ING4 alleviated lipopolysaccharide-induced inflammation by regulating the NF-κB pathway via a direct interaction with SIRT1

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Abstract
Sepsis is a complex inflammatory disorder in which high mortality is associated with an excessive inflammatory response. Inhibitor of growth 4 (ING4), which is a cofactor of histone acetyltransferase and histone deacetylase complexes, could negatively regulate this inflammation. However, the exact molecular signaling pathway regulated by ING4 remains uncertain. As a pivotal histone deacetylase, Sirtuin1 (SIRT1), which is widely accepted to be an anti-inflammatory molecule, has not been found to be linked to ING4. This study investigated how ING4 is involved in the regulation of inflammation by constructing lipopolysaccharide (LPS)-induced macrophage and mouse sepsis models. Our results revealed that ING4 expression decreased, whereas the levels of proinflammatory cytokines increased in LPS-stimulated cultured primary macrophages and RAW 264.7 cells. ING4 transfection was confirmed to alleviate the LPS-induced upregulation of proinflammatory cytokine expression both in vitro and in vivo. In addition, ING4-overexpressing mice were hyposensitive to an LPS challenge and displayed reduced organ injury. Furthermore, immunoprecipitation indicated a direct interaction between ING4 and the SIRT1 protein. Moreover, ING4 could block nuclear factor-kappa B (NF-κB) P65 nuclear translocation and restrict P65 acetylation at lysine 310 induced by LPS treatment. These results are the first to clarify that the anti-inflammatory role of ING4 is associated with SIRT1, through which ING4 inhibits NF-κB signaling activation. Our studies provide a novel signaling axis involving ING4/SIRT1/NF-κB in LPS-induced sepsis.

INTRODUCTION
Sepsis is an unbalanced systemic inflammatory reaction associated with severe injury or infection. Sepsis-associated multiple organ dysfunction syndrome and septic shock severely reduce the survival of patients; the mortality rate for multiple organ dysfunction syndrome is between 25% and 30%, and the mortality rate increases to 40% for septic shock. After infection, innate immune cells “sense” invading pathogens by recognizing pathogen-associated molecular patterns via an assortment of pattern recognition receptors, such as Toll-like receptors; the inflammatory cytokines secreted in response to this signaling are pivotal in the process of sepsis. The imbalance between excessive inflammation mediated by pro-inflammatory cytokines and immune suppression mediated by anti-inflammatory cytokines initiates the inflammation cascade.

As one of the most important members of innate immunity, macrophages can be polarized to assume different phenotypes, a pro-inflammatory M1 phenotype or an anti-inflammatory M2 phenotype, during the
occurrence, development and resolution of inflammation. Thus, macrophages function as crucial coordinators in the regulation of the balance between pro-inflammatory events and anti-inflammatory events. Lipopolysaccharide (LPS) has been identified as the poorly defined causative agent of Gram-negative bacteria-induced sepsis, and it stimulates cells through Toll-like receptor 4 to trigger inflammatory signaling cascades. In an LPS-associated sepsis model, nuclear factor-kappa B (NF-κB) signaling is activated and thus initiates the transcription of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein 1, which are all markers of systemic inflammatory response syndrome or multiple organ dysfunction syndrome. Hence, how to precisely and effectively equilibrate the innate immune reaction is a key point that needs to be addressed in sepsis therapy to avoid self-injury.

Inhibitor of growth 4 (ING4) is a member of the conserved ING family, which comprises five evolutionarily conserved proteins containing a bipartite nuclear localization signal and a carboxy-terminal plant homeodomain-like zinc finger. ING4 has been reported to interact with histone acetyltransferase and histone deacetylase complexes and thereby plays important roles in multiple biological functions, such as chromatin remodeling, gene transcription, cell cycle arrest, proliferation and apoptosis. It has been reported that ING4 can suppress NF-κB binding activity by physically interacting with the P65 subunit and serve as an E3 ubiquitin ligase to cause P65 ubiquitination and degradation, which can terminate NF-κB activation. Furthermore, ING4 can decrease P65 phosphorylation and P65 combination with P300 to favor the P65–HDAC1 interaction and thus attenuate NF-κB activity at specific NF-κB-targeted promoters. In addition, ING4-null mice show hypersensitivity to intraperitoneal LPS injection, and macrophages isolated from ING4-null mice reveal that ING4 inhibits innate inflammation by boosting the NF-κB-mediated activation of IkBα promoters, accordingly reducing the levels of nuclear P65 and pro-inflammatory cytokines targeted by NF-κB.

The silent mating type information regulation 2 homolog (Sirtuin) 1 (SIRT1), a member of a group of highly conserved NAD+-dependent deacetylases, is highly correlated with multiple biochemical functions. SIRT1 has been defined as a pivotal inflammatory suppressor of the innate immune response. In mouse macrophages, SIRT1 knockout significantly increases LPS-stimulated pro-inflammatory factor secretion. SIRT1 has powerful anti-inflammatory properties through its direct inhibition of NF-κB activation by deacetylating the P65 subunit of the NF-κB complex at lysine 310. Moreover, SIRT1 mediates the deacetylation of hypoxia-inducible transcription factor-1 alpha subunit, which is also a key switch in the pro-inflammatory response. It has been demonstrated that ING1 and ING2 can recruit SIRT1 to the R2 domain of RBP1 and inhibit R2-associated mSin3A/HDAC1 transcriptional repression. Moreover, ING4 can enhance P53 acetylation at lysine 382 via a physical interaction with P300 and strengthen the interplay between P65 and HDAC1. However, whether SIRT1 and ING4 have a direct link with each other in the inflammatory response is unknown.

Here, we performed coimmunoprecipitation studies using an anti-SIRT1 antibody and anti-ING4 antibody and confirmed the interplay between these two proteins. Based on this result, we hypothesized that ING4 may negatively regulate the inflammatory response by interacting with SIRT1. Our results provide new insight into the role of ING4 in the LPS-induced inflammatory response.

**RESULTS**

**ING4 levels were associated with a reduction in the LPS-induced inflammatory response**

To assess whether ING4 is involved in sepsis, we used LPS to stimulate macrophages to establish a cell model. As multiple studies have reported that at an LPS concentration of 1 μg mL⁻¹, macrophages could be polarized to the M1 phenotype and activated in septic inflammation, we chose 1 μg mL⁻¹ LPS to treat macrophages. Besides, cell counting kit-8 assays, Hoechst and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining were performed which showed that the 1 μg mL⁻¹ LPS treatment had no effect on cell viability or numbers (Supplementary figure 1). The RAW 264.7 cell line and peritoneal macrophages (PMs) were exposed to 1 μg mL⁻¹ LPS, and pro-inflammatory cytokine and ING4 levels were detected at different times. Quantitative real-time polymerase chain reaction results showed that in both RAW 264.7 cells and PMs challenged with LPS, the levels of pro-inflammatory cytokines including IL-1β, IL-6, TNF-α and monocyte chemoattractant protein 1 were remarkably increased with prolonged treatment time (P < 0.01) but peaked at different times between 2 and 12 h (Figure 1a, c). These data illustrated that the macrophage inflammation model was successfully constructed. We further performed quantitative real-time polymerase chain reaction and immunoblotting to examine ING4 expression and found that the ING4 level progressively decreased with prolonged LPS treatment (Figure 1b, d). Together, these results indicated that ING4 might participate in LPS-induced inflammation.
ING4 alleviated the LPS-induced upregulation of pro-inflammatory cytokine expression in RAW 264.7 macrophages

To investigate the role of ING4 in regulating inflammation, pcDNA3.1-ING4 and pLVX-shING4 plasmids were utilized to overexpress and knockdown ING4 in RAW 264.7 macrophages. After transient transfection for 48 h, ING4 expression was successfully increased or decreased at both the transcriptional and translational levels (Figure 2a, b). Along with the enhanced ING4 expression, the expression of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and monocyte chemoattractant protein 1 induced by LPS in the macrophages decreased significantly (Figure 2c). Conversely, ING4 inhibition mediated by pLVX-shING4 transfection dramatically increased pro-inflammatory cytokine release by RAW 264.7 macrophages (Figure 2d). These results indicated that ING4 reduced LPS-induced inflammation in RAW 264.7 macrophages.

ING4-overexpressing mice were hyposensitive to LPS challenge and displayed reduced organ injury

To further verify the protective effect of ING4 in vivo, a sepsis mouse model induced by intraperitoneal LPS injection was established. BALB/C mice were transfected in vivo with pcDNA3.1-ING4 or pcDNA3.1-EGFP-C2
ING4 in LPS-induced inflammation

Y Yang et al.

Figure 2. Inhibitor of growth 4 (ING4) alleviated the lipopolysaccharide (LPS)-induced upregulation of pro-inflammatory cytokine expression in RAW 264.7 macrophages. RAW 264.7 cells were transfected with 2 µg pcDNA3.1-ING4, pLVX-shING4 or negative control (NC) plasmid with or without LPS treatment. (a, b) ING4 messenger RNA (mRNA) and protein levels were evaluated 48 h after transfection. (c, d) Inflammatory cytokine transcripts, including those encoding interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), IL-6 and monocyte chemoattractant protein 1 (MCP-1), were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) 48 h after transfection with or without LPS treatment and normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript level. The data are presented as the mean ± s.e.m. and represent at least three independent experiments. **P < 0.01 compared with the NC. Statistical significance was determined by a Student’s t-test.

[...]

Interaction between ING4 and SIRT1 in macrophages

Considering the role of ING4 as an important cofactor in the regulation of protein acetylation and deacetylation,15,17 we wondered whether a direct
interaction between ING4 and SIRT1, one of the most important protein deacetylases, existed. We first examined expression in response to LPS stimulation at different time points and found that SIRT1 expression showed a decreasing tendency, especially at 2 h post-treatment, in RAW 264.7 macrophages (Figure 4a). To determine whether the hypothesized interplay existed, we implemented RAW 264.7 cell immunoprecipitation to evaluate the interaction. The interaction of SIRT1 with ING4 was confirmed by blotting complexes containing endogenous ING4 precipitated with ING4-specific antibodies and probing for SIRT1 (Figure 4b). Conversely, an anti-SIRT1 antibody was used for precipitation, and the isolated complexes were blotted with an anti-ING4 antibody (Figure 4b). Thus, an endogenous interaction assay studying RAW 264.7 cells demonstrated that ING4 interacts with SIRT1 directly. Furthermore, immunofluorescence was employed to determine the cellular distributions of the two proteins, and the superimposition of the red fluorescence indicating SIRT1 over the green fluorescence indicating ING4 confirmed the association between ING4 and SIRT1 (Figure 4c).

**ING4 reduced the acetylation and nuclear translocation of NF-κB P65**

It has been reported that ING4 enhances P53 acetylation at the Lys382 residue, physically interacts with P300 and strengthens P65–HDAC1 interactions, thus attenuating...
NF-κB activity. Hence, based on the confirmed endogenous interaction between ING4 and SIRT1 in RAW 264.7 cells, we wondered whether ING4 affects P65 acetylation by interacting with SIRT1 to interfere with the NF-κB pathway during LPS stimulation. Accordingly, considering that NF-κB signaling activation depends on P65 nuclear translocation, we separated the cytoplasmic and nuclear components of RAW 264.7 cells in the context of ING4 overexpression and knockdown and LPS treatment at the indicated time points (0, 0.5, 1 and 2 h). Immunoblotting for cytoplasmic and nuclear proteins showed that LPS stimulation resulted in obvious P65 nuclear translocation (Figure 5a, b). It is widely accepted that SIRT1-mediated regulation of the inflammatory response mediated by the NF-κB pathway relies on altered acetylation of P65 at lysine 310. Therefore, we examined ace-P65 (Lys310) and found that compared with NC expression, ING4 overexpression restricted the translocation and acetylation of P65 after LPS stimulation.
stimulation (Figure 5a). By contrast, silencing ING4 led to relatively increased P65 nuclear translocation and acetylation (Figure 5b).

DISCUSSION

Sepsis, as a complex disorder, is highly associated with acute organ dysfunction and high lethality and therefore requires urgent and effective treatment based on accurate awareness of the presenting characteristics. The excessive inflammatory response characterized by systemic inflammatory response syndrome is correlated with sepsis. Undoubtedly, it is paramount to find effective therapeutic targets to avoid organ injuries induced by excessive inflammation. In this study, we explored the intrinsic functions of ING4 in inflammation and inflammation-related organ trauma. On the basis of sepsis models established by LPS treatment of macrophages in vitro or in vivo, we found that ING4 expression decreased with LPS treatment and that ING4 transfection led to a significant decrease or increase in the expression of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and monocyte chemoattractant protein 1. Furthermore, in a mouse sepsis model, ING4-overexpressing mice showed hyposensitivity to LPS treatment, reduced pro-inflammatory cytokine IL-1β, TNF-α and IL-6 secretion into the serum and presented alleviated LPS-induced organ injury. ING4 has been described to interact with the P65 subunit of NF-κB and enhance HDAC1–P65 interactions, thus attenuating NF-κB activity. One of the most important histone deacetylase enzymes, SIRT1, might also be a target of ING4 upstream of the NF-κB pathway. Through coimmunoprecipitation experiments with RAW 264.7 macrophages, we first affirmed the endogenous interaction between ING4 and SIRT1 in macrophages. Moreover, ING4-overexpressing RAW 264.7 cells displayed decreased nuclear translocation and acetylation of P65, which might be mediated by enhanced SIRT1 activity.

Our results confirmed the suppressive function of ING4 in regulating LPS-induced innate inflammation in a pro-inflammatory cytokine-dependent manner, which is in agreement with a previous report that ING4 negatively regulates the cytokine-mediated inflammatory response. ING4 is negatively correlated with the expression of nuclear P65 and MMP9. Furthermore, Byron et al. found that in breast cancer cells, ING4 could decrease the expression of NF-κB-targeted genes. In line with these previous reports, our study found that ING4 overexpression in macrophages stimulated with LPS led to significantly decreased nuclear translocation. However, Hou et al. demonstrated that ING4 could induce...
NF-κB/P65 ubiquitination by delivering Lys48-linked polyubiquitin to the Lys62 residue of P65, resulting in NF-κB activation inhibition.\(^{19}\) However, our results showed that after ING4 or shING4 transfection into RAW 264.7 macrophages, no obvious changes in P65 protein levels were observed. This discrepancy might be because in different disease or cell models, NF-κB undergoes multiple postinduction modifications, which would cause pivotal changes in the physical interactions of NF-κB with other NF-κB-related proteins.\(^{34,35}\) Dialectically, the reason why the role of ING4 as an E3 ubiquitin ligase did not appear to affect the LPS-induced inflammatory response requires further exploration.

In this study, we revealed an indirect mechanism through which ING4 regulates the NF-κB pathway via the intermediary SIRT1. We discovered for the first time that ING4 could regulate P65 acetylation by an endogenous interaction with SIRT1 in macrophages, especially during LPS treatment. These results complementarily expanded on previous studies showing that ING4 negatively regulates the NF-κB pathway. Likewise, it is widely accepted that SIRT1 plays a negative role in regulating the process of inflammation by blocking P65 nuclear translocation via P65 deacetylation at the Lys310 residue.\(^{24,26,36}\) In addition, regarding deacetylases such as SIRT1, Li et al. reported that RGFP109, a selective inhibitor of HDAC1 and HDAC3, upregulated ING4 expression and enhanced ING4 recognition and combination with NF-κB/P65.\(^{37}\) However, whether SIRT1 can influence the expression or function of ING4 in LPS-induced inflammation remains unanswered in our study and thus requires further investigation.

In addition, although we discovered that ING4 interacted with SIRT1 and enhanced SIRT1 activity in LPS-induced inflammation, we still have been exploring the reverse regulation of SIRT1 impacting ING4, and the result, whether SIRT1 could directly deacetylate ING4, might be presented in our subsequent research. Furthermore, according to the findings of Zhang et al., the nuclear localization signal of ING4 is essential for its binding to p53.\(^{38}\) In addition, five novel aberrantly spliced variant forms of ING4_v1 and ING4_v2 identified by Li et al. were found to cause nuclear localization signal or PHD domain deletion, which contributed to faulty localization of p53 and/or histone acetyltransferase/histone deacetylase complexes in gastric cancer.\(^{39}\) However, in this study, the specific domain mediating the interaction between the two proteins was not determined. To address this issue, further experiments are required to investigate the underlying mechanisms.

In summary, our results showed that in the LPS-induced inflammatory response, ING4 expression was downregulated in macrophages both in vivo and in vitro. ING4 significantly alleviated excessive inflammation and organ injury by directly binding to the SIRT1 protein, leading to decreased NF-κB P65 acetylation and nuclear translocation. Our research presents novel insight into the ING4/SIRT1/NF-κB axis in LPS-induced sepsis (Figure 6) and shows that ING4 might be a promising target for treating sepsis.

**METHODS**

**Cell line and primary cell culture**

RAW 264.7 cells (ATCC, Manassas, VA, USA; #TIB-71) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (Excell Bio, Shanghai, China; #FSP500). Mouse PMs were isolated from wild-type male C57B6/L mice and cultured in RPMI (Roswell Park Memorial Institute) 1640 medium with 10% fetal bovine serum. After allowing adherence for 2 h, the medium was changed, and the PMs were collected for LPS treatment the next day. For LPS stimulation of both RAW 264.7 cells and PMs, the cells were treated with 1 \(\mu\)g mL\(^{-1}\) LPS for 0.5, 1, 2, 4, 6, 12 or 24 h.

**Mice experiments**

Specific pathogen-free male BALB/C and C57B6/L mice were purchased from the Experimental Animal Centre of the Fourth Military Medical University. All the procedures were carried out in accordance with the ARRIVE principles and approved by the Ethics Committee of the Fourth Military Medical University (No. XJYYLL-2013109). Each BALB/C
mouse was transfected in vivo with 60 μg pcDNA3.1-ING4 or pcDNA3.1-EGFP-C2 (NC) plasmid dissolved in 60 μL Micropolyp-transfecter Tissue Reagent (Micropoly, Nantong, China; #MT215) through caudal vein injection. Three days later, the transfected mice were injected intraperitoneally with 15 mg kg⁻¹ LPS (Sigma Aldrich, Shanghai, China; #L2880) to establish a mouse sepsis model. Serum and tissue samples were collected from BALB/C mice at 6 h after LPS treatment. The survival rate was evaluated for a 5 or 15 mg kg⁻¹ injection of LPS and monitored every 12 h for 72 h.

**Cell counting kit-8 assay**

Cell counting kit-8 (Dalian Meilun Biotechnology, Dalian, China; #MA0218) assays were performed according to the manufacturer’s protocol. Two sets of the repeatedly treated cell groups were seeded into 96-well plates at a density of 10 000 cells per well. The number of viable cells was then measured at a wavelength of 450 nm according to the experimental procedure.

**TUNEL staining**

Cells were fixed with 4% paraformaldehyde for 30 min and then labeled with 20 μL labeling buffer for 2 h at 37°C in a humidified box according to the TUNEL Apoptosis Detection Kit (Bosterbio, Pleasanton, CA, USA; #MK1023) protocol. After incubation with the blocking reagent from the kit for 30 min, the cells were incubated with anti-DIG-biotin for 30 min, incubated with SABC-FITC and mounted with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL, USA; #0100-20). The cells were then observed with an FSX100 microscope (Olympus, Tokyo, Japan).

**Hoechst staining**

After RAW 264.7 cells were treated with or without LPS for 24 h, Hoechst 33342 staining solution (Solarbio, Peking City, China; #C0030) was added to coat the cells. After incubation at 37°C for 20 min, the cells were washed with phosphate-buffered saline for three times and then observed with an FSX100 microscope (Olympus).

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted using TRIzol reagent (Takara, Kusatsu, Japan; #9109). In total, 1000 ng of isolated RNA was reverse transcribed into complementary DNA using a PrimeScript RT Reagent Kit (Takara; #RR036A-1). Quantitative PCR was performed with a CFX Connect real-time system (BIO-RAD, Shanghai, China) using UltraSYBR Mixture (CWBIO, Beijing, China; #CW0957M) and specific primers (Supplementary table 1). The amplification conditions were 95°C and 10 min for pre-denaturation, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Relative expression was calculated by the $2^{-\Delta\Delta C_t}$ method and normalized to the glyceraldehyde 3-phosphate dehydrogenase expression level.

**Immunoprecipitation and western blotting**

Cell pellets were collected by centrifugation at 1000 g and lysed in an IP lysis buffer (Beyotime, Shanghai, China; #P0013) for 1 h. After centrifugation at 13 680 g, the extracts were incubated with an anti-SIRT1 antibody, an anti-ING4 antibody, a normal rabbit IgG antibody or a normal mouse IgG antibody with gentle rotation overnight at 4°C. After incubation with protein A/G magnetic beads (Thermo Scientific, Waltham, MA, USA; #88802) for 4 h, the complexes were washed with phosphate-buffered saline three times and lyse buffer two times, resuspended in a 2× loading buffer (Beyotime; #P0015L), boiled at 100°C for 10 min on heat blocks and then subjected to western blot analysis. The same amount of protein (20–40 μg) was loaded for each sample, separated via sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, #IPVH00010). The membranes were blocked in 5% non-fat milk dissolved in tris-buffered saline–TWEEN 20 for 3 h and then incubated overnight at 4°C with primary antibodies. The next day, the membranes were probed with an horseradish peroxidase–conjugated secondary antibody for 1 h and visualized with chemiluminescence (Millipore, #WBKLS0500). The antibodies used in this study were as follows: anti-SIRT1 (Abcam, Abcam Trading (Shanghai) Company Ltd., Pudong, Shanghai, China; #ab96723), anti-ING4 (Proteintech, Proteintech Group, Inc., Rosemont, IL, USA; #10617-1-AP), anti-P65 (CST; #8242), anti-aceP65 (Lys310; CST; #12629), anti-H2A (Proteintech; #17030-1-AP), anti-α-tubulin (Proteintech; #11224-1-AP) and anti-GAPDH (Bioss, Beijing Biosynthesis Biotechnology Co., Ltd., Peking City, China; #bs-2188R).

**Nuclear and cytoplasmic protein extraction**

After the indicated treatment, cell pellets were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Keygenbio, Nanjing, China; #KGP150/KGP1100), and the extracts were then detected by western blot analysis using the following primary antibodies: anti-P65 (CST, Danvers, MA, USA; #8242), anti-aceP65 (Lys310; CST; #12629), anti-H2A (Proteintech; #17030-1-AP) and anti-α-tubulin (Proteintech; #11224-1-AP).

**Transient transfection**

RAW 264.7 cells were seeded into 6-well plates at 20% confluence. On the following day, the cells were transfected with 2 μg pcDNA3.1-ING4, pLVX-shING4 or NC with X-tremeGENE HP DNA Transfection Reagent (Roche, Shanghai, China; #0636623600). Forty-eight hours later, the cells were stimulated with 1 μg mL⁻¹ LPS to establish an LPS-induced macrophage model.

**Immunofluorescence**

Cells were plated at 10% confluence on glass coverslips. After treatment with or without LPS, the cells adhered to the slides...
were washed with phosphate-buffered saline three times and fixed in 4% paraformaldehyde at room temperature for 15 min. After fixation, Triton X-100 was used to permeabilize the membrane for 15 min. After blocking in 4% bovine serum albumin for 1 h, the cells were incubated with primary antibodies against ING4 and SIRT1 overnight at 4°C, incubated with DyLight 488-conjugated goat anti-rabbit IgG (Abbkine, Wuhan, China; #A23220) and IFKine red-conjugated donkey anti-mouse IgG (Abbkine, #A24411) antibodies for 1 h in the dark and mounted with DAPI Fluoromount-G (Southern Biotech, Birmingham, AL, USA; #0100-20) according to the manufacturer’s instructions. Fluorescence images were acquired using an FSX100 microscope (Olympus).

Hematoxylin–eosin staining

Mouse organ tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4-µm-thick sections. The sections were stained with hematoxylin–eosin and observed and imaged under an FSX100 microscope (Olympus). Five high-magnification images were selected randomly.

Enzyme-linked immunosorbent assay

Mouse blood samples were collected retro-orbitally and centrifuged at 860 g for 20 min. The upper layer of serum was assayed using enzyme-linked immunosorbent assay kits to measure the levels of IL-1β (Bosterbio; #EK0394), IL-6 (Bosterbio; #EK0411) and TNF-α (Bosterbio; #EK0527).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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