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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Brucella-induced activation of AIM2 inflammasome and Caspase-1 enhances Interleukin-18 secretion in THP-1 cells

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SUMMARY

Introduction/Objective The purpose was to investigate the role of the inflammasome absent in melanoma 2 (AIM2) in *Brucella*-induced inflammatory responses in macrophages.

Methods A cell model of *Brucella* infection was established using a human macrophage cell line. Real-time quantitative polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA) kits were employed to measure the expression of the inflammasome AIM2 and the inflammatory cytokine Interleukin-18 (IL-18) at different time points post-infection, as well as to analyze the correlation between AIM2 and IL-18. Experiments involving the activation and inhibition of AIM2 were conducted to verify its effect on the production of the cytokine IL-18. Western blotting was conducted to determine the expression of AIM2 and IL-18.

Results It is found that IL-18 mRNA and concentration levels increased post-infection, peaking at 48 hours before decreasing by using RT-qPCR and ELISA ($p < 0.05$). Western blot analysis confirmed a similar temporal pattern for IL-18 protein expression ($p < 0.05$). Additionally, AIM2 and Caspase mRNA and protein expression were evaluated, showing a peak at 48 hours ($p < 0.05$), indicating inflammasome activation. The critical role of the AIM2 inflammasome and caspase-1 in IL-18 secretion was further demonstrated with AIM2 activators, which increased IL-18 mRNA and protein levels, and AIM2 inhibitors or Caspase-1 knockout, which reduced them ($p < 0.05$).

Conclusion *Brucella* infection induces the activation of the AIM2 inflammasome and caspase-1, leading to the secretion of the pro-inflammatory cytokine IL-18 in THP-1 cells.

Keywords: brucellosis; macrophage; AIM2; caspase-1; IL-18

INTRODUCTION

Human brucellosis is a zoonotic infectious disease caused by *Brucella* infection, which is widespread globally, with more than 500,000 new cases of human brucellosis reported by the World Health Organization annually [1, 2]. Human brucellosis is characterized by early nonspecific flu-like symptoms. Without timely antibiotic treatment, chronic infection may follow, leading to debilitating sequelae, including recurrent fever, osteomyelitis, arthritis, neurologic symptoms, and endocarditis [3]. The interaction between *Brucella* infection and the host immune response is key to the pathogenesis of the disease. Clarifying the pathogenic mechanisms of *Brucella* is crucial for the effective prevention and control of brucellosis; however, these mechanisms have not been well characterized.

The genus *Brucella* comprises gram-negative bacteria that cause disease in the host by surviving and replicating inside cells, primarily targeting macrophages, dendritic cells, and trophoblasts [4]. Macrophages are the first cellular barrier against external danger signals to the body. Studies have shown that the survival of *Brucella* within macrophages is due to its ability to modulate innate and adaptive immune signaling pathways. Macrophages employ various defense mechanisms, including phagocytosis, antigen presentation to immune effector cells, and inflammasome activation, and play a pivotal role in both inflammatory and chronic diseases. Macrophages are described as the primary cells expressing genes for inflammasomes, whose activation contributes not only to the secretion of pro-inflammatory cytokines but also to the induction of pyroptosis [5–8].

The innate immune system is the body's first line of defense [9]. It can recognize various endogenous and exogenous stimuli in the body through pattern recognition receptors, including pathogen-associated molecular patterns and danger-associated molecular patterns, and activate further immune responses [10, 11]. Inflammasomes are multi-protein complexes formed by the participation of pattern recognition receptors [12]. The absent in melanoma 2 (AIM2) inflammasome is one of the most studied and best described groups, acting as a cytosolic dsDNA sensor [13]. Upon host infection by bacteria, viruses, fungi, and parasites, the AIM2 inflammasome is typically activated. As an important cellular macromolecular signaling platform, the inflammasome converts the

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precursor of caspase-1 into active caspase-1, promoting the maturation and secretion of inflammatory cytokines: interleukin-1 β (IL-1β) and IL-18, while the activators also participate in the formation and cleavage of Gasdermin D, directly mediating pyroptosis [14, 15, 16]. Although the AIM2 inflammasome plays a critical protective role when the body is infected with viruses or bacteria, studies have shown that its overactivation causes damage to normal cells. The body has negative regulatory mechanisms for the activation of the AIM2 inflammasome that keep cells in a dormant state when not stimulated. Therefore, regulating the activation of the AIM2 inflammasome is key to controlling inflammatory responses and pyroptosis, enhancing the body's defenses, and reducing harm to the body. In recent years, the impact of AIM2 inflammasome-mediated pyroptosis on disease has received increasing attention, with scant research in the context of brucellosis. Thus, we undertook this work, using human-derived macrophage lines to explore the regulation of the AIM2 inflammasome and inflammatory cytokines during *Brucella* infection.

METHODS

Main materials and reagents

Human monocyte-derived macrophage line (THP-1 cells), *Brucella* suis strain M28, Roswell Park Memorial Institute (RPMI) medium or RPMI 1640, *Brucella* IL-18 enzyme-linked immunosorbent assay (ELISA) kit, *Brucella* total RNA extraction kit.

Establishment of the *Brucella* cell infection model

The *Brucella* cell infection model was established in a P3 laboratory. Human monocyte-derived THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM pyruvate, 1% glutamine, 1% sodium pyruvate, and 1% penicillin/streptomycin. Cells were seeded at a density of 5×10^5 cells/well in a six-well plate and maintained at 37°C in a humidified 5% CO_2 incubator. Human-derived macrophages (THP-1) were infected with *Brucella* strains at various multiplicity of infections and cells were harvested at different time points (0, 24, 48, 72 hours).

Real-time quantitative polymerase chain reaction (RT-qPCR) for cytokine and inflammasome AIM2 expression levels

First, total RNA was extracted and purified from THP-1 cells using a total RNA extraction kit according to the manufacturer's instructions. RNA concentration and purity were measured with a spectrophotometer, and RNA integrity was assessed by agarose gel electrophoresis. RNA samples that passed quality control were used immediately for reverse transcription or stored at -80°C. Second, qualified RNA samples were reverse transcribed into cDNA using reverse transcriptase according to the manufacturer's instructions. Next, RT-qPCR reaction was performed. PCR primers were designed based on the published human AIM2 and caspase-1 gene sequences using Primer software. For the RT-qPCR reaction, cDNA was mixed with PCR master mix and aliquoted into tubes containing the primers. The final reaction volume was 20 μL, including 10 μL of PCR master mix, 0.8 μL of forward primer (10 μM), 0.8 μL of reverse primer (10 μM), 0.4 μL of ROX reference dye, and 2 μL of cDNA solution. Each gene in each sample was tested in triplicate. Tubes were gently mixed and briefly centrifuged to ensure that all components were at the bottom. The reaction mixture was then placed in RT-qPCR instrument for amplification according to the manufacturer's protocol. Finally, the relative mRNA expression levels were calculated. Ct values were automatically generated by the RT-qPCR instruction analysis software. Relative expression of mRNA levels was calculated using the 2-ΔΔCt method. The PCR primers involved in this study are listed in Table 1.

Table 1. Primer sequences of genes detected by RT-qPCR

Gene	Primer sequences
AIM2	F-TATCGGCACAGTGGTTTCTTAGAGG
	R-GGGCTGAGTTTGAAGCGTGTTG
Caspase-1	F-CCCACATCCTCAGGCTCAGAAG
	R-TGCGGCTTGACTTGTCCATTATTG
IL-18	F-TGGCTGCTGAACCAGTAGAAGAC
	R-GAGGCCGATTTCCTTGGTCAATG
GAPDH	F-TGCACCACCAACTGCTTAGC
	R-GGCATGGACTGTGGTCATGAG

RT-qPCR – real-time quantitative polymerase chain reaction; AIM2 – absent in melanoma 2; IL-18 – interleukin-18; GAPDH – glyceraldehyde 3-phosphate dehydrogenase

ELISA for IL-18 secretion cytokine levels

To quantify secreted cytokines, supernatants were collected from the 6-well plates and stored at -80°C. An ELISA kit was used to measure cytokine levels according to the manufacturer's instructions. After the reaction, a standard curve was plotted using a spectrophotometer, and the absorbance was measured to determine the level of the cytokine IL-18 in the serum samples.

Western blotting

Total protein of each cell group was extracted with RIPA lysate, and protein concentration was quantified using BCA protein concentration assay kit. Take 50 μg protein samples were separated by SDS-PAGE electrophoresis, and then wet transferred to PVDF membrane, 5% skimmed milk powder was sealed at room temperature for two hours. Diluted primary antibody was added respectively and incubated at 4°C overnight. The next day, the membrane was incubated with horseradish peroxidase-labelled secondary antibody at room temperature for two hours. The membrane was washed three times with tris-buffered saline with 0.1% Tween® 20 detergent. The enhanced chemiluminescence ultrasensitive chemiluminescent solution was added for

exposure in a gel imaging analysis system, and the gray values of the bands were measured by Image J software (Bethesda, MD: U.S. National Institutes of Health).

Data statistics

Data was analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and presented as mean ± standard deviation (mean ± SD). Comparisons between the two groups were made using the independent samples t-test and between multiple groups using one-way ANOVA, with $p < 0.05$ being considered a statistically significant difference.

This work was approved by the Biomedical Research Ethics of Inner Mongolia Medical University (YKD202401097).

RESULTS

IL-18 mRNA levels and concentrations at different time points after THP-1 cells were infected with *Brucella*

After THP-1 cells were inoculated with *Brucella*, RT-qPCR and Elisa were used to evaluate the mRNA expression of the cytokine IL-18 at different time points. As shown in Figure 1A, it was observed that the mRNA expression of the cytokine IL-18 gradually increased over time after bacterial inoculation but began to decrease after 48 hours ($p < 0.05$). Likewise, as shown in Figure 1B, ELISA testing of the supernatant revealed that the concentration of the cytokine IL-18 increased significantly until reaching a peak at 48 hours and then began to decrease ($p < 0.05$).

Differences in IL-18 protein expression at different time points after THP-1 cells were infected with *Brucella*

To clarify the expression of IL-18 protein in THP-1 cells caused by *Brucella* infection, Western blotting was used to detect the protein expression of IL-18 in THP-1 cells at different time points (0, 24, 48, and 72 hours). The results showed that the expression of IL-18 in HP-1 cells increased in a time-dependent manner. At 48 hours, the protein expression of IL-18 in HP-1 cells reached a maximum ($p < 0.05$), as shown in Figure 2.

Analysis of inflammasome AIM2-related gene expression at different time points after THP-1 cells were infected with *Brucella*

After inoculation with *Brucella*, RT-qPCR was used to measure the relative expression of AIM2 and Caspase-1 mRNA at different time points. As shown in Figure 3A, the mRNA expression of cytokines AIM2 and Caspase changed over time after inoculation with *Brucella*. gradually increased over time and reached a peak at 48 hours

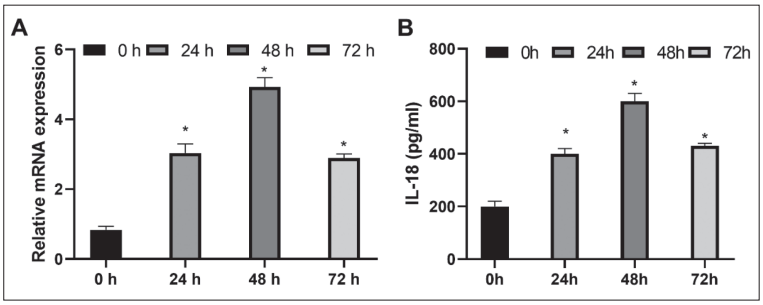

Figure 1. Expression of interleukin-18 (IL-18) at different time points after human monocyte-derived macrophage line (THP-1 cells) were infected with *Brucella*; A – relative expression of IL-18 mRNA in THP-1 cells after infection with Brucella; B – concentration of IL-18 in THP-1 cells after infection with *Brucella*;
compared to control $p < 0.05$

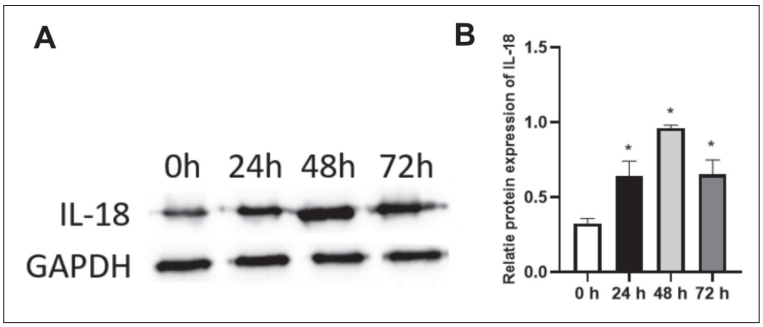

Figure 2. The secretion concentration of interleukin-18 (IL-18) at different time points after THP-1 cells were infected with *Brucella*; GAPDH – glyceraldehyde 3-phosphate dehydrogenase;
compared to control $p < 0.05$

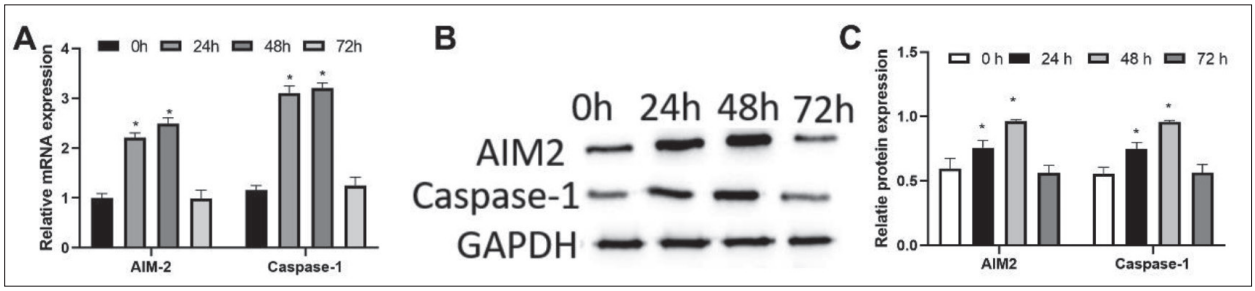

Figure 3. Relative levels of absent in melanoma 2 (AIM2) and Caspase-1 genes at different time points after human monocyte-derived macrophage line (THP-1 cells) were infected with Brucella; A – relative levels of AIM2 and Caspase-1 genes after THP-1 cells were infected with Brucella; B-C – relative protein expression of AIM2 and Caspase-1 in THP-1 cells after infection with Brucella; GAPDH – glyceraldehyde 3-phosphate dehydrogenase;
compared to control $p < 0.05$

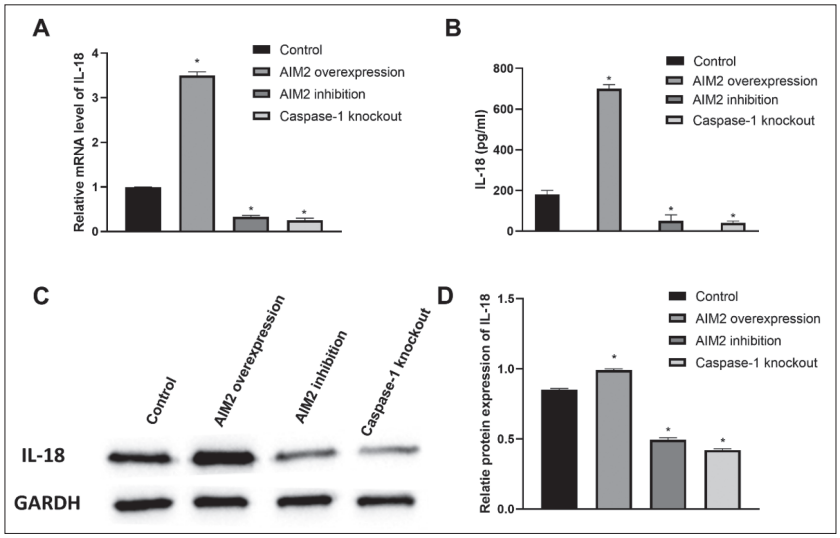

Figure 4. Absent in melanoma 2 (AIM2) and Caspase-1 mediate the secretion of cytokine interleukin-18 (IL-18); A – relative expression of IL-18 mRNA after interfering with the expression of AIM2 and Caspase-1; B – changes in IL-18 concentration after interfering with the expression of AIM2 and Caspase-1; C-D – changes in relative protein expression of IL-18 after interfering with the expression of AIM2 and Caspase-1; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; compared to control $p < 0.05$

($p < 0.05$). As shown in Figure 3B–3C, Western blotting, after infection with *Brucella*, the relative protein expression of AIM2 and downstream caspase-1 genes in THP-1 cells reached a peak at 48 hours and then declined ($p < 0.05$). The results showed that the expression levels of AIM2 receptor and downstream caspase-1 activating genes increased early in infection, reached a peak after 48 hours, and gradually decreased.

Caspase-1 mediates the secretion of Cytokine IL-18

To confirm the role of inflammasome AIM2 in regulating cytokine secretion, THP-1 cells infected with *Brucella* were treated with AIM2 activators and inhibitors. Given the critical role of caspase-1 in classical inflammasome-mediated secretion of pro-inflammatory cytokines, we conducted gene knockout experiments to verify the role of caspase-1. RT-qPCR results found that treatment with AIM2 activator led to an increase in the relative expression of the cytokine IL-18 mRNA. On the contrary, after treatment with an AIM2 inhibitor and Caspase-1 knockout, the mRNA expression of IL-18 increased. The relative expression of mRNA decreased ($p < 0.05$), as shown in Figure 4 A. ELISA test found that the release concentration of cytokine IL-18 increased significantly after AIM2 activator treatment ($p < 0.05$), while after AIM2 inhibitor treatment and Caspase-1 knockout, the secretion level of cytokine IL-18 was decreased compared to the control group ($p < 0.05$), as shown in Figure 4 B. Western blotting results showed the same trend, as shown in Figures 4 C-D. The above results indicate that *Brucella* activates caspase-1 by activating inflammasome AIM2, thereby inducing the secretion of IL-18.

DISCUSSION

Inflammation plays a vital role in the pathogenesis and progression of brucellosis. Macrophages serve as the first line of defense against pathogens by sensing damage stimuli and producing various cytokines. The innate immune system includes pattern recognition receptors that can detect pathogen-associated molecular patterns such as lipopolysaccharides, peptidoglycans, or viruses [17]. The abnormal presence of these substances can activate the innate immune system, leading to the production of inflammatory cytokines to combat exogenous or endogenous threats. They also act as alarm signals to antigen-presenting cells, thereby initiating the adaptive immune response and serving as a bridge between innate and adaptive immunity. AIM2, a recently identified inflammasome receptor, is a member of the hematopoietic interferon-inducible HIN200 protein family. It binds to DNA through the HIN200 domain and forms a complex with ASC to initiate caspase-1, which promotes secretion of the cytokines IL-1β and IL-18 [18]. Studies have shown that the AIM2 inflammasome pathway is critical for host detection of stealth bacterial pathogens such as *Brucella*, which lack highly stimulatory ligands such as flagellin proteins and classical lipopolysaccharides. Due to the heterogeneity of tissue cells, the inflammatory cell necrosis induced by the inflammasome may have both promoting and inhibitory effects.

In present study, we characterized the role of the AIM2 inflammasome in induction of macrophage inflammatory responses to *Brucella* using human macrophage cell lines. By monitoring IL-18 production, we revealed the important role of AIM2 in the mediating of caspase-1 activation and IL-18 production in macrophages.

Currently, IL-18-mediated inflammation is mainly studied in animal models of bacterial, viral, parasitic, and fungal infections. The immune microenvironment shaped by IL-18 can further induce the differentiation of naive T cells into effector and memory T cells, activating the adaptive immune response and playing an important biological role in inflammation-related diseases. The study by Lin et al. [19] investigated the relationship between caspase-1-related inflammasome expression and serum inflammatory cytokine levels during acute brucellosis. In the acute phase of the disease, the inflammasomes are fully activated, inducing cytokines such as IFN-γ and IL-18, thereby triggering a cellular immune response. Our research also indicates that the production of IL-18 increases in the early stages of *Brucella* infection, which may be a crucial mechanism in the acute phase inflammatory response. We hypothesize that during the transition to the chronic phase of brucellosis, excessive production of IL-18 may lead to increased inflammatory burden and tissue damage, with deleterious consequences for the host. However, our results show that

the IL-18 secretion begins to decline 72 hours after *Brucella* infection. According to the 2019 *Brucella* guidelines, a diagnosis of chronic brucellosis is only confirmed after at least six months. Therefore, future experiments could extend the infection period to observe changes in IL-18 secretion to verify our hypothesis.

Studies have confirmed that the expression of the AIM2 inflammasome is increased in patients with systemic lupus nephritis and psoriasis, suggesting that the AIM2 inflammasome may be involved in autoimmune diseases [20, 21]. By measuring the expression of the AIM2 inflammasome at different time points during *Brucella* infection, we found a significant increase in AIM2 expression in the early stages of infection. We then treated *Brucella*-infected macrophages with AIM2 inflammasome activators and inhibitors. Compared to normal conditions, treatment with activators increased the release of the cytokine IL-18, while inhibitors significantly reduced it. These results confirm the important role of the AIM2 inflammasome in regulating the secretion of cytokine IL-18, and its involvement in the immune response to *Brucella*. By recognizing foreign or intracellular dsDNA, AIM2 triggers inflammasome activation, leading to the release of pro-inflammatory cytokines such as IL-1β, IL-18, and the induction of a heat metastasis response. In addition, AIM2 interacts with signaling pathways independent of inflammasome activation, such as AKT and NF-κB, to regulate cancer progression [22], which is consistent with our findings.

The upregulation of AIM2 and IL-18 expressions in the early stages of infection, followed by a downregulation after 72 hours, and the positive correlation between AIM2 expression and IL-18, may be attributable to the early recognition of *Brucella* by the AIM2 receptor. The pathogen is initially controlled through the generation of inflammatory cytokines. However, as the disease progresses into the chronic phase, *Brucella* may employ mechanisms to suppress or downregulate the AIM2 receptor response to evade clearance by the host immune system. In a research conducted by Su et al. [23] of patients with acute and chronic brucellosis, it was found that gene expression levels of the AIM2 receptor were higher in the acute-phase group than in the healthy control group, and lower in the chronic phase group, highlighting the significant role of the inflammasome AIM2 in the pathogenesis and development of brucellosis.

The inflammasome AIM2, upon binding to the precursor of caspase-1, facilitates the cleavage and activation of caspase-1. Activated caspase-1 promotes the production and secretion of IL-1β and IL-18, and can also cause cell membrane rupture, leading to the release of intracellular inflammatory cytokines and triggering a robust inflammatory response [24]. Using caspase-1 knockout experiments, we discovered that caspase-1 plays a critical role in the generation of IL-18, which is essential for the early innate immune response to *Brucella* infection. Fernanda and others have investigated the role of NLRP3 and AIM2 in inflammasome activation following *Brucella* infection and have shown that after *Brucella* infection of glial cells, it is predominantly the NLRP3 and AIM2 inflammasomes that coordinate to induce caspase-1 activation and cytokine secretion [25]. It is believed that the activation of the inflammasome AIM2 and caspase-1 plays an important role in the generation of the inflammatory response.

Limitations of the study

Based on our study findings, we hypothesize that the transition of brucellosis to its chronic phase may be due to the activation of AIM2, which triggers the overproduction of cytokines, leading to tissue damage. These insights and understandings pave the way for further investigation into the pathogenic mechanisms of *Brucella*, which will contribute to the development of novel therapeutic strategies. However, present research has its limitations; as a multifactorial disease, focusing solely on a single inflammasome within brucellosis does not provide a comprehensive picture, and a more holistic approach to the disease is warranted. It should also be noted that the role of AIM2 has been primarily studied in animals, and the impact on the immune capabilities of other human organs and tissues has not been thoroughly investigated. Emphasis on the local suppression of excessive and persistent activation of various inflammasomes may represent a future therapeutic direction for the treatment of brucellosis.

CONCLUSION

Brucella infection induces the activation of the AIM2 inflammasome and caspase-1, leading to the secretion of the pro-inflammatory cytokine IL-18 in THP-1 cells.

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Contribution of authors: We declare that this work was done by the authors named in present article. Li Yupu conceived and designed the study; Zhaojing Zhang, Pengfei Zhao and Pengfei Qiao collected and analyzed the data, while Yupu Li wrote the manuscript. All authors read and approved the manuscript.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest: None declared.

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Активација инфламазома *AIM*2 и каспазе-1 изазвана бруцелом повећава секрецију ИЛ-18 у *THP*-1 ћелијама

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САЖЕТАК

Увод/Циљ Циљ овог истраживања био је да се испита улога инфламазома одсутног у меланому 2 (*AIM*2) у инфламаторном одговору макрофага изазваном инфекцијом бруцелом.

Методе Модел инфекције ћелија бруцелом успостављен је коришћењем људске макрофагне ћелијске линије. Квантитативна полимеразна ланчана реакција (*RT-qPCR*) и ензимски повезани имуносорбентни тест (*ELISA*) коришћени су за мерење експресије инфламазома *AIM*2 и инфламаторног цитокина интерлеукина-18 (ИЛ-18) у различитим интервалима после инфекције, као и за анализу корелације између *AIM*2 и ИЛ-18. Спроведени су експерименти активације и инхибиције *AIM*2 како би се истражила његова улога у продукцији цитокина ИЛ-18. За одређивање експресије *AIM*2 и ИЛ-18 коришћена је метода Вестерн блот.

Резултати Утврђено је да су нивои мРНК ИЛ-18 и његова концентрација повећани после инфекције, достигавши максимум после 48 сати, пре него што су се смањили, што је потврђено *RT-qPCR* и *ELISA* тестом ($p < 0,05$). Анализом Вестерн блот потврђен је сличан временски образац експресије протеина ИЛ-18 ($p < 0,05$). Додатно, испитани су нивои мРНК и експресија протеина *AIM*2 и каспазе-1, који су достигли врхунац 48 сати после инфекције ($p < 0,05$), што указује на активацију инфламазома. Кључна улога инфламазома *AIM*2 и каспазе-1 у секрецији ИЛ-18 додатно је потврђена, јер је активација *AIM*2 довела до повећања нивоа мРНК и протеина ИЛ-18, док су инхибицијом *AIM*2 или инактивацијом каспазе-1 ти нивои смањени ($p < 0,05$).

Закључак Инфекција бруцелом изазива активацију инфламазома *AIM*2 и каспазе-1, што доводи до секреције проинфламаторног цитокина ИЛ-18 у *THP*-1 ћелијама.

Кључне речи: бруцелоза; макрофаг; *AIM*2; каспаза-1; ИЛ-18