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Short communication

Inflammasome activation restrains the intracellular *Neospora caninum* proliferation in bovine macrophages

Xiaocen Wang[a,1], Pengtao Gong[a,1], Nan Zhang[a], Lu Li[a], Sining Chen[a], Lijun Jia[b], Xianyong Liu[c], Jianhua Li[a,*], Xichen Zhang[a,*]

[a] Key Laboratory of Zoonosis Research by Ministry of Education, Institute of Zoonosis, College of Veterinary Medicine, Jilin University, Changchun, 130062, China
[b] Laboratory of Veterinary Microbiology, Department of Veterinary Medicine, Yanbian University, Yanji, 133002, China
[c] National Animal Protozoa Laboratory, Key Laboratory of Animal Epidemiology of The Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing, 100083, China

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ABSTRACT

Neospora caninum is an intracellular parasite that causes neosporosis in cattle. Bovine neosporosis is considered a major cause of bovine abortion worldwide. Rapid replication of *N. caninum* tachyzoites within host cells is responsible for the acute phase of *N. caninum* infection. Evidence shows that the host immune response plays an essential role in recognizing and regulating the replication of invading pathogens. Nucleotide-binding oligomerization domain receptors (NLRs) are a class of cytoplasmic sensors that can sense pathogens and induce the formation of the inflammasome complex. Activation of the inflammasome promotes restriction of microbial replication. Our previous study revealed NLRP3 inflammasome activation in *N. caninum*-infected murine macrophages. However, the role of inflammasome activity in *N. caninum*-infected bovine cells is unknown. To address this question, a bovine peritoneal macrophage cell line was used to investigate the role of inflammasome activation in regulating intracellular *N. caninum* replication. The results showed that inflammasome mediated activation of caspase-1 occurs in *N. caninum*-infected bovine macrophages, and caspase-1-dependent cell death was considered to be induced in *N. caninum*-infected bovine macrophages because *N. caninum* induced cell death decreased following pretreatment with zVAD-fmk and VX765. Meanwhile, the inhibition of caspase-1 in *N. caninum*-infected bovine macrophages led to the presence of more parasites in the parasitophorous vacuole. In contrast, inflammasome activation induced by ATP treatment in *N. caninum*-infected bovine macrophages contributed to the clearance of *N. caninum*. In addition, pyroptotic cell supernatant collected from ATP-stimulated bovine macrophages also impaired the ability of this parasite to infect new cells. In conclusion, this study is the first report on the role of the bovine inflammasome in restraining intracellular *N. caninum* replication and suggests that the bovine inflammasome may be a potential target for future development of drugs or vaccines against *N. caninum* infection in cattle.

1. Introduction

Neospora caninum belongs to the phylum Apicomplexa and is a tissue cyst-forming parasite that causes neosporosis especially in both dairy and beef cattle. Bovine neosporosis mainly leads to reproductive failure and has a global distribution and causes significant economic losses (Guido et al., 2016). However, treatment or vaccine against this disease in cattle is not currently available (Horcajo et al., 2016). Knowledge of the immune response during bovine neosporosis could improve the control of this disease (Innes et al., 2002). When *N. caninum* invades the host, the innate immune response plays a critical role in initial recognition and elimination of this pathogen, as well as mediating the appropriate adaptive immune response against infection (Brake, 2002). The utilization of these immune-relevant molecules can be important for vaccine control strategies (Brake, 2002; Innes et al., 2002).

The inflammasome is a cytosolic multiprotein complex that can assemble during detecting infection (Gurung and Kanneganti, 2016). Activation of the inflammasome mediates caspase-1-dependent inflammatory responses: maturation of the proinflammatory cytokines IL-1β and IL-18 and rapid cell death termed pyroptosis (Jo et al., 2016). These responses facilitate the restriction of pathogen replication and

* Corresponding authors.
E-mail addresses: jianhuali7207@163.com (J. Li), xczhang@jlu.edu.cn (X. Zhang).
[1] Equal contributors.

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mediate the host defense and the adaptive immune response (Evavold and Kagan, 2018). During *Toxoplasma gondii* infection, NLRP1 or NLRP3 inflammasomes can be activated in human, mouse or rat macrophages. These inflammasome responses are protective to the host by restricting parasites and triggering host defense (Cavailles et al., 2014; Cirelli et al., 2014; Ewald et al., 2014; Gorfu et al., 2014). Furthermore, P2X 7-mediated NLRP3 inflammasome activity not only restrains the growth of *T. gondii* in macrophages by extracellular ATP treatment (Moreira-Souza et al., 2017) but also inhibits *T. gondii* proliferation in small intestinal epithelial cells (Quan et al., 2018). Our previous study showed that in murine peritoneal macrophages, *N. caninum* could induce NLRP3 inflammasome activation (Wang et al., 2017), and this inflammasome response was essential to promote host defenses against *N. caninum* infection (Wang et al., 2018). However, the role of the bovine inflammasome in controlling intracellular *N. caninum* needs to be explored.

2. Materials and methods

2.1. Parasites and cells

N. caninum Nc-1 tachyzoites and Nc-GFP (Nc-1 strain) tachyzoites were maintained in Vero cells as previously described (Wang et al., 2017). A bovine peritoneal macrophage cell line was kindly provided by Professor Aizhen Guo from Huazhong Agricultural University, Wuhan, China, and cultured as described previously (Stabel and Stabel, 1995). Our previous study showed that *N. caninum* can induce extracellular traps when exposed to bovine macrophages (Wei et al., 2018).

2.2. Infection assays

Bovine macrophages were seeded in 12-well plates or in 96-well plates at 5×10^5 or 8×10^4 cells/well, respectively. Following LPS (200 ng/ml; Sigma, Shanghai, China) treatment for 2 h, cells were washed twice with PBS to remove the LPS. Then, the cells were stimulated with ATP (5 mM; Sigma, Shanghai, China) for 30 min as a positive control or infected with Nc-1 or Nc-GFP tachyzoites at a multiplicity of infection (MOI = 3 or 1; parasite: cell) for 2 h, and non-attached parasites were removed by PBS. Finally, infected cells were incubated for the indicated times.

To verify inflammasome activation in *N. caninum* infection, bovine macrophages were pretreated with 50 μM VX-765 (an inhibitor of caspase-1 and -4; Selleck, Shanghai, China) and 50 μM zVAD-fmk (an inhibitor of pan-caspase; Selleck, Shanghai, China) for 1 h, then cells were washed twice with PBS before stimulation. When required, *N. caninum*-infected bovine macrophages were incubated with 5 mM ATP for 30 min.

To explore the effect of the pyroptotic cell supernatant on *N. caninum*, pyroptotic cell supernatant was collected from ATP-stimulated bovine macrophages, and supernatant from negative control bovine macrophages was also acquired. A total of 1×10^6 purified Nc-1 tachyzoites were incubated with both kinds of supernatants at 37 °C for 1 h respectively, and then used to infect fresh bovine macrophages at a MOI = 1. Extracellular parasites were removed with PBS washes after 2 h, and the infected cells were maintained at 37 °C until 24 h post infection (p.i.).

2.3. Detection of active caspase-1

With or without LPS (200 ng/ml) pretreatment, bovine macrophages were infected with Nc-1 tachyzoites (MOI = 3) for 3 h, or stimulated with 5 mM ATP for 30 min. Active caspase-1 in bovine macrophages was detected using a FAM-FLICA Caspase-1 Assay kit (ImmunoChemistry Technologies, Bloomington, USA) and analyzed by confocal microscopy according to the manufacturer's instructions.

2.4. Quantitative real-time PCR (qPCR)

Parasite load in infected cells was measured by qPCR as previously described (Wang et al., 2017), and 200 ng of total DNA from infected cells was used as a template in qPCR analyses.

2.5. Cell viability assay

Cell viability of infected bovine macrophages was assessed using a TransDetect Cell Counting kit (Trans, Beijing, China) according to the manufacturer's instructions.

2.6. Replication assay

For light microscopy, Nc-1 tachyzoite-infected bovine macrophages (MOI = 1) on the glass coverslips were fixed with methanol for 1 min and stained with a Wright-Giemsa Stain kit (Baso, Zhuhai, China) according to the manufacturer's instructions. The number of parasites in each vacuole was counted, and at least 100 vacuoles were counted in each group.

For confocal microscopy, Nc-GFP tachyzoite-infected cells were fixed with 4% paraformaldehyde for 10 min and then stained with TRITC-phalloidin (YEASEN, Shanghai, China) for F-actin and DAPI (Thermo, Rockford, USA) for mammalian nuclei according to the manufacturer's instructions. The cells were analyzed on an Olympus FV1000 Laser Scanning Confocal microscope (Japan) with a 100× objective.

2.7. Statistics

Data analysis was performed using Prism 5.0 (GraphPad Software, Inc.) and data were expressed as the means ± SEM. To evaluate the differences between two groups, the two-tailed *t*-test was used, or data from multiple groups were analyzed using ANOVA test. Significance is shown by $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

3. Results and discussion

To investigate inflammasome activation caused by *N. caninum* infection in bovine macrophages, active caspase-1 was detected. When caspase-1 is cleaved, active caspase-1 is released into the supernatant, and the most reliable results of active caspase-1 detection are assessed by western blot. However, during this study, available commercial antibodies or reagents used to detect bovine molecules were limited. Therefore, caspase-1 activity in this study was measured with a fluorescent peptide that specifically binds activated caspase-1 within cells as previously described (Wang et al., 2014). Although may fail to detect active caspase-1 released from dead cells with a lytic form, this method can detect active caspase-1 in live cells and then can indicate the activation of caspase-1. When compared with the negative control, caspase-1-activated bovine macrophages were observed in the ATP treatment group, as well as in both *N. caninum* infection groups for 3 h with or without LPS pretreatment (Fig. 1A). Caspase-1 can induce rapid cell death termed pyroptosis, so the cell viability of bovine macrophages was measured. The results showed that the viability of bovine macrophages was not altered with LPS treatment but was significantly reduced with ATP treatment, and was also greatly decreased in both *N. caninum* infection groups for 3 h with or without LPS pretreatment. Notably, with LPS pretreatment, the cell viability of the *N. caninum*-infected bovine macrophages was lower than that of the bovine macrophages infected with *N. caninum* infection alone (Fig. 1B). This indicates that the cell death of *N. caninum*-infected bovine macrophages primed with LPS was more obvious, and this obvious cell death was thought to be mediated by caspase-1. To further confirm caspase-1 activation in *N. caninum*-infected bovine macrophages at 3 h p.i., inhibitors of pan-caspase and caspase-1 were used to detect cell viability.

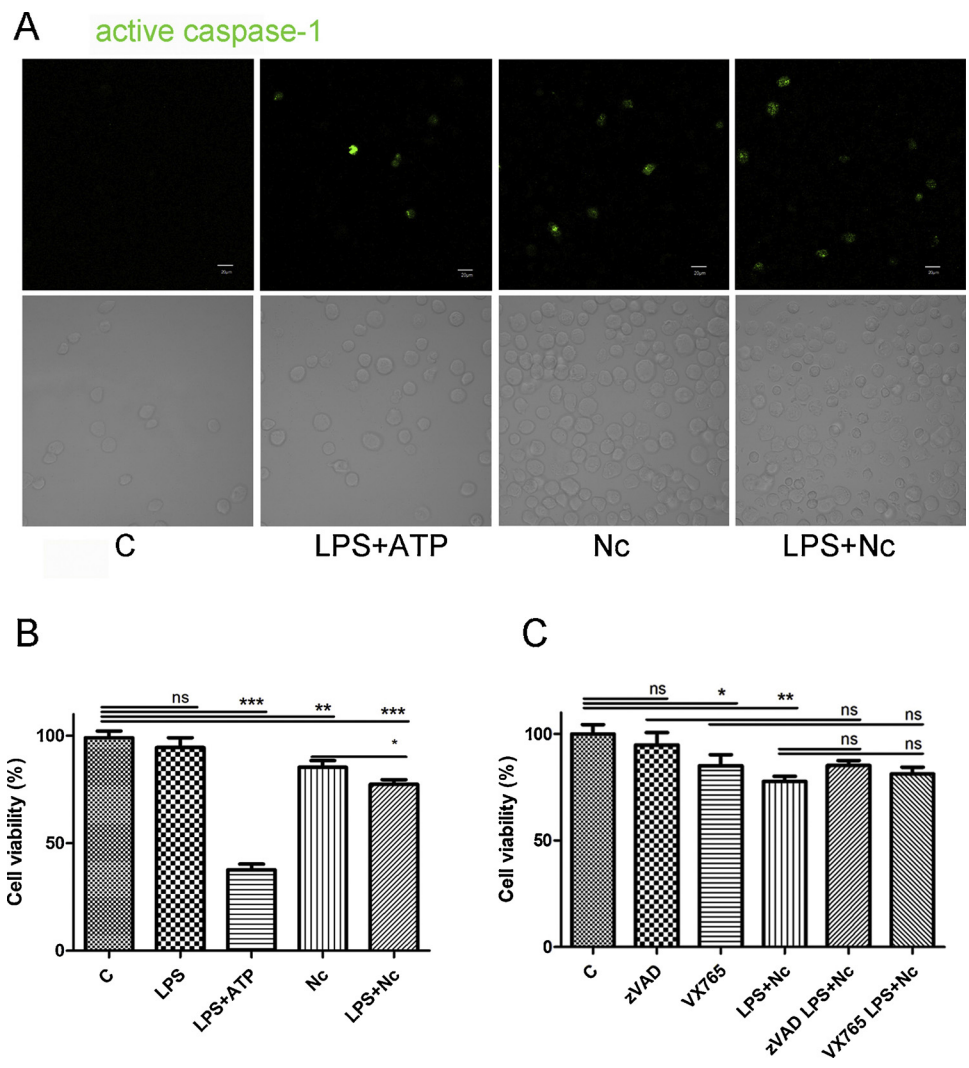

Fig. 1. Caspase-1 participates in *N. caninum*-induced cell death in bovine macrophages.With or without LPS (200 ng/ml) pretreatment bovine macrophages were infected with *N. caninum* (MOI = 3) for 3 h, or stimulated with 5 mM ATP for 30 min or treated with LPS alone. **A** Active caspase-1 was detected using a FAM-FLICA Caspase-1 Assay kit and analyzed by confocal microscopy. **B** Cell viability of infected bovine macrophages was detected using TransDetect Cell Counting kit. **C** Following pretreatment with 50 μM pan-caspase inhibitor zVAD-fmk (zVAD) or 50 μM caspase-1 inhibitor VX765, cell viability of infected bovine macrophages was detected. C, control; MOI, multiplicity of infection (parasite: cell); ns, not significant. The data are representative of three independent experiments and are presented as the means ± SEM. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) *Scale bars*: 20 μm.

The obvious cell death in infected bovine macrophages primed with LPS was thought to be inhibited following the pretreatment with pan-caspase and caspase-1 inhibitors, because *N. caninum* did not decrease the viability of the pan-caspase inhibitor zVAD or caspase-1 inhibitor VX765-pretreated bovine macrophages compared with that of the zVAD- or VX765-treated cells (Fig. 1C). These results indicate that inflammasome activation can be induced and mediate caspase-1-dependent cell death in *N. caninum*-infected bovine macrophages.

Cleavage of caspase-1 indicates activation of the inflammasome, and inflammasome activation requires two signals. The first signal can be provided experimentally by LPS, and the second signal can be provided by stimuli. LPS alone fails to stimulate caspase-1 activation in murine macrophages (Wang et al., 2017) and in bovine monocytes (Hussen et al., 2012). As a second stimulus, *N. caninum* could induce inflammasome activation in bovine macrophages in this study, and these results were similar to those obtained in murine macrophages (Wang et al., 2017). Caspase-1-dependent cell death is called pyroptosis. This programmed cell death is regarded as proinflammatory, is characterized by rapid loss of cell membrane integrity and release of cytosolic contents and is one of the efficient mechanisms of pathogen clearance deployed by the innate immune system (Miao et al., 2010).

To test the role of the inflammasome in controlling parasite replication, bovine macrophages were pretreated with inhibitors of pan-caspase and caspase-1 and then infected with *N. caninum* Nc-1 or Nc-GFP tachyzoites (MOI = 1) for 24 h. In addition, ATP treatment is able to activate the NLRP3 inflammasome in infected cells (Moreira-Souza et al., 2017), and *N. caninum*-infected cells were stimulated by ATP treatment. Because *N. caninum* can induce obvious caspase-1-dependent cell death in LPS-pretreated bovine macrophages (Fig. 1B), *N. caninum*-infected bovine macrophages in the replication assay were pretreated with LPS. When compared with Nc-GFP-infected cells, more parasites in vacuoles were observed in bovine macrophages pretreated with pan-caspase and caspase-1 inhibitors, while reduced parasites were discovered in bovine macrophages treated with ATP by confocal microscopy (Fig. 2A). To further demonstrate the results of confocal microscopy, light microscopy-based quantification of parasites in each vacuole of Nc-1-infected cells was measured. The results showed that the percentage of infected cells was greatly increased in the groups pretreated with pan-caspase and caspase-1 inhibitors but decreased in the group treated with ATP (Fig. 2B). Intracellular replication of Nc-1 tachyzoites was measured by counting the parasite number in each vacuole. The results showed that pretreatment with pan-caspase and caspase-1 inhibitors increased the parasite load in bovine macrophages while treatment with ATP decreased the parasite load (Fig. 2C). Furthermore, the number of *N. caninum* in cells was measured by qPCR, and the results also showed that parasite loads in the groups pretreated with pan-caspase or caspase-1 inhibitors were greatly increased but significantly decreased in the ATP treatment group (Fig. 2D). *N. caninum* proliferation of both Nc-1 and Nc-GFP in HFF cells showed no differences (Ma et al., 2017). These data suggest that activation of the NLRP3 inflammasome induced by ATP in infected cells contributes to the clearance of *N. caninum* and indicate that the inflammasome plays

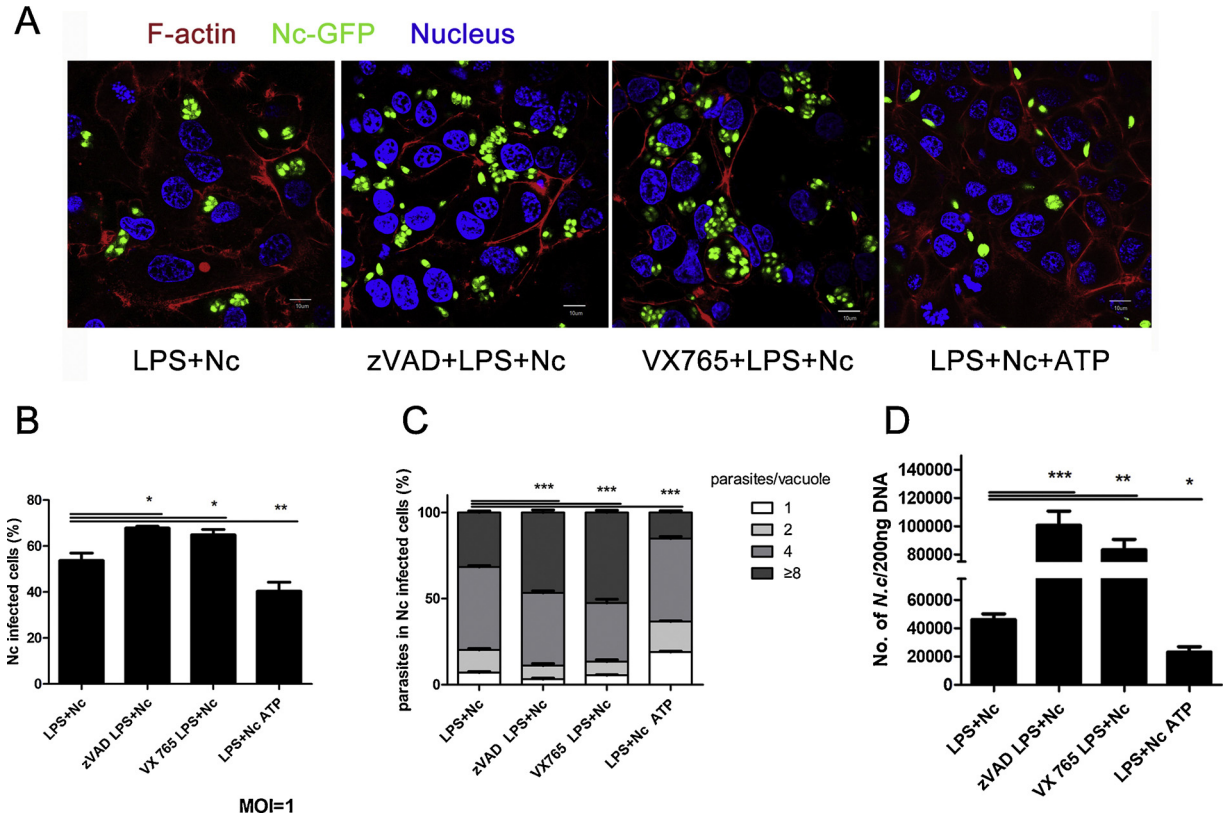

Fig. 2. Role of the inflammasome in regulating intracellular *N. caninum* replication.
After pretreatment with the pan-caspase inhibitor zVAD-fmk (zVAD) or caspase-1 inhibitor VX765, the LPS-pretreated bovine macrophages were infected with *N. caninum* Nc-GFP or Nc-1 tachyzoites (MOI = 1) for 24 h. In the ATP (an inducer of NLRP3 inflammasome) treatment group, LPS-pretreated bovine macrophages were infected with parasites for 2 h, treated with 5 mM ATP for 30 min, then washed with PBS to remove ATP and finally incubated for a total of 24 h. **A** Bovine macrophages infected with Nc-GFP tachyzoites were stained with TRITC-phalloidin (F-actin) and DAPI (Nucleus) for confocal microscopy observation. Nc-1 tachyzoite-infected bovine macrophages were stained with Giemsa, and then the percentage of infected cells (**B**) and quantification of parasites in each parasitophorous vacuole (**C**) were estimated by light microscopy. **D** The number of Nc-1 tachyzoites in infected bovine macrophages was determined by qPCR. MOI, multiplicity of infection (parasite: cell). The data are representative of two independent experiments and are presented as the means ± SEM. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) *Scale bars*: 10 μm.

an important role in eliminating *N. caninum*. We inferred that release of *N. caninum* into the extracellular space was forced when cells underwent pyroptosis; consequently, the released *N. caninum* must invade other cells in order to replicate, so this process may delay the rapid replication of *N. caninum* within cells. In addition, these released pathogens from pyroptotic cells could also be killed by neutrophils in the host (Miao et al., 2010). Similarly, another study showed that *T. gondii* growth in macrophages can be inhibited after treatment with ATP (Moreira-Souza et al., 2017). Recent studies have revealed that pyroptosis is directly mediated by gasdermin D (GSDMD) (Shi et al., 2015). Further studies have also shown that pyroptotic cell supernatant, including the N-terminal cleavage product of GSDMD, has a direct bactericidal effect (Liu et al., 2016).

Pyroptotic cells (such as those induced by ATP treatment) can release cytosolic contents into the surrounding media, and we next explored whether pyroptotic cell supernatant also contributed to the clearance of *N. caninum*. Nc-1 tachyzoites were incubated with pyroptotic cell supernatant from ATP-stimulated bovine macrophages (termed ATP sn in Fig. 3) or supernatant from bovine macrophages and then used to infect fresh bovine macrophages for 24 h. The parasite load in bovine macrophages was detected by qPCR, and the results showed that pretreatment with pyroptotic cell supernatant (ATP sn in the figure) can significantly reduce the number of intracellular *N. caninum* (Fig. 3A). We next determined whether pyroptotic cell supernatant impaired the ability of *N. caninum* to infect or replicate in bovine macrophages. The results revealed that pyroptotic cell supernatant mainly reduced the percentage of infected cells (Fig. 3B) but did not alter the proliferation ability of *N. caninum* in bovine macrophages (Fig. 3C). These data indicate that pyroptotic cell supernatant released from bovine macrophages can promote the clearance of *N. caninum* by impairing the ability of this parasite to infect new cells after inflammasome activation. Furthermore, these data indicate that pyroptotic cell supernatant not only has an antibacterial effect (Liu et al., 2016) but also exerts an anti-parasitic function. However, the mechanism of pyroptosis in clearing *N. caninum* should be further explored in GSDMD -deficient mice in future studies. These results affirm the importance of inflammasome activation in clearing invading pathogens. It seems that drugs or inducers that can activate inflammasomes may be potential therapeutic agents for bovine neosporosis.

4. Conclusion

This study demonstrated that the inflammasome can be activated by *N. caninum* in bovine macrophages. Activation of the inflammasome helped to restrict the proliferation of intracellular *N. caninum*. These findings suggest that the inflammasome could act as a potential target for therapeutic options or vaccine strategies for the control of *N. caninum* infection in cattle in the future.

Conflict of interest statement

The authors declared that they have no conflicts of interest to this

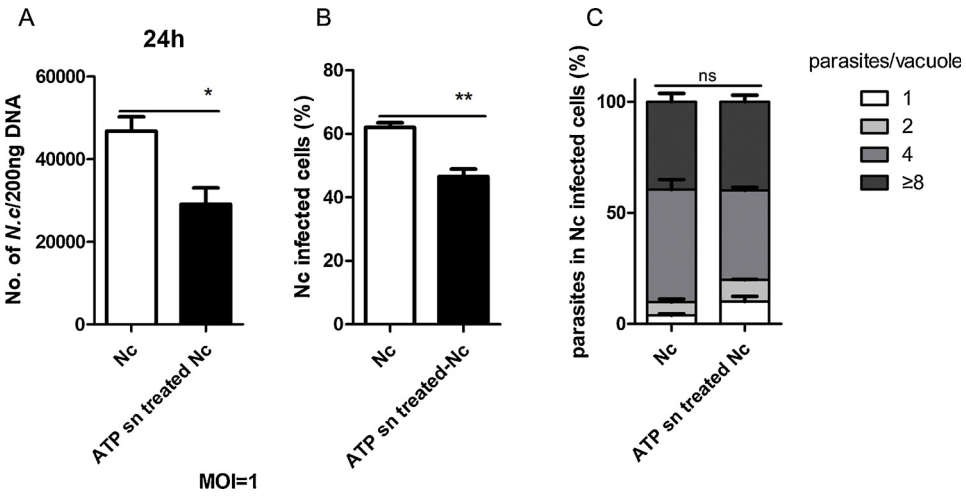

Fig. 3. Pyroptotic cell supernatant has an anti-parasitic effect on *N. caninum*.
N. caninum was incubated with pyroptotic cell supernatant collected from ATP-stimulated bovine macrophages (termed ATP sn in the figure) or with supernatant acquired from blank bovine macrophages at 37 °C for 1 h. Then, these two kinds of *N. caninum* were used to infect fresh bovine macrophages at a MOI = 1 for 24 h. **A** The number of *N. caninum* in infected bovine macrophages was determined by qPCR. Infected bovine macrophages stained with Giemsa were estimated for the percentage of infected cells (**B**) and quantification of parasites in each vacuole (**C**) by light microscopy. MOI, multiplicity of infection (parasite: cell); ns, not significant. The data are representative of two independent experiments and are presented as the means ± SEM. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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