

High Interleukin 17 Expression Is Correlated With Better Cardiac Function in Human Chagas Disease

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This study was designed to investigate whether the expression of interleukin 17 (IL-17) is associated with the indeterminate or cardiac clinical forms of Chagas disease and whether IL-17 expression can be correlated with patients' cardiac function. Our results demonstrated that cardiac Chagas patients have a lower intensity of expression of IL-17 by total lymphocytes and lower frequency of circulating T helper 17 cells. Correlative analysis showed that high IL-17 expression was associated with better cardiac function, as determined by left ventricular ejection fraction and left ventricular diastolic diameter values. Therefore, IL-17 expression can be a protective factor to prevent myocardial damage in human Chagas disease.

Keywords. cardiomyopathy; Chagas disease; IL-17; immunoregulation; pathology; *T. cruzi*; Th17.

Progressive chronic heart disease is the leading cause of debilitation in individuals with Chagas disease, often leading to death. Chagas disease is caused by infection with the parasite *Trypanosoma cruzi* and affects approximately 10 million people in Latin America, although recent findings have shown

increasing incidence in nonendemic countries [1]. Although most infected individuals remain asymptomatic for years, between 20% and 30% of the patients develop cardiac disease during the chronic phase of the disease.

A plethora of data have demonstrated that the host's immune response, especially immunoregulatory mechanisms, plays a key role in the differential clinical evolution of Chagas disease. It has been shown that the heart inflammation observed in cardiac Chagas patients is associated with the presence of CD8⁺ T cells, granzymes, and inflammatory cytokines [2]. Moreover, whereas the balance between inflammatory and anti-inflammatory cytokines produced by circulating cells is shifted toward the latter in indeterminate patients, cardiac patients display a predominance of the inflammatory environment [3].

Interleukin 17 (IL-17) is known as a proinflammatory cytokine mainly produced by activated CD4⁺ T cells [4]. IL-17 responses have been linked to the pathogenesis of several inflammatory and autoimmune diseases [5]. It has been shown that infection with *T. cruzi* in murine models leads to the production of IL-17 by CD4⁺ T cells, CD8⁺ T cells, natural killer T cells, and gamma delta T cells [6]. IL-17A^{-/-} mice infected with *T. cruzi* have increased mortality compared with wild-type mice [6]. In addition, blockade of IL-17 resulted in greater recruitment of inflammatory cells to the heart tissue of infected mice, despite a reduction in cardiac parasitism [7].

Although IL-17 has been associated with inflammatory and autoimmune diseases, data from experimental *T. cruzi* infection suggest that this cytokine is associated with protective rather than pathogenic responses. Given this controversy, this study was designed to evaluate the expression of IL-17 by T cells from patients with the indeterminate and cardiac forms of Chagas disease and investigate whether there is a correlation between IL-17 expression and cardiac function. The identification of markers related to susceptibility and resistance is critical for the identification of patients with greater potential to progress toward the cardiac form of Chagas disease, which would allow for possible interventions to prevent disease development or improve treatment choices.

METHODS

Patients

This cross-sectional study involved patients from endemic areas within Minas Gerais, Brazil, under the medical care of one of us (MOCR). A total of 12 Chagas patients (6 men and

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6 women; age range, 34–68 years) who had positive specific serology for *T. cruzi*, were in the chronic phase of the disease, and had well-defined clinical forms were enrolled in this study. Detailed evaluations that included physical examinations, electrocardiogram, chest x-rays, and echocardiogram were performed to classify patients into different groups as previously defined [8]. The 12 Chagas patients were divided into two clinical groups: Patients in the indeterminate (I; n = 7) group were defined by a normal chest radiograph and electrocardiogram, a normal barium swallow and enema, and the absence of clinical manifestations of the disease. Patients with dilated cardiomyopathy (DC; n = 5) presented with right and/or left ventricular dilation, global left ventricular dysfunction, and alterations in the cardiac electric impulse generation and conduction. Left ventricular ejection fraction and left ventricular diastolic diameter were used as echocardiographic parameters for assessing ventricular function for the Chagas patients [8]. We also included in our analysis individuals without Chagas disease (N; n = 7; 3 men and 4 women; age range, 19–43 years), as determined by negative specific serological tests for *T. cruzi* infection. We excluded from our study individuals with any other chronic inflammatory diseases, valvular heart disease, coronary artery disease, arterial hypertension, diabetes mellitus, alcoholism, and bacterial infections. All individuals included in this study were volunteers, and treatment and clinical care was offered to all patients according to current practice guidelines, regardless of their enrollment in this research project. This study was approved by the Research Ethics Committee of the Federal University of Minas Gerais (COEP-UFMG-ETIC006/05). Peripheral blood was collected by venipuncture, and informed consent was obtained from all individuals.

Parasites

Trypomastigotes of the Y strain of *T. cruzi* were grown in Vero cell lines, as previously performed [9], and were used for infecting peripheral blood mononuclear cells from patients and non-Chagasic individuals.

Infection of Peripheral Blood Cells

Infection of peripheral blood mononuclear cells was performed using 10 trypomastigotes per cell, as previously described [9]. Briefly, cells and parasites were incubated at 37°C with 5% carbon dioxide for a period of 3 hours. Cells were then washed by centrifugation with phosphate-buffered saline for removal of free trypomastigotes. Supernatant was removed, and a volume of Roswell Park Memorial Institute medium supplemented with antibiotic/antimicotic (amphotericin B 0.25 µg/mL, penicillin 200 U/mL, and streptomycin 0.1 mg/mL) and l-glutamine (1 mM) (all from Sigma) equal to the amount of blood initially incubated was added to the tubes. Infected cells were incubated at 37°C with 5% carbon dioxide for a

period of 14 hours. After this period, brefeldin A (1 µg/mL; Sigma) was added to prevent protein secretion, and cultures were reincubated for an additional 4 hours. For all individuals, we carried out cultures submitted to the same procedures described above in the absence of parasites as nonstimulated control.

Evaluation of IL-17 Expression by Total Lymphocytes and CD4⁺ and CD8⁺ T Cells

Frequencies of CD4⁺ and CD8⁺ T cells, as well as the expression of IL-17A by these cell subpopulations, were determined by flow cytometry. Infected or noninfected peripheral blood mononuclear cells were harvested after the final 18 hours of culture and submitted to specific surface (CD4 and CD8) and intracellular (IL-17) staining, as previously done [10, 11]. Anti-CD4-Cychrome, anti-CD8-Cychrome, and anti-IL17-Phycoerythrin were purchased from Becton-Dickinson. Cells were read in a flow cytometer, and a minimum of 40 000 gated events from each sample was acquired using a FACScan (Becton-Dickinson). IL-17 expression was analyzed by gating on lymphocytes, and the frequency of total lymphocytes, CD4⁺ lymphocytes, or CD8⁺ lymphocytes expressing IL-17, as well as the mean intensity of expression of IL-17 by these cells, was determined. Unlabeled cells and Phycoerythrin-labeled isotype controls were present in all experiments.

Statistical Analysis

The means of the different groups were compared using Tukey–Kramer all-pair comparison analysis of variance contained within the Graph Pad Prism software. Paired *t* test was used to ascertain differences among noninfected vs infected cultures from the same patient. Correlation analysis between IL-17 expression and left ventricular ejection fraction or left ventricular diastolic diameter were performed using linear regression and Spearman rank correlation test. Differences that returned *P* values ≤.05 were considered statistically significant.

RESULTS

Cardiac Chagasic Patients Display Lower Frequency and Intensity of Expression of IL-17, as Compared With Non-Chagasic Individuals and Indeterminate Patients

To determine the presence of IL-17-producing lymphocytes in chronic Chagas patients and non-Chagasic individuals, we analyzed nonstimulated cells, to provide information about IL-17 expression by cells freshly isolated from patients. We also analyzed cells after *in vitro* infection with trypomastigote forms of *T. cruzi*, to determine whether contact with the parasite led to the expansion of these cells and, if so, to what extent in the different groups. Our analysis showed that chronic Chagasic patients, regardless of clinical form, had a lower frequency of expression of IL-17 by lymphocytes

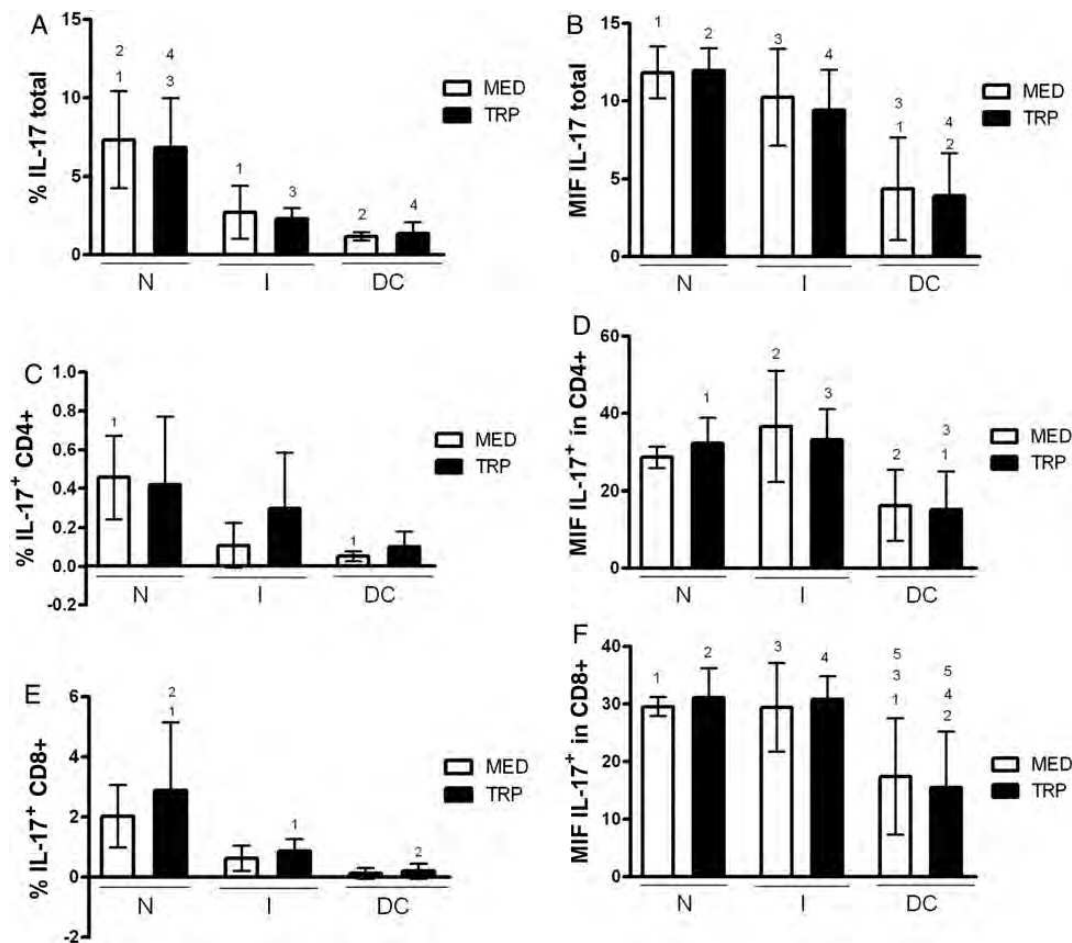


Figure 1. Analysis of the frequency and mean intensity of expression of interleukin 17 by total lymphocytes (A and B), CD4⁺ cells (C and D), and CD8⁺ cells (E and F) in circulating cells from non-Chagasic individuals (N), indeterminate (I), and cardiac (DC) Chagas patients. Data is presented as average per group ± standard deviation. The presence of the same numbers in different bars represent statistically significant differences between them. (P < .05). Abbreviations: IL-17, interleukin 17; MED, media; MIF, mean intensity of fluorescence; TRP, trypanostigote forms of *Trypanosoma cruzi* in vitro.

compared with non-Chagasic individuals in both nonstimulated and stimulated cultures (Figure 1A). This shows a unique behavior of IL-17 in Chagas disease because most cytokines are increased in patients, rather than decreased (reviewed by [3]). However, the intensity of IL-17 expression was only lower in total lymphocytes from cardiac patients, before and after stimulation, when compared with lymphocytes from non-Chagasic individuals and indeterminate patients (Figure 1B).

We then analyzed the expression of IL-17 by CD4⁺ and CD8⁺ T-cell subpopulations. Our data showed that the expression of IL-17 by CD4⁺ lymphocytes was substantially lower in cardiac patients than in non-Chagasic individuals before stimulation with *T. cruzi* (Figure 1C). Differences observed comparing unstimulated cells from indeterminate patients with non-infected individuals and comparing indeterminate with cardiac patients were not statistically significant (P = .1 and P = .2, respectively). Moreover, similar frequencies of CD4⁺IL-17⁺ cells were observed comparing amongst all groups after *in vitro*

stimulation. The intensity of expression of IL-17 in CD4⁺ cells was lower in cells from cardiac patients than in cells from indeterminate patients and noninfected individuals (Figure 1D). It is possible that regulatory T cells are producing IL-17 because these cells were increased in indeterminate but not cardiac patients [12]. However, our data does not permit us to answer this question, which would require further analysis.

Although the frequency of CD8⁺ lymphocytes expressing IL-17 was lower in cells from indeterminate and cardiac patients after stimulation than in cells from noninfected individuals (Figure 1E), the intensity of expression of IL-17 was only lower in CD8⁺ T cells from cardiac patients, but not indeterminate patients, as compared with noninfected individuals (Figure 1F). Furthermore, stimulated CD8⁺ T cells showed lower intensity of expression of IL-17 when compared with nonstimulated cells in the cardiac group (Figure 1F). Interestingly, <60% of IL-17 comes from CD4⁺ and CD8⁺ T cells. Although our data do not allow for a definitive conclusion, it

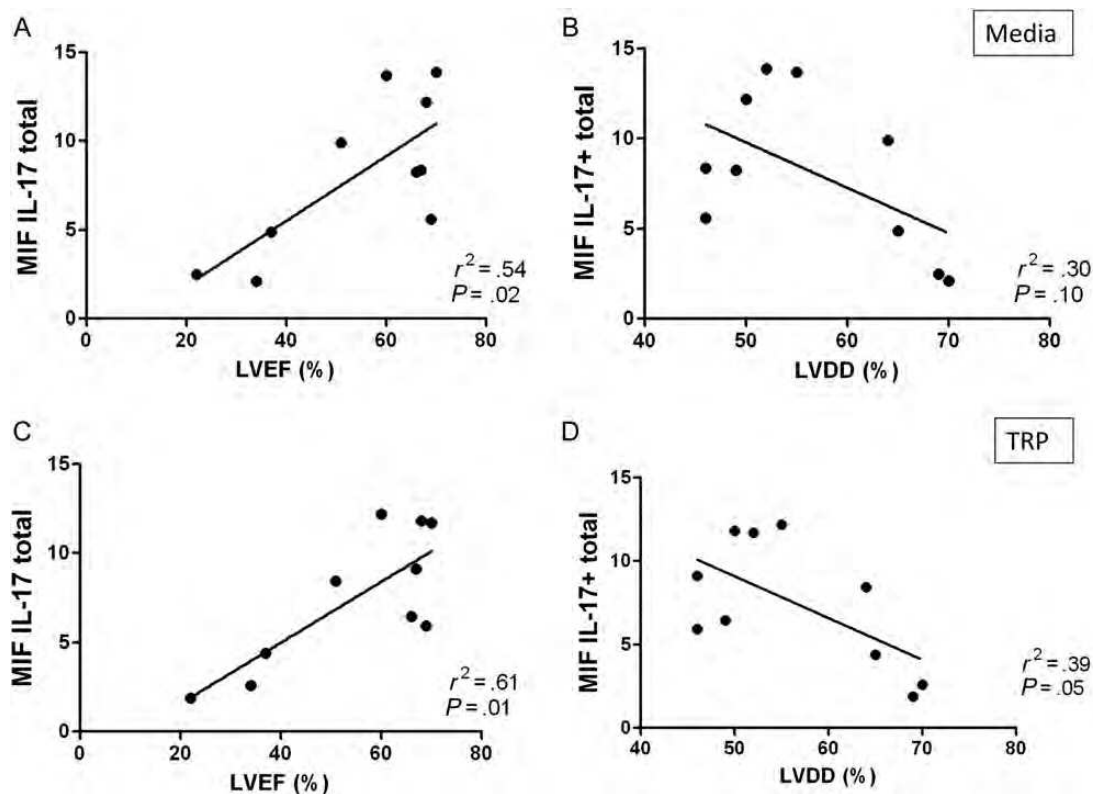


Figure 2. Higher intensity of interleukin 17 (IL-17) expression is correlated with improved cardiac function. Correlation analysis was performed between mean intensity of IL-17 expression and left ventricular diastolic volume (LVDD) (A and C) or left ventricular ejection fraction (LVEF) (B and D) in peripheral blood mononuclear cells from Chagas patients non-stimulated (Media) or exposed to trypomastigote forms of *Trypanosoma cruzi* in vitro. LVDD and LVEF values were taken by echocardiographic exams. Intensity of IL-17 expression by patient's peripheral blood mononuclear cells stimulated or not was determined by flow cytometry, as described in the Methods section. P values were calculated using Spearman correlation test as described. Abbreviations: IL-17, interleukin 17; MIF, mean intensity of fluorescence; LVDD, left ventricular diastolic volume; LVEF, left ventricular ejection fraction; TRP, trypomastigote forms of *Trypanosoma cruzi* in vitro.

is tempting to hypothesize that CD4⁺CD8⁺ T cells are an important source of this cytokine. We have shown that CD4⁺CD8⁺ T cells can indeed produce IL-17 [11].

Lower IL-17 Expression Is Correlated With Worse Cardiac Function in Human Chagas Disease

To further determine if IL-17 expression is correlated with improved cardiac function and thus with a possible protective role in human Chagas disease, we performed a correlative analysis between the frequency of IL-17-expressing cells and 2 echocardiographic parameters of cardiac function: left ventricular ejection fraction and left ventricular diastolic diameter. These distinct clinical measurements are directly and inversely correlated with better cardiac function, respectively [8]. Strikingly, a significant negative correlation between lower left ventricular diastolic diameter and higher mean intensity of fluorescence (MIF) of IL-17-producing T cells was observed both in media and upon stimulation with the parasite (Figure 2). Conversely, a significant positive correlation ($P = .01$) was seen between higher left ventricular ejection fraction and higher mean

intensity of fluorescence (MIF) of IL-17-producing T cells (Figure 2). These data clearly showed a correlation between the expression of IL-17 and better cardiac function and suggest that IL-17-producing T cells may display a protective role in human Chagas disease.

DISCUSSION

The establishment of an inflammatory reaction in the myocardium of individuals infected with *T. cruzi* leads to progressive cardiomyopathy, heart failure, and death. Most studies about IL-17 in human diseases have associated the expression of this cytokine with inflammation and autoimmune processes [5]. Although increased IL-17 expression was associated with susceptibility to *T. cruzi* infection in a murine model using bradykinin receptor 2^{-/-} mice [13], other studies have shown that mice treated with anti-IL-17 and IL-17^{-/-} mice exhibited earlier mortality compared with controls and that inhibition of IL-17 also resulted in greater heart inflammation [6, 7]. Our data showed that cardiac patients have lower frequency of

IL-17A-expressing cells than non-Chagasic individuals. Also, the intensity of expression of IL-17 by lymphocytes is lower in cardiac patients than in non-Chagasic individuals and indeterminate Chagas patients. We have shown that the use of captopril, an angiotensin-converting enzyme inhibitor that displays cardioprotective effects [14], which is frequently administered to cardiac Chagas patients, leads to an increase in the expression of IL-17 [10]. Thus, it is tempting to speculate that the mechanisms of cardioprotection of the angiotensin-converting enzyme inhibitors could be related to the expression of IL-17.

Our previous studies have shown a correlation between high expression of the immunomodulatory cytokine interleukin 10 and better cardiac function [15], whereas others have shown that interferon gamma and tumor necrosis factor alpha are correlated with worse cardiac function (reviewed in [3]). To gain insight into the establishment of the immunoregulatory networks in Chagas patients and its influence in pathology development, we are now performing studies to determine whether interleukin 10 and IL-17 act synergistically and whether there is an inverse expression of these cytokines and the ones with opposite function mentioned above. Moreover, we are expanding these studies to a larger cohort of patients to verify the use of these molecules as clinical biomarkers.

Notes

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