STIM1 Deficiency In Intestinal Epithelium Attenuates Colonic Inflammation and Tumorigenesis by Reducing ER Stress of Goblet Cells

Xiaojing Liang,1,* Jiansheng Xie,1* Hao Liu,1 Rongjie Zhao,1 Wei Zhang,1 Haidong Wang,1 Hongming Pan,1 Yubin Zhou,2 and Weidong Han1

1Department of Medical Oncology, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, People's Republic of China; 2Center for Translational Cancer Research, Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, Texas

SUMMARY

Stromal interaction molecule 1 deficiency in intestinal epithelium reduces goblet cell endoplasmic reticulum stress and subsequent cell loss induced by stressors through attenuating Ca2+ overload, maintaining the mucus layer, and decreasing microbial exposure, therefore, rendering intestinal epithelium-specific stromal interaction molecule 1 conditional knockout mice less susceptible to colitis and colitis-associated colorectal cancer.

BACKGROUND & AIMS: As an indispensable component of store-operated Ca2+ entry, stromal interaction molecule 1 (STIM1) is known to promote colorectal cancer and T-cell–mediated inflammatory diseases. However, whether the intestinal mucosal STIM1 is involved in inflammatory bowel diseases (IBDs) is unclear. This study aimed to investigate the role of intestinal epithelial STIM1 in IBD.

METHODS: Inflammatory and matched normal intestinal tissues were collected from IBD patients to investigate the expression of STIM1. Intestinal epithelium-specific STIM1 conditional knockout mice (STIM1<sup>ΔIEC</sup>) were generated and induced to develop colitis and colitis-associated colorectal cancer. The mucosal barrier, including the epithelial barrier and mucus barrier, was analyzed. The mechanisms by which STIM1 regulate goblet cell endoplasmic reticulum stress and apoptosis were assessed.

RESULTS: STIM1 could regulate intestinal epithelial homeostasis. STIM1 was augmented in the inflammatory intestinal tissues of IBD patients. In dextran sodium sulfate–induced colitis, STIM1 deficiency in intestinal epithelium reduced the loss of goblet cells through alleviating endoplasmic reticulum stress induced by disturbed Ca2+ homeostasis, resulting in the maintenance of the integrated mucus layer. These effects prevented commensal bacteria from contacting and stimulating the intestinal epithelium of STIM1<sup>ΔIEC</sup> mice and thereby rendered STIM1<sup>ΔIEC</sup> mice less susceptible to colitis and colitis-associated colorectal cancer. In addition, microbial diversity in dextran
sodium sulfate–treated STIM1\textsuperscript{IEC} mice slightly shifted to an advantageous bacteria, which further protected the intestinal epithelium.

**CONCLUSIONS:** Our results establish STIM1 as a crucial regulator for the maintenance of the intestinal barrier during colitis and provide a potential target for IBD treatment. (Cell Mol Gastroenterol Hepatol 2022;14:193–217; https://doi.org/10.1016/j.jcmgh.2022.03.007)

Keywords: IBD; Mucosal Barrier; Goblet Cells; ER Stress.

Under normal conditions, the intestinal tract is filled with kilograms of microbes, and the luminal commensal bacteria is separated from the intestinal mucosa, especially from the lamina propria, which is involved in innate and adaptive immune responses to defend against commensal bacteria.\textsuperscript{1} The complete separation of commensal bacteria from intestinal epithelium is mainly dependent on the mucosal barrier provided by intestinal epithelial cells (IECs), including a monolayer epithelial barrier and an extracellular mucus barrier. The epithelial barrier is formed by IECs that are arranged in a single layer with apical junctional complexes, including tight junctions and adherens junctions, that constitute strong adhesive bonds to seal the paracellular space and defend against bacteria passage.\textsuperscript{2} IECs are covered with a 2-layer mucus barrier, which forms the first line of defense in the intestinal mucosal barrier. The loose outer mucus layer provides a natural habitat for commensal bacteria. The dense inner mucus layer is firmly attached to epithelial cells and does not allow commensal microbes to penetrate, forming the primary structure of the mucus barrier.\textsuperscript{3}

Inflammatory bowel diseases (IBDs), including Crohn’s disease and ulcerative colitis, are characterized by dysfunction of the mucosal barrier and alterations in microbial community.\textsuperscript{4} The inner mucus layer is attenuated and can be penetrated by commensal bacteria in IBD patients and dextran sodium sulfate (DSS)-induced colitis mouse models. The variable bacteria that attached to and penetrated IECs can activate immune responses to produce inflammatory cytokines, including interferon-\(\gamma\) and tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)), which can increase epithelial permeability and induce epithelial barrier dysfunction by disrupting epithelial cell junctions.\textsuperscript{5} The immune responses activated by defective mucosal barriers and microbial dysbiosis promote the accumulation of inflammatory signals, causing chronic or recurrent relapsing colonic inflammation, pathologic proliferation and apoptosis in epithelium, and even triggering tumorigenesis of colon cancer.\textsuperscript{6–9}

As the major component of the mucus layer, Mucin-2 (MUC2) is generated by goblet cells. An appropriate number of goblet cells and their proper functions in MUC2 synthesis, folding, and secretion are essential for maintaining the mucus layer. Indeed, dysfunctions in goblet cells are the known characteristics of IBD.\textsuperscript{10–13} The glycosylation and folding of MUC2 in endoplasmic reticulum (ER) are controlled by calreticulin and calnexin, which require steady

ER–Ca\textsuperscript{2+} homeostasis.\textsuperscript{14–17} Disturbed ER–Ca\textsuperscript{2+} homeostasis can affect the calnexin/calreticulin pathway,\textsuperscript{18} causing MUC2 misfolding in ER. Furthermore, aberrant MUC2 folding induces ER stress in goblet cells.\textsuperscript{19} Studies in murine models have shown that severe or chronic ER stress can lead to goblet cell apoptosis, which further impairs the mucus layer and increases microbial exposure, confirming that abnormal ER stress is one of the triggers for the pathogenesis of IBD.\textsuperscript{20–22} Hence, ameliorating goblet cell ER stress by maintaining ER–Ca\textsuperscript{2+} homeostasis is regarded as a potential interventional strategy to alleviate colitis.

Stromal interaction molecule 1 (STIM1) is the major component of store-operated Ca\textsuperscript{2+} entry (SOCE) to mediate Ca\textsuperscript{2+} influx by sensing Ca\textsuperscript{2+} reductions in ER and gating ORAI calcium release-activated calcium modulator (ORAI) channels in plasma membrane. Previous studies have shown that STIM1 is overexpressed in many cancers, including colorectal cancer.\textsuperscript{23} STIM1 boosts the production of proinflammatory cytokines, especially cyclooxygenase 2, to enhance the progression and motility of colorectal cancer, indicating that STIM1 promotes the inflammatory response during tumorigenesis.\textsuperscript{24} In lung endothelial cells, blockade of STIM1 significantly reduced lipopolysaccharide-induced lung inflammation. Ca\textsuperscript{2+} oscillations and nuclear factor of activated T Cells (NFAT) - nuclear accumulation were abrogated in STIM1\textsuperscript{IEC} mice, thereby leading to the decrease in proinflammatory signaling and oxidative stress.\textsuperscript{24} Moreover, STIM1 is essential for the differentiation and function of T cells. STIM1-deficient T cells were incapable of inducing autoimmunity in autoimmune diseases, including IBD, owing to impaired helper T cell 17 effector functions. Other studies further confirmed that STIM1 could facilitate pathogenic helper T cell 17 cells to exacerbate intestinal inflammation.\textsuperscript{25–28} Consistently, the STIM1-mediated SOCE in T cells isolated from the lamina propria (LP) of IBD patients was increased when compared with that of healthy individuals.\textsuperscript{29}

In this study, we collected intestinal tissues from IBD patients and confirmed the overexpression of STIM1 in inflammatory tissues. To investigate the effects of intestinal

\textsuperscript{*}Authors share co-first authorship.

**Abbreviations used in this paper:** CAC, colitis-associated colorectal cancer; CdH1, Cadherin 1; DSS, dextran sodium sulfate; ER, endoplasmic reticulum; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HBSS, Hank’s balanced salt solution; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; iHCl, immunohistochemistry; IL, interleukin; LP, lamina propria; mLN, mesenteric lymph node; mRNA, messenger RNA; MUC2, Mucin-2; ORAI, ORAI calcium release-activated calcium modulator; PAS, periodic acid–Schiff; PBS, phosphate-buffered saline; qPCR, quantitative polymerase chain reaction; sIgA, secretory IgA; SOCE, store-operated Ca\textsuperscript{2+} entry; STIM1, stromal interaction molecule 1; THP, \(\text{\textsuperscript{1}}\text{H}_{\text{\textsuperscript{3}}}\)-labeled STIM1\textsuperscript{IEC} mice; STIM1\textsuperscript{IEC}, intestinal epithelium-specific STIM1 conditional knockout mice; TG, thapsigargin; Tn, tunicamycin; TNF–\(\alpha\), tumor necrosis factor \(\alpha\); UEA-1, ulex europaeus agglutinin-1; WB, Western Blots; Z01, Zonula Occludens 1.

© 2022 The Authors. Published by Elsevier Inc. on behalf of the AGA Institute. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
mucosal STIM1 in IBD, we generated intestinal epithelium-specific STIM1 conditional knockout mice (STIM1\textsuperscript{ΔIEC}) and identified the important role of intestinal epithelial STIM1 in promoting colitis. STIM1 deficiency specifically in intestinal epithelium significantly reduced goblet cell loss through the inhibition of ER stress, maintaining the integrity of the inner mucus layer, protecting intestinal epithelium from the stimulation by commensal bacteria, and thereby alleviating DSS-induced colitis and tumorigenesis of colitis-associated colorectal cancer (CAC).

Results

Enhanced Expression of STIM1 in IBD Patients

STIM1 has been confirmed to be overexpressed in various cancers, but the expression level of STIM1 protein in IBD patients has not been reported. We collected inflammatory and matched-adjacent normal intestinal tissues from IBD patients. Western Blots (WB) results showed that STIM1 was expressed at significantly higher level in inflammatory tissues than that in matched normal tissues (Figure 1A), immunohistochemistry (IHC) and immunofluorescence results showed that the overexpression of STIM1 was found in both IECs and LP (Figure 1B–F).

STIM1 Deficiency in Intestinal Epithelium Has No Effect on Mouse Growth or Epithelial Differentiation

Previous results have confirmed that STIM1-mediated SOCE in LP of IBD patients was increased,\textsuperscript{25} but enhanced STIM1 in IECs of IBD patients was found. To identify the role of epithelia STIM1 in intestinal inflammation, we generated STIM1\textsuperscript{ΔIEC} mice with conditional deletion of STIM1 in intestinal epithelia. Quantitative polymerase chain reaction (qPCR), WB and IHC assays were performed to verify STIM1 knockout. We found that STIM1 expression level was substantially and specifically reduced in intestinal epithelium of STIM1\textsuperscript{ΔIEC} mice (Figure 2A–D). Next, we detected the body weight, colonic villus height, ORAI1 expression, and genes related to epithelial differentiation between STIM1\textsuperscript{ΔIEC} mice and their wild-type littersmates, and observed no significant difference (Figure 2E–K). These results indicate that STIM1 ablation in intestinal epithelium has no effect on mouse growth or epithelial differentiation.

STIM1 Deficiency in Intestinal Epithelium Alleviates DSS-Induced Colitis

To identify the role of STIM1 in colitis development, STIM1\textsuperscript{ΔIEC} mice and their wild type littersmates (STIM1\textsuperscript{IIEC}) were administered 2% DSS to induce colitis. STIM1 depletion in intestinal epithelium led to a protective effect during DSS treatment. The weight loss of STIM1\textsuperscript{ΔIEC} mice was lower than that of STIM1\textsuperscript{IIEC} mice after DSS removal. On day 9, the body weights of most STIM1\textsuperscript{ΔIEC} mice began to recover, whereas all STIM1\textsuperscript{IIEC} mice still suffered sustained weight loss (Figure 3A). In addition, the disease activity index, which reflected weight loss, stool consistency, and fecal blood presence, also was decreased in STIM1\textsuperscript{ΔIEC} mice (Figure 3B). Other clinical parameters that indicate colitis severity, such as splenomegaly and colon length, were assessed, and the results further indicated that STIM1 deficiency in intestinal epithelium reduced the susceptibility to DSS-induced colitis (Figure 3C–E). Histologically, the colon tissues of STIM1\textsuperscript{ΔIEC} mice maintained basic structure, with mild inflammatory cell infiltration and ulceration. By contrast, STIM1\textsuperscript{IIEC} mice showed severe ulceration, massive inflammatory cell infiltration, and complete loss of epithelial structure, resulting in a higher histologic activity index (Figure 3F and G).

To confirm the role of STIM1, we examined the inflammatory cytokines of experimental mice. A previous study reported increased serum secretory IgA (slgA) in IBD patients\textsuperscript{30}; in this study, we detected a 4-fold reduction of serum slgA level in STIM1\textsuperscript{ΔIEC} mice (Figure 3H). Interleukin (IL)6 and TNF-α were decreased in the sera, colon tissues, spleen, mesenteric lymph node (mLN), and thymus of STIM1\textsuperscript{ΔIEC} mice, although the difference in TNF-α levels was not statistically significant (Figure 4A–E). In addition, qPCR analyses of inflammatory cytokines in colon tissues showed that STIM1\textsuperscript{ΔIEC} mice produced lower levels of IL1β, IL6, TNF-α, inducible nitric oxide synthase, cyclooxygenase 2, and cathelicidin antimicrobial peptide (Camp) than STIM1\textsuperscript{IIEC} mice (Figure 4F).

Collectively, these results suggest that STIM1 deficiency in intestinal epithelium alleviates DSS-induced colitis.

STIM1 Deficiency in Intestinal Epithelium Facilitates Epithelial Barrier Maintenance

To explore the mechanism by which colitis is alleviated in STIM1\textsuperscript{ΔIEC} mice, we analyzed differential gene expression of colon tissues from DSS-treated mice by RNA sequencing. The results showed great distinction between STIM1\textsuperscript{IIEC} and STIM1\textsuperscript{ΔIEC} mice (Figure 5A). Gene Ontology analysis of differentially expressed genes between STIM1\textsuperscript{IIEC} and STIM1\textsuperscript{ΔIEC} mice identified significant enrichment in cell adhesion and cell junction among biological processes and cellular components (Figure 5B and C). Kyoto Encyclopedia of Genes and Genomes pathway analysis showed enriched cell adhesion molecules and extracellular matrix–receptor interaction (Figure 5D). Furthermore, genes related to cell junctions, which are essential for epithelial barrier, including Cadherin 1 (Cdh1), Zonula Occludens 3 (ZO3), occludin, Cldn2, Cldn3, Cldn7, Cldn8, and Cldn15, were upregulated in STIM1\textsuperscript{ΔIEC} mice (Figure 5E). These results indicated that STIM1 deficiency in the intestinal epithelium is beneficial for the maintenance of integrated epithelial barrier.

To further confirm the role of STIM1 in the epithelial barrier, fluorescein isothiocyanate (FITC)–dextran was used to examine intestinal permeability. The decreased FITC–dextran level in the serum of STIM1\textsuperscript{ΔIEC} mice showed a more integrated epithelial barrier in STIM1\textsuperscript{ΔIEC} mice than that in STIM1\textsuperscript{IIEC} mice with DSS administration (Figure 6A).
Figure 1. Enhanced expression of STIM1 in IBD patients. (A) Protein expression of STIM1 in inflammatory and matched-adjacent normal intestinal tissues from IBD patients. (B–D) IHC staining and IHC scores showing STIM1 expression in IECs and LP of IBD patients (n = 25). Scale bars: 100 μm, boxed pullout: 50 μm. Twenty-five random numbers between 0 and 0.2 were generated and added to the IHC score of each sample to avoid an overlap of points. (E) Representative immunofluorescence of CD45 (green) and STIM1 (red) in intestinal tissues from IBD patients. Scale bars: 100 μm. (F) Representative immunofluorescence of EpCAM (green) and STIM1 (red) in intestinal tissues from IBD patients. Scale bars: 100 μm. The results are presented as the means ± SEM. ****P < .0001, **P < .01, and *P < .05. (C and D) Paired Student t test for IHC scores without random numbers. DAPI, 4',6-diamidino-2-phenylindole.
Consistently, with DSS treatment, messenger RNA (mRNA) levels of cell adhesion genes, including occludin, Cdh1, zonula occludens, and claudins, were up-regulated in STIM1ΔIEC mice IECs, which corresponded with the significantly up-regulated protein levels of occludin, Cdh1, and Zonula Occludens 1 (ZO1) in STIM1ΔIEC mice IECs.
IHC was used to further confirm the expression levels of Cdh1 and occludin in intestinal epithelial tissues. The results showed that STIM1 deficiency in intestinal epithelium did not affect the epithelial barrier in the steady state. However, on day 10 after exposure to DSS, there was a significant difference in the expression of Cdh1 and occludin between STIM1f/f and STIM1ΔIEC mice (Cdh1, 1.06% vs 18.95%; occludin, 1.83% vs 14.14%) (Figure 6F–I). These data show that STIM1 deficiency in the intestinal epithelium effectively protects the epithelial barrier against DSS-induced dysfunction.

### STIM1 Deficiency in Intestinal Epithelium Protects Colonic Inner Mucus Against DSS-Induced Damage

Colonic IECs are covered by 2 layers of mucus, the outer mucus layer is loose and forms a habitat for commensal bacteria, while the inner mucus layer is dense and tightly attached to epithelial cells, forming the first line of defense to prevent contact between epithelium and luminal flora.3

Because adherens and tight junction molecules of the epithelial barrier were altered in DSS-treated STIM1ΔIEC mice (Figures 5 and 6), we examined the inner mucus layer in DSS-treated mice to investigate whether the first line of defense was affected. Strikingly, results of Alcian blue–periodic acid–Schiff (PAS) staining showed that STIM1ΔIEC mice had thicker mucus than STIM1f/f mice on day 7 (Figure 7A and B). To further verify the alterations in the inner mucus layer, we visualized the mucosal surface using scanning electron microscopy and found that the mucosal surface of STIM1ΔIEC mice was covered with rich mucus, in contrast to the bare surface and exposed microvilli of STIM1f/f mice (Figure 7C). Immunostaining of ulex europaeus agglutinin-1 (UEA-1), which is specific for fucose lectin in mucus secreted by goblet cells and can be used to indicate the mucus layer, also showed a dense and integrated mucus layer in STIM1ΔIEC mice, while mucus in STIM1f/f mice was interrupted and weakly stained (Figure 7D and E), indicating that STIM1 deficiency in intestinal epithelium protects colonic mucus against DSS-induced damage.

### STIM1 Deficiency in Intestinal Epithelium Sustains the Integrity of Inner Mucus Layer by Alleviating Goblet Cell Loss

Mucins are generated by goblet cells, for which we hypothesized that STIM1 may affect the function of goblet cells.

First, we examined the mRNA levels of SAM pointed domain containing ETS transcription factor (Spdef), atonal
BHLH transcription factor 1 (Atoh1), kruppel like factor 4 (Klf4), and hes family BHLH transcription factor 1 (Hes1), the key factors for differentiation and maturation of goblet cells,13 and found no difference between DSS-treated STIM1DIEC and STIM1f/f mice (Figure 8A). IL10 can promote the production of inner mucus by suppressing the misfolding of mucins in goblet cells31; however, although a wide range of cytokines were decreased in DSS-treated STIM1DIEC mice (Figure 4), the expression level of IL10 was not altered significantly (Figure 8B), refuting the surmise that STIM1 deficiency in intestinal epithelium promotes the correct mucin folding by affecting the expression of IL10. Next, we explored whether STIM1 had effects on goblet cell secretion. However, transmission electron microscopy images showed that the release of mucin granules in apical goblet cells of STIM1ΔIEC mice was similar to that of STIM1f/f mice (Figure 8C).

These results showed that STIM1 deficiency in the intestinal epithelium had no effect on goblet cell differentiation, maturation, or secretion in the development of colitis. However, the number of PAS-positive cells per crypt was significantly higher in STIM1ΔIEC mice than in STIM1f/f mice (5.9 vs 1.5 per crypt, respectively) (Figure 8D and E), indicating that STIM1 affects the number of goblet cells during colitis. Consistently, PAS assay also showed more PAS-positive products in the colon tissues of STIM1ΔIEC mice.
Collectively, these results indicate that STIM1 deficiency in the intestinal epithelium maintains the inner mucus layer integrity in DSS-induced colitis by alleviating goblet cell loss.
Figure 6. STIM1 deficiency in intestinal epithelium facilitates epithelial barrier maintenance. (A) Intestinal permeability was assessed by FITC–dextran assays on day 7 of DSS administration (n = 6 per group). (B) The expression of tight junctions was assessed using WB (n = 5 per group) for occludin, Cdh1, and ZO1 in IECs on day 7. (C–E) Relative mRNA expression of occludin, Cdh1, zonula occludens, and claudins in the IECs of STIM1f/f and STIM1ΔIEC mice on day 7 (n = 7 per group). (F–I) IHC staining and quantification of Cdh1- and occludin-positive cells on day 0 and day 10 (n = 4 per group). Scale bars: 100 μm. Data are presented as the means ± SEM. ****P < .0001, ***P < .001, and **P < .01; NS at > .05.
STIM1 Deficiency in Colonic Goblets Cells Alleviates ER Stress and Subsequent Cell Apoptosis by Reducing Ca\textsuperscript{2+} Overload

Mucins, especially MUC2, are large and complex glycoproteins that can increase the probability of protein misfolding during ER biosynthesis in goblet cells. Murine models show that MUC2 misfolding within ER causes ER stress in goblet cells, and prolonged ER stress leads to goblet cell loss and spontaneous colitis. The high expression of MUC2 makes goblet cells the initial IECs that undergo ER stress and subsequent apoptosis during DSS treatment, eventually leading to depleted and defective mucus and driving colitis susceptibility.\textsuperscript{11,19–22,33}

Because STIM1\textsuperscript{ΔIEC} mice showed reduced goblet cell loss during DSS-induced colitis, we hypothesized that this effect was the consequence of ER stress inhibition in goblet cells. Consistent with our speculation, during DSS treatment, the mRNA levels of genes related to ER stress induced by protein misfolding, especially IRE1 and CHOP, the fundamental ER stress signals that initiate and promote apoptosis,\textsuperscript{34} were significantly lower in the colons of STIM1\textsuperscript{ΔIEC} mice (Figure 9A). WB analysis also showed decreased IRE1\(\alpha\) and CHOP protein expression in STIM1\textsuperscript{ΔIEC} mice (Figure 9B). In addition, UEA-1 and CHOP protein levels were detected on day 7 of DSS administration, as shown by immunofluorescence staining, and the decreased expression of CHOP in goblet cells (UEA-1–positive cells) of STIM1\textsuperscript{ΔIEC} mice showed reduced ER stress in goblet cells (Figure 9C). The decreased expression of cleaved-Caspase 3 (C-Caspase 3) in goblet cells further suggested the inhibited apoptosis of goblet cells in STIM1\textsuperscript{ΔIEC} mice (Figure 9D).

To further confirm the role of STIM1 in ER stress and prolonged ER stress–induced apoptosis of goblet cells, we used human colonic LS174T cells that produce large amounts of MUC2 as an in vitro model of intestinal goblet cells.
The ER stress of cells with or without STIM1 deficiency was induced by tunicamycin (Tm) or thapsigargin (TG). The results showed that STIM1 knockdown significantly decreased the up-regulation of ER stress markers induced by Tm or TG at both protein and mRNA levels, including CHOP, IRE1α, Bip, PERK, and ATF4 (Figure 10A and B). Furthermore, STIM1 deficiency effectively reduced the apoptosis of LS174T cells induced by ER stressors (Figure 10C and D). ER stress is elicited by disturbance of ER-Ca²⁺ homeostasis accompanied by intracellular Ca²⁺ overload, which further exacerbates ER stress and results in apoptosis. Hence, we examined changes in intracellular Ca²⁺ influx induced by Tm or TG in LS174T cells and determined that STIM1 knockdown significantly reduced ER stressor–induced Ca²⁺ overload (Figure 10E and F), suggesting that STIM1 deficiency alleviates ER stress by reducing Ca²⁺ overload.

Both in vivo and in vitro models showed that STIM1 deficiency in goblet cells decreased their susceptibility to ER stress and inhibited subsequent cell apoptosis, suggesting that STIM1 deficiency in intestinal epithelium alleviates goblet cell loss by inhibiting ER stress.

**STIM1 Deficiency in Intestinal Epithelium Protects Intestinal Epithelium Against Stimulation of Commensal Bacteria**

Under physiological conditions, the inner mucus layer prevents contact between the intestinal epithelium and commensal bacteria and the outer mucus layer acts as a habitat and food source for commensal bacteria. During DSS treatment, bacteria could penetrate the inner mucus layer immediately before inflammatory reactions and attach to the intestinal epithelium to aggravate colonic inflammation and even trigger the development of colon cancer. Moreover, microbial community is altered in colitis, which can affect mucus glycosylation and promote the development of intestinal inflammation.

Therefore, we examined the intestinal microbial community to investigate whether the commensal bacteria is the inducer of colitis distinction in STIM1ΔIEC and STIM1ΔIEC mice. A 16S sequence analysis of stool samples showed similar β-diversity between DSS-treated STIM1ΔIEC and STIM1ΔIEC mice using principal component analysis (Figure 11A). However, at the phylum level, Bacteroidetes were slightly more abundant and Proteobacteria were slightly less abundant in STIM1ΔIEC mice compared with STIM1ΔIEC mice (Figure 11B), consistent with the fecal bacterial changes reported in healthy controls compared with IBD patients. At the genus level, STIM1ΔIEC mice showed a decreasing trend in potential pathogenic bacteria, including Escherichia–Shigella and Helicobacter, and an increasing trend in beneficial bacterial species, including Lactobacillus and Ruminococcus (Figure 11C and D).

Although these data suggested that STIM1 deficiency in intestinal epithelium provided a stable environment for promoting beneficial bacteria and hindering potential pathogenic species during DSS treatment, the slight alteration with no statistical difference in commensal bacteria indicated that the change of their species was not a potent inducer of the colitis distinction between STIM1ΔIEC and STIM1ΔIEC mice. Next, we applied an EUB338 DNA probe to detect the distances between bacteria and intestinal epithelium in DSS-treated mice and observed that commensal bacteria were prevented from penetrating the mucus layer or attaching to the epithelium of STIM1ΔIEC mice (Figure 12A), thereby leading to significant inhibition of pathogenic bacterial translocation from the bowel lumen to colon tissue, liver, spleen, and mLN (Figure 12B), and the inhibition was benefit from integrated inner mucus layer (Figure 7).

These results indicated that STIM1 deficiency in the intestinal epithelium protects the intestinal epithelium against stimulation of commensal bacteria through regulating the inner mucus layer rather than changing the bacterial community, and thereby alleviates colonic inflammation exacerbated by bacterial stimulation.

**STIM1 Deficiency in Intestinal Epithelium Promotes the Recovery From Colitis and Renders Mice Less Susceptible to Colitis-Associated Colorectal Cancer**

The integrated inner mucus layer can protect intestinal epithelium against chronic inflammation stimulated by commensal bacteria and thereby prevent subsequent colorectal tumorigenesis. Hence, we explored whether STIM1 deficiency in intestinal epithelium decreased the occurrence of CAC developed from chronic inflammation.

We examined the colitis convalescence of STIM1ΔIEC and STIM1ΔIEC mice to investigate whether the process of chronic inflammation was altered. In STIM1ΔIEC mice, the epithelium was stimulated continuously by commensal bacteria during colitis because of the defective inner mucus layer (Figures 7 and 12), leading to further exacerbated colitis and slow
Figure 9. STIM1 deficiency in intestinal epithelium alleviates the ER stress and subsequent cell apoptosis of goblet cells. (A) Relative mRNA expression of the indicated genes related to ER stress in DSS-treated colon tissues on day 7 (n = 7 per group). (B) Analysis of protein levels of IRE1α and CHOP in DSS-treated colon tissues on day 7 (n = 6 per group). (C) DSS-treated colon sections on day 7 were stained for UEA-1 (green) and CHOP (red) to visualize ER stress in goblet cells (n = 3 per group). Scale bars: 20 μm. Arrows indicate goblet cells undergoing ER stress. (D) DSS-treated colon sections on day 7 were stained for UEA-1 (green) and C-Caspase 3 (red) to visualize apoptosis in goblet cells (n = 3 per group). Scale bars: 20 μm. Arrows indicate goblet cells undergoing apoptosis. Results are presented as the means ± SEM. ***P < .001; *P < .05.
**Figure 10.** STIM1 knockdown in LS174T cells alleviates ER stress and prolonged ER stress–induced apoptosis by reducing Ca^{2+} overload. LS174T cells were treated with 10 μg/mL Tm or 5 μmol/L TG for 6 hours, 12 hours, or 24 hours with or without STIM1 knockdown. (A) ER stress was examined by WB at 12 hours. (B) ER stress was quantified by qPCR at 6 hours. (C and D) Apoptosis was examined by WB and immunostaining for C-Caspase 3 at 24 hours. Scale bars: 200 μm. (E) Tm- or (F) TG-induced intracellular Ca^{2+} concentrations were measured by Fluo-4 AM in 20 minutes (left), and representative changes in intracellular Ca^{2+} fluorescence intensity at 15 minutes (right) in LS174T cells with or without STIM1 knockdown were shown (n = 40 cells per group). Results are presented as the mean ± SEM. ****P < .0001, ***P < .001, **P < .01, and *P < .05; NS at P > .05. DAPI, 4′,6-diamidino-2-phenylindole; F/Fbase, Ca^{2+} fluorescence intensity / base of Ca^{2+} fluorescence intensity; siCtrl, siRNA-Control; siSTIM1, siRNA-STIM1.
recovery. As anticipated, STIM1f/f mice did not recover to 95% of normal body weight throughout the convalescence (Figure 13A). Although colon length and the mRNA levels of inflammatory cytokines (except for IL1β) were similar between STIM1ΔIEC and STIM1f/f mice (Figure 13B and C), histopathology showed that STIM1f/f mice suffered chronic inflammation manifested as ulceration, inflammatory cell infiltration, and partial loss of epithelium structure, while the epithelium of STIM1ΔIEC mice almost recovered to normal (Figure 13D and E).

Next, we administered STIM1ΔIEC and STIM1f/f mice azoxymethane followed by 3 cycles of DSS to sustain chronic inflammation and induce CAC. STIM1ΔIEC mice showed decreased body weight loss (Figure 14A), accompanied by significantly higher survival rates and longer colon lengths (Figure 14B and C), indicating the inhibition of recurring colitis in STIM1ΔIEC mice. Statistical analysis of tumors in the colon showed that STIM1ΔIEC mice showed decreased numbers and sizes of tumors, and the tumor loads were significantly lower than those of STIM1f/f mice (tumors per mouse: 5.889 vs 9.056; large tumors (>3 mm) per mouse: 2.833 vs 4.278) (Figure 14D–G). Furthermore, histopathologic images showed that STIM1ΔIEC mice had low-grade tumor dysplasia with almost normal adjacent mucosa, in contrast to STIM1f/f mice, which showed high-level dysplasia with massive mucosal hyperplasia (Figure 14H and I).

Overall, these results indicate that STIM1 is essential for the tumorigenesis of CAC by promoting acute and chronic inflammation.

Discussion

STIM1 has been confirmed to promote the formation of colorectal cancer.23,42 As the initial stage of CAC, IBD patients have shown increased STIM1-mediated SOCE in lamina propria, and STIM1 also has been shown to be essential for T-cell–mediated immunoinflammatory diseases in mouse models.26–28 However, the specific mechanisms by which intestinal STIM1 regulates the development of IBD...
still are unclear. Here, we found that STIM1 was highly expressed in the inflammatory intestine of IBD patients. To investigate the role of intestinal epithelial STIM1 in IBD, we generated STIM1<sup>ΔIEC</sup> mice with specific STIM1 deficiency in intestinal epithelium and discovered previously unknown roles of STIM1 in the regulation of mucosal inflammation, mucus barrier, and goblet cell ER stress.

The intestinal epithelia with active inflammation in IBD patients show ER stress signals. Studies have confirmed that ER stress in IECs is the cause of intestinal inflammation, and high ER stress in IECs with high secretory activity could directly initiate and promote intestinal inflammation. The expression levels of ER stress signals were decreased significantly in STIM1<sup>ΔIEC</sup> mouse intestines, indicating that activated ER stress may be the major mechanism by which STIM1 promotes colitis. The colonic mucus layer is formed by large and complex mucins produced by goblet cells, among which MUC2 is the most essential. This function of goblet cells makes it susceptible to protein misfolding induced by environmental stimulation, including DSS treatment, and the accumulation of misfolded and aberrantly assembled MUC2 can cause ER stress in goblet cells. Our studies indicated that the ER stress of goblet cells in STIM1<sup>ΔIEC</sup> mice was decreased during colitis. Disturbances of ER–Ca<sup>2+</sup> homeostasis induce ER stress and consequent cell apoptosis. As the major component of SOCE, STIM1 is involved in the maintenance of Ca<sup>2+</sup> homeostasis and has been confirmed to play complicated roles in ER stress. In our study, STIM1 deficiency in intestinal epithelium was first observed to ameliorate goblet cell ER stress. In addition, in STIM1-silenced LS174T cells, the induction of ER stress with Tm or TG was reduced by attenuating Ca<sup>2+</sup> overload (Figure 10).

The prolonged stimulation of ER stressors can result in irremediable ER stress, activate the terminal unfolded protein response, and trigger caspase-dependent apoptosis. Previous evidence has shown that irremediable ER stress-induced massive goblet cell loss is the cause of thinner and permeable mucus; furthermore, goblet cell loss is the defining feature of IBD patients and murine colitis. A defective mucus barrier cannot effectively protect the intestine from various environmental stimuli. Mice with misfolded MUC2 in goblet cells lack mature MUC2 and cannot produce mucus, which lead to spontaneous colitis. Mice with impaired secretory function of goblet cells also showed defective mucus, suffered mild proinflammatory response, and were more susceptible to DSS-induced colitis. In STIM1<sup>ΔIEC</sup> mice, prolonged ER stress–induced goblet cell apoptosis and loss was inhibited, which ensured the normal production of MUC2 by goblet cells and the maintenance of the integrated inner mucus layer during colitis (Figure 7). In summary, the integrated mucus barrier protected STIM1<sup>ΔIEC</sup> mice from DSS-induced colitis, suggesting a potential therapy for IBD by ameliorating ER stress in goblet cells to maintain an integrated mucus layer.

The outer mucus layer acts as a natural habitat and food source for commensal bacteria, of which the mucin

Figure 12. STIM1 deficiency in intestinal epithelium protects the intestinal epithelium against stimulation by commensal bacteria. (A) In situ EUB338 DNA probe hybridization of colon sections on day 7 of DSS administration, commensal bacteria are shown in red, nuclei are shown in blue (n = 5 per group). Scale bars: 20 μm, boxed pullout: 10 μm. (B) On day 7 of DSS administration, pathogenic bacterial translocation from intestinal lumina to colon tissues, liver, spleen, and mesenteric lymph nodes, as indicated by MacConkey agar plates (n = 6 per group). Data are presented as the means ± SEM. **P < .01 and *P < .05. CFU, colony-forming unit.
components can interact with the bacterial community. Perturbation of genetic and environmental factors on bacterial community affects mucus glycosylation and promotes the development of intestinal inflammation. However, in our study, although the mucus in STIM1^ΔIEC mice is rich as a consequence of decreased goblet cell loss, the alteration of bacterial community showed no statistical difference. The bacterial community of STIM1^ΔIEC mice had only slight differences at the genus level, including the contraction of pathogenic bacteria and the expansion of beneficial bacteria. Because the bacterial changes were not statistically significant, and the bacteria in STIM1^ΔIEC mice were completely prevented from contacting intestinal epithelium, we determined that the slight alteration in commensal bacteria was a consequence, but not the cause, of alleviated colitis in STIM1^ΔIEC mice. Interestingly, Ahuja et al. found that mice lacking acinar ORAI1 showed impaired antimicrobial pancreatic secretion, ultimately causing bacterial outgrowth and inflammation in the intestine. Patients with ORAI1 mutations also showed immunodeficiency and increased infections of viral, bacterial, and fungal pathogens. Because STIM1 deficiency in intestinal epithelium alleviates colitis mainly by affecting the function and loss of goblet cells, we speculated that it was the discrepancy in cell-specific results that caused our contrary results to those reported previously.

In patients and mouse models of IBD, the defective mucus barrier allows massive numbers of bacteria to penetrate and reach the epithelium to activate the immune system. Mononuclear phagocytes mediate the initiation of proinflammatory effects. Through the toll-like receptor - myeloid differentiation primary response 88 (TLR–MyD88) signaling pathway, TLRs recognize bacterial molecules and promote immune responses to produce inflammatory cytokines, including IL1, IL23, IL6, and IL10, which are essential in inducing T-cell responses. Moreover, the increase of interferon-γ and TNF-α can disrupt epithelial cell junctions to induce epithelial barrier dysfunction, which further increase epithelial permeability and enhance bacteria-induced immune response.
generated by plasma cells in lymphoid follicles and intestinal lamina propria in response to bacteria stimulation. The integrated mucus layer protects STIM1 mice against bacterial stimulation, thereby decreasing the production of sIgA and inflammatory cytokines and preventing acute or chronic inflammation. Recurrently chronic mucosal inflammation stimulated by bacteria can lead to epithelial apoptosis and subsequent pathologic epithelial proliferation, which are known to be essential for the development of CAC. Therefore, it is not surprising that the number and size of tumors in STIM1 mice were decreased significantly (Figure 14), which further supports the previous evidence that commensal microbiota penetrating the defective mucus barrier contribute to the tumorigenesis of CAC.

The precise mechanism by which STIM1 regulates ER stress of goblet cells needs further clarification. ER is the primary store of Ca\(^{2+}\), steady ER-Ca\(^{2+}\) homeostasis can control intracellular Ca\(^{2+}\) homeostasis to ensure normal ER function, for example, protein glycosylation and protein folding in ER require Ca\(^{2+}\)-dependent calnexin and calreticulin. ER-Ca\(^{2+}\) homeostasis is perturbed easily by ER stressors, and disturbed ER-Ca\(^{2+}\) homeostasis accompanied by intracellular Ca\(^{2+}\) overload can elicit protein misfolding, which results in ER stress. The prolonged intracellular Ca\(^{2+}\) overload further aggravates ER stress and causes cell death. STIM1 is the activator of SOCE to supply intracellular Ca\(^{2+}\), and its knockdown has been shown to protect cells against ER stress and apoptosis through abrogating cytoplasmic Ca\(^{2+}\) overload induced by stressors. Our study confirmed that STIM1 deficiency reduced intracellular Ca\(^{2+}\) overload induced by ER stressors in goblet cells, leading to a striking decrease in ER stress and apoptosis of goblet cells during colitis.

**Figure 14.** STIM1 deficiency in intestinal epithelium renders mice less susceptible to CAC. (A) Percentage change in body weight and (B) Kaplan–Meier survival curves throughout azoxymethane/DSS-induced CAC (n = 12 per group). (C) Analysis of colon length in mice with colorectal tumors (n = 18 per group). (D) Representative images of colonic tumors. (E) Number of colon tumors per mouse (n = 18 per group). (F) Number of large tumors (diameter, >3 mm) per mouse (n = 18 per group). (G) Tumor loads indicated by the sum of tumor diameters per mouse (n = 18 per group). (H) Representative H&E-stained colon sections and tumor morphology (n = 6 per group). Scale bars: 400 μm. (I) Percentages of mice with tumor dysplasia. Kaplan–Meier survival curves were analyzed by the log-rank test, while other differences were verified using a 2-tailed Student t test. Data are depicted as the means ± SEM. ****P < .0001, **P < .01, and *P < .05.
In conclusion, our results show that STIM1 is overexpressed in LP and IECs of IBD patients, which further confirms the previous theory that STIM1 is involved in immunoinflammatory diseases. Meanwhile, our study provides evidence that STIM1 in intestinal epithelium regulates ER stress and subsequent apoptosis. Goblet cell depletion diminishes mucus synthesis, defective mucus layer would allow massive bacteria to reach to intestinal epithelium, driving further impairment to mucosal barrier and activating immune responses to trigger mucosal inflammation and tumorigenesis. STIM1 deficiency in intestinal epithelium alleviates the disturbance of Ca$^{2+}$ homeostasis to inhibit ER stress and subsequent depletion of goblet cells, maintaining the integrity of mucus barrier, therefore alleviating colitis and tumorigenesis in mice.

**Materials and Methods**

**Patients**

Human intestinal inflammatory tissues and matched-adjacent normal tissues were collected from IBD patients at the Department of Pathology, Sir Run Run Shaw Hospital, Zhejiang University (ethics approval 20210125-30).

**LS174T Cell Culture and Treatment**

LS174T colon carcinoma cells, which were differentiated by goblet cells, were purchased from the Cell Bank of the Chinese Academy of Science and maintained in Dulbecco's modified Eagle medium (#MA0212; Meilunbio, Dalian, China) with 10% fetal bovine serum (FBS) (#NFBS-2500A; Noverse, Uruguay, South America) at 37°C and 5% CO$_2$. LS174T cells were treated with tunicamycin (#HY-A0098; MCE, South Brunswick Township, NJ) at 10 μg/mL, and thapsigargin (#T9033; Sigma-Aldrich, St. Louis, MO) at 5 μmol/L for 6 or 12 hours. The small interfering RNA sequence against human STIM1 is 5'-GGGAA-GACCTCAATTACCA-3'.

**Mice**

Mice carrying STIM1 floxed alleles were generated as previously described, and they were crossed with Villin-Cre mice (purchased from Shanghai Model Organisms, Shanghai, China) to obtain STIM1$^{flx}$ mice. All mice were
bred and housed in specific pathogen-free conditions, and sex- and age-matched mice (age, 8–10 wk; weight, 23–25 g) were used for each experiment (ethics approval ZJU2015-220-01).

Experimental Colitis and CAC
To induce acute colitis, mice were administered 2% DSS (molecular weight, 36,000–50,000 Daltons; MP Biomedicals, Irvine, CA) in drinking water for 7 days, followed by regular drinking water for 3–4 days until the mice lost more than 25% of their body weight. The body weight and disease activity index (including weight loss, stool consistency, and the presence of fecal blood) were recorded every day until the mice were killed.

To induce CAC, mice were injected with azoxymethane (10 mg/kg; MP Biomedicals) intraperitoneally, and after 7 days of regular water they were administered 3 cycles of 2% DSS in drinking water for 6 days, followed by regular water for 15 days.

Intestinal Permeability
Four hours before the mice were killed, the mice were orally gavaged with FITC-dextran (0.6 mg/kg body weight; Sigma-Aldrich), and blood samples were collected via cardiac puncture. After clotting and centrifuging, the supernatant of the blood sample was analyzed at 485/535 nm on a Synergy H4 Hybrid Reader (Biotek, Winooski, VT).

Bacterial Translocation
After the mice were killed, whole mouse mLNs, spleens, and livers were removed, weighed, and collected in 1 mL sterile phosphate-buffered saline (PBS) with EDTA-free protease inhibitor cocktail. Colons were opened longitudinally and washed in sterile PBS several times to completely remove stool. Then, 0.1 g colon tissue was removed and collected in 0.9 mL sterile PBS with EDTA-free protease inhibitor cocktail. All tissues were homogenized in a bead miller (JXFSTPRP-CL, Jing xin, Shanghai, China) for 2 minutes at 4°C. Then, 200 mL tissue homogenate was plated on MacConkey agar plates (#M8560; Solarbio, Beijing, China) for 24 hours at 4°C and 5% CO2 for 48 hours, and the number of bacterial colonies on the plates was counted and normalized to the tissue weight (per gram).

Cytokine Determination
The supernatants of blood and tissue homogenates were analyzed with enzyme-linked immunosorbent assay kits according to the manufacturer’s recommendations to detect the levels of IL6, TNF-α (Invitrogen, Carlsbad, CA), and sIgA (MultiSciences, Hangzhou, China).

Histopathology, Immunostaining, and Fluorescence In Situ Hybridization
Colon sections (approximately 2-cm long) were fixed in neutral-buffered formalin (#G1101; Servicebio, Wuhan, China) overnight at 4°C, and embedded in paraffin, and then 5-um-thick tissue sections were stained with H&E (Servicebio) for histopathology. The histologic activity index of colon sections was scored blindly based on the extent and severity of the inflammation, ulceration, and hyperplasia of mucosa as previously described.57

For immunohistochemistry, colon tissue sections were stained with STIM1 (#5668; CST, Danvers, MA), Cdh1 (#3195; CST), and occludin (#71-1500; Thermo Fisher, Waltham, MA) according to the manufacturer’s recommendations. Immunohistochemical scores of STIM1 were assigned as previously described58: 0, negative; 1, faint and barely discernable cytoplasmic staining; 2, moderate and smooth cytoplasmic staining; 3, intense and granular cytoplasmic staining; and 4, no staining. The proportion of positive cells in tissue sections was determined using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD), and 4–5 regions of interest were assessed per mouse.

For immunofluorescence, tissue slides were stained with CD45 (#40763; Abcam, Waltham, MA), EpCAM (#A1177; Abclonal, Wuhan, China), FITC-conjugated UEA-1 (#L9006; Sigma), CHOP (#2895; CST), and C-Caspase 3 (#9664; CST).

To detect whether the bacteria were in contact with intestinal epithelium, an EUB338 DNA probe (5’-GCTGCTCCCGTAGGAGTC-3’; Servicebio) was used for fluorescence in situ hybridization.

Goblet Cells and Mucus Layer Preservation
Ex Vivo
The terminal colons with stool were excised and immediately submerged in Carnoy fixative at 4°C for 2 hours and then placed into 100% ethanol. Fixed colon tissues were embedded in paraffin and stained with PAS or Alcian blue/PAS. The number of goblet cells and the thickness of the inner mucus layer were measured by Image-Pro Plus 6.0, 4–5 regions of interest were measured per mouse and the mean was calculated.

PAS Assay
A total of 100 μL colon tissue homogenate supernatant was prepared as described earlier, and incubated with 200 μL of 0.1% periodic acid (0.1 g/100 mL; Solarbio) at room temperature for 2 hours. Then, 200 μL Schiff reagent (Solarbio) was added and incubated for 30 minutes at room temperature. The resulting mixture was measured at 555 nm and the PAS optical density value was normalized to that of STIM1f/f mice.

Isolation of IECs
Colons from experimental mice were freshly isolated, opened longitudinally, and washed using ice-cold sterile PBS several times to completely remove stool. Clean colon tissues were cut into 2-mm segments and incubated in Hank’s balanced salt solution (HBSS) (without calcium chloride, magnesium sulfate, or sodium bicarbonate; Sigma-Aldrich) with 30 mmol/L EDTA (Sigma-Aldrich) and 1.5 mmol/L dithiothreitol (Sigma-Aldrich) at 4°C for 20 minutes to remove mucus. Then, the colon pieces were washed in HBSS and incubated in HBSS with 30 mmol/L EDTA and 2% FBS in a shaker at 200
| Name            | Sequences               |
|-----------------|-------------------------|
| Mouse GAPDH     | F: TCACCACCATGGAGAAGGC  |
|                 | R: GCTAAGCAATTGGTGCTCA  |
| Mouse STIM1     | F: TGAAGAGTCTACGAGACGA  |
|                 | R: AGGTGCTATTTTTTCATG    |
| Mouse Chga      | F: ATCCCTCTATCTGGAGACAC |
|                 | R: GGGGCTCTTCTCAAAACT    |
| Mouse Lyz1      | F: AATGATGATCTACCAGTG    |
|                 | R: TACCTGTGCTCCTGCTCT    |
| Mouse Spdef     | F: TTGCTGTACTAGTTCACCAG |
|                 | R: CCAGGAGCTGCTGATGTT    |
| Mouse NFATc1    | F: TGCTTCTTGAGAGCAGTACT |
|                 | R: CAGGGAGGATGAGGGCTCAT  |
| Mouse NFATc2    | F: GAATCCCCTCCAGATAGTG   |
|                 | R: CCCCCAGAACCTACAGTAG   |
| Mouse NFATc3    | F: TGACTCTCAGAGGCTCTCT   |
|                 | R: GAGGTGCGAAGGAGATG     |
| Mouse NFATc4    | F: GAGGAGCCCTACAGACTC    |
|                 | R: AGGGTTTCTCTGGAGATG    |
| Mouse IL6       | F: GAGGATACCACTCCCAAGA   |
|                 | R: AAGTGCATCATGGTATG     |
| Mouse IL10      | F: GTGGCCAGAGCCTAGGAG    |
|                 | R: ACCTGCTCCAGCTGGTCT    |
| Mouse IL1β      | F: GCTGAAAGCTCTCACCAG    |
|                 | R: AGGCCCAAGCTATTGTGG    |
| Mouse TNFα      | F: TACTGAACTTGCGGTACG    |
|                 | R: CAGCCTTCTTCCTTTGAG    |
| Mouse iNOS      | F: CAGCTGGGCTGTAACACCT   |
|                 | R: CATTGGAAGCATGAGGCTTG  |
| Mouse cyclooxygenase 2 | F: AAGGAACTCAGCAGCTGATCC |
|                 | R: ACAAGGATTGGAAACAGGA   |
| Mouse Camp      | F: CAGCCCTTTTGCGGAGAAGA |
|                 | R: CCCCCTTTTGGAGGAGAAG   |
| Mouse Cdh1      | F: TCCTGTGCTGCATGCTATG   |
|                 | R:GGCATGCCATCTAAGATCG    |
| Mouse occludin  | F: TTGAAAGTCCACCTCCATTACA |
|                 | R: CCGGATAAAAAGAGATGCCTGG |
| Mouse ZO1       | F: GCCGCTAAGAGCAGACGAA  |
|                 | R: GCCCTTCTTTAACAATCAAG  |
| Mouse ZO2       | F: ATGGAGACAGTACACGCTG   |
|                 | R: TGAACACCTGCTATTTTCCT |
| Mouse ZO3       | F: TGGGTAAGCTGCTGTCTGGA  |
|                 | R: GGTGCTGTATTGGTGAGG    |
| Mouse Cldn2     | F: GGGCTGTAGCACATTCAT    |
|                 | R: TGGCAACACTGAGGAACACT  |
| Mouse Cldn3     | F: AAGCCAATGGGCAAAAGA   |
|                 | R: CTGGCAAGTAGCTGAGTG    |
| Mouse Cldn7     | F: AGGGTTCTGTCTGGTCTTT   |
|                 | R: GTAGCGACTTTTGCTTTCA  |
| Mouse Muc2      | F: CCTGAAAGCTGTGCTGCTGT |
|                 | R: GGGTATGGTAACCTCAGCTC |
| Mouse AGR2      | F: CGGATGGGAGGAGACATG    |
|                 | R: CGGGTGGGAGGAGCTGCTGA |
| Mouse Atoh1     | F: AAAGAGGCCTGGCACGCAA  |
|                 | R: TGGTTTCAACCCGCTCAT   |
| Mouse Klf4      | F: AGGCCACACCTGCCAGA     |
|                 | R: CAGCCCTCCAGCTCAAGT    |
rpm and 37°C for 60 minutes. The resulting supernatants contained IECs, and were collected for RNA extraction and Western blot.

**16S Sequencing**

After the mice were killed, the fresh colonic contents were collected from colitis mice, and DNA was extracted using the Stool DNA Kit (#D4015; Omega, Norcross, GA) according to the manufacturer's recommendations. Primers for 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') were used to amplify the bacterial V3–V4 region of the 16S ribosomal RNA gene as previously described.59 The amplicon libraries of samples were sequenced on an Illumina NovaSeq platform (San Diego, CA) according to the instructions provided by LC-Bio Technology (Hangzhou, China).

**Measurement of Intracellular Calcium**

LS174T cells seeded in 35-mm diameter, glass-bottom dishes (D35-20-0-N; Coelvis, Mountain View, CA) were loaded with 5 μmol/L Flu-4/AM (#S1060; Beyotime, Shanghai, China) for 30 minutes in Dulbecco’s modified Eagle medium with 10% FBS at room temperature. Then, cells were washed and immersed in HBSS (#C0218; Beyotime) with 2 mmol/L Ca²⁺, and images were recorded and analyzed every 4 seconds at an excitation wavelength of 488 nm using the Leica Application Suite X confocal imaging system (Solms, Germany). Changes in intracellular Ca²⁺ fluorescence intensity (F) were normalized to baseline Ca²⁺ fluorescence intensity (Fbase) and were presented as F/Fbase.

| Name      | Sequences  |
|-----------|------------|
| Mouse Hes1 | F: CCCAGCCAGTGTCGAACAC  |
|           | R: T7GTGTCAAGGCGCTCTT |
| Mouse CHOP| F: AACAGAGTGCACAGGATCAT |
|           | R: T7CTCTCTCTCCTCCTGG |
| Mouse IRE1| F: GCCCAACGCACATGGGAGGA |
|           | R: TACCCCGGGAGCAAAACAGGT |
| Mouse ATF4| F: T7CTGTGGCGGTGTTAAGT |
|           | R: CTGCTGGTCTTAATACGGA |
| Mouse ATF6| F: GGGCAGGGGACATCTTGCTGA |
|           | R: AGGCCCAGGCCGAAACAGGT |
| Human actin| F: GAGCTACAGAGTCGGCTGAC |
|           | R: GGATGTTCTCAGTGATGCCAAGAG |
| Human CHOP| F: GGCGATGAGGAGAAGAAGAC |
|           | R: CCAATTGTTCTAGTGTTG |
| Human IRE1| F: GAGACAGGCCTAATCAATGGG |
|           | R: CGGTAGAGGCTACTAAACA |
| Human PERK| F: CGGATGAGCAGAGTTGGCAC |
|           | R: T7CCTCTACGGCTCTTGTC |
| Human Bip | F: GCCTGTATTCTCTAGAACCTG |
|           | R: TTACTTGGCAGGCCAGTTG |
| Human ATF4| F: AGTTCAGTCTGATGCGCCTG |
|           | R: CCAAGCTGCTAGAGGAGGTC |
| Human XBP1| F: GCTCAGACTGCCAGATCG |
|           | R: GCATCTGTCTTAACTCTGGT |

AGR2, anterior gradient 2; ATF, activating transcription factor; Atoh1, atonal BHLH transcription factor 1; Chga, chromogranin A; Camp, cathelicidin antimicrobial peptide; F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hes1, hes family BHLH transcription factor 1; INOS, inducible nitric oxide synthase; Il4, kruppel like factor 4; Lyz1, lysozyme 1; NFAT, nuclear factor of activated T Cells; R, reverse; Spdef, SAM pointed domain containing ETS transcription factor; XBP1, X-box binding protein 1.
cocktails (Bimake, Houston, TX). Lysate samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gels, transferred to polyvinylidene fluoride membranes, and incubated with the following antibodies: Actin (#8457, CST), STIM1 (#5668, CST), Cdh1 (#3195; CST), occludin (#71-1500; Thermo Fisher), ZO1 (#96587; Abcam), ATF4 (#11815; CST), Bip (#3177; CST), CHOP (#2895; CST), PERK (#5683; CST), IRE1α (#3294; CST), and C-PARP (#9546; CST). After being incubated with anti-rabbit/mouse horse-radish-peroxidase antibodies, signals were analyzed by enhanced chemiluminescence (Amersham Imager 600, GE, Boston, MA).

RNA Sequencing, Extraction, Reverse Transcription, and qPCR
Total RNA of colon tissues collected from experimental mice were extracted by Total RNA Extraction Reagent (#R401-01-AA; Vazyme, Nanjing, China). The mRNA was purified by oligodeoxythymidylate (oligo(dT))-attached magnetic beads, and then the purified mRNA was fragmented into small pieces to amplify and generate DNA nanoballs with more than 300 copies of 1 molecule. The DNA nanoballs were loaded into the patterned nanoarray, and paired-end 100 base reads were generated using the BGISeq500 platform (BGI, Shenzhen, China).

Total RNA of IECs, mouse tissues (colon, spleen, and thymus), and LS174T cells was extracted by the Total RNA Extraction Reagent, and reverse transcription was performed using the HiScript II 1st-Strand complementary DNA Synthesis Kit (#R312-02; Vazyme) according to the manufacturer’s instructions. qPCR was performed using MagicSYBR Mixture (#CW3008M; CWBIO). Glyceraldehyde-3-phosphate dehydrogenase and actin were used for normalization of mouse samples and human samples, respectively. The primer sequences used to analyze the targeted gene expression are shown in Table 1.

Statistical Analysis
The data are presented as the means ± SEM. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). The Student t test (unpaired or paired Student t test) and 1-way analysis of variance were used for comparisons between 2 groups or multiple groups, respectively. Survival analysis was performed by the log-rank (Mantel–Cox) test. Statistical significance was considered to be ****P < .0001; ***P < .001; **P < .01; *P < .05; and NS at P > .05.

References
1. Pickard JM, Zeng MY, Caruso R, Núñez G. Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. Immuno Rev 2017; 279:70–89.
2. Odenwald MA, Turner JR. The intestinal epithelial barrier: a therapeutic target? Nat Rev Gastroenterol Hepatol 2017;14:9–21.
3. Pelaseyed T, Bergström JH, Gustafsson JK, Ermund A, Birchenough GM, Schütte A, van der Post S, Svensson F, Rodríguez-Piñeiro AM, Nyström EE, Wising C, Johansson ME, Hansson GC. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. Immuno Rev 2014; 260:8–20.
4. Yu LC. Microbiota dysbiosis and barrier dysfunction in inflammatory bowel disease and colorectal cancers: exploring a common ground hypothesis. J Biomed Sci 2018;25:79.
5. Wang F, Graham WV, Wang Y, Witkowski ED, Schwarz BT, Turner JR. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol 2005; 166:409–419.
6. Johansson ME, Gustafsson JK, Holmén-Larsson J, Jabbar KS, Xia L, Xu H, Ghishak FK, Carvalho FA, Gewertz AT, Sjövall H, Hansson GC. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. Gut 2014;63:281–291.
7. Johansson ME, Gustafsson JK, Sjöberg KE, Petersson J, Holm L, Sjövall H, Hansson GC. Bacteria penetrate the inner mucus layer before inflammation in the dextran sulfate colitis model. PLoS One 2010;5:e12238.
8. Neurath MF. Targeting immune cell circuits and trafficking in inflammatory bowel disease. Nat Immunol 2019;20:970–979.
9. Velichc A, Yang W, Heyer J, Fragale A, Nicholas C, Viani S, Kucherlapati R, Lipkin M, Yang K, Augenlicht L. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science 2002;295:1726–1729.
10. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature 2011; 474:298–306.
11. Van der Sluis M, De Koning BA, De Bruijn AC, Velichc A, Meijerink JP, Van Goudoever JB, Büsser HA, Dekker J, Van Seueningen I, Renes IB, Einerth AW. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology 2006;131:117–129.
12. Cornick S, Kumar M, Moreau F, Gaisano H, Chadee K. VAMP8-mediated MUC2 mucin exocytosis from colonic goblet cells maintains innate intestinal homeostasis. Nat Commun 2019;10:4306.
13. Lian Q, Yan S, Yin Q, Yan C, Zheng W, Gu W, Zhao X, Fan W, Li X, Ma L, Ling Z, Zhang Y, Liu J, Li J, Sun B. TRIM34 attenuates colon inflammation by sustaining barrier integrity. Cell Mol Immunol 2021;18:350–362.
14. McCool DJ, Okada Y, Forstner JF, Forstner GG. Roles of calreticulin and calnexin during mucin synthesis in LS180 and HT29/A1 human colonic adenocarcinoma cells. Biochem J 1999;341:593–600.
15. Corbett EF, Michalak M. Calcium, a signaling molecule in the endoplasmic reticulum? Trends Biochem Sci 2000;25:307–311.
16. Vassilakos A, Michalak M, Lehrman MA, Williams DB. Oligosaccharide binding characteristics of the molecular
chaperones calnexin and calreticulin. Biochemistry 1998; 37:3480–3490.

17. Tanikawa Y, Kanemura S, Ito D, Lin Y, Matsusaki M, Kuroki K, Yamaguchi H, Maenaka K, Lee YH, Inaba K, Okumura M. Ca(2+) Regulates ERp57-calnexin complex formation. Molecules 2021;26:2853.

18. Di Jeso B, Ulianich L, Pacifico F, Leonardi A, Vito P, Consiglio E, Formisano S, Arvan P. Folding of thyroglobulin in the calnexin/calreticulin pathway and its alteration by loss of Ca2+ from the endoplasmic reticulum. Biochem J 2003;370:449–458.

19. Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Comalli RJ, Adams R, Kato M, Neels KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA. Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med 2008;5:e54.

20. Coleman OI, Haller D. ER stress and the UPR in shaping intestinal tissue homeostasis and immunity. Front Immunol 2019;10:2825.

21. Tawiah A, Cornick S, Moreau F, Gorman H, Kumar M, Tiwari S, Chadee K. High MUC2 mucin expression and secretion, and apoptosis in goblet cells. Am J Pathol 2018; 188:1354–1373.

22. Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, Tawiah A, Cornick S, Moreau F, Gorman H, Kumar M, Tiwari S, Chadee K. High MUC2 mucin expression and misfolding induce cellular stress, reactive oxygen production, and apoptosis in goblet cells. Am J Pathol 2018; 188:1354–1373.

23. Wang JY, Sun J, Huang MY, Wang YS, Hou MF, Sun Y, He H, Krishna N, Chiu SJ, Lin S, Yang S, Chang WC. STIM1 overexpression promotes colorectal cancer progression, cell motility and COX-2 expression. Oncogene 2015;34:4358–4367.

24. Gandhirajan RK, Meng S, Chandramoorthy HC, Mallilankaraman K, Mancarella S, Gao H, Razmpour R, Yang XF, Houser SR, Chen J, Koch WJ, Wang H, Soboloff J, Gill DL, Madesh M. Blockade of NOX2 and STIM1 signaling limits lipopolysaccharide-induced vascular inflammation. J Clin Invest 2013; 123:887–902.

25. Oh-Hora M, Yamashita M, Hogan PG, Sharma S, Lamperti E, Chung W, Prakriya M, Teske S, Rao A. Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. Nat Immunol 2008;9:432–443.

26. Ma J, McCaill CA, Khalil S, Lüthy K, Teske S. T-cell-specific deletion of STIM1 and STIM2 protects mice from EAE by impairing the effector functions of Th1 and Th17 cells. Eur J Immunol 2010;40:3026–3042.

27. Schuhmann MK, Stegner D, Berna-Erro A, Bittner S, Braun A, Kleinschitz C, Stoll G, Wiendl H, Meuth SG, Nieswandt B. Stromal interaction molecules 1 and 2 are key regulators of autoreactive T cell activation in murine autoimmune central nervous system inflammation. J Immunol 2010;184:1536–1542.

28. Kaufmann U, Kahlfuss S, Yang J, Ivanova E, Koralov SB, Teske S. Calcium signaling controls pathogenic Th17 cell-mediated inflammation by regulating mitochondrial function. Cell Metab 2019;29:1104–1118.e6.

29. Schwarz A, Tutsch E, Ludwig B, Schwarz EC, Stallmach A, Hoth M. Ca2+ signaling in identified T-lymphocytes from human intestinal mucosa. Relation to hyperreactivity, proliferation, and inflammatory bowel disease. J Biol Chem 2004;279:5641–5647.

30. Engstrom JF, Arvantakis C, Sagawa A, Abdou NI. Secretory immunoglobulin deficiency in a family with inflammatory bowel disease. Gastroenterology 1978; 74:747–751.

31. Hasnain SZ, Tauro S, Das I, Tong H, Chen AC, Jefferson PL, McDonald V, Fiorin TH, McGuckin MA. IL-10 promotes production of intestinal mucus by suppressing protein misfolding and endoplasmic reticulum stress in goblet cells. Gastroenterology 2013;144:357–368.e9.

32. Herp S, Brugiroux S, Garzetti D, Ring D, Jochum LM, Beutler M, Eberl C, Hussain S, Walter S, Gerlach RG, Ruscheweyh HJ, Huson D, Sellin ME, Slack E, Hanson B, Loy A, Baines JF, Rausch P, Basic M, Bleich A, Berry D, Stecher B. Mucispirillum schaeudleri antagonizes salmonella virulence to protect mice against colitis. Cell Host Microbe 2019;25:681–694.e8.

33. Hetz C, Zhang K, Kaufman RJ. Mechanisms, regulation and functions of the unfolded protein response. Nat Rev Mol Cell Biol 2020;21:421–438.

34. Siwecka N, Rozpedek-Kamirska W, Wawryzkiewicz A, Pytel D, Diehl JÁL, Majsterek I. The structure, activation and signaling of IRE1 and its role in determining cell fate. Biomedicines 2021;9:156.

35. Bahar E, Kim H, Yoon H. ER Stress-mediated signaling: action potential and Ca(2+) as key players. Int J Mol Sci 2016;17:1558.

36. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe 2007;2:119–129.

37. Nagalingam NA, Kao JY, Young VB. Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis. Inflamm Bowel Dis 2011;17:917–926.

38. Nadeem MS, Kumar V, Al-Abbasi FA, Karnal MA, Anwar F. Risk of colorectal cancer in inflammatory bowel diseases. Semin Cancer Biol 2010;20:64–70.

39. Martens EC, Neumann M, Desai MS. Interactions of commensal and pathogenic microorganisms with the intestinal mucosal barrier. Nat Rev Microbiol 2018;16:457–468.

40. Packey CD, Sartor RB. Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Curr Opin Infect Dis 2009;22:292–301.

41. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, Snapper SB, Huttenhower C. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol 2012;13:R79.

42. Xia J, Wang H, Huang H, Sun L, Dong S, Huang N, Shi M, Bin J, Liao Y, Liao W. Elevated Orai1 and STIM1
expressions upregulate MACC1 expression to promote tumor cell proliferation, metabolism, migration, and invasion in human gastric cancer. Cancer Lett 2016;381:31–40.

43. Patergnani S, Danese A, Bouhamed E, Aguiari G, Previati M, Pinton P, Giorgi C. Various aspects of calcium signaling in the regulation of apoptosis, autophagy, cell proliferation, and cancer. Int J Mol Sci 2020;21:8323.

44. Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. Ann Rev Pathol 2015;10:173–194.

45. Strugala V, Dettmar PW, Pearson JP. Thickness and continuity of the adherent colonic mucus barrier in active and quiescent ulcerative colitis and Crohn’s disease. J Clin Pract 2008;62:762–769.

46. Pullan RD, Thomas GA, Newcombe RG, Williams GT, Allen A, Rhodes J. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. Gut 1994;35:353–359.

47. Nowarski R, Jackson RJ, Gagliani N, de Zoete MR, Palm NW, Bailis W, Low JS, Harman CC, Graham M, Elinav E, Flavell RA. Epithelial IL-18 equilibrium controls barrier function in colitis. Cell 2015;163:1444–1456.

48. Okamoto R, Watanabe M. Role of epithelial cells in the pathogenesis and treatment of inflammatory bowel disease. J Gastroenterol 2016;51:11–21.

49. Jakobsen HE, Rodriguez-Piñeiro AM, Schütte A, Ermund A, Boysen P, Bemark M, Sommer F, Bäckhed F, Hansson GC, Johansson ME. The composition of the gut microbiota shapes the colon mucus barrier. EMBO Rep 2015;16:164–177.

50. Ahuja M, Schwartz DM, Tandon M, Son A, Zeng M, Swaim W, Eckhaus M, Hoffman V, Cui Y, Xiao B, Worley PF, Muallern S. ORAI1-mediated antimicrobial secretion from pancreatic acini shapes the gut microbiome and regulates gut innate immunity. Cell Metab 2017;25:635–646.

51. Lian J, Cuk M, Kahlffus S, Kozhaya L, Vaeth M, Rieux-Laucat F, Picard C, Benson MJ, Jakovecovic A, Bilic K, Martinac I, Stathopoulos P, Kacskovics I, Vraetz T, Speckmann C, Ehl S, Issekutz T, Unutmaz D, Feske S. ORAI1 mutations abolishing store-operated Ca(2+) entry cause anhidrotic ectodermal dysplasia with immunodeficiency. J Allergy Clin Immunol 2018;142:1297–1310.e11.

52. Pabst O. New concepts in the generation and functions of IgA. Nat Rev Immunol 2012;12:821–832.

53. Bravo R, Parra V, Gatica D, Rodriguez AE, Torrealba N, Paredes F, Wang ZV, Zorzano A, Hill JA, Jaimovich E, Quest AF, Lavandero S. Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration. Int Rev Cell Mol Biol 2013;301:215–290.

54. Ly LD, Xu S, Choi SK, Ha CM, Thoudam T, Cha SK, Wiederkehr A, Wollheim CB, Lee IK, Park KS. Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes. Exp Mol Med 2017;49:e291.

55. Gombedza FC, Shin S, Kanaras YL, Bandyopadhyay BC. Abrogation of store-operated Ca(2+) entry protects against crystal-induced ER stress in human proximal tubular cells. Cell Death Discovery 2019;5:124.

56. Li B, Xiao L, Wang ZY, Zheng PS. Knockdown of STIM1 inhibits 6-hydroxydopamine-induced oxidative stress through attenuating calcium-dependent ER stress and mitochondrial dysfunction in undifferentiated PC12 cells. Free Radical Res 2014;48:758–768.

57. Zaki MH, Vogel PM, Malireddi RK, Body-Malapel M, Anand PK, Bertin J, Green DR, Lamkanfi M, Kanneganti TD. The NOD-like receptor NLPR12 attenuates colon inflammation and tumorigenesis. Cancer Cell 2011;20:649–660.

58. Yi ES, Boland JM, Maleszewski JJ, Roden AC, Oliveira AM, Aubry MC, Erickson-Johnson MR, Caron BL, Li Y, Tang H, Stoddard S, Wampfler J, Kulig K, Yang P. Correlation of IHC and FISH for ALK gene rearrangement in non-small cell lung carcinoma: IHC score algorithm for FISH. J Thorac Oncol 2011;6:459–465.

59. Logue JB, Stedmon CA, Kellerman AM, Nielsen NJ, Andersson AF, Laudon H, Lindström ES, Kritzberg ES. Experimental insights into the importance of aquatic bacterial community composition to the degradation of dissolved organic matter. ISME J 2016;10:533–545.

Received September 29, 2021. Accepted March 24, 2022.

Correspondence
Address correspondence to: Weidong Han, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, 3# East Qingchun Road, Hangzhou, Zhejiang 310016, China. e-mail: hanwd@zju.edu.cn; fax: 86-571-86436673.

CRediT Authorship Contributions
Weidong Han (Conceptualization: Equal; Funding acquisition: Lead; Resources: Lead)
Jiaojiao Liang (Conceptualization: Equal; Data curation: Lead; Formal analysis: Lead; Investigation: Lead; Writing – original draft: Lead; Writing – review & editing: Equal)
Jiansheng Xie (Conceptualization: Equal; Writing – review & editing: Supporting)
Hao Liu (Investigation: Supporting; Methodology: Supporting)
Rongjie Zhao (Formal analysis: Equal; Investigation: Supporting; Software: Equal)
Wei Zhang (Writing – review & editing: Supporting)
Haidong Wang (Investigation: Supporting)
Hongming Pan (Conceptualization: Supporting; Funding acquisition: Equal)
Yubin Zhou (Conceptualization: Supporting; Writing – review & editing: Supporting)

Conflicts of interest
The authors disclose no conflicts.

Funding
This work was supported by the National Natural Science Foundation of China (81572361 and 81972745), the Ten Thousand Plan Youth Talent Support Program of Zhejiang Province (JWKR0108009), and the Zhejiang Medical Innovative Discipline Construction Project-2016.

Data Transparency
The experimental data underlying this article are available in the article. The raw sequence data will be shared on reasonable request.