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Original Article BASIC MEDICAL SCIENCES

The relationship between caspase-1 related inflammasome expression and serum inflammatory cytokine levels during acute brucellosis

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ABSTRACT

OBJECTIVE: Brucellosis is a zoonotic disease caused by *Brucella* in domestic and wild animals. It also causes systemic diseases with the involvement of different parts of the human body. An efficient innate immune response is crucial to cure brucellosis with optimum antibiotic treatment. The inflammasomes are innate immune system receptors and sensors that regulate the activation of cysteine-dependent aspartate specific protease-1 (caspase-1) and caspase-1-induced cell death process known as pyroptosis. The aim of the present study was to investigate the expression levels of *CASPASE-1* and associated inflammasomes *AIM2*, *NLRP3*, and *NLRC4* to analyze their relationship with the inflammatory cytokine interleukin (IL)-1β, IL-18, and interferon-gamma (IFN-γ) in peripheral blood samples of patients with acute brucellosis with healthy controls.

METHODS: Peripheral blood samples were obtained from 20 healthy volunteers and 20 patients with acute brucellosis. RNA and serum samples were isolated to examine the expression levels of *AIM2*, *NLRP3*, *NLRC4*, and *CASPASE-1* by real-time polymerase chain reaction, and IL-1β, IL-18, and IFN-γ were measured by enzyme-linked immunosorbent assay.

RESULTS: In the acute brucellosis group, *AIM2* and *NLRC4* expressions were significantly higher than in healthy volunteers. A significant increase on caspase-1 expression in patients with acute brucellosis was not observed. Serum IL-18 and IFN-γ levels were significantly higher in patients with acute brucellosis than in healthy controls.

CONCLUSION: Caspase-1-related inflammasomes are sufficiently activated to induce the secretion of cytokines, such as IFN-γ and IL-18, to induce cellular immune response. Caspase-1 activation level should be investigated at different periods of disease in a group with high number of patients to understand the role of pyroptosis and caspase-1 in brucellosis.

Keywords: Acute brucellosis; caspase-1; cytokines; inflammasomes.

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Brucella spp. are Gram negative facultative intracellular bacterium that causes zoonotic disease. It causes infection in humans after the consumption of contaminated foods, especially unpasteurized milk, milk products, and occupational hazards. It can cause serious economic losses in developing countries due to disease effect in both animals and human subjects [1, 2].

Brucellosis is a systemic disease in which any organ and system can be involved in some mammals. Bacteria create a unique intracellular niche in macrophages that cause chronic granulomatous infection and require combined protracted antibiotic treatment in humans. The clinical forms of human brucellosis are determined according to the duration of symptoms as acute (<8 weeks), subacute (from 8 to 52 weeks), and chronic (>1 year). Immune response varies according to those clinical forms [1, 3–5]. In acute brucellosis, the overproduction of T helper-1 (Th1) cytokines, mainly interferon-gamma (IFN-γ) and interleukin (IL)-2, has been detected in serum samples and intracytoplasmic niche. Adequate antibiotic treatment reduces or normalizes those levels. However, in chronic brucellosis, lower $CD3^{+}IFN\text{-}\gamma^{+}$ levels before antibiotic treatment indicate defective Th1 response in those patients. The deterioration of immune response may affect the development of acute immune response and may cause the formation of chronic and relapse form of disease [5–7].

Both innate and acquired immune responses together are responsible for an efficient immune response. The deficiency that can develop in only one will reduce the impact of the immune response [8]. In innate immune response, inflammasomes are playing a crucial role not only to abolish the pathogen by inducing cysteine-dependent aspartate specific protease-1 (caspase-1)-associated pyroptosis but also to induce the adaptive cellular immune response by inducing the secretion of pro-inflammatory cytokines IL-1β and IL-18 [9]. In 2002, Martinon et al. discovered that a multiprotein complex named as inflammasome is responsible for activating caspase-1. In their study, nucleotide-binding oligomerization domain (NOD)-like receptors and the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) play crucial roles in the structure of inflammasomes [10]. Active caspase-1 subsequently functions to cleave the proinflammatory IL-1 family of cytokines into their bioactive forms, IL-1β and IL-18, as well as induce pyroptosis, a type of inflammatory cell deaths. Canonical inflammasomes, such as NLRP1, NLRP3, NLRC4, and absent in melanoma 2 (AIM2), can activate caspase-1 in response to pathogens and danger signals in ASC dependent or independent manner [11].

Nowadays, it is still not known which type of inflammasomes is activated or not during acute brucellosis and their relationship with inflammatory cytokine levels. Recently, IL-18 levels are observed to significantly increase during acute brucellosis in comparison with healthy controls [7]. Thus, investigating the expression levels of caspase-1-associated inflammasomes that are related with the secretion of IL-18 and IL-1β can be helpful to answer the question of which inflammasomes are activated during brucellosis. In the canonical inflammasome group, only NLRP1 is activated by the induction of toxins. Since *Brucella* spp. do not produce toxins, it is worth to investigate the expression level of AIM2, NLRP3, and NLRC4 during brucellosis [12].

The aim of the present study was to investigate the expression levels of caspase-1 and caspase-1-associated inflammasomes, such as AIM2, NOD-like receptor family pyrin domain-containing 3 (NLRP3), and NOD-like receptor family caspase recruitment domain-containing 4 (NLRC4), in peripheral blood samples of patients with acute brucellosis and the levels of inflammatory cytokines, such as IL-1β, IL-18, and IFN-γ, in systemic circulation.

MATERIAL AND METHOD

Patients

The study was approved by the ethical committee for human clinical investigations which conforms to protocols in accordance with the Declaration of Helsinki (form no. 2013/74). All participants were volunteers. Written informed consent was obtained from the patients.

A total of 20 patients with acute brucellosis were enrolled in the study. The study included 8 male and 12 female patients. The mean age of the patients was 38 (19–65) years. Acute brucellosis is defined as patients consistently having the signs and symptoms of brucellosis for <8 weeks along with the presence of one of the following: serum *Brucella* agglutination titer ≥1/160 or isolation of *Brucella* spp., either from the blood or other clinical sample [1].

The controls were selected from 20 age-matched healthy volunteers who were negative for brucellosis according to serologic tests and clinical data. The study included 9 male and 11 female controls. The mean age

of the controls was 38 (20–64) years. All controls were free from common infectious diseases or any chronic or autoimmune disorders, and female volunteers were not pregnant.

Real-time Polymerase Chain Reaction (RT-PCR) Analysis

Peripheral blood samples were collected from healthy controls and patients with acute brucellosis. Total RNA samples were extracted from peripheral blood by using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of RNA samples were evaluated by MaestroNano Spectrophotometer (Maestrogen Inc., Taiwan), and the integrity was confirmed by electrophoresis (1.5% agarose gel at 100 mV with and without heating procedures) (Fig. 1A).

One microgram of RNA was used for cDNA synthesis, and reverse transcription was performed by using RT² HT First Standard Kit (Qiagen) according to the manufacturer's protocol. For RT²-qPCR reactions, cDNA was mixed with SYBR® Green qPCR FAST Mastermix (RT² SYBR® Green FAST Mastermixes; Qiagen) and then aliquoted into the tubes containing commercially provided primers for *AIM2, NLRP3, NLRC4, CASPASE-1*, and housekeeping gene *GAPDH* (Qiagen) (Table 1). Reactions were performed at a final volume of 25 L containing 12.5 L RT² SYBR Green Mastermix, 6.5 L RNAase free water, 5 L cDNA, and 1 L RT2-qPCR Primers. PCR conditions were performed according to the manufacturer's instructions and repeated for 40 cycles. The array was run on a QIAGEN-Rotor Gene Q (Qiagen) (Fig. 1B), and data were analyzed using the 2−ΔCt method, where ΔCt is calculated as ΔCt=(Ct gene of interest−Ct housekeeping). Samples were normalized to housekeeping gene *GAPDH* by using RT² Profiler PCR Array Data Analysis version 3.5 software analyze program.

Cytokine Detection

Serum samples were isolated from peripheral blood of all controls and patients with acute brucellosis and were kept at −80 °C until the study day. IL-1β, IL-18, and IFN-γ levels were analyzed in serum samples by commercially provided ELISA kits, and the study was performed according to the manufacturer's instructions (eBioscience, La Jolla, CA, USA).

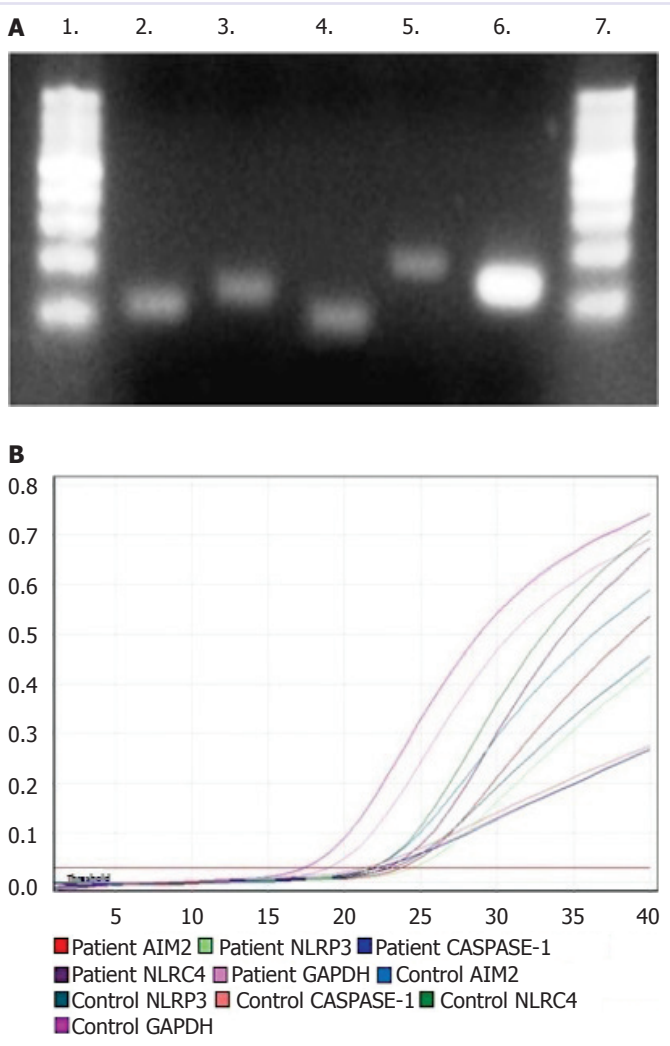

FIGURE 1. **(A)** DNA samples after RT-PCR and visualization of those PCR products at gel electrophoresis (at 2% gel electrophoresis, 45 min, 90 V running conditions). 1st and 7th lines 100 bp DNA marker (Biomatik), 2nd line RT2-PCR product for *AIM2* (114 bp), 3rd line RT2-PCR product for *NLRP3* (142 bp), 4th line RT2-PCR product for *Caspase-1* (81 bp), 5th line RT2-PCR product for *NLRC4* (183 bp), and 6th line is the RT2-PCR product of housekeeping gene *GAPDH*. **(B)** Representative amplification curves of cDNA from the healthy and patient groups for each gene.

Statistical Analysis

Statistical analysis was performed by using the Statistical Package for Social Sciences version 10.0 software (SPSS 10.0; SPSS Inc., Chicago, IL, USA). Data are presented as mean values with standard deviations for continuous variables. A comparison of continuous variables among the groups was performed using Student's t test for variables with a normal distribution and the Mann–Whit-

Table 1. Properties of primers

Gene	Reference segment no	Reference position	Band range (bp)
AIM2; Absent in melanoma 2	NM_004833.1	1221	114
NLRP3; NOD-like receptor family, pyrin domain containing 3	NM_183395.2	3365	142
CASPASE-1; Cysteine dependent aspartate specific protease-1	NM_033292.2	1052	81
NLRC4; NOD-like receptor family, caspase recruitment domain-containing 4	NM_021209.4	3273	183
GAPDH; Glyceraldehyde 3-phosphate dehydrogenase	NM_002046.3	756	130

Table 2. Standard agglutination test results of patients diagnosed as acute brucellosis

Brucella standard agglutination titer	*Acute Brucellosis*	
	n	%
1/80	–	–
1/160	7	35
1/320	9	45
1/640	3	15
1/1280	1	5

ney U test for variables with a non-normal distribution. Normality for continuous variables in groups was determined using the Kolmogorov–Smirnov test. Additionally, the Pearson correlation test was performed to analyze the relationship between two parametric variables. A p value <0.05 was considered statistically significant.

RESULTS

Standard agglutination assay is one of the methods to diagnose brucellosis. Patients with acute brucellosis had a titer of ≤1/160 in that test (Table 2). Blood culture tests were performed for 12 out of 20 patients with acute brucellosis, and 11 of them were positive.

Inflammasome Genes and Caspase-1 Expression Levels

The expression levels of *AIM2*, *NLRP3*, and *NLRC4* were investigated in patients with acute brucellosis and healthy controls. A significant increase was observed on *AIM2* and *NLRC4* expressions in comparison with healthy controls (Fig. 2A, B). *NLRP3* expression level was not as high as *AIM2* and *NLRC4* in patients with acute brucellosis (Fig. 2C). A significant positive correlation was observed between the expression levels of *AIM2* and *NLRC4* in patients with brucellosis (p=0.035, r=0.487).

Interestingly, *CASPASE-1* expression level was not significant in comparison with the healthy group (Fig. 2D). There was no any correlation between inflammasome genes and caspase-1 gene expression levels in patients with brucellosis. In case of relative fold change while NLRC4 increased approximately 4 times and AIM2 increased 2 times in comparison with the control group (Fig. 3).

IL-1β, IL-18, and IFN-γ Levels

IFN-γ, IL-1β, and IL-18 levels were investigated in the serum samples of each patient and healthy control. IFN-γ, the cytokine representing cellular immune response, significantly increased in patients with acute brucellosis (Fig. 4A). In case of cytokines IL-1β and IL-18 secreted after pro-caspase-1 activation, there was no significant difference on IL-1β levels between patients with acute brucellosis and healthy controls (Fig. 4B). However, IL-18 levels increased dramatically in patients with acute brucellosis (Fig. 4C).

The correlations between cytokine and inflammasome gene expression, caspase-1 expression, were also analyzed. There was a positive correlation between

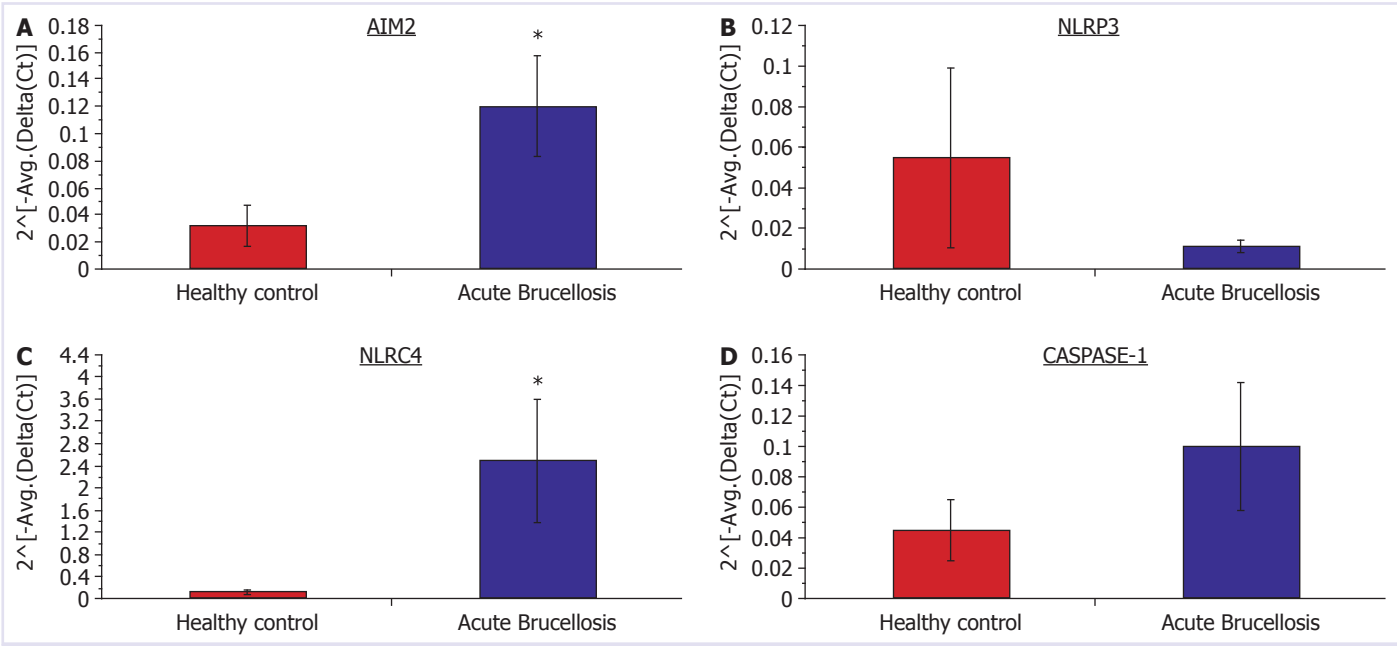

FIGURE 2. Average ΔCt values of *AIM2* **(A)**, *NLRP3* **(B)**, *NLRC4* **(C)**, and *CASPASE 1* **(D)** in the healthy control and acute brucellosis groups. GAPDH has been used as a housekeeping gene. *symbolizes statistically significant elevation in the acute brucellosis group in comparison with healthy control. Average ΔCt values were presented with ±SD.

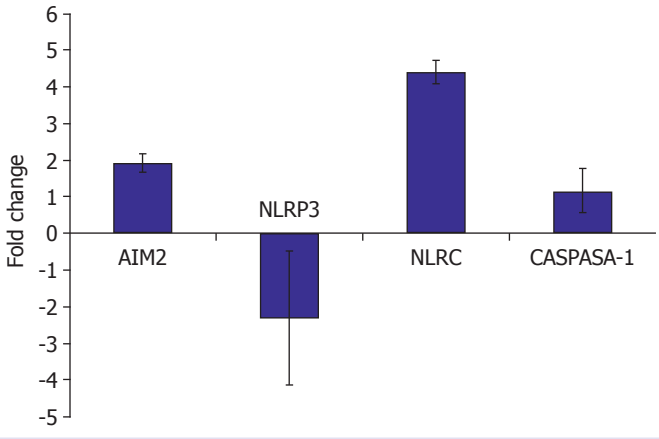

FIGURE 3. Relative fold changes of *AIM2*, *NLRP3*, *NLRC4*, and caspase-1 expression levels.

NLRC4 and IL-18 levels in patients with brucellosis ($p<0.01$, $r=0.883$). In addition, there was a positive correlation between AIM2 and IFN-γ in the brucellosis group ($p<0.01$, $r=0.896$). There was no any correlation between caspase-1 expression and cytokine levels.

DISCUSSION

Brucellosis is a healing disease with adequate antibiotic therapy. However, after treatment, some patients can develop the infection again. At that point, whether or not the innate immune response is active enough during acute brucellosis is still being discussed [13–15]. The aim of our study was to investigate the levels of expression of caspase-1-associated inflammasomes and -related inflammatory cytokines, IL-1β and IL-18, during acute brucellosis that play roles in inducing the inflammatory immune response.

Brucella can release molecules like its own DNA, which subsequently induces the production and release of pro-inflammatory cytokines in the host immune system, as detected by cytosolic AIM2 inflammasome. Gomes et al. supported the idea that *Brucella* genomic DNA is a ligand for AIM2 inflammasome. In experimental studies, they have observed that AIM2 is activated by *Brucella* DNA in mouse macrophages, leading to caspase-1 activation and release of IL-1β. In addition, *AIM2* knockout mice have been shown to be more susceptible to *Brucella* infection than wild-type control mice [16]. However, how AIM2 perceives DNA due to *Brucella* infection remains unclear.

There are similar observations on other samples of intracellular pathogens as in *Brucella*. Rathinam et al. showed that while IL-18 production decreases, bacterial burden increases during *Francisella tularensis* infection in AIM2$^{-/-}$ mouse macrophages. Researchers have concluded that AIM2 inflammasome is a crucial complex to

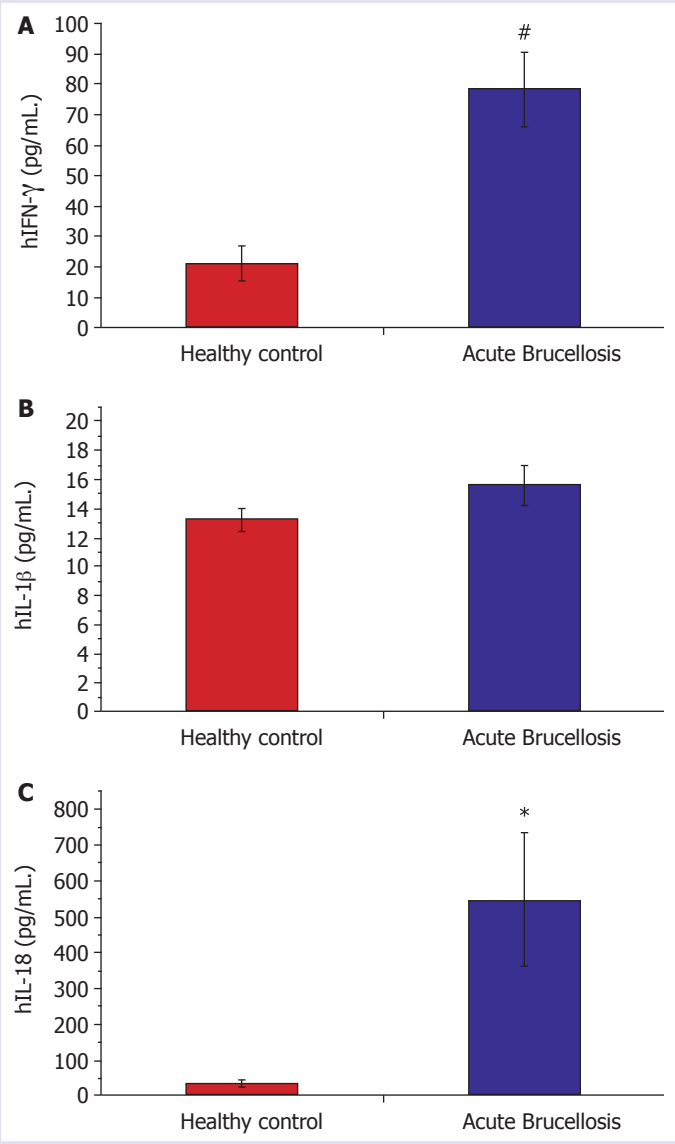

Figure 4. Serum IL-1β, IL-18, and IFN-γ mean values in healthy control and acute brucellosis volunteers. Mean±SD were presented in each group. * and # symbolize statistical differences of IL-18 and IFN-γ values between the healthy group and acute brucellosis group, respectively.

stimulate the pro-inflammatory immune response in *F. tularensis* infection [17].

In spite of all those data, the condition of AIM2 inflammation has not yet been investigated in humans during brucellosis. In our study, *AIM2* expression was observed to increase during acute brucellosis. However, that elevation was only correlating with IFN-γ level in serum samples. These results led us to consider that AIM2 may play a crucial role in the activation of the adaptive immune response during brucellosis.

An inflammatory immune response initiated by NLRP3 inflammasome triggers various host-induced danger signals. It has been shown that IL-1β is secreted at lower levels in *NLRP3* knockout mouse macrophages, and that the group was more susceptible to *Brucella* infection than control mice [16]. NLRP3-induced inflammatory immune response triggers various conditions, such as metabolic disturbance and infection in the host.

It has not been clarified yet how these highly variable stress signals are triggered by a single inflammasome. One of the important elements for NLRP3 activation is reactive oxygen species (ROS) [18, 19]. Li et al. determined that *Brucella* spp. induce mitochondrial ROS, and that induction is required for NLRP3/AIM2 inflammasome activation and IL-1β and IL-18 secretion during the bacterial induction of RAW264.7 macrophages [20]. Petrielli et al. showed that the K^{+2} flux triggers the activation of NLRP3 inflammasome [21]. Additionally, Yang et al. demonstrate that high K^{+2} flux inhibits caspase-1 maturation and release of IL-1β in cell culture by using virulent *Mycobacterium bovis* strain [22]. In contrast to all these studies, there was no increase on NLRP3 expression level, and there was no increase on IL-1β serum levels in patients with brucellosis.

Bacterial flagellin, a monomeric subunit of the flagellar filament, is considered as a pathogen-associated molecular pattern. Flagellin is detected by NLRC4 in *Brucella* infected macrophage cytoplasm via bacterial virulence-associated secretion systems. The activation of caspase-1 via NLRC4 inflammasome leads to the maturation and release of biologically active proinflammatory cytokines IL-1β and IL-18. In addition, it may trigger cell death known as pyroptosis [23–25]. However, *Brucella* flagellin component is regarded as a "host protective factor." Terwagne et al. investigated whether *Brucella* FliC flagellin, the monomeric subunit of flagellar filament, is sensed by the host during infection or not. The most interesting observation in their study was the use of a flagellin-deficient mutant *Brucella melitensis* in their studies, and they explored that infecting mice with those mutant *B. melitensis* caused histologically demonstrable injuries in the spleen of infected mice in comparison with wild type ones. According to their data, they suggested that the recognition of FliC via NLRC4 inflammasome plays a role in the immunologic standoff between *Brucella* and host [26]. In our study, there was a significant increase on the expression of *NLRC4* in patients with acute brucellosis. That elevation was significantly correlating with serum IL-18 level in the serum samples of those patients.

Inflammasome-dependent caspase-1 activation can result in cell death known as pyroptosis. Pyroptosis usually occurs due to infection with intracellular pathogens and is likely to be part of the antimicrobial response. Active caspase-1 allows the host to control various microbial infections. It has been demonstrated that *Brucella* abortus-induced caspase-1 activation did not cause pyroptosis in macrophages [16]. There was no significant increase on the expression level of *CASPASE-1* during acute brucellosis, and that expression did not show any correlation with IFN-γ, IL-1β, and IL-18. That may be a strategy of *Brucella* not to lose its niche.

Overall, the elevation on *AIM2* and *NLRC4* expression levels during acute brucellosis can be a sign for the activation of the innate immune response, and those genes can be responsible for the release of pro-inflammatory cytokine IL-18 and inflammatory cytokine IFN-γ. However, studies with high number of patients and also investigating those parameters in patients with chronic, especially relapse, brucellosis would be helpful to understand the exact mechanism of the immune response in brucellosis.

Conflict of Interest: The authors whose names are listed in the title page have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

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