Characterization of Epithelial IL-8 Response to Inflammatory Bowel Disease Mucosal *E. coli* and Its Inhibition by Mesalamine

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Background: Mucosally adherent *E. coli* are found in inflammatory bowel disease (IBD) and colon cancer. They promote release of the proinflammatory cytokine interleukin-8 (IL-8). We explored mechanisms for this release and its inhibition by drugs.

Methods: IL-8 release from colon epithelial cells in response to mucosal *E. coli* isolates from IBD, colon cancer, and controls was characterized at the cellular and molecular level.

Results: IL-8 response of HT29 cells was greater with Crohn's disease (689 ± 298 [mean ± SD] pg IL-8/mL at 4 hours, $n = 7$) and colon cancer isolates (532 ± 415 pg/mL, $n = 14$) than with ulcerative colitis (236 ± 58 pg/mL, $n = 6$) or control isolates (236 ± 100 pg/mL, $n = 6$, $P < 0.0001$). Bacterial supernatants contained shed flagellin that triggered IL-8 release. For whole bacteria the IL-8 response to *E. coli* that agglutinate red blood cells (548 ± 428 pg IL-8/mL, $n = 16$), a function that correlates with epithelial invasion, was greater than for nonhemagglutinators (281 ± 253 pg/mL, $n = 17$; $P < 0.0001$). This was particularly marked among *E. coli* that, although flagellate, could not release IL-8 from TLR5-transfected HEK293 cells. IL-8 release was mediated by extracellular-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) and inhibited by mesalamine, but not hydrocortisone, at therapeutic concentrations.

Conclusions: Mucosa-associated *E. coli* shed flagellin that elicits epithelial IL-8 release but this may only become relevant when the mucosal barrier is weakened to expose basolateral TLR5. Adherent and invasive IBD and colon cancer *E. coli* isolates also elicit a flagellin-independent IL-8 response that may be relevant when the mucosal barrier is intact. The IL-8 release is MAPK-dependent and inhibited by mesalamine.

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Key Words: *Escherichia coli*, inflammatory bowel disease, colon cancer, IL-8, flagellin, MAPK, mesalamine

Inflammatory bowel disease (IBD) is thought to result from an aberrant mucosal immune response to the gut microbiota. This is supported by evidence that various animal models of IBD all require the presence of commensal bacteria to develop inflammation.[1] At least 7 independent studies have now reported an increased presence of adherent and invasive *E. coli* (AIEC) strains in Crohn's disease (CD)[2–8] and they have also been found in ulcerative colitis (UC)[3,6,9] and colon cancer.[4,6] Similar *E. coli* have recently been shown to be associated with granulomatous colitis in boxer dogs.[10] They are present in both inflamed and uninflamed mucosa,[4,6] suggesting that they are not just recruited as a consequence of inflammation and it is looking increasingly likely that they play an important role in IBD pathogenesis.

Although there is evidence of tissue invasion by *E. coli* in CD, where they have been identified within macrophages[11] and inside granulomata,[12] there is no evidence that they invade the mucosa in UC and their ability to elicit an IL-8 response from intestinal epithelial cells,[4] and thus to promote the superficial neutrophil recruitment[13] that typifies UC, is a likely alternative mechanism for a pathogenic effect. Moreover, the demonstration that unequivocal pathogens such as *Salmonella* spp, *Shigella* spp, *Yersinia* spp, and *Vibrio chol-*

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Conflicts of interest: J.M.R. is a past/present member of advisory boards for Procter and Gamble, Schering-Plough, Chiesi, Falk, and Celltech. With the University of Liverpool and Provexis Plc UK, J.M.R. holds a patent for use of a soluble fiber preparation as maintenance therapy for Crohn's disease. H.M.M. is presently an employee of Provexis Plc, Biosciences Building, Crown Street, Liverpool, L69 7ZB, UK. MV has filed a patent on the recombinant CEACAM binding polypeptide rD-7.

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erae can only invade the gut mucosa via the specialized M cells that overlay Peyer's patches in the small intestine and lymphoid follicles in the colon and cannot invade the mucosa via normal colon epithelial cells[14] makes it unlikely that the AIEC found in IBD and colon cancer invade normal colon epithelial cells, although it is plausible that these bacteria might invade M cells and thus provoke the initial lesions of CD which probably occur at Peyer's patches or lymphoid follicles.[15]

In this study we set out to characterize the mechanisms involved in the epithelial IL-8 response to IBD and colon cancer mucosal *E. coli* isolates with particular emphasis on assessing the relative importance of the AIEC phenotype in mediating this response, the downstream signaling mechanisms involved, and the effects of drugs used to treat IBD.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Escherichia coli were previously isolated from colonic mucosal biopsies of patients with CD, colon cancer, UC, and a control population (irritable bowel syndrome [IBS]/sporadic polyposis).[4] Thirty-three isolates were studied including 7 from CD, 6 from UC, 14 from colon cancer, and 6 control patient (irritable bowel or sporadic polyp) *E. coli*. The CD *E. coli* were isolated from 7 CD patients (2 were receiving corticosteroids and azathioprine, respectively, and the remainder were on 5-aminosalicylic acid [5-ASA]); UC *E. coli* were isolated from 5 patients (all 5 on 5-ASA and 2 were receiving corticosteroids); control *E. coli* were isolated from 2 patients (IBS and sporadic polyps), and colon cancer *E. coli* were isolated from 11 patients (2 patients undergoing resection received intravenous cephalosporin and metronidazole). The phylogenetic group of each *E. coli* (A, B1, B2, or D) was defined by triplex polymerase chain reaction (PCR) as described previously.[16] The characteristics of the bacterial strains used in this study are summarized in Table 1.

Isolates were cultured on Columbia agar with overnight incubation in air at 37°C. Prior to infection of cultured epithelial cells, bacteria were washed in sterile PBS and adjusted to an OD_{550nm} of 0.125 (equivalent to 150×10^6 CFU/mL). In some studies *E. coli* were heat-inactivated at 60°C for 30 minutes. To demonstrate whether *E. coli* possessed flagellae, isolates were stab-inoculated into 0.3% motility agar. To assess for the presence of curli fimbriae, *E. coli* isolates were grown on nutrient agar supplemented with 30 μg/mL Congo red for 48 hours at 26°C.

Reagents

SP600125 (an inhibitor of c-Jun N-terminal kinase [JNK]), SB203580 (a pyridinyl imidazole inhibitor of p38-MAPK pathway), and Bay11-7082 (an irreversible inhibitor of NF-κB activation, through inhibition of IκB-α phosphorylation) were all obtained from Calbiochem (Nottingham, UK). U0126 (a MEK inhibitor that inhibits the kinase activity of ERK1/2) was obtained from Promega (Madison, WI). Monoclonal antibodies to JNK1 (SC1648), phospho-JNK1/2 (SC6254), and polyclonal antibodies to ERK2 (SC154G) and TLR5 (SC10742) were all from Santa Cruz Biotechnology (San Francisco, CA). Other monoclonals included anti-phosphoERK1/2 (Cell Signaling Technology, Beverley, MA), pan-actin antibody (Neomarkers, Fremont, CA) and anti-Ras (Calbiochem). Polyclonal rabbit anti-flagellar serum was a kind gift from Dr. J. Giron (Microbiology & Immunology, University of Arizona, Tucson). An NF-κB p65 subunit siRNA kit (Cell Signaling Technology) included anti p65 and ERK2 antibodies.

Recombinant proteins included human IL-8 (Peprotech EC, London, UK), TNF-α (R&D Systems, Abingdon, UK), and *E. coli* flagellin (Inotek, Beverley, MA). *E. coli* O127:B8 lipopolysaccharide (LPS), polymixin B, hydrocortisone, methyl α-D-mannopyranoside, and protease type XIV were all from Sigma (Poole, UK). Human leukocyte elastase was from Elastin Products (Owensville, MO). 5-ASA (mesalamine) was a kind gift from Dr. S. Andersson (Pharmacia, Uppsala, Sweden). The carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-binding polypeptide rD-7 was produced using the pQE30 expression system as described previously.[17] The rD-7 polypeptide had been shown to inhibit the binding of respiratory and urogenital pathogens (*M. catarrhalis, H. influenzae, N. gonorrhoeae,* and *N. meningitidis*) via inhibition of CEACAM-dependent bacterial adhesion.[17]

Cell Culture

Human colon cancer cell lines HT29 and T84 were obtained from the European Collection of Animal Cell Culture (Public Health Laboratory Service, Wiltshire, UK). HT29 cells were cultured in DMEM containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 4 mM glutamine. HEK293 uroepithelial cells, stably transfected with pUNO-hTLR5 (encoding human toll-like receptor 5), pUNO-LacZ (encoding β-galactosidase) or vector alone were obtained from Invitrogen (San Diego, CA). HEK293 were cultured in complete DMEM supplemented with 10 μg/mL blasticidin. Polarized T84 model epithelia were cultured in Ham's F12 medium (10% FCS, penicillin and streptomycin) on 0.45 μm collagen-coated permeable supports (Millipore, Watford, UK). Monolayers with a transepithelial electrical resistance (TEER) of >800 °/cm^2 were used for assessment of flagellin transcytosis. All cell lines were maintained at 37°C in 5% CO_2, 95% air.

TABLE 1. Characteristics of *E. coli* Strains Used

E. coli Isolate	Disease Group	Hemagglutination Status	Afa/Dr Cluster	Curli Fimbriae	Adherence to I407 Cells	Invasion of I407 Cells	Adherence to HT29 Cells	Invasion of HT29 Cells	Phylotype	Replication Within Macrophages/ adherent Monocytes
HM95[a]	Crohn's disease	-	-	+	+	+	+	+	A	+
HM154[a]	Crohn's disease	-	-	+	-	-	+	-	B1	+
HM427[a]	Crohn's disease	+	+	+	+	+	+	+	B2	+
HM580[a]	Crohn's disease	+	+	-	+	+	+	+	D	+
HM605[a]	Crohn's disease	+	-	+	+	+	+	-	B2	+
HM615[a]	Crohn's disease	+	-	+	+	-	+	-	B2	+
HM670	Crohn's disease	+	-	-	+	+	+	-	B2	+
LF82	Crohn's disease	-	n/a	n/a	+	+	n/a	n/a	B2	+
HM44[a]	Colon cancer	+	-	+	+	+	+	-	B2	n/a
HM164[a]	Colon cancer	-	-	-	+	+	+	-	B2	n/a
HM196	Colon cancer	-	-	-	-	-	+	-	D	n/a
HM213	Colon cancer	+	-	-	+	+	+	-	B2	n/a
HM229	Colon cancer	+	-	+	+	+	+	-	B2	n/a
HM288[a]	Colon cancer	+	+	+	+	+	+	-	B2	n/a
HM329	Colon cancer	+	-	-	+	-	+	-	B2	n/a
HM334	Colon cancer	+	-	-	+	+	+	-	B2	n/a
HM356[a]	Colon cancer	-	-	-	+	+	+	-	B1	n/a
HM358	Colon cancer	+	+	-	+	+	+	+	D	n/a
HM497	Colon cancer	-	-	-	+	-	+	+	A	n/a
HM544[a]	Colon cancer	+	-	-	+	+	+	-	B2	n/a
HM545	Colon cancer	+	-	+	+	+	+	-	B2	n/a
HM550[a]	Colon cancer	-	-	+	+	+	+	-	D	n/a
HM250[a]	Ulcerative colitis	+	-	+	n/a	n/a	n/a	n/a	B2	n/a
HM295[a]	Ulcerative colitis	-	-	-	n/a	n/a	n/a	n/a	B2	n/a
HM378[a]	Ulcerative colitis	-	-	-	n/a	n/a	n/a	n/a	B2	n/a
HM380[a]	Ulcerative colitis	-	-	+	n/a	n/a	n/a	n/a	B2	n/a
HM387[a]	Ulcerative colitis	-	-	+	n/a	n/a	n/a	n/a	B2	n/a
HM394[a]	Ulcerative colitis	-	-	+	n/a	n/a	n/a	n/a	B2	n/a
HM452[a]	Control	-	-	-	n/a	n/a	n/a	n/a	B2	n/a
HM454[a]	Control	+	-	+	n/a	n/a	n/a	n/a	B2	+
HM455[a]	Control	-	-	-	n/a	n/a	n/a	n/a	B2	n/a
HM456[a]	Control	+	-	+	n/a	n/a	n/a	n/a	B2	+
HM488[a]	Control	-	-	-	n/a	n/a	n/a	n/a	B2	+
HM489[a]	Control	-	-	+	n/a	n/a	n/a	n/a	B2	n/a

[a]Denotes strains used for generation of pooled bacteria-free *E. coli* culture supernatant.
n/a indicates data not available.

Assessment of IL-8 Release from HT29 Cells in Response to Mucosal *E. coli* Isolates and Bacteria-free *E. coli* Culture Supernatants

HT29 cells (8×10^5 per well) were infected, in triplicate, for up to 8 hours, with apical addition of whole *E. coli* isolates from each disease group (7 CD, 14 colon cancer, 6 UC, and 6 controls) at a multiplicity of infection (MOI) of 10 ($\approx 7 \times 10^6$ CFU/mL) in keeping with previous studies of bacterial adherence to intestinal cell lines.[2,4] IL-8 released to the medium was measured by enzyme-linked immunosorbent assay (ELISA). TNF-α (2 ng/mL, 4 hours) was used as a control stimulus.

Pooled bacteria-free *E. coli* culture supernatants were generated from *E. coli* isolates, 6 each pooled separately from

4 disease groups (UC, CD, colon cancer, and control patients). Bacterial suspensions (OD_{550nm} 0.125) were 0.2 μm-filtered and supernatants confirmed bacteria-free by overnight culture on Columbia agar. Epithelial cells were treated with 50 μL supernatant/mL culture medium and IL-8 release compared to supernatants predigested (3 h, 37°C) with either 0.25 μM leukocyte elastase or 20 μg/mL Streptococcal protease XIV to remove flagellin, as described previously.[18] Heat-inactivated elastase and protease (90°C, 15 min) were used as controls.

Quantification of IL-8 Release

Following treatment, culture medium and cell monolayers were harvested and processed as previously described.[19] IL-8 was measured in triplicate using a solid-phase sandwich ELISA with antihuman IL-8 capture and detection antibodies (790A 28G2 and 893C 4G2; Biosource, Camarillo, CA). Within and between assay coefficients of variation were 10.1% ($n = 3$) and 13% ($n = 7$), respectively.

Investigation of the Role of Flagella, Outer Membrane Vesicles (OMV), and LPS in Mediating the Release of IL-8

Flagella from CD (HM427) and colon cancer (HM288) *E. coli* isolates were purified by ammonium sulfate precipitation.[20] Purity was monitored by SDS-PAGE, immunoblotting, and transmission electron microscopy (TEM). OMVs were isolated by ultracentrifugation from a 0.2 μm-filtered *E. coli* culture supernatant, pooled from 6 mucosal *E. coli* (5 hemagglutination-positive and 1 negative isolate, from 3 UC patients) or *E. coli* HM427 supernatant, as described previously.[21] In separate experiments, pretreatment of bacterial suspensions with polymixin B (PMB; 10 μg/mL, 1 h) was used to block any action of LPS. IL-8 release evoked by LPS derived from *E. coli* serotype O127:B8 (1 μg/mL) was used as a control for the inhibitory action of polymixin B.

Assessment of Flagellin Transcytosis

Polarized T84 model epithelia were apically colonized with 10^8 CFU/mL CD *E. coli* HM427. After 4 hours, flagellin present within the apical and basolateral media was analyzed by SDS-PAGE as per Lyons et al[22] followed by immunoblotting with anti-flagellar serum.

Analysis of Flagellin-TLR5 and Fimbrial-receptor Interactions Involved in Mucosal *E. coli*-mediated IL-8 Release

To assess the role of TLR5, HT29, and HEK293 cells (expressing TLR5, LacZ, or vector alone) were apically infected (MOI 10), for 4 hours with each of 33 *E. coli* and also with pooled bacteria-free *E. coli* culture supernatants, purified flagella (1 μg/mL), recombinant flagellin (1 μg/mL), or OMVs (from pooled UC isolates). TNF-α (2 ng/mL) was used as a positive control.

To investigate the role of CEACAMs and Type 1 (mannose-dependent) fimbriae, HT29 cell monolayers were pretreated with anti-CEACAM peptide rD-7 (1 μg/mL) or methyl α-D-mannopyranoside (10 mM) for 60–90 minutes. Subsequently, cells were incubated for 4 hours with apical addition of whole *E. coli* isolates. Inhibitors were maintained in the medium throughout the period of infection. IL-8 released into the medium was measured by ELISA and compared to untreated controls.

Amplification of *fliC* and Sequence Alignment of Flagellin (FliC) Protein Monomers from Mucosa-associated *E. coli*

Amplification of *fliC* was performed by PCR in a total reaction volume of 50 μL containing 1 μL of bacterial supernatant, 100 nM each of *fliC* oligonucleotide primers (sense 5′ ATGGCACAAGTCATTAAT 3′ and anti-sense 5′ TTAACCCTGCAGTAGAGA 3′) and 1.1x PCR Master Mix (ABgene, Surrey, UK). Thermal cycling conditions were 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, with a final extension cycle of 72°C for 10 minutes. Amplification was verified using agarose gel electrophoresis (10% reaction volume) and the remaining 90% purified using MicroSpin S-400 HR columns (Amersham Biosciences, Little Chalfont, UK). DNA sequencing was performed by Lark Technologies (Essex, UK) and deduced amino-acid residues from *fliC* amplicon sequences were analyzed using CLUSTAL W (1.83) multiple sequence alignment software. *E. coli* flagellin (FliC) sequences were compared with respect to those regions previously determined to be important for TLR5 recognition.[23–25]

Analysis of Intracellular Signal Pathways Involved in Mucosal *E. coli*-mediated IL-8 Release

Following apical infection with each of the 33 *E. coli* isolates, IL-8 release was assessed in HT29 cells pretreated (all 10 μM) with U0126 (to block ERK1/2 activity), SB203580 (to inhibit p38 MAPK pathway), or SP600125 (to inhibit JNK activity) for 60–90 minutes. Inhibitors were present throughout the 4-hour infection. In separate experiments, MAPK activation by CD and colon cancer isolates, for up to 60 minutes, was confirmed by SDS-PAGE and immunoblotting as described previously.[26] Phorbol 12-myristate 13-acetate (PMA) (0.25 μM, 20 min) was used as a positive control for MAPK activation.

Activation of Ras, the upstream guanine-nucleotide binding protein that stimulates the Raf-MEK-MAPK pathway,[27] was assessed over a 30-minute infection. Glutathione S transferase (GST)-K85A RBD (Ras-binding domain of Raf1) fusion protein was used to pull down active (GTP-

bound) Ras, as described elsewhere.[28] Total lysates and pull-down samples were analyzed by SDS-PAGE and immunoblot using antibody against Ras. Equal loading was confirmed using ERK2 antibody.

To assess the role of NF-κB, IL-8 release was assessed in response to each of the 33 *E. coli* isolates from HT29 cells pretreated with IκBα phosphorylation inhibitor Bay11-7082 (10 μM, 60 min) to block NF-κB activation. Inhibitor was present throughout the 4-hour infection. Downregulation of NF-κB was also performed using p65 subunit siRNA duplexes introduced to 50% confluent HT29 cells in the presence of 8 μL/mL TransIT-TKO (Cambridge Bioscience) 24 hours before treatment with flagella or apical addition of whole *E. coli*. Suppression of the p65 subunit was confirmed by immunoblotting.

Effect of 5-ASA (Mesalamine) and Hydrocortisone on Mucosal *E. coli*-mediated IL-8 Release

HT29 cells were pretreated for 1 hour with 5-ASA (2.5–20 mM) or hydrocortisone (10–100 μM), followed by addition of 0.2 μm-filtered pooled UC *E. coli* culture supernatant. After 4 hours IL-8 release was measured by ELISA.

Quantification of Cellular Protein and DNA

Protein and double-stranded DNA concentrations of cell lysates were determined using the bicinchoninic acid assay (Sigma) and PicoGreen (Molecular Probes, Paisley, UK) as per the manufacturer's instructions.

Cytotoxicity Assay

5-ASA and hydrocortisone were assessed for cytotoxicity toward the cultured epithelial cells using the adenylate kinase assay (ToxiLight Non-Destructive Cytotoxicity Bio-Assay Kit, Cambrex Bio, Cambridge, MA) as per the manufacturer's instructions.

Statistical Analysis

Independent sample groups were assessed for both normality and for equality of variances. As appropriate, treatment groups were analyzed using Student's *t*-test or Mann–Whitney *U*-test. For multiple treatment groups, 1-way or Kruskall–Wallis analysis of variance (ANOVA) was employed, followed by pairwise comparisons of treatment means (StatsDirect v2.3.1; Sale, UK). Differences were considered significant when $P < 0.05$.

RESULTS

Mucosal *E. coli* Isolates Stimulate IL-8 Release from Colonic Epithelial Cells

All mucosal *E. coli* isolates from CD ($n = 7$) and colon cancer ($n = 14$) were shown to stimulate the release of IL-8 from HT29 cells in a time-dependent manner (Fig. 1A). A lower IL-8 release was seen in response to *E. coli* isolates from control patients (IBS/sporadic polyposis) (236 ± 100 pg IL-8/mL at 4 hours [all mean ± SD unless indicated otherwise], $n = 6$) and those from UC (236 ± 58 pg/mL, $n = 6$) compared with isolates from CD (689 ± 298 pg/mL, $n = 7$) and colon cancer (532 ± 415 pg/mL, $n = 14$; $P < 0.0001$, ANOVA) (Fig. 1B).

Intracellular IL-8 content was also increased in response to treatment with CD and colon cancer *E. coli*, rising from a median of 2.4 pg/mL (range 1.2–51 pg/mL) at 0.5 hours to a median of 261 pg/mL (range 108–601 pg/mL) at 8 hours as compared to uninfected control cells: 2 pg/mL (1.3–2.2) and 60 (59.5–69.5) pg/mL at 0.5 and 8 hours, respectively ($P < 0.01$ ANOVA; $n = 21$, i.e., 7 CD and 14 colon cancer isolates). No significant changes were seen in cellular double-stranded DNA levels in the HT29 cells when infected with mucosal *E. coli* isolates over the 8-hour incubation period (201 ± 73 ng/mL DNA) at the end of the treatment period compared to 207 ± 85 ng/mL in untreated controls ($n = 21$).

Magnitude of IL-8 Release Correlates with Ability of *E. coli* to Cause Hemagglutination

Our previous work had shown a strong correlation between the ability of mucosal *E. coli* to hemagglutinate human red blood cells and their ability to adhere to intestinal epithelial cells in culture.[4] *E. coli* that cause hemagglutination ($n = 16$) induced a greater IL-8 response from HT29 cells (548 ± 428 pg IL-8/mL) than nonhemagglutinators (281 ± 253 pg/mL, $n = 17$; $P < 0.0001$ Mann–Whitney *U*) (Fig. 1C); heat-inactivation of CD *E. coli* isolate HM427 only caused a modest reduction in its ability to induce an IL-8 response: 203 ± 53 pg/mL at 4 hours compared with 323 ± 84 pg/mL for live *E. coli* HM427 ($n = 3$, $P = 0.01$, Mann–Whitney *U*).

Pooled Bacteria-free *E. coli* Culture Supernatant Induces a Substantial IL-8 Response

Twenty-four *E. coli* isolates, 6 each from control patients (including 1 hemagglutinator), CD (including 4 hemagglutinators), UC (including 1 hemagglutinator), and colon cancer (including 3 hemagglutinators) were pooled to form a bacterial suspension and a bacteria-free supernatant was derived by passage through a 0.2-μm filter. Filtered supernatants caused an IL-8 response, (911 ± 292 pg/mL) that was 51.3 ± 10.9% of that produced by an equivalent preparation of whole bacteria (1763 ± 314 pg/mL) ($n = 24$, $P < 0.0001$, Mann–Whitney *U*).

IL-8 Response to Bacteria-free *E. coli* Culture Supernatants Is Reduced by Protease and Leukocyte Elastase Digestion

Pretreatment of bacteria-free *E. coli* supernatants with either protease XIV (20 μg/mL for 3 h at 37°C) or leukocyte

elastase (0.25 μM for 3 h at 37°C) caused a substantial reduction in the IL-8 response from HT29 cells compared with untreated *E. coli* supernatant. IL-8 response to pooled bacteria-free *E. coli* culture supernatant was reduced by 46 ± 19% with protease XIV ($P = 0.003$, ANOVA) and 68 ± 30% with elastase ($P < 0.0001$, ANOVA) (Fig. 2). These findings would be compatible with flagellin as the stimulus for IL-8 release.

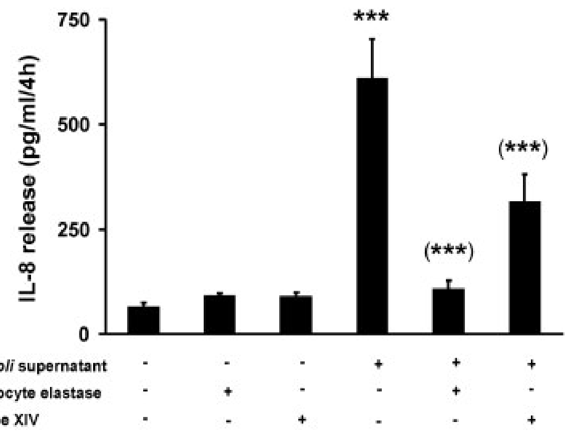

FIGURE 2. IL-8 response to pooled bacteria-free *E. coli* culture supernatant is largely abolished by protease and human leukocyte elastase digestion. Pooled 0.2 μM filtered *E. coli* culture supernatant from CD ($n = 6$), UC ($n = 6$), colon cancer ($n = 6$), and control patient isolates ($n = 6$) was digested with either 20 μg/mL protease type XIV or 0.25 μM human leukocyte elastase, followed by heat inactivation at 90°C for 15 minutes. HT29 cells were treated in triplicate for 4 hours with digested or nondigested supernatant derived from 7×10^6 pooled *E. coli*/mL. IL-8 concentrations in the supernatant were determined by ELISA. The bars represent the overall means of values derived for IL-8 responses to supernatant from each disease group. Significant differences from basal IL-8 release, ***$P < 0.001$ and from pooled supernatant-induced IL-8 release, (***)$P < 0.001$ (ANOVA).

Purified Flagella Stimulate Comparable IL-8 Release to Whole Bacteria

Flagella, isolated from 7×10^6 *E. coli*, either colon cancer isolate HM288 or the CD isolate HM427 (Fig. 3A), caused an IL-8 release of 178 ± 40 pg/mL and 148 ± 38

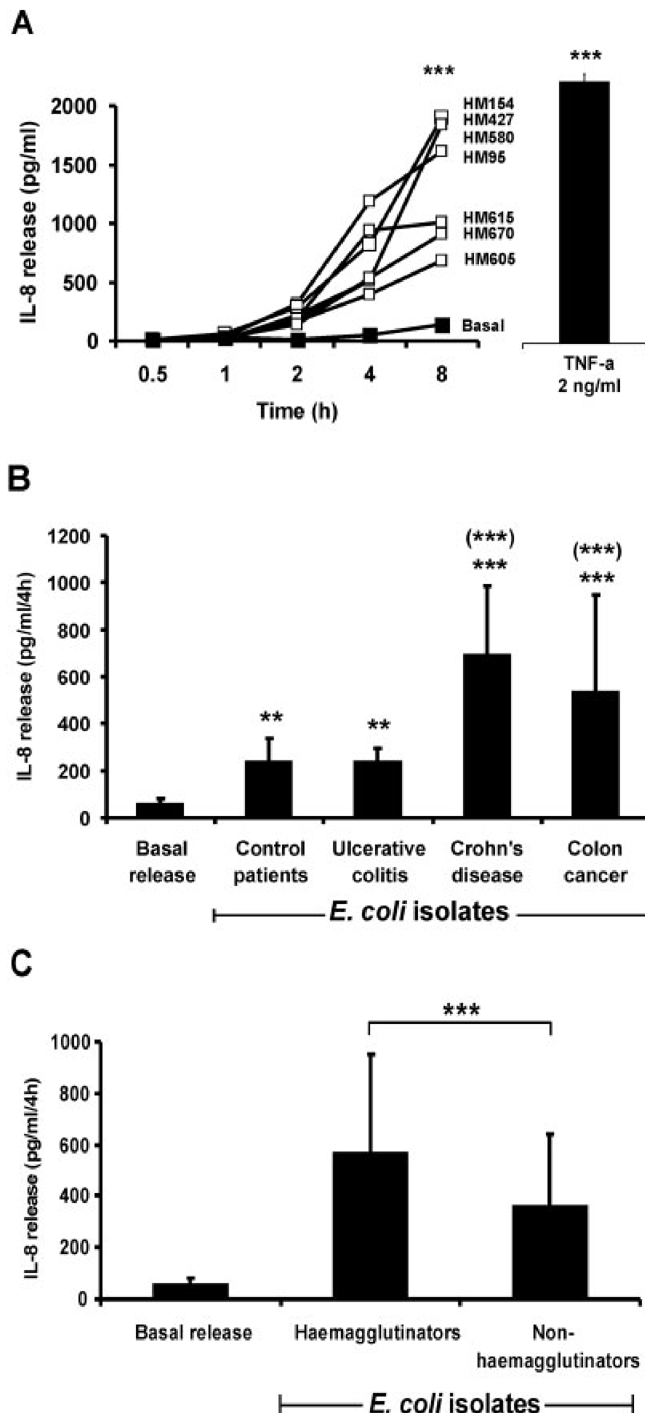

FIGURE 1. Stimulation of IL-8 release from HT29 colon epithelial cells by mucosal *E. coli*. A: CD mucosal *E. coli* ($n = 7$) stimulate IL-8 release from confluent HT29 cells in a time-dependent manner. IL-8 release was measured by ELISA and data expressed as pg/mL (mean ± SEM). Solid bar represents IL-8 release at 8 hours induced by 2 ng/mL TNF-α ($n = 6$). Significant differences from 8 hours basal release, ***$P < 0.0001$ (ANOVA). B: A significantly greater IL-8 response was seen following treatment using mucosal *E. coli* isolated from patients with CD ($n = 7$) and colon cancer ($n = 14$) compared to *E. coli* isolated from UC ($n = 6$) or control patients ($n = 6$). Significant differences from basal IL-8 response, **$P < 0.01$, ***$P < 0.001$ and significant differences from control and UC patient responses, (***)$P < 0.001$ (Kruskal-Wallis ANOVA). C: Hemagglutinating *E. coli* isolates ($n = 16$) elicited a greater IL-8 response from HT29 cells than nonagglutinating *E. coli* strains ($n = 17$), ***$P < 0.0001$ (Mann–Whitney *U*).

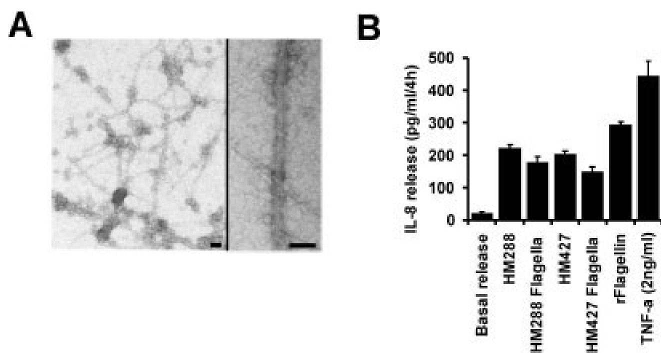

FIGURE 3. Flagellin induces a comparable IL-8 release to whole bacteria. A: TEM of purified flagella from CD mucosal *E. coli* HM427. Scale bar = 50 nm. B: Purified flagella from *E. coli* isolates, HM427 (CD), HM288 (colon cancer) stimulated IL-8 release from HT29 cells at a level comparable to the IL-8 response elicited by the same starting amount (7×10^6) of whole bacteria. Recombinant *E. coli* flagellin was used at 1 μg/mL. HT29 cells were treated in triplicate for 4 hours.

pg/mL, respectively, as compared to 220 ± 34 pg/mL and 201 ± 29 pg/mL in response to equivalent amounts of whole bacteria (Fig. 3B). Almost all (99 ± 5%, $n = 3$) of the IL-8-stimulatory activity of the bacterial supernatant was shown to be attributable to an OMV preparation (Fig. 4A). OMV are known to contain proteins relevant to pathogenicity[29] but immunoblotting showed flagellin to be present within the purified OMV preparations (Fig. 4B) and TEM confirmed the presence of flagella associated with OMVs (Fig. 4C), again compatible with flagellin as the major stimulant for IL-8 response to the bacterial supernatants.

LPS from Mucosal *E. coli* Contributes Little to the IL-8 Response

Inhibition of LPS by preincubation of bacterial suspension with 10 μg/mL polymyxin B for 1 hour only caused a reduction of 7 ± 4% in IL-8 response to 2 mucosal *E. coli* isolates from CD.

TLR-5 Dependence of IL-8 Response to 19/33 *E. coli* Isolates

Having shown that flagellin was a major determinant of the IL-8 response to the mucosal *E. coli,* we investigated the role of the known flagellin-receptor, TLR5, by testing the IL-8 response in HEK293 cells with and without transfected TLR5. Nineteen of the 33 *E. coli* isolates tested (8/14 colon cancer isolates and 5/7 CD and 2/6 each of UC and 4/6 control *E. coli*) elicited IL-8 release in a TLR5-dependent fashion, defined as a response to TLR5-expressing HEK293 cells ≥20% of that seen with HT29 cells (Fig. 5A). For these *E. coli* the median IL-8 response was 394 ± 253 pg/mL in HEK-hTLR5 cells compared to 32 ± 42 pg/mL in HEK293-LacZ cells. For *E. coli* where the IL-8 release was not mediated by flagellin-TLR5 interaction, the median IL-8 response was only 23 ± 18 and 38 ± 37 pg/mL, respectively, from HEK293-hTLR5 and HEK293-LacZ cells ($n = 14$). TNF-α (2 ng/mL, for 4 h) evoked a similar IL-8 response from TLR5- and β-galactosidase-expressing HEK293 cells. All 33 *E. coli* isolates were able to induce an IL-8 release from HT29 cells, with a median IL-8 release of 405 ± 346 pg/mL. HT29 cells were also shown to express TLR5 (Fig. 5B).

Amplification of *fliC* from representative mucosa-associated *E. coli* isolates, able ($n = 4$) or unable ($n = 5$) to induce TLR5-dependent IL-8 production in HEK293-hTLR5 cells, showed some variation in amplicon length but no correlation with IL-8 response in HEK293-hTLR5 cells (data not shown). In addition, deduced amino-acid sequences (aligned using the CLUSTAL W algorithm) demonstrated no significant differences in flagellin residues within those regions previously determined to be important for TLR5 recognition,[23–25] indicating that all our mucosa-associated *E. coli* isolates are likely to make flagellin molecules that can be recognized by TLR5 (see Table 2). All 33 isolates were shown to possess flagella and to be motile on 0.3% agar plates.

Among the 14 *E. coli* isolates that did not mediate IL-8 release via TLR5, the IL-8 response from HT29 cells was considerably greater for hemagglutinators (median 513 pg/mL, range 277–813) than for nonhemagglutinating strains (median 175 pg/mL, range 48–295; $P = 0.004$ Mann–Whitney *U*). Study of pooled bacterial supernatants from each disease group and isolated OMV confirmed the TLR5-mediated IL-8 response (see Fig. 5C).

Curli fimbriae, an extracellular matrix component of Enterobacteriaceae (including commensal *E. coli*), have recently been shown to be involved in IL-8 release from HT29 cells via interaction with flagellin.[30,31] We therefore assessed all 33 *E. coli* isolates in this study for the presence of curli fimbriae and its association with TLR5-dependent epithelial cell IL-8 release. Overall, 17/33 *E. coli* isolates possessed curli fimbriae. There was a good association between presence of curli and the magnitude of IL-8 response from HEK293-hTLR5 cells ($P = 0.05$, unpaired *t*-test). However, there was no relationship seen between curli and hemagglutination status of the *E. coli*, nor any association with isolates from specific patient groups.

It is noteworthy that supernatants from CD, colon cancer, and UC *E. coli* isolates, unlike the whole bacteria, all cause similar IL-8 responses, suggesting that the enhanced IL-8 responses to whole bacteria from CD and colon cancer are not explained by flagellin-TLR5 interaction.

Following a 4-hour incubation of polarized T84 epithelium with CD mucosa-associated *E. coli* HM427, an AIEC,[4] applied to the apical aspect, flagellin was undetectable in the basolateral medium as determined by immunoblot analysis (*n*

= 3; Fig. 5D), suggesting that trancytosis of flagellin had not occurred even though a significant IL-8 response was seen (249.1 ± 26.4 pg Il-8/mL) compared with control untreated cells (43.6 ± 19.8 pg IL-8 /mL, $P < 0.0001$).

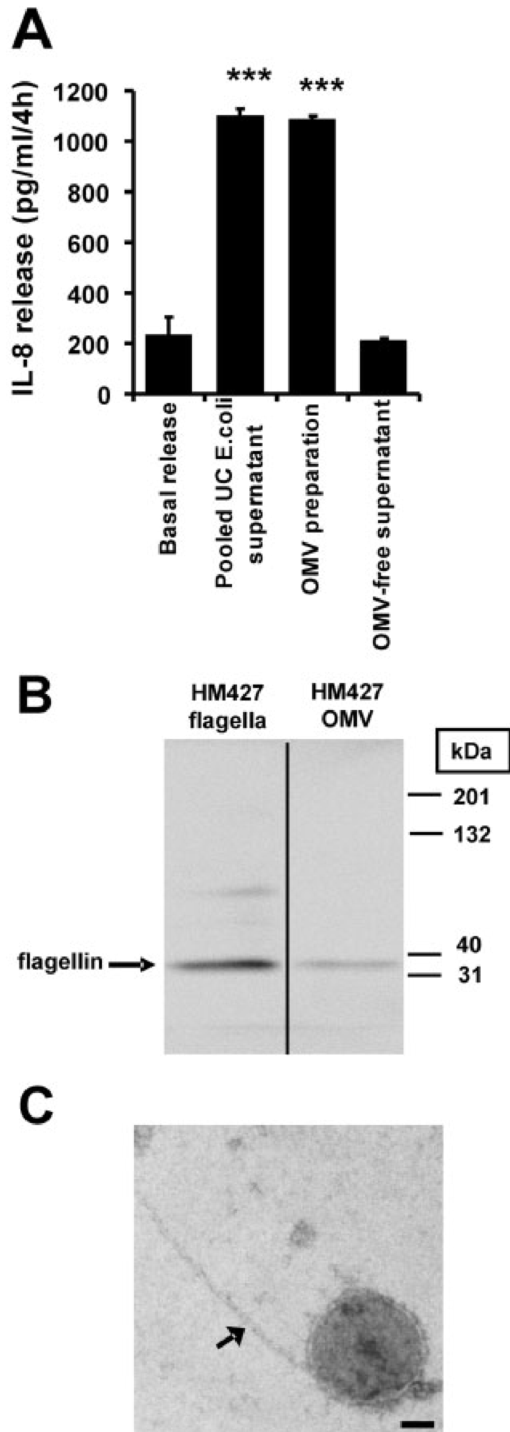

FIGURE 4. IL-8 release induced by pooled bacteria-free *E. coli* culture supernatant is mediated via a shed outer membrane vesicle (OMV) preparation. A: All of the IL-8 response evoked by a bacteria-free *E. coli* culture supernatant can be accounted for by a sedimented OMV fraction prepared by ultracentrifugation from the same 6 pooled UC *E. coli* isolates (both ***$P < 0.0001$ [ANOVA], compared with basal IL-8 release). The supernatant, free of pelleted OMV (as confirmed by TEM), did not significantly stimulate IL-8 release above basal levels. B: Immunoblot of OMV and flagella, both isolated from a CD *E. coli* isolate HM427, demonstrating flagellin immunoreactivity in the OMV fraction. C: TEM revealed that *E. coli* OMVs are ≈100–150 nm in size, electron-dense, and surrounded by an outer membrane. Flagella (arrow) were also associated with OMVs. Scale bar = 50 nm.

IL-8 Response to *E. coli* Is Neither Dependent on Interaction with Host CEACAMs Nor Involves Bacterial Mannose-dependent Fimbrial Adhesins

CEACAM6 was recently shown to act as a receptor for AIEC adhesion and was found to be upregulated in the ileal mucosa of CD patients.[32] Pretreatment of HT29 cells with 1 μg/mL recombinant polypeptide rD-7, an antimicrobial agent that blocks the interactions of a number of mucosal pathogens with CEACAM-expressing epithelial cells,[17] failed to reduce the overall IL-8 response to all 33 *E. coli* isolates (IL-8 release in presence of rD-7: 298 ± 66 pg IL-8/mL, compared to untreated controls 323 ± 72 pg/mL). Only 3/33 *E. coli* isolates showed a reduction in IL-8 response of >20% (range 21%–32%) with rD-7 but there was neither any disease group association, relation to hemagglutination status, nor correlation with possession of Afa/Dr. We also assessed the possible inhibitory effect of addition of 10 mM α-methyl mannopyrannoside; however, this showed a similar IL-8 response (301 ± 68 pg/mL, n = all 33 *E. coli* isolates) compared with untreated controls. These studies suggest little or no role for Afa/CEACAM interaction in the IL-8 response.

IL-8 Response to *E. coli* Isolates Is Mediated via ERK1/2 and p38 MAPK Activation but Not NF-κB Activation

We next investigated the intracellular signaling pathways responsible for the IL-8 response. Inhibition of ERK1/2 activation by pretreatment with 10 μM U0126 for 1 hour reduced IL-8 release in response to whole bacteria by 77 ± 14%, from 610 ± 355 to 194 ± 107 pg IL-8/mL across all 33 *E. coli* isolates ($P < 0.0001$, ANOVA) (Fig. 6A). Similarly, inhibition of p38 MAPK activation by SB203580 (10 μM) reduced IL-8 release by 84 ± 18%, from 509 ± 322 to 149 ± 81 pg IL-8/mL ($P < 0.0001$, ANOVA; $n = 33$) (Fig. 6A). By contrast, inhibition of c-Jun N-terminal kinase (JNK) activation by pretreatment of HT29 cells with 10 μM SP600125 for 90 minutes caused a more modest reduction in

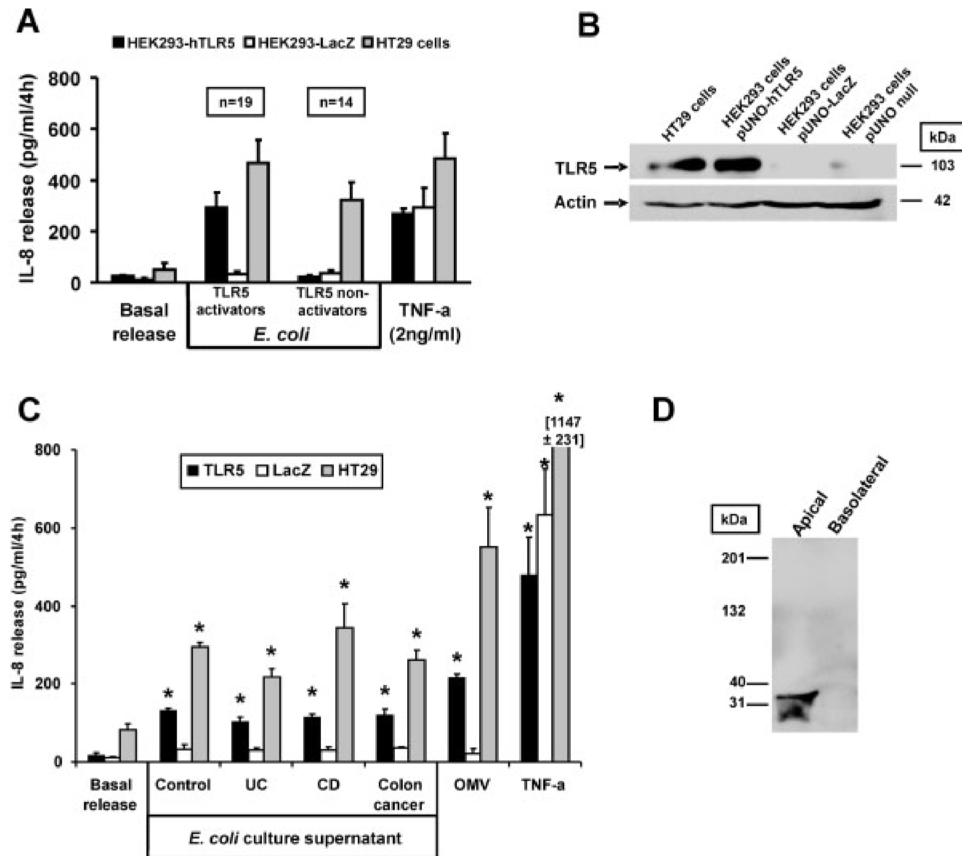

FIGURE 5. The IL-8 response to 19/33 *E. coli* isolates is mediated via flagellin-TLR5 interaction. A: IL-8 release from HEK293-hTLR5, HEK293-LacZ, and HT29 cells was measured in response to all 33 *E. coli*. All cell lines showed an IL-8 response to TNF-α. *E. coli* that did not stimulate an IL-8 response from TLR5-transfected HEK293 cells ($n = 14$) were still capable of eliciting an IL-8 response from HT29 cells. B: HT29 colon epithelial cells express TLR5. Representative immunoblot of HT29 cells and HEK293 cells expressing TLR5, β-galactosidase (LacZ) or with vector alone, using anti-TLR5 or pan-actin antibody. C: Study of pooled bacterial supernatants from each disease group (each $n = 6$ isolates, with triplicate determinations) and an OMV preparation (from pooled UC isolates) confirmed the TLR5-mediated IL-8 response seen with whole *E. coli* isolates. Significant differences from basal IL-8 release for all cell lines *$P < 0.001$ (ANOVA). D: Assessment of flagellin transcytosis in polarized T84 model epithelia colonized on the apical aspect with 10^8 CFU/mL CD *E. coli* HM427. After 4 hours, although flagellin was easily detected in the apical medium, no flagellin was found in the basolateral medium (concentrated 20-fold) as analyzed by SDS-PAGE and immunoblotting with anti-flagellar serum.

IL-8 response. IL-8 release was reduced by only 36 ± 28%, from 402 ± 277 to 285 ± 228 pg IL-8/mL across all 33 *E. coli* isolates ($P < 0.0001$, ANOVA) (Fig. 6A). Treatment of HT29 cells with mucosal CD *E. coli* HM427 resulted in the activation of Ras between 1 and 5 minutes, returning to basal levels 30 minutes after infection. Peak activation occurred at 2 minutes, with a 1.8 ± 0.62 (mean ± SEM; $n = 3$) fold increase in active (GTP-bound) Ras (Fig. 6B,C). This was followed by subsequent activation of ERK1/2 within 5 minutes of infection (Fig. 6D,E). Peak activation was seen at 15 minutes, with 1.15 ± 0.05 and 1.94 ± 0.25-fold increase in phosphorylation of ERK1 (p44) and ERK2 (p42), respectively (mean ± SEM; $n = 3$). Phosphorylation of ERK1/2 was sustained for at least 60 minutes (Fig. 6D,E).

Blockade of NF-κB activation using the IκBα phosphorylation inhibitor Bay 11-7082 (10 μM for 1 h) reduced *E. coli*-induced IL-8 release by 29 ± 23%, from 349 ± 108 to 255 ± 129 pg/mL across all 33 *E. coli* isolates ($P < 0.0001$, ANOVA). A greater than 50% reduction in IL-8 release by NF-κB inhibition was only observed in response to 11/33 *E. coli* isolates. For 2 of these isolates, colon cancer *E. coli* HM288 and CD *E. coli* HM427, both hemagglutinators, coincubation with Bay 11-7082 caused >80% inhibition of IL-8 response. Pretreatment of HT29 cells for 24 hours with siRNA to down-regulate the p65 subunit of NF-κB resulted in a 48 ± 8% and 45 ± 19% reduction in IL-8 release in response to HM288 and HM427, respectively ($P < 0.001$, ANOVA; $n = 3$) (Fig. 7A,B). These studies show that the IL-8 response is largely ERK/MAPK-dependent, with relatively little input from JNK or NF-κB activation.

TABLE 2. Sequence Alignment of Flagellin (FliC) Protein Monomers from Mucosal *E. coli*

Isolate	Gene	Start	Sequence	End	Start	Sequence	End
E. coli able to induce TLR5-dependent IL-8 production[a]							
HM550	fliC	57	QTTEGALSEINNNLQRIRELTVQASTGTNSDSDLDSIQDEIKSR	100	474	TTNPLAALDDAISSIDKFRSSLGAIQNRLDSAV	507
HM387	fliC	60	QTTEGALSEINNNLQRIRELTVQASTGTNSDSDLDSIQDEIKSR	103	477	TTNPLAALDDAISSIDKFRSSLGAIQNRLDSAV	510
HM164	fliC	54	QTTEGALSEINNNLQRIRELTVQASTGTNSDSDLDSIQDEIKSR	97	481	TKDPLKALDEAISSIDKFRSSLGAIQNRLDSAV	514
HM095	fliC	54	QTAEGALSEINNNLQRVRELTVQATTGTNSDSDLSSIQDEIKSR	97	318	TQNPLALLDKAIASVDKFRSSLGAVQNRLSSAV	351
			********* * *			*****	
E. coli unable to induce TLR5-dependent IL-8 production[a]							
HM544	fliC	54	QTTEGALSEINNNLQRIRELTVQASTGTNSDSDLDSIQDEIKSR	97	434	TSDPLAALDDAISQIDKFRSSLGAVQNRLDSAV	467
HM489	fliC	61	QTTEGALSEINNNLQRIRELTVQASTGTNSDSDLDSIQDEIKSR	104	478	TTNPLAALDDAISSIDKFRSSLGAIQNRLDSAV	511
HM394	fliC	60	QTTEGALSEINNNLQRIRELTVQASTGTNSDSDLDSIQDEIKSR	103	487	TKDPLKALDEAISSIDKFRSSLGAIQNRLDSAV	520
HM358	fliC	56	QTTEGALSEINNNLQRIRELTVQASTGTNSDSDLDSIQDEIKSR	99	473	TTNPLAALDDAISSIDKFRSSLGAIQNRLDSAV	506
HM295	fliC	54	QTTEGALSEINNNLQRIRELTVQASTGTNSDSDLDSIQDEIKSR	97	481	TKDPLKALDEAISSIDKFRSSLGAIQNRLDSAV	514
			********** * *			*****	

Flagellin sequences were aligned using CLUSTAL W (1.83) multiple sequence alignment software. Shaded amino acids indicate sequence differences. Asterisks indicate equivalent amino acid residues previously determined by others to be important for TLR5 recognition.[23–25]
[a]*E. coli* evoked IL-8 release was determined in HEK293-hTLR5 cells.

IL-8 Response to Pooled Bacteria-free *E. coli* Culture Supernatant Is Inhibited by Mesalamine (5-ASA) but Not by Hydrocortisone at Therapeutically Relevant Concentrations

Mesalamine, a drug effective in the treatment of UC, is known to inhibit MAPK.[33] We assessed the effect of mesalamine on the *E. coli* IL-8 response and also the effect of hydrocortisone, a drug that is also effective in UC but that probably has its main action via inhibition of NF-κB activation. Pretreatment of HT29 cells with 5-ASA dose-dependently inhibited the IL-8 response to a bacteria-free *E. coli* culture supernatant pooled from 6 UC isolates (Fig. 8A). Maximal inhibition of IL-8 release (97 ± 1%) was seen with 20 mM 5-ASA and a plausible therapeutic concentration, 5 mM 5-ASA,[34] inhibited IL-8 release by 45 ± 17%. A similar effect was also observed in response to whole *E. coli* isolates (data not shown). Hydrocortisone also reduced the *E. coli* supernatant-induced IL-8 response but only by 49 ± 13% at 100 μM, a concentration ≈20-fold greater than that likely to occur at usual therapeutic doses[35] ($P < 0.05$ ANOVA) (Fig. 8B). At the maximal concentrations tested, 5-ASA (20 mM) and hydrocortisone (100 μM), the adenylate kinase levels released in to the culture medium was within 90%–110% of vehicle-treated control cells.

DISCUSSION

In this study we demonstrate that mucosal *E. coli* isolates from IBD and colon cancer induce an IL-8 response from HT29 colon epithelial cells that is mediated by activation of the ERK1/2 and p38 MAPK pathways but not by activation of NF-κB. This activity is inhibited by therapeutic concentrations of mesalamine but not hydrocortisone. For many of the *E. coli* isolates, similar IL-8 stimulatory activity is present in the bacteria-free culture supernatants. This is shown to be due to flagellin, shed along with OMVs, interacting with TLR5 on the epithelial cells. Some *E. coli* isolates, however, are unable to induce IL-8 release via flagellin-TLR5 interaction and for these bacteria the IL-8 response correlates with their ability to agglutinate red blood cells and to adhere to epithelial cells. The whole bacteria-mediated IL-8 response consequently tends to be greater with the more adherent *E. coli* isolated from CD and colon cancer than with the UC and control *E. coli* isolates.

This study has shown a substantial IL-8 response to *E. coli* isolated from the control groups used (IBS/sporadic polyps). It needs to be recognized that these are not completely healthy controls and that there is increasing evidence for subtle inflammatory changes in some patients with IBS.[36] There is also growing interest in the possibility that bacteria may have a role in the development of sporadic adenomas and colon cancer.[37]

Flagellin initiates the inflammatory response to several pathogenic organisms including enteropathogenic *E. coli*

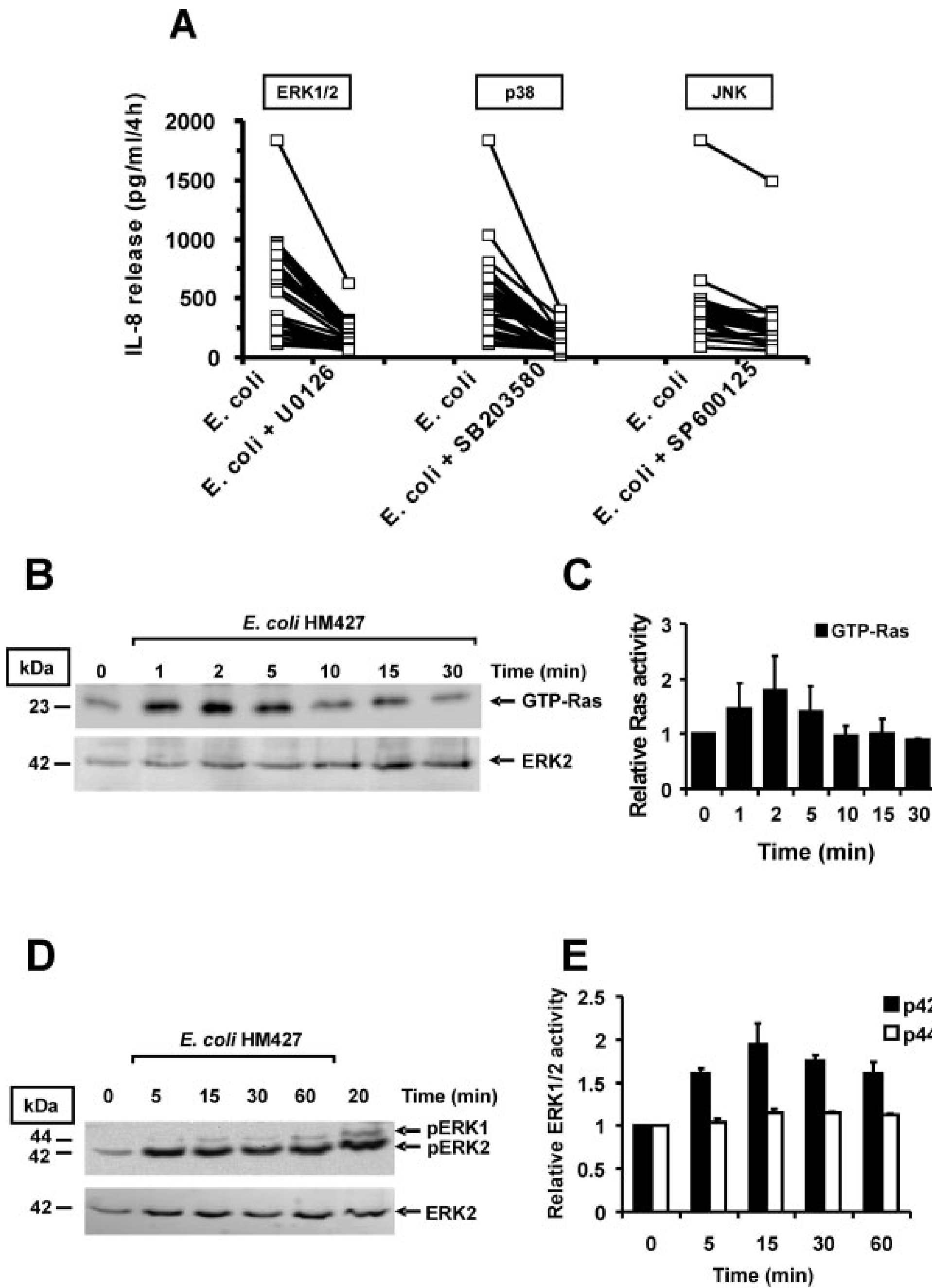

FIGURE 6. The IL-8 response to mucosal *E. coli* is mediated via the Ras-ERK1/2 and p38 MAPK pathways. A: IL-8 release in response to all 33 mucosal *E. coli* isolates was markedly reduced in the presence of inhibitors of the ERK and p38 MAPK pathway (U0126 and SB203580, respectively). In contrast, inhibition of JNK activation with 10 μM SP600125 only caused a modest reduction in IL-8 response. All $P < 0.001$ (ANOVA). B: Mucosal *E. coli* isolates cause rapid activation of Ras within 1 minute in HT29 cells, returning to basal levels 30 minutes after infection. C: Densitometric analysis of immunoblots for active (GTP-bound) Ras. Ras activation was expressed relative to control ($n = 3$ experiments; mean $\pm$ SEM). D: Subsequent phosphorylation of ERK1/2 (p44 and p42) occurred within 5 minutes of infection with *E. coli*. Peak activation was observed at 15 minutes and phosphorylation sustained for at least 60 minutes. E: Densitometric analysis of the p44/p42 immunoblots. ERK1/2 activity was expressed relative to control ($n = 3$ experiments; mean $\pm$ SEM).

(EPEC), enteroaggregative *E. coli* (EAggEC), and *Salmonella* spp and aflagellate mutants of these organisms are unable to induce an IL-8 response.[38–40] The present study illustrates that intestinal bacteria need not possess classical pathogenicity genes in order to elicit this response and also shows that epithelial IL-8 release may be promoted by such bacteria without any need for cellular invasion. This finding is consistent with previous reports of nonpathogenic *E. coli* strains capable of inducing cytokine response from explanted colonic mucosa[41] and from intestinal epithelial cells in vitro.[42]

The flagellin receptor, TLR5, is largely expressed on the basolateral aspect of intestinal epithelial cells,[43] so this raises the question of how this receptor will be accessed by the mucosal *E. coli* without cellular invasion. Although there is some evidence from human colonic epithelium[44] and murine ileal epithelium[42] of low-level expression of TLR5 on the apical surface, the healthy colonic epithelium is probably not responsive to luminal flagellar stimulation.[45] This situation changes in the presence of inflammation, however. Mice cotreated with dextran sulfate sodium (DSS) and flagellin

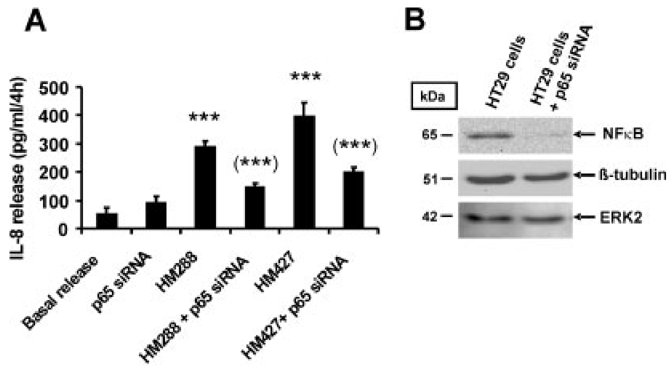

FIGURE 7. A: Downregulation of NF-κB by p65 siRNA reduced IL-8 release in response to representative CD and colon cancer mucosa-associated *E. coli* isolates HM427 and HM288 (both inhibited by >80% using Bay 11-7082). All experiments were conducted in triplicate. Significant differences from basal IL-8 release, ***$P < 0.001$; and from *E. coli*-induced IL-8 release, (***)$P < 0.001$ (ANOVA). B: Immunoblot showing downregulation of NF-κB p65 subunit by siRNA. Equal loading was confirmed using pan actin and ERK2 monoclonal antibodies. p65 downregulation was calculated to be 62 ± 18% ($n = 3$; $P < 0.0001$, Student's *t*-test).

develop a much more florid colitis than those treated with DSS alone, implying that flagellin plays an important role in the development and progression of colitis once the mucosal barrier is impaired.[45] Thus, the impaired barrier that probably exists as a result of altered tight junction structure and function in UC[46] and CD[47] is likely to be sufficient for flagellin to access basolateral TLR5 in vivo. The weakened mucus barrier in ulcerative colitis would be an additional factor.[48]

In mammals, 3 major MAPK pathways have been identified: MAPK-ERK1/2, stress-activated p38 MAPK (α, β, γ, and δ), and JNK1/2/3.[49] In most cases activation of MAPK signaling networks occurs by small G proteins such as Ras, Rac, and Rap1, but also involves other enzymes.[27] Our finding that the epithelial IL-8 response to mucosal *E. coli* isolates is mediated via Ras-ERK1/2 and p38 MAPK is consistent with the reported involvement of MAPK in the host cell responses to infection with pathogenic *E. coli* such as enterohemorrhagic *E. coli* (EHEC),[50] EPEC,[51] and EAggEC.[38] Phosphorylation of MAPK leads to downstream activation of transcription factors including NF-κB and AP-1, both of which upregulate IL-8 transcription. It has been shown that p38 MAPK, JNK1/2, and ERK1/2 are activated in the inflamed colonic mucosa of IBD patients, with the p38α isoform exhibiting the greatest increase in phosphorylation.[52] There is preliminary evidence that MAPK inhibitors may be therapeutically effective in IBD.[53]

5-ASA (mesalamine)-based compounds are used widely in the therapy of IBD and have a variety of effects that include inhibition of MAPK. They act locally at the mucosal level and efficacy is related to mucosal concentrations of the drug.[54] In vitro effects of 5-ASA include inhibition of prostaglandin and leukotriene biosynthesis,[55] proinflammatory cytokine production, and inhibition of both ERK and NF-κB pathways.[33] The antiinflammatory effect of 5-ASA has also been shown to be dependent on its interaction with the peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear receptor superfamily.[34] Some of these effects are complementary; inhibition of ERK phosphorylation, for example, will result in reduced phosphorylation and hence reduced degradation of PPARγ.[56] Here, we demonstrate that clinically and biologically relevant concentrations of 5-ASA inhibit the IL-8 release triggered by mucosal *E. coli* isolates. In contrast to 5-ASA, hydrocortisone was only able to abolish epithelial IL-8 release at 20-fold higher concentrations than are relevant clinically. This is consistent with reports that hydrocortisone only reduces cytokine levels at supratherapeutic concentrations in the respiratory epithe-

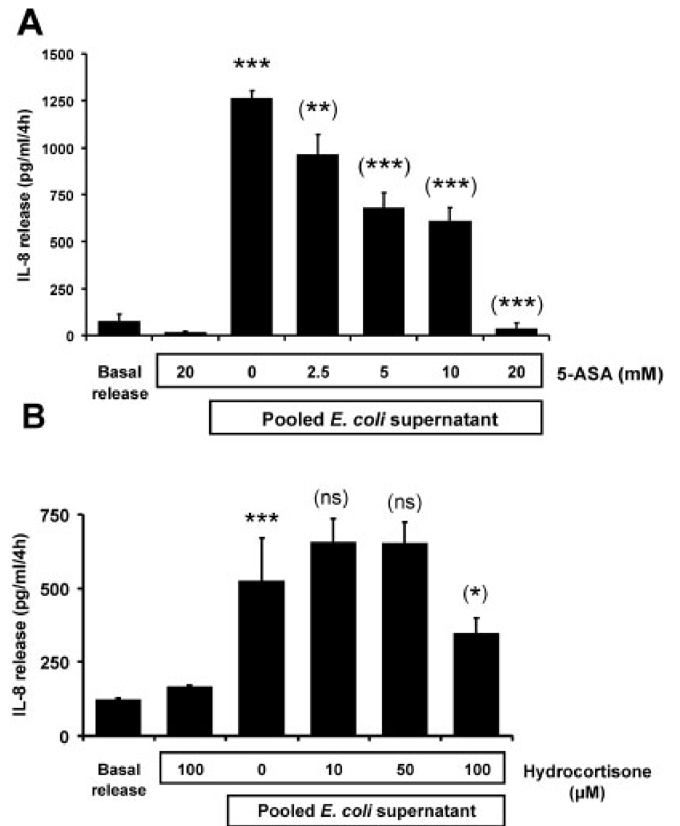

FIGURE 8. IL-8 response to pooled bacteria-free *E. coli* culture supernatant is inhibited by 5-ASA (mesalamine) but not by hydrocortisone at therapeutically relevant concentrations. A: 5-ASA dose-dependently inhibits IL-8 release in response to a pooled *E. coli* supernatant derived from 6 mucosal isolates from 5 UC patients. B: In contrast, hydrocortisone only inhibits IL-8 release in response to a pooled *E. coli* supernatant at supratherapeutic concentrations. All experiments were conducted in triplicate. Significant differences from basal IL-8 release, ***$P < 0.001$; and from *E. coli* supernatant-induced IL-8 release, (*)$P < 0.05$, (**)$P < 0.01$, and (***)$P < 0.001$ or (ns) $P > 0.05$ (ANOVA).

lium in response to coronoviral infection.[57] Thus, the epithelial cell is the main target cell for 5-ASA therapy, whereas hydrocortisone exerts its effect mainly by acting on other cells such as T cells,[58] monocytes,[59] macrophages,[60] and dendritic cells.[61] It is also consistent with the fact that inhibition of NF-κB activation, which is a principal target for corticosteroids, only had a minor effect on epithelial IL-8 response to mucosal *E. coli* in the current study.

The potential role of adhesive and invasive *E. coli* in colon cancer is intriguing. There are similarities between the biology of sporadic colon cancer and IBD-associated cancer.[37] Epithelial activation of NF-κB is critical in the development of inflammation-associated cancer, probably acting via suppression of apoptosis.[62] The adherent and adhesive mucosal *E. coli* isolates are able to invade colon cancer cell lines in vitro[4] and the present study also suggests that at least some of these bacteria are able to activate NF-κB in these colon cancer cells. It is therefore reasonable to speculate that adherence to or invasion of dysplastic epithelial cells by such *E. coli* could have an important role in determining progression from dysplasia to cancer.

Inhibition of the mechanisms that are essential for the epithelial IL-8 response to mucosa-associated *E. coli* represents a logical approach to therapy in IBD, perhaps particularly in UC, where the inflammation is more superficial and where there is little evidence of bacterial invasion.

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