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ORIGINAL ARTICLE

Frequencies of CD4+ T Regulatory Cells and their CD25^{high} and FoxP3^{high} Subsets Augment in Peripheral Blood of Patients with Acute and Chronic Brucellosis

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Abstract
Objectives: Brucellosis remains one of the most common zoonotic diseases worldwide. In humans, brucellosis can be a serious, debilitating, and sometimes chronic disease. Different mechanisms can be postulated as to the basis for the induction of the chronic status of infectious diseases that T regulatory cells are one of the most important related mechanisms. The current study was designed to determine whether percentage of CD4+Treg cells and their CD25^{high} and FoxP3^{high} subpopulations in peripheral blood are changed in human brucellosis samples in comparison to a control group.
Methods: In total, 68 brucellosis patients (acute form: $n = 43$, chronic form: $n = 25$) and 36 healthy volunteers entered our study. After isolating of peripheral blood mononuclear cells, heparinized venous blood samples were obtained from both patients and healthy donors, CD4, CD25, and FoxP3 molecules were evaluated by two- and three-color flow cytometric methods.

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Results: The results revealed a new finding in relation to Treg cells and human brucellosis. The numbers of $CD4^{+}$Treg cells and their $CD25^{high}$ and $FoxP3^{high}$ subsets increase significantly in the peripheral blood of acute and chronic forms of brucellosis samples compared with healthy groups, with this increase being greater in the chronic group.
Conclusion: There seems to be a correlation between increase of CD4+Treg cells and their subsets and the disease progress from healthy state to acute and chronic brucellosis.

1. Introduction

Brucellosis is an intracellular bacterial infection with worldwide distribution with > 500,000 human cases reported annually [1,2]. The disease is caused by organisms of the genus *Brucella*, especially by *Brucella melitensis*, and is known as the most common zoonotic disease [3]. In humans, the disease culminates in high clinical morbidity and diverse clinical manifestations, as any organ can be affected [4]. Despite early diagnosis and treatment, approximately 10−30% of patients develop chronic disease [5,6].

Host protection against intracellular pathogens such as *Brucella* spp. depends on cell-mediated immunity (CMI) [7,8]. *Brucella* can survive within macrophages of the host and invades the normal mechanisms of bacterial killing, causing chronic disease [6,7]. Th1 related cytokines [interleukin (IL)-12 and interferon-γ], which are fundamental for the clearance of brucellosis agent, and percentages of *ex vivo* and phytohemagglutinin (PHA)-cultured CD4+ T lymphocytes are found to be diminished in chronic patients [9−11].

In recent years, regulatory T cells (Tregs) have become a popular subject for immunological research. Numerous reports shed light on the major aspects of Treg biology in humans, with the characterization of different T-cell subpopulations, including naturally occurring CD4+ CD25+Tregs, induced Tregs [IL-10 producing CD4+ type I regulatory T cells (Tr1) and T helper type 3 (Th3) cells], and CD4+ CD25+ T cells that develop in the periphery by conversion of CD4+ CD25− T cells. All these different T-cell populations with regulatory function coexist and contribute to immune suppression [12−14].

There is now considerable evidence that CD4+ CD25+ Tregs represent a stable population of peripheral lymphocytes. These CD4+ CD25+ Tregs represent about 5−10% of human CD4+ T cells, and are commonly identified by the constitutive expression of IL-2 receptor α (IL-2Ra; CD25), as well as the transcription factor scurfin, encoded by the forkhead family transcription factor 3 (Foxp3) gene [15], and are also characterized by their very low levels of proliferation on T-cell receptor (TCR) stimulation *in vitro*. The CD4+ $CD25^{high}$ subset (which has high levels of CD25 expression) in normal individuals comprises about 1−3% of human circulating CD4+ T cells. Unlike the total population of CD4+ CD25+ T cells, these CD4+ $CD25^{high}$ cells constitutively expressing FoxP3 molecules at high levels can significantly inhibit the proliferation and cytokine secretion induced by TCR cross-linking of CD4+ CD25− responder T cells, CD8+ T cells, dendritic cells, natural killer cells, and B cells [16−20]. However, FoxP3 is emerged transiently in $CD25^{low}$ CD4+T lymphocytes, which may be without suppressive activities; but they contain effector functions [21].

The role of CD4+$CD25^{hi}$T and CD4+$FoxP3^{hi}$T cells in immunopathology of *Brucella* infection has not yet been elucidated [22]. So, in our study these cells were evaluated to determine two substantial questions: is the frequency of CD4+$CD25^{high}$T, CD4+$FoxP3^{high}$T, CD4+$CD25^{high}$FoxP3+, and CD4+CD25+$FoxP3^{high}$ cells increased in the peripheral blood (PB) of patients with acute or chronic brucellosis as a direct result and, if so, can these Treg cells impinge on antibacterial immune responses as an inductive result, like other infectious diseases.

2. Materials and methods

2.1. Participants

There were 104 unrelated participants enrolled in the study: 68 brucellosis patients; and 36 healthy age- and gender-matched volunteers who were used as controls. According to disease history, clinical picture, and laboratory findings, patients were divided into acute (AB) and chronic (CB) brucellosis groups (Table 1).

The AB group included 43 consecutive patients. All AB patients had a disease duration ≤8 weeks (mean ± standard deviation, 3.5 ± 2.3 weeks). The diagnosis was based on compatible clinical picture (Table 2) in combination to high serum titers of anti-brucellar antibodies or fourfold increase of the initial titers in two paired samples drawn 2 weeks apart. In addition, in 19 out of 43 AB patients, *Brucella melitensis* was isolated in blood culture. In 33 patients brucellar DNA was detected by PCR analysis in the blood and the serum.

The CB group was composed of 25 patients. All CB patients had disease duration ≥6 months [23]. Thirteen of 22 CB patients had also positive PCR analysis in the blood.

Table 1. Demographic data of the groups (acute, chronic, and control) studied

	AB	CB	Healthy control
N	43	25	36
Female	14	8	13
Male	29	17	23
Age (mean ± standard deviation, y)	43.5 ± 19.2	48.7 ± 14.3	42.6 ± 15.2

AB = acute brucellosis; CB = chronic brucellosis.

High specific titers were defined by anti-brucellar antibodies, which were considered for the Wright agglutination test ≥1:320, for the Coombs' agglutination test ≥1:320, and for the complement fixation test ≥32. Healthy volunteers were tested serologically for brucellosis and found to be negative.

2.2. Flow cytometry

Surface CD4 and CD25, and intracellular FoxP3 expression were assessed by flow cytometry using the eBioscience human regulatory T cell staining kit Number 3 (eBioscience, San Diego, CA, USA). In brief, PB mononuclear cells (PBMCs) were obtained by Ficoll–Hypaque density gradient centrifugation. Cells were incubated with FITC anti-human CD4 (RPA-T4) and PE anti-human CD25 (BC96) for 30 minutes at 4°C.Then, after twice washing and fixing with fixation/permeabilization solutions, the cells were stained with PE-Cy5 anti-human FoxP3 (PH101), and incubated similarly. Finally, two- and three-color fluorescence flow cytometric analysis by a BD FACS Calibur system were used to demonstrate the frequency of CD4+CD25+, CD4+ FoxP3+ and CD4+CD25+FoxP3+ cells in PB samples.

Table 2. Clinical characteristic of the patient groups studied

	AB (n = 43)	CB (n = 25)
Symptoms		
Fever	43	6
Sweating	27	3
Chills	30	5
Malaise/fatigue	24	17
Arthalgias	19	10
Lumbar pain	*16*	6
Headache	13	
Myalgias	11	10
Focal disease		
Spondylitis	5	3
Sacroiliitis		3
Epididymoorchitis	3	
Meningoencephalitis	6	

AB = acute brucellosis; CB = chronic brucellosis.

2.3. Statistical analysis

Parametric statistical tests were applied as the variables were distributed normally (Kolmogorov–Smirnov test). Data were analyzed by one-way analysis of variance using SPSS version 21 (SPSS Inc., Chicago, IL, USA) and were represented as mean ± SD. A p value $<0{\cdot}05$ was considered to be statistically significant.

2.4. Ethics statement

Our study was confirmed by the ethical committee of Tehran University of Medical Sciences, Tehran, Iran. The participants were informed about the study and they chose whether or not to participate in this study.

3. Results

3.1. CD4+Treg cells and their subsets

The percentages of Treg cells were calculated as the frequencies of CD4+CD25+, CD4+CD25^{high}, CD4+FoxP3+, CD4+FoxP3^{high}, CD4+CD25+FoxP3+, CD4+CD25highFoxP3+, and CD4+CD25+FoxP3^{high} cells in the CD4+ population of PBMCs, and their frequencies were 6.7%, 2.0%, 1.5%, 0.4%, 1.4%, 0.41%, and 0.4% of CD4+ cells, respectively, in PBMCs of healthy age-matched controls (Table 3, Figures 1 and 2). FoxP3+ cells, similar to CD25+cells, were classified into two subsets (FoxP3^{high} and FoxP3^{low}) based on the intensity of FoxP3 expression (Figure 3).

The proportions of CD4+CD25+ cells included 16.6% in AB and 27.6% in CB patients. Also, the CD25^{high} subset of these cells contained 3.9% in AB and 6.8% in CB patients (Table 3, Figures 1 and 2).

CD4+FoxP3+ cells accounted for 5.7% of CD4+T-cells in AB and 10.6% of CD4+T cells in CB patients. In total CD4+lymphocytes, FoxP3^{high} subpopulation consisted of 1.6% and 2.9% in AB and CB, respectively.

In patients with AB, CD4+CD25+FoxP3+cells comprised 5.06% of total CD4+ lymphocytes; in CB patients, percentage of these cells was 9.5% of total CD4+ lymphocytes (Table 3).

CD4+CD25^{high}FoxP3+cells subtended 1.28% of CD4+ lymphocytes in AB and 2.45% in CB patients. Moreover, frequencies of CD4+CD25+FoxP3^{high} cells encompassed 1.41% and 2.03% of CD4+ lymphocytes in attribution of AB and CB patients, respectively (Table 3, Figure 3).

3.2. Correlation between the level of CD25 expression in CD4+ lymphocytes and CD4+FoxP3+cells

Correlation coefficients of CD4+CD25^{high} cells and CD4+FoxP3+ cells in the three groups consisted of R = 0.792, $p < 0.001$ (healthy control), R = 0.817,

Table 3. Frequencies of Treg cell subsets determined by surface markers (CD4 and CD25) and intracellular FoxP3 levels ($p < 0.01$)

	Control	Acute	Chronic
Expression of surface markers (%)			
CD4+CD25+ cells	6.7 ± 1.8	16.6 ± 4.2	27.6 ± 6.4
CD4+CD25^{low} cells	4.7 ± 1.3	12.6 ± 3.4	21.2 ± 4.6
CD4+CD25^{high} cells	2.0 ± 0.5	3.9 ± 1.1	6.8 ± 2.1
Expression of intracellular Foxp3 molecule (%)			
CD4+Foxp3+cells	1.5 ± 0.6	5.7 ± 1.8	10.6 ± 2.5
CD4+Foxp3^{low} cells	1.2 ± 0.5	4.0 ± 1.2	7.7 ± 1.5
CD4+Foxp3^{high} cells	0.4 ± 0.2	1.6 ± 0.4	2.9 ± 0.60
CD4+CD25+FoxP3+cells	1.4 ± 0.48	5.06 ± 1.37	9.5 ± 2.06
CD4+CD25^{low}FoxP3+cells	1.11 ± 0.5	3.76 ± 0.94	7.09 ± 1.25
CD4+CD25^{high}FoxP3+cells	0.41 ± 0.18	1.28 ± 0.47	2.45 ± 0.57
CD4+CD25+FoxP3^{low}cells	1.18 ± 0.48	3.57 ± 1.11	5.28 ± 1.32
CD4+CD25+FoxP3^{high}cells	0.4 ± 0.2	1.41 ± 0.38	2.03 ± 0.4

Data are presented as mean ± SD.

$p < 0.001$ (AB), and R = 0.661, $p < 0.001$ (CB) versus correlation between CD4+CD25^{low} cells and CD4+FoxP3+ cells with coefficients including R = 0.680, $p < 0.001$ (healthy control), R = 0.586, $p < 0.001$ (AB), and R = 0.483, $p = 0.014$ (CB; Figure 4).

4. Discussion

Regulatory T cells (Treg cells) were originally identified through their function in controlling autoimmunity, where they mediate immunological tolerance to self-antigens [24]. However, it later became apparent

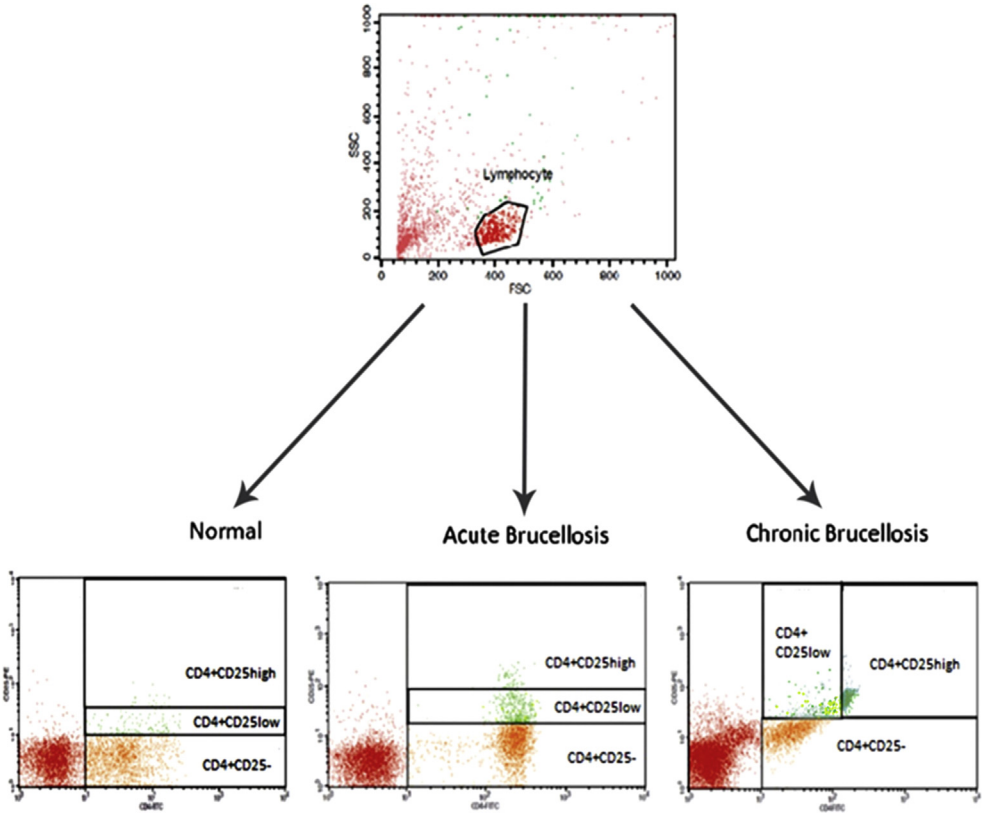

Figure 1. Two-color flow cytometric analysis of CD4+CD25+ cells and subdivision of these cells tool into CD4+CD25^{high} Treg cells and CD4+CD25^{low} cell subsets. First, lymphocytes gated in FSC (forward light scatter)−SCC (side light scatter) dot plot and then CD4+CD25- and CD4+CD25+ (high and low) gated in CD4-FITC intensity versus CD25-PE intensity.

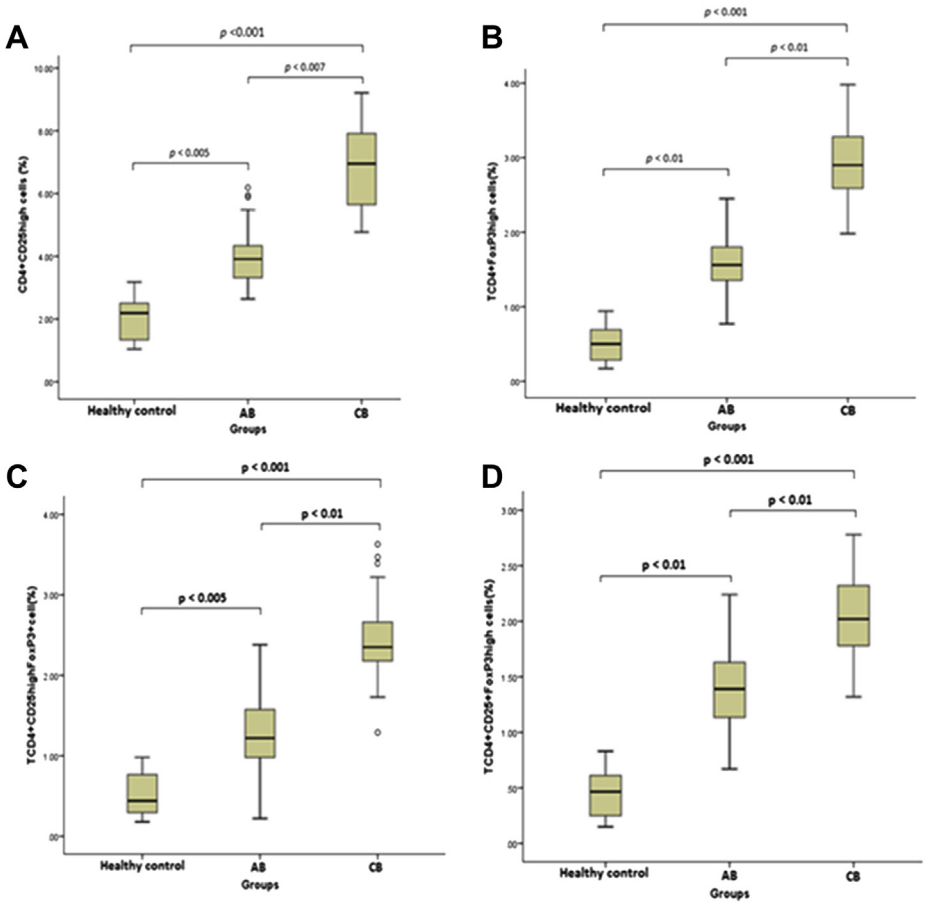

Figure 2. Percentages of (A) CD4+CD25^{high}, (B) CD4+FoxP3^{high}, (C) CD4+CD25^{high}FoxP3+, and (D) CD4+CD25+FoxP3^{high}. Treg cells within lymphocytes in the peripheral blood of healthy control ($n = 36$), AB ($n = 43$), and CB ($n = 25$) groups. Difference among groups was analyzed through the one-way analysis of variance test; and p values are shown on the figure; ° represents extreme values.

that Treg cells also control immune responses to foreign antigens on pathogens [25]. The function of Treg cells during infection appears to be primarily to prevent collateral damage from unrestrained immune responses to the pathogen, with a number of studies demonstrating enhanced immunopathology with defective or depleted Treg cells. It also seems that the induction of Treg cells during infection is an immune subversion strategy engaged by certain pathogens in order to last their survival in the host [26].

In agreement with other studies [11,27], we found that the percentage expression of IL-2 receptor α on peripheral CD4+lymphocytes in the healthy group was <10% (data not shown). Also, in the current study, > 90% of FoxP3+ cells in all three groups were CD4+FoxP3+ (data not shown), which implies that the majority of FoxP3+Treg cells in healthy individuals, AB, and CB patients manifest surface CD4 molecules.

In our study, the percentage of peripheral CD4+CD25+(low or high) T-lymphocytes was increased in AB patients, as compared to controls, suggesting that helper T-cells are activated in AB. This finding adds further support to data presented by other researchers [11,28,29] and it approves that CD25 is upregulated in AB patients. CB patients showed significantly increased percentage of CD25 expression on peripheral CD4+CD25^{high} lymphocytes in comparison to AB patients. This seems to be in contrast with a previous study by Skendros et al [11], which showed diminished percentage in CD25 expression on peripheral CD4+T cells in compared with AB. But, they use PHA in the culture of obtained PBMC for activating and proliferating of lymphocytes and for this the acute brucellosis group has the higher level of CD4+CD25^{low} cells as CD4+T-helper cells, were activated and proliferated more than other groups. By contrast, the increase of CD4+CD25^{high} cells in PB of CB patients and induced suppression of T cells by this Treg cells, appears to be a substantial reason for the significant reduction in the percentage of PHA-proliferating TCD4+CD25+ cells and anergy induction in CB [30].

In addition, in our research, there were significant increases in the proportions of CD4+FoxP3+cells and their FoxP3^{high} subpopulation, as well as CD4+CD25+FoxP3+ cells and their CD25^{high} or FoxP3^{high} subsets in CB in comparison to AB patients

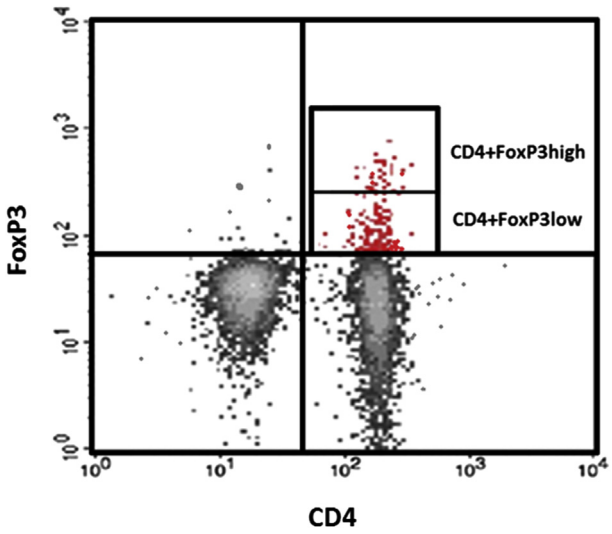

Figure 3. Classification of CD4+FoxP3+ cells based on levels of intracellular FoxP3 expression (FoxP3^{high} and FoxP3^{low}) via flow cytometric two-color method.

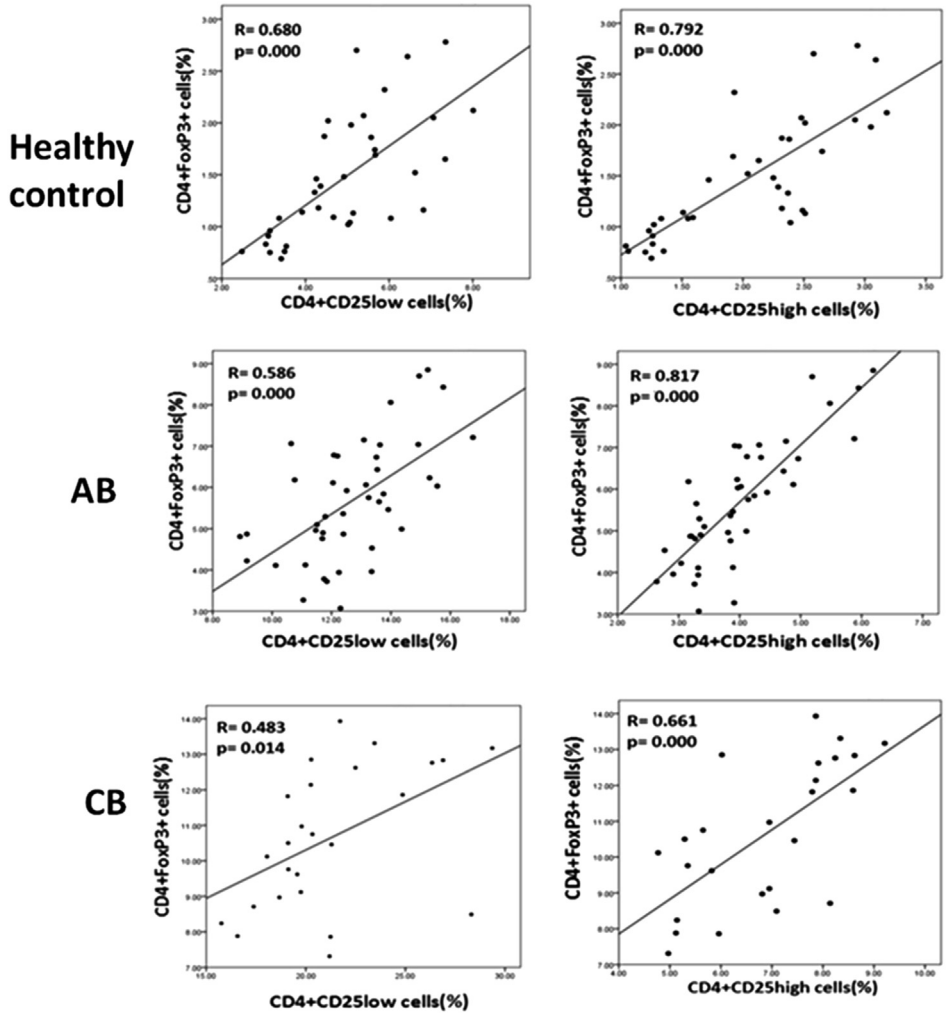

Figure 4. Correlation of CD4+FoxP3+ cells with CD4+CD25+ cells. CD4+CD25^{high} cells exhibited more vigorous power of coefficient to CD4+FoxP3+ cells than to CD4+CD25^{low} T cells in all three groups (control, acute brucellosis, chronic brucellosis). Each row was assigned to the one group, in which the left and the right diagrams depict the correlation among frequencies of CD4+FoxP3+ cells with CD4+CD25^{low} or CD4+CD25^{high} cells, respectively.

and healthy controls. Also, CD4+CD25^{high} Treg cells in the AB group showed a significant increase in frequencies compared with the control group.

According to Curiel et al [31], FoxP3 expression in low levels can cause differentiation of Treg cells to Th2 or Th17 cells. So, the intensity of FoxP3 expression is crucial in the state of Treg suppressive activity. In fact, the presence of FoxP3 molecule maybe not only function as an on-and-off switch to offer suppressive activity of Treg cells but also control the regulatory capacity of these cells owing to the decreased function of Treg cells due to attenuated FoxP3 expression [32]. Consequently, our report about an increase of FoxP3+CD4+Treg subsets in AB and CB patients versus control volunteers indicates a significant rising trend in suppressive activities of the immune response in parallel with an ongoing infection such as brucellosis.

By contrast, we found the relationship between CD4+CD25^{high} and CD4+FoxP3+ cells displayed a stronger power of correlation coefficient than CD4+CD25^{low} and CD4+FoxP3+ cells in all three groups. This positive correlation verifies the direct relationship between the high-level intensity of CD25 (CD25^{high}) and FoxP3 expression. Also, the AB group ($R = 0.817$) showed a better correlation of CD4+CD25^{high} and CD4+FoxP3+ cells compared with the two other groups [$R_{(healthy\ group)} = 0.792$; $R_{(CB)} = 0.661$], which may be due to the larger sample size of the AB group (Figure 4).

In conclusion, our results demonstrate that there is a marked increase in circulating CD4+ CD25+, CD4+FoxP3+, and CD4+CD25+FoxP3+ Tregs and their CD25^{high} or FoxP3^{high} subpopulations in AB and CB patients. Tregs play a negative role not only in modulating the effectors of immune responses by inhibiting interferon-γ secretion and cellular proliferation upon *Brucella* antigen stimulation, but also in influencing the disease persistence in CB. These findings suggest that modulation of CD4+ CD25+ Tregs might be one potential therapeutic strategy for the treatment of the chronic form of this infection. However, this study was limited by analysis of the PB compartment only, and further detailed investigation of the level and function of CD4+ CD25+ and CD4+FoxP3+Tregs in brucellosis needs to be carried out.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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