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Chronic Human Infection with *Trypanosoma cruzi* Drives CD4+ T Cells to Immune Senescence[1]

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Abstract

Previously we found that the frequency of IFN-γ-producing $CD8^+$ T cells specific for *Trypanosoma cruzi* inversely correlates with disease severity in chronic human Chagas disease along with low levels of IL-2-secreting $CD8^+$ T cells in all clinical stages. This impairment of the parasite-specific T cell responses was associated with phenotypic features of immune senescence of the $CD8^+$ T cell compartment. These data prompted us to address the question of whether the $CD4^+$ T cell compartment also experiences signs of exhaustion. Thus, we performed a functional and phenotypical characterization of *T. cruzi*-specific and overall $CD4^+$ T cells in chronically infected subjects with different degrees of cardiac dysfunction. The results show an inverse association between disease severity and the frequency of *T. cruzi*-specific IFN-γ-producing $CD4^+$ T cells. The high expression of CD27 and CD28 with a relative low expression of CD57 found on $CD4^+$IFN-γ^+ T cells suggests that the effector T cell pool in chronic *T. cruzi* infection includes a high proportion of newly recruited T cells, but a low frequency of long-term memory cells. The total $CD4^+$ T cell compartment shows signs of senescence and later stages of differentiation associated with more severe stages of the disease. These findings support the hypothesis that long-term *T. cruzi* infection in humans might exhaust long-lived memory T cells.

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is one of the most important public health problems in Latin America (1). The disease evolves through an acute to a chronic phase, wherein subjects may be clinically asymptomatic or show progressive heart disease leading to an end-stage dilated cardiomyopathy in 20–30%.

The relevance of both $CD4^+$ and $CD8^+$ T cell compartments in the control of *T. cruzi* infection has been demonstrated in human infection with *T. cruzi* and in experimental models (2–5). Mice deficient in T cell subsets display high systemic and tissue parasite loads and, succumb to acute infection (6–8). Immunosuppression in recipients of organ transplant

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or as a result of coinfection with HIV results in exacerbation of parasite load (3,4). The finding of $CD4^+$ and $CD8^+$ T cell infiltrates in endomyocardial biopsies from acute and chronically infected chagasic patients further supports the important role of both T cell populations in the immune control of *T. cruzi* (9,10).

Supporting the notion that clinical disease in subjects with chronic *T. cruzi* infection might worsen in the presence of ineffective immune responses, we previously reported that individuals with more severe clinical disease have significantly lower frequencies of *T. cruzi*-specific $CD8^+$ IFN-γ^+ T cells than subjects in the asymptomatic stage of infection or with only mild chronic chagasic heart disease (11,12). This apparent impairment in $CD8^+$ T cell responses specific for *T. cruzi* was also associated with an increased frequency of fully differentiated memory ($CD45RA^-CD27^-CD28^-$) cells and an increased rate of apoptosis in the total peripheral $CD8^+$ T cell population, possibly reflecting a progressive exhaustion in the $CD8^+$ T cell compartment in these subjects with long-term *T. cruzi* infection (11).

Chronic exposure to Ags may cause functional defects of pathogen-specific $CD8^+$ T cells and eventually of the whole T cell population (13–16). Persistent viral infections have been suggested to cause chronic activation of the immune system as evidenced by high expression of markers of cell activation and cell division (17,18), leading to the differentiation of T cells with low self-renewal capacity (19,20). The loss of $CD8^+$ T cell function in the presence of Ag persistence appears to be hierarchical beginning with cytolytic activity followed by IL-2, TNF-α, and IFN-γ production, total immune exhaustion, and finally cell deletion (16). Persistent infections in humans are thought to have an important role in immune exhaustion of pathogen-specific $CD8^+$ T cells, as reported in infections with HIV (21–23) and hepatitis C virus (24).

$CD4^+$ Th cells play a critical role in the formation and maintenance of competent $CD8^+$ T cell memory during chronic infections (14,25–27). Thus, it can be reasoned that inadequate $CD4^+$ Th activity may also contribute to the impairment of $CD8^+$ T cell responses, resulting in a less efficient control of the pathogen multiplication and promoting the pathogen persistence.

These data prompted us to address the question of whether the $CD4^+$ T cell compartment also experiences functional and phenotypic exhaustion in subjects in the chronic phase of *T. cruzi* infection. Our results demonstrate that the total $CD4^+$ T cell compartment reflects the impact of long-term constant activation of the immune system driven by persistent *T. cruzi* infection in chronically infected subjects, while the *T. cruzi*- specific IFN-γ-producing $CD4^+$ T cell compartment is dominated by recently recruited T cells, supporting the model that long-term *T. cruzi* infection in humans might exhaust long-lived memory T cells.

Materials and Methods

Selection of study population

Subjects were recruited at the Instituto Nacional de Parasitología “Dr. M. Fatala Chaben” (INP) and at the Chagas Disease Section, Cardiology Department, Hospital Interzonal General de Agudos “Eva Perón.” Signed informed consent was obtained from all individuals before inclusion in the study. *T. cruzi* infection was determined by a combination of indirect immunofluorescence assay, hemagglutination, and ELISA tests performed in the Diagnosis Department of INP. Infected subjects positive on at least two of these tests were considered to be infected. Chronic chagasic subjects were evaluated clinically and grouped according to the Kuschnir grading system (28). Group 0 (G0, $n = 13$; mean age ± SD = 42 ± 10 years) included seropositive individuals exhibiting a normal electrocardiogram (ECG)[3] and a normal chest x-ray; group 1 (G1, $n = 17$; mean age ± SD = 65 ± 5 years) seropositive

patients with a normal chest x-ray but abnormalities in the ECG; group 2 (G2, $n = 4$; mean age ± SD = 53 ± 3 years) seropositive patients with ECG abnormalities and heart enlargement as determined by chest x-ray; and group 3 (G3, $n = 13$; mean age ± SD = 63 ± 8 years) seropositive patients with ECG abnormalities, heart enlargement, and clinical or radiological evidence of heart failure. The uninfected control group ($n = 15$; mean age ± SD = 42 ± 14 years) consisted of aged-matched healthy Caucasian natives from Argentina who have always resided in nonendemic areas and who were serologically negative for *T. cruzi*. Infected chagasic subjects and noninfected controls with hypertension, ischemic heart disease, cancer, HIV infection, syphilis, diabetes, arthritis, or serious allergies were excluded from this study. This study was approved by the Institutional Review Boards of the Hospital Interzonal General de Agudos “Eva Perón” and INP “Dr. M. Fatala Chaben” (Buenos Aires, Argentina).

Collection of PBMCs

Approximately 50 ml of blood was drawn by venipuncture into heparinized tubes (Vacutainer; BD Biosciences). PBMCs were isolated by density gradient centrifugation on Lymphocyte Separation Medium (Valeant Pharmaceuticals) and resuspended in RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated FCS (HyClone).

Monoclonal Abs

mAb anti-CD27-PE, anti-CD57-FITC, anti-caspase 3-FITC, anti-CD122-FITC, anti-CD28-allophycocyanin, and anti-CD8-allophycocyanin were purchased from BD Pharmingen. Anti-IFN-γ-PE or FITC and anti-CD4-allophycocyanin-Cy7 were obtained from Caltag Laboratories. Other sources of mAbs were Serotec (anti-CD45RA-PE-Cy5) and eBioscience (anti-IL-7R-PE).

T. cruzi lysate

Protein lysate from *T. cruzi* amastigotes was obtained by four freeze/thaw cycles followed by sonication as previously reported (29). Briefly, trypomastigotes from the Brazil strain were cultured overnight in pH 5 DMEM (Mediatech) to transform trypomastigotes into amastigotes. After washing, the parasites were frozen at −20°C and thawed twice. Thereafter, the sample was subjected to two freeze/thaw cycles at −70°C followed by sonication. The supernatant of a 12,000 rpm centrifugation was collected, filter sterilized, and the protein concentration was determined.

Stimulation of PBMCs with *T. cruzi* amastigote lysate

PBMCs isolated from *T. cruzi*-infected subjects and controls were stimulated with 15 μg/ml *T. cruzi* amastigote lysate or medium alone in 48-well plates at 37°C in a CO_2 incubator for 16–20 h. Ten micrograms of brefeldin A per ml was added to the samples for the last 6 h of incubation. After stimulation, PBMCs were removed from the plates and stained for intracellular and cell surface markers. The magnitude of *T. cruzi*-specific responses was calculated by subtracting the percentage of $CD4^+IFN\text{-}\gamma^+$ or $CD8^+IFN\text{-}\gamma^+$ T cells in nonstimulated cultures from the percentage of $CD4^+IFN\text{-}\gamma^+$ or $CD8^+IFN\text{-}\gamma^+$ responding T cells to *T. cruzi* amastigote lysate. The cutoff value for a positive $CD4^+$ or $CD8^+$ T cell response was set by calculating the mean percentage of the specific response plus 2 SDs of 10 noninfected controls.

[3]Abbreviation used in this paper: ECG, electrocardiogram.

Intracellular and cell surface staining for phenotypic markers

One million uncultured PBMCs or PBMCs stimulated with *T. cruzi* amastigote lysate were stained with anti-CD4 (APC-Cy7), anti-CD28 (APC), anti-CD45RA (PE-Cy5), anti-CD27 (PE), anti-IL-7R (PE), anti-CD122 (FITC), or anti-CD57 (FITC) 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-γ (FITC) or anti-caspase 3 (FITC) for 30 min at 4°C. Cells were then washed twice with Perm/Wash solution and resuspended in PBS containing 2% paraformaldehyde. Data were acquired on a CyAn (DakoCytomation). Acquired data were further analyzed with FlowJo version 4.2 (Tree Star) software.

Statistical analysis

Differences between groups were evaluated by ANOVA followed by the Bonferroni test for multiple comparisons. Correlation analysis was done by the Spearman test. Differences were considered statistically significant when $p \leq 0.05$.

Results

T. cruzi-specific CD4$^+$ IFN-γ-producing T cells in chronically *T. cruzi*-infected subjects have a less differentiated phenotype

Previously, we reported that *T. cruzi*-infected subjects with no or mild heart disease were more likely to retain T cells responsive to HLA-A2.1-binding *trans*-sialidase peptides or *T. cruzi*-infected dendritic cells, whereas subjects with more advanced cardiac disease do not (12,29). To address whether the *T. cruzi*-specific CD4$^+$ T cell function is also compromised in association with disease status during chronic *T. cruzi* infection, IFN-γ responses were compared in the CD4$^+$ and CD8$^+$ T cell compartment of PBMCs from chronically infected subjects with different degrees of cardiac dysfunction. As previously reported for CD8$^+$ T cells, the frequencies of CD4$^+$IFN-γ-producing T cells are lowest in those subjects with more severe disease (groups G2 and G3), confirming an inverse association between disease severity and functionality for T cells in general in these subjects (Fig. 1).

One likely mechanism of T cell dysfunction in chronic infections like *T. cruzi* is Ag-driven exhaustion (16,26,30). To determine whether this remarkable attrition in parasite-specific CD4$^+$ T cells in subjects with more severe disease might be attributable to a shift of this T cell population toward a more differentiated senescent state, *T. cruzi*-specific IFN-γ-producing CD4$^+$ T cells were analyzed for the expression of the T cell differentiation markers CD27 and CD28 and for CD57, a molecule indicative of replicative senescence. Surprisingly, the population of *T. cruzi*-specific CD4$^+$ T cells producing IFN-γ in response to *T. cruzi* Ags was dominated by cells with an early (CD27$^+$CD28$^+$) and intermediate (CD27$^-$CD28$^+$) differentiation phenotype irrespective of the clinical status (Fig. 2*A*). The relatively low level of CD57 expression on CD4$^+$IFN-γ^+ T cells (in most subjects <30% of the CD4$^+$IFN$^+$ T cells irrespective of the clinical status, Fig. 2*B*) and the inverse correlation between the expression of CD57 and CD27/CD28 on IFN-γ^+ T cells (Fig. 2, *C* and *D*) further suggest that the majority of parasite-specific CD4$^+$ T cells have been recently recruited into the T cell response to *T. cruzi* and have undergone only a modest number of Ag-induced rounds of proliferation (Fig. 2*B*).

Analysis of T cell differentiation status of the total peripheral CD4$^+$ T cell population during chronic Chagas disease

Increasing evidence indicates that persistent exposure of T cells to infectious agents results not only in the loss of the functional capability of pathogen-specific T cells but also in a phenotypic change in the total T cell compartment, with decreased number of naive T cells,

reduction in the diversity of naive TCR, and increased frequencies of memory T cells (30). In agreement with this notion, we (11) and others (31,32) have previously reported that chronic *T. cruzi* infection leads to alterations in the total peripheral $CD8^+$ T cell compartment.

To determine whether the decreased frequency of *T. cruzi*-specific $CD4^+$ T cells in individuals with more severe disease symptoms is reflected in the phenotype of the total $CD4^+$ T cell compartment, we next examined the expression of markers of Ag experience/memory (CD45RA), maturation/exhaustion (CD27, CD28), apoptosis (caspase 3), and replicative senescence (CD57) on the overall $CD4^+$ T cell population. The number of more differentiated $CD4^+$ T cells ($CD27^-CD28^-$) is higher on average in those subjects with the most severe disease (Table I). This was the case with both late differentiated memory ($CD45RA^-CD27^-CD28^-$) and terminally differentiated effector $CD4^+$ T cells ($CD45RA^+CD27^-CD28^-$) (Table I). The total $CD4^+$ T cell compartment in individuals with chronic *T. cruzi* infection also exhibits evidence of higher levels of TCR-triggering events, as seen by the high number of cells expressing CD57 (Fig. 3*A*) and greater spontaneous apoptosis of $CD4^+$ T cells compared with uninfected controls (Fig. 3*B*). Additionally, "naive"-like $CD4^+$ T cells appear to be decreased in subjects with cardiac disease (groups G1, G2, and G3; Table I) and these cells also exhibit signs of exhaustion with high expression of CD57 and caspase 3 (Fig. 3, *C* and *D*).

Thus overall, in contrast to the *T. cruzi*-specific $CD4^+$ T cell population, the overall $CD4^+$ T cell compartment shows signs of senescence and later stages of differentiation associated with more severe forms of the disease, indicative of the chronic activation of the host immune system driven by decades of exposure to the parasite.

Expression of cytokine receptors involved in T cell homeostasis

The maintenance of stable naive and memory T cell compartments is dependent on homeostatic proliferation of T cells. Memory T cells up-regulate antiapoptotic molecules that promote their survival and express receptors for the homeostatic cytokines IL-7 and IL-15, which allow for their maintenance independently of the presence of Ag (33). To determine whether long-term infection with *T. cruzi* leads to alterations in T cell homeostasis, we analyzed the expression of IL-7R and the IL-2 and IL-15 receptor (CD122) on peripheral T cells from chronically infected subjects. A tendency to higher levels of cells expressing IL-7R in the effector ($CD45RA^+CD28^-$) $CD4^+$ T cell compartment in subjects with no or mild cardiac disease (G0 and G1) compared with those with more advanced clinical disease (G2–G3) was found (Fig. 4). Conversely, the expression of IL-7R among naive-like ($CD45RA^+CD27^+CD28^+$) and memory ($CD45RA^-CD27^{+/-}CD28^{+/-}$) $CD4^+$ T cells remain unaltered in chronically *T. cruzi*-infected subjects (data not shown). Likewise, the expression of CD122 did not vary in naive-like, memory and effector $CD4^+$ T populations (data not shown).

Discussion

Immunity to *T. cruzi* involves multiple effector mechanisms but as *T. cruzi* invades and replicates in essentially all types of mammalian cells, T cell-mediated immunity is particularly important for the control of the infection. Although host infection by *T. cruzi* appears to be well controlled, sterilizing immunity is apparently rare, resulting in decade-long infections in most human cases.

We have previously found that the frequency of IFN-γ-producing $CD8^+$ T cells specific for *T. cruzi* inversely correlates with disease severity in chronic human Chagas disease (11,29) along with low levels of IL-2-secreting $CD8^+$ T cells in all clinical stages (12). This

impairment of the parasite-specific T cell responses was associated with phenotypic features of immune senescence of the $CD8^+$ T cell compartment (11).

Memory T cells can persist for extended periods in the absence of Ag, as observed after complete resolution of acute infections (26). However, during persistent infections, Ag-specific T cells appeared to be dependent on Ag for their maintenance (13,34). One of the mechanisms proposed for the maintenance of pathogen-specific T cells during long-term infections is the Ag-driven recruitment of new T cells (34–37).

In this study, we demonstrate that the frequency of Ag-experienced IFN-γ-producing $CD4^+$ T cells specific for *T. cruzi* decreases in conjunction with $CD8^+$ T cells, confirming an inverse association between disease severity and functionality for not only $CD8^+$ T cells. The high expression of CD27 and CD28 with a relative low expression of CD57, a marker associated with a greater number of cell divisions and short telomeres (38–39), on *T. cruzi*-specific $CD4^+$IFN-γ^+ T cells suggests that the effector T cell pool in chronic *T. cruzi* infection includes a high proportion of newly recruited T cells but a low frequency of long-term memory cells. The maintenance of T cell responses in chronic *T. cruzi* infection is likely Ag dependent, as suggested by our previous studies showing a predominant functional profile of IFN-γ-only secreting T cells, characteristic of effector/effector memory cells (12), and that parasite-specific IFN-γ-producing T cells decrease following treatment of subjects with the anti-*T. cruzi* drug benznidazole (S. Laucella, manuscript in preparation). Whether the decreased frequency of effector functional $CD8^+$ T cells in chronic *T. cruzi* infection might be in part the result of deficient $CD4^+$ T cell help is at present unclear.

These data could fit with a model in which prolonged exposure to *T. cruzi* Ags results in the failure of memory T cells to acquire the properties of Ag-independent T cells. In this context, it is possible that subjects who progress toward disease have not only slowly exhausted their memory populations over time but also that they lose the ability to recruit new cells into the response. Consistent with this model, the heterogeneity in the function and phenotype of Ag-specific $CD4^+$ and $CD8^+$ T cells (11,12) even among subjects in the same clinical stage and without signs of heart disease might be indicating a higher risk for progression, an issue that should be further explored in long-term follow-up of infected subjects. However, we cannot rule out the possibility that other regulatory pathways might have an effect on the impairment of T cell responses as previously suggested (40–45).

As in the human infection, the majority of $CD4^+$ and $CD8^+$ T cells in the experimental *T. cruzi* infection in mice also exhibited an effector memory-like phenotype (46) but a stable population of the Ag-independent parasite-specific central memory $CD8^+$ T cell population has been identified (47). These differences might account for the long-term Ag exposure in humans (>20 years) compared with mice. It is also notable that in other chronic human infections where T cell exhaustion is a common occurrence, this process is generally associated with high Ag load (48). However, since parasite load is extremely low in subjects chronically infected with *T. cruzi*, the long-term parasite persistence rather than the high parasite load is likely to be responsible for driving the parasite-specific T cell population to immunosenescence.

Additionally, the overall $CD4^+$ T cell compartment in chronic chagasic subjects also showed several features compatible with a process of immune exhaustion, with increased frequencies of late differentiated memory T cells and increased levels of senescent and apoptotic effector T cells in the more severe forms of the disease. Further evidence of the importance of maintaining $CD4^+$ T cells in the long-term infection is the higher levels of the homeostatic IL-7R on $CD4^+$ T cells in subjects that have no developed cardiomyopathy.

The expression of CD57 on naive-like $CD4^+$ ($CD45RA^+CD27^+CD28^+$) T cells stands out because CD57 has been associated with repeated Ag stimulation and replicative senescence. One possible explanation for the presence of CD57 on otherwise naive-like T cells is that the processes of extrathymic differentiation might compensate for the decrease in naive $CD4^+$ T cells, as previously reported for naive $CD8^+$ T cells in Hodgkin's disease patients who received mediastinal irradiation (49). In that case, the naive $CD8^+$ T cells also display high expression of CD57 (49). It is also likely that these $CD57^+$ cells are not truly naive T cells but are memory T cells that had regained the expression of CD45RA, a hypothesis that merits further investigation.

The plausible picture emerging from our results is that among persistent infections, *T. cruzi* infection constitutes a distinctive example of a process of immunosenescence due to long-term stimulation with low parasite load. The data presented in this study might be useful for the monitoring of the disease status supporting that treatment early in the infection might prevent the aging of the T cell immune system triggered by chronic *T. cruzi* persistence.

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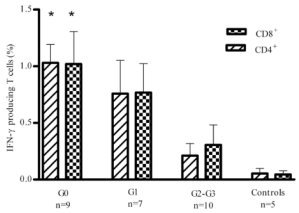

FIGURE 1.

A decrease in the frequencies of *T. cruzi*-specific IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells is associated with a more severe clinical status in chronic *T. cruzi* infection. PBMCs were cultured for 16–20 h with *T. cruzi* lysate or medium alone. Intracellular and surface markers were stained after fixation and permeabilization of cells. Lymphocytes were gated in side scatter vs forward scatter light. The number of *T. cruzi*-specific T cells was determined by subtracting the percentage of IFN-γ^+ T cells in unstimulated cultures from the percentage of IFN-γ^+ cells upon stimulation with *T. cruzi* lysate. Bars represent the mean percentages of *T. cruzi*-specific $CD4^+$IFN-γ^+ and $CD8^+$IFN-γ^+ T cell responses; error bars represent SD. *, $p < 0.05$ compared with G1–G3 and controls.

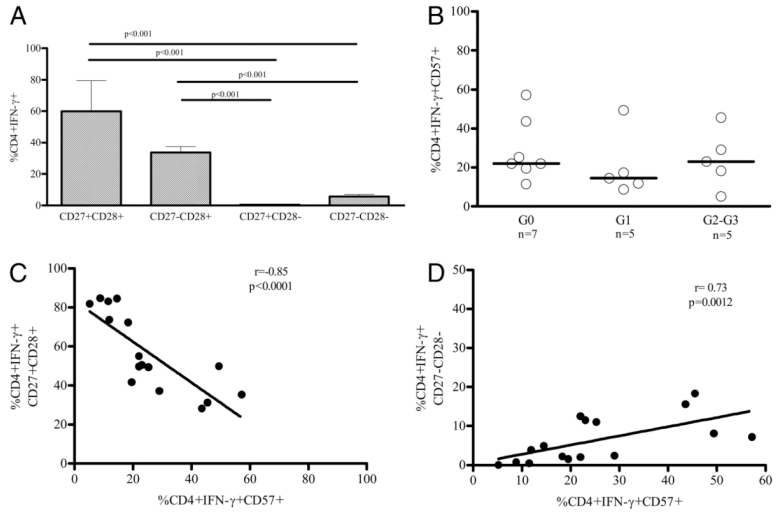

FIGURE 2.

T. cruzi-specific CD4$^+$ T cells in chronically infected subjects are primarily recently recruited T cells. The CD4 IFN-γ double-positive compartment was analyzed for the expression of CD27, CD28, and CD57 by flow cytometry (*A*). Bars represent the mean frequencies of CD4$^+$ IFN-γ^+ T cells expressing CD27 and CD28 in chronically *T. cruzi*-infected subjects; error bars indicate SD. Number of subjects studied in each patient group: G0 = 7, G1 = 7, G2 = 2, and G3 = 4. *B*, CD57 expression on CD4$^+$IFN-γ^+ T cells in the different clinical stages of the disease. Median values are represented by horizontal lines. *C* and *D*, Correlation between the expression of CD27, CD28, and CD57 on *T. cruzi*-specific IFN-γ-producing CD4$^+$ T cells was evaluated by the Spearman correlation test.

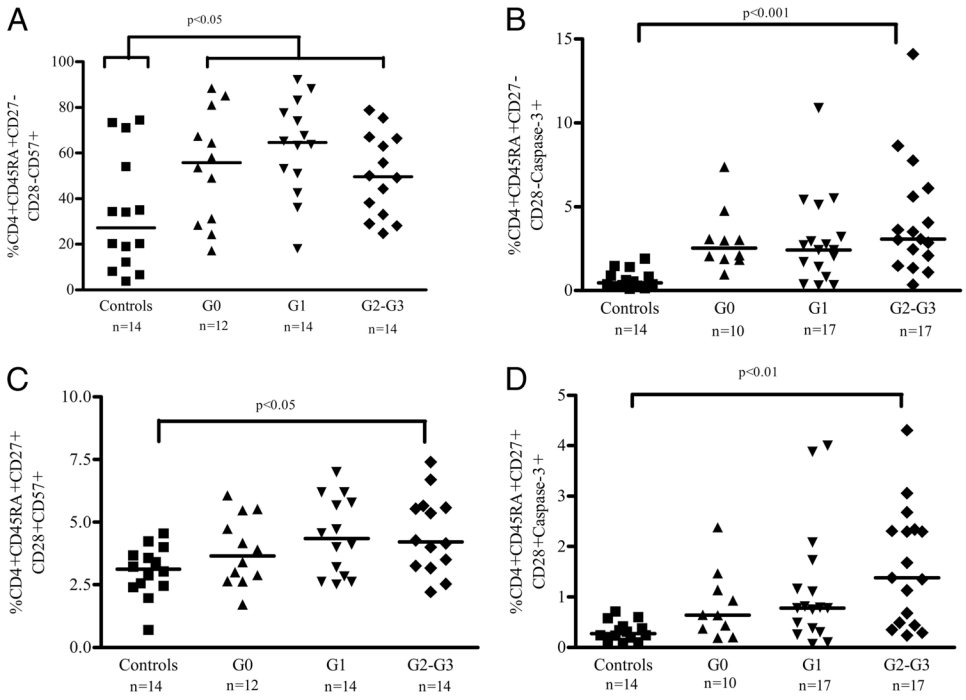

FIGURE 3.

The total peripheral $CD4^+$ T cell population displays features of immune senescence in subjects with chronic *T. cruzi* infection. PBMCs were stained for CD4, CD45RA, CD27, CD28, CD57, and caspase 3. Lymphocytes were gated in side scatter vs forward scatter light. Each point represents the expression of CD57 (*A* and *C*) and caspase 3 (*B* and *D*) on total terminally differentiated effector ($CD45RA^+CD27^-CD28^-$) (*A* and *B*) and naive-like ($CD45RA^+CD27^+CD28^+$ (*C* and *D*) $CD4^+$ T cells. Median values are represented by horizontal lines.

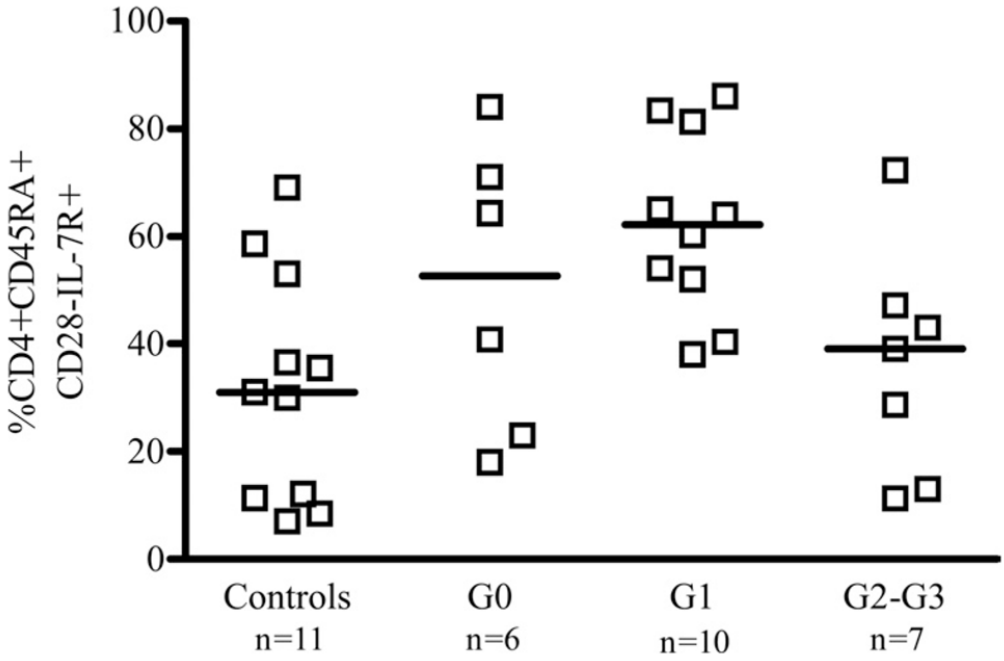

FIGURE 4.

IL-7R expression in effector $CD4^+$ T cells in chronic Chagas disease patients. PBMCs were stained for CD4, CD45RA, CD28, and IL-7R. Lymphocytes were gated in side scatter vs forward scatter light and analyzed by flow cytometry. Each point represents the expression of IL-7R on the total $CD4^+CD45RA^+CD28^-$ T cell population. Median values are represented by horizontal lines.

Table I

Differentiation profile of $CD4^+$ T cells in chronically T. cruzi-infected subjects with different degrees of heart involvement[a]

		% $CD45RA^-$		
	% $CD45RA^+CD27^+CD28^+$	$CD27^+CD28^+$	$CD27^-CD28^-$	% $CD45RA^+CD27^-CD28^-$
G0 (n = 13)	86.36 ± 8.75	75 ± 11.4*	10.4 ± 8.6*	7.8 ± 6*
G1 (n = 17)	78.29 ± 13.84*	67.1 ± 14.8*	21.4 ± 15.7*	14.6 ± 12.6*
G2–G3 (n = 17)	78.46 ± 14.51*	68 ± 14*	20.5 ± 18.3*	13.2 ± 16.1*
Controls (n = 14)	93.02 ± 4.2	83.1 ± 7.1	7.1 ± 6.3	3.2 ± 3.4

[a] Data are presented as mean ± SD.

* $p < 0.001$ compared with controls.