The relationship between caspase-1 related inflammasomes 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 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.
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 quantity of RNA samples were evaluated by MaestroNano Spectrophotometer (Maestrogen Inc. 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.
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).

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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-γ Levels

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; r=0.487).

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.
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].

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
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 virulent 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 NLRC4 expression 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-

**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.
brial 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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