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Inhibitory Receptor Expression on CD8⁺ T Cells Is Linked to Functional Responses against Trypanosoma cruzi Antigens in Chronic Chagasic Patients

Paola Lasso,*†,‡ Jose Mateus,*† Paula Pavia,† Fernando Rosas,§ Nubia Roa,¶ M. Carmen Thomas,§ Manuel C. López,¶ John M. González,‖ Concepción J. Puerta,‖ and Adriana Cuéllar*

In mammals, chronic diseases resulting from infectious agents have been associated with functional T cell response deficiency, a high frequency of terminally differentiated T cells, the presence of monofunctional Ag-specific T cells, and increased expression of inhibitory receptors. Similar to other chronic diseases, the progressive loss of certain functional activities during Trypanosoma cruzi infection might result in the inability to control replication of this parasite. To examine this hypothesis, we evaluated the differentiation and cell effector function of CD8⁺ T cells and characterized the expression of inhibitory receptors and the presence of the parasite in the bloodstream of chagasic patients. The results showed that patients at an advanced severe disease stage had a higher frequency of terminally differentiated CD8⁺ T cells than patients at an early stage of the disease. A monofunctional CD8⁺ T cell response was observed in patients at an advanced stage, whereas the coexpression of markers that perform three and four functions in response to parasite Ags was observed in patients at a less severe disease stage. The frequency of CD8⁺ T cells producing granzyme B and perforin and those expressing inhibitory receptors was higher in symptomatic patients than in asymptomatic patients. Taken together, these findings suggest that during the course of Chagas disease, CD8⁺ T cells undergo a gradual loss of function characterized by impaired cytokine production, the presence of advanced differentiation, and increased inhibitory receptor coexpression. The Journal of Immunology, 2015, 195: 3748–3758.

Chagas disease, which results from infection with Trypanosoma cruzi, primarily occurs in Latin America. However, because of population migration from Latin America, this disease has spread to the United States, Canada, Europe, and Western Pacific countries in recent decades (1, 2). Approximately 10 million people are infected worldwide, and >25 million are at risk for the disease (2). In Colombia, an estimated 436,000 individuals are infected, with 11% of the population at risk for contracting the disease (1). The course of Chagas disease involves two clinical phases: acute and chronic. In the acute phase, control of the parasite is highly influenced by the patient’s humoral and cellular immune responses, although these responses do not completely control the progression of the disease (3). Thus, most infected patients remain in an indeterminate chronic phase, without symptoms and clinical manifestations. In the absence of treatment, 30–40% of these patients develop a determinate chronic disease phase that is characterized by cardiac or digestive alterations associated with persistence of the parasite (1, 2). Heart involvement frequently progresses to cardiomegaly, cardiac failure, severe arrhythmia, and even death (4).

Trypanosoma cruzi is an obligate intracellular parasite that invades and replicates in mammalian cells. Similar to other intracellular infectious agents, T. cruzi induces a CD8⁺ T cell immune response resulting in the secretion of cytokines and the release of cytotoxic granules (5, 6), and studies in experimental models have shown that CD8⁺ T cells play a crucial role in the course of this disease. Indeed, increased parasite burden and mortality have been observed postinfection in mice depleted of CD8⁺ T cells compared with infected wild-type animals (7–10).

In some models of chronic infections, such as those caused by viruses, T cells progress toward several stages of dysfunction, with phenotypic modifications (11, 12). T cell functions, including IL-2 production, increased cell proliferation, and ex vivo killing, are the most sensitive functions initially lost. Other properties, including TNF-α secretion, are often lost during the intermediate phase of this process, whereas IFN-γ production

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The online version of this article contains supplemental material.

Abbreviations used in this article: CCD, chronic Chagas disease; CCP, chronic chagasic patient; ECG, electrocardiogram; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, healthy donor; LVEF, left ventricular ejection fraction; MFI, mean intensity fluorescence; NYHA, New York Heart Association; PD-1, programmed cell death-1; SEB, staphylococcal enterotoxin B; TCM, T cell population of central memory; TSAs, T. cruzi soluble Ag; TCM, T cell population of effector memory cells; TIM-3, T cell Ig mucin-3.

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appears to be more resistant to early functional loss. The results of recent studies demonstrate that the stage of Ag-specific CD8 T cell dysfunction correlates with a progressive increase in the amount and diversity of inhibitory receptors expressed and coexpressed on these cells. These receptors include programmed cell death-1 (PD-1), CTLA-4/CD152, T cell Ig mucin-3 (TIM-3), and CD160 and CD244 (2B4) (13–16).

Several studies have described CD8 T cell dysfunction in humans within the context of chronic viral infections, such as HIV, hepatitis C virus (HCV), and hepatitis B virus (HBV) (16–19), as well as in malignant tumors (20–22). More recently, this phenomenon was reported in visceral leishmaniasis, a parasitic infection caused by *Leishmania donovani* (23). The frequency of CD8 T cells producing IFN-γ was found to be higher in chronic asymptomatic *T. cruzi*-infected individuals than in those presenting a severe form of the disease (24, 25). Nonetheless, when two cytokines were evaluated, *T. cruzi*-specific CD8 T cells in clinically compromised subjects displayed a functional profile of T cells secreting IFN-γ alone with a low frequency of dual IFN-γ cytokine expression (IL-2, IL-7–secreting T cells (26)). In addition, it has been shown that CD8 T cell proliferation is diminished in chronic chagasic patients (CCP) compared with healthy donors (HD) and patients with nonchagasic cardiomyopathy (27). Moreover, an increase in the frequency of fully differentiated CD8 T cells with attenuated cytokine effector functions has been observed in subjects presenting a severe form of this disease and in mouse models of chronic Chagas disease (CCD) (25, 28, 29). These data suggest that, similar to other chronic infections (16–19), CD8 T cells undergo a progressive dysfunction during *T. cruzi* infection that is characterized by impaired functional activity, advanced cell differentiation, and increased inhibitory receptor coexpression. To assess whether CD8 T cells exhibit dysfunction and increased differentiation during CCD, effector functions in response to *T. cruzi* Ags and the expression of inhibitory receptors on the cell surface were evaluated.

### Materials and Methods

#### Selection of study population

Forty-three CCP aged 22–76 y were recruited at Fundación Clínica Abroad Shiao, Instituto Nacional de Salud and Hospital Universitario San Ignacio in Bogotá, Colombia. All chagasic donors were positive for anti-*T. cruzi* Abs by both indirect immunofluorescence and ELISA assays (30) and classified according to the American College of Cardiology and American Heart Association Staging (31–33). Fourteen patients were classified as group A (normal electrocardiogram (ECG) findings, normal heart size, normal LVEF, NYHA class I), 10 patients were classified as group B (abnormal ECG findings, normal heart size, normal LVEF, NYHA class I), 12 patients were classified as group C (abnormal ECG findings, increased heart size, decreased LVEF, NYHA class II–III), and 7 patients were classified as group D (abnormal ECG findings, increased heart size, decreased LVEF, NYHA class IV). For purposes of analysis, 24 of these chagasic patients were classified as asymptomatic (A and B stages), and 19 were classified as symptomatic (C and D stages). The group of HD comprised 12 seronegative individuals aged 23–60 y who had always resided in nonendemic areas and exhibited normal ECG results, heart size, LVEF, and clinical examination. The characteristics of the study population are summarized in Table I. 

#### Blood collection

Approximately 24 ml blood was obtained from each individual through venipuncture and collected in heparinized tubes for PBMC isolation; 4 ml blood was collected in EDTA-containing tubes for DNA extraction and WBC counts, and 6 ml blood was collected in tubes without anticoagulant for serological testing (BD Vacutainer, San Jose, CA). PBMCs were isolated through density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bioscience, Uppsala, Sweden). The absolute blood CD8+ T cells count (CD8+ T cells/µl) was calculated from the percentage of CD8+ T cells using flow cytometry. The total WBC count was determined using a standard differential cellular blood count.

### Isolation of *T. cruzi* soluble Ags

Green Monkey renal fibroblast-like cells (VERO cells) (ATCC CCL-81, Manassas, VA) were grown in DMEM (Eurobio, Les Ulis, France) supplemented with 10% FBS (Eurobio), 2.0 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.01 M HEPES (Eurobio). The cells were grown at 37°C in a humid atmosphere at 5% CO2. After reaching semiconfluence, the VERO cells were incubated for 10 h with *T. cruzi* I trypomastigotes (MHOM/CO/01/DA human isolate) (34), and the parasites were recovered at 96 h postinfection (55, 36). A trypomastigote lysate was obtained as previously described (37). In brief, the parasite culture was washed twice with cold 1× PBS (Eurobio), pH 7.0, and suspended at 1× 10^6 parasites/µl in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, and 1.4% Triton X-100) containing 2% protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). The samples were incubated on ice for 30 min until cells lysis was complete (30 min), and the supernatant containing the protein lysate was collected by centrifugation for 15 min at 4°C and stored until further use at −80°C.

The soluble protein [*T. cruzi* soluble Ag (TcSA)] concentration was determined using the Bradford assay. The protein profile was analyzed after resolving the samples by 10% NaDodSO4-PAGE (SDS-PAGE), followed by Coomassie blue and silver nitrate staining (38). A soluble total protein extract from *T. cruzi* amastigotes/trypomastigotes (ratio 3:1) was obtained as previously described (39). *T. cruzi* KMP-11 recombinant protein

Purified recombinant *T. cruzi* KMP-11 (KMP-11r) was obtained as previously described (40, 41), reconstituted in 0.1× PBS (Eurobio) and stored at −20°C.

### Conventional PCR and quantitative PCR strategies for parasite detection

DNA was extracted from EDTA-treated blood samples using the guanidine hydrochloride method. The DNA was purified using a commercial high-purity PCR template preparation kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. The DNA was eluted with 100 µl elution buffer and stored at −20°C. The integrity of the purified DNA was analyzed through PCR amplification of the human β-globin fragment, as previously described (42). Quantitative PCR experiments were performed according to Pirón and coworkers (43) using the primers cru1 5′-ASTCCGCTGATCTGTTTTCGA-3′, cru2 5′-AATCCCT-CCAAGCAGCGGATA-3′, and the probe cru3 3′ (6FAM-5′-CAACACAGGACGATACAA-3′-BHQ); which amplify a 166-bp fragment of satellite DNA of the parasite. Each sample was performed in duplicate using a LightCycler 1.5 Instrument (Roche). The parasite load was estimated through the extrapolation of values obtained in a standard curve generated using DNA purified from the blood of HD and reconstituted with serial dilutions of *T. cruzi* DNA at a range of 10^1 to 10^4 parasite equivalents per milliliter (Roche) (44). In addition, all DNA samples were evaluated using the primers S35 5′-AAATAATGGGG(T/G)GAGATGCATGA-3′ and S36 5′-GGGTTCGATTGGGGTTGGTGT-3′, which are based on the conserved regions of minicircles from *T. cruzi* kinetoplast DNA (45). The expected 330-bp amplified product was resolved by electrophoresis through 1.5% agarose gels containing ethidium bromide. Each set of reactions included the following controls: reaction (water added in the place of the reaction mixture), gray (water added in the place of the DNA sample addition to the reaction), negative (genomic DNA from an HD), and positive (genomic DNA of *T. cruzi* Tcl. 1 ng for conventional PCR, and 10^3 and 10^4 parasite equivalents per milliliter for quantitative PCR).

### Abs

The following Abs were used for cell surface staining: anti-CD3 Pacific blue (clone UCHT1), anti-CD8 allophycocyanin-H7 (clone SK1), anti-CD127 PE-Cy7 (clone HIL-7-R-M21), anti-CCR7 PE-Cy7 (clone 3D12), anti-CD62L allophycocyanin (clone DREG-56), anti-CD27 FITC (clone L28), anti-CD4 FITC (clone 2.69), anti–PD-1 PE (clone MIH4) (BD Biosciences, San Jose, CA), anti-CD160 allophycocyanin (clone DREG-56), anti-CD27 FITC (clone L28), anti-2B4 FITC (clone 2.69), anti–PD-1 FITC (clone MIH4) (BD Biosciences, San Jose, CA), anti-CD161 Alexa Fluor 647 (clone BY55), anti–PD-1 PE (clone eBio105) (BioSource, San Diego, CA), and anti–TIM-3 PerCP (clone 344823) (R&D Systems, Minneapolis, MN). The Abs for intracellular staining included anti-perforin PE (clone B-D48;...
However, at least 50,000 events, gated on live CD3+ CD8+ T cells, were acquired through flow cytometry using a FACSAria II flow cytometer (BD Immunocytometry Systems, San Jose, CA), and the results were subsequently analyzed by BD FACSuite (1 µg/ml) or KMP-11 recombinant protein (10 µg/ml) in the presence of CD82 (1 µg/ml) and CD49d (1 µg/ml) for 6 h at 37°C (46). The last 5 h of culture were performed in the presence of brefeldin A (1 µg/ml) and monensin (1 µg/ml) (BD Pharmingen). The PBMCs were adjusted to 1 × 106 cells/tube and labeled with LIVE/DEAD Fixable Aqua for 20 min in the dark at room temperature. The cells were subsequently stained with anti-CD3 Pacific blue, anti-CD8 allophycocyanin-H7, anti-CD127 PE-Cy7, anti-CD160 Alexa Fluor 647, anti-2B4 FITC, and anti-TIM-3 PerCP Abs. To determine the T cell phenotype and function, we cultured PBMCs with Tc1-SA (1 µg/ml) or Tc2-SA (1 µg/ml) staphylococcal enterotoxin B (SEB; Sigma-Aldrich) as a positive control. Because of the limitations of the cell numbers available, not all samples were analyzed for all multicolor panels. However, at least 50,000 events, gated on live CD3+ CD8+ T cells, were acquired through flow cytometry using a FACSAria II flow cytometer (BD Immunocytometry Systems, San Jose, CA), and the results were subsequently analyzed by FlowJo software (Tree Star, Ashland, OR). The gating strategy is provided in Supplemental Fig. 1. The gates for positivity in multicolor panels were determined using fluorescence minus one control stains, as recommended (47). A positive cytokine response was defined as a frequency >0.05%, determined as the average frequency of CD8+ T cell response obtained from HD after stimulation with Tc1-SA plus 3 SDs after background subtraction (the values for cells from HD cultured without Ag). In addition, the mean intensity fluorescence (MFI) of each functional parameter was also determined using FlowJo software. Coexpression of inhibitory receptors and multifunctional analyses were performed using a Boolean gating strategy. The data are represented using Pestle version 1.7 and SPICE version 5.3 software (the National Institutes of Health, Bethesda, MD) (48).

Statistical analysis

Parametric or nonparametric statistics were applied according to the particular distribution of the analyzed datasets. The statistical analysis of the significance between two groups was calculated using the Mann–Whitney U test. Differences among subject groups were evaluated using Kruskal–Wallis and Dunn’s posttest for multiple comparisons. A linear regression test was used for trend analysis. The tests were two-tailed, and the differences were considered statistically significant when p < 0.05. GraphPad Prism version 6.0 for Mac OS X statistics software (GraphPad Software, San Diego, CA) was used for the statistical analyses. The coexpression pie charts were compared using 10,000 permutations calculated with the software SPICE version 5.3.

### Results

#### Memory CD8+ T cell distribution in CCP

Memory T cells can be divided into distinct subsets based on their differentiation stage through the identification of molecules involved in cellular homing to lymphoid tissues (CCR7 and CD62L) and markers implicated in the generation and maintenance of T cell memory (CD27 and CD127) (49, 50). Two typing strategies were used to evaluate the CD8+ T cell memory phenotype from CCP showing different degrees of disease severity (stages A–D) (Table I). The CD8+ T cell memory phenotype from HD was analyzed as a control. The expression of CCR7 and CD62L is required for the differentiation of T cell populations of central memory (Tcm; CCR7+ CD62L+) from effector memory cells (Tem; CCR7− CD62L−); Fig. 1A, upper panel), which have a distinct homing capacity and effector function (50). In addition, the expression of CD27 and CD127 discriminates CD8+ T cells from early (CD27+ CD127+ to late (CD27− CD127−) stages of cellular differentiation (Fig. 1A, lower panel). In general, the percentage of CD8+ T cells with a Tcm phenotype was lower in CCP than in HD, regardless of the antigenic stimulus (KMP-11 or Tc1-SA), as summarized in Table II. Divergence in the differentiation stage was also observed, as the percentage of CD8+ cells in the late stage of differentiation increased in CCP compared with HD (Table II). When the degree of pathology was considered (Fig. 1B), patients with severe disease (groups C and D) presented a higher frequency of Tem compared with patients with mild disease (group A). In addition, patients at an advanced stage of the disease (groups C and D) had a lower frequency of Tem compared with patients from group A (Fig. 1B). Similarly, Tem significantly decreased as the disease severity increased (p < 0.002; Fig. 1B). When cell differentiation phenotypes were compared among patients with different degrees of disease severity, patients from group D had a higher frequency of CD8+ T cells at a late differentiation stage and a significantly lower frequency of CD8+ T cells at an early differentiation stage than patients from group A (Fig. 1C). With increasing disease severity, early-differentiated CD8+ T cells decreased and late-differentiated cells became more apparent (p < 0.002; Fig. 1C). No differences were observed between HD and group A patients with a mild disease according to both analysis strategies (Fig. 1B, 1C). Considering the possible variations in CD8+ T cell counts among individuals, the total number of CD8+ T cells in each memory phenotype subpopulation was also determined, and the total number of CD8+ Tcm and Tem

### Table I. Clinical characteristics of studied individuals

<table>
<thead>
<tr>
<th>Variable</th>
<th>Asymptomatic Chagasic Patients</th>
<th>Symptomatic Chagasic Patients</th>
<th>HD</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individuals, n (%)</td>
<td>24 (43.6%)</td>
<td>19 (34.6%)</td>
<td>12 (21.8%)</td>
<td>—</td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>44 (22–67)</td>
<td>44 (34–80)</td>
<td>45.5 (28–60)</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>9 (36%)</td>
<td>12 (63%)</td>
<td>7 (58%)</td>
<td>—</td>
</tr>
<tr>
<td>ACC/AHAS classificationa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C</td>
<td></td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median LVEF (range)</td>
<td>61.35 (50–78)</td>
<td>28 (10–50)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*aComparison between chagasic patients and HD and between asymptomatic and symptomatic chagasic patients were estimated with the nonparametric Mann–Whitney U test.

Classification by the American College of Cardiology (ACC) and American Heart Association Staging (AHAS).

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Abcam, Cambridge, MA), anti-CTLA-4 allophycocyanin (clone BN3), anti-granzyme B Alexa Fluor 647 (clone GB11), anti-IFN-γ Alexa Fluor 700 (clone B27), anti-CD107α FITC (clone HA43), anti-CD107β FITC (clone H4B4), anti–IL-2 PerCP-Cy5.5 (clone MQ1-17H12), and anti–TNF-α allophycocyanin (clone 6401.1111) (BD Biosciences). A LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen Molecular Probes, Eugene, OR) was used for dead cell exclusion.

Flow cytometry and intracellular cytokine staining assays

All conjugated Abs were titrated as previously reported (46). A total of 1 × 106 PBMCs were stained with LIVE/DEAD Fixable Aqua for 20 min in the dark at room temperature. After washing with 2 ml 1× PBS, the cells were stained with anti-CD3 Pacific blue, anti-CD8 allophycocyanin-H7, anti-CD127 PE-Cy7, anti-CD160 Alexa Fluor 647, anti-2B4 FITC, and anti-TIM-3 PerCP Abs. To determine the T cell phenotype and function, we cultured PBMCs with Tc1-SA (1 µg/ml) or KMP-11 recombinant protein (10 µg/ml) in the presence of CD82 (1 µg/ml) and CD49d (1 µg/ml) for 6 h at 37°C (46). The last 5 h of culture were performed in the presence of brefeldin A (1 µg/ml) and monensin (1 µg/ml) (BD Pharmingen). The PBMCs were adjusted to 1 × 106 cells/tube and labeled with LIVE/DEAD Fixable Aqua for 20 min in the dark at room temperature. The cells were subsequently stained with anti-CD3 Pacific blue, anti-CD8 allophycocyanin-H7, anti-CD127 PE-Cy7, anti-CD27 FITC, anti–CCR7 PE-Cy7, anti–CD62L allophycocyanin, and anti–PD-1 PE Abs, followed by fixation and permeation for intracellular staining with anti–CTLA-4 allophycocyanin, anti–IL-2 PerCP-Cy5.5, anti–IFN-γ Alexa Fluor 700, anti–TNF-α allophycocyanin, anti–Perforin PE, and anti–granzyme B Alexa Fluor 647 for 30 min at 4°C. To evaluate cytotoxic activity, we added anti–CD107α FITC and anti–CD107β FITC to the PBMCs before stimulation. In each experiment, nonstimulated cells were included as a negative control, and the cells were stimulated with 1.5 µg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich) as a positive control. Because of the limitations of the cell numbers available, not all samples were analyzed for all multicolor panels. However, at least 50,000 events, gated on live CD3+ CD8+ T cells, were acquired through flow cytometry using a FACSAria II flow cytometer (BD Immunocytometry Systems, San Jose, CA), and the results were subsequently analyzed by FlowJo software. Coexpression of inhibitory receptors and multifunctional analyses were performed using a Boolean gating strategy. The data are represented using Pestle version 1.7 and SPICE version 5.3 software (the National Institutes of Health, Bethesda, MD) (48).
and CD8+ T cells at the early and late stages of differentiation was correlated with the frequency of CD8+ T cell memory subpopulations (data not shown). Altogether, these findings indicate that patients with an intense disease severity have the highest frequency and number of CD8+ T cells with effector memory phenotypes and late differentiation among the assayed CCD patients.

**Functional activity of CD8+ T cells according to the memory differentiation profile in CCP**

To evaluate the functional activity of CD8+ T cells according to the memory phenotype, we measured the cytolytic machinery (granzyme B and perforin) and cytokine secretion (IFN-γ and IL-2) in cells from CCP at different stages of the disease and HD as a control. Differences were observed depending on the stimulating agent. When cells were stimulated with SEB, no differences between CCP and HD were observed in terms of perforin and granzyme B production (Fig. 2A, panel SEB). However, after stimulation with TcSA, patients with more severe disease (group D) showed a higher frequency of CD8+ T cells producing perforin and granzyme B compared with the patients from groups A and B (p < 0.05; Fig. 2A). After stimulation with the recombinant KMP-11 protein, the observed effect was stronger, with the highest percentage of CD8+ T cells producing perforin and granzyme B. This effect was more pronounced in patients in C and D stages than in those in A and B stages. As expected, CD8+ T cells from HD did not secrete perforin or granzyme B after stimulation with KMP11 or TcSA Ags. To determine the functional activity of CD8+ T cells according to the cellular stage of differentiation, we analyzed the expression of perforin and granzyme B in early- and late-differentiated cells. The highest frequency of CD8+ T cells producing perforin and granzyme B was observed during late differentiation (Fig. 2B), independent of the stimulus used, TcSA or recombinant KMP-11. In addition, the MFI of these molecules was evaluated in the early- and late-differentiated CD8+ T cells. In both cases, similar results were obtained (Supplemental Fig. 2A). When total CD8+ T cells producing IFN-γ and IL-2 were evaluated after stimulation with SEB, CCP showed a similar profile of cytokine secretion compared with HD, asymptomatic chagasic patients (groups A and B), and symptomatic chagasic patients (groups C and D). (C) Frequency of early (CD27+ CD127+) and late (CD27− CD127−) CD8+ T cell differentiation stages in HD and asymptomatic (groups A and B) and symptomatic (groups C and D) chagasic patients. Median values are indicated with horizontal lines. A positive or negative trend in the CD8+ T cell phenotype with increasing disease severity is shown as a dotted line. The p values were calculated using a one-way ANOVA nonparametric Kruskal–Wallis test with Dunn’s posttest. *p < 0.05, **p < 0.01, ***p < 0.001.

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**Table II. Phenotypic characteristics of CD8+ T cells**

<table>
<thead>
<tr>
<th>Subsets</th>
<th>Chagasic Patients (CCP, n = 34)</th>
<th>HD (n = 10)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of CD8+ T Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Memory phenotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR7+ CD62L+ (TCM) median (range)</td>
<td>20.10 (11.52–44.86)</td>
<td>36.08 (22.98–45.88)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CCR7− CD62L− (TEM) median (range)</td>
<td>30.67 (11.49–60.00)</td>
<td>21.66 (14.49–31.64)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Differentiation state</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD27+ CD127+ (early) median (range)</td>
<td>32.68 (15.87–65.28)</td>
<td>45.54 (30.30–68.56)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD27− CD127− (late) median (range)</td>
<td>29.47 (12.24–48.78)</td>
<td>21.55 (7.72–39.54)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Data shown correspond to cells cultured with TcSAs.

*Comparison between chagasic patients and HD were estimated with the nonparametric Mann–Whitney U test.
IL-2 from TEM and TCM CD8+ cells was analyzed. As shown in charts, the production of IFN-γ and IL-2 was evaluated in both types of cells, the highest amount of IFN-γ and IL-2 per cell was also observed in TCM (Supplemental Fig. 2E) regardless of the used parasite stimulus, TcSA or recombinant KMP-11.

When the relative amount of perforin, granzyme B, IFN-γ, or IL-2 produced by CD8+ cells was assessed according to the disease severity, it was found that CD8+ T cells from asymptomatic patients (groups C and D) tend to produce more perforin and granzyme B (Supplemental Fig. 2C, 2D) compared with asymptomatic patients (groups A and B). Nevertheless, statistical differences were significant only when the relative amount of granzyme B was compared between groups B and D and between groups A and C (Supplemental Fig. 2D). In contrast, it was observed that CD8+ T cells from patients of group D tend to produce a lower level of IFN-γ and IL-2 (Supplemental Fig. 2E, 2F) than the asymptomatic patients. Despite this, significant differences were detected only when the MFI of IL-2 between groups B and D was compared (Supplemental Fig. 2F). Interestingly, within the group of asymptomatic patients, a significant increase in the relative amount of IFN-γ was found in patients of group B relative to that found in patients of group A (Supplemental Fig. 2E).

**Polyfunctional profile of the T. cruzi-specific CD8+ T cell response**

Previous reports have suggested that the quality of the T cell response, as defined by polyfunctional activity, is crucial for determining the clinical outcome of chronic infections (51–53). Therefore, we extended the evaluation of the functional activities of CD8+ T cells from CCP stimulated with TcSA or KMP-11 by simultaneously measuring the production and expression of IFN-γ, TNF-α, IL-2, CD107a/b, and perforin. When these five markers were assessed after stimulation with SEB, a similar functional profile was observed in CCP, regardless of the disease severity, and HD (Fig. 3A). However, after stimulation with TcSA or KMP-11, the CD8+ T cells from chagasic patients did exhibit a differential functional profile depending on the disease severity. A decrease in polyfunctional CD8+ T cells (expressing three or four markers) and an increase in the frequency of monofunctional CD8+ T cells were observed with increasing disease severity (Fig. 3A). The percentages of the 12 most prevalent combinations of functional markers in TcSA-stimulated CD8+ T cells from chagasic patients at different disease severities is shown in Fig. 3B. CD8+ T cells performing five functions were not detected in either group of patients. The most prevalent population of parasite-specific CD8+ T cells simultaneously expressing three (CD107a/b, IFN-γ, and TNF-α) or four (CD107a/b, IFN-γ, perforin, and TNF-α) markers was observed in patients from the B

**FIGURE 2.** Functional activity and memory profile of CD8+ T cells in CCP. (A) The CD8+ T cell functional activity in CCP, as determined by perforin and granzyme B expression after stimulation with SEB, TcSA, or recombinant KMP-11 protein. The functional profiles are grouped and color-coded according to the number of functions, as shown in pie charts. (B) The frequencies of early and late CD8+ T cell differentiation stages expressing perforin and granzyme B (Supplemental Fig. 2C, 2D) compared with asymptomatic patients (groups A and B). Nevertheless, statistical differences were significant only when the relative amount of granzyme B was compared between groups B and D and between groups A and C (Supplemental Fig. 2D). In contrast, it was observed that CD8+ T cells from patients of group D tend to produce...
cruzi–specific CD8+ T cells with increasing disease severity. Suggested an impairment in the production of cytokines from perforin or CD107a/b (Fig. 3B). Taken together, these results from patients from the D group, with these cells primarily expressing 12 distinct combinations of 5 functions after stimulus with Tc:SA in CCP with different degrees of disease severity.

Inhibitory receptor expression from CD8+ T cells in CCP

To determine whether the deficient polyfunctional response of parasite-specific CD8+ T cells from chagasic patients is associated with the expression of inhibitory receptors, we evaluated the expression of PD-1, CTLA-4, 2B4, CD160, and TIM-3 ex vivo in PBMCs from CCP and HD. A higher frequency of CD8+ T cells expressing these inhibitory receptors was observed in CCP compared with HD (Fig. 4A). When the coexpression of these molecules was evaluated, a higher frequency of CD8+ T cells coexpressing two or three inhibitory receptors was observed in CCP than in HD (Fig. 4B, 4C). In addition, a higher frequency of CD8+ T cells expressing CD160, PD-1, and CTL-4 in symptomatic versus asymptomatic patients (Fig. 4D) was detected, and a higher frequency of CD8+ T cells coexpressing two or three inhibitory receptors was observed in symptomatic patients compared with asymptomatic patients (Fig. 4E, 4F). When the frequency of CD8+ T cells expressing PD-1 and CTLA-4 in chagasic patients at different disease stages was evaluated, a significant positive correlation between these two inhibitors markers and the progression of the disease (p < 0.005; Fig. 5A, 5B) was observed. A higher frequency of CD8+ T cells coexpressing these inhibitory receptors was observed in chagasic patients from group D (p < 0.05) compared with patients with milder disease and HD (Fig. 5C). However, the expression levels of PD-1 and CTLA-4 in PBMCs from CCP and HD were not altered after stimulation with SEB (polyclonal stimulus), Tc:SA, or KMP-11 (data not shown).

Parasite detection in peripheral blood of chagasic patients

We evaluated whether the impaired cytokine secretion of CD8+ T cells and the increased expression of inhibitory receptors observed in this study (Fig. 6) are associated with the persistence of T. cruzi in the peripheral blood of CCP. The presence of parasite DNA was detected in 5 of 15 patients from group A, 2 of 8 patients from group B, 2 of 8 patients from group C, and 2 of 5 patients from group D.

Discussion

During acute viral and bacterial infections, Ag-specific T cells clear the pathogen and differentiate into long-lived memory T cells capable of maintaining themselves for long term without Ag stimulation. Upon reinfection, these cells mount a rapid recall response and reactivate their polyfunctional effector response (54–56). In contrast with acute infections, in chronic viral, bacterial (Mycobacterium tuberculosis and Salmonella spp.), and parasitic (Toxoplasma gondii and Trypanosoma cruzi) infections, there is a delay in the generation of the CD8+ T cell immune response. In addition, Ag-specific T cells become dysfunctional, express inhibitory receptors, and even fail to control the infection (57–64). Although this process was initially described for models of chronic viral infections, such as lymphocytic choriomeningitis, HIV, simian immunodeficiency, and HBV and HCV (12), recent studies have shown that this phenomenon also occurs in protozoan infections (65, 66). Chagas disease is associated with a poor functional T cell response and is characterized by increased levels of terminally differentiated T cells and monofunctional Ag-specific T cells (24–26, 28). Thus, the aim of this study was to determine whether CD8+ T cells lose polyfunctionality with an advanced stage of cell differentiation and sustained inhibitory receptor expression during CCD. Indeed, patients with severe disease have a higher frequency of terminally differentiated CD8+ T cells and a lower frequency of polyfunctional parasite-specific CD8+ T cells compared with patients without symptoms or with mild disease. In addition,
functional alterations in cytokine production by CD8+ T cells are accompanied by an increased frequency of CD8+ T cells coexpressing inhibitory receptors as the disease becomes more severe. Notably, the frequency of CD8+ T cells observed in this study was similar to that observed using absolute CD8+ T cell counts.

In this study, the phenotype of memory CD8+ T cells was assessed using two strategies: one for determining CD8+ TCM and TEM and another for evaluating CD8+ T cells in the early or late differentiation stage. In accordance with previous studies in which experimental mouse models of *T. cruzi* infection and patients with CCD (28, 67–69) were used, we observed that CCP have a higher frequency of CD8+ TEM than TCM. As disease becomes more severe, an increase in CD8+ TEM cells was observed. Failure in the control of infection observed in patients with severe disease may correlate with the efficient protective capacity of TCM compared with TEM (70). In persistent viral infections, CD8+ T cells fail to acquire and retain certain memory T cell characteristics, such as Ag-independent self-renewal and robust recall responses, likely reflecting Ag persistence; this also occurs in Chagas disease (12).

The CD27 and CD28 costimulatory molecules, which are involved in the generation of Ag-primed cells and in the regulation of T cell activation, respectively, have been commonly used to distinguish subsets of CD8+ T cells at different stages of cellular differentiation (71). However, given the similarity between CD28 and CD127 (the IL-7R α-chain) expression during cellular differentiation,

**FIGURE 4.** Inhibitory receptor expression from CD8+ T cells in CCP. (A) The frequency of CD8+ T cells expressing 2B4, CD160, PD-1, CTLA-4, or TIM-3 in HD and CCP. (B) Coexpression of CTLA-4 and PD-1 from CD8+ T cells in HD and CCP. (C) Coexpression of 2B4, CD160, and TIM-3 from CD8+ T cells in HD and CCP. (D) Frequency of CD8+ T cells expressing 2B4, CD160, PD-1, CTLA-4, or TIM-3 in asymptomatic (groups A and B) and symptomatic (groups C and D) chagasic patients. Coexpression of PD-1 and CTLA-4 (E) or coexpression of 2B4, CD160, and TIM-3 (F) in asymptomatic (groups A and B) and symptomatic (groups C and D) chagasic patients is presented in pie charts. The color in the pie charts depicts the coexpression of inhibitory receptors. The p values of the permutation test in the coexpression analysis (B, C, E, and F) are shown in the pie charts. The p values were calculated using the Mann–Whitney *U* test (A and D). *p < 0.05, **p < 0.01, ***p < 0.001.
in this study, the expression of CD27 and CD127 molecules was used to distinguish the differentiation stages of CD8+ T cells. Because CD127 is downregulated on CD8+ T cells after TCR stimulation (72), the CD8+ T cells in chronic infection generally do not express this cytokine receptor because of constant antigenic stimulation (11, 73). During chronic HIV infection, the repeated expansion of CD8+ T cells leads to a loss of the molecules needed for costimulation, Ag presentation, and memory maintenance in a large population of late-differentiated and primarily dysfunctional cells (74). Thus, the stage of cellular differentiation in persistent infections depends on the Ag and the type of infection; however, it is not possible to claim similar mechanisms during all types of chronic infections (71). Nonetheless, a correlation between memory phenotype (TCM and TEM) and stages of CD8+ T cell differentiation (early and late) were observed in this study. As previously reported (25), patients with severe disease have a higher frequency of total CD8+ T cells during the late differentiation stage and a lower frequency of total CD8+ T cells during the early differentiation stage compared with patients with no clinical findings or mild disease.

CD8+ T cells secrete cytokines and display cytotoxic activity via the release of granules containing perforin and granzymes. Conversely, the functional activity of CD8+ T cells depends on the memory phenotype, that is, CD8+ T cells at the late differentiation stage secrete little IL-2 and have potent cytotoxic function (49, 70, 71, 75). In this study, cytotoxic activity was higher in CD8+ T cells at the late stage of differentiation in patients with more severe forms of cardiac disease, whereas the production of IL-2 in CD8+ T cells was lower. Studies have shown that the severity of the disease, that is, tissue damage, resulting from *Leishmania braziliensis* infection is directly correlated with more granzyme-producing CD8+ T cells (76) and that CD8+ T cell–mediated cytotoxicity promotes lesions in cutaneous leishmaniasis (77). In addition, CD8+ T cells producing perforin might play a detrimental role in experimental *T. cruzi* infection in mice, leading to heart injury (78). Hence the infection severity in CCP might be associated with the increased frequency of CD8+ T cells with potent cytotoxic profiles corroborated by the high amount of granzyme B produced by the CD8+ T cells from patients classified

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as group D (Supplemental Fig. 2D). Likewise, the infection severity in CCP may be associated with a low frequency of CD8+ T cells producing cytokines. Thus, these markers could be evaluated for possible use in determining the state of disease severity. In the same way, the finding of an increased amount of IFN-γ in patients of group B (abnormal ECG findings) compared with those of group A (normal ECG findings) (Supplemental Fig. 2E), suggests that the measurement of the MFI of this molecule can be used as a marker of disease progression, at least in the asymptomatic group of patients.

In several studies, polyfunctional T cells have been associated with an enhanced immune response in infection models and vaccinations (49). Long-term nonprogressor HIV patients show higher frequencies of polyfunctional HIV-specific CD8+ T cells compared with progressed HIV patients (51), and both polyfunctional CD4+ and CD8+ T cells have been associated with an enhanced control of HIV replication (79). Even more, polyfunctional CD8+ T cells can provide protective immunity in vaccination studies against experimental T. cruzi infection (80–82). In Chagas disease, children with short-term T. cruzi infections exhibit a higher frequency of polyfunctional T cells in comparison with adults with long-term infections (83). We observed that asymptomatic patients have a higher frequency of polyfunctional parasite-specific CD8+ T cells, which could lead to more effective parasite control, whereas the monofunctional cytotoxic parasite-specific CD8+ T cells predominant in symptomatic patients could result in tissue damage. This result correlates with that presented earlier, wherein it was shown that IFN-γ production by parasite-specific cells was higher in asymptomatic patients, whereas these cells tended to degranulate more in symptomatic donors (84). In addition, in a previous study, we showed that the functional alterations in CD8+ T cells during Chagas disease are Ag specific (85). This finding was confirmed in this study, because both HD and CCP displayed the same functional profile against the polyclonal stimulus SEB. Taken together, these results suggest that polyfunctional CD8+ T cells play a key role in controlling T. cruzi infection; however, studies should be conducted to determine whether the activities of these cells are correlated with protection in Chagas disease.

Although differential protein expression has been described between the trypanastigote and amastigote stages, common major Ags are expressed between these stages. In this study, the soluble proteins of T. cruzi trypanastigotes were used to induce CD8+ T cell responses against total parasite Ags. Although a potential limitation of this study, the frequency of CD8+ T cells producing IFN-γ and TNF-α after stimulation with soluble trypanastigote proteins did not vary when stimulated with soluble Ags from T. cruzi amastigotes/trypanastigotes (ratio 3:1) (Supplemental Table 1).

Concomitant with the loss of polyfunctional T cell responses, CD8+ T cells upregulate the expression of inhibitory receptors, such as PD-1, CTLA-4, CD160, 2B4, LAG3, and TIM-3 (12). In some chronic infections, such as HIV, HCV, and HBV, the simultaneous expression of inhibitory receptors can substantially affect the functional activity of CD8+ T cells (12, 13, 15, 16, 65, 86). Accordingly, the increased expression of inhibitory receptors on CD8+ T cells from patients with severe disease could be implicated in the alteration of CD8+ T cell function. Consistently, in Chagas disease, although the levels of CD8+ T cells expressing the inhibitory receptor LIR-1 did not differ between chagasic patients and healthy volunteers, LIR-1 expression was found to increase with increasing disease severity (87). In contrast, the frequency of CD8+ T cells expressing CTLA-4 was lower, regardless of the disease severity. Because a higher frequency of CD4+ T cells expressing LIR-1, which decreases after antiparasitic treatment, was observed in chagasic patients (87), a reduction in the level of parasite Ags could improve CD8+ T cell function. Although inhibitory receptor expression on T cells is an indicator of Ag elimination failure, these receptors are important for self-tolerance and the prevention of autoimmunity (12). The increased expression of CTLA-4 and PD-1 in lymphocytes has been described in acute T. cruzi mouse infection, and as expected (15, 88–90), the blockade of these inhibitory receptors decreases parasitemia and parasitism and increases inflammation and mortality (91, 92). In this article, we report increased inhibitory receptor expression on CD8+ T cells in patients with severe forms of the disease, which, consistent with other chronic infections (12), could be implicated in the alteration of CD8+ T cell polyfunctional activity.

The dysfunction of T cells during chronic viral infection has been associated with initial common effector differentiation, followed by a progressive loss of function over time because of the persistent contact with viral Ags (93). In contrast, even though some chronic viral infections with persistent high viremia can be found, in chronic parasitic infections, such as Chagas disease, there is a low parasite burden (65). Although the parasite burden was nonquantifiable in this study, parasite DNA was found in the peripheral blood of all groups of chagasic patients, indicating parasite persistence. During chronic infection, the parasite remains in tissues, such as the heart and other reservoir tissues (94–97); thus, the parasite load in the blood is low or not detectable in some patients. Indeed, the antigenic load is an important difference between viral and parasitic models of CD8+ T cell dysfunction. Nonetheless, it is likely that a low yet persistent level of parasite Ags leads to a state of functional impairment.

During acute T. cruzi infection, the activation of the immune response controls parasite replication (1); at this point, inhibitory receptor expression plays a regulatory role to avoid marked inflammation (91). As the parasite persists at low levels, the expression of inhibitory receptors on T cells is maintained during the early chronic stage (Fig. 6) (87). Thus, we propose the following scenario: in asymptomatic donors (group A), the CD8+ T cell profile is balanced, but the presence of the parasite induces polyfunctional CD8+ T cells, as observed in patients with mild disease (group B). This antigenic persistence increases the frequency of CD8+ T cells expressing inhibitory receptors, which, in turn, limits the polyfunctional response of parasite-specific CD8+ T cells, as observed in patients with increased heart involvement (groups C and D; Fig. 6). In summary, the results obtained in this study support the hypothesis that during CCD, CD8+ T cells undergo gradual dysfunction characterized by: 1) impaired cytokine effector activity; 2) late-stage cell differentiation; 3) increased inhibitory receptor coexpression; and 4) increased expression of cytolytic mediators, that is, granzyme B and perforin; all of these features are associated with the progression of chronic cardiac myocarditis. Altering these progressive changes in CD8+ T cell function could improve the prognosis of chronic chagasic myocarditis. Indeed, the results of studies using mouse models have suggested that the application of genetic vaccination with recombinant adenoviruses can reprogram this deleterious immune response and attenuate chronic chagasic myocarditis (98).

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Disclosures
The authors have no financial conflicts of interest.
References
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20. Mumprecht, S., C. Sch
Identification of CD8+ T cells

Phenotype characterization
Gated on CD8+ T cells

Functional characterization
Gated on CD8+ T cells or on CD8+ T cells with early and late differentiation stage

Inhibitory receptors expression
Gated on CD8+ T cells
FIGURE S1. Gating strategy. The gating strategy is a representative flow cytometry analysis from a chagasic patient with stage D disease severity. The lymphocytes were selected and cell doublets excluded from the analysis for the identification of CD8$^+$ T cells based on the forward scatter area (FSC-A) and forward scatter height (FSC-H) (Singlets 1), the forward scatter width (FSC-W) and forward scatter height (FSC-H) (Singlets 2), and the side scatter width (SSC-W) and SSC-H characteristics (Singlets 3). Moreover, live CD3$^+$ CD8$^+$ cells were selected. The phenotypic characterization of total CD8$^+$ T cells was performed as shown in the figure. The CD8$^+$ T cells of the representative functional characterization analysis were stimulated with TcSA. Gates applied for the identification of cytokine-positive cells were defined according to unstimulated samples for each subject. The evaluation of inhibitory receptor expression was gated on CD8$^+$ T cells.
FIGURE S2. Amount of perforin, granzyme B, IFN-γ and IL-2 produced from a CD8+ T cell following stimulation with TcSA or recombinant KMP-11 protein of T. cruzi. (A) Early and late CD8+ T cell functional activity in CCP determined through MFI of granzyme B and perforin following stimulation with TcSA or recombinant KMP-11 protein. (B) TCM and TEM CD8+ T cell functional activity in CCP determined through
MFI of IFN-γ and IL-2 produced following stimulation with TcSA or KMP-11 protein. The perforin (C), granzyme B (D), IFN-γ (E), and IL-2 (F) MFI s of antigen-specific CD8⁺ T cells in chronic chagasic patients (CCP) at different degrees of disease severity are shown. The results are shown as box and whisker (min to max) plots. The p values were calculated using a one-way ANOVA nonparametric Kruskal-Wallis test with Dunn’s post-test; *p < 0.05, **p < 0.01.
Table S1. Induction of cytokine production after the stimulation with soluble proteins of trypomastigotes or amastigotes and trypomastigotes of *T. cruzi*

<table>
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<tr>
<th>Patients</th>
<th>Frequency of CD8⁺ T cells producing IFN-γ</th>
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<tr>
<td></td>
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<td># 1  # 2  # 3</td>
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<td><em>Antigenic stimulation</em></td>
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<td>0.32  0.26  0.28</td>
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<td>trypomastigotes</td>
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