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IL-10 Is Required to Prevent Immune Hyperactivity During Infection with *Trypanosoma cruzi*[1]

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Previous studies have associated the production of IL-10 with suppression of the protective cell-mediated immune response to *Trypanosoma cruzi*. To further understand the role of IL-10 in the resistance to and pathogenesis of Chagas' disease, we infected C57BL/6 wild-type (IL-10 +/+) or C57BL/6 IL-10 knockout (IL-10 −/−) mice with the virulent Tulahuen strain of *T. cruzi*. IL-10 −/− mice had a lower parasite burden and higher levels of serum TNF-α, IL-12, and IFN-γ compared with infected IL-10 +/+ mice. However, infection resulted in earlier mortality of IL-10 −/− mice compared with IL-10 +/+ controls. The earlier mortality of IL-10 −/− mice could be reversed by administering rIL-10 or a neutralizing Ab specific for IL-12. A role for T cells in the early mortality of IL-10 −/− mice was suggested by experiments in which SCID IL-10 −/− mice infected with *T. cruzi* had a delay in time to death and significantly lower serum levels of IFN-γ compared with IL-10 −/− mice. Furthermore, treatment of infected IL-10 −/− mice with a mAb specific for CD4 resulted in reduced serum levels of IFN-γ and a delay in time to death. Altogether, our results demonstrate for the first time that during infection with *T. cruzi* there is a critical requirement for IL-10 to prevent the development of a pathologic immune response associated with CD4$^+$ T cells and overproduction of IL-12. *The Journal of Immunology*, 1997, 158: 3311–3316.

Interleukin-10 was initially identified by its capacity to inhibit the production of IFN-γ by Th1 CD4$^+$ T cell clones (1, 2). Subsequent studies demonstrated that this activity was indirect and due to the ability of IL-10 to inhibit production of cytokines (TNF-α, IL-1 and IL-12) by macrophages (3, 4) which are required for optimal stimulation of IFN-γ production by T cells (5, 6). Furthermore, IL-10 can inhibit macrophage expression of MHC class I and class II molecules as well as expression of the B7 costimulatory molecules (7–9). In addition, IL-10 can antagonize the ability of IFN-γ to activate the antimicrobial effector mechanisms of macrophages (10, 11). Taken together, these studies demonstrate that IL-10 down-regulates many of the factors required for the development of a cell-mediated immune response. The critical role of IL-10 in controlling cell-mediated immunity was revealed by studies with IL-10-deficient mice (IL-10 −/−). These mice develop a severe inflammatory bowel disease (12), apparently as a consequence of a pathogenic Th1-type response (13). In addition, IL-10 −/− mice are extremely susceptible to the development of septic shock and deleterious skin reactions when exposed to contact-sensitizing agents (14).

Previous studies have correlated the production of IL-10 in infected mice with either susceptibility (15, 16) or resistance (17) to *Trypanosoma cruzi*. The ability of IL-10 to inhibit the killing of *T. cruzi* by macrophages (11) as well as macrophage production of cytokines (IL-12, TNF-α) important in the resistance to *T. cruzi* (18, 19) is a likely explanation for the association of IL-10 production with susceptibility to these parasites. In the present studies, we have used C57BL/6 IL-10 −/− mice to determine the role of IL-10 in the immune response to *T. cruzi*. Our results demonstrate that, although IL-10 is a potent inhibitor of the mechanism(s) involved in resistance to *T. cruzi*, the absence of IL-10 results in a pathogenic immune response that involves CD4$^+$ T cells and the overproduction of IL-12.

Materials and Methods

Mice and parasites

Adult male C57BL/6 (IL-10 +/+; Simonsen Labs, Gilroy, CA) and C57BL/6 SCID mice (IL-10 +/+; Jackson ImmunoResearch Laboratories, West Grove, PA) and C57BL/6 IL-10 −/− and C57BL/6 SCID IL-10 −/− mice, 6 to 8 wk old, were used for these experiments. The IL-10 −/− deficient mice were bred and maintained in the animal facilities of DNAX (Palo Alto, CA). The C57BL/6 mice used in this study do not develop the severe colitis associated with IL-10 deficiency in other strains of mice (20), and no sign of autoimmune disease was apparent in these mice when they were used. Mice were maintained with access to food and water ad libidum. The Tulahuen strain of *T. cruzi* was used for these studies (21). Infection is routinely maintained by transfer of blood from infected to uninfected mice. For these experiments, the blood of infected animals was collected using PBS containing 3.8% sodium citrate and the numbers of trypomastigotes counted (21). Mice were infected by i.p. injection of 10^4 parasites in a volume of 0.2 ml. Parasitemia was determined as previously described (22) using blood diluted 1:10 in PBS with 3.8% sodium citrate.

Histopathology

In experiments to determine parasite burden in infected mice, we used the heart as a representative tissue. Tissue was collected and processed for histopathology as described previously (23). Paraffin-embedded sections were stained with hematoxylin-eosin. Two sections per heart were stained with hematoxylin-eosin and the number of foci of pseudocysts per section

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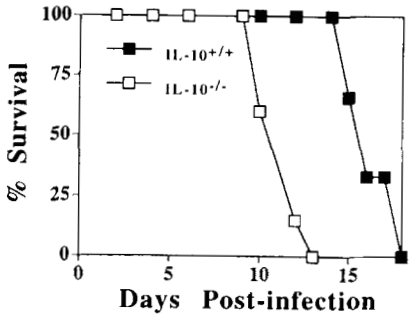

FIGURE 1. Survival of IL-10 −/− and IL-10 +/+ mice infected with *T. cruzi*. Results are the pooled data from four separate experiments with IL-10 +/+ (n = 12) and IL-10 −/− (n = 20) mice.

were counted in a blinded fashion at ×400 magnification. Foci of inflammatory cells were excluded from the counts unless parasites were clearly visible.

Cytokines and Abs

Rat anti-mouse IFN-γ (XMG1.2) and rat anti-mouse TNF-α (MP6-XT22) were supplied by John Abrams (DNAX), and rat anti-mouse IL-12 p40 (C17.A) was provided by Dr. Phil Scott (University of Pennsylvania, Philadelphia, PA). These Abs were administered i.p. to mice on days 5 and 7 postinfection at a dosage of 1 mg/mouse per treatment. Rat mAbs specific for β-galactosidase were used as isotype controls. Recombinant murine IL-10 was prepared as described (14), and 10 μg was injected i.p./mouse/day from day 5 to 17 postinfection. Previous studies have demonstrated that 10 μg IL-10 administered i.p. to IL-10 knockout mice protected them from LPS-induced endotoxic shock (14). Monoclonal Abs specific for CD4 (GK1.5) or CD8 (2.43) were purified from ascites and administered at a concentration of 200 μg/mouse on days 5, 6, and 7 postinfection. This protocol has previously been shown to be efficient in depleting these T cell populations (24, 25).

Assays for IL-12, IFN-γ, and TNF-α

A two-site ELISA was employed to assay levels of IFN-γ using reagents supplied by DNAX, as previously described (26). The sensitivity of this assay was 19 to 38 pg/ml. TNF-α was assayed using a two-site ELISA (PharMingen, San Diego, CA). The sensitivity of this assay was 39 to 78 pg/ml. IL-12 (p40) levels were measured using a two-site ELISA with mAb C17.8 and biotinylated C15.6 (mAbs provided by Dr. Dale Umetsu, Stanford University, Stanford, CA) following the PharMingen protocol. The sensitivity of this assay was typically 300 pg/ml.

Statistics

Statistical analysis (unpaired t test, Mann-Whitney test) was performed using INSTAT software (GraphPad, San Diego, CA). A p value of less than 0.05 was considered significant.

Results

Infection of IL-10 −/− mice results in a reduced parasite burden but earlier mortality

Infection of IL-10 +/+ mice with *T. cruzi* resulted in death 15 to 18 days postinfection, whereas infection of IL-10 −/− mice resulted in death 10 to 13 days after infection (Fig. 1). To determine whether the earlier mortality of IL-10 −/− mice was associated with increased numbers of parasites, we measured the parasitemia in the blood and counted the number of foci of parasite replication in the hearts of infected mice. As shown in Table I, IL-10 +/+ mice developed a high level of parasitemia and large numbers of parasites in the heart. In contrast, IL-10 −/− mice had a significantly lower level of parasitemia and few, if any, foci of parasite replication in the heart. Histopathologic examination of the hearts of infected mice revealed that IL-10 +/+ mice developed foci of inflammation and necrosis within the endocardium and interstitium of the myocardium, which is often associated with the presence of parasites (Fig. 2*A*). This was not observed in IL-10 −/− mice (Fig. 2*B*). Histolopathologic examination of the livers from IL-10 +/+ mice infected with *T. cruzi* revealed the presence of multiple small foci of inflammation composed of lymphocytes and histiocytes. Small numbers of amastigotes were observed within the hepatocytes and found free within the inflammatory foci. Interestingly, examination of the livers of IL-10 −/− mice infected with *T. cruzi* revealed a more severe inflammatory process. There were numerous prominent foci of necrosis and inflammation, composed of lymphocytes, macrophages, and necrotic cellular debris (Fig. 2).

Table I. *Parasite burden in IL-10 +/+ and IL-10 −/− mice infected with* T. cruzi[a]

	Parasitemia ×10³/ml	Number of Foci of Parasite Replication
Expt. 1		
IL-10 +/+	322 (15)	50.7 (13.7)
IL-10 −/−	128 (14)	1[b]
Expt. 2		
IL-10 +/+	266 (34)	32.5 (9.4)
IL-10 −/−	128 (27)	1.4 (1.0)

[a] Groups of three to five infected mice were killed 10 days after infection, and parasitemia and parasite replication in the heart were assessed as described in *Materials and Methods*. IL-10 −/− mice had significantly fewer parasites in the blood and the heart compared with IL-10 +/+ mice. Similar results were observed in two additional experiments. Data are presented as means (n = 3–4 mice per group) with SD given in parentheses.

[b] Of the three hearts examined, only one parasite pseudocyst was present.

Role of cytokines in the early mortality observed in IL-10 −/− mice infected with T. cruzi

To determine whether the earlier mortality of infected IL-10 −/− mice was due simply to a lack of IL-10 rather than a developmental problem associated with deletion of the IL-10 gene, we treated IL-10 −/− mice with 10 μg per day of recombinant murine IL-10 starting 5 days after infection. This treatment regimen resulted in a significant delay in time to death of these mice (Fig. 3), which was comparable to the time to death of infected IL-10 +/+ mice.

Analysis of sera from infected mice revealed elevated levels of IL-12, TNF-α, and IFN-γ for both IL-10 −/− and IL-10 +/+ mice compared with uninfected mice (Fig. 4). However, levels of all three cytokines in the sera of infected IL-10 −/− mice were significantly higher than in the sera of infected IL-10 +/+ mice. There was an approximately 2-fold increase in serum TNF-α and a remarkable 5- to 10-fold increase in serum IFN-γ and IL-12.

To determine whether elevated cytokine levels were involved in the early mortality of infected IL-10 −/− mice, we treated these mice with anti-cytokine Abs starting 5 days after infection. The administration of anti-IL-12 resulted in a 4- to 5-day delay in time to death (Fig. 5A). The administration of anti-TNF-α or anti-IFN-γ resulted in a 1- to 2-day delay in time to death (Fig. 5*B*). Analysis of the blood and hearts from IL-10 −/− mice on day 10 postinfection revealed that treatment with anti-IL-12 resulted in a significant increase in parasitemia in the blood (control = $1.22 \times 10^5 \pm 8600$/ml; anti-IL-12 $2.9 \times 10^5 \pm 3700$/ml). Similar results were observed in mice treated with anti-IFN-γ or anti-TNF-α (data not shown). These results demonstrate that the mechanism of resistance to *T. cruzi* in IL-10 −/− mice, as in IL-10 +/+ mice, is dependent on IL-12 and IFN-γ (19, 27). Taken together, these results suggest that overproduction of IL-12 is a critical determinant in the early mortality experienced by infected IL-10 −/− mice.

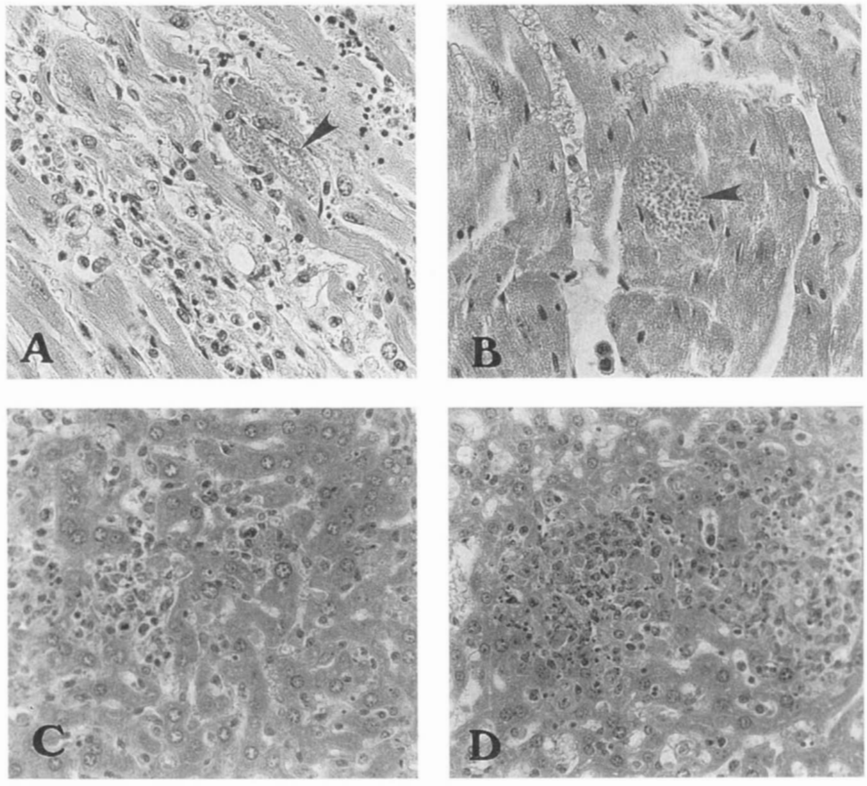

FIGURE 2. Histopathology of heart in IL-10 +/+ mice (*A*) and IL-10 −/− mice (*B*) infected with *T. cruzi*. Arrows point to parasite foci. Histopathology of liver in IL-10 +/+ mice (*C*) and IL-10 −/− mice (*D*) infected with *T. cruzi*. Infected mice were killed 10 days after infection and tissues processed for histopathology. Original magnification, ×400.

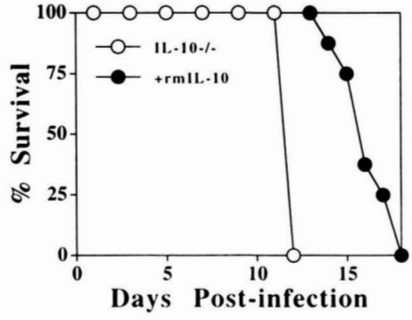

FIGURE 3. Effect of administering murine rIL-10 to IL-10 −/− mice infected with *T. cruzi*. Mice were treated i.p. with 10 μg/day of murine rIL-10 or diluent from day 5 until day 17 of infection. Results are the pooled data from two separate experiments with eight mice per experimental group.

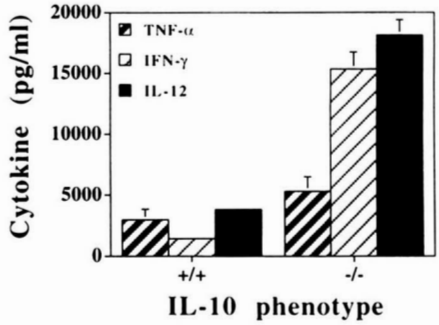

FIGURE 4. Serum levels of IFN-γ, TNF-α, and IL-12 in IL-10 +/+ and IL-10 −/− mice infected with *T. cruzi*. Groups of three infected mice were killed 10 days after infection and their sera analyzed for levels of cytokines. Results presented are representative of four experiments. Sera from uninfected mice did not contain detectable levels of these cytokines.

T cells are required for the early mortality of IL-10 −/− mice infected with T. cruzi

The availability of IL-10 −/− mice on a SCID background allowed us to assess the role of lymphocytes in the early mortality and elevated cytokine production observed in IL-10 −/− mice infected with *T. cruzi*. Infected SCID IL-10 −/− and SCID IL-10 +/+ mice survived significantly longer than IL-10 −/− mice (Fig. 6). Analysis of serum from these mice revealed that SCID IL-10 +/+ and SCID IL-10 −/− mice had significantly lower levels of IFN-γ than IL-10 −/− mice (Table II). In contrast, the levels of IL-12 detected in the sera of SCID IL-10 +/+, SCID IL-10 −/−, and IL-10 −/− mice were comparable (Table II vs Fig. 4). The decreased IFN-γ production by the SCID IL-10 −/− mice coincided with an increased parasite burden relative to that detected in the IL-10 −/− mice (Table II).

These results obtained with SCID IL-10 −/− mice suggested that the unregulated activities of T cells contribute to the early mortality of IL-10 −/− mice. Therefore, experiments were performed to assess the role of $CD4^+$ vs $CD8^+$ cells. As shown in Figure 7, administration of anti-CD4 Ab to infected IL-10 −/− mice resulted in a significant delay in time to death of 5 to 6 days. The results with anti-CD8 Ab were less striking, with a typical delay in time to death of 1 day, which was not statistically significant. Analysis of the sera of infected IL-10 −/− mice revealed that these treatments did not affect serum levels of IL-12 (Fig. 8). In contrast, although anti-CD8 Ab did not affect levels of IFN-γ, anti-CD4 Ab resulted in a remarkable decrease in levels of IFN-γ (Fig. 8). This decrease in IFN-γ production coincided with a log fold increase in the parasitemia (control = 4.8×10^4/ml ± 4.8×10^4; anti-CD4 = 4.83×10^5/ml ± 8×10^4).

When the same experiments were performed with IL-10 +/+ mice, we obtained the opposite results. Administration of anti-CD4

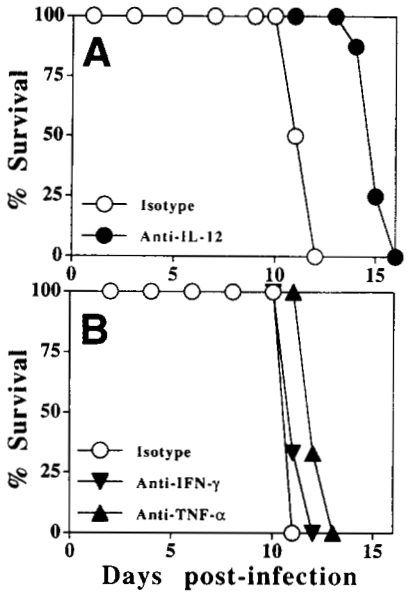

FIGURE 5. Effect of anti-cytokine Abs on time to death of IL-10 −/− mice infected with *T. cruzi*. Mice were infected with *T. cruzi* and treated i.p. with 1 mg/mouse of an isotype control or anti-IL-12 (*A*), anti-TNF-α, or anti-IFN-γ (*B*) on days 5, 7, and 9 postinfection. The results presented are the pooled data from two separate experiments with eight mice per experimental group. Administration of anti-TNF-α or anti-IL-12, but not anti-IFN-γ, resulted in a statistically significant delay in time to death.

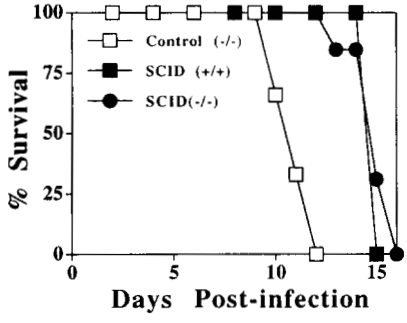

FIGURE 6. Time to death of IL-10 −/− (n = 12), SCID IL-10 −/− (n = 13), and SCID IL-10 +/+ (n = 8) mice infected with *T. cruzi*. Results presented are the pooled data from three separate experiments.

or anti-CD8 Ab resulted in earlier mortality (Fig. 7). In these experiments, administration of anti-CD4 or anti-CD8 Ab also resulted in an increase in the parasitemia (control = 1.3×10^5/ml ± 2.7×10^4; anti-CD4 = 8.6×10^5 ml ± 6×10^4; anti-CD8 = 6×10^5/ml ± 3.6×10^4, respectively), confirming the importance of T cells in the anti-parasite response in IL-10 +/+ mice. Analysis of the sera of infected IL-10 +/+ mice revealed that anti-CD4 or anti-CD8 Ab did not significantly affect levels of IL-12 or IFN-γ (Fig. 8).

Discussion

Our data clearly show that infection of IL-10 −/− mice with a virulent strain of *T. cruzi* resulted in reduced parasitemia and reduced numbers of parasites in the heart as compared with infected IL-10 +/+ mice. Similar results were observed in studies in which IL-10 −/− mice infected with the avirulent Y strain of *T. cruzi* were shown to have a mechanism of resistance to *T. cruzi* that is dependent on IL-12, IFN-γ, and TNF-α (28). Collectively, the

Table II. *Serum levels of IL-12 and IFN-γ of IL-10 −/−, SCID IL-10 −/−, and SCID IL-10 +/+ mice infected with* T. cruzi[a]

Phenotype	IL-12 (pg/ml)	IFN-γ (pg/ml)	Parasitemia $\times 10^3$/ml
Expt. 1			
IL-10 −/−	7,113 (1,423)	42,927 (2,306)	48.3 (52)
SCID IL-10 −/−	11,211 (2,396)	6,152 (1,533)	195 (60)
SCID IL-10 +/+	9,812 (2,846)	1,309 (292)	192 (66)
Expt. 2			
IL-10 −/−	15,628 (2,089)	13,350 (2,306)	ND
SCID IL-10 −/−	11,882 (1,750)	2,600 (731)	ND
SCID IL-10 +/+	7,188 (634)	1,537 (440)	ND

[a] Mice were infected as described in *Materials and Methods*; groups of three to four mice were killed on day 10 postinfection and analyzed for parasitemia and serum levels of IL-12 and IFN-γ. Data presented are means with SD given in parentheses.

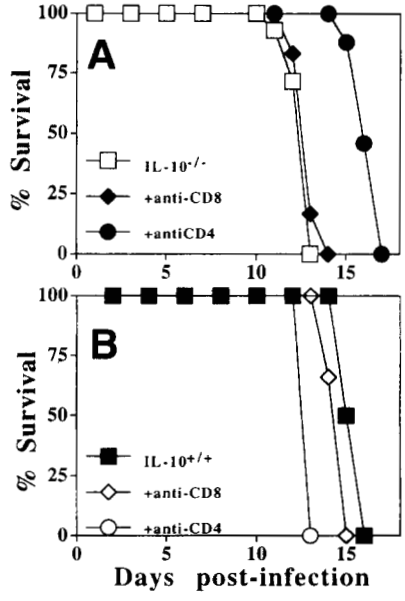

FIGURE 7. Effect of administration of antisera for CD4 or CD8 to IL-10 −/− (*A*) or IL-10 +/+ mice (*B*) infected with *T. cruzi*. The results presented in *A* are the pooled data from three separate experiments with IL-10 −/− treated with an isotype control Ab (n = 14); anti-CD4 Ab (n = 17); or anti-CD8 Ab (n = 12). The results presented in *B* are from a single experiment with IL-10 +/+ mice treated with anti-CD4 or anti-CD8 (n = 6 mice per group). Mice were treated with 200 μg/day anti-CD4, anti-CD8, or an isotype control on days 5, 6, and 7 postinfection.

studies with IL-10 −/− mice support the hypothesis that endogenous IL-10 is an inhibitor of the protective immune response to *T. cruzi* (16). However, in our studies we observed that despite the reduced numbers of parasites, IL-10 −/− mice died earlier than wild-type mice. The early mortality of IL-10 −/− mice, characterized most notably by high levels of serum IL-12 and IFN-γ, could be prevented by treatment with anti-IL-12 or anti-CD4 Abs. These data demonstrate that although production of IL-10 may result in an increased parasite burden, IL-10 is required to prevent a pathogenic $CD4^+$ T cell response associated with overproduction of IL-12 and IFN-γ.

In IL-10 −/− mice, the high levels of IFN-γ produced after infection appears to be derived from $CD4^+$ T cells. However, $CD4^+$ T cells are not the major source of IFN-γ in infected IL-10 +/+ mice. The serum levels of IFN-γ observed in infected IL-10 +/+ mice were comparable to those detected in

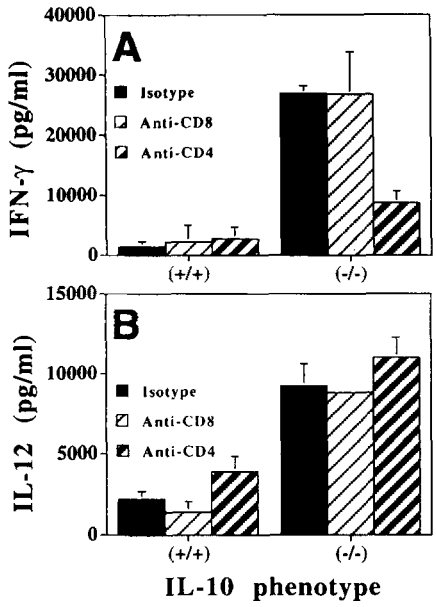

FIGURE 8. Effect of administration of mAbs specific for CD4 or CD8 to mice infected with *T. cruzi*. Groups of three to four infected mice were treated with 200 μg/day anti-CD4, anti-CD8, or an isotype control on days 5, 6, and 7 postinfection. Mice were killed on day 10 postinfection and serum levels of IFN-γ (*A*) and IL-12 (*B*) were measured by ELISA. Results presented are representative of three experiments.

the sera of SCID IL-10 +/+ and SCID IL-10 −/− mice. Moreover, depletion of $CD4^+$ or $CD8^+$ T cells in IL-10 +/+ mice did not result in a reduction of serum levels of IFN-γ. Taken together, these results suggest that NK cells are the major source of IFN-γ in infected IL-10 +/+ mice. These data are in agreement with Cardillo and colleagues (18), who demonstrated that NK cells are the source of most of the IFN-γ produced in mice during acute infection with *T. cruzi*.

Our studies, which demonstrated that $CD4^+$ T cells and IL-12 are important in the early mortality of infected IL-10 −/− mice, suggested that IL-12-driven production of IFN-γ by $CD4^+$ T cells may be involved in the premature deaths of these mice. However, in the studies presented here, treatment of infected IL-10 −/− mice with anti-IFN-γ did not result in a significant delay in time to death. Although incomplete neutralization cannot be ruled out, there are other explanations for the lack of protective effect of anti-IFN-γ treatment. IFN-γ is a major mediator of resistance to *T. cruzi* (15, 18, 27), and while neutralization of IFN-γ may prevent the cytokine-mediated pathology, it could lead to early mortality due to increased parasite replication (see Table II). Alternatively, the early mortality of IL-10 −/− mice may be dependent on IL-12 and $CD4^+$ T cells but independent of IFN-γ.

Interestingly, our findings indicate that high levels of IL-12 alone are not sufficient for the early mortality of infected IL-10 −/− mice and that it is the combination of $CD4^+$ T cells and IL-12 that proves pathogenic in IL-10 −/− mice. This was shown by our studies in which infected SCID IL-10 −/− and SCID IL-10 +/+ mice had a delay in time to death compared with IL-10 −/− mice but had levels of serum IL-12 similar to those observed in IL-10 −/− mice. Thus, in the absence of lymphocytes, high levels of IL-12 did not result in early mortality. It was also surprising that IL-10 +/+ SCID mice had levels of IL-12 comparable to those observed in IL-10 −/− mice, since the IL-10 +/+ SCID mice still have macrophages as a source of IL-10. Previous work has shown that infection of SCID mice with *T. cruzi* results in increased macrophage expression of IL-10 mRNA (16). These results suggest that in IL-10 +/+ mice it is lymphocytes, not macrophages, that produce the IL-10 that inhibits overproduction of IL-12. In this regard, recent studies have shown that IL-12 can stimulate human Th0, Th1, and Th2 cell clones to produce IL-10 (29, 30). Thus, it appears likely that IL-12 induces T cells to produce IL-10, which can inhibit further production of IL-12 by macrophages and so limit a potentially pathologic immune response. Further studies are required to more precisely define the source of the IL-10 that regulates IL-12 production during infection with *T. cruzi*.

In the studies presented here, we have confirmed the importance of IL-10 as a negative regulator of the cell-mediated immune response to *T. cruzi* and demonstrated that IL-10 is required to prevent the development of an infection-induced pathologic immune response. These latter results would not have been expected based on studies with neutralizing Abs, and they highlight the critical role of IL-10 in maintaining the balance between a pathogenic and a protective immune response during Chagas' disease. Importantly, our studies with IL-10 −/− mice give several novel insights into the role of IL-10 in the regulation of the immune response during this infection. The striking phenotype we have observed in the IL-10 −/− mice suggests the possibility that other immunosuppressive cytokines such as TGF-β, IL-4, or IL-13 do not compensate for the loss of IL-10 in this system. Moreover, since T cell production of IFN-γ is apparent only in the absence of IL-10, this indicates the important role of IL-10 in the inhibition of T cell production of IFN-γ during Chagas' disease in immunocompetent mice. Interestingly, infection of IL-10 −/− mice with *Toxoplasma gondii* results in early mortality, which is dependent on $CD4^+$ T cells and is characterized by overproduction of IL-12 and IFN-γ (31); and preliminary studies with *Plasmodium chabaudi* appear to show similar findings (32). However, our studies with *T. cruzi* have demonstrated that IL-12 alone is not sufficient for the early mortality observed in IL-10 −/− mice but that it is the combination of IL-12 and $CD4^+$ T cells that is required for the development of this pathologic immune response. Nevertheless, it seems that the general principle that IL-10 is a critical regulator of the balance between a protective and a pathogenic cellular immune response during infection is true for at least three important parasitic diseases.

Acknowledgments

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