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

María Cecilia Albareda,* Gabriela Carina Olivera,* Susana A. Laucella,* María Gabriela Alvarez,† Esteban Rodrigo Fernandez,* Bruno Lococo,† Rodolfo Viotti,† Rick L. Tarleton,‡ and Miriam Postan2*

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. *The Journal of Immunology*, 2009, 183: 4103–4108.

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 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 \textit{T. cruzi} infection in chroni-
cally infected subjects, while the \textit{T. cruzi}-specific IFN-\gamma-producing
CD4\(^{+}\) T cell compartment is dominated by recently recruited T
cells, supporting the model that long-term \textit{T. cruzi} infection in
humans might exhaust long-lived memory T cells.

\textbf{Materials and Methods}

\textbf{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 De-
partment, Hospital Interzonal General de Agudos “Eva Perón.” Signed in-
formed consent was obtained from all individuals before inclusion in the
study. \textit{T. cruzi} infection was determined by a combination of indirect im-
munofluorescence 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 Kuschin grading
system (28). Group 0 (G0, \(n = 13\); mean age ± SD = 42 ± 10 years)
included seropositive individuals exhibiting a normal electrocardiogram
(ECG)\(^{1}\) 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 abnor-
malities 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 enlarge-
ment, 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
\textit{T. cruzi}. Infected chagasic subjects and noninfected controls with hyperten-
sion, ischemic heart disease, cancer, HIV infection, syphilis, diabetes, ar-
thritis, 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).

\textbf{Collection of PBMCs}

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

\textbf{Monoclonal Abs}

mAb anti-CD27-Pe, anti-CD57-FITC, anti-caspase 3-FITC, anti-CD122-
FITC, anti-CD28-allophycocyanin, and anti-CD58-allophycocyanin were
purchased from BD Pharmingen. Anti-IFN-\gamma-Pe or FITC and anti-CD4-
allophycocyanin-Cy7 were obtained from Caltag Laboratories. Other
sources of mAbs were Soropec (anti-CD45RA-FITC-Cy5) and eBioscience
(anti-IL-7R-PE).

\textbf{T. cruzi lysate}

Protein lysate from \textit{T. cruzi} amastigotes was obtained by four freeze/thaw
cycles followed by sonication as previously reported (29). Briefly, trypo-
mastigotes 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 sam-
ple was subjected to two freeze/thaw cycles at −70°C followed by soni-
cation. The supernatant of a 12,000 rpm centrifugation was collected, filter
sterilized, and the protein concentration was determined.

\textbf{Stimulation of PBMCs with \textit{T. cruzi} amastigote lysate}

PBMCs isolated from \textit{T. cruzi}-infected subjects and controls were stimu-
lated with 15 \(\mu\)g/ml \textit{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 brefel-
din 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 intra-
cellular and cell surface markers. The magnitude of \textit{T. cruzi}-specific re-
 sponses was calculated by subtracting the percentage of CD4\(^{+}\)IFN-\gamma\(^{-}\)
or CD8\(^{+}\)IFN-\gamma\(^{-}\) T cells in nonstimulated cultures from the percentage of
CD4\(^{+}\)IFN-\gamma\(^{-}\) or CD8\(^{+}\)IFN-\gamma\(^{-}\) responding T cells to \textit{T. cruzi} amastigote

\(^{1}\) Abbreviation used in this paper: ECG, electrocardiogram.

\textbf{Intracellular and cell surface staining for phenotypic markers}

One million uncultured PBMCs or PBMCs stimulated with \textit{T. cruzi} amas-
tigote 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-\gamma (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% paraformal-
dehyde. Data were acquired on a CyAn (DakoCytomation). Acquired data
were further analyzed with FlowJo version 4.2 (Tree Star) software.

\textbf{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 \(p < 0.05\).

\textbf{Results}

\textit{T. cruzi}-specific CD4\(^{+}\) IFN-\gamma-producing T cells in chronically
\textit{T. cruzi}-infected subjects have a less differentiated phenotype

Previously, we reported that \textit{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 \textit{T. cruzi}-infected dendritic
cells, whereas subjects with more advanced cardiac disease
dose not (12, 29). To address whether the \textit{T. cruzi}-specific
CD4\(^{+}\) T cell function is also compromised in association with
heart enlargement during chronic \textit{T. cruzi} infection, IFN-\gamma responses
were compared in the CD4\(^{+}\) and CD8\(^{+}\) T cell compartment of
PBMCs from chronically infected subjects with different diseases.
As previously reported for CD8\(^{+}\) T cells, the frequencies of CD4\(^{+}\) IFN-\gamma-producing T cells are lowest in
those subjects with more severe disease (groups G2 and G3), con-
firming an inverse association between disease severity and func-
tional properties of these cells in general (Fig. 1). One likely mechanism of T cell dysfunction in chronic infections like \textit{T. cruzi} is Ag-driven exhaustion (16, 26, 30). To deter-
mine 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 se-
nescent state, \textit{T. cruzi}-specific IFN-\gamma-producing CD4\(^{+}\) T
 cells were compared for the expression of the T cell differentiation mark-
ers CD27 and CD53 and have undergone only

\textbf{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 ca-
20°C and thawed twice. Thereafter, the sam-
ple was subjected to two freeze/thaw cycles at −70°C followed by soni-
cation. The supernatant of a 12,000 rpm centrifugation was collected, filter
sterilized, and the protein concentration was determined.
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.

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 naive T cells, reduction in the diversity of naïve 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.

Expression of cytokine receptors involved in T cell homeostasis

The maintenance of stable naïve 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 naïve-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 naïve-like, memory and effector CD4+ T populations (data not shown).


**Table I. Differentiation profile of CD4+ T cells in chronically T. cruzi-infected subjects with different degrees of heart involvement**

<table>
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<tbody>
<tr>
<td>G0 (n = 13)</td>
<td>86.36 ± 8.75</td>
<td>75 ± 11.4*</td>
<td>10.4 ± 8.6*</td>
<td>7.8 ± 6*</td>
</tr>
<tr>
<td>G1 (n = 17)</td>
<td>78.29 ± 13.84*</td>
<td>67.1 ± 14.8*</td>
<td>21.4 ± 15.7*</td>
<td>14.6 ± 12.6*</td>
</tr>
<tr>
<td>G2-G3 (n = 17)</td>
<td>78.46 ± 14.51*</td>
<td>68 ± 14*</td>
<td>20.5 ± 18.3*</td>
<td>13.2 ± 16.1*</td>
</tr>
<tr>
<td>Controls (n = 14)</td>
<td>93.02 ± 4.2</td>
<td>83.1 ± 7.1</td>
<td>7.1 ± 6.3</td>
<td>3.2 ± 3.4</td>
</tr>
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</table>

* Data are presented as mean ± SD.

**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 CD4+ 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 explanation for the presence of CD57 on otherwise naive-like T cells stands out because CD57 has been associated with Ag-independent parasite-specific central memory CD8+ T cells in Hodgkin’s disease patients (49). It is also associated with Ag-independent parasite-specific central memory 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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Disclosures
The authors have no financial conflict of interest.

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