

# Reduced IFN- $\gamma$ Production in Chronic Brucellosis Patients

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## ABSTRACT

**Background:** Brucella is a well-known intracellular bacterium entailing acute and chronic illnesses in humans and domestic animals. The infection chronicity may be affected by the cell-mediated immunity and cytokine patterns. **Objective:** To evaluate the patterns of T-helper cytokines in patients suffering from chronic and acute brucellosis. **Methods:** In this cross-sectional study, 22 individuals with acute brucellosis, 21 individuals with chronic brucellosis, and 21 healthy individuals with the same genetic background were recruited from October 2015 to April 2016. Peripheral lymphocytes were isolated and stimulated by phytohemagglutinin (PHA) and brucella antigen in cell culture. The lymphocyte proliferation was detected by MTT assay. After collecting the supernatants, and through the use of ELISA method, we quantified the interferon gamma (IFN- $\gamma$ ), interleukin (IL)-5, IL-17 and transforming growth factor-beta (TGF- $\beta$ ). **Results:** Patients with chronic brucellosis had a lower level antigen-specific stimulation index compared to those suffering from acute brucellosis ( $p=0.0001$ ). Cases with chronic brucellosis had a lower level of IFN- $\gamma$  compared to cases with acute brucellosis ( $p=0.001$ ). Finally, patients with chronic brucellosis had higher levels of IL-5 and TGF- $\beta$  in comparison with the acute group ( $p=0.01$  and  $p=0.04$ , respectively). **Conclusion:** Chronic brucellosis reduces lymphocyte proliferation and TH1 cytokine secretion, but it enhances IL-5 and TGF- $\beta$  production. Polarizing the immune responses plays a crucial part in the progression and development of chronic diseases.

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**Keywords:** Acute Brucellosis, Chronic Brucellosis, Cytokines, Lymphocyte Proliferation

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## INTRODUCTION

*Brucella* species are gram-negative, facultative anaerobic and intercellular bacteria inducing severe brucellosis in humans and animals (1). Brucellosis is endemic in many developing countries and is the most common zoonotic disease afflicting more than half a million people annually (1-3). *Brucella* species often attack the reticuloendothelial system cells and remain in the infected macrophages in different parts of the body such as the spleen, brain, heart, liver, and bone marrow (4). Following infection, most patients enter the acute phase of the disease accompanied by undulant fever (5) and, in most cases, they gradually go into remission; chronic disease occurs in 10-30% of patients (4, 5). The cellular and humoral immunity plays a major role as far as protection and recovery from infection is concerned (4, 6). The response of T helper-1 cells (TH1) is critical for the resolution of the infection (7). Nevertheless, *B. abortus* can persist for many years inside macrophages, evading host cell-mediated immune responses and establishing a chronic infection (8). The way *brucella* interferes with adaptive T cell immunity is not fully known. Research, on the other hand, has shown that human monocytes/macrophages infected with *brucella* downregulate the expression related to major histocompatibility complex class II (MHC-II) molecules, resulting in impaired MHC-II presentation pathway with induced apoptosis in CD4<sup>+</sup>T lymphocytes (8-10). It has further been documented that cases suffering from chronic brucellosis have fewer peripheral regulatory T lymphocytes (11-13). Accordingly, *brucella* infection affecting cell-mediated immunity since it reduces the T regulatory (Treg) lymphocytes over chronic infection. Based on the previous studies done on animal models, TH1 response protects against brucellosis, while TH2 response intensifies the severity of the disease (4, 10, 14-16). The role of cytokines in developing resistance against brucellosis has been mostly studied in mice (17). However, the pathophysiology of brucellosis is different in ruminants and humans.

As mentioned earlier, TH1 response has an important role in the improvement of patients' health condition. Therefore, it can be stated that the synergistic interactions of TH17 and TH1 response lead to an optimal protection (18). On the other hand, TH2 cytokines such as interleukin (IL)-5 and IL-10 enhance the pathogenesis of *brucella*, via repression of a protective TH1 response (19). Certain studies have shown high levels of transforming growth factor-beta (TGF- $\beta$ ) and IL-10 cytokines in patients infected with brucellosis (19). Therefore, the increase in TGF- $\beta$ , reduces the performance of T lymphocytes, prolongs the disease, and gradually leads to a chronic phase (16, 19). In order to better fathom the immune response, cytokine profiles, chronicity and recurrence of *brucella* infection; we evaluated the cytokine secretion in peripheral blood mononuclear cells (PBMCs) in cases suffering from acute and chronic brucellosis.

## MATERIALS AND METHODS

**Patients.** The cases of the current cross-sectional study were recruited from October 2015 to April 2016. All participants were informed of the nature and objectives of the study; they filled out a consent form based on the instructions provided by the medical ethics committee. We enrolled 22 patients with acute brucellosis and 21 patients with chronic brucellosis. The type of brucellosis was verified based on laboratory and clinical tests under the supervision of a specialist on infectious diseases. The controls

were selected from healthy volunteers whose brucella serologic tests were negative. Brucellosis was diagnosed based on clinical signs, symptoms, and bacteriological and serological tests. The diagnosis of acute brucellosis was confirmed based on the course of the disease (less than six months), clinical signs, symptoms, and laboratory findings. For this purpose, we employed positive blood cultures, antibody titers equal to or greater than 1:80 in the Wright test, or a two-fold increase in standard tube agglutination (STA) titers or in 2-ME (2-mercaptoethanol) over a period of two weeks. The chronic stage of the disease was identified in terms of low-grade fever, local symptoms of the disease, extreme weakness or fatigue, the course of the disease (more than one year), laboratory findings, and the lack of proper response to common treatments. Demographic, clinical, and laboratory data obtained from the patients are presented in Table 1.

**Table 1. Demographic data and clinical characteristic of the brucellosis patients.**

Variables	Groups	Acute	Chronic	Healthy
		brucellosis	brucellosis	control
Female		8	6	6
Male		14	15	15
Age (mean ± standard deviation)		37±17	43±10	30±4
Disease Duration(mean ± standard deviation)/month		12±6	3±2	-
Wright Titers>1: 80 (number)		16	3	0

**PBMCs separation.** The blood samples were collected under sterile conditions using sterile vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. The samples were tested less than 4 hours following collection. The blood samples were used for measuring the proliferation of lymphocytes in the whole blood. Briefly, PBMCs were separated with Ficoll-Isopaque. The dilution of blood specimens were carried out with a ratio of 1:1 in RPMI-1640 containing 5mM HEPES, 100 U/mL penicillin, 100µg /mL streptomycin, and 10% heat deactivated fetal bovine serum (FBS), all acquired from Gibco Life Technologies Inc., NY, USA. In order to separate PBMCs, they were centrifuged (600×g for 20min) using 1.077 g/mL Ficoll-Isopaque (Lymphoprep, Oslo, Norway); once separated, they were washed in RPMI-1640. The trypan blue exclusion was used to count the number of suitable cells which were further resuspended in RPMI supplemented with 10% FBS and applied to evaluate the proliferation and determine cytokine as explained in the following.

**MTT assay.** Proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) evaluation approach(20). 1µg/mL PHA or 10 µg/mL brucella antigen (100µL cell suspension/well) was used to stimulate a total number of  $2 \times 10^6$  cells/ ml in RPMI-1640 supplemented with 10% FBS. The whole

*brucellaabortus* strain 544 antigen was purchased from Pasture Institute of Iran. Subsequently, plates were incubated in a 5% CO<sub>2</sub> at 37°C for 72 hours. Next, 20 µL of 5 mg/mL MTT (5 mg/mL) was added to the cells, with the resulting mixture incubated for 4 hours. The medium was separated after centrifugation and 200 µL of Dimethyl sulfoxide (DMSO) was added to all of them. A microtiter plate reader (Stat Fax2100, USA) at 540 nm was utilized to measure the values of the optical density (OD) of the stimulated and non-stimulated cells. All tests were repeated in triplicate. The proliferation responses pertaining to MTT were stated in terms of the mean stimulation index (SI); these responses were obtained by separating the optical density (OD) values of the stimulated cells using the corresponding OD values of the non-stimulated ones.

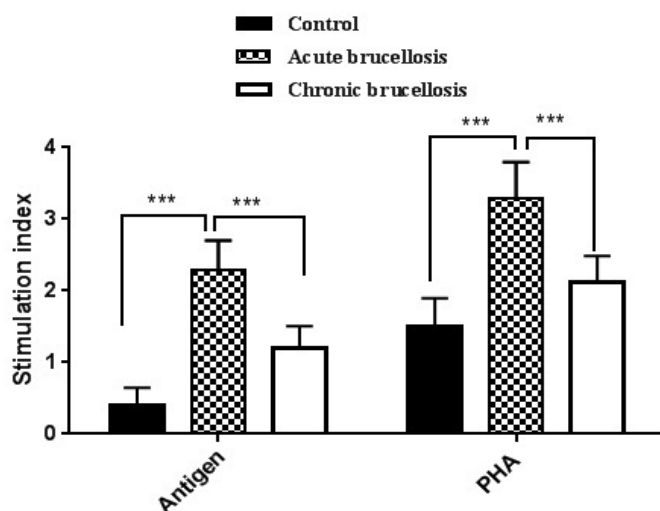
**Cytokine analysis.** To detect the cytokines, PBMC was incubated at a density of  $2 \times 10^6$  cells/mL in 1mL cultures in the presence or absence of brucella antigen (10µg /mL) and cultured under cell culture condition for 72 hours. The upper phase was collected and IFN- $\gamma$ , IL- 5, IL-17, and TGF- $\beta$  were quantified by ELISA kit (Peprotech EC Ltd, London, UK) based on the protocol proposed by the manufacturer. All specimens were examined two times and their values were measured with the microplate reader (Stat Fax 2100, USA) at wavelengths of 450 and 650 nm. The sensitivity related to IFN- $\gamma$ , IL-10, and TGF- $\beta$  was 2, 4.1, and 16pg /ml, respectively.

**Statistical analysis.** Results of statistical analysis were expressed as mean  $\pm$  SD. Mann-Whitney U test was used to evaluate the statistical significance (to compare two sample means), and student's t-test was made use of so as to assess whether the means of the two groups were statistically different from each other; a P value less than 0.05 indicated a significant difference.

## RESULTS

### Lymphocyte proliferation.

The level of SI in the presence of PHA in the chronic brucellosis patients ( $1.2 \pm 0.45$ ) was considerably lower compared to cases with acute brucellosis ( $2.35 \pm 0.33$ ), (Fig. 1). Also, there was a significant difference in SI values concerning the brucella antigens of the acute and chronic brucellosis patients ( $p=0.003$ , Fig. 1).



**Figure 1. Cell proliferation assay.** Culturing of PBMC related to the brucellosis cases was performed three times in the existence of PHA (1 µg/mL) and brucella antigen (10 µg/mL). After 72h, MTT reduction was applied to evaluate the proliferation. Data were presented as mean  $\pm$  standard deviation. \*\*\*;  $p \leq 0.0001$ .

### Cytokines production.

The concentrations of IL-5, IL-17, TGF- $\beta$  and IFN- $\gamma$  in the brucellosis cases (acute and chronic) were recognizably higher than that in the control group ( $p=0.0001$ ). Moreover, the chronic brucellosis group had lower concentrations of IFN- $\gamma$  ( $410 \pm 130$  pg/mL) compared to patients suffering from acute brucellosis ( $893 \pm 683$  pg/mL,  $p=0.0001$ ). Finally, chronic brucellosis cases showed more concentrations of IL-5 and TGF- $\beta$  compared with acute cases ( $p=0.01$  and  $p=0.04$ , respectively). No significant difference, on the other hand, was observed between acute and chronic brucellosis patients regarding the level of IL-17 (Table 2).

**Table 2. Concentrations of IFN- $\gamma$ , IL-5, IL-17 and TGF- $\beta$  in supernatant of PBMCs of brucellosis patients cultured in the presence of brucella antigen.**

Cytokines	Groups			p value*
	Control (n=21)	Acute brucellosis (n=22)	Chronic brucellosis (n=21)	
IFN- $\gamma$ (pg/mL)	189 $\pm$ 134**	893 $\pm$ 683	410 $\pm$ 130	0.0001
TGF- $\beta$ (pg/mL)	31 $\pm$ 18	137 $\pm$ 38	194 $\pm$ 72	0.0400
IL-5 (pg/mL)	55 $\pm$ 32	292 $\pm$ 77	371 $\pm$ 103	0.0100
IL-17 (pg/mL)	90 $\pm$ 69	150 $\pm$ 92	190 $\pm$ 132	NS

\*Comparison between acute and chronic brucellosis. \*\* Mean  $\pm$  standard deviation.

## DISCUSSION

*Brucella* species usually multiply in reticuloendothelial cells and the emergence of immunity relies on appropriate responses to host cells and TH1 cytokine production (4). It is well known that the components of cellular immunity, especially TH1 cells, play a vital role in early stages of infection (14, 19) where TH1 immune response is essential for the clearance of brucellosis (14). Brucellosis can occur in acute, focal and chronic forms (21). However, it is not yet clear why certain individuals have the acute form while others develop the chronic form of the disease. Recent studies have demonstrated that *B. abortus* can both directly and indirectly modulate T-cell function and *B. abortus* downregulates the MHC-II molecules expression on antigen presenting cells. Research has documented that that patients with chronic brucellosis present diminished peripheral Treg cells percentages as well as inversion of the CD4/CD8 ratio (10-13, 22). *B. abortus* can prompt T lymphocytes apoptosis in an APC-independent manner (9). The results of our study indicated that PBMCs of chronic brucellosis patients had a lower proliferative response to particular brucella antigen or mitogen (PHA), compared to acute brucellosis patients. In addition, lymphocytes in cases suffering from chronic brucellosis had a low blastogenic response to brucella antigen compared with PHA and PPD (23). Such reduction in the response of T cell proliferative to PHA in chronic brucellosis cases may be associated with T-cell anergy.

Skendroset *al.* studied the cellular immunity responses that may describe the defective TH1 immunity responses and the T cell anergy expressed in cases suffering from chronic brucellosis (11). However, exposure of lymphocytes to brucella for a long time in chronic cases may induce anergy in peripheral T lymphocytes. It is noteworthy that untreated infection in cases with chronic brucellosis may be related to cytokine network imbalance (17). More specifically, the PBMCs of chronic anergic brucellosis cases, when stimulated by brucella protein, have a considerably low production of IFN- $\gamma$  (10). Previous studies have shown that LPS, PrpA and Btp1/TcpB are the three important immunomodulatory molecules with the capacity to interfere with host immune response (24). Moreover, these molecules are able to inhibit the secretion of IFN- $\gamma$  or to increase the production of IL-10 (19, 24). Our results show that PBMCs from chronic brucellosis patients secrete low IFN- $\gamma$  to specific brucella antigens compared to acute brucellosis patients. Also, the levels of TGF- $\beta$  and IL-5 in the chronic brucellosis patients were significantly higher than the acute group. No prominent difference was seen between the acute and chronic cases as far as the level of IL-17 is concerned. There exist a myriad reports as to the fact that TH1 cytokines produce a large amount of serum or intracytoplasmic (IFN- $\gamma$  mainly) in acute brucellosis that was decreased or normalized after sufficient treatment with antibiotic (21, 25, 26). Lately, it has also been posited that TGF- $\beta$ 1 mediates unresponsiveness to brucella antigens in cases with chronic brucellosis (16). These outcomes suggest that at the beginning of human brucellosis, the domineering factor is the response of TH1; nonetheless, it diminishes with the prolongation of the illness, possibly because TH2 switches and/or releases immune-regulative cytokines including TGF- $\beta$ 1 (16). However, the change in T-cell function, the reduction in TH1 cytokines such as IFN- $\gamma$ , and possibly IL-17 and the increase in TH2 cytokine production is probably owing to the clinicopathological manifestations of chronic or relapsing brucellosis (21, 27). It has to be noted that IL-10 plays an important role in the induction TH2 response. IL-4 promotes the TH-2 response and suppresses TH1 cell responses through the upregulation of IFN $\gamma$  transcriptional repressor and IL-17 production. The main limitation of this study was that the levels of IL-4 and IL-10 were not measured. We strongly recommend measuring the levels of IL-4 and IL-10 cytokines in chronic brucellosis patients in the future studies. In conclusion, comparing the acute and chronic cases, the latter has a lower proliferation index. The *in vitro* detection of cytokine profile in chronic and acute cases indicates a shift in TH2 immune response. However, the polarization of the immune responses to TH1 may play an important role in prevention of chronic infection.

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