Running title: CD4-CD8- T cells in human Chagas disease

Title: Trypanosoma cruzi-induced activation of functionally distinct αβ and γδ CD4-CD8- (double negative) T cells in individuals with polar forms of Chagas disease

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Abstract

CD4-CD8- (double negative- DN) T-cells have recently been shown to display important immunological functions in human diseases. They express γδ or αβ T-cell receptors that recognize lipid/glycolipid antigens presented via the non-classical MHC molecules of the CD1 family. We recently demonstrated that while αβ DN T-cells are mainly committed to the expression of inflammatory cytokines, γδ DN T-cells express mainly IL-10 in patients with cutaneous leishmaniasis. We also demonstrated a correlation between DN T-cells and the expression of IFN-gamma in the acute phase of T. cruzi experimental infection. In this work, we sought to investigate whether αβ or γδ DN T-cells display distinct immunoregulatory potentials in patients with polar forms of human Chagas disease. Our data showed that in vitro infection with Trypanosoma cruzi leads to expansion of DN T-cells in patients of the indeterminate and severe cardiac clinical forms. However, while αβ DN T-cells are more committed to the production of inflammatory cytokines in both forms, γδ DN T-cells display a marked significant increase in antigen-specific IL-10 expression in indeterminate as compared to cardiac patients. Finally, higher frequencies of the IL-10-producing γδ DN T-cells were correlated with improved clinical measures of cardiac function in the patients, suggesting a protective role for these cells in Chagas disease. Taken together, these data show distinct functional characteristics for αβ and γδ DN T-cells associated with distinct morbidity and clinical forms in human Chagas disease.
Introduction

T cell activation is a key event in the establishment of immune responses directed toward intracellular pathogens. Depending on the functional capacity of the activated T cells, the fate of the infection may take different paths either towards a protective or a pathogenic outcome. While it is important that a strong, activated immune response is elicited early on in the infection in order to eliminate (or control) the pathogen, the further control of this activation is necessary to re-establish homeostasis, avoiding tissue damage (18, 26).

One hallmark of most parasitic infections is that the great majority of individuals are able to trigger innate immunity and elicit an activated T cell response during the acute infection, leading to the control of the parasite and establishment of a chronic infection. Interestingly, while many individuals develop severe forms of parasitic diseases once infection progresses to the chronic phase, most patients develop relatively mild forms, allowing for a host-parasite co-existence. One such example is observed upon human infection with the protozoan parasite Trypanosoma cruzi, which leads to Chagas disease. As a result of thousands of years of co-evolution between human host and the parasite (7), most infected individuals develop an asymptomatic form of Chagas disease, named indeterminate. This form is characterized by a lack of clinical signs and symptoms and has been associated predominantly with a modulatory cellular immune response based on cytokine profiles and down regulatory molecule expression (6, 21, 49, 50, 52). Chronic patients may also develop symptomatic clinical forms, mainly with digestive or cardiac alterations. Differential geographical prevalence of Chagas disease clinical forms has been reported. In Brazil, 15-30% of Chagas patients display the cardiac form, which is present in twenty states, while the digestive cases, observed in about 10% of infected individuals, have been reported in four states in the central region of the country (53). The digestive form is
frequently found in Chile, but is practically absent in Central America (43). These geographical differences might be related, in part, to host genetics and immune response of local human populations, but it is believed that they are also related to the genetic diversity of *T. cruzi* (12). Different strains of parasite display tropism for different tissues and thus, an important factor determining the clinical course of disease might be the specific pool of infecting clones and their specific tropisms (30). However, a possible role for environmental, nutritional and immunological aspects of the host cannot be discarded. While digestive and cardiac forms present significant morbidity, the cardiac form is the one associated with highest mortality. It is caused by neuronal and cardiomyocyte damage, ultimately resulting in ventricular dilation and subsequent functional heart failure, which can lead to death (45). Cardiac patients display a T cell-mediated inflammatory response *in situ* (14, 25, 42), which is responsible for the pathology; this inflammatory profile is also observed in circulating activated T cells found at high frequencies in these patients (2, 17, 20, 33). Although it is clear that a plethora of parasite and host factors influence the clinical outcome of Chagas disease, recent studies have suggested that activation of functionally distinct T cell populations in *T. cruzi* infected individuals may be responsible for the establishment of different clinical forms (18, 21). Thus, identifying these populations and the factors responsible for their activation will be critical for driving immune-based interventions to prevent pathology.

While the great majority of T cells express either the CD4 or the CD8 molecules, which are important for stabilizing the peptide-MHC complex and favor T cell activation, a minority population of T cells that do not express CD4 or CD8 molecules has been identified in humans (9, 11, 28, 38). These double-negative (DN) T cells have been shown to be important sources of immunoregulatory cytokines in human leishmaniasis (4), to display modulatory functions (39)
but also, under different circumstances, to display cytolytic activity (11, 37). A subpopulation of DN T cells are activated through the engagement of their αβ or γδ T cell receptor (TCR) when recognizing non-classical MHC molecules of the CD1 family, presenting lipid or glycolipid antigens (37). This particular lipid/glycolipid antigenic recognition, as well as their immunoregulatory potential and susceptibility to chronic stimulation, highlighted these cells as playing an important role in parasitic infections.

In our work by Bottrel et al., we determined that DN lymphocytes were the second most prevalent cell type producing IFN-gamma in human cutaneous leishmaniasis, and that this IFN-gamma production was seen after short term cultures with media alone, as well as after stimulation with soluble *Leishmania* antigen (SLA) (10). The novel work of Antonelli et al. went on to demonstrate that DN T cells composed of two different cell populations are present in the blood of individuals infected with *Leishmania braziliensis* and that DN T cells expressing the αβ TCR displayed a profile consistent with activation of leishmaniacidal and inflammatory activities (higher IFN-gamma and TNF-alpha), while the DN subpopulation expressing γδ TCR had a modulatory potential via higher production of IL-10 (5). Interestingly, IFN-gamma production has been associated with pathogenic responses in human leishmaniasis in more than one clinical form (3, 8, 23). We recently demonstrated that rats infected with the CL-Brenner clone of *T. cruzi* displayed a marked increase in the frequency of circulating DN T cells during the acute phase of infection (34). Taken together, these data led to the question of the role that DN T cell subpopulations play in the clinical dichotomy of chronic human Chagas disease.

To answer these questions, we investigated the immunoregulatory potential of DN T cells in patients with the two polar forms of Chagas disease: indeterminate and dilated cardiac. Our data demonstrated that, although no quantitative differences were seen with regards to the non-
stimulated frequency of DN αβ and γδ T cell sub-populations when comparing patients and non-chagasic individuals, *in vitro* infection with trypomastigote forms of *T. cruzi* induced a marked increase in the frequency of these cells in chagasic patients. Moreover, the expanded αβ DN T cells displayed a greater inflammatory potential in cardiac patients than in indeterminate patients, which was accompanied by a greater down modulatory ratio of IL-10 to inflammatory cytokine frequencies by γδ DN T cells from indeterminate individuals, suggesting distinct roles for these cells in modulating the response in chronic Chagas disease. Finally, we observed a correlation between higher frequencies of IL-10 producing γδ DN T cells and improved clinical measures of cardiac function, suggesting a protective role for these cells in human Chagas disease. These data indicate that functionally distinct DN T cells are present in Chagas disease patients, and that they are associated with the resulting morbidity of the disease.

**Patients, Material and Methods**

**Patients:** This study employed a cross-sectional design involving patients from endemic areas within Minas Gerais, Brazil, under the medical care of Dr. Manoel O. C. Rocha. A total of twelve patients with positive specific serology for *T. cruzi*, within the chronic phase of the disease and with well-defined clinical forms were enrolled in this study. Detailed evaluations, including physical examinations, electrocardiogram, chest X-rays and echocardiogram were performed in order to classify patients into different groups as previously defined by us (44). Clinical groups were: indeterminate (I; n=7), which did not present with any clinical manifestations or alterations upon clinical, radiological and echocardiographic examination. Patients with dilated cardiomyopathy (DC; n=5) presented with right and/or left ventricular dilation, global left ventricular dysfunction, as well as alterations in the cardiac electric impulse generation and
conduction. These alterations were evident in electrocardiogram, chest x-rays and echocardiography, which showed the occurrence of heart enlargement in all cardiac patients analyzed. Left ventricular ejection fraction (LVEF) and left ventricular diastolic diameter (LVDD) were used as clinical parameters of ventricular function for the Chagas patients (45). We also included in our analysis individuals without Chagas disease (N; n=7), as determined by negative specific serological tests for Chagas disease. Individuals with the digestive form of Chagas disease were not included in this study due to low incidence of well-documented cases in our geographical location in Brazil. Characteristics of the study groups are summarized in Table 1. 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 work were volunteers and treatment and clinical care was offered to all patients, as needed, despite their enrollment in this research project. This study is part of an extended project evaluating risk factors for cardiac damage/involvement in Chagas’ disease, which has the approval of the Research Ethics Committee from Federal University of Minas Gerais (COEP-UFMG-ETIC006/05) and are in accordance with the Declaration for Helsinki. 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 or L929 cell lines, as previously performed by us (50). Briefly, cells were infected with ten trypomastigotes/cell and, after removal of free trypomastigotes by washing with culture media, were maintained in RPMI enriched with 5% fetal calf serum and antibiotic (penicillin 500U/ml and streptomycin 0.5mg/ml) for approximately 5 days. After this period, trypomastigotes ruptured the cells and were collected from the supernatant. The contamination with amastigote forms was always below 3%. Parasites obtained in such a manner were used for infecting blood cells from patients and non-chagasic individuals.
Infection of blood cells from patients and non-chagasic individuals with *T. cruzi* trypomastigotes:

Infection of peripheral blood cells was performed using ten trypomastigotes/cell, as previously described (50). Briefly, cells and parasites were incubated at 37°C, 5% CO₂, for a period of 3 hours. After this time, cells were washed by centrifugation with phosphate-buffered saline (PBS) for removal of free trypomastigotes. After centrifugation, supernatant was removed and an equal volume to the amount of blood initially incubated of RPMI supplemented with antibiotic/antimicotic (amphotericin B 0.25µg/ml, penicillin 200U/ml and streptomycin 0.1mg/ml) and l-glutamine (1mM) was added to the tubes. Infected cells were incubated at 37°C, 5% CO₂ for a period of 14 hours. After this period, Brefeldin A (1µg/ml) was added to prevent protein secretion and cultures re-incubated for additional 4 hours. For all individuals, we carried out cultures of blood submitted to the same procedures described above, in the absence of parasites, as non-stimulated control.

Determination of the frequencies of DN T cells and expression of cytokines by αβ and γδ DN T cells: Frequencies of αβ and γδ DN T cells, as well as expression of IFN-gamma, TNF-alpha, IL-17, and IL-10 by these DN T cell subpopulations were determined by flow cytometry. Infected cells (treated as described above) or non-infected blood cells were harvested after the final 18 hours of culture and submitted to specific staining for the above-mentioned molecules. We used a combination of Cyochrome-labeled anti-CD4 and CD8 to detect DN T cells, as previously done by us (4). FITC-labeled anti-αβ or anti-γδ were also used in the staining to identify the specific sub-populations. Cells were harvested and plated at a concentration of 200,000 cells/well and incubated with a 20µl mixture of the surface antibodies (anti-CD4+anti-CD8-Cyochrome + anti-αβ or anti-γδ - FITC) for 15 minutes at 4°C. Samples were washed three times in PBS/1%BSA and fixed by a twenty-minute incubation with a 2% formaldehyde solution. After removal of the fixing solution
by centrifugation, and washing with PBS, we permeabilized the cells by incubation for 10 minutes with a 0.5% saponin solution, and proceeded to the intracellular cytokine labeling. Samples were incubated with PE-labeled anti-cytokine monoclonal antibodies for 20 minutes at room temperature, washed twice with 0.5% saponin solution, resuspended in PBS and read in a flow cytometer. A minimum of 40,000 gated events from each sample were collected and analyzed using the FlowJo program. Analysis were performed by gating on the lymphocyte population and further gating on the CD4-CD8- αβ or γδ+ T cells to determine the expression of the different molecules, as previously done by us (4).

Statistical analysis: The means of the different groups were compared using Tukey-Kramer all pair comparison analysis of variance contained within the JMP software from SAS. Pared T test was used to ascertain differences amongst non-infected versus infected cultures within the same group of patients. Correlation analysis were done using Pearson’s correlation coefficient. Differences that returned p values of less than or equal to 0.05 were considered statistically significant from one another.

Results

In vitro infection with trypomastigote forms of T. cruzi induces an expansion of αβ and γδ DN T cells in chronic Chagas disease patients: To determine the frequency of DN T cell subsets in chronic Chagas patients and non-chagasic individuals, we performed flow cytometric analysis of peripheral blood cells from these individuals, as described above. The analysis were carried out in non-stimulated cells, to provide information of the frequency of these cells ex vivo from the patients, as well as after in vitro infection with trypomastigote forms do 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 the frequency of αβ and γδ DN T cells in non-stimulated cultures were similar among groups (Figure 1 A, white bars). Moreover, we observed that within the total DN T cells, the frequency of αβ and γδ subpopulations were similar among groups (αβ: N= 23+/−8, I= 30+/−11, DC= 25+/−7; γδ: N= 77+/−8, I= 70+/−11, DC= 75+/−7).

Exposure of the cells from indeterminate and cardiac patients, as well as non-chagasic individuals to trypomastigote forms of T. cruzi led to an expansion of αβ and γδ DN T cells in all groups (Figure 1 A, dark bars; representative FACS plots in Figure 1 B).

αβ and γδ DN T cells from chagasic patients display distinct immunoregulatory profiles: In order to determine the functional characteristics of αβ and γδ DN T cells from patients with polar clinical forms of Chagas disease, we investigated the expression of inflammatory (IFN-gamma, TNF-alpha and IL-17) and anti-inflammatory (IL-10) cytokines by these cells in cultures exposed or not to the parasite. We observed that, whereas there was no difference in the frequency of αβ and γδ DN T cells expressing any of the cytokines in non-stimulated cultures of the different groups (Figures 2 and 3, white bars), exposure to the parasite revealed dramatic differences among them. In contrast, stimulation of peripheral blood cells with T. cruzi led to a significant increase in frequency of αβ DN T cells expressing IFN-gamma, TNF-alpha and IL-17 from chagasic patients, but not from non-infected individuals (Figure 2, left panels). The induction of inflammatory cytokines was more evident in cells from DC patients exposed to the parasite than from I patients, as demonstrated by the significantly higher frequency of cytokine producing cells in the DC group as compared to the N group for all cytokines (Figure 2, left panels). When analyzing the expression of inflammatory cytokines within the γδ DN T cells, a T. cruzi induced increase in cells expressing IFN-gamma, TNF-alpha and IL-17 was seen for both clinical forms, but not non-chagasic individuals (Figure 2, right panels). In this sub-population,
the induction of inflammatory cytokines was significantly higher in cells from I and DC as compared to N, after exposure to the parasite (Figure 2, right panels).

Analysis of expression of the down modulatory cytokine IL-10 within the αβ and γδ DN T cell populations showed that this cytokine was dramatically induced in *T. cruzi* cultures with cells from indeterminate patients (Figure 3 A; representative FACS plot in figure 3 B). This increase in the frequency of IL-10 producing cells from indeterminate patients was seen when comparing unstimulated and *T. cruzi* stimulated cultures, as well as when comparing *T. cruzi* stimulated cultures of I versus N and DC within the γδ DN T cell subpopulation (Figure 3). To determine the regulatory potentials of each subpopulation of αβ and γδ DN T cells from I and DC patients we calculated regulatory ratios by dividing the frequency of cells producing IL-10 by the frequency of cells producing either IFN-gamma, TNF-alpha or IL-17. These results show that γδ DN T cells from I have a much greater down modulatory profile as compared to γδ DN T cells from DC patients (Table 2). An analysis of the relative contribution by DN and other T cells to the overall frequency of IL-10 producing lymphocytes demonstrates that, on average, DN T cells account for at least 32% of the total IL-10 expression. This is a striking contribution, given that DN T cells represent a minority population of T cells. Interestingly, while the contribution of DN T cells for the overall IL-10 expression by lymphocytes in non-stimulated cultures from non-chagasic individuals was lower than that of CD4+/CD8+ T cells (33±/8 versus 51±/13, p<0.05), it was higher in non-stimulated cultures from indeterminate patients (49±/22 versus 27±/12, p<0.05). No statistically significant changes were observed comparing the DN T cells and CD4+/CD8+ T cells from cardiac patients.
Higher frequencies of IL-10 producing γδ DN T cells are correlated with improved clinical parameters of heart function in human Chagas disease: Previous studies performed by our group have suggested that IL-10 has a protective role in human Chagas disease through an association between the indeterminate clinical form of Chagas disease and high producing genotypes for IL-10 (13). In order to further determine if the IL-10 producing γδ DN T cell subpopulation is correlated with improved cardiac function, and thus a possible protective role in human Chagas disease, we performed a correlative analysis between the frequency of these cells and two clinical parameters of cardiac function: left ventricular ejection fraction (LVEF) and left ventricular diastolic diameter (LVDD). These distinct clinical parameters are directly and inversely correlated with better cardiac function, respectively (45). Strikingly, a significant positive correlation was seen between higher LVEF and higher frequencies of IL-10+ γδ DN T cells (Figure 4 A). Moreover, a highly significant negative correlation between lower LVDD and higher frequencies of IL-10+ γδ DN T cells was also seen (Figure 4 A). Interestingly, although αβ DN T cells also express IL-10 upon stimulation with the parasite, we did not observe correlations between the frequency of these cell subpopulation and the clinical parameters analyzed (Figure 4 B). These data suggest that IL-10 producing γδ DN T cells display an important immunoregulatory role that leads to maintenance of better cardiac function in chagasic patients.

Discussion

Human infection with T. cruzi is the cause of Chagas disease, an illness that currently affects approximately 18 million people in Latin America, where it is considered endemic. In addition, it is estimated that one-hundred million people are at risk of infection with T. cruzi.
Although treatment is available and relatively effective (41, 48), toxicity and lack of widely distributed pediatric formulations is still a major problem in human Chagas disease. While vector transmission was controlled in certain areas of South America, disease transmission via blood transfusion and organ transplant has brought the disease to attention of health professionals in Latin America and other non-endemic countries such as the United States and other countries (29). Moreover, cases of acute Chagas disease have been described in areas where acute cases were not reported for over 15 years (51). Despite the fact that most Chagas patients display a relatively mild, asymptomatic, clinical form, about 30% of the patients develop severe disease, leading to cardiac involvement and often, death (45). Thus, the social and economic burden caused by Chagas disease places it amongst the most morbid of all parasitic diseases.

The mechanisms behind the development of the severe cardiac form of Chagas disease have not been completely elucidated. However, it is well accepted that T cells are key players in mounting an immune response during the chronic phase of the disease (18). Thus, T cell activation and function are critical in determining the clinical outcome of Chagas disease. Cardiac patients display a highly activated, inflammatory T cell response both \textit{in situ} (14, 25, 42), as well as in the peripheral blood (2, 17, 20, 33). Interestingly, however, patients who do not develop pathology and remain asymptomatic, also display high frequency of activated T cells in their bloodstream (19). This apparent contradiction has been better understood more recently, mainly due to the use of two important approaches: (1) clear definition of patient clinical forms by performing refined clinical analysis and (2) identification and characterization of T cell subpopulations that display distinct functional activities. Thus, recent studies, using patients with well-defined clinical forms have shown that, although T cell activation is observed in severe and asymptomatic Chagas patients, these cells have distinct functional potentials (18). Most studies
have focused on the analysis of expression of factors that control the establishment of inflammatory responses in Chagas disease, such as inflammatory cytokines and chemokines (21, 22). Studies performed by us and other groups have shown that major T cell populations, defined by the expression of CD4 and CD8 display phenotypic and functional differences in individuals with different clinical forms of Chagas disease. To this end, the frequency of memory cells, as well as senescent cells have been associated with the chronic cardiac form of Chagas disease (1, 2, 24). While these studies have provided critical information, the determination of the contribution of distinct sub-populations to the immunoregulation and functional activities, as well as the antigens that lead to their activation, is critical for the understanding the mechanisms of generation of pathogenic versus protective responses in Chagas disease.

A quantitatively small subpopulation of T cells that do not express CD4 or CD8 molecules have been identified and, because of their ability to tolerate chronic stimulation due to the lack of the stabilizing CD4 or CD8 molecules, have been shown to be critical in the chronic immune diseases, specially auto-immune processes (9, 31). Furthermore, a large portion of these cells are activated by recognizing lipid/glycolipid antigens presented via CD1 molecules (37). Glicolipid determinants from *T. cruzi* have been shown to be important in the activation of cellular immune responses in experimental infection (35). Although previous studies in murine infection with *T. cruzi* suggested that CD1 molecules were not critical in eliciting cellular responses to parasite components (35, 40), others have shown that CD1 presentation is important for natural killer T cell activation (15, 16, 32).

The role of DN T cells in *T. cruzi* infection has not yet been clarified. It has been shown that mice infected with the parasite display a 40-100 fold increase in the frequency of liver γδ+CD4-CD8- lymphocytes, associated with expression of IFN-gamma (46). Interestingly, the
same group later showed that the liver is an important organ for parasite clearance in the chronic infection (47). An increase in the DN T cell frequency in the liver of animals infected with *Plasmodium* was also associated with parasite inhibition (36). Infection of rats with the highly virulent CL-Brener clone of *T. cruzi* was associated with an expansion of CD4-CD8- T cells and IFN-gamma production (34).

Recent studies have also pointed to important roles of DN T cells in human parasitic diseases. We have shown that αβ and γδ DN T cells display distinct immunoregulatory profiles in human cutaneous leishmaniasis (4, 26). Moreover, a high frequency of DN T cells was observed in the peripheral blood of individuals with *P. falciparum* malaria (54). In this work, we performed an analysis of the frequency of DN T cell αβ and γδ subpopulations in individuals with polar clinical forms of Chagas disease. Our results showed that, although there were no quantitative differences in the frequencies of these cells freshly isolated from chagasic patients and non-infected individuals, *T. cruzi* infection led to an expansion of DN T cells in vitro and these cells were quite different in their immunoregulatory potential. Although a parasite induced expansion of DN T cells was observed in cultures of cells from patients as well as non-infected individuals, the DN T cells from non-infected individuals did not express parasite induced cytokines; compatible with a primary response. On the other hand, expanded cells from patients produced high levels of cytokines, indicative of antigenic-specific recall response, and also showed different cytokine profile expression in indeterminate and cardiac patients. We observed that αβ DN T cells from individuals of the cardiac clinical form of Chagas disease display a higher expression of inflammatory cytokines upon *in vitro* stimulation with *T. cruzi*. Interestingly, γδ DN T cells from indeterminate patients displayed a markedly high expression of IL-10 following *T. cruzi* stimulation, which was not observed in cardiac patients. Analysis of the
ratio IL-10/inflammatory cytokines revealed a clear down modulatory environment associated with γδ DN T cells in indeterminate patients and not cardiac. Given that we do not know the exact nature of the antigen responsible for the activation of these cells, we did not yet focus on any specific DN T cell subpopulation, such as the DN NKT cells. Further studies are being carried out in our laboratory to clarify these questions. However, the observed functional differences presented here are clearly associated with important clinical features of the patients and continue to support earlier findings by our group and others defining key differences in the immunoregulatory environment between indeterminate and cardiac chagasic patients (21).

Monitoring cardiac function is an important procedure that permits one to follow the course of pathology development and worsening of human Chagas disease. Unfortunately, due to the high costs of several of the required exams, these procedures are not always possible to perform. We evaluated a group of clinically well-defined Chagas patients, in which two measures of cardiac function were performed: left ventricular ejection fraction (LVEF) and left ventricular diastolic diameter (LVDD). These clinical characteristics, although physiologically related, reflect different levels of cardiac lesion. The greater the LVEF and the smaller the LVDD, the better the cardiac function. A positive correlation between a higher frequency of IL-10 producing γδ DN T cells and improved cardiac function as measured by LVEF was seen. Moreover, the higher the frequency of IL-10 producing γδ DN T cells, the lower the LVDD, which again indicates the association of IL-10 producing γδ DN T cells with better cardiac function. Previous studies performed by us showed that a down modulatory profile, as accessed mainly by IL-10 and CTLA-4 expression, was predominant in indeterminate patients (49, 50). Moreover, we demonstrated that IL-10 promoter gene polymorphism, which leads to high IL-10 expression, is associated with the occurrence of the indeterminate clinical form. Here, we suggest
that IL-10 derived from γδ DN T cells may also be involved in protection. This is an important 
finding, since these cells are likely activated via distinct mechanisms, as compared to the other 
cell populations studied to date. This could aid in the development of novel antigen-based 
prophylactic or therapeutic interventions.

An important question still unanswered is why these cell populations display distinct 
functional capabilities in patients with indeterminate and cardiac clinical forms. This is 
particularly intriguing when we remember that indeterminate patients, who apparently display a 
modulated response that may be important for avoiding tissue inflammation, may develop 
cardiac disease in the future. The hypothesis is that these individuals would undergo cellular 
functional changes, which would lead to pathology establishment. Assuming that these changes 
would be a cause and not consequence of pathology, identifying such differences and 
determining what are their causes will provide critical information for preventing cardiac damage 
and clinical worsening pathology.

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are reliable markers of autoimmune lymphoproliferative syndrome (ALPS) associated with FAS loss of function. Blood 113:3027-3030.


TABLE 1: Individuals analyzed in the study and their clinical status.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serology for Chagas disease</th>
<th>Clinical form</th>
<th>Age (Years old)</th>
<th>Sex</th>
<th>LVEF (%)</th>
<th>LVDD (mm)</th>
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<td>I7</td>
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<td>66</td>
<td>49</td>
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<tr>
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<tr>
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<td>ND</td>
<td>Male</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>DC4</td>
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<td>Male</td>
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<tr>
<td>DC5</td>
<td>Positive</td>
<td>Cardiac</td>
<td>63</td>
<td>Male</td>
<td>37</td>
<td>65</td>
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TABLE 2: Indeterminate patients maintain a regulatory ratio of IL-10 producing cells*.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Ratio IL-10/IFN-gamma</th>
<th>Ratio IL-10/TNF-alpha</th>
<th>Ratio IL-10/IL-17</th>
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<tr>
<td>Indeterminate</td>
<td>1.01 +/- 0.26</td>
<td>1.26 +/- 0.21</td>
<td>1.66 +/- 0.21</td>
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<tr>
<td>Cardiac</td>
<td>0.32 +/- 0.07**</td>
<td>0.55 +/- 0.14**</td>
<td>0.89 +/- 0.09**</td>
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</table>

* The frequency of γδ DN T cells expressing the above mentioned cytokines following *in vitro* stimulation with *T. cruzi* was determined as described in Materials and Methods for each patient and then used to calculate regulatory ratios by dividing the frequency of IL-10 (a down regulatory cytokine) producing cells by the frequency of IFN-gamma, TNF-alpha or IL-17 producing cells.

**The values represent the average of ratios for IL-10/given cytokine +/- the S.E. for 7 Indeterminate and 5 Cardiac patients. In all cases the comparison between the ratios for Indeterminate vs. Cardiac groups returned a p <0.01.
Figure 1: Trypanosoma cruzi activation of peripheral blood cells induces expansion of αβ and γδ double negative (CD4-CD8-) T cells. Whole blood cells from non-infected controls (N), indeterminate chagasic patients (I) and dilated cardiac chagasic patients (DC) were incubated overnight as described in Materials and Methods with either media alone (MED) or with live T. cruzi (TRP) and then analyzed for the frequency of αβ and γδ CD4-CD8- T cells using flow cytometry. Panel A shows the average frequencies for each group +/- S.D. The number of individuals in each group were; N = 7, I =7 and DC =5. Statistical significance is indicated in each graph with differences between groups indicated by common numbers. Comparisons between groups was performed using Tukey-Kramer comparison of all pairs, and within groups (MED vs. TRP) using the paired t-test as described in Materials and Methods. All patients were meticulously classified based on clinical criteria as described in Materials and Methods. Panel B shows representative dot plots from an indeterminate patient and gating for analysis of αβ and γδ CD4-CD8- T cells. Both anti-CD4 and anti-CD8 antibodies were conjugated with CyChrome allowing identification of the double negative (DN) T cells using specific antibodies against either αβ and γδ T cell receptors conjugated with FITC. The gates used for determining the percent of DN T cells and further analysis of cytokine expression in the DN T cell populations are shown.
Figure 2. Trypanosoma cruzi activation of peripheral blood cells induces specific inflammatory cytokine production by αβ and γδ double negative (CD4-CD8-) T cells from both indeterminate and dilated cardiac chagasic patients. Whole blood cells from non-infected controls (N), indeterminate chagasic patients (I) and dilated cardiac chagasic patients (DC) were incubated overnight with either media alone (MED) or with live T. cruzi (TRP) and then analyzed for the frequency of αβ or γδ double negative (DN) T cells producing specific cytokines using flow cytometry as described in Materials and Methods. The data represents the average for each group +/- S.D. The number of individuals in each group were; N = 7, I =7 and DC =5. The top Panel shows the average percent of IFN-gamma producing cells within αβ or γδ DN T cells from individual cultures without (MED) or with (TRP) stimulus. The middle Panel shows the same for TNF-alpha producing cells within αβ or γδ DN T cells, and the bottom Panel shows the values for IL-17 producing cells within αβ or γδ DN T cells. Statistical significance is indicated in each graph with differences between groups indicated by common numbers. Comparisons between groups was performed using Tukey-Kramer comparison of all pairs, and within groups (MED vs. TRP) using the paired t-test as described in Materials and Methods. All patients were meticulously classified based on clinical criteria as described in Materials and Methods.
Figure 3: γδ double negative (CD4-Cd8-) T cells from indeterminate chagasic patients display a biased down modulatory profile following stimulation with Trypanosoma cruzi. Whole blood cells from non-infected controls (N), indeterminate chagasic patients (I) and dilated cardiac chagasic patients (DC) were incubated overnight with either media alone (MED) or with live T. cruzi (TRP) and then analyzed for the frequency of αβ or γδ double negative (DN) T cells producing IL-10 using flow cytometry as described in Materials and Methods. The data represents the average for each group +/- S.D. The number of individuals in each group were; N = 7, I = 7 and DC = 5. Panel A shows the average percent of IL-10 producing within αβ or γδ DN T cells from individual cultures without (MED) or with (TRP) stimulus for each group. Statistical significance is indicated in each graph with differences between groups indicated by common numbers. Comparisons between groups was performed using Tukey-Kramer comparison of all pairs, and within groups (MED vs. TRP) using the paired t-test as described in Materials and Methods. Panel B shows representative dot plots and gating for analysis of αβ and γδ CD4-Cd8- T cells producing IL-10. Both anti-CD4 and anti-CD8 antibodies were conjugated with Cyochrome allowing identification of the double negative (DN) T cells using specific antibodies against either αβ and γδ T cell receptors conjugated with FITC. The gates used for determining the percent of DN T cells producing IL-10 is then determined in a histogram using anti-IL-10 conjugated with PE. Above is the percent of cells producing IL-10 from cultures of media alone and below percent of cells producing IL-10 from cultures after stimulation with T. cruzi as described in Materials and Methods. All patients were meticulously classified based on clinical criteria as described in Materials and Methods.
Figure 4: Higher frequencies of IL-10 producing $\gamma\delta$ double negative (CD4-CD8-) T cells are correlated with better heart function in chagasic patients. The frequency $\gamma\delta$ double negative (CD4-CD8-) T cells producing IL-10 following stimulation with *Trypanosoma cruzi* was calculated from a group of chagasic patients who had associated detailed clinical data measuring ventricular function. These measurements, the left ventricular ejection fraction (LVEF) and left ventricular diastolic diameter (LVDD). The higher the LVEF, the better the ventricular function, and the lower the LVDD, the better the ventricular function. Panel A shows Person’s correlation plots between the frequency of IL-10$^+$ $\gamma\delta$ double negative (CD4-CD8-) T cells and measurements of ventricular function. In contrast, in Panel B, no correlation is seen between IL-10$^+$ $\alpha\beta$ double negative (CD4-CD8-) T cells and measurements of ventricular function. A total of nine chagasic patients were used in this analysis for which this clinical data was available. Statistical significance (p value) is indicated in each graph together with the $r^2$ value.