

# Evaluation of Regulatory T Cells in Patients with Acute and Chronic Brucellosis

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## Abstract

**Background:** Brucellosis is one of the most common chronic diseases, with widespread distribution. In spite of cell-mediated immunity (CMI) modulated mainly via activated T-helper type 1 (Th1) cells, brucellosis can advance to chronic disease in about 10-30% of cases. Regulatory T cells (Treg cells) are involved the immune response to brucellosis; however, their role, particularly in the change from the acute to the chronic phase, have not yet been elucidated. The main hypothesis of this study was that Treg cells play critical roles in the progression of brucellosis from the acute to the chronic phase.

**Methods:** Forty-eight unrelated subjects participated in this case-control study. The percentages of CD4+, CD25+, FoxP3+, and CD25/FoxP3+ T cells in the peripheral blood mononuclear cells (PBMCs) of acute (AB) and chronic brucellosis (CB) patients and healthy controls were determined by flow cytometry. The mean fluorescence intensities (MFIs) of CD4+, CD25+, and FoxP3+ T cells were also measured.

**Results:** We found a significantly lower percentage of CD25/FoxP3+ Treg cells in CB than in the AB and control groups ( $p < 0.05$ ). Also, CD4 and CD25 MFIs were significantly less in CB than in AB and controls ( $p < 0.05$ ).

**Conclusions:** We propose that the reduced number of CD25/FoxP3+ Treg cells in the CB group leads to T cell anergy and this contributes to the development of chronic infection.

**Keywords:** Brucellosis, CD25 Antigen, FoxP3 Protein, Regulatory T cells

## Introduction

Brucellosis is one of the most common chronic diseases, with widespread distribution. It is caused by the genus *Brucella* as a facultative intracellular bacteria (1, 2). Brucellosis is transmitted to humans through skin contact with infected animals or consumption of *Brucella*-infected dairy products (2). In the Middle East, brucellosis is endemic; however, control and eradication projects are underway (1-3).

The immune response against *Brucella* occurs via cell-mediated immunity (CMI), mainly involving activated antigen-presenting cells (APCs) and T-helper type 1 (Th1) cells (4). *Brucella* avoids activation of innate and adaptive immune responses through various mechanisms

that result in chronic infection in about 10-30% of cases (5-7). Patients with chronic brucellosis show impaired immune response via the Th1 cells because of T cell anergy or diminished CD4+ T cell numbers (7). Furthermore, regulatory T cells (Treg cells), which are components of the immune system, are involved in immune responses to brucellosis; however, their role, particularly in the change from the acute to the chronic phase, have not yet been elucidated.

Treg cells, which contribute to immunomodulation and suppression, have been a popular research topic in the field of immunology (8-10). Treg cells are characterized by CD4, CD25, and Foxp3 expression and represent about 5-10%

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of peripheral CD4<sup>+</sup> T cells (11). The peripheral circulation of patients with chronic brucellosis (CB) contains fewer IL-2 alpha receptor (CD25) – positive CD4<sup>+</sup> T cells than that of patients with acute brucellosis (AB), even after in vitro mitogen-stimulation (7, 12, 13).

Although Treg cell play important roles in the immunopathology of various infectious diseases, their role in brucellosis has not yet been clarified (14, 15). In different studies, brucellosis as one of the most common chronic diseases shows contradictory results regarding to Treg cells (7, 14). So, the main hypothesis of this study was that Treg cells play critical roles in the progression of brucellosis from the acute to the chronic phase. In this regard, the percentage of Treg cells, based on CD4, CD25, and FoxP3 expression, were analyzed

in the peripheral blood of AB and CB patients and healthy controls.

## Materials and Methods

### Study populations

Forty-eight unrelated subjects participated in this case-control study. Our study was performed at the Emergency Department of Vali-e-Asr Hospital, Arak, Iran. Written consents were received from all participants and the experimental protocol for blood collection for was approved by the Human Ethics Committee of Arak University of Medical Sciences, Arak, Iran (No. 961). Sixteen patients had confirmed AB, 16 had CB, and 16 age-matched healthy subjects were classified as controls (Table 1).

**Table 1.** Demographic data and clinical characteristic of the studied groups

Groups	AB (n=16)	CB (n=16)	Control (n=16)
Age (years), mean $\pm$ SD	42 $\pm$ 16.16	49.4 $\pm$ 13.9	36.6 $\pm$ 5.7
Fever	16	6	0
Sweating	12	6	0
Chills	16	7	0
Malaise/fatigue	12	10	0
Arthralgias	12	4	0
Lumbar pain	13	9	0
Myalgias	12	10	0

AB: acute brucellosis; CB: chronic brucellosis; SD: standard deviation

### Inclusion and exclusion criteria

The AB and CB groups were included and categorized in this study based on disease history, clinical data (Table 1), and laboratory findings. The laboratory tests for diagnoses of brucellosis were based on *Brucella* species isolation from blood cultures and anti-*Brucella* antibodies detection. Patients positive in the past year for the Wright agglutination test ( $\geq 1:320$ ) or the Coombs test ( $\geq 1:160$ ) were defined as CB, while patients whose

antibody titers peaked two weeks after the onset of infection were defined as AB. Subjects in the healthy control group were all negative for the Wright and Coombs tests, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR). Patients with other diseases such as infectious, neoplastic, or autoimmune were excluded from the study. Patients receiving antibiotics, immuno-stimulating agents, or vaccinations were also excluded from the study.

### Sample preparation

At least 3 ml venous blood samples were collected from the patients and controls and stored in tubes containing ethylenediamine tetraacetic acid (EDTA). After mixing, 50 µl of the blood samples were transferred into round-bottomed centrifuge tubes for staining using surface antibodies including peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 and phosphatidylethanolamine (PE)-conjugated anti-CD25. Following 30 min incubations, the erythrocytes were lysed by adding 1 ml of BD FACS Lysing Solution (BD Biosciences, Bedford, MA), followed by incubation for 10 min at room temperature. After washing with phosphate-buffered saline (PBS), the cells were fixed using BD CellFix Solution (BD Biosciences, Bedford, MA), permeabilized using 0.5% saponin (Sigma-Aldrich, Germany), and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-FoxP3. In the flow cytometry analysis, appropriate PE-, PerCP-, and FITC-conjugated antibodies were included for isotype controls. All antibodies were obtained from BD Biosciences (BD Biosciences, Bedford, MA).

### Flow cytometry analysis

Ten thousand prepared and stained cells were analyzed using a FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA) and Flowjo

(Treestar, Ashland, OR) software. Lymphocytes were gated on the basis of forward and side scatter, and the percentages of CD4+, CD25+, FoxP3+, and CD25/FoxP3+ cells were determined. Furthermore, the mean fluorescence intensities (MFI) of the CD4+, CD25+, and FoxP3+ T lymphocytes were separately calculated.

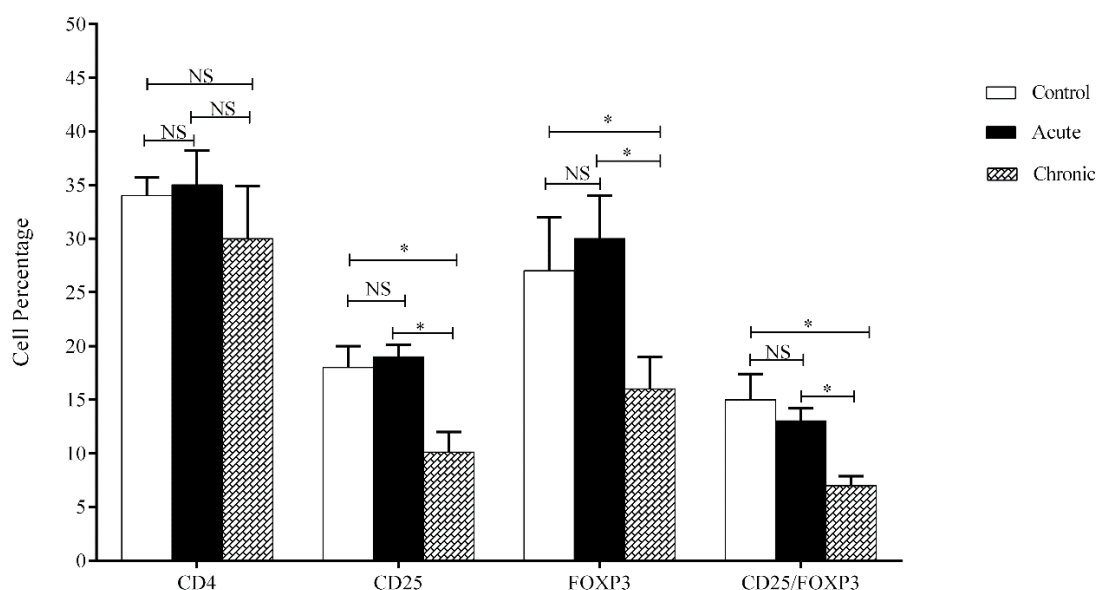
### Statistical analysis

Statistical analyses included the independent sample t-test to evaluate differences between variables in the groups. Groups were compared using the Mann-Whitney U-test. Data were analyzed by SPSS software version 16.0 (SPSS, Inc., Chicago, Illinois, USA) and represented as means  $\pm$  standard deviations (SDs).  $P$  values  $< 0.05$  were considered statistically significant.

### Results

#### Percentages of CD4+, CD25+, FoxP3+, and CD25/FoxP3+ T lymphocytes

The percentages of CD4+, CD25+, FoxP3+, and CD25/FoxP3+ T lymphocytes were analyzed by flow cytometry. No significant differences in CD4+ percentages were seen between the three groups ( $P > 0.05$ ). The percentages of CD25+, FoxP3+, and CD25/FoxP3+ T lymphocytes were all significantly less in the CB than in the AB and control groups ( $P < 0.05$ , Fig 1).

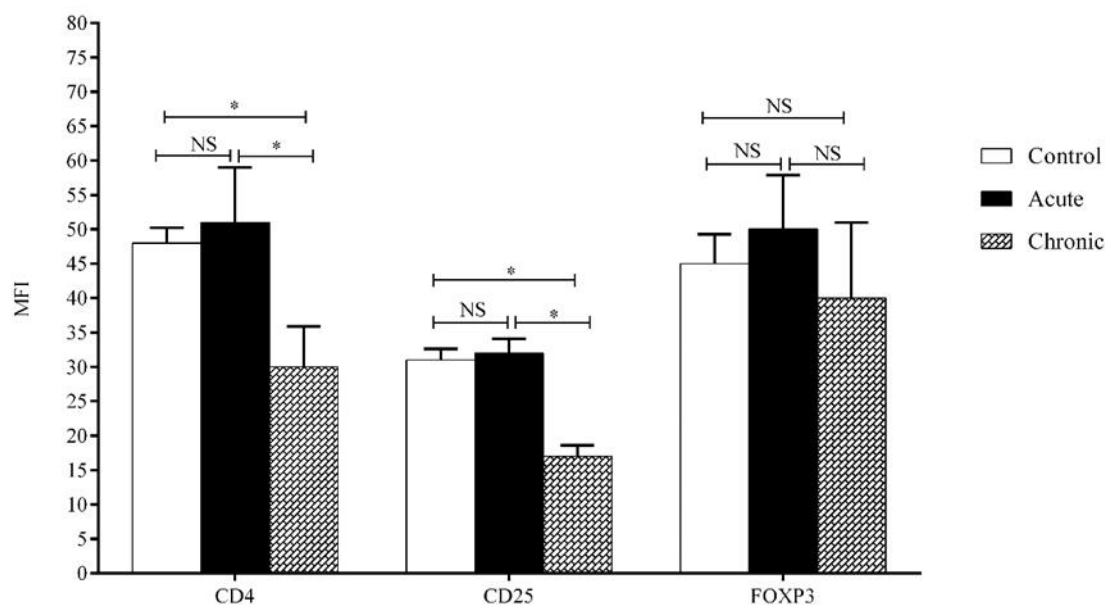


**Fig. 1.** Percentages of lymphocytes with different molecular patterns in the studied groups. The bar graph represents the percentages of CD4+, CD25+, FoxP3+, and CD25/FoxP3+ T lymphocytes in acute, chronic, and control groups (\*:  $P < 0.05$ ; NS:  $P > 0.05$ ).

### The MFIs of CD4+, CD25+, and FoxP3+ T lymphocytes

The MFIs of CD4+, CD25+, and FoxP3+ T lymphocytes were determined after flow cytometry. The MFIs of the CD4+ and CD25+ T cells in the CB group were significantly less

than those of the AB and control groups ( $P < 0.05$ ). The MFIs of the FoxP3+ T cells in the three groups were not significantly different (Fig. 2).



**Fig. 2.** Mean fluorescence intensities (MFIs) of lymphocytes with different molecular patterns in the studied groups. The bar graph represents the MFIs of CD4+, CD25+, and FoxP3+ T lymphocytes in acute, chronic, and control groups (\*:  $P < 0.05$ ; NS:  $P > 0.05$ ).

## Discussion

*Brucella* is an intracellular organism whose eradication requires adaptive CMI. In response, brucellosis can convert from acute to chronic form via modulation of host immune responses that have not yet been specifically characterized. Therefore, in this study, to evaluate the role of Treg cells in patients with different clinical forms of brucellosis, especially those with chronic infections, percentages and MFIs of T cells expressing important Treg cell markers, including CD4, CD25, and FoxP3, were determined. In agreement with other studies (7, 13, 16-19), we found the MFI of CD4+ T cells to be significantly less in CB than in the AB and control groups; however, the percentage of CD4+ T lymphocytes was not significantly different in CB from the other groups. Previous studies also showed that patients with CB display lower CD4+ T-cell proliferation, CD4+/CD25+ T cell counts, Th1 responses to PHA, and IL-2 and IFN $\gamma$  production,

than patients with AB (7, 13). Although there are published contradictory results regarding to Treg cells. For example, Bahador et al. showed that Treg cells in acute and chronic brucellosis are increased compare to healthy control (14).

Another study showed that the functional impairment of CD4+ T lymphocytes in CB could be responsible for the relapses displayed in CB patients (18). According to Sridevi et al. (13), stimulation indices of PHA-stimulated CD4+ T cells of patients with CB were significantly less than those of the AB and control groups. Furthermore, upregulation of CD152 (CTLA-4) on CD4+ T lymphocytes in patients with CB could mirror the impairment of CD4+ T lymphocytes (19). Based on these and other data, we suggest that the reduced number of CD4+ T lymphocytes with lower MFIs, as the most important immune response against brucellosis,

results in the shift to the from the acute to the chronic form of infection.

In agreement with other studies, we found a lower percentage of CD25/FoxP3<sup>+</sup> Treg cells in CB than in the other groups. For example, Skendros et al. (2007) found that the percentage of Treg cells was less in CB patients, especially those with relapsing brucellosis, than in AB patients (7). Other studies showed that the percentage of regulatory T lymphocytes was greater in AB patients than in healthy controls (7, 20, 21). These results suggest that Treg cells are inactivated in CB patients, and continuous Treg cell inactivation would allow the establishment of chronic of infection.

Based on previous studies, one of the most important mechanisms that could explain the reduction of Treg cells in infected patients with CB is anergy induction, because of a deficiency in costimulatory signals (7, 13, 22, 23). In line with this hypothesis, lower IL-2 production by PBMCs after stimulation with PHA or Brucella antigens

has been correlated with induced anergy in patients with CB (16, 24).

In conclusion, in agreement with other studies, we found a lower percentage of CD25/FoxP3<sup>+</sup> Treg cells in CB than in the AB and control groups. We also found that the MFIs of CD4<sup>+</sup> and CD25<sup>+</sup> T cells were significantly less in CB than in the AB and control groups. Based on the our findings, we propose that reduced MFI of CD4<sup>+</sup> T lymphocytes and Treg cells lead to T-cell anergy, which may be responsible for the shift from acute to chronic brucellosis.

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The authors declare that they have no conflicts of interest.

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