

Pivotal Role of Interleukin-12 and Interferon- γ Axis in Controlling Tissue Parasitism and Inflammation in the Heart and Central Nervous System during *Trypanosoma cruzi* Infection

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The role of cytokines in the control of tissue parasitism and pathogenesis of experimental Chagas' disease was investigated. Wild-type and different cytokine as well as inducible nitric oxide synthase (iNOS) knockout mice were infected with the Colombian strain of *Trypanosoma cruzi*, and the kinetics of tissue parasitism, inflammatory reaction, parasitemia, and mortality were determined. We demonstrate the pivotal role of the interleukin (IL)-12/interferon (IFN)- γ /iNOS axis and the antagonistic effect of IL-4 in controlling heart tissue parasitism, inflammation, and host resistance to acute infection with *T. cruzi*. Further, the heart and central nervous system were shown the main sites of reactivation of *T. cruzi* infection in mice lacking functional genes for IFN- γ and IL-12, respectively. Our results also show that in contrast to IFN- γ knockout (KO) mice, splenocytes from IL-12 KO mice infected with *T. cruzi* produced low levels of IFN- γ upon stimulation with antigen. Consistently, high levels of anti-*T. cruzi* IgG2a antibodies were detected in the sera from IL-12 KO, but not from IFN- γ KO mice, infected with the Colombian strain of *T. cruzi*. Thus, our results suggest that the level of IFN- γ deficiency is a major determinant of the site of reactivation of *T. cruzi* infection in immunocompromised host. (Am J Pathol 2001, 159:1723–1733)

Trypanosoma cruzi is the etiological agent of Chagas' disease.^{1,2} In humans, the acute infection with *T. cruzi* lasts for 2 to 4 months and is characterized by the presence of parasites in the blood stream and different host tissues as well as variable degrees of nonspecific symptoms and myocarditis.^{3,4} After development of immunity,

although somewhat persistent,⁵ both tissue parasitism and myocarditis are controlled and most symptoms of acute *T. cruzi* infection are resolved. However, 10 to 20 years after the initial infection, associated with increased tissue parasitism and inflammation,^{6–8} the digestive and/or cardiac forms of the disease become symptomatic in 10 to 30% of the chronic chagasic patients.^{9,10} Furthermore, immunodeficiency induced by either treatment with immunosuppressive drugs or infection with HIV-1 leads to reactivation of *T. cruzi* infection mainly in the central nervous system (CNS).^{11–13}

The symptoms presented by patients with either acute or chronic Chagas' disease are highly variable, and thought to be dependent on both parasite and host-related factors.^{3–14} Similarly, the pathology observed in mice infected with *T. cruzi* is highly dependent on parasite strain,^{15–17} size of parasite inoculum,¹⁸ host genetic background,^{19,20} and the action of various components of host immune system.^{10,14} Different studies indicate the crucial role of cytokines, such as interferon (IFN)- γ ,²¹ tumor necrosis factor- α ,²² and interleukin (IL)-12²³ as well as nitric oxide^{24–26} in host resistance to infection with *T. cruzi*. Soon after the first rounds of replication in the vertebrate host tissues, *T. cruzi* parasites are thought to trigger the synthesis of proinflammatory cytokines that initiate the production of IFN- γ by natural killer (NK) cells,²⁷ CD4⁺CD8⁻ $\alpha\beta$ ⁺ and CD4⁻CD8⁺ $\alpha\beta$ ⁺ T lymphocytes.²⁸ IFN- γ combined with tumor necrosis factor- α will activate macrophages to produce high levels of nitric oxide that are primarily responsible for control of parasite replication during the acute phase of infection.²²

However, scanty information is available regarding the role of cytokines controlling parasite replication and pathology in the heart tissue and CNS, the main sites of parasite replication and pathology during Chagas' dis-

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ease in immunocompetent and immunodeficient hosts, respectively. The lack of such information is mainly because various studies using the experimental murine model of Chagas' disease used parasite strains with low tropism to the cardiac tissue and CNS. Secondly, most studies using genetically engineered immunodeficient mice infected with *T. cruzi*, have limited their evaluation to measurements of parasitemia and mortality rates. The present study was undertaken to determine the role of different cytokines on the kinetics of tissue parasitism and inflammatory cell infiltrates in the heart tissue and CNS of mice infected with the Colombian strain, a *T. cruzi* strain with high tropism for the cardiomyocytes and previously shown to induce encephalitis in susceptible mice.²⁹⁻³¹ Our results show the essential role of IL-12/IFN- γ /inducible nitric oxide synthase (iNOS) axis, and the antagonistic effect of IL-4, in regulating parasite replication in the heart tissue and host resistance to *T. cruzi* during acute phase of infection. Moreover, we demonstrate that the deficiency of endogenous IL-12 and IFN- γ is determinant of the reactivation of *T. cruzi* infection in CNS and heart tissues, respectively. Finally, this study provides the first experimental model that resembles the parasite-elicited mass encephalitis, which is often found in chronic chagasic patients with AIDS, and contributes with new understanding of the pathogenesis of *T. cruzi* infection in immunodeficient hosts.

Materials and Methods

Animals

Female BALB/c and C57BL/6 mice were obtained from the Oswaldo Cruz Foundation-FIOCRUZ (Rio de Janeiro, Brazil). The IL-4-KO in the BALB/c genetic background, IFN- γ -KO, IL-12-KO, and iNOS-KO mice all in the C57BL/6 genetic background were provided by the Laboratory of Gnotobiology, ICB-UFMG (Belo Horizonte, Brazil). Female mice, 6 to 8 weeks of age, were maintained under standard conditions with environmental barriers in the animal house of the Centro de Pesquisas René Rachou, FIOCRUZ (Belo Horizonte, Brazil), and used for the experiments described below.

Parasites and Experimental Infection

The Colombian strain of *T. cruzi* was isolated by Frederici and colleagues³¹ and maintained by serial passages from mouse to mouse in the Laboratory of Chagas' Disease, CPqRR-FIOCRUZ (Belo Horizonte, Brazil). BALB/c, C57BL/6, and different KO mice were infected intraperitoneally with 5000 blood trypomastigote forms of the Colombian strain of *T. cruzi*. In some experiments, the animals were treated with a subcurative dose of benznidazole (100 mg/kg/day) from day 10 to day 17 after infection. The levels of parasitemia were evaluated using 5 μ l of blood in an optical microscope as previously described.³² All experiments were repeated three times and conducted according to the institutional guidelines for animal ethics of the Oswaldo Cruz Foundation.

Histological Evaluation

The myocardium and CNS were fixed in neutral 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Tissue parasitism was scored by counting the total number of amastigote nests in 25 microscope fields (1×200 magnification) per histopathological section. Four sections were counted for each animal and the individual data determined as the mean result of the four sections.

An inflammatory infiltrate was considered when we detected 50 leukocytes or more in each inflammatory area. The inflammatory infiltrate was subdivided into focal and diffuse, depending on how close the inflammatory cells were associated. Most cells from the focal inflammatory infiltrate were in direct contact with each other, forming a continuous site of inflammation. In contrast, the diffuse inflammatory infiltrate was defined as a high density of mononuclear cells scattered throughout the cardiac or nervous tissue or composed of one or more small inflammatory foci containing less than 50 inflammatory cells. Considering that we found a high positive correlation between the intensities of focal and diffuse inflammatory infiltrates, for the sake of simplicity the results are presented as the average of the intensity of focal and diffuse inflammatory infiltrates. For the inflammatory infiltrate score, the total numbers of focal or diffuse inflammatory foci were counted in 25 microscope fields (1×200 magnification) per tissue section. Four sections were counted for each animal and individual data determined as the mean result of the four sections.

For both tissue parasitism and inflammation scores, the quantification was performed in four noncontiguous sections (40- μ m distance between them) in 25 fields (1×200) magnification in a blind manner by three researchers.

Parasite-Specific Immunocytochemistry in the Cardiac Tissue and CNS from Infected Mice

For immunocytochemistry, deparaffinized sections were incubated for 30 minutes at 37°C in 1% bovine serum albumin to reduce nonspecific binding and then incubated in immune anti-*T. cruzi* mouse serum diluted 1:300 at 4°C overnight. Secondary biotinylated antibodies were goat anti-mouse antibodies. The sensitivity was improved with the avidin-biotin technique (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, CA). The reaction was visualized by incubating the section with 3,3'-diaminobenzidine tetrahydrochloride (Amresco, Solon, OH).

Parasite Antigen Preparations

The epimastigote forms of the Colombian strain of *T. cruzi* were grown at 28°C in cell-free liver infusion tryptose medium supplemented with 10% fetal calf serum. Live parasites were harvested, washed three times in phosphate-buffered saline (PBS), resuspended at a final concentration of 10^9 parasites/ml and then submitted into three cycles of freeze and thawing at -70°C and 37°C,

respectively, followed by five 30-second rounds of sonication. The resulting extract was centrifuged at $10,000 \times g$ for 30 minutes and the supernatant, named soluble *T. cruzi* antigens (TcAg), frozen at -70°C until use in the stimulation of cytokine synthesis by spleen cells or enzyme-linked immunosorbent assay assays to measure serum levels of anti-*T. cruzi* antibodies.

Quantification of Parasite-Specific IgG1 and IgG2a

A Maxsorp enzyme-linked immunosorbent assay plates (Nalge Nunc International, Rochester, NY) were covered overnight at 4°C with $10 \mu\text{g}/\text{ml}$ of TcAg in carbonate-bicarbonate buffer ($0.1 \text{ mol}/\text{L}$, $\text{pH } 9.6$). Serum from individual animals were diluted 1:40 in PBS-0.05% Tween 20 (PBS-Tween) and incubated for 1 hour at 37°C after blocking with 1% albumin in carbonate-bicarbonate buffer for 2 hours at 37°C . One hundred μl of 1:1000 of nonconjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) diluted in PBS-Tween was added to each well and the plate incubated for 1 hour at 37°C . For the detection of anti-IgG1 and IgG2a antibodies, a peroxidase-conjugated anti-goat IgG (1:5000) was incubated for 1 hour at 37°C . The assay was developed using the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (Sigma Chemical Co., St. Louis, MO), and the reaction was stopped with $20 \mu\text{l}$ of 20% sulfuric acid solution.

Spleen Cell Preparations and IFN- γ Assay

Spleens were removed aseptically and single-cell suspensions were prepared in complete medium after lysis of red cells. Splenocytes were then cultured in the presence of medium alone, with $20 \mu\text{g}$ of TcAg, or $5 \mu\text{g}/\text{ml}$ Con-A. After incubation for 72 hours, supernatants were removed and assayed for IFN- γ .²⁹ IFN- γ was assayed in a two-site enzyme-linked immunosorbent assay using a rat anti-IFN- γ mAb R46A2 (ATCC, Rockville, MD) and a polyclonal rabbit serum specific for the cytokine. IFN- γ levels were calculated by reference to a standard curve constructed with recombinant cytokine (Genzyme, Cambridge, MA). Sensitivity of this method was $100 \text{ pg}/\text{ml}$.

Statistical Analysis

Arithmetic means (parasitemia, amastigote nests, inflammatory foci, and antibody levels) and standard deviations of the means were calculated. The Student's *t*-test was used to determine the statistical significance of the differences observed. Differences were considered statistically significant when $P < 0.05$. The Kruskal-Wallis test was used to compare the mouse survival rates, and differences were considered statistically significant when $P < 0.05$.

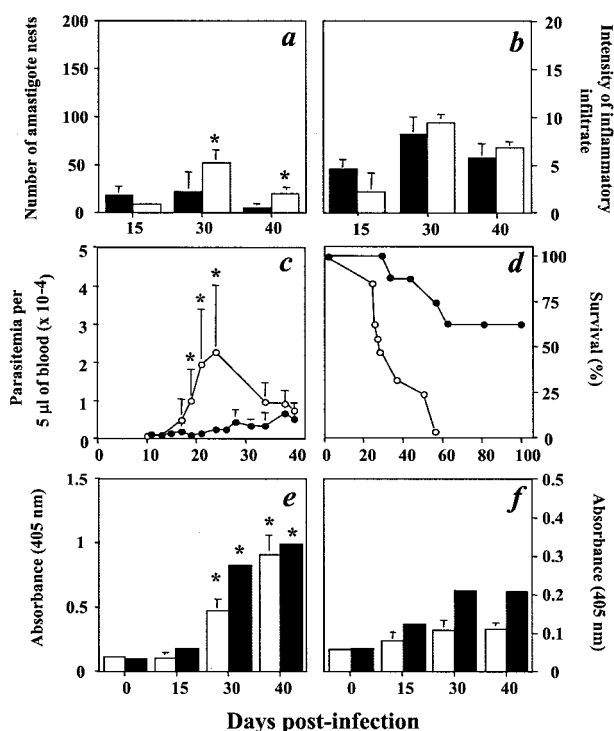


Figure 1. Course of infection with the Colombian strain of *T. cruzi* in C57BL/6 and BALB/c mice. **a:** The number of amastigote nests in 25 histopathological fields (original magnification, 1×200) of cardiac sections obtained from either C57BL/6 (■) or BALB/c (□) mice at different times after infection. **b:** Number of inflammatory infiltrates present in 25 histopathological fields (original magnification, 1×200) of cardiac sections obtained from C57BL/6 (■) and BALB/c (□) mice at different times after infection. **c** and **e:** The parasitemia (**c**) and survival (**d**) curves of either C57BL/6 (—●—) or BALB/c (—○—) mice infected with the Colombian strain of *T. cruzi* are shown. The serum levels of *T. cruzi*-specific IgG1 (□) and IgG2a (■) isotypes are shown for both BALB/c (**e**) and C57BL/6 (**f**) mice. Each value is the average and SD of at least four animals. Similar results were obtained in three experiments. One asterisk indicates that differences are statistically significant ($P < 0.01$), when comparing different parameters from C57BL/6 and BALB/c mice obtained at the same time after infection. The differences between survival rates of C57BL/6 and BALB/c mice were considered statistically significant ($P < 0.01$).

Results

Comparison of BALB/c and C57BL/6 Mice in Resistance/Susceptibility to Infection with the Colombian Strain of *T. cruzi*

The tissue parasitism (Figure 1a, and Figure 2, D and E), parasitemia (Figure 1c), and rate of mortality (Figure 1d) were all higher in the BALB/c as compared to C57BL/6 mice infected with *T. cruzi*. In contrast, the intensity and the kinetics of diffuse and focal inflammatory infiltrates were similar in the heart tissue of both isogenic mouse strains infected with *T. cruzi* parasites (Figure 1b and Figure 2, D and E). Very rare isolated amastigote nests and no or only mild inflammation were found in the CNS of either BALB/c or C57BL/6 mice infected with the Colombian strain of *T. cruzi* (data not shown). The serum levels of both parasite-specific IgG1 and IgG2a isotypes were higher in BALB/c (Figure 1e) than in C57BL/6 (Figure 1f) mice. Further, the IgG1/IgG2a ratio was also

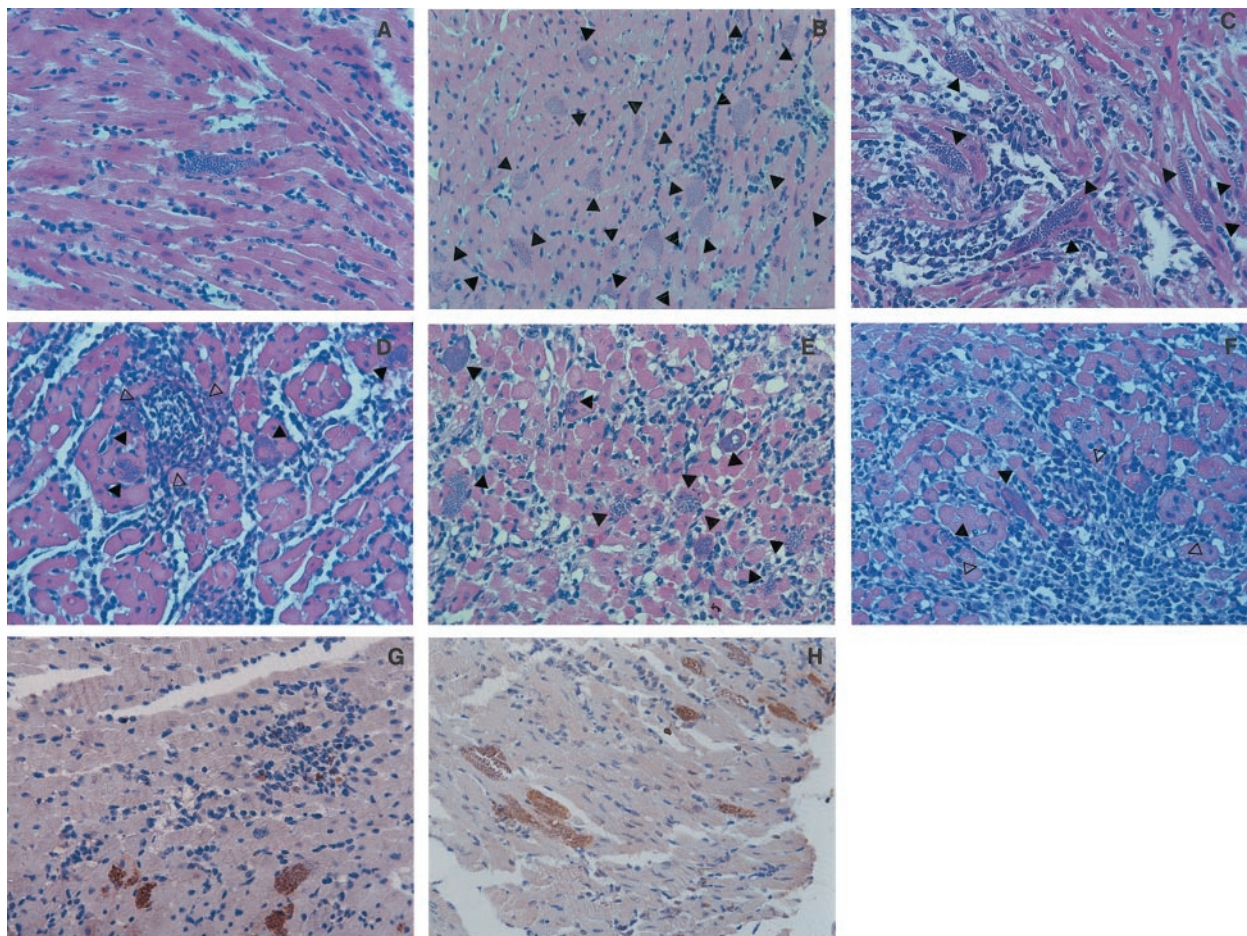


Figure 2. Illustration of *T. cruzi* parasitism and inflammation in cardiac tissue from WT and different KO mice infected with the Colombian strain of *T. cruzi*. **A:** One amastigote nest and mild inflammation is shown in one field of cardiac tissue from a C57BL/6 mouse at 15 days after infection (H&E; original magnification, 1×285). **B:** Twenty amastigote nests (**closed arrow**) and mild inflammation are shown in one field of cardiac tissue from an IFN- γ KO mouse at 15 days after infection (H&E; original magnification, 1×565). **C:** Seven amastigote nests (**closed arrow**) and intense diffuse inflammatory reaction are shown in one field of cardiac tissue from an iNOS KO mouse at 15 days after infection (H&E; original magnification, 1×285). **D:** Four amastigote nests (**closed arrow**) and intense diffuse as well as focal inflammatory reactions (**open arrows**) are shown in one field of cardiac tissue from a C57BL/6 mouse at 30 days after infection (H&E; original magnification, 1×285). **E:** Nine amastigote nests (**closed arrow**) and intense diffuse inflammatory reaction are shown in one field of cardiac tissue from a BALB/c mouse at 30 days after infection (H&E; original magnification, 1×285). **F:** Two amastigote nests (**closed arrow**) and intense diffuse as well as focal inflammatory (**open arrows**) reactions are shown in one field of cardiac tissue from an IL-4 KO mouse at 30 days after infection (H&E; original magnification, 1×285). Parasite-specific immunocytochemistry (brown staining) in cardiac tissue from IL-12 KO mouse at 50 days after infection (**G**) and IFN- γ KO mouse at 40 days after infection (**H**) with the Colombian strain of *T. cruzi* and treated with subcurative dose of benznidazole.

higher in BALB/c as compared to C57BL/6 mice infected with the Colombian strain of *T. cruzi*.

IL-4 Knockout (KO) Mice Are More Resistant to Infection with the Colombian Strain of T. cruzi

Because the BALB/c mice are normally high producers of IL-4, which has been shown to antagonize various IFN- γ -mediated protective immune responses in various systems,³³ we evaluated the course of infection with the Colombian strain of *T. cruzi* in IL-4-KO BALB/c mice. Accordingly, IL-4 KO mice were more resistant to *T. cruzi* infection as indicated by heart tissue parasitism (Figure 2, E and F, and Figure 3a), parasitemia (Figure 3c), and lower mortality ratio (Figure 3d). No major differences were observed in the intensity of diffuse and focal inflammation in the heart tissue when comparing IL-4 KO and wild-type (WT) animals (Figure 2, E and F, and Figure 3b).

The histopathology sections presented in Figure 2, E and F, show an intense inflammatory reaction in the heart tissue of a BALB/c and an IL-4 KO mouse at 30 days after infection containing various and few amastigote nests, respectively. Figure 3e shows that the levels of anti-*T. cruzi*-specific IgG1 antibodies were reduced in sera of IL-4 KO mice infected with *T. cruzi*. In contrast, no alteration in the serum levels of parasite-specific IgG2a antibodies was observed when comparing IL-4 KO (Figure 3e) and WT BALB/c (Figure 3f) mice infected with *T. cruzi*.

IL-12 KO, IFN- γ KO, or iNOS KO Are Highly Susceptible to Infection with the Colombian Strain of T. cruzi

We also infected IL-12 and IFN- γ C57BL/6 KO mice with the Colombian strain of *T. cruzi*. Our results show that

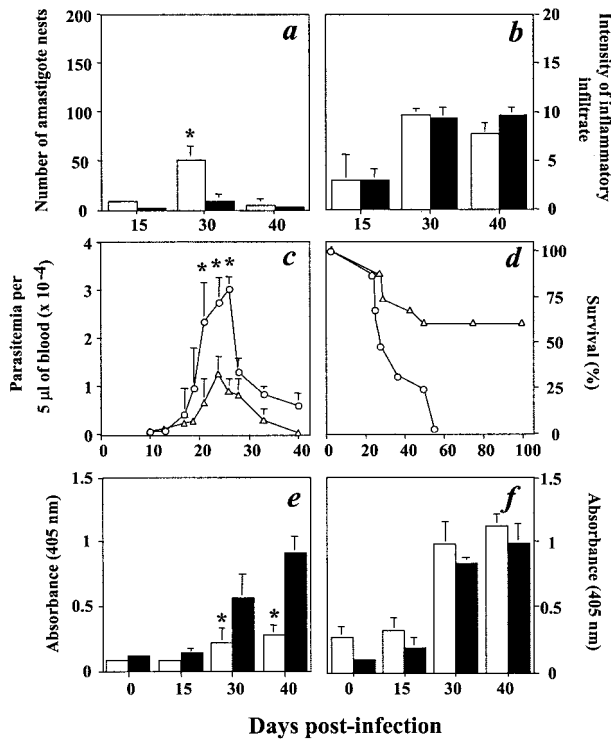


Figure 3. Course of infection with Colombian strain of *T. cruzi* in BALB/c and IL-4 KO mice. **a:** The number of amastigote nests in 25 histopathological fields (original magnification, 1×200) of cardiac sections obtained from either BALB/c (\square) or IL-4 KO (\blacksquare) mice at different times after infection. **b:** The number of inflammatory infiltrates present in 25 histopathological fields (original magnification, 1×200) of cardiac sections obtained from BALB/c (\square) or IL-4 KO (\blacksquare) mice at different times after infection. **c** and **d:** Shown are the parasitemia (**c**) and survival (**d**) curves in either BALB/c ($\text{---}\circ\text{---}$) or IL-4 KO ($\text{---}\triangle\text{---}$) mice infected with the Colombian strain of *T. cruzi*. The serum levels of *T. cruzi*-specific IgG1 (\square) and IgG2a (\blacksquare) isotypes are shown for both IL-4 KO (**e**) and BALB/c (**f**) mice. Each value is the average and SD of at least four animals. Similar results were obtained in three experiments. One asterisk indicates that differences are statistically significant ($P < 0.01$), when comparing different parameters from IL-4 KO and BALB/c mice obtained at the same time after infection. The differences between survival rates of IL-4 KO and BALB/c mice were considered statistically significant ($P < 0.01$).

both IFN- γ KO and IL-12 KO were highly susceptible to infection displaying a high number of amastigote nests (Figure 4a), parasitemia (Figure 4c), and 100% of mortality by 20 days after infection (Figure 4d). In contrast to the intensity of tissue parasitism, we observed limited inflammatory reaction in the heart tissue from IFN- γ KO and IL-12 KO, at 15 days after infection (Figure 4b). No enhanced tissue parasitism and/or inflammation in the CNS was observed, when comparing IFN- γ KO and IL-12 KO to WT mice acutely infected with the Colombian strain of *T. cruzi* (data not shown).

Enhanced susceptibility was also observed in iNOS KO mice that presented higher tissue parasitism (Figure 4e), parasitemia (Figure 4g), and accelerated mortality (Figure 4h) as compared to C57BL/6 mice. These results differed from those obtained from IL-12 KO and IFN- γ KO, in that the iNOS KO survived more than 30 to 40 days of infection presenting the peak of heart tissue parasitism (Figure 4e) and parasitemia (Figure 4g) at day 30 after infection. Differences were observed in the intensity of diffuse and focal inflammation in the cardiac tissue, when

comparing iNOS KO and WT other KO mice at 15 days after infection, but not at the latter time point.

The histopathology sections show one amastigote nest in the cardiac tissue from C57BL/6 mice (Figure 2A) contrasting with multiple amastigote nests in the heart from IFN- γ KO (Figure 2B) and iNOS KO (Figure 2C) mice, all at 15 days after infection. Note mild inflammation observed in IFN- γ KO mice, contrasting with more intense inflammation in iNOS KO mice (Figure 2C).

Heart Tissue and CNS as the Major Sites of Reactivation of *T. cruzi* Infection in IFN- γ KO and IL-12 KO Mice

We next tested the role of endogenous IFN- γ , IL-12, and iNOS in the control of tissue parasitism, parasitemia, and mortality in mice that have latent infection with the Colombian strain of *T. cruzi*. WT and the various KO mice were infected and submitted to treatment with benznidazole, beginning at 10 days after infection, soon after parasitemia become patent. The mice received 100 mg/kg/day of benznidazole from days 10 to 17 after infection. This therapeutic protocol, although highly efficient in controlling tissue parasitism and parasitemia, is not curative for mice infected with the Colombian strain of *T. cruzi*. After cessation of chemotherapy, the mice were followed for tissue parasitism, parasitemia, and mortality.

Benznidazole therapy resulted in 100% and 80% of survival (and no measurable parasitemia) in WT and iNOS KO mice, respectively. In contrast, greater parasitemia (Figure 5a) and accelerated mortality (Figure 5b) became apparent in the IFN- γ KO and IL-12 KO mice were after cessation of chemotherapy. Although increased, the parasitemia (Figure 5a) and heart tissue parasitism (Figure 5c and Figure 2G and 2 hours) of IL-12 KO was relatively small when compared to that observed in the IFN- γ KO mice. One hundred percent mortality was observed at 40 and 55 days after infection of IFN- γ KO and IL-12 KO mice, respectively (Figure 5b). The small increase in tissue parasitism observed in IL-12 KO mice was accompanied by a dramatic increase in heart tissue inflammation at day 40 after infection and after (Figure 5e). In contrast, a weak inflammatory process was observed in the heavily parasitized heart tissue from IFN- γ KO mice at 40 days after infection or earlier (Figure 5e). The WT and iNOS KO mice were relatively resistant to reactivation of infection up to 100 days after infection (Figure 5, a and b). These findings are in agreement with those published by Saefel and colleagues³⁴ showing that mouse treatment with iNOS inhibitors does not change the course of late acute and chronic infection with *T. cruzi*.

The extensions of CNS tissue damage, probably the main cause of death in IL-12 KO mice infected with the Colombian strain of *T. cruzi* and treated with benznidazole, were analyzed by histopathology and immunocytochemistry. From day 45 after infection IL-12 KO mice start showing signs of neurological disturbances, marked by complete or partial hind limb paralysis as well as unbalanced movements. Consistently, the tissue parasitism (Figure 5d and Figure 6, E and F), inflammation (Figures

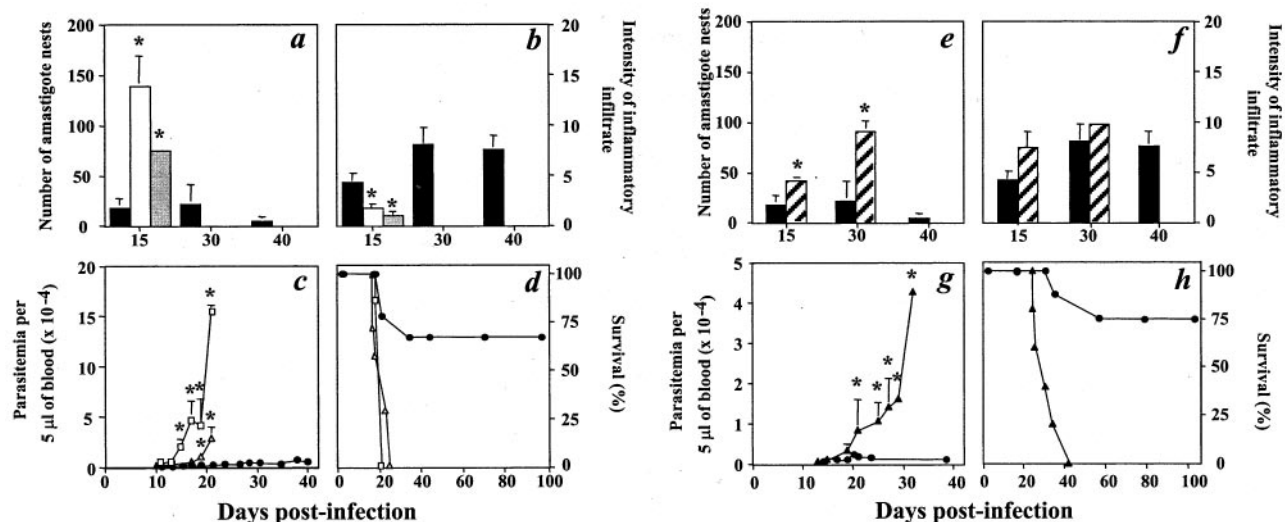


Figure 4. Course of infection with the Colombian strain of *T. cruzi* in C57BL/6, IFN- γ KO, IL-12 KO, and iNOS KO mice. **a:** The number of amastigote nests in 25 histopathological fields (original magnification, 1×200) of cardiac sections obtained from either C57BL/6 (■), IFN- γ KO (□), or IL-12 KO (▤) mice at different times after infection. **b:** The number of inflammatory infiltrates present in 25 histopathological fields (original magnification, 1×200) of cardiac sections obtained from C57BL/6 (■), IFN- γ KO (□), or IL-12 KO (▤) mice at different times after infection. **Bottom left:** Shown are the parasitemia (**c**) and survival (**d**) curves in either C57BL/6 (—●—), IFN- γ KO (—□—), or IL-12 KO (—▲—) infected with the Colombian strain of *T. cruzi*. **e:** The number of amastigote nests in 25 histopathological fields (original magnification, 1×200) of cardiac sections obtained from either C57BL/6 (■) or iNOS KO (▨) mice at different times after infection. **f:** The number of inflammatory infiltrates present in 24 histopathological fields (original magnification, 1×200) in cardiac sections obtained from either C57BL/6 (■) or iNOS KO (▨) mice at different times after infection. **Bottom right:** Shown are the parasitemia (**g**) and survival (**h**) curves in either C57BL/6 (—●—) or iNOS KO (—▲—) infected with Colombian strain of *T. cruzi*. Each value is the average and SD of at least four animals. Similar results were obtained in three experiments. One asterisk indicate that differences are statistically significant at $P < 0.01$ level, respectively, when comparing different parameters from C57BL/6 to IFN- γ KO, IL-12 KO (**left**), or to iNOS KO (**right**) mice obtained at the same time after infection. The differences between survival rates of C57BL/6 and IFN- γ KO, IL-12 KO, or iNOS KO mice infected with *T. cruzi* were considered statistically significant ($P < 0.01$).

5f and 6E), and lesions in the brain (Figures 6, E and G, and 6 hours) of IL-12 KO progressively increased from day 40 to 55 after infection, as indicated by analysis at days 40, 45 (not shown), 50, and 55 (not shown) days after infection. The inflammatory reaction in the IL-12 KO mice was characterized by glial nodules and necrotic lesions (Figure 6E), vascular cuffing by lymphocytes (Figure 6G), and meningitis (Figure 6H). An intense tissue parasitism accompanying the inflammatory reaction in the brain of IL-12 KO mice at 50 to 55 days after infection, can be observed in either histopathological section (Figure 6E) or immunocytochemistry (Figure 6F). Although less intense than in IL-12 KO mice, a gradual increased tissue parasitism and inflammation in the brain was also observed during reactivation of infection in IFN- γ KO (Figure 5d and Figure 6, C and D) mice, at 30, 35 (not shown), and 40 days after infection. Inflammation (Figures 5e and 6A) and amastigote nests (Figures 5d and 6B) were a rare finding in the brain of WT mice infected with Colombian strain of *T. cruzi*. It is noteworthy that under a similar protocol, infection with Y strain of *T. cruzi* resulted in reactivation of infection in the IFN- γ KO but not in the IL-12 KO mice (data not shown), indicating a parasite strain dependency for the reactivation of *T. cruzi* in the CNS.

We then determined the composition of inflammatory infiltrate both in the heart and brain from IL-12 KO mice infected with *T. cruzi* and submitted to early treatment with benznidazole. Heart and brain were harvested after cessation of chemotherapy at 50 days after infection. Our immunocytochemistry results show that the inflammatory infiltrate in the heart of IL-12 KO presented a ratio of 0.1:1.0:3.4 for macrophages: CD4⁺ T cells: CD8⁺ T cells. In contrast, in the brain the inflammatory infiltrate was

mostly devoid of CD8⁺ T cells presenting the ratio of 3.7:3.7:1.0 for macrophages: CD4⁺ T cells: CD8⁺ T cells. It is noteworthy that in advanced lesions the inflammatory infiltrates in the brain, but not in the heart, from IL-12 KO were also very rich in polymorphic nuclear cells, mainly neutrophils, also reflected by the higher expression of MAC-1 (granulocytes and macrophage markers) relative to F4/80 (macrophage marker). Our immunocytochemistry results also demonstrate the presence of cells producing IFN- γ in the inflammatory processes found in the heart from IL-12 KO mice at 45 to 55 days after infection. IFN- γ -producing cells were a rare finding in the CNS of IL-12 KO mice at 45 to 55 days after infection with the Colombian strain of *T. cruzi* (data not shown).

We also measured the levels of anti-*T. cruzi* IgG1 and IgG2a antibodies in the sera of mice infected with Colombian strain and treated with benznidazole. Our results show that the parasite-specific IgG1 (Figure 5g) and IgG2a (Figure 5h) levels were higher in IFN- γ KO and IL-12 KO, respectively. In agreement with these results, we found that when stimulated with TcAg, spleen cells from IL-12 KO mice produced ~20% of the total IFN- γ produced by splenocytes from WT animals (Figure 5i). Together these results indicate that an IL-12-independent pathway for IFN- γ synthesis is operating during *T. cruzi* infection.

Discussion

The precise mechanisms involved in the pathogenesis of Chagas' disease are primarily unknown. Nevertheless, follow-up studies of chagasic patients⁹ and in experimen-

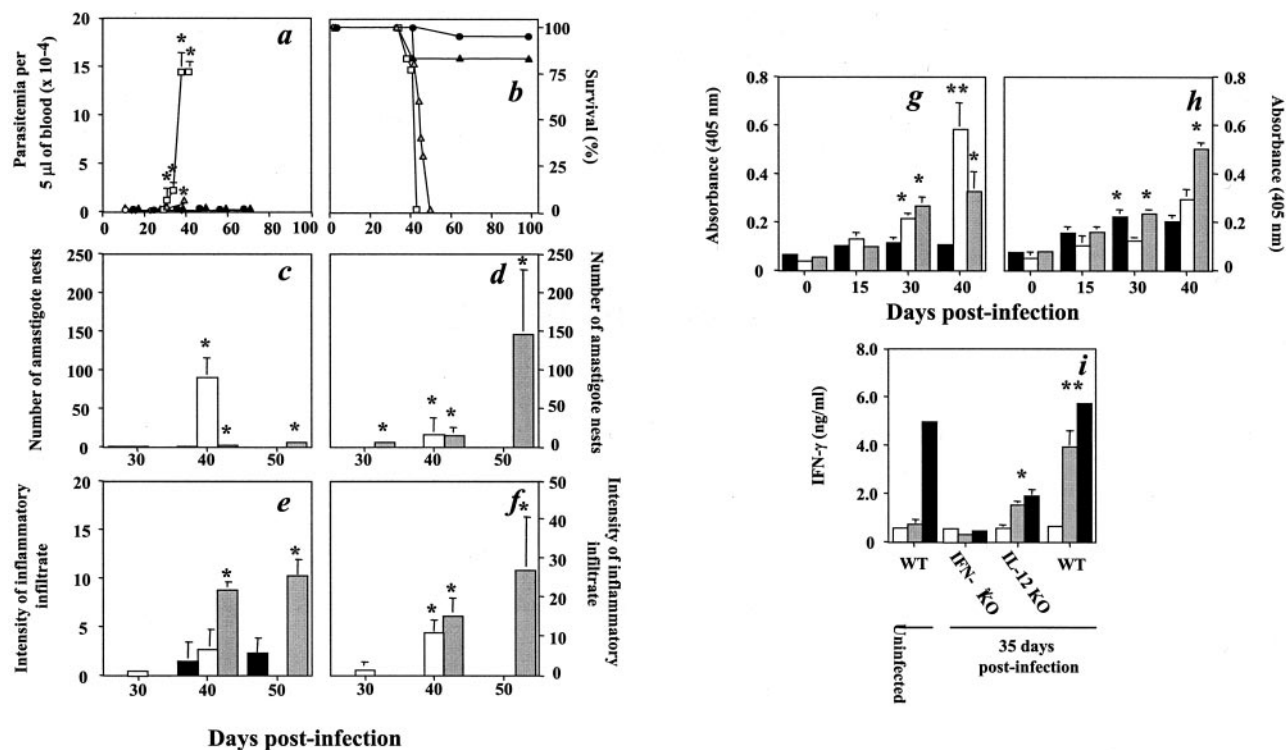


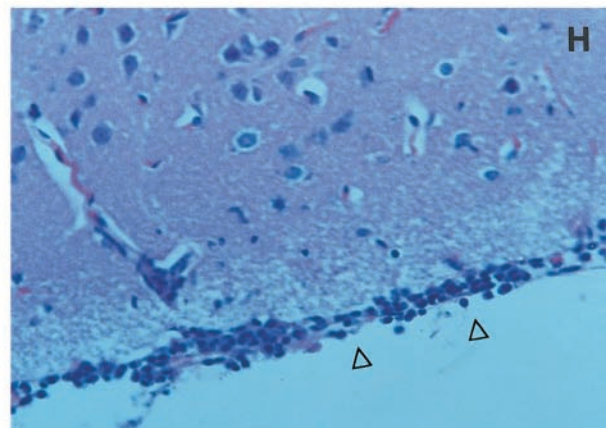
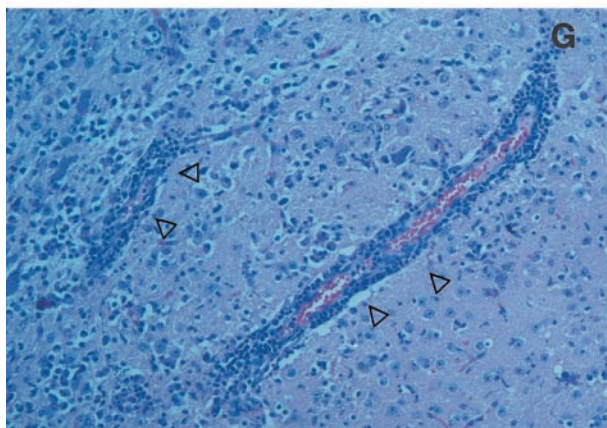
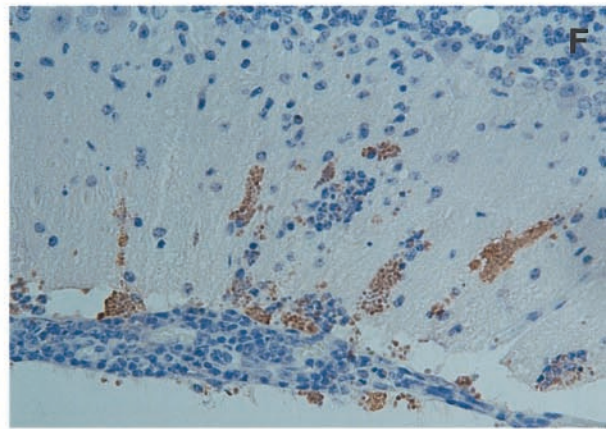
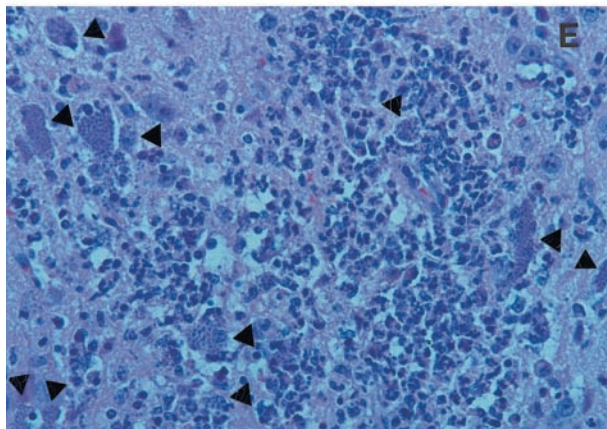
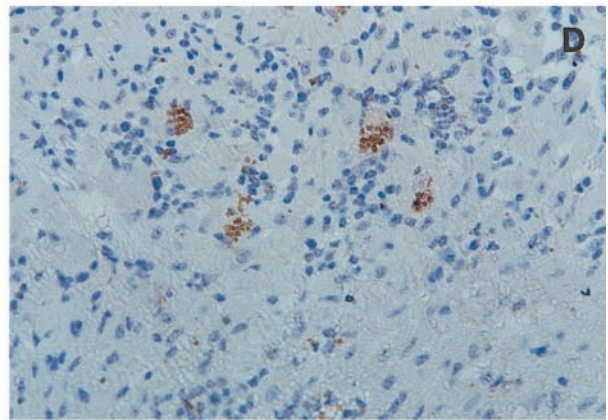
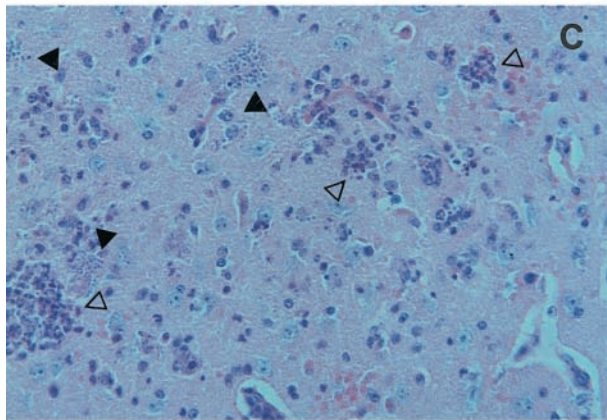
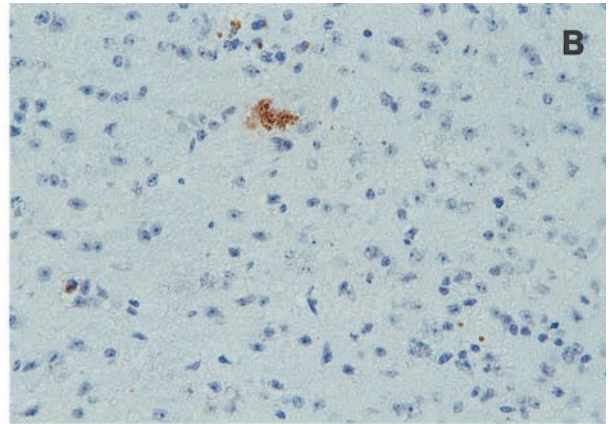
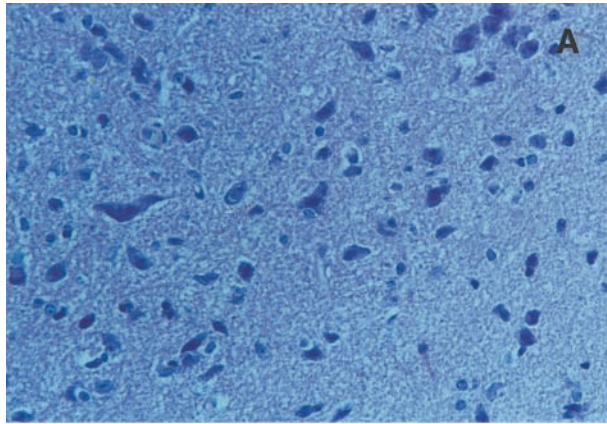
Figure 5. Reactivation of infection with the Colombian strain of *T. cruzi* in C57BL/6, IFN- γ KO, IL-12 KO, and iNOS KO mice. **Top left:** Shown are the parasitemia (a) and survival (b) curves in either C57BL/6 (—●—), IFN- γ KO (—□—), or IL-12 KO (—▲—) or iNOS KO (—◆—) mice infected with the Colombian strain of *T. cruzi* and treated with a subcurative dose of benznidazole. The number of amastigote nests in 25 histopathological fields (original magnification, 1 \times 200) of cardiac (c) or brain (d) sections obtained from either C57BL/6 (■), IFN- γ KO (□), or IL-12 KO (▨) mice at different times after infection and treatment with a subcurative dose of benznidazole. **e:** The number of inflammatory infiltrates present in 25 histopathological fields (original magnification 1 \times 200) of cardiac sections obtained from C57BL/6 (■), IFN- γ KO (□), or IL-12 KO (▨) mice at different times after infection and treatment with a subcurative dose of benznidazole. **f:** The number of inflammatory infiltrates present in 25 histopathological fields (original magnification, 1 \times 200) of brain sections obtained from C57BL/6 (■), IFN- γ KO (□), or IL-12 KO (▨) mice at different times after infection and treatment with a subcurative dose of benznidazole. The serum levels of *T. cruzi*-specific IgG1 (g) and IgG2a (h) isotypes are shown for C57BL/6 (■), IFN- γ KO (□), and IL-12 KO (▨) mice at different times after infection and treatment with a subcurative dose of benznidazole. **i:** IFN- γ levels produced by splenocytes cultured in the presence of medium alone (□), TcAg (▨), or Con A (■) derived from C57BL/6, IFN- γ KO, or IL-12 KO mice at 35 days after infection and treatment with a subcurative dose of benznidazole. Each value is the average and SD of at least four animals. Similar results were obtained in three experiments. One and two asterisks indicate that differences are statistically significant at $P < 0.05$ and $P < 0.01$ levels, respectively, when comparing different parameters from C57BL/6 to IFN- γ KO and IL-12 KO mice obtained at the same time after infection. The differences between survival rates of C57BL/6 and iNOS KO to IFN- γ KO and IL-12 KO mice were considered statistically significant ($P < 0.01$).

tal models^{15,18} suggest a positive correlation between severity of disease and/or heart alterations during acute disease and chronic cardiac pathology. Moreover, when compared to asymptomatic individuals, tissue parasitism is higher both in the heart and in esophagus of patients displaying the severe forms of chronic Chagas' disease.⁶⁻⁸ Additional evidences for the role of parasite in the pathogenesis of Chagas' disease are the different tissue tropism and disease outcome in experimental models¹⁵⁻¹⁷ infected with distinct *T. cruzi* strains. Finally, various components of the host immune system have been shown to primarily contribute to host resistance to *T. cruzi* infection and suggested to play a crucial role in the immunopathogenesis of Chagas' disease.^{10,14}

In the present study, we used the Colombian strain of *T. cruzi* that causes an intense cardiomyopathy^{29,31,35} as well as encephalitis³⁰ during experimental infection in mice. Our previous study showed that inflammation, elicited by the infection with Colombian strain in mice, is accompanied by enhanced expression of a mixed profile of type 1 (ie, IL-12 and IFN- γ) as well as type 2 cytokine (ie, IL-4 and IL-10) mRNAs in the heart.²⁹ Herein, by using isogenic as well as cytokine and iNOS KO mice, we

investigated the role of different cytokines on control of tissue parasitism and inflammation during infection with the Colombian strain of *T. cruzi* in mice.

Initially, we used the C57BL/6 and BALB/c isogenic mice strains that, on various stimuli or microbial infections, have been shown to develop a dominant type 1 and type 2 immune response, respectively.^{36,37} Consistent, with studies performed with other *T. cruzi* strains,^{19,20} BALB/c mice were shown to be more susceptible than C57BL/6 when infected with the Colombian strain. The enhanced susceptibility of BALB/c mice to *T. cruzi* infection was associated with enhanced production of IL-4 (data not shown) as also indicated by the higher parasite-specific IgG1/IgG2a ratios, when compared to C57BL/6 mice. More importantly, we could identify IL-4 as a major immunological factor mediating susceptibility of BALB/c mice, as suggested by the lower number of amastigote nests, parasitemia, and lower rate of mortality of IL-4 KO BALB/c mice acutely infected with the Colombian strain of *T. cruzi*. In contrast to our results, early reports demonstrate no differences of resistance in IL-4 KO or STAT6 KO as compared to WT mice infected with other *T. cruzi* strains (ie, Y and Brazil), that present low tropism for the



cardiac tissue.^{38,39} Indeed, we have previously observed that in contrast to the Colombian strain, the Y strain of *T. cruzi* induces lower levels of IL-4 synthesis and a more dominant IL-12 and IFN- γ immune response during infection in the murine model.^{29,32}

In the conditions used here, the levels of cardiac inflammation were similar when comparing BALB/c and C57BL/6 or IL-4 BALB/c KO mice. Other studies have demonstrated the important anti-inflammatory role of IL-4⁴⁰ and IL-10^{41,42} in preventing excessive inflammation in animals infected with *T. cruzi*. The anti-inflammatory effect of IL-10 during infection with *T. cruzi* also seems to be dependent on parasite strain, because IL-10 does not influence the course of infection with the Y strain of *T. cruzi*.⁴³ Nevertheless, these studies suggest that IL-4 and IL-10 may act in synergy to control myocarditis elicited during infection with *T. cruzi*. However, as shown here, if the immunoregulatory role of IL-4 is dominant it will favor parasite replication leading to enhanced host susceptibility to infection. It is noteworthy that, a study performed by Reis and colleagues⁴⁴ show a close association of IL-4-producing T cells and the presence of amastigote nests in the myocarditis elicited by *T. cruzi* parasites in humans.

Different studies have demonstrated the importance of IL-12/IFN- γ /iNOS axis in resistance to *T. cruzi* infection.^{21–26} Therefore, our next set of experiments evaluated the role of endogenous IL-12, IFN- γ , and iNOS on host resistance to infection with the Colombian strain of *T. cruzi*. Our results indicate that either IL-12 or IFN- γ are essential for host resistance to acute infection with this parasite. According to a previous study,²⁶ our experiments show that iNOS is also essential to control parasite replication in the cardiac tissue and host resistance to infection with *T. cruzi*. It is noteworthy that the iNOS KO mice were somewhat more resistant than either IL-12 KO or IFN- γ KO mice, indicating that an additional IFN- γ -dependent mechanism, but iNOS-independent may also contribute to the control of parasite replication in heart tissue and host resistance to the acute infection with the Colombian strain of *T. cruzi*.

Regarding the reactivation of *T. cruzi* infection in IFN- γ KO mice was characterized by extremely high levels of parasitemia and cardiac tissue parasitism, and relatively low levels of inflammation. In contrast, a small increase in parasitemia and cardiac tissue parasitism was associated to a dramatic increase in inflammation in cardiac tissue of the IL-12 KO mice after cessation of chemotherapy. Interestingly, similar findings are observed in heart tissue of AIDS patients during reactivation of chronic *T. cruzi* infection.⁴⁵ More importantly, reactivation of *T. cruzi* infection in IL-12 KO mice was associated with signs of

neurological disturbances, encephalitis, and areas of the CNS with intense parasite replication, inflammation, and tissue damage. The essential role of endogenous IL-12 on control of *Toxoplasma gondii* and *Cryptococcus neoformans* infection in the CNS from the murine model has also been reported.^{46,47}

Based on parasite-elicited IFN- γ production by spleen cells and parasite-specific IgG isotype measurements in sera from WT, IL-12 KO, and IFN- γ KO mice, we favor the hypothesis that the level of endogenous IFN- γ is a major determinant of the site of parasite replication during reactivation of *T. cruzi* infection. Indeed, previous studies have suggested that the induction of IFN- γ during *T. cruzi* infection may also occur via an IL-12-independent manner.^{23,48} Thus, in the IFN- γ KO mice, where virtually no IFN- γ is produced, the reactivation of infection occurred in peripheral organs mainly in the heart tissue. In contrast, in the IL-12 KO mice, the levels of IFN- γ and IFN- γ -dependent effector mechanisms, although lower than in the WT mice, are sufficient to control parasite replication in the peripheral organs. However, the IL-12-independent IFN- γ -based immunity seems not to be sufficient to control parasite replication in the CNS. Our studies with iNOS KO mice indicate that the main mechanism responsible for the control of reactivation of *T. cruzi* infection is dependent on IFN- γ , but relatively independent of iNOS.

Importantly, during reactivation of infection the inflammatory processes in the CNS from IL-12 KO mice were rich in polymorphic nuclear cells and mostly devoid of CD8⁺ T cells. Further, the CD4:CD8 ratio in the CNS and heart from benznidazole-treated IL-12 KO mice, at 50 days after infection, were 3.7: 1 and 1:3.4, respectively. Consistent with the hypothesis that CD8⁺ T lymphocytes are major sources of IFN- γ during *T. cruzi* infection, we found IFN- γ -producing cells in the heart, but not in the CNS from these animals. Thus, considering the important role of CD8⁺ T lymphocytes in resistance to *T. cruzi*,^{28,49} the increased susceptibility to parasite replication and mass encephalitis could be explained by the lack of CD8⁺ T lymphocyte migration and local production of IFN- γ in the CNS from IL-12 KO mice. The parasite-specific IgG2a antibodies could be an alternative IFN- γ -inducible effector mechanism responsible for controlling parasite replication and preventing reactivation of *T. cruzi* infection. However, we found that in contrast to IFN- γ KO, the IL-12 KO mice produce high levels of parasite-specific IgG2a when infected with *T. cruzi*. Thus, it is possible that parasite-specific antibodies from IgG2a isotype play an important role in controlling parasitemia and tissue parasitism in the peripheral organs but not in the CNS.

Finally, this study provides for the first time an experimental model and new basis to understand the patho-

Figure 6. Illustration of *T. cruzi* parasitism and inflammation in the brain from WT, IFN- γ KO, and IL-12 KO mice infected with the Colombian strain of *T. cruzi* and treated with benznidazole. Brain section from C57BL/6 at 50 days after infection with no inflammation (H&E; original magnification, 1 \times 285) (A) and a rare amastigote nest (B) as detected by immunocytochemistry (brown staining). Brain section from IFN- γ KO at 40 days after infection with focal inflammations/glial nodules (open arrows) and three amastigote nests (closed arrow) (H&E; original magnification, 1 \times 285) (C). Several amastigote nests (D) are evidenced in the CNS from IFN- γ KO at 40 days after infection by immunocytochemistry (brown staining). Brain section from IL-12 KO at 50 days after infection with an intense focal inflammation (glial nodule) and 10 amastigote nests (closed arrow) containing various number of parasites (H&E; original magnification, 1 \times 285) (E). Various amastigote nests (F) in the CNS from IL-12 KO at 50 days after infection are stained with a parasite-specific antibody (brown staining). G (H&E; 1 \times 285) and H (H&E; original magnification, 1 \times 1000) show, respectively, the intense cuffing of brain arterioles (open arrows) and meningitis (open arrows) observed in IL-12 KO mouse at 50 days after infection with the Colombian strain of *T. cruzi*.

genesis of the *T. cruzi*-induced mass encephalitis in immunodeficient hosts. Thus, our results indicate that host resistance to *T. cruzi* replication in the CNS (an immunologically privileged site) as compared to peripheral organs, requires higher levels of endogenous IFN- γ and/or IFN- γ -mediated effector functions. Considering the gradual decline in IL-12 and IFN- γ production in HIV-1-infected patients,^{50–52} these findings may explain, in part, why the CNS is the main site of reactivation of infection with *T. cruzi*^{12,13} in AIDS patients.

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