

Modulation of Chagasic Cardiomyopathy by Interleukin-4

Dissociation between Inflammation and Tissue Parasitism

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Chronic chagasic cardiomyopathy (CChC) is characterized by an inflammatory reaction which may eventually lead to heart enlargement, arrhythmia, and death. As described herein, interleukin-4-deficient mice mount increased specific T helper (Th) 1 immune responses when infected with *Trypanosoma cruzi*, as compared to wild-type mice. Interestingly, these mice had reduced parasitism and mortality and exacerbated inflammation in their hearts, demonstrating a clear dissociation between inflammation and parasite load. The modulation of these phenomena so as to maximize host and parasite survivals may depend on a fine balance between Th responses, in which a Th1 response will, on one hand, control parasitism and, on the other hand, enhance heart inflammation throughout the course of the infection. (Am J Pathol 2001, 159:703–709)

Perhaps the most intriguing feature of Chagas' disease is the fact that only about 25% of *Trypanosoma cruzi*-infected individuals develop chronic chagasic cardiomyopathy (CChC), a debilitating disease which is the leading cause of heart failure in several Latin American countries.¹ The factors determining the progression from asymptomatic individuals to patients with chagasic cardiomyopathy are unknown. Moreover, no currently available treatment prevents disease development. CChC occurs in a time point when tissue parasitism is very scarce or virtually absent.^{2,3} This observation has prompted many authors to consider the participation of autoallergic phenomena in the pathogenesis of the disease,^{4–9} in opposition to the alternative hypothesis that CChC is totally due to *T. cruzi*-specific immune responses.^{10,11}

The DTH-like aspect of the inflammatory lesions in CChC suggests the participation of T helper (Th) 1 (Th1) cells in its pathogenesis, as this lymphocyte subset

strongly promotes the activation of macrophages by the secretion of cytokines such as interferon- γ (IFN- γ). The expansion and function of Th1 cells are regulated by cytokines produced by Th2 cells, such as interleukin (IL) -4, -10 and -13, potent promoters of humoral immune responses.¹² In several disease models, it has been shown that cross-regulations between these two T-cell subsets are critical for the determination of disease outcome. In fact, certain pathogens can induce the preferential expansion of one Th subset, while suppressing the other.¹³ *T. cruzi* infection, however, induces a non-polarized response: in different murine experimental models, both resistant and susceptible mice had a mixed pattern of response to the parasite.^{14,15} On the other hand, an association between progression to severe CChC and high IFN- γ levels has been demonstrated in human beings.^{16,17} If Th1 responses indeed mediate disease in *T. cruzi*-infected individuals, cytokines such as IL-4 could play a beneficial, anti-pathogenic role during *T. cruzi* infection. Thus, we investigated a possible modulating role of IL-4 in a CChC model in which IL-4-deficient mice are infected with *T. cruzi*, as described below.

Materials and Methods

Mice

Specific-pathogen-free, 6- to 8-week-old female or male wild-type, IL-4^{-/-} and nu/nu BALB/c mice were raised and maintained at the animal facilities at the Gonçalo Moniz Research Center-FIOCRUZ, and provided with rodent diet and water *ad libitum*.

Parasites, Infection and T. cruzi Antigen Preparation

Trypomastigotes of Colombian-strain¹⁸ *T. cruzi* were obtained by infection of LCC-MK2 cell line. Infection was

Supported by grants from the Brazilian National Research Council (CNPq) and FIOCRUZ.

Accepted for publication April 30, 2001.

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performed by inoculation of 100 trypomastigotes by intraperitoneal route. Parasitemia was evaluated at different time points after infection by counting the number of trypomastigotes in peripheral blood aliquots contained between a glass slide and a coverslip, with a determined blood volume per microscopic field.¹⁹ Epimastigotes of Colombian and PF strain²⁰ were obtained by axenic culture in liver infusion tryptase medium. *T. cruzi* antigen was prepared by subjecting epimastigotes to five cycles of freezing and thawing. *T. cruzi* lysates were centrifuged at $30,000 \times$ for 30 minutes; supernatants were then aliquoted and stored at -70°C until use.

Histopathological Evaluation

Groups of IL-4^{+/+} or IL-4^{-/-} mice were sacrificed at different time points after infection. Heart, liver, spleen, and striated muscle were removed and fixed in buffered 10% formalin. Sections were analyzed by optical microscopy after paraffin embedding followed by standard hematoxylin/eosin staining. Inflammatory cells infiltrating heart tissue were counted using digital morphometric evaluation. Images were digitalized using a JVC TK-1280 color video camera adapted to an Axioskop 2 microscope (Carl Zeiss, Göttingen, Germany). The images were analyzed using the AxioVision II program (Carl Zeiss), with which the inflammatory cells were manually counted and integrated by area. The number of parasite nests was also determined by counting in the acquired images. One 100 fields per section were counted in five to ten sections per heart.

Immunohistochemistry

Sections of 5 μm from frozen heart fragments were fixed with cold acetone and incubated with one of the following antibodies: rat anti-mouse CD4 or CD8, (PharMingen, San Diego, CA); rat anti-mouse Fc receptor (2.4G2 cell culture supernatant); peroxidase-conjugated goat anti-mouse IgM (Sigma, St. Louis, MO); *T. cruzi*-hyperimmunized rat antiserum. The sections pre-incubated with rat antibodies were incubated with horseradish-peroxidase conjugated to anti-rat IgG (Sigma). Reaction was developed using the peroxidase substrate diaminobenzidine (Vector, Burlingame, CA), followed by counterstaining with hematoxylin. The percentage of CD4⁺ or CD8⁺ cells was determined by counting the number of positive cells over the total number of inflammatory cells determined as described above.

Reconstitution and Infection of BALB/c nu/nu Mice

BALB/c nu/nu mice received adoptive transfer of thymocytes obtained from newborn wild-type mice or IL-4^{-/-} mice (one thymus per mouse). After 15 days, reconstituted and non-reconstituted control nude mice were infected with 100 Colombian strain trypomastigotes.

Mice were sacrificed 30 days later for histopathological evaluation of the hearts, as described above.

In Vitro Stimulation and Measurement of Proliferative Response

Spleen cell suspensions were prepared in RPMI medium (Life Technologies, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah), L-glutamine (2 mmol/L), vitamins, sodium pyruvate (1 mmol/L), Hepes (10 mmol/L), 5×10^{-5} mol of 2-mercaptoethanol, and gentamicin (50 $\mu\text{g/ml}$) (Sigma). For cytokine determination, spleen cells were cultured in 24-well plates and stimulated with 1 $\mu\text{g/ml}$ of concanavalin A (Con A) (Sigma) or Colombian strain *T. cruzi* antigen (50 $\mu\text{g/ml}$). Cell-free supernatants were collected after 72 hours and stored at -20°C for cytokine analysis. To evaluate the proliferative response, splenocytes were plated in 96-well plates at 4×10^5 /well in 200 μl and triplicate wells were stimulated with Con A or *T. cruzi* antigen for 120 hours, as described in the figure legends. After pulsing with 1 μCi of methyl-[³H]thymidine (Amersham, Little Chalfont, England) for 14–18 hours, proliferation was assessed by measurement of [methyl-³H]thymidine uptake in a β -plate counter (Packard, Meriden, CT).

Cytokine and Anti-T. cruzi Antibody Quantification

Supernatants of splenocyte cultures were tested for IFN- γ , IL-2, and IL-4 contents by ELISA, using antibody pairs from PharMingen and following the manufacturer's instructions. Reaction was developed using the 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Kinkergaard & Perry Laboratories, Gaithersburg, MD) and read at 450 nm.

Anti-*T. cruzi* isotype production was evaluated by ELISA using PF-strain epimastigote lysate as antigen and biotinylated isotype-specific anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 antibodies (PharMingen), followed by streptavidin-peroxidase conjugate (Sigma). Reaction was developed using TMB substrate as described above.

Statistical Analyses

Data were analyzed using Student's *t*-test, Wilcoxon's rank sum test, or Fisher's exact probability test, as indicated in the text. Differences were considered significant when $P < 0.05$.

Results

IL-4^{-/-} Mice Are More Resistant to Infection by Colombian-Strain T. cruzi

Parasitemia levels in *T. cruzi*-infected wild-type BALB/c mice were about five times higher than in IL-4 knockout

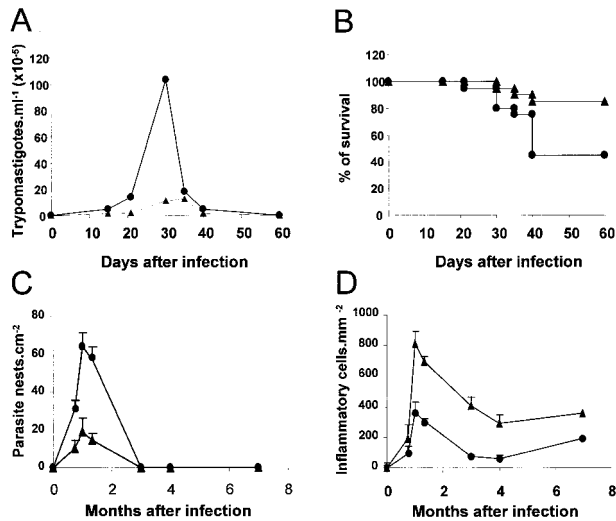


Figure 1. Infection and heart inflammation in IL-4^{-/-} and wild-type BALB/c mice. Groups of 20 IL-4^{-/-} (triangles) or wild-type (circles) mice were infected with *T. cruzi*. **A:** Parasitemia. **B:** Percentage of surviving mice. **C:** Number of parasite nests in the heart. **D:** Number of inflammatory cells in the heart. Each symbol in **A** represents the median of the 9 to 20 surviving mice and in **C** and **D** represents the mean value of three to five surviving mice. **Vertical bars** represent the standard deviations of the means.

mice (Figure 1A; $P < 0.02$, < 0.01 and < 0.002 on the 15th, 21st, and 25th or 30th days postinfection, respectively; Wilcoxon's rank sum test; statistical analysis was not meaningful after the 30th day postinfection due to bias arising from the death of many wild-type animals). Moreover, whereas 55% of the animals in the wild-type mouse group died before or on the 40th day of infection, only 15% of infected IL-4^{-/-} mice did not reach the 60th day after infection (Figure 1B; $P = 0.019$ on the 60th day postinfection; Fisher's exact probability test). Tissue parasitism in spleen, liver and striated muscle was also lower in IL-4^{-/-} mice than in IL-4^{+/+} mice. Heart tissues from IL-4^{-/-} mice had three- to fourfold less parasite nests than those of wild-type mice ($P < 0.05$, Wilcoxon's rank sum test; Figure 1C). After the fourth month of infection, no intact parasite, and only a single macrophage containing parasite antigenic material, was detected in those heart tissues by immunohistochemistry.

Myocarditis Is Exacerbated in *T. cruzi*-Infected IL-4^{-/-} Mice

Inflammatory infiltrates in tissues of all infected mice were composed mainly of mononuclear cells. In the acute phase of the disease (up to 40 days after infection), foci of inflammatory infiltration of similar intensity were found in the liver and in striated muscles of animals from both groups, causing intense myocytolysis, in the presence of parasites. Large spleens, with intense cell proliferation and myeloid metaplastic reaction with megakariocytes were found in mice from both groups. Infected wild-type mouse spleens, however, were significantly larger than those of IL-4^{-/-} mice (mean weights \pm SD were 625 ± 63 and 373 ± 60 mg, respectively; $P < 0.0001$, Student's *t*-test).

Interestingly, the intensity of myocarditis was higher, with 3- to 10-fold more mononuclear cells, in IL-4-deficient than in wild-type mice, despite the lower heart parasitism ($P < 0.05$, Wilcoxon's rank sum test; Figures 1D and 2, A and B). Inflammation was multifocal and mononuclear cells were frequently found attached to myocardial fibers.

After the acute phase of infection (namely around three to four months after infection), hearts of wild-type mice had a healed appearance, with scarce inflammatory foci (Figures 1D and 2C). In contrast, a conspicuous multifocal inflammatory reaction, with or without associated fibrosis, was observed in hearts from IL-4^{-/-} mice (Figure 2D).

Myocarditis increased in severity from the fourth month to the seventh month of infection in mice from both groups. Wild-type mice, however, still had a mild disease, with little fibrosis (Figure 2E), whereas IL-4^{-/-} mice had severe multifocal myocarditis, with mononuclear cells frequently adhering to cardiac fibers undergoing myocytolysis (Figure 2F). In the latter mice fibrosis was very prominent, particularly in the atria, both around intact heart fibers and in areas of active inflammation.

Increased Proportion of CD4⁺ T Cells in Hearts of IL-4^{-/-} Mice

The mononuclear infiltrate in hearts of mice from both groups was mainly composed by macrophages (Fc receptor⁺ and surface IgM⁻ cells), B cells (surface IgM⁺ cells; these constituted no more than 5% of the infiltrate), and T lymphocytes (CD8⁺ or CD4⁺ cells; these constituted no more than 25% of the infiltrate), with higher numbers of CD8⁺ cells in relation to CD4⁺ cells 30 days after infection (Figure 3). At four and seven months after infection, however, CD4⁺ cells predominated, inverting the CD8⁺/CD4⁺ ratio. This was significantly more intense in IL-4^{-/-} mice than in wild-type mice at seven months after infection (Figure 3, $P < 0.05$, Wilcoxon's rank sum test). The majority of CD8⁺ cells in the acute phase of infection surrounded parasite nests (Figure 2G), whereas CD4⁺ cells were frequently found in intimate association with damaged fibers (Figure 2H).

Increased Th1 Response in *T. cruzi*-Infected IL-4^{-/-} Mice

At 30 days after infection, IL-4^{-/-} mouse splenocytes produced two to three times more IFN- γ in response to *T. cruzi* antigen *in vitro* than those of wild-type mice (Figure 4A). Moreover, non-specific stimulation by concanavalin A (Con A) led splenocytes from IL-4^{-/-} mice to produce IL-2 (not shown) and proliferate at normal levels, whereas splenocytes from wild-type mice had markedly reduced responses (Figure 4C; $P < 0.0357$, Student's *t*-test).

After the acute phase of infection (4 to 7 months after infection), spleen cells from infected and uninfected wild-type mice and from IL-4^{-/-} mice had identical re-

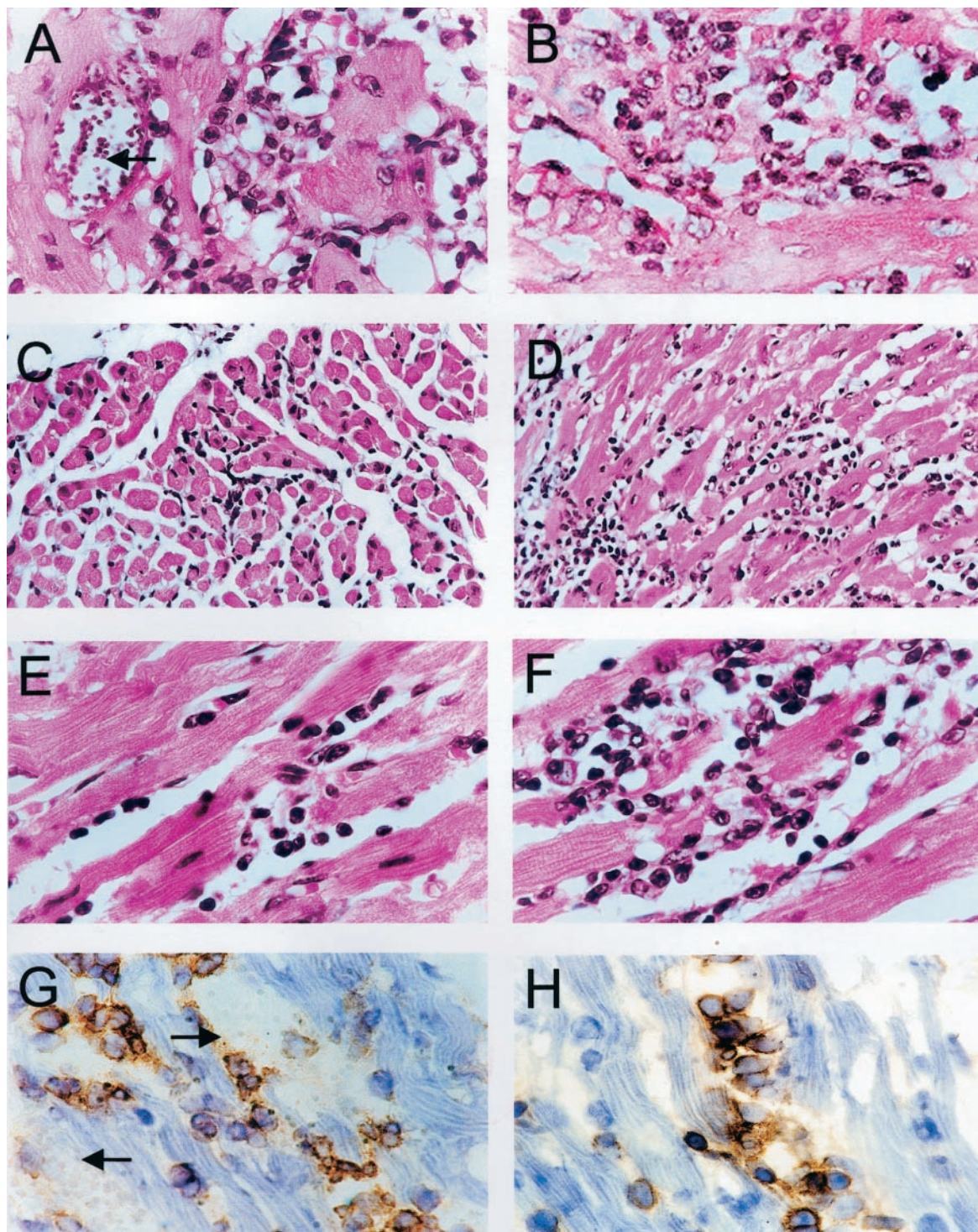


Figure 2. Pathology of hearts from *T. cruzi*-infected BALB/c mice. **A, C and E:** Heart sections of wild-type mice. **B, D and F:** Heart sections of IL-4^{-/-} mice, 1 (**A and B**), 4 (**C and D**) and 7 (**E and F**) months after infection, stained by H&E. **G and H:** Immunoperoxidase staining (DAB) in heart sections of IL-4^{-/-} mice using an anti-CD8 antibody 1 month after *T. cruzi* infection (**G**) or an anti-CD4 antibody 7 months after infection (**H**). Magnifications: **C and D**, $\times 200$; **A, B, E-H**, $\times 400$. **Arrows** indicate parasite nests.

sponses to Con A and *T. cruzi* antigen, in terms of proliferation (Figure 4C and data not shown). However, splenocytes of IL-4^{-/-} mice still produced higher levels of IFN- γ on *in vitro* *T. cruzi*-antigen stimulation than wild-type mouse splenocytes (Figure 4A; $P < 0.0309$, Student's *t*-test). This was associated with increased IgG3

($P < 0.0207$, Student's *t*-test) and reduced IgG1 ($P < 0.0021$, Student's *t*-test) anti-*T. cruzi* antibody levels (Figure 4D). The production of IL-2 by splenocytes obtained from both groups of mice on stimulation with *T. cruzi* antigen was similar in all time points analyzed (Figure 4B).



Figure 3. CD4⁺ and CD8⁺ cells in heart of wild-type or IL-4^{-/-} mice. Numbers of CD4⁺ (solid bars) and CD8⁺ (open bars) cells were evaluated in sections of hearts obtained from surviving mice at the indicated times after *T. cruzi* infection by immunohistochemistry. Each bar represents the mean of results from six sections per heart obtained from three to six mice per group.

T. cruzi Infection Causes Intense Myocarditis in nu/nu BALB/c Mice Reconstituted with IL-4^{-/-} Thymocytes

T. cruzi-infected BALB/c nu/nu mice had intense parasitemia and succumbed to the acute infection. Their hearts, 30 days after infection, had a remarkable high parasite load without any inflammation (Figure 5A). In contrast, hearts from infected nu/nu mice, previously reconstituted with thymocytes from both wild-type and IL-4^{-/-} mice, had inflammation (Figure 5B). The inflammation, which was diffuse and composed by mononuclear cells, similar to that observed in infected wild-type or IL-4^{-/-} mice, was more intense in nu/nu mice reconstituted with IL-4^{-/-} than with wild-type mouse thymocytes (two-fold more inflammatory cells). In addition, the num-

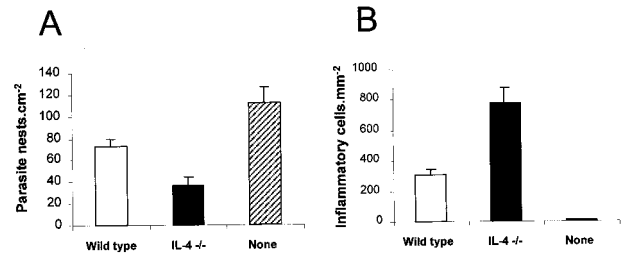


Figure 5. Parasitism and inflammation intensities in hearts of *T. cruzi*-infected nude mice. Mice were reconstituted with wild-type (Wild type) or IL-4^{-/-} (IL-4^{-/-}) BALB/c splenocytes or were not reconstituted (None). Each bar represents the mean number of parasites nests per cm² (A) or the mean number of inflammatory cells per mm² (B) in heart sections of 2–4 mice. Vertical bars represent the standard deviations of the means.

ber of parasite nests was lower in IL-4-reconstituted nu/nu mice (Figure 5).

Discussion

The results described herein, obtained when BALB/c mice were infected with 100 myotropic, Colombian-strain *T. cruzi*, revealed an enhancing effect of IL-4 on the infection: IL-4^{-/-} mice had reduced tissue parasitism and mortality when compared to control mice. Infection of mice with low numbers of parasites, mimicking the natural transmission,²¹ reproduces well the three phases of *T. cruzi* infection in human beings, constituting an adequate model of the human disease.^{4,22} These results apparently conflict with a recent report that IL-4^{-/-} and control BALB/c mice, when infected with high numbers (5000 parasites) of reticulotropic, Y strain *T. cruzi* trypomastigotes, did not differ in terms of parasitemia and mortality.²³ In that experiment, in which the intensity of myocarditis was not evaluated, IL-4^{-/-} mouse splenocytes also produced increased levels of IFN- γ in response to *T. cruzi* antigens. The infective inoculum used, however, by being higher than that which occurs in nature,²¹ may not reproduce closely what happens in natural infection. Moreover, *T. cruzi* strains differ markedly in their biological behavior,²⁴ a fact that has to be taken into account when interpreting conflicting observations in experimentally or naturally infected mammals.

As described in the literature,²⁵ wild-type mice were immunosuppressed in the acute phase of *T. cruzi* infection, as demonstrated by their reduced lymphoproliferative response to Con A *in vitro*. *T. cruzi*-infected IL-4^{-/-} mice, with reduced parasitism, on the other hand, had more intense *in vitro* lymphoproliferative response to Con A (Figure 4C) and less marked splenomegaly than wild-type mice. Whether these two latter phenomena could be ascribed to the reduction in parasitism or could result from a putative effect of IL-4 on the non-specific immunosuppression or on the polyclonal B-cell activation of *T. cruzi*-infected mammals is open to speculation.

The reduced acute-phase parasitemia and mortality of *T. cruzi*-infected IL-4^{-/-} mice, in relation to infected normal mice, can be ascribed to an enhanced parasite-specific Th1 immune response. This enhanced response was demonstrated in this paper by the detection of in-

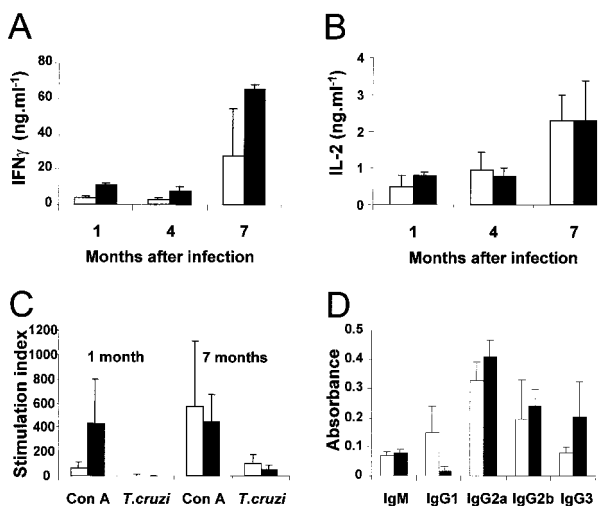


Figure 4. Immune response in *T. cruzi*-infected wild-type (open bars) and IL-4^{-/-} (closed bars) mice. A–C: Levels of IFN- γ (A) and IL-2 (B) in culture supernatants, and DNA synthesis (C) in *T. cruzi* antigen- or Con A-stimulated spleen cell cultures. Spleen cell suspensions were prepared from organs collected at the indicated times after infection. D: Levels of *T. cruzi*-specific IgM, IgG1, IgG2a, IgG2b, and IgG3 antibodies in sera from individual mice, prepared from blood samples collected seven months after *T. cruzi* infection. Each bar represents the mean of data obtained from three to six mice. Vertical bars represent standard deviations of means.

creased amounts of IFN- γ in supernatants of antigen-stimulated splenocytes and increased levels of IFN- γ -dependent IgG3 anti-*T. cruzi* antibodies *in vivo*. As expected, while splenocytes from wild-type mice produced IL-4 on *in vitro* stimulation with Con A and *T. cruzi* antigen in all time points analyzed, no IL-4 was produced *in vitro* by IL-4 $^{-/-}$ splenocytes (not shown) and IL-4 $^{-/-}$ mice had reduced levels of IL-4-dependent IgG1 anti-*T. cruzi* antibodies. In fact, IFN- γ has been shown to control parasitism *in vivo* and *in vitro*.²⁶⁻³¹

On the other hand, the development of heart inflammatory foci in *T. cruzi* infection was intensified in IL-4 $^{-/-}$ mice. This, to our knowledge, has not been previously reported. That it was not due to a putative abnormality in the target organ, and indeed depended on T-cell activity was demonstrated by the passive transfer of the severe myocarditis-susceptibility trait to athymic nude mice by IL-4 $^{-/-}$ thymocytes. In addition, the development of inflammation in thymocyte-reconstituted athymic mice, as also reported for the first time herein, is probably the most conclusive demonstration that the heart disease in *T. cruzi*-infected mammals is immune mediated.

The inverse relationship between tissue parasitism and inflammatory response in hearts of IL-4 $^{-/-}$ mice was striking. In fact, only a single macrophage containing *T. cruzi* antigen could be observed in several sections of heart tissue obtained from four mice four months after infection. These findings, however, cannot be used as a conclusive evidence that CChC is mediated by non-parasite-specific immune responses,³² since very little parasite antigen, undetectable by immunohistochemical analysis, could theoretically maintain T cells specifically activated and recruiting large numbers of effector macrophages. Evidence for autoimmune phenomena in the pathogenesis of CChC comes from elsewhere.⁴⁻⁹

At the acute phase of the infection, when parasitism was brought under control, heart-infiltrating lymphocytes were predominantly CD8 $^{+}$. These lymphocytes, rather than CD4 $^{+}$ cells, were frequently found in the vicinity of parasites or parasite antigens. These findings indicate that CD8 $^{+}$ cells may be directly involved in parasite control, either by releasing IFN- γ and/or by lysing infected cells before the full differentiation of amastigotes into trypomastigotes, and are consistent with data showing severe, lethal infection in β 2-microglobulin or TAP-1 knockout mice.³³ Later on, during the intermediary and chronic phases of the infection, the percentage of CD4 $^{+}$ cells increased and supplanted the percentage of CD8 $^{+}$ cells, mainly in IL-4 $^{-/-}$ mice. Contrasting to what was found for CD8 $^{+}$ cells in the acute phase, these cells were frequently found in close association with myocytes undergoing degenerative changes. These findings are consistent with the hypothesis that CD4 $^{+}$ T cells mediate CChC and with the fact that anti-CD4-antibody treatment, and not anti-CD8, cures experimental CChC in mice.^{5,22}

As for the reduction of parasitism, the enhanced heart alterations in IL-4 $^{-/-}$ mice could be easily ascribed to an intensified Th1 response, since Th1 responses have been shown to be more aggressive than Th2 responses to host tissues in several situations.¹³ In fact, an associ-

ation of severity of myocarditis and increased IFN- γ levels has been described in human beings.^{16,17}

IL-4, by regulating an IFN- γ -producing response, may play an important role in preventing the development of incapacitating heart disease in *T. cruzi*-infected animals. In fact, the intense carditis and extensive fibrosis found in hearts of IL-4 $^{-/-}$ mice during the chronic phase of *T. cruzi* infection closely resembles the severe CChC found in human patients.² The modulating activity of IL-4-producing T cells may maintain a balance between parasitism and tissue integrity in the indeterminate phase of the infection, during which mild inflammatory foci resolve into focal fibrosis and perhaps a contained Th1 response would keep parasitism under relative control.³ The progressive destructive process in CChC could therefore result from a failure of a pathogenic Th1 response to be down-regulated by IL-4. This failure could, in its turn, depend on host genetic characteristics, on age-dependent changes of the immune system,³⁴ superposition of infections by unrelated microorganisms, and/or by *T. cruzi* re-infection.³⁵

Acknowledgments

We thank Dr. José O. Mengel, who kindly provided the breeding pairs of BALB/c IL-4 $^{-/-}$ mice.

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