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Genistein protects against DSS-induced colitis by inhibiting NLRP3 inflammasome via TGR5-cAMP signaling

Yu Chen[a,1], Thi Ha Le[a,1], Qianming Du[b], Zheng Zhao[c], Yunxin Liu[c], Jianjun Zou[c], Weiwei Hua[a], Chao Liu[c,*], Yubing Zhu[c,*]

[a] Department of Clinical Pharmacy, School of Basic Medicine & Clinical Pharmacy, China Pharmaceutical University, Nanjing 210009, PR China
[b] General Clinical Research Center, Nanjing First Hospital, Nanjing Medical University, Nanjing 210006, PR China.
[c] Department of Pharmacy, Nanjing First Hospital, Nanjing Medical University, Nanjing, 210006, PR China

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ABSTRACT

NLRP3 inflammasome has been reported to be associated with inflammatory bowel disease including colitis due to its potential ability to induce IL-1β secretion. Emerging studies have demonstrated that Genistein, a major isoflavone, has potential anti-inflammatory effects in murine model colitis. However, its anti-inflammatory mechanism remains unclear. The effects of Genistein in dextran sulfate sodium (DSS)-induced murine colitis via targeting NLRP3 inflammasome was investigated in this study. Also, the mechanisms of protective action of Genistein in DSS-induced colitis may relate to TGR5 signaling. Genistein treatment not only remarkably attenuated loss of body weight and shortening of colon length but also significantly reduced inflammatory cells infiltration and pro-inflammatory mediator production in serum and colon. Moreover, Genistein treatment down-regulated production of caspase-1 and IL-1β and increased intracellular cAMP level, which were similar to the treatment for INT-777, a semi-synthetic TGR5 agonist, in phorbol myristate acetate (PMA)-differentiated monocytic THP-1 cells and U937 cells. These protective effects of Genistein might be attributed by ubiquination of NLRP3 which was induced due to interaction of cAMP with NLRP3. Furthermore, the effects of Genistein on NLRP3 inflammasome disappeared in TGR5-silenced U937 cells. In conclusion, our study unveils that Genistein was able to inhibit NLRP3 inflammasome via TGR5-cAMP signaling in macrophages. It therefore might be a potential effective drug for inflammatory bowel diseases.

1. Introduction

Ulcerative colitis (UC) is an idiopathic, chronic and relapsing inflammatory bowel disease characterized by repeated abdominal pain and diarrhea [1]. Although its mechanism remains unknown, it appears to be correlated with activation of mucosal immune system and production of consecutive pathologic cytokine [2]. NLRP3 inflammasome, a multiprotein complex which is composed of Nod-like receptors (NLRs), adaptor protein ASC and caspase-1, plays a key role in the host defense against inflammation and the development of immune responses [3–5]. The activation of NLRP3-inflammasome is regulated by two signals. The first signal (also called the priming step) includes microbial molecules or endogenous cytokine, which is required to up-regulate expression of NLRP3 and pro-IL-1β [6,7]. The second signal is provided by various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) such as extracellular ATP, alum and bacterial toxins including nigericin [8]. When being activated by nigericin, NLRP3 proteins were polymerized, and thereby binding to the adaptor protein ASC, which in return promotes recruitment of pro-caspase-1 [9,10]. Then, NLRP3 inflammasome induces auto-cleaves of pro-caspase-1 into activated form caspase-1, which cleaves pro-IL-1β and pro-IL-18 into biologically active forms of IL-1β and IL-18 [11,12]. These DSS-treated mice are characterized by a general inflammatory condition associated with weight loss and

Abbreviations: DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; UC, ulcerative colitis; PMA, phorbol-12-myristate-13-acetate; LPS, lipopolysaccharide; ATP, adenosine triphosphate; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1 < beta > ; TNF-α, tumor necrosis factor- < alpha > ; MPO, myeloperoxidase; NLRP3, NOD-like receptor protein 3; ASC, apoptosis-associated speck-like protein containing a *C*-terminal caspase recruitment domain; ROS, reactive oxygen species; GPCR, G-protein-coupled receptors; cAMP, cyclic adenosine monophosphate; DAI, disease activity index; H&E, hematoxylin and eosin

* Corresponding authors.
E-mail addresses: 13311010047@stu.cpu.edu.cn (C. Liu), zyb86052002@163.com (Y. Zhu).
[1] These authors contributed equally to this work.

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histopathological features that mimic some of clinical observations of UC. In previous studies, it has been demonstrated that NLRP3 inflammasome in an acute colitis model induced by dextran sulfate sodium (DSS) and NLRP3 gene knockout exerted protective effects on mice [13,14], This suggests that NLRP3 inflammasome might serve as a potential therapeutical target for the inflammatory bowel diseases.

Genistein, a major isoflavone, serves as a potent agent due to its potential beneficial effects and anti-inflammatory effects on several degenerative diseases and various other chronic diseases [15–18]. The protective effects of Genistein on a rat model of TNBS-induced colitis have been confirmed [19,20], however, to our knowledge, the effects of Genistein on DSS-induced colitis on mice remain unclear. Multiple cells were considered in the process of the UC such as macrophages and colon epithelial cells. Previous studies illustrated that Genistein improved cell viability and cellular permeability in intestinal epithelial cells and alleviated DSS-caused colonic injury [21]. In this study, we indicated that Genistein protected DSS-induced colitis via inhibiting activation of NLRP3 inflammasome which was expressed and hyperactive in macrophages. The inhibition effects of Genistein on caspase-1 and IL-1β activity were dependent on the increased intracellular cAMP level in PMA-induced THP-1 macrophages. Therefore, these data allow an assumption that Genistein might have a protective effect on DSS-induced colitis in mice and could be a vital candidate in prevention or treatment of UC.

TGR5 (also known as GPBAR1 or GPR131), as a bile acid membrane receptor, belongs to the G-protein-coupled receptor (GPCR) family which transduces extracellular signals via heterotrimeric G proteins [22–24]. Activation of TGR5 signaling regulates metabolic homeostasis including glucose metabolism, bile acid and energy homeostasis [25,26]. In addition, highest expression of TGR5 was conformed in monocytes and macrophages among inflammatory cells [27,28]. Interestingly, activation of TGR5 decreased pro-inflammatory cytokine production and phagocytic activity through increasing intracellular concentrations of cAMP, suggesting an immunomodulatory action through TGR5 [29,30]. In agreement with these observations, we hypothesized that TGR5 might have a role in inhibition of NLRP3 inflammasome in macrophages.

It is seen from the above observations that whether Genistein has an effect on TGR5 activation pathway or not is still unclear. Here, we demonstrate that Genistein is a negative regulator of inflammasome activation through TGR5-cAMP signaling and suggest TGR5 as a potential target for treatment of NLRP3 inflammasome-related diseases.

2. Materials and methods

2.1. Reagents

Genistein, DSS, LPS (E.coli:SerotypeO55:B5), ATP (A5394), Nigericin and phorbol-12-myristate-13-acetate (PMA, P8139) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of Genistein, at 20 mM in dimethylsulfoxide (DMSO), were stored at −80 °C before using. KH7 and INT-777 were purchased from Tocris Bioscience (Bristol, U.K.). Primary antibody ASC was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against TGR5 and caspase-1 were purchased from Abcam (Cambridge, UK); antibodies against NLRP3 and GAPDH were purchased from CST Technology Inc. (CST Technology Inc., MA). ELISA kits for mouse IL-1β, TNF-α, caspase-1, MPO activity and human IL-1β, caspase-1 was purchased from Boster Biotech Co. Ltd. (Wuhan, China). Intracellular cAMP quantification and LDH release were performed by using EIA kits from Enzo Life Sciences. TGR5 siRNA and control siRNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz Biotechnology Inc., CA).

2.2. Cell culture and cell transfection

THP-1 cells and U937 cells were obtained from the American Type Culture Collection and cultured in RPMI-1640 medium with 10% fetal calf serum under a humidified 5% (v/v) CO_2 atmosphere at 37 °C. Both THP-1 cells and U937 cells were differentiated into macrophages in RPMI-1640 medium containing PMA (100 ng/ml) for 12 h, then THP-1-Ms and U937-Ms were primed with LPS (200 ng/ml) for 4 h, followed by stimulation of inflammasome activators ATP (5 mM, 30 min) or Nigericin (10 μM, 30 min). Then, THP-1-Ms cells and U937-Ms were collected for the experiments. TGR5 interfering RNA (siRNA) was used to perform knockdown experiments. U937-Ms were plated in 12-well plates and then were transfected with 50 nM TGR5 siRNA or control siRNA using Lipofectamine 2000TM reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. All experiments on transfected cells were performed after 48 h.

2.3. Animal experiments

Male C57BL/6 mice (6–8 weeks, 18–20 g) were obtained from Comparative Medicine Centre of Yangzhou University and group-housed at SPF facility under controlled temperature (22 ± 2 °C) and photoperiods (12:12-h light-dark cycle). All the operations was carried out according to the Chinese Community Guidelines and the Institutional Animal Care and Use Committee of China Pharmaceutical University (Nanjing, China). Mice were allowed to acclimate to these conditions for at least 5 days before conducting the experiments. According to age and body weight, the animals were divided into five groups with ten mice in each group. The first group of mice were orally administered with tap water without DSS from day 1 to day 10 as control, the second received 2.5% (wt/vol) DSS from day 3 to day 10 inducing acute colitis in the mice, the remaining three groups of mice were given DSS plus Genistein with Genistein used at doses 5, 15, 45 mg/kg from day 1 to day 10, respectively. Genistein was dissolved in 0.5% methyl cellulose solution. Mice were observed once daily for water/food consumption, weight, morbidity, stool consistency, and blood in feces and at the anus. At day 11, mice were euthanized and rapidly dissected, entire colon was quickly removed and colonic length was recorded. Segments of the colon taken for histopathological essay were fixed in 10% normal buffered formalin and frozen in liquid nitrogen for further analysis.

Disease activity index (DAI) was scored according to the average of three parameters: stool consistency, fecal blood and percentage weight loss. The scoring system was as follows: percentage of body weight loss: none = 0, 1–5% = 1, 5–10% = 2, 10–20% = 3, and ≥ 20% = 4; stool consistency: 0 = well-formed pellets, soft but still formed = 1; very soft = 2; diarrhea = 3; and fecal blood: 0 = no blood, 1 = positive hemoccult, 2 = blood traces in stool visible, 3 = gross rectal bleeding.

2.4. Western blots and co-immunoprecipitation

THP-Ms or U937 cells were seeded in 6-well plates. Then stimulated with Genistein (5 μM, 10 μM, 20 μM) for 2 h before the treatment or not with LPS (200 ng/ml) for 4 h, ATP (5 mM) or Nigericin (10 μM) were added to LPS-stimulated cells during the last 30 min. Cells were washed by cold PBS and resuspended in RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with a complete protease inhibitor cocktail (Roche). The cell lysates were placed on ice for 30 min and then centrifuged at 12,000 rpm for 10 min. Protein concentration of cell lysates was determined by a BCA kit (Pierce, Rockford, IL). For ASC pyroptosome, the pellets were cross-linked with2mM disuccinimidyl suberate (DSS) for 30 min at 37 °C and then mixed with 50 μl × 2 SDS sample buffer, boiled and fractionated on 12% SDS-polyacrylamide gel followed by immunobloting with anti-mouse ASC antibody. An equal amount of protein was separated with 8%–12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Proteins were

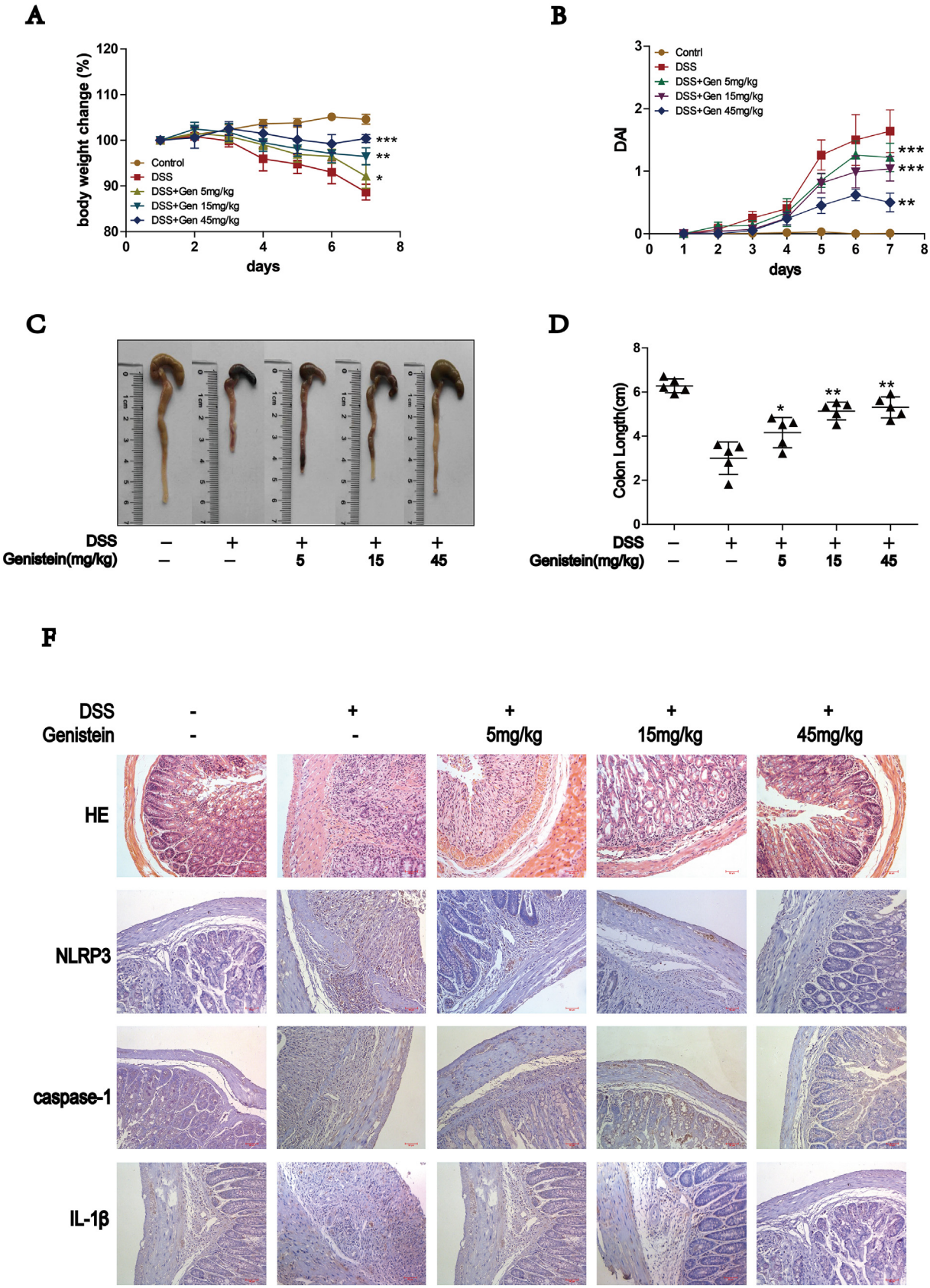

Fig. 1. Genistein inhibited activation of NLRP3 inflammasome in DSS-induced mice.
(A) Changes of body weight in each group (n = 10 per group) after DSS induction of colitis. (B) The disease activity index (DAI) of each group was measured (n = 5). (C, D) Lengths of colons was evaluated (n = 5). (E) Serial sections of colon tissues were stained with H&E. Immunohistochemistry of IL-1β, caspase-1 and NLRP3 in colonic tissues of each group was measured. Brown colored is positive (scale bar, 50 μm). Statistical analysis was performed using one-way ANOVA. Data are averages ± SD (n = 10 or n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. DSS group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detected using specific antibodies overnight at 4 °C followed by HRP-conjugated secondary antibodies for 1 h at 37 °C. Enhanced chemiluminescent reagents (Beyotime, Jiangsu, China) were used to detect the HRP on the immunoblots, and the visualized bands were captured by film. Co-Immunoprecipitation (Co-IP) was performed overnight at 4 °Cusing NLRP3 antibody. Immunocomplexes were collected with Protein G Sepharose beads (GE Healthcare) for 1 h at 4 °C prior to western blotting.

2.5. Elisa

Colons from mice in each group were homogenized with RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche) to extract total protein. The homogenate was centrifuged at 12,000 rpm at 4 °C for 20 min. The amount of total extracted protein was determined by BCA TM protein assay kit (Pierce, Rochford, IL). The amount of IL-1β, TNF-α and MPO activity in colon tissues, serum were quantified by ELISA kit from Cayman Chemical. THP-Ms were seeded on a six-well and stimulated with Genistein (5 μM, 10 μM, 20 μM) or INT-777 (30 μM) in the presence of or not KH7 (25 mM) for 1 h. The cells were treated with LPS (200 ng/ml) for 4 h. ATP (5 mM) or Nigericin (10 μM) was added to LPS-stimulated cells during the last 30 min. Supernatants were collected, IL-1β and caspase-1 were measured by ELISA kit (R&D Systems), according to the manufacturer's Instruction. cAMP in cell lysates was assayed using the Human cAMP Parameter Assay Kit (R&D Systems) following the manufacturer's instructions. Pyroptosis and necroptosis were quantitated by Assaying the activity of LDH released into cell culture supernatants after various treatments using the CytoTox96 LDH release kit (Promega) according to the manufacturer's protocol. The LDH activity in the culture supernatant was expressed as a percentage of total LDH in the cell lysate.

2.6. Immunofluorescence

To investigate colocalization of ASC and NLRP3, THP-Ms cells on cover slips were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100 for 30 min and blocked with 5% BSA for 1 h. Cells were co-immunostained with anti-ASC and anti-caspase-1 antibodies overnight at 4 °C. Then cells were immunostained with Alexa Fluor 488-conjugated anti-rabbit IgG and 620-conjugated anti-rabbit IgG (Life technology, CA) for 2 h. The cover slips were counterstained with DAPI and imaged with a confocal laser scanning microscope (Olympus, Lake Success, NY).

2.7. Immunohistostaining

Immunohistochemical stains against NLRP3, IL-1β and caspase-1 were performed by using immunohistochemistry kit (KeyGEN, Nanjing, China). Briefly, paraffin-embedded slides were deparaffinized, rehydrated by serially dipping into 100–70% ethanol, distilled water and PBS. Then they were treated with 3% hydrogen peroxide and blocked with 10% goat serum for 1 h at 37 °C. Slides were incubated with primary antibodies in PBS containing 1% BSA (1:200) overnight at 4 °C. Biotinylated secondary anti-rabbit antibodies were added and incubated at room temperature for 1 h. Streptavidin-HRP was added, and after 40 min the sections were stained with DAB substrate and counterstained with hematoxylin.

2.8. Statistical analysis

For statistical analysis, data obtained from independent experiments were analyzed with the *t*-test for two groups or one-way ANOVA (GraphPad Software) for more than three groups. The results are expressed as mean ± SD and P values < 0.05 were considered significant.

3. Results

3.1. Genistein ameliorated colitis induced by DSS

To investigate effects of Genistein on acute colitis, mice were given daily administration of Genistein during DSS-induced acute colitis. It has been shown that DSS induced severe inflammation in mice characterized by a dramatic loss of body weight. Compared to vehicle-treated group, Genistein significantly attenuated body weight loss in a dose-dependent manner (Fig. 1A), and also evidently reduced disease activity index (DAI), a clinical parameter reflecting the severity of colitis (Fig. 1C). DSS-induced colon shortening was markedly improved at doses of 15 and 45 mg/kg Genistein (Fig. 1B and D). The severity of colonic ulceration and inflammation was further evaluated by histopathological analysis using Haematoxylin & eosin (H&E) staining (Fig. 1E). Damage of crypts, loss of goblet cells, infiltration of mononuclear cells, and severe mucosal destruction were observed in the colon specimens of DSS-treated mice. Strikingly, Genistein-treated mice exhibited low inflammatory cell infiltration and intact colonic architecture with no apparent ulceration.

3.2. Genistein inhibited activation of NLRP3 inflammasome in DSS-induced mice

To analyze effects of Genistein on the inflammatory mediators involved in DSS-induced colitis, levels of TNF-α, MPO activity, IL-1β and IL-18 in colon serums and homogenates were measured by ELISA. As can be seen in Fig. 2A and B, Genistein remarkably inhibited DSS-induced elevated levels of TNF-α and MPO activity and strongly suppressed production of IL-1β and IL-18. Moreover, previous studies showed that NLRP3 inflammasome may play crucial roles in DSS-induced colitis. We found that Genistein significantly decreased the expression of NLRP3 inflammasome-dependent cytokine (Fig. 2A and B). Thus, we hypothesized that Genistein could inhibit the activation of DSS-induced NLRP3 inflammasome on acute colitis. As expected, the activation of DSS-induced NLRP3 inflammasome was significantly suppressed by Genistein as shown by western blotting (Fig. 2C). Genistein exhibited a markedly inhibition on NLRP3, cleaved-IL-1β and cleaved-caspase-1 (caspase-1 p20) in colon tissues. Furthermore, the same results were also observed by immunohistochemical assays (Fig. 1E). Taken together, these findings suggested that Genistein markedly suppressed the activation of NLRP3 inflammasome in DSS-induced mice.

3.3. Genistein inhibited activation of NLRP3 inflammasome in Macrophages

In vivo, Genistein inhibited activation of NLRP3 inflammasome and decreased production of IL-1β. To investigate the underlying mechanism, we also examined whether Genistein could suppress the activation of NLRP3 in vitro. As showed in Fig. 3A and B, pretreatment of LPS and ATP resulted in activation of NLRP3 inflammasome in THP-Ms cells and U937-Ms cells, while Genistein dose-dependently decreased NLRP3-dependent caspase-1 activation and mature IL-1β secretion. The increased protein levels of cleaved caspase-1 (p10), cleaved-IL-1β and NLRP3 induced by LPS plus ATP treatment were dramatically suppressed by Genistein (Fig. 3C). When macrophages have been commandeered by microbial pathogens, they can be lysed by pyroptosis, a cell death mechanism [31]. Pyroptosis is triggered by the activated form of caspase-1, and results in lysis of the affected cell. Therefore, activation of caspase-1 was associated with induction of pyroptosis as determined by LDH release assay. As showed in Fig. 3D, Genistein did not affect LDH release in LPS-primed macrophages after ATP treatment, suggesting that pyroptosis did not involve in inhibition effects of Genistein on caspase-1 activation and IL-1β secretion. In addition, immunoprecipitation results revealed that interaction between NLRP3 and ASC, which is critical for ASC nucleation, was increased after ATP

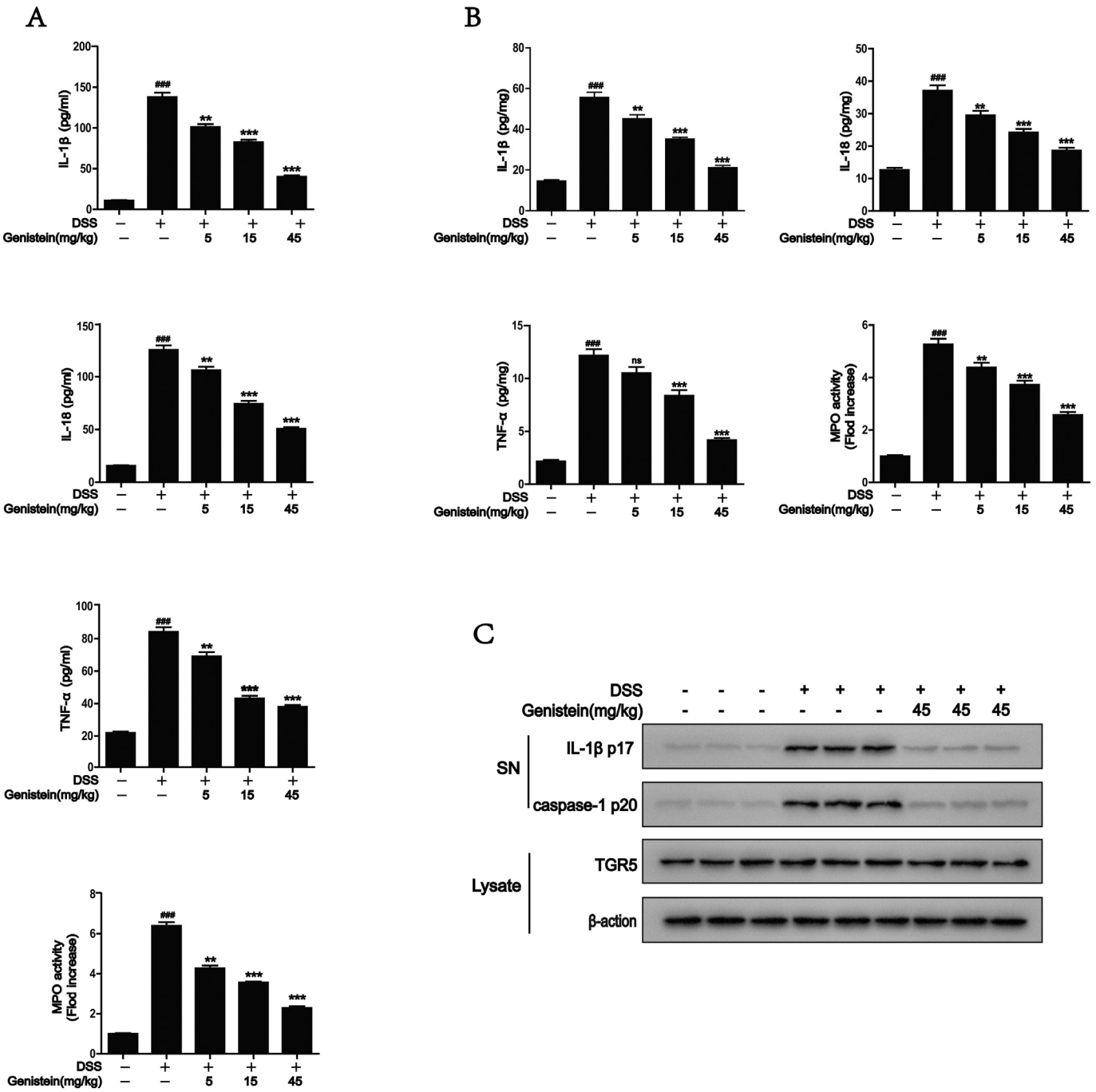

Fig. 2. Genistein inhibited activation of NLRP3 inflammasome in DSS-induced mice.
(A) Secretion of IL-1β, IL-18, TNF-α and MPO activity in serum was determined by ELISA. (B) Secretion of IL-1β, IL-18, TNF-α and MPO activity in colonic tissue were determined by ELISA. Statistical analysis was performed using one-way ANOVA. Data are averages ± SD ($n = 10$). ### $P < 0.01$ vs. Control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. DSS group.

stimulation, showing that Genistein specifically inhibited the association of ASC with NLRP3.

ASC nucleation-induced oligomerization is considered to be a common mechanism of NLRP3 inflammasome activation. We found that the rise of nigericin-induced ASC dimerization and oligomerization was markedly attenuated by Genistein (Fig. 4B). One proposed mechanism of NLRP3 inflammasome activation is accumulation of damaged mitochondria and generation of reactive oxygen species (ROS) in mitochondria [32,33]. Increased mROS release induced by ATP is associated with mitochondrial membrane permeability. Consistent with this, we used a fluorescence-based assay and flow cytometry to measure mitochondrial damage. Genistein, however, did not affect ATP-induced loss of mitochondrial membrane potential and ROS production in U937-Ms (Fig. 4C). Collectively, these results determined that Genistein inhibited the activation of NLRP3 inflammasome through disrupting the interaction between NLRP3 and ASC, leading to NLRP3-mediated ASC nucleation.

3.4. Genistein inhibited NLRP3 inflammasome by activation of TGR5 through cAMP Pathway

TGR5, as a bile acid membrane receptor, belongs to the G-protein-

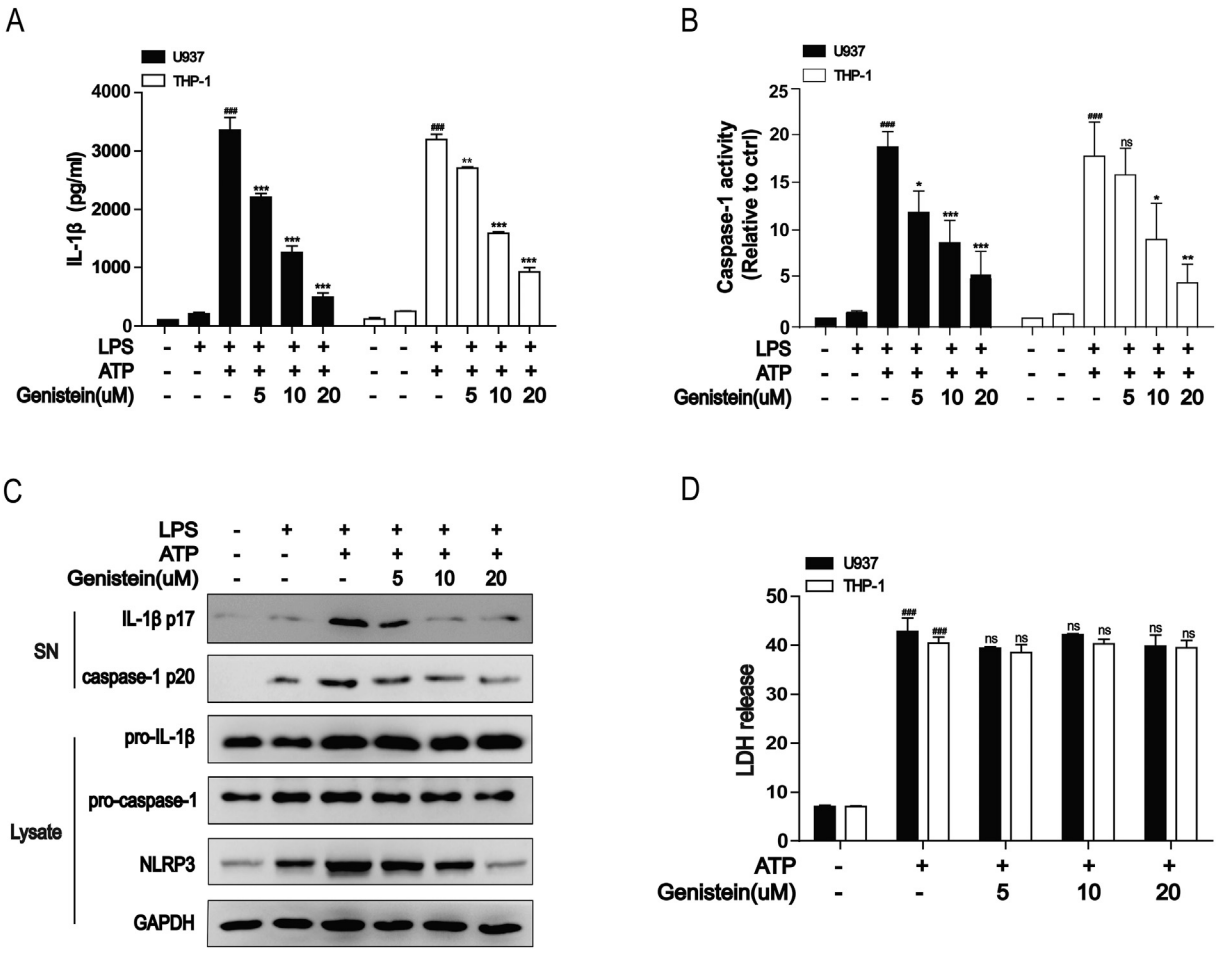

Fig. 3. Genistein inhibited activation of NLRP3 inflammasome in Macrophages.
(A, B) THP-1-Ms and U937-Ms were treated with indicated concentrations of Genistein for 2 h before stimulated with LPS for 4 h followed by 30 min incubation of ATP. Released IL-1β and caspase-1 in the cell supernatant were analyzed by ELISA. (C) Protein levels of cleaved caspase-1, cleaved IL-1β and NLRP3 in THP-1-Ms were determined by Western Blot. (D) LDH release in the culture supernatants of THP-1-Ms and U937-Ms were analyzed by ELISA. Statistical analysis was performed using one-way ANOVA. Data are averages ± SD ($n = 3$). ### $P < 0.01$ vs. Control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. LPS/ATP group.

coupled receptor (GPCR) family. Activation of TGR5 in macrophages has been reported to reduce pro-inflammatory cytokine production and elevate the cyclic AMP (cAMP) [34]. Recently, cAMP has been reported as a central signaling molecule in various cellular systems and plays an important role in down-regulating NLRP3 inflammasome activation via different mechanisms [35]. Data from previous studies demonstrate that Genistein exerts beneficial anti-inflammatory effects through a cAMP-dependent pathway in ECs [36,37]. In this study we examined the effect of Genistein on intracellular cAMP levels in LPS-treated macrophages. We found that Genistein dose-dependently elevated intracellular cAMP level both in THP-Ms cells and U937-Ms cells (Fig. 5A). As TGR5 may be involved in the increased cAMP level in macrophages, THP-Ms and U937-Ms were exposed to INT-777, a semi-synthetic TGR5 agonist, and subsequently stimulated by LPS and Nigericin. As a result, INT-777 significantly elevated intracellular cAMP level (Fig. 5A) and decreased the secretion of caspase-1 and IL-1β (Fig. 5B) in a dose-dependent manner, similar to Genistein treatment (Fig. 5C and D). These results indicate that TGR5 activation inhibits NLRP3 inflammasome in macrophages.

To determine whether cAMP contributes to the inhibition effects of Genistein on NLRP3 inflammasome in human macrophages, we measured intracellular cAMP level in THP-Ms. After treating by KH7, an AC inhibitor, intracellular cAMP level significantly decreased (Fig. 6A). In addition, KH7 treatment induced secretion of IL-1β and caspase-1 in the presence of Genistein or INT-777 (Fig. 6B and C). In addition, the oligomerized ASC diminished by Genistein was reversed by KH7 (Fig. 4B). These results clearly demonstrated that cAMP is the direct mechanism of Genistein-induced NLRP3 inhibition. In addition, we knocked out TGR5 by using TGR5-specific siRNA in U937-Ms and investigated the role of TGR5 in modulating cAMP levels. We found that LPS and Nigericin-stimulated U937-Ms transfected with control siRNA produced much less IL-1β and caspase-1 upon Genistein exposure (Fig. 6E). In contrast, IL-1β and caspase-1 secretion from the U937-Ms transfected with TGR5 siRNA were substantially increased in response to Genistein orINT-777 (Fig. 6F). Meanwhile, U937-Ms transfected with TGR5 siRNA had no effect on intracellular cAMP elevation induced by Genistein (Fig. 6D). Genistein effects on NLRP3 inflammasome activation was completely abrogated by TGR5 siRNA (Fig. 6E and F), suggesting that upregulation of cAMP by Genistein is TGR5-dependent in NLRP3 inflammasome inhibition.

3.5. Genistein promoted NLRP3 ubiquitination via TGR5-cAMP signaling

Recent studies have shown that ubiquitination of NLRP3 negatively regulate NLRP3 inflammasome activation [38,39]. Deubiquitination of NLRP3 is critically involved in NLRP3 inflammasome activation. Our studies suggested that Genistein could significantly elevate the intracellular cAMP level which is proposed to negatively regulate NLRP3 inflammasome activation (Fig. 6A and B). To figure out how cAMP suppresses NLRP3 inflammasome activation, we investigated the

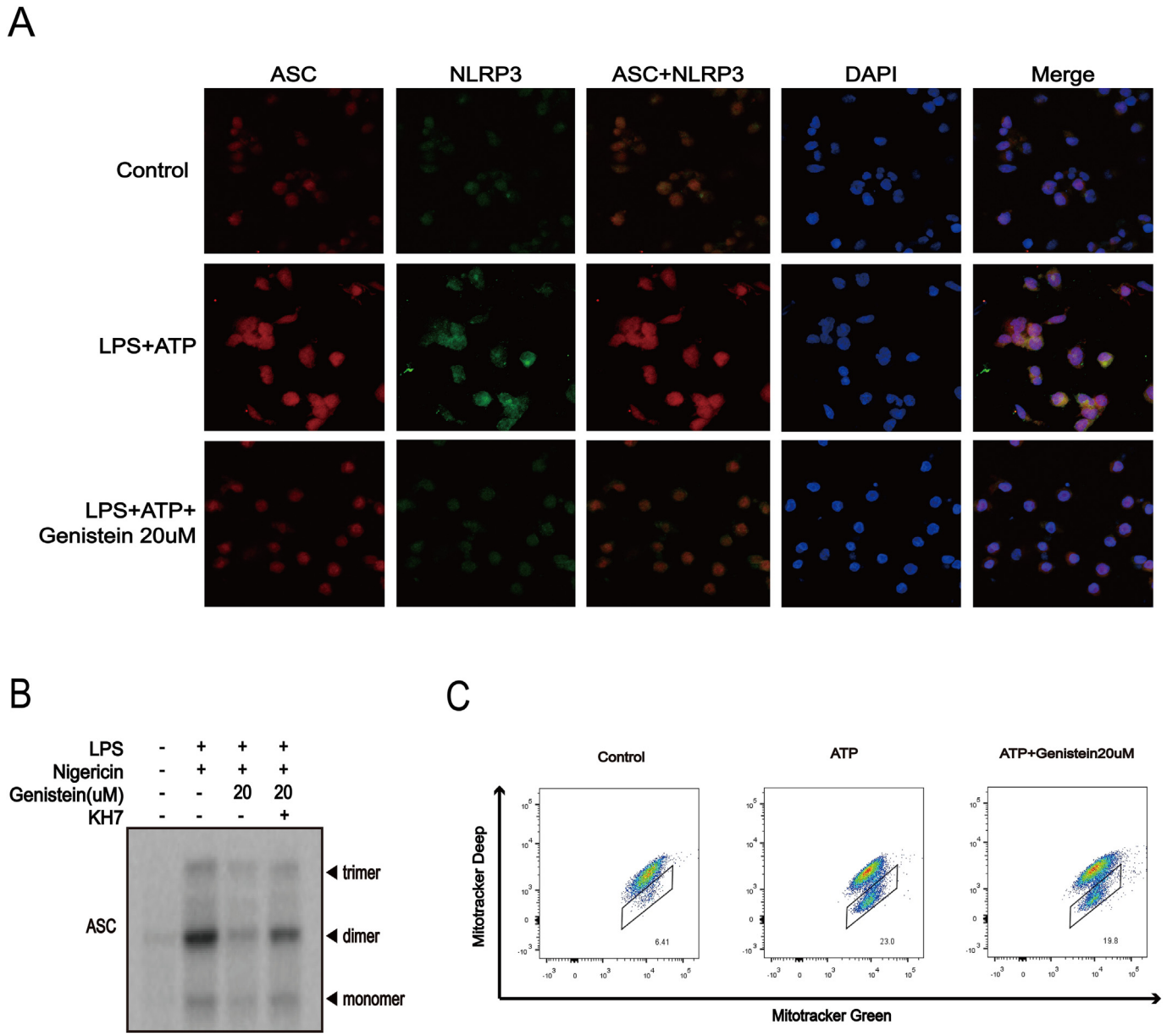

Fig. 4. Genistein inhibited activation of NLRP3 inflammasome in Macrophages.
(A) Immunofluorescence was performed to analyze interaction between ASC and NLRP3 (scale bar, 20 μm). (B) Immunoblotting of ASC was performed in cross-linked pellets and in cell lysates. (C) Flow cytometric analysis of mitochondrial status in U937-Ms challenged with ATP or Genistein. Gates represent cells with damaged mitochondria. Statistical analysis was performed using one-way ANOVA. Data are averages ± SD ($n = 3$). ### $P < 0.01$ vs. Control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. LPS/ATP group.

interaction of cAMP with NLRP3. NLRP3 were immunoprecipitated and the precipitates were examined with anti-cAMP antibody by CO-IP and western blotting analysis in U937-Ms. Endogenous CO-IP studies demonstrated that cAMP interacts with endogenous NLRP3 by Genistein treatment, which can completely ablated upon the addition of KH7 (Fig. 6G), suggesting that cAMP binds to NLRP3 can directly inhibit the assembly of inflammasome agents. Possible mechanism of cAMP may be through promoting the ubiquitination and degradation of NLRP3. We noted that Genistein treatment triggered ubiquitination of NLRP3 in U937-Ms (Fig. 6H), whereas ubiquitination of NLRP3 was blocked in present of KH7. We confirmed that Genistein negatively mediated NLRP3 inflammasome via cAMP, which binds with NLRP3 and promotes its ubiquitination and degradation (Fig. 6H). Importantly, binding of NLRP3 to cAMP and the elevated ubiquitination level of NLRP3 induced by Genistein were all rescued by TGR5 siRNA (Fig. 6H), suggesting this pattern heavily depended on TGR5. These results indicate that Genistein promotes NLRP3 ubiquitination via TGR5-cAMP signaling.

4. Discussion

Ulcerative colitis is an inflammatory condition of the intestine and a vital pathogenic factor of colorectal cancer [40], which has been a major health problem in our country. Currently, therapeutic options for IBD continue to evolve such as immunosuppressive drugs. However, these immunosuppressants have also limited efficacy and safety [41]. Therefore, novel strategies with high safety and efficacy are urgently needed. Over the past few years, increasing evidences support that NLRP3 inflammasome is associated with inflammatory bowel disease and chronic colitis [2]. NLRP3 inflammasome is the most extensively studied cytosolic multiprotein complexes, which might be involved in inflammation-related cytokine production. Experiments in mice have

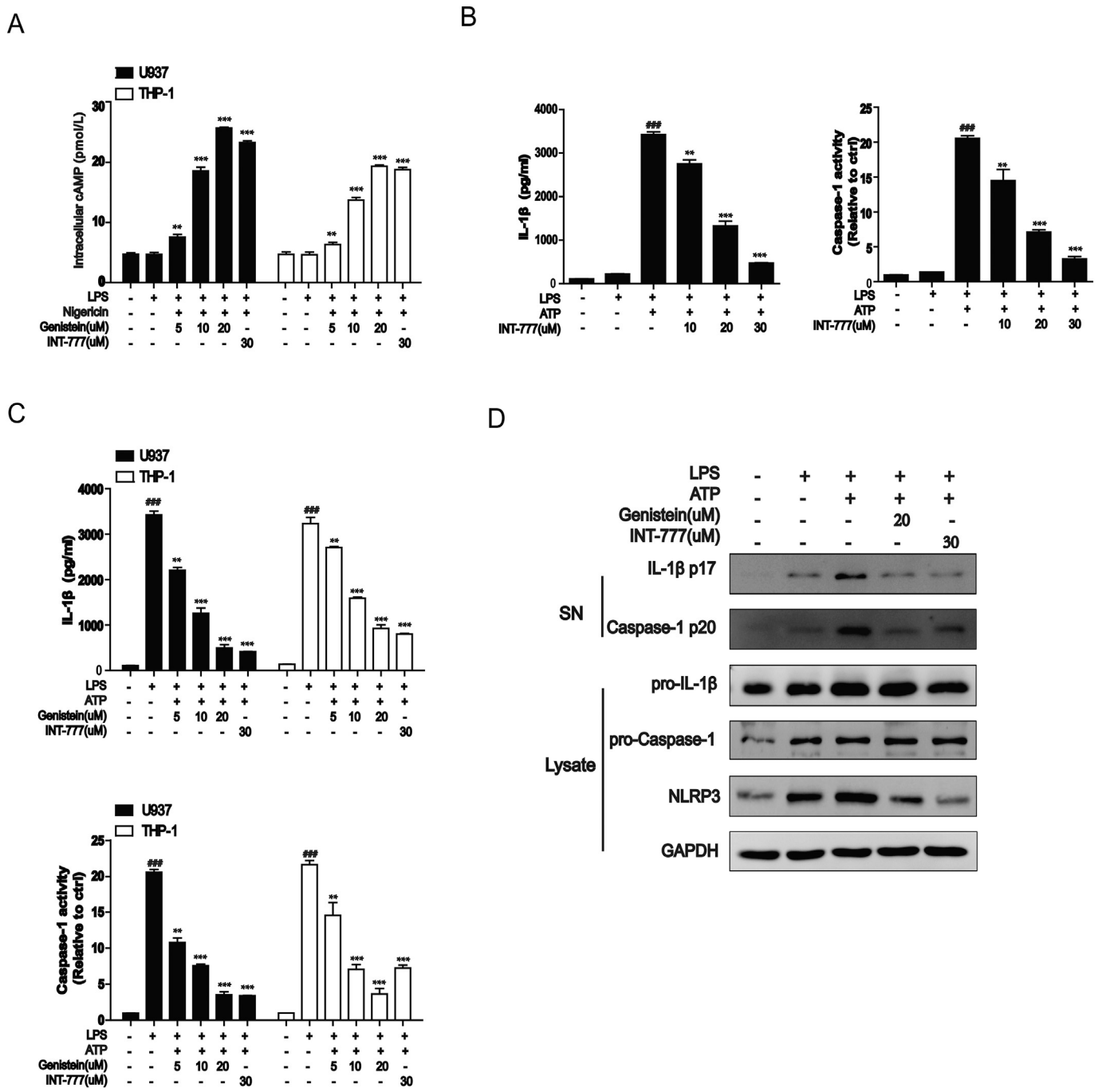

Fig. 5. Genistein inhibited NLRP3 inflammasome by activation of TGR5 through cAMP Pathway.
(A) The cAMP level in THP-1-Ms and U937-Msl lysates were analyzed. (B) THP-1-Ms were treated with INT-777 for 1 h before stimulated with LPS and ATP. Released IL-1β and caspase-1 in the cell supernatant were analyzed by ELISA. (C) THP-1-Ms and U937-Ms were treated with Genistein or INT-777, the levels of IL-1β and caspase-1 in the cell supernatant were analyzed. (D) The supernatants and lysates of THP-1-Ms were collected for WB. Statistical analysis was performed using one-way ANOVA. Data are averages ± SD ($n = 3$). ### $P < 0.01$ vs. Control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. LPS/ATP group or LPS/Nigericin group.

shown that DSS-induced ulcerative colitis models are mediated by the NLRP3 inflammasome [13]. Thus, acute colitis models induced by DSS were used to evaluate the role of the NLRP3 inflammasome on colitis. Genistein, serves as a major isoflavone, has potential anti-inflammatory effect on some degenerative diseases and various other chronic diseases [25–28]. Previous publications have reported that Genistein has significantly protective effects in a rat model of TNBS-induced colitis [29,30], and DSS-induced colitis [21]. However, its mechanism has not yet been clarified and examined. In this study, we demonstrated that targeting on NLRP3 inflammasome by Genistein ameliorated the severity of DSS-induced colitis. Generally, Genistein administrated at the dose of 5-45 mg/kg significantly recovered the loss in body weights while alleviated pathological symptoms and damage of colons. We found that Genistein at the dose of 45 mg/kg remarkably decreased TNF-α level and MPO activities in colon serum or homogenates. Furthermore, Genistein can protect colon from damage induced by inflammatory cells infiltration. IL-1β, a proinflammatory cytokine, is mainly produced by activated macrophages, and it also plays an

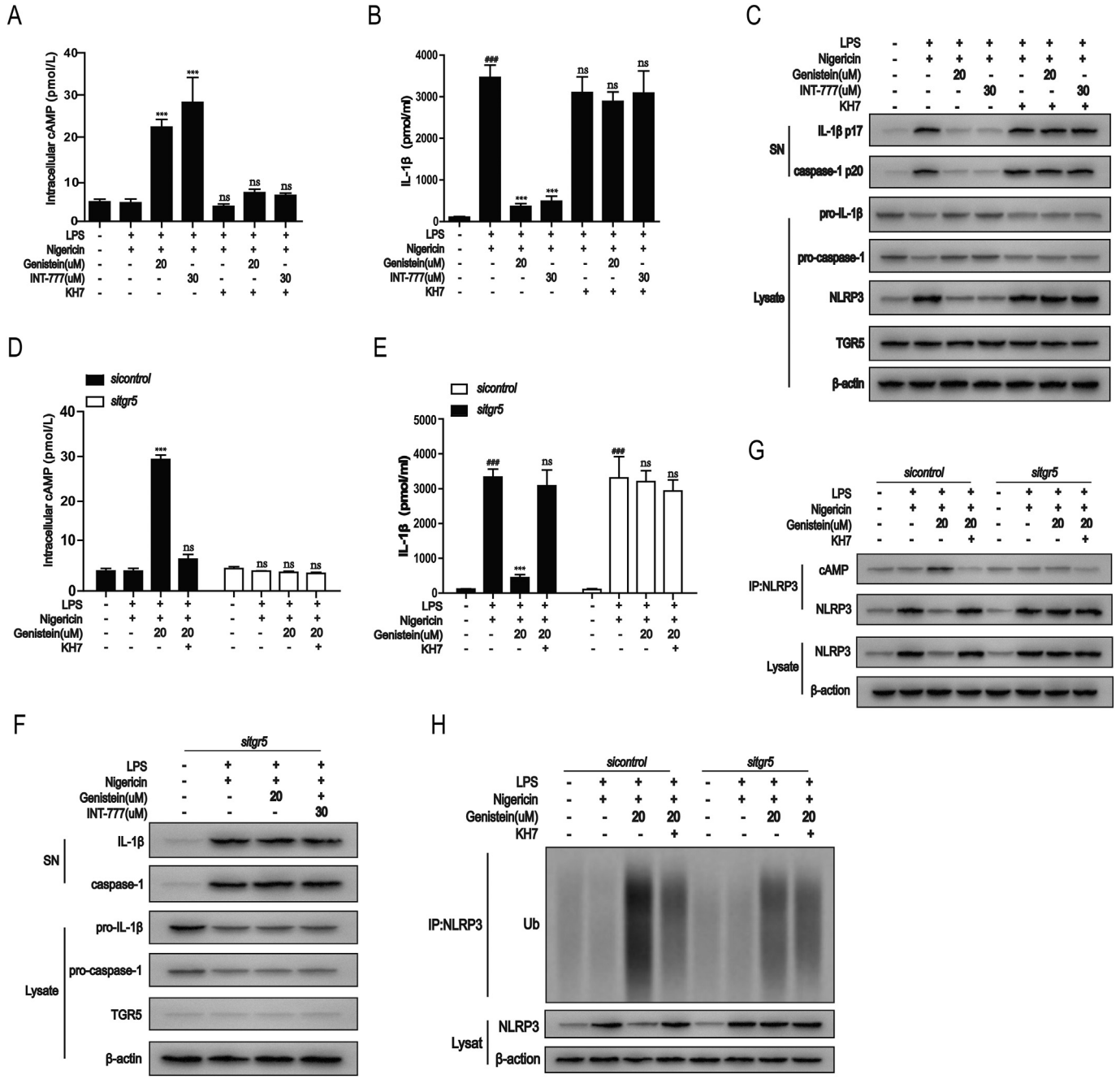

Fig. 6. Genistein promoted NLRP3 ubiquitination via TGR5-cAMP signaling.
The cAMP level and released IL-1β, (B) in cell lysates were analyzed by ELISA. (C) The IL-1β and caspase-1 in the cell supernatant and lysates were analyzed by WB. (D,E) The cAMP level and released IL-1β in U937-Ms from siTGR5 or sicontrol lysates were analyzed by ELISA and WB (F). (G) Immunoblot analysis of cAMP-NLRP3 interaction was analyzed. LPS primed-U937-Ms from siTGR5 or siControl. (H) Immunoblot analysis of Ub and in cell lysates immunoprecipitated with NLRP3 antibody. Statistical analysis was performed using one-way ANOVA. Data are averages ± SD ($n = 3$). ### $P < 0.01$ vs. Control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. LPS/ATP group or LPS/Nigericin group.

important role in intestinal inflammation [42]. The mature and secretion of IL-1β and caspase-1 were dependent on NLRP3 inflammasome activation [43]. Genistein successfully suppressed the production of IL-1β, caspase-1 and the protein level of NLRP3 in colon, suggesting that down-regulation of NLRP3 inflammasome activation contributed to the beneficial effect of Genistein in DSS-induced colitis.

To confirm the conclusion of our study in vivo, anti-inflammatory effect of Genistein was evaluated in two types of macrophages: PMA-differentiated THP-1 cells and U937 cells. Genistein has been reported to improve cellular permeability in Caco-2 cells and inhibit DSS-induced activation of TLR4/NF-κB signal [21]. Here, we have shown that Genistein has protective effect on DSS-induced colitis via inhibiting activation of NLRP3 inflammasome in macrophages. NLRP3 inflammasome assembly is critical for the activation of NLRP3 inflammasome which composed of NLRP3, adaptor protein ASC and caspase-1. Genistein specifically inhibited the association of ASC with NLRP3 and ASC nucleation. Besides, Genistein did not affect LDH release in LPS-primed macrophages and the ATP-induced loss of mitochondrial membrane potential and ROS production, which suggests that Genistein has little effect on pyroptosis or damaged mitochondria in macrophages. These results indicate that Genistein inhibited activation of NLRP3 inflammasome by interrupting ASC-mediated NLRP3

inflammasome assembly. Data from previous studies demonstrate that Genistein exerts beneficial anti-inflammatory effects through a cAMP-dependent pathway in ECs [15–18]. We demonstrate that Genistein dose-dependently elevated intracellular cAMP level both in THP-Ms cells and U937-Ms cells, suggesting that Genistein could boost intracellular cAMP level in macrophages and down-regulate the NLRP3 inflammasome through cAMP-dependent pathway, which in conjunction with early findings [37].

G-protein-coupled receptors (GPCRs) are believed to exert signal transduction by generating a number of second messengers cAMP. Previous studies have proved that GPCR is crucial in signaling and is accomplished by a series of biochemical modifications such as ubiquitination, a posttranslational modification which typically result in protein degradation [44]. TGR5, as a bile acid membrane receptor, is a member of GPCRs. In addition, TGR5 is expressed in the immune system especially in macrophages and is responsive to INT-777, a semi-synthetic TGR5 agonist that selectively activates TGR5 [22,23]. In the present study, we have found that INT-777 significantly inhibited IL-1β and caspase-1 secretion with concomitant decreased the levels of cAMP in macrophages, but these phenomenons were significantly blocked by KH7, an AC inhibitor that decreases cAMP levels. These data are in line with the finding that TGR5 has immunomodulatory action through cAMP [24,25]. Meanwhile, present findings raised questions that whether upregulation of cAMP by Genistein is TGR5-dependent. Consequently, effects on NLRP3 inflammasome in TGR5-deficient U937-Ms cells showed completely contrary effects in response to Genistein compared to control U937-Ms cells. We found that LPS and Nigericin-stimulated U937-Ms transfected with TGR5 siRNA produced more IL-1β and caspase-1 upon Genistein exposure. These observations together implicate that inhibition effect of Genistein on NLRP3 inflammasome is dependent on TGR5-cAMP signaling pathway.

According to published reports, ubiquitination of NLRP3 has been proposed to negatively regulate NLRP3 inflammasome activation, implying that ubiquitination may be a critical brake on NLRP3 inflammasome activation [38,39]. In this regard, we speculated that TGR5-cAMP signaling was involved in ubiquitination of NLRP3. Our observations that the interaction of cAMP with NLRP3 and the ubiquitination of NLRP3 were triggered by Genistein treatment can be ablated completely by KH7. In conclusion, our findings indicated that Genistein modulates NLRP3 inflammasome via cAMP, which binds to NLRP3 and promotes its ubiquitination and degradation. It is notable that the binding of cAMP to NLRP3 and the ubiquitination of NLRP3 induced by Genistein were all rescued by silenced TGR5, showing that TGR5 is necessary for down-regulation of NLRP3 inflammasome. In addition, the expression of TGR5 was also detected in intestines of mice model. In summary, here we have demonstrated that Genistein inhibit NLRP3 inflammasome activation via the TGR5-cAMP signaling. In light of our findings, Genistein will be a vital candidate in the prevention or treatment inflammation-related diseases.

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Conflicts of interest

The author declared that there is no conflict of interest.

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