Purinergic P2X7 Receptor Mediates the Elimination of *Trichinella spiralis* by Activating NF-κB/NLRP3/IL-1β Pathway in Macrophages

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**ABSTRACT** Trichinellosis is one of the most neglected foodborne zoonoses worldwide. During *Trichinella spiralis* infection, the intestinal immune response is the first line of defense and plays a vital role in the host’s resistance. Previous studies indicate that purinergic P2X7 receptor (P2X7R) and pyrin domain-containing protein 3 (NLRP3) inflammasome are involved in the intestinal immune response in *T. spiralis* infection. However, the precise role of P2X7R and its effect on NLRP3 remains largely underdetermined. In this study, we aimed to investigate the role of P2X7R in the activation of NLRP3 in macrophages during the intestinal immune response against *T. spiralis*. We found that *T. spiralis* infection upregulated expression of P2X7R and activation of NLRP3 in macrophages in mice. In vivo, P2X7R deficiency resulted in increased intestinal adult and muscle larval burdens, along with decreased expression of NLRP3/interleukin-1β (IL-1β) in macrophages from the infected mice with *T. spiralis*. In vitro experiments, P2X7R blockade inhibited activation of NLRP3/IL-1β via NF-κB and thus reduced the capacity of macrophages to kill newborn larvae of *T. spiralis*. These results indicate that P2X7R mediates the elimination of *T. spiralis* by activating the NF-κB/NLRP3/IL-1β pathway in macrophages. Our findings contribute to the understanding of the intestinal immune mechanism of *T. spiralis* infection.

**KEYWORDS** P2X7R, NF-κB, NLRP3, IL-1β, macrophages, *Trichinella spiralis*

Trichinellosis, caused by the parasitic nematode *Trichinella spiralis*, is one of the most neglected foodborne zoonoses worldwide (1). Transmission to humans involves the consumption of raw or undercooked meat and its derivatives from infected animals (1, 2). Upon ingestion, larvae in infected meat are released and migrate to the small intestine, where they mature and then develop into adults (the enteral phase). After mating, adults release newborn larvae (NBL), and NBL migrate into striated muscle, where they encapsulate and form encapsulated larvae (the muscle phase). The intestinal mucosa is the first line of defense in the host’s resistance to *T. spiralis* infection (3). Therefore, the intestinal immune response of hosts plays a vital role in *T. spiralis* infection.

In the enteral phase of *T. spiralis* infection, adults and larvae feed on intestinal villi and invade intestinal tissue, which results in damage of intestinal tissue and cell death. Intestinal tissue damage and necrotic cells in infected hosts release a number of damage-associated molecular pattern molecules (DAMPs), including extracellular ATP. Extracellular ATP is sensed by the ATP-gated purinergic P2X receptors (P2XRs). Among them, the most involved receptor in infection and inflammation is P2X7 receptor (P2X7R) (4, 5). It has been demonstrated that many pathogens, such as *Mycobacterium tuberculosis*, *Leishmania*, 

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Heligmosomoides bakeri, and Toxoplasma gondii, can evade the killing effects of infected hosts through interrupting the P2X7R pathway, which indicates that P2X7R acts as a trigger receptor of immune protection in infectious diseases (6–9). A previous study shows that both P2X7R expression and levels of interleukin-1β (IL-1β) increase in the jejunum of infected mice with T. spiralis, and IL-1β is one of the most important effector molecules to clear the parasite in infected hosts (10). However, the related immunological mechanism by which P2X7R mediates immune responses during T. spiralis infection has not been further elucidated.

Pyrin domain-containing protein 3 (NLRP3) in inflammasome, a member of the Nod like-receptor (NLR) protein family, is widely expressed in macrophage and has the function of recognizing pathogens (10). There has been increasing research interest in the role of NLRP3 activation during parasitic infections (4, 11). It is reported that activated resident macrophages trigger an inflammatory response through NLRP3 inflammasome (12). Many macrophages accumulate in abdominal cavity during the enteral phase in murine trichinellosis, which indicates that macrophages serve as an important component in the intestinal immune response (13). Macrophages play an essential role in the control of some parasite infections, and P2X7R expression is upregulated in macrophages in models of chronic inflammatory diseases (9, 14). P2X7R in macrophages promotes the elimination of the intracellular parasite in vitro and plays an important role in regulation of NLRP3 inflammasome activation (12, 15). Protein NLRP3 and Pro-caspase-1 are two main components in peritoneal macrophages. NLRP3 is critical for innate immunity and for inducing the release of IL-1β (16). Nevertheless, there is little evidence of the effect of P2X7R on NLRP3 activation in the infection with the extracellular parasite T. spiralis.

In this study, we explored the effect of P2X7R on NLRP3 activation in macrophages and its protective mechanism during the intestinal immune response in murine T. spiralis infection. We found that T. spiralis infection upregulated expression of P2X7R and induced activation of NLRP3/IL-1β in macrophages in mice. P2X7R deficiency resulted in increased intestinal adult and muscle larval burdens, accompanied by depressed activation of NLRP3 in macrophages from the infected mice with T. spiralis. Moreover, P2X7R blockade inhibited activation of NLRP3/IL-1β via NF-κB and thus reduced the capacity of macrophages to kill NBL of T. spiralis in vitro.

RESULTS

P2X7R expression on macrophages was upregulated in the enteral phase of murine T. spiralis infection. The effect of T. spiralis infection on P2X7R expression in mice was examined. T. spiralis-infected mice showed a significantly elevated percentage of P2X7R+ F4/80 macrophages in mesenteric lymph nodes (MLN) (Fig. 1A and B) and higher P2X7R expression in peritoneal macrophages (Fig. 1C and D), indicating that T. spiralis infection induced increased expression of P2X7R on macrophages in murine trichinellosis.

NLRP3 in macrophages was activated in the enteral stage of murine T. spiralis infection. Protein NLRP3 and Pro-caspase-1 in peritoneal macrophages were detected, and concentrations of IL-1β in serum and peritoneal fluid were also investigated. As shown in Fig. 2A to C, the infected mice had higher protein levels of NLRP3 and Pro-caspase-1 in peritoneal macrophages than the uninfected mice. Concentrations of IL-1β in serum and peritoneum increased significantly in the infection group (Fig. 2D and E). Taken together, these results indicated that T. spiralis infection resulted in activation of NLRP3 in mice.

P2X7R deficiency increased the worm burdens in murine T. spiralis infection. Since T. spiralis upregulated the expression of P2X7R, to verify the role of P2X7R in trichinellosis, we infected wild-type (WT) and P2X7R knockout (P2X7R−/−) mice with T. spiralis. Compared with the infected WT mice, the infected P2X7R−/− mice showed decreased activity, arched back, and towering hair (data not shown). P2X7R deficiency led to heavy worm burdens of intestinal adults (Fig. 3B) and muscle larvae in the infected mice (Fig. 3A and C). Furthermore, P2X7R deficiency aggravated pathological damage in the small
P2X7R deficiency increased the infection intensity and then exacerbated parasitological lesions in murine *T. spiralis* infection. P2X7R deficiency inhibited activation of NLRP3 in macrophages in the enteral stage of murine *T. spiralis* infection. To investigate the effect of P2X7R on the activation of NLRP3 in murine trichinellosis, we compared the activated markers of NLRP3 in macrophages from the infected WT and P2X7R+/− mice with *T. spiralis*. As shown in Fig. 4A to C, P2X7R deficiency resulted in decreased expression levels of NLRP3 and Pro-caspase-1 in peritoneal macrophages from the infected mice, and levels of IL-1β in serum and peritoneal fluid decreased in the infected P2X7R+/− mice (Fig. 4D and E). Collectively, P2X7R deficiency inhibited activation of NLRP3 in macrophages in murine trichinellosis.

**P2X7R Alleviates Trichinellosis by Activating NLRP3 Infection and Immunity**

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**FIG 1** P2X7R 1 expression on macrophages was upregulated in the enteral phase of murine *T. spiralis* infection. Wild-type mice were administered orally with 300 larvae of *T. spiralis* in 0.2 ml of sterilized 0.9% saline. Uninfected control mice were administered orally with 0.2 ml of sterilized 0.9% saline. At 7 days postinfection (dpi), 10 mice from each group were sacrificed by cervical dislocation. (A) Proportions of P2X7R+F4/80+ cells in mesenteric lymph nodes (MLN) were detected by flow cytometry. (B) Ratios of P2X7R+F4/80+ cells to lymphocytes in MLN were calculated. (C and D) Expression of P2X7R on peritoneal macrophages was detected by Western blotting (C) and quantified (D). Data are expressed as means ± SEMs based on 10 mice in each group and from two independent combined experiments. Asterisks mark significant differences between different groups (***, *P* < 0.001).
P2X7R blockade inhibited the capacity of macrophages to kill NBL of *T. spiralis* in vitro. To explore the effect of P2X7R on the capacity of macrophages to kill NBL, we cocultured the NBL with supernatants from cultured PMA-U937. We found that activated macrophages with ATP and *T. spiralis* ES antigen treatment killed more than 60% of NBL in vitro. However, blockade of P2X7R and inhibition of NF-κB resulted in a significant decrease of the killing capacity of macrophages (Fig. 6A and B). These results indicated that both P2X7R and the NF-κB pathway played a vital role in the parasiticidal capacity of macrophages.

**DISCUSSION**

A number of studies have demonstrated that P2X7R has a central role in the inflammatory responses in some parasite infections, especially in the intestinal immune responses (9, 11, 17). The previous work shows that P2X7R expression is elevated in the small intestine of mice infected with *T. spiralis* (10), and P2X7R was proved to be located predominantly on macrophages in models of inflammatory diseases (18, 19). Our data showed that *T. spiralis* infection was associated with increased expression of P2X7R on macrophages, which indicates that P2X7R might play an important role in establishing an innate immune response against *T. spiralis* infection.

To verify the role of P2X7R in the elimination of *T. spiralis* in hosts, we compared the worm burdens of the infected P2X7R−/− mice and the infected WT control. P2X7R deficiency resulted in much higher worm burdens in the infected mice (evidenced by increased worm adults and muscle larvae numbers). Therefore, the pathological damage in the small intestine and muscle became deteriorated. Loss of function P2X7R has been linked to increased susceptibility to intracellular pathogens (20, 21). The macrophages infected with *T. gondii* and *Leishmania amazonensis* led to enhanced expression of P2X7R, which helps in the elimination of the parasites (22, 23). It has been reported that inhibition or deletion of P2X7R result in an increase in parasite load of *T. gondii* (14, 22). Our results, together with the previous studies, indicate that P2X7R participates in the elimination of both intracellular and extracellular parasites, although further studies are needed to investigate the detailed cellular and molecular mechanisms.

The inflammasome is a key line of immune defense against invading pathogens. Accumulating evidence indicates that NLRP3 activation is essential for the control of
various parasitic infections (4, 11, 24). Our results showed that the *T. spiralis*-infected mice had higher expression of NLPR3 in peritoneal macrophages than the uninfected control, which is consistent with the previous studies (25, 26). Nevertheless, the cellular mechanism of NLRP3 activation in the parasite infection remains largely unknown. Many studies have identified that P2X7R is one of the most potent activators of the NLRP3 inflammasome (11, 27, 28). Our results showed that P2X7R deficiency inhibited NLRP3 activation in the infected mice, evidenced by decreased protein expressions of NLRP3 and Pro-caspase and lower concentrations of IL-1β in serum and peritoneum. In *in vitro* experiments, P2X7R blockade with the antagonist also led to consistent results. It has been demonstrated that under pathophysiological conditions caused by parasite infections, ATP released from dying cells enhances P2X7R activation to upregulate the NLRP3 inflammasome, subsequently promoting IL-1β secretion (11). The NLRP3 inflammasome is closely associated with P2X7R in *T. gondii* infection and thus inhibits parasite proliferation in small intestinal epithelial cells (9). P2X7R is one of the most potent activators of the NLRP3 inflammasome and, therefore, of mature IL-1β release (29). IL-1β is a crucial factor of host defense in response to infections and injuries (11, 29, 30). IL-1β production is strictly regulated at both the transcriptional and posttranslational levels through the activity of P2X7R-mediated NLRP3 inflammasome (9, 29). Many studies have reported that parasite infections produce significant amounts of IL-1β in different immune cells, and concentrations of IL-1β are closely associated with parasite elimination in hosts (9, 11, 21). The NLRP3 inflammasome is activated in response to Leishmania infection and is important for the restriction of parasite replication *in vitro* and *in vivo* (31). Our data showed that inhibition of the NLRP3/IL-1β pathway mediated by P2X7R decreased the capacity of macrophages to kill *T. spiralis* parasites both *in vivo* and *in vitro*. Our findings, consistent with previous studies, strongly suggest that P2X7R plays a pivotal part in facilitating IL-1β release for its important role in NLRP3 inflammasome activation and in mediating the resistance to parasites, and the mechanism needs to be further clarified.
Production of IL-1β is a multistep process, including synthesis of pro-IL-1β, proteolytic cleavage to mature IL-1β, and release into the extracellular environment. The activation of the nuclear factor NF-κB induces the synthesis of pro-IL-1β in many inflammatory diseases (30). The seminal studies demonstrate that P2X7R is a potent stimulus for activation of the NF-κB pathway in microglia, osteoclasts, and osteoblasts (32–34). Previous studies found that NLRP3 is one of the downstream pathways of NF-κB (27), and we wondered whether the NF-κB pathway played a role in the effect of P2X7R on activation of NLRP3 in T. spiralis infection. It is reported that stimulation of monocyte-derived macrophages U937 in vitro may provide a useful model for further study of mechanisms of macrophage cytotoxicity and its activation in T. spiralis infection (35, 36). Thus, we investigated the role of NF-κB in P2X7R-mediated NLRP3 activation in U937 in response to T. spiralis ES antigens. The data showed that the expression of P-NF-κB in the treated macrophages decreased significantly after blockade of P2X7R. Furthermore, blockade of NF-κB inhibited both activation of NLRP3 and synthesis of IL-1β. As a result, the capacity of macrophages to kill T. spiralis larvae had a significant decrease after NF-κB blockade in vitro. Our results fit with the view that NF-κB plays an important role in mediation of P2X7R in the NLRP3/IL-1β pathway and then has an effect on the capacity of macrophages to kill parasites.

Taken together, our findings reveal the role of P2X7R in the induction of NLRP3 inflammasome activation in T. spiralis-induced macrophages. To the best of our knowledge, this is the first report that P2X7R plays an important role in the activation of NLRP3 during T. spiralis infection. P2X7R deficiency or blockade decreased the capacity of macrophages to kill the parasite by inhibiting NLRP3 activation in vivo and in vitro. Furthermore, our data showed that P2X7R mediated the parasiticidal activation of NLRP3 via NF-κB. These results may contribute to our better understanding of the intestinal immune mechanism of T. spiralis infection but also offer new insight into the identification of innate resistance during the enteral stage of trichinellosis.

MATERIALS AND METHODS

Mice and parasites. P2X7R knockout (P2X7R−/−) mice in the C57BL/6 genetic background were purchased from The Jackson Laboratory (USA). Wild-type C57BL/6 mice were provided by the Hubei Province Center for Disease Control and Prevention (Wuhan, China). All mice were maintained in a standard specific-pathogen-free animal facility at Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Our experiment was reviewed and approved by the Institutional Animal Care...
and Use Committee at Tongji Medical College (SCXK2016-0011). The life cycle of *T. spiralis* (ISS534) was maintained by serial passage in Kunming mice at 6-month intervals (37).

**Experimental infection.** *P2X7R* 

2/2 and WT mice, aged 6 to 8 weeks, were administered orally with 300 larvae of *T. spiralis* in 0.2 ml of sterilized 0.9% saline, following a previously published protocol (38). Uninfected control mice were administered orally with 0.2 ml of sterilized saline. At 7 days postinfection (dpi), 10 mice from each group were sacrificed by cervical dislocation. Blood was collected from the oculomotor sinus of mice under ether anesthesia. Small intestine, MLN, and peritoneal macrophages were collected for further analysis. At 42 dpi, 6 mice from each group were sacrificed; skinned and eviscerated mouse carcasses were collected for determination of skeletal muscle larvae.

**Collection of intestinal adult worms.** To facilitate the recovery of adult worms from the intestine, the infected mice were not fed for 1 day prior to sacrifice. The small intestines were opened longitudinally and washed thrice in ice-cold 0.9% saline and then cut into 2-cm-long fragments with sharp scissors and cultured in normal saline at 37°C for 2.5 h. Then, adults released from the small intestine into saline were collected and counted under a microscope (39).

**Determination of skeletal muscle larvae burden.** *T. spiralis* muscle larvae were collected and counted as previously described (40). In brief, the mice were sacrificed with euthanasia, and skinned and eviscerated mouse carcasses were cut into pieces and digested in 1% pepsin-hydrochloride digestion fluid for 1.5 h at 37°C. A magnetic spin bar was utilized to provide continual mixing during digestion, and then the entire digest from the beaker were poured through an 80 sieve (180-μm mesh) into a 2-
killing PMA-U937 with 5% CO2 for 24 h at 37°C, following the procedure described previously with some modifications. Macrophages were isolated by adherence to plastic wells and confluence, then, cells were stimulated with ATP for 30 min (the ATP group), followed by treatment with T. spiralis ES antigens for 24 h (the ATP + ES antigen group). For the ATP + A740003 + ES antigen group, A740003 (antagonist of P2X7R) was added before stimulation with ATP. Cells incubated with cell culture medium were used as the control. For the ATP + PTDC + ES antigen group, an NF-κB inhibitor, PTDC, was added before stimulation with ATP. Supernatants were collected from each group and cocultured with 100 newborn larvae. The death of larvae was determined under direct microscope by two independent observers in a blind fashion. (A and B) The percentages of death rate were calculated. Each experiment had three replicates. Data were expressed as means ± SEMs from four independent combined experiments. Asterisks mark significant differences between different groups (*, P < 0.05; ***, P < 0.001).

Preparation of excretory-secretory (ES) antigens of adults and larvae. The ES antigens of adults and larvae were prepared as previously described (41, 42). Adults and larvae were pooled and cultured at 37°C in RPMI 1640 (Invitrogen) and 5% CO2, respectively, for 18 h. The supernatant containing ES antigens was collected after using an Amicon Ultra-3 centrifugal filter unit (Millipore, USA) at 4°C and centrifuged at 5,000 × g for 1 h. The protein concentration was detected with the bicinchoninic acid assay (BCA; Pierce, USA) according to the manufacturer’s protocol.

Isolation of peritoneal macrophages. Peritoneal macrophages from the uninfected and infected mice were collected after washing the peritoneal cavity with 3 ml sterile phosphate-buffered saline (PBS). Macrophages were isolated by adherence to plastic wells and confirmed with F4/80 immunostaining (10).

Flow cytometry. Flow cytometry analysis was performed to assess expression levels of P2X7R in macrophages in MLN from mice. Briefly, live cells were isolated from MLN of T. spiralis-infected mice at 7 dpi. The cell surfaces were stained with anti-F4/80-FITC and anti-P2X7R-PE for 30 min at 4°C. All antibodies were purchased from eBioscience (San Diego, CA). Data were analyzed with FlowJo version 10.0.7 (Tree Star).

Histological analysis. The small intestine (duodenum and jejunal segments) was obtained from the uninfected and infected mice at 7 dpi. All segments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C. Then, samples were embedded in paraffin and cut into 5-μm sections. For the histopathological study, the samples were stained with hematoxylin and eosin (H&E).

Cell culture and in vitro treatment. Human macrophage cell line U937 (Cell Bank, Chinese Academy of Sciences) was cultured with 5% CO2 for 24 h at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Invitrogen). Phorbol myristate acetate (PMA) (100 ng/ml) was added to stimulate U937 into the adherent macrophage phenotype (PMA-U937). Then, cells were stimulated with ATP for 30 min, followed by treatment with 10 μM/ml of T. spiralis ES antigens for 24 h. To evaluate the effect of P2X7R blockade on NLRP3 signals, 10 μM A740003 (antagonist of P2X7R; Sigma) was added before stimulation with ATP. Cells incubated with cell culture medium were used as the control. To determine the role of NF-κB in the activation of NLRP3 by P2X7R, an NF-κB inhibitor pyrrolidine dithiocarbamate (50 μM; Selleck Chemicals, USA) was added before stimulation with ATP. Supernatants and cultured cells were collected for further use.

Determination of parasitoidal capacity of macrophages in vitro. To confirm the effect of P2X7R on killing T. spiralis larvae by activated macrophages in vitro, NBL were incubated with supernatants from cultured PMA-U937 with 5% CO2 for 24 h at 37°C, following the procedure described previously with some modifications (40, 43). Briefly, NBL were separated from female adult worms by filtration over nylon gauze and then washed thrice in sterilized saline. The number of NBL was adjusted to 1,000/ml with RPMI 1640 medium. For each well, 100 μl of larva suspension and 1 ml of supernatants from cultured PMA-U937 were added. The death of larvae was determined under direct microscope by two independent observers in a blind fashion with the following standard: worm body shown as “C” shape or completely straightened, no longer curled up, with a blurred interior. Each larva was observed for 30 s until the worm body no longer moved.

Western blot analysis. Tissues and cells were lysed in RIPA lysis buffer (Beyotime Biotechnology, Nanjing, China) containing a cocktail of protease inhibitors (Roche, Basel, Switzerland). Samples were centrifuged at 12,000 × g for 15 min, and supernatants were collected for further analysis. The protein concentrations were detected with the BCA according to the manufacturer’s protocol. Western blot analysis was performed using primary antibodies against P2X7R and β-actin (Cell Signaling Technology, USA). Bovine serum albumin (BSA) was added as a standard. The bands were quantified by scanning densitometry, and the relative expression level was calculated.

FIG 6 P2X7R blockade inhibited the capacity of macrophages to kill NBL of T. spiralis in vitro. Human macrophage cell line U937 was cultured, and PMA was added to stimulate U937 into the adherent macrophage phenotype (PMA-U937). Then, cells were stimulated with ATP for 30 min (the ATP group), followed by treatment with T. spiralis ES antigens for 24 h (the ATP + ES antigen group). For the ATP + A740003 + ES antigen group, A740003 (antagonist of P2X7R) was added before stimulation with ATP. Cells incubated with cell culture medium were used as the control. For the ATP + PTDC + ES antigen group, an NF-κB inhibitor, PTDC, was added before stimulation with ATP. Supernatants were collected from each group and cocultured with 100 newborn larvae. The death of larvae was determined under direct microscope by two independent observers in a blind fashion. (A and B) The percentages of death rate were calculated. Each experiment had three replicates. Data were expressed as means ± SEMs from four independent combined experiments. Asterisks mark significant differences between different groups (*, P < 0.05; ***, P < 0.001).
TABLE 1. Primer sequences of target mRNA

| Genes   | Primer 5' (→ 3')                        | Primer 3' (→ 5')                        |
|---------|----------------------------------------|----------------------------------------|
| NLRP3   | TTTCTTTCTGTTTGCTGCAATTTTTG            | TTCTGCGCATATCACGAGGG                   |
| Pro-caspase-1 | GAAGGACAAAAACGAGGTGA               | TGGAGAGCACAAAGGCTGATA                  |
| IL-1β   | AACACAGTAGGTTGCTCCTCCAC              | TGGAGAAACACCACTTGTTGTCCTCA             |
| GAPDH   | GTCAGTGTTGACCTGACCT                   | AGGGGTCTACATGGCAACTG                   |

Sample collection for cytokine analysis and ELISA. Serum was collected from blood by centrifuging at 6,000 × g for 5 min following coagulation of 0.5 ml whole blood. The peritoneal lavage was centrifuged at 800 × g for 5 min, and the supernatant was collected for enzyme-linked immunosorbent assay (ELISA) analysis. Concentrations of IL-1β in mouse serum and peritoneal lavage fluids were measured with ELISA kits (R&D Systems) according to the manufacturer’s instructions.

Quantitative real-time PCR. Total RNA from tissues and cells was extracted with a TRIzol reagent kit (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. After being quantified using a Nanodrop 2000 instrument (Thermo Scientific, USA), RNA was reverse-transcribed to cDNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific). Then, real-time PCR was performed using Power Sybr green PCR master mix (Applied Biosystems, USA) with gene-specific primers as listed in Table 1. The amplification reactions were carried out with an initial hold step (95°C for 5 min), followed by 40 cycles of a three-step PCR (95°C for 1 min, 60°C for 45 s, and 72°C for 30 s). Relative mRNA expression of the target gene was calculated in terms of the comparative cycling threshold (Ct) normalized by GAPDH with the 2−ΔΔCt method.

Statistical analysis. Each experiment was performed at least twice with 6 to 10 mice or samples per group. All results were presented as means ± standard error of the means (SEMs) of two independent experiments. Statistical significance was determined using an unpaired Student’s t test or one-way analysis of variance (ANOVA) (GraphPad Prism version 7.0 software). The significant difference levels are indicated by *; P < 0.05; **; P < 0.01, and ***; P < 0.001.

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