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UNCORRECTED PROOF

The relationship between caspase-1 related inflammasomes expressionand 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 involvement of different parts of body in human. 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 caspase-1 and caspase-1 induced cell death process known as pyroptosis. We aimed to investigate expression levels of *CASPASE-1* and associated inflammasomes *AIM2*, *NLRP3*, *NLRC4* to analyze their relationship with the inflammatory cytokine IL-1β, IL-18 and IFN-γ in peripheral blood samples of acute brucellosis patients with healthy controls.

METHODS: Peripheral blood samples were obtained from 20 healthy volunteers and 20 acute brucellosis patients. RNA and serum samples were isolated to examine the expression levels of *AIM2*, *NLRP3*, *NLRC4*, *CASPASE-1* by RT-PCR and IL-1β, IL-18, IFN-γ were measured by ELISA.

RESULTS: In acute brucellosis group *AIM2* and *NLRC4* expressions significantly increased in comparison to healthy volunteers. We did not observe significant elevation on caspase-1 expression in acute brucellosis patients. Serum IL-18 and IFN-γ levels significantly increased in acute brucellosis patients in comparison to healthy controls.

CONCLUSION: Caspase-1 related inflammasomes are sufficiently activated to induce secretion of cytokines, 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 human after consumption of contaminated foods, especially unpasteurized milk, milk products and occupational hazards. *Brucella* 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 causes chronic granulomatous infection and requires combined protracted antibiotic treatment in human. The clinical forms of human brucellosis are determined according to duration of symptoms as acute (less than 8 weeks), sub-acute (from 8 to 52 weeks) and chronic (more than 1 year). Immune response varies according to those clinical forms [1, 3–5]. In acute brucellosis; overproduction of T helper-1 (Th1) cytokines [mainly Interferon-gamma (IFN-γ) and IL-2] has been detected in serum samples and intra-cytoplasmic niche. Adequate antibiotic treatment reduces or normalizes those levels. However, in chronic brucellosis lower CD3+IFN-γ+ levels before antibiotic treatment, indicates defective Th1 response in those patients. 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 from an efficient immune response. The deficiency that can develop in only one will reduce the impact of immune response [8]. In innate immune response inflammasomes are playing a crucial role not only to abolish the pathogen by inducing Cystein dependent Aspartate Specific Proteinase-1 (Caspase-1) associated pyroptosis but also to induce adaptive cellular immune response by inducing secretion of pro-inflammatory cytokines IL-1β and IL-18 [9]. In 2002 Martinon et al. has discovered that a multiprotein complex named as inflammasome was responsible from activating Caspase-1. In that duty NOD like receptors and the adaptor protein apoptosis associated speck-like protein containing a caspase recruitment domain (ASC) play crucial role in 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; 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].

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

In this study; we aimed to investigate the expression levels of caspase-1 and caspase-1 associated inflammasomes; Absent in melanoma 2 (AIM2), NOD-like receptor family pyrin domain containing 3 (NLRP3), NOD-like receptor family Caspase Recruitment Domain-Containing 4 (NLRC4) in peripheral blood samples of acute brucellosis patients and levels of inflammatory cytokines, IL-1β, IL-18 and IFN-γ, in systemic circulation.

MATERIALS AND METHODS

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 number 2013/74). All participants were volunteers and provided written informed consent.

Twenty patients with acute brucellosis (8 men and 12 women, mean age 38 years, range 19-65 years) were enrolled in that study. Acute brucellosis is defined as patients consistently having the signs and symptoms of brucellosis for less than eight 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 (9 men and 11 women, mean age 38 years, range 20-64 years) who were negative for brucellosis according to serologic tests and clinical data. All controls were free from common infectious diseases or any chronic or autoimmune disorders, and female volunteers were not pregnant.

Table 1. Properties of primers

Gene	Reference segment number	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; Cystein dependent Aspartate Specific Proteinase-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

Real-time PCR analysis

Peripheral blood samples were collected from healthy controls and acute brucellosis patients. Total RNA samples were extracted from peripheral blood by using QIAamp RNA Blood Mini Kit (QiaGen) according to manufacturer's instructions. The quality and quantitiy of RNA samples were evaluated by MaestroNano Spectrophotometer (Maestrogen İnc. Taiwan) and integrity was confirmed by electrophoresis (1.5% agarose gel at 100mV with and w/o heating procedures).

One microgram (1µg) of RNA was used for cDNA synthesis, and reverse transcription were 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 (QIAGEN; RT2 SYBR® Green FAST Mastermixes), then aliquoted into the tubes containing commercially provided primers for *AIM2*, *NLRP3*, *NLRC4*, *CASPASE-1* and house keeping gene GAPDH (QiaGen) (Table 1). Reactions were performed at final volume 25µL. containing 12.5 µL RT2 SYBR Green Mastermix, 6.5µL RNAase free water, 5µL cDNA and 1µL RT2qPCR Primers. PCR conditions were performed according to manufacturer's instructions and repeated for 40 cycles. The array was run on a QIAGEN-Rotor Gene Q (Qiagen) and data were analyzed using 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 all control and acute brucellosis patient's peripheral blood and were kept at -80 till to study day. IL-1β, IL-18 and IFN-γ levels were analyzed in serum samples by commercially provided ELISA kits and study performed according to manufacturer's instructions (eBioscience).

Statistical analysis

Statistical analysis was performed by using the Statistical Package for the Social Sciences 10.0 (SPSS 10.0) (SPSS Inc., Chicago, USA) software. The results of continuous variables are presented as mean values with standard deviations. A comparison of continuous variables among groups was performed using Student's t test for variables with a normal distribution and the Mann-Whitney U test for variables with a non-normal distribution. Normality for continuous variables in groups was determined using the Kolmogrov-Smirnov test. Additionally, the Pearson correlation test was performed to analyze the relationship between two parametric variables, and p values <0.05 were considered statistically significant.

RESULTS

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

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

Inflammasome Genes and Caspase-1 Expression Levels

Expression levels of *AIM2*, *NLRP3*, *NLRC4* were investigated in acute brucellosis patients and healthy controls. We observed significant elevation on *AIM2* and *NLRC4* expressions in comparison to healthy controls (Figure 1A, B). *NLRP3* expression level was not as high as *AIM2* and *NLRC4* in acute brucellosis patients (Figure 1C). We observed significant positive correlation between expression levels of *AIM2* and *NLRC4* in brucellosis patients ($p=0.035$; $r=0.487$).

Interestingly; *CASPASE-1* expression level was not significant in comparison to healthy group (Figure 1D). We did not observe any correlation between inflammasome genes and Caspase-1 gene expression levels in brucellosis patients. In case of relative fold change while *NLRC4* increased approximately 4 times and *AIM2* increased 2 times in comparison to control group (Figure 2).

IL-1β, IL-18 and IFN-γLevels

IFN-γ, IL-1β and IL-18 levels were investigated in serum samples of each patients and healthy controls. IFN-γ, the cytokine representing cellular immune response significantly increased in acute brucellosis patients (Figure 3A). In case of the cytokines IL-1β and IL-18, secreted after pro-Caspase 1 activation, we did not observe significant difference on IL-1β levels between acute brucellosis patients and healthy controls (Figure 3B). However, IL-18 levels increased dramatically in acute brucellosis patients (Figure 3C).

We also analyzed the correlations between cytokines and inflammasome genes expression, Caspase-1 expression, also. We observed positive correlation between *NLRC4* and IL-18 level in brucellosis patients ($p<0.01$, $r=0.883$). In addition we determined positive correlation between *AIM2* and IFN-γ in brucellosis group ($p<0.01$;

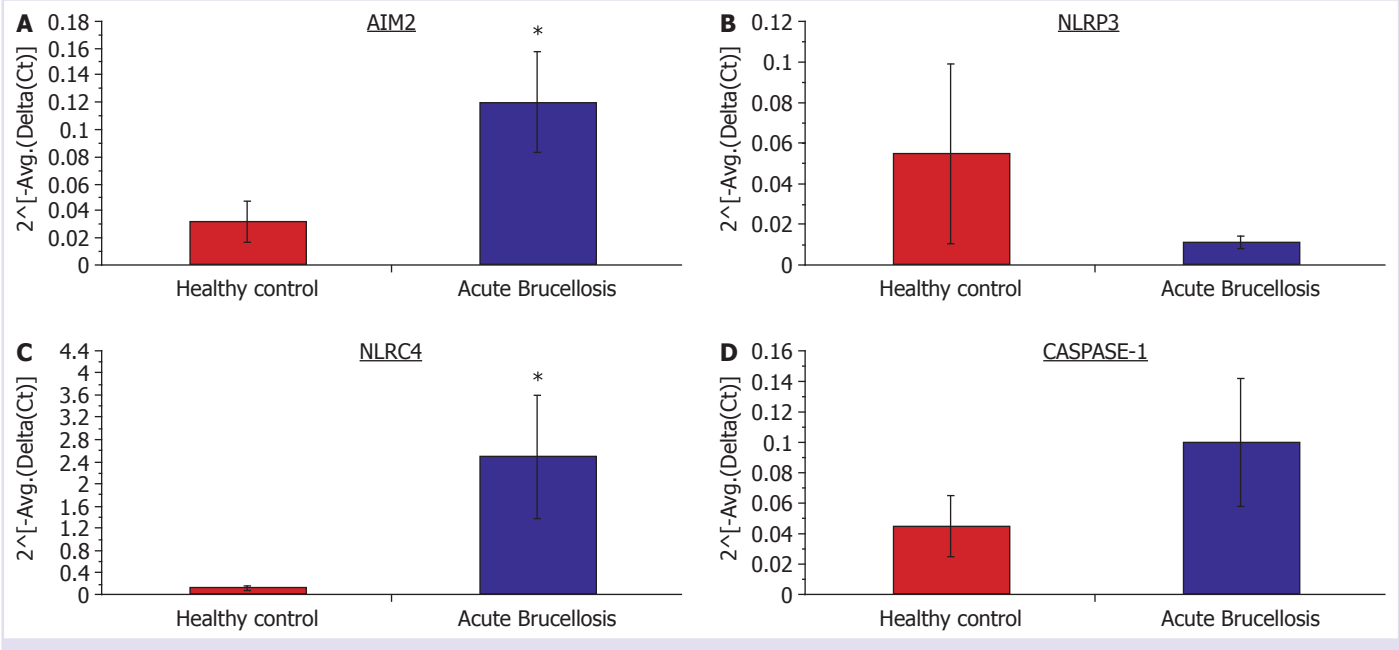

Figure 1. Average delta Ct values of AIM2 **(A)**; NLRP3 **(B)**, NLRC4 **(C)** and CASPASE 1 **(D)** in healthy control and acute brucellosis groups. GAPDH has been used as house-keeping gene. *Symbolizes statistical significant elevation in acute brucellosis group in comparison to healthy control. Average delta Ct values were presented with ±S.D.

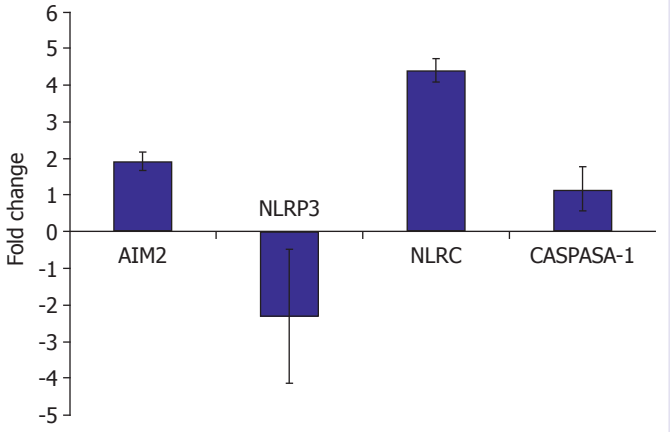

FIGURE 2. Relative fold changes of AIM2, NLRP3, NLRC4 and Caspase-1 expression levels.

r=0.896). We did not observe 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 role 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 host immune system, is detected by cytosolic *AIM2* inflammasome. Marko et al. has 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, which leads 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. has shown that while IL-18 production decreased, bacterial burden increased during F. tularensis infection in *AIM2*-/- mouse macrophages. Researchers have concluded that *AIM2* inflammasome is a crucial complex to stimulate pro-inflammatory immune response in F. tularensis infection [17].

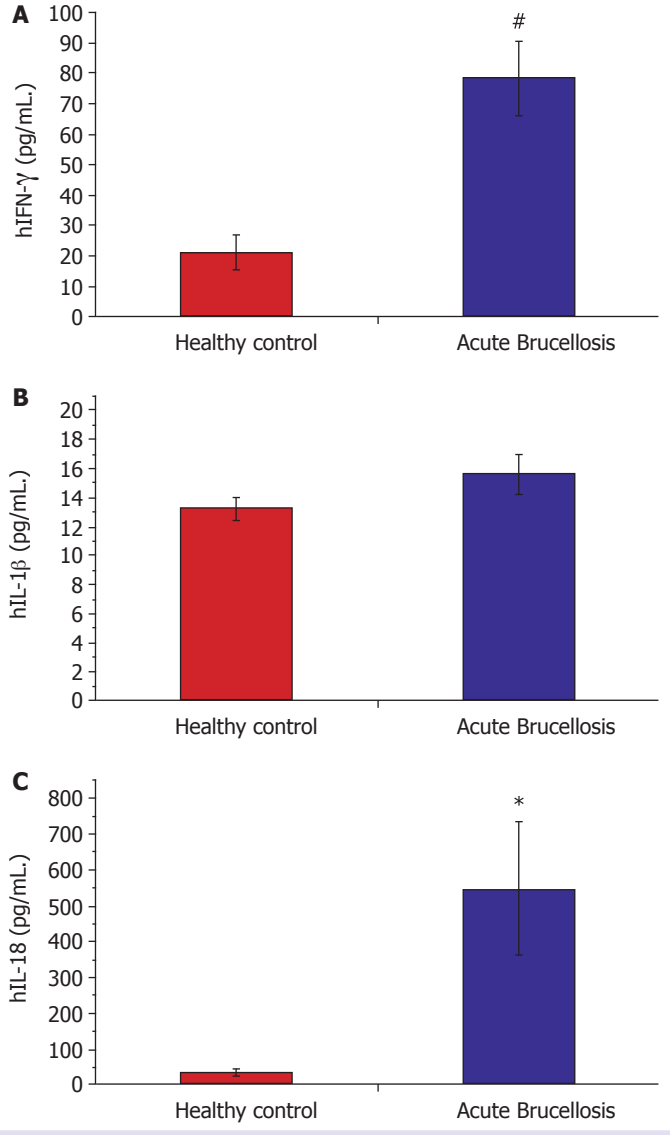

FIGURE 3. Serum IL-1β, IL-18 and IFN-γ mean values in healthy control and acute brucellosis volunteers. Mean values ±S.D. were presented in each group. *And # symbolize statistical differences of IL-18 and IFN-γ values between healthy group and acute brucellosis group, respectively.

In spite of all those data, the condition of *AIM2* inflammation has not yet been investigated in human during brucellosis. In our study we observed that *AIM2* expression increases during acute brucellosis. However, that elevation was only correlating with IFN-γ level in serum samples. These results led us to consider that

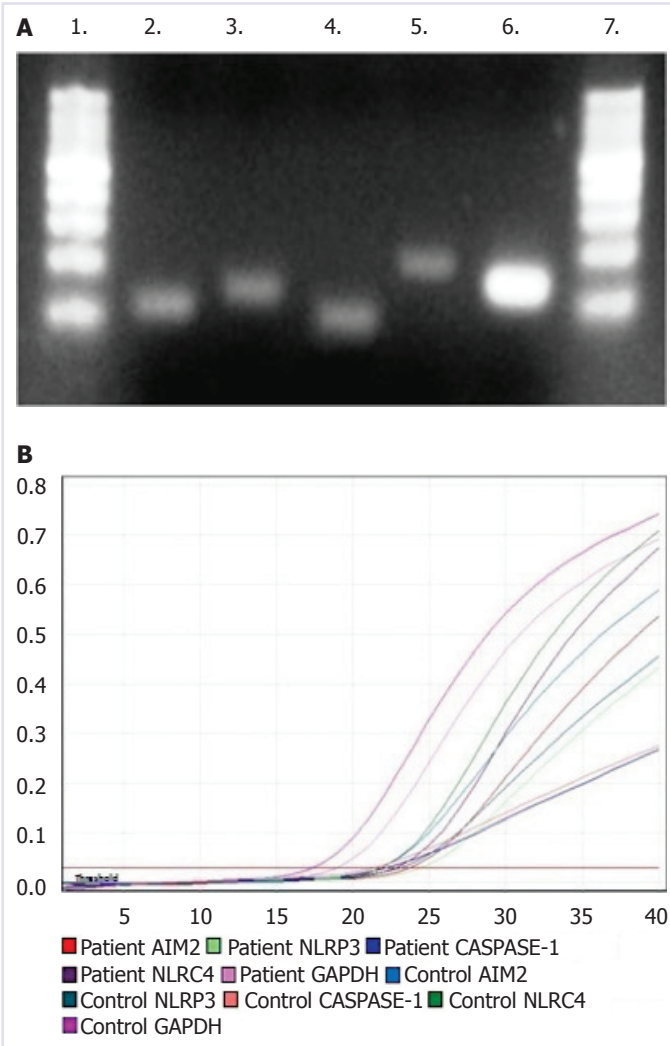

Figure 4. **(A)** DNA samples after real-PCR and visualization of those PCR products at gel electrophoresis (at 2% gel electrophoresis, 45 minutes, 90 Volt running conditions). 1st and 7th lines 100bp DNA marker (Biomatik); 2nd line RT2-PCR product for AIM2 (114bp), 3rd line RT2-PCR product for NLRP3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

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* knock-out mouse macrophages and that group was more susceptible to *Brucella* infection when compared to 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. induced mitochondrial ROS and that induction was required for *NLRP3*/*AIM2* inflammasome activation and IL-1β, IL-18 secretion during bacterial induction of RAW264.7 macrophages [20]. Petrielli et al. showed that the K+2 flux triggers 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 virulant M. bovis strain [22]. In contrast to all these studies we did not observe an elevation on *NLRP3* expression level and we did not detect an increase on IL-1β serum levels in brucellosis patients.

Bacterial flagellin, a monomeric subunit of the flagellar filament, is considered as a Pathogen Associated Molecular Pattern (PAMP). Flagellin is detected by *NLRC4* in *Brucella* infected macrophage cytoplasm via bacterial virulence-associated secretion systems. Activation of caspase-1 via *NLRC4* inflammasome; leading to the maturation and release of the 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 has 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 B.mellitensis in their studies and they explored that, infecting mice with those mutant B.mellitensis caused histologically demonstrable injuries in the spleen of infected mice in comparison to wild type ones. According to their data, they suggested that recognition of FliC via *NLRC4* inflammasome plays a role in the immunologic standoff between *Brucella* and host [26]. In our study we observed significant elevation on expression of *NLRC4* in acute brucellosis patients. That elevation was significantly correlating with serum IL-18 level in 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 micro-

bial infections. It has been demonstrated that B. abortus induced Caspase-1 activation did not cause pyroptosis in macrophages [16]. We did not observe significant elevation on 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 loose its niche.

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

Conflict of Interest: The authors whose names are listed in 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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