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The Paneth Cell α-Defensin Deficiency of Ileal Crohn's Disease Is Linked to Wnt/Tcf-4[1]

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Ileal Crohn′s disease (CD), a chronic mucosal inflammation, is characterized by two pertinent features: a specific decrease of Paneth cell-produced antimicrobial α-defensins and the presence of mucosal-adherent bacteria. A mutation in NOD2, the muramyl dipeptide recognition receptor, is found in some patients, which leads to an even more pronounced α-defensin decrease. However, the underlying mechanism remains unclear for the majority of patients. In this study, we report a reduced expression in ileal CD of the Wnt-signaling pathway transcription factor Tcf-4, a known regulator of Paneth cell differentiation and α-defensin expression. Within specimens, the levels of Tcf-4 mRNA showed a high degree of correlation with both HD5 and HD6 mRNA. The levels of Tcf-4 mRNA were decreased in patients with ileal disease irrespective of degree of inflammation, but were not decreased in colonic CD or ulcerative colitis. As a functional indicator of Tcf-4 protein, quantitative binding analysis with nuclear extracts from small intestine biopsies to a Tcf-4 high-affinity binding site in the *HD-5* and *HD-6* promoters showed significantly reduced activity in ileal CD. Furthermore, a causal link was shown in a murine Tcf-4 knockout model, where the comparably reduced expression of Tcf-4 in heterozygous (+/−) mice was sufficient to cause a significant decrease of both Paneth cell α-defensin levels and bacterial killing activity. Finally, the association between Paneth cell α-defensins and Tcf-4 was found to be independent of the NOD2 genotype. This new link established between a human inflammatory bowel disease and the Wnt pathway/Tcf-4 provides a novel mechanism for pathogenesis in patients with ileal CD. *The Journal of Immunology,* 2007, 179: 3109–3118.

Inflammatory bowel disease (IBD)[3] is a chronic inflammation of the intestine. Details of disease pathogenesis are emerging and the current concept holds that in genetically susceptible individuals, an inappropriate and ongoing activation of the mucosal immune system driven by normal luminal bacterial flora results in chronic intestinal inflammation (1–3). On the basis of its clinical features and histopathology, IBD is commonly classified into two major entities: ulcerative colitis (UC) and Crohn's disease (CD) (3). Whereas UC is typically restricted to the colon, CD can occur at many sites, predominantly in the small intestine (ileum) the colon, or in both locations. Recent studies have supported a link between CD and reduced expression of defensins, a group of endogenous antimicrobial peptides (for review, see Ref. 4). CD of the colon is associated with a low-gene copy number polymorphism of the human β-defensin locus resulting in an attenuated epithelial induction of β-defensin antimicrobial peptides in the colonic mucosa (5, 6). In contrast, CD of the ileum is associated with a deficiency in another group of defensins, Paneth cell α-defensins (7, 8).

Paneth cells reside in the base of small intestinal crypts of Lieberkühn where they secrete high levels of antimicrobial peptides and proteins in response to bacterial stimuli such as muramyl dipeptide, CpG DNA, and LPS (8–13). Among their broad arsenal of antimicrobials, the α-defensins human defensin (HD) 5 and HD6 are the most abundant products (14, 15). These Paneth cell antimicrobials protect the host from food and waterborne pathogens and likely help regulate the composition of bacterial colonization in the small intestinal lumen. CD of the small intestine is characterized by a specific reduction of Paneth cell α-defensins, which results in decreased antimicrobial mucosal barrier function and a shift in the composition of the luminal microbiota (7, 8).

Paneth cells also express NOD2, an intracellular receptor of MDP (for review, see Refs. 16 and 17). A pivotal advance for the field came with the discovery that mutations in the gene encoding NOD2 (*CARD 15*) confers genetic susceptibility to approximately one-third of patients with CD (18, 19). Clinical analysis has revealed that this mutation is not linked to all patients with CD, but rather CD with ileal involvement (20). In the subset of ileal CD patients harboring *NOD2* mutation, the aforementioned decrease in Paneth cell α-defensins is particularly pronounced (7, 8). Consistent with, and extending this correlative finding, NOD2 knockout mice have a selective decrease in Paneth cell α-defensin expression (21). Even though a causal link between NOD2 and Paneth cell defensins is now generally accepted, the question remains what other factors are responsible for the decrease in Paneth cell

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; CD, Crohn's disease; HD, human defensin; UC, ulcerative colitis.

Table I. *Oligonucleotide sequences used in real-time RT-PCR analysis*

	Sense	Antisense
Human		
GAPDH	5′-CCAGCCGAGCCACATCGCTC -3′	5′-ATGAGCCCCAGCCTTCTCCAT- 3′
IL-8	5′- ATGACTTCCAAGCTGGCCGTGGC- 3′	5′- TCTCAGCCCTCTTCAAAAACTTC- 3′
HD5	5′-GCCATCCTTGCTGCCATTC- 3′	5′-AGATTTCACACACCCCGGAGA- 3′
HD6	5′- CCTCACCATCCTCACTGCTGTTC- 3′	5′-CCATGACAGTGCAGGTCCCATA- 3′
Tcf-4	5′-ATCGTCCCAGAGTGATGTCG- 3′	5′-CGGGCCAGCTCGTAGTATTT- 3′
β-Catenin	5′-GCCAAGTGGGTGGTATAGAG- 3′	5′-GCTGGGTATCCTGATGTGC- 3′
Mouse		
GAPDH	5′- TCATCAACGGGAAGCCCATCAC- 3′	5′- AGACTCCACGACATACTCAGCACCG- 3′
β-Actin	5′- GCTGAGAGGGAAATCGTGCGTG-3′	5′-CCAGGGAGGAAGAGGATGCGG- 3′
P-lysozyme	5′- GCCAAGGTCTACAATCGTTGTGAGTTG- 3′	5′- CAGTCAGCCAGCTTGACACCACG- 3′
CRS-1C	5′- CACCACCCAAGCTCCAAATACACAG- 3′	5′- ATCGTGAGGACCAAAAGCAAATGG- 3′
Crypt-1	5′- TCAAGAGGCTGCAAAGGAAGAGAAC- 3′	5′- TGGTCTCCATGTTCAGCGACAGC- 3′
Crypt-4	5′- CCAGGGGAAGATGACCAGGCTG-3′	5′- TGCAGCGACGATTTCTACAAAGGC- 3′
NOD-2	5′- CGACATCTCCCACAGAGTTGTAATCC- 3′	5′- GGCACCTGAAGTTGACATTTTGC- 3′

In all cases, control plasmids were cloned to allow assessment of absolute mRNA copy numbers.

α-defensins in the majority of patients with ileal CD who do not carry mutations in *NOD2*.

Paneth cells, like other cells of the small intestine are derived from a compartment of progenitor cells residing in the crypts adjacent to the basal location of the Paneth cells (22). Wnt signaling, which can be transduced through β-catenin/Tcf-4, helps maintain the undifferentiated state of the progenitor cells (23, 24) and, paradoxically, also induces positioning, differentiation, and maturation of Paneth cells in intestinal crypts (25, 26). The Paneth cell gene program is critically dependent on Tcf-4 (also known as transcription factor 7-like 2) in the embryonic mouse intestine (25). Activation of the Wnt pathway results in the formation of an intracellular β-catenin-Tcf-4 complex which translocates into the nucleus, where Tcf-4 acts as a transcription factor to control the expression of several downstream target genes. In the differentiation of Paneth cells, the known targets include matrix metalloproteinase MMP-7 and Paneth cell α-defensins (25). Using *Tcf-4* null mice, it has been demonstrated that the direct link between Tcf-4 and cryptdin expression occurred irrespective of the other known developmental roles of Tcf-4 in the gut epithelium (25). Because α-defensins HD5 and HD6 are the most abundant Paneth cell products controlled by Tcf-4, we hypothesized that a dysfunction of this signaling pathway could explain the observed decrease of Paneth cell α-defensins in small intestine CD. In this study, we report that CD of the small intestine is associated with a decrease of the Wnt signaling transcription factor Tcf-4 resulting in the observed decrease of Paneth cell α-defensins. Furthermore, the effects of Tcf-4 levels on α-defensin expression were independent of the influence of the *NOD2* genotype.

Materials and Methods

Patients and patient material

Ileal biopsies from healthy individuals (controls) and CD patients were obtained and processed at the Robert Bosch Hospital (Stuttgart, Germany).

Table II. *Tcf-4 potential binding sites in HD5 and HD6 promoter region*

	Original sequence for gel shift (5′→3′)	Position
HD5 number		
1st	CTTGAG**AACAAAG**GCAGTC	−120→-102
2nd	AGGAGC**ATCAAAG**GGATCT	**−137→-119**
2nd mut	AGGAGC**GCCAAAG**GGATCT	−137→-119
3rd	CTCACG**CTTTGAT**GAAAGC	−166→-148
4th	AAATTA**CTTTGAT**ATTCCC	−672→-654
5th	TTGATA**TACAAAG**TGCCAA	−886→-868
6th	TTATTT**TTCAATG**AATAAA	−1409→-1391
7th	CAAGAG**TACAAAG**TTTCAG	−1641→-1623
8th	CTAGAG**TACAAAG**GTGGTC	−1707→-1689
9th	CACTGT**ATCAAAG**AAGAAT	−2311→-2293
HD6 number		
1st	AGGAGC**ATCAAAG**GGACAT	**−137→-119**
2nd	TTGGAG**AACAAAG**GAGCAT	−148→-130
3rd	TCACAC**TACAATG**AAGCTT	−165→-147
4th	ATTATT**CATTGAA**AGCACT	−304→ -286
5th	TTTACC**CTTTGAA**TGGTCC	−759→-741
6th	AGAAAAA**ACAAAG**CACAAA	−856→-838
7th	TTGAGC**TACAAAG**TATTCA	−891→-873
8th	TTATAT**TACAAAG**CTACGG	−1214→-1196
9th	AAAAAAA**ACAAAG**TTGAAG	−1356→-1238
10th	GACACC**CTTTGAT**GTTCTA	−2158→-2140
10th mut	GACACC**CTTTGGC**GTTCTA	−2158→-2140
11th	CATGAT**CATTGAA**AGCTGG	−2300→-2282

The *Tcf-4* potential binding sequence is **(A/T)(A/T)CAA(A/T)G.** The analysis was performed in the *HD5* and *HD6* promoter region minus 3 kb. For the competitor, the core binding sequence for Tcf-4 was mutated from **(A/T) (A/T)** CAA (A/T)G→**GC** CAA (A/T)G. Numbering of binding sites was performed from proximal to distal with respect to transcriptional start site. This order is consistent with the order in Fig. 6. mut, Mutation.

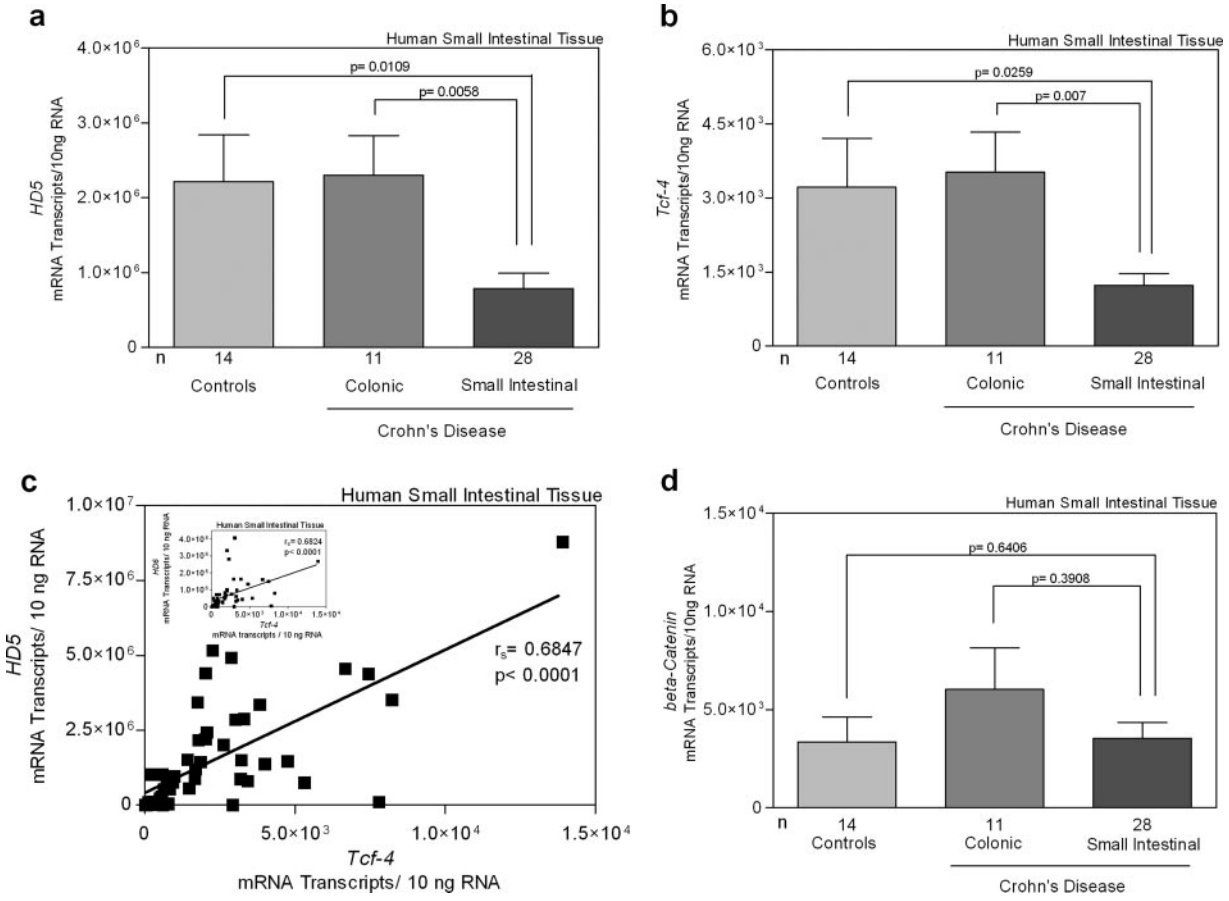

FIGURE 1. Expression of Paneth cell α-defensin HD5 and Wnt signaling molecules in the ileal mucosa. Paneth cell α-defensin mRNA expression (HD5, *a*), Wnt signaling molecule Tcf-4 (*b*), and β-catenin (*d*) was quantitated in ileal biopsies of three groups: controls ($n = 14$), CD patients with solely colonic disease ($n = 11$), and CD patients with ileal disease ($n = 28$). The mRNA copy number per 10 ng of total RNA was determined with quantitative real-time RT-PCR using external standards as described previously (8, 14). Mean values are presented along with their SE. Statistical analyses of quantitative real-time PCR were performed nonparametrically by using the *U* test of Wilcoxon and Mann-Whitney. Tcf-4 and HD5 (or HD6, *inset*) mRNA were correlated *c*). Linear regression using the Spearman analysis for nonparametric data was performed to assess the relationship between the transcript of HD5, HD6, Tcf-4, and other gene products. Values of $p < 0.05$ were considered to be statistically significant.

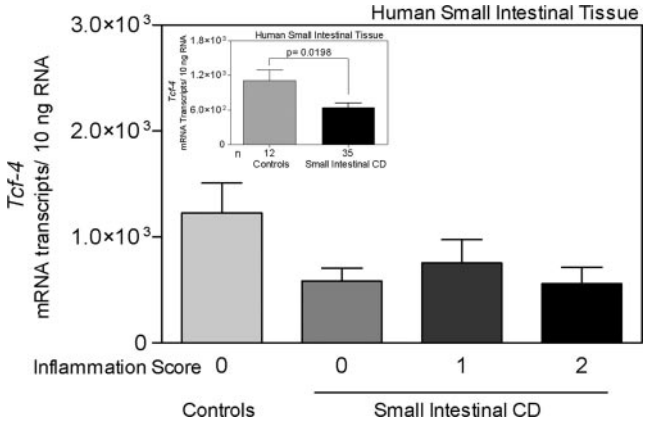

FIGURE 2. Nondependence of Tcf-4 expression on level of tissue inflammation. Expression of Tcf-4 mRNA in specimens from surgical samples of non-IBD controls and patients with ileal CD is shown with respect to level of histological inflammation. H&E-stained paraffin sections from specimens were assessed for mucosal inflammation by a blinded gastrointestinal pathologist (no inflammation, 0; moderate, 1; and severe, 2, in a previous study (8). Expression levels of Tcf-4 mRNA (means ± SE is shown for each group.

As a second cohort, surgical specimens of ileal mucosa were obtained at the Cleveland Clinic Foundation (Cleveland, OH). This cohort was previously described (8). 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. Exclusion criteria included the diagnoses of backwash ileitis, indeterminate colitis, concurrent CMV or *Clostridium difficile* infection. Samples were immediately snap frozen in liquid nitrogen.

Real-time PCR

For quantification of the mRNA, real-time PCR was performed by using a fluorescence detection monitor (LightCycler; Roche Diagnostics). Briefly single-stranded cDNA from human and mouse tissue corresponding to 10 ng of RNA (or gene-specific plasmids as controls) served as a template with specific oligonucleotide primer pairs (Table I) as described previously (7, 8).

NOD2 mutation analysis

Genotyping of genomic DNA for the functionally relevant *NOD2* mutations (SNP8, SNP12, and SNP13) was performed in all patient samples using TaqMan technology (Applied Biosystems) as described previously (7, 8).

Histological analyses

Surgical specimens from CD and non-IBD controls from a previous study (8) with known histological inflammation score were used to assess the

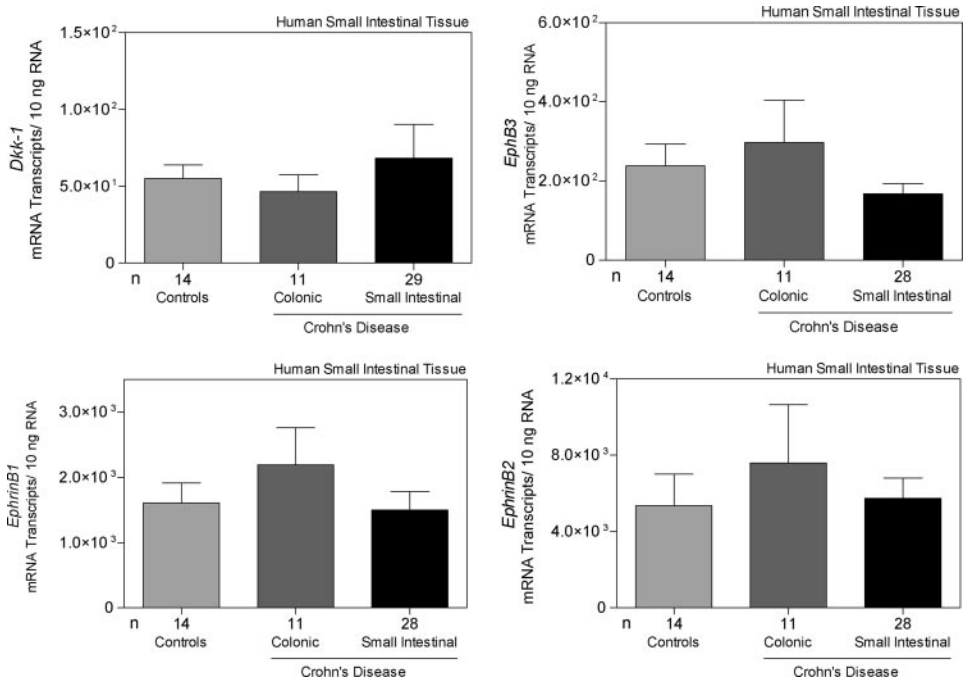

FIGURE 3. Expression of Wnt/Tcf-4-related genes (Dkk-1, EphB3, EphrinB1, EphrinB2) in the ileal mucosa of healthy controls and CD samples. The mRNA copy number per 10 ng of total RNA was determined with quantitative real-time RT-PCR using external standards as described. Mean values are presented along with their SE.

possible association of small intestinal inflammation and Wnt pathway expression.

Promoter analysis

Previous studies (25, 27) have shown that there are multiple consensus Wnt signaling Tcf-4 binding sites in the promoter regions of mouse and human Paneth cell cryptdins/defensins (*HD5, HD6*) genes, respectively. Other potential binding sites in these promoter regions were identified using Lasergene 7.0 DNA analysis GeneQuest software (DNASTAR). In the 3-kb upstream flanking regions, we found 9 potential Tcf-4 binding sites for *HD5* and 11 consensus sequences (A/T A/T CAA A/T G) in the *HD6* gene promoter region (Table II).

Nuclear extracts

Nuclear protein extracts were obtained from both Caco-2 cells and small intestine biopsies of healthy controls and CD patients using the NE-PER kit from Pierce (catalog no. 78833).

EMSA

Gel mobility shift assays were performed as previously described (28). Human Tcf-4 protein was synthesized from expression vector pK-Myc-FLH-Tcf-4 (generously provided by V. Korinek, Institute of Molecular Genetics, Academy of Sciences of the Czech. Republic) using the TNT T7 Quick-Coupled Transcription/Translation System (Promega). Nuclear response elements were prepared by annealing 10 μl each of two complementary oligonucleotide stocks (100 μM) in 180 μl of 25 mM NaCl, 25 mM Tris-Cl (pH 7.5), and 5 mM $MgCl_2$. For radioactive labeling, 2 μl of the annealed oligonucleotides, 5 μl of 10× buffer (500 mM NaCl, 500 mM Tris-Cl (pH 7.5), 100 mM $MgCl_2$, 25 μCi of [α-^{32}P]dCTP, 5 μl of 2 mM dATP, dGTP, dTTp, 2 U of Klenow fragment, and H_2O to a final volume of 50 μl were incubated at 37°C for 1 h and then purified through Sephadex columns (MincroSpin G-25 columns; GE Health). Binding reaction contained 10 mM HEPES (pH 7.8), 60 mM KCl, 0.2% Nonidet P-40, 6% glycerol, 2 mM DTT, 0.25 μg of poly(dI:dC), 2 μl of 10 μM nonspecific oligonucleotides (5′-AGC TTG CGA AAA TTG TCA CTT CCT GTG TAC ACC CA-3′), 50,000 cpm of labeled probe, and 4 μl of recombinant Tcf-4 in a final volume of 20 μl. Samples were incubated on ice for 20 min after the recombinant protein (or nuclear extract) had been added. For supershift experiments, 4 μg of mouse anti-Tcf-4 mAb (Upstate Biotechnology) was preincubated with the recombinant protein or the nuclear extract 1 h at room temperature before the addition of the oligonucleotide. In competition experiments, unlabeled competitor oligonucleotides were added before the addition of the in vitro-translated protein. The protein-DNA complex was resolved on a pre-electrophoresed 5% polyacrylamide gel in 44.5 mM boric acid and 1 mM EDTA (pH 8.3) at 200 V at 4°C. Gels were dried, autoradiographed overnight at room temperature, and then analyzed with the BAS-1800 II phosphor-storage scanner (Fuji) and AIDA software (Raytest). For comparison, the mean of *Tcf-4* transcription activity in healthy controls was defined as 100.

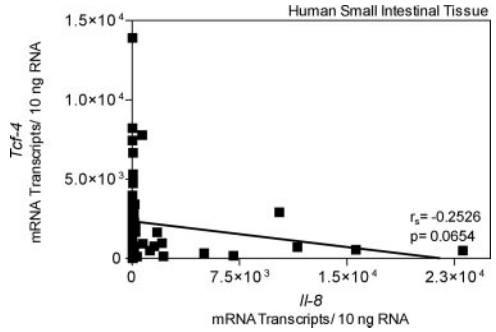

FIGURE 4. Absence of correlation between Tcf-4 expression and proinflammatory cytokine (IL-8) in ileal CD patients. The correlation of Tcf-4 and proinflammatory cytokine IL-8 is shown in individuals with small intestinal CD corresponding to Fig. 1 (ileal biopsies). A linear regression using the Spearman analysis for nonparametric data was performed. The value of $p < 0.05$ was considered to be statistically significant.

Analysis of Tcf-4 in heterozygous knockout mice

Heterozygous Tcf-4 knockout (strain Tcf7l2) and wild-type littermate mice were genotyped as described previously (23). The reduced levels of Tcf-4 in the heterozygous (vs no expression in homozygous knockout mice) resembles the in vivo decreases as observed in human disease. Tissue samples from the distal (ileum) and middle (jejunum) part of the small intestine were taken from *Tcf7l2*$^{+/-}$ mice and control *Tcf7l2*$^{+/+}$ mice. The samples were used for real-time PCR gene expression analysis and functional assessment of antimicrobial activity.

Antimicrobial activity in mouse small intestinal mucosa

Cationic proteins from mouse small intestine mucosal biopsies were isolated by weak cation exchange matrix chromatography as described elsewhere (8, 29). Mid-logarithmic growth phase suspensions of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were incubated with the cationic protein fraction at 37°C in a final volume of 100

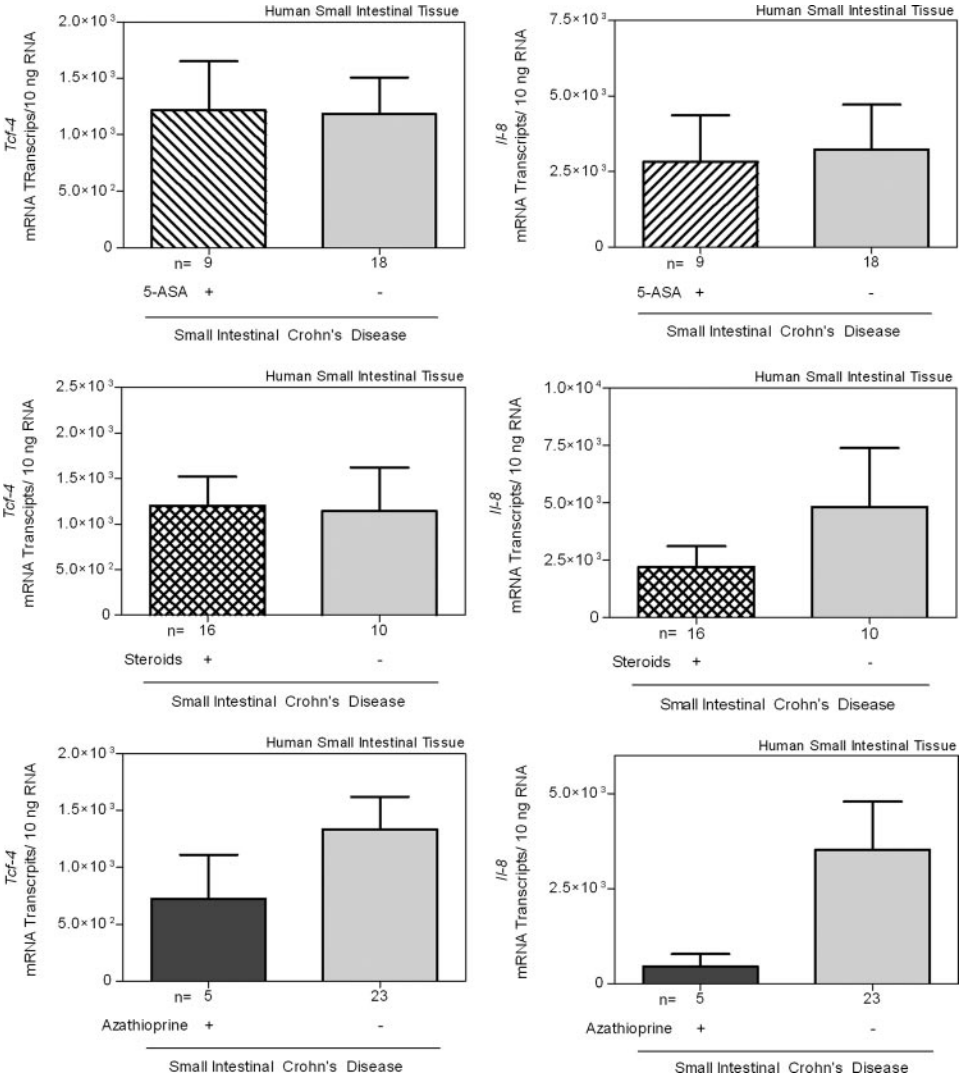

FIGURE 5. Possible effects of common CD treatments on Tcf-4 expression in small intestine tissue. The expression of Tcf-4 (*left panels*) and proinflammatory cytokine IL-8 (*right panels*) is shown for 5-aminosalisylate, oral steroids, and azathioprine. None of the observed changes between the treatment groups were statistically significant.

μl of 1/6 diluted Schaedler broth (BD Biosciences). Bacterial suspensions incubated with vehicle (0.01% acetic acid) served as negative controls. After 120 min, [bis-(1,3-dibutylbarbituric acid)*t*-rimethine oxonol] (Molecular Probes), a dye sensitive to membrane potential, was added at a concentration of 1 μg/ml. Bacterial pellets were isolated by centrifugation, resuspended in 300 μl of FACSFlow (BD Biosciences),and analyzed by flow cytometry by using a FACSCalibur (BD Biosciences) as described previously (8, 29). A total of 30,000 events was analyzed in each sample. The flow cytometry enabled us to distinguish viable from nonviable bacteria at the single-cell level. The antimicrobial activity was determined as percentage of depolarized bacteria compared with untreated controls.

Statistics

Statistical analyses of quantitative real-time PCR were performed nonparametrically or parametrically (in case of normal distribution) by using the *U* test of Wilcoxon, Mann-Whitney, or Student *t* test, respectively. Tcf-4 relative binding ability in the *HD5*, *HD6* promoter region was subjected to *t* test and ANOVA analysis by using GraphPad Prism version 4.0 software. Values of $p < 0.05$ were considered to be statistically significant. Regression using the Spearmen rank analysis was performed to assess the relationship between the transcript of *HD5*, *Tcf-4*, and other gene products. For illustration, mean values are presented along with their SE.

Results

Tcf-4 expression is specifically reduced in ileal CD

We measured Wnt signaling protein Tcf-4, Paneth cell α-defensin, and control mRNA expression in the ileal mucosa of three groups: controls, CD patients with solely colonic disease, and CD patients with ileal disease. Consistent with previous findings (7, 8), we confirmed in ileal biopsy specimens from ileal CD patients that there was reduced expression of HD5 (Fig. 1*a*) and HD6 mRNA (data not shown) compared with controls. We previously reported that this decrease reflected a corresponding decrease in α-defensin peptide levels (8). In these specimens, we observed that Tcf-4 mRNA levels were also significantly reduced compared with controls ($p = 0.026$; Fig. 1*b*). Compared with patients with an inflammatory course, those with progressive disease according to the Vienna classification (fibrostenosing and penetrating disease) showed a trend of even lower Tcf-4 expression (40% reduction, $p = 0.06$; data not shown). Similar decreases of Tcf-4 mRNA were observed in an independent cohort of surgical specimens from the small intestines of CD patients ($n = 35$) vs controls ($n = 12$, $p = 0.0198$; Fig. 2, *inset*). Within specimens, the levels of Tcf-4 and HD5 and HD6 mRNA showed a high degree of correlation (Fig. 1*c*). In contrast, both Tcf-4 and Paneth cell α-defensin expressions were unchanged in ileal biopsies from patients with colonic CD compared with controls (Fig. 1, *b* and *a*, respectively). The small intestine mRNA expression of β-catenin, the key adaptor for activation of Tcf-4, did not show any significant differences in any of the groups (Fig. 1*d*). Four Tcf-4/Wnt-related genes known to be important in other tissues were unchanged and showed no correlation with Tcf-4 (Fig. 3).

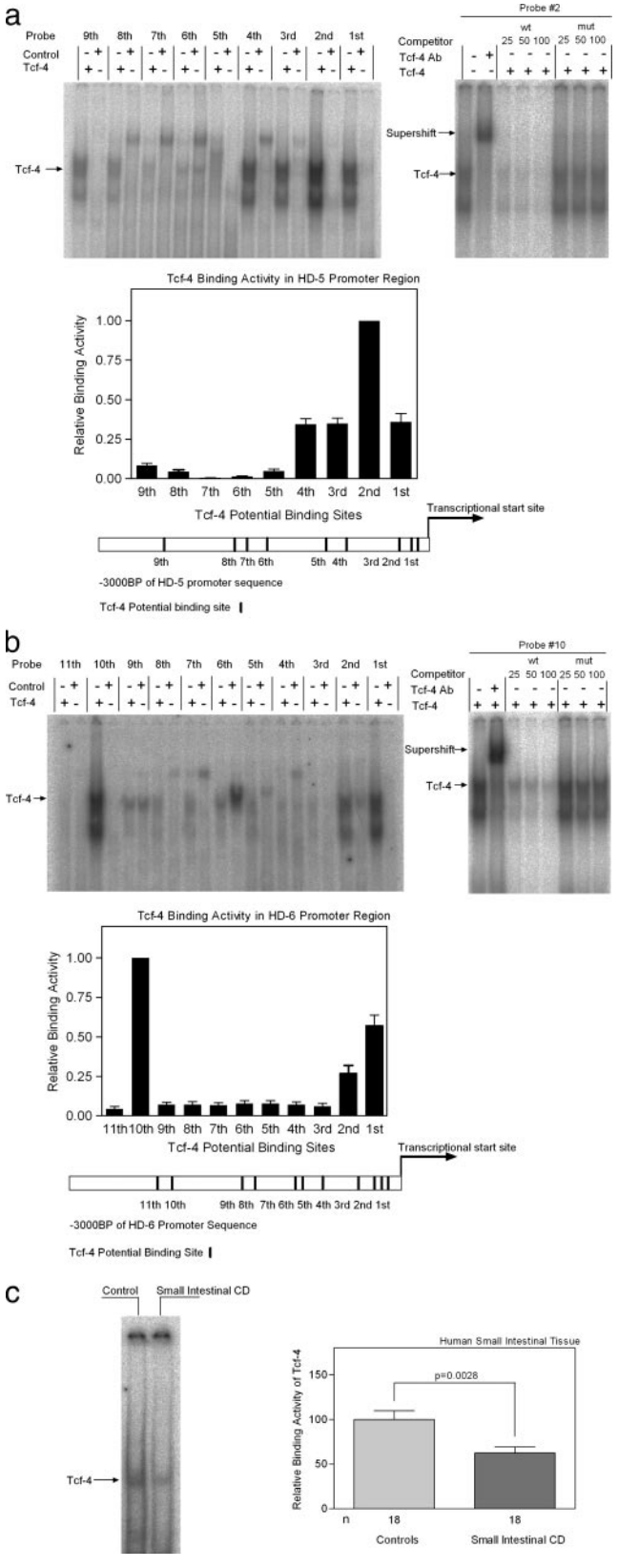

FIGURE 6. EMSA analysis of the Tcf-4 binding in Paneth cell α-defensin gene promoters. EMSA was performed using recombinant Tcf-4 protein and radiolabeled double-stranded oligonucleotides (probes) corresponding to the Tcf-4 motif in the *HD5* and *HD6* promoters (*a* and *b*). Location of putative Tcf-4 binding sites (■) are presented in the graph of *HD5* and *HD6* promoter regions. The binding reactions contained (+) or lacked (−) indicated recombinant Tcf-4 protein, Tcf-4 Ab, unlabeled wild-type (wt) oligonucleotide, or competitor oligonucleotide with scrambled sequence as indicated. Migration of bound complexes of protein and oligonucleotide are marked by an arrow. The relative binding activity of each probe to human Tcf-4 protein was quantified by densitometry and relative values were normalized to the highest value which was defined as 1.0. Mean values are presented along with their SE (three independent experiments for HD5 and HD6 promoters, respectively). Representative (*left*) and quantitative comparisons (*right*) of Tcf-4-binding activity in extracts of small intestine tissue of healthy control and ileal CD are shown in *c*. For these experiments, probe #2 of *HD5*, which is nearly identical to probe #1 of *HD6*, was used. The value of $p < 0.05$ was considered to be statistically significant. mut, Mutation.

To test whether the observed decrease in Tcf-4 might be a direct consequence of inflammation, we examined Tcf-4 mRNA expression levels in a group of specimens that were previously categorized by a gastrointestinal pathologist based on degree of histological inflammation and which showed a significant correlation with the expression of the proinflammatory cytokine IL-8 (8). In these specimens (data not shown), as well as in ileal biopsies corresponding to Fig. 1, Tcf-4 did not show any correlation with IL-8 in contrast to the transcription factor's tight link with Paneth cell α-defensin levels (Fig. 4). Moreover, the levels of Tcf-4 mRNA were similarly decreased in all patients with ileal CD, irrespective of whether the mucosal inflammation in intestinal biopsies was absent, moderate, or severe (Fig. 2). Furthermore, we tested Tcf-4 levels in macroscopically uninflamed as well as inflamed UC pouch biopsies as an inflammatory non-CD control. Like HD5 and HD6 (8), the levels were similar in both groups ($n = 8$ normal UC pouch, $n = 10$ inflamed UC pouch; data not shown). Together, these data indicate that the inflammation itself does not affect expression levels of Tcf-4.

To examine whether the observed effect was due to therapy, we analyzed the possible effects of different common treatments on Tcf-4 expression in the group of ileal CD. Neither treatment with 5-aminosalicylate or oral steroids nor azathioprine had a significant effect on Tcf-4 expression levels (Fig. 5). Also, we compared the treatment schemes between ileal and colonic CD. The treatment in ileal and colonic CD was almost identical (data not shown) even though Tcf-4 and Paneth cell α-defensins were significantly decreased in ileal disease. These observations argue against a systematic change caused by one of these drugs.

Reduced binding activity of Tcf-4 in ileal CD

Complementing previous studies that reported 5 potential binding sites of Tcf-4 in the *HD5* promoter and a total of 11 in the *HD6* promoter region (25, 27), we identified 4 additional putative sites in the 3-kb upstream region of the *HD5* gene. Using recombinant Tcf-4 protein, a comparison of each potential binding site for the quantity of Tcf-4-binding activity by EMSA revealed that binding site 2 in the *HD5* promoter region has the strongest binding capacity (Fig. 6*a*). In the *HD6* promoter region, binding sites 1 and 10 bound the recombinant Tcf-4 protein most avidly (Fig. 6*b*); the same results were obtained in independent experiments using nuclear extracts of Caco-2 cells as a source of Tcf-4 (data not shown). In each case, the specificity of the Tcf-4 binding was confirmed by supershift experiments using a Tcf-4 Ab (Fig. 6, *a* and *b*). Because binding site 2 in *HD5* and binding site 1 in *HD6* contained nearly the same sequence, we selected this probe for further analysis in human tissue. We used this EMSA-binding activity as a functional indicator of Tcf-4 protein levels, since available Abs were not sufficiently sensitive to provide satisfactory quantitation of the protein by Western blot analysis. Quantitative binding analysis of this common Tcf-4 high-affinity binding site probe using EMSA

showed significantly reduced activity ($p = 0.0028$) in nuclear extracts from small intestine biopsies in ileal CD as compared with healthy control tissue (Fig. 6*c*), which is consistent with the reduced expression of Tcf-4 mRNA (Fig. 1*b*). Two patients with heterozygote NOD2 SNP13 mutations were in the range of the ileal CD patients (data not shown).

Reduced Tcf-4 levels cause decreased Paneth cell α-defensin expression

To directly assess whether modestly reduced Tcf-4 levels could cause a decrease in Paneth cell α-defensins, we turned to a Tcf-4 knockout murine model. We reasoned that the reduced levels of this transcription factor in heterozygous (+/−) knockout mice compared with wild-type littermates would be comparable to the differences in Tcf-4 expression observed in ileal CD compared with controls (Fig. 1). Similar to human ileal CD, there was no decrease in Paneth cell numbers in the Tcf-4 heterozygote (+/−) mice (data not shown). The Tcf-4$^{+/-}$ heterozygous knockout mice had a significant reduction of both Paneth cell α-defensin-4 (called cryptdin-4), an abundant mouse Paneth cell α-defensin (Fig. 7*a*), as well as cryptdin-1 (Fig. 7*a*). The expression levels of cryptdin-related sequence (CRS-1C) (Fig. 7*a*) and mouse lysozyme (data not shown) were also diminished, but the decreases were not statistically significant. To test whether the decrease of Paneth cell antimicrobial products in this murine model is functionally relevant, we performed an antimicrobial killing assay using small

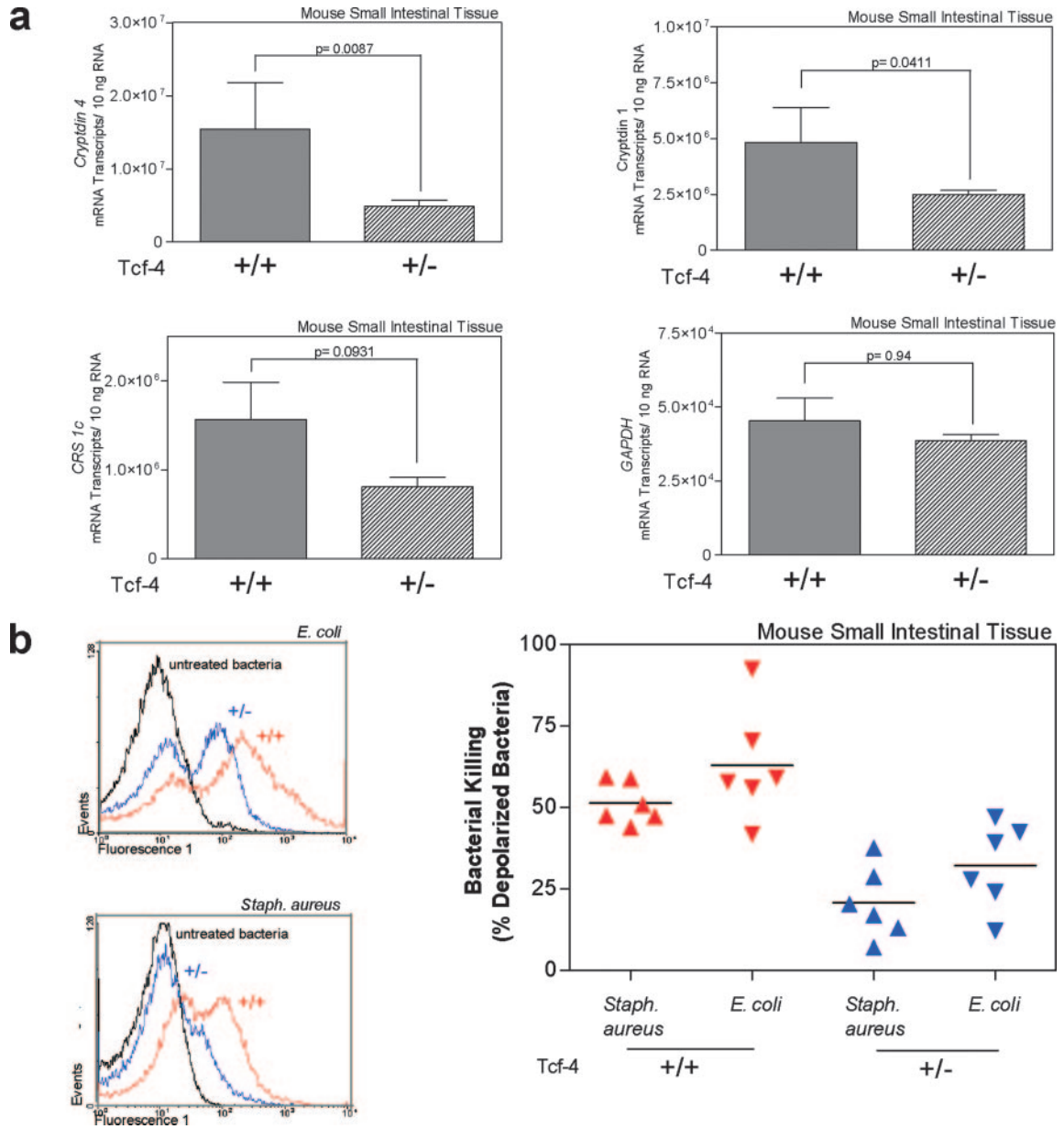

FIGURE 7. Expression of Paneth cell α-defensins (cryptdins) and mucosal antimicrobial activity in Tcf-4-heterozygous knockout and wild-type mice. The absolute mRNA copy number of murine Paneth cell α-defensins (cryptdins) 4 and 1, cryptdin-related sequence (CRS1C) in heterozygous Tcf-4 knockout and wild-type mouse small intestine was determined by quantitative real-time RT-PCR using external standards (*a*) (six mouse samples in each group corresponding to *b*). Representative FACS analysis of antimicrobial activity in small intestine tissue from the same mice with Gram-positive (*Staph. aureus*) and Gram-negative (*E. coli*) bacteria (*b*, *left side*). The small intestinal mucosal killing activity against both strains (*S. aureus* and *E..coli*) is shown in *b*, *right side*. Mean values are presented along with their SE. Values of $p < 0.05$ were considered to be statistically significant.

intestine tissue samples from the same mice. Total antimicrobial activity against *E. coli* and *S. aureus* was dependent on the *Tcf-4* genotype and reduced by half in heterozygote mice (Fig. 7*b*).

Reduced Tcf-4 is independent of NOD2 genotype

Because NOD2 significantly affects human and mouse Paneth cell α-defensin expression (7, 8, 21), we tested whether Tcf-4 expression was also directly dependent on NOD2. In patient biopsies, the expression level of Tcf-4 was independent of whether the patients had a *NOD2* mutation or not (Fig. 8*a*). Conversely, in heterozygous (+/−) Tcf-4 knockout mice, NOD2 expression levels were unchanged compared with wild-type control mice (Fig. 8*a, inset*). To further test whether the decrease of Tcf-4 is independent of NOD2, we assessed whether the Tcf-4 and HD5 levels correlated in two groups: ileal CD patients with the common heterozygote *NOD2* L1007fs mutation (SNP13) and individuals without this mutation. Included in this correlation were patients with SNP8 or SNP12 missense mutations, which we previously showed had HD5 levels indistinguishable from those of wild-type *NOD2* (8). Consistent with the patient cohort from Stuttgart with *NOD2* wild-type ileal CD patients (Fig. 1*c*), there was a strong correlation of Tcf-4 with HD5 ($r_s = 0.74$, $p < 0.0001$) and HD6 ($r_s = 0.6824$, $p < 0.0001$; data not shown) in the surgical group of ileal CD patients not harboring the SNP13 mutation (Fig. 8*b*). Strikingly, the correlation was absent in the ileal CD patients with *NOD2* SNP13 mutation (Fig. 8*b*). Specimens we previously characterized with SNP13 mutations have a significantly more pronounced decrease of Paneth cell α-defensins (8) at given Tcf-4 levels. Together, these data in mice and human specimens indicate that *NOD2* genotype and reduced Tcf-4 levels represent independent mechanisms for decreased expression of Paneth cell α-defensins.

Discussion

Many studies point to a complex interplay between commensal microbes and the intestinal mucosa that result in establishing a delicate balance under normal healthy conditions (for review, see Refs. 30 and 31). In IBD pathogenesis, a perturbation of this balance is proposed to underlie the disease, whereby intestinal microbes contribute to the development and perpetuation of chronic mucosal inflammation in genetically susceptible individuals (32). We had initially reported in European patients that ileal CD is associated with decreased expression of Paneth cell α-defensins (7) and this decrease was prospectively confirmed in patient cohorts from the United States (8), Australia (G. Radford-Smith, unpublished observations), and Japan (33). Interestingly, the latter population does not exhibit *NOD2* mutations (34). Furthermore, this diminished expression was not observed in CD of the colon or in UC in any of these studies. The decrease in α-defensins was even more pronounced in patients with a frameshift (SNP13) mutation in NOD2, as compared with those with either missense mutations (SNP8 and SNP12) or the majority of ileal CD patients with a normal NOD2 genotype (8). In this study, we report an explanation for these findings where a decrease of the Wnt pathway transcription factor Tcf-4 is responsible for the reduced expression of both Paneth cell α-defensins HD5 and HD6. CD patients with ileal disease showed a consistent decrease in Tcf-4 levels and activity, whereas patients with strictly CD of the colon had normal levels of ileal Tcf-4 and downstream Paneth cell α-defensins. Furthermore, the decreased expression of Tcf-4 in ileal CD was not a secondary effect of inflammation itself. We propose that the Tcf-4 dependent decrease of Paneth cell α-defensins in ileal CD leads to the changes in the luminal and/or surface bacteria composition, which have been implicated by others (35–38) as triggers of inflammation in CD (Fig. 9).

The current data show, both in the mouse and humans, that the decrease in Paneth cell α-defensins/cryptdins is Tcf-4 dependent and weakens enteric antimicrobial defense. Previous studies showed that compared with wild-type mice, HD5-transgenic mice were resistant to the otherwise lethal concentrations of orally administered *Salmonella*, indicating that HD5 had a biologically significant effect on pathogenic microbes in the intestinal lumen (9). Furthermore, using this same transgenic model, we reported that modest changes in HD5 expression levels, similar in magnitude to those observed in ileal CD compared with controls, significantly altered the composition of the luminal microbiota (8). Other studies in murine models have elegantly demonstrated that decreased Paneth cell α-defensin expression weakens mucosal defense against pathogenic bacteria (21, 39). In patients, the decrease of Paneth cell α-defensins in ileal CD results in a significantly

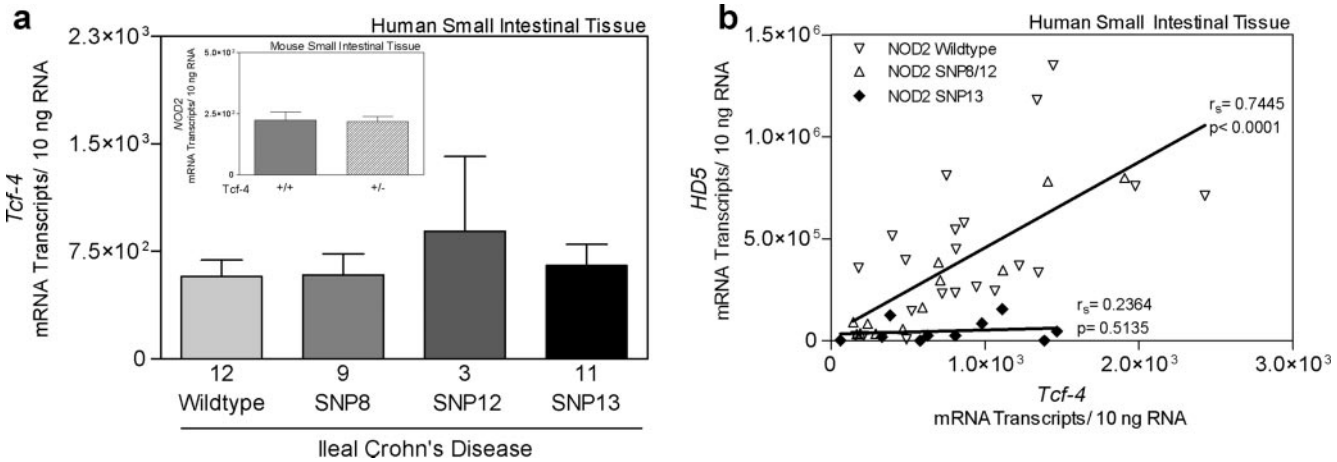

FIGURE 8. Analysis of interdependence of NOD2, Tcf-4, and Paneth cell α-defensin expression levels in human and mouse ileal tissue. The expression of Tcf-4 mRNA is shown with respect to NOD2 mutation status in a cohort of surgical specimens with ileal CD, where adequate numbers of patients harboring SNP13 mutations were available (*a*). The expression of NOD2 mRNA was determined in Tcf-4 heterozygous (+/−) knockout and wild-type mice (*inset*, *a*). The correlation of Tcf-4 and HD5 mRNA levels is shown in individuals harboring the common SNP13 NOD2 frameshift mutation (◆) as well as in individuals without this mutation (NOD2 wild type and SNP 8 and 12; *b*). A linear regression using the Spearman analysis for nonparametric data was performed. The value of $p < 0.05$ was considered to be statistically significant.

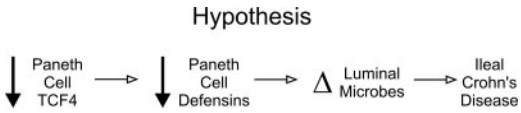

FIGURE 9. Proposed hypothesis for the role of decreased Tcf-4 and Paneth cell α-defensins in the pathogenesis of ileal CD. A decrease of the Wnt signaling transcription factor Tcf-4 causes a reduction of Paneth cell α-defensins, weakened mucosal defenses, and alterations in the commensal microbiota. These changes may allow attachment and mucosal invasion of lumenal bacteria, which then triggers and/or perpetuates inflammation in genetically susceptible individuals. This progression leads to ileal CD.

reduced mucosal killing activity toward Gram-positive and Gram-negative bacteria (8). In this study, we show that modest changes in murine Tcf-4 levels (+/+ vs +/−) result in a 2-fold drop in Paneth cell α-defensins that leads to a compromised epithelial defense toward bacteria with a magnitude assessed in vitro comparable to that in ileal CD patients (8). Together, these data support the notion that Tcf-4-dependent Paneth cell α-defensin expression has significant effects on intestinal mucosal defense and that relatively small changes in expression levels can impact on host-microbe balance at the mucosa.

We and others have shown that a frameshift (SNP13) mutation in the pattern recognition receptor NOD2 linked to reduced levels of α-defensins in some patients with ileal CD (7, 8, 21). The dependence of Paneth cell α-defensin expression on Tcf-4 levels reported here was found to be independent of the NOD2 genotype. This is significant, because our data provide an explanation for the observed decreased levels of α-defensins in the majority of CD patients with ileal disease and defines the subgroup of patients with ileal CD on a molecular level.

In conclusion, Tcf-4 function is deficient in ileal CD, and this can then elegantly explain the decrease in two different α-defensins (HD5 and HD6), both target genes of this transcription factor. In contrast, neither the Tcf-4 deficiency nor associated decreases in α-defensins was evident in either colonic CD or UC. We are confident that future studies will show whether this is due to a genetic variant in the Tcf-4 gene or alternatively a Tcf-4 regulatory gene or pathway. Such scenarios will provide new genetic susceptibility links to ileal CD that would nicely complement our knowledge about the ramifications of NOD2 mutations (40). When looking downstream from these different underlying regulatory defects, the weakened antimicrobial defenses characteristic of CD may provide an attractive focus for the development of effective new therapies to protect the mucosa and resolve inflammation.

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Disclosures

Jan Wehkamp, Edward F. Stange, and Charles L. Bevins have a pending patent on treatment with defensins.

References

1. Strober, W. 2006. Immunology: unraveling gut inflammation. *Science* 313: 1052–1054.
2. Korzenik, J. R. 2007. Is Crohn's disease due to defective immunity? *Gut* 56: 2–5.
3. Podolsky, D. K. 2002. Inflammatory bowel disease. *N. Engl. J. Med.* 347: 417–429.
4. Wehkamp, J., K. Fellermann, K. Herrlinger, C. Bevins, and E. F. Stange. 2005. Mechanisms of disease: defensins in gastrointestinal diseases. *Nat. Clin. Pract.* 2: 406–415.
5. Wehkamp, J., K. Fellermann, K. R. Herrlinger, S. Baxmann, K. Schmidt, B. Schwind, M. Duchrow, C. Wohlschlager, A. C. Feller, and E. F. Stange. 2002. Human β-defensin 2 but not β-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. *Eur. J. Gastroenterol. Hepatol.* 14: 745–752.
6. Fellermann, K., D. E. Stange, E. Schaeffeler, H. Schmalzl, J. Wehkamp, C. L. Bevins, W. Reinisch, A. Teml, M. Schwab, P. Lichter, et al. 2006. A chromosome 8 gene-cluster polymorphism with low human β-defensin 2 gene copy number predisposes to Crohn disease of the colon. *Am. J. Hum. Genet.* 79: 439–448.
7. Wehkamp, J., J. Harder, M. Weichenthal, M. Schwab, E. Schaeffeler, M. Schlee, A. Stallmach, F. Noack, P. Fritz, J. M. Schroder, et al. 2004. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal α-defensin expression. *Gut* 53: 1658–1664.
8. Wehkamp, J., N. H. Salzman, E. Porter, S. Nuding, M. Weichenthal, R. E. Petras, B. Shen, E. Schaeffeler, M. Schwab, R. Linzmeier, et al. 2005. Reduced Paneth cell α-defensins in ileal Crohn's disease. *Proc. Natl. Acad. Sci. USA* 102: 18129–18134.
9. Salzman, N. H., D. Ghosh, K. M. Huttner, Y. Paterson, and C. L. Bevins. 2003. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 422: 522–526.
10. Porter, E. M., C. L. Bevins, D. Ghosh, and T. Ganz. 2002. The multifaceted Paneth cell. *Cell. Mol. Life Sci.* 59: 156–170.
11. Ayabe, T., D. P. Satchell, C. L. Wilson, W. C. Parks, M. E. Selsted, and A. J. Ouellette. 2000. Secretion of microbicidal α-defensins by intestinal Paneth cell response to bacteria. *Nat. Immunol.* 1: 113–118.
12. Rumio, C., D. Besusso, M. Palazzo, S. Selleri, L. Sfondrini, F. Dubini, S. Menard, and A. Balsari. 2004. Degranulation of Paneth cells via Toll-like receptor 9. *Am. J. Pathol.* 165: 373–381.
13. Ouellette, A. J. 2004. Defensin-mediated innate immunity in the small intestine. *Best Pract. Res. Clin. Gastroenterol.* 18: 405–419.
14. Wehkamp, J., H. Chu, B. Shen, R. W. Feathers, R. J. Kays, S. K. Lee, and C. L. Bevins. 2006. Paneth cell antimicrobial peptides: topographical distribution and quantification in human gastrointestinal tissues. *FEBS Lett.* 580: 5344–5350.
15. Bevins, C. L. 2004. The Paneth cell and the innate immune response. *Curr. Opin. Gastroenterol.* 20: 572–580.
16. Inohara, N., M. Chamaillard, C. McDonald, and G. Nunez. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu. Rev. Biochem.* 19: 355–383.
17. Peyrin-Biroulet, L., C. Vignal, R. Dessein, M. Simonet, P. Desreumaux, and M. Chamaillard. 2006. NODs in defence: from vulnerable antimicrobial peptides to chronic inflammation. *Trends Microbiol.* 14: 432–438.
18. Hugot, J.-P., C. Chamaillard, H. Zouali, S. Lesage, J.-P. Cezard, J. Belaiche, S. Almer, C. Tysk, C. A. O'Morain, M. Gassull, et al. 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411: 599–603.
19. Ogura, Y., D. K. Bonen, N. Inohara, D. L. Nicolae, F. F. Chen, R. Ramos, H. Britton, T. Moran, R. Karalluskas, R. H. Duerr, et al. 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411: 603–606.
20. Gasche, C., and P. Grundtner. 2005. Genotypes and phenotypes in Crohn's disease: do they help in clinical management? *Gut* 54: 162–167.
21. Kobayashi, K. S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R. A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307: 731–734.
22. Gregorieff, A., and H. Clevers. 2005. Wnt signaling in the intestinal epithelium: from endoderm to cancer. *Genes Dev.* 19: 877–890.
23. Korinek, V., N. Barker, P. Moerer, D. E. van, G. Huls, P. J. Peters, and H. Clevers. 1998. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat. Genet.* 19: 379–383.
24. Gregorieff, A., D. Pinto, H. Begthel, O. Destree, M. Kielman, and H. Clevers. 2005. Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology* 129: 626–638.
25. van Es, J. H., P. Jay, A. Gregorieff, M. E. van Gijn, S. Jonkheer, P. Hatzis, A. Thiele, M. van den Born, H. Begthel, T. Brabletz, M. M. Taketo, and H. Clevers. 2005. Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nat. Cell Biol.* 7: 381–386.
26. Batlle, E., J. T. Henderson, H. Beghtel, M. M. van den Born, E. Sancho, G. Huls, J. Meeldijk, J. Robertson, W. M. van de, T. Pawson, and H. Clevers. 2002. β-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 111: 251–263.
27. Andreu, P., S. Colnot, C. Godard, S. Gad, P. Chafey, M. Niwa-Kawakita, P. Laurent-Puig, A. Kahn, S. Robine, C. Perret, and B. Romagnolo. 2005. Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. *Development* 132: 1443–1451.
28. Geick, A., M. Eichelbaum, and O. Burk. 2001. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J. Biol. Chem.* 276: 14581–14587.
29. Nuding, S., K. Fellermann, J. Wehkamp, H. A. Mueller, and E. F. Stange. 2006. A flow cytometric assay to monitor antimicrobial activity of defensins and cationic tissue extracts. *J. Microbiol. Methods* 65: 335–345.
30. Backhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon. 2005. Host-bacterial mutualism in the human intestine. *Science* 307: 1915–1920.

31. Hooper, L. V., and J. I. Gordon. 2001. Commensal host-bacterial relationships in the gut. *Science* 292: 1115–1118.
32. Schreiber, S., P. Rosenstiel, M. Albrecht, J. Hampe, and M. Krawczak. 2005. Genetics of Crohn disease, an archetypal inflammatory barrier disease. *Nat. Rev. Genet.* 6: 376–388.
33. Ayabe, T., H. Tanabe, T. Kono, T. Ito, A. Maemoto, A. Watari, T. Ashida, A. J. Ouelette, and Y. Kohgo. 2005. The innate intestinal immunity by Paneth cells and their α-defensins in patients with Crohn's disease. *Gastroenterology* 128: A500–A501.
34. Inoue, N., K. Tamura, Y. Kinouchi, Y. Fukuda, S. Takahashi, Y. Ogura, N. Inohara, G. Nunez, Y. Kishi, Y. Koike, et al. 2002. Lack of common NOD2 variants in Japanese patients with Crohn's disease. *Gastroenterology* 123: 86–91.
35. Swidsinski, A., A. Ladhoff, A. Pernthaler, S. Swidsinski, V. Loening-Baucke, M. Ortner, J. Weber, U. Hoffmann, S. Schreiber, M. Dietel, and H. Lochs. 2002. Mucosal flora in inflammatory bowel disease. *Gastroenterology* 122: 44–54.
36. Darfeuille-Michaud, A., J. Boudeau, P. Bulois, C. Neut, A. L. Glasser, N. Barnich, M. A. Bringer, A. Swidsinski, L. Beaugerie, and J. F. Colombel. 2004. High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. *Gastroenterology* 127: 412–421.
37. Duchmann, R., E. May, M. Heike, P. Knolle, M. Neurath, and K.-H. Meyer zum Büschenfelde. 1999. T cell specificity and cross reactivity towards enterobacteria, *Bacteroides*, *Bifidobacterium*, and antigens from resident intestinal flora in humans. *Gut* 44: 812–818.
38. Cash, H. L., C. V. Whitham, C. L. Behrendt, and L. V. Hooper. 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science* 313: 1126–1130.
39. Wilson, C. L., A. J. Ouellette, D. P. Satchell, T. Ayabe, Y. S. Lopez-Boado, J. L. Stratman, S. J. Hultgren, L. M. Matrisian, and W. C. Parks. 1999. Regulation of intestinal α-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 286: 113–117.
40. Wehkamp, J., and E. F. Stange. 2005. NOD2 mutation and mice: no Crohn's disease but many lessons to learn. *Trends Mol. Med.* 11: 307–309.