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A heart-specific CD4$^+$ T-cell line obtained from a chronic chagasic mouse induces carditis in heart-immunized mice and rejection of normal heart transplants in the absence of *Trypanosoma cruzi*

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SUMMARY

*To study the role of autoreactive T cells in the pathogenesis of cardiomyopathy in Chagas' disease, we generated a cell line by repeated in vitro antigenic stimulation of purified splenic CD4$^+$ T lymphocytes from a chronically Trypanosoma cruzi-infected mouse. Cells from this line were confirmed to be CD4$^+$ CD8$^-$ and proliferated upon stimulation with soluble heart antigens from different animal species, as well as with T. cruzi antigen, in the presence of syngeneic feeder cells. In vitro antigen stimulation of the cell line produced a Th1 cytokine profile, with high levels of IFN*γ *and IL-2 and absence of IL-4, IL-5 and IL-10. The cell line also terminated the beating of fetal heart clusters in vitro when cocultured with irradiated syngeneic normal spleen cells. In situ injection of the cell line into well established heart transplants also induced the cessation of heart beating. Finally, adoptive transfer of the cell line to heart-immunized or T. cruzi-infected BALB/c nude mice caused intense heart inflammation.*

Keywords *T-cell line,* Trypanosoma cruzi*, autoreactivity, carditis, Chagas' disease*

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INTRODUCTION

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi,* is one of the leading causes of death in many countries of Latin America. Development of cardiopathy occurs in 30% of the infected individuals, in most cases as severe carditis (1). Mice infected with *T. cruzi* have been widely employed as models for the investigation of chronic chagasic cardiomyopathy. The disease is characterized by foci of myocytolysis accompanied by an inflammatory infiltrate predominantly composed of mononuclear cells and by interstitial fibrosis. Myofibres containing parasites are rarely observed (2,3).

Different mechanisms have been proposed to explain the pathology of the cardiomyopathy that occurs in chronic Chagas' disease (4–6). It has been suggested that the lesions result from recurrent parasitic invasion. However, the very rare finding of tissue parasites strongly argues against this hypothesis (3). Second, microvascular abnormalities have been postulated to play a significant role in the pathogenesis of chronic Chagas' heart disease (7,8). Third, many studies indicate that autoimmune mechanisms play a critical role in the physiopathology of Chagas' chronic cardiopathy (9–11).

Supporting the autoimmunity hypothesis, we have demonstrated that mice chronically infected with *T. cruzi* reject syngeneic heart grafts (12). This was in striking contrast to hearts grafted into normal or *T. cruzi*-immunized syngeneic recipients, which are not rejected and can persist for more than 6 months. The study of heart tissue grafted into chagasic mice revealed a persistent and intense mononuclear inflammatory infiltrate, quite similar to the pattern obtained with allogeneic grafts, suggesting that cellular mechanisms are implicated in the rejection. These studies have also shown that splenic CD4$^+$ T cells from chronically

infected mice mediate syngeneic heart graft destruction when injected *in situ*, whereas $CD8^+$ or non-T-cells are not effective. Kinetic studies transferring $CD4^+$ cells showed the presence of autoreactivity as early as 15 days after infection and maximal capacity of graft destruction on day 30 (12). A study not using the same mouse and *T. cruzi* strains that produced the data described above failed to show a role for autoreactivity in the rejection of transplanted hearts (13). We have recently confirmed, however, that in the cardiomyopathy model employing Colombian strain *T. cruzi*-infected BALB/c or DBA mice, which is used in the work described herein, the rejection of normal heart transplants indeed depends on the presence of autoreactive T cells and not of parasites (Soares *et al*. submitted for publication). The production of these apparently conflicting results is not surprising, since it has been extensively shown that *T. cruzi* pathogenicity and virulence markedly varies when different parasite or mouse strains are used (11,14,15), something that may account for the fact that only approximately 30% of chronically *T. cruzi*-infected human beings develop heart disease (1).

In order to characterize the antigens involved in the autoreactive process in chronic Chagas' disease, as well as the mechanisms leading to cardiac tissue damage, we have generated a $CD4^+$ T-cell line from a *T. cruzi* infected mouse. The results presented here constitute strong evidence for an autoimmune component participating in the development of Chagas' heart disease and open new perspectives for the design of rational immunointervention and novel therapies.

MATERIALS AND METHODS

Animals and parasites

BALB/c (H-2^d), DBA/2 (H-2^d), C57Bl6 (H-2^b), and BALB/c nu/nu mice, as well as Wistar rats and New Zealand rabbits, were raised and maintained at the animal facilities of Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Trypomastigotes of the Colombian strain of *T. cruzi* (16,17) were obtained by infection of the LCCMK2 cell line. Animal numbers in experimental groups were the minimum necessary to permit statistical analysis. All animals were sacrificed under anaesthesia.

Antigen preparations

Supernatants of *T. cruzi*-infected LCCMK2 cultures were centrifuged at 2500 ***g*** for 30 min to collect free trypomastigotes. Parasites were washed three times in phosphate buffered saline (PBS) at 4°C and lysed by suspension in distilled water followed by five cycles of freezing and thawing. After addition of 1 volume of 10-fold concentrated PBS, the lysate was centrifuged at 30 000 ***g*** for 30 min. The supernatant was then aliquoted and stored at − 70°C. Hearts were removed from sacrificed mice (from different strains), rats or rabbit and washed thoroughly with saline to remove residual blood. Heart antigens were prepared under sterile conditions by Potter homogenization of hearts or heart fragments in PBS at 4°C. Heart lysates were centrifuged at 30 000 ***g*** for 30 min. Supernatants were aliquoted and stored at − 70°C.

Reagents and culture media

The anti-heart T-cell line and myoblast cultures were raised and maintained in RPMI (Life Technologies, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% FCS (Hyclone, Logan, UT, USA), L-glutamine (2 mM), vitamins, sodium pyruvate (1 mM), Hepes (10 mM), 5×10^{-5} M of 2-mercaptoethanol, and 50 μg/ml of gentamycin (Sigma, St Louis, MO, USA). Amphoterycin B, collagenase, and trypsin were purchased from Life Technologies. Antibodies and recombinant cytokines for cytokine ELISA and flow cytometry were purchased from PharMingen (San Diego, CA, USA). Concanavalin A (Con A), Ficoll-Hypaque, and complete Freund's adjuvant (CFA) were purchased from Sigma.

Generation of the anti-heart T-cell line

$CD4^+$ T cells were purified from spleens of DBA/2 mice chronically infected with the Colombian strain of *T. cruzi* (6 months postinfection). Purification was carried out by removing $CD8^+$ cells and B cells with anti-CD8 and anti-IgM-coated magnetic beads (Dynal, Oslo, Norway). The $CD4^+$-enriched cell population in RPMI was repeatedly treated with amphoterycin B (25 μg/ml) in order to eliminate any living *T. cruzi* in the culture. The purified population was then stimulated with syngeneic heart antigen (100 μg/ml) in the presence of feeder cells (4000 rad-irradiated syngeneic spleen cells) and 10 ng/ml of recombinant murine IL-2 (PharMingen) in 6-well plates. Cycles of stimulation (3–5 days) were followed by short resting periods (1–2 days) over 8 months. To determine the cell phenotype, blast cells from the T-cell line culture were purified by centrifugation in a discontinuous gradient of Ficoll-Hypaque and subjected to flow cytometry using phycoerythrin-conjugated anti-CD4 and fluorescein-conjugated anti-CD8 antibodies in a double staining technique. Analyses were carried out using a FACScalibur cytometer (Becton & Dickinson, San Jose, CA, USA).

Proliferative response of the T-cell line

To test the reactivity of the T-cell line, Ficoll-Hypaque purified blasts (3×10^5/well) or normal lymphocytes (nonadherent DBA splenocytes) and syngeneic feeder cells (5×10^5/well) were plated in 96-well plates in a final volume of 0·2 ml of supplemented RPMI medium. Triplicate wells were stimulated with *T. cruzi* and various heart antigens for 72 h. Plates were pulsed with 1 μCi of [methyl-^{3}H] thymidine (Amersham, Little Chalfont, Bucks, UK) for 14–18 h. Proliferation was assessed by measurement of ^{3}H thymidine uptake in a β-plate counter (Packard, Meriden, CT, USA).

Cocultures of the T-cell line with myoblasts

Myoblast cultures were obtained as described previously (18). Briefly, DBA/2, C57Bl6, foetal hearts (18–20th day) and rat newborn hearts were removed and fragmented with the aid of scissors. Heart cells were gently dissociated with 0·05% collagenase and 0·125% trypsin in PBS, washed three times and plated with supplemented RPMI in 24-well plates. Clusters of beating cells could be observed after 4–7 days of culture. Feeder cells (2×10^6) and Ficoll-Hypaque-purified blast cells from the T-cell line, normal splenocytes or Con A-activated splenocytes (8×10^5) were added to established myoblast cultures. After 24 h and 48 h, the number of beating clusters was determined in an inverted microscope. To determine the proliferative response of the T-cell line against myoblasts, myoblast cultures were obtained as described above. After 5–7 days of culture, 3×10^5 irradiated syngeneic feeder cells and 10^5 purified T-cell line blasts or 10^5 Con A-activated spleen cells were added to each well. Wells were homogenized 48 h later and 100 μl aliquots were plated in 96-well plates in triplicates. ^{3}H-thymidine was added for a period of 12 h to allow the quantification of DNA synthesis.

Transfer of the T-cell line into BALB/c nu/nu mice

The functional role of the T-cell line was evaluated by intravenous adoptive transfer of 10^6 purified T-cell blasts into BALB/c nu/nu mice. Groups of 10 mice were simultaneously immunized with syngeneic heart antigen preparation in complete Freund's adjuvant, subcutaneously, or infected with 10^2 trypomastigotes of the Colombian strain. After 15 or 30 days, animals were sacrificed and their hearts histologically evaluated by optical microscopy of haematoxylin and eosin-stained sections.

Cytokine determination

To determine the cytokine profile of the T-cell line, BALB/c nu/nu mice were inoculated with the T-cell line or normal spleen cells. After 60 days of reconstitution, mice were sacrificed and their spleens removed. Spleen cells were stimulated *in vitro* with heart or *T. cruzi* antigens (100 μg/ml of protein). Cell-free supernatants were collected after 72 h and stored at − 20°C for cytokine analysis. Amounts of IFNγ, IL-2, IL-4, IL-5 and IL-10 were measured by capture ELISA, using antibody pairs from PharMingen, following manufacturer's instructions. Reaction was developed using the 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Plates were read at 450 nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Inoculation of the T-cell line in syngeneic heart transplants

Syngeneic hearts were transplanted into the ears of BALB/c mice, according to a previously described methodology (14). Briefly, hearts from newborn DBA mice were removed and transplanted into the pinna of the ears of syngeneic mice (8-week-old). Beating of hearts started 7–10 days after the transplant. Blasts from the T-cell line (10^5) were injected into well-established heart transplants. The double-blind observation of heart beating by two different researchers was used as the criterion of engraftment. Heart grafts were also injected with CD4$^+$ T cells from syngeneic normal or chronic chagasic mice, as controls.

RESULTS

A CD4$^+$ T-cell line obtained from chronic chagasic mice reacts with heart and *T. cruzi* antigens even after long-term cultivation in the absence of *T. cruzi* antigens

To better demonstrate the role of autoreactive CD4$^+$ T cells in the chronic carditis induced by *T. cruzi* infection, we generated an anti-heart T-cell line from cells of chronic chagasic mice. Blast cells purified from the T-cell line culture after 8 months of repeated *in vitro* stimulation with heart antigen were 95% CD4$^+$, as shown in Figure 1(a). To investigate the reactivity of the T-cell line, purified blasts were stimulated by various antigens in the presence of irradiated syngeneic spleen cells. As shown in Figure 1(b), after 8 months of culture, the T-cell line still showed reactivity against *T. cruzi* antigen. The T-cell line also reacted against xenogeneic heart antigens, but only in the

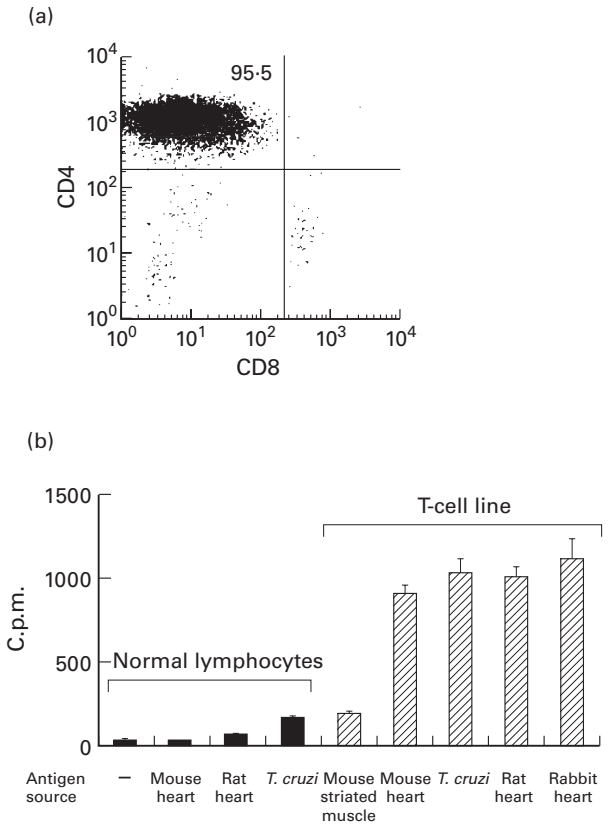

Figure 1 Characterization of the anti-heart autoreactive T-cell line. The T-cell line was obtained from splenocytes of chagasic DBA mice repeatedly stimulated *in vitro* with heart homologous antigen. (a) After centrifugation in Ficoll-Hypaque gradient, a blast cell-enriched population was stained with phycoerythryn-conjugated anti-CD4 antibodies or fluorescein-conjugated anti-CD8 antibodies. Analysis was carried out in a Becton-Dickinson FACScalibur. (b) Ficoll-Hypaque purified T-cell line blasts, maintained for 8 months *in vitro* by stimulation with heart antigens, were stimulated *in vitro* with 100 μg/ml of *T. cruzi* antigen or syngeneic, allogeneic or xenogenic heart antigens, in the presence of feeder cells in 96-well plates. After 3 days of culture, proliferation was assessed by measurement of ^{3}H-thymidine uptake. Results are from one experiment, which is representative of five others.

presence of syngeneic feeder cells (data not shown). In contrast, normal lymphocytes had a weak proliferative response to rat heart and to *T. cruzi* antigens (4–20 times less than the T-cell line). The reactivity against *T. cruzi* was not due to persistence of *T. cruzi* antigens in the T-cell line culture due to residual parasite infection, since the culture was repeatedly treated with amphoterycin B during the first month of culture in order to eliminate any *T. cruzi* parasites. The absence of *T. cruzi* infection in the T-cell line was confirmed by microscopic observations of the T-cell culture and by cultivation in LIT medium for axenic growth of parasites.

The anti-heart T-cell line destroys heart cells *in vitro*

We next tested the ability of the anti-heart T-cell line to promote damage in intact cardiac cells *in vitro*. Figure 2 shows representative results of 10 independent experiments in which myoblasts were cocultured with Con A-activated splenocytes (Figure 2a) or with the T-cell line (Figure 2b). Figure 2(c) shows the percentage of beating myoblast clusters after 48 h of coculture with different lymphocyte populations. After coculture with the T-cell line, all clusters of myoblasts were completely destroyed, whereas in control cultures most of the myoblasts remained beating ($P < 0{\cdot}02$, Wilcoxon's rank sum test). The T-cell line proliferated and induced destruction of clusters when cocultured with syngeneic (DBA/2), allogeneic (C57Bl6) or xenogeneic (rat) myoblasts and syngeneic feeder cells (Figure 2c,d). Syngeneic feeder cells were required for the destruction of myoblasts mediated by the T-cell line (Figure 2c).

Adoptive transfer of the autoreactive T-cell line promotes strong inflammatory infiltrates in hearts and death of immunized nude mice

T. cruzi-infected nude mice have massive parasitism of myocytes in the absence of inflammation (19) (Figure 3a). We therefore investigated whether the heart-specific T-cell line would cause inflammation in BALB/c nu/nu mice infected with *T. cruzi*. Intense inflammation, as well as parasite nests, was observed in hearts from mice injected with the T-cell line and simultaneously infected with *T. cruzi* (Figure 3b). Intense inflammation could also be observed in hearts of animals injected with the T-cell line and immunized with syngeneic heart antigen preparation in CFA (Figure 3c,d). In fact, 100% mortality was observed 1–2 months after the T-cell transfer in these immunized, uninfected mice. No inflammation or death was observed in hearts of nonimmunized, uninfected mice injected with the T-cell line (not shown).

The anti-heart T-cell line has a Th1 cytokine profile

To better understand the mechanisms by which these autoreactive T cells promote disease, we analysed the cytokine production upon heart and *T. cruzi* antigen stimulation. BALB/c nu/nu mice were adoptively transferred with normal spleen cells or with cells from the T-cell line. Spleen cells from the two groups of animals and from nonreconstituted BALB/c nu/nu mice were stimulated *in vitro* with heart or *T. cruzi* antigens. Splenocytes from the T-cell line recipients stimulated with heart or *T. cruzi* antigens produced high levels of IFNγ (Figure 4a) and IL-2 (Figure 4b), whereas IL-4, IL-5 and IL-10 were not

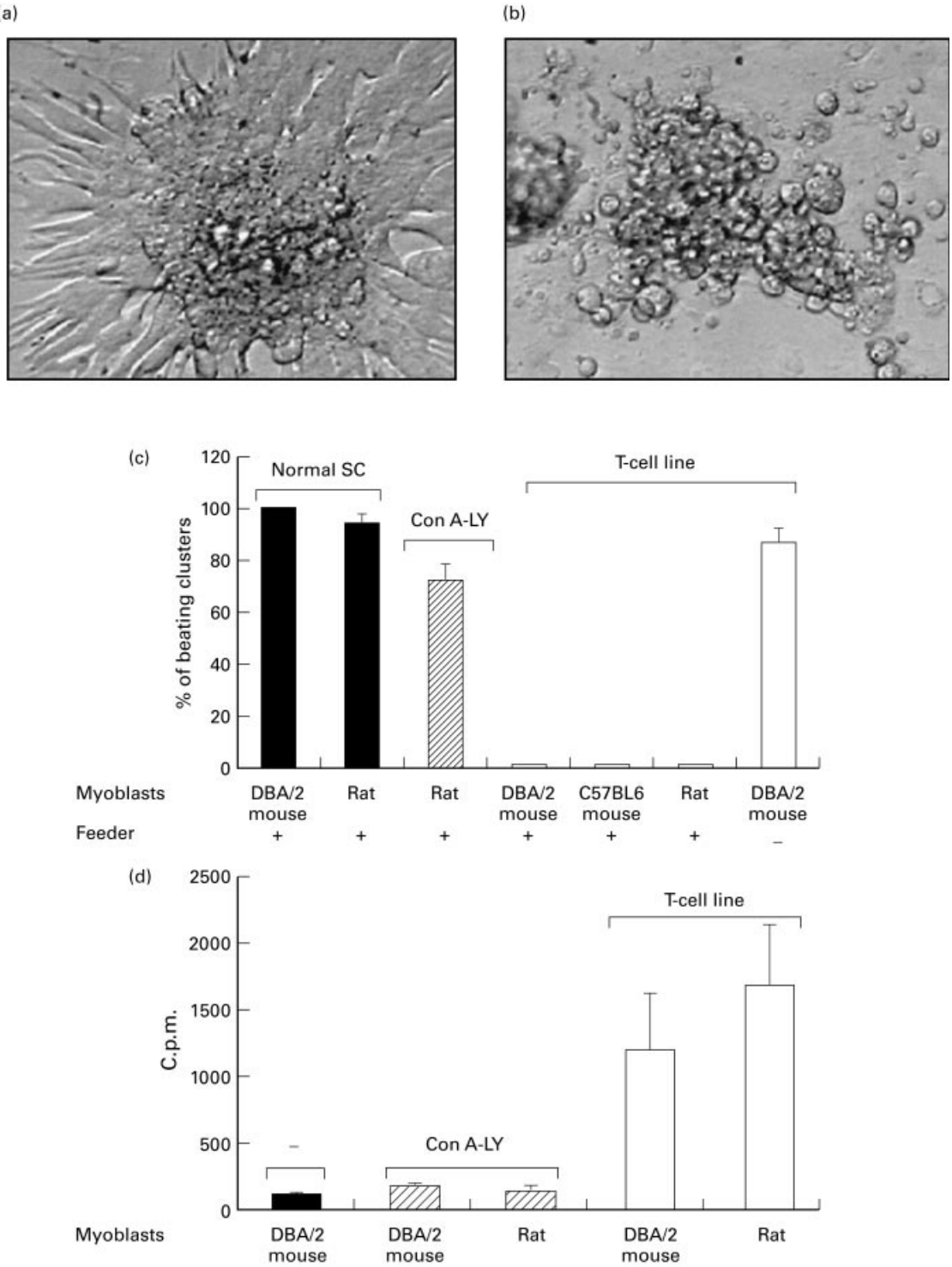

Figure 2 $CD4^+$ anti-heart T-cell line obtained from a chronic chagasic mouse is stimulated by and destroys syngeneic myoblast culture. Fetal heart cells were cultured *in vitro and* incubated with feeder cells. After 24 h of coculture, all heart cells had ceased to beat and were destroyed in the presence of the $CD4^+$ anti-heart T-cell line (b), whereas no damage was observed in the presence of Con A-activated cells (Con A-LY) (a). Myoblast cultures from different origins were cocultured with 3×10^5 irradiated syngeneic feeder cells and 10^5 purified T-cell line blasts, normal spleen cells (normal SC) or Con A-activated spleen cells. (c) The percentage of beating clusters was determined by counting them after 48 h of coculture. (d) Proliferation of cells in the cocultures described above was assessed by ^{3}H-thymidine uptake, as described in Material and Methods section. Data shown are representative of five experiments. Columns represent the mean results obtained from triplicate cultures, and vertical bars represent the SD of the means.

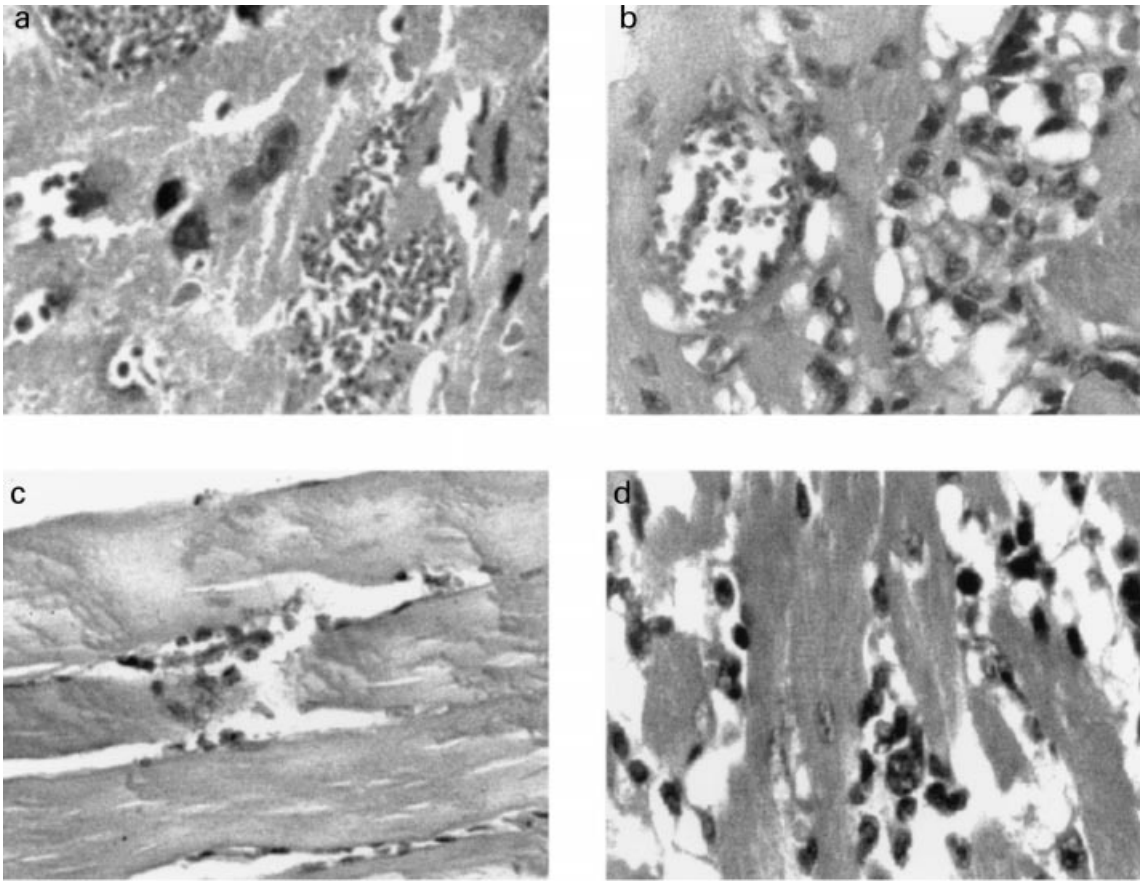

Figure 3 Inflammatory response in hearts of BALB/c nu/nu mice inoculated with the autoreactive T-cell line. (a) Control BALB/c nu/nu mice infected with *T. cruzi*. (b,c,d) BALB/c nu/nu mice, injected intravenously with 10^6 T-cell line cells, were also injected subcutaneously with syngeneic heart antigen (200 μg/mouse) in complete Freund's adjuvant (c,d), or infected with 10^2 trypomastigotes of the Colombian strain of *T. cruzi* (b). Hearts were removed 15 days (a,b,c) or 30 days (d) after the T-cell transfer for histopathological evaluation. Note the appearance of isolated inflammatory foci and myocytolysis 15 days after T-cell line transfer (c), spreading throughout the organ 15 days later (d). (a,b,d) H&E, × 400, (c) × 200.

detected in these cultures (data not shown). Splenocytes from nonreconstituted or reconstituted controls produced low or undetectable levels of the tested cytokines upon stimulation with any of the two antigens (Figure 4).

The autoreactive $CD4^+$ T-cell line induces syngeneic heart transplant rejection

We have demonstrated that purified $CD4^+$ T cells from chronically chagasic mice can induce rejection of heart transplants (12). To test whether the T-cell line was also capable of inducing graft rejection, purified blasts from the T-cell line were injected *in situ* into well-established heart transplants (Table 1). Control transplants were injected with purified $CD4^+$ T cells from normal mice or chronic chagasic mice. Similar to the observations made with purified chronic $CD4^+$ T cells, injection of the T-cell line into well-established grafts induced cessation of beating, necrosis in 24–48 h and disappearance of the grafts 1–2 weeks after cell transfer (Table 1). Histopathology of heart transplants 48 h after injection of the T-cell line demonstrates the presence of a diffuse mononuclear inflammatory infiltrate and intense myocytolysis (data not shown). Twelve days after the T-cell line transfer, when most of the heart tissue was reabsorbed, inflammamatory infiltrates and intense fibrosis were observed (data not shown).

DISCUSSION

We show here that an anti-heart $CD4^+$ T-cell line obtained from a chronic chagasic mouse is sufficient to induce a heart inflammatory response, death and syngeneic heart transplant rejection in the absence of *T. cruzi*. This finding

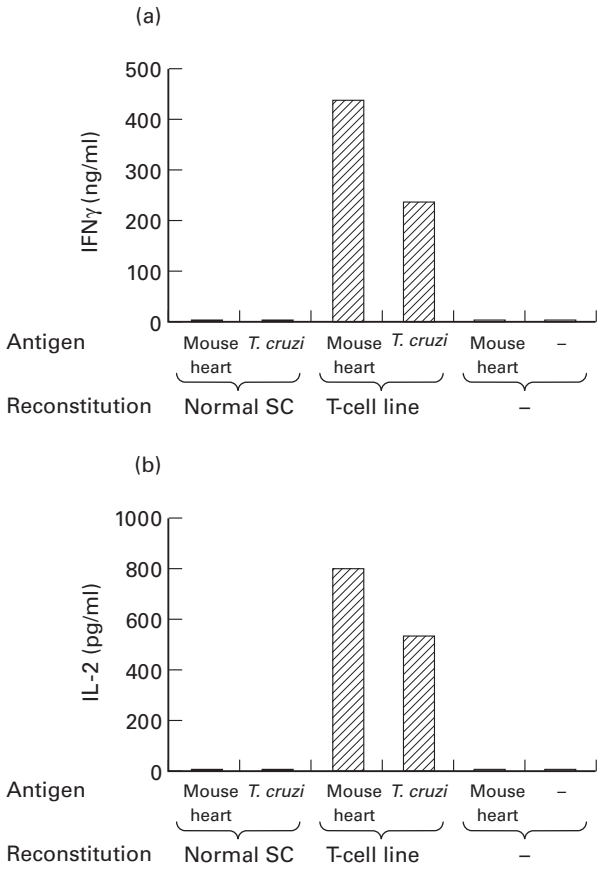

Figure 4 Cytokine production by spleen cells from nu/nu mice reconstituted with the anti-heart T-cell line. Groups of three BALB/c nu/nu mice were transferred with the T-cell line or with normal spleen cells (SC). After 60 days of reconstitution, spleen cells were stimulated *in vitro* with heart or *T. cruzi* antigens, as described in Material and Methods section. Spleen cells from nonreconstituted nu/nu mice were also stimulated as controls. (a) Interferon-γ and (b) IL-2 production was assessed 72 h after *in vitro* stimulation by ELISA.

is in accordance with previous observations regarding the role of $CD4^+$ T cells in the development of chronic carditis (12). Moreover, these results clearly support the hypothesis that chronic myocarditis in Chagas' disease has an autoimmune component. The low parasitism in the chronic phase of infection may be necessary and sufficient to generate a persistent state of inflammation capable of attracting and activating autoreactive T cells in the heart. Since a previous study has failed to correlate chronic carditis and heart transplant rejection with an autoreactive response (13), we suggest, based on the observations described in this paper and in others (12), that the contribution of antiparasite and anti-heart responses to the generation of chronic chagasic cardiomyopathy may vary according to the experimental model used.

The mere intravenous injection of the T-cell line into mice was unable to cause carditis. This suggests that further *in vivo* activation is necessary to allow migration of the T cells, through expression of adhesion molecules, to peripheral tissues, including the heart. *T. cruzi* infection or immunization of the T-cell recipients, as carried out in this work, could be promoting this activation. This phenomenon would be similar to that observed in mice transgenic for anti-basic myelin protein TCR, which only develop disease if immunized with antigen in complete Freund's adjuvant (20). The direct injection of the T-cell line into a heart transplant, or its coculture with myocytes *in vitro*, as also performed in this work, would of course obviate the need for migration.

Anti-*T. cruzi* cell lines and $CD4^+$ and $CD8^+$ T-cell clones have been previously characterized (21–24). These T cells were able to mediate protection against *T. cruzi* infection. Human T-cell clones cross-reacting with *T. cruzi* and myocardial antigens have also been described (25,26). However, to our knowledge, this is the first report of a T-cell line reactive with heart and *T. cruzi* antigens with the

Table 1 Rejection of syngeneic heart transplant injected *in situ* with the autoreactive T-cell line

Group	Recipient mice	Origin of $CD4^+$ T lymphocytes[a]	No. of mice with rejected graft/total no. of mice in group
I	Normal	–	0/10
II	Chronic chagasic[b]	–	9/10[c]
III	Normal	Normal mice	0/6
IV	Normal	Chronic chagasic mice[b]	6/6[d]
V	Normal	T-cell line	11/12[e]

[a]10^5 purified $CD4^+$ T cells were injected into the engrafted heart tissue 30 days after the transplants. [b]DBA-2 mice, 6 months after infection with Colombian strain *T. cruzi*. [c]Significantly different in relation to group I ($P = 0{\cdot}00012$, Fisher's exact probability test). [d]Significantly different in relation to group III ($P = 0{\cdot}0022$, Fisher's exact probability test). [e]Significantly different in relation to group III ($P = 0{\cdot}00004$, Fisher's exact probability test).

potential of promoting carditis. The finding of reactivity against heart antigens of different animal species suggests that the antigens recognized are well conserved. We are currently determining whether this T-cell line shows reactivity against human cardiac antigens as well as identifying the *T. cruzi* and heart antigens that it recognizes.

The chronic chagasic cardiomyopathy has been described as a delayed-type hypersensitivity (DTH) inflammatory reaction (2). Mononuclear phagocytes and T cells are the main components of the inflammatory infiltrate present in the heart during this phase of infection. Our anti-heart $CD4^+$ T-cell line shows a Th1 cytokine profile. This profile is compatible with the pathology of Chagas' disease, since Th1 cells are strong promoters of DTH-type responses. The requirement of feeder (normal spleen) cells for the cell line to effect destruction of heart myoblasts *in vitro* is consistent with a DTH effector mechanism, as macrophages activated by the $CD4^+$ T-cell line would mediate the lesion. In agreement with this observation, recent reports have shown an association between Th1 response and cardiac disease in chagasic patients (27,28).

Although we did not work with a clonal population, our T-cell line reacted with *T. cruzi* antigens even after being kept for more than 8 months *in vitro* under exclusive stimulation with heart antigens, strongly supporting the view that *T. cruzi*-specific lymphocytes were maintained by heart antigen stimulation (i.e. that they cross-react). Moreover, we performed fusion of BW-5 thymoma cells with spleen cells from the same *T. cruzi*-infected mice used to raise the T-cell line. Two out of four clones obtained from this fusion showed reactivity against both *T. cruzi* and heart antigens, indicating the presence of cross-reactive T cells in the spleen cell population (Ribeiro dos Santos, unpublished data).

The findings that a heart-specific $CD4^+$ T-cell line induces carditis *in vivo* and myocyte destruction *in vitro* are consistent with the fact that anti-CD4-antibody treatment, and not anti-CD8, aborts the development of experimental CChC in *T. cruzi*-infected mice (29). Notwithstanding the CD phenotype of the autoreactive cell, the possibility that it mediates carditis in the chronic phase of infection implies that the low parasitism found in that phase, even in tissues other than the heart, may be critical for triggering and/or maintaining a pathogenic autoimmune response. It is also possible that parasites of different strains display distinct degrees of molecular mimicry with heart antigens, explaining conflicting reports on their pathogenicities.

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