1. Introduction

Ulcerative colitis, characterized by chronic, relapsing and remitting inflammation, not only impairs patients’ quality of life, but also increases the risk of colon cancer (1,2). A lot of evidence has shown that the risk of colorectal cancer development in patients with IBD is much higher than that in the general population. The pathogenesis of ulcerative colitis is complicated and may involve genetic, environmental and immunological factors (3). It is suggested that dysfunctional of the epithelial barrier, provoked by physical/chemical damage or genetic mutations, will caused inappropiate responses to microbiota or related products thus resulting in chronic inflammation (4,5). To investigate this disease in mice, a chemical-induced model of acute colonic inflammation has been introduced by oral administration of dextran sulfate sodium (DSS) and characterized by a general inflammatory process associated with weight loss and histopathologic features that mimic some clinical demonstrations of ulcerative colitis. By oral administration of DSS, mice develop an inflammatory condition in the intestine associated with clinical symptoms (diarrhea, abdominal pain, and weight loss), histopathological features and changes in cytokine profile, which resembles ulcerative colitis in human (6,7). Here we use this model to search for potential therapeutic compounds for ulcerative colitis.

Fumigaclavine C, an alkaloidal metabolite, was isolated from the culture of Cephalosporium sp. IFB-018, an endophytic fungus from the rhizoma of a salinity-tolerant medicinal plant Imperata cylindrical by a column chromatography fraction from the chloroform–methanol (1:1) extract and was identified by a combination of spectroscopic methods as reported previously by us (8). Our previous study has reported its immunosuppressive activity against Con A-induced hepatitis and TNBS-induced colitis in mice via down regulating Th1 cytokine production (9,10), which suggests that this compound may have a characteristic to inhibit the T-cell mediated immune response. In this study, we tried to test whether Fumigaclavine C can be benefit for ulcerative colitis treatment and search for its possible mechanism.
2. Materials and methods

2.1. Mice

Six-to-eight-week-old female C57BL/6 mice were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals’ suffering and to reduce the number of animals used.

2.2. Reagents

Fumigaclavine C (more than 90% of purity, isolated and identified as reported. Phorbol myristate acetate (PMA), lipopolysaccharide (LPS) and adenosine triphosphate (ATP) were purchased from Sigma–Aldrich (St. Louis, MO). Dextran sulfate sodium (DSS, 36–50 kDa) was bought from MP Biomedical (Aurora, OH). RPMI-1640 and Fetal bowel serum were purchased from Life technology. Anti-p-STAT1 and human IL-1β were purchased from Santa Cruz (Santa Cruz, CA). Anti-p-STAT1 and anti-p-STAT3 were bought from Cell Signaling Technology. Caspase-1 assay Kit was bought from Beyotime (Nantong, China). ELISA kits for murine TNF-α, IL-1β, IL-17A and human IL-1β were purchased from Dakewe Biotech Co. Ltd (Beijing, China). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

2.3. Cell culture

Human THP-1 cells were purchased from Shanghai Institute of Cell Biology (Shanghai, China) and maintained in RPMI 1640 medium, supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO₂ atmosphere at 37 °C. Peritoneal macrophages were obtained from the peritoneal cavity by injection of PBS. Cells were washed twice in PBS and suspended in RPMI-1640 medium containing 10% FCS, 10,000 U/ml penicillin and 10 mg/ml streptomycin. The macrophages suspended in culture medium were cultured in 24-well microplates for 40 min at 37 °C in a moist atmosphere of 5% CO₂. Non adherent cells were removed by washing the plate twice with PBS. The adherent macrophages were used for experiments.

2.4. Induction of colitis and treatment

Colitis was induced in C57BL/6 mice with 2.5% DSS (molecular weight 36–50 kDa) dissolved in drinking water (days 1–7). Normal mice were given water. Vehicle control (PBS), Fumigaclavine C (3, 10, 30 mg/kg) and Cyclosporine A (CSA) (20 mg/kg) were given orally from day 1 to day 10, respectively.

2.5. Clinical scoring and histological analysis

Body weight, stool consistency and the presence of gross blood in feces and at the anus were observed everyday. The disease activity index (DAI) was calculated by assigning well-established and validated scores. Briefly, the following parameters were used for calculation: a) diarrhea (0 points = normal, 2 points = loose stools, 4 points = watery diarrhea); b) hematochezia (0 points = no bleeding, 2, slight bleeding, 4 points, gross bleeding) [11]. At day 10 following induction of colitis, animals were sacrificed, the colon was removed and pieces of colonic tissue were used for ex vivo analysis. For histological analysis, part of the colon was fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with H&E (hematoxylin-eosin) according to standard protocols. For histological morphometry, colonic tissues were embedded in paraffin, and cut into 5 μm of sections and stained with hematoxylin and eosin. Histopathological grading system was used in a blinded manner: Grade 0: histological findings identical to normal mice; Grade 1: mild mucosal and/or submucosal inflammatory infiltrate and edema, punctuate mucosal erosions often associated with capillary proliferation, muscularis mucosae intact; Grade 2: 50% of the specimen display Grade 1 changes; Grade 3: prominent inflammatory infiltrate and edema frequently with deeper areas of ulceration extending through the muscularis mucosa into the submucosa, rare inflammatory cells invading the muscularis propria but without muscle necrosis; Grade 4: 50% of the specimen display Grade 3 changes; Grade 5: extensive ulceration with coagulative necrosis bordered underneath by numerous neutrophils and lesser numbers of mononuclear cells, necrosis extends deeply into the muscularis mucosae; Grade 6: 50% of the specimen display Grade 5 changes.

2.6. Cytokine analysis by ELISA

Total protein of colons from mice in each group was extracted by homogenated with lysis buffer. The homogenate was centrifuged at 12,000 g at 4 °C for 15 min. The amount of total extracted protein was determined by BCA™ protein assay kit (Pierce, Rochford, IL). The amount of IL-1β, IL-17A and TNF-α in the colon homogenate was quantified by ELISA kit (Dakewe, Beijing, China) and normalized to the protein quantity.

2.7. Real-time quantitative PCR

RNA samples were reverse transcribed to cDNA and subjected to quantitative PCR, which was performed with the BioRad CFX96 Touch™ Real-Time PCR Detection System (BioRad, CA) using IQ™ SYBR® Green Supermix (BioRad) and threshold cycle numbers were obtained using BioRad CFX Manager software. The program for amplification was 1 cycle of 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 95 °C for 10 s. The primer sequences used in this study were as follows: tnf-α forward 5’-CGATGACAGCCTGTAGGCC-3’; tnf-α reverse 5’-GTCTTTCAGATCGATCCGTGG-3’; il-1β forward 5’-CTTACGGCCAGCATACTC-3’; il-1β reverse 5’-TCCAGTTCTTATGGAAGACTC-3’; il-17A forward 5’-TGGAGAAGTGGTGTGGGTG-3’; il-17A reverse 5’-CTCCTTTGAGTGCTTCGCG-3’; β-actin forward 5’-TGCCTTCCGTATGGCTCTT-3’; β-actin reverse 5’-TTTATGTACGACGACATTT-3’

2.8. Western blot

The protein lysates were separated by 10% SDS-PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with 5% nonfat milk for 1 h at room temperature. The blocked membrane was incubated with the indicated primary Abs, and then with a horse-radish peroxidase-conjugated secondary Ab. Protein bands were visualized using Western blotting detection system according to the manufacturer’s instructions (Cell Signaling Technology, MA).

2.9. Statistical analysis

Results were expressed as mean ± SEM of three independent experiments and each experiment included triplicate sets. Data were statistically evaluated by one-way ANOVA followed by
Dunnett’s test between control group and multiple dose groups. The level of significance was set at a $P$ value of 0.05.

3. Results

3.1. Fumigaclavine C attenuated DSS-induced experimental colitis

In the present study, a mouse model of DSS-induced experimental colitis was used to evaluate the therapeutic effect of Fumigaclavine C. Mice were challenged with DSS for 7 days thus lead to inflammatory conditions in the colon. Oral intake of DSS induces a severe illness in mice characterized by a dramatic loss of body weight, evident rectal bleeding and diarrhea. As shown in Fig. 1A, compared with normal group, mice in DSS group lose about 20% bodyweight, while mice from the 30 mg/kg Fumigaclavine C-treated group just lose 10 percent. Fumigaclavine C also significantly reduced the DAI, a clinical parameter reflecting the severity of weight loss, rectal bleeding and stool consistency (Fig. 1B). DSS typically causes colonic shortening while such change was also improved by 10 and 30 mg/kg of a Fumigaclavine C and 20 mg/kg of CsA (Fig. 1C and D). Histological analysis showed distortion of crypts, loss of goblet cells, infiltration of mononuclear cells, and severe mucosal damage in the colon specimens of colitis mice (Fig. 2A). The results of standard pathological examination in mice showed much improvement in pathological changes in mice treated with 30 mg/kg of Fumigaclavine C and 20 mg/kg of CsA (Fig. 2B).

3.2. Fumigaclavine C inhibited inflammatory cytokines expression in colons of mice with DSS-induced colitis

To determine the effect of Fumigaclavine C on cytokine production in mice with DSS-induced colitis, cytokine expression at both mRNA and protein levels in colons were measured in parallel following induction of colitis. Total RNA of colons were extracted and analyzed for cytokine mRNA expression using quantitative RT-PCR method. As shown in Fig. 3, the mRNA expressions of TNF-α, IL-1β, and IL-17A were remarkably increased after DSS challenge. Fumigaclavine C significantly inhibited the elevated expression of these cytokines after DSS challenge (Fig. 3A). Moreover, we analyzed the cytokine levels in colonic homogenated protein from each group via ELISA analysis. Administration of Fumigaclavine C to mice also significantly down-regulated the inflammatory cytokines at protein level (Fig. 3B).

3.3. Fumigaclavine C reduced DSS-induced activation of NF-κB, STAT3 and STAT1 signaling

Activations of NF-κB, STAT3 and STAT1 all play essential roles in transcriptional induction of various genes involved in inflammation, such as TNF-α, IL-1β, IFN-γ and IL-17A (12). As shown in Fig. 4, DSS treatment caused different levels of phosphorylations of p65, STAT3 and STAT1 in the injured colons from mice. Both Fumigaclavine C and CsA treatment markedly reduced the phosphorylations of p65, STAT3 and STAT1.

3.4. Fumigaclavine C reduced cleaved caspase-1 expression of peritoneal macrophages in DSS-induced colitis mice

To further investigate the mechanism of protection from colitis by Fumigaclavine C, we examined the regulation of caspase-1 activation in murine peritoneal macrophages in vivo from each group. We observed that expression of activated caspase-1 (caspase-1 p10) was significantly elevated in macrophages from vehicle-treated DSS mice. In contrast, little cleaved caspase-1...
activation was detected in either Fumigaclavine C-treated or untreated colonic samples (Fig. 5A).

3.5. Fumigaclavine C inhibited the activation of NLRP3 inflammasome in vitro

Caspase-1 is an enzyme that responsible for IL-1β maturation. IL-1β was processed as an inactive cytoplasmic precursor (pro-IL-1β) which has to be cleaved by to produce the mature active form. Evidence indicates that a complex-named NLRP3 inflammasome (contain NLRP3, ASC and pro-caspase-1) are responsible for the activation of caspase-1 maturation (caspase-1 p10 and p20) (13). In order to verify the in vivo results that caspase-1 activation was suppressed by Fumigaclavine C, we build up a cell model by using LPS and ATP which mimics the process of NLRP3 inflammasome activation. As shown in Fig. 5C, Fumigaclavine C exhibited a concentration-dependent inhibition of IL-1β secretion from LPS-treated human monocytic THP-1 cells by the ELISA assay without affecting the survival of macrophages (data not shown). Consistent with the results we got in the animal model, our results showed that activation of caspase-1 (as indicated by the presence of the cleaved form p10 and enzyme activity) was also significantly inhibited by Fumigaclavine C (Fig. 5B and D).

4. Discussion

Generally, ulcerative colitis is treated as an autoimmune disease. Treatments including anti-inflammatory drugs, immunosuppres-
immune response have been proved effective in controlling the symptoms. However, they also have potential side effects including steroid dependence (14) or serious infections (15). Therefore, new therapeutic strategy and candidates for ulcerative colitis need to be developed. We report herein that reducing IL-1β release from macrophages via NLRP3 inflammasome inhibition by using Fumigaclavine C protects mice against DSS-induced colitis which suggest target NLRP3 inflammasome for ulcerative colitis therapy.

The NLRP3 inflammasome is multi-protein complex which recognize microbial and danger components and serve as a platform for caspase-1 activation and pro-inflammatory cytokine IL-1β maturation (16). NLRP3 inflammasome is increasingly being

Fig. 4. Fumigaclavine C decreased activations of inflammatory signaling pathways in colon tissues from DSS-colitis mice. (A) Colonic homogenate from each group of mice were subjected to Western blot. (B) Phosphorylations of p65, STAT1 and STAT3 were normalized to ACTIN. Data are presented as means ± SEM. *P < 0.05, **P < 0.01 vs. DSS-treated group.

Fumigaclavine C inhibited NLRP3 inflammasome activation in vivo and in vitro. (A) Peritoneal macrophages were isolated from normal, vehicle-treated and Fumigaclavine C-treated colitis mice (30 mg/kg) at day 7. Caspase-1 activation in peritoneal macrophages was examined by western blot. (B) LPS-primed THP-1 cells were treated with Asiatic acid (12.5, 25, 50 μM) for 1 h, following by 1 h incubation of 5 mM ATP. Protein levels of pro-caspase-1, cleaved caspase-1, ASC and NLRP3 were determined by Western blot (B). Released IL-1β in the supernatant was analyzed by ELISA (C). Caspase-1 activity was measured (D). Data are presented as means ± SEM (n = 6 per group). *P < 0.05, **P < 0.01 vs. DSS-treated group.
recognized because of its clinical importance in autoimmune, infectious and metabolic diseases. NLRP3 inflammasome activation has a crucial role in host defense against infection while excessive activation will lead to various auto-inflammatory conditions (17). Still the role of inflammasome in colitis is controversial. On one hand, work by Zaki et al. showed that the NLRP3 inflammasome protected against loss of epithelial integrity and mortality during experimental colitis (18). On the other hand, On the other hand, Bauer et al. believed that colitis induced in mice with DSS was mediated by the NLRP3 inflammasome (19). Furthermore, IL-1β neutralization (20,21) or chemical CASP1 inhibitor (22,23) effectively reduced severity in murine colitis. Therefore, inhibition of CASP1-mediated IL-1β secretion may serve as a useful therapeutic option for patients with IBD (24,25). Thus target for NLRP3 inflammasome complex may be a novel therapy option for patients with inflammatory related diseases.

In this study, we demonstrated that Fumigaclavine C attenuated the severity of DSS-induced colitis in mice by inhibited NLRP3 inflammasome activation. Generally, intragastric administration of Fumigaclavine C at the dose of 3–30 mg/kg significantly recovered the decrease in body weights, and the diarrhea in colitis mice was also notably alleviated. Meanwhile, Fumigaclavine C administration markedly prevented the inflammatory damage of colons and the activation of caspase-1 in peritoneal macrophage cells. These findings showed the beneficial effect of Fumigaclavine C on DSS-induced colitis.

It is known that IL-1β is a proinflammatory cytokine mainly produced by activated macrophages, and it is likely to be essential in the early phase of the inflammatory cascade leading to the inflamed colon. In colitis patients, high levels of the IL-1β are detected and correlated with the severity of inflammation (26). Moreover, IL-1β modulates the functions of dendritic cells, macrophages, neutrophils, as well as the differentiation of TH17 cells (27,28). IL-1β is secreted in the early phase of the inflammatory cascade in macrophages. It is known that IL-1β in monocytes and macrophages can activate the NLRP3 inflammasome-activated caspase-1. And mechanism study has proved that NLRP3/ASC/caspase-1-mediated maturation of IL-1β after toll like receptor stimulation (signaling I), and this precursor is cleaved to its activated 17-kDa form by the NLRP3 inflammasome-activated caspase-1. And mechanism study has proved that NLRP3/ASC/caspase-1-mediated maturation of IL-1β is essential for experimental colitis induced by (19). Our vivo experiments here showed that treatment with Fumigaclavine C significantly reduced prevented the activation of caspase-1 in peritoneal macrophage cells of mice with DSS-induced colitis. Meanwhile, the in vitro study suggested that Fumigaclavine C could inhibit NLRP3 activation thus suppressing the activation of caspase-1. We hypothesized that the beneficial effect of Fumigaclavine C on DSS-induced colitis might be attributed to its inhibition of inflammasome activation. Still the detail mechanism for how Fumigaclavine C works on the NLRP3 inflammasome activation need to be explored in the further study.

In conclusion, our work explored a novel therapeutic strategy for ulcerative colitis by targeting NLRP3 inflammasome with Fumigaclavine C. Administration of Fumigaclavine C significantly attenuated DSS-induced experimental colitis in mice. The mechanism of Fumigaclavine C’s action involved inhibition of NLRP3-caspase-1-IL-1β cascade in macrophages.

Conflict of interest

The authors declared that no competing interests exist.

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References