Original Article

Transient receptor potential melastatin 7 aggravates necrotizing enterocolitis by promoting an inflammatory response in children

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Background: As a rare disease in children, necrotizing enterocolitis (NEC) leads to high morbidity and mortality. However, its pathophysiology is largely unclear. Transient receptor potential melastatin 7 (TRPM7) is a membrane protein, which plays key roles in the inflammatory response. This study sought to examine the promoting effect of TRPM7 on NEC in children and explore the therapeutic effect of a TRPM7 inhibitor NS8593.

Methods: First, we detected *TRPM7* and NLR family pyrin domain containing 3 (*NLRP3*) expression and the state of inflammation in children with NEC through quantitative real-time polymerase chain reaction (RT-PCR), Western blot, and enzyme-linked immunosorbent assays. Next, Human intestinal epithelial cell lines were induced to NEC by lipopolysaccharides (LPSs). The level of cytokines and reactive oxygen species (ROS) were tested by RT-PCR and flow cytometry. The TRPM7 mediated calcium flux were determined by fluorescence. In addition, we used the TRPM7 inhibitor NS8593 to treat the *in vivo* rat model. The mRNA and protein expression were determined by real-time PCR and Elisa analysis, respectively.

Results: *TRPM7* and *NLRP3* expression was more increased in the samples from children with NEC compared to the control samples. Additionally, the elevated secretion of interleukin-1β, interleukin-6, and tumor necrosis factor alpha was also detected in the serum of children with NEC. These results showed that TRPM7 had a promoting effect on NEC development, possibly via the activation of NLRP3. To test our hypothesis, the TRPM7 inhibitor NS8593 was used to treat the LPS-stimulated IEC-6 cells. We found that the TRPM7 inhibitor NS8593 inhibited LPS-induced cytokine production and exhibited an anti-inflammatory effect by alleviating TRPM7-mediated NLRP3 inflammasome activation. Through *in-vivo* experiments, we found that TRPM7 was involved in the occurrence of NEC, and its inhibitor NS8593 played a certain therapeutic role in the rat model.

Conclusions: Our study revealed TRPM7 inhibitors attenuated LPS-induced ROS and reduced the release of pro-inflammatory cytokines. It also exhibited protective effects on the NEC model.

Keywords: NS8593; necrotizing enterocolitis (NEC); transient receptor potential melastatin 7 (TRPM7); NLR family pyrin domain containing 3 (NLRP3); inflammation

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Introduction

As a rare disease in children, necrotizing enterocolitis (NEC) leads to high morbidity and mortality. However, its pathophysiology remains largely unclear. Epidemiologic observations strongly suggest that a series of predisposing factors contribute to ischemic deficiency, including imbalances in the microvascular and excessive inflammatory response (1,2). These risk factors lead to hypoxia-ischemia, which triggers a series of inflammatory responses in the gut. Thus, excessive inflammation and aberrant reactive oxygen species (ROS) accumulation are key factors in the pathological process of NEC (3-6).

Transient receptor potential melastatin 7 (TRPM7) belongs to the membrane protein family, which works in inflammation (7-9). As an ion channel protein, TRPM7 is involved in Mg^{2+}, Zn^{2+}, and Ca^{2+} homeostasis (7,10,11). Recently, the function of TRPM7 in immune response has attracted much attention (12). TRPM7 channel activity is critical for macrophage proliferation and polarization, and its inhibitor NS8593 reduces pro-inflammatory cytokine tumor necrosis factor alpha (*TNF*-α) expression (13). Conversely, the TRPM7 channel is associated with lipopolysaccharide (LPS)-induced inflammatory responses. Interleukin-1β (IL-1β) secretion was reduced in TRPM7-deficient macrophages via TRPM7-mediated calcium ion influx (14,15). More importantly, *TRPM7* has been shown to be widely expressed in the intestine; however, its function in NEC has not been well defined. NLR pyrin domain containing 3 (NLRP3) inflammasome has been shown to participate in the innate immune system (16,17). It is reported that NLRP3 inflammasome is abnormally activated in severe acute colitis (18). Recently, it was found that TRPM7 contributed to NLRP3 inflammasome activation in cardiac myoblasts injury (19). Our study explored the underlying mechanism on TRPM7 and NLRP3 in NEC, and showed that TRPM7 aggravates NEC by promoting the NLRP3-dependent inflammatory response in children, and its inhibitor NS8593 might be a potential small molecule for NEC treatment. We present the following article in accordance with the ARRIVE and MDAR reporting checklists (available at https://tp.amegroups.com/article/view/10.21037/tp-22-633/rc).

Highlight box

Key findings

- TRPM7 aggravates NEC by promoting the NLRP3-dependent inflammatory response in children.

What is known and what is new?

- TRPM7 was involved in miR-129-5p-regulated NLRP3 inflammasome activation;
- TRPM7-mediated calcium flux contributes to NLRP3 inflammasome activation.

What is the implication, and what should change now?

- TRPM7 is a novel therapeutic target for NEC in children, and inhibitor NS8593 might be a potential small molecule for NEC treatment.

Methods

Patients

NEC intestinal tissue specimens were collected from 15 children diagnosed with NEC at The Affiliated Hospital of Zunyi Medical University, and their adjacent normal tissue specimens were also collected as a control. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The Affiliated Hospital of Zunyi Medical University (No. KLL-2019-016) and informed consent was taken from all the patients' legal guardians.

Cell culture

Mouse small intestinal cells IEC-6 were purchased from the American Type Culture Collection. The cells were routinely cultured in Roswell Park Memorial Institute Medium-1640 (Meilunbio, China) supplemented with 10% fetal bovine serum (Gibco) under 37 ℃ with 5% carbon dioxide.

Quantitative real-time polymerase chain reaction

The total ribonucleic acid (RNA) was extracted from the cells using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and a complementary deoxyribonucleic acid synthesis was then undertaken (DRR047A, Takara, Tokyo, Japan). The relative expression of the target genes was calculated using the relative quantification ($2^{-\Delta\Delta Ct}$) method and normalized. The primers used in this study are listed in *Table 1*.

Western blot and enzyme-linked immunosorbent assays (ELISAs)

The total protein was extracted from the tissues or cells

Table 1 The primers used in this study

Name	Sequence (5'–3')
TNF-α	CTCAAAACTCGAGTGACAAGC
	CCGTGATGTCTAAGTACTTGG
IL-6	CCAATTTCCAATGCTCTCCT
	ACCACAGTGAGGAATGTCCA
IL-1β	ATGATGGCTTATTACAGTGGCAA
	GTCGGAGATTCGTAGCTGGA
NLRP3	GATCTTCGCTGCGATCAACAG
	CGTGCATTATCTGAACCCCAC
TRPM7 (human)	ACTGGAGGAGTAAACACAGGT
	TGGAGCTATTCCGATAGTGCAA
GAPDH (human)	GGAGCGAGATCCCTCCAAAAT
	GGCTGTTGTCATACTTCTCATGG
TRPM7 (rat)	AGGATGTCAGATTTGTCAGCAAC
	CCTGGTTAAAGTGTTCACCCAA
GAPDH (rat)	AGGTCGGTGTGAACGGATTTG
	GGGGTCGTTGATGGCAACA

TNF-α, tumor necrosis factor α; IL, interleukin; NLRP3, NLR family pyrin domain containing 3; TRPM7, transient receptor potential melastatin 7; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and quantified using a BCA kit. Next, Western blot was performed using the antibodies [TRPM7 (ab245408), NLRP3 (CST 15101)]. The content of the cytokines in the serum samples and cell supernatants were detected using the corresponding ELISA kits (R&D, USA).

ROS assays

The cells were collected after being washed twice with pre-cold phosphate buffered solution (PBS) and stained by a DCFH-DA probe (Yeasen, China) solution for half an hour at 37 ℃. Next, the stained cells were collected by centrifugation and re-suspended in 1 mL of pre-cooled PBS. A FACSCanto II flow cytometer was used for ROS detection (20).

Animal model

Neonatal Sprague-Dawley (SD) rats (one day old, male or female, weighing 7–8 g, feed manually) were used to establish the NEC model using a method described in a previous study (21). All the rat pups were housed on a 12/12-h light/dark cycle at a moderate temperature (23±2 ℃). The investigators were as gentle as possible with the rat pups. If the rat pups became stressed, they were put into the incubator to warmup rapidly. Briefly, the rat pups were fed a formula supplemented with LPS [10 μg/g body weight (BW)] (Sigma-Aldrich, St. Louis, MO, USA) and exposed to hypothermia and hypoxia as previously described. On day 1, the SD rat pups (6–8 g) were hand-fed every 4 h with 0.1 mL of cow's milk-based rat milk substitute formula. The feeding amount was subsequently increased 0.1 mL every 24 h until it reached 0.3 mL. The rat pups were then stressed twice every day with a hypoxic-reoxygenation treatment (whereby they breathed 100% nitrogen gas for 3 min then 100% oxygen gas for 3 min immediately thereafter) followed by a cold stress (4 ℃ for 10 min) for 3 days. The rat pups were monitored every day for clinical signs of NEC, such as abdominal distension, apnea, rectal bleeding, and lethargy. BW was assessed every day. The rat pups were randomly divided into the following 3 groups (n = at least 5 per group): the healthy control group, the NEC group, and the NS8593 group. Three mg/kg NS8593 were mixed with milk and treated to NEC rats until the end of the experiment. All animals were euthanized. Experiments were performed under a project license (No. KLL-animal-2019-20) granted by the Committee on Animal Experiments of the Affiliated Hospital of Zunyi Medical University, in compliance with the Affiliated Hospital of Zunyi Medical University institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration.

Statistical analysis

Data are representative of at least three independent experiments. The results are presented as the mean ± standard error of the mean. GraphPad Prism (version 8.3.0) was used to generate all the figures and conduct the statistical analysis. The analysis was performed using a one-way analysis of variance (ANOVA) or a two-way ANOVA or a *t*-test. A P value <0.05 was considered statistically significant.

Results

TRPM7 and NLRP3 expression levels are increased in NEC patients

To test the *TRPM7* and *NLRP3* expression in the NEC

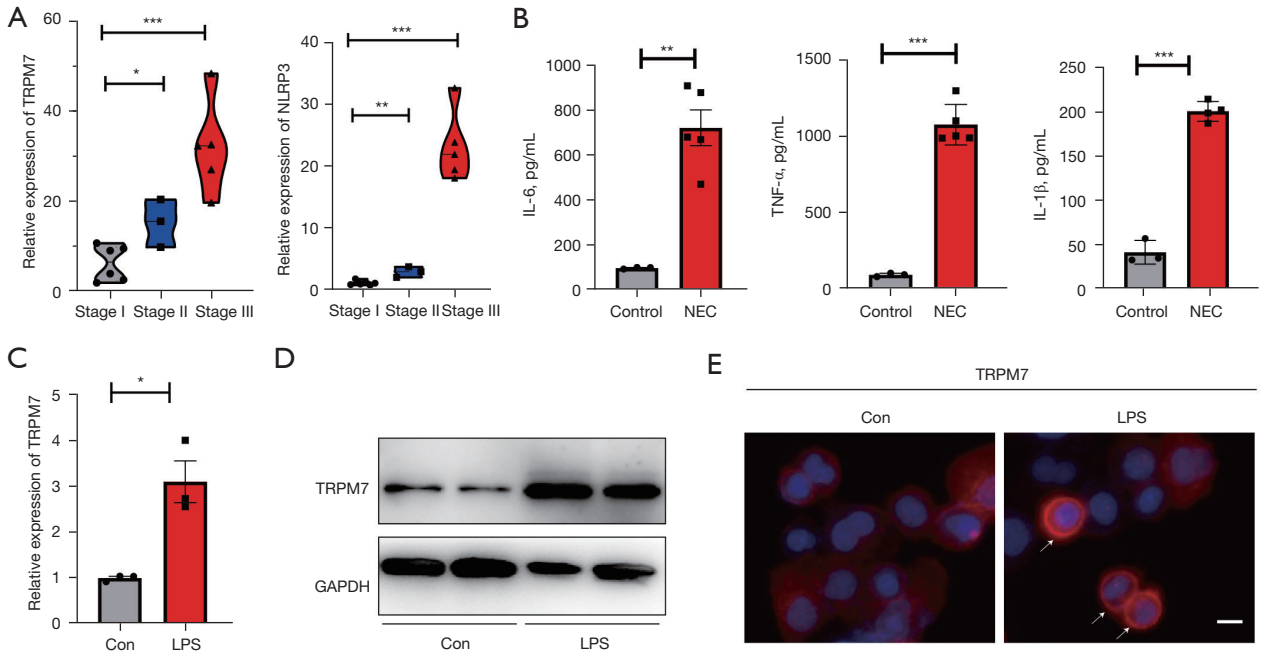

Figure 1 TRPM7 and NLRP3 are upregulated in NEC patients. (A) TRPM7 and NLRP3 was increased in NEC patients. (B) The cytokines in the serum of the NEC patients were examined by ELISAs. (C,D) The expression of TRPM7 as detected in the ICE-6 cells treated with 50 μg/mL LPS for 4 h. (E) The location of TRPM7 was observed via immunostaining (arrows). Scale bar =5 μm. The data are representative of at least 3 independent experiments. The analysis was performed using an unpaired Student's *t*-test; *, P<0.05; **, P<0.01; ***, P<0.001. TRPM7, transient receptor potential melastatin 7; NLRP3, NLR family pyrin domain containing 3; IL, interleukin; TNF-α, tumor necrosis factor α; Con, control; LPS, liposaccharide; NEC, necrotizing enterocolitis; ELISA, enzyme-linked immunosorbent assay.

patients, qRT-PCR was performed. We found that the expression of both of these 2 genes was significantly increased in patients with NEC, accompanied with severity of NEC (*Figure 1A*). These results hinted that TRPM7 might involve in the development of NEC. We also detected the inflammatory state by measuring the concentration of interleukin-6 (IL-6), IL-1β, and TNF-α in the serum of the NEC patients. We found that all these cytokines were significantly increased in the NEC patients (*Figure 1B*). We then stimulated the IEC-6 cells with 50 μg/mL LPS for 4 h and found that the *TRPM7* expression levels were upregulated 2-fold after LPS treatment (*Figure 1C,1D*). The immunostaining results demonstrated that TRPM7 was gradually transferred from the cytoplasm to the membrane (*Figure 1E*). The findings suggested that TRPM7 might play a functional role in NEC development.

TRPM7 inhibitor NS8593 attenuated the pro-inflammatory cytokine release

To further verify the role of TRPM7 in NEC development, we used its inhibitor NS8593 to treat the cell model and detected the level of TNF-α, IL-6, and IL-1β in the cell culture supernatant. As expected, LPS strongly upregulated the messenger RNA (mRNA) and secretion levels of these cytokines in the IEC-6 cells, which revealed the increased transcriptional activity of the pro-inflammatory cytokines (*Figure 2A,2B*). After adding NS8593 (20 μM), the expression levels of these cytokines decreased (*Figure 2A,2B*). The above results indicated that the TRPM7 inhibitor NS8593 inhibited LPS-induced cytokine production and exhibited anti-inflammatory activity. The accumulation of free oxygen and free radicals is another major feature in the process of NEC. Thus, we examined

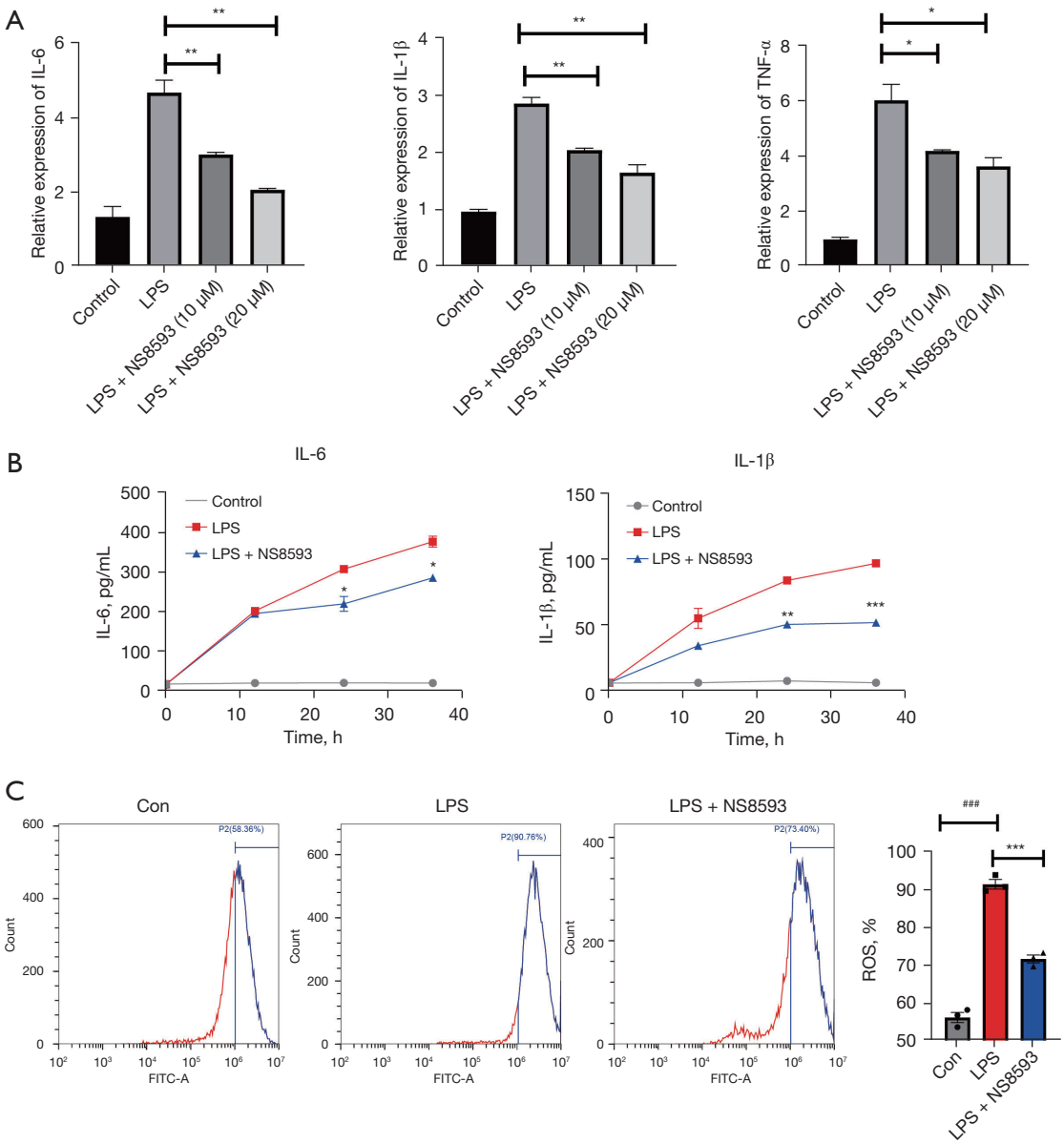

Figure 2 TRPM7 antagonist NS8593 attenuated the release of pro-inflammatory cytokines and scavenged the free. (A) The IEC-6 cells pre-treated with NS8593 for 1 h were exposed to LPS (50 μg/mL) for 4 h and then sent for qPCR. (B) The culture supernatant was collected for the ELISA analysis of IL-6 and IL-1β. (C) The ROS level was measured by flow cytometry. The data are representative of at least 3 independent experiments. The analysis was performed using a one-way ANOVA or one-way ANOVA. *, P<0.05; **, P<0.01; ***, P<0.001; ###, P<0.0001. IL, interleukin; LPS, liposaccharide; FITC-A, fluorescein isothiocyanate A; ROS, reactive oxygen species; Con, control; TRPM7, transient receptor potential melastatin 7; NLRP3, NLR family pyrin domain containing 3; qPCR, quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor α; ANOVA, analysis of variance.

the level of ROS using dichloro-dihydro-fluorescein diacetate (DCFH-DA) assays. It was demonstrated that the ROS levels in the LPS-treated IEC-6 cells were significantly increased but decreased significantly when NS8593 was introduced (*Figure 2C*). In sum, NS8593 was shown to inhibit the inflammatory response and maintain redox homeostasis by targeting TRPM7.

TRPM7 activated NLRP3 inflammasome as an ion channel protein

To investigate whether TRPM7 participates in NLRP3 inflammasome activation, the IEC-6 cells were pre-treated with NS8593, and then treated with LPS for 4 h, followed by adenosine triphosphate (ATP) challenged for 30 minutes. We found that NS8593 significantly blocked the secretion of IL-1β (*Figure 3A*). Additionally, NS8593 inhibited ATP-induced NLRP3 expression (*Figure 3B*). Next, we explored the inhibiting effect of NS8593 on nigericin, which activated NLRP3 inflammasome. Similarly, NS8593 effectively blocked the release of IL-1β and decreased NLRP3 expression (*Figure 3C*,*3D*). These findings suggest that the inhibition of TRPM7 alleviates NLRP3 inflammasome activation in intestinal epithelial cells. Next, we detected cytosolic Ca^{2+} signaling in the IEC-6 cells after 4 h of LPS treatment. As *Figure 3E* shows, incubation with LPS significantly increased the intracellular Ca^{2+} level compared to that of the untreated cells. Additionally, fluorescence was reduced after NS8593 treatment (*Figure 3F*). These findings suggest that TRPM7 mediates NLRP3 inflammasome activation via Ca^{2+} signaling.

NS8593 alleviated NEC development in vivo

Finally, we evaluated whether TRPM7 was involved in the occurrence of NEC *in vivo*, and whether its inhibitor played a certain therapeutic role. At the beginning of the modeling, the BW of the NEC group and the NS8593-treated NEC group did not differ significantly. During the modeling period, the NEC group lost more BW than the NS8593-treated NEC (NEC + NS8593) group (*Figure 4A*). Before sacrifice, the BW of the NEC + NS8593 group was significantly higher than that of the NEC group. The final survival rates of the 3 groups differed significantly as follows: 25% for the NEC group, 50% for the NEC + NS8593 group, and 100% for the control group (*Figure 4B*). There were distinct NEC-like pathological changes in the intestinal tissue of the NEC group. However, these phenotypes were obviously relieved in the NEC + NS8593 group (*Figure 4C*). The mRNA results showed that *TRPM7* and *NLRP3* expression were significantly upregulated in the NEC group, the expression of pro-inflammatory cytokines was also increased (*Figure 4D*), while the inflammatory levels were decreased after administration NS8593 (*Figure 4E*). Taken together, the results suggest that TRPM7 inhibitors might be potential small molecules for the NEC treatment.

Discussion

NEC is characterized by excessive inflammatory responses and LPS-induced ROS accumulation (2,3). Our current work revealed that TRPM7, a calcium-permeable ion channel, ameliorated inflammation in NEC by reducing NLRP3 activation. In the pathogenesis of NEC, LPS-activated inflammation is well described (22). Briefly, LPS triggers inflammatory cytokine release by stimulating nicotinamide adenine dinucleotide phosphate oxidase and mitochondrial ROS production (23). Accumulated ROS play a key role in toll-like receptor 4 signaling, and the inhibition of ROS production or the direct scavenging of ROS as antioxidants has been shown to attenuate intestinal mucosa damage (24,25). Recent studies have shown that ROS is considered a downstream signal of NLRP3 activation, which controls pro-inflammatory cytokine production. Excess pro-inflammatory cytokines were detected in NEC development and were found to be correlated with the severity of inflammation (21). It has been examined that the effect of Ca^{2+} influx on NLRP3 inflammasome activation and cytokine release (26). However, the mechanism mediating calcium imbalance remains unclear.

The members of the transient receptor potential (TRP) family work as cellular Ca^{2+} channel proteins to mediate Ca^{2+} current (27). TRPM2 is reported to be particularly crucial for inflammasome activation in a ROS-dependent manner in macrophages and monocytes (28,29). However, the effect of TRPM7 on inflammasome activation is not yet known. To date, only 1 study has reported that miR-129-5p targets TRPM7 to attenuate the hypoxia/reoxygenation injury to cardiac myocytes, along with the inhibition of NLRP3 inflammasome activation (19). Once TRPM7 expression is rescued, the miR-129-5p-induced inhibition of NLRP3 inflammasome activation is counteracted, which indirectly suggests that TRPM7 regulates NLRP3 inflammasome activation. We found that *TRPM7* and *NLRP3* expression was upregulated in NEC patients and

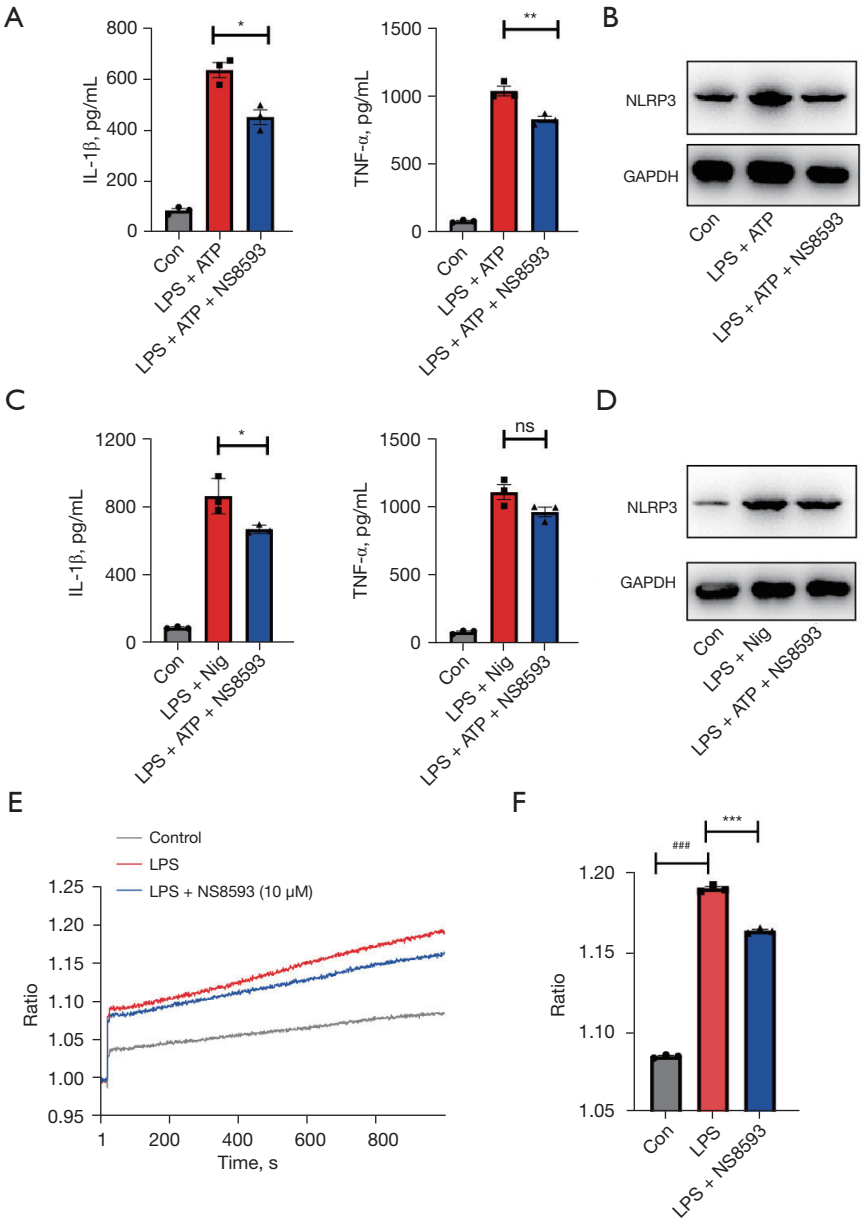

Figure 3 TRPM7 mediates NLRP3 inflammasome activation via Ca^{2+} signaling. (A-D) The IEC-6 cells were pre-treated with 20 μM of NS8593 for 1 h and then primed with 50 ng/mL of LPS for 4 h and subsequently challenged with 5 mM of ATP for 30 min or 10 μM of nigericin for 1 h to activate NLRP3 inflammasome. (A,C) IL-1β and TNF-α release in the supernatants were measured using the ELISA method. (B,D) NLRP3 expression level were immunoblotted with antibody. (E,F) The cytosolic Ca^{2+} was stained with Fluo-4AM and detected by fluorescence spectrophotometry. The data are representative of at least 3 independent experiments. The analysis was performed using a one-way or two-way ANOVA. *, P<0.05; **, P<0.01; ***, P<0.001; ###, P<0.0001. IL, interleukin; TNF-α, tumor necrosis factor α; Con, control; LPS, liposaccharide; ATP, adenosine triphosphate; ns, not significant; nig, nigericin; TRPM7, transient receptor potential melastatin 7; NLRP3, NLR family pyrin domain containing 3; ELISA, enzyme-linked immunosorbent assay.

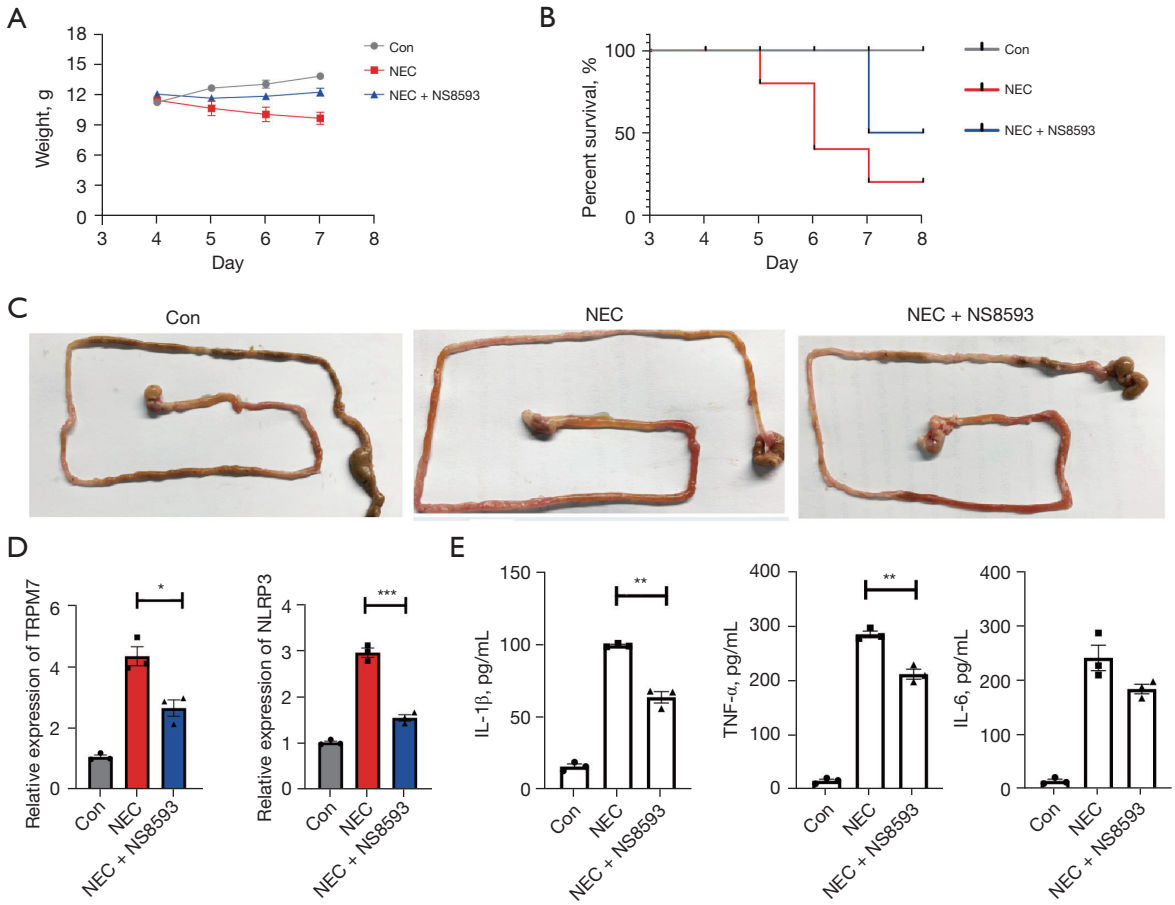

Figure 4 NS8593 ameliorated the intestinal injury in the rat NEC model. (A) Body weight changes in the control group (n=5), NEC group (n=6), and NEC + NS8593 group (n=6). (B) Survival curves of newborn rat in the 3 groups. (C,D) The TRPM7 and NLRP3 mRNA level in the terminal ileum. (E) The protein concentrations of inflammatory cytokines in the 3 groups. The data are representative of at least 3 independent experiments. The analysis was performed using a one-way or two-way ANOVA, *, P<0.05; **, P<0.01; ***, P<0.001. Con, control; NEC, necrotizing enterocolitis; TRPM7, transient receptor potential melastatin 7; NLRP3, NLR family pyrin domain containing 3; IL, interleukin; TNF-α, tumor necrosis factor α; ANOVA, analysis of variance.

NEC models. Blocking TRPM7-mediated calcium efflux could reduce the release of cytokines and extenuate damage by inhibiting NLRP3 inflammasome activation. Our work suggests that TRPM7-mediated calcium flux might contribute to inflammasome activation.

Taken together, our findings revealed TRPM7 inhibitors attenuated LPS-induced ROS and reduced the release of pro-inflammatory cytokines. It also exhibited protective effects on the NEC model. Although NS8593 has not been used in clinical, it provides a candidate molecule for drug development. Our findings suggest that TRPM7 inhibitors represent a promising strategy for NEC effective therapeutics.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://tp.amegroups.com/article/view/10.21037/tp-22-633/rc

Data Sharing Statement: Available at https://tp.amegroups.

com/article/view/10.21037/tp-22-633/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-22-633/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of The Affiliated Hospital of Zunyi Medical University (No. KLL-2019-016) and informed consent was taken from all the patients' legal guardians. All the animal experiments were performed under a project license (No. KLL-animal-2019-20) granted by the Committee on Animal Experiments of the Affiliated Hospital of Zunyi Medical University, in compliance with the Affiliated Hospital of Zunyi Medical University institutional guidelines for the care and use of animals.

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