

Short Communication

Ex vivo T-lymphocyte chemokine receptor phenotypes in patients with chronic Chagas disease

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Abstract

Introduction: Elucidating the molecules involved in the inflammatory process of chronic Chagas disease may allow identification of treatment targets. **Methods:** The *ex vivo* phenotypic expression of chemokine receptors CCR1, CCR3, CCR4, CCR5, CXCR2, CXCR3, CXCR4, and CXCR5 on the CD4⁺ and CD8⁺ T-cells of patients with chronic Chagas cardiomyopathy of varying severity was evaluated using flow cytometry. **Results:** Differential expression of CD4⁺CCR3⁺ and CD8⁺CCR4⁺ T-cells was observed in patients with mild cardiac involvement compared, respectively, with patients with severe cardiac and asymptomatic forms of Chagas disease. **Conclusions:** These receptors are possibly involved in the pathogenesis of chronic Chagas cardiomyopathy.

Keywords: Chronic Chagas disease. Cardiomyopathy. Chemokine receptors.

Chagas disease, caused by the hemoflagellate protozoan *Trypanosoma cruzi*, presents with systemic features and follows a chronic evolution; it is a serious public health problem throughout Latin America¹. The chronic phase of this disease comprises three main clinical forms: asymptomatic or indeterminate, cardiac, and digestive forms². The cardiac form is associated with high morbidity and mortality, occurring mostly in symptomatic patients in the chronic phase of the disease. One of the main immunologic features of intracellular protozoan infections such as *T. cruzi* is polarization to a TH1 response. The TH1 response profile is characterized by a repertoire of proinflammatory cytokines, mainly tumor necrosis factor- α and interferon- γ , that play a role in parasite elimination and host survival³. However, if the inflammatory environment is modulated immunologically by the expression of anti-inflammatory cytokines such as interleukin-10, this may trigger a balanced response with consequent attenuation of inflammation⁴. Therefore, modulatory mechanisms are essential to prevent exacerbated inflammatory responses against the parasite, with consequent tissue injury. Evaluation of the inflammatory milieu in patients with chronic Chagas disease, by quantifying chemokine production and the expression of chemokine receptors, could further our understanding of the mechanisms causing heart damage and they could help to

explain the absence of clinical alterations among individuals with the indeterminate form of the disease.

Patients with chronic Chagas disease attending the *Ambulatório de Doença de Chagas e Insuficiência Cardíaca do Pronto Socorro Cardiológico de Pernambuco* (PROCAPE), *Universidade de Pernambuco*, Brazil, were selected. Patients were included according to following criteria: 1) positive serology for Chagas disease on enzyme-linked immunosorbent assay and immunofluorescence reaction (RIFI); 2) results of clinical tests and complementary examinations (physical examination, electrocardiography, chest radiography, Doppler echocardiography, and esophagography) were available to allow characterization of cardiac stages A, B (B1 and B2), C and D, according to the I Latin American Guidelines for the diagnosis and treatment of Chagas cardiomyopathy⁵; 3) absence of gastrointestinal symptoms and comorbidities (hypertension, diabetes, thyroid disease, and inflammatory and/or infectious diseases); and 4) absence of previous etiological treatment with benznidazole.

In total, 33 individuals (13 men and 20 women) were included in the study. The group of patients classified as having the indeterminate form of the disease ($n = 12$, aged ~56 years) comprised those with stage A cardiac involvement, i.e. without current or previous symptoms of heart failure and whose clinical tests were normal. Patients were classified as having a mild cardiac form of the disease ($n = 10$, aged ~68 years) if they were in stage B1, i.e. had electrocardiographic abnormalities (conduction disorders or arrhythmias) but had normal global ventricular function. Patients were classified as having a severe

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cardiac form of the disease ($n = 11$, age ~ 58 years) if they were in stage C, i.e. a previous or current clinical diagnosis of heart failure according to the Framingham criteria (simultaneous presence of two major criteria or one major criterion and two minor criteria)⁶ and evidence of ventricular dysfunction (systolic heart failure with a left ventricular ejection fraction $< 50\%$, as determined by Doppler echocardiography).

After collecting 10mL samples of blood in Becton Dickinson Vacutainers® containing sodium heparin, 100 μ L were used directly in the execution of the phenotypic expression protocol. Samples were immunostained by incubation for 30 min with fluorescein isothiocyanate-conjugated anti-CD8 (clone: 3B5, Caltag, Burlingame, CA, USA); peridinin-chlorophyll protein complex-conjugated anti-CD4 (clone: S3.5, Invitrogen™, Carlsbad, CA, USA); Alexa Fluor-conjugated anti-CCR1 (clone: 5354), anti-CCR3 (clone: SF8), and anti-CXCR5 (clone: PF8B2; Becton Dickinson, Franklin Lakes, NJ, USA); and phycoerythrin-conjugated anti-CCR4 (clone: 161), anti-CCR5 (clone: 2D7/CCR5), anti-CXCR2 (clone: 6C6), anti-CXCR3 (clone: 1C6/CXCR3), and anti-CXCR4 (clone: 1D9; Becton Dickinson, Franklin Lakes, NJ, USA). After incubation, erythrocytes were lysed (FACS Lysing Solution; Becton Dickinson Bioscience), followed by washing in phosphate buffered saline containing 0.5% bovine serum albumin and 0.1% sodium azide (Sigma-Aldrich) and centrifugation (300 \times g for 5 min).

Samples were processed using a BD FACSCalibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), available on the Core Technology Platforms (NPT1) of *Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz* (CPqAM/FIOCRUZ). Sample acquisition and analysis were performed using BD Cell Quest™ Pro software. For each analysis, 20,000 events were acquired, gated on *cluster of differentiation 4* (CD4+) and *cluster of differentiation 8+*

(CD8+) T-cell subpopulations expressing the chemokine receptors. The analysis was conducted by obtaining two-dimensional graphics of fluorescent punctual distributions.

The graphics were developed and the statistical tests performed using GraphPad Prism 6.0 software. Bartlett's test for homogeneity of variance was first applied. To compare the median values of the phenotypic expression of chemokine receptors on the surface of T-cells among the groups of patients, the Kruskal-Wallis test was applied, followed by the multiple-comparison Dunn's test when differences between medians were demonstrated. Statistical significance was set at 5% ($\alpha = 0.05$).

The group of patients with mild cardiac disease had higher levels of CD4+ T-cells expressing the CCR3 receptor than did the group with severe cardiac disease ($p = 0.03$; **Figure 1**). In a study investigating associations between Chagas disease morbidity and the expression of cytokines and cytokine receptors, Gomes et al.⁷ described the CCR3 receptor as being a marker of the TH2 response; the expression of CCR3 was highest when the level of myocardial inflammation was low, as higher levels of CD4+ T-cell expression of CCR3 were observed in individuals with the mild cardiac form of Chagas disease than in those with severe cardiac involvement. Thus, in the present study, this receptor may be involved in the recruitment of and possibly in the mechanisms underlying the activation of CD4+ T-cells in individuals with the mild cardiac form of the disease, in a mechanism to compensate the progressive myocardial inflammation. The lower levels of CCR3 observed in patients with the severe cardiac form of Chagas disease might be due to the complete loss of immunoregulation, as this receptor is more closely associated with the TH2 response. In the present study, the expression of other chemokine receptors on the surface of CD4+ T-cells did not vary significantly between the different groups (**Figure 1**).

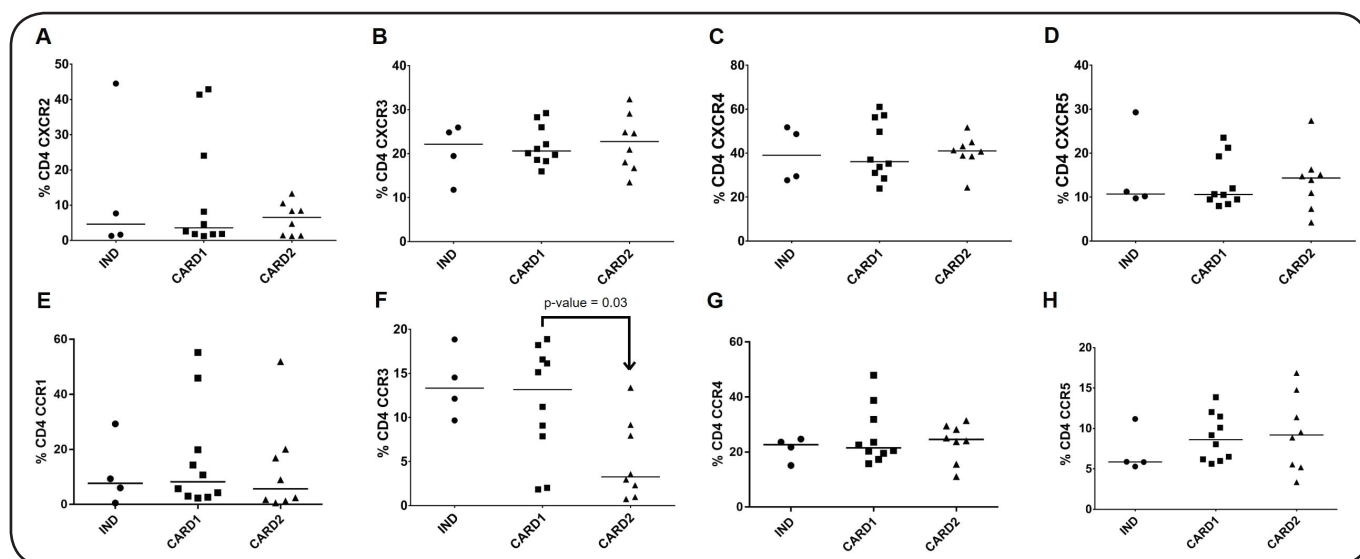


FIGURE 1 - *Ex vivo* phenotype expression of chemokine receptors on the surface of CD4+ T-cells of patients with chronic Chagas disease. **A**: %CD4 CXCR2. **B**: %CD4 CXCR3. **C**: %CD4 CXCR4. **D**: %CD4 CXCR5. **E**: %CD4 CCR1. **F**: %CD4 CCR3. **G**: %CD4 CCR4. **H**: %CD4 CCR5. IND: indeterminate form ($n = 12$); CARD1: mild cardiac form ($n = 10$); CARD2: severe cardiac form ($n = 11$). CD4: *cluster of differentiation 4*; CXCR2, CXCR3, CXCR4, CXCR5, CCR1, CCR3, CCR4, CCR5: chemokine receptors. Median values are indicated by the horizontal lines. The Kruskal-Wallis test was performed, followed by Dunn's test. Significant differences are indicated by arrows and exact p-values.

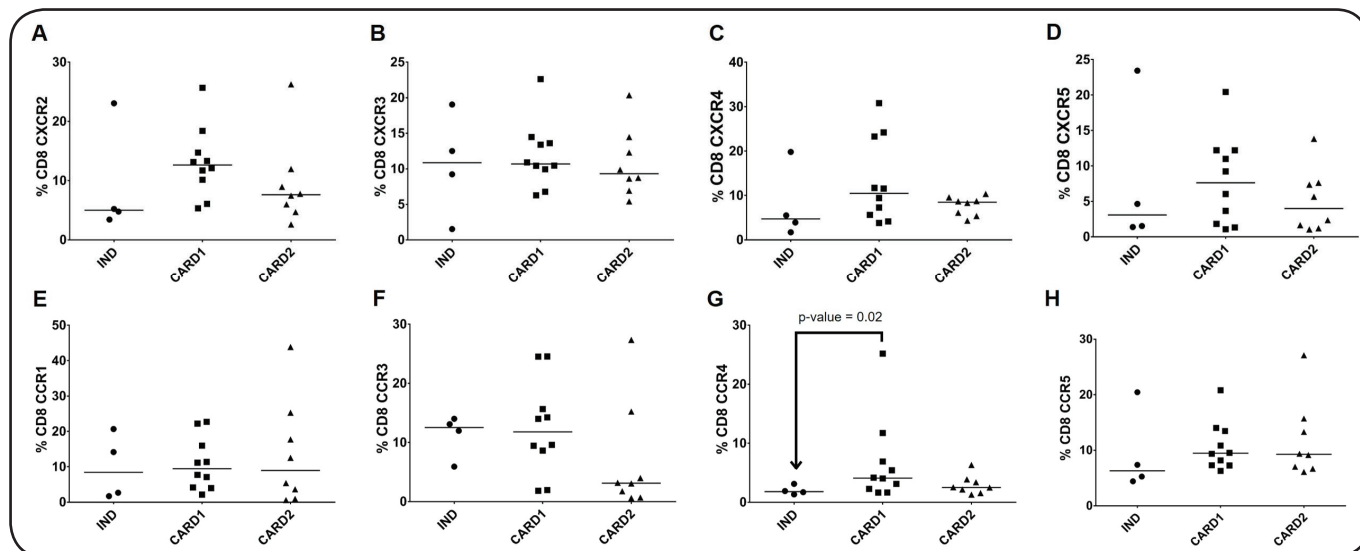


FIGURE 2 - *Ex vivo* phenotype expression of chemokine receptors on the surface of CD8+ T-cells of patients with chronic Chagas disease. **A:** %CD8 CXCR2. **B:** %CD8 CXCR3. **C:** %CD8 CXCR4. **D:** %CD8 CXCR5. **E:** %CD8 CCR1. **F:** %CD8 CCR3. **G:** %CD8 CCR4. **H:** %CD8 CCR5. **IND:** indeterminate form ($n = 12$); **CARD1:** mild cardiac form ($n = 10$); **CARD2:** severe cardiac form ($n = 11$). **CD8:** cluster of differentiation 8; **CXCR2, CXCR3, CXCR4, CXCR5, CCR1, CCR3, CCR4, CCR5:** chemokine receptors. Median values are indicated by the horizontal lines. The Kruskal-Wallis test was performed, followed by Dunn's test. Significant differences are indicated by arrows and exact p-values.

The mild cardiac disease group had higher levels of CD8+ T-cells expressing the CCR4 receptor than did the indeterminate group ($p = 0.02$; **Figure 2**). Induction of cellular immunity and immune responses, mediated by CD8+ T-cells, is essential for the control of *T. cruzi* proliferation, as shown in murine models and in human infections⁸. The concomitant finding of the presence of Chagas disease and high numbers of CD8+ T-cells in experimental models has led some researchers to suggest that CD8+ T-cells cause the observed tissue damage⁹. Although CCR4 is a predominant receptor on TH2 cells, the fact that it has been observed to have greater expression in CD8+ T-cells suggests that CD8+ T-cells might use CCR4 for migrating to sites of inflammation. Hence, it is necessary to evaluate the production of cytokines as well as the production of perforins and granzymes in this particular subpopulation, to determine their possible role in the establishment of the disease their role is likely to be proinflammatory. Additionally **Figure 2**, demonstrates an apparent increase in the expression of CD8+CCR4+ T-cells in patients with the mild cardiac form of Chagas disease, compared with patients with the severe cardiac form. In this group of patients, with the severe cardiac form, especially the older patients, it is not uncommon to observe immune response dysfunction and exhaustion, possibly as a result of chronic antigen exposure and myocardial remodeling¹⁰. Therefore, the level of some immunological markers, notably memory cells, could be in lower in patients with the severe cardiac form of Chagas disease, supporting the hypothesis that the severity of disease is inversely correlated with the quality of immune responses to *T. cruzi*¹¹. Thus, with further analysis, this marker may also provide a useful tool for identifying patients who are likely to progress from the mild to a more severe cardiac form of the disease. The expression of other

chemokine receptors on the surface of CD8 + T-cells did not vary significantly among the different groups (**Figure 2**).

To conclude, our findings suggest that in patients with the cardiac form of chronic Chagas disease, the chemokine receptors CCR3 and CCR4 are involved in the mechanisms of anti-inflammatory and pro-inflammatory immune responses, respectively.

Ethical considerations

The approaches conducted in this study were approved by the Ethics Committee on Research in Human Beings of CPqAM/FIOCRUZ (CAEE: 0032.0.095.000-10) as per the 1975 Declaration of Helsinki, revised in 1983. All subjects provided written informed consent prior to their inclusion in the study.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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