# *Trypanosoma cruzi* modulates the profile of memory CD8<sup>+</sup> T cells in chronic Chagas' disease patients

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#### Abstract

We present a cross-sectional analysis of the maturation and migratory properties of the memory CD8<sup>+</sup> T cell compartment, in relation to the severity of heart disease in individuals with chronic *Trypanosoma cruzi* infection removed from endemic areas for longer than 20 years. Subjects with none or mild heart involvement were more likely to mount *T. cruzi*-specific memory IFN- $\gamma$  responses than subjects with more advanced cardiac disease, and the *T. cruzi*-specific CD8<sup>+</sup> T cell population was enriched in early-differentiated (CD27<sup>+</sup>CD28<sup>+</sup>) cells in responding individuals. In contrast, the frequency of CD27<sup>+</sup>CD28<sup>+</sup>CD8<sup>+</sup> T cells in the total memory CD8<sup>+</sup> T cell population decreases, as disease becomes more severe, while the proportion of fully differentiated memory (CD27<sup>-</sup>CD28<sup>-</sup>) CD8<sup>+</sup> T cells increases. The analysis of CCR7 expression revealed a significant increase in total effector/memory CD8<sup>+</sup> T cells (CD45RA<sup>-</sup>CCR7<sup>-</sup>) in subjects with mild heart disease as compared with uninfected controls. Altogether, these results are consistent with the hypothesis of a gradual clonal exhaustion in the CD8<sup>+</sup> T cell population, perhaps as a result of continuous antigenic stimulation by persistent parasites.

#### Introduction

Chagas' disease, caused by the protozoan parasite Trypanosoma cruzi, is characterized by cardiac and digestive abnormalities, which develop in 20-30% of infected individuals many years after the acute infection has been controlled. Trypanosoma cruzi is an obligate intracellular parasite, which invades and replicates within a wide variety of mammalian cells. As with other intra-cytoplasmatic parasites, T. cruzi proteins released into the host cell cytosol are processed and presented on MHC-class I molecules, leading to the recognition of parasite components by CD8<sup>+</sup> T cells (1, 2). The important role of CD8<sup>+</sup> T lymphocytes in resistance to T. cruzi has been demonstrated by studies showing the predominance of CD8<sup>+</sup> T cell in inflammatory infiltrates in infected tissues (3–6), the ability of CD8<sup>+</sup> T cells from chronic chagasic patients and infected mice to act as CTLs against T. cruziinfected cells (7) and the increased tissue parasite burden, the absence of inflammatory response and the rapid mortality of mice lacking CD8<sup>+</sup> T cells (8, 9).

Previous studies from our laboratories have shown that the frequency of IFN-y-producing memory CD8<sup>+</sup> T cells specific for T. cruzi-derived peptides is very low regardless of the clinical status but in patients with severe disease is essentially undetectable (10). Moreover, the analysis of a wider range of T cell targets, by stimulation with a *T. cruzi* amastigote lysate, revealed a higher frequency of responders among patients with mild or no clinical disease and a low frequency of responders among those with the more severe form of the disease, indicating an inverse correlation between T. cruzi-specific T cell responses and disease severity. In spite of the large amount of data available on the characteristics of the immune response in Chagas' disease, the mechanisms involved in the maintenance of T. cruzi-specific memory T cell responses and their role in the transition from the asymptomatic phase to clinically manifest cardiomyopathy remains poorly understood.

Recently, based on the combined expression of CD27 and CD28, Appay *et al.* (11) proposed a linear differentiation model

#### 466 CD8<sup>+</sup> T cell sub-populations in human Chagas' disease

for memory CD8<sup>+</sup> T cells, in which CD27<sup>+</sup>CD28<sup>+</sup>, CD27<sup>-</sup>CD28<sup>+</sup> or CD27<sup>+</sup>CD28<sup>-</sup> and CD27<sup>-</sup>CD28<sup>-</sup> cells are considered to be 'early', 'intermediate' and 'late' stages of memory CD8<sup>+</sup> T cells differentiation, respectively. These distinct memory T cell populations appear to dominate in different virus infections depending on the differentiation phenotype. The expression of the chemokine receptor CCR7 for lymph node homing permits further discrimination into central memory T cells (CD45RA<sup>-</sup>CCR7<sup>+</sup>), which lack immediate effector functions and have the capacity to migrate to lymph nodes, and effector memory T cells (CD45RA<sup>-</sup>CCR7<sup>-</sup>) that circulate to peripheral tissues and exhibit rapid effector function upon encounter with the antigen (12).

Herein, we have examined the impact of chronic *T. cruzi* infection on the maturation and migratory properties of memory CD8<sup>+</sup> T cells in relation to the severity of the heart disease in chronic chagasic subjects.

#### Methods

#### Selection of study population

Chronic chagasic subjects were recruited at the Instituto Nacional de Parasitología Dr. Mario Fatala Chabén and at the Chagas disease Section, Cardiology Department, Hospital Interzonal General de Agudos Eva Perón. Signed informed consent was obtained from all individuals prior to inclusion in the study. Trypanosoma cruzi infection was determined by a combination of indirect immunofluorescence assay, hemagglutination and ELISA tests, performed in the Diagnostic Department of the Instituto Nacional de Parasitología Dr. Mario Fatala Chabén and in the Clinical Laboratory of Parasitology, Hospital Interzonal General de Agudos Eva Perón. Subjects positive on at least two of these tests were considered to be infected. Subjects were evaluated clinically and grouped according to the Kuschnir grading system (13). Group 0 (G0) included seropositive individuals exhibiting a normal electrocardiogram (ECG) and a normal chest X-ray, group 1 (G1) seropositive patients with a normal chest X-ray but abnormalities in the ECG, group 2 (G2) seropositive patients with ECG abnormalities and heart enlargement as determined by chest X-ray and group 3 (G3) seropositive patients with ECG abnormalities, heart enlargement and clinical or radiological evidence of heart failure. A total of 25 out of 92 patients had been previously treated with 5 mg benznidazole kg<sup>-1</sup> day<sup>-1</sup> for 30 days, at some point during adulthood. Benznidazole was provided by the Ministry of Health of Buenos Aires Province, and administered by the patient in ambulatory conditions (Table 1).

The control group consisted of age-matched Caucasian natives from Argentina who have always resided in nonendemic areas and were serologically negative for *T. cruzi*. Chagasic subjects and non-infected controls with hypertension, ischemic heart disease, cancer, HIV infection, syphilis, diabetes, arthritis or serious allergies were excluded from this study. The data on the number, sex and age of the subjects included in this study are summarized in Table 1.

#### Collection of PBMCs

Approximately 50 µl blood was drawn from patients and control subjects by venipuncture into heparinized tubes

#### Table 1. Characteristics of study population

Clinical status	Number	Previously treated <sup>a</sup>	Age <sup>b</sup>	Gender	
				Male	Female
G0 G1 G2 G3	29 29 13 21 26	9 9 4 3	46.3 (31–66) 56.3 (37–71) 54.5 (41–74) 61.8 (43–82) 37.4 (18, 70)	12 15 5 15	17 14 8 6

G0, seropositive individuals with normal ECG and chest X-rays; G1, seropositive patients with normal chest X-ray but abnormal ECG; G2, seropositive patients with abnormal ECG and heart enlargement on chest X-rays; G3, seropositive patients with abnormal ECG and heart enlargement on chest X-rays and clinical or radiological evidence of heart failure. <sup>a</sup>Number of patients previously treated for *Trypanosoma cruzi* infection. <sup>b</sup>Median years (range).

(Vacutainer, Becton Dickinson, San Jose, CA, USA). PBMCs were isolated by density gradient centrifugation on lymphocyte separation medium (ICN, Ohio, OH, USA) and resuspended in RPMI-1640 (Mediatech, Herndon, VA, USA) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT, USA), 20 mM HEPES, 2 mM L-glutamine, 1 mM pyruvate, 0.1 mM non-essential amino acids, 50 U penicillin per 50 μg ml<sup>-1</sup> streptomycin and 50 μM 2-mercaptoethanol (complete RPMI).

#### mAbs

mAbs anti-CCR7–PE, anti-Ki-67–FITC and anti-CD28– allophycocyanin (APC) were purchased from BD Pharmingen (San Diego, CA, USA). Anti-IFN-γ–PE or –FITC, anti-CD38 Tri-color (TC) and anti-CD8–APC-Cy7 were obtained from Caltag Laboratories (Burlingame, CA, USA). Other sources of mAbs were Serotec (Oxford, UK), anti-CD45RA–PE-Cy5; Bioscience (San Diego, CA, USA), anti-CD27–FITC, and Becton Dickinson, anti-CD8–PerCP.

#### Stimulation of PBMCs with T. cruzi-infected dendritic cells

Dendritic cell (DC)-enriched populations were generated from PBMCs of chronic chagasic subjects and non-infected individuals, as described by Ponsaerts et al. (14). Briefly,  $2 \times 10^7$  PBMCs per well were incubated in six-well plates at 37°C in 5% CO<sub>2</sub> atmosphere for 2 h, to allow monocytes to adhere. Non-adherent cells were then removed and frozen at -80°C for later use, and the adherent cell fraction was cultured in serum-free AIM-V medium (GIBCO, Invitrogen, CA, USA) supplemented with 1000 U ml<sup>-1</sup> recombinant human granulocyte macrophage colony-stimulating factor (ID Labs Inc., Ontario, Canada) for 2 days. Afterward, the cells were harvested, washed and re-suspended in complete RPMI at  $1 \times 10^5$  DCs ml<sup>-1</sup>. Two hundred microliters of the DC suspension was seeded per well in 48-well plates and cocultured with VERO cell culture-derived trypomastigotes (Brazil strain) at a parasite to DC ratio of 5 : 1 for 48 h. Then, free parasites in the supernatants were removed and frozen autologous monocyte-depleted PBMCs thawed and added to T. cruzi-infected DC cultures at a DC to autologous cell ratio of

1 : 20 and cultured overnight, with the addition of brefeldin A (10  $\mu$ g ml<sup>-1</sup>) during the last 5 h of incubation. After stimulation, PBMCs were stained for intracellular and cell-surface markers. The magnitude of *T. cruzi*-specific responses was calculated by subtracting the percentage of CD8<sup>+</sup>IFN- $\gamma^+$  T cells in non-stimulated cultures from the percentage of CD8<sup>+</sup>IFN- $\gamma^+$  responding to *T. cruzi*-infected DCs. The cut off value for a positive CD8<sup>+</sup> T cell response was set by calculating the mean percentage of the specific response plus 2 SD of five non-infected controls.

# Intracellular and cell-surface staining for phenotypic markers

One million uncultured PBMCs or PBMCs cultured with *T. cruzi*-infected DCs were stained with anti-CD8 (APC-Cy7), anti-CD28 (APC), anti-CD45RA (PE-Cy5), anti-CD27 (PE), anti-CCR7 (PE) or anti-CD38 (TC) for 1 h at 4°C. After incubation, the cells were washed and permeabilized with Cytofix/ Cytoperm solution (BD Pharmingen) for 15 min at 4°C followed by two washes with Perm/Wash solution (BD Pharmingen) and then stained with anti-IFN- $\gamma$  (FITC) or Ki-67 (FITC) for 30 min at 4°C. Cells were then washed twice with Perm/Wash solution and re-suspended in PBS containing 2% PFA. Data were acquired on a FACSCalibur (Becton Dickinson) or a CyAn (DakoCytomation, Ft Collins, CO, USA). Acquired data were further analyzed with CellQuest or Flowjo version 4.2 (Tree Star, San Carlos, CA, USA) software.

#### Apoptosis detection

Cell apoptosis was assessed by Annexin V staining with the flow cytometric apoptosis detection kit (BD Pharmingen, Becton Dickinson). A total of 2 million PBMCs ml<sup>-1</sup> were cultured with media alone for 20 h. Afterward,  $1 \times 10^6$  cells were stained with anti-CD8 (PE) for 45 min at 4°C. After incubation, the cells were washed and re-suspended in binding buffer and stained with 5 µl of Annexin and 5 µl of propidium iodine for 15 min at room temperature in the dark. Afterward, 400 µl of binding buffer was added and the samples were run within 1 h. Data were acquired on a FACSCalibur (Becton Dickinson) flow cytometer. Acquired data were analyzed with Flowjo version 4.2 software.

#### Statistical analysis

Differences between groups were evaluated by analysis of variance followed by Bonferroni test for multiple comparison. A simple lineal regression test was used for trend analysis. Differences were considered statistically significant when  $P \le 0.05$ .

#### Results

#### Maturation profile of T. cruzi-specific memory CD8<sup>+</sup> T cells in chronic chagasic subjects

The previously reported (10) low frequency of IFN- $\gamma$ -producing memory CD8<sup>+</sup> T cells specific for *T. cruzi* in subjects with chronic *T. cruzi* infection prompted us to characterize *T. cruzi*-specific responses using infected autologous DCs to enhance *in vitro* antigen presentation (15). To assess the maturation

#### CD8<sup>+</sup> T cell sub-populations in human Chagas' disease 467

status of responding *T. cruzi*-specific memory CD8<sup>+</sup> T cells, we measured the expression of CD27 and CD28 surface markers on IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells stimulated with *T. cruzi*infected DCs. Confirming our previous observations, subjects in the G0 and G1 clinical groups were more likely to mount *T. cruzi*-specific IFN- $\gamma$  responses (16 of 19; 84%) than subjects classified as G2 and G3 (four of eight; 50%; Fig. 1). Interestingly, the *T. cruzi*-specific CD8<sup>+</sup> T cell population was, on average, enriched in CD27<sup>+</sup>CD28<sup>+</sup> cells, characteristic of an early-differentiated phenotype in responding subjects, irrespective of clinical status (Fig. 2).

# Maturation profile of the total CD8<sup>+</sup> T cell compartment in PBMCs of chronic chagasic subjects

In the case of HIV infection, Appay et al. (16) had shown that the phenotype of the total CD8<sup>+</sup> T cell compartment reflected well the phenotype of the HIV-specific T cells. Because of the relatively low frequency of *T. cruzi*-specific IFN-γ-producing memory CD8<sup>+</sup> T cells and our previous failure to identify memory CD8<sup>+</sup> T cells using MHC-tetramers (10), we evaluated the impact of *T. cruzi* infection on the total memory CD8<sup>+</sup> T cell differentiation status, measuring the expression of CD27 and CD28 in peripheral CD45RA<sup>-</sup>CD8<sup>+</sup> (antigen experienced) T cells from 51 chronic chagasic subjects with different degrees of cardiac dysfunction. Seventeen non-chagasic subjects were used as controls. Although similar percentages of peripheral CD8<sup>+</sup>CD45RA<sup>-</sup> T cells were found in chagasic subjects and the controls (mean percentage  $\pm$  SD = 9.1  $\pm$  4.1 and 7.5  $\pm$  -4.4, respectively), the pattern of CD27 and CD28 expression on the antigen-experienced CD8<sup>+</sup> T cells was distinct in these two groups. In chagasic subjects, the percentages of CD27<sup>+</sup>CD28<sup>+</sup> cells were lower and the percentages of CD27<sup>-</sup>CD28<sup>-</sup> cells higher in the CD8<sup>+</sup>CD45RA<sup>-</sup> T cell population relative to control subjects (Table 2). Furthermore, as disease becomes more severe, a negative trend in the percentages of early-differentiated (CD27<sup>+</sup>CD28<sup>+</sup>) (P < 0.0001) and a positive trend in the percentages of fully



**Fig. 1.** IFN- $\gamma$  production specific for *Trypanosoma cruzi* after stimulation with *T. cruzi*-infected DCs. Monocyte-depleted PBMCs were cultured for 6 h with an enriched DC population infected with *T. cruzi* or uninfected. Intracellular and surface markers were stained after fixation and permeabilization of cells. Data are expressed as the percent of *T. cruzi*-specific CD8<sup>+</sup>IFN- $\gamma^+$  T lymphocytes. The horizontal line indicates the cut off value for positive responses.

#### 468 CD8<sup>+</sup> T cell sub-populations in human Chagas' disease

differentiated (CD27<sup>-</sup>CD28<sup>-</sup>) (P < 0.0001) memory CD8<sup>+</sup> T cells was found (Table 2). The proportions of the CD27<sup>+</sup>CD28<sup>-</sup> and CD27<sup>-</sup>CD28<sup>+</sup> subsets do not change in any consistent way with disease severity. These findings suggest that the memory CD45RA<sup>-</sup>CD8<sup>+</sup> T cell compartment differentiates toward a more mature phenotype with increased severity of the disease in chronic *T. cruzi* infection.

The percentage of CD8<sup>+</sup>CD45RA<sup>+</sup> T cell population, composed of naive and effector T cells, was similar in chronic chagasic subjects and uninfected controls (mean percentage  $\pm$  SD = 17.6  $\pm$  7.6 and 18.7  $\pm$  4.9, respectively). However, evaluation of the expression of CD27 and CD28 in CD8<sup>+</sup>CD45RA<sup>+</sup> T cells showed significantly lower percentages of CD27<sup>+</sup>CD28<sup>+</sup> (naive) and higher percentages of CD27<sup>-</sup>CD28<sup>-</sup> (effector) T cells (mean percentage  $\pm$  SD = 17.8  $\pm$  11.4 and 44.9  $\pm$  19.4, respectively) in comparison with the controls (mean percentage  $\pm$  SD = 40.2  $\pm$  16.3 and 23.0  $\pm$ 



**Fig. 2.** Phenotypic characterization of *Trypanosoma cruzi*-specific CD8<sup>+</sup> T cells in chronically infected subjects. (A–C) Representative data for IFN- $\gamma$  production by T cells from a responder subject, co-cultured with uninfected autologous DCs (A) or with DCs infected with *T. cruzi* (B). The CD8, IFN- $\gamma$  double-positive compartment in (B) was then analyzed for the expression of CD27 and CD28 (C). (D) CD27/CD28 expression profile among the *T. cruzi*-specific CD8<sup>+</sup>IFN- $\gamma^+$  T lymphocytes in chronic chagasic subjects. Median values are indicated by the horizontal lines.

16.1, respectively, P < 0.001; Fig. 3). However, no significant differences were observed in the percentages of effector and naive CD8<sup>+</sup> T cells among the four clinical groupings within chronically infected individuals. The higher levels of effector CD8<sup>+</sup> T cells were associated with a predominantly resting phenotype of the total CD8<sup>+</sup> T cell compartment, as observed by the low expression of CD38 and Ki-67, markers for recently activated and proliferating T cells, respectively (data not shown). This distinctive phenotypic profile of chronic chagasic subjects indicates an association between chronic *T. cruzi* infection and a more mature phenotype of the total CD8<sup>+</sup> T cell compartment.

### Assessment of central and effector total memory CD8<sup>+</sup> T cells in chronic chagasic subjects

Memory CD8<sup>+</sup> T cells were further characterized for the expression of the CCR7 chemokine receptor associated with the homing of memory T cells to lymph nodes. The results of this analysis showed that the percentage of CD8<sup>+</sup>CD45RA<sup>-</sup>CCR7<sup>-</sup> (effector memory) T cells in G1 subject group was significantly higher (mean percentage  $\pm$  SD = 31.8  $\pm$  21.1; Fig. 4) than in the uninfected group (mean percentage  $\pm$  SD = 9.9  $\pm$  8.8, *P* < 0.05). In contrast, the percentage of CD8<sup>+</sup>CD45RA<sup>-</sup>CCR7<sup>+</sup> (central memory) T cells in chagasic subjects is similar to the controls, and does not vary with respect to clinical group (Fig. 4). These results show that the number of CD8<sup>+</sup> T cells capable of rapid effector function is highest in subjects who are in the early stages of heart disease development, and is low and at control levels in subjects with more severe disease.

# Levels of spontaneous apoptosis in the peripheral CD8<sup>+</sup> T cell compartment

The low frequency of *T. cruzi*-specific IFN- $\gamma$ -secreting CD8<sup>+</sup> T cells (10; Fig. 1) and the highly differentiated profile of the total memory CD8<sup>+</sup> T cell compartment (Table 2) found in chronic chagasic subjects with the most severe clinical forms of the disease suggest that CD8<sup>+</sup> T cells during persistent *T. cruzi* infection were being driven to senescence. To investigate this possibility, the level of Annexin V, a marker of apoptosis, was examined in CD8<sup>+</sup> T cells from 23 chronic chagasic subjects and six uninfected controls. The results of this analysis showed higher levels of spontaneous apoptosis of CD8<sup>+</sup> T cells in subjects with heart dysfunction as compared with asymptomatic subjects and uninfected controls (Fig. 5),

**Table 2.** Memory phenotype of CD8<sup>+</sup> T cells in chronic chagasic patients

Cell phenotype	Clinical group					
	G0 ( <i>n</i> = 16)	G1 ( <i>n</i> = 14)	G2 ( <i>n</i> = 7)	G3 ( <i>n</i> = 12)	Control ( $n = 20$ )	
CD27 <sup>+</sup> CD28 <sup>+</sup> * CD27 <sup>+</sup> CD28 <sup>-</sup> CD27 <sup>-</sup> CD28 <sup>+</sup> CD27 <sup>-</sup> CD28 <sup>+</sup>	$\begin{array}{r} 28.76 \pm 12.9^{**} \\ 7.94 \pm 6.42 \\ 26.28 \pm 10.45 \\ 36.82 \pm 18.08^{**} \end{array}$	$33.31 \pm 17.31^{**}$ $5.05 \pm 2.19$ $26.94 \pm 15.3$ $34.67 \pm 20.46^{**}$	$24.58 \pm 9.41^{**}$ $8.96 \pm 3.79$ $15.24 \pm 9.45$ $51.21 \pm 15.78^{**}$	$\begin{array}{l} 19.52 \pm 8.51^{**} \\ 7.21 \pm 4.85 \\ 16.81 \pm 7.76 \\ 56.45 \pm 12.59^{**} \end{array}$	$\begin{array}{r} 49.89 \pm 15.85 \\ 7.81 \pm 4.08 \\ 22.64 \pm 12.79 \\ 22.66 \pm 12.83 \end{array}$	

Data are expressed as the mean  $\pm$  SD of the percentage of CD8<sup>+</sup>CD45RA<sup>-</sup> T lymphocytes of each phenotype. \*Negative trend P < 0.0001; \*\*P < 0.0001 respect to the controls; \*\*\*positive trend P < 0.0001.



**Fig. 3.** Differentiation status of the total CD8<sup>+</sup> T cell population in individuals with chronic Chagas' disease. PBMCs were stained for CD8, CD45RA, CD27 and CD28 and analyzed by flow cytometry. Each point represents the expression of CD27 and CD28 on the total CD8<sup>+</sup>CD45RA<sup>+</sup> T cell population for 16 G0, 15 G1, 8 G2, 12 G3 and 17 non-infected controls. Median values are represented by horizontal lines. \**P* < 0.01 versus G0, G1, G2 and G3 (analysis of variance).



**Fig. 4.** Immunophenotyping of memory CD8<sup>+</sup> T cells in chronic chagasic subjects. PBMCs were stained for CD8, CD45RA and CCR7 and analyzed by flow cytometry. Each point represents the expression of CD45RA and CCR7 on the total CD8<sup>+</sup> T cell population for each subject. The median value for each clinical group (11 G0, 14 G1, 5 G2, 8 G3 and 13 non-infected controls) is represented by horizontal lines. \**P* < 0.01 versus G0 and control (analysis of variance).

consistent with a higher rate of terminally differentiated CD8<sup>+</sup> T cells from patients with severe cardiac dysfunction.

#### Discussion

The primary clinical consequence of *T. cruzi* infection is a chronic cardiomyopathy which becomes manifest many years after the initial infection. There is a large body of data from human and animal model systems providing evidence for a strong connection between parasite persistence and the



**Fig. 5.** Flow cytometric analysis of spontaneous apoptosis in chronic chagasic subjects. The data represent the percentage of CD8<sup>+</sup> Annexin V<sup>+</sup> T lymphocytes for seven G0 (asymptomatic); six G1, four G2 and six G3 (heart dysfunction) and 5 uninfected. Median values are indicated by the horizontal lines. \*P < 0.01 versus G0 and controls.

presence of disease (reviewed in 17-19). It can be reasoned that an immune response of sufficient strength, sustained in time, and directed to the appropriate targets would efficiently control T. cruzi infection, preventing disease progression. Supporting the notion that the clinical disease status relates to the level of T. cruzi-specific T cell responses, we recently showed that subjects removed from the endemic area for longer than 20 years with advanced disease have significantly lower levels of IFN- $\gamma$ -secreting memory T cells specific for the parasite, compared with those with none or only modest disease symptoms (10). In this study, we used autologous DCenriched populations infected with T. cruzi to stimulate PBMCs in order to improve antigen presentation and CD8<sup>+</sup> T cell stimulation and the results of this analysis were consistent with those found with a parasite lysate as well as with peptides predicted to be targets of CD8<sup>+</sup> T cell responses in T. cruzi infection (10, 36).

The lower frequency of *T. cruzi*-specific CD8<sup>+</sup> T cells observed in subjects with more severe disease supports a less efficient generation of memory CD8<sup>+</sup> T cells or a more marked decline in memory T cell function over time in these individuals. Interestingly, a predominantly early-differentiation phenotype of T. cruzi-specific memory CD8<sup>+</sup> T cells capable of rapid production of IFN- $\gamma$  was found in all clinical groups. This phenotypic profile of the CD8<sup>+</sup> T cells responding to infected DCs contrasts with that of the total antigen-experienced memory CD8<sup>+</sup> T cell population in chronically infected subjects. In the latter, the number of early-differentiated memory (CD45RA-CD27+CD28+) CD8+ T cells is low in subjects with more severe disease, while the proportion of fully differentiated memory (CD45RA-CD27-CD28-) CD8+ T cells is high. This apparently disparate phenotype of the T. cruzi-specific, IFN-y-producing cells relative to the total T cell population may not be surprising, given that the T cells responding to *T. cruzi* with IFN-γ production represent only a small fraction of the total CD8<sup>+</sup> T cell compartment in chronically infected chagasic subjects (<3%) and likely underestimate the number of *T. cruzi*-specific CD8<sup>+</sup> T cells. We believe that this minor population of the IFN- $\gamma$ -producing, T. cruzi-responsive cells is a more recently developed effector

#### 470 CD8<sup>+</sup> T cell sub-populations in human Chagas' disease

memory CD8<sup>+</sup> T cell population, as evidenced by the expression of markers of early differentiation (CD27 and CD28) and their high capacity for rapid effector function. In contrast, the bulk of the total CD8<sup>+</sup>, antigen-experienced population appears to reflect the effects of persistent exposure to parasite antigen and is composed primarily of late-differentiated cells that are largely incapable of effector function and exhibit signs of senescence (Fig. 5). Such late-differentiated memory CD8<sup>+</sup> T cells, with reduced proliferation capacity and low T cell competence, have been documented in other persistent infections (20).

The homing capacity of memory CD8<sup>+</sup> T cells to peripheral tissues is likely to be important for the targeting of the immune response to the sites where T. cruzi localizes and replicates in the chronic stages of the disease. CD8<sup>+</sup> T cells isolated from the muscle tissue of mice with chronic T. cruzi infections were demonstrated to express surface markers reflecting an effector/memory phenotype but have attenuated effector function (21). In our study, we found a significantly higher level of total effector/memory CD8<sup>+</sup> T cells (CD45RA<sup>-</sup>CCR7<sup>-</sup>) in G1 subjects relative to G0 and control subjects, and decreased levels of this subpopulation in the more severe G2 and G3 subjects. This result is interesting in that an increase in the overall effector memory is not reflected in an increase in CD8<sup>+</sup> T cells with rapid effector response to T. cruzi-infected DCs (Fig. 1). This observation may reflect the failure of the host immune response to control parasite replication at target tissues, concomitant with the onset of heart symptoms.

Peripheral blood T lymphocytes from subjects with indeterminate and cardiac clinical forms of chronic Chagas' disease living in endemic areas of Brazil have been shown to be 'activated', as determined by the expression of HLA-DR and CD45RA (22–24). However, based on the expression of CD38 and Ki-67, CD8<sup>+</sup> T cells from chronic chagasic subjects in the current study did not display a recently activated phenotype, consistent with reports for other chronic infections (11, 25). The higher numbers of effector peripheral CD8<sup>+</sup> T lymphocytes with lower levels of naive CD8<sup>+</sup> T cells found in chronic chagasic subjects removed from endemic areas for longer than 20 years suggest that the immune profile in chronic chagasic subjects is independent of continuous reexposure to new infections.

In combination with our previous study, these results suggest that protection from disease associates with an increased frequency of highly competent CD8<sup>+</sup> T cells enriched in early-differentiated cells, as observed in the subjects with no or mild disease symptoms (10; Fig. 2 and Table 2). Early-differentiated T cells with potent proliferation capacity have been associated with protective immunity in HIV-infected long-term non-progressors as well as in the experimental infection with Leishmania major (26, 27). Conversely, in the face of the persistence of stimulating antigen, the overall T cell compartment is eventually driven to exhaustion, exhibits a low frequency of competent parasitespecific CD8<sup>+</sup> T cells and predisposes the subject for disease progression. Prolonged antigen exposure during chronic parasitic, viral and bacterial infections results in the failure of memory CD8<sup>+</sup> T cells to acquire the properties of antigenindependent memory T cells (28-31) and eventual clonal exhaustion (32-35).

This model might predict that all individuals with chronic *T. cruzi* infection, and thus persisting antigen, will eventually suffer immune exhaustion and develop severe disease. However, the parasite load, which is influenced by both the genetics of the infecting parasites as well as the quality of the host immune response, may determine the rate at which immune exhaustion occurs, and perhaps the rate of disease progression. This model also suggests that measurement of the frequency of early-differentiated T cells may be useful in predicting which asymptomatic subjects are most likely to show progression to more severe disease. Longitudinal studies to monitor disease progression in G0 and G1 subjects with different frequencies of rapid responding early-differentiated CD8<sup>+</sup> T cells should allow testing of this hypothesis.

Finally, this model predicts that parasite load will be higher in subjects with low levels of recently differentiated effector/ memory parasite-specific CD8<sup>+</sup> T cells. This prediction will be difficult to test as methods for the consistent quantitative assessment of parasite load in chronically infected subjects are currently unavailable.

In conclusion, our data suggest a gradual clonal exhaustion perhaps as a result of continuous antigenic stimulation by persistent parasites and associated with increased disease severity. Additional longitudinal studies of changes in CD8<sup>+</sup> T cell sub-populations in chronically infected subjects may reveal specific markers for the propensity for progression to severe disease.

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#### Abbreviations

APC	allophycocyanin
DC	dendritic cell
ECG	electrocardiogram
G0	group 0
G1	group 1
G2	group 2
G3	group 3
TC	Tri-color

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