Type 1 Chemokine Receptor Expression in Chagas’ Disease Correlates with Morbidity in Cardiac Patients

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Chagas’ disease, caused by Trypanosoma cruzi, is one of the most important public health problems in Latin America. The overall prevalence of this human infection is estimated at 16 to 18 million cases. Approximately 120 million people, i.e., 25% of the inhabitants of Latin America, are at risk of contracting the infection (37).

It is estimated that 25 to 30% of T. cruzi-infected individuals will progress to chronic irreversible cardiac, esophageal, and colonic pathology, causing considerable mortality and morbidity, which imposes a heavy socioeconomic burden on countries with weakened or deteriorating economies (10, 11).

Regardless of the primary mechanism involved on the development of chronic cardiomyopathy i.e., whether due to parasite persistence or a contributing role of autoimmunity, several studies have suggested a predominance of gamma interferon (IFN-γ) production in tissue and peripheral cells of chagasic cardiac patients compared to infected asymptomatic indeterminate individuals (1, 4, 15, 16, 19, 26, 27).

Chemokines and their receptors are considered important among the different factors that may affect the immune response in the different tissues. These inflammatory molecules play a role in mediating the extravasation and accumulation of specific effector and memory cells in chronic inflammatory processes in several diseases. The chemokine receptors CXCR3 and CCR5 are markers for T cells associated with certain inflammatory reactions, particularly those of type 1 profile (25). In the peripheral blood of individuals with progressive multiple sclerosis, CXCR3+ T cells and CCR5+ T cells were increased in comparison with healthy controls, suggesting that these chemokine receptors are important for the immunopathological process developed by patients with multiple sclerosis (5). As for Chagas’ disease, the functional roles of particular chemokines and chemokine receptors in host resistance to infection and on the pathogenesis of this disease remain largely unknown. Recently, it was reported that patients with mild cardiac disease presented elevated leukocyte expression of CCR5 and lower expression of CXCR4 in comparison with normal individuals, suggesting that chemokines are involved in the development of the early forms of chagasic cardiomyopathy (33). In the present study we investigated the ex vivo expression of chemokine receptors on CD4+ and CD8+ T cells producing type 1 or 2 cytokines in unstimulated peripheral blood mononuclear cells (PBMC) from chagasic patients and in PBMC cultures stimulated in vitro with T. cruzi antigens. CCR5 and CXCR3 expression were considered as markers of type 1, and CCR3 as a marker of a type 2 immune response.

MATERIALS AND METHODS

Patient selection. Patient selection was conducted at the Referral Outpatient Center for Chagas disease of the Federal University of Minas Gerais, Brazil. Positive serology for Chagas disease was determined by two or more tests...
producing cells after 6 days of in vitro culture (15). During the last 4 h of culture, monoclonal antibodies reactive with the following cytokines: IL-10 (JES3-9D7), antibodies to the chemokine receptors CCR2 (48607), CCR3 (61828), CCR5 (Ind), CD4 (RPA-T4) or CD8 (RPA-T8) all conjugated with PerCP and also with phycoerythrin. Phenotypic analyses were performed by three-color flow cytometry using a Becton Dickinson FACScan flow cytometer. A minimum of 50,000 events was acquired for each assay. This number was required due to the low frequency of positive events being analyzed. Controls of nonstimulated cells versus those stimulated with phorbol myristate acetate (10 ng/ml) and ionomycin (1 μg/ml) were used to standardize the antibodies as positive controls. Analysis of fluorescence-activated cell sorting. Lymphocytes were ana- lyzed for their intracellular cytokine and chemokine receptors expression patterns and frequencies and for surface markers using the CellQuest Software (Becton Dickinson). The frequency of positive cells was analyzed in two gates for each staining protocol as gate 1 (R1), lymphocyte gate; gate 2 (R2) and large lymphocyte blast gate as shown in Fig. 1A. Limits for the quadrant markers were always set based on negative populations and isotype controls. This approach allows for the determination of the frequency of populations in subregions of mononuclear cells, taking advantage of the known position of mononuclear cells that have characteristic size and granularity profiles. For the analysis of CD4+ and CD8-positive lymphocytes, the quadrants were always set for CD4 and CD8 high populations so as not to include CD4 low-positive monocytes and macrophages and CD8 low-positive NK cells, respectively (Fig. 1B). For analysis of the third color we performed multiparametric analysis using fluorescence 1 for the surface marker (e.g., CD4) versus granularity. The analyzed population presented low granularity and strong positivity for this cell marker, therefore delineating gate 3 (R3) (Fig. 1C). After this region was selected, we analyzed the fluorescence intensity of these cells in R3 (Fig. 1D). This analytical approach allowed us to identify simultaneously the population of cells expressing at the same time the chemokine receptor and the cytokine in the cytoplasm.

Statistical analysis. Analyses were performed using GraphPad Prism version 3.0 software. The following nonparametric tests were performed: (i) Mann-Whitney test to compare two groups (NI versus IND, NI versus CARD, or IND versus CARD), (ii) Kruskal-Wallis test to compare the three groups (NI versus IND versus CARD), and (iii) Spearman correlation to determine the association between two variables (chemokine receptors and cytokine production). Differences were considered significant when the P value was < 0.05. The error bar is presented as the standard error of the mean.

RESULTS
Chemokine receptor and intracellular cytokine expression on PBMC from chagasic patients. The results obtained after in vitro stimulation of PBMC cultures for all the chemokine receptors investigated in this study were qualitatively very similar to those obtained before in vitro stimulation (ex vivo); the latter findings are not shown. Figure 2 shows the expression of chemokine receptors and intracellular cytokines in the large lymphocyte blast gate in PBMC from IND and CARD chagasic patients and control patients (NI) stimulated in vitro with T. cruzi antigens. CCCR5 and CXCXR3 were expressed at higher percentages in PBMC from CARD patients than from IND and NI individuals (P < 0.05) (Fig. 2A). The CCCR3 expression was elevated in IND patients compared to NI individuals, but no differences for the expression of CCCR2 and CXCR4 were observed (Fig. 2A). No statistically significant differences were observed in CCCR2 and CXCR4 expression on PBMC from chagasic patients (Fig. 2A). The frequencies of IL-10-producing cells were higher in the IND group compared to the NI and CARD groups (P < 0.05) (Fig. 2B). The percentage of IFN-γ+ cells is higher in the CARD group compared to the NI and IND groups (P < 0.05) (Fig. 2B). No statistically significant differences were observed in IL-4 and TNF-α-producing cells in PBMC from chagasic patients compared to the other groups (Fig. 2B).

Spearman correlation was carried out in order to evaluate the relationship between the expression of the type 1 (CCR5 and CXCR3) or type 2 (CCR3) chemokine receptors and the type 1 (IFN-γ and TNF-α) and type 2 (IL-4 and IL-10) cyto- kines on PBMC as described in Materials and Methods. The
values are shown in Table 1. We observed positive correlation for CARD patients between (i) the percentages of cells expressing CCR5 or CXCR3 and the percentages of cells expressing IFN-γ/H9253 and (ii) the percentages of cells expressing CXCR3 and the percentages of cells expressing TNF-α/H9251. Furthermore, negative correlation between the percentages of cells expressing CCR5 and that of cells expressing IL-10 was observed for CARD patients (Table 1). A positive correlation between the percentages of cells expressing CCR3 and the that of cells expressing IL-10 or IL-4 was observed for IND patients. (Table 1).

Chemokine and cytokine receptors coexpression in subsets of PBMC from chagasic patients. The majority of the cells that expressed CCR5, CXCR3, and CCR3 were CD4⁺ or CD8⁺ T-cells (data not shown). The coexpression of chemokine receptors and intracellular cytokines by CD4⁺ or CD8⁺ T-cells after specific antigen stimulation was evaluated by multiparametric fluorescence-activated cell sorting analysis (Fig. 3). The percentage of CD4⁺ T-cells coexpressing both CCR3 and IL-4 was significantly higher in NI patients compared to CARD and IND groups (Fig. 3A). The IND group presented significantly higher percentage of CD8⁺ T-cells coexpressing both CCR3 and IL-10 compared to CARD and NI groups (Fig. 3B). The percentage of CD8⁺ T-cells coexpressing both CCR3 and IL-10 was significantly higher in NI and IND patients compared to the group of CARD patients (Fig. 3B).

The percentage of CD4⁺ or CD8⁺ T-cells coexpressing CCR5 or CXCR3 and IFN-γ was significantly higher in patients in the CARD group than in IND and NI individuals (Fig. 3C and E and 3D and F, respectively). The CARD group presented significantly higher percentages of CD4+ T-cells or CD8⁺ T-cells coexpressing CCR5 or CXCR3 and TNF-α compared to patients NI and IND groups (Fig. 3C and E and 3D and F, respectively).

**DISCUSSION**

An exacerbated type 1 specific response against *T. cruzi* has been associated with cardiomyopathy in human disease, as well as in experimental infections (1, 4, 15, 16, 26, 27, 31). Recent studies with human T-cell clones suggest that CCR5 or...
CXCR3 chemokine receptors may be markers for type 1 response, and CCR3, CCR4, and CCR8 are corresponding markers for type 2 immune response (6, 28, 29, 38). The diversity of chemokine receptor expression during the development and after cell activation can explain the complex migratory pathways taken by cells and has provided new insights into the mechanisms that control cell effector functions.

There is much evidence from experimental models of *T. cruzi* infection that chemokines and their receptors may be relevant mediators of leukocyte influx, which is involved in the pathogenesis of the disease (2, 21, 22, 32–35). The expression of chemokine receptors (CKRs) in this study provides a new set of evidences that type 1 specific immune response to *T. cruzi* antigens is exacerbated in CARD patients and that a specific type 1 immune response developed by IND individuals is probably regulated by a specific type 2 response, as previously reported by our group (4, 15) and others (1, 16, 26, 27).

The profile of cytokine and chemokine receptor expression observed here differs strongly between the CARD and IND group of patients and reflects an imbalance toward a type 1

![Graph A](image1)

![Graph B](image2)

**FIG. 2.** Chemokine receptor (CCR2, CCR5, CXCR3, CXCR4, and CCR3) (A) and intracellular cytokine (IL-10, IL-4, TNF-α, and IFN-γ) (B) expression by PBMC from chagasic patients was evaluated after in vitro stimulation with *T. cruzi* antigens. The results given were calculated as the percentage of blast-gated cells. NI, noninfected individual (*n* = 10); IND, indeterminate patients (*n* = 15); CARD, cardiac patients (*n* = 15). The asterisks indicate statistical differences (*P* < 0.05) between groups.

**TABLE 1.** Spearman correlation analysis between intracellular cytokines and CKR expression on PBMC from Chagasic patients

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Cytokine</th>
<th>CCR3 (r value)*</th>
<th>CCR5 (r value)*</th>
<th>CXCR3 (r value)*</th>
</tr>
</thead>
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<tr>
<td>IND</td>
<td>IL-10</td>
<td>0.911**</td>
<td>0.343</td>
<td>0.285</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>0.714*</td>
<td>0.452</td>
<td>0.396</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>0.159</td>
<td>0.142</td>
<td>0.173</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>−0.285</td>
<td>0.881**</td>
<td>0.489</td>
</tr>
<tr>
<td>CARD</td>
<td>IL-10</td>
<td>0.738*</td>
<td>−0.916**</td>
<td>−0.381</td>
</tr>
<tr>
<td></td>
<td>IL-4</td>
<td>0.687*</td>
<td>0.415</td>
<td>0.287</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>0.476</td>
<td>0.523</td>
<td>0.881**</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>−0.874**</td>
<td>0.998**</td>
<td>0.727*</td>
</tr>
</tbody>
</table>

*The asterisk and double asterisks show the statistically significant correlations at *P* < 0.05 or 0.01 (respectively) determined by Spearman rank correlation. A total of 15 patients with IND or CARD clinical forms of chronic Chagas disease were evaluated. The correlation analyses between chemokine receptors (CCR3, CCR5, and CXCR3) and intracellular cytokine expression (IL-10, IL-4, TNF-α, and IFN-γ) were evaluated in the large lymphocyte blast gate (see Fig. 1B).
response in the former group of patients. Therefore, the expression of CCR5, as well as CXCR3, correlating positively with IFN-γ and/or TNF-α expression in CARD patients (Table 1) and the coexpression of CCR5 and IFN-γ, CCR5 and TNF-α, CXCR3 and IFN-γ, and CXCR3 and TNF-α by CD4⁺ or CD8⁺ T cells in PBMC after antigenic stimulation (Fig. 3) suggest that the potential colocalization of these trafficking cells in the heart tissues of T. cruzi-infected patients are likely under a process of recruitment by the inflamed myocardium. The accumulation of these T cells could lead to the establishment/maintenance/perpetuation of the chronic chagasic myocarditis observed in these individuals. Furthermore, the negative correlation between IL-10 and CCR5, observed only for patients in the CARD group (Table 1) may be a reflection of their unbalanced (exacerbated toward type 1) response as hypothesized above.

In T. cruzi experimental infection, TNF-α, IFN-γ, and the IFN-γ-induced chemokines RANTES (CCL5), MIG (CXCL9), and CRG-2/IP-10 (CXCL10), as well as JE/MCP-1 (CCL2) and MIP-1α (CCL3), were found to be dominant factors expressed in situ during acute infection, persisting during the chronic phase of parasite-elicited myocarditis and contributing to the intense recruitment of activated T cells to the heart (12).

We propose that the inflamed hearts of patients in the CARD group create a favorable environment for the migration of CD4⁺ or CD8⁺ T cells coexpressing CCR5 and IFN-γ, CCR5 and TNF-α, CXCR3 and IFN-γ, and CXCR3 and TNF-α by CD4⁺ or CD8⁺ T cells in PBMC after antigenic stimulation (Fig. 3) suggest that the potential colocalization of these trafficking cells in the heart tissues of T. cruzi-infected patients (33) might be a reflection of the recruitment of circulating CD8⁺ T cells coexpressing CCR5 and IFN-γ, CCR5 and TNF-α, CXCR3 and IFN-γ, and CXCR3 and TNF-α. In this context, the predominance of CD8⁺ IFN-γ⁺ T cells observed in the myocardium of T. cruzi-infected patients (33) might be a reflection of the recruitment of circulating CD8⁺ T cells coexpressing CCR5 and IFN-γ, CCR5 and TNF-α, CXCR3 and IFN-γ, and CXCR3 and TNF-α.

*NI, noninfected individuals (n = 10); IND, indeterminate patients (n = 15); CARD, cardiac patients (n = 15). Asterisks indicate statistical differences (P < 0.05) between groups.

**FIG. 3.** The percentage of CD4⁺ or CD8⁺ T cells coexpressing chemokine receptors and cytokines was determined by multiparametric fluorescence-activated cell sorting analysis as described in Materials and Methods. The results given were calculated as the percentage of R3 gated cells. (A) Percentage of CD4⁺ cells coexpressing CCR3–IL-4 or CCR3–IL-10; (B) percentage of CD8⁺ cells coexpressing CCR3–IL-4 or CCR3–IL-10; (C) percentage of CD4⁺ cells coexpressing CCR5–IFN-γ or CCR5–TNF-α; (D) percentage of CD8⁺ cells coexpressing CCR5–IFN-γ or CCR5–TNF-α; (E) percentage of CD4⁺ cells coexpressing CXCR3–IFN-γ or CXCR3–TNF-α; (F) percentage of CD8⁺ cells coexpressing CXCR3–IFN-γ or CXCR3–TNF-α. NI, noninfected individuals (n = 10); IND, indeterminate patients (n = 15); CARD, cardiac patients (n = 15).
In fact, the pattern of CKR expression appears to depend on the state of activation of T-cell and may be up- or downregulated after in vitro treatment of cells with cytokines (36). In this context, it was demonstrated that proinflammatory (TNF-α and IL-12) and type 1 cytokines (IFN-γ and IL-2) significantly upregulate CCR5 mRNA expression in cells from HIV patients, whereas IL-10 downmodulates CKR expression, suggesting that changes in the cytokine milieu influences CKR expression and may explain the tropism specificity of some strains facilitating human immunodeficiency virus transmission and disease progression (24). In apical periodontitis, CXCR3+ cells and IFN-γ-producing cells were found to be present in human periapical granulomas, whereas CCR3+ cells and IL-4-producing cells could not be detected, suggesting that type 1 cells may play an important role in pathological processes (18).

The patients in the IND group presented significantly lower expression of CCR5 when compared to patients in the CARD group (Fig. 2 and 3 and Table 1). These data are consistent with recent findings in Peruvian T. cruzi-seropositive patients, demonstrating the higher frequency of the CCR5 promoter point mutation (CCR5 59029 A/G) in asymptomatic individuals in comparison with CARD patients (7, 13) and provide a genetic explanation for the differential expression of this chemokine receptor between the two groups of T. cruzi-infected individuals, with or without cardiac disease. The authors of these suggest that the presence of this point mutation duet 59029-G may result in reduced expression of CCR5 on the cells membrane of individuals bearing this allele.

The enhanced secretion of chemokines by T. cruzi-infected macrophage may drive the selective migration of CCR5+ cells such as type 1 cells toward the affected tissues, facilitating the development of severe cardiac lesions (2, 3, 7, 9, 20). These data suggest that the level of expression of CCR5 on the cell membrane could determine not only the number and cell type in the infected organs but also the degree of the inflammatory response in T. cruzi infections. It is possible that a smaller number of type 1 cells into the inflamed cardiac tissue, due to a decrease in CCR5 membrane expression, protects against the development of severe chagasic myocardopathy. Similar data have been previously reported in genetic associations of CCR5 variants and development of AIDS in human immunodeficiency virus type 1-infected individuals (8, 21).

Talvani et al. (33) have also found an elevated expression of CCR5 in PBMC from cardiac chagasic Brazilian patients. The explanation of Talvani et al. for this fact, in the context of chagasic pathology, diverges from that presented here and discussed by Calzada and coworkers (7, 13). The information on the cytokine production profile by CCR5+ T cells provided in this study (not approached in the Talvani study) support the interpretation of the Calzada group that the mutated CCR5 genotype protects T. cruzi-seropositive individuals from developing chagasic cardiomyopathy.

Besides the lower levels of CCR5 expression observed in IND compared to patients in the CARD group, the asymptomatic T. cruzi-infected individuals presented the highest percentages of CD4+ or CD8+ cells coexpressing CCR3–IL-4 or CCR3–IL-10 compared to CARD patients, which is consistent with previous studies of a probable key role for IL-10 and probably IL-4 in regulating the necessary (type 1) inflammatory response that participates in the control of parasite replication in heart tissues (4, 15, 16, 26, 27, 31).

The mechanisms regulating the differential expression of chemokine receptors in activated cells producing cytokines in T. cruzi-infected individuals remains unclear. However, recent observations suggest that some organ-specific autoimmune disease might be provoked by systemic immune deviation (28), and it is well known that vigorous Th1-type immune response leads to an inhibition of Th2 cells. The progressive destructive process in CARD patients could therefore result from a pathogenic Th1 response that is not downregulated by IL-10. This immunoregulation failure could, in turn, depend on many factors such as: host genetics, age-dependent changes of the immune response, coinfections by unrelated microorganisms, and/or by T. cruzi reinfection.

In conclusion, we have demonstrated in this study one more set of significant differences between patients in CARD and IND groups concerning the predominance of CD4+ and CD8+ T cells coexpressing chemokine receptors and cytokines such as elevated expression of CCR5 and/or CXCR3 by CD4+ and CD8+ T cells producing IFN-γ and/or TNF-α in CARD patients in comparison with IND patients, and in contrast, an elevated percentage of CD4+ or CD8+ T cells coexpressing CCR3 and IL-4 or CCR3 and IL-10 in IND patients compared to CARD individuals. This set of results support the concept that an exacerbated type 1 immune response is involved on the genesis and/or maintenance of heart pathology in Chagas disease. Experimental data from the murine model reinforce aspects of our hypothesis (12, 23, 31); however, no model of disease pathogenesis incorporates our findings. Since patients infected with T. cruzi must be continuously and closely evaluated to investigate their clinical development, parallel evaluation of the immune response give us a unique opportunity to identify its putative role in disease development and to shed light on the possible mechanisms of disease control displayed by IND patients who do not evolve clinically to CARD illness. Epidemiological data have shown that a percentage of T. cruzi-infected asymptomatic individuals remain indefinitely in this latent stage, while a small percentage develop to the severe clinical form every year. Investigating the specific immune response to the parasite in this group of patients will be relevant for the development of an adequate model to test the hypothesis.

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