

CCR5 Plays a Critical Role in the Development of Myocarditis and Host Protection in Mice Infected with *Trypanosoma cruzi*

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The pathogenesis of myocarditis during *Trypanosoma cruzi* infection is poorly understood. We investigated the role played by chemokine receptor 5 (CCR5) in the influx of T cells to the cardiac tissue of *T. cruzi*-infected mice. mRNA and protein for the CCR5 ligands CCL3, CCL4, and CCL5 were detected in the hearts of infected mice in association with CD4⁺ and CD8⁺ T cells. There was a high level of CCR5 expression on CD8⁺ T cells in the hearts of infected mice. Moreover, CCR5 expression on CD8⁺ T cells was positively modulated by *T. cruzi* infection. CCR5-deficient mice infected with *T. cruzi* experienced a dramatically inhibited migration of T cells to the heart and were also more susceptible to infection. These results suggest that CCR5 and its ligands play a central role in the control of T cell influx in *T. cruzi*-infected mice. Knowledge of the mechanisms that trigger and control the migration of cells to the heart in patients with Chagas disease may help in the design of drugs that prevent myocarditis and protect against the development of severe disease.

Trypanosoma cruzi is an obligatory intracellular protozoan parasite that causes Chagas disease, an important public-health problem in Latin America. In studies of infection, there is significant blood parasitemia, tissue parasitism, and tissue inflammation and a great degree of lethality, all of which are dependent on the strain of the parasite, the dose of the inoculum, and the species being studied. Control of the infection is eventually achieved. Several studies have reported an essential role for CD4⁺ [1–3] and CD8⁺ [3, 4] T cells and for interferon (IFN)- γ and tumor necrosis factor (TNF)- α [5–8] in the control of acute infection. However, it is still not known how these types of cells migrate to sites of tissue infection and interact with inflam-

matory mediators at these sites to mediate the control of parasite replication.

We recently reported that *T. cruzi*-infected macrophages and cardiomyocytes expressed mRNA for a range of chemokines, including CCL3 (macrophage inflammatory protein [MIP]-1 α), CCL4 (MIP-1 β), and CCL5 (RANTES) [9, 10]. In these studies, not only were these chemokines expressed, but *T. cruzi*-infected macrophages and cardiomyocytes also expressed NO, to mediate NO-dependent killing in vitro [9–13]. Moreover, we showed that mRNA for these chemokines was also expressed during acute infection in mice and that cytokines (i.e., TNF- α and IFN- γ) known to participate in the control of infection modulated the expression of chemokines [14–16]. Given that chemokines are well known for their ability to induce the migration of leukocytes and the activation of immune cells, these previous studies suggested that, in addition to facilitating the production of NO, the expression of chemokines could drive the migration of inflammatory and immune cells to sites of parasite infection.

The biological activities of chemokines are mediated by their interactions with cell-surface receptors that belong to the structurally related 7-transmembrane-

Received 25 June 2004; accepted 15 August 2004; electronically published 13 January 2005.

Financial support: Fundação de Amparo à Pesquisa do Estado de São Paulo; Conselho Nacional de Desenvolvimento Científico e Tecnológico; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

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The Journal of Infectious Diseases 2005; 191:627–36

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domain superfamily of proteins [17]. CCR5 mediates the activity of the chemokines CCL3, CCL4, and CCL5, whose mRNA is found *in vitro* after infection of macrophages and cardiomyocytes with *T. cruzi* [9, 10] and *in vivo* after infection of macrophages and cardiomyocytes with the Colombiana strain [15]. As such, these chemokines may have a relevant role in driving the selective migration of CCR5⁺ immune cells toward affected tissues. Here, we confirm the expression of CCL3, CCL4, and CCL5 in mice infected with the Y strain of *T. cruzi*. Moreover, using CCR5-deficient (CCR5^{-/-}) mice, we evaluate the role played by the interaction between CCR5 and its ligands in protection against *T. cruzi* infection and in the development and maintenance of myocarditis.

MATERIALS AND METHODS

Mice. A breeding pair of mice with disruption of the CCR5 gene [18] was obtained from Jackson Laboratories. Breeding stock backcrossed on C57BL/6 was obtained, and the genotype of CCR5 in mice was determined by polymerase chain reaction (PCR) of DNA [19]. Female C57BL/6 wild-type (*wt*) and CCR5^{-/-} mice, 6–8 weeks old, were bred and maintained in microisolator cages. All procedures were in accordance with international guidelines for the use of animals and received prior approval by the animal ethics committee of the School of Medicine of Ribeirão Preto.

Parasites and experimental infection. The Y strain of *T. cruzi* was used in all experiments. For *in vitro* experiments, trypomastigote forms were grown and purified from a fibroblast cell line (LLC-MK₂). In all experiments, the mice were inoculated intraperitoneally with 10³ blood-derived trypomastigote forms, which had been obtained from previously infected mice.

Total RNA extraction and cDNA preparation by reverse-transcription (RT) PCR. Total RNA was isolated from the cardiac tissue of mice by use of Trizol LS (Life Technologies) reagent, in accordance with the manufacturer's instructions. Briefly, the samples were homogenized, and 0.2 mL of chloroform (Sigma) was added to each 1 mL of Trizol LS reagent. The samples were then centrifuged at 12,000 *g* for 15 min at 4°C, and the aqueous phase was transferred to a clean tube. The same volume of isopropyl alcohol was added, and the samples were mixed in a vortex and incubated for 15 min at –20°C, to precipitate the RNA from the aqueous phase. After further centrifugation, the RNA pellets were washed in 75% ethanol, and the samples were suspended in water at a concentration of 0.5 µg of RNA/µL. cDNA was synthesized by use of Superscript II reverse transcriptase (Gibco).

Chemokine mRNA detection. The expression of mRNA for chemokines (CCL3, CCL4, and CCL5) and β-actin was analyzed by RT-PCR, which was performed with *Taq* polymerase (Gibco) in a PTC-100 thermal cycler (MJ Research). The reaction conditions were 35 cycles of 1 min at 94°C, 1 min at

54°C, and 2 min at 72°C, with a final extension step of 7 min at 72°C. For each set of primers, a negative sample (water) was run in parallel. The PCR products were separated by acrylamide gel electrophoresis and stained with silver nitrate. The primer sequences and PCR product sizes have been published elsewhere [9].

Spleen-cell cultures. Suspensions of splenocytes from uninfected mice were washed in Hanks' balanced salt solution (HBSS) and treated with lysis buffer (9 parts 0.16 mol/L ammonium chloride and 1 part 0.17 mol/L Tris-HCl [pH 7.5]) for 4 min. The erythrocyte-free cells were then washed 3 times in HBSS and adjusted to 3 × 10⁶ cells/mL of RPMI 1640 (Flow Laboratories) supplemented with 10% fetal calf serum (Hyclone), 2-mercaptoethanol (5 × 10⁻⁵ mol/L), L-glutamine (2 mmol/L), and antibiotics (all from Sigma). The cell suspension was distributed (1 mL/well) in 24-well tissue-culture plates (Corning) and cultured for 40 h at 37°C in a humidified 5% CO₂ atmosphere, in the presence or absence of trypomastigote forms of *T. cruzi* (5 parasites/cell) or concanavalin A (ConA; 2 µg/mL).

Isolation of inflammatory cells from cardiac tissue of *T. cruzi*-infected mice. To isolate mononuclear cells from myocardia, 15–20 mouse hearts removed 17 days after infection were washed (to remove blood clots), pooled, minced with scissors into 1–2-mm fragments, extensively washed, and subjected to enzymatic digestion with a solution containing 0.05% trypsin (Gibco) and 0.01% collagenase type II (Worthington) for 10 min at 37°C. The cell suspension was spun, the supernatant was removed, and the pellet was suspended in 2 mL of 100% Percoll (Pharmacia). Aliquots (2 mL) of Percoll (70% and 40%) were added to the top of the cell suspension, and the suspension was centrifuged at 1500 *g* for 30 min. The leukocytes (between the 70% and 40% fractions) were removed and washed with RPMI 1640 (5%), and their phenotypes were evaluated by flow cytometry.

Flow-cytometry analysis. Both inflammatory cells obtained from the cardiac tissue of *T. cruzi*-infected mice and splenocytes were stained with fluorescein isothiocyanate (FITC)- or phosphatidylethanolamine-labeled antibodies against CD3, CD4, CD8, CCR5 (PharMingen), or CCR3 (Santa Cruz Biotechnology) molecules. Viable cells (3 × 10⁵) were analyzed by use of a flow cytometer and CellQuest software (version 3.3; Becton Dickinson).

Histological evaluation. Groups of 3–4 mice were killed on different days after infection with *T. cruzi*. Hearts were fixed in neutral 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined by light microscopy.

Immunohistochemical analysis. Cardiac tissues of mice were removed on days 17 and 20 after infection with *T. cruzi*, placed in compound-embedding medium (Sakura Finetek),

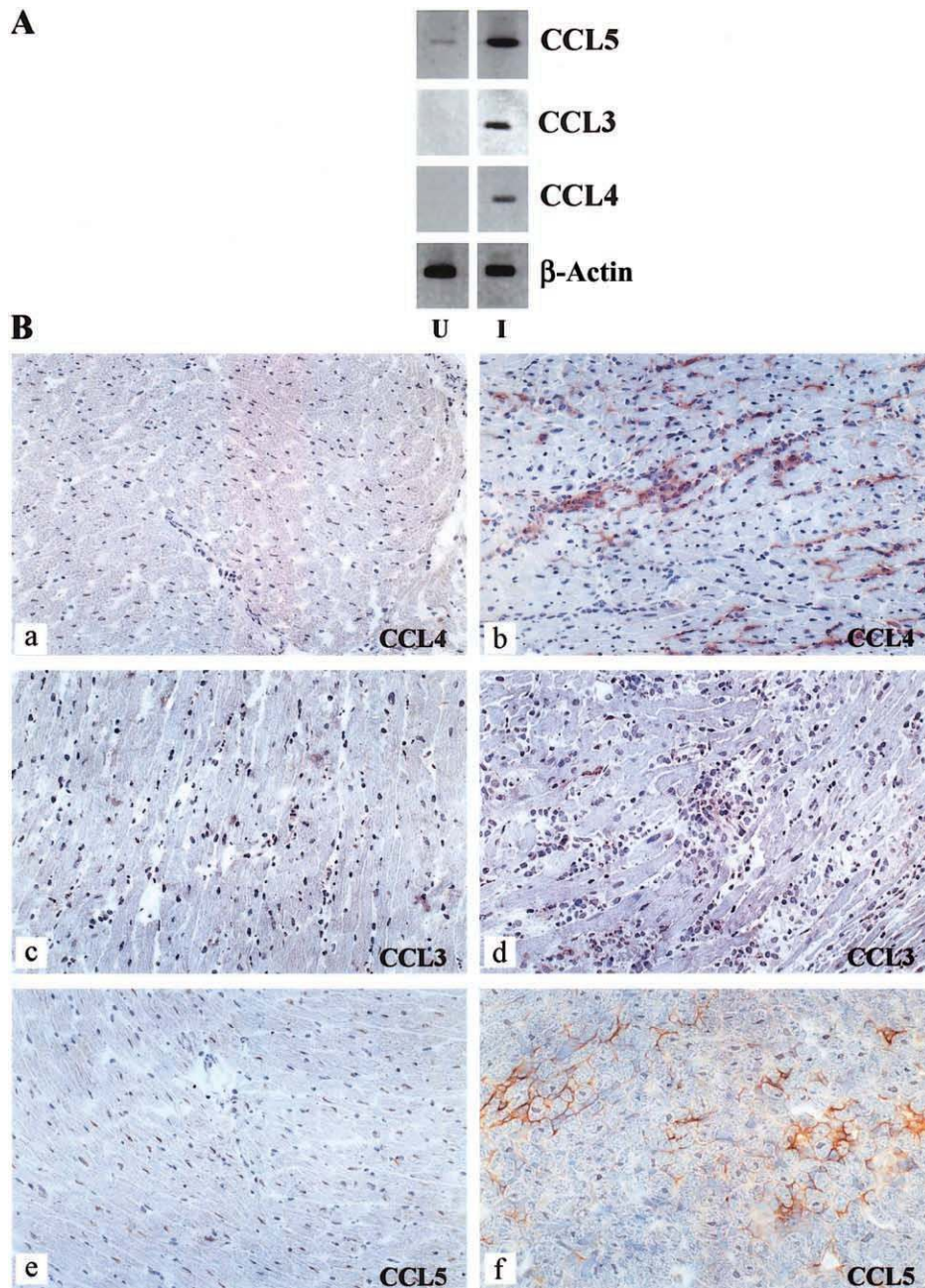


Figure 1. Expression of CCL3, CCL4, and CCL5 in the myocardia of *Trypanosoma cruzi*-infected mice. **A**, Total myocardial RNA was extracted from uninfected (U) and infected (I) mice on day 15 after infection, and expression of CCL3, CCL4, CCL5, and β -actin was assessed by reverse-transcription polymerase chain reaction (PCR). The PCR products were electrophoresed in polyacrylamide gels and stained with silver nitrate. **B**, Hearts of uninfected (*a*, *c*, and *e*) and infected (*b*, *d*, and *f*) mice on day 20 after infection with 1000 trypomastigote forms of *T. cruzi* were prepared for immunohistochemical analysis, and the presence of CCL4 (*a* and *b*), CCL3 (*c* and *d*), and CCL5 (*e* and *f*) were evaluated by use of the immunoperoxidase method (see Materials and Methods). The presence of the chemokines was revealed by use of diaminobenzidine tetrahydrochloride as the substratum for the peroxidase, which generated a brown coloration. Results shown are representative of 3 different experiments. Original magnification for all microphotographs, $\times 200$.

snap-frozen by use of liquid nitrogen, and stored at -70°C . Ten-micrometer sections were prepared in a cryostat, collected on polylysine-coated slides, fixed with cold acetone, and allowed to air dry.

The endogenous peroxidase activity was blocked by use of 3% hydrogen peroxide for 20 min, and nonspecific binding was blocked by use of goat serum (diluted 1:200 in PBS) for 45 min. The slides were washed and incubated overnight with

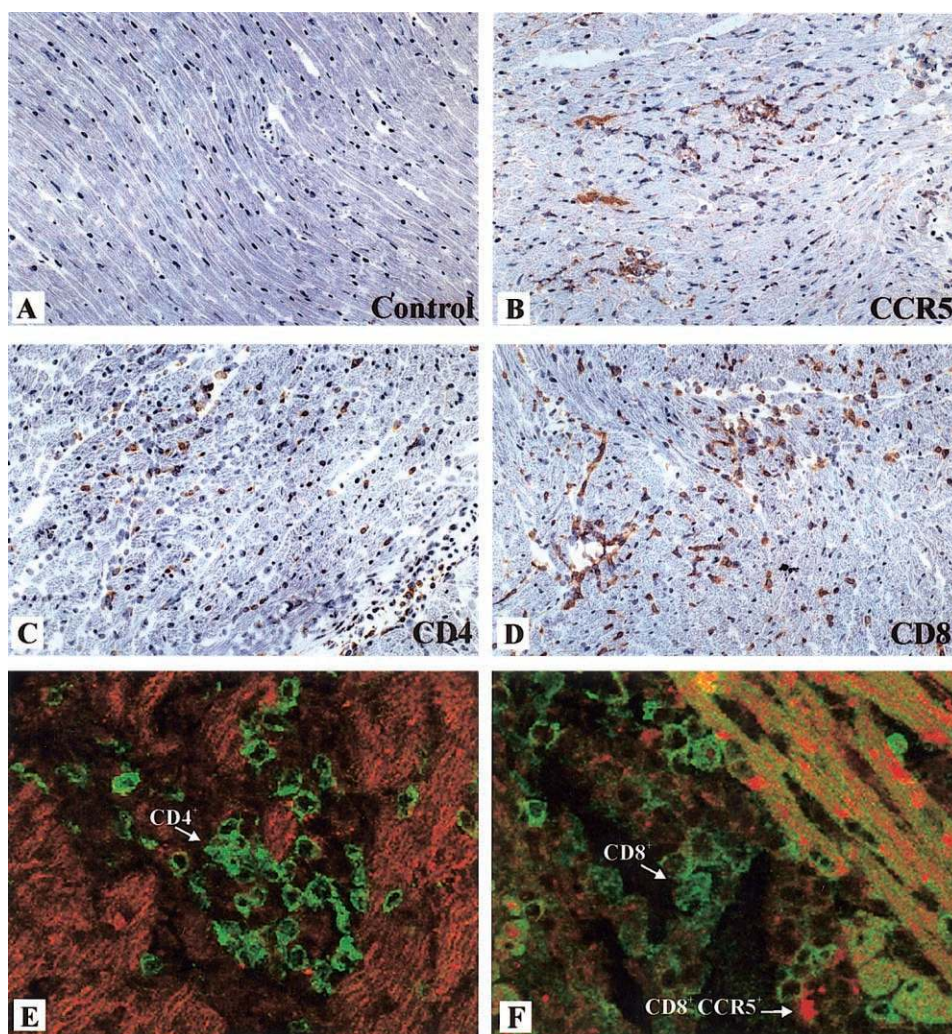


Figure 2. Expression of CCR5, CD8, and CD4 in the myocardia of uninfected or *Trypanosoma cruzi*-infected mice. Hearts of uninfected (A) or infected (B–F) mice on day 17 after infection with 1000 trypomastigote forms of *T. cruzi* were obtained and prepared for analysis by immunohistochemistry and immunofluorescence. Expression of CCR5 (A and B), CD4 (C), and CD8 (D) in myocardia was evaluated by use of the immunoperoxidase method (see Materials and Methods). Diaminobenzidine tetrahydrochloride was used as the substratum for the peroxidase, which generated a brown coloration. Panels E and F show the results of double immunofluorescence labeling, in which CD4 (E) and CD8 (F) were stained with fluorescein-labeled specific antibody and CCR5 (E and F) was stained with antibody conjugated with Texas red. Original magnification for all microphotographs, $\times 200$.

goat anti-mouse MIP-1 α , MIP-1 β , RANTES, or CCR5 (Santa Cruz Biotechnology) or with rat anti-mouse CD4 or CD8 (Pharmingen) diluted 100 times in PBS–1% bovine serum albumin (BSA). After extensive washes and incubation for 30 min with biotin-labeled rabbit anti-goat or goat anti-rat antibody (Pharmingen), the reaction product was detected by use of avidin-biotin–peroxidase complex (Vector Laboratories), the color of the reaction was developed by use of diaminobenzidine tetrahydrochloride (DAB; Sigma), and the reaction was counterstained with Mayer’s hematoxylin. Controls were performed by incubation of cells with nonimmune goat or rat IgG.

Immunofluorescent staining and confocal analysis. Cryostat sections obtained and fixed as described above were in-

cubated overnight at 4°C with anti-CD16/CD32 monoclonal antibody (MAb; Pharmingen) in PBS–1% BSA and then were blocked with avidin-biotin. Sections were incubated with the appropriate dilutions of rat anti-mouse MAb against CD4 and CD8 (labeled with FITC) and biotinylated anti-CCR5 or normal rat IgG for 1 h. Immunostaining for CCR5 was detected by incubation with streptavidin–Texas red (Pharmingen) for 1 h. Between each incubation step, slides were washed 3 times in PBS and mounted in aqueous medium. The slides were analyzed by fluorescent microscopy (Leica), and the images were processed by use of Adobe Photoshop software (version 7.0).

Statistical analysis. Results are expressed as the mean \pm SE of the triplicate cultures or experiments. Statistical analysis

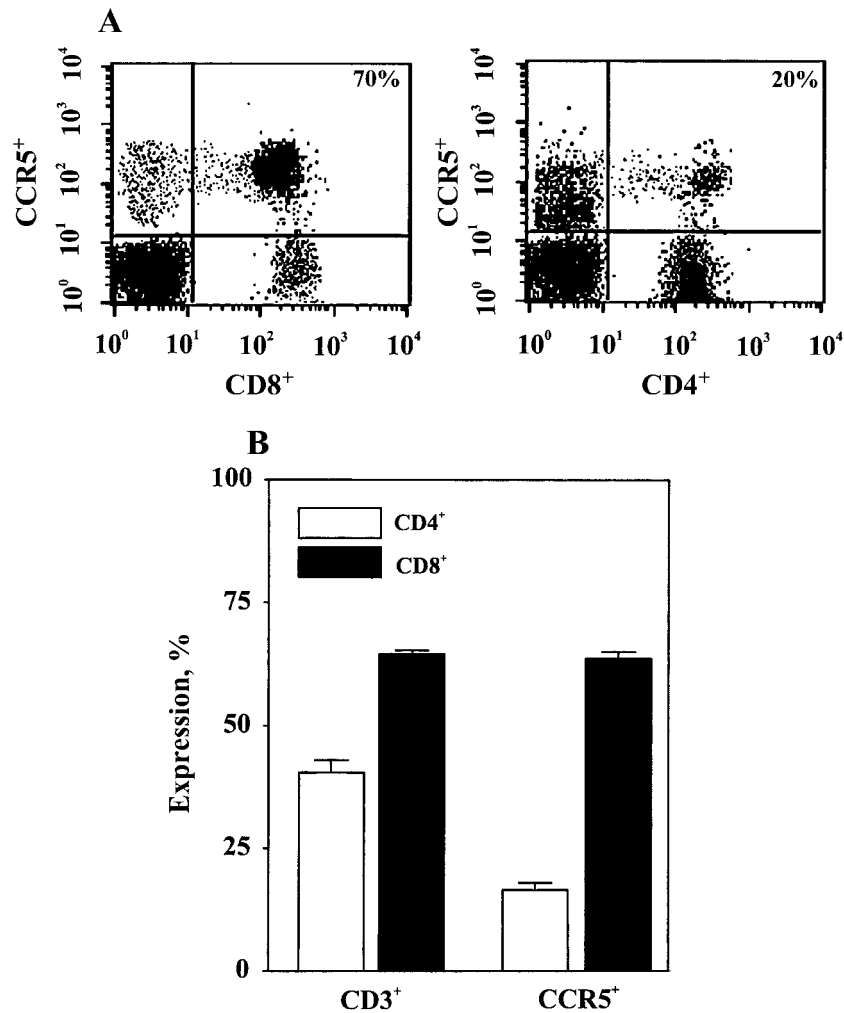


Figure 3. Predominance of CCR5⁺CD8⁺ T cells in the myocardia of *Trypanosoma cruzi*-infected mice. C57BL/6 mice were infected with 1000 trypomastigote forms; 17 days after infection, hearts were harvested and subjected to chemical (2750 U/mL of collagenase) and mechanical (shaking) dissociation of the cells. The lymphocytes were isolated by use of Percoll gradient; were identified by use of antibodies against CD4, CD8 (fluorescein isothiocyanate labeled), CD3, and CCR5 (phosphatidylethanolamine labeled); and were subjected to flow-cytometry analysis. *A*, An example of the dot plots obtained. *B*, Percentages of CD4⁺ (white bars) and CD8⁺ (black bars) T cells that expressed CD3 and CCR5. Each bar represents the mean \pm SD of triplicate samples and is representative of 3 separate experiments.

was performed by use of analysis of variance, followed by the Student-Newman-Keuls test (InStat software; GraphPad). $P < .05$ was considered to be statistically significant.

RESULTS

Detection of CCL3, CCL4, and CCL5 in the myocardia of *T. cruzi*-infected mice. Detection of mRNA for CCL3, CCL4, and CCL5 in cardiomyocytes from mice infected with *T. cruzi* has been previously reported [10]. Here, we examined whether these chemokines were also expressed in the cardiac tissue of mice infected with *T. cruzi*. No detectable expression of CCL3 and CCL4 mRNA and only a very low expression of CCL5

mRNA were found in the myocardia of uninfected *wt* mice. Infection with *T. cruzi* resulted in significant mRNA for CCL3 and CCL4 and enhanced mRNA for CCL5 in the myocardia on day 15 after infection (figure 1A). Moreover, CCL3, CCL4, and CCL5 were also detected by immunohistochemistry in the myocardia of infected mice but not in the myocardia of uninfected mice (figure 1B). Presence of the chemokines was clearly associated with infiltrated cells in cardiac tissue. As shown in figure 1B, there was a mononuclear interstitial infiltrate in the myocardia of infected mice that was positive for the presence of CCL3, CCL4, and CCL5 chemokines. The staining was most intense in the cardiac tissue obtained on days 17–20 after infection. In the myocardia of uninfected mice, no

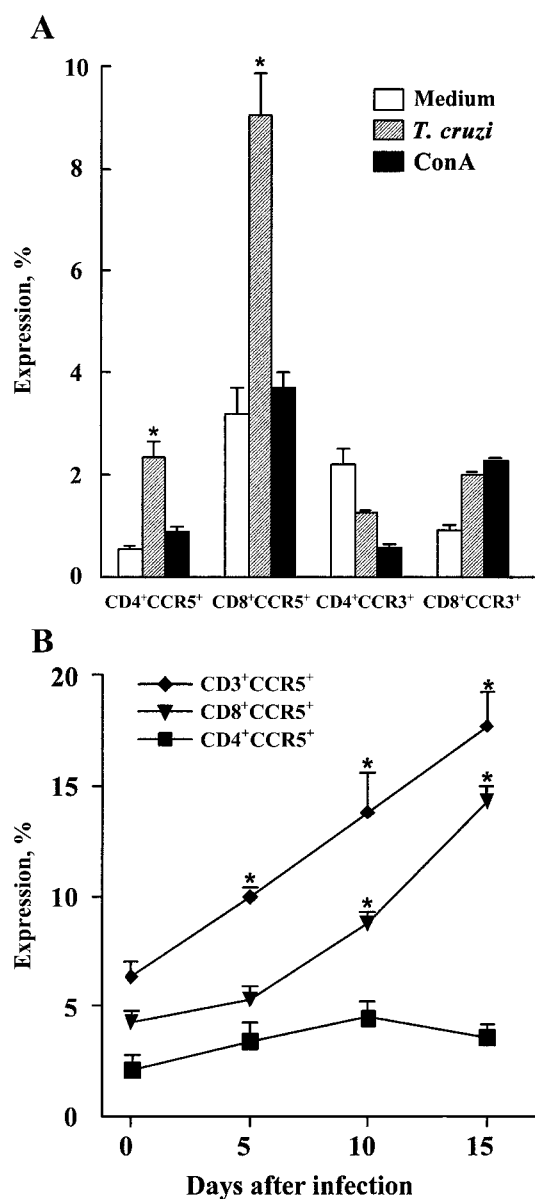


Figure 4. Induction of CCR5 expression in lymphocytes by trypomastigote forms of *Trypanosoma cruzi*. CCR5 expression was determined in *T. cruzi*-cultured T cells from C57BL/6 mice (A) and in T splenocytes from mice experiencing the acute phase of infection (B). In panel A, the spleen cells were cultured with medium only, trypomastigote forms of *T. cruzi* (5 parasites/cell), or concanavalin A (ConA; 2 μ g/mL). The cells were harvested; labeled with antibodies specific for CCR5, CCR3, CD4, and CD8; and subjected to flow-cytometry analysis. In panel B, CCR5 expression was determined by flow-cytometry analysis in T splenocytes from uninfected or infected mice on days 5, 10, and 15 after infection with 1000 trypomastigote forms of *T. cruzi*. Each point represents the mean \pm SD of triplicate samples and is representative of 3 separate experiments. * $P < .05$, compared with the values obtained in the absence of *T. cruzi* (A) or with the values obtained for cells from uninfected mice (B). Statistical analysis was performed by use of analysis of variance, followed by the Student-Newman-Keuls test.

inflammatory infiltrate was present, and no staining for CC chemokines was observed.

Phenotype of inflammatory cells in the cardiac tissue of *T. cruzi*-infected mice. Because CCL3, CCL4, and CCL5 bind to CCR5 on the surface of leukocytes, we examined whether the inflammatory cells in the myocardia of infected mice expressed CCR5. These experiments showed that there were very few CD4⁺ and CD8⁺ T cells in uninfected mice (figure 2A) and that the number of these cells increased markedly after infection (figure 2C and 2D). When we examined mouse cardiac tissue obtained during the acute phase of infection, we found that the inflammatory infiltrate was predominantly composed of CD8⁺ T cells (figure 2C and 2D). The quantification of T cells infiltrating the cardiac tissue (figure 3A) revealed that the majority of CD3⁺ T cells in the cardiac tissue of infected mice were also CD8⁺ (figure 3B). Other cells found in cardiac tissue expressed CD11c (11%), CD14 (7%), and CD56 (20%). Expression of CCR5 was found to be positive in the majority of leukocytes in inflammatory infiltrate (figure 2B), indicating that a significant amount of CD8⁺ T cells expressed CCR5. Indeed, double immunofluorescence labeling revealed that the majority of CD8⁺ T cells (figure 2F), but not of CD4⁺ T cells (figure 2E), expressed CCR5. These data were confirmed by flow-cytometry analysis, which showed that 70% of CD8⁺ and 20% of CD4⁺ T cells that were isolated from myocardia during acute infection expressed CCR5 (figure 3A and 3B).

Inducement of enhanced expression of CCR5 in lymphocytes by infection with *T. cruzi*. Because there was a high expression of CCR5 in T cells found in cardiac tissue, we evaluated whether the parasites could modulate the expression of CCR5 in lymphocytes. Splenocytes were incubated with trypomastigote forms or with ConA, and the expression of CD4, CD8, CCR5, and CCR3 was evaluated. As shown in figure 4A, incubation with trypomastigote forms, but not with ConA, induced a significant enhancement of the expression of CCR5 in CD4⁺ and CD8⁺ T cells. Incubation of splenocytes with the parasites or ConA did not alter the expression of CCR3 (figure 4A). These results show that incubation of splenocytes with trypomastigote forms of *T. cruzi* for 40 h results in enhanced expression of CCR5 on the surface of murine T cells.

We found a linear increase in the expression of CCR5 on T cells after infection, on the basis that the increase was significant as soon as 5 days after infection (figure 4B). The increased expression of CCR5 was almost exclusively on CD8⁺ T cells; expression on CD4⁺ T cells was not significantly increased (figure 4B). These data indicate that *T. cruzi* is able to induce expression of CCR5 on CD8⁺ T splenocytes.

Greater susceptibility of CCR5^{-/-} mice to infection with *T. cruzi*, which mediates the cell influx to cardiac tissue. To investigate the relevance of CCR5 to *T. cruzi* infection, CCR5^{-/-} mice were infected with *T. cruzi*. The results showed a significantly

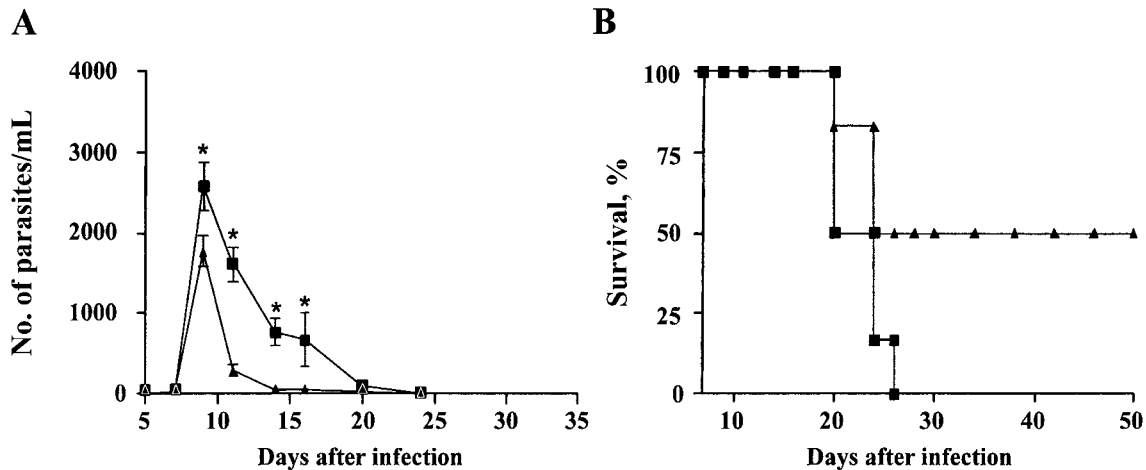


Figure 5. Natural course of *Trypanosoma cruzi* infection, as measured by parasitemia (A) and survival rate (B) of wild-type (wt) (▲) and CCR5-deficient (CCR5^{-/-}) (■) mice infected with 1000 trypomastigote forms of *T. cruzi* strain Y. CCR5^{-/-} mice were found to be more susceptible to *T. cruzi* infection than wt mice. In panel A, the mean \pm SD no. of parasites per microliter of blood for 1 of 4 representative experiments (10 mice/group) is shown. * $P < .05$, for the comparison of CCR5^{-/-} vs. C57BL/6 wt mice infected with *T. cruzi*.

increased parasitemia during the acute phase of infection in CCR5^{-/-} mice (between days 7–15 after infection) (figure 5A). Moreover, although the mortality of wt mice was 50% on day 26 after infection, all CCR5^{-/-} had died by that day ($P < .05$) (figure 5B). Interestingly, the intensity and kinetics of diffuse and focal inflammatory infiltrates were different in the cardiac tissue of CCR5^{-/-} and wt mice infected with the parasite. Although, on day 20 after infection, significant inflammatory infiltrates were found in the myocardia of wt mice infected with *T. cruzi* (figure 6C), these were almost absent in the myocardia of CCR5^{-/-} mice infected with *T. cruzi* (figure 6D). Instead, we found very large amounts of parasite nests (figure 6D), such that in several microscopy fields the amounts of parasites in the tissue were virtually uncountable. Also noticeable was the finding that the sizes of parasite nests in CCR5^{-/-} mice were bigger than those in wt mice. In contrast, rare, isolated parasite nests were found in the cardiac tissue of wt mice (figure 6C). Absence of cell infiltrate was found in uninfected wt (figure 6A) and CCR5^{-/-} (figure 6B) mice.

DISCUSSION

It is now clear that IFN- γ - and TNF- α -activated macrophages control of the replication of *T. cruzi* in various hosts [20]. The mechanism by which macrophages are able to kill intracellular parasites has been shown to be dependent on NO [8–13, 20], although a recent study using *T. cruzi* strains Tulahuén and Brazil suggested that inducible NO synthase (iNOS) is not required to control parasite replication in vivo [21]. However, much less is known about the mechanisms governing the migration of leukocytes to sites of tissue infection, especially to hearts of infected hosts. More recently, a group of mediators,

the chemokines, have been described in some detail and have been shown to participate in the recruitment of leukocytes in several models of inflammatory diseases [14, 22–24]. In the present study, we demonstrate an important role for CCR5-acting chemokines in the control of leukocyte migration and parasite replication during the acute phase of infection with the Y strain of *T. cruzi*. This strain induces a parasitemia that peaks around days 9–11 of infection and causes an intense inflammatory reaction in cardiac tissue that is more intense between days 13 and 20 [25].

Infection of macrophages [9] and cardiomyocytes [10] with *T. cruzi* trypomastigote forms is accompanied by the significant production of several chemokines, including CCL3, CCL4, and CCL5. Not only can the latter cells produce chemokines, but chemokines can also act on the cells to induce iNOS expression, NO expression, and the killing of parasites [9–13]. Thus, in the present study, it was important to ascertain whether chemokine expression would also occur in vivo after infection of mice with the Y strain of *T. cruzi*. Indeed, both mRNA and protein for CCL3, CCL4, and CCL5 could be found in the cardiac tissue of *T. cruzi*-infected mice, in close correlation with tissue inflammation; these results are in agreement with the findings of other studies [14–16]. Notably, these latter studies showed that the expression of chemokines was greatly under the control of IFN- γ and that the modulation of chemokine expression was accompanied by the modulation of the recruitment of leukocytes to sites of *T. cruzi*-induced inflammation [26]. Altogether, these studies and the present data demonstrate the close correlation between the expression of chemokines and the development of tissue inflammation after *T. cruzi* infection in vivo.

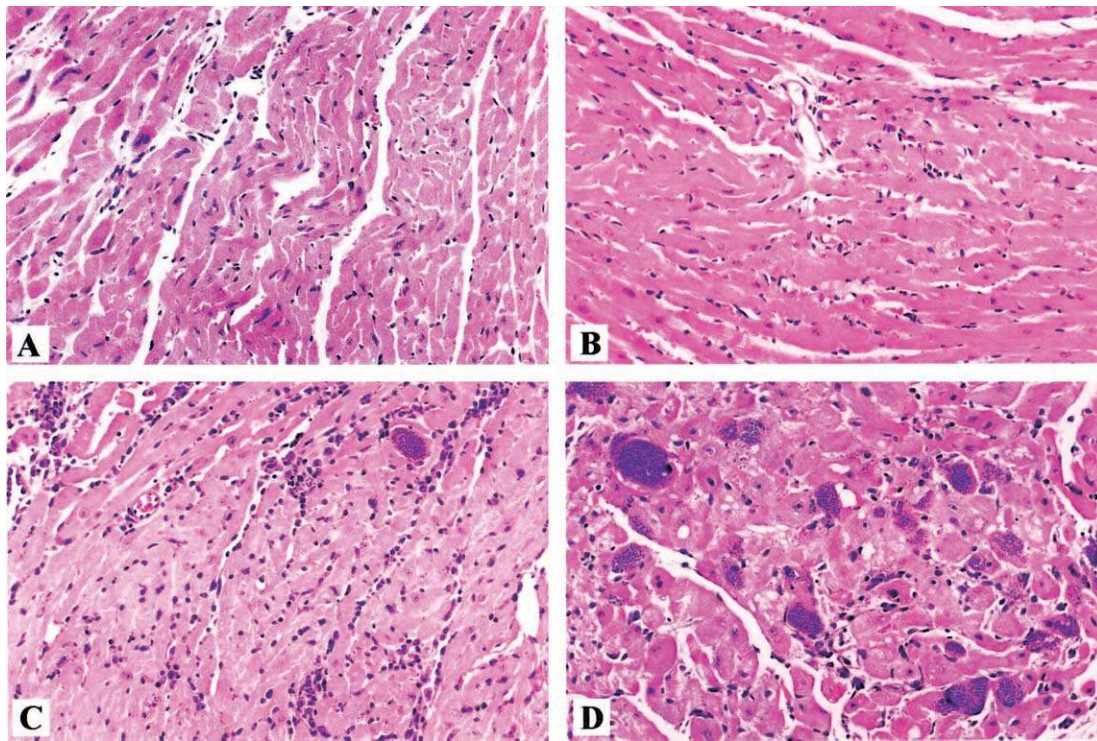


Figure 6. Inflammatory infiltrate and parasite nests in wild-type (*wt*) and CCR5-deficient (CCR5^{-/-}) mice. Histological sections of cardiac tissue from *wt* (A and C) and CCR5^{-/-} (B and D) mice that were either uninfected (A and B) or infected (C and D) with 1000 trypomastigote forms of *Trypanosoma cruzi* were obtained and stained with hematoxylin-eosin. Hearts were harvested on day 20 after infection. Note the absence of inflammatory cells and the increased no. of parasite nests in the cardiac tissue of CCR5^{-/-} mice. Original magnification for all microphotographs, ×200.

Not only was the expression of chemokines enhanced, but the expression of CCR5, a receptor for CCL3, CCL4, and CCL5, was also enhanced on infiltrating leukocytes in the hearts of *T. cruzi*-infected mice. More specifically, there was a marked increase in the number of CD8⁺ T cells expressing CCR5, as assessed by both immunohistochemistry and flow-cytometry analysis. The number of CD4⁺ T cells expressing CCR5 in the cardiac tissue of infected mice also increased, but to a lesser extent. Interestingly, the increased expression of CCR5 on tissue lymphocytes was mirrored by a significant increase in the expression of this receptor on circulating lymphocytes. Indeed, infection with *T. cruzi*, but not stimulation with ConA, also enhanced the expression of CCR5 on both CD4⁺ and CD8⁺ T cells cultivated in vitro. These results are in agreement with those of a recent study that showed that both CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells obtained from the peripheral blood of infected patients expressed CCR5 to a greater degree than did those obtained from uninfected individuals [27]. In the latter study, the enhanced CCR5 expression on T cells was lost only when heart failure supervened chronic Chagas disease. Thus, infection with *T. cruzi* was accompanied by a parasite-associated increase in the expression of CCR5 and migration of T cells to infected tissues, in which several CCR5-acting chemokines are produced. The increased expression of CCR5 could be due to

the production of IFN- γ [28, 29], TNF- α [8, 30], interleukin (IL)-10 [7, 31], and/or IL-12 [30, 32, 33] that occurs after the addition of trypomastigote forms of *T. cruzi* to cultures of splenocytes or after the infection of mice. Indeed, CCR5 expression is positively regulated by these cytokines [34–37].

The role of CCR5 in the control of *T. cruzi* infection and in the disease associated with the infection was evaluated in CCR5^{-/-} mice. In these mice, a higher level of parasitemia and greater mortality was observed, compared with those in *T. cruzi*-infected *wt* mice. Moreover, the amount of parasites in the cardiac tissue of CCR5^{-/-} mice was greatly enhanced. The reduced control of parasite replication was associated with a dramatic reduction of the migration of T cells and overall disease, indicating that CCR5 was crucial to the migration of lymphocytes to the hearts of infected mice. Importantly, there was no defect in the ability of macrophages from CCR5^{-/-} mice to produce NO and IL-12 or to kill the parasite in vitro (data not shown). Similar to our results in intracellular adhesion molecule-1-deficient mice [38], the decreased number of lymphocytes in cardiac tissue was associated with decreased local production of IFN- γ (data not shown) and an increased level of parasitemia, suggesting that there was a defect in the migration of cells capable of producing cytokines (such as IFN- γ) that activate the production of NO. Thus, the lack of CCR5

is accompanied by the inability of CCR5⁺ T cells to migrate to the hearts of infected mice and the inability of the host to control parasite replication. Similarly, absence of CCR5 leads to increased susceptibility to *Cryptococcus neoformans* infection, because of the absence of the migration of cells to the brain [18]. On the contrary, a defective trafficking of leukocytes in CCR5^{-/-} mice has been associated with (1) resistance to the development of cerebral malaria in mice infected with *Plasmodium berghei* [39] and (2) demyelination after infection with mouse hepatitis virus [40].

CD8⁺ T cells compose the great majority of cells observed in the inflammatory infiltrate in the hearts of patients with chagasic cardiomyopathy [41–44]. These cells may play a role in the damage to the heart that is observed during the chronic phase of disease [41–44]. Indeed, cardiac lesions observed during the chronic phase of infection with *T. cruzi* have been associated with the local inflammatory response that is driven by host immune cells [45, 46]. It is not known, however, whether the CD8⁺ T cells that infiltrate the hearts of patients with Chagas disease also express CCR5 or whether there is a functional relevance for the observed increase of CCR5 expression on the CD8⁺ T cells that are obtained from the peripheral blood of patients with Chagas disease [27]. A role for CCR5 in human disease has also been suggested by a study that showed that a point mutation of the CCR5 promoter was significantly increased in asymptomatic patients, compared with that in patients with chagasic cardiomyopathy [47]. It is clear that further studies are necessary to understand the role played by CCR5 in the pathogenesis of Chagas disease.

In summary, our results demonstrate that CCR5-acting chemokines are expressed in the hearts of *T. cruzi*-infected mice and that CCR5 is most relevant to the control of the lymphocyte migration and parasite replication. Future studies should be conducted to evaluate whether CCR5 is also relevant to the pathophysiology of chronic infection and whether the blockade of CCR5 could be a viable target for the prevention of the migration of lymphocytes to inflamed cardiac tissue.

References

- Russo M, Starobinas N, Minoprio P, Coutinho A, Hontebeyrie-Joskowicz M. Parasitic load increases and myocardial inflammation decreases in *Trypanosoma cruzi*-infected mice after inactivation of helper T cells. *Ann Inst Pasteur Immunol* **1988**; 139:225–36.
- Rottenberg ME, Riarte A, Sporrang L, et al. Outcome of infection with different strains of *Trypanosoma cruzi* in mice lacking CD4 and/or CD8. *Immunol Lett* **1995**; 45:53–60.
- Tarleton RL, Grusby MJ, Postan M, Glimcher LH. *Trypanosoma cruzi* infection in MHC-deficient mice: further evidence for the role of both class I- and class II-restricted T cells in immune resistance and disease. *Int Immunol* **1996**; 8:13–22.
- Tarleton RL, Koller BH, Latour A, Postan M. Susceptibility of β 2-microglobulin-deficient mice to *Trypanosoma cruzi* infection. *Nature* **1992**; 356:338–40.
- McCabe RE, Meagher SG, Mullins BT. Endogenous interferon- γ , macrophage activation, and murine host defense against acute infection with *Trypanosoma cruzi*. *J Infect Dis* **1991**; 163:912–5.
- Torrico F, Heremans H, Rivera MT, Van Marck E, Billiau A, Carlier Y. Endogenous IFN- γ is required for resistance to acute *Trypanosoma cruzi* infection in mice. *J Immunol* **1991**; 146:3626–32.
- Silva JS, Morrissey PJ, Grabstein KH, Mohler KM, Anderson D, Reed SG. Interleukin 10 and interferon γ regulation of experimental *Trypanosoma cruzi* infection. *J Exp Med* **1992**; 175:169–74.
- Silva JS, Vespa GN, Cardoso MA, Aliberti JC, Cunha FQ. Tumor necrosis factor α mediates resistance to *Trypanosoma cruzi* infection in mice by inducing nitric oxide production in infected γ interferon-activated macrophages. *Infect Immun* **1995**; 63:4862–7.
- Aliberti JC, Machado FS, Souto JT, et al. β -Chemokines enhance parasite uptake and promote nitric oxide-dependent microbiostatic activity in murine inflammatory macrophages infected with *Trypanosoma cruzi*. *Infect Immun* **1999**; 67:4819–26.
- Machado FS, Martins GA, Aliberti JC, Mestriner FL, Cunha FQ, Silva JS. *Trypanosoma cruzi*-infected cardiomyocytes produce chemokines and cytokines that trigger potent nitric oxide-dependent trypanocidal activity. *Circulation* **2000**; 102:3003–8.
- Vespa GN, Cunha FQ, Silva JS. Nitric oxide is involved in control of *Trypanosoma cruzi*-induced parasitemia and directly kills the parasite in vitro. *Infect Immun* **1994**; 62:5177–82.
- Lima MF, Zhang Y, Villalta F. β -Chemokines that inhibit HIV-1 infection of human macrophages stimulate uptake and promote destruction of *Trypanosoma cruzi* by human macrophages. *Cell Mol Biol (Noisy-le-grand)* **1997**; 43:1067–76.
- Villalta F, Zhang Y, Bibb KE, Kappes JC, Lima MF. The cysteine-cysteine family of chemokines RANTES, MIP-1 α , and MIP-1 β induce trypanocidal activity in human macrophages via nitric oxide. *Infect Immun* **1998**; 66:4690–5.
- Aliberti JC, Souto JT, Marino AP, et al. Modulation of chemokine production and inflammatory responses in interferon- γ - and tumor necrosis factor-R1-deficient mice during *Trypanosoma cruzi* infection. *Am J Pathol* **2001**; 158:1433–40.
- Talvani A, Ribeiro CS, Aliberti JC, et al. Kinetics of cytokine gene expression in experimental chagasic cardiomyopathy: tissue parasitism and endogenous IFN- γ as important determinants of chemokine mRNA expression during infection with *Trypanosoma cruzi*. *Microbes Infect* **2000**; 2:851–66.
- dos Santos PV, Roffe E, Santiago HC, et al. Prevalence of CD8⁺ $\alpha\beta$ T cells in *Trypanosoma cruzi*-elicited myocarditis is associated with acquisition of CD62L^{low}LFA-1^{high}VLA-4^{high} activation phenotype and expression of IFN- γ -inducible adhesion and chemoattractant molecules. *Microbes Infect* **2001**; 3:971–84.
- Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokines grammar for immune cells. *Annu Rev Immunol* **2004**; 22:891–928.
- Huffnagle GB, McNeil LK, McDonald RA, et al. Cutting edge: role of C-C chemokine receptor 5 in organ-specific and innate immunity to *Cryptococcus neoformans*. *J Immunol* **1999**; 163:4642–6.
- Zhou Y, Kurihara T, Ryseck RP, et al. Impaired macrophage function and enhanced T cell-dependent immune response in mice lacking CCR5, the mouse homologue of the major HIV-1 coreceptor. *J Immunol* **1998**; 160:4018–25.
- Silva JS, Machado FS, Martins GA. The role of nitric oxide in the pathogenesis of Chagas disease. *Front Biosci* **2003**; 8:314–25.
- Cummings KL, Tarleton RL. Inducible nitric oxide synthase is not essential for control of *Trypanosoma cruzi* infection in mice. *Infect Immun* **2004**; 72:4081–9.
- Souto JT, Aliberti JC, Campanelli AP, et al. Chemokine production and leukocyte recruitment to the lungs of *Paracoccidioides brasiliensis*-infected mice is modulated by interferon- γ . *Am J Pathol* **2003**; 163: 583–90.
- Ji J, Sun J, Soong L. Impaired expression of inflammatory cytokines and chemokines at early stages of infection with *Leishmania amazonensis*. *Infect Immun* **2003**; 71:4278–88.

24. Godessart N, Kunkel SL. Chemokines in autoimmune disease. *Curr Opin Immunol* **2001**; 13:670–5.
25. Silva JS, Rossi MA. Intensification of acute *Trypanosoma cruzi* myocarditis in BALB/c mice pretreated with low doses of cyclophosphamide or γ irradiation. *J Exp Pathol (Oxford)* **1990**; 71:33–9.
26. Teixeira MM, Gazzinelli RT, Silva JS. Chemokines, inflammation and *Trypanosoma cruzi* infection. *Trends Parasitol* **2002**; 18:262–5.
27. Talvani A, Rocha MO, Ribeiro AL, Correa-Oliveira R, Teixeira MM. Chemokine receptor expression on the surface of peripheral blood mononuclear cells in Chagas disease. *J Infect Dis* **2004**; 189:214–20.
28. Cardillo F, Voltarelli JC, Reed SG, Silva JS. Regulation of *Trypanosoma cruzi* infection in mice by γ interferon and interleukin 10: role of NK cells. *Infect Immun* **1996**; 64:128–34.
29. Graefe SE, Jacobs T, Gaworski I, Klauenberg U, Steeg C, Fleischer B. Interleukin-12 but not interleukin-18 is required for immunity to *Trypanosoma cruzi* in mice. *Microbes Infect* **2003**; 5:833–9.
30. Hunter CA, Slifer T, Araujo F. Interleukin-12-mediated resistance to *Trypanosoma cruzi* is dependent on tumor necrosis factor alpha and gamma interferon. *Infect Immun* **1996**; 64:2381–6.
31. Holscher C, Mohrs M, Dai WJ, et al. Tumor necrosis factor α -mediated toxic shock in *Trypanosoma cruzi*-infected interleukin 10-deficient mice. *Infect Immun* **2000**; 68:4075–83.
32. Aliberti JC, Cardoso MA, Martins GA, Gazzinelli RT, Vieira LQ, Silva JS. Interleukin-12 mediates resistance to *Trypanosoma cruzi* in mice and is produced by murine macrophages in response to live trypomastigotes. *Infect Immun* **1996**; 64:1961–7.
33. Antunez MI, Cardoni RL. IL-12 and IFN- γ production, and NK cell activity, in acute and chronic experimental *Trypanosoma cruzi* infections. *Immunol Lett* **2000**; 71:103–9.
34. Croitoru-Lamoury J, Guillemin GJ, Boussin FD, et al. Expression of chemokines and their receptors in human and simian astrocytes: evidence for a central role of TNF α and IFN γ in CXCR4 and CCR5 modulation. *Glia* **2003**; 41:354–70.
35. Takayama T, Morelli AE, Onai N, et al. Mammalian and viral IL-10 enhance C-C chemokine receptor 5 but down-regulate C-C chemokine receptor 7 expression by myeloid dendritic cells: impact on chemotactic responses and in vivo homing ability. *J Immunol* **2001**; 166:7136–43.
36. Iwasaki M, Mukai T, Nakajima C, et al. A mandatory role for STAT4 in IL-12 induction of mouse T cell CCR5. *J Immunol* **2001**; 167: 6877–83.
37. Juffermans NP, Paxton WA, Dekkers PE, et al. Up-regulation of HIV coreceptors CXCR4 and CCR5 on CD4⁺ T cells during human endotoxemia and after stimulation with (myco)bacterial antigens: the role of cytokines. *Blood* **2000**; 96:2649–54.
38. Michailowsky V, Celes MRN, Marino AP, et al. ICAM-1 deficiency leads to impaired recruitment of T lymphocytes and enhanced host susceptibility to infection with *Trypanosoma cruzi*. *J Immunol* **2004**; 173:463–70.
39. Belnoue E, Kayibanda M, Deschemin JC, et al. CCR5 deficiency decreases susceptibility to experimental cerebral malaria. *Blood* **2003**; 101:4253–9.
40. Glass WG, Liu MT, Kuziel WA, Lane TE. Reduced macrophage infiltration and demyelination in mice lacking the chemokine receptor CCR5 following infection with a neurotropic coronavirus. *Virology* **2001**; 288:8–17.
41. Higuchi Mde L, Gutierrez PS, Aiello VD, et al. Immunohistochemical characterization of infiltrating cells in human chronic chagasic myocarditis: comparison with myocardial rejection process. *Virchows Arch A Pathol Anat Histopathol* **1993**; 423:157–60.
42. Reis MM, Higuchi Mde L, Benvenuti LA, et al. An in situ quantitative immunohistochemical study of cytokines and IL-2R⁺ in chronic human chagasic myocarditis: correlation with the presence of myocardial *Trypanosoma cruzi* antigens. *Clin Immunol Immunopathol* **1997**; 83:165–72.
43. Reis DD, Jones EM, Tostes S Jr, et al. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor- α ⁺ cells and dominance of granzyme A⁺, CD8⁺ lymphocytes. *Am J Trop Med Hyg* **1993**; 48:637–44.
44. Higuchi MD, Ries MM, Aiello VD, et al. Association of an increase in CD8⁺ T cells with the presence of *Trypanosoma cruzi* antigens in chronic, human, chagasic myocarditis. *Am J Trop Med Hyg* **1997**; 56: 485–9.
45. Brener Z, Gazzinelli RT. Immunological control of *Trypanosoma cruzi* infection and pathogenesis of Chagas' disease. *Int Arch Allergy Immunol* **1997**; 114:103–10.
46. Abel LC, Rizzo LV, Ianni B, et al. Chronic Chagas' disease cardiomyopathy patients display an increased IFN- γ response to *Trypanosoma cruzi* infection. *J Autoimmun* **2001**; 17:99–107.
47. Calzada JE, Nieto A, Beraun Y, Martin J. Chemokine receptor CCR5 polymorphisms and Chagas' disease cardiomyopathy. *Tissue Antigens* **2001**; 58:154–8.