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# Potential role of $CD4^+CD25^{HIGH}$ regulatory T cells in morbidity in Chagas disease

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## 1. ABSTRACT

Several immunoregulatory mechanisms are proposed to be effective both in human and experimental *Trypanosoma cruzi* infection. However, the role of $CD4^+CD25^{high}$ T cells in Chagas disease has not yet been elucidated. These cells are critical for the regulation of immune response to infectious agents and in the control of autoimmune diseases. In this study, the presence of $CD4^+CD25^{high}$ regulatory T cells in the whole blood of non-infected individuals (NI), and patients with the indeterminate (IND) and cardiac form (CARD) of Chagas disease was evaluated. To further characterize this population of regulatory cells, the co-expression of CTLA-4, CD62L, CD45RO, CD45RA, HLA-DR, CD40L, CD69, CD54, IL-10R and the intracellular molecules FOXP3 and IL-10 on the $CD4^+CD25^{high}$ T lymphocytes was examined. FOXP3 was expressed by the majority of $CD4^+CD25^{high}$ when compared with the other $CD4^+$ T cells subsets in patients with Chagas disease. Patients with the IND form of the disease had a higher frequency of circulating regulatory $CD4^+CD25^{high}$ T cells than patients with the CARD form. Moreover, there was an increase in $CD4^+CD25^{high}FOXP3^+$ cells that were also $IL\text{-}10^+$ in the IND group whereas, in the CARD group, there was an increase in the percentage of $CD4^+CD25^{high}$ $FOXP3^+$ cells that expressed CTLA-4. These data suggest that IL-10 produced by regulatory T cells is effective in controlling disease development in patients with the IND form. However, in individuals with the CARD form of the disease, the same regulatory mechanism, mediated by IL-10 and CTLA-4 expression is not sufficient to control the progression of the disease. The data suggest that $CD4^+CD25^{high}FOXP3^+$ regulatory T cells in patients with Chagas disease might play a role in the immune response against *T. cruzi* infection although with distinct effects in patients with the IND and CARD forms of disease.

## 2. INTRODUCTION

The protozoan parasite, *Trypanosoma cruzi*, is the etiological agent of American trypanosomiasis or Chagas disease. The disease is endemic throughout Latin America where 18-20 million people are infected (1). The acute phase of infection with *T. cruzi* is characterized by intense parasitism and evident blood parasitemia and may result in death. In survivors, the acute phase is followed by an asymptomatic or indeterminate phase, during which there are no clinical symptoms. The asymptomatic phase may last anywhere between months to decades. About 30% of those individuals who experience the indeterminate phase of the disease go on to develop clinical symptoms of Chagas disease (2).

The scarce parasitism during the chronic phase, the prolonged latent period that precedes morbidity, and the intriguing existence of different clinical forms suggest an autoimmune basis for the pathology of chronic Chagas disease. The demonstration of *T. cruzi* or its antigens by immunohistochemical techniques or of *T. cruzi* DNA by polymerase chain reaction (PCR) in inflamed myocardial tissues suggests that parasite antigens may be necessary to trigger the inflammatory process (3,4,5). Several studies of Chagas disease have demonstrated that immunoregulatory mechanisms control the intense immune activity in the chronic phase. These immunoregulatory mechanisms are necessary to prevent a deleterious effect of the excessive stimulation of immune system and fatality (6,7). In our laboratory, we demonstrated that patients with the cardiac (CARD) form of chronic Chagas disease develop a strong immune response against the parasite, with high levels of IFN-gamma and low levels of IL-10 (8,9) and this response is associated with cardiac pathology. These data suggest that an increase in the secretion of IL-10 during the chronic phase may be associated with protection of the host against the severe pathology induced by type 1 immune response (8,9).

Recently the role of $CD4^+CD25^+$ regulatory T cells has become the focus of increased study because of the putative importance of these cells in maintaining self tolerance and in defense against infections. It has been suggested that these cells are important in perpetuating the persistence of the infection and consequent long-term resistance against re-infection (10,11,12,13). $CD4^+CD25^+$ cells are naturally generated in the thymus and constitute 5-10% of peripheral $CD4^+$ T cells in naive animals and humans (14). However, by itself the expression of CD25 is not a definitive marker of natural regulatory T cells. CD25 is an activation marker for T cells and is therefore also expressed by effectors Type 1 and Type 2 cells. For these reasons, characterization of the $CD4^+CD25^+$ regulatory T cells population with additional markers is crucial for improved characterization of the cells. Among the described markers are CD38, CD62L, and CTLA-4 or intracellular expression of the transcriptional repressor FOXP3 (forkhead box P3) (15,16).

The suppressive mechanisms of $CD4^+CD25^+$ T regulatory cells are not yet clear. However, there is increasing evidence that cell-cell contact is required to trigger these suppressive mechanisms and that the expression of CTLA-4 might be involved (17). Furthermore, other studies have shown that IL-10 is an important cytokine involved in the suppressive function of regulatory T cells (18). Although there is no evidences for the effector function of this cell population in human Chagas disease, we have previously demonstrated that IL-10 is significantly involved on the control of morbidity during the chronic phase of the infection with *T.cruzi* (8). Thus, it is possible that in human Chagas disease, $CD4^+CD25^+$ T cells may be an important population in the control of morbidity development. In this study we present the first evidence suggesting that in humans, control of morbidity may be occurring via IL-10 induction/secretion.

## 3. PATIENTS, MATERIALS AND METHODS

### 3.1. Recruitment of Subjects

The initial cohort of study subjects was recruited 8 years ago from the Referral Outpatient Center for Chagas Disease of the Hospital das Clínicas, Federal University of Minas Gerais, Brazil. All study participants provided written informed consent following the guidelines of the Ethics Committee of the Federal University of Minas Gerais. The study protocol complied with the regulations of the Brazilian National Council on Research in Humans and was approved by the Ethics Committee of the Federal University of Minas Gerais under protocol COEP-ETIC 372/04. Individuals with systemic arterial hypertension, diabetes mellitus, thyroid dysfunction, renal insufficiency, chronic obstructive pulmonary disease, hydroelectrolytic disorders, alcoholism, previous clinical history suggesting coronary artery obstruction and rheumatic disease, or who were unable to fulfill the study requirements for annual examinations were excluded from the study. Individuals were considered seropositive for *T. cruzi* infection if two or more standard tests (indirect immunofluorescence, ELISA, indirect haemagglutination) were positive. Study participants were evaluationed annually for a range of clinical and immunological parameters related to Chagas disease (8).

For this study, we investigated the immune responses of 27 patients in the chronic phase of the infection who had completed the protocol described above. The patients infected with *T. cruzi* were classified as being in the indeterminate phase of Chagas disease (IND) or having the cardiac (CARD) form of the disease as previously reported (19). Individuals in the IND group ($n$=14) ranged in age from 30 to 67 years. These individuals had no significant alterations in the electrocardiography, chest x-ray, echocardiogram, esophagogram and barium enema. The CARD group ($n$=13) ranged in age from 25 to 70 years, and presented echocardiographic and/or clinical and radiological signs of heart enlargement, with a final diastolic diameter of the left ventricle of more than 55mm. The cardiac patients that participated in this study were classified as belonging to the group CARD V as previously reported (19). Eleven normal healthy individuals, ranging in age from 29 to 55 years, from an area that is not endemic for

Chagas disease and who had negative serology for Chagas disease, were included as a control group (NI).

### 3.2. Antigens

Epimastigote (EPI) antigens were prepared by using the CL strain of *T. cruzi*. EPI were washed three times in cold phosphate-buffered saline (PBS), disrupted by repeated freezing at -70°C and thawing, and homogenized at 4 to 6°C in a Potter-Elvejem centrifuge at 20,000 rpm five times for 60 seconds, with 30 seconds intervals at 4ºC. The suspension was subsequently centrifuged at 40,000 x *g* for 60 minutes on ice. The clear supernatant was dialyzed for 24 hours at 4°C against PBS, filter sterilized on 0.2-µm-pore-size membranes, assayed for protein concentration, aliquoted, and stored at -20°C until use.

### 3.3. Flow cytometric analysis of peripheral blood

Whole blood was collected in Vacutainer tubes containing EDTA (Becton Dickinson) and 100µl samples were mixed in tubes with 2µl of undiluted monoclonal antibodies conjugated with fluorescein isothiocyanate(FITC), phycoerythrin(PE), or allophycocyanin(APC) for the following cell surface markers: CD4(SK3), CD25(MA251), HLA-DR(G466), CD45RA(HI100), CD45RO(UCHL-1),CD62L(DREG56), CD40L(TRAP1), CD54(HA58), CD69(FN50), IL-10R(3F9), CD152[CTLA-4 (BNI3)]. The tubes were incubated in the dark for 30 minutes at room temperature. Following incubation, erythrocytes were lysed using 2ml of fluorescence-activated cell sorter (FACS) Lysing Solution. After incubation, the cells were washed twice with 2ml of phosphate-buffered saline containing 0.01% sodium azide. The cells were then fixed in formaldehyde (4%) and permeabilized in saponin buffer (0.5%) (Sigma) for 15 minutes. Finally, the cells were incubated with monoclonal antibody reactive to FOXP3(PCH101). After incubation the cells were fixed in 200µl of FACS fix solution (10g/l paraformaldehyde, 1% sodium-cacodylate, 6.65g/l sodium chloride, 0.01% sodium azide). Phenotypic analyses were performed by flow cytometry with a Becton Dickinson FACScalibur flow cytometer, collecting data on 5 x $10^4$ lymphocytes (gate by forward and side scatter properties) and analyzed using CellQuest software (BD Biosciences).

### 3.4. Flow cytometric analysis of cells culture

Whole blood was stimulated with either medium alone (RPMI 1640 supplemented with 1.6% L-glutamine, 3% antibiotic-antimycotic, 5% of AB Rh-positive heat-inactivated normal human serum) or EPI (25 µg/ml) for 22 hours at 37°C and 5% $CO_2$. During the last 4 hours of culture, Brefeldin A (SIGMA, St. Louis, MO, USA) (10µg/ml) was added to the cultures (20). Cultured cells were washed twice in PBS containing 1% bovine serum albumin and stained with monoclonal antibodies specific for several cell-surface markers conjugated with FITC or PE or APC (same described above). The cells were then fixed in formaldehyde (4%) and permeabilized in saponin buffer (0.5%) (Sigma) for 15 minutes. Finally, the cells were incubated with monoclonal antibodies reactive to IL-10(JES3-9D7), IL-2(MQ1-17H12), IFN- gamma(B27), TNF-alpha(Mab11) and FOXP3(PCH101). Phenotypic analyses were performed by flow cytometry with a Becton Dickinson FACScalibur flow cytometer, and data on 5 x $10^4$ lymphocytes collected (gate by forward and side scatter properties) and analyzed using CellQuest software (BD Biosciences).

### 3.5. FACS analysis of surface markers and intracellular cytokine

Lymphocytes were analyzed for their intracellular cytokine expression patterns and frequencies as well as for cell surface markers using Cell Quest software. The frequency of cells was analyzed in four gates for each staining: gate 1 (R1), lymphocyte gate (Figure 1A); gate 2 (R1 and R2), T $CD4^+CD25^{high}$ lymphocytes gate; gate 3 (R1 and R3), T $CD4^+CD25^{low}$ gate, and gate 4 (R1 and R4), T $CD4^+CD25^-$ gate (Figure 1B). Limits for the quadrant markers were always set based on negative populations and isotype controls. This approach allowed us to determine the frequency of populations in sub regions of mononuclear cells, taking advantage of the known position of T $CD4^+CD25^{high}$ lymphocytes based on their size and granularity profiles. The analyzed regulatory population presented strong positivity for CD25 cell marker. Moreover, this analytical approach allowed us to obtain a homogeneous and well-defined population. The following step was to build histograms used to quantify the percentage of the following markers: CD45RA, CD45RO, CD62L, CD40L, CD69, HLA-DR, CD54, CD152, IL10R, intracellular cytokines and FOXP3 on $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ e $CD4^+CD25^-$ T cells subpopulations (Figure 1C).

### 3.6. Statistical analysis

Analyses were performed using GraphPad Prism version 3.0 software. The following nonparametric tests were performed: 1) Mann-Whitney test to compare two groups (NI x IND or NI x CARD or IND x CARD), 2) Kruskal-Wallis test to compare the three groups (NI x IND x CARD), and 3) Wilcoxon test to compare cultures stimulated and non stimulated. Differences were considered significant when a *P* value of less than 0.05 was obtained. The statistical analyses were performed using the median values of each group.

## 4. RESULTS

### 4.1. Human $CD4^+CD25^{high}$ regulatory T cells are elevated in individuals with the indeterminate form of Chagas disease

It has been demonstrated that within the $CD4^+CD25^+$ T cells, both sub-populations expressing either low or high levels of CD25 exhibit regulatory functions in mice. However, in humans only $CD4^+$ T cells expressing high levels of CD25 exhibit similar strong regulatory function (21). We utilized peripheral blood samples from patients with Chagas disease to determine whether $CD4^+CD25^+$ cell sub-populations are correlated with the clinical forms of the disease. We evaluated the expression of this marker both before and after in vitro stimulation with parasite antigens. In Figure 2 we show the percentage of CD4+CD25high, CD4+CD25low and CD4+CD25- cells in the peripheral blood of patients with the different clinicalforms of the disease as well as in non-infected controls.

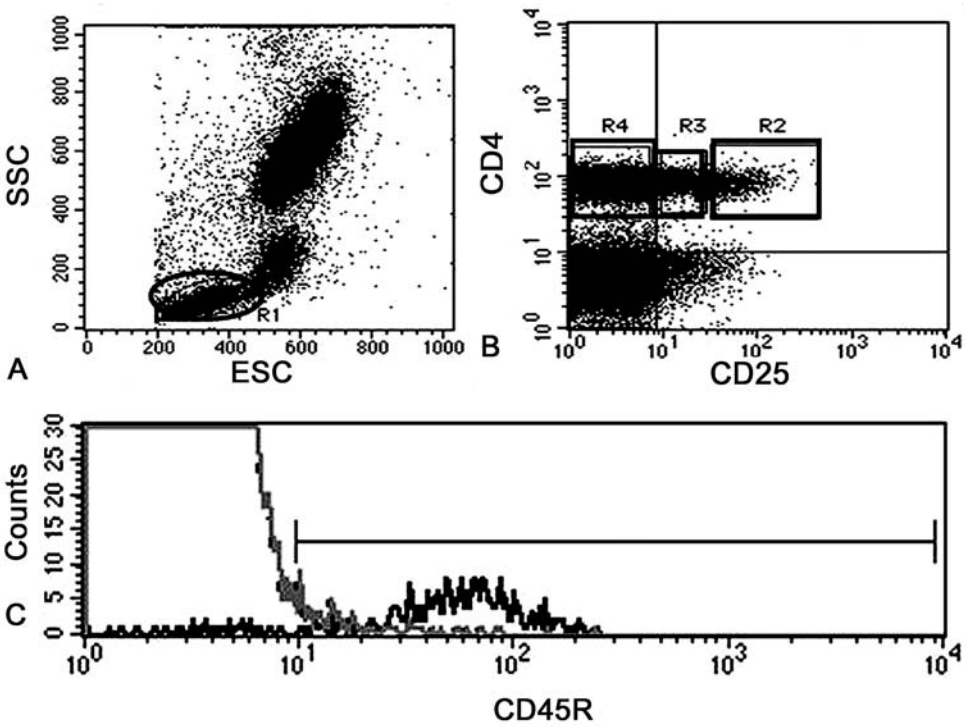


**Figure 1.** Identification of peripheral lymphocytes population in diagram of FSC x SSC (A). Dot plot of FL-3 x FL-4, displaying the frequency of $CD4^+CD25^+$ cells, after stimulation with EPI. The $CD4^+CD25^{high}$(R2), $CD4^+CD25^{low}$(R3) and $CD4^+CD25^{negative}$(R4) populations were sorted using the indicated sorting gates (B). The histograms represent the expression of CD45RO in $CD4^+CD25^{high}$ T cells (C).

Our results show that $CD4^+CD25^{high}$ T cells comprise 1-5% of the total $CD4^+$ T cell population in all individuals studied. After *in vitro* antigenic stimulation, we observed that patients with IND form of the disease had significantly higher percentages of $CD4^+CD25^{high}$ (p less than 0.05) when compared with non-infected individuals(NI) (Figure 2). No statistically significant differences were observed in $CD4^+CD25^{low}$ or $CD4^+CD25^-$ T cells between the groups studied (Figure 2).

Figure 3 shows the expression of FOXP3, a transcriptional repressor associated with the function of regulatory T cells, in blood from patients with the IND and CARD forms of Chagas disease and the NI group before and after *in vitro* stimulation with *T. cruzi* antigens. Flow cytometric analysis showed that FOXP3 was expressed by the majority of $CD4^+CD25^{high}$ T cells from the IND, CARD and NI groups when compared with the percentages of expression by $CD4^+CD25^{low}$ and $CD4^+CD25^-$ T cells.

### 4.2. Peripheral blood $CD4^+CD25^{high}$ cells from Chagasic patients express different levels of surface molecules

$CD4^+CD25^{(high/low/negative)}$ T cells from Chagasic patients were evaluated for expression of different cell surface markers in order to fully characterize this regulatory population in these patients. The different levels of expression of cell surface markers on T $CD4^+CD25^{high}$, T $CD4^+CD25^{low}$, T $CD4^+CD25^-$ subsets are shown in Table 1.

Some human T cells express CD45RA surface markers characterizing them as naive, whereas memory T cells express the CD45RO isoform. Our results show that the $CD4^+CD25^{high}$ T cells from Chagasic patients and non-infected individuals express low levels of CD45RA when compared to $CD4^+CD25^{low}$ or $CD4^+CD25^-$ subsets (Table 1). In contrast, CD45RO was expressed at significantly higher levels by $CD4^+CD25^{high}$ T cells than the $CD4^+CD25^{low}$ or $CD4^+CD25^-$ subsets. This higher expression of CD45RO on $CD4^+CD25^{high}$ regulatory cells was previously observed in healthy individuals (21).

We also observed that the expression of activation markers (HLA-DR, CD40L and CD69), the L-selectin CD62L, IL-10R, and the adhesion molecule (CD54/ICAM-1) on $CD4^+CD25^{high}$ T cells surface was increased when compared with $CD4^+CD25^{low}$ T cells and $CD4^+CD25^-$ T cells in all groups studied (Table 1). After stimulation with *T. cruzi* antigens we observed a decreased in the percentage of $CD4^+CD25^{high}$ T cells in Chagasic patients expressing CD62L and IL-10R. However, the levels of HLA-DR, CD40L, CD54 and CD69 were higher on the surface of these cells in Chagasic patients when compared with non-infected individuals (Table 1). Our results show that $CD4^+CD25^{high}$ regulatory T cells of Chagasic patients express a number of cell surface markers that are associated with activation, migration, and antigen presentation.

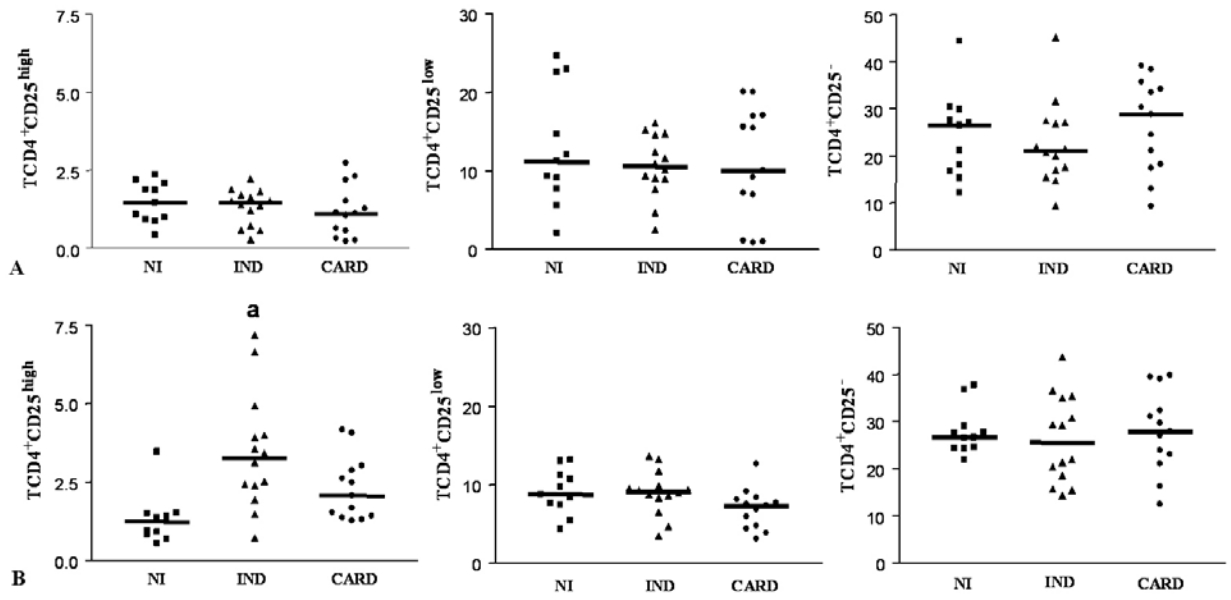


**Figure 2.** Percentages of $CD4^{+}CD25^{high}$ or $CD4^{+}CD25^{low}$ or $CD4^{+}CD25^{negative}$ T cells, evaluated by flow cytometry as described in Material and Methods. A) Representative *ex vivo* context. B) After stimulation with *T. cruzi* antigens (EPI). The differences between the groups are considered significant at p less than 0.05 and are represented by letters: a = different when compared to NI group

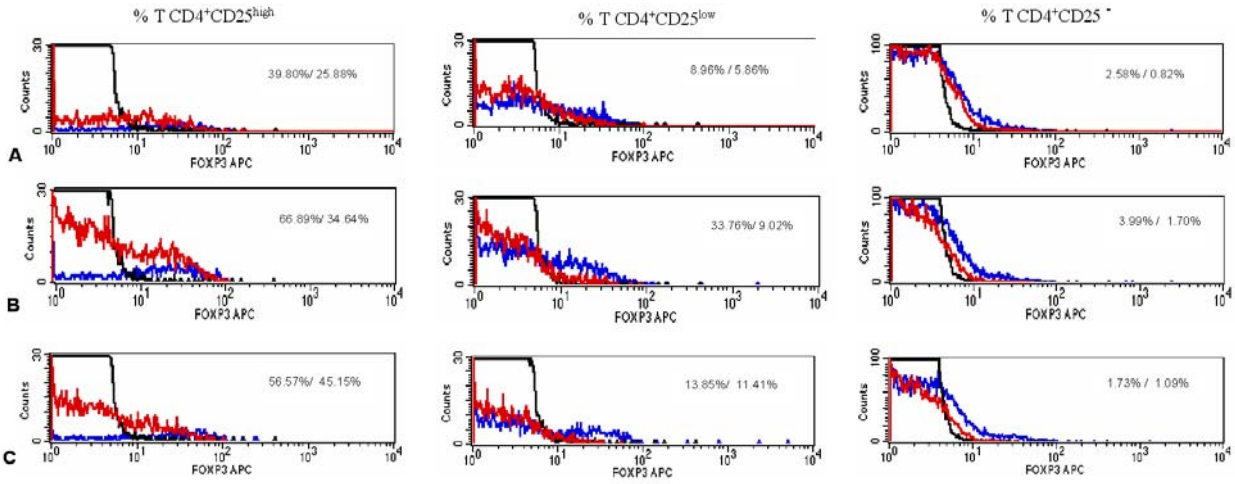


**Figure 3.** FOXP3 expression by $CD4^{+}CD25^{high}$ or $CD4^{+}CD25^{low}$ or $CD4^{+}CD25^{negative}$ T cells. A) Non-infected individuals. B) Patients with indeterminate form of the disease. C) Patients with cardiac form of the disease. The black line represent the isotype control, the blue line represents the *ex vivo* context and the red line represents the state after stimulation with *T.cruzi* antigens (EPI). The numbers represent the FOXP3 % of one individual or patient in *ex vivo* context and after stimulation with EPI antigens, respectively

### 4.3. Regulatory T cells from IND and CARD clinical forms of Chagas disease present different effector mechanism

The suppressive mechanisms by which $CD4^{+}CD25^{+}$ T regulatory cells act are not yet clear. Some studies suggest that the immune suppressive activity is due to the secretion of cytokines (14). However, other investigators have suggested that suppression is due to cell-to-cell contact via CTLA-4 (21). In Figure 4, we show the expression of CTLA-4(CD152) and IL-10 on $CD4^{+}CD25^{(high/low/negative)}$ T cells from Chagasic patients after in vitro stimulation with T. cruzi antigens. Our results revealed ahigher expression of IL-10 by $CD4^{+}CD25^{high}$ T cells in Chagasic patients, mainly in the IND group, when compared to the NI group (Figure 4). We also observed a higher frequency of $CD4^{+}CD25^{high}CTLA\text{-}4^{+}$ T cells in the CARD group when compared with NI group (Figure 4). There was no statistically significant difference in intracellular expression of IL-2, TNF-alpha and IFN-gamma in $CD4^{+}CD25^{(high/low/negative)}$ T cells between Chagasic patients and NI individuals (data not shown).

**Table 1.** Phenotypic analyses of $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and $CD4^+CD25^{negative}$ T cells subsets

| Cell marker | *Ex vivo/In vitro* | $CD25^{high}$ | $CD25^{low}$ | $CD25^{negative}$ |
|---|---|---|---|---|
| CD45RA | ***Ex vivo*** | | | |
| | NI | 5.97 ± 1.62 | 17.71 ± 3.53 | 35.55 ± 4.93 |
| | IND | 7.57 ± 1.91 | 21.47 ± 3.80 | 35.41 ± 4.97 |
| | CARD | 6.56 ± 2.15 | 13.66 ± 2.20 | 29.35 ± 3.79 |
| | ***In vitro*** | | | |
| | NI | 5.25 ± 2.07 | 15.50 ± 2.63 | 28.49 ± 4.00 |
| | IND | 5.10 ± 1.26 | 18.45 ± 3.01 | 31.60 ± 3.68 |
| | CARD | 4.74 ± 1.06 | 14.16 ± 2.52 | 25.7 ± 3.59 |
| CD45RO | ***Ex vivo*** | | | |
| | NI | 90.77 ± 2.08 | 77.59 ± 3.09 | 55.29 ± 3.58 |
| | IND | 90.37 ± 2.39 | 80.05 ± 4.33 | 60.56 ± 4.75 |
| | CARD | 92.34 ± 1.83 | 82.95 ± 2.91 | 60.58 ± 3.75 |
| | ***In vitro*** | | | |
| | NI | 89.88 ± 2.45 | 76.52 ± 3.11 | 59.07 ± 3.87 |
| | IND | 88.91 ± 2.47 | 73.55 ± 5.10 | 57.06 ± 5.70 |
| | CARD | 90.94 ± 1.67 | 80.36 ± 2.31 | 62.95 ± 4.08 |
| HLA-DR | ***Ex vivo*** | | | |
| | NI | 14.14 ± 1.80 | 3.04 ± 0.55 | 3.07 ± 1.29 |
| | IND | 14.05 ± 1.44 | 4.11 ± 1.15 | 4.52 ± 1.55 |
| | CARD | 16.82 ± 3.57 | 6.03 ± 2.37 | 4.34 ± 1.21 |
| | ***In vitro*** | | | |
| | NI | 11.77 ± 1.33 | 2.28 ± 0.34 | 1.68 ± 0.34 |
| | IND | 14.44 ± 2.07 | 4.65 ± 0.96 | 2.98 ± 1.01 |
| | CARD | 15.81 ± 2.96 | 6.09 ± 1.32 | 4.36 ± 1.20 |
| CD62L | ***Ex vivo*** | | | |
| | NI | 91.24 ± 1.87 | 81.58 ± 2.17 | 83.03 ± 1.95 |
| | IND | 92.33 ± 1.34 | 79.35 ± 2.46 | 73.89 ± 4.28 |
| | CARD | 91.83 ± 2.02 | 77.85 ± 2.12 | 72.09 ± 3.08 |
| | ***In vitro*** | | | |
| | NI | 93.30 ± 1.54 | 85.79 ± 1.62 | 86.87 ± 1.66 |
| | IND | 76.33 ± 4.16 [1] | 81.67 ± 2.07 | 79.92 ± 3.44 |
| | CARD | 79.92 ± 3.02 [1] | 84.69 ± 1.71 | 78.07 ± 2.36 [1] |
| CD40L | ***Ex vivo*** | | | |
| | NI | 3.02 ± 1.52 | 1.47 ± 0.35 | 0.88 ± 0.18 |
| | IND | 5.17 ± 2.28 | 1.53 ± 0.41 | 0.92 ± 0.15 |
| | CARD | 4.12 ± 1.58 | 2.18 ± 0.59 | 1.03 ± 0.12 |
| | ***In vitro*** | | | |
| | NI | 5.12 ± 1.24 | 4.44 ± 0.94 | 3.06 ± 0.71 |
| | IND | 12.34 ± 2.17 [1] | 5.63 ± 1.09 | 2.70 ± 0.54 |
| | CARD | 11.08 ± 1.83 [1] | 6.11 ± 1.25 | 2.55 ± 0.60 |
| CD54 | ***Ex vivo*** | | | |
| | NI | 35.92 ± 5.70 | 23.43 ± 5.00 | 12.71 ± 3.19 |
| | IND | 47.62 ± 5.42 | 38.10 ± 5.33 | 30.95 ± 4.68 [1] |
| | CARD | 49.74 ± 7.13 | 40.41 ± 5.17 | 28.37 ± 3.27 [1] |
| | ***In vitro*** | | | |
| | NI | 34.34 ± 5.43 | 23.58 ± 5.82 | 15.22 ± 4.60 |
| | IND | 76.76 ± 4.59 [1] | 47.72 ± 5.45 | 33.94 ± 6.13 [1] |
| | CARD | 73.13 ± 6.86 [1] | 47.42 ± 5.84 | 30.52 ± 4.31 [1] |
| CD69 | ***Ex vivo*** | | | |
| | NI | 0.52 ± 0.13 | 0.41 ± 0.07 | 0.23 ± 0.03 |
| | IND | 0.86 ± 0.19 | 0.44 ± 0.08 | 0.37 ± 0.07 |
| | CARD | 0.88 ± 0.40 | 0.94 ± 0.41 | 0.46 ± 0.13 |
| | ***In vitro*** | | | |
| | NI | 1.47 ± 0.38 | 0.54 ± 0.15 | 0.22 ± 0.03 |
| | IND | 36.96 ± 5.03 [1] | 16.03 ± 2.09 [1] | 6.49 ± 1.10 [1] |
| | CARD | 26.62 ± 4.46 [1] | 13.13 ± 4.17 [1] | 3.61 ± 0.57 [1] |
| IL-10R | ***Ex vivo*** | | | |
| | NI | 93.59 ± 1.66 | 72.67 ± 4.24 | 49.48 ± 4.29 |
| | IND | 91.18 ± 2.09 | 68.45 ± 4.91 | 54.73 ± 3.98 |
| | CARD | 93.76 ± 1.79 | 74.88 ± 4.17 | 52.28 ± 3.94 |
| | ***In vitro*** | | | |
| | NI | 20.69 ± 2.45 | 9.60 ± 1.28 | 6.48 ± 1.10 |
| | IND | 13.46 ± 2.80 [1] | 10.73 ± 2.51 | 5.43 ± 1.48 |
| | CARD | 11.92 ± 2.02 [1] | 9.16 ± 2.46 | 4.33 ± 0.79 |

Values of human surface markers are expressed as % medium for each group. *Ex vivo* = before stimulation and *In vitro* = after stimulation. [1]Significantly different from NI (p less than 0.05).

## 5. DISCUSSION

The immunological mechanisms involved in the control of *T. cruzi* replication and in the development of cardiac pathologies in Chagas disease are not well understood. Recent studies have determined that regulatory T cells are contained within the $CD4^+$ T cell subset which control excessive or misdirected immune responses. Regulatory T cells represent approximately 10% of

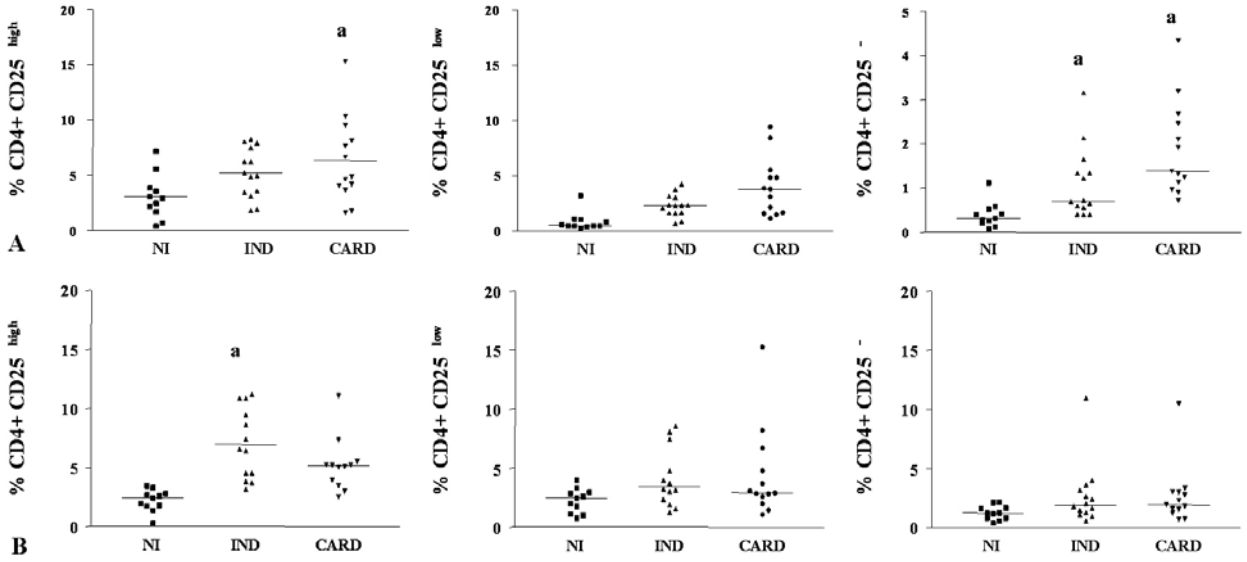


**Figure 4.** Percentage of $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and $CD4^+CD25^{negative}$ T cells, evaluated by flow cytometry after a short *in vitro* stimulation with *T.cruzi* antigen (EPI) as described in Material and Methods. A) CTLA-4 expression. B) Intracellular cytokine IL-10 expression. The difference between a given group and the NI group is considered significant at p less than 0.05 and is indicated by the letter a placed on the graph above the group in question.

peripheral $CD4^+$ cells in humans. These cells which express high levels of the cell-surface marker CD25 develop in the thymus and later enter peripheral tissues where they suppress the activation of other cells (13,22,23). Although the role of regulatory T cells has been assessed for a number of human diseases, little is known about their role in Chagas disease. Here, we show that there is an increase in the frequency of $CD4^+CD25^{high}$ T cells in the peripheral blood of patients with the IND,form of the disease, suggesting that these cells could be involved in the control of morbidity in Chagas disease.

We also show that a higher percentage of $CD4^+CD25^{high}$ T cell population from patients with the IND form of the disease express FOXP3 and IL-10 when compared to the other groups of individuals evaluated (Figure 3 and 4). As previously described by Gomes *et al*., 2003, IL-10 has a key role in regulating type 1 inflammatory response to infection and in the control of parasite replication in the heart and other host tissues during the chronic phase of the disease. This immunoregulatory cytokine has also been suggested to be involved in the mechanisms by which regulatory T cells exert their suppressive activity *in vivo* (24). The ability of regulatory T cells to inhibit Type 1 responses, especially IFN-gamma production, further support our previous results showing that PBMC from patients with the IND form of the disease produce low levels of IFN-gamma (8). This suggests that regulatory T cells may be involved in the regulation of the excessive Type 1 immune response observed in patients with the CARD form of the disease. Based on our results, and those previously published, it is possible that the regulatory role of IL-10 is due to an increase in the number of regulatory T cells.

Among the most important markers of regulatory T cells is the forkhead transcription factor FOXP3 (25). This marker plays a key role in regulatory T cell function and is a specific marker for these cells (26,27). Previously published studies have consistently reported that FOXP3 is predominantly expressed by both human and murine $CD4^+CD25^+$ T regulatory cells. We analyzed FOXP3 protein expression in peripheral blood before and after stimulation with *T. cruzi* antigens. Our data confirm that the majority of FOXP3 cells are indeed $CD4^+CD25^{high}$. Although $CD4^+CD25^{high}$ have higher expression of FOXP3, low expression of this protein was also observed in $CD4^+CD25^{low}$ and $CD4^+CD25^-$ T cells. Similar results have been found for other infections (16,28,29). Whether low FOXP3 expression can modulate/suppress T cell and/or antigen-presenting cell activation or can convert non-regulatory $CD4^+$ T cells into regulatory T cells is still not clear, and future studies are required to answer these questions. We also observed that, besides expression of FOXP3, the $CD4^+CD25^{high}$ cell population in Chagasic patients also expresses several other cell-surface markers that included CD45RO and CTLA-4 (Table 1, Figure 2 and 3), further supporting the role of $CD4^+CD25^+$ T cells as regulatory T cells.

Although $CD4^+CD25^+$ regulatory T cells are selected by high affinity interactions with self-peptide in the thymus, it is now apparent that these cells respond to numerous environmental stimuli in the periphery (30). Currently, the antigenic specificity of $CD4^+CD25^+$ regulatory T cells remains unknown. However, there is evidence that they can mount a recall response to foreign antigens (30,31). It has been shown that $CD4^+CD25^+$ T cells isolated from the skin of *Leishmania major*-infected

mice suppressed IFN-gamma production by *L. major* reactive $CD4^+CD25^-$ effectors cells in the presence of *L. major* infected dendritic cells but not in uninfected animals (12). In the present study we observed that the percentage of $CD4^+CD25^{high}FOXP3^+$ regulatory T cells, after *in vitro* stimulation with specific *T. cruzi* antigens, was higher in Chagasic patients. More importantly we show that this marker increase occurred mainly in cells from patients with the IND form of the disease also suggesting a role for $CD4^+CD25^{high}FOXP3^+$ regulatory T cells in the control of pathology in *T. cruzi* infected individuals (Figure 2). To demonstrate the specificity of this, response cells from the same individuals were stimulated with unrelated antigens from *Schistosoma mansoni* or *L. major,* and the preliminary data show that the percentage of $CD4^+CD25^{high}$ regulatory T cells was lower (data not shown). Although these results support previous suggestions that regulatory T cells have a large repertoire capable of recognizing both self and foreign peptides, one cannot rule out the possibility that they react to heart specific antigens whose expression is regulated/elicited by infection with the *T. cruzi*.

It has been demonstrated that human T cells from patients with indeterminate and cardiac forms of Chagas disease present an increased expression of class II MHC molecules in the peripheral blood (32).We have shown that $CD4^+CD25^{high}$ T cells from Chagasic and NI individuals express HLA-DR. It has also been demonstrated that MHC-II expression on human $CD4^+CD25^{high}$ T cells identifies a functionally distinct population of T regulatory cells that induces early contact-dependent suppression associated with high FOXP3 expression (21).

The expression of CTLA-4 occurs in regulatory T cells (17,34,35) and it may be functionally important. Indeed, anti-CLTA-4 treatment abrogates the ability of regulatory T cells to inhibit colitis (17,34) and also induces the development of gastritis in normal mice (35). In our study we observed a high percentage of $CD4^+CD25^{high}$ T cells from patients with the CARD form of the disease expressing CTLA-4 (Figure 4A). Our results suggest that Type 1 immune response is the main immune mechanism involved in the genesis and/or maintenance of heart pathology in Chagas disease (8,9). Therefore, it is possible that the elevated expression of CTLA-4 may be the mechanism by which inhibition of IL-10 secretion by macrophages occurs, leading to development of severe heart disease.

In conclusion, $CD4^+CD25^{high}$ regulatory T cells from the IND patients seem to reduce the severity of type 1 responses to *T. cruzi* infection. Thus, regulatory T cells, may be beneficial to patients with the IND form of the disease through expression of IL10 and maintenance of a balance among efficient effectors that kill the parasites. However, $CD4^+CD25^{high}$ regulatory T cells from patients with the CARD form of the disease could contribute to the development of severe heart pathology through CTLA-4 and suppression of anti-pathogen protective immune responses. Therefore, the differences in host-parasite interaction may be influenced by the ratio of regulatory to effector T cells.

The results presented in this paper provide the first evidence that *T. cruzi* infection induces the presence of regulatory T cells. This finding raises several important questions such as how do regulatory mechanisms by $CD4^+CD25^{high}$ abrogate the development of heart damage in some patients and allow the development of pathology in others. Functional studies to characterize this population are being conducted and may provide a better understanding of the immunological complexities that control the course of indeterminate clinical form of Chagas disease and determine whether a patient will remain at the indeterminate stage of the disease or progress to the cardiac and/or digestive forms of the disease.

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