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# Reduced Paneth cell $\alpha$ -defensins in ileal Crohn's disease

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The pathogenesis of Crohn's disease (CD), an idiopathic inflammatory bowel disease, is attributed, in part, to intestinal bacteria that may initiate and perpetuate mucosal inflammation in genetically susceptible individuals. Paneth cells (PC) are the major source of antimicrobial peptides in the small intestine, including human  $\alpha$ -defensins HD5 and HD6. We tested the hypothesis that reduced expression of PC  $\alpha$ -defensins compromises mucosal host defenses and predisposes patients to CD of the ileum. We report that patients with CD of the ileum have reduced antibacterial activity in their intestinal mucosal extracts. These specimens also showed decreased expression of PC  $\alpha$ -defensins, whereas the expression of eight other PC products either remained unchanged or increased when compared with controls. The specific decrease of  $\alpha$ -defensins was independent of the degree of inflammation in the specimens and was not observed in either CD of the colon, ulcerative colitis, or pouchitis. The functional consequence of  $\alpha$ -defensin expression levels was examined by using a transgenic mouse model, where we found changes in HD5 expression levels, comparable to those observed in CD, had a pronounced impact on the luminal microbiota. Thus, the specific deficiency of PC defensins that characterizes ileal CD may compromise innate immune defenses of the ileal mucosa and initiate and/or perpetuate this disease.

innate immunity | intestine | bacteria | inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic inflammation of the intestine often grouped into two major entities, Crohn's disease (CD) and ulcerative colitis (UC), based on clinical features and histopathology. While inflammation in UC is typically restricted to the colon, that of CD occurs at many sites, most commonly in the ileum of the small intestine and in the colon (1). In all cases, intestinal microbiota are thought to trigger the disease in genetically susceptible individuals (2). However, variations in both inherited susceptibility and clinical phenotypes suggest that neither UC nor CD is a homogeneous disorder (1, 3, 4).

Multiple lines of evidence support that genetic factors significantly contribute to the pathogenesis of IBD (2), and numerous genome-wide screens have identified several susceptibility loci, including those referred to as IBD1–8. A seminal advance came with characterization of IBD1, which revealed that approximately one-third of CD patients have loss-of-function mutations in *CARD15*, which encodes the nucleotide-binding oligomerization domain 2 (NOD2; for review, see ref. 2). NOD2/*CARD15* mutations are especially associated with localization of CD in the small intestine (for reviews, see refs. 3 and 4). NOD2 is an intracellular receptor for muramyl dipeptide, a component of bacterial peptidoglycan (5, 6), consistent with the notion that defective responses to luminal bacteria are important in CD pathogenesis.

NOD2 expression has been found in monocytes, monocyte-derived cells, and epithelial cells (5, 7). In monocytic cells, muramyl

dipeptide activation of NOD2 leads to NF- $\kappa$ B-dependent proinflammatory cytokine expression (5), and this pathway is attenuated in blood monocytes from CD patients with NOD2 mutations (6). In the intestinal mucosa, NOD2 is predominantly expressed in Paneth cells (PCs) (7), which are secretory epithelial cells of the small intestinal crypts. PCs are known to synthesize and secrete several antimicrobial peptides, including lysozyme, secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>), and human  $\alpha$ -defensins 5 and 6 (HD5 and HD6) (8–10), but the function of NOD2 in these cells is unclear. Recent studies of NOD2-deficient mice show both a reduced expression in PCs of human  $\alpha$ -defensin homologues (called cryptidins in mice) and increased susceptibility to ingested *Listeria monocytogenes*, a Gram-positive bacterial pathogen (11). It should be noted that expression of all cryptidins is not equally affected by the NOD2 deficiency in this study, consistent with the complexity of PC gene regulation that is the focus of much current study (12–14).

In addition to genetic factors in IBD, numerous studies have implicated a key role of the intestinal microbiota in disease pathogenesis, both in patients with IBD (15–20) and in rodent models of IBD (for reviews, see refs. 21 and 22). Although a specific causative pathogen seems unlikely, it appears that intestinal microbes normally present as commensal microbiota may trigger disease development in genetically susceptible hosts (23). The contribution of luminal microbes to the pathogenesis of IBD is highlighted by reports that surgical diversion of the fecal stream effectively resolves CD inflammation distal to the surgical site (15), and that in some cases antibiotics ameliorate IBD (20). In addition, the intestinal mucosa of CD patients is covered by adherent strains of *Escherichia coli* and other bacteria from the lumen, whereas these bacteria are absent from the normal small bowel mucosa (16, 18, 19). Furthermore, a loss in immunological tolerance toward luminal microbiota is observed in patients with CD (17). Thus, IBD pathogenesis likely results from a breach in effective mucosal barrier functions to constituents of the commensal microbiota (24).

PCs are key contributors to mucosal innate immunity in the small intestine by secretion of antimicrobial peptides including  $\alpha$ -defensins, cationic peptides with a broad spectrum of antimicrobial activity (25–27). PC granules are released into the intestinal lumen upon stimulation with bacterial products, including muramyl

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Abbreviations: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; NOD2, nucleotide-binding oligomerization domain 2; PC, Paneth cell; sPLA<sub>2</sub>, secretory phospholipase A<sub>2</sub>; HD5/6, human  $\alpha$ -defensins 5/6; TG, transgenic.

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dipeptide and lipopolysaccharide (28). Two murine models have underscored the functional importance of PC  $\alpha$ -defensins in innate immunity. Mice rendered deficient in their ability to process PC  $\alpha$ -defensins precursors do not produce mature  $\alpha$ -defensins peptides and are highly susceptible to challenges with orally administered bacterial pathogens (29). Similarly, a protection against a normally lethal *Salmonella* infection in HD5 transgenic (TG) mice demonstrates that this peptide has a biologically significant effect on pathogenic microbes in the intestinal lumen (30).

In view of the prominent role of defensins as mucosal antibiotics, the highly abundant expression of  $\alpha$ -defensins in PCs, the mucosal expression of the CD susceptibility gene product NOD2 in PCs, and the likely role of bacteria in IBD pathogenesis, we hypothesized that  $\alpha$ -defensins may play a central role in CD pathophysiology (10, 31). A deficiency in the innate immune defense provided by defensins might allow bacteria to adhere to the CD mucosa and trigger an inflammatory response. When we initially tested this hypothesis by comparing PC  $\alpha$ -defensin mRNA in ileal biopsies from controls with those from CD patients, but without regard for location of disease, no statistical differences in levels were observed (32). However, segregation of the CD specimens according to the clinical phenotype of either ileal or colonic involvement revealed a significant difference in defensin mRNA expression, such that only patients with ileal involvement, especially those harboring NOD2/*CARD15* mutations, had decreased ileal levels of HD5 and HD6 mRNA (32).

Herein, we report a decrease in antimicrobial activity and a specific reduction of PC  $\alpha$ -defensins peptides in CD of the ileum. The decrease of PC  $\alpha$ -defensins could not be attributed simply to a nonspecific response to inflammation in ileum and was not observed in ileal mucosa of patients with either CD limited to the colon (Crohn's colitis) or UC. We established a functional consequence of changes in PC  $\alpha$ -defensin expression by examining the composition of inherent microbiota in a HD5 TG mouse model and observed changes in the microbiota attributable to HD5 expression levels. We propose that a PC  $\alpha$ -defensin deficiency may be a key factor in the pathogenesis of ileal CD through its compromise of innate immunity. This view of disease pathophysiology would provide a justification for seeking alternative therapeutic strategies (10, 20, 33) aimed to bolster protective innate immune mechanisms and restore the host-microbe balance at the intestinal mucosa.

## Methods

**Patients and Patient Material.** Surgical specimens of ileal mucosa and pouch biopsies were obtained at the Cleveland Clinic Foundation. Ileal biopsies were obtained and processed at the Robert Bosch Hospital. The protocols were approved by the respective Institutional Review Boards at these locations. The diagnosis at both institutions was based on standard criteria using clinical, radiological, endoscopic, and histopathological findings (34). Exclusion criteria included the diagnoses of backwash ileitis, indeterminate colitis, concurrent cytomegalovirus or *Clostridium difficile* infection, CD of the pouch, chronic pouchitis, and nonsteroidal antiinflammatory drug-induced pouchitis.

**Supporting Information.** Further details are provided in *Supporting Text*, Tables 1–4, and Fig. 5, which are published as supporting information on the PNAS web site.

**Real-Time PCR.** Real-time PCR was performed by using single-stranded cDNA from tissue (or gene-specific plasmids as controls) with specific oligonucleotide primer pairs (Table 2) in a temperature cycler equipped with a fluorescence detection monitor (LightCycler, Roche Diagnostics, Mannheim, Germany), as described (32).

**Protein Isolation and Immunoblot Analysis.** Protein extracts from ileal mucosa were isolated from randomly selected controls, CD

NOD2/*CARD15* wild-type and CD NOD2/*CARD15* SNP13 patients, as described (35). Protein expression of HD5,  $\alpha$ -1-trypsin inhibitor, lysozyme, and sPLA<sub>2</sub> in the patient samples was quantified by immunoblotting, as described (36).

**Antimicrobial Activity in Ileal Mucosal Biopsies.** Cationic proteins from ileal mucosal biopsies were isolated by using a weak cation exchange matrix, as described (35). Assays were normalized to protein concentration, as determined by Bradford assay. Midlogarithmic growth phase suspensions of *E. coli* (American Type Culture Collection 25922) and *Staphylococcus aureus* (American Type Culture Collection 25923) were incubated with the cationic protein fraction at 37°C in a final volume of 100  $\mu$ l of 1:6 diluted Schaedler Broth (BD Biosciences, Sparks, CA) (37). Bacterial suspensions incubated with vehicle (0.01% acetic acid) served as negative controls. After 120 min, bis-(1,3-dibutylbarbituric acid)-trimethine oxonol Molecular Probes), a dye sensitive to membrane potential, was added at a concentration of 1  $\mu$ g/ml. Bacterial pellets were isolated by centrifugation, resuspended in 300  $\mu$ l of FACS-Flow (BD Biosciences) and analyzed by flow cytometry by using a FACSCalibur (BD Biosciences). A total of 30,000 events were analyzed in each sample. The antimicrobial activity was determined as percentage of depolarized bacteria compared with untreated controls (37).

**Histologic Analyses.** HD5 immunohistochemistry and histologic staining were performed in parallel sections. Immunohistochemistry on ileal tissue was performed as described (38) and phloxine tartrazine histologic staining as described (39). Hematoxylin/eosin-stained paraffin sections from CD and non-IBD controls were blindly scored for inflammation by a gastrointestinal pathologist (R.E.P.).

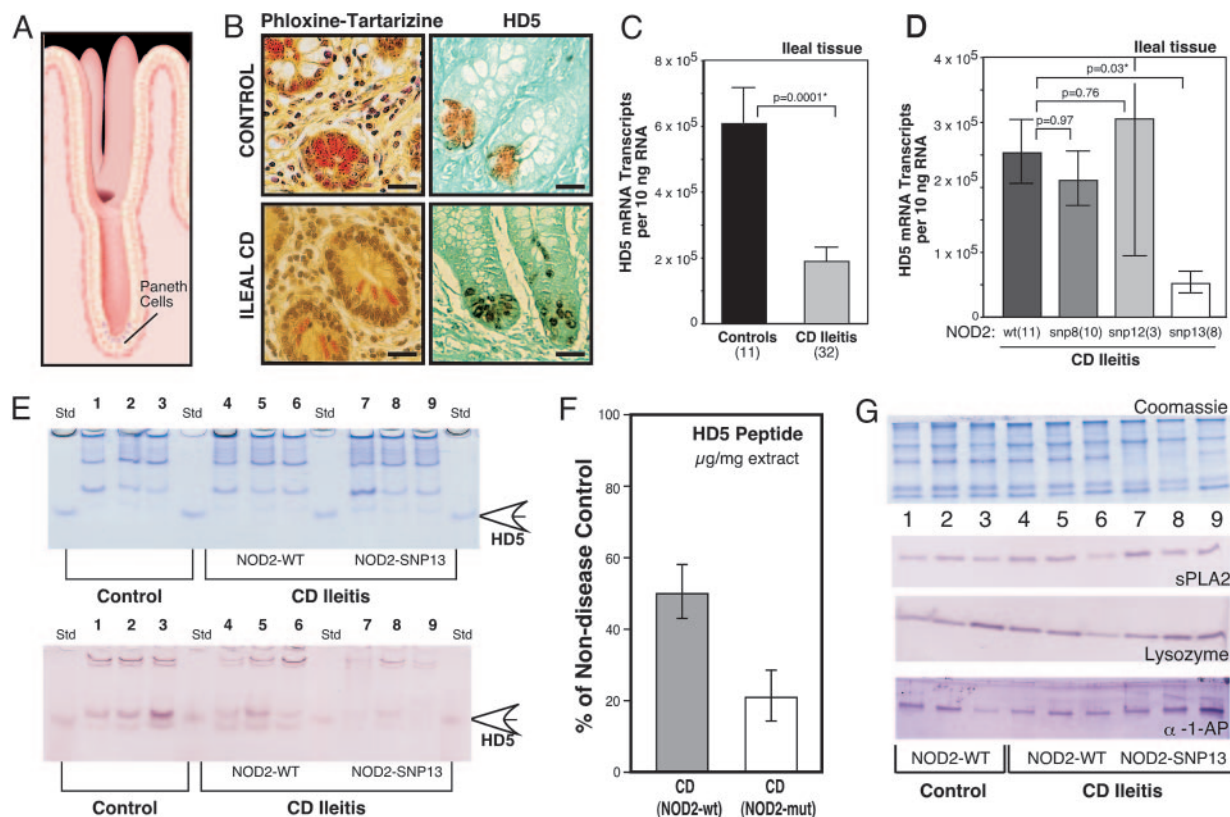
**Analysis of HD5 TG Mice.** Bacterial microbiota was examined in an HD5 TG mouse model (30). All animal studies were approved by the involved Institutional Animal Care and Use Committees. *In situ* hybridization on representative samples of HD5 TG mice and human small intestine was performed as described (30). Intestinal bacteria from the mouse intestine were fixed and analyzed as described (40). Briefly, an aliquot of fixed bacteria was hybridized to a Texas red-labeled Bact338 oligonucleotide probe (an oligonucleotide sequence common to all bacteria). The bacteria were then washed and mounted for viewing under oil by using an epifluorescence photomicroscope. Fluorescent images were captured by using METAMORPH software (Universal Imaging, Downingtown, PA).

**Statistics.** All statistical analyses of quantitative RT-PCR grouped data were performed nonparametrically by using the *U* test of Wilcoxon, Mann, and Whitney. Values of  $P < 0.05$  were considered statistically significant. For illustration, mean values are presented together with their standard error. HD5 protein expression data were subjected to *t* test and ANOVA analysis by using SIGMASTAT software, Ver. 2.0 (SPSS, Chicago).

## Results

We measured PC antimicrobials and other PC products in the ileal mucosa of four groups: controls, UC patients, CD patients with solely colonic disease (colitis), and CD patients with ileal disease (ileitis). PCs are located at the base of the crypts of Lieberkühn (Fig. 1A), and cross sections of these crypts show prominent eosinophilic PC granules (Fig. 1B), known to be rich in antimicrobial peptides (9), including HD5 (Fig. 1B). In surgical resection specimens from CD ileitis patients, the ileal expression of HD5 (Fig. 1C) and HD6 mRNA (Fig. 5) was significantly reduced compared with non-IBD controls. Similar decreases were observed in endoscopic biopsy specimens from CD ileitis patients versus controls (data not shown). In contrast, PC defensin expression in ileal biopsies was unchanged in patients with either colonic CD (32) or UC (E.F.S., unpublished





**Fig. 1.** PC  $\alpha$ -defensins in controls and IBD patients. (A) Illustration of the position of PCs at the base of the crypt of Lieberkühn in the small intestinal mucosa (illustration by David R. Schumick Illustration, Elyria, OH). (B) Phloxine tartrazine staining of small intestinal ileal mucosa (Left), showing antimicrobial peptide-rich granules. Immunohistochemical localization of the PC  $\alpha$ -defensin HD5 (Right). (Upper) Normal control; (Lower) CD. (Scale bars, 25  $\mu$ m.) (C) Expression of HD5 mRNA in surgical specimens from controls and patients with ileal CD. The mRNA copy number per 10 ng of total RNA was determined with quantitative real-time RT-PCR using external standards. [Scale bars represent means ( $\pm$  standard error).] The significance values are based on the Mann-Whitney test (\*,  $P < 0.05$ ). (D) Expression of HD5 in patients with ileal CD with respect to NOD2/CARD15 genotype. Note that compared with non-IBD controls, all NOD2/CARD15 genotype subgroups (wild type and mutated) of ileal CD presented here have significantly reduced HD5 mRNA levels, as shown in C. Data expressed and analyzed as in C. (E) (Upper) Coomassie blue-stained SDS gel containing protein extracts (12  $\mu$ g per lane) from ileal mucosa of controls (lanes 1–3) and ileal CD patients (lanes 4–9). NOD2/CARD15 genotype analysis detected SNP-13 mutation in ileal CD patients (lanes 7–9); wild-type sequence was found in other samples (lanes 1–6). Standards are recombinant proHD5 (open arrow). (Lower) Immunoblot analysis of HD5 peptide in ileal tissue samples. Blot using HD5 antibody (36, 39) was from replicate gel as in A with 0.6  $\mu$ g per lane protein loading. The difference in mean values between controls (lanes 1–3) and ileal CD specimens (lanes 4–9) was significant by *t* test analysis ( $P = 0.038$ ). (F) Quantification of HD5 peptide in ileal tissue samples. Scale bars represent the percentage ( $\pm$  standard error) of HD5 peptide amounts in CD specimens as compared with nondisease control samples, which was set as 100%. HD5 peptide concentrations in ileal samples were determined by quantitative comparison of immunoblot bands from tissue to serial dilutions of recombinant HD5 peptide on the same gel/membrane (data not shown). Signal was quantified by using a VersaDoc 1000 BioRad imaging system. (G) Immunoblot blot analysis of sPLA<sub>2</sub>, lysozyme, and  $\alpha$ -1-antiprotease. (Upper) Coomassie blue staining of an SDS-Tricine PAGE gel (12  $\mu$ g/lane), as described in E. (Lower) Analysis from replicate gel as in E with 1.2  $\mu$ g per lane protein loading with sPLA<sub>2</sub>, lysozyme, and  $\alpha$ -1-antiprotease antibodies.

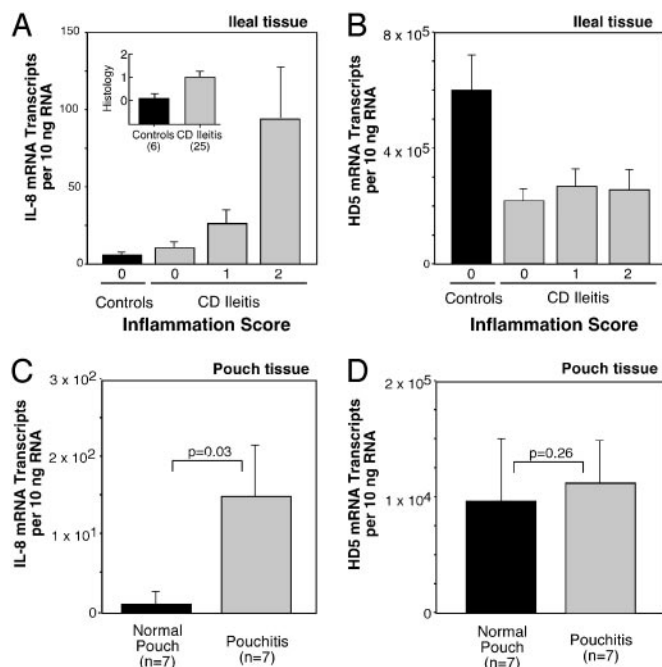
data). The ileal mRNA expression of two other PC antimicrobials (lysozyme and sPLA<sub>2</sub>) and five other PC products [ $\alpha$ -1-antitrypsin, pancreatic secretory trypsin inhibitor, hepatoma-specific protein/pancreatitis associated protein, trypsin 2 (anionic trypsin), and trypsin 3 (mesotrypsin)] was not significantly changed in CD ileitis patients versus controls (Fig. 5), whereas pancreatic stone protein was increased (Fig. 5).

Because CD patients with NOD2/CARD15 mutations are predisposed to ileal involvement (3, 4), we analyzed the expression of the ileal PC products in samples with different NOD2/CARD15 genotypes at three loci (SNP8, SNP12, and SNP13). The ileal levels of HD5 mRNA were comparably low in ileal CD either with SNP8, SNP12, or wild-type NOD2/CARD15 genotypes as compared with non-IBD controls (Fig. 1C vs. D), with a consistent 3-fold reduction in each group. In contrast, patients with NOD2/CARD15 SNP13 mutations had further reduced expression levels of HD5 mRNA versus the other genotypes ( $P = 0.03$ ), resulting in a 9-fold difference compared with non-IBD controls ( $P = 0.0005$ ). HD6 expression in CD ileitis was similarly reduced to  $\approx$ 5-fold lower than

non-IBD controls ( $P = 0.018$ ), but the difference within the CD group (between wild-type and SNP13 NOD2/CARD15 genotypes) was not statistically significant ( $P = 0.15$ ). None of the other PC products showed significant differences in expression levels when comparing subgroups of NOD2/CARD15 genotypes (Table 3).

Compared with non-IBD controls, Western blot analysis of mucosal tissue extracts (Fig. 1E) showed less HD5 peptide in patients with ileal CD ( $P = 0.038$ ). Along with reductions of HD5 mRNA (Fig. 1C and D), tissue concentrations of HD5 peptide are reduced in ileal CD with wild-type NOD2/CARD15, but even further diminished in case of a SNP13 mutation (Fig. 1E and F). In contrast to HD5, Western blot analysis of  $\alpha$ -1-antitrypsin, lysozyme, and sPLA<sub>2</sub> showed no decrease in the ileal CD samples as compared with non-IBD controls (Fig. 1G). Immunohistochemistry in nine representative samples (three controls and six ileal CD) identified PCs as the source of HD5 (as shown in Fig. 1B).

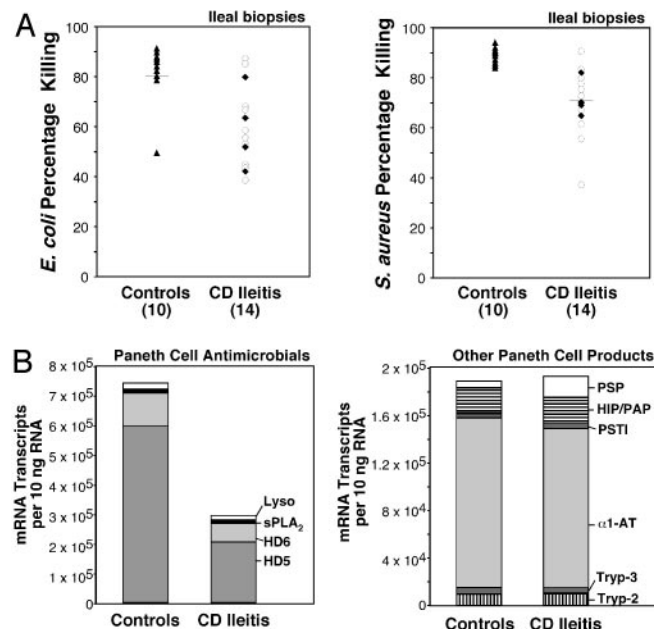
To test whether the observed decrease in PC  $\alpha$ -defensins is a direct consequence of inflammation, we examined HD5 and HD6 expression levels with respect to mucosal inflammation. Histologic



**Fig. 2.** Mucosal inflammation and expression of PC defensin HD5. (A) Correlation of mucosal inflammation and the proinflammatory cytokine IL-8 mRNA levels in ileal CD and non-IBD controls. Hematoxylin/eosin-stained paraffin sections from specimens were assessed for mucosal inflammation by a gastrointestinal pathologist who was uninformed about samples [no inflammation (0), moderate (1), and severe (2)]. IL-8 mRNA expression levels, expressed as mRNA copies per 10 ng of RNA [means ( $\pm$  standard error)] shown for each group. *Inset* shows the average inflammation score for controls ( $n = 8$ ) and ileal CD patients ( $n = 25$ ). (B) Expression of HD5 mRNA in specimens from non-IBD controls and patients with ileal CD grouped as in A. Data expressed as mRNA copy number per 10 ng of RNA [means ( $\pm$  standard error)], from same specimens as analyzed in Fig. 1C. (C) Expression of IL-8 mRNA in specimens from normal and inflamed ileal pouches. Biopsy specimens were from normal ( $n = 7$ ) and inflamed pouch mucosa (pouchitis,  $n = 7$ ). IL-8 mRNA expression levels are expressed as in A. The significance values based on the Mann-Whitney test (\*,  $P < 0.05$ ). (D) Expression of HD5 mRNA in the same specimens as in C. A similar pattern was observed for HD6 (data not shown).

assessment of the degree of inflammation by a gastrointestinal pathologist (R.E.P.) showed clear correlation with the expression of the proinflammatory cytokine IL-8 (Fig. 2A), an indicator of mucosal inflammation. As compared with non-IBD controls, the levels of HD5 mRNA were similarly decreased in all patients with ileal CD, irrespective of whether the mucosal inflammation was absent, moderate, or severe (Fig. 2B). Similarly, HD6 showed the same pattern of decrease, independent of inflammation status (data not shown). We next examined the concentrations of PC  $\alpha$ -defensin mRNA in mucosal biopsies of ileal pouches, which are ileal stool reservoirs surgically constructed after the total removal of the colon. The ileal pouch mucosa occasionally becomes inflamed, a condition called pouchitis (41). PC  $\alpha$ -defensin mRNA did not show significant changes in pouchitis specimens compared with those from noninflamed pouches (Fig. 2C and D), thus showing that this inflammation does not affect expression levels.

In ileal CD compared with non-IBD controls, we found reduced total antimicrobial activity against *E. coli* and *S. aureus* in ileal mucosal biopsies (Fig. 3A). The magnitude of the reduced activity paralleled the lower levels of PC antimicrobial peptide expression in CD ileitis (Fig. 3B Left), which is principally attributable to the reduction in HD5 expression. We were not able to unequivocally attribute the reduced antimicrobial activity in mucosal biopsies observed in these assays to HD5 content, however, because cur-



**Fig. 3.** Quantitative analysis of antimicrobial activity and PC mRNA transcript copy numbers in ileal mucosa. (A) Antimicrobial activity in mucosal biopsies of ileal CD and non-IBD controls. Protein extracts were incubated with cultures of either *E. coli* (Left) or *S. aureus* (Right), and bacterial killing was assessed by using a flow cytometric assay. Biopsies from ileal CD (all NOD2 wild type) were obtained from either macroscopically inflamed (open circles) or uninfamed (filled diamonds) regions of the ileum. (B) Expression levels of four PC antimicrobials (on the left) and six nonantimicrobial PC products in ileal specimens of controls and ileal CD. Data are compiled from experiments described in Figs. 1 and 5 and are expressed as mRNA copy number per 10 ng of RNA.

rently available antibodies do not neutralize HD5 activity (data not shown).

In view of recently reported locus polymorphisms that result in variable numbers of defensin genes per genome (42, 43), we sought to determine whether the numbers of HD5 or HD6 genes were reduced in any of 20 CD patients (NOD2/*CARD15* wild-type,  $n = 10$ ; and NOD2/*CARD15* SNP13 mutation,  $n = 10$ ), who showed diminished defensin levels. The analysis showed two gene copies per diploid genome for both  $\alpha$ -defensins in all tested CD patients, identical to what was seen in non-IBD controls ( $n = 10$ ) (data not shown).

To study possible *in vivo* consequences of the changes in PC  $\alpha$ -defensin expression levels we observed in ileal CD, we turned to our recently described murine HD5 TG model (30). We reasoned that by comparing heterozygous and homozygous HD5 TG mice, we could test whether a 2-fold difference in HD5 expression, similar to that observed in Crohn's ileitis versus controls ( $\approx 3$ -fold), had a biological effect on luminal microbes. The TG mice express HD5 in mouse PCs (Fig. 4A) at levels comparable to those of human PCs (Fig. 4B), while not appearing to alter the expression of endogenous mouse PC defensins (30). Luminal microbiota obtained from the small intestines of wild-type, heterozygous, and homozygous HD5 TG mice were labeled with a fluorescent oligonucleotide probe designed to hybridize to all known bacterial species (TR-Bact 338, ref. 40). Morphologically, we observed a graded transition in the composition of bacterial microbiota, from predominantly small bacilli and cocci in the wild-type mice, to a mixed population of bacilli and fusiform bacterial species in the heterozygous TG mice, and finally a population of predominantly fusiform bacteria in the homozygous TG mice (Fig. 4C). These findings, together with unpublished studies (N.H.S., unpublished observations), suggest that the composition of the commensal bacterial population in the







## **SUPPLEMENTARY MATERIAL**

### ***Methods***

#### **Surgical Samples**

Surgically-resected ileal specimens were obtained from individuals undergoing surgery for either CD, or, for controls, cancer, bowel obstruction, or other non-inflammatory conditions. These samples were obtained through the Cooperative Human Tissue Network (funded by the National Cancer Institute). Immediately after surgery, ileal mucosa was separated from the adjacent tissue as described (36), snap frozen in liquid nitrogen, and stored at -80°C. A portion of each specimen was processed for routine histologic evaluation. Ileal surgical specimens were obtained from a total number of 11 controls with normal NOD2/*CARD15* genotype and 60 CD patients. After isolation of DNA from a portion of ileal tissue and determination of the NOD2/*CARD15* status (32), we selected 32 CD specimens for further study (11-NOD2/*CARD15* wt, 8-NOD2/*CARD15* SNP13, 3-NOD2/*CARD15* SNP12, 10-NOD2/*CARD15* SNP8; Suppl. Table 1).

#### **Biopsy Specimens**

For study of specimens from a population that was not pre-selected by a required surgical intervention, we studied ileal biopsies from controls (n=7), CD patients with (n= 14) and without (n=7) macroscopic ileal involvement, and ulcerative colitis patients (n=10). Control and CD samples were randomly selected from a group of samples that were used in a previous study (32). Ileal pouch biopsy specimens were taken from patients whose indication for prior colectomy was UC. Samples were taken from inflamed (n=7) and non-inflamed (n=7) pouches, diagnosed according to published criteria (42).



### **Protein isolation and Immunoblot analysis**

Briefly, for each specimen equal amounts of protein extract (ranging from 0.6 – 12 µg/lane depending on the experiment), or standard (recombinant HD5 peptide and human lysozyme previously purified,  $\tilde{\alpha}$ -1-antitrypsin from Sigma, Saint Louis, MO, or molecular weight marker for sPLA<sub>2</sub>) were subjected to AU-PAGE or SDS-Tricine PAGE, blotted onto Millipore PSQ membrane (Millipore Corporation, Bedford, MA) using a semidry transfer protocol, and fixed to the membrane with 0.05% glutaraldehyde in Tris buffered saline (TBS) for 30 min. After blocking in 0.75 % nonfat milk in PBS for 30 min, the membrane was incubated with polyclonal rabbit sera against HD5 (at 1:1000; (39)), human  $\tilde{\alpha}$ -1-antitrypsin (1:100; Sigma), human lysozyme (1:500, DakoCytomation, Carpinteria, CA) or sPLA<sub>2</sub> (1:1000, Cayman Chemical, Ann Arbor, MI) in 0.25% non-fat milk powder in PBS supplemented with 0.01% thiomerosal for 18 hours, washed with 0.1% BSA in TBS, incubated for 1 hour with alkaline phosphatase-conjugated goat anti-rabbit IgG (at 1:2000, Immunopure, Pierce, Rockford, IL), washed as above, immersed for 5 minutes in TBS, and then developed with NBT/ BIPD (Research Products Inc., Mt. Prospect, IL). Resulting membranes were scanned and quantified using the VersaDoc 1000 imaging system, and Quantum One software (Bio-Rad Laboratories, Hercules, CA). HD5 peptide concentrations in patient samples were determined by comparison to a standard curve generated from serial dilutions of HD5 and data were graphed using SigmaPlot version 7.0

### **HD5 and HD6 DNA Copy Number Analysis by Quantitative Real-Time PCR**

To determine the number of HD5 and HD6 genes per diploid genome, a Bio-Rad iCycler Thermal Cycler instrument in the 96-well plate format was used for quantitative real-time PCR analysis. The PCR reaction conditions were 1X iQ SYBR Green Supermix (Bio-Rad), 250nM each primer, and genomic DNA at 4, 2 or 1 ng. The PCR cycling parameters were 1 cycle 95°C for 3 min, 40 cycles 95°C for 30 sec and 54°C for 30 sec, and 1 cycle 95°C for 1 min, followed by a melt curve initiating at 55°C, and increasing in 0.5°C increments for 80 repetitions. For each experiment, data points were run in triplicates, three different DNA concentrations were used, and two reference gene primer sets (Suppl. Table 4) were included. In addition, three primer sets, annealing to different areas of the target gene HD5, were used (Suppl. Table 4). Threshold cycle was plotted against the log of DNA concentration using a scatter plot, and the slope of the line is the efficiency of the PCR reaction. The efficiency of the PCR reactions for the different primer sets were similar to those of the target defensin genes, and reference genes allowing a valid computation of  $\alpha$ -defensin gene copy number. Defensin gene copy numbers were calculated by first subtracting the threshold cycle number for the defensin gene from that of the average number of the reference genes cycle number. From the difference in threshold cycle,  $\Delta C_t$ , gene copy number per diploid genome was calculated using the formula: copy number =  $2^{(\Delta C_t) * 2}$ . Reported gene copy number is the average of the results obtained for three different DNA concentrations from each sample.

**Supplementary Table 1. NOD2/*CARD15* genotypes from patients with ileal Crohn's disease.**

	<b>SNP8</b>	<b>SNP12</b>	<b>SNP13</b>
<b>Ileal CD (n=60)</b>	<b>9 heterozygous</b>	<b>3 heterozygous</b>	<b>7 heterozygous</b>
	<b>1 homozygous</b>	<b>0 homozygous</b>	<b>1 homozygous</b>



**Supplementary Table 2.** Oligonucleotide sequences used in quantitative real-time RT-PCR analysis.

	Sense	Antisense	T (°C)
HD5	5'GCCATCCTTGCTGCCATT 3'	5'GCTTCTGGGTTGTAGCCTCATC 3'	60° C
HD6	5'CCTATGGGACCTGCACTGTCATGG 3'	5'GATGGCAATGTATGGGACACACGAC 3'	58° C
sPLA <sub>2</sub>	5'CGCACTCAGTTATGGCTTCTACG 3'	5'AGGTGATTCTGCTCCCCGAG 3'	58° C
Lysozyme	5'AAAACCCCAGGAGCAGTTAAT 3'	5'CAACCCTCTTTGCACAAGCT 3'	60° C
HIP/PAP	5' CCCACTGCTATGCCTTGTTTTTGTG 3'	5' ACTGCTACTCCACTCCCAACCTTCTC 3'	60° C
PSP	5' AAGCATGGCTCAGACCAACTCGTTC 3'	5' GCGGTTCTTTTTTGGGTCATGGAG 3'	56° C
Trypsin 2	5' AACAGCCGGACTCTGGACAA 3'	5' GACACGCGGGAATTGATGAC 3'	60° C
Trypsin 3	5' CCACCCTAAATACAACAGGGACAC 3'	5' TGGACACGCGGGCATT 3'	60° C
PSTI	5' GACGCAGAACTTCAGCCATGA 3'	5' TCTTGGTGCATCCATTAAGTTCA 3'	60° C
$\alpha$ -1-antitrypsin	5' CTCTCCAAGGCCGTGCAT 3'	5' GGATAGACATGGGTATGGCCTCTA 3'	60° C
IL-8	5' ATGACTTCCAAGCTGGCCGTGGC 3'	5' TCTCAGCCCTCTTCAAAAACTTC 3'	60° C
GAPDH	5' TGACCACAGTCCATGCCATCACTG 3'	5' ATGACCTTGCCCACAGCCTTGG 3'	60° C

**Supplementary Table 3. PC mRNA levels in patients with ileal CD and non-IBD controls.** Mean count of mRNA copies per 10ng mRNA (SEM). Samples are from the same specimens as in Figures 1C and 1D. Grey box designates segregated NOD2 genotype subgroups of ileitis specimens. The significance values, based on the Mann Whitney test, are shown (\* significant,  $p < 0.005$ , and † not significant). p-values were subject to Bonferroni's adjustment for multiple testing.

PC gene products	Controls  n=11	CD ileitis				
		Total n=32	NOD2 wt n=11	SNP8 n=10	SNP12 n=3	SNP13 n=8
<b>sPLA-2</b>	4,258 (1,134)	2,964 † (769)	2,834 (811)	2,357 (756)	5,965 (4,700)	2,776 (2,308)
<b>Lysozyme</b>	10,625 (3,743)	11,645 † (2,032)	12,359 (3,157)	16,210 (4,592)	8,221 (5,402)	6,242 (2,818)
<b>HIP/PAP</b>	26,595 (6,859)	28,777 † (5,990)	23,393 (8,917)	35,732 (12,858)	26,627 (20,556)	27,454 (11,792)
<b>PSP</b>	322 (79)	8,734 * (2,781)	8,436 (3,072)	6,029 (2,415)	6,205 (5,849)	13,472 (10,029)
<b>Tryp-2</b>	8,759 (2,284)	7,352 † (1,323)	6,927 (1,845)	9,275 (3,271)	7,781 (4,477)	5,370 (1,930)
<b>Tryp-3</b>	4,583 (1,217)	2,845 † (354)	3,130 (569)	2,496 (564)	4,507 (2,052)	2,264 (623)
<b>PSTI</b>	2,266 (505)	2,440 † (426)	2,716 (585)	2,272 (762)	4,391 (2,587)	1,538 (747)
<b>α1-AT</b>	144,271 (30,151)	137,860 † (19,301)	124,045 (23,421)	116,440 (32,927)	275,667 (106,246)	131,954 (37,784)

**Supplementary Table 4.** Oligonucleotide sequences used in real-time PCR analysis for Gene copy number determination. Human genomic DNA was screened with primers for each HD5 exon sequence used to determine the HD5 and HD6 DNA copy number.

Primer Names	Primer Sequences	Sequence Accession #	Position in Sequence	Location in Gene
TBP F	TGAGAAGATGGATGTTGAGTTG	NM_003194.2	1344 – 1365	Exon 9
TBP R	AGATAGCAGCACGGTATGAG	NM_003194.2	1491 -1471	Exon 9
MPO F	CCAGCCCAGAATATCCTTGG	X15377	12516-12535	Exon 12
MPO R	GGTGATGCCTGTTGTCG	X15377	12647-12665	Exon 12
Defa5 #1	AAAGGGATCTTGAGAACAAA	M97925	1231 – 1250	Upstream Exon I
Defa5 #1	GAGAGCAGGAGTGGATATGT	M97925	1357 – 1376	Exon I
Defa5 #2	GACTGCTTCTGGGTTGTAG	M97925	1502 – 1484	Exon I
Defa5 #2	ATATCCACTCCTGCTCTCC	M97925	1359 – 1377	Exon I
Defa5 #3	TTTTCCCCATGTGAGTCTAT	M97925	2360 – 2341	Intron
Defa5 #3	CTTGTTAGGACCTGGAAATC	M97925	2210 – 2229	Intron



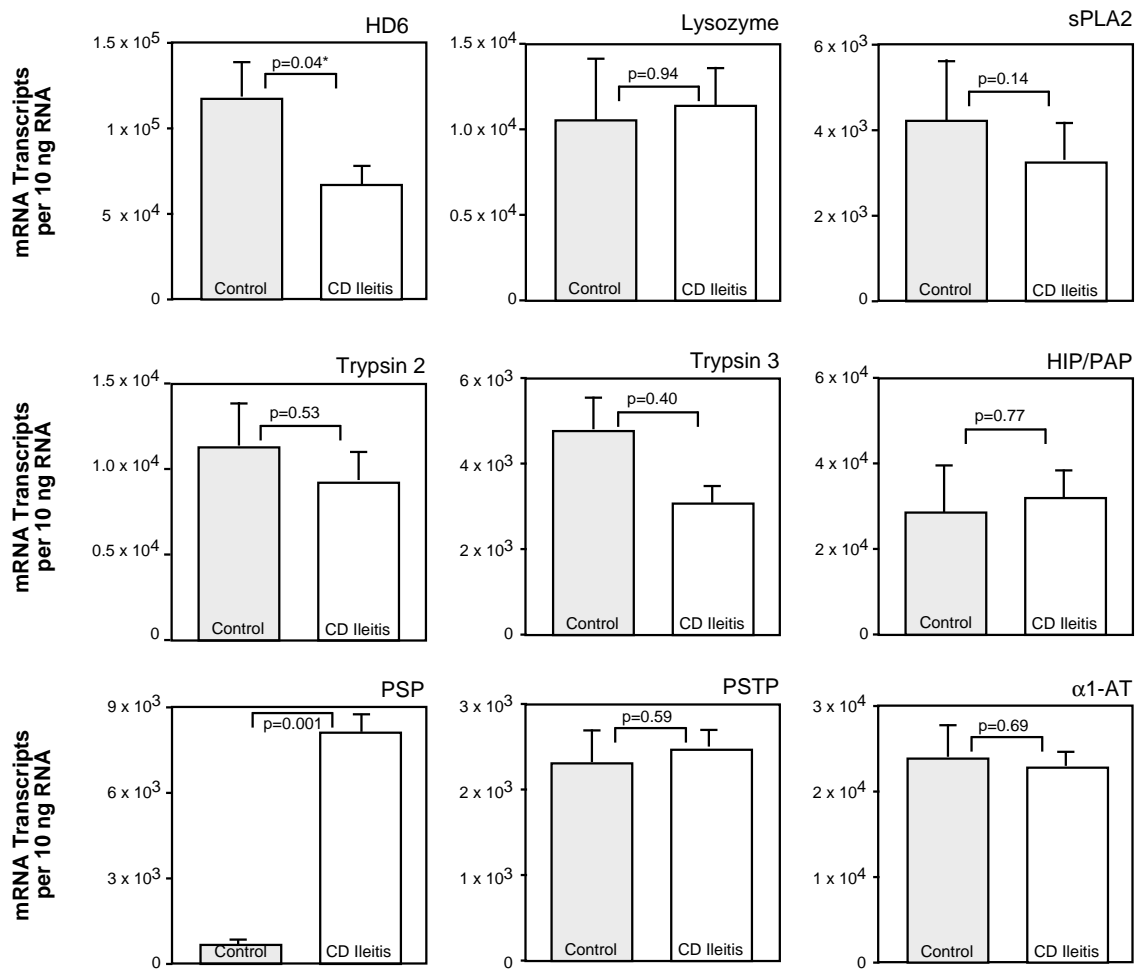


Figure 5: Paneth cell mRNA levels in controls and patients with ileal CD. mRNA levels of the antimicrobial peptides HD6, lysozyme and sPLA, HIP/PAP and PSP, the proteases trypsin 2 and 3, and the anti-proteases PSTI and alpha-1-antiprotease ( $\alpha 1$ -AT). All samples are from the same surgical specimens as in Figure 1C. All values are expressed as transcript copy number per 10ng RNA. The bars represent means  $\pm$  1 standard error. Analysis of GAPDH mRNA as a control, showed no difference between the two groups (data not shown). The significance values, based on the Mann Whitney test, are shown with  $p < 0.05$  (\*) as statistically significant.