Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) Antagonist (Met-RANTES) Controls the Early Phase of Trypanosoma cruzi–Elicited Myocarditis

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Background—Comprehension of the pathogenesis of Trypanosoma cruzi–elicited myocarditis is crucial to delineate strategies aimed at ameliorating the inflammation associated with heart dysfunction. The augmented expression of CC chemokines, especially CCL5/RANTES and CCL3/MIP-1α, in the hearts of infected mice suggests a role for CC chemokines and their receptors in the pathogenesis of T cruzi–elicited myocarditis.

Methods and Results—We report that during the early phase of infection in C3H/HeJ mice infected with 100 blood trypomastigotes of T cruzi, most of the inflammatory cells invading the heart tissue were CD8+ cells and expressed CCR5, a CCL5/RANTES, and CCL3/MIP1-α receptor. Furthermore, peripheral blood CD8+ T lymphocytes displayed increased expression of CCR5. These findings led us to use Met-RANTES, a selective CCR1 and CCR5 antagonist, to modulate the acute T cruzi–elicited myocarditis. Met-RANTES treatment did not interfere with parasitism but significantly decreased the numbers of CD4+ and CD8+ T cells, CCR5+, and interleukin-4+ cells invading the heart, paralleling the diminished deposition of fibronectin. Moreover, Met-RANTES treatment resulted in increased survival of infected animals, compared with saline treatment.

Conclusions—These results indicate that the massive influx of CCR5+ cells into cardiac tissue is not crucial for cell-mediated anti–T cruzi immunity but appears to be critical for pathogenesis of T cruzi–elicited myocarditis. Thus, CC chemokine receptors might become an attractive therapeutic target for further evaluation during T cruzi infection.

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Key Words: heart disease • myocarditis • inflammation • infection • receptors

Chagas’ disease, caused by the protozoan Trypanosoma cruzi, is the major cause of pathologic changes of the heart in Latin America. Establishment of an inflammatory process is crucial for parasite control. However, in ∼30% to 40% of patients, inflammation becomes progressive, resulting in chronic disease that is mainly characterized by myocarditis associated with prominent fibrosis and organ dysfunction.1

The recruitment of leukocytes depends on the nature and state of activation of these cells, being mainly coordinated by chemokines.2 These small proteins can be grouped into 4 subfamilies (CXC, or α; CC, or β; C, or γ; and CX.C, or δ) that act on G protein–coupled serpentine receptors on target cells.3 Besides playing a crucial role in leukocyte recruitment and migration, chemokines and their receptors have been shown to affect various biologic events, including T-cell proliferation,4 Th1/Th2 differentiation,5 and resistance to infection.2,6,7 High levels of CC chemokines, especially CCL5/RANTES (regulated on activation, normal T cell expressed and secreted) and CCL3/macrophage inflammatory protein-1α (MIP-1α), are detected in experimental T cruzi–elicited chronic myocarditis.8,9 Interestingly, there is enhanced expression of CCR5 on leukocytes from patients with mild Chagas’ disease.10 Moreover, a CCR5-promoter allele that results in lower expression of CCR5 is present at a higher frequency in asymptomatic compared with cardiomyopathic chagasic patients.11 These results led us to consider that CC chemokines, mainly CCL5/RANTES and CCL3/MIP-1α, and the CC chemokine receptor CCR5 could be involved in the immunopathogenesis of T cruzi–triggered myocarditis.9,12

Herein, we evaluated the expression of CCR5 on heart-infiltrating and peripheral blood leukocytes of T cruzi–infected mice and used the N-terminal–methionylated RANTES (Met-RANTES), a selective CCR1 and CCR5

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to modulate the early phase of \textit{T cruzi}–elicited myocarditis.

**Methods**

**Animals**

Female C3H/HeJ (H-2\(^b\)) mice 5 to 7 weeks old obtained from the animal facilities of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) were maintained under standard conditions and treated according to institutional guidelines regarding ethics of animal use of the Oswaldo Cruz Foundation.

**Experimental Infection**

Mice were infected intraperitoneally with 100 blood trypomastigotes of the Colombian strain of \textit{T cruzi} isolated from a cardiac chagasic patient\(^1\) and maintained by serial passages from mouse to mouse. Parasitemia was estimated from 5 \(\mu\)L of blood obtained from the tail vein\(^2\) and was used as a parameter to establish acute and chronic phases.

**Treatment of \textit{T cruzi}–Infected Mice With Met-RANTES**

Groups of 8 mice were injected daily with 0.1 mL SC of in vivo injection-grade saline (BioManguinhos) or saline containing 10 \(\mu\)g Met-RANTES\(^3\) from day 0 to day 14 or 14 to 28 days after infection. Met-RANTES was a kind gift of Dr Amanda Proudfoot (Serono Pharmaceuticals, Geneva, Switzerland). The parasitemia and survival rate were estimated daily. The animals were killed by exsanguination under ophthalmic anesthesia (tetracaine chloride) followed by spinal cord disruption.

**Antibodies**

Polyclonal antibody recognizing \textit{T cruzi} antigens was a gift from Dr Rosa Pinho (IOC-Fiocruz, Brazil). Polyclonal antibody anti-mouse fibronectin was obtained from Gibco. Purified biotin- and fluorescein isothiocyanate–conjugated anti-mouse CD8a (53-6.7), purified phycocerythrin- and biotin-conjugated anti-mouse CD4 (GK1.5), purified anti-mouse Mac-1 (CD11b, M1/70.15.11.5.HL), phycocerythrin-conjugated anti-mouse CCR5 (C34-3448), purified anti-mouse interferon-\(\gamma\) (IFN-\(\gamma\); R4-6A2), purified anti-mouse tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\); MP6-XT22), purified anti-mouse interleukin-4 (IL-4; 11B11), and CyChrome-streptavidin were purchased from PharMingen. Biotinylated anti-rat immunoglobulin was purchased from Dako. Biotinylated anti-rabbit immunoglobulin and peroxidase-streptavidin complex were purchased from Amersham. Appropriate controls were prepared by replacing primary antibodies with purified rat immunoglobulin or normal rabbit serum.

**Immunohistochemistry**

Groups of 5 to 8 infected and 3 to 6 age-matched control mice were killed under anesthesia at various time points after infection. The heart was removed, embedded in tissue-freezing medium (Tissue-Tek, Miles Laboratories), and stored in LN\(_2\). Serial 5- to 7-\(\mu\)m-thick sections were fixed in cold acetone and subjected to indirect immunoperoxidase staining or immunofluorescence, as previously described.\(^4\) Sections of spleen were used as positive controls for lymphocyte staining. The cells that stained for CD4, CD8, IFN-\(\gamma\), TNF-\(\alpha\), and IL-4 were counted by light microscopy. For detection of CCR5\(^*\) cells in the heart tissue, immunofluorescence was used,\(^5\) and analysis was performed by confocal microscopy (LSM 410, Zeiss). Three sections were counted for each animal.

**Flow-Cytometry Analysis**

Suspensions of peripheral blood mononuclear cells (PBMCs) were prepared by pooling 1.5 mL heparinized individual samples (3 animals per group) and performing Ficoll-Hypaque (\(d=1.077g/mL\)) separation. To isolate mononuclear cells invading the cardiac tissue, 10 to 15 hearts were minced with scissors into 1- to 2-mm fragments and subjected to trypsin and collagenase A digestion, as previously described.\(^6\) The cells recovered were washed, resuspended in phosphate-buffered saline containing 2% fetal calf serum, and labeled as previously described.\(^6\) Controls for specific labeling were prepared with isotype-matched controls. One-color labeled samples were prepared to set compensation values. Samples were analyzed by flow cytometry using a FACScalibur (FACScalibur, Becton-Dickinson) by gating the mononuclear cells and using a narrow, forward-angle, light-scatter parameter to exclude dead cells from analysis. At least 12 000 cells were acquired inside this gate. Fluorescence gates were cut in accordance with labeling controls, respecting curve inflections. Cytometric analyses were performed with the program WinMDI, version 2.5.

**RT-PCR Assay for Detection of Chemokine mRNA**

RNA was isolated from the hearts of mice by acid guanidinium–thiocyanate-phenol-chloroform extraction (RNA STAT-60). Reverse transcriptase–polymerase chain reaction (RT-PCR) conditions; primer sequences used for detection of CCL2/monocyte chemotactic protein-1 (MCP-1), CCL3/MIP-1\(\alpha\), CCL4/MIP-1\(\beta\), and CCL5/RANTES; and PCR product sizes have been published elsewhere.\(^8\) The PCR products and molecular-weight markers were electrophoresed in 6% polyacrylamide gels and stained with silver nitrate. Densitometry of gels was carried out on a CS-9301PC densitometer (Shimadzu). The PCRs were standardized with hypoxanthine phosphoribosyltransferase (HPRT).

**Statistical Analysis**

Data are expressed as arithmetic or geometric mean±SE. Student’s \(t\) test was used to analyze the statistical significance of the observed differences. The Kaplan–Meier method was used to compare survival times of the study groups. All statistical tests were performed with SPSS 8.0 software. Differences were considered statistically significant when \(P<0.01\).

**Results**

**CD8\(^+\) T Cells Predominate in Cardiac Tissue of Colombian Strain–Infected C3H/HeJ Mice**

Histopathologic examinations showed that inflammation was not detected in the cardiac tissue of \textit{T cruzi}–infected C3H/HeJ mice at 14 days after infection, although a few mononuclear cells, mainly CD4\(^+\) T cells as characterized by immunohistochemistry and flow cytometry, were detected at 21 days after infection (data not shown). The CD8\(^+\) T lymphocytes supplanted CD4\(^+\) cells by day 28 after infection (Figure 1A), and the predominance of CD8 cells by a factor of 2 to 3, characteristic of the chronic phase,\(^9\) was achieved by day 42 after infection (CD4, 20.6±1.2% and CD8, 14.7±0.6% in control vs CD4, 14.3±2.2% and CD8, 56.7±4.0% in infected mice).

**CC Chemokines and CCR5\(^+\) Mononuclear Cells Are Detected in Cardiac Tissue of \textit{T cruzi}–Infected Mice**

Increased expression of CCL5/RANTES (7.01±0.70-fold increase), CCL3/MIP-1\(\alpha\) (5.7±0.40-fold increase), CCL2/MCP-1 (3.49±0.37-fold increase), and CCL4/MIP-1\(\beta\) (1.36±0.3-fold increase) mRNAs was detected in the hearts of C3H/HeJ mice at 28 days after infection compared with noninfected mice (Figure 1B). Furthermore, in the heart tissue of acutely infected mice, most of the inflammatory cells were CCR5\(^+\), whereas the hearts of control mice were devoid of CCR5\(^+\) cells (Figure 1C).


CCR5 Expression Is Upregulated in PBMCs of T cruzi–Infected Mice

To investigate the differential accumulation of CD8+ and CCR5+ cells in the heart tissue during T cruzi infection, we characterized the expression of CCR5 on CD4+ and CD8+ PBMCs of infected mice. A significant decrease in the proportion of circulating CD4+ T cells and a clear increase in the percentage of CD8+ T cells were observed at day 28 after infection (CD4, 32.4±1.6% and CD8, 20.1±1.0% in control vs CD4, 18.9±1.2% and CD8, 44.2±1.7% in infected mice). Furthermore, the proportion of CCR5+PBMCs was increased in infected mice (Figure 2A). Moreover, the predominance of CD8+ T cells in the myocardium at day 28 after infection (Figure 1A) paralleled a differential upregulation in the proportion of CCR5+CD8+ PBMCs in infected mice (Figure 2B).

Met-RANTES Treatment of T cruzi–Infected Mice Results in a Beneficial Effect

The accumulation of CCR5+ cells in the hearts and the upregulation of CCR5 expression on PBMCs, particularly CD8+ T lymphocytes, found in infected mice led us to use Met-RANTES to modulate the acute T cruzi–elicited myocarditis. Independently of the therapeutic scheme used, no significant difference was observed when the parasitemia levels at day 28 after infection of Met-RANTES–treated mice (8.1±1.0×10^5 parasites/mL for treatment from days 0 to 14 after infection and 6.9±2.3×10^5 parasites/mL for treatment from days 14 to 28 after infection) and vehicle-injected animals (7.3±2.2×10^5 parasites/mL) were compared. Furthermore, Met-RANTES administered from 14 to 28 days after infection did not interfere significantly with the numbers and size of parasite nests in the hearts of infected mice (Figure 3A). Indeed, there was a nonsignificant decrease in the numbers of parasite nests in Met-RANTES–treated mice compared with untreated mice (1.25±0.73 vs 2.0±0.91 nests per microscopic field, respectively). Parasitism and inflammation were unaltered in the hearts of animals that received Met-RANTES from days 0 to 14 after infection (data not shown). Importantly, Met-RANTES administration from days 14 to 28 after infection resulted in a significant decrease in cardiac inflammation, mainly due to a reduction in the numbers of CD4+ (>50%) and CD8+ (60%) lymphocytes (Figure 3A and 3B). A nonsignificant decrease (~21%) in the numbers of macrophages was also observed (data not shown). Furthermore, in animals treated with Met-RANTES from days 14 to 28 after infection, a significant reduction of CCR5+ cell numbers (55%) was observed in comparison with saline-injected mice (Figure 4A), consistent with the 50% to 60% inhibition of CD4+ and CD8+ cell recruitment (Figure 3B).

The Th1/Th2 cytokine status inside the cardiac tissue may drive the fate of parasitism control and myocarditis in T cruzi–infected
mice. Interestingly, Met-RANTES treatment did not induce significant alterations in the numbers of cells producing TNF-α (Figure 4B) and IFN-γ (5 ± 2 and 3 ± 0.8 cells per 50 microscopic fields in saline-treated vs 5 ± 2 and 4 ± 1.2 cells per 50 microscopic fields in Met-RANTES-treated mice in 2 independent experiments) in the hearts of infected mice. In contrast, the numbers of IL-4+ cells were reduced by 70% in Met-RANTES–treated mice, compared with vehicle-injected animals (Figure 4B).

An important feature of chagasic myocarditis is the increased deposition of extracellular matrix components, including fibronectin, in damaged areas. In T. cruzi–infected mice treated with Met-RANTES from days 14 to 28 after infection, decreased fibronectin deposition around cardiomyocytes and inflammatory cells was observed, compared with vehicle-injected animals (Figure 5A).

Importantly, both therapeutic schemes with Met-RANTES increased the survival rates of infected animals. Daily administration of Met-RANTES from days 0 to 14 after infection resulted in survival of 88% of infected animals, whereas treatment from days 14 to 28 after infection led to a survival rate of 100% when compared with a 57% survival by day 28 after infection in vehicle-injected animals (Figure 5B).

**Discussion**

The major challenge in designing an efficacious treatment for T. cruzi–elicited myocarditis is to develop a strategy able to reduce the intense inflammation associated with tissue damage without hampering parasitism control. Herein we provide evidence for a putative mechanism involved in the pathophysiology of T. cruzi–elicited myocarditis and raise the possibility of developing a new therapeutic intervention that targets the cell migration process.

The selective recruitment and migration of leukocytes to injured sites are proposed to be controlled by locally produced chemokines. In experimental T. cruzi infection, we found that establishment of the myocarditis, mainly composed of CD8+ cells and mimicking the pathology described in chagasic patients, is a rather early process paralleled by enhanced expression of the CC chemokines CCL2/MCP-1, CCL5/RANTES, CCL3/MIP-1α, and to lesser extent CCL4/...
MIP-1β. Of interest, the latter chemokines have been shown to induce the recruitment of CD8+ and CD4+ T cells.4,20 Thus, it is possible that the CC chemokines present in the inflamed hearts of T cruzi–infected mice might create a favorable environment for preferential migration of macrophages, activated CD4+, and especially CD8+ T cells.

The T cruzi–elicited myocarditis was mainly characterized by cells bearing the CC chemokine receptor CCR5, a receptor for CCL3/MIP-1α and CCL5/RANTES.3 Importantly, the proportion of CCR5+ PBMCs, mainly CD8+ T cells, was also increased in infected animals. Thus, not only are there ligands for CCR5 expressed in the hearts of infected mice but also there is an increase in the number of CCR5+CD8+ PBMCs capable of migrating to the heart. The compound effect of chemokine ligand and receptors is the increased presence of CCR5+CD8+ T cells in the hearts of infected mice. Interestingly, CD8+ T cells are important sources of CCL3/MIP-1α, and an increase in CCR5 expression on CD8+ T cells, differentiation of primed CD8+ T cells into effector cells, and their release into the circulation are dependent on CCL3/MIP-1α.21 Altogether, these findings raise the possibility that during early acute T cruzi infection, the CD8+ cells infiltrating cardiac tissue may produce CCL3/MIP-1α (or other CCR5-active chemokines) that could contribute to regulating CCR5 expression and preferential recruitment of the CD8+CCR5+ T cells, creating a positive-feedback loop. Thus, the interactions of CCL3/MIP-1α and/or CCL5/RANTES with CCR5 may take part in the establishment of T cruzi–elicited myocarditis. Supporting this, CCR5 expression is increased on PBMCs obtained from cardiopathic chagasic patients compared with uninfected individuals.10 Furthermore, asymptomatic patients have a higher frequency of the CCR5Δ32–59029G allele, associated with CCR5 low expression, compared with cardiopathic patients.11 Altogether, these data favor a relevant pathophysiologic role for CCR5 and its ligands in T cruzi–triggered myocarditis. It was thus important to investigate the effects of an agent capable of inhibiting or antagonizing CCR5 in T cruzi–elicited myocarditis.

Previous studies have shown the efficacy of Met-RANTES in the control of inflammation in diverse pathologies.3 Herein we show that Met-RANTES treatment of T cruzi–infected mice did not interfere significantly with parasitemia or heart parasitism, although recent studies have supported a role for CC chemokines as inhibitors of parasite replication in vitro.6,22 Thus, it appears that action on CCR1/CCR5 may not play a crucial endogenous role in the control of T cruzi parasitism. Also, administration of CCR5 antagonists appears to be safe in the setting of acute T cruzi infection. It is unclear whether endogenous activation of other chemokine receptors may be relevant for T cruzi control. In this sense, CCL2/MCP-1 (and hence, its CCR2 receptor) may be the most active chemokine to elicit parasite uptake and death.23 However, further studies are required to unravel the role of CCL2/CCR2 in T cruzi infection.

Importantly, a significant decrease in the numbers of CCR5+, CD4+, CD8+ cells, and to a lesser extent macro-
phages was observed in the hearts of Met-RANTES–treated, infected mice. Although not observed, one could expect an increase in parasite burden coinciding with a reduction in the numbers of macrophages, CD4", and CD8 T cells after Met-RANTES treatment, considering the crucial role of these cells in parasitism control and protective immunity. However, in T cruzi infection, CD8 and CD4 lymphocytes are also involved in cardiac tissue destruction and autoimmunity, respectively. Thus, Met-RANTES treatment results in inflammation control in the absence of parasite burden, showing that the massive influx of CCR5 inflammatory cells into cardiac tissue is not crucial for anti–T cruzi immunity.

The proinflammatory cytokines TNF-α and IFN-γ, critical for T cruzi replication control, are present in the inflamed hearts of chronic chagasic patients and T cruzi–infected mice. Furthermore, the ability to mount a vigorous IFN-γ response is associated with cardiomyopathy in chagasic patients. On the other hand, the presence of IL-4 cells is correlated with the presence of T cruzi antigens in severe chagasic cardiomyopathy, suggesting that IL-4 is involved in parasite dissemination. Moreover, IL-4–deficient mice had reduced heart parasitism and mortality during acute infection. In Met-RANTES–treated, infected mice, the numbers of IFN-γ- and TNF-α+ cells were unaltered. This is in agreement with the relevance of these cytokines for parasite control and the lack of effect of Met-RANTES on tissue parasitism. In contrast, the intense reduction in the numbers of IL-4+ cells paralleled the reduction in the numbers of CCR5+ cells. Thus, in the animals treated with Met-RANTES, there was a reduction of CD4+ and CD8+ T cells and IL-4+ cells but not of IFN-γ+ cells, suggesting that Met-RANTES treatment favored the balance toward a Th1 response in the heart. The decreased numbers of Th2+ cells after Met-RANTES treatment could be the result of the differential expression of CCR5high by Th1 and CCR5low by Th2 cells. It is possible that the Th2 cell population (CCR5low) could be blocked by Met-RANTES first. In this selective property could reside the beneficial effect of Met-RANTES compared with the broader effect of the anti-inflammatory drugs previously used in T cruzi infection that resulted in increased parasitemia, parasitism, and mortality.

Fibrosis is associated with T cruzi–triggered heart dysfunction. Our results show that the enhanced fibronectin expression is associated with inflammation and the presence of IL-4+ cells in the hearts of infected mice. Thus, considering the profibrotic properties of this cytokine, it is tempting to propose that IL-4, besides favoring parasite dissemination, is also inducing cardiac fibrosis in T cruzi infection, although other profibrotic mediators may also participate. Nevertheless, fibronectin also favors parasite dissemination. Furthermore, fibronectin-bound cytokines, particularly TNF-α, act as chemotactic and proadhesive factors on activated T cells. Therefore, the decreased fibronectin deposition may contribute to the beneficial effect of Met-RANTES during T cruzi infection, hindering parasitism and inflammation establishment. Most important, Met-RANTES treatment was accompanied by increased survival of infected animals compared with saline treatment. It is not known whether this effect of Met-RANTES on survival rates relies on the establishment of a Th1/Th2 balanced inflammation in the absence of fibrosis, and further studies must be carried out to clarify this issue.

Altogether, our results show that treatment with Met-RANTES clearly diminished the infiltration of T cells and fibronectin deposition, critical aspects of chagasic cardiomyopathy, in the hearts of T cruzi–infected mice. Although additional studies are required to clarify the contribution of cytokines and chemokines to parasitism control, myocarditis perpetuation, and organ dysfunction, the presented results suggest that CC chemokine receptors might become an attractive therapeutic strategy for further evaluation during T cruzi infection.

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