Down-Regulation of Human Enteric Antimicrobial Peptides by NOD2 during Differentiation of the Paneth Cell Lineage

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Ileal Crohn’s disease (CD) arising from the alteration of intestinal homeostasis is characterized by two features, namely a decrease in Paneth cell-produced antimicrobial peptides that play a key role in maintaining this balance and an increase in NOD2, an intracellular sensor. Although mutations in NOD2 are highly correlated with the incidence of CD, the physiological role of NOD2 in intestinal immunity remains elusive. Here, we show that NOD2 can down-regulate the expression of human enteric antimicrobial peptides during differentiation of the Paneth cell lineage. This finding, which links the decrease of human enteric antimicrobial peptides to increased NOD2 in ileal CD patients, provides a new view into the pathogenesis of ileal CD.

Crohn’s disease (CD), the main clinical phenotype of inflammatory bowel disease (IBD)1, is a chronic, relapsing inflammatory disorder2. Although CD can occur anywhere in the gastrointestinal tract, it primarily affects the terminal ileum where as many as 75% of CD patients have inflammation3. The terminal ileum is characterized by two relevant features: the greatest number of Paneth cells4 that are generally absent from the colon and rectum, except in IBD5, and the highest microbial density, which is low in healthy proximal small intestine3,6. Human Paneth cells serve as a key arm of innate mucosal immunity to maintain the intestinal homeostasis between a host and its colonizing microbes by secreting antimicrobial peptides7,8. These antimicrobial peptides are composed predominantly of human enteric α-defensin 5 and 6 (HD5 and HD6) as well as lysozyme and secretory phospholipase A2 (sPLA2), to a lesser extent9. These peptides not only have a strong antibacterial function against Gram-positive and Gram-negative bacteria, but they also have activity against viruses, fungi and protozoa10–12. Their antimicrobial activities contribute to their roles in intestinal innate immunity. In addition, human Paneth cells express NOD213, a member of the nucleotide-binding oligomerization domain-leucine-rich repeat (NOD-LRR) proteins14, and the Paneth cell expression of NOD2 is increased in CD patients13. Although mutations in NOD2 are highly correlated with a diminished expression of human enteric α-defensin15 and the incidence of CD16,17, the physiological role of NOD2 in intestinal immunity remains elusive.

The purpose of this study was to determine whether NOD2 may regulate the expression of human enteric antimicrobial peptides. For this purpose, we should choose a suitable cell line because human Paneth cells do not survive under in vitro culture conditions13,18. Because Caco2 intestinal epithelial cells can display characteristics of small intestinal epithelial differentiation in vitro19,20 and constitutively express the NOD2 gene13, they are suitable for in vitro studies to investigate the physiological role of NOD2 in specialized intestinal epithelial cells such as Paneth cells.

Results

Activation of FGFR-3-mediated signaling induces in vitro differentiation of Caco2 cells along the Paneth cell lineage. Although Caco2 cells can spontaneously differentiate along the enterocyte lineage in vitro21,22, they also express abundant FGFR-323, which is a critical regulator of Paneth cell differentiation during mouse gut development24. Therefore, we treated Caco2 cells with FGF9, a high affinity ligand for FGFR-324, and determined whether the activation of FGFR-3-mediated signaling induces in vitro differentiation of Caco2 cells along the Paneth cell lineage. We found that the mRNA expression of SI and APOA1, which encode two enterocyte differentiation markers25, was greatly decreased. These significant decreases were sustained for at least 72 h after a consecutive 3-day treatment with FGF9 (Fig. 1A and 1B), suggesting that the differentiation of Caco2 cells along the enterocyte lineage is suppressed and that this differential inhibition is stable. In contrast, we found...
that the mRNA expression of HD5, HD6, lysozyme and sPLA2, which encode four Paneth cell differentiation markers, was greatly increased after a consecutive 3-day treatment with FGF9 (Fig. 1C–1F). In addition, we found that these significant increases were sustained for at least 24 h after a consecutive 3-day treatment with FGF9 (Fig. 1C–1F). These results indicate that the activation of FGFR-3-mediated signaling can induce the in vitro differentiation of Caco2 cells along the Paneth cell lineage and maintain this induction of differentiation for a period of time.

NOD2 signaling down-regulates the expression of human enteric antimicrobial peptides during differentiation of the Paneth cell lineage. To determine the effect of the NOD2 gene on the expression of human enteric antimicrobial peptides, we first asked whether NOD2 regulates FGF9-induced expression of human enteric antimicrobial peptides during differentiation of the Paneth cell lineage. We utilized FGF9 with or without MDP, an agonist for NOD2, to stimulate Caco2 cells for 3 consecutive days and examined the mRNA expression of HD5, HD6, lysozyme and sPLA2 using real-time PCR. We found that the mRNA expression of HD5, HD6, lysozyme and sPLA2 was decreased approximately 10.6-, 9.6-, 2.7- and 2.3-fold, respectively, in Caco2 cells treated with MDP plus FGF9 compared with FGF9 only (Fig. 2A, 2C, 2E and 2G). This result indicates that MDP-NOD2 signaling can down-regulate the expression of human enteric antimicrobial peptides, especially enteric α-defensin, during differentiation of the Paneth cell lineage.

To further substantiate the role of NOD2 in regulating FGF9-induced expression of human enteric antimicrobial peptides, we transfected Caco2 cells with a NOD2-specific siRNA, followed by a consecutive 3-day stimulation with FGF9 or FGF9 plus MDP. We found that the mRNA expression of HD5, HD6, lysozyme and sPLA2 was significantly higher in Caco2 cells transfected with NOD2-siRNA than in untransfected or mock transfected cells (Fig. 2B, 2D, 2F and 2H), suggesting that NOD2 signaling can down-regulate the expression of human enteric antimicrobial peptides during differentiation of the Paneth cell lineage; however, we found no significant differences in the mRNA expression of HD5 and HD6 was significantly increased in FGF9-stimulated NOD2-siRNA-transfected cells compared with untransfected or mock transfected cells (Fig. 3A and 3B), thus further confirming that NOD2 signaling can down-regulate the expression of human enteric antimicrobial peptides during differentiation of the Paneth cell lineage.

NOD2 itself differentially regulates the expression of human enteric antimicrobial peptides. We next determined whether NOD2 itself can affect the expression of human enteric antimicrobial peptides. We treated Caco2 cells with the NOD2 agonist MDP and then determined the mRNA expression of HD5, HD6, lysozyme and sPLA2 using real-time PCR. We found that the mRNA expression of HD5 and HD6 was increased approximately 2.8- and 1.7-fold, respectively, in Caco2 cells after a consecutive 3-day treatment with MDP compared with untreated control (Ctrl).
cells (Fig. 2A and 2C). This result demonstrates that NOD2 itself can slightly up-regulate the expression of human enteric α-defensin 5 and 6, which is consistent with the report showing the decreased expression of Paneth cell α-defensins in NOD2-knockout mice; however, the mRNA expression of lysozyme and sPLA2 was not significantly different between MDP-treated cells and untreated control (Ctrl) cells (Fig. 2E and 2G), suggesting that NOD2 itself does not affect the expression of lysozyme and sPLA2.

**FGF9 does not regulate NOD2 expression during differentiation of the Paneth cell lineage.** Finally, we determined whether FGF9 treatment affected NOD2 expression during differentiation of the Paneth cell lineage. We treated Caco2 cells with FGF9 and determined the protein expression of NOD2 via immunoblotting at different times of induction of cell differentiation. We found that NOD2 protein expression was not significantly different between FGF9-treated cells and untreated control (Ctrl) cells (Fig. 4). This result indicates that FGF9 does not regulate NOD2 expression during differentiation of the Paneth cell lineage.

**Discussion**

This study assessed whether NOD2 can regulate the expression of human enteric antimicrobial peptides. Our data in Caco2 cells show that NOD2 can down-regulate the expression of human enteric antimicrobial peptides, specifically α-defensins 5 and 6. This finding is consistent with previous reports showing decreased expression of Paneth cell α-defensins in NOD2-knockout mice. However, the mRNA expression of lysozyme and sPLA2 was not significantly different between MDP-treated cells and untreated control cells, suggesting that NOD2 itself does not affect the expression of these proteins.

Additionally, the study investigated the role of FGF9 in regulating NOD2 expression during differentiation of the Paneth cell lineage. Treatment with FGF9 did not significantly affect NOD2 expression, indicating that FGF9 does not regulate NOD2 expression during this process.

Overall, these findings contribute to our understanding of the complex interplay between NOD2 and the expression of antimicrobial peptides in the gut epithelium, highlighting the importance of NOD2 in modulating the immune response and maintaining intestinal health.
Although NOD2 can down-regulate FGF9-induced expression of HD5, HD6, Lysozyme and sPLA2, NOD2 itself can up-regulate the expression of HD5 and HD6. We found that the mRNA expression of HD5 and HD6 was increased in MDP-stimulated cells compared with untreated control cells (Fig. 2). This result is in line with the previous report showing the decreased expression of Paneth cell α-defensins in NOD2-knockout mice. Because FGF9 does not affect NOD2 expression during differentiation of the Paneth cell lineage (Fig. 4), it is extremely interesting to explore the mechanism by which NOD2 dually regulates the expression of human enteric α-defensins under the different types of NOD2 stimuli.

In this study, we used Caco2 cells, serving as a functional model, to investigate whether NOD2 regulates the expression of human enteric antimicrobial peptides in the Paneth cell lineage. This cell line is suitable because primary Paneth cells do not survive in vitro, however, Caco2 cells can display characteristics of small intestinal epithelial differentiation in vitro, suggesting that they maintain intestinal stem cell functions. In addition, they constitutively express the NOD2 gene and also express abundant FGF-3, which is a critical regulator of Paneth cell differentiation during gut development. Finally, we found that Caco2 cells activated by FGF-3-mediated signaling for 3 consecutive days express Paneth cell lineage-specific genes. Thus, Caco2 cells are suitable for this in vitro study to investigate the role of the NOD2 protein in the Paneth cell lineage.

In summary, our results indicate that NOD2 can down-regulate the expression of human enteric antimicrobial peptides during differentiation of the Paneth cell lineage. In light of NOD2 over-expression in CD patients, our data provide a plausible explanation for the diminished levels of human enteric antimicrobial peptides in ileal CD patients. This finding is significant because a strongly advocated view is that the ineffective bacterial clearance that results from the reduced expression of human enteric antimicrobial peptides induces and sustains the abnormal adaptive immune responses observed in CD patients. In addition, T-helper 1 (Th1) cytokines such as TNFα and interferon-γ (IFNγ) can up-regulate NOD2 expression in intestinal epithelial cells. Based on these findings, a hypothesis for the role of NOD2 in the pathogenesis of CD is proposed (Fig. 5) in which NOD2 constitutes a critical link between the innate and adaptive immunity in the intestinal tract. Indeed, anti-TNFα, anti-IFNγ or anti-interleukin-12 administration is an effective therapeutic strategy in CD, although none are a permanent cure for CD. As discussed above, we speculate that if these cytokine-based therapies work by interrupting the over-expression of NOD2, the most effective therapy for CD patients will be directed at antagonism of NOD2-mediated inhibition of human antimicrobial peptides.

**Methods**

**Cell culture and stimulation.** Caco2 cells (ATCC) were cultured in Dulbecco’s modified Eagle medium (HyClone) supplemented with 20% fetal calf serum (HyClone), 2 mM L-glutamine, 100 U/ml Penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO2. The cells were used between passages 15 and 30. For all of the experiments, to better mimic the steric conditions existing in the intestine in vivo, the cells were plated at a subconfluent cell density onto 6-well...
Millicell hanging fiber inserts (3 mm pore size, Polyethylene Terephthalate, Millipore) were placed in low-access of media to their apical and basolateral sides. Media were changed every 24 h. To determine the role of NOD2, fibroblast growth factor 9 (FGF9) (10 ng/ml; R&D Systems), a high affinity ligand for the fibroblast growth factor receptor-3 (FGFR-3; muramyl dipeptide (MDP) (10 µg/ml; InvivoGen), an agonist for intracellular NOD2; and FGF9 (10 ng/ml) plus MDP (10 µg/ml) were added to both sides of the inserts daily starting at 24 h post-plating and ending at 72 h post-plating.

**SIRNA.** After 24-h culture with antibiotic-free normal growth medium containing 20% fetal calf serum, Caco2 cells that were approximately 60% confluent were transfected with the NOD2 siRNA (50 nM; Santa Cruz Biotechnology) and a non-targeting control siRNA. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were lysed for 30 min on ice in RIPA lysis buffer (10 mM Tris-pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.5% deoxycholate, supplemented with the protease inhibitor PMSE). After being centrifuged at 14,000 u for 30 min at 4°C, the supernatants were collected. SDS-polyacrylamide gel electrophoresis and western blotting were performed in accordance with standard protocols. Monoclonal mouse anti-HIS5 (Millipore), anti-HIS6 (Biorey) and polyclonal goat anti-NOD2 (Santa Cruz Biotechnology) were diluted at 1:1000 and 1:200, respectively. Monoclonal rabbit anti-GAPDH (Cell Signaling Technology) was diluted at 1:1000. Secondary antibodies were all diluted at 1:4000. Image J software was used to quantify and analyze the density of the protein bands.

**Statistical Analysis.** The results are shown as the mean ± standard deviation. Statistical significance was determined by one-way analysis of variance with Tukey's multiple comparisons under equal variances or with Dunnett T3's multiple comparisons under unequal variances; a value of P < 0.05 was considered statistically significant.

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**Author contributions**

G.T. designed the studies, performed the experiments, wrote the manuscript and prepared the table and figures. F.Z. conceived the studies and reviewed the manuscript. R.L., C.L., X.Z., F.W., J.M., S.L. and W.Z. reviewed the manuscript.

**Additional information**

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