Antagonism of Adherent Invasive *E. coli* LF82 With Human α-defensin 5 in the Follicle-associated Epithelium of Patients With Ileal Crohn's Disease

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Background: The first visible signs of Crohn's disease (CD) are microscopic erosions over the follicle-associated epithelium (FAE). The aim of the study was to investigate the effects of human α-defensin 5 (HD5) on adherent-invasive *Escherichia coli* LF82 translocation and HD5 secretion after LF82 exposure in an in vitro model of human FAE and in human FAE ex vivo.

Methods: An in vitro FAE-model was set up by the coculture of Raji B cells and Caco-2-cl1 cells. Ileal FAE from patients with CD and controls were mounted in Ussing chambers. The effect of HD5 on LF82 translocation was studied by LF82 exposure to the cells or tissues with or without incubation with HD5. The HD5 secretion was measured in human FAE exposed to LF82 or *Salmonella typhimurium*. The HD5 levels were evaluated by immunofluorescence, immunoblotting, and ELISA.

Results: There was an increased LF82 translocation across the FAE-model compared with Caco-2-cl1 ($P < 0.05$). Incubation of cell/tissues with HD5 before LF82 exposure reduced bacterial passage in both models. Human FAE showed increased LF82 translocation in CD compared with controls and attenuated passage after incubation with sublethal HD5 in both CD and controls ($P < 0.05$). LF82 exposure resulted in a lower HD5 secretion in CD FAE compared with controls ($P < 0.05$), whereas *Salmonella* exposure caused equal secretion on CD and controls. There were significantly lower HD5 levels in CD tissues compared with controls.

Conclusions: Sublethal HD5 reduces the ability of LF82 to translocate through FAE. The HD5 is secreted less in CD in response to LF82, despite a normal response to *Salmonella*. This further implicates the integrated role of antimicrobial factors and barrier function in CD pathogenesis.

Key Words: inflammatory bowel disease, antimicrobial peptides, barrier function

INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by intestinal inflammation and infiltration of immune cells that can affect any part of the gastrointestinal tract.[1] The etiology of the disease is not completely understood; however, it is recognized that environmental factors, genetic predisposition, microbial flora, barrier dysfunction, and immune dysregulation all play a part in the development of the disease.[2,3] Early inflammation is frequently located in the distal ileum, and the first visible signs of CD are microlesions localized in the follicle-associated epithelium (FAE) overlying the Peyer's patches.[4,5] The FAE is the interface between the intestinal lymphoid system and the luminal environment and contains about 10% of microfold or membranous (M) cells[6] that are specialized in transportation of luminal contents to underlying immune cells.[6,7] In patients with CD localized in the small intestine, the disease is associated with different pathophysiological and genetic defects that put focus on the Paneth cells.[8] Paneth cells are mainly located in the bottom of the crypts of small intestinal mucosa and are the main source of antimicrobial peptides (AMPs) that exert their antimicrobial function by damaging the

membranes of microbes.[9] Paneth cells secret AMPs such as human α-defensin 5 (HD5) and human α-defensin 6 (HD6)[10, 11] which are one of the oldest elements of the immune system[12] and play a key role in the antimicrobial defense in humans and other mammals.[13] However, HD5 and HD6 are functionally different, and we previously showed that they display differential degradation in active antimicrobial fragments.[14] Very little is known about the Paneth cells in relation to the FAE; however, Mantani et al[15] showed that in rats the relative frequency of Paneth cells was significantly less in the crypts next to the FAE compared with the crypts situated further away from the dome.

Patients with ileal CD have been shown to exhibit a reduced expression of defensins, which in turn results in a weakened ability to perform antimicrobial function.[16, 17] Mechanisms of Paneth cell defensin deficiency are complex, including genetics, epigenetics, and environmental factors like smoking.[18] Altered gut microbiota is now considered a hallmark of CD, leading to overgrowth of enterobacteria, such as *Escherichia coli*.[19, 20] A specific strain of adherent invasive *E. coli* (AIEC) has been associated with CD, specifically AIEC LF82, that has been isolated from the ileal mucosa of patients with CD.[21, 22] The AIEC strain is characterized by its ability to adhere and invade intestinal epithelial cells, and it also has the capacity to survive and replicate within infected macrophages, causing them to release large quantities of tumor necrosis factor (TNF), thus initiating inflammatory processes.[23–25] In addition, Mazzarella and colleagues[26] recently demonstrated that CD biopsies cultured with *E. coli* LF82 produces more pro-inflammatory cytokines and increases the expression of the LF82 receptor carcinoembryonic antigen-related cell-adhesion molecule 6 (CEACAM6) compared with biopsies treated with nonpathogenic *E. coli*, confirming the involvement of LF82 in CD pathogenesis.

We recently showed[27] an increased translocation of *E. coli* LF82 in FAE of patients with CD compared with controls, and translocation was dependent on bacterial long polar fimbriae and the expression of CEACAM6 on the ileal brush border. To further elucidate the mechanisms involved in the interactions between LF82 and the intestinal epithelium, the aim of the present study was to (1) evaluate the involvement of HD5 in the defense mechanism against *E. coli* LF82 in an in vitro model of FAE and ex vivo in FAE from patients with CD; (2) study the secretion of HD5 from FAE tissues of CD after exposure to LF82 and *Salmonella typhimurium* as a control pathogen; and (3) estimate the levels of HD5 in the epithelial crypts by immunofluorescence and in tissue lysates by immunoblotting.

MATERIALS AND METHODS

Antimicrobial Effect of HD5 on *E. coli* LF82

The AIEC reference strain *E. coli* LF82-expressing green fluorescent protein (GFP) was prepared and used as previously described.[27] To confirm the antimicrobial effect of HD5 on *E. coli* LF82, bacteria were cultured overnight in Luria-Bertani (LB) medium supplemented with 100 µg/mL ampicillin for a positive selection of LF82. The overnight culture was diluted 1:20 in LB medium and grown to an optical density of 0,6 (OD_{600}). The culture was centrifuged and pellet resuspended, and 1×10^6/mL of bacteria suspension was incubated with or without 1 µM or 4 µM of HD5 (PeptaNova, Sandhausen, Germany) in 37°C. These concentrations were chosen from a previous publication[28] showing that 1 µM was sublethal the *E. coli* strain American Type Culture Collection (ATCC) 25922. After 3 hours, ice-cold Phosphate-buffered saline (PBS) was added to stop the incubation, and samples were diluted to 10^{-3} and plated in duplicates on LB agar plates supplemented with 100 µg/mL of ampicillin. Plates were air dried for 30 minutes until incubated at 37°C. After 18 hours, colonies on the plates were counted, and the antimicrobial activity of HD5 was calculated by comparing the colony-forming units (CFUs) for wells containing *E. coli* LF82 with or without HD5. Experiments were run in triplicates and repeated twice. Results showed (Supplementary Fig. 1) that 1 µM and even 4 µM of HD5 had only a mild (15%) effect on LF82 survival, which is in concordance with the notion that LF82 may be more resistant to HD5 than other strains.[29] Therefore, the sublethal concentration of 1 µM was used in subsequent experiments.

In Vitro Experiments

Cell cultures

Human colorectal epithelial cells clone one (Caco-2-cl1; originally obtained from Dr. Maria Rescigno, Milan, Italy) were cultured at passages 86 to 98 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (Gibco Invitrogen Corporation, UK) at 37°C in 5% CO_2. Raji B cells (ATCC, Manassas, VA, USA) were cultured in RPMI-medium (Gibco) supplemented similar to DMEM at 37°C in 5% CO_2. Both Caco-2-cl1 and B-cells were split upon reaching confluence.

In vitro follicle-associated epithelium coculture model

For in vitro studies of bacterial translocation through the FAE, an in vitro FAE coculture model was introduced as described previously.[27] In brief, Caco-2-cl1 cells (1×10^6 cells/mL) were grown for 14 to 17 days on 3.0 µm polycarbonate filters (Costar, Baedvenhorp, NL, USA) coated with Matrigel Matrix (Corning, Kennebunk, USA) until reaching confluence. Confluence was determined by measuring transepithelial resistance (TER), and cells that reached 400–500 Ω were considered confluent. Raji B cells were added on the basolateral side to the confluent monolayers of Caco-2-cl1 cells. As controls, corresponding monocultures of Caco-2-cl1 cells (in vitro villus epithelium [VE] model) on matched filters were used. The

coculture was maintained for 4 to 6 days until M cells were generated. Transepithelial resistance was measured to confirm the transformation of epithelial cells to M-like cells. If the TER showed a decrease by at least 10%, the transformation was confirmed.[30]

To further verify the transformation to in vitro FAE model, transcytosis of live GFP-*Salmonella typhimurium* was studied. *S. typhimurium* is known to translocate from the apical to the basolateral side of M cell monolayers at an increased rate compared with Caco-2-cl1 monolayers.[30] Preparation of GFP-expressing *S. typhimurium* was done as described previously for *E. coli* HB101.[31] Passage was determined by measuring the fluorescence intensity of the basolateral media at 488 nm using Vicor³V multilabel reader (PerkinElmer, Hägersten, Sweden) and by plating samples with translocated *S. typhimurium* from the basolateral side on LB-agar (supplemented with ampicillin) overnight at 37°C, 5% CO_2, followed by a semiquantitative CFU-determination through a modified scoring system,[27] where score 1 represents the least CFUs and 5 represents the most.

Bacterial translocation in the in vitro follicle-associated epithelium and villus epithelium model

Once the transformation of Caco-2-cl1 cells to M-like cells was verified, media was changed to Hanks' balanced salt solution supplemented with 10% v/v fetal bovine serum. Transepithelial resistance was monitored before the start of the experiment at time 0. To let HD5 reach the epithelium where it normally is released, cells were pre-incubated with HD5 before adding bacteria. After the addition of bacteria, HD5 was left in the buffer to exert its effect also on the bacteria. Cells were either pretreated for 20 minutes with 1 µM of HD5 (PeptaNova) or left as control wells, with added PBS only before bacteria were added.

Cells were added 10^8 live GFP-expressing LF82, and after 3 hours, TER was measured, and the media on the basolateral side was collected. The number of bacteria that had translocated through the barrier to the basolateral side was semiquantitative, determined by fluorimetry and CFU counting as described previously. Experiments were performed in 6 to 8 independent settings, and each experiment was implemented in triplicates. Control experiments were performed with cell media only (vehicle control) to verify the absence of background fluorescence.

Bacterial viability assay

To further study the effect of HD5 on viability/motility of *E. coli* LF82, experiments were set up to determine the number of viable bacteria with and without treatment of HD5 based on quantification of adenosine triphosphate (ATP). In addition, LF82 passage through the cells was measured simultaneously by fluorimetry for comparisons of viability vs passage.

In brief, cells were cultured as described previously. After 20 minutes of pretreatment with HD5 or PBS, cells were added 10^8 live GFP-expressing LF82, and after 3 hours, the media on the basolateral side was collected. The number of passing bacteria was determined by fluorimetry as described previously, and ATP from bacteria was quantified using the BacTiter-Glo Microbial Cell Viability Assay (Promega Biotech AB, Nacka, Sweden) according to manufacturer's instructions. The luminescence signal of the ATP present in each basolateral sample was measured in Chameleon Multilabel Microplate Reader (Hidex Oy, Åbo, Finland). Experiments were performed in 3 independent settings, and each experiment was implemented in triplicates.

Human Tissue Experiments (Ex Vivo)

Patients, samples, and ethics

Noninflamed specimens from the terminal ileum next to the ileocaecal valve or from the neoterminal ileum in patients with previous resection were taken during surgery from a total of 20 patients with CD at the University Hospital of Linköping. Details of anti-inflammatory medication, primary or recurrent surgery, indication for surgery, Montreal classification, and pre-operative C-reactive protein for the CD patients (median age 46 years, range 20–71 years, 14 men) are given in Table 1. As non-IBD controls, macro- and microscopically normal ileal specimens were achieved from 26 patients (74 years, range 52–89 years, 13 men) during surgery for colonic cancer at the University Hospital of Linköping or at Vrinnevi Hospital, Norrköping, Sweden. The patients did not have any generalized disease, and no one had received preoperative chemo- or radiotherapy.

Ussing chamber experiments

After dissection, tissues were directly put in ice-cold oxygenated Krebs buffer, and the external muscle and myenteric plexus were stripped off the mucosa. Segments of FAE were microscopically identified[27] before being mounted on modified Ussing chambers (Harvard Apparatus Inc., Holliston, MA, USA) as previously described.[31] Briefly, the mucosal compartments were filled with 1.5 mL of cold 10 mM mannitol in Krebs buffer and the serosal compartments with 10 mM glucose in Krebs buffer and were continuously oxygenated at 37°C while circulated by gas flow. The tissues were equilibrated for 40 minutes to stabilize all conditions. After equilibration, samples were redrawn from the serosal sides, and compartments were filled with fresh Krebs buffer.

Effects of HD5 on E. coli LF82 translocation

Surgical specimens from 6 CD patients (59 years, range 45 to 70, 4 men) and 6 non-IBD controls (75 years, range 62–81 years, 3 men) were mounted in Ussing chambers, and

TABLE 1. Patient Characteristics of the 20 Patients Included During Surgery for Crohn's Disease

Age (y)	Sex	Anti-inflammatory Medication	Primary Surgery	Indication for Surgery	Montreal Classification	Pre-op p-CRP
45	M	Infliximab	Yes	Stricture	A2L1B2	<10
70	M	Low dose of steroids	Yes	Stricture	A3L1B2	<10
57	F	None	Yes	Stricture	A2L1B2	<10
47	F	None	Yes	Stricture	A2L1B2	<10
61	M	None	Yes	Stricture	A2L3B2	13
63	M	None	No	Stricture, abscess	A2L3B3	<10
42	M	None	Yes	Stricture, fistulas	A2L1B3	12
20	F	Azathioprine	Yes	Stricture, fistulas	A2L1B3	<10
38	F	None	Yes	Stricture	A2L1B2	10
67	M	5-ASA	No	Stricture	A3L1B2	<10
42	F	Purinethol	No	Stricture	A1L3B3	<10
29	M	Azathioprine, 5-ASA	No	Stricture, weight loss	A1L3B3	<10
71	F	Azathioprine	Yes	Stricture	A2L3B2	<10
28	M	Azathioprine	Yes	Stricture	A1L1B2	<10
53	M	None	No	Stricture	A2L3B3	<10
46	M	None	Yes	Stricture	A2L1B2	<10
44	M	5-ASA, infliximab	Yes	Fistula	A2L3B3	<10
29	M	Thiopurine, adalimumab	No	Fistula	A2L3B3	<10
50	M	None	No	Stricture	A2L1B3	<10
20	F	Budesonide, azathioprine, infliximab	Yes	Stricture	A1L3B2	<10

Abbreviations: 5-ASA, 5-aminosalicylic acid. Age at diagnosis, A1: <16, A2: 16–40, A3: >40. Location of disease, L1: ileum, L3: ileum and colon; behavior of disease, B2: strictures, B3: perforations. Pre-op p-CRP, pre-operative plasma-C-reactive protein.

2 FAE segments were pretreated with 1 µM of HD5 on both sides, whereas 4 other segments served as controls. After 20 minutes, 10^8 live GFP-expressing *E. coli* LF82 was added to all 4 chambers, and tissues pretreated with HD5 were further incubated in the continued presence of HD5. Studies of paracellular permeability were performed by adding 34 µCi/mL of ^{51}Chromium-EDTA (^{51}Cr-EDTA; PerkinElmer, Boston, MA, USA), mw 384 Da, to 4 chambers. Two additional chambers only received added Krebs as controls. After 120 minutes, serosal samples were collected. Bacterial translocation was measured at 488 nm in VICTOR X3 multileader plate reader and recalculated as bacteria/chamber x10^3. Blanks and basolateral samples were measured in duplicate against a standard curve. The ^{51}Cr-EDTA was measured in a gamma-reader (1282 Compugamma, LKB, Bromma, Sweden), and permeability was given as 60 to 120 minutes P_{app} (apparent permeability coefficient; cm/s × 10^{-6}). The transepithelial potential difference (PD) and the TER were continuously monitored, and after experiments, 10 µM of forskolin was added to the apical side of each chamber to verify tissue viability.

Effects of E. coli LF82 and S. typhimurium on HD5 secretion

Surgical specimens from 6 CD patients (45 years, range 38–63 years, 4 men) and 8 controls (82 years, range 72–83 years,

5 men) were used to study the influence of *E. coli* LF82 on secretion of HD5 from tissues. Segments of FAE were mounted on Ussing chambers as described previously. Two chambers each received 10^8 *E. coli* LF82 to the apical side or Krebs buffer as vehicle. As an additional control, 10^8 *S. typhimurium* was added to 2 more chambers. After 3 and 15 minutes, respectively, FAE tissues and mucosal buffers were collected and snap-frozen in liquid nitrogen before stored at −70°C.

Measurements of HD5 by ELISA

Before analysis, frozen FAE segments from Ussing chamber experiments were treated as previously described.[27] Briefly, tissues were immersed in lysis buffer and homogenized. After centrifugation, supernatants were removed, and protein concentrations were measured.

To examine HD5 levels in FAE tissue lysates and mucosal buffers from Ussing chambers, the Defensin Alpha 5, Paneth Cell Specific ELISA kit was used following manufacturer's instructions (USCNK, Cloud Clone Corp, Houston, TX, USA). Briefly, wells were precoated with primary antibody specific to HD5, and unspecific binding was blocked. Undiluted tissue lysates, mucosal buffers, positive and negative controls, and standard curve were added in duplicates. Secondary biotinylated antibody specific to HD5, avidin-conjugated streptavidin-horseradish peroxidase, tetramethylbenzidine enzyme substrate, and HCl were added.

Absorbance at 450 nm was measured in VERSAmax Tunable Microplate-Reader (Molecular Devices, San Jose, CA, USA) utilizing Softmax pro 5 (Molecular Devices) which generated a standard curve, from which the samples concentrations were calculated. Levels of HD5 in FAE lysates and mucosal buffers were pooled together, and results are presented as ng/mL of total HD5 secretion.

Quantification of HD5 in tissue lysates by western blotting

Tissues were identified as VE or FAE as described previously. Approximately 20 to 30 μg of frozen tissue from 8 CD patients (49 years, range 22–78 years, 4 men) and 8 controls (69 years, range 46–82, 4 men) were further treated as previously described.[27] In brief, tissues were immersed in lysis buffer, homogenized, and centrifuged. Supernatants were redrawn, and after protein concentration determination, tissue lysates were diluted to a final concentration of 1 mg protein/mL and heated for 5 minutes at 95°C.

The western blotting was performed as previously described.[27] Briefly, tissue lysates (20 μg/μL protein) were loaded on gels, separated, and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with nonfat milk for 1 hour at room temperature and then incubated overnight at 4°C with rabbit-anti-HD5 (Millipore, Solna, Sweden) 1:200 (Pierce) and mouse-anti-human β-actin (Cell Signaling, BioNordika, Sweden), 1:10,000 in 5% bovine serum albumin. Membranes were washed and incubated at room temperature with 1:20,000 secondary antibodies donkey anti-mouse Alexa Fluor 790 and donkey anti-rabbit Alexa Fluor 680 (Invitrogen, Sweden) for 1 hour. After washing, fluorescent bands were detected and quantified by Odyssey CLx and Image Studio software (LI-COR Biosciences). To compensate for variations in β-actin (which probably arises from the nature of the samples), HD5 protein levels were normalized to the β-actin loading control. Values are presented as fluorescence units.

Immunofluorescence of HD5

To identify potential variances in expressions of HD5 in the crypts of different epithelial types, paired segments of regular VE and VE adjacent to the FAE, so-called adjacent-VE, were evaluated. The adjacent-VE is defined as the VE covering the first villus next to the FAE. We previously showed[27] that the adjacent-VE to FAE is different from VE further away from the dome. For example, the cell adhesion protein and bacterial coreceptor CD9 was expressed in adjacent-VE, but villi further away from the dome had none or very low CD9 expression.

Tissue segments from 5 CD patients (42 years, range 28–67, 3 men) and 5 non-IBD controls (71 years, range 52–81, 2 men) were fixed directly after dissection and identification of epithelial types in 4% paraformaldehyde for 2 hours at room temperature. Samples were moved to a 30% solution of sucrose in PBS and then put in plastic forms containing OCT mounting medium (Histolab, Gothenburg, Sweden). Embedded tissues were cryosectioned at 7 μm (Leica Biosystems CM 3050, Kista, Sverige), blocked for 10 minutes with universal Protein Blocking Solution (Dako Sweden Ab, Solna Sweden), washed in PBS, and incubated with 1:50 primary monoclonal antibody mouse-anti-HD5 (Millipore, Solna, Sweden) overnight at 4°C in a humid chamber. After rinsing, sections were incubated for 1 hour in room temperature with 1:100 secondary antibody goat-anti-mouse IgG Alexa Fluor 488 (Thermo Scientific, Hillsboro, Oregon, USA) and mounted with ProLong Gold containing DAPI (Life Technologies, Stockholm, Sweden). Sections were incubated with secondary antibody only as negative controls. The expressions of HD5 in crypts were blindly evaluated by 2 independent researchers in a Nicon Eclipse E800 fluorescence microscope with a Nikon DS-Ri1 digital camera. In total, 20 images/section were taken and further processed using ImageJ software (National Institutes of Health). The expression of HD5 in the crypts of VE and adjacent-VE was measured by encircling the crypts in each image, and a mean fluorescence intensity was calculated by the program. From each individual, a mean value was calculated from VE and adjacent-VE, respectively. Finally, a median value was calculated for all samples.

Statistical Analysis

According to the D'Agostino-Pearson omnibus normality test, data were judged as non-normally distributed and presented as median (25–75 interquartile range). Statistical significance between 2 groups was tested by Mann-Whitney U test. Wilcoxon matched-pairs signed rank test was used for paired comparisons. Differences with $P < 0.05$ were considered as significant. Statistical analyses were performed using GraphPad's Prism software 7 (San Diego, CA, USA).

ETHICAL CONSIDERATIONS

The regional human ethics review board approved the study and all patients and controls gave their written informed consent.

RESULTS

Confirmation of Follicle-associated Epithelium Coculture Model

Measurements showed a 14.7% lower TER in the FAE coculture model compared with the Caco-2-cl1 cells and thus verified the transformation. Scanning electron microscopy further confirmed the transformation of Caco-2-cl1 cells to M-like cells, as previously described[27] (Fig. 1A).

Experiments with *S. typhimurium* confirmed the transformation to model FAE by demonstrating increased passage of *S. typhimurium* into the basolateral medium of coculture

monolayers compared with Caco-2-cl1. Fluorimetry showed a 30.3% median increased passage of *S. typhimurium* in the coculture model compared with Caco-2-cl1 (*P* < 0.05; Fig. 1B), and semiquantitative counting of CFU completed these findings by showing a 33.3% median increase of *S. typhimurium* passage in the coculture model compared with the Caco-2-cl1 (*P* < 0.05; Fig. 1C). Exposure to *S. typhimurium* significantly decreased TER compared with unexposed cells,in both Caco-2-cl1 model (34.4% median decrease in TER) and coculture (34.1%; *P* < 0.05).

FAE Coculture Model Studies (In Vitro)

No effect on TER by LF82 and HD5

Measurements of TER showed no difference in cocultures of Caco-2-cl1 and Raji B cells exposed to LF82 for 3 hours compared with unexposed filters (Table 2A). The same pattern was seen in Caco-2-cl1 monolayers (Table 2A). Incubation with only HD5 had no significant effect on TER nor had incubation of the cells with HD5 before addition of LF82 (Table 2A).
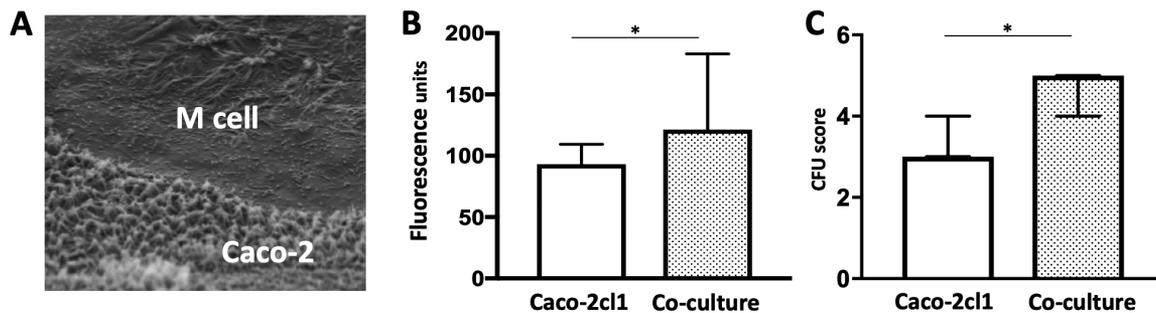

FIGURE 1. (A) Scanning electron microscopy showing areas of sparse irregular microvilli on the apical surface indicating the presence of M-like cells. Passage experiments with live gfp-expressing *Salmonella typhimurium* further confirmed the transformation to model FAE by showing increased translocation of *S. typhimurium* into the basolateral medium of coculture monolayers compared with Caco-2-cl1, measured by (B) fluorimetry, and by (C) colony forming units (CFU) determination through a semiquantitative scoring system where score 1 represents the least CFUs and score 5 represents the most, and the plates in between were scored as 2, 3, or 4. Data are presented as median (25–75 interquartile range) and statistical significance was estimated using Mann-Whitney *U* test. *P < 0.05.

TABLE 2. Effects of *E. coli* LF82 and Human α-defensin 5 (HD5) on Transepithelial Resistance in vitro (A) and ex vivo in Ussing Chambers (B)

(A) Cell Culture Model	Time Point	Vehicle	HD5	LF82	LF82 + HD5
Caco-2-cl1	0 h	610.3 (587.4–981.6)	599.5 (564.5–997.8)	605.0 (550.3–654.0)	533.5 (463.0–1015.0)
Caco-2-cl1	3 h	601.4 (535.5–998.2)	484.7 (410. –928.5)	473.3 (439.7–511.8)	434.0 (370.0–921.0)
Coculture	0 h	612.3 (498.3–997.5)	593.6 (501.0–711.4)	506.6 (450.0–560.5)	623.0 (587.0–1015.0)
Coculture	3 h	599.4 (445.2–972.6)	533.8 (522.5–716.5)	475.5 (410.3–523.5)	693.8 (621.0–776.0)

(B) Treatment	TER (Ohm × cm²)	
	Non-IBD	CD
Vehicle	81.2 (72.7–83.3)	77.3 (65.5–81.9)
HD5 control	80.5 (75.8–85.1)	78.4 (69.2–81.3)
LF82	77.5 (71.4–81.5)	71.9 (69.7–79.8)
LF82 +HD5	80.5 (72.8–84.9)	78.1 (67.8–82.9)

(A) Cells were cultured as Caco-2-cl1 monocultures or cocultures constituting of Caco2-cl1 and Raji B cells. TER was measured at 0 h and 3 hours after 20 minutes of pre-incubation and subsequent incubation with cell media only (vehicle), HD5, infection with *E. coli* LF82, or HD5 pre-incubation before LF82 infection. Values are presented as median (25–75 interquartile range) ohm x cm². Comparisons between 2 groups were done with Mann Whitney *U* test and Wilcoxon matched pairs signed rank test was used for paired comparisons. There were no significant differences in TER between any of the conditions and time points. (B) Macro- and microscopically normal ileal specimens were achieved from 6 patients during surgery for colonic cancer, as noninflammatory bowel disease controls, and from 6 patients with Crohn's disease. Segments of follicle-associated epithelium were mounted in Ussing chambers and exposed to Krebs buffer (vehicle), only HD5 as control, *E. coli* LF82, or incubation with HD5 plus addition of LF82. Comparisons between 2 groups with Mann Whitney *U* test showed no significant differences in TER between any of the conditions or time points. Values, median (25–75 interquartile range), represent recordings at 90 minutes, but there were no effects on TER at any time point.
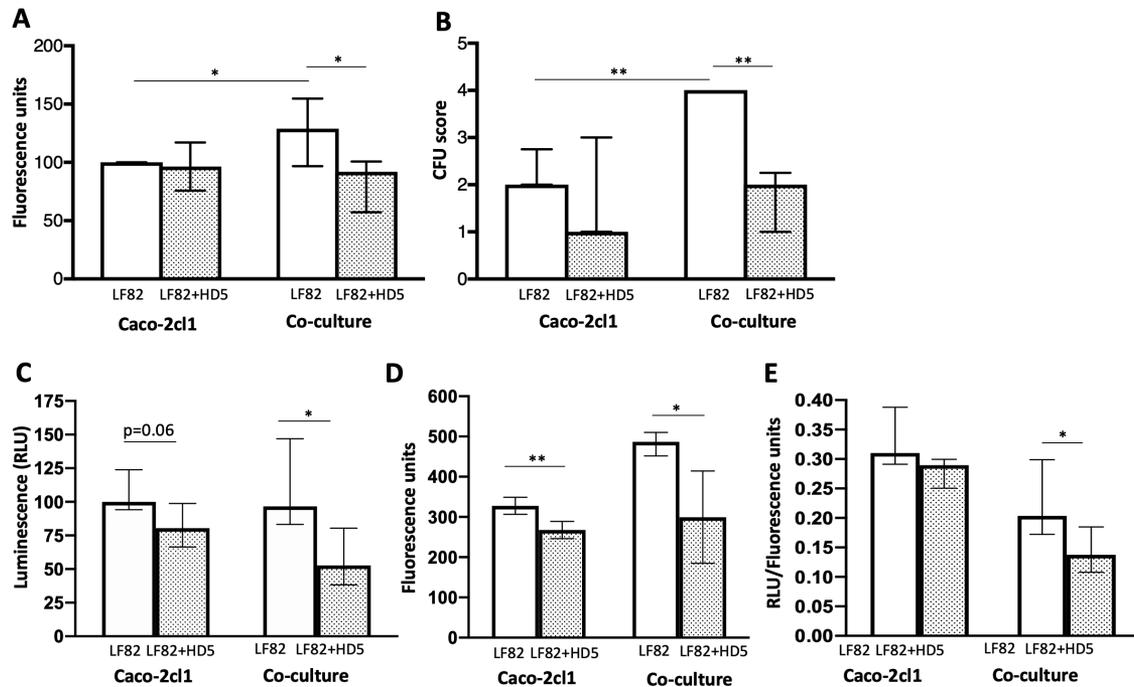
FIGURE 2. Effects of human α-defensin 5 on translocation and viability of live gfp-expressing *E. coli* LF82 in Caco-2-cl1 monoculture and follicle-associated epithelium coculture model. Cells were either exposed to LF82 only or pretreated for 20 minutes with HD5. (A) *E. coli* LF82 translocation measured by fluorimetry. (B) LF82 translocation measured (in the same basolateral samples as in A) by a semiquantitative colony forming units (CFU) scoring system where score 1 represents least CFUs and score 5 represents most, and the plates in between were scored as 2, 3 or 4. (C) Measurements of ATP content in bacteria by luminescence viability assay. (D) *E. coli* LF82 translocation measured by fluorimetry (in the same basolateral samples as in C). (E) The ratio between ATP content and translocating bacteria as an approximation of ATP content per translocated LF82. Data are presented as median (25–75 interquartile range) and statistical significance was estimated using Mann-Whitney *U* test. *$P < 0.05$. **$P < 0.005$. Abbreviation: RLU, relative light unit.

HD5 decreases LF82 translocation through FAE coculture model

The translocation of LF82 as measured by fluorescence in the in vitro model FAE was 28.9% higher compared with passage in the in vitro VE model ($P < 0.05$; Fig. 2A). Furthermore, LF82 passage was unchanged in the Caco-2-cl1 model pretreated with HD5 compared with cells that did not receive pretreatment (Fig. 2A). However, in the coculture model, HD5-treatment had a significant effect, with a decrease of 28.7% in translocation of LF82 in cocultures pretreated with HD5 compared with untreated ($P < 0.05$; Fig. 2A). There was no measurable fluorescence in vehicle control wells (data not shown).

To confirm the results obtained by the fluorimetry, basolateral media was cultured on agar plates overnight, and the numbers of colonies were semiquantified. Results confirmed the fluorimetry data, showing a 50% higher translocation of LF82 through the cocultures compared with the monoculture monolayers ($P < 0.005$; Fig. 2B). Moreover, there was a 50% decrease in the passage of LF82 through cocultures pretreated with HD5 compared with untreated cells ($P < 0.005$). In the Caco-2-cl1 monoculture, the median

of LF82 translocation after pretreatment with HD5 was numerically lower, but it was not statistically significant (Fig. 2B).

HD5 decreases LF82 viability through FAE coculture model

Experiments using the BacTiter-Glo viability assay showed a significant decrease in ATP in *E. coli* LF82 passing through the cocultures after pretreatment with HD5 ($P < 0.05$; Fig. 2C). In Caco-2-cl1 monocultures, a similar pattern was seen; however, the decrease was not statistically significant ($P = 0.06$; Fig. 2C). Fluorimetry measurements from the same basolateral samples as in Figure 2C revealed a similar pattern as shown in Figure 2A, with a significantly decreased passage of LF82 after HD5 pretreatment (Fig. 2D). Calculations of the ratio between ATP content and passage of LF82, as an approximation of ATP content per translocated LF82, indicated significantly lower levels in LF82 bacteria after passage through cocultures with HD5 treatment ($P < 0.05$; Fig. 2E). In Caco-2-cl1 monocultures, the ratio was equal with and without HD5 pretreatment of LF82 (Fig. 2E).
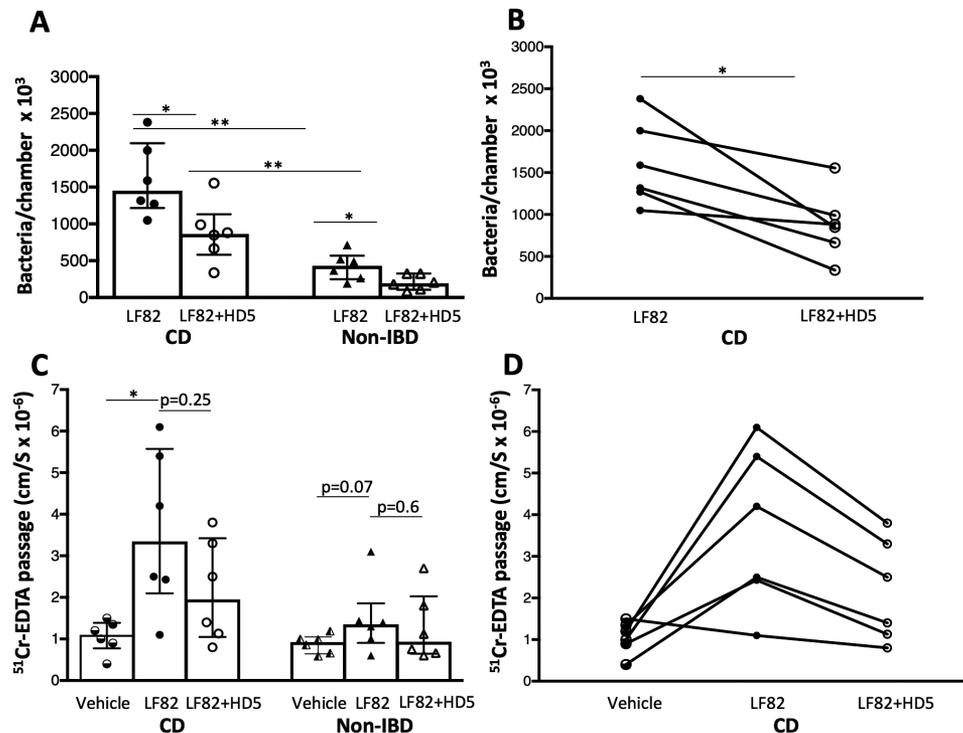
FIGURE 3. Effects of human α-defensin 5 on passage of live gfp-expressing *E. coli* LF82, and effects of *E. coli* LF82+HD5 on paracellular permeability in the follicle-associated epithelium of patients with Crohn's disease and noninflammatory bowel disease controls. (A) Segments of FAE were mounted in Ussing chambers and exposed to LF82 with or without 20 minutes pretreatment with HD5. Translocation of LF82 was measured by fluorimetry and recalculated as bacteria/chamber x10^3. (B) Individual effects on LF82 translocation by HD5 pre-incubation. (C) Segments of FAE were mounted in Ussing chambers, added [51]Chromium (Cr)-EDTA, and exposed to LF82 (or Krebs buffer only as vehicle) with or without 20 minutes of pretreatment with HD5. Permeability to ^{51}Cr-EDTA was calculated during the 60 to 120 minute period. (D) Individual effects on ^{51}Cr-EDTA permeability by HD5 pre-incubation. Data are presented as median (25–75 interquartile range), and comparisons between 2 groups were carried out using the Mann–Whitney *U* test. Comparisons of individual effects were performed using the Wilcoxon matched-pairs signed-rank test. *$P < 0.05$. **$P < 0.005$.

Human Studies (Ex Vivo)

Analyses showed that primary or recurrent surgery (ie, whether terminal or neoterminal ileum were studied) had no significant impact on the different results.

No effect on TER by LF82 and HD5

Following equilibration, PD was stable in all tissues mounted on Ussing chambers (data not shown). Addition of *E. coli* LF82 to the mucosal sides of tissues had no significant effect on TER at any time point nor did HD5 (Table 2B).

HD5 decreases LF82 translocation through human FAE

Interestingly, there was an increased translocation of *E. coli* LF82 in FAE of patients with CD compared with non-IBD controls ($P < 0.005$; Fig. 3A), which is in line with recently published data.[27] Tissues pre-incubated 20 minutes with HD5 showed a significantly lower translocation of LF82 compared with tissues not pretreated, in both CD patients

($P < 0.05$) and controls ($P < 0.05$; Fig. 3A). Figure 3B illustrates the individual effects of HD5 on LF82 translocation in CD patients using the Wilcoxon matched-pairs signed-rank test ($P < 0.05$). Serosal buffer from tissues exposed to Krebs buffer only (vehicle controls) did not show any measurable fluorescence (data not shown).

Paracellular permeability is increased by LF82, and the increase is diminished by HD5

Increased paracellular permeability to ^{51}Cr-EDTA was demonstrated in CD tissues exposed to LF82 compared with unexposed tissues ($P < 0.05$); however, a significant increase could not be seen in controls ($P = 0.07$; (Fig. 3C), which agrees with what we have shown recently.[27] The passage of ^{51}Cr-EDTA was numerically lower in tissues that were pretreated with HD5, but the effects were not significant, neither in CD nor in controls, when comparing data as groups (Fig. 3C). When comparing the effect of HD5 individually with Wilcoxon matched-pairs signed-rank test, there was a decrease in translocation of LF82 in patients with CD (Fig. 3D; $P < 0.05$) but not in controls (data not shown).
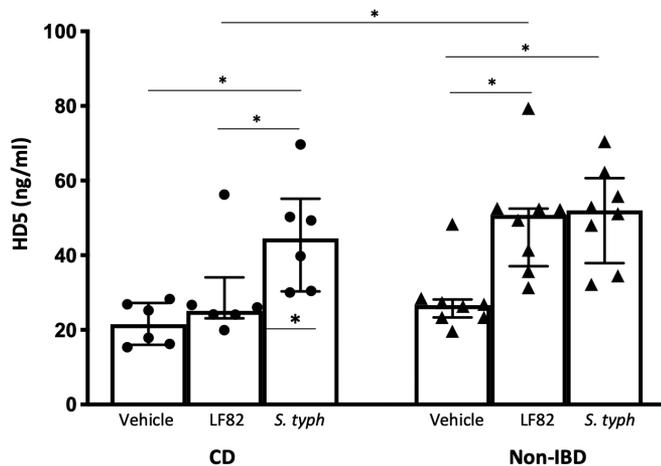
FIGURE 4. Levels of human α-defensin 5 after stimulation of tissues with *E. coli* LF82 and *Salmonella typhimurium* in Ussing chambers. Segments of follicle-associated epithelium from patients with Crohn's disease and noninflammatory bowel disease controls were mounted in Ussing chambers, and tissues were stimulated for 15 minutes with *E. coli* LF82 or *S. typhimurium* or Krebs buffer as vehicles. Secretion of HD5 was measured by ELISA. Values represent median (25–75 interquartile range). Comparisons between 2 groups were done with Mann-Whitney *U* test. *P < 0.05.

Decreased HD5 secretion in response to LF82 but not S. typhimurium in CD compared with controls

Measurements of HD5 by ELISA showed that FAE from CD secreted lower levels of HD5 after 3 minutes exposure to LF82 in Ussing chambers (22.4 (19.6–43.6) ng/mL) compared with non-IBD controls (42.7 (29.0–62.1); P = 0.06), and at 15 minutes (P < 0.05; Fig. 4). Interestingly, *S. typhimurium* exposure caused equal HD5 secretion in CD and controls, both at 3 minutes (CD, 49.7 (40.0–59.1) vs controls, 57.7 (38.0–67.5) and at 15 minutes (Fig. 4), suggesting a disturbed secretion in CD in response to *E. coli* LF82.

Further measurements showed no difference between CD exposed to vehicle or LF82, neither at 3 minutes (vehicle 18.7 (16.5–28.2) vs LF82 22.4 (19.6–43.6); P = 0.2) nor at 15 minutes (P = 0.2; Fig. 4). However, exposure to *S. typhimurium* caused an increased HD5 secretion compared with vehicle, both at 3 minutes (*S. typhimurium* 49.7 (39.9–59.1) vs vehicle 18.7 (16.5–28.2); P < 0.05) and at 15 minutes (P < 0.05; Fig. 4). In contrast to CD, non-IBD controls secreted higher levels of HD5 after 3 minutes of exposure of both LF82 (42.7 (29.0–62.1); P < 0.05) and *S. typhimurium* (57.7 (38.0–67.5); P < 0.05) compared with vehicle (26.6 (23.3–28.2)). The same pattern was seen after 15 minutes of bacterial exposure, with a significant increase of HD5 secretion both after exposure to LF82 and *S. typhimurium* (Fig. 4). Data together support that CD patients have a disturbed secretion of HD5 in response to *E. coli* LF82.

Lower expression of HD5 in the crypts and tissue lysates of FAE and VE from CD patients

Analysis of tissue lysates by western blotting revealed lower levels of HD5 in both FAE and VE of CD compared with non-IBD controls (P < 0.05; Fig. 5A). There was no significant difference between FAE and VE within the patient groups.

Immunofluorescence showed lower levels of HD5 in the crypts of patients with CD compared with controls (P < 0.05; Fig. 5B). No difference in expression levels between crypts in regular VE and VE adjacent to the FAE could be observed.

DISCUSSION

Reduced production of HD5 is a central feature of ileal CD in humans and may result in both altered mucosal antimicrobial defense[32] and variations in the microbiota composition.[33] Adherent invasive *E. coli* LF82 has been highly associated with CD,[19–21] but the relation between HD5 and LF82 in CD pathogenesis has not, to our knowledge, previously been reported.

In the present study, the increased translocation of LF82 across coculture monolayers was lowered by pretreatment with HD5, suggesting that HD5 has the ability to influence the uptake of LF82 *in vitro*. In addition, HD5 pretreatment of cocultures caused a lower ATP content in basolateral LF82, which suggests that the HD5-induced reduction of LF82 passage primarily involves effects on bacterial viability. To deepen the knowledge of the interaction between HD5 and LF82 bacteria, ex vivo studies on FAE tissue of patients with CD and non-IBD controls were carried out. A higher LF82 translocation was observed in FAE of CD patients compared with controls, and LF82 passage was significantly decreased in FAE of both CD and non-IBD controls after pretreatment with HD5 in the Ussing chambers. Likewise, the increased passage of the paracellular probe ^{51}Cr-EDTA by LF82 was diminished by the addition of HD5, showing that HD5 diminishes both the transcellular and paracellular effects of LF82. In contrast to the effect of LF82 on ^{51}Cr-EDTA permeability, TER was not affected by LF82 (or HD5) in human FAE. Most likely, this refers to diverse regulations of permeability pathways (ie, the leak and the pore pathways).[34]

Studies have shown that HD5 has the ability to kill *E. coli* MG1655 by concentrating on the cytoplasm and that it possibly interacts with DNA.[35, 36] Moreover, HD5 has the ability to permeabilize the inner membrane of *E. coli* bacteria; however, a period of 40 minutes was required to achieve membrane perturbation.[37] Rajabi et al have shown that a concentration of 1 µM of HD5 was sublethal to the *E. coli* strain ATCC 25922.[28] Moreover, Chileveru and colleagues[35] demonstrated that the oxidized form of HD5 causes morphological changes to *E. coli*, including bleb formation, cellular elongation, and clumping. In the present study, a concentration of 1 µM was used, shown to be sublethal in LF82, but still limiting the ability for the bacteria to translocate across the epithelium.
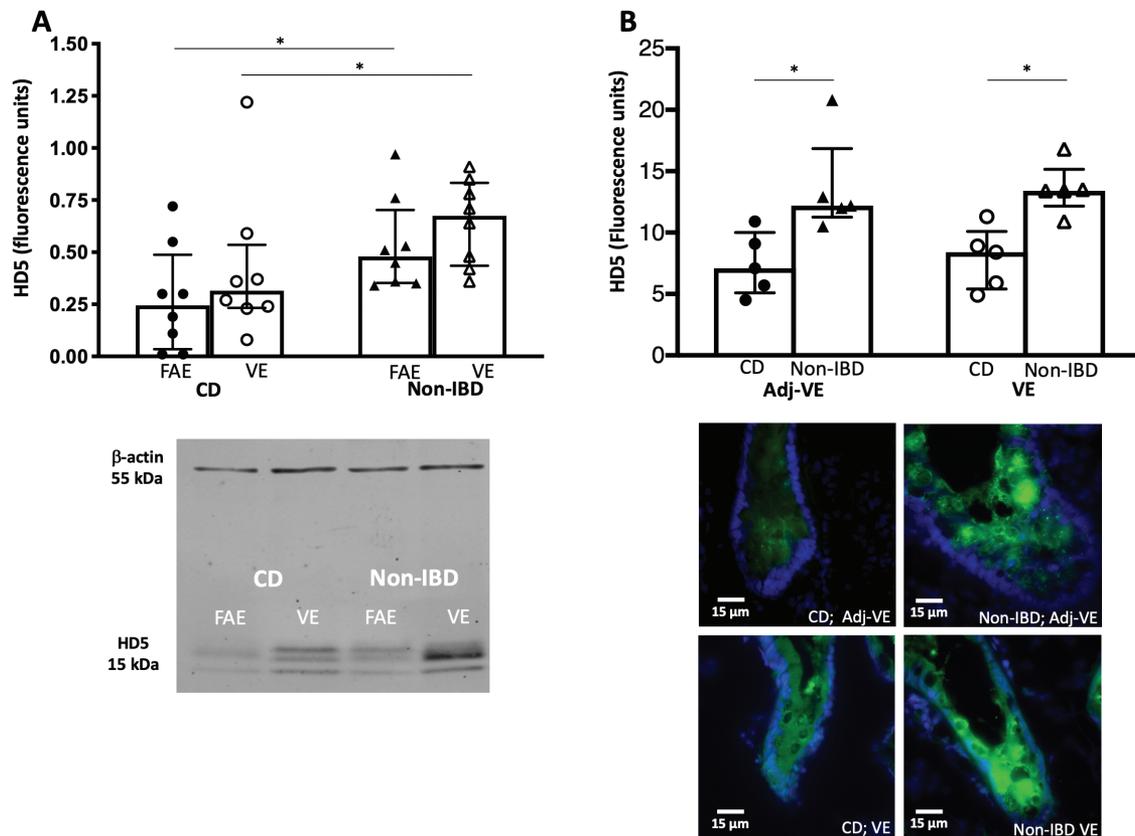
FIGURE 5. Expression of human α-defensin 5 by western blotting and immunofluorescence staining in samples from patients with Crohn's disease and noninflammatory bowel disease controls. (A) HD5 expressions analysed by western blotting in lysates of follicle-associated epithelium and villus epithelium from 8 CD patients and 8 controls. The image shows a representative blot (converted into a black and white density blot) from 1 CD and 1 control patient, respectively. Expressions of HD5 were normalized according to the β-actin loading control. (B) Immunofluorescence staining in the ileal crypts of VE adjacent to the FAE and regular VE from 5 patients with CD and 5 controls. Expression of HD5 was identified by measuring the fluorescence intensity of HD5 in the crypts using Image J software. Photographs show representative staining of HD5 (green) at 400x magnification in crypts in Adj-VE and VE from a patient with CD and non-IBD, respectively. Blue, cell nuclei stained with DAPI. Values represent median (25–75 interquartile range). Comparisons between 2 groups were done with Mann-Whitney *U* test. *$P < 0.05$.

Previous research has shown decreased expressions of HD5 in CD patients with ileal disease,[16, 17, 38] leading to a decreased antimicrobial activity in those patients and alteration of the microbial flora in the lumen.[21, 39] In the present study, we studied the secretion of HD5 after exposure to *E. coli* LF82 and showed that tissues from CD patients secreted significantly less HD5 compared with controls. In contrast, exposure to *S. typhimurium* resulted in a similar HD5 secretion response in CD and controls. These data suggest that CD patients may have a reduced mucosal defense toward AIEC, and thereby a dysfunctional immune response, as previously demonstrated in organ cultures of biopsies from patients with CD,[26] despite having a normal defensive response toward pathogens such as *S. typhimurium*. The fact that experiments with pretreatment of CD tissues with HD5 significantly decreased LF82 translocation suggests that it is mainly the patient's own ability to secrete HD5 in response to LF82 that is disturbed. However, more studies are needed to elucidate the exact mechanisms.

The mechanism for granule secretion from Paneth cells is not completely understood, although it is known that granules are immediately released when the apical surface of the epithelial cells is exposed to live bacteria,[40] and the mechanism seems to be calcium-dependent.[41] The decreased HD5 secretion in response to LF82 in CD could refer to a weakened capacity of the secretory response of Paneth cells to LF82 stimuli, but the underlying mechanisms for this disturbance need to be further investigated. One of the limitations of this study is that we focused only on HD5, because HD6, with its dual mechanism of action combining net formation[42] and antibiotic activity,[43] may be relevant as well and is also decreased in CD. However, the finding that incubation with HD5 alone is protective argues in favor of its dominant role.

In the present study, immunofluorescence revealed significantly lower expressions of HD5 in the ileal crypts of CD patients compared with the non-IBD controls, which confirms previous findings.[10, 17, 38] However, our study is first to report a

decrease in HD5 expressions in the crypts of VE adjacent to the FAE of CD patients, where important bacterial-epithelial interactions take place.[44] In addition, our data and the data from Cerrillo et al[38] involve tissues achieved from the terminal ileum. Cerrillo and colleagues[38] compared HD5 expressions in ileal biopsies from inactive and active CD patients and from healthy controls. Immunohistochemistry revealed reduced HD5 expressions in both inflamed and noninflamed mucosa compared with controls and reduced expressions in inflamed biopsies compared with noninflamed, indicating that the lower HD5 expressions seen in the noninflamed mucosa in patients with inactive disease are partially recovered. It has been hypothesized[45] that the lower levels of α defensins in CD ileum might refer only to the loss of epithelium as a consequence of inflammation. However, other Paneth cell products like lysozyme, α-1-antitrypsin, and pancreatic secretory trypsin inhibitor are not diminished, arguing against a general Paneth cell loss.[17]

The impact of inflammation on Paneth cell function is controversial,[10, 17, 45] and it is unknown whether the low expression of defensins is reversible or irreversible. The mutation in the pattern recognition receptor nucleotide-binding oligomerization domain containing 2 (NOD2) has been associated with the reduction of HD5 and 6 in ileal CD.[10] Previous studies have shown that a mutation in granule exocytosis pathway factor ATG16L1[46, 47] or in the endoplasmic reticulum stress response gene XBP1[48,49] can lead to an increased apoptosis of Paneth cells, which is associated with CD. Furthermore, increased Paneth cell autophagy and a reduced number of secretory granules in patients with CD have also been noted.[50] Our findings of reduced expressions in macroscopically noninflamed tissue support an inflammation-independent suppression of HD5 formation. It is plausible that the genetic factors mentioned previously are stable and limit defensin formation and secretion even in the absence of inflammation. However, epigenetic mechanisms may well be altered in an inflammatory environment. In future studies, it would therefore be important to investigate details of the relationship between bacterial sensing and HD5/defensin release in the ileum under different conditions, such as terminal and neoterminal ileum in CD and non-IBD controls, as well as in disease controls such as backwash ileitis in ulcerative colitis. Finally, a recent study has shown that HD5 can be used as a base to design new potent antibiotic peptides, which have shown to be effective against *E. coli* and *Staphylococcus aureus*.[37] It could be of interest to further explore the therapeutic benefits of HD5, for example, in different conditions in vitro and in animal models.

CONCLUSION

Our findings bring new insight in the effects of HD5 on the uptake of AIEC LF82, which is highly associated with CD, thus supporting previous studies showing that HD5 plays an important role in CD. Moreover, the present study deepens the knowledge of HD5 in ileal tissue of CD patients by demonstrating less HD5 secretion after LF82 exposure, despite normal reactivity to *S. typhimurium,* and decreased expressions in the crypts of both VE and VE adjacent to the FAE of CD patients compared with controls. The decrease in HD5 observed in CD patients might lead to a loss of tolerance toward invasion of luminal CD-associated AIECs such as *E. coli* LF82. Further studies are, however, needed to elucidate how HD5 can be implicated in future drug development and translated to clinical practice.

SUPPLEMENTARY DATA

Supplementary Data are available at *Inflammatory Bowel Diseases* online.

ACKNOWLEDGMENTS

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