Evaluation of Gene Expression of TGF-B and FOXP3 in Individuals Chronically Infected with *Trypanosoma Cruzi*

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**Abstract**

Chagas diseases till poses a serious endemic problem despite the fact that it was discovered over 100 years ago. The mechanisms that lead to the development of clinical manifestations may be attributed to the host immune response against the parasite. Individuals with the indeterminate (IND) form have regulatory mechanisms that limit pathology development. The aim of the present study was to assess the gene expression of TGF-β, a cytokine with an immune regulatory profile, and FOXP3, a transcription factor, in patients with chronic Chagas disease after in vitro stimulation of Peripheral Blood Mononuclear Cells (PBMCs) with the recombinant antigens Cytoplasmatic Repetitive Antigen (CRA) and the Flagellar Repetitive Antigen (FRA) from *Trypanosoma cruzi*. We selected 37 patients with Chagas disease in the Clinics of Chagas Disease and Heart Failure at the Heart Emergency Unit of Pernambuco (Pronto Socorro Cardiológico de Pernambuco-PROCAPE) at the University of Pernambuco (Universidade de Pernambuco-UPE), Recife-PE, Brazil.

**Abbreviations**

CARD1 : Mild Cardiac Alterations
CARD2: Severe Cardiac Alterations
IND: Asymptomatic, Indeterminate form
CRA: Cytoplasmatic Repetitive Antigen
NI: Non-infected
FRA: Flagellar Repetitive Antigen
PBMCs: Peripheral Blood Mononuclear Cells

**Introduction**

Chagas disease is caused by the protozoan *Trypanosoma cruzi* and it is a major public health problem, affecting millions of people in Central and South Americas. There are an estimated 9.8 million people infected with *T. cruzi* throughout the world, mainly in Latin America [1]. In Brazil, approximately three million people are infected [2]. In humans, the disease is characterized by an acute phase with high parasitemia levels, followed by a chronic, continuous phase, when detection of circulating parasites through direct blood tests is difficult.

Most infected people develop the chronic phase and either remain with the asymptomatic form (also called indeterminate-IND) throughout their lives, or progress to symptomatic clinical forms after 10 to 20 years of infection. Approximately 30% of patients in the chronic phase develop the cardiac form (CARD) of the disease. This form has a wide range of symptoms that may be manifested as mild (CARD1) or severe (CARD2) cardiac alterations [4,5].

Several studies on Chagas diseases how that although individuals who develop the CARD form are able to control parasitemia, they may not be able to mount an efficient immune regulatory response and may present an exacerbated inflammatory response, leading to tissue damage. Conversely, individuals that remain asymptomatic may be able to reduce the number of parasites in the early stage of infection through regulatory mechanisms that limit pathology development [6,7].

The immune mechanisms by which individuals with the IND form start to present heart-associated clinical manifestations after 10-20 years of infection have not been fully elucidated [8]. One of
these mechanisms may be through the activity of regulatory T cells, characterized phenol typically in humans as CD4+ CD25 High FOXP3+ T cells [9]. These cells are an important source of regulation of the immune response, controlling the local inflammatory response and avoiding severe tissue destruction [10]. According to Araujo et al., [11], patients presenting the CARD form have specific populations of CD8+ T cells, which results in the production of inflammatory cytokines. These cytokines may be involved in the establishment of an exacerbated inflammatory process, leading to an uncontrolled immune response. The latter is characterized by the absence of or in ability of action of regulatory T cells (Treg cells) through the production of their main cytokine, TGF-β, which modulates the expression of FOXP3 by Treg and is also able to transform peripheral CD4+ CD25 - T cells in CD4+ CD25+ cells [12]. On the contrary, patients with the IND form have an effective, possibly transient, immune regulatory response that controls the response to infection mediated by regulatory effector T cells [11].

Several studies have used specific, specific antigens of T. cruzi, known as Cytoplasmatic Repetitive Antigen (CRA) and Flagellar Repetitive Antigen (FRA) [13,14] with aim to help clarifying the immune pathology of Chagas disease [15]. The role of CRA and FRA in the immune response of patients with chronic Chagas disease has been studied by our research group and promising results have been generated [16-18]. Here, the immune response of patients with chronic Chagas disease was assessed after in vitro stimulation of Peripheral Blood Mononuclear Cells (PBMCs) with CRA and FRA.

The present study aimed to describe the gene expression profiles of TGF-β and FOXP3 in patients with chronic Chagas disease after in vitro stimulation with the recombinant T. cruzi antigens CRA and FRA. The results obtained here may contribute to a better understanding of the development of symptomatic forms of the disease.

Material and Methods

Study population

Individuals with Chagas disease were selected in the Clinics of Chagas Disease and Heart Failure at the Heart Emergency Unit of Pernambuco (Pronto Socorro Cardiologico de Pernambuco-PROCAPE) at the University of Pernambuco (Universidade de Pernambuco-UPE), Recife-PE, Brazil. The inclusion criteria were three: 1) positive serology for Chagas infection in two tests with different methodology principles (one test using recombinant antigens and the other test using crude antigens of T. cruzi); 2) patients that have undergone clinical exams to characterize the clinical form (physical examination, electrocardiogram, echocardiogram, and chest and esophagus X-rays), and 3) patients that have not undergone gienetic treatment. The CARD group was further divided into CARD1 (n = 10, 50-74 years old; 4 women and 6 men), which included patients without cardiac dilation and with an ejection fraction > 55% on the echocardiogram, and CARD2 (n = 10, 43-73 years old; 4 women and 6 men), which included patients with clinical and or echo cardiac graphic and radiological signs of fast heart rate and an ejection fraction < 40% on the echocardiogram. Patients with the Indeterminate form (IND) (n = 17, 32-68 years old; 9 women and 8 men) were selected by not having any heart and digestive alterations, but with positive serology for T. cruzi infection. A group of Non-Infected (NI) volunteers (n = 7) was included for comparison with individuals with Chagas disease by using the following criteria: 1) people that have in inhabited an endemic Chagas disease area; 2) people that have never received blood transfusion, and 3) people with non-reactive serologic tests for Chagas disease. The individuals included in the study signed an Informed Consent Form (ICF), which was previously approved by the Ethics Committee of the Centro de Pesquisas Aggeu Magalhães-Fiocruz (CPqAM/Fiocruz) under the ID No.03/2009.

PBMCs culture and stimulation with recombinant T.cruzi antigens

PBMCs were obtained from samples collected in tubes containing sodium heparin through Ficoll-Paque density centrifugation (GE Healthcare, Uppsala, Sweden), as previously described [19]. The cell culture was performed as described by Melo et al., [20]. CRA and FRA antigens were obtained as described by Krieger et al., [14]. They were then analyzed by electrophoresis in a poly acryl amide gel in the presence of sodium dodecyl sulfate [21] and by silver staining [22] to assess Escherichia coli contamination.

Total RNA extraction, reverse transcription reaction and real-time PCR

Total RNA was isolated from PBMCs using Trizol reagent (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. Next, total RNA was incubated with deoxyribonucleic acid amplification in the Amplification Kit Grade (Invitrogen™) at a final RNA concentration of 100 ng/µL in order to ensure complete elimination of genomic DNA in the samples. Reverse Transcription (RT) to obtain cDNA was performed using the High Capacity cDNA Reverse Transcription with RNase Inhibitor kit (Applied Biosoysm®, Foster City, CA, USA), according to manufacturer's instructions. The cDNA was then used in Real-Time PCR assays, as described by Melo et al., [20]. The sequences of primers and probes (200nM) to amplify β-actin (endogenous gene) and TGF-β genes were described by Mocellin et al., [23] and Mwacharo et al., [24], respectively, and were: β-Actin sense-5'-GGCACCCGCAAAATGAAG-3' and antisense-5'-GGCGATCCACCGAGGATC-3', probe-VIC TCAAAGATCTGCCCTCCCTAGAGAACCG-GMB; TGF-β sense-5'-GGCAAGGGTACCCTGAACGAGGAGGA-3' and antivense-5'-TGAGGTATCGCCAGGAATTGT-3', probe-6FAM-CAGCGTGTGGAGGTCTTAGGACT-GMB. Primers and probes for the FOXP3 gene were obtained directly from the custom primers and probes by manufacturer (Applied Bio systems®-Product Number: 4304971). Standard curves of the targets showed similar efficiency (>99%). The relative quantitation was calculated according by Wilhelm et al. [25]. The relative quantification was established by the ratio between the mean CT (cycle threshold) values of the target gene and the reference gene (β-actin) in each stimulated sample / reference sample (negative controls). In addition, the efficiency correction was performed according by Pfaff [26].

Statistical analysis

A descriptive analysis was performed to expose the obtained results. To test data homogeneity assumption the Levene test was used and homogeneity was confirmed by the Bartlet test. To compare the gene expression mean of TGF-β cytokine and FOXP3 among the groups and for each stimulus, ANOVA followed by Tukey test were used. To assess “high” and “low” producers of the cytokine and FOXP3, the chi-square test of proportions was applied. All conclusions were based on a significance level of 5%. Excel 2000 was used to build the database and Graph Pad Prism 5.0 was used to perform the statistical tests.
Results

Gene expression of TGF-β cytokine and FOXP3 transcription factor after in vitro stimulation with recombinant *T. cruzi* antigens.

Analysis of the differences in gene expression levels of TGF-β and FOXP3 was performed after in vitro stimulation of cells with *T. cruzi* recombinant antigens (Rec-Ags) CRA and FRA in comparison to cultures without stimulation, where no statistically significant differences were found for the two genes in any group. The results of the ANOVA test for CRA (TGF-β (p = 0.13) and FOXP3 (p = 0.38)) and FRA (TGF-β (p = 0.13) and FOXP3 (p = 0.67)) antigens showed no difference between groups of individuals were found (Table 1).

Frequency of high-and low-producers of the transcription factor FOXP3 after in vitro stimulation with the recombinant *T. cruzi* antigens CRA and FRA.

In order to improve the detection of gene expression changes of TGF-β cytokine and FOXP3 transcription factor, a cut-off value was set corresponding to the global average expression values from all individuals (IND, CARD1, CARD2, and NI). The individual values of each group were inserted above or below the cut-off value and used to investigate the frequency of cytokine expression among groups. Although there was no statistically significant difference in gene expression, it was observed that most patients with the IND form (70%) were classified as “high-producers” of TGF-β, when compared to CARD 1 (30%), CARD 2 (50%) and NI (57.14%) individuals after stimulation with CRA. When we used the FRA, we found that 63.16% of individuals in the IND form were above the cut-off, compared to CARD1 (40%), CARD2 (40%) and NI (72.43%) (Table 1).

As for FOXP3 gene, we found that a small proportion of individuals from all groups. After stimulation with CRA: IND (21.05%), CARD1 (10%), CARD2 (33.33%) and NI (0%). When we the FRA: IND (21.05%), CDAR1 (20%), CARD2 (33.33%) and NI (12.5%). No statistically significant difference was observed for FOXP3 gene with both Rec-Ags used (Table 1).

Discussion

Few studies have investigated the role of soluble factors involved in the immune response against Chagas disease. Similar to the results reported here, studies such as Rezende-oliveira et al., [27] in which TGF-β levels were measured in the supernatant of mononuclear cells infected with *T. cruzi*, and Nogueira et al., [28], who analyzed TGF-β gene expression through real time PCR in patients with chronic Chagas disease, reported no statistically significant differences in TGF-β expression. This allows us to conclude that TGF-β synthesis assessed through qPCR after cultivation with CRA and FRA Rec-Ags may not be used as a prognostic marker of the evolution of the clinical forms of chronic Chagas disease.

Thus, despite the fact that a few studies suggest an association of the IND form with a high production of anti-inflammatory and regulatory cytokines such as IL-4 and IL-10 and TGF-β, and of the CARD form with high levels of pro-inflammatory cytokines such as IFN-γ and TNF-α [29,30] here we found results similar to other studies, where no relationship was observed between the production profile of a certain cytokine and Chagas disease clinical symptoms [17,20,31].

The FOXP3 gene, unlike other genes associated to regulatory T cells (Tregs), is specific of the Treg lineage [33]. In mice, the ectopic expression of FOXP3 in naive T cells is able to convert them into Tregs with similar phenotype and function of innate Tregs, conferring them an *in vivo* and *in vitro* suppress or activity and increased expression of molecules characteristic of Tregs such as CD25+, CTLA-4 (T Lymphocyte-Associated Protein 4) and GITR (Glucocorticoid-Induced TNFR-Related Protein) [34]. In humans, it is still unclear whether FOXP 3 is a specific marker for Tregs, as activated T cells may transiently express FOXP3 (even if at low levels when compared with T CD4+ CD25 high) [35]. Our results are consistent with data obtained previously by our group. The frequency of FOXP3 molecule in CD4+ CD25+ T cells was evaluated among individuals with different clinical forms of Chagas disease through flow cytometry. No statistical difference was observed among groups in the presence of CRA or FRA, despite the fact that 75% and 70% of IND individuals expressed FOXP3 after stimulation with CRA and FRA, respectively [36]. Thus, the expression of FOXP3 under the conditions studied here may not be used as a prognostic marker for the evolution of chronic clinical forms of Chagas disease.

Various factors such as, for example, the strain of the parasite, time of infection and reinfection potential may be important in the progression of Chagas disease. The fact that the experimental groups were made up of individuals from the same endemic area of the country and the median age between the groups were similar, we believe that our sample has undergone minimal interference by these factors. However, we know that the genetic background and consequently the development of the immune response of the host during infection is a limiting factor in any immunological study.

The categorization of patients in regard to the production of cytokines has been used to assess individuals that within a certain group exhibit high production levels of cytokines [37,38]. Gomes et al., [37], when studying IFN-γ production, divided the groups of patients with cardiac changes according to disease severity. In this way, it was possible to establish a correlation between “high” and “low”
IFN-γ producers and the clinical status of the patient. Lorena et al., Melo et al., and Vitelli-Avellar et al., [16,20,38], established a cut-off value based on the overall mean among all study subjects and were able to separate them into “high” and “low” cytokine producers.

In the present study, we evaluated the gene expression patterns of TGF-β cytokine by establishing a cut-off value and consequently identifying high expression levels. Although we did not observe statistically significant differences, we found an increased production of TGF-β mostly in individuals with the IND form, with frequencies of 70% and 63.16% after stimulation with CRA and FRA, respectively.

The fact that most IND individuals are TGF-β producers suggests that this cytokine may play a role in the immunomodulatory response, controlling morbidity in this group of patients, as previously described in the literature [12]. However, further evaluation of cells producing this cytokine through flow cytometry (cell phenol typing) may help to clarify the possible role of a Th1-type inflammatory response by these cells in controlling the disease.

Detection of FOXP3 in regulatory T cells has been the subject of intense study aimed to elucidate the immune regulatory mechanism of these cells in Chagas disease [11,39]. In the present work, when analysis of patient distribution was performed, no difference was observed among the groups IND, CARD1, CARD2 and NI when PBMCs were stimulated in vitro with the Rec-Ags CRA and FRA. However, it would be interesting to assess the association of FOXP3 expression data with other regulatory T cells markers such as CD103, CD122 and CD152 (CTLA-4).

Conclusion

We believe that the immunological mechanisms involved, in the joint interaction of immune cells, and the differential genetic susceptibility of the host, lead to a highly complex pathology, imposing difficulties in the detection of a specific immune response. Thus, further follow-up studies of these individuals are necessary to help clarifying the immune pathological mechanisms behind Chagas disease and to offer data to support the development of vaccines and efficient immune therapies against this disease.

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Reference


