data are expressed as the percentage and SEM.

Results

Characterization of *E. coli* O83:H1 as an adhesive and invasive organism as compared to AIEC LF82

To evaluate the characteristics of the CD-associated *E. coli* strain serotype O83:H1 strain as a potential AIEC, Caco-2BBe and T-84 cells were infected with *E. coli* strains O83:H1, other CD-associated *E. coli* strains (strains 6604 and 6655), or the prototype AIEC strain LF82 at an MOI of approximately 100:1 for 3 h to determine bacterial adhesion to and invasion of IEC. The commensal *E. coli* strain HS was used as a negative control. Interestingly, *E. coli* HS was able to adhere to both IECs (Fig. 1A) but invasion was minimal (Fig. 1B). Likewise, all tested *E. coli* strains adhered to Caco-2BBe and T-84 cells to varying degrees with *E. coli* stain 6655 adhering significantly lower to Caco-2BBe cells compared to all other strains ($p<0.05$; Fig. 1A). *E. coli* O83:H1 adhered and invaded both Caco-2BBe and T-84 equally as well as LF82 (Fig. 1B) where invasion of both IEC by LF82 and O83:H1 was significantly better than all other *E. coli* strains ($p<0.05$; Fig. 1B).

These data established that *E. coli* O83:H1 possessed characteristics similar to the AIEC LF82 strain with respect to their ability to adhere to and invade IEC. Therefore, for this particular study we selected *E. coli* O83:H1 to perform further analysis.

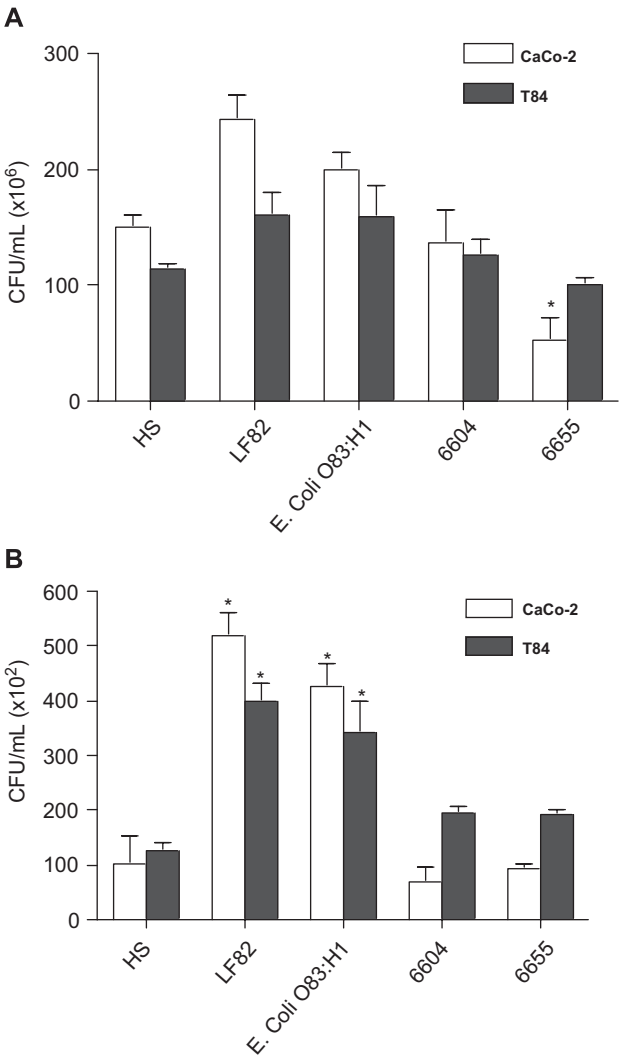

Fig. 1. CD-associated *E. coli* adhere and invade Caco-2BBe and T-84 cells. (A) Following a 3-h incubation with commensal HS, the prototype AIEC LF82, or not yet characterized CD-associated *E. coli* strains O83:H1, 6604 or 6655 (MOI of approximately 100:1), adhesion to and invasion of Caco-2BBe and T-82 cells were determined. (A) Adhesion of LF82 and *E. coli* O83:H1 to Caco-2 and T-84 cells was high compared to HS but did not reach statistical significance. However, the adhesion of *E. coli* strain 6655 to Caco-2BBe cells was significantly less than all other strains ($p<0.05$). (B) *E. coli* LF82 and O83:H1 invaded Caco-2BBe and T-84 cells significantly better than all other strains ($p<0.05$). Data are expressed as mean ± standard deviation of triplicate experiments measuring CFU of bacteria. *$p<0.05$, ANOVA.

E. coli O83:H1 flagella play a crucial role in the adhesion and invasion of IEC

Previous studies have linked flagella with the ability of bacteria to adhere and invade IEC contributing to the organism's virulence (Barnich et al., 2003; Giron et al.,

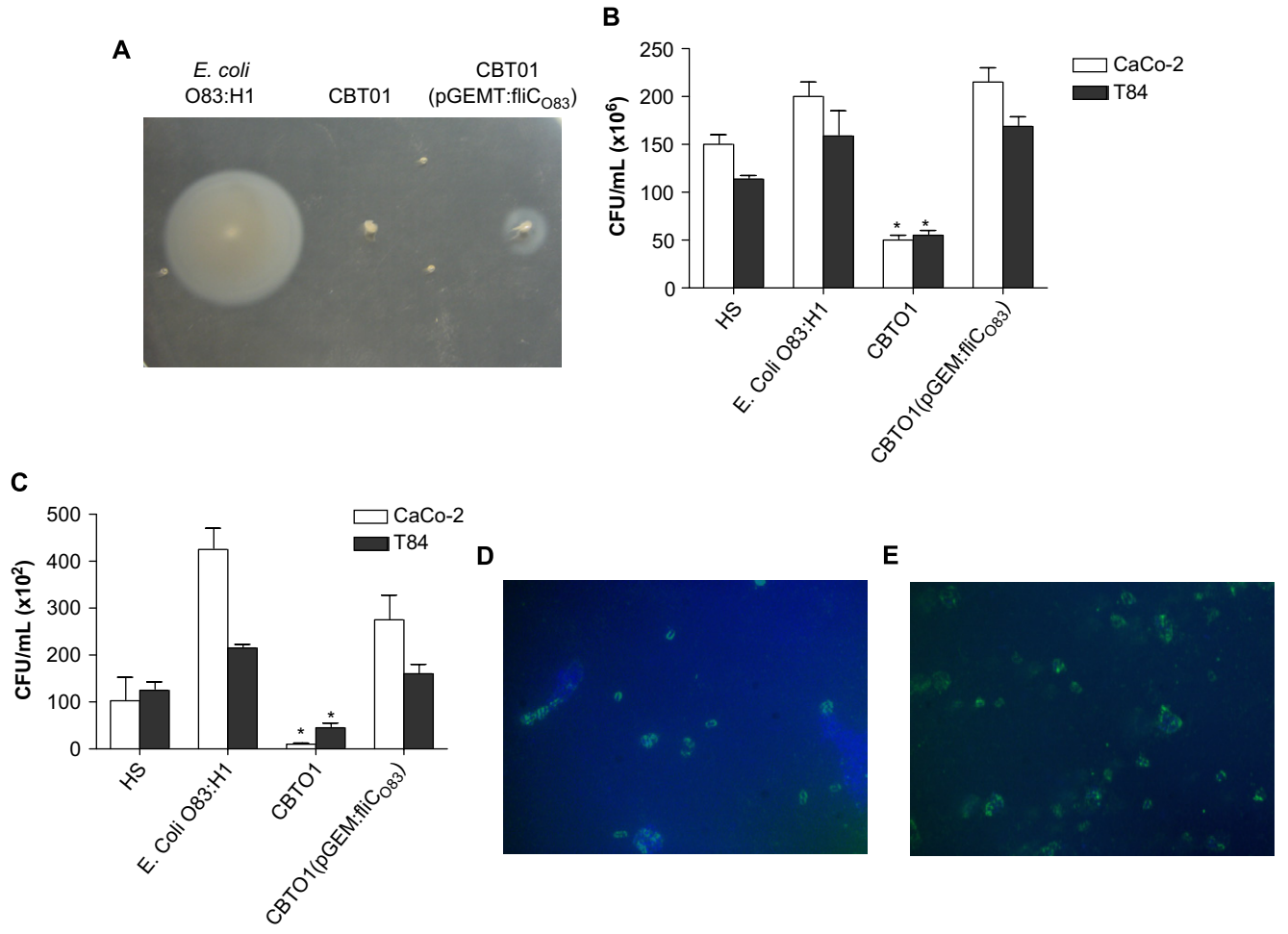

Fig. 2. Non-flagellated *E. coli* O83:H1 (expressing type I fimbriae) does not adhere to or invade IEC. (A) Motility test of *E. coli* strains O83:H1, CBT01 (*fliC*), and CBT01 (pGEMT:fliC$_{O83}$) in LB medium plus 0.3% agar after 16 h of incubation at 37 °C. (B) Adhesion of the non-flagellated CBTO1 was significantly lower in both Caco-2BBe and T-84 cells compared to all other strains (*, $p<0.01$). Further, restoration of flagella to CBTO1 (pGEMT:fliC$_{O83}$) restored adhesion to both cell types equal to that of wild type O83:H1. (C) Likewise, the lack of flagella on CBTO1 significantly decreased its ability to invade Caco-2BBe and T-84 cells (*, $p<0.01$) compared to the wild type *E. coli* O83:H1. *E. coli* O83:H1 invaded Caco-2BBe cells significantly better vs. all other groups (#, $p<0.01$). Data are expressed as mean ± standard deviation of duplicate experiments measuring CFU bacteria. Fluorescent staining of type I fimbriae (green) showed type I fimbriae expression remained intact on CBTO1 (D) as compared to wild type O83:H1 (E). The nucleus was stained with DAPI (blue).

2002; Dibb-Fuller et al., 1999; Schmitt et al., 2001). Therefore, to further characterize the CD-associated *E. coli* strain O83:H1, and determine the contribution of its flagella to adhesion and invasion of IEC, Caco-2BBe and T-84 cells were infected with the non-flagellated CBT01 strain (O83:H1 *fliC::cat*), and the complemented strain CBTO1 (pGEMT:fliC$_{O83}$) (Fig. 2A). Results showed that at an MOI of approximately 100:1 the non-flagellated CBTO1 strain significantly reduced the organism's ability to adhere to Caco-2BBe and T-84 cells (Fig. 2B) compared to all other strains ($p<0.01$). However, when the ability to produce flagella was restored to the organism, CBTO1 (pGEMT:fliC$_{O83}$) adhered to both IEC as well as the wild type O83:H1 (Fig. 2B). Likewise, the absence of flagella significantly reduced the organism's ability to invade (Fig. 2C) both IEC, as compared to that of the wild-type strain O83:H1 ($p<0.01$) and the complementation CBTO1 (pGEMT:fliC$_{O83}$) strain ($p<0.05$).

Previous data indicate that flagella and type I fimbriae cooperate during adhesion and invasion of IECs (10). Therefore, to ensure that the elimination of flagella expression by *E. coli* O83:H1 did not interfere with the expression type I fimbriae, fluorescent staining with anti-type I fimbriae specific anti-sera showed CBTO1 expression of type 1 fimbriae remained intact (Fig. 2D)

similar to that of the wild type *E. coli* O83:H1 (Fig. 2E) as visualized by fluorescent microscopy. These data further demonstrate the crucial role flagella plays in the pathogenesis of *E. coli* O83:H1.

E .coli O83:H1 and LF82 induced differential secretion of IL-8 and CCL20 from polarized IEC

To determine whether the CD-associated *E. coli* strains induced an inflammatory response, polarized Caco-2BBe and T-84 cells, grown on transwell filters, were infected apically with *E. coli* O83:H1, CBTO1, CBTO1 (pGEMT:fliC$_{O83}$), 6604, 6655, LF82 or *E. coli* HS (MOI of approximately 100:1) for 4 h followed by the quantization of IL-8 secretion in cell supernatants as analyzed by ELISA. As expected, un-stimulated Caco-2BBe and T-84 cells induced minimal to no detectable IL-8 secretion (Fig. 3A and B). Similarly, HS, 6604 and 6655 stimulated minimal IL-8 secretion from both cell lines (Fig. 3A and B). Conversely, *E. coli* O83:H1 induced higher secretion of IL-8 in the basolateral supernatant of Caco-2BBe (Fig. 3A) and T-84 (Fig. 3B) cells compared to the apical supernatants with significantly higher basolateral IL-8 secretion by T-84 cells compared to un-stimulated cells or cells stimulated with non-flagellated CBTO1 ($p<0.05$, Fig. 3B). The non-flagellated CBTO1 induced little or no IL-8 secretion equivalent to that of un-stimulated cells (Fig. 3A and B). However, the restoration of flagella in the CBTO1 (pGEMT;fliC) strain increase IL-8 secretion from the basolateral surface of both cell lines with a significant increase in IL-8 secretion from the basolateral surface of T-84 cells compared to the basolateral CBTO1 supernatants ($p<0.05$; Fig. 3B). LF82 induced significantly higher IL-8 levels in the apical supernatant of both IEC compared to basolateral supernatant of all tested organisms ($p<0.05$, Fig. 3A). Similarly, the apical supernatants from LF82-stimulated T-84 cells were significantly higher when compared to un-stimulated cells, *E. coli* HS and CBTO1 ($p<0.05$, Fig. 3B).

To further characterize the inflammatory capabilities of the CD-associated *E. coli* strain, supernatants from *E. coli*-stimulated Caco-2BBe and T-84 cells were tested for CCL20. Consistent with the IL-8 secretion, results showed that *E. coli* LF82 and O83:H1 secreted significantly high levels of CCL20 from Caco-2BBe and T-84 (Fig. 3C) cells compared to all other strains tested ($p<0.05$).

The results from these studies, thus far, have established *E. coli* O83:H1 and LF82 stimulated IEC to induce a distinct inflammatory response. Differential secretion of IL-8 and CCL20 by IEC was dependent on the CD-associated *E. coli* strain. Further, the contribution of the *E. coli* O83:H1 flagella is crucial to the pathogenicity of this organism in order to induce an inflammatory response in IEC.

CD-associated *E. coli* strains induced immune cell migration through polarized IEC

During intestinal inflammation in IBD, there is a strong migration of DC and neutrophils (Stagg et al., 2003; Ina et al., 2002; Kucharzik et al., 2001; Bell et al., 2001). Because IEC responded to *E. coli* O83:H1 and LF82 by eliciting an inflammatory response, we examined the migration of these immune cells through polarized IEC in response to *E. coli*-stimulation.

To mimic the *in vivo* physiological situation of bacteria stimulating the apical surface of IEC, these experiments again utilized polarized Caco-2BBe and T-84 cells grown on transwell filters. The IEC apical surface was stimulated with *E. coli* O83:H1, CBTO1, LF82, or HS 2 h prior to the addition of immune cells to the basolateral surface of IEC. The addition of immune cells to un-stimulated IEC and HS-stimulated cells served as negative controls. Following 4 h of migration, supernatants were collected from each chamber and immune cells were counted. Results showed that both *E. coli* O83:H1- and LF82-stimulated Caco-2BBe and T-84 cells induced significantly high levels of DC migration compared to un-stimulated controls, commensal HS, or non-flagellated CBTO1 ($p<0.05$, Fig. 4A). Further, DC migration was minimal when both cell lines were stimulated with the non-flagellated CBTO1 (O83:H1 *fliC::cat*) strain (Fig. 4A). Interestingly, the overall DC migration was higher in T-84 cells stimulated with *E. coli* O83:H1 and LF82 (Fig. 4A).

Similarly, neutrophil migration was significantly elevated in both cell lines following stimulation with O83:H1 or LF82 as compared to the other tested *E. coli* strains and un-stimulated cells (Fig. 4B, $p<0.05$). Corresponding to the DC migration results, our findings showed that there was minimal neutrophil migration detected in both cell lines stimulated with the non-flagellated CBTO1 strain (Fig. 4B) and compared to *E. coli* O83:H1 and LF82 stimulated IEC. Neutrophil migration was significantly lower in T-84 cells stimulated with CBTO1 as compared to *E. coli* O83:H1 and LF82 ($p<0.05$, Fig. 4B). However, these results showed the reverse of DC activation, in that the induction of neutrophil migration was higher overall in O83:H1- or LF82-stimulated Caco-2BBe cells than in T-84 cells (Fig. 4B).

These results demonstrate for the first time the ability of two different CD-associated *E. coli* strains, O83:H1 and LF82, to induce immune cell migration in two polarized intestinal cell lines. These data further demonstrate these organisms' potential qualifications to contribute to bowel inflammation.

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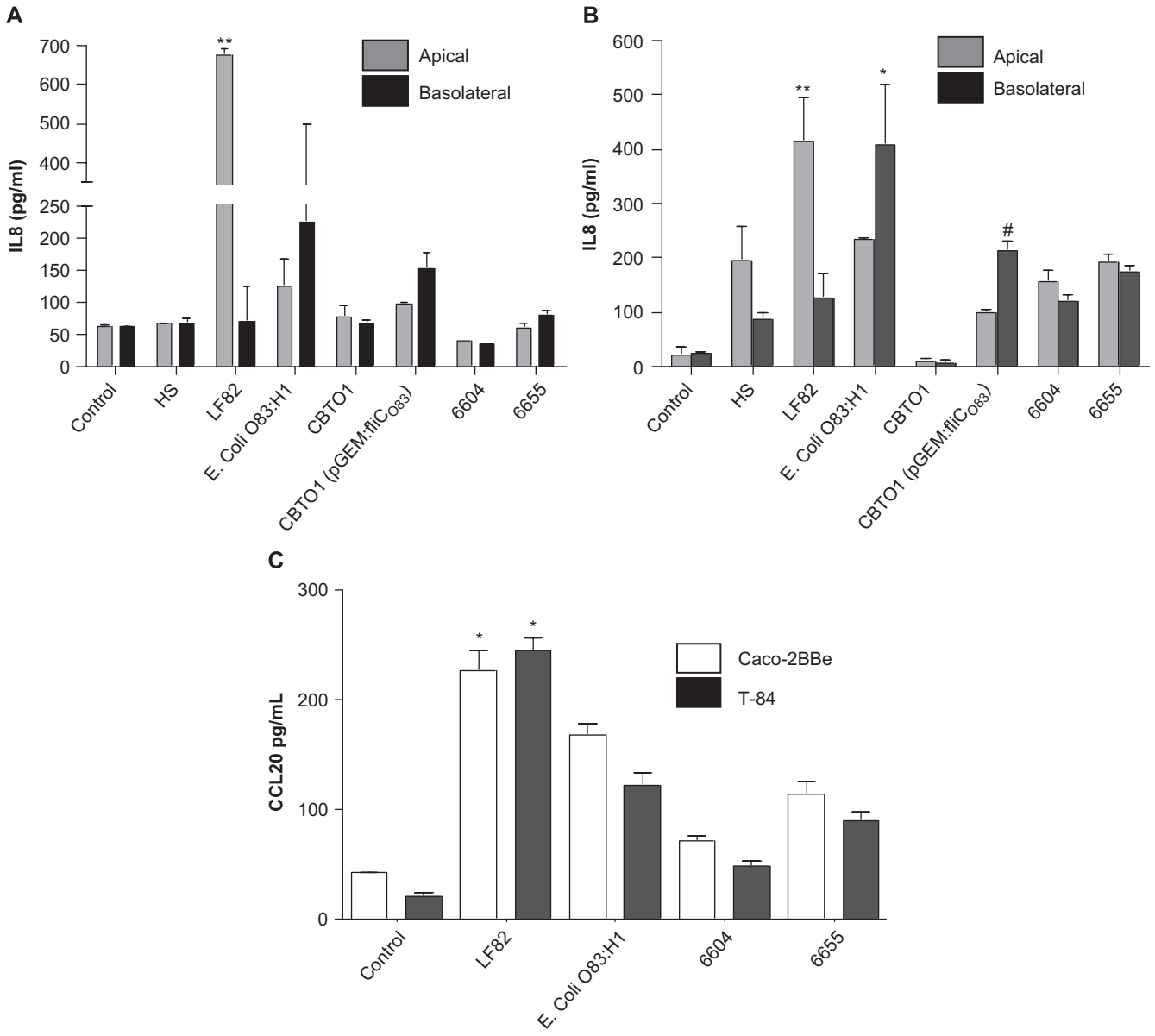

Fig. 3. CD-associated *E. coli* strains induced IL-8 secretion in polarized IEC. Polarized Caco-2BBe (A) and T-84 (B) cells were stimulated with commensal *E. coli* HS (HS), LF82, O83:H1, CBTO1, CBTO1(pGEMT:fliC), 6604 or 6655 (MOI of approximately 100:1) for 4 h and then supernatants were analyzed for IL-8 secretion by ELISA. *E. coli* O83:H1 induced IL-8 secretion primarily from the basolateral surface of Caco-2BBe (A) and T-84 (B) cells with a significant increase of basolateral IL-8 secretion from T-84 cells (B, $*p<0.05$ vs. all other basolateral supernatants). IEC stimulated with LF82 showed significant IL-8 secretion from the apical surface of both cell lines (A and B, $**p<0.05$ vs. all other apical supernatants). The non-flagellated CBTO1 and HS induced minimal levels of IL-8 secretion from both cell lines. However, the complementation of flagella in the CBTO1 (pGEMT;fliC$_{O83}$) strain increase IL-8 secretion from the basolateral surface of both cell lines with a significant increase in IL-8 secretion from the basolateral surface of T-84 cells (B) compared to the basolateral CBTO1 supernatants (# $p<0.05$). Supernatants from *E. coli*-stimulated Caco-2BBe and T-84 cells were also tested for CCL20 (also known as MIP-3α). *E. coli* LF82 and O83:H1 secreted significantly high levels of CCL20 from Caco-2BBe and T-84 cells (C) compared to all other strains tested ($*p<0.05$). Data are expressed as mean ± standard deviation of three experiments measuring IL-8 secretion by bacterially stimulated IEC as analyzed by ELISA. $p<0.05$, ANOVA.

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E. coli O83:H1 does not harbor virulence factors found in various pathogenic *E. coli* strains or *Shigella flexneri*

Because CD-associated O83:H1 *E. coli* can adhere, invade and induced inflammatory responses from IEC and immune cell migration, we further examined *E. coli* O83:H1 for the presence of several well-known virulence factors from various intestinal pathogenic *E. coli* and *Shigella flexneri* strains, using multiplex PCR with DNA primers to amplify the different virulence genes (Aranda et al., 2007). The PCR data showed that *E. coli* O83:H1, 6604, and LF82 did not harbored sequences homologous to the following virulence genes: (1) *bfpA* (found in enteropathogenic *E. coli*, EPEC); (2) *ipaH* (from *S. flexneri* M90T); (3) *aggR* (gene from enteroaggregative *E. coli*, EAEC), (4) *eae* and *stx* (found in enterohemorrhagic *E. coli*, EHEC), or (5) *elt* (gene from enterotoxigenic *E. coli*, ETEC) (Fig. 5). Interestingly, the CD-associated *E. coli* strain 6655 tested positive for the *elt*

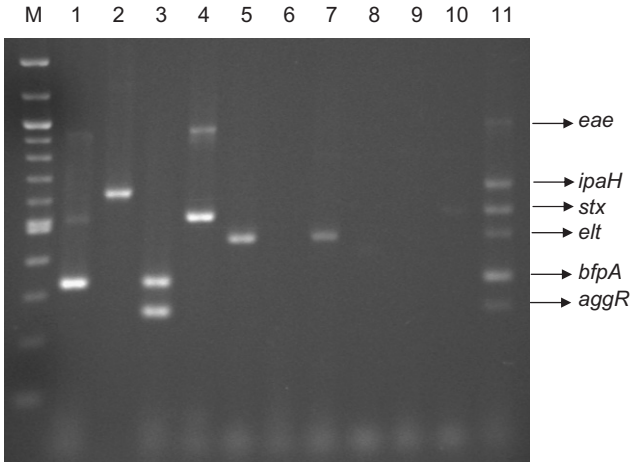

Fig. 5. Determination of Crohn's Disease (CD) *E. coli* virulence factors by multiplex PCR. DNA primers from known virulence genes of various enteric pathogens (*E. coli* strains and *S. flexneri*) were used to perform the PCR reaction. Lanes: M, DNA molecular weight markers (100-bp ladder); 1, *bfpA* and *eae* from EPEC E2348/69; 2, *ipaH* found in *S. flexneri* M90T; 3, *aggR* from EAEC O42; 4, *stx* and *eae* found in EHEC EDL933; 5, *elt* of ETEC H10407; 6, CD isolate 6604; 7, CD isolate 6655; 8, AIEC O83:H1; 9, AIEC LF82; 10, *E. coli* K-12 MG1655; 11, positive control (mix reaction using EPEC, *Shigella*, EAEC, EHEC and ETEC bacterial cultures). *E. coli* LF82 and O83:H1 did not harbor any of the tested virulence genes.

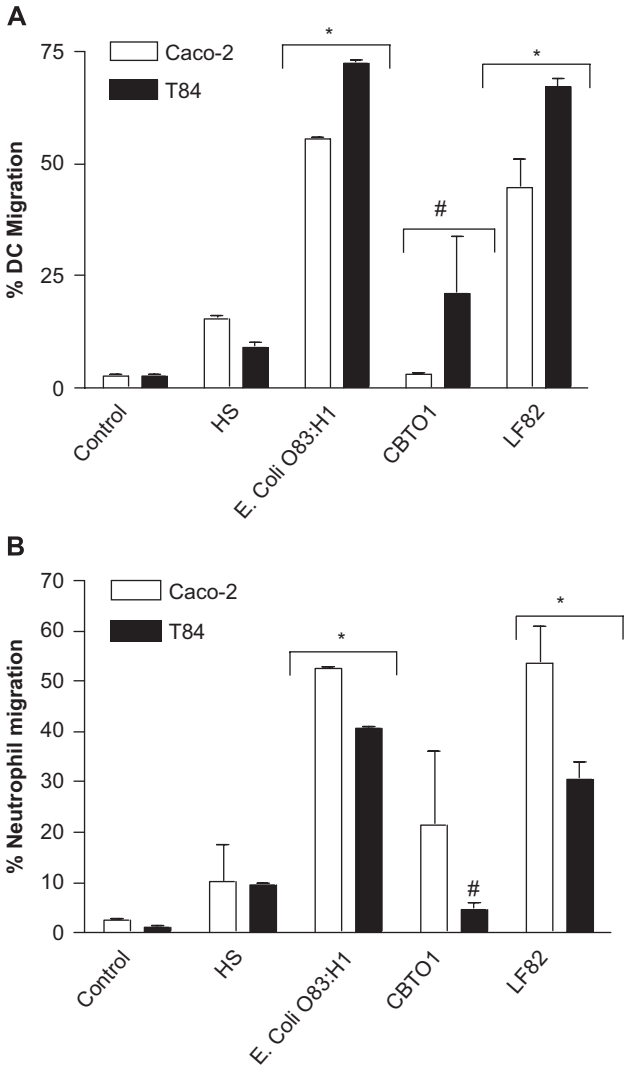

Fig. 4. Immune cell migration in response to *E. coli* O83:H1 and LF82 stimulated polarized IEC. Polarized IEC were apically stimulated with HS, *E. coli* O83:H1, non-flagellated CBTO1, or LF82 (MOI of approximately 100:1) for 2 h. Freshly isolated human DC or neutrophils were then added to the basolateral surface of cells and incubated for 4 h. Apical and basolateral supernatants were collected to determine immune cell migration. (A) Overall DC migration was somewhat higher in bacterially stimulated T-84 cells than Caco-2BBe cells. However, both cell lines stimulated with *E. coli* O83:H1 or LF82 showed a significant increase in DC migration compared to un-stimulated controls, HS and CBTO1 (*, $p<0.05$). Further, the non-flagellated CBTO1 strain was significantly impaired in its ability to induce DC migration in both cell lines as compared to O83:H1and LF82 (#, $p<0.05$). (B) Bacterial stimulation of Caco-2BBe cells showed an overall increase in neutrophil migration compared to that in T-84 cells. However, the trend remains the same in that both cell lines stimulated with *E. coli* O83:H1 or LF82 induced significant neutrophil migration as compared to un-stimulated cells and HS (*, $p<0.05$). The lack of flagella dramatically decreased CBTO1's ability to induce neutrophil migration in both cells lines as compared to that in the flagellated wild-type O83:H1 with migration being significantly lower through T-84 cells (#, $p<0.05$). Data are expressed as percent activation or migration ± standard deviation of three experiments. * or # $p<0.05$, ANOVA.

gene. The non-pathogenic *E. coli* strain, K-12 was used as a negative control and as expected, did not possess any of the virulence factors tested above.

Discussion

A variety of clinical and laboratory observations have suggested that intestinal microflora contribute to and/or perpetuate IBD (Liu et al., 1995; Schultsz et al., 1997, 1999; Burke and Axon, 1988; Lamps et al., 2003; Martin et al., 2004; Darfeuille-Michaud, 2002; Darfeuille-Michaud et al., 2004; Boudeau et al., 1999). Mucosal flora have been shown to play a vital role in the inflammatory processes in IBD, as demonstrated by isolates of *E. coli* from Crohn's lesions that are able to adhere and invade IEC and are classified as AIEC strains (Martin et al., 2004; Darfeuille-Michaud, 2002; Darfeuille-Michaud et al., 2004; Boudeau et al., 1999). Further evidence that gut flora play a vital role in the IBD is that IL-10 knockout mice only develop IBD if they are not kept in a sterile, pathogen-free environment (Rennick and Fort, 2000). In this study, we have identified an *E. coli* strain (sereotype O83:H1) isolated from a CD lesion, which share characteristics with the prototype AIEC strain LF82. Interestingly, strain LF82 and *E. coli* O83:H1 are the same serotype. Therefore, we have demonstrated for the first time that our *E. coli* O83:H1 isolate and the AEIC LF82 play an important role in activating inflammatory responses and immune cell migration in two well-established human intestinal epithelial cell lines.

Importantly, this study utilized two polarized human IEC that were differentiated, forming tight junctions and distinct apical and basolateral surfaces. This tissue culture model is more representative of an *in vivo* situation and lends more relevance to the *in vivo* physiological positioning of IEC. Additionally, polarized IEC differentially express cell surface proteins and/or factors on their apical and basolateral surfaces that cannot be distinguishable in monolayers of IEC cultured on plastic. These two intestinal cell lines were utilized in this study to ensure that the interaction between the CD-associated *E. coli* strains was not cell-line specific.

In our study we determine whether other *E. coli* strains isolated from Crohn's disease lesions have similar AIEC characteristics as the already identified LF82 strain (Boudeau et al., 1999; Darfeuille-Michaud et al., 1998) and to further investigate the inflammatory potential of these CD-associated *E. coli* strains. Our experiments demonstrated that the *E. coli* O83:H1 isolate had the ability to adhere to and invade polarized IEC at levels comparable to that of AIEC LF82 (Darfeuille-Michaud et al., 2004). Moreover, we observed similarities between these two strains of CD-associated *E. coli* in that they both induced IL-8 and CCL20 secretion from Caco-2BBe and T-84 cells and the subsequent migration of immune cells through these cells. Further, this study demonstrated that *E .coli* O83:H1 flagella expression is necessary to induce inflammation in IEC and plays a central role in the pathogenesis of this organism.

It was shown previously that the adhesion and invasion of Gram-negative bacteria are largely dependent on flagella that extend from the outer membrane of the bacterium (Barnich et al., 2003; Giron et al., 2002; Dibb-Fuller, 1999; Schmitt et al., 2001). These organelles allow the bacteria to attach and gain close contact to the host cell, aiding bacteria in the invasion of host cells. Therefore, to further characterize the role of flagella in *E. coli* O83:H1, a non-flagellated mutant of this organism was generated and tested for its adhesion and invasion capabilities. Our findings show that the *E. coli* O83:H1 flagellin-mutant was unable to adhere to and/or invade IEC as effectively as the wild-type strain. Additionally, the non-flagellated mutant was only able to induce minimal levels of IEC IL-8 secretion. These observations are consistent with those from other studies that have demonstrated the contribution of bacterial flagella as a crucial player in the adhesion and/or invasion of the bacterium to the host cells (Barnich et al., 2003; Schmitt et al., 2001). Important findings by Barnich et al. (2003) demonstrated that flagella play a direct role in adhesion of the AIEC strain LF82 and could be related to the coordinated expression of invasive determinants. The significance of flagella is also observed in other intestinal pathogens. A non-flagellated mutant of *Salmonella* showed a 50-fold reduction in invasion of cultured intestinal epithelial cells compared to that in flagellated wild-type *Salmonella* (Schmitt et al., 2001). A more and/or equally important role for flagella in addition to enhancing bacterial adhesion and invasion may be emerging in that it appears that flagella are also contributing to the virulence of *E. coli* O83:H1 because the lack of flagella significantly decreased, and in some cases eliminated, the ability of *E. coli* O83:H1 to induce an inflammatory response and immune cell migration in IEC.

To further characterize additional factors that contribute to the virulence of *E. coli* O83:H1, we utilized multiplex-PCR to determine if O83:H1 harbored virulence genes found in bacteria that are known to infect the gastrointestinal tract. We found that *E. coli* O83:H1 did not posses common virulence factors found in EPEC, *S. flexneri*, EAEC, EHEC or ETEC. Our results are consistent with groundbreaking work from Darfeuille-Michaud et al. (1998) that showed AIEC LF82 did not harbor virulence genes from similar enteric bacterial strains. Because no clearly defined virulence factors have been identified in LF82 and *E. coli* O83:H1, more research is needed to investigate this significant area.

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Our findings, as well as those of others (Martin et al., 2004; Darfeuille-Michaud, 2002; Darfeuille-Michaud et al., 2004; Boudeau et al., 1999; Schultsz et al., 1999), support the significance of *E. coli* adherence to and/or invasion of IEC. *E. coli* isolates have been shown to colonize CD lesions and can trigger signal transduction pathways, resulting in a chronic, low-grade inflammatory response (Darfeuille-Michaud, 2002; Boudeau et al., 1999; Schultsz et al., 1999; La Ferla et al., 2004). In this study, *E. coli* O83:H1 demonstrated the ability to differentially secrete an inflammatory cytokine, IL-8, from the basolateral surface of both intestinal cell lines. Interestingly, LF82 induced IEC to secrete IL-8 primarily from their apical surface. Although *E. coli* O83:H1 and LF82 show similar abilities to induce inflammation in IEC, these CD-associated *E. coli* strains differ in their apparent stimulation of IEC as demonstrated by differential IL-8 secretion from IEC surfaces. IL-8 is a potent chemoattractant for neutrophils (Kucharzik and Williams, 2003), where CCL20 is a potent chemoattractant for DC (Stagg et al., 2003). Further, IL-8 and CCL20 production involves the activation of the transcription factor NF-κB that is known to be responsible for the production of various other inflammatory cytokines (Xiao and Ghosh, 2005; Tak and Firestein, 2001; Kucharzik and Williams, 2003; Stagg et al., 2003). Therefore, it is anticipated that IEC are secreting a number of cytokines in response to *E. coli* O83:H1 and LF82 that influence immune cell migration. Caco-2BBe and T-48 cells responded in a similar manner to LF82 and *E. coli* O83:H1 in regard to IL-8 and CCL20 secretion and immune cell migration. These results further confirmed the similarities and consistency between these two human intestinal cell lines as well as showing that the response to *E. coli* O83:H1 and LF82 is not limited to one intestinal cell line.

Characteristically cytokine secretion is accompanied by the subsequent recruitment of immune cells, specifically first responders such as DC and neutrophils, to the site of infection. Niess et al. (2005) showed that luminal sampling by DC occurred by globular structures formed at the end of transepithelial dendrites that served as luminal sensors to monitor intestinal content. A number of studies have shown that the migration of neutrophils and luminal sampling/processing of IEC antigens by DC is linked to disease activity in IBD (Kucharzik et al., 2003a, 2005b; Stagg et al., 2003; Eksteen et al., 2004; Murakami et al., 2002). Hence, in our study, it is likely that cytokine secretion from *E. coli* O83:H1- and LF82-stimulated IEC recruited DC and neutrophils (Zen and Parkos, 2003) as demonstrated by immune cell migration through IEC in our transwell filter model. Even though we detected significantly higher IL-8 secretion from the apical surface of LF82-stimulated IEC, immune cell migration was equal to that of *E. coli* O83:H1-stimulated cells. As stated above, this indicates that there are likely other cytokines, other than IL-8, being secreted from the basolateral surface of IEC that influence immune cell migration.

In conclusion, increasing evidence shows that the diseased activity in IBD, including CD, is linked to an influx of immune cells and is likely due to stimulation of the intestinal epithelium by luminal bacterial flora. Significant progress and new insight into the pathogenesis of CD, specifically with respect to Darfeuille-Michaud et al. (2002, 2004) and Boudeau et al. (1999), whose work demonstrated that CD-associated *E. coli* strains, such as the AIEC LF82 strain, may play a major role in the initiation and perpetuation of intestinal inflammation. Our study showed for the first time that LF82 induced cytokine secretion and immune cell migration in polarized IEC that further expand the work of Darfeuille-Michaud, 2002; Darfeuille-Michaud et al., 2004; Boudeau et al., 1999). Likewise, we have identified a strain of *E. coli* isolated from a CD patient that has the characteristics of an AIEC isolate in that it can invade polarized IEC. But equally important is that we showed that the *E. coli* O83:H1 and LF82 isolate stimulated inflammatory responses in IEC and induced immune cell migration. We are not suggesting that *E. coli* O83:H1 is of the same caliber of intestinal pathogen as is *S.* Typhimurium that induces a rapid, vigorous and usually self-limiting innate immune response. But rather we propose, as our data suggest, that *E. coli* O83:H1 can induce a low-grade, chronic inflammation of the intestine. The characteristics of *E. coli* O83:H1 likely cause and/or contribute to a continuous low-grade inflammation of the gut in CD patients. Equally important is that *E. coli* O83:H1 flagella play a vital role in bacterial invasion and the induction of inflammatory responses in polarized IEC. Further characterization of *E. coli* O83:H1 strain, as well as other CD isolated *E. coli* strain is necessary to determine what makes some CD-associated *E. coli* strains AIEC and not others. These future studies and the contribution of flagella are underway to advance our understanding and treatment strategies regarding CD.

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