Inhibition of RIPK3 Pathway Attenuates Intestinal Inflammation and Cell Death of Inflammatory Bowel Disease and Suppresses Necroptosis in Peripheral Mononuclear Cells of Ulcerative Colitis Patients

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ABSTRACT

Receptor-interacting serine/threonine-protein kinase (RIPK) 3 is a member of the TNF receptor-I signaling complex and mediates necroptosis, an inflammatory cell death. Ulcerative colitis (UC) is an excessive inflammatory disease caused by uncontrolled T cell activation. The current study is aimed to determine whether RIPK3 inhibitor attenuates UC development inhibiting inflammation and necroptosis using experimental colitis mice model. Dextran sulfate sodium-induced colitis mice were administered RIPK3 inhibitor (3 mg/ml) 3 times and their tissues were analyzed by immunohistochemistry. RIPK3, mixed lineage kinase domain-like (MLKL), phosphorylated MLKL, IL-17, and CD4 in colitis patient colon tissues were detected using confocal microscopy. Protein levels were measured using immunohistochemistry and ELISA. The differentiation of Th17 cells was evaluated using flow cytometry. The expression of proinflammatory cytokines and necroptosis in peripheral blood mononuclear cells from UC patients was decreased markedly by RIPK3 inhibitor treatment. We also observed that the injection of RIPK3 inhibitor improves colitis severity and protects intestinal destruction. RIPK3 inhibitor reduced necroptosis factors and proinflammatory cytokines in the colon and consequently protected colon devastation. The expression of inflammatory mediators in experimental colitis mice splenocytes was decreased significantly by RIPK3 inhibitor treatment. These results suggest that RIPK3 inhibitor ameliorates severity of experimental colitis and reduces inflammation through the inhibition of inflammatory response and necroptosis and support RIPK3-targeting substances for treatment of UC.

Keywords: RIPK3 protein; Inflammatory bowel diseases; Necroptosis; Ulcerative colitis; Inflammation
INTRODUCTION

Receptor-interacting serine/threonine-protein kinase (RIPK) 3, a member of the TNF receptor-I signaling complex, causes necroptosis that is inflammatory cell death and a rigidly programmed type of necrosis. Necroptosis is related to a severe inflammatory response. It has been suggested that necroptosis is a programmed fashion and initiates a colossal exposure of inflammatory mediators (1,2). During necroptosis, cells endure rupture and release their matters into the extracellular space (3). Indeed, necroptosis leads to enormous release of inflammatory mediated and exacerbates inflammation (2,3). The inflammatory cell death is involved in many inflammatory disorders and pathophysiological conditions (4,5).

Ulcerative colitis (UC) is an inflammatory disease and causes imbalance of the human gut and a dysregulated immune response in the gastrointestinal tract. UC induces inflammation and ulcers of the colon and rectum (6). This relapsing disease can be characterized by various symptoms including diarrhea, weight loss and rectal bleeding (7,8). There are enormous factors such as genes, nutrients and cells that are involved in the pathogenesis of UC because the gastrointestinal tract conducts a key function in the regulation of immune-inflammatory responses against pathogens (9-11).

Although the cause of UC is unclear, dysfunction of immune inflammatory system is related with UC pathogenesis. It has been demonstrated that Th17 cells and TNF related genes are involved in UC susceptibility (12). Recently, there is a report that necroptosis is involved in UC development. It is well documented that the expression of RIPK3 is upregulated in the UC-involved colon tissues (13).

We hypothesized that the inhibition of RIPK3 attenuates necroptosis and inflammatory response in colitis. To identify whether RIPK3 inhibitor improves colitis progression, first, we measured the expression of necroptosis markers in colonic tissues from UC patients and animal models of UC. Second, we examined the anti-necroptosis and anti-inflammatory activity of RIPK3 inhibitor in vitro. Finally, we evaluated the therapeutic function of RIPK3 inhibitor in vivo using an experimental colitis.

MATERIALS AND METHODS

Immunohistochemistry

Formalin-fixed paraffin-embedded colon tissue sections (7-µm thickness) from UC patients were stained with H&E. Sections were treated with 3% (v/v) H2O2 in methanol to block endogenous peroxidase activity. Immunohistochemistry (IHC) was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Tissue sections were incubated with Abs against IL-17, RIPK3 and mixed lineage kinase domain-like (MLKL) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Sections were then incubated with a biotinylated secondary Ab and a streptavidin–peroxidase complex for 1 h. Color complexes were developed using 3,3′-diaminobenzidine (Dako, Carpinteria, CA, USA). Histological assessments were conducted by 2 independent blinded observers. The independent observers involved in analysis of immunohistochemistry staining counting 3 microscopy photos. Images were captured using a DP71 digital camera (Olympus, Center Valley, PA, USA) attached to an Olympus BX41 microscope (Olympus, Tokyo, Japan) at a magnification of ×3,400. Mice colon tissues were stained and analyzed by identical protocol.
Histopathological analysis

The biopsy specimens of UC patients were then reviewed by a blinded expert gastrointestinal pathologist and graded using the Geboes grading system (14). The Geboes score ranges from 0 to 5.4, with higher scores indicating more severe inflammation, and we defined UC as active histological inflammation with a Geboes score of ≥3.1.

Mice colon tissue assessment included reporting of edema, extent of injury, leukocyte infiltration, crypt abscesses, and loss of goblet cells. In this grading system, inflammation severity was scored using a scale of 0–3 (0, no inflammation; 1, slight inflammation; 2, moderate inflammation; and 3, severe inflammation), as the extent of injury (0, no injury; 1, mucosal injury; 2, mucosal and submucosal injury; and 3, transmural injury). Crypt damage was scored using a scale of 0–4 (0, no damage; 1, basal third was damaged; 2, basal two-thirds was damaged; 3, only the surface epithelium was intact; and 4, loss of entire crypt and epithelium). Each value was multiplied by an extent index, ranging from 1–4, that reflected the amount of involvement for each section (1, 0%–25%; 2, 26%–50%; 3, 51%–75%; and 4, 76%–100%). At least three sections from each colon were analyzed.

Confocal microscopy

Colon tissue sections from UC patients tissue (7-µm thickness) were incubated with phycoerythrin-conjugated Abs against IL-17, phosphorylated MLKL (p-MLKL), RIPK3, a fluorescein isothiocyanate-conjugated Ab against CD4 (eBioscience, San Diego, CA, USA) overnight at 4°C. Tissue sections were then washed with PBS and incubated with the appropriate secondary Abs conjugated with fluorescent molecules where necessary. Sections were analyzed using a Zeiss microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany) at a magnification of ×3,400. Mice spleens were stained and analyzed by identical protocol.

Isolation of peripheral blood mononuclear cells

PBMCs from UC patients were isolated from buffy coats in heparinized blood samples by Ficoll-Hypaque (Amersham Biosciences, Pittsburgh, PA, USA) density-gradient centrifugation. PBMCs were cultured in RPMI 1640 medium (GibcoBRL, Carlsbad, CA, USA) containing penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS (GibcoBRL) that had been inactivated by heating to 55°C for 30 min. The cell suspensions were dispensed into 48-well plates (Nunc, Roskilde, Denmark) at 37°C/5% CO2.

Preparation of mice splenocytes

Mouse spleens were collected for cell preparation and washed twice with PBS. The spleens were minced and the red blood cells were lysed with 0.83% ammonium chloride. The total splenocytes fraction was filtered through a cell strainer and centrifuged at 1,300 rpm at 4°C for 5 min. The cells were cultured RPMI 1640 medium (GibcoBRL) containing penicillin (100 U/ml), streptomycin (100µg/ml) and 5% FBS (GibcoBRL) and seeded into 48-well plates (Nunc) at 37°C/5% CO2.

Flow cytometry

PBMCs of UC patients were immunostained with propidium iodide (PI) and annexin V (Biocompare, South San Francisco, CA, USA). Before intracellular staining, cells were restimulated for 4 h with phorbol myristate acetate (25 ng/ml) and ionomycin (250 ng/ml) in the presence of GolgiStop™ (BD Biosciences, San Diego, CA, USA). Intracellular staining was performed using the kit (eBiosciences, San Diego, CA, USA) following the manufacturer’s protocol. Then the PBMCs were stained with various combinations of fluorescent Abs against...
Flow cytometry was performed on a FACSCalibur™ flow cytometer (BD Biosciences). Mice splenocytes were stained and analysed by identical protocol.

**ELISA**

Cytokine levels in culture supernatants from UC patients PBMCs or mice splenocytes were measured by sandwich ELISA. Abs directed against mouse TNF-α, IL-6, and IL-17 and biotinylated anti-mouse TNF-α, IL-6, and IL-17 (R&D Systems, Minneapolis, MN, USA) were used as the capture and detection Abs, respectively. The concentration of cytokines present in the test samples was determined from standard curves established with serial dilutions of recombinant TNF-α, IL-6, and IL-17 (R&D Systems). The absorbance was measured using an ELISA microplate reader at 405 nm (Molecular Devices, Sunnyvale, CA, USA).

**Cell treatment**

We treated 1 or 10 μM GSK’872 to 5×10⁵ PBMCs from UC patients which were stimulated with anti-CD3 (0.5 μg/ml) or zVAD (20 μM) and TNF-α (10 ng/ml).

The cells were stimulated with 0.5 μg/ml plate-bound anti-CD3 mAb (BD Biosciences), 1 μg/ml soluble anti-CD28 mAb (BD Biosciences), 2 μg/ml anti-IFN-γ Ab, 2 μg/ml anti-IL-4 Ab, 2 μg/ml anti-IL-2 Ab (all from R&D Systems), 2 ng/ml recombinant TGF-β (R&D Systems), and 20 ng/ml recombinant IL-6 (R&D Systems) for 3 days to establish Th17 polarization.

**Animals**

We purchased C57BL/6 mice (8-wk-old) from SLC Inc. (Shizuoka, Japan) and maintained them under specific pathogen-free conditions at the Institute of Medical Science (Catholic University of Korea). Mice were provided standard mouse chow (Ralston Purina, St. Louis, MO, USA) and water ad libitum. All surgeries were performed under isoflurane anesthesia and we made an effort to minimize the suffering of all animals. Mice were euthanized at the end of a study for the purpose of sample collection and histologic examination by CO₂ chamber. The experimental protocol was approved by Animal Research Ethics Committee at the Catholic University of Korea (CUMC-2016-0250-01), and all animals were treated and sacrificed in accordance with the guidelines of the Catholic University of Korea on Use and Care of Animals.

**Ethics statement**

All the subjects (12 patients) were diagnosed as UC at least 1 year before the time of study inclusion. All the clinical, endoscopic and histologic findings of the subjects were consistent with those of UC. Using biopsy forceps, 2 pieces of colonic mucosal tissue were obtained at the UC-involved rectosigmoid and UC-uninvolved proximal colon during surveillance colonoscopy, respectively. The 10 mL of peripheral blood was sampled at the time of colonoscopic examination. All patients gave informed written consent. This study was approved by the Institutional Review Board of Seoul St. Mary’s Hospital (KC16TISI0005) and was performed in accordance with the Helsinki II Declaration.

**Induction of dextran sulfate sodium (DSS) induced colitis and RIPK3 inhibitor treatment**

Colitis was induced in C57BL/6 mice through the oral ingestion of 3% DSS (MP Biomedicals, Santa Ana, CA, USA) for 4 days. Mice were intraperitoneally injected with RIPK3 inhibitor (GSK’872; Calbiochem, La Jolla, CA, USA) 3 mg/kg three times a week for 13 days after inflammatory bowel disease induction. During the experimental period, severity of...
colitis was assessed daily by measuring the percentage of body weight change and disease activity index (DAI). The DAI was calculated as previously described (15), with the score incorporating indicators of body weight loss, stool consistency, and visible gross bleeding.

**Statistical analysis**

All experiments were iterated three times at least. The data were analyzed using the nonparametric Mann-Whitney *U* test to compare two groups or one-way analysis of variance with Bonferroni’s *post hoc* test for multiple comparisons. GraphPad Prism® (ver. 5.01, GraphPad Software, La Jolla, CA, USA) was used for all analyses. A value of *p*<0.05 was considered to indicate significance. Data are expressed as mean±SD.

**RESULTS**

**Inflammatory cell infiltration and necroptosis were increased in involved tissues from patients with UC**

We conducted histologic analysis to measure inflammation and the expression of necroptosis factors. Excessive immune cell infiltration and tissue damage were observed especially in involved colonic tissues (Fig. 1A). The expression of RIPK3 and MLKL was increased in involved colon tissue compared to uninvolved tissue. IL-17A production was also promoted in involved colon tissue compared to uninvolved tissue (Fig. 1B and C). Moreover, confocal scanning revealed that necroptosis and inflammation in CD4⁺ T cells are associated with UC development. In involved tissue from UC patients, the expression of RIPK3, p-MLKL, and IL-17A in CD4⁺ T cells were increased compared to uninvolved tissue (Fig. 2A and B). These results suggest that involved colonic tissues show severe inflammation and necroptosis, and UC is related to inflammatory cell death in CD4⁺ T cells.

**RIPK3 inhibitor reduced necroptosis and inhibited the expression of proinflammatory cytokines**

We pretreated 1 μM RIPK3 inhibitor PBMCs from UC patients stimulated by anti-CD3. Treatment with RIPK3 inhibitor decreased the percentage of PI-positive cells (Fig. 3A). Although the concentration of IFN-γ had no significant difference, the levels of IL-17, IL-6, and TNF-α in the supernatant were downregulated significantly by RIPK3 inhibitor treatment (Fig. 3B). We also induced necroptosis in PBMCs obtained from UC patients using zVAD and TNF-α. RIPK3 inhibitor reduced the percentage of PI-positive and annexin V-negative cells at 36 h (Fig. 3C and Supplementary Fig. 1). These results suggest that RIPK3 inhibitor can reduce necroptosis and the production of proinflammatory cytokines.

**RIPK3 inhibitor decreased the inflammatory response and Th17 cell differentiation**

Since Th17 cells are related with the pathogenesis of colitis and downregulation of Th17 cells differentiation can improve experimental colitis (16,17), we examined Th17 cell differentiation and the inflammatory response in mice splenocytes. Normal splenocytes were pretreated with DMSO or RIPK3 inhibitor and the cells were stimulated with anti-CD3. Treatment with RIPK3 inhibitor decreased the expression of proinflammatory cytokines (Fig. 4A). There were no markedly difference of Th1 differentiation, however, RIPK3 inhibitor decreased the differentiation of Th17 cells significantly (Fig. 4B). The expression of IL-17 and TNF-α in the supernatant was also reduced significantly by RIPK3 inhibitor treatment (Fig. 4C). These findings suggest that RIPK3 inhibitor can decrease the inflammatory response and Th17 cells differentiation.
Experimental colitis induced the expression of key factors in necroptosis

We performed a histological analysis of colon tissue from naïve and DSS-induced colitis mice. Experimental colitis increased immune cell infiltration and tissue damage (Fig. 5A). The expression of RIPK3 and MLKL was also promoted significantly in DSS-induced colitis mice (Fig. 5B). Additionally, we analyzed that the relative RIPK3 and MLKL mRNA expression in colon tissue of normal controls and DSS-induced colitis using information contained in the National Center for Biotechnology Information Gene Expression Omnibus database (GSE34874). In our analysis, gene expression of RIPK3 was higher than that in normal controls (Supplementary Fig. 2). We also analyzed the relative RIPK3 and MLKL mRNA expression in mucosal tissue contained in this database and found that the relative mRNA expression of RIPK3 was increased slightly whereas gene expression of MLKL was enhanced significantly in mucosal tissue from UC compared to that from healthy controls (Supplementary Fig. 3A). The relative RIPK3 mRNA was decreased slightly whereas gene expression of MLKL was upregulated.

Figure 1. Colitis induces the expression of necroptosis and IL-17. (A) Colon tissues from UC patients were stained with H&E (original magnification, ×200, n = 3). (B and C) Immunohistochemical visualization of RIPK3, MLKL, and IL-17 in the colon tissue of UC patients (n=3). *p<0.05, **p<0.01, ***p<0.001.
in uninvolved colonic mucosa from UC patients compared to involved colonic mucosa from UC patients (Supplementary Fig. 3B). We also observed that the relative gene expression of RIPK3 was decreased slightly whereas gene level of MLKL was increased significantly in intestinal mucosa from active UC patients compared to that from healthy controls. However, the relative mRNA expression of RIPK3 and MLKL was significantly reduced in remission UC patients (Supplementary Fig. 3C). In the investigation, we observed the upregulation of key necroptosis factors and proinflammatory cytokines in involved colon tissues from experimental colitis and UC patients. Thus, necroptosis may be involved in the pathogenesis of colitis.

RIPK3 inhibitor reduced the progression of DSS induced colitis, necroptosis factors and proinflammatory cytokines

To investigate whether RIPK3 inhibitor can improve colitis, the mice with DSS induced colitis were injected intraperitoneally with either RIPK3 inhibitor three times a week or with vehicle only. RIPK3 inhibitor treatment decreased DSS induced colitis onset improving weight loss (Fig. 6A), survival (Fig. 6B) and colon length (Fig. 6C and D). Treatment with RIPK3 inhibitor was also related with a significant decrease in the DAI score (Fig. 6E) and

Figure 2. Colitis induces the expression of necroptosis and IL-17 in CD4+ T cells. (A and B) Confocal scanning detection of RIPK3, MLKL, and IL-17 by staining in the colon tissue from UC patients. Images were obtained for each UC patients (n=3), and the representative images are shown (original magnification, ×200). *p<0.05, **p<0.01.
in colon tissue damage with H&E staining (Fig. 6F and G). We also performed a histological analysis of colon tissue from the mice with DSS induced colitis. The expression of RIPK3 and p-MLKL was downregulated significantly by RIPK3 inhibitor (Fig. 7A). We also measured the expression of necroptosis factors in the colon from vehicle and RIPK3 inhibitor-treated mice with experimental colitis. We found that RIPK3 inhibitor reduced the expression of IL-17 and TNF-α significantly in the colon tissue. Additionally, RIPK3 inhibitor reduced the expression of RIPK3, p-MLKL, and IL-17 in CD4+ T cells (Fig. 8A and B). These results suggest that RIPK3 inhibitor can improve colon tissue destruction and colitis through downregulation of inflammation and key factors in necroptosis.
Figure 4. RIPK3 inhibitor treatment downregulates Th17 cell differentiation and inflammatory response. (A) Normal splenocytes were treated with DMSO or RIPK3 inhibitor and stimulated with anti-CD3 for 72 h. The expression of IL-6, IL-17, and TNF-α in culture supernatants was measured using ELISA. Statistical analyses were conducted using the nonparametric Mann-Whitney U test. (B) Splenocytes from mice with collagen-induced arthritis were cultured under Th17 conditions for 72 h and then the population of CD4\(^+\)IL-17\(^+\) cells was quantified. (C) IL-17 and TNF-α expression in culture supernatants was measured using ELISA. The data represent the mean±SD from 3 independent experiments. 

SSC, side scatter. 

\( ^* p<0.05 \), \( ^{**} p<0.01 \) (n = 5).
DISCUSSION

Though RIPK3 activity in necroptosis has been investigated extensively and RIPK3 production was increased in colon tissue from UC patients (2,13), there is no report suggesting therapeutic effect of RIPK3 inhibitor in colitis. In this study, we found that RIPK3 inhibition attenuated DSS-induced colitis severity. Since little is known about the function of RIPK3 in T cell-mediated immune responses and inflammatory colitis, the most valuable observation of the current study is that RIPK3 inhibitor improved experimental colitis by downregulating necroptosis. To the best of our knowledge, this is the first report to demonstrate evidence indicating that RIPK3 inhibitor could be treated as a therapeutic agent in colitis. Previous reports have suggested that experimental colitis was exacerbated or not improved by the loss of RIPK3 (18,19). However, RIPK3 deficiency improved ischemia injury and increased survival through suppression of necrotic lesions in allogeneic kidney transplantation (20). Moreover, necrostatin-1, RIPK1 inhibitor which can reduce necroptosis shows protective activity on intestinal inflammation and attenuates tumor progression mediated by colitis (21). Thus, genetic deletion of necroptosis factors can be related with intestinal tissue repair. However, this study showed that RIPK3 inhibitor ameliorated necroptosis in CD4+ T cells and reduced Th17 cells differentiation and that are important pathogenic factors in colitis. Our results demonstrate that RIPK3 inhibition in CD4+ T cells can be used as a treatment of UC.

Currently, colitis is characterized by uncontrolled intestinal inflammation and many inflammatory diseases are associated with necroptosis (2,22). Additionally, CD4+ T cells are involved in the pathogenesis of colitis (23). Though necroptosis is involved in the pathogenesis of inflammatory autoimmune arthritis in recent (24), however, the role of necroptosis in colitis pathogenesis is not apparent. We found that the expression of RIPK3 and MLKL was promoted in the involved colon tissue of UC patient and DSS-induced colitis mice. The expression of necroptosis mediators was also increased in CD4+ T cells in the involved colon tissue of UC patients. These results suggest that necroptosis may perform a significant role in colitis pathogenesis.

Figure 5. DSS-induced colitis increased the expression of necroptosis factors. (A) Colon tissues from normal and DSS-induced colitis mice were stained with H&E, and safranin O (n=4). (B) Immunohistochemical visualization of RIPK3 and MLKL in the colon of normal and DSS-induced colitis mice (n=4).
Necroptosis is related with various inflammatory disorders because it causes excessive inflammatory response (2,3). Indeed, the expression of RIPK3 and MLKL was increased by experimental autoimmune arthritis (24). It is also well reported that the expression of RIPK3 and MLKL was increased in the UC-involved colon tissues (13). We observed that treatment with RIPK3 inhibitor reduces the percentage of PI-positive cells in PBMCs of UC patients which were stimulated by anti-CD3 and necroptosis conditions. Moreover, RIPK3 inhibitor decreased proinflammatory cytokines production from UC patients PBMCs and splenocytes stimulated by anti-CD3 in vitro. RIPK3 inhibitor decreased the expression of RIPK3 and p-MLKL in colon and CD4+ T cells in vivo. The results of current investigation demonstrate that RIPK3 inhibition can be a promising therapeutic strategy in the treatment of UC.

In this study, we observed that RIPK3 inhibitor reduces Th17 cells differentiation and the expression of proinflammatory cytokines in vitro and in vivo. Th17 cells are significant factors...
in excessive and chronic inflammatory response (25). Recently, the inhibition of Th17 cells differentiation can improve experimental colitis and intestinal inflammation (17). Our data revealed that RIPK3 inhibitor treatment prevented colitis through the inhibition of Th17 cells differentiation and inflammation.

The study has a limitation, in vitro assays and histologic data were performed using relatively small number of samples of UC patients. However, this study is the first evidence to suggest the possible therapeutic potential of RIPK3 inhibitor in experimental colitis. Experimental colitis using conditional knock-out mice is also needed to confirm that RIPK3 deletion in CD4⁺ T cells can improve colitis without any adverse effect. Future studies with a large number of UC patient’s samples and RIPK3 deficiency in CD4⁺ T cells are required to validate our hypothesis more precisely.

There has been little evidence indicating the involvement of necroptosis in colitis pathogenesis and therapeutic activity of RIPK3 inhibitor. Our results may shine light on the pathogenesis of colitis demonstrating a significant role of necroptosis. The present study demonstrated that the expression of key molecules in necroptosis was increased by colitis. RIPK3 inhibitor also improves intestinal inflammation by downregulating Th17 cells differentiation and the expression of RIPK3 and MLKL in the colon. Notably identified RIPK3 inhibitor activity effect suggests that RIPK3 inhibitor treatment leads to reduction of intestinal inflammation. In conclusion, necroptosis factors can be considered a therapeutic candidate for colitis and RIPK3 inhibitor ameliorates colitis through the inhibition of intestinal inflammation.
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SUPPLEMENTARY MATERIALS

Supplementary Figure 1
The percentage of PI-positive cells was decreased by RIPK3 inhibitor treatment.

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Supplementary Figure 2
The level of RPIK3 of DSS-induced colitis group was slightly higher than control group. MLKL levels of control and DSS-induced colitis group had no significant differences.

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Supplementary Figure 3
Relative mRNA level of RPIK3 was measured. (A) RIPK3 and MLKL levels of UC were compared with healthy controls. (B) The levels of RIPK3 and MLKL expression of inflamed patients was compared with healthy control. (C) The levels of RIPK3 and MLKL were measured in groups of normal controls, UC active and UC remission respectively.

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