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Inflammatory Bowel Disease Susceptibility Gene C1ORF106 Regulates Intestinal Epithelial Permeability

Paolo Manzanillo,*,1 Maria Mouchess,*,1 Naruhisa Ota,* Bingbing Dai,* Ryan Ichikawa,† Arthur Wuster,‡ Benjamin Haley,§ Gabriela Alvarado,* Youngsu Kwon,* Roger Caothien,§ Meron Roose-Girma,§ Soren Warming,§ Brent S. McKenzie,§ Mary E. Keir,† Alexis Scherl,|| Wenjun Ouyang,*,1 and Tangsheng Yi*  

*Department of Immunology Discovery, Genentech Inc., South San Francisco, CA 94080; †Department of Biomarker Discovery, Genentech Inc., South San Francisco, CA 94080; ‡Department of Human Genetics, Genentech Inc., South San Francisco, CA 94080; §Department of Molecular Biology, Genentech Inc., South San Francisco, CA 94080; Department of Translational Immunology, Genentech Inc., South San Francisco, CA 94080; and ||Department of Pathology, Genentech Inc., South San Francisco, CA 94080

ABSTRACT

Intestinal epithelial cells form a physical barrier that is tightly regulated to control intestinal permeability. Proinflammatory cytokines, such as TNF-α, increase epithelial permeability through disruption of epithelial junctions. The regulation of the epithelial barrier in inflammatory gastrointestinal disease remains to be fully characterized. In this article, we show that the human inflammatory bowel disease genetic susceptibility gene C1ORF106 plays a key role in regulating gut epithelial permeability. C1ORF106 directly interacts with cytohesins to maintain functional epithelial cell junctions. C1orf106-deficient mice are hypersensitive to TNF-α–induced increase in epithelial permeability, and this is associated with increased diarrhea. This study identifies C1ORF106 as an epithelial cell junction protein, and the loss of C1ORF106 augments TNF-α–induced intestinal epithelial leakage and diarrhea that may play a critical role in the development of inflammatory bowel disease. ImmunoHorizons, 2018, 2: 164–171.

INTRODUCTION

Inflammatory bowel disease (IBD), composed of Crohn’s disease and ulcerative colitis, is characterized by chronic intestinal inflammation and affects nearly 1 in 200 people in the United States. Twin studies, familial risk data, and genomewide association studies of IBD patients have implicated pathways involved in inflammatory response, host–pathogen interactions, and epithelial barrier function (1, 2). How these genetic variants, gene pathways, and the intestinal microbiome interact and affect the cause of IBD remains poorly understood.

The intestinal epithelial cells maintain a selectively permeable barrier under homeostatic conditions through the interactions of tight junction proteins, such as ZO-1 and occludin (3). TNF-α, a proinflammatory cytokine secreted by activated T cells and monocytes, disrupts the epithelial barrier function through disrupting tight junctions and directly inducing epithelial cell death (4). Compromised epithelial barrier function leads to increased exposure of the immune system to gut microbiota, subsequently leading to further aberrant and chronic activation of innate and adaptive immunity within the gut. TNF-α inhibitors...
were highly effective in a subset of IBD patients with rapid restoration of mucosal epithelial integrity. Mechanisms by which TNF-α affects other tight junction proteins to regulate epithelial barrier integrity and IBD pathobiology remain to be further understood.

**MATERIALS AND METHODS**

**Mice**

All of the mice used in this study were bred and maintained at Genentech under specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Committee. C1orf106 (5730559C18Rik) knockout (KO) mice were generated at Genentech. A targeting vector was used to flank exons 2 and 3 by loxP sites in C57BL/6N embryonic stem (ES) cells (derived from C2) by standard gene-targeting methods. The 1.2-kb floxed region corresponds to GRCm38/mm10 chr1:136226612-136227834. The KO allele was generated from the conditional KO allele by Cre treatment at the ES cell stage, and C1orf106 KO mice were obtained using standard methods and maintained on the C57BL/6N genetic background.

**RNA isolation and quantitative real-time PCR analysis**

RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad). Data were analyzed with software to obtain cycle threshold (Ct) values. Quantitative real-time PCR analysis was performed using Applied Biosystems 7500 Real-Time PCR System. The relative abundance (ΔΔCt) to rpl19 was calculated as follows: 2^−(average Ct gene − average Ct Rpl19). TaqMan probes were purchased from Life Technologies.

**Western blotting and immunoprecipitation**

Cells were rinsed with 1× PBS and lysed in radioimmunoprecipitation assay lysis buffer containing a protease inhibitor mixture. Lysates were cleared, and the supernatant was collected for protein concentration estimation using a Pierce bicinchoninic acid assay. For immunoprecipitation, lysates were incubated with FLAG agarose beads (Sigma-Aldrich). Reduced samples were separated by SDS-PAGE and transferred onto nitrocellulose using iBlot, per recommended protocol. Blots were incubated overnight at 4°C with Ab prepared in blocking buffer. After washes, the membrane was incubated with appropriate HRP-conjugated secondary Ab.

**Immunofluorescence staining and microscopy**

Cells were washed with PBS, incubated in 4% (w/v) paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with blocking buffer (0.2% Triton X-100, 0.05% Tween 20, 5% milk in PBS). Cells were incubated in appropriate concentrations of primary Ab in blocking buffer, washed, and incubated with secondary host-specific anti-IgG Alexa Abs (Invitrogen). Cells were mounted on coverslips containing ProLong Gold mounting medium with DAPI. Images were acquired with a Leica TCS SPE confocal microscope (ZEISS) using a 63× or 40× objective and presented as a single section.

**Colon and fecal water determination**

Fresh stools or colons were collected in presterilized 1.5-ml tubes and immediately sealed. After weighing, tubes were uncapped and completely desiccated by incubation in a dry oven at 60°C for 24 h. Tubes were then reweighed, and water was determined as the fraction of total mass lost upon desiccation.

**FITC-dextran measurement and cytokine treatment**

C1ORF106 wild-type (WT) and KO mice were denied access to food but allowed water for 16 h prior to gavage with 0.2 ml of saline containing 250 mg FITC–4 kDa dextran or 20 mg recombinant murine TNF-α (purified in Genentech) and analyzed 4 h posttreatment.

**Infection of Citrobacter rodentium and Salmonella typhimurium**

*C. rodentium* was cultured in Luria broth overnight, and mice were inoculated orally with 2 × 10⁹ CFUs of bacteria, as described previously (5). The highly virulent *S. Typhimurium* strain SL1344 was grown by previously established methods (6). A single inoculum of bacteria was grown in Lysogeny broth under aerated (shaking) conditions at 37°C for 8 h. Cultures at late log phase (OD600 of 0.45–0.65) were collected, washed, and resuspended in sterile PBS at a concentration of 5 × 10⁹ CFUs/ml. Mice were made to fast for 3–4 h before oral gavage of *S. Typhimurium* at a dose of 1 × 10⁹ CFUs in 200 µl sterile saline.

**Helicobacter hepaticus and anti–IL-10R Ab–induced experimental colitis**

Experimental colitis was induced as described previously (7). Briefly, mice were fed 1 × 10⁹ CFUs of *H. hepaticus* by oral gavage delivered with a 22 gauge curved, blunted needle on days 0 and 1 of the experiment. One milligram of an IL-10R–blocking Ab (clone 1B1.2; Bio X Cell) was administered as an i.p. injection once weekly starting at day 0 every week for 4 wk.

**Quantification and statistical analysis**

All data are representative of at least three independent experiments with 3–10 mice per group. Specific numbers of mice per group are annotated in the corresponding figure legends. Data are presented as mean ± SEM. Statistical significance was determined by two-tailed Student t test, two-tailed Mann–Whitney U test, or Kaplan–Meier log-rank test. The *p* values are indicated in the figures (*p* < 0.05, **p** < 0.01, ***p*** < 0.001).
Colon histology and immunohistochemistry

Colons were prepared as a “Swiss roll,” fixed in formalin, and processed and embedded into paraffin blocks. Five-micron sections were cut and stained with H&E for histological assessment. Immunohistochemistry for cleaved caspase 3 (#9661L; Cell Signaling Technology) was performed on formalin-fixed, paraffin-embedded intestines using a standard automated protocol.

Yeast two-hybrid

Yeast two-hybrid (Y2H) assays were performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech) according to the manufacturer’s instructions. For the Y2H screen, C1orf106(NM_018265.3) was cloned into the bait vector PGBKT7 and transformed into the Y2H gold strain. The bait strain was mated to the Universal Human Matchmaker Library (Clontech) and selected on SD/-Leu/-Trp agar plates containing Aureobasidin A and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-a-Gal). Positive blue colonies were then repatched on higher stringency quadruple dropout SD/-Ade/-His/-Leu/-Trp agar plates. Positive hits were sequenced using Matchmaker AD LD-Insert Screening Amplimer Set (Clontech).

RESULTS

IBD susceptibility single-nucleotide polymorphisms are associated with reduced C1ORF106 expression in epithelial tissues

Common risk alleles within the C1ORF106 locus have been associated with gastrointestinal disease in both IBD and celiac disease cohorts (Fig. 1A). Fine mapping of the loci reveals multiple missense, intronic, and intergenic single-nucleotide polymorphisms (SNPs) linked to increased disease susceptibility within the C1ORF106 locus (6, 9–12). C1ORF106 is highly expressed in epithelial-rich tissues in both human and mouse, particularly in the gastrointestinal tract, as well as in other tissues with key roles in barrier function, such as skin (Fig. 1B).
To determine which cell types of the gastrointestinal tract express C1ORF106, we isolated CD45+ leukocytes and epithelial cells from human colonic biopsy specimens. Sort-purified Epcam1+ epithelial cells, but not CD45+ leukocytes, express high levels of C1orf106 transcript (Fig. 1C) and protein (Fig. 1D). Immunoﬂuorescence staining of C1ORF106 on human colon epithelial cell line Caco2 revealed expression of C1ORF106 in the epithelial junctions through colocalization with occludin (Fig. 1E).

A recent study revealed a reduction of C1orf106 protein expression with a rare Tyr->Phe coding variant (rs41313912, minor allele frequency <1%), which is associated with increased risk of IBD (13). We therefore assessed the gene expression of C1orf106 in various tissues from healthy volunteers bearing either the C allele or A allele at rs7554511 (Fig. 1F). Reduced gene expression of C1ORF106 is associated with the IBD risk rs7554511 C allele in esophagus, skin, and colon of healthy subjects (Supplemental Fig. 1) as well as in colonic biopsy specimens from IBD patients (Fig. 1F). The enriched expression of C1ORF106 in colonic tissues and at epithelial junctions suggested a potential role of this protein in regulating epithelium function in IBD.
C1ORF106 directly interacts with cytohesins through its DUF3338 domain

C1ORF106 has no previously characterized functional domains except for a DUF3338 (domain of unknown function) motif at the N-terminus. To elucidate the function of C1ORF106, we performed a Y2H screening using full-length human C1ORF106 as bait against a universal human Y2H prey library. Eighty-four hits were identified in the screen, with the majority (58 of 84, 69%) belonging to the cytohesin family of proteins (Fig. 2A). The interaction between C1ORF106 and cytohesins was validated through pairwise direct Y2H β-galactosidase assays, which showed activity of CYH1, CYH2, and CYH3 against full-length C1ORF106 (Fig. 2B). Truncated versions of C1ORF106 demonstrated that the DUF3338 domain was essential and sufficient to interact with cytohesin proteins in the Y2H assay (Fig. 2C). NanoBRET assays measuring direct protein interactions in transfected 293T cells showed association of cytohesins and C1ORF106 in mammalian cells (Fig. 2D). Two additional cytohesin adherens junction scaffold proteins, FRMD4A and FRMD4B, share sequence homology with C1ORF106 and have also been shown to contain DUF3338 domains (14). Similar interaction between C1ORF106 and cytohesins was also revealed by an independent mass spectrometry–based approach (13). These data suggest that DUF3338 is a novel cytohesin interaction domain and mediates C1ORF106 interaction with cytohesins.

Impaired cytohesin localization and cell junction formation in C1ORF106-deficient human colonic epithelial cells

Cytohesins belong to a family of protein-sorting molecules involved in vesicle trafficking and activation of the ADP ribosylation factor (ARF)–guanine exchange factor (GEF) complex (15). Within epithelial cells, cytohesins have been shown to play a key role in the assembly of tight and adherens junctions important for maintaining cell–cell adhesions critical to the intestinal barrier. Inhibition of cytohesin using a small molecule antagonist impairs proper cell junction formation and delays epithelial polarization (14). In addition, other DUF3338-containing and cytohesin-interacting proteins (e.g., FRMD4A/B) have been implicated in the formation of cell junctions (14). Therefore, we next asked whether C1ORF106 interacts with cytohesins to maintain cell–cell junctions and epithelial barrier function. Accordingly, we generated primary colonic organoids from human colon biopsy specimens, and lentiviral CRISPR guide RNAs against C1ORF106 were used to ablate C1ORF106. Genetic deletion of C1ORF106 was further confirmed by Western blotting (Fig. 3A). Epithelial integrity was significantly compromised in C1ORF106-deficient epithelial cells as measured by transepithelial electrical resistance assays using monolayer cultures generated from primary cells (Fig. 3B). Cytohesin-1 colocalizes with ZO-1 and other cell junction proteins and is critical for primordial junction formation in C1ORF106-depleted epithelia (Fig. 3C).
formation (6). To test whether C1ORF106 may function in directing the localization of cytohesin-1 to ZO-1–containing primordial junctions, FLAG-tagged cytohesin-1 was transfected into WT or C1ORF106-deficient human colon epithelial cells. Immunostaining of endogenous ZO-1 and cytohesin-1 in epithelial monolayers derived from WT and C1ORF106-deficient human colon primary cells confirmed changes in the localization and association of these proteins in the absence of C1ORF106 (Fig. 3C, 3D). Whereas cytohesin-1 and ZO-1 are colocalized to the cell membrane in control human colon epithelial cells, reduced cell membrane association of both proteins was observed in C1ORF106-deficient cells. The presence of intracellular stress granules suggests incomplete formation of cell junctions and barrier function in the absence of C1ORF106 (Fig. 3D). Taken together, our data suggest C1ORF106 regulates cytohesin localization at cell junctions and is essential to maintain intestinal epithelial barrier function.

**C1orf106-deficient mice exhibit normal baseline epithelial barrier function and innate host defense mechanisms**

C1ORF106 has a mouse homolog of 5730559C18rik, and we generated a global KO mouse of 5730559C18rik by targeting the DUF3338 domain containing exons 2 and 3 to cause a frame shift in the open reading frame (designated C1orf106 KO mice). C1orf106-deficient mice were born in normal Mendelian ratios, and expression of cell junction proteins was largely unchanged (data not shown). Histopathological analyses of small intestine and colon from C1orf106 KO mice did not reveal obvious abnormalities (data not shown). Because the above data supported a potential role of C1ORF106 in maintaining intestinal barrier function, we next evaluated the potential in vivo defects in epithelial barrier integrity. WT or C1orf106 KO mice were gavaged with 4 kDa FITC-dextran or creatinine, which have hydrodynamic diameters of 28 or 6 Å, respectively. Neither FITC-dextran nor creatinine were found at increased levels in the serum 4 h after gavage in C1orf106 KO mice.
KO mice (Fig. 4A, 4B). These data suggest that in the absence of epithelial injury, C1ORF106 does not play a critical role in intestinal epithelial barrier function. To assess the role in the context of epithelial injury, we used the dextran sulfate sodium (DSS)–induced colitis model, which is triggered by chemically induced epithelial damage, to further evaluate whether lack of C1orf106 results in increased sensitivity to epithelial damage. Similar body weight loss and histological epithelial damage by 1.5% and 3% DSS was seen in mice with or without C1orf106 deficiency (Fig. 4C, 4D). Intestinal epithelial cells also exert essential innate defense mechanisms to control luminal commensal pathogens, such as C. rodentium, and work together with innate immune cells to control S. typhimurium. C1orf106-decient mice did not exhibit defective bacterial clearance of the enteric pathogens C. rodentium and S. typhimurium (Fig. 4E–H), indicating that antibacterial responses are grossly normal. In the anti–IL-10/H. hepaticus colitis model with IL-23–driven immune responses, no difference in pathologic condition was observed between WT and C1orf106-deficient mice (Fig. 4I). These data suggested that deficiency of C1orf106 is not sufficient to induce IBD pathologic condition, but the possibility remains that C1ORF106 may have synergistic effects with other inflammatory mediators.

C1orf106-deficient mice showed exacerbated barrier defect upon inflammatory insults such as TNF-α and LPS

The proinflammatory cytokine TNF-α has been shown to increase epithelial permeability and disrupt the barrier function. TNF-α inhibitors have been widely used in human IBD, with evidence of mucosal healing in patients that shows clinical benefit (16–18). We next asked whether C1ORF106 deficiency could exacerbate TNF-α–induced epithelial permeability. Accordingly, we injected recombinant murine TNF-α into C1orf106-deficient mice. TNF-α administration induced an increase in the weight-to-length ratio of the jejunum and increased fecal water content in C1orf106-deficient mice (Fig. 5A, 5B). Gross examination of the colon of WT and C1orf106 KO mice following TNF administration revealed apparent diarrhea, with loose stool in the rectums of KO mice (Fig. 5C). The diarrhea and increased luminal fluid is likely driven by the disruption of epithelial junctions because increased FITC-dextran leakage was seen with C1orf106 KO mice injected with TNF-α (Fig. 5D). C1orf106-deficient mice also exhibited defects in response to bacterial pathogens and/or stimuli, such as LPS. After LPS injection, increased epithelial apoptosis was present in the ileum of the C1orf106 KO mice (Fig. 5E), as indicated by increased numbers of apoptotic cell bodies on H&E-stained slides and increased numbers of epithelial cells stained positively for cleaved caspase 3 by immunochemistry (Fig. 5E).

DISCUSSION

The current study reveals C1ORF106 as a cell junction protein regulating epithelial junction formation and permeability. Reduced C1ORF106 expression is associated with increased IBD risk. C1ORF106 interacts with ARF–GEF cytohesins through its DUF3338 domain. Deficiency of C1orf106 compromised barrier function under certain proinflammatory conditions and led to increased fecal water content and diarrhea. During the preparation of this article, an independent study also demonstrated a role for C1ORF106 in regulating epithelial adherens junctions (13).
additional data provided in this study support that C1orf106 deficiency may have synergic effects with other proinflammatory stimuli such as TNF-α, a clinically validated therapeutic target, for IBD disease pathogenesis. We also determined that the common risk SNP rs755451I reduces C1ORF106 expression in human colon biopsy specimens. Anti-TNF-α may be particularly effective in IBD patients with reduced C1ORF106 expression or mutations, and future studies should investigate whether C1ORF106-dependent epithelial barrier function is related to clinical responsiveness to TNF-α inhibition. Contrary to the report by Mohanan et al. (12), we did not observe that C1ORF106 deficiency directly influences the enteric bacterial infection or experimental colitis induced by DSS or H. hepaticus. This could be due to the different microbiome environment between two animal facilities, and future works are needed to understand additional environmental factors that may cooperate with C1ORF106 in intestinal inflammation regulation. Our work adds human genetic evidence that impaired barrier function contributes to the IBD pathophysiology, and targeted therapy for epithelial barrier restoration may provide benefit for IBD patients without general immunosuppression.

DISCLOSURES

All authors are current or previous employees of Genentech, Roche and have stock in Roche. P.M. and W.O. are current employees of Amgen, Inc.

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