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*Cs*NOSIP Reverses NLRP3 Inflammasome Activation Induced by *Cs*ESPs via Inducing iNOS and NO Expression and Inhibiting NF-κB Signaling Pathway in Macrophages

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Abstract

Excretory/secretory products from parasites (ESPs) can act as pathogen-associated molecular patterns (PAMPs) to activate innate immunity. Parasites may achieve immune evasion by modulating the interaction between PAMPs and the nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing three (NLRP3) inflammasome. Previous studies have suggested that some components of ESPs from *Clonorchis sinensis* (*Cs*ESPs) can induce the host's immune responses, but the components that balance immunopathology and maintain chronic infection in chronic *Clonorchis sinensis* (*C. sinensis*) remain unclear. We previously found that the iNOS-interacting protein from *C. sinensis* (*Cs*NOSIP), a component of *Cs*ESP, stimulates macrophages to produce reactive oxygen species (ROS) and nitric oxide (NO), both of which inhibit NLRP3 inflammasome activation. Therefore, this study investigated the effects of *Cs*ESP and *Cs*NOSIP on inflammasome activation using RT-PCR, Western blot, and ELISA. This study showed that *Cs*ESPs promoted NLRP3 inflammasome activation in RAW264.7 cells, while *Cs*NOSIP inhibited LPS-induced IL-1β secretion through an NLRP3-caspase-1-dependent pathway and reversed the *Cs*ESPs-induced activation through the iNOS/NO–NF-κB pathway. These results reveal the antagonistic effects of *Cs*ESPs and *Cs*NOSIP in inflammasome regulation, suggesting that this balance contributes to the regulation of the host's immunity and the promotion of chronic infection of *C. sinensis*, providing potential targets for prevention and treatment.

Keywords: *Clonorchis sinensis*; NLRP3 inflammasome; *Cs*NOSIP; iNOS/NO pathway

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1. Background

Clonorchiasis, a prevalent foodborne parasitic disease caused by *Clonorchis sinensis* (*C. sinensis*), is predominantly endemic in East Asia, affecting countries such as China, South Korea, Thailand, and northern Vietnam. Globally, it is estimated that around 13.24 million

people are currently infected [1–3]. Individuals infected with *C. sinensis* in the early stages typically show no symptoms. However, repeated and chronic infections may eventually lead to various liver- and biliary-related symptoms and complications. Multiple studies have shown that *C. sinensis* continuously release excretory/secretory products (*Cs*ESPs). Some components of *Cs*ESPs can induce the host's immune response, which may be closely associated with the occurrence of cholangitis, liver fibrosis, and cholangiocarcinoma (CCA) [4,5]. However, the specific components of *Cs*ESPs that regulate immune pathology and contribute to the establishment of chronic *C. sinensis* infection remain unidentified.

Molecules in ESPs as pathogen-associated molecular patterns (PAMPs) can activate innate immune responses [6,7]. Recent studies have shown that molecules secreted or presented on the surface of parasites (such as glycolipids, glycoproteins, DNA, and RNA) can activate innate immune responses via Pattern Recognition Receptors (PRRs). For example, the circumsporozoite protein of *Plasmodium falciparum* enhance inflammatory responses in the host [8,9], while glycoproteins released by *Trypanosoma* induce a strong Th1-type immune response [10], thereby strengthening the host's ability to clear parasites [11]. Conversely, the glycosylated antigens of *Schistosoma* shift the host's immune response toward a Th2-type, thereby avoiding excessive Th1-mediated cytotoxicity [12–14]. Similarly, the lipophosphoglycan of *Leishmania* could disrupt macrophage activation, facilitating its intracellular survival [15]. These findings indicate that ESPs have dual roles in parasitic infections: they may assist in parasite clearance or be exploited by parasites for immune evasion and long-term survival.

Recent studies further suggest that parasites might regulate the interaction between PAMPs and the NLRP3 inflammasome to promote immune evasion. For instance, *Leishmania* can evade the host's immune response by targeting NLRP3 inflammasome activation, leading to the suppression of the inflammatory response [16,17]. During chronic infection, *Toxoplasma gondii* products can activate the NLRP3 inflammasome. However, the absence of the inflammasome or any of its components leads to enhanced parasitic growth, suggesting that NLRP3 activation plays a protective role in controlling the infection [18]. However, whether similar mechanisms are involved in *C. sinensis* infections remains unclear. Our previous studies identified the nitrite oxide synthase-interacting protein from *C. sinensis* (*Cs*NOSIP) as being predominantly localized in the gut, eggs, and vitelline glands of *C. sinensis* adults. It showed that the *Cs*NOSIP stimulates macrophages to produce a reactive oxygen species (ROS) and NO, and NO/nitrite oxide synthase (iNOS) has been shown to suppress NLRP3 inflammasome activation through multiple pathways in vitro. Thus, it remains to be determined whether the *Cs*NOSIP facilitates chronic infection by regulating NLRP3 inflammasome activity and the specific mechanisms involved.

The present study aims to investigate the activation of the NLRP3 inflammasome during a *C. sinensis* infection, providing a theoretical basis for understanding the interactions between parasites and their hosts and the mechanisms maintaining long-term homeostasis.

2. Materials and Methods

2.1. Expression and Purification of Recombinant CsNOSIP

A purified *Cs*NOSIP protein and *Cs*ESPs were produced, as previously described [19,20]. Briefly, pET-30a (+)-*Cs*NOSIP was constructed and transformed into *Escherichia coli* (DE3) (Promega, Shanghai, China). Induced by isopropyl-β-D-thiogalactopyranoside (IPTG), the protein was expressed at 30 °C for 3 h in Luria–Bertani medium and purified with a His Bind Purification kit (Novagen, Darmstadt, Germany). The purified *Cs*NOSIP was analyzed by 12% SDS-PAGE, stained with Coomassie brilliant blue G-250, and the final recombinant soluble protein concentration was detected by the bicinchoninic acid (BCA) protein assay kit (Cwbiotech, Beijing, China). The protein was treated by AffinityPak™ Detoxi-Gel™

Endotoxin Removing Gel (Thermo Fisher Scientific, Waltham, MA, USA) to remove the endotoxin. Then, the concentration of *Cs*NOSIP was determined by a BCA protein assay kit again, to avoid the loss of protein during the process of endotoxin elimination.

2.2. Culture and Treatment of Macrophage Cell Line RAW264.7 Cells

RAW264.7 cells are a mouse macrophage-like cell line that is widely used to study immune function, inflammation, and cancer biology. RAW264.7 cells were purchased from Experimental Animal Center of Sun Yat-sen University. RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. Cells were seeded at 5×10^5 cells/well in a 12-well plate or 1×10^6 in a 6-well plate and firstly, primed with LPS from *Escherichia coli* (055: B5) (Sigma-Alrich, Steinheim, Germany) (500 ng/mL) for 3 h. To investigate the effects of *Cs*ESPs and *Cs*NOSIP, LPS-primed RAW264.7 were co-incubated with *Cs*ESPs 10 μg/mL for 12 h or/and different concentrations of *Cs*NOSIP (5, 10, 20 μg/mL) for 6 or 12 h, in the presence or absence of 100nM N-Nitro-L-Arginine Methyl Ester (L-NAME), an inhibitor of iNOS (Sigma-Alrich, Steinheim, Germany). Finally, the NLRP3 inflammasome activator ATP (Sigma-Alrich, Steinheim, Germany) (5 mM) was added for 1 h before the cells were harvested.

2.3. Reverse Transcription and Quantitative Real-Time PCR

Total RNA was isolated from cells by using TRIzol reagent according to the manufacturer's protocol, and cDNA was synthesized using a TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR Kit (TransGen Biotech, Beijing, China). Quantitative real-time PCR were performed to evaluate the transcriptional levels of NLPR3 inflammasome-related genes (NLPR3, ASC, caspase-1, IL-1β, etc.). mRNA levels of the target proteins were calculated against that of β-actin, according to the $2^{-\Delta\Delta Ct}$ method. All experiments were performed independently at least three times and were replicated by independent researchers. Primer sequences are listed in Table 1.

Table 1. Primer sequences for quantitative real-time PCR.

Gene	Forward	Accession Number	Product Size (bp)
β-actin	TCATGAAGTGTGACGTTGACATCCGT TTGCGGTGCACGATGGAGGGGCCGGA	NM_007393.5	188
NLRP3	AGCCTTCCAGGATCCTCTTC CTTGGGCAGCAGTTTCTTTC	NM_145827.3	192
Caspase-1	AGATGGCACATTTCCAGGAC GATCCTCCAGCAGCAACTTC	NM 009807.2	245
ASC	GAAGCTGCTGACAGTGCAAC GCCACAGCTCCAGACTCTTC	NM 023258.4	226
IL-1β	TGTCCTCATCCTGGAAGGTCCACG CGCAGCAGCACATCAACAAGAGC	NM_008361.4	158
iNOS	TCCTCACTGGGACAGCACAGAATG GTGTCATGCAAAATCTCTCCACTGCC	NM_010927.4	214

2.4. ELISA

IL-1β levels in supernatants of cell cultures were measured according to the manufacturer's protocol in ELISA kits (Elabscience, Wuhan, China). The concentration of IL-1β was determined using a standard curve according to the kit instructions. All experiments were performed in triplicate wells and repeated in at least three independent experiments.

For immunofluorescence staining, RAW264.7 cells cultured on slides were fixed with 4% paraformaldehyde for 20 min at room temperature (RT), then washed with PBS and permeabilized in PBS containing 0.2% Triton X-100 for 5 min. The slides were blocked with PBS containing 5% bovine serum albumin for 30 min and incubated with a primary monoclonal antibody against NLRP3 (Cell signaling technology, Boston, MA, USA), caspase-1 p10 (M-20) (Santa Cruz, Santa Cruz, CA, USA) or ASC (ABclonal, Boston, MA, USA) overnight at 4 °C. The slides were then incubated with a Cy3-conjugated or/and FITC conjugated secondary antibody (Proteintech, Rosemont, IL, USA) for 1 h in darkness, and finally stained with 4′,6-diamidino-2-phenylindole (DAPI, SouthernBiotech, Homewood, AL, USA). Images were obtained using a microscope (Olympus BX63) and cell Sens Dimension (Version1.8) software (Olympus, Tokyo, Japan).

2.5. Western Blot Analysis

Protein lysates for immunoblotting from treated cells, as described above, were prepared with RIPA buffer (Cwbiotech, Beijing, China) supplemented with protease inhibitors (Beyotime, Shanghai, China). Protein lysates for Western blotting from treated cells as described above were prepared with RIPA buffer (Cwbiotech, Beijing, China) supplemented with phosphatase inhibitors (Beyotime, Shanghai, China). Cells lysates were denatured in loading buffer containing SDS and 100 mM DTT and boiled for 5 min. Equal amounts of total protein (40 μg) were loaded onto SDS-PAGE-separated gels and then transferred to 0.22 μM PVDF membranes (Millipore, Burlington, MA, USA), followed by incubation in blocking buffer (25 mM Tris, pH 7.4, 0.15 M NaCl, 0.1% Tween-20, 5% nonfat milk) for 1 h at RT. The membranes were incubated with primary antibodies against NLRP3 (ABclonal, Boston, MA, USA), CASP1 (ABclonal, Boston, MA, USA), iNOS (DB Biotech, Košice, Slovak), Phospho-NF-κB p65 (Ser536) (Cell signaling technology, Boston, MA, USA), or GADPH (Ray antibody Biotech, Beijing, China) at 4 °C overnight. After washing, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h on RT. Proteins were visualized with an ECLkit (Advansta, San Jose, CA, USA). GADPH was used as a loading control. All experiments were performed independently at least three times and were replicated by independent researchers. The intensity of Western blotting bands was analyzed with Image J software (NIH, Bethesda, MD, USA, 2022).

2.6. Measurement of NO Level

The Griess reactions were used to detect the accumulation of NO in the culture medium. RAW264.7 cells were treated as previously described and the culture medium was collected to evaluate the NO according to the protocol of the Nitric Oxide Detection Kit (Beyotime, Shanghai, China).

2.7. Statistical Analysis

All data are presented as the mean ± SEM. Data were analyzed by performing independent Student's *t*-tests and ANOVA, followed by a Bonferroni multiple comparisons test using SPSS software for Windows (version 16.0; SPSS, Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. CsESPs Facilitated Activation of NLRP3 Inflammasome in RAW264.7 Cells

IL-1β is a key product of NLRP3 inflammasome activation and plays an important role during numerous inflammatory processes. Compared with the positive group, 10 μg/mL of *Cs*ESPs stimulation increased IL-1β transcription levels ($p < 0.01$, Figure 1a) in 500 ng/mL lipopolysaccharide (LPS)-primed RAW264.7 cells for 12 h, followed by treatment with 5 mM

ATP (an activator of the NLRP3 inflammasome). Consistent with its facilitation effect on IL-1β, *Cs*ESPs could facilitate the immunofluorescence intensity of NLRP3 inflammasome-related components—NLRP3 and caspase-1, but not ASC (Figure 1b).

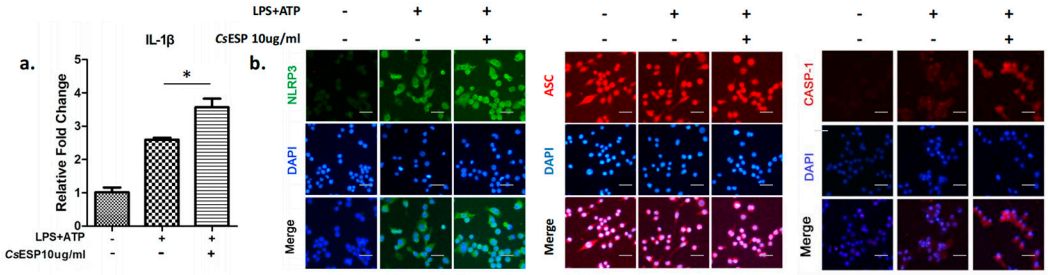


Figure 1. The activation of NLRP3 inflammasome in the treated cells. A total of 500 ng/mL of LPS-primed RAW264.7 cells were treated with 10 μg/mL *Cs*ESPs for 12 h, followed by treatment with 5 mM ATP for 1 h. (**a**) Quantitative real-time PCR analysis of IL-1β levels. Data are shown as mean ± SEM. * $p < 0.01$. (**b**) Immunofluorescence staining analysis. NLRP3 (green), ASC (red) and Caspase-1 (red). Nuclei were stained with DAPI (blue). The images were magnified at 200× (scale bar: 25 μm).

3.2. CsNOSIP Suppressed NLRP3 Inflammasome Mediated IL-1β Secretion in LPS-Primed RAW264.7 via NLRP3- and Caspase-1-Dependent Manner

*Cs*NOSIP dramatically inhibited transcription levels of IL-1β in LPS-primed RAW264.7 cells in a dose-dependent manner, whether treating cells for 6 h ($p < 0.01$, Figure 2a) or 12 h ($p < 0.001$, Figure 2a). Consistent with the results at the mRNA levels, the expression of IL-1β in the culture supernatants ($p < 0.0001$, Figure 2b) and the total cell lysates (Figure 2c) were also decreased. However, *Cs*NOSIP only suppressed the transcriptional levels of NLRP3 in a dose-dependent manner at the 6 h ($p < 0.001$) and 12 h ($p < 0.001$) treating points, but this had no effect on those of caspase-1 and ASC (Figure 2d–f). The protein levels of NLRP3 in stimulated RAW264.7 cells were consistent with Figure 2d (Figure 2g). However, although *Cs*NOSIP had no inhibitory effect on caspase-1, a high concentration (20 μg/mL) of *Cs*NOSIP could dramatically suppress the expression of cleaved caspase-1 (p20) (Figure 2h), a regulating component of inflammasome to facilitate the secretion of IL-1β.

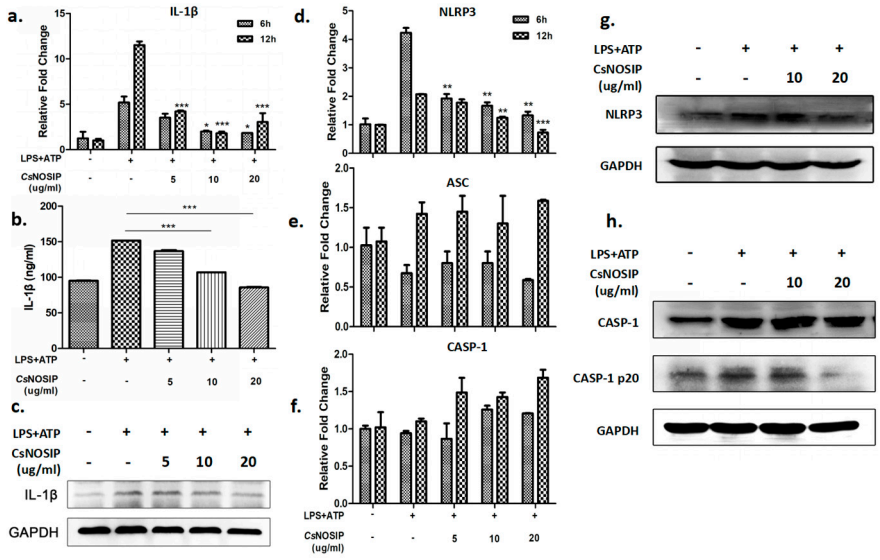


Figure 2. *Cs*NOSI-inhibited NLRP3 inflammasome mediated caspase-1 activation and IL-1β secretion. A total of 500 ng/mL of LPS-primed RAW264.7 cells were treated with different concentration

of *Cs*NOSIP (5, 10, 20 μg/mL) for 6 h and 12 h, followed by treatment with 5 mM ATP for 1 h. (**a**) Quantitative real-time PCR analysis of IL-1β expression. ELISA detection (**b**) and Western blotting (**c**) analysis of IL-1β expression in culture supernatant of treated cells for 12 h. (**d**–**f**) Quantitative real-time PCR analysis of mRNA levels of NLRP3 inflammasome-related genes (NLRP3, ASC, caspase-1) in the treated cells. Western blotting analysis of NLRP3 (**g**), caspase-1 and caspase-1 p20 (**h**) in 12 h treated cells. In panel (**a**,**b**,**d**–**f**), the levels were, respectively, compared to those in LPS+ATP-alone-treated positive control cells. Data are shown as mean ± SEM. In panel (**d**,**g**,**h**), GAPDH was a loading control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. CsNOSIP Reversed the Facilitation Effect of NLRP3 Inflammasome Activation Induced by CsESPs in LPS Primed-RAW264.7

The immunofluorescence staining results showed that, when compared with *Cs*NOSIP treatment alone, the immunofluorescence intensity of NLRP3 and caspase-1 dramatically decreased in LPS-primed RAW264.7 cells treated with *Cs*ESPs and *Cs*NOSIP in response to ATP treatment (Figure 3).

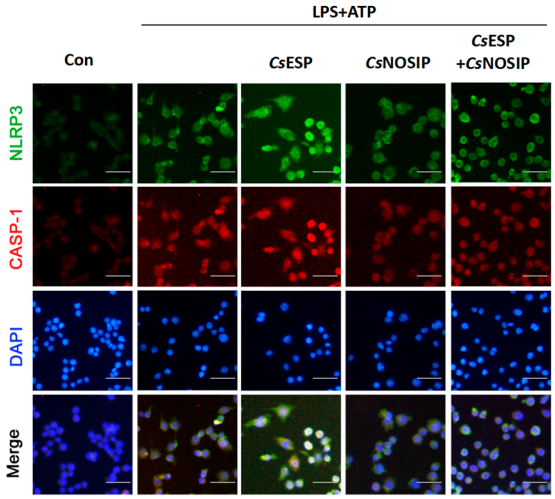


Figure 3. *Cs*NOSIP dampened the NLRP3 inflammasome activation induced by *Cs*ESPs in RAW264.7 cell. Immunofluorescence staining analysis of NLRP3 (green) and total caspase-1 (red) expression in 500 ng/mL LPS primed-RAW264.7 cells treated with 10 μg/mL *Cs*ESPs or/and 20 μg/mL *Cs*NOSIP for 12 h followed by treatment with 5mM ATP for 1 h. Nuclei were stained with DAPI (blue). The images were magnified at 200× (scale bar: 25 μm).

3.4. Cellular Mechanisms Involved in CsNOSI-Mediated Reverse Effect on NLRP3 Inflammasome Activation Induced by CsESPs

Upon activation with LPS and ATP, the NO secretion was significantly increased when the RAW264.7 cells were additionally co-cultured with *Cs*NOSIP for 6 h ($p < 0.001$, Figure 4a) and 12 h ($p < 0.001$, Figure 4a). The effect was accentuated with increasing doses of *Cs*NOSIP and longer stimulation time. *Cs*NOSIP stimulation facilitated the expression of iNOS in LPS-primed RAW264.7 cells in a concentration-dependent manner at different time points—6 h ($p < 0.001$) and 12 h ($p < 0.001$) (Figure 4b,c)—in both transcription and translation levels. The *Cs*NOSIP prevention effect was reversed in the presence of L-NAME, an inhibitor of iNOS (Figure 4e). Furthermore, NF-κB p65 expression was suppressed in the stimulated RAW264.7 cells in a dose-dependent manner (Figure 4d).

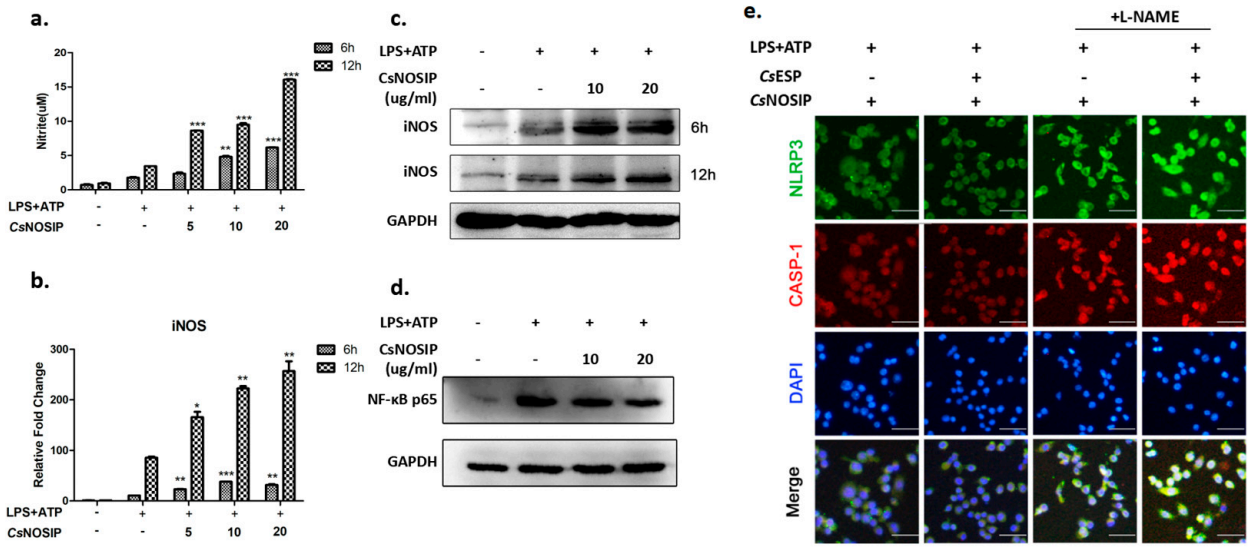


Figure 4. The expression of iNOS, NO and NF-κB in treated cells. A total of 500 ng/mL LPS primed-RAW264.7 cells were treated with different concentrations of *Cs*NOSIP (5, 10, 20 μg/mL) for 6 h and 12 h, followed by treatment with 5mM ATP for 1 h. (**a**) Griess reaction analysis of NO expression in culture supernatant of the cells. Data are shown as mean ± SEM. Quantitative real-time PCR (**b**) and Western blotting (**c**) analysis of iNOS expression levels were compared to that in positive control. Data are shown as mean ± SEM. (**d**) Western blotting analysis of NF-κB p65 expression. The GAPDH was a loading control for Western blotting. (**e**) Immunofluorescence staining analysis of NLRP3 (green) and total caspase-1 expression in 500 ng/mL LPS-primed RAW264.7 cells treated with *Cs*ESPs (10 μg/mL) or/and *Cs*NOSIP (20 μg/mL) for 12 h, followed by treatment with 5 mM ATP for 1 h in the presence or absence of 100 uM L-NAME. Nuclei were stained with DAPI (blue). The images were magnified at 200× (scale bar: 25 μm). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

The inflammasome is a type of intracellular multi-protein complex and an important component of the innate immune system [21]. Among them, NLRP3 inflammasome is the most extensively studied, and plays a central role in regulating the inflammatory response during the host's defense against various pathogens [17]. In the host's immune response to infection, inflammation must be balanced within an appropriate range: excessive inflammation can lead to cell and tissue damage in the host, while insufficient inflammation may provide favorable conditions for pathogen survival and spread.

As an important secretion of the parasite that directly interacts with the host, ESP plays a crucial role in the interaction between the parasite and the host. *Cs*ESPs is also involved in liver damage and the development of cholangiocarcinoma (CCA) triggered by *C. sinensis* in the bile ducts [22]. In the present study, *Cs*ESPs could induce the activation of the NLRP3 inflammasome in vitro (Figure 1). In the endemic area, chronic infections of *C. sinensis* are more common. This suggests that *Cs*ESPs might regulate the host's inflammatory response by activating the NLRP3 inflammasome during the chronic infection.

Activation of the NLRP3 inflammasome triggered by PAMPs from pathogens plays an important role in the immune defense against parasitic infections [23]. Related reports have indicated that in some parasitic infections, activation of the NLRP3 inflammasome may have dual effects. For example, in *Trypanosoma cruzi* [24] and *Leishmania* [25] infections, activation of the NLRP3 inflammasome helps control the parasite load and reduce tissue damage in the host. However, in malaria, excessive activation of the NLRP3 inflammasome may exacerbate disease severity and decrease the likelihood of host survival [26,27]. Moreover, in *Leishmania* infections, NLRP3 inflammasome activation not only promotes the maturation and secretion of inflammation-related factors, such as IL-1β and IL-18, but may also enhance

local inflammation through the secretion of interferon-γ (IFN-γ), thus helping to clear the infection [28]. However, excessive activation of the inflammasome can also lead to tissue damage and inflammatory diseases. Studies have found that overactivation of NLRP3 limits the spread of *Toxoplasma gondii* within the host, but by regulating this pathway, *T. gondii* can persist long term within the host while avoiding fatal pathological responses [29,30].

These findings suggest that the role of the NLRP3 inflammasome in parasitic infections is complex. On the one hand, it plays a protective role in the host's immune response; on the other hand, dysregulated activation may suppress immune clearance and delay the host's response to the parasite. *C. sinensis* adults can keep living in the host's body long term: even for 20–30 years. *Cs*ESPs activated the NLRP3 inflammasome, raising the possibility that there may be molecules that could maintain a stable parasitic environment to balance the immune response activated by NLRP3 inflammasome.

*Cs*ESPs consist of various substances secreted or excreted at different stages of the parasite's life cycle, including enzymes, toxins, and other functional molecules with different functions during the interaction between the worm and the host [20]. Among them, calcium-binding protein [31] and alkaline phosphatase [32] enhance the function of the host's immune cells, promoting the immune response and helping to eliminate the parasite. In contrast, trypsin, elastase, and matrix metalloproteinases [20,33] degrade the host's tissues, assisting the parasite in migrating, parasitizing, and developing within the host.

Research data suggest that an increase in NO generation stabilized intracellular potassium levels and inhibits the activity of NLRP3 [34]. Typically, a high expression of iNOS is accompanied by the release of large amounts of NO [35,36]. Our previous studies have confirmed that *Cs*NOSIP could stimulate macrophages to produce ROS and NO in vitro [37]. *Cs*NOSIP predominantly localized in the gut and vitelline glands of *C. sinensis* adults [37]. *Cs*NOSIP could appear in *Cs*ESPs and act as a molecule playing a role in the interaction between the worm and host, accompanied by renewal or degradation of the worm. Thus, we proposed that *Cs*NOSIP might also have an inhibitory effect on NLRP3 activation. The activation of the NLRP3 inflammasome depends on the oligomerization of ASC and activation of Caspase-1, with IL-1β being a hallmark product of NLRP3 inflammasome activation, whose secretion relies on Caspase-1 cleavage [38]. Our data showed that *Cs*SONIP significantly reduced the expression of ASC, IL-1β, and Caspase-1 when stimulating macrophages in vitro, with a concentration-dependent effect (Figure 2), suggesting that *Cs*SONIP interferes with the formation of the NLRP3 inflammasome.

Since *C. sinensis* may persist in the host for 20–30 years, it raises the question of whether the interference of *Cs*SONIP with NLRP3 inflammasome activation contributes to the long-term chronic infection. We found that when *Cs*SONIP and *Cs*ESPs were co-stimulated in macrophages, *Cs*SONIP attenuated the activation effect of *Cs*ESPs on the inflammasome (Figure 3). It suggested that *Cs*SONIP might play a negative feedback regulatory role, preventing excessive activation of the inflammasome. We hypothesized that during a *C. sinensis* infection, when inflammasome activation reached a certain threshold, *Cs*SONIP might suppress NLRP3 activation to protect the parasite from the host's immune responses, ensuring its long-term chronic infection.

Moreover, similar interactions have been observed in other parasitic infections. For instance, *Toxoplasma gondii* secretes effector molecules such as GRA15, which activate NF-κB to upregulate NLRP3 expression [30], but ROP16 limits excessive activation of NLRP3 by inhibiting ROS generation [29,39]. Eggs or adult worm's antigens from *Schistosoma* or adult worms secrete glycoproteins that trigger the initial activation of NLRP3 inflammasome through the toll-like receptor four and other pathways, while also limiting the complete activation of NLRP3 by inhibiting potassium ion efflux and ROS generation [40]. These findings suggest that during parasitic infections, such interactions can trigger mild inflam-

mation that helps maintain host survival, while avoiding excessive inflammation that could lead to pathogen clearance.

The mechanism by which *Cs*SONIP weakened the activation of the NLRP3 inflammasome in a *C. sinensis* infection deserves further investigation. The activation of the NLRP3 inflammasome typically requires initial activation of the NF-κB signaling pathway [41], and NO has been shown to inhibit NF-κB activity through multiple mechanisms, including the suppression of IκB kinase activation [42]. Research also shows that inhibition of the NF-κB pathway reduces the expression levels of NLRP3 and its downstream effectors, thereby reducing the inflammatory response [43]. Our data also indicate that *Cs*SONIP significantly inhibited the expression of NF-κB in macrophages, suggesting that *Cs*SONIP might suppress NLRP3 activation by inhibiting NF-κB activity (Figure 4). This further implied that *C. sinensis* might utilize this strategy to suppress the host's immune killing effects, establish chronic infection, and maintain long-term survival.

In summary, our findings indicate that *Cs*ESP, as a major pathogenic component, promotes NLRP3 inflammasome activation and IL-1β secretion in LPS-primed RAW264.7 cells. We also uncovered that *Cs*NOSIP could suppress the NLRP3 inflammasome activation and IL-1β secretion, which was probably through upregulating the expression of iNOS and NO and downregulating the NF-κB signaling pathway. All these results indicated that *Cs*NOSIP was attributable, at least in part, to the inhibition of inflammatory responses induced by a *C. sinensis* infection, which in turn, conferred the chronicity of *C. sinensis* infections. However, further studies should be investigated in vivo models to validate our in vitro data.

Thus, the opposing effects of *Cs*ESPs and *Cs*SONIP on the NLRP3 inflammasome during a *C. sinensis* infection help the parasite persist long-term within the host, but it was well worth investigating the involved mechanisms of how the worm regulates or renews the generation of *Cs*SONIP in *Cs*ESPs, and then initiates a system of checks and balances related to activating the NLRP3 inflammasome. This complex immune regulation mechanism not only helps the pathogen survive but may also provide potential targets for parasitic infection research and the design of immunotherapies.

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