Chemokine CC Receptor 2 Is Important for Acute Control of Cardiac Parasitism but Does Not Contribute to Cardiac Inflammation after Infection with *Trypanosoma cruzi*

Jenny L. Hardison,[1] William A. Kuziel,[3] Jerry E. Manning,[1,2] and Thomas E. Lane[1,2]

[1]Department of Molecular Biology and Biochemistry and [2]Center for Immunology, University of California, Irvine, and [3]Protein Design Labs, Inc., Fremont, California

The CC chemokine ligand 2 (CCL2) and CC chemokine receptor 2 (CCR2) are expressed in the heart after infection with *Trypanosoma cruzi*, suggesting that they play an important role in host defense. Infection of CCR2-deficient ($CCR2^{-/-}$) mice with *T. cruzi* resulted in increased cardiac parasitism, yet the severity of cardiac inflammation was not affected. In addition, expression of interferon-γ and inducible NO synthase in the heart, which are associated with effective killing of trypomastigotes, was not affected in $CCR2^{-/-}$ mice. These observations reveal that CCR2 signaling plays a distinct role that is separate from that of influencing either chemotaxis or previously defined anti-trypomastigote mechanisms for the control of *T. cruzi*'s replication in the heart.

Trypanosoma cruzi is the causative agent of Chagas disease, which is endemic to Central and South America. Currently, 18–20 million people are infected, and >100 million are at risk of infection [1]. Of those infected, ~30% will develop chronic cardiac pathology ⩾10 years after infection. Chronic Chagas disease, or cardiomyopathy, is characterized by inflammatory infiltrates, necrosis, and fibrosis in the heart [1]; inflammatory infiltrates are composed primarily of $CD8^+$ T cells but also contain $CD4^+$ T cells and macrophages [2]. As the disease progresses, the heart becomes enlarged, and, over time, this condition can result in congestive heart failure and sudden death [1, 2]. Therefore, an understanding of the mechanisms participating in promoting and maintaining inflammation in the heart may foster identification of new therapeutic targets in the treatment of chronic Chagas disease.

During the past several years, there has been increased interest in characterizing the expression of proinflammatory chemokine genes in the hearts of *T. cruzi*–infected mice [3–5]. Chemokines and their cognate receptors direct the extravasation of leukocytes and monocytes from the bloodstream into tissues and also regulate the positional migration of these cells in tissues. In addition, in numerous disease models, chemokines have been shown to play a significant role in the influx of cells, where they participate in the control of pathogens and/or contribute to chronic inflammation. Chemokine genes are expressed in the hearts of *T. cruzi*–infected mice, suggesting that chemokines may play a role in host defense and/or disease. In support of this idea, we and others have determined that CXC chemokine ligand 9 (CXCL9) and CXCL10, as well as CC chemokine ligand 5 (CCL5), are detectable in the heart during both acute and chronic cardiomyopathy [3, 5]. When mice are treated with neutralizing antibodies specific for CXCL9 and CXCL10, the result is increased parasitemia, indicating that these chemokines play an important role in generating a protective immune response [5]; however, blocking these chemokines does not ultimately alter the severity of chronic cardiomyopathy, as characterized by both the parasitic burden and chronic inflammation in the heart. One factor controlling the response to chemokine-ligand expression is the corresponding expression of the appropriate chemokine receptor(s). We have recently shown that *T. cruzi*–infected mice lacking CC chemokine receptor 5 (CCR5), which recognizes CCL5, results in an increase in both parasitemia and parasitic burden in the heart, an increase that correlates with a paucity of infiltration of T cells and macrophages [4].

In addition to inducing the directional migration of targeted populations of leukocytes during periods of inflammation in response to infection or injury, chemokines are now recognized to be important in numerous biological processes, one of which is to exert antimicrobial effects against various types of pathogens, including parasites [6]. Indeed, after a series of in vitro studies, Aliberti et al. [7] reported that CCL2 signaling both increased the macrophage uptake of *T. cruzi* and enhanced the

Received 5 December 2005; accepted 9 January 2006; electronically published 26 April 2006.

Potential conflicts of interest: none reported.

Financial support: National Institutes of Health (grant AI18873 to J.E.M. and grants NS41249 and NS18146 to T.E.L.); National Multiple Sclerosis Society (postdoctoral fellowship 1562-A-1 to J.L.H.).

Reprints or correspondence: Dr. Thomas E. Lane, Dept. Molecular Biology and Biochemistry, 3205 McGaugh Hall, University of California, Irvine, Irvine, CA 92697-3900 (tlane@uci.edu).

The Journal of Infectious Diseases 2006; 193:1584–8

0022-1899/2006/19311-0016$15.00

production of NO, which resulted in the killing of this parasite. These observations suggest that, by controlling this parasite's replication, CCL2 may play a role in resistance to infection with *T. cruzi*. Previous studies in our own and other laboratories have demonstrated that, in response to infection with *T. cruzi*, CCL2 is expressed in the heart [3, 5]. In addition, the CCL2 receptor, CCR2, also is expressed in the heart after infection with *T. cruzi*, which suggests that it plays an important role in the CCL2:CCR2 signaling axis, thereby promoting host defense. To characterize the in vivo role that CCL2 signaling plays in leukocyte trafficking, CCR2-deficient ($CCR2^{-/-}$) mice were infected with *T. cruzi*, and both the ability to control this parasite's replication in the heart and the severity of cardiac inflammation were evaluated.

Materials and methods. Female CCR2 wild-type ($CCR2^{+/+}$) mice (B6129F2/J, obtained from The Jackson Laboratory in Bar Harbor, ME), and female $CCR2^{-/-}$ mice ($129B6F2\text{-}Cmkbr2^{tm1Kuz}$), all of which were 6–8 weeks old, were bred and housed in pathogen-free conditions, in enclosed filter-top cages. Mice were infected subcutaneously with 50 blood-derived Colombiana-strain trypomastigotes, and parasitemia was monitored weekly, as described elsewhere [5]. Mice were killed at days 0, 15, 30, 60, 120, and 200 after infection, and their hearts were collected for histological and RNA analysis by RNase protection assay. The hearts were fixed in 10% formalin and were embedded in paraffin, and 5-μm sections were cut in 100-μm increments, for staining with hematoxylin-eosin. Each section was scored for inflammation, by an investigator blinded as to its source; and infected cardiomyocytes were counted according to a method described elsewhere [5]. Significant differences in parasitism were determined by Mann-Whitney *U* test; $P \leq .05$ was considered to be significant.

Total RNA was extracted from hearts by use of TRIzol reagent (Invitrogen). A minimum of 3 mice were evaluated at each examination time. Chemokine, chemokine-receptor, cytokine, and cell-surface marker transcripts were analyzed by use of custom multitemplate probe sets (BD PharMingen), and individual transcripts were normalized to housekeeping gene L32 [4, 5]. Normalized units are obtained by dividing the band intensity of each individual transcript by the band intensity of L32 and are expressed as percentages. Data are expressed as means $\pm$ SD, and significant differences between $CCR2^{+/+}$ mice and $CCR2^{-/-}$ mice, at each examination time, were determined by Student's *t* test; $P \leq .05$ was considered to be significant.

Results and discussion. At days 30–60 after infection, the hearts of *T. cruzi*–infected $CCR2^{+/+}$ mice had detectable mRNA transcripts for both CCL2 and CCR2, suggesting that CCL2 and CCR2 potentially play a role in the regulation of host defense and/or inflammation in the heart (figure 1*A*). Evaluation of parasitemia revealed no difference between $CCR2^{-/-}$ mice ($n = 11$) and $CCR2^{+/+}$ mice ($n = 12$), and all mice in both groups survived to day 200 after infection, the last point at which survival was measured (data not shown). Collectively, these data indicate that (1) CCR2 signaling is not required for control of *T. cruzi*'s replication in the blood and (2) CCR2 is not an important influence in survival. However, at day 30 after infection, cardiac parasitism in $CCR2^{-/-}$ mice was ~6-fold higher than that in $CCR2^{+/+}$ mice (table 1 and figure 1*B*). Infected cardiomyocytes in $CCR2^{-/-}$ mice were often found in close proximity and were frequently quite large, a result that contrasts with what was observed in $CCR2^{+/+}$ mice (figure 1*B*). However, the deficiency in the $CCR2^{-/-}$ mice's control of this parasite's replication in the heart was eventually overcome, and, at days 60–200 after infection, there were no differences between the 2 groups of mice (table 1).

To determine whether the increase in cardiac parasitism in $CCR2^{-/-}$ mice correlated with a muted inflammatory response, the severity of cardiac inflammation was determined by scoring of the hematoxylin-eosin–stained tissues and, by use of the RNase protection assay, examination of the mRNA transcripts for the presence of infiltrating immune cells. As shown in table 1, at no examination time after infection was there any difference between the severity of inflammation in $CCR2^{-/-}$ mice and that in $CCR2^{+/+}$ mice. Consistent with these results is the finding that the mRNA transcript levels for CD4, CD8, and F4/80 detected in the hearts of $CCR2^{+/+}$ mice were similar to those detected in the hearts of $CCR2^{-/-}$ mice (figure 1*C* and *D*). Both interferon (IFN)–γ and inducible NO synthase (iNOS) have been shown to be important in reducing the parasitic burden in infected tissues [2]; in the present study, however, at no examination time after infection were the transcript levels for either IFN-γ or iNOS in $CCR2^{-/-}$ mice different from those in $CCR2^{+/+}$ mice (figure 1*C* and *D*). Similarly, there were no deficiencies in expression of chemokine transcripts in the hearts of $CCR2^{-/-}$ mice, compared with that in the hearts of $CCR2^{+/+}$ mice (figure 1*E*); rather, at days 60 and 120 after infection, expression of CCL5 was increased in $CCR2^{-/-}$ mice during chronic infection. Expression of some chemokine receptors was intermittently reduced in $CCR2^{-/-}$ mice after infection (figure 1*F*); for example, at day 30 after infection, expression of CXCR3 in $CCR2^{-/-}$ mice was ~3-fold lower than that in wild-type control mice, and, at day 60 after infection, expression of CCR5 in these mice was ~5-fold lower than that in wild-type control mice. Evaluation either by histological analysis or on the basis of expression of markers for T cells and macrophages showed that at no examination time after infection was the infiltration of leukocytes into the heart decreased in $CCR2^{-/-}$ mice, a finding that implies that signaling through CCR2 may play a role in both cell activation and expression of other chemokine receptors. The immune response in peripheral tissue also did not appear to be decreased in $CCR2^{-/-}$ mice: at days 30 and 60 after infection, expression of chemokines in the spleen of

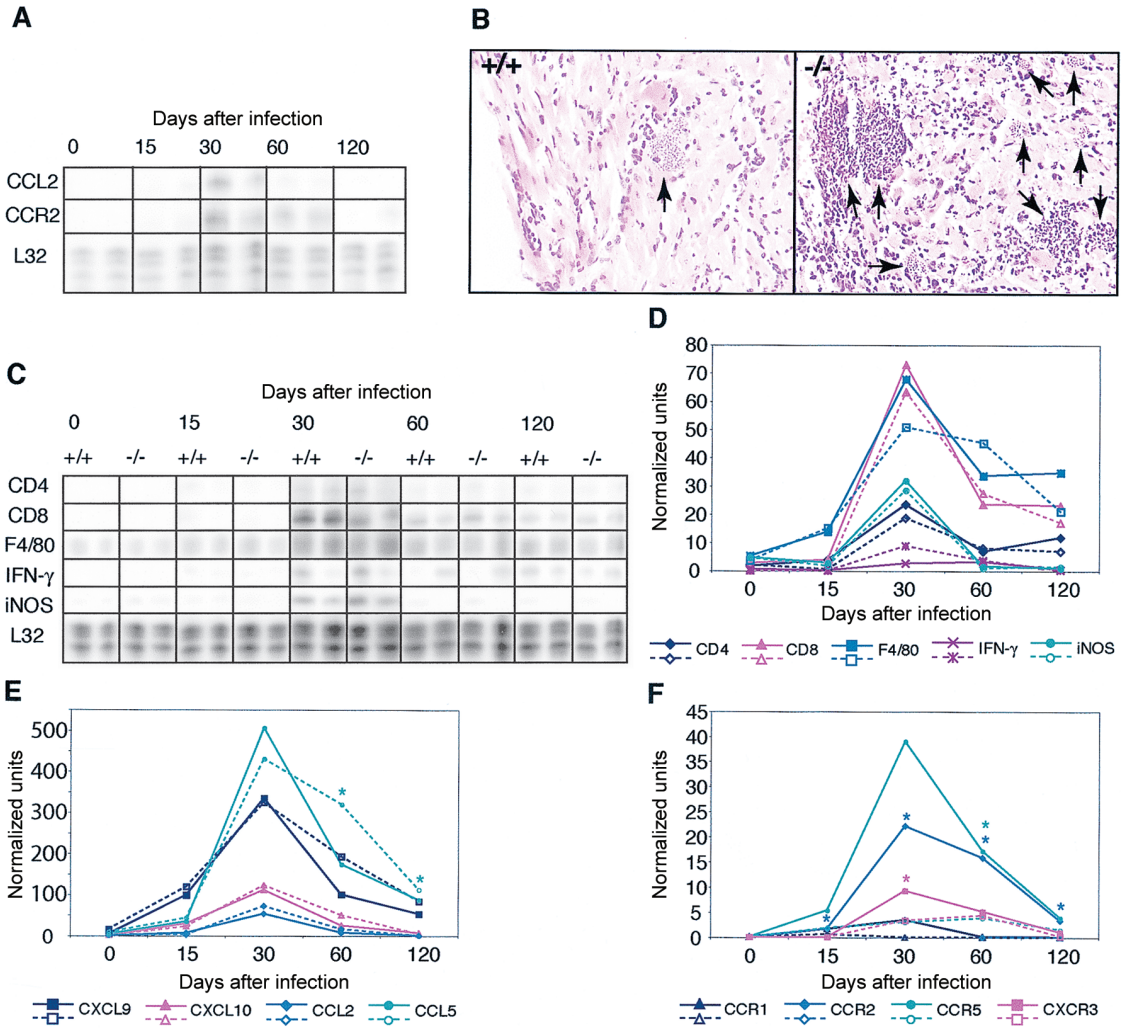

Figure 1. CC chemokine receptor 2 (CCR2)'s enhancement of host defense after infection with *Trypanosoma cruzi*. *A,* Expression of chemokine mRNA and chemokine-receptor mRNA, as determined on the basis of RNase protection assay (RPA), in the hearts of CCR2 wild-type ($CCR2^{+/+}$) mice infected with *T. cruzi*. CCR2 is expressed at days 30 and 60 after infection; its ligand, CC chemokine ligand 2 (CCL2), is expressed at day 30 after infection. *B,* Infected cardiomyocytes in the heart, primarily at day 30 after infection. Infected cells are more numerous and larger in CCR2-deficient ($CCR2^{-/-}$) mice (−/−) than in $CCR2^{+/+}$ mice (+/+). *C,* Analysis of mRNA transcripts for T cell (CD4 and CD8) and macrophage markers (F4/80), interferon-γ (IFN–γ), and inducible NO synthase (iNOS), by RPA. The levels in the hearts of $CCR2^{+/+}$ mice are similar to those in the hearts of $CCR2^{-/-}$ mice. *D,* Average expression of each transcript, normalized as a percentage of housekeeping gene L32. Results in $CCR2^{+/+}$ mice are represented by solid lines; those in $CCR2^{-/-}$ mice are represented by dotted lines. *E* and *F,* Expression of chemokine *(E)* and chemokine-receptor *(F)* mRNA transcripts in the heart, on the basis of RPA. Results in $CCR2^{+/+}$ mice are represented by solid lines; those in $CCR2^{-/-}$ mice are represented by dotted lines. A minimum of 3 animals were evaluated at each examination time. An asterisk (*) indicates that the average expression in $CCR2^{-/-}$ mice is significantly different from that in $CCR2^{+/+}$ mice ($P \leq .05$; Student's *t* test).

$CCR2^{-/-}$ mice was increased, compared with that in the spleen of $CCR2^{+/+}$ mice; however, at days 15, 30, 60, and 120 after infection, expression of chemokine receptors was similar in both groups of mice (data not shown). Collectively, these data (1) demonstrate that CCR2 neither enhances immune-cell infiltration into the heart nor influences antiparasitic effector responses such as expression of either IFN-γ or iNOS and (2) imply that CCR2 is important in the control of *T. cruzi*'s replication in the heart. Therefore, the mechanism by which CCR2 controls this parasite's replication is not dependent on the regulation of cardiac inflammation.

CCR2 is the primary receptor for CCL2 and is found on monocytes, activated T cells, B cells, and NK cells [8]. Recent studies have indicated that, in response to stimuli or infection, $CCR2^{-/-}$ mice display deficiencies in trafficking of T cells and macrophages [9, 10]. Previous studies have also demonstrated that CCL2 can activate T cells and macrophages, presumably through binding to CCR2 expressed on the surface of these

Table 1. Cardiac inflammation and tissue parasitism.

Mouse strain, days after infection	Average inflammation score, mean ± SD		Tissue parasitism, mean ± SE, no. of infected cells/100 mm^2
	Atrium	Ventricle	
CCR2$^{+/+}$			
0 (n = 3)	0.67 ± 1.15	0.67 ± 1.15	0.00
15 (n = 5)	2.00 ± 0.00	2.00 ± 0.00	0.00
30 (n = 5)	4.00 ± 0.00	3.60 ± 0.55	11.33 ± 2.94
60 (n = 3)	2.67 ± 1.53	3.00 ± 1.00	1.06 ± 1.06
120 (n = 4)	2.75 ± 0.96	3.00 ± 0.00	0.00
200 (n = 3)	2.67 ± 0.58	2.67 ± 0.58	0.00
CCR2$^{-/-}$			
0 (n = 3)	1.33 ± 0.58	2.00 ± 0.00	0.00
15 (n = 5)	2.00 ± 0.00	2.00 ± 0.00	0.92 ± 0.92
30 (n = 8)	3.88 ± 0.35	3.75 ± 0.46	75.05 ± 33.61[a]
60 (n = 4)	3.33 ± 0.58	3.67 ± 0.58	1.40 ± 0.70
120 (n = 3)	2.00 ± 0.00	2.33 ± 0.58	0.00
200 (n = 4)	3.25 ± 0.50	3.00 ± 0.00	0.00

NOTE. CCR2$^{+/+}$, positive for CC chemokine receptor 2; CCR2$^{-/-}$, negative for CC chemokine receptor 2.

[a] Significantly different ($P \leq .05$; Mann-Whitney U test) from CCR2$^{+/+}$, at the same time after infection.

cells [10, 11]. Indeed, leukocyte populations from CCR2$^{-/-}$ mice do not bind CCL2 and exhibit defects both in activation and in recruitment to sites of inflammation [9]. Infection of CCR2$^{-/-}$ mice with various microbial pathogens has increased our understanding of how this receptor contributes to immunological events involved in host protection; for example, CCR2$^{-/-}$ mice with lungs infected with *Cryptococcus neoformans* exhibit a decreased protective Th1 response accompanied by a switch to a strong Th2 response [12]. This switch in Th responses correlates with both prolonged infection and the infecting agent's greater dissemination throughout the body. Analysis of CCR2$^{-/-}$ mice either infected with *Leishmania donovani* or immunized with shistosomal antigen has revealed an impaired cellular immune response characterized both by reduced T cell and macrophage recruitment to sites of antigen localization and by decreased expression of IFN-γ [11, 13]. Collectively, these studies strongly suggest that expression of CCR2 is required for the development of protective Th1 responses after microbial challenge. The findings of the present study indicate that, in *T. cruzi*–infected mice, the absence of CCR2 does not decrease expression of IFN-γ (figures 1*C* and *D*); nor were there increased numbers of transcripts for the Th2-associated cytokines IL-4 and IL-10 (data not shown). Therefore, it is unlikely that the increased parasitic burden in the hearts of CCR2$^{-/-}$ mice is a result of a muted Th1 response accompanied by increased expression of Th2-associated cytokines.

Alternately, differential expression of chemokine receptors in CCR2$^{-/-}$ mice may contribute to the inability to control *T. cruzi*'s replication in the heart during acute infection, in light of in vitro studies that have shown that chemokines play a role in promoting the control of this parasite's replication [7, 14] (figure 1*F*). Chemokines such as CCL2 and CXCL10 increase nitrite production by infected cardiomyocytes to a level that is sufficient for control of this parasite's replication [14]. Moreover, by virtue of their enhancement of both production of NO and phagocytosis of trypomastigotes, CCL2, CCL3, CCL4, and CCL5 have been implicated in promoting the trypanocidal activities of macrophages, with CCL2 being the most potent chemokine promoting these activities [7]. In addition to promoting these trypanocidal activities, CCL2 may also be important in promoting the effective survival responses of cardiomyocytes. Indeed, in a model of myocardial trauma, CCL2 signaling through CCR2 has been found to be associated with increased cardiomyocyte survival [15]. These findings highlight the possibility that CCR2 plays a previously unappreciated role in host defense after infection with *T. cruzi*.

The results of the present study show that the contribution that CCR2 makes to host defense is transient and is limited to the acute stages of infection, which coincide both with increased expression of CCL2 and with cardiac parasitism (table 1 and figure 1*A*). This finding suggests that, during this time, CCR2 is used to inhibit the spread of parasites and to promote the killing of these parasites in the heart. Potential mechanisms by which CCR2 signaling enhances tissue-specific host defense may include regulation of phagocytosis of trypomastigotes and/or control of *T. cruzi*'s intracellular replication, possibly through

the sequestering of required growth factors. Ongoing studies are under way to further characterize the mechanisms by which CCR2 promotes the killing of this parasite.

References

1. Tanowitz HB, Kirchhoff LV, Simon D, Morris SA, Weiss LM, Wittner M. Chagas' disease. Clin Microbiol Rev **1992**; 5:400–19.
2. Brener Z, Gazzinelli RT. Immunological control of Trypanosoma cruzi infection and pathogenesis of Chagas' disease. Int Arch Allergy Immunol **1997**; 114:103–10.
3. Talvani A, Ribeiro CS, Aliberti JC, et al. Kinetics of cytokine gene expression in experimental chagasic cardiomyopathy: tissue parasitism and endogenous IFN-gamma as important determinants of chemokine mRNA expression during infection with Trypanosoma cruzi. Microbes Infect **2000**; 2:851–66.
4. Hardison JL, Wrightsman RA, Carpenter PM, Kuziel WA, Lane TE, Manning JE. The CC chemokine receptor 5 (CCR5) is important in control of parasite replication and acute cardiac inflammation following infection with Trypanosoma cruzi. Infect Immun **2006**; 74:135–43.
5. Hardison JL, Wrightsman RA, Carpenter PM, Lane TE, Manning JE. The chemokines CXCL9 and CXCL10 promote a protective immune response but do not contribute to cardiac inflammation following infection with Trypanosoma cruzi. Infect Immun **2006**; 74:125–34.
6. Kahn IA, MacLean JA, Lee FS, et al. IP-10 is critical for effector T cell trafficking and host survival in Toxoplasma gondii infection. Immunity **2000**; 12:483–94.
7. Aliberti JC, Machado FS, Souto JT, et al. beta-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.
8. Mack M, Cihak J, Simonis C, et al. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. J Immunol **2001**; 166:4697–704.
9. Kuziel WA, Morgan SJ, Dawson TC, et al. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. Proc Natl Acad Sci USA **1997**; 94:12053–8.
10. Chen BC, Kuziel WA, Lane TE. Lack of CCR2 results in increased mortality and impaired leukocyte activation and trafficking following infection of the central nervous system with a neurotropic coronavirus. J Immunol **2001**; 167:4585–92.
11. Sato N, Kuziel WA, Melby PC, et al. Defects in the generation of IFN-g are overcome to control infection with *Leishmania donovani* in CC chemokine receptor (CCR) 5-, macrophage inflammatory protein-1-a-, or CCR2-deficient mice. J Immunol **1999**; 163:5519–25.
12. Traynor TR, Kuziel WA, Toews GB, Huffnagle GB. CCR2 expression determines T1 versus T2 polarization during pulmonary *Cryptococcus neoformans* infection. J Immunol **2000**; 164:2021–7.
13. Warmington KS, Boring L, Ruth JH, et al. Effect of C-C chemokine receptor 2 (CCR2) knockout on type-2 (schistosomal antigen-elicited) pulmonary granuloma formation: analysis of cellular recruitment and cytokine responses. Am J Pathol **1999**; 154:1407–16.
14. 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.
15. Tarzami ST, Cheng R, Miao W, Kitsis RN, Berman JW. Chemokine expression in myocardial ischemia: MIP-2 dependent MCP-1 expression protects cardiomyocytes from cell death. J Mol Cell Cardiol **2002**; 34:209–21.