Mystery of Immune Response in Relapsed Brucellosis: Immunophenotyping and Multiple Cytokine Analysis

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Mystery of Immune Response in Relapsed Brucellosis: Immunophenotyping and Multiple Cytokine Analysis Running

Relaps Brusellozda İmmün Yanıtın Gizemi: İmmünfenotipleme ve Çoklu Sitokin Analizi

Running Title: Immune Response in Relapsed Brucellosis
Türkçe Kısa Başlık: Relaps Brusellozda İmmün Yanıt

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Abstract

Introduction: Brucella spp. are intracellular bacteria that may cause acute, subacute and chronic infections. Although optimum antibiotic treatment is available, relapse of brucellosis occurs in some patients. There isless amount of knowledge about immune response in relapse of brucellosis.

Materials and Methods: Twenty patients with acute brucellosis and sixteen patients with relapse brucellosis were enrolled in this study to explore the immune response variation during relapse of brucellosis. The distribution of peripheral blood mononuclear cells investigated by flow cytometry and various cytokines levels involved in inflammatory and anti-inflammatory response, measured by ELISA in serum samples.

Results: The most prominent data in phenotyping examination was the significant 1.45 times reduction at the percentage of activated T cell ($CD3^+HLA-DR^+$) population in the relapse group in comparison to acute brucellosis. However, percentage of activated T cell population in the relapse group was 2.59 times higher than the healthy group ($p<0.01$). In case of cytokine levels; we observed significant reduction at inflammatory cytokines IL-6, IL-18, IFN-g and IL-17 in relapsed patients in comparison to patients with acute brucellosis. While there was no significant difference in IL-15 and TNF-a levels between relapse and acute brucellosis group; the levels of these two cytokines were significantly higher in the relapse group than healthy subjects. Interestingly, we observed 2.87 times elevation ofIL-4 levels in the relapse group in comparison to acute brucellosis ($p<0.01$). Similarly; IL-10 levels increased 2.09 times in patients with relapsed brucellosis patients in comparison to acute brucellosis ($p<0.01$).

Conclusion: Elevation of regulatory cytokinesin systemic immune system, and reduction of activated T cell frequency occur during the relapse of brucellosis. These results may have important consequences to understand the immunopathology in the systemic circulation during relapse of brucellosis.

Key Words: Brucellosis, Cytokines, Immune Response, Relapse

Özet

Giriş: Brucella spp., akut, subakut ve kronik enfeksiyonlara neden olabilen hücre içi bakteridir. Optimum antibiyotik tedavisine rağmen, bazı hastalarda brusellozun relapsı ortaya çıkmaktadır. Brusellozun relapsındaki immün yanıt hakkında çok az bilgi bulunmaktadır.

Gereç ve Yöntemler: Bu çalışmada, brusellozun relapsı esnasındaki immün yanıt değişimlerini araştırmak için akut brusellozlu yirmi hasta ve relaps brusellozlu on altı hasta kaydedilmiştir. Periferik kan mononükleer hücrelerin dağılımı akım sitometri ile araştırılmıştır ve inflamatuar ve anti-inflamatuar yanıtta işe karışan çeşitli sitokin seviyeleri serum örneklerinde ELISA ile ölçülmüştür.

Bulgular: Fenotipleme araştırmasında en dikkat çeken veri; akut brusellozla karşılaştırıldığında relaps grubundaki aktive olmuş T hücre (CD3$^+$HLA-DR$^+$) yüzdesindeki 1.45 kat anlamlı azalmaydı. Ancak, relaps grubundaki aktive olmuş T hücre populasyonunun yüzdesi sağlıklı gruptan 2.59 kat daha yüksekti (p<0.01). Diğer taraftan, akut brusellozlu hastalarla karşılaştırıldığında relaps olmuş hastalardaki IL-6, IL-8, IFN-g ve IL-17 inflamatuar sitokinlerde anlamlı azalma gözlemledik. Relaps ve akut bruselloz grupları arasında IL-15 ve TNF-a seviyelerinde anlamlı fark bulunmazken, relaps grubunda bu iki sitokinin seviyesi sağlıklı örneklere göre anlamlı bir biçimde daha yüksekti. İlginç bir şekilde, akut bruselloz ile karşılaştırıldığında relaps grubundaki IL-4 seviyelerinde 2.87 kat yükselme gözlemledik (p<0.01). Benzer şekilde, IL-10 seviyeleri de akut bruselloz hastaları ile karşılaştırıldığında relaps olmuş brusellozlu hastalarda 2.09 kat artmıştı (p<0.01).

Sonuç: Regülatör sitokinler sistemik immün sistemde yüksektir ve brusellozun relapsı esnasında azalmış aktive T hücre sıklığı ortaya çıkmaktadır. Bu sonuçlar brusellozun relapsı esnasındaki sistemik dolaşımdaki immünopatolojiyi anlamada öneme sahip olabilir.

Anahtar kelimeler: Bruselloz, Sitokinler, İmmün Yanıt, Relaps

Introduction

Brucella spp. are gram-negative, facultative intracellular bacteria that cause acute and chronic zoonotic disease. The consumption of contaminated foods, especially unpasteurized milk and dairy products, and more rarely occupational exposures can result in infection in humans. It not only is a huge economic burden in developing countries, but it is also a potential bio-terrorism agent in the United States since it is a class III pathogen [1-3]. Historically, only Brucella melitensis, B. abortus, B. suis and B. canis were considered human pathogens; however, the majority of human disease is caused by B. melitensis and B. abortus [1, 4]. Similarly, infections caused by different Brucella spp. have been shown in different reports. De et al. reported an infection caused by B. inopinata in a patient with prosthetic breast implants, and humans infected with a murine strain, B. ceti, have been reported [5-7].

Brucellosis is a systemic disease involving many organs or systems in some mammals. Bacteria create a unique intracellular niche in macrophages, which causes granulomatous infection and requires combined protracted antibiotic treatment in humans. Additionally, the presence of neutrophilic infiltrates has been reported and can cause a necrotizing inflammatory response, such as arthritis, meningitis, peritonitis, and abscesses in different human organs and placentitis in some mammals [8-10]. The clinical forms of human brucellosis are determined according to the duration of symptoms and are described as acute (less than 8 weeks), sub-acute (from 8 to 52 weeks) and chronic (more than 1 year). The immune response varies according to the clinical forms [1, 8, 11, 12]. In acute brucellosis, overproduction of T helper-1 (Th1) cytokines, primarily interferon-gamma (IFN-g) and IL-2, has been detected in serum samples and in the intra-cytoplasmic niche. Adequate antibiotic treatment reduces or normalizes these levels. However, in chronic brucellosis, detection of lower CD3$^+$IFN-g$^+$ levels prior to antibiotic treatment, indicates a defective Th1 response in those patients. This defect causes an imbalance between Th1 and T helper-2 (Th2) T cell responses. It is estimated that the diminished production of Th1 cytokines in these patients occurs because of the Th2 switch and/or the release of immune-regulatory cytokines, such as TGF-b1 [12, 13-15].

In the case of relapsed brucellosis, there is less amount of knowledge about the type and kinetics of the immune response. The cause of relapse in brucellosis can be due to either a lack of an immunological response or a genetic deficiency in these patients. To understand the differences in immune responses during the relapsed brucellosis compared with acute brucellosis and healthy volunteers, we investigated the type of immune cells in systemic circulation using immuno-phenotyping of peripheral blood samples. Second, we analyzed the levels of inflammatory and anti-inflammatory cytokines in peripheral blood circulation.

Materials and Methods

Study Population

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 2011/19). All participants were volunteers and provided written informed consent.

Twenty patients with acute brucellosis (9 men and 11 women, mean age 37 years, range 19-55 years) and sixteen patients with relapsed brucellosis (9 men and 7 women, mean age 40 years, range 22-56 years) were enrolled in this 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]. Relapse is defined as the reappearance of symptoms or signs of the disease, assessed by physician, or a new positive blood culture, or demonstration of specific IgG rising by Brucella tube agglutination test with 2-Mercapthoethanol (2ME)-result within the 12-month period after the end of therapy [16]. Data were collected until time of relapse or conclusion of the trial, or whichever came first. All blood samples were obtained before antibiotic treatment in acute and relapsed brucellosis groups.

The controls were selected from 20 age-matched healthy volunteers (9 men and 11 women, mean age 37 years, range 28-52 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.

Measurement of Cytokines

Inflammatory cytokines, IL-15 (Immunoleader, Boster Biological Technology, Pleasonton, CA), IL-17 and IL-18 (eBioscience, Bendermed system, Vienna, Austria) were measured by ELISA according to the manufacturer's instructions. The inflammatory cytokines IL-6, IFN-g, TNF-a, and anti-inflammatory cytokines IL-4 and IL-10 were measured by FlowCytomix bead assay (BenderMed Systems, GmbH, Vienna, Austria) using flow cytometry as described previously [17]. Briefly; serum samples or serially diluted standards were resuspended with fluorescent beads coated with monoclonal antibodies specific to the cytokines together with biotin-conjugated monoclonal antibodies specific for each cytokine in 96-well plates. Following that, the beads were incubated with phycoerythrin (Pe)-labeled streptavidin. Fluorescent 4 with 675 nm detector was used to analyze and quantitate beads. Standard curves were determined for each cytokine from a range of 27-20.000 pg/mL. Samples and standards were analyzed with a FACSCanto[TM] II flow cytometer (BD BioScience San Jose, CA, U.S.A.). The concentrations were measured by using the FlowCytomix Pro 2.2 software (BenderMed Systems). For each analysis, 10.000 beads were collected.

Flow Cytometry Analysis

Mouse anti-human monoclonal antibodies specific for IgG$_{1,2a}$, CD14, CD45, CD3, CD19, CD4, CD8, HLA-DR, and CD16$^+$56$^+$ were purchased from BD Bioscience (San Jose, Ca, U.S.A.). Peripheral blood lymphocyte phenotypes and activated T cell (CD3$^+$HLA-DR$^+$) frequencies were analyzed following an immunofluorescence procedure described previously. Anti-human monoclonal antibodies specific for IgG$_{1,2a}$ were used as a negative control to detect non-specific binding. Briefly, peripheral blood samples were incubated with 10 ml fluorescein isothiocyanate (FITC)- or Pe-labeled mouse anti-human monoclonal antibodies in the dark for 30 minutes at 4°C [18]. Then, the erythrocytes were lysed in BD FACS Lysis solution according to the manufacturer's instructions. A BD BioScience FACSCanto[TM] II six-color flow cytometer equipped with the computer software FASC Diva (BD BioScience, U.S.A.) was used to assess the phenotype of the mononuclear cells. For each analysis 20.000 cells were collected. The percent of positive cells obtained according to gating with isotype control for each sample.

Statistical Analysis

Statistical analysis was performed 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 Kolmogorov-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

Blood culture tests were performed for 14 of 20 acute brucellosis patients, and 10 of them were positive. In case of relapsed brucellosis, blood culture tests were performed for 12 of 16 patients and two of them were positive.

Phenotype Distribution of Peripheral Blood Mononuclear Cells

Before analyzing the lymphocytes and sub-type of lymphocytes in the peripheral blood, granulocytes and lymphocytes were separated with different gates as shown in Figure 1. Since the monocyte population was dragged through granulocyte population in several samples of the acute and relapsed brucellosis groups based on flow cytometry, it was difficult to separate monocytes from granulocytes by using forward and side scatter analysis only. Therefore, monocytes were identified by using anti-CD14 monoclonal antibody as shown in figure 2A-2B. We did not observe significant difference in ratio of CD14 population between healthy and brucellosis groups.

Since B-lymphocytes provide an infectious niche for Brucella [19]; investigating the frequency of CD19$^+$ B cell population makes sense to understand the immunopathology of brucellosis. As shown in figure 2B we did not observe a significant difference on percentage of CD19$^+$ B cell population in three groups.

Patients with acute brucellosis had a significantly higher percentage of CD3$^+$ T lymphocytes compared with healthy individuals and relapse patients (p<0.001 and p=0.01, respectively) (Figure 3). The analysis of the T-lymphocyte sub-groups showed a significant reduction in CD4$^+$ T cell frequency in relapsed brucellosis patients compared with the acute brucellosis group (p=0.028) (Figure 3). We observed a significant increase in CD8$^+$ T cell frequency in acute and relapsed brucellosis compared with the healthy group (p<0.05) (Figure 3). Furthermore, the CD4/CD8 ratio significantly increased toward to CD8$^+$ T cells only in the relapsed brucellosis group compared with the healthy individuals (p<0.001) (Table). Activated T cells were defined as the CD3$^+$HLA-DR$^+$ population in peripheral blood samples. During acute brucellosis, the percentage of activated T cells increased significantly compared with the healthy and relapsed brucellosis groups (Figure 4A and figure 4B, p<0.01 and p=0.01, respectively). Although relapse of infection reduced the percentage of activated T cells, it did not reach the levels of the healthy group and was significantly higher than healthy controls (Figure 4A, 4B) (p<0.01). We also determined whether that elevation correlated with the titer values of the agglutination tests or not. We did not observe any correlation between these two variables (p>0.05 for both variables) (r=0.430 and r=0.260 for acute and relapsing brucellosis groups, respectively).

Other lymphoid group members, Natural Killer (NK) and NK-T lymphocytes, which are phenotypically and functionally distinct from B and T lymphocytes, were investigated. NK cells were identified as CD3$^-$CD16$^+$CD56$^+$, and NK-T cells were identified as CD3$^+$CD16$^+$CD56$^+$. We did not observe significant differences for the frequency of NK and NK-T cells for the three groups (Table).

Inflammatory and Anti-inflammatory Cytokine Levels

For inflammatory cytokines, we analyzed the cytokines responsible from innate immune response IL-6, TNF-a, IL-15 and the cytokines responsible from adaptive immune response IFN-g, IL-17, IL-18 in all samples. Acute brucellosis induced a significant increase in IL-6 and TNF-a compared with healthy individuals (p=0.001 for IL-6 and p<0.001 for TNF-a) (Figure 5A and 5B, respectively). As shown, relapsed brucellosis patients had significantly lower IL-6 levels compared with acute brucellosis

(Figure 5A). We observed a significant increase in TNF-a levels in acute and relapsed brucellosis patients compared with healthy individuals (p<0.001) (Figure 5B). We did not observe a reduction in IL-15 levels in relapsed patients, a cytokine that induces the proliferation of NK cells and promotes the innate and adaptive immune responses. The IL-15 levels were significantly higher in the relapsed group than in healthy individuals (Figure 5C) (p=0.002). However, this increase was not significant when compared with the acute brucellosis group (p>0.05).

Acute brucellosis induced a significant increase in IFN-g and IL-17 levels compared with healthy individuals. Interestingly, IFN-g and IL-17 levels in relapsed brucellosis patients were significantly lower than in acute brucellosis patients (p<0.001) (Figure 5D and figure 5E). However, IFN-g levels in the relapsed brucellosis group were significantly higher than in healthy individuals (p=0.014) but statistical significance was not reached for IL-17 levels in relapsed and healthy subjects.

Acute brucellosis induced significant elevation of IL-18 level in serum samples in comparison to healthy objects. That elevation reached to 3.2 times in acute brucellosis group in comparison to relapse group (p<0.001) (Figure 5F).

An opposite effect was observed for the anti-inflammatory cytokines IL-4 and IL-10 (Figure 6A and figure 6B). Acute brucellosis did not induce a significant increase in IL-4 compared with healthy individuals. For IL-10, we observed a significant increase of IL-10 in the serum samples of acute brucellosis patients compared with healthy individuals (p=0.007). There was a significant elevation of both IL-4 and IL-10 levels in patients with relapsed brucellosis when compared to acute patients (p<0.001 for IL-4 and IL-10).

Discussion

The clinical forms of human brucellosis are determined according to the duration of symptoms as acute, sub-acute and chronic. Although adequate antibiotic treatment reduces or normalizes immunpathological view of these clinical forms, it is not known why brucellosis causes relapse in some patients. Is that because of a genetic deficiency in host or a defect on a part of immune response? In this study we investigated the latter possibility by phenotyping the immune cells and measuring the levels of inflammatory and anti-inflammatory cytokines in systemic circulation.

According to our data; a significant reduction in the percentages of activated T lymphocytes, resembling an anti-inflammatory immune response, with high concentrations of IL-4 and IL-10 in the peripheral blood of relapsed brucellosis patients were detected when compared with acute brucellosis patients.

Host protection against Brucella spp. depends on cell-mediated immunity, including activated macrophages, dendritic cells and activated T cells. The T-helper 1 (Th1) immune response and the production of IFN-g from these cells are essential for the clearance of Brucella infection in humans. Kocoglu et al. demonstrated a significant increase in circulating activated T cell numbers (CD3+HLA-DR+) without significant alterations in the subpopulations of peripheral blood lymphocytes of acute brucellosis patients [20]. Brucella spp. avoid or manipulate host immunity for their own benefit, and according to our findings, in the case of relapse, there is a decrease in the frequency of both CD4+ T cells and activated T cells (CD3+HLA-DR+) in the peripheral blood. Chronic brucellosis patients display a defective Th1 response and a decreased percentage of CD4+ T cells expressing CD25 [21, 22]. We hypothesize that the suppression of naïve or activated T cells or a defective Th1 response are either a function of regulatory T cells or suppressor T cells, which leads to the progression of infection both in relapse and chronic brucellosis patients. Corresponding with the phenotypic observations, the limited expansion of CD4+ T cells affects the production of inflammatory cytokines in the Th1 and Th17 T cell groups. However, the significant decrease of IFN-g and IL-17 levels and the significant increase of IL-10 in relapse patients suggest a change in the frequency of Treg cells during brucellosis relapse compared with acute brucellosis. Pasquali et al. observed that regulatory T cells suppress the effector functions of CD4+ T cells in infected mice, and they showed that Treg cells allow the expansion of brucellosis [23]. In a clinical investigation, Skendros et al. observed that chronic brucellosis patients retain low percentages of CD4+CD25+ and CD4+CD28+ T lymphocytes after potent stimulation with phytohemagglutinin-cultured peripheral blood cells using Escherichia coli lipopolysaccharide (LPS). Accordingly, they concluded that Brucella spp. modulate the functional arms of the human immune system [22].

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Similar to the observations of Çelik et al., we determined that the CD8^{+} T cell population increases in acute patients compared with healthy subjects [24]. Interestingly, that elevation also occurs in relapse patients. Unfortunately, we do not know whether these CD8^{+} T cells are functional and whether their expansion is limited or not.Therefore, additional studies are necessary on that case. Beside that; Skendros et al. observed a significant elevation on CD8^{+} T cell population in chronic brucellosis patients and also relapsed chronic brucellosis patients in comparison to acute brucellosis. They concluded that, elevation of CD8^{+} T cell population in peripheral blood mononuclear cells of chronic brucellosis and relapsed patients probably compensates ineffectual CD4^{+}T cell response in chronic brucellosis patients [25].

Brucellosis causes a Th1 type inflammatory response and induces the production of inflammatory cytokines during acute infection. The majority of studies indicate that the major source of IFN-g is CD4^{+} T cells; however, CD8^{+} T cells, gd T cells and NK cells also produce IFN-g [26, 27]. Interestingly, during relapse of brucellosis, we observed a significant decrease in inflammatory cytokines, except for TNF-a and IL-15, in the systemic circulation. Recently, Feldman et al. performed an ex vivo study to measure the expression and secretion of several inflammatory cytokines in peripheral blood mononuclear cells after stimulation with LPS or heat-killed B. melitensis. They concluded that acute and relapse patients were largely identical by their cytokine gene expression profiles; however, there was a strong cytokine secretion that accurately discriminates acute from relapse patients which may allow for better follow up care of brucellosis patients through improved identification of patients at risk of relapse [28]. We have found several similar results to those of Feldman et al., particularly in the case of TNF-a levels, in which they observed higher expression after LPS stimulation compared with both acute brucellosis and control donors. They also observed increased IFN-g expression after heat-killed B. melitensis (HKBM) stimulation in relapse patients and concluded that relapse of brucellosis in patients can induce the expression of cytokines necessary to mount a Th1 response. However, they found increased IL-10 gene expression after stimulation with HKBM in both acute and relapsed brucellosis patients. The main question that remains unanswered is, 'Does the relapse of brucellosis cause an inflammatory or anti-inflammatory immune response?' It has been observed that protective immunity against B. abortus was down-regulated by IL-10, and the dominant Th1 cytokine production switched toward to Th2 cytokine production during long term of the disease [29, 30].

According to our data, a reduction of inflammatory cytokines in relapsed patients is related to increases in immune suppressor cytokines, such as IL-10 and IL-4, which down-regulate the inflammatory immune response and the number of activated T cells. We propose several explanations for this type of down regulation of the inflammatory immune response. One is related to the expansion of regulatory T cells, and the second is brucellosis-acquired cellular anergy. Accordingly, Pasquali et al. observed that CD4^{+}CD25^{+} regulatory T cells are responsible for the maintenance and progression of B. abortus infection in mice [23]. Moreover, Xavier et al. showed that the persistent intracellular pathogen B. abortus prevents the immune activation of macrophages by inducing production of an anti-inflammatory cytokine IL-10 from CD4^{+}CD25^{+} T cells during early infection time point that modulates macrophage function and contributes to an initial balance between pro-inflammatory and anti-inflammatory cytokines which is beneficial to the pathogen, thereby promoting enhanced bacterial survival [31]. Anergy occurs primarily for chronic brucellosis patients, which represent approximately 10% to 30% of brucellosis patients. It is predicted that patients with chronic brucellosis display defective Th1 responses and T cell anergy, which is identified by decreased Th1 cytokine production by peripheral blood cells, increased proportion of CD8 cells in the CD4/CD8 ratio, reduced CD69^{+} early activated T lymphocytes, and low proliferative responses of lymphocytes to mitogens or Brucella antigens [11, 12].

Whether or not all intracellular bacteria cause a regulatory immune response during relapse of disease is not known. In case of Mycobacterium tuberculosis, an intracellular bacteria, may cause relapse tuberculosis after primary disease. De Steeenwinkel et al . investigated the cytokine response of immune system in relapse tuberculosis patients in comparison to primary disease patients. They observed that; levels of TNF-a, IFN-g, IL-6, IL-17 levels were significantly lower in relapse tuberculosis patients in comparison to primary tuberculosis. In contrast to our observation in relapse of brucellosis; they observed that in relapse of tuberculosis memory Th1 cells locally and systemically expanded and congregated in lung , permitting an efficient control of Mycobacterium tuberculosis growth [32].

In conclusion, during relapse of brucellosis CD4/CD8 ratio was decreased and regulatory cytokines are dominant in the systemic immune system. Additionally, activated T cell frequency reduced during the relapse of brucellosis. Determining triple cytokine (IL-4, IL-10 and IFN-g) levels can be beneficial to

follow up acute and relapse brucellosis patients. These results may have important consequences for understanding the immunopathology in the systemic circulation during brucellosis relapse.

Declaration of Interest

We authors declared no potential competing interests about that manuscript.

This study has been approved by a Local Ethics Committee.

A consent form was filled out by all participants.

Surgical and Medical Practices: Y.B.; Ü.K.; S.K.
Concept = B.K.; Y.B.; Ü.K.; S.K.
Design = B.K.; Y.B.
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Analysis or Interpretation = B.K.; Y.B.; Ü.K.
Literature Search = B.K.; Y.B.; Ü.K.
Writing = B.K.; Y.B.

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Table

Population	Healthy (n=20)	Acute Brucellosis (n=20)	Relapse Brucellosis (n=16)
CD4/CD8 Ratio	1.82 ± 0.179	1.39 ± 0.136	$1.11 \pm 0.180^{*}$
NK cells	9.96 ± 1.27 (%)	8.35 ± 0.75 (%)	7.42 ± 0.63 (%)
NK-T cells	4.96 ± 0.93 (%)	6.28 ± 1.52 (%)	6.96 ± 2.01 (%)

Table: Comparison of CD4/CD8 ratio and percentages (%) of NK and NK-T cells in peripheral blood samples of healthy individuals, acute and relapse brucellosis subjects. NK cells were determined as $CD3^{-}CD16^{+}CD56^{+/-}$; NK-T cells were determined as $CD3^{+}CD16^{+}CD56^{+/-}$ in flow cytometry analysis. Results presented as mean ±SD.
*; symbolizes the significant difference between relapse brucellosis and healthy groups (p<0.001).

Figure Legends
Figure 1.
Distribution of lymphocytes and granulocytes in peripheral blood samples of healthy (white bar), acute brucellosis (black bar) and relapsed brucellosis patients (grey bar). According to forward and side scatter analysis in flow cytometry, the mean percentages of lymphocytes and granulocytes were calculated. Data are shown as the mean ± Standart error
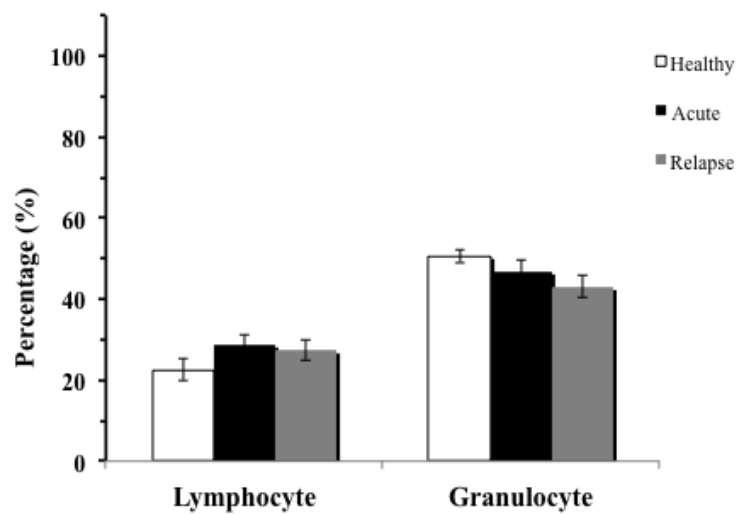
FIGURE 1.

Figure 2.
CD14+ monocytes population was presented among peripheral blood cells. Figure A is a representative flow cytometry diagram of a peripheral blood sample from an acute brucellosis patient. Figure B shows the percentages of CD14+ and CD3-/CD19+ B-lymphocyte populations among lymphocyte gate. Data are shown as the mean ± Standart error.
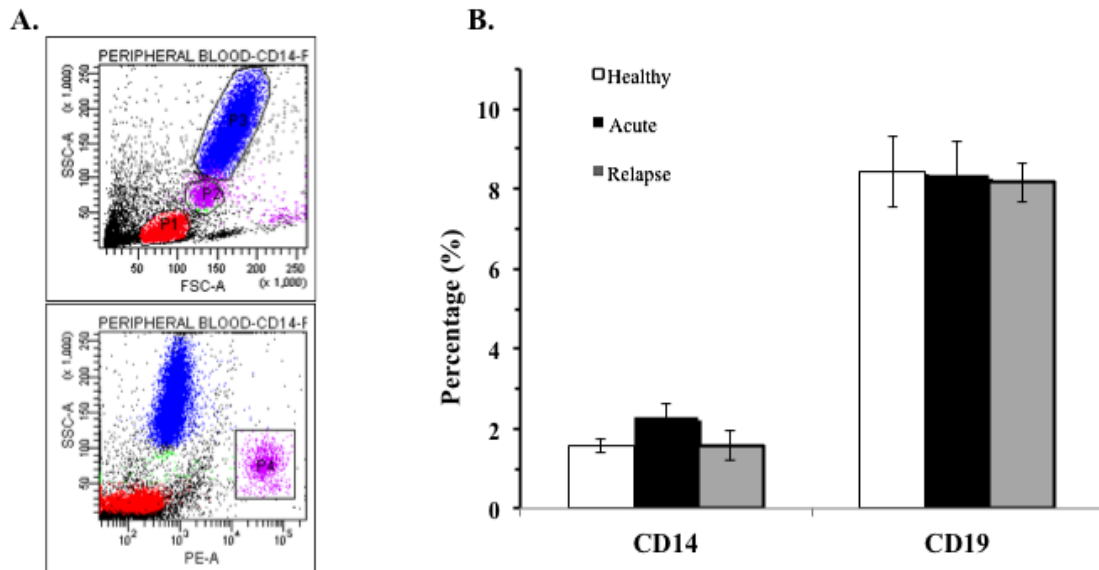
FIGURE 2.

Figure 3

Immunophenotyping profile of peripheral blood lymphocytes T- (CD3+/CD19−) were presented among lymphocyte gate and T helper cells (CD3+CD4+) and cytotoxic T cells (CD3+CD8+) percentages were presented after gating among CD3+ T-lymphocytes. Data are shown as the mean ± Standart error. * indicates statistical significance of CD3 T cell percentage in acute brucellosis patients in comparison to healthy (p<0.001) and relapsed (p=0.01) group. # indicates statistical difference between acute and relapsed groups. $ indicates statistical difference of CD8+ T cell percentage in healthy individuals in comparison to acute (p=0.009) and relapse brucellosis patients (p=0.005).
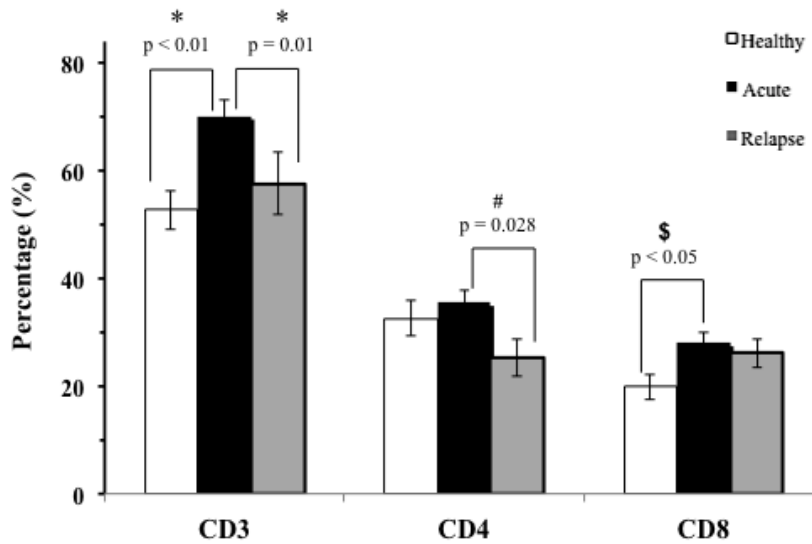
FIGURE 3.

Figure 4. A-B

Distribution of activated T cells in peripheral blood samples of healthy (white bar), acute (black bar) and relapsed (grey bar) brucellosis patients. Activated T cells were identified as $CD3^+HLA-DR^+$ T cells in flow cytometry analysis. $CD3^+HLA-DR^+$ activated T cells were analyzed after gating $CD3^+$ T cells in lymphocyte gate Data are presented as the mean value of percentages ± Standart Error *indicates statistical significance of activated T cell level in acute brucellosis in comparison to healthy group. #, indicates significant difference between relapsed and acute brucellosis groups. ¶, represents the significant difference between relapsed brucellosis and healthy groups (A). Representative flow cytometry analysis of activated T cells from healthy, acute brucellosis, relapsed brucellosis groups were presented. (B).
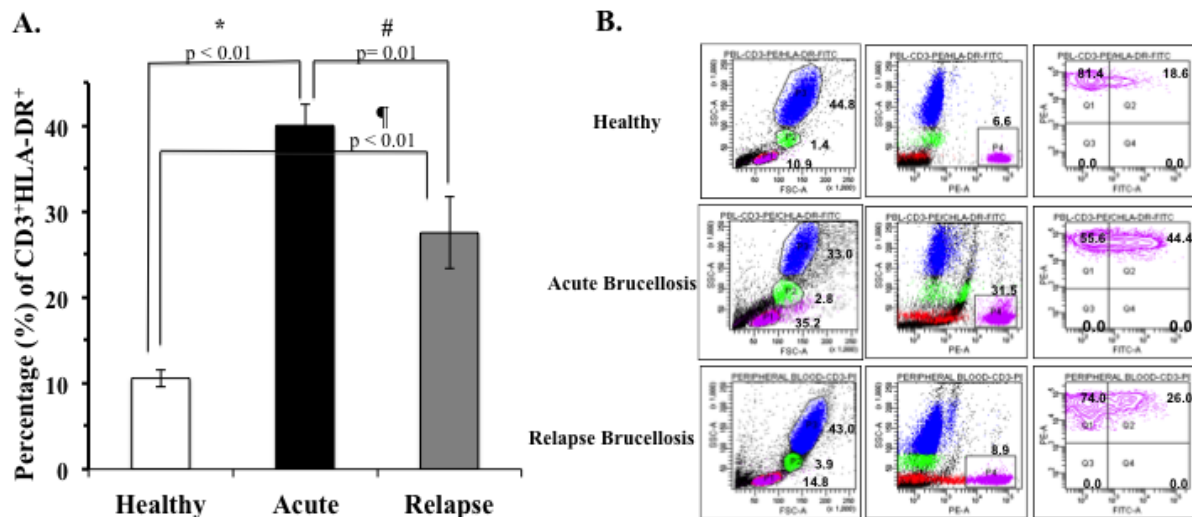
FIGURE 4.

Figure 5. A-F
Concentrations of inflammatory cytokines role in innate immune response IL-6 (A), TNF-□□□B□□□IL-15 (C) and role in adaptive immune response IFN-□ (D), IL-17 (E), IL-18 (F) in peripheral blood serum samples of healthy (white bar) donors, acute (black bar) and relapsed (grey bar) brucellosis patients. Data are shown as mean values of 20 healthy donors, 20 acute and 16 relapsed brucellosis patients ± Standart Error. * and # symbolize statistical difference between mentioned groups.
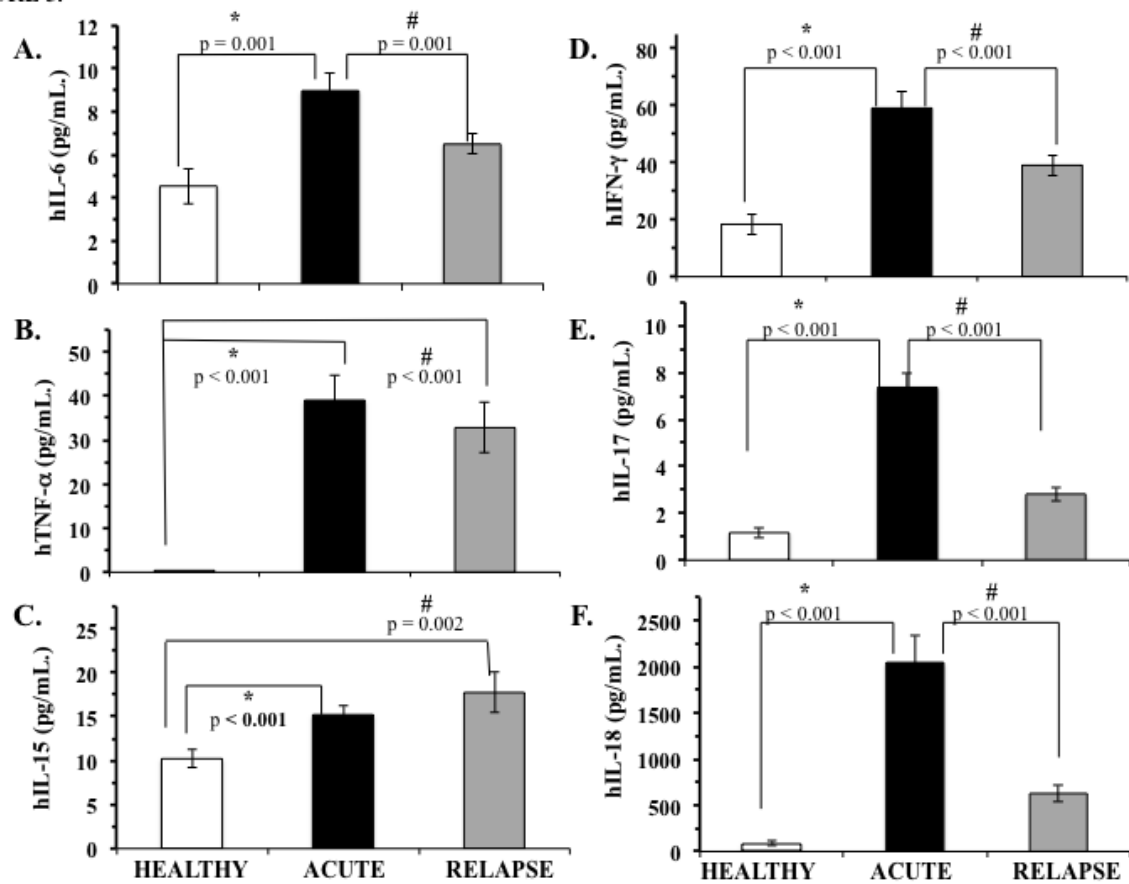

FIGURE 5.

Figure 6. A-B
Concentrations of anti-inflammatory cytokines IL-4 (A) and IL-10 (B) in peripheral blood serum samples of healthy (white bar) donors, acute (black bar) and relapsed (grey bar) brucellosis patients. Data are shown as mean values of 20 healthy donors, 20 acute and 16 relapsed brucellosis patients ± Standart Error . *, # and $ symbolize statistical difference between mentioned groups.