Presence of Antigen-Experienced T Cells with Low Grade of Differentiation and Proliferative Potential in Chronic Chagas Disease Myocarditis

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Abstract

Background: The main consequence of chronic Trypanosoma cruzi infection is the development of myocarditis in approximately 20–30% of infected individuals but not until 10–20 years after the initial infection. We have previously shown that circulating interferon-γ-secreting T cells responsive to Trypanosoma cruzi antigens in chronic Chagas disease patients display a low grade of differentiation and the frequency of these T lymphocytes decreases along with the severity of heart disease. This study sought to explore the expression of inhibitory receptors, transcription factors of type 1 or regulatory T cells, and markers of T cell differentiation, immunosenescence or active cell cycle in cardiac explants from patients with advanced Chagas disease myocarditis.

Methodology/Principal Findings: The expression of different markers for T and B cells as well as for macrophages was evaluated by immunohistochemistry and immunofluorescence techniques in cardiac explants from patients with advanced chronic Chagas disease submitted to heart transplantation. Most infiltrating cells displayed markers of antigen-experienced T cells (CD3+, CD4+, CD8+, CD45RO+) with a low grade of differentiation (CD27+, CD57+, CD45RA−, PD-1−). A skewed T helper1/T cytotoxic1 profile was supported by the expression of T-bet; whereas FOXP3+ cells were scarce and located only in areas of severe myocarditis. In addition, a significant proliferative capacity of CD3+ T cells, assessed by Ki67 staining, was found.

Conclusions/Significance: The quality of T cell responses and immunoregulatory mechanisms might determine the pattern of the cellular response and the severity of disease in chronic Trypanosoma cruzi infection.

Introduction

The main consequence of chronic Trypanosoma cruzi (T. cruzi) infection is the development of myocarditis in approximately 20–30% of infected individuals but not until 10–20 years after the initial infection [1]. Advanced chronic Chagas heart disease (cChHD) is characterized by dilated cavities with high degree of fibrosis and inflammation [2,3]. The analysis by immunohistochemical, but mainly by molecular techniques, of cardiac samples from chronically T. cruzi-infected subjects provided evidence of the association between parasite persistence and tissue damage in cChHD [2,4,5]. Reis et al. showed that inflammatory lesions were dominated by CD8+ lymphocytes, many of which expressed granzyme A [6]. Lymphocytes in these lesions express lymphocyte function antigen-a (LFA-1), CD44, very late antigen-4 (VLA-4) [7] and cytotoxic lymphocyte antigen 4 (CTLA-4) [8]. A Th1 cytokine pattern predominated in the cardiac inflammatory cell infiltrate of Chagas disease patients with heart failure [9]. Whereas some authors have shown increased peripheral levels of IFN-γ in patients with severe heart disease [10–12], other studies have demonstrated an inverse association between disease severity and IFN-γ production [8,13,14]. We have previously shown that most IFN-γ-secreting T cells in response to T. cruzi display a low grade of differentiation but high expression of the inhibitory receptor CTLA-4 in the circulation of subjects with chronic T. cruzi infection [8,15,16]. Conversely, the total T cell compartment in Chagas disease patients is enriched in highly differentiated T cells compared to uninfected controls [15–17].
Analysis of heart explants

Eight explanted hearts were weighed and fixed for 72 h in 10% phosphate-buffered formaldehyde. After fixation, transmural sections of the whole circumference of the left and right ventricle at a plane equidistant from the base to the apex were collected and embedded in paraffin. A 5-mm-thick section from each region was stained with hematoxylin and eosin and Masson’s trichrome solution. The interventricular septum of each heart was selected for histological and immunohistochemistry analysis. The diagnosis of myocarditis was defined according to the Dallas criteria taking into account the inflammatory infiltrate of the myocardium and the presence of necrosis and/or degeneration of adjacent myocytes [18]. The distribution of the inflammatory infiltrate was classified as focal, confluent or diffuse [19].

The median number of lymphocytes plus macrophages identified by the expression of CD3 and CD68, respectively, was calculated for 8 tissue samples from cChHD. 2 GCM samples and 1 IDCM sample assessed, as described in “Quantification of cells” [20]. Myocarditis recorded in each tissue sample was considered as severe when the number of lymphocytes plus macrophages was over the median number of these cell types in cChHD, moderate when the number of lymphocytes plus macrophages was between 25th and 50th percentile, and mild when the number of lymphocytes plus macrophages was under percentile 25th.

For quantitative assessment of fibrosis an interventricular septum block that was embedded in paraffin and sectioned at 5 μm was stained with picrosirius red. After obtaining digital images with a digital scanner (UMAX Technologies Inc., USA) at 2 μm magnification and a 1200 ppp resolution, the percentage of the surface area occupied by collagen was established by morphometric analysis using the digital analysis system Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, USA) [21]. The percentage of fibrosis was semi-quantified as mild (<10%), moderate (10%–20%), or severe (>20%) [22]. Molecular detection of T. cruzi in the same interventricular tissue sections by PCR was previously performed [23]. The presence of T. cruzi was also analyzed by direct observation of intracellular amastigotes.

Immunohistochemistry

Formalin fixed paraffin embedded tissue sections were rehydrated. Heat induced antigen retrieval, incubation time and antibody concentrations were selected following the manufacturer’s recommendations. For CD3 (Mouse monoclonal, Santa Cruz Biotechnology, USA), CD8 (Leica Microsystems, Germany), CD68 (Biogenex, USA), CD20 (Biogenex, USA), CD45RA (Biogenex, USA), CD21 (Biogenex, USA), PD-1 (Abcam plc, UK), CD27 (Leica Microsystems, Germany), Ki67 (Rabbit monoclonal from Abcam plc, UK, and mouse monoclonal HLA-G from Leica Microsystems, Germany) and T-bet (BD, Biosciences, USA), heat induced antigen retrieval was done using Antigen Retrieval Citra Plus (Citrate buffer buffered Ag retrieval solution pH = 6; Biogenex, USA). Antigen retrieval solution EZ-AR21H (EDTA based retrieval buffer pH = 9; Biogenex, USA) was used to detect CD4 (Leica Microsystems, Germany) and FOXP3 (BD, Biosciences, USA) expression. For CD37 (BD, Biosciences, USA) and CD45RO expression (Biogenex, USA), no antigen retrieval after rehydration was done. Biotinylated anti mouse immunoglobulin G, peroxidase labeled streptavidin, and AEC (3-amino-9-ethyl carbazole) as chromogen were used as secondary detection systems, (Biogenex, USA). All immunohistochemistry slides were counterstained with hematoxylin. The list of antibodies and clones used, as well the function of each marker are depicted in Table S1 [24–37].

Immunofluorescence

Tissue sections with high inflammation from patients 1 to 4 were selected for double labeling studies by immunofluorescence.
| Case | Gender | Age | Etiology | NYHA FC* | LVEDD (mm) | LVEF (%) | Heart weight/body weight (g/Kg)) | Myocarditis Median number of CD3+CD68+ cells (IQR) | Fibrosis (%) | Parasite nests |
|------|--------|-----|----------|----------|------------|----------|-------------------------------|--------------------------------|-------------|---------------|
| 1    | M      | 42  | cChHD    | IV       | 63         | 15       | 410/75                        | 287 (144–394)                | 45          | Yes           |
| 2    | F      | 48  | cChHD    | IV       | 72         | 33       | 420/48.9                      | 116 (79–185)                 | 23          | No            |
| 3    | M      | 45  | cChHD    | III      | 75         | 13       | 495/61                        | 117 (63–166)                 | 11          | No            |
| 4    | M      | 46  | cChHD    | III      | 77         | 14       | 460/66.6                      | 72 (42–83)                   | 21          | No            |
| 5    | M      | 59  | cChHD    | III      | 65         | 21       | 460/54.5                      | 20 (18–54)                   | 12          | No            |
| 6    | F      | 51  | cChHD    | IV       | 47         | 26       | 555/71                        | 33 (25–47)                   | 7           | No            |
| 7    | F      | 61  | cChHD    | III      | 61         | 14       | 335/54.7                      | 18 (13–26)                   | 22          | No            |
| 8    | F      | 59  | cChHD    | IV       | 76         | 18       | 465/51.9                      | 14 (6–26)                    | 20          | No            |
| A    | M      | 28  | GCM      | IV       | 59         | 25       | 395/87                        | 217 (187–266)                | 31          | No            |
| B    | M      | 47  | GCM      | IV       | 61         | 20       | 355/105                       | 185 (101–243)                | 61          | No            |
| C    | M      | 46  | iDCM     | IV       | 78         | 30       | 510/79.5                      | 9 (4–12)                     | 11          | No            |

*The degree of myocarditis was determined according to the number of lymphocytes plus macrophages in cChHD: Severe >48 median CD3+CD68+/HPF, Moderate 20–48 median CD3+CD68+/HPF, Mild <20 median CD3+CD68+/HPF.

The percentage of fibrotic area was determined by morphometric analysis as described in Materials and Methods [21].

Parasite DNA by PCR was detected in heart sections of all cChHD, except for patient 6 [23]. Abbreviations:
* NYHA FC, New York Heart Association, functional class;
* LVEDD, left ventricular end-diastolic diameter;
* LVEF, left ventricular ejection fraction;
* cChHD, chronic Chagas heart disease;
* GCM, Giant Cell Myocarditis;
* iDCM, idiopathic dilated cardiomyopathy;
* IQR, interquartile range; HPF, high power field.

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Table 2. Phenotypic and functional profile of inflammatory cells in heart tissues from cChHD, GCM and IDCM.

| Case | CD3  | CD8  | CD4  | CD68 | CD20 | CD57 | PD-1 | CD45RO | CD27 | Ki67 | T-bet | FOXP3 | HLA-G |
|------|------|------|------|------|------|------|------|--------|------|------|-------|-------|-------|
| 1    | 203/407 | 122/306 | 104/397 | 65/403 | 12/177 | 3/279 | 1/291 | 172/282 | 74/241 | 40/350 | 80/296 | 0/180 | 0/408 |
|      | (49.9) | (39.9) | (26.2) | (16.1) | (6.8) | (1.1) | (0.3) | (61.0) | (30.7) | (11.4) | (27.0) | (0)   | (0)   |
| 2    | 87/299 | 85/168 | 40/198 | 39/226 | 1/320 | 3/285 | 0/241 | 75/189 | 65/199 | 4/156 | 5/233 | 3/340 | 0/205 |
|      | (29.1) | (50.6) | (20.2) | (17.3) | (0.3) | (1.0) | (0)   | (39.7) | (32.7) | (2.6) | (2.1) | (0.9) | (0)   |
| 3    | 78/175 | 72/209 | 85/290 | 37/174 | 3/233 | 12/224 | 0/199 | 82/181 | 25/169 | 15/218 | 56/234 | 0/130 | 0/97  |
|      | (38.1) | (53.2) | (28.5) | (13.8) | (0.6) | (2.4) | (0)   | (35.7) | (8.2)  | (12.3) | (3.2) | (0)   | (0)   |
| 4    | 5/4/2 | 26/48  | 5/43  | 15/74  | 0/77  | 2/44  | 0/33  | 15/89  | 4/65  | 15/116 | 22/144 | 0/70  | 0/42  |
|      | (14.3) | (54.2) | (11.6) | (20.3) | (0)   | (4.5) | (0)   | (16.8) | (6.1)  | (12.9) | (15.3) | (0)   | (0)   |
| 5    | 14/76 | 12/37  | 1/50  | 22/89  | 0/56  | 2/62  | 0/37  | 1/64  | 7/79  | 0/68  | 0/50  | 1/92  | 0/65  |
|      | (18.4) | (32.4) | (2)   | (24.7) | (0)   | (3.2) | (0)   | (1.5)  | (8.9)  | (0)   | (0)   | (1.1) | (0)   |
| 6    | 5/4/3 | 2/18   | 1/56  | 14/64  | 0/48  | 1/52  | 0/53  | 4/52  | 0/24  | 0/33  | 0/50  | 0/53  | 0/37  |
|      | (11.6) | (11.1) | (1.8) | (21.9) | (0)   | (1.9) | (0)   | (7.7)  | (0)   | (0)   | (0)   | (0)   | (0)   |
| 8    | 4/4/2 | 3/12   | 0/55  | 8/53   | 0/46  | 1/36  | 0/31  | 3/54  | 0/31  | 0/44  | 0/35  | 0/42  | 0/37  |
|      | (9.5)  | (32.4) | (0)   | (24.7) | (0)   | (3.2) | (0)   | (1.5)  | (8.9)  | (0)   | (0)   | (1.1) | (0)   |
A    | 160/358 | 96/320  | 102/344 | 90/322 | 32/308 | 0/412 | 0/174 | 112/243 | 112/353 | 1/447 | 0/432 | 0/161 | 3/455 |
|     | (44.7) | (30)   | (29.6) | (27.9) | (10.4) | (0)   | (0)   | (46.1) | (31.7) | (0.2) | (0)   | (0)   | (0.7) |
B    | 68/264 | 98/383  | 163/439 | 112/288 | 8/263  | 2/242 | 1/282 | 150/386 | 210/436 | 4/365 | 5/250 | 0/298 | 52/461 |
|     | (25.7) | (25.6) | (37.1) | (38.9) | (3.0)  | (0.8) | (0.3) | (38.9) | (48.2) | (1.1) | (2)   | (0)   | (11.5) |
C    | 1/24  | 0/19   | 0/16  | 9/42   | 0/14  | 0/36  | 0/37  | 0/26  | 0/31  | 0/19  | 0/16  | 0/15  | 0/23  |
|     | (4.2)  | (0)    | (0)   | (21.4) | (0)   | (0)   | (0)   | (0)   | (0)   | (0)   | (0)   | (0)   | (0)   |

Data are shown as the median number of positive cells for each marker/median number of total mononuclear cell count in 10 high power field (×400) for each marker for each patient. Percentages are shown in brackets. Chronic Chagas disease patients with advanced heart disease (cChHD) [1–8]; Patients suffering from Giant Cell Myocarditis (GCM) [A and B]; Patient with idiopathic Dilated Cardiomyopathy (IDCM) [C].

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Double labeling assays were carried out by staining with a combination of anti-Ki67 (rabbit monoclonal antibody)/anti-CD8; anti-Ki67/anti-CD20 (mouse mAbs); anti-Ki67 (mouse mAb)/anti-CD3 (rabbit polyclonal) and anti-Ki67/anti-CD21 (rabbit monoclonal) (Table 1). Fluorescein labeled anti-rabbit goat immunoglobulin (Vector, USA) and Alexa fluor 594 labeled anti-mouse immunoglobulin (Invitrogen, USA) were used as secondary detection system. Nuclei staining were done with ready to use mounting medium for fluorescence with DAPI (Vectorshield, Vector, USA). Antibody dilutions were used according to the manufacturer’s instructions. Observations were made with a 100 W ultraviolet lamp and photographed with an AXIOCAM camera (Carl Zeiss AG, Germany).

Quantification of cells

For quantification of the total number of mononuclear inflammatory cells (i.e. cells with positive and negative staining for each marker assessed), 10 High Power Field (HPF) at 400× were counted for each section (1 section for each one of the 13 markers assessed). The median number of mononuclear inflammatory cells in 130 HPF was calculated for each patient.

The total number of mononuclear inflammatory cells with positive staining for each marker assessed was counted in 10 HPF, Magnification 400×. The percentage of cells expressing each marker was calculated by the ratio between the median number of positive cells for each marker and the median number of total mononuclear cell count in 10 HPF.

Cell counting was manually implemented using the Cell Counter plug-in of Image J 1.43b software (National Institutes of Health, USA). The position of the cells in each HPF was determined by Cell Counter plug-in and utilized thereafter for spatial histological analysis. This analysis was carried out in each one of the eight samples from Chagas disease patients, as well as in control samples.

Statistical analysis

Continuous variables are reported as means (SDs) or medians (interquartile range –IQR–), while categorical variables are presented as the percentage of subjects out of total subjects evaluated. Continuous variables with non-Gaussian distribution were analyzed by Mann-Whitney U test. Correlation analysis between the percentage of cells positive for each marker and the total number of mononuclear inflammatory cells was done using the Spearman correlation test.

In order to estimate the spatial pattern of infiltrating cells surrounding a T. cruzi–infected cardiomyocyte, a spatial point pattern analysis was applied. A focal analysis of the distribution of CD4+CD8− cells around an infected cardiomyocyte was performed by the bivariate Wiegand-Moloney O-ring statistic [38]. This test allows the characterization of a spatial pattern around a point at varying distances to the point, detecting aggregation, repulsion or randomness. The analysis was implemented with software Programita, using a random labeling null model. Confidence envelopes were calculated using 999 Montecarlo simulations [39]. A p value <0.05 (2-tailed) was considered statistically significant. For statistical analysis SPSS 11.0 statistical software (SPSS Inc, USA) was used.

Results

Clinical and demographical characteristics of cChHD patients

The clinical and demographical characteristics of cChHD patients are depicted in Table 2. Patients 1–5 and 7–8 were in end-stage cChHD, whereas patient 6 displayed cChHD and concomitant valvular heart disease. This patient suffered from rheumatic fever during her childhood, but no evidence of active endocarditis was observed by the time of the study. All patients were in the New York Heart Association classes III and IV by the time of transplantation. Echocardiographic studies revealed moderate to severe dilation of the cavities with a mean left ventricular end-diastolic diameter of 67.0±10.2 mm. The mean left ventricular ejection fraction, determined by radionuclide ventriculography, was 19.2±7.1%.

Analysis of cardiac explants specimens

The mean weight of cChHD explanted hearts was 450±65 g. Four of cChHD interventricular septum samples showed severe diffuse myocarditis, 2:8 moderate myocarditis and 2:8 had mild focal myocarditis. Interstitial fibrosis was moderate to severe in all except for one interventricular septum sample. Amastigote nests were only recorded in 3 sections with severe diffuse myocarditis of patient 1 (Table 1, Figure 1A). However, the presence of T. cruzi by PCR in the interventricular septum of patients 1–5 and 7–8 but not in patient 6 was demonstrated in a previous report [23]. The mean weight of hearts with GCM was 375 g and exhibited severe diffuse myocarditis and severe fibrosis. The IDCM heart weighted 510 g, presenting moderate fibrosis and absence of myocarditis (Table 1).

Characterization of the inflammatory cell infiltrate in the hearts of patients with severe chronic Chagas disease

Inflammatory mononuclear cell infiltrates in heart tissue samples from cChHD were evaluated by immunohistochemistry. As observed in Figure 1A and Table 2, patients 1 to 4 exhibited severe diffuse inflammatory infiltrate, while patients 5 to 8 presented lower number of total infiltrating cells within the interventricular septum. The percentage of T (CD3+, CD4+, CD8+) and B cells (CD20+) was correlated with the total cell count in inflammatory cell infiltrates (Figure 1B and 1C). Conversely, the percentage of macrophages/dendritic cells (CD68+ cells) in regions with the highest levels of infiltration was similar to those observed in areas with low inflammation (Figure 1B and 1C). In 7 out of the 8 cChHD, CD68+ T cells were prevalent among infiltrating mononuclear cells compared to CD4+, CD68+ and CD20+ cells (Table 2).
T cell differentiation of infiltrating cells in the heart of patients with Chagas disease myocarditis

Since T cells were the prevalent cell population in heart tissues with high degree of inflammation, the expression of markers of antigen-experienced T cells and T cell differentiation (Table S1) was evaluated in heart samples. A high percentage (>30%) of infiltrating cells in heart tissues from cChHD with severe myocardiitis express CD45RO, a marker of antigen-experienced T cells (Figure 2A, patients 1–4 in Table 2). Likewise, CD27 expression, a marker of low grade T cell differentiation was higher in cChHD with severe myocardiitis than in patients with lower degree of inflammation (Figure 2B, patients 1–4 vs. patients 5–8 in Table 2). In contrast, the expression of the inhibitory receptor PD-1, the immunoosenescence marker CD57 and CD45RA (i.e. expressed by naive and terminally differentiated T cells; Table S1), was generally very low in heart tissues from cChHD (Figure 2C, 2D, Figure S1, Table 2). Since CD57 is expressed in terminally differentiated effector T cells and also in NK, the low expression of CD57 confirms the scarcity of NK in heart tissue samples from chronic Chagas disease patients [3]. PD-1 expression was detected in intramyocardial lymphoid follicles (tertiary lymphoid follicles) in the heart of patients with severe Chagas myocardiitis (i.e. patients 1–4) [Figure 2G and 2F, left panel]. Of note, tertiary lymphoid follicles were randomly observed in myocardial tissues, as confirmed by the expression of CD21 and PD-1. These findings indicate that antigen-experienced T cells with low grade of differentiation are abundant in Chagas disease myocarditis.

Infected cardiac myocytes are surrounded by cells bearing memory markers

To better characterize the phenotype of the cell infiltrate recruited by infected myocytes, tissue samples bearing amastigotes nests along with high inflammation were selected and analyzed for the expression of CD45RO, CD45RA, CD57, CD68 and CD20 (Figure S1). Most cells surrounding the infected cardiomyocyte were CD45RO+, CD45RA-, CD57-, CD68+ and CD20- (Figure S1A and S1B). With the aim to evaluate whether the different cell types were recruited with the same efficacy towards the infected cardiomyocyte, the spatial pattern of CD45RO+ and CD45RO− cells was analyzed by using the O-ring test for 2-dimensional point patterns. A statistically significant aggregation of CD45RO+ cells (blue line in Figure S1D) was observed near the infected cardiomyocyte (i.e. CD45RO+ cells are over the confidence envelope) [Figure S1C and S1D, P<0.001]. The proportion of CD45RO+ cells decreases at longer distances from the infected cell.

Functional profile of T cells in Chagas disease myocarditis: High number of T-bet+ and Ki67-expressing lymphocytes and low number of FOXP3+ cells

The functional profile of the cell infiltrate was assessed by the expression of T-bet, a marker of type 1 T cell responses, the regulatory molecules FOXP3 and HLA-G, and Ki67, a marker of proliferative potential (Table S1). T-bet expression was broadly detected in cell nuclei in areas of high cell infiltrate and was correlated with the degree of cell infiltration (P<0.001, rho = 0.669) (Figure 3A; Table 2). In contrast, HLA-G+ and FOXP3+ cells were found in a very low proportion or were absent in cChHD (Figure 3B, Table 2). Cells with proliferative potential were observed in tissue samples from patients with severe myocardiitis (median percentage of proliferating cells = 9%) in areas with high inflammation (Figure 3C, Table 2 and Table 3). In order to identify the proliferating lymphocyte subsets, double immunofluorescence staining was carried out using Ki67, CD3, CD8 and CD21 antibodies (Figure 4). Most cells expressing Ki67+ were CD3+ cells [median percentage (interquartile range) = 74 (66–89)], while a minor proportion of Ki67+ cells expressed CD8+ (median percentage (interquartile range) = 21 [15–19]), as shown in Figure 4A, 4B and 4C. In contrast, a very low proportion of Ki67+ cells expressed CD21 (data not shown), suggesting that Ki67+/CD21− proliferating cells belong to the T cell compartment.

Comparison of phenotypic and functional profiles between chronic Chagas disease myocarditis and Giant Cell Myocarditis

To assess whether the phenotypic and functional profiles found in Chagas disease myocarditis were associated with parasite persistence, the same set of markers were assessed in heart tissues from patients suffering from idiopathic GCM (patients A and B, Table 2), and compared to the profile in cChHD with severe myocardiitis (patients 1–4, Table 2). GCM is characterized by an intense myocardiitis with multifocal cardiomyocyte damage. Although T lymphocytes were a major cell type in both severe cChHD and GCM, the latter was enriched in macrophages/dendritic cells and CD4+ T cells (Table 3), whereas CD8 was prevalent in cChHD with severe myocardiitis.

Regarding the differentiation status of the cell infiltrate in these two types of myocardiitis, no differences were found in PD-1 and CD45RO expression, whereas CD57 expression was low in both types of myocardiitis. cChHD with severe myocardiitis presented lower levels of CD27+ cells (i.e. 2/4 patients with severe myocardiitis in Table 2) compared to GCM (Table 2 and Table 3). Higher counts of Ki67+ and T-bet+ cells were found in cChHD as compared with GCM, whereas the expression of FOXP3 was very low in both myocarditis. HLA-G expression was only recorded in cardiomyocytes and mononuclear inflammatory cells from GCM (Table 2 and Table 3). Altogether, these findings support the notion that chronic infection with T. cruzi drives T cell differentiation and proliferation in chronic Chagas disease myocarditis.

Discussion

Most studies concerning the characterization of inflammatory cells in heart tissues in chronic Chagas disease have comprised the evaluation of different cell types, and the cytokine profile, while the differentiation status of T cells is less known. Herein, we report that antigen-experienced (CD45RO+) T cells with a low degree of...
Figure 3. T-bet, FOXP3 and Ki67 expression in cChHD myocarditis. A) Representative photo (O.M. 400×) of T-bet⁺ cells (arrows) in cChHD, left panel. Percentage of T-bet⁺ cells vs. total mononuclear inflammatory cells counted in 10 representative HPFs for each cChHD (n = 8), right panel. B) Representative photo (O.M. 400×) of FOXP3 in cChHD, left panel. Arrows indicate FOXP3⁺ cells. Percentage of FOXP3⁺ cells vs. total mononuclear inflammatory cells counted in 10 representative HPFs for each cChHD (n = 8), right panel. C) Representative photo (O.M. 400×) of Ki67 in cChHD, left panel. Arrows indicate Ki67⁺ lymphocytes in cChHD. Percentage of Ki67⁺ cells vs. total mononuclear inflammatory cells counted in 10 representative HPFs for each cChHD (n = 8), right panel. Correlation analysis for all markers was carried out by Spearman correlation test.

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**Table 3.** Comparative analysis between inflammatory cell infiltrates of cChHD and GCM.

| Markers | cChHDsM (n = 4) | GCM (n = 2) | P value cChHDsM vs. GCM |
|---------|---------------|------------|------------------------|
| Cell type |               |            |                        |
| CD3     | 87/206 (42.2) | 137/330 (41.5) | 0.374                  |
| CD8     | 92/205 (44.9) | 97/269 (36.1) | 0.483                  |
| CD4     | 76/272 (27.9) | 132/392 (33.7) | 0.009                  |
| CD68    | 36/224 (16.1) | 101/312 (32.4) <0.001 |
| CD20    | 3/211 (1.4)   | 7/275 (2.5)   | 0.147                  |
| Differentiation |             |            |                        |
| CD57    | 3/222 (1.3)   | 2/313 (0.6)   | 0.001                  |
| PD-1    | 0/231 (0)     | 1/229 (0.4)   | 0.146                  |
| CD45RO  | 82/203 (40.4) | 120/355 (33.8) | 0.541                  |
| CD27    | 45/187 (24.1) | 154/425 (36.2) <0.001 |
| Functional |            |            |                        |
| Ki67    | 16/222 (7.2)  | 1/408 (0.2)   <0.001  |
| T-bet   | 30/206 (14.6) | 1/426 (0.2)   <0.001  |
| FOXP3   | 0/182 (0)     | 0/261 (0)     0.092   |
| HLA-G   | 0/137 (0)     | 24/455 (5.3)  <0.001  |

Data are shown as the median number of positive cells for each marker/median number of total mononuclear cell count in 10 HPF (400 x) for each marker. Percentages are shown in brackets.

Significant differences between groups were assessed by Mann-Whitney U test. cChHDsM, chronic Chagas heart disease with severe myocarditis (patients 1–4 of Table 3); GCM, Giant Cell Myocarditis; HPF, high power field.

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**Text:**

...differentiation (CD27+/CD57−), a Th1 profile (Tbet+) and proliferative capacity (Ki67+) are recruited into the heart of patients with chronic advanced Chagas disease myocarditis. As previously described, the inflammatory cell infiltrate in most patients was dominated by CD8+ T cells [8,16]. Although less prominent than CD8+ T cells, appreciable counts of CD4+ T cells were also found in intracardiac infiltrates.

The T cell phenotype in heart tissues concurs with that observed in circulating CD8+ and CD4+ T cells responsive to T. cruzi antigens in patients with chronic Chagas disease, regardless the clinical status [13,16], but it is at odds with the phenotype of total peripheral CD4+ and CD8+ T cells [13–17]. [Table S2]. CD8+ T cells [13–17], [Table S2], 8,10–13,13–17,40–43. Whereas most circulating IFN-γ-producing CD8+ and CD4+ T cells responsive to T. cruzi display a low degree of differentiation (CD27+/CD28+/CD57−/LIR-1−) [8,16], high differentiated CD27+/CD28+/CD57+/LIR-1+ CD8+ and CD4+ T cells are increased in the total peripheral T cell compartment of patients with severe cardiomyopathy [8,15–17]. Subjects with chronic T. cruzi infection with severe cardiomyopathy also displayed lower frequencies of T. cruzi-responsive IFN-γ-producing T cells and lower levels of IFN-γ production compared to subjects with no signs of heart disease [8,13,16], leading us to propose that long-term parasite persistent might dampen parasite specific T cell responses [44]. Thus, recently developed effector T cells that bear a low degree of differentiation appear to be an important source of T. cruzi-specific T cells in the periphery of chronic Chagas disease patients. In contrast, the bulk of the total CD8+ and CD4+ T cells might reflect the effect of persistent exposure to the parasite.

The low degree of differentiation of T cells found in the heart of chronically T. cruzi-infected subjects was somewhat unexpected, since T cells at target tissues are more likely to be stimulated by antigen and induced to further differentiation. However, CD27− T cells also comprised a substantial fraction of the inflammatory infiltrate, indicating a heterogeneous composition of T cells with different degree of differentiation in heart tissues. The presence of CD27+/CD57− cells might account for proliferating CD3+ T cells, as proliferation is a hallmark of T cells with low grade of differentiation (CD27+ cells) and low rounds of antigen stimulation (CD57− T cells) [45].

We also demonstrate that antigen-experienced (CD45RO+) T cells were more efficiently recruited than other mononuclear cells towards the infected cardiomyocyte, supporting that T. cruzi is a driving force for T cell recruitment. Activated VLA-4/LFA-1+ granzyme A+ T lymphocytes have been observed in cardiac infiltrates of patients with chagasic heart failure [7], while the local production of IL-7 and IL-15 was claimed to be associated with the maintenance and predominance of CD8+ T cells in heart tissues [46]. However, the recruitment of early effector T cells might be also involved in the maintenance of inflammatory cells at sites of chronic localized infection.

Tertiary lymphoid organs associated to severe myocarditis might have been developed in the context of chronic inflammatory conditions [47–49]. Since the formation of TLO involves the recruitment of lymphocytes not normally associated with inflammatory infiltrates, notably naïve T cells and central memory T cells, these structures might be a source of recently recruited effector T cells from the naive or central memory pool. To the best of our knowledge, there are not reports describing these structures in chronic chagasic cardiomyopathy. TNF-α has been pointed out as a key molecule in the generation of ectopic TLO which might facilitate the perpetuation of inflammation in the heart. Cells expressing TNF-α and IFN-γ have been identified in heart tissues from patients with chronic chagasic cardiomyopathy [9,50]. Our findings showing T-bet expression in areas of high inflammation and proliferation further confirmed a type 1 T cell response in...
Figure 4. Proliferation of T and B cells in Chagas disease myocarditis. A) Representative photos (O.M. 400 ×) of double immunofluorescence staining of Ki67 with CD3 (upper row); CD8 (middle row); or CD21 (lower row) cell. All images correspond to patient 1. Nuclei were stained with DAPI (blue). Single CD3 (green), single Ki67 (red) and double CD3 and Ki67 staining (merge) are shown in the upper row (inset, arrows). Single CD8 (red), single Ki67 (green) and double CD8 and Ki67 staining are shown in the middle row (inset, arrow). Single CD21 (green) and single Ki67 (red) expression is shown in the lower row (inset, arrow). Proportion of total Ki67+ cells showing CD3 (B) or CD8 (C) expression (filled columns), in 10 HPFs (numbered from 1 to 10) of patient 1.

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patients with severe heart disease. The low levels of PD-1 counts concur with the presence of T-bet+ cells, since T bet inhibits PD-1 expression [51].

Recent reports have demonstrated that regulatory T cells (FOXP3+) are absent in Chagas disease myocarditis, indicating that deficiency in IL-10 producing T regs may led to a regulatory imbalance, perhaps rendering heart tissues increasingly susceptible to type 1-dependent pathology [9,52]. In line with these findings, scarce FOXP3+ cells were found in heart tissues from patients with severe heart disease. Nevertheless, we have shown that CD3+ T lymphocytes infiltrating heart tissues express CTLA-4, another negative regulator of effector T cell responses [8]. In addition to be involved in the regulatory T cell function of T regs, CTLA-4 is also upregulated on activated T cells [53]. This might explain the concomitant presence of proliferating and CTLA-4+ T cells. However, it is likely that proliferating T lymphocytes do not CTLA-4 or likely express dysfunctional CTLA-4.

The predominant role for T. cruzi in the differentiation and functional T cell profile observed in the heart of subjects with chronic T. cruzi infection becomes apparent at comparing the inflammatory cell infiltrates between chagasic and GCM myocarditis which is a rare and frequently fatal type of myocarditis with features of autoimmune disease [54,55]. Higher levels of infiltrating cells with low degree of differentiation (i.e. CD27− expressing cells), lower T-bet expression and lower proliferative capacity were observed in GCM compared with severe Chagas disease myocarditis. Of note, HLA-G, a non-classical class I major histocompatibility complex molecule playing a tolerogenic role in innate and adaptive responses [56] was not expressed in inflammatory cell infiltrates of chronic Chagas disease patients, while it was expressed by cardiomycocytes in GCM. These findings support that immunoregulation is different between an infectious and a non-infectious myocarditis. Coxsackievirus infection is another common cause of infectious myocarditis, in which a pathogen driven inflammatory process was supported by the reduction in inflammation and resolution of the infection after treatment with IFN-beta [57,58].

The development of tissue damage might depend on parasite burden; the effectiveness of the host immune response in controlling parasite replication, and the effectiveness of the host immune response in limiting peripheral damage. The effectiveness of the host immune response in controlling parasite replication without the induction of tissue damage will largely rely on the quality of T cell responses [59]. We have recently reported that children in early stages of T. cruzi infection maintain polyfunctional and stronger T cell responses to T. cruzi in their circulation, contrasting with the prevalent monofunctional T cell profile in long-term T. cruzi-infected adults [60]. Although, we cannot rule out that recruitment of IFN-γ-producing T cells into the heart might account for the decrease of IFN-γ-producing T cells from the periphery, it is also possible that clonal exhaustion occurs overtime. Consequently, in the phase of an ineffective T cell response, the development of tissue damage might occur in order to get parasites under control. Similarly, immunoregulatory pathways might also be dampened overtime.

In summary the quality of T cell responses and immunoregulatory mechanisms might determine the pattern of the cellular response and the severity of disease in chronic T. cruzi infection.

Limitation of the study

The main limitation of the study is the sample size. However, the detailed analysis conducted and the difficulty to get tissue samples can mitigate, at least partially, such limitation.

Supporting Information

Figure S1 Spatial analysis of infiltrating cells surrounding T. cruzi-infected cardiomyocyte in heart tissues from patients with chronic Chagas disease. A) Immunohistochemistry staining of the infected cardiomyocyte showing CD45RO, CD45RA, CD57 and CD68 expression [O.M. 400×]. Open arrows show amastigote nests while black arrows indicate positive staining for the corresponding marker. B) Immunohistochemistry assay [O.M. 100×] showing antigen-experienced CD45RO+T cells surrounding the infected cardiomyocyte. C) Spatial analysis of CD45RO+T cells surrounding an infected human cardomycocyte. Cell positioning dot plot showing 933 CD45RO+ (red dots) and 428 CD45RO− (blue dots) T cells. D) Statistics of the spatial distribution of CD45RO+ cells at different distances (r) from the infected cardiomyocyte was performed using the O-ring test [24–25]. The statistic values are depicted as O12 (r) (blue line). The confidence envelopes (P<0.001, grey lines, E12+) were determined by 999 Monte Carlo simulations. The upper limit of the confidence envelope (upper grey line, E12+) delimits statistically significantly aggregation of CD45RO+ cells; while the lower limit (E12−) delimits statistically significant repulsion of these cells. Values between E12+ and E12− do not differ significantly from randomness. (TIF)

Table S1 Note: * Unless otherwise stated, antibodies were unconjugated mouse monoclonal antibodies. † CD8α is also expressed in some dendritic cell subsets, and can be induced in NKs and HDE CD4 T cells. ‡ CD4 is expressed by monocytes and can be induced in macrophages and dendritic cells. DE, Terminally Differentiated Effector T cells; TCM, Central Memory T cells; TEM, Effector Memory T cells. (DOCX)

Table S2 ^ T. cruzi-infected vs. uninfected controls. PBMC were stimulated with T. cruzi antigens, E, effector; CM, central memory; EM, effector memory; TET, terminally differentiated effector; cChHD, advanced chronic Chagas heart disease. (DOCX)

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Author Contributions

Conceived and designed the experiments: SAL CV. Performed the experiments: RJA PCM. Analyzed the data: SAL CV RJA RV FG LEF RRF. Contributed reagents/materials/analysis tools: SAL RL FG LEF RRF. Wrote the paper: SAL CV RJA RL.

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