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# Cytotoxicity in patients with different clinical forms of Chagas' disease

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## SUMMARY

There are few studies on cell-mediated cytotoxicity in human Chagas' disease. In the present study, we evaluated peripheral blood mononuclear cell (PBMC) cytotoxicity activity from chagasic patients with different clinical forms of disease. To verify the cytotoxic response, we performed cell lysis assays using $^{51}$Cr-labelled K562 cells as targets. Results are reported as lytic units (LU = number of cells required for 30% lysis) per million PBMC. Exposure of patients' PBMC to *Trypanosoma cruzi* antigen led to an increase in cytotoxic activity compared with unstimulated patient cells against K562. Asymptomatic cardiomyopathy patients had higher responses ($37{\cdot}8 \pm 5{\cdot}0$ LU/$10^6$ PBMC; mean $\pm$ s.d.) than indeterminate ($11{\cdot}5 \pm 3{\cdot}6$ LU/$10^6$) and symptomatic cardiomyopathy ($7{\cdot}8 \pm 2{\cdot}5$ LU/$10^6$). Normal control PBMC stimulated with *T. cruzi* antigen had $4{\cdot}36 \pm 1{\cdot}31$ LU/$10^6$ PBMC against K562. Addition of recombinant interferon-gamma (IFN-$\gamma$) did not lead to significant increase in cytotoxicity in any group of patients. On the other hand, recombinant human IL-12 significantly increased cytotoxic responses from symptomatic cardiomyopathy patients and normal controls who presented low levels of cytotoxicity induced by *T. cruzi* antigen. The combined use of IL-12 and a neutralizing anti-IFN-$\gamma$ antibody did not change IL-12-induced cytotoxic responses, showing the direct role of this cytokine on natural killer (NK) cells. NK cells were the main cells responsible for the lysis of K562 target cells as evidenced by testing cell subsets following magnetic cell sorting. These data demonstrate that chagasic patients with different clinical forms of disease have PBMC which respond to *T. cruzi* antigen with a cytotoxic response, and this response is up-regulated by IL-12.

**Keywords** Chagas' disease cytotoxicity interferon-gamma IL-12

## INTRODUCTION

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a major health problem in Latin America, where an estimated 18 million people are infected and an additional 90 million are at risk of infection [1]. Most patients survive the initial acute stage of the infection, but some go on to develop the chronic manifestations of the disease years later. Morbidity and mortality in patients with chronic Chagas' disease is secondary to an inflammatory cardiomyopathy with attendant arrhythmias, conduction defects, congestive heart failure and/or thromboembolic events [2,3].

Immunity against protozoa has traditionally been thought to depend exclusively on CD4$^+$T helper functions controlling B cell (i.e. antibody) and lymphokine-mediated effector mechanisms. Recently, the importance of CD8$^+$ lymphocytes in the host immune response has been shown in parasitic infections. An important role for cytotoxic CD8$^+$ cells has been shown in malaria [4,5], murine leishmaniasis [6,7] and toxoplasmosis [8]. We have also reported the presence of cytotoxic cells in the peripheral blood of patients with cutaneous and mucosal leishmaniasis [9]. Depletion of CD8$^+$ cells in mice infected with *T. cruzi* has been shown to increase susceptibility to acute infection and abolish the protective immunity induced by an attenuated vaccine [10]. Moreover, recent observations [11] indicate that $\beta_2$-microglobulin-deficient mice are highly susceptible to infection, and die during the acute stage of the disease.

There are few studies on cell-mediated cytotoxicity in human Chagas' disease. Cytotoxic mechanisms may be implicated in either protective aspects by impairing the intracellular parasite multiplication, or in pathological mechanisms contributing to the destruction of myocardial fibres or other infected cells.

In this study we investigated the ability of peripheral blood mononuclear cells (PBMC) isolated from patients with different clinical forms of Chagas' disease to generate cytotoxic cell activity

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**Table 1.** Main features of patients with Chagas' disease

| Group* | Clinical findings | Patient No. | ECG | Chest x-ray | Echocardiogram |
|---|---|---|---|---|---|
| II | None | 1 | LBBB | Normal | Normal |
| | | 2 | LBBB | Cardiomegaly | ND |
| | | 3 | Normal | Cardiomegaly | Sclerosis of the aortic valve |
| | | 4 | Normal | Ventricular hypertrophy | ND |
| | | 5 | LBBB | Normal | Left ventricular dysfunction |
| III | Congestive heart failure | 6 | RBBB + LAHB | Cardiomegaly | ND |
| | | 7 | PVB + RBBB | Cardiomegaly | ND |
| | | 8 | LBBB | Cardiomegaly | ND |
| | | 9 | LVH + LAE | Cardiomegaly | ND |
| | | 10 | PVB | Cardiomegaly | ND |
| | | 11 | Sinus bradi or tachycardia | Cardiomegaly | Sinoatrial block |
| | | 12 | RBBB + LBBB | Cardiomegaly | ND |
| | | 13 | PVB + RBBB + LAHB | Cardiomegaly | ND |
| | | 14 | Atrial fibrilation | Cardiomegaly | ND |
| | | 15 | LBBB + PVB | Cardiomegaly | Artificial pacemaker |
| | | 16 | LBBB | Cardiomegaly | ND |
| | | 17 | PVB + RBBB | Cardiomegaly | Sinoatrial block + atrial arrythmia |

All patients in Group I (indeterminate) had no clinical findings and negative test results.

*Group II, asymptomatic cardiomyopathy patients; Group III, symptomatic cardiomyopathy patients.

RBBB, Right bundle branch block; LBBB, left bundle branch block; LAHB, left anterior hemiblock; PVB, premature ventricular beats; LAE, left atrial enlargement; LVH, left ventricular hypertrophy.

against K562 cells using *T. cruzi* antigen for T cell stimulation. We have also evaluated the role of IL-12 and interferon-gamma (IFN-$\gamma$) by adding these exogenous products in this antigen-driven cytotoxic system.

## PATIENTS AND METHODS

*Patient selection and characterization*

A detailed evaluation, including a complete physical examination, electrocardiogram (ECG), chest x-ray and echocardiogram, was performed in each patient. Clinical diagnosis was confirmed in all patients by laboratory tests (serology by ELISA and immunofluorescence). Patients' findings are summarized in Table 1.

According to these results, patients were divided into three groups: (i) indeterminate: patients with a positive serology for *T. cruzi* but no signs or symptoms of Chagas' disease as well as absence of cardiac alterations (normal ECG, normal chest x-ray and normal echocardiogram); (ii) asymptomatic cardiomyopathy: patients with absence of clinical complaints but with evidence of alterations in the echocardiogram or ECG, such as: (a) first degree A. V. block; (b) second degree A. V. block; (c) total A. V. block; (d) right bundle branch block; (e) left bundle branch block; (f) intraventricular block; (iii) symptomatic cardiomyopathy: those with congestive heart failure (alterations in ECG, chest x-ray and/or echocardiogram).

Indeterminate patients and those with asymptomatic cardiomyopathy were seen in the Out-Patient Clinic of the Hosp. Univ. Prof. Edgard Santos. Symptomatic cardiomyopathy patients were hospitalized at the same institution. Informed consent was obtained from all patients, and the study was approved by the Committee on Human Rights of the Hospital Universitário Edgard Santos (University of Bahia).

*Reagents*

All cytokines were human recombinant products. IFN-$\gamma$ (a gift from Dr Maud Brandley, Institute Roussel Uclaf, Paris, France) was used at 40 U/ml; IL-12 (a gift from Dr Stanley Wolf, Genetics Institute, Cambridge, MA) was used at 500 U/ml. Anti-IFN-$\gamma$ MoAb (kindly provided by Dr Robert Coffman, DNAX Research Institute, Palo Alto, CA) was used at 20 $\mu$g/ml. All reagents were free of endotoxin as indicated by the manufacturers.

*Parasite and antigen*

Soluble antigen was prepared from the 12SF strain of *T. cruzi* [12]. Briefly, epimastigotes cultivated in liver-infusion tryptose (LIT) medium were washed three times in PBS, re-suspended in PBS with $10^9$/ml epimastigotes, and rapidly frozen ($-70^\circ$C) and thawed ($37^\circ$C) six times. This material was centrifuged (16 000 ***g***) for 10 min, after which the supernatant was collected, the protein content was determined by Lowry's method, adjusted to 1 mg/ml and stored at $-20^\circ$C.

*Effector cells*

Patients' PBMC were obtained from heparinized venous blood over a Ficoll–Hypaque gradient. Cultures were performed in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10% heat-inactivated pooled AB serum, at a concentration of $2 \times 10^6$ cells/ml and stimulated with *T. cruzi* antigen (10 $\mu$g/ml) for 5 days at $37^\circ$C and 5% $CO_2$ in a humid incubator.

**Table 2.** Effect of *Trypanosoma cruzi* antigen alone or combined with IFN-$\gamma$ on the cytotoxic activity of Chagas' disease patients' peripheral blood mononuclear cells (PBMC) against K562 target cells

| Patient group | Age (years) | *n* | Lytic units/million PBMC (geometric mean ± s.e.m)* | | |
|---|---|---|---|---|---|
| | | | Unstimulated | Tc Ag† | IFN-$\gamma$‡ + Tc Ag |
| Indeterminate (I) | 33–47 | 5 | $2{\cdot}4 \pm 2{\cdot}34$ | $11{\cdot}5 \pm 3{\cdot}6$ | $13{\cdot}5 \pm 2{\cdot}1$ |
| Asymptomatic cardiomyopathy (II) | 35–52 | 5 | $3{\cdot}2 \pm 2{\cdot}2$ | $37{\cdot}8 \pm 5{\cdot}0$ | $36{\cdot}2 \pm 3{\cdot}8$ |
| Symptomatic cardiomyopathy (III) | 34–66 | 10 | $2{\cdot}2 \pm 2{\cdot}0$ | $7{\cdot}8 \pm 2{\cdot}5$ | $13{\cdot}5 \pm 3{\cdot}2$ |
| Normal volunteers | 37–42 | 4 | $2{\cdot}4 \pm 2{\cdot}7$ | $4{\cdot}36 \pm 1{\cdot}31$ | $7{\cdot}65 \pm 1{\cdot}63$ |

*Comparisons among different conditions in the same group of patients (in rows): repeated measures ANOVA followed by Tukey's test. Group I, unstimulated × Tc Ag $P < 0{\cdot}05$; Group II, unstimulated × Tc Ag $P < 0{\cdot}001$; Group III, unstimulated × Tc Ag $P < 0{\cdot}001$. In all groups of patients Tc Ag × Tc Ag + IFN-$\gamma$ $P > 0{\cdot}05$.

†Epimastigote lysate used at 10 $\mu$g/ml.

‡Recombinant human IFN-$\gamma$ used at 40 U/ml for 5 days in culture.

*Tumour target cells*

Target cells were prepared from suspension cultures of the natural killer (NK)-susceptible tumour cell line K562 (American Type Culture Collection, Rockville, MD) maintained in RPMI 1640 with 10% fetal calf serum (FCS). Target cells were radiolabelled by suspension at a concentration of $10^7$ cells/ml in medium containing 200 $\mu$Ci/ml of $^{51}$Cr sodium chromate (Instituto de Pesquisa Energética e Nuclear, São Paulo, Brazil) for 90 min at 37°C. After washing three times they were re-suspended in medium at a concentration of $4 \times 10^4$ cells/ml.

*Cytotoxicity assay*

Radiolabelled tumour target cells, at $2 \times 10^3$ cells/well, were dispensed in 50 $\mu$l aliquots into wells of round-bottomed microtitre plates (Corning, Corning, NY), and incubated in RPMI 1640 medium with 10% FCS with different numbers of effector cells (ranging from 3 : 1 to 100 : 1 effector : target ratio) for 4 h at 37°C, and 5% $CO_2$ in a humid incubator. Cells were incubated for 4 h allowing for target lysis. Supernatants were harvested (Skatron Instruments, Harvesting Press, Dolasletta, Norway) and radioactivity counted using a gamma counter (Beckman, Meriden, CT). Percent specific lysis was calculated using the formula: $100 \times$ ((experimental release − spontaneous release)/(maximum release − spontaneous release)) for a mean of triplicate wells. Spontaneous release was obtained by target cells in the presence of medium alone, and maximum release by addition of 0·1 M HCℓ. Specific cytotoxicity is expressed in lytic units. One lytic unit was defined as the number of cells required for 30% specific target lysis (determined from dose–response curves with $r \geqslant 0{\cdot}95$), and results are expressed as number of lytic units per million PBMC.

*Separation of different cells populations responsible for cytotoxicity*

Unfractionated PBMC were stimulated with *T. cruzi* antigen (10 $\mu$g/ml) for 5 days in a humid atmosphere with 5% $CO_2$. These cells were then separated by magnetic cell sorting (Mini Macs Miltenyi Biotec, Sunnyvale, CA). The material was first magnetically stained using MoAbs conjugated to magnetic beads (Macs microbeads). Magnetic staining was done directly with specific Macs microbeads for $CD4^+$ and $CD8^+$ subpopulations. In order to separate NK cells, we used negative enrichment NK cell isolation kit. Cells were labelled with a cocktail of anti-CD3, CD4, CD19, CD33 antibodies of mouse IgG1 isotype to label non-NK cells and mixed with colloidal supermagnetic microbeads recognizing mouse IgG1. The stained material was passed through separation column. Unstained material passed through the column from the magnetic source. The purity of the populations obtained after purification was confirmed by flow cytometry analysis (FACScan, Becton Dickinson, Mountain View, CA), using MoAbs anti-CD8, CD4, CD3 and CD56 labelled with PE or FITC. We obtained 90% of purity in T $CD4^+$ and $CD8^+$ cell populations and 82% purity in relation to NK cell populations.

*Statistical analysis*

Curves for determining lytic units in each experiment were calculated from dose–response determinations with four different effector : target ratios, by simple linear regression. Comparisons of PBMC cytotoxic responses were performed in log transformed data. Comparisons among the three groups and controls were performed by one-way ANOVA followed by Tukey's test. Comparisons among different conditions in the same group of individuals were made by repeated measures ANOVA followed by Tukey's test. In all tests, $P < 0{\cdot}05$ was considered significant.

## RESULTS

*Cytotoxicity against tumour target cells*

Cytotoxic responses of the three groups of patients, as well as normal volunteers, are shown in Table 2. *Trypanosoma cruzi* antigen stimulation induced the highest increase in cytotoxic response in asymptomatic cardiomyopathy patients. A cytotoxic response of $3{\cdot}2 \pm 2{\cdot}2$ LU/million PBMC in unstimulated cultures increased to $37{\cdot}8 \pm 5{\cdot}0$ LU/million PBMC ($P < 0{\cdot}001$) following antigen stimulation in these patients. Indeterminate individuals ($2{\cdot}4 \pm 2{\cdot}4$ *versus* $11{\cdot}5 \pm 3{\cdot}6$ LU/million PBMC for unstimulated and *T. cruzi* antigen-stimulated cultures respectively; $P < 0{\cdot}05$) and patients with symptomatic cardiomyopathy ($2{\cdot}2 \pm 0{\cdot}3$ *versus* $7{\cdot}8 \pm 2{\cdot}5$ LU/million PBMC for unstimulated and *T. cruzi* antigen-stimulated cultures respectively; $P < 0{\cdot}001$) also increased their cytotoxic activity in response to antigen stimulation.

*Trypanosoma cruzi* antigen did not stimulate PBMC from normal individuals ($2{\cdot}4 \pm 2{\cdot}7$ *versus* $4{\cdot}36 \pm 1{\cdot}31$ for unstimulated and antigen-stimulated cultures, respectively; $P > 0{\cdot}05$).

**Table 3.** Effect of IL-12 on *Trypanosoma cruzi* antigen-stimulated cytotoxic activity of peripheral blood mononuclear cells (PBMC) from symptomatic cardiomyopathy patients against K562 target cells

| | Lytic units/million PBMC | | |
|---|---|---|---|
| Patient no. | Unstimulated | Tc Ag* | IL-12† + Tc Ag |
| 8 | 2·4 | 5·2 | 7·1 |
| 12 | 0·3 | 1·7 | 7·7 |
| 16 | 0·4 | 5·0 | 14·3 |
| 17 | 0 | 0·8 | 15·6 |
| Normal controls‡ | 0·34 ± 0·56 | 1·44 ± 1·68 | 10·47 ± 1·66## |

*Epimastigote lysate used at 10 $\mu$g/ml.
†Recombinant human IL-12 used at 500 U/ml.
‡Mean ± s.d. ($n = 5$).
§IL-12 without antigen gave 8·75 ± 1·91 LU/$10^6$ (not significant in comparison with IL-12 + *T.cruzi* Ag).

Addition of bovine serum albumin to patients' PBMC at a concentration similar to that used for *T. cruzi* antigen did not induce cytotoxic responses (data not shown).

*Role of IFN-$\gamma$ in the cytotoxic response*
Addition of IFN-$\gamma$ (40 U/ml) to *T. cruzi* antigen (10 $\mu$g/ml)-stimulated cultures did not lead to significant increase in cytotoxicity in any group of patients (Table 2).

*Role of IL-12 in the cytotoxic response*
Freshly isolated PBMC from four symptomatic cardiomyopathy patients (shown in Table 1) were stimulated with *T. cruzi* antigen (10 $\mu$g/ml) during 5 days with or without IL-12 (500 U/ml) and cytotoxic activity was measured against K562 target cells. IL-12 significantly increased ($P = 0{\cdot}005$) *T. cruzi* antigen-induced cytotoxic responses of PBMC from symptomatic cardiomyopathy patients (Table 3). However, this effect was also observed in normal individuals. The addition of rIL-12 to the culture of PBMC from normal donors, without antigen, led to an increase of cytotoxic responses against K562 target cells ($P < 0{\cdot}05$).

In order to investigate if the effect of IL-12 on the cytotoxic response was due to indirect action through the production of IFN-$\gamma$ we added anti-IFN-$\gamma$ MoAb to IL-12-stimulated cultures. Cells from three symptomatic cardiomyopathy patients were stimulated with *T. cruzi* antigen (10 $\mu$g/ml) and IL-12 (500 U/ml) in the presence of anti-IFN-$\gamma$ antibody (20 $\mu$g/ml) for 5 days and tested against K562 tumour target cells. No significant change was observed with the use of the anti-IFN-$\gamma$ MoAb (Fig. 1).

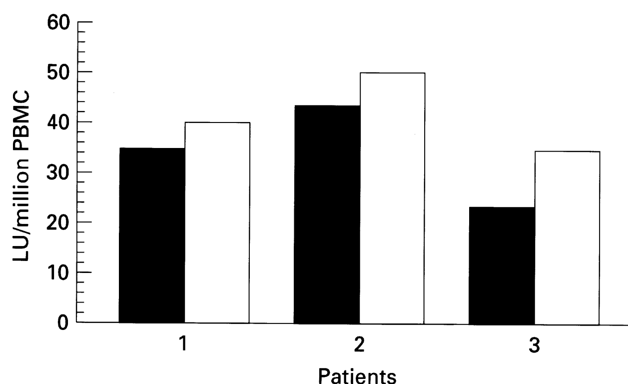


**Fig. 1.** Evaluation of the participation of IFN-$\gamma$ on the IL-12-mediated enhancement of cytotoxic activity. Peripheral blood mononuclear cells (PBMC) from symptomatic cardiomyopathy patients were stimulated with *Trypanosoma cruzi* antigen (10 $\mu$g/ml) and IL-12 (500 U/ml) in the presence of anti-IFN-$\gamma$ antibody (20 $\mu$g/ml) for 5 days and tested against K562 tumour target cells. The neutralization of IFN-$\gamma$ did not change the cytotoxic activity. Each pair of bars represents a different patient. ■, IL-12; □, IL-12 + anti-IFN-$\gamma$. LU, Lytic units.

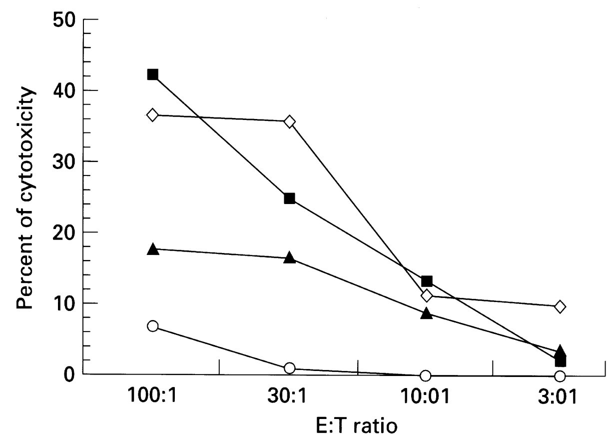


**Fig. 2.** Phenotype of cells responsible for cytotoxicity activity against K562 tumour target cells. Peripheral blood mononuclear cells (PBMC) from chagasic patients were stimulated with *Trypanosoma cruzi* antigen (10 $\mu$g/ml) for 5 days followed by cell subset separation using Mini Macs magnetic sorting (see Patients and Methods). Natural Killer (NK) cells present cytotoxic response similar to total cells. Results from one out of three similar experiments, with cells from three different patients, are shown. ■, PBMC; ○, $CD4^+$; ▲, $CD8^+$; ◇, NK.

*Phenotype of cells responsible for cytotoxicity against tumour cells*
To determine the phenotype of cells responsible for the cytolytic activity, cells subsets were obtained by magnetic sorting, and tested against K562 target cells. A representative experiment out of three (Fig. 2) shows that $CD4^+$ and $CD8^+$ T cells were not able to kill target cells, while NK cells still presented cytotoxic response similar to total cells. Similar results were obtained with cells from asymptomatic cardiopathy or indeterminate patients (data not shown). The purity of the NK cell population was checked by flow cytometry analysis using FITC- or PE-labelled anti-CD56 and anti-CD3 MoAb. Cells with the $CD56^+/CD3^-$NK phenotype corresponded to 82% of purified cells.

## DISCUSSION

The results of the present study demonstrate that *T. cruzi* antigen is able to stimulate cytotoxic activity of PBMC from patients with Chagas' disease. PBMC from indeterminate or asymptomatic cardiomyopathy patients are more active as cytotoxic effectors against tumour target cells than cells from symptomatic cardiomyopathy patients. It is difficult to distinguish whether the decreased cytotoxic response is implicated in the development of cardiac pathology, by allowing parasite growth, or if a previously

higher response is modulated in the later stages of disease. It is also possible that decreased cytotoxic activity in PBMC from cardiac patients represents a compartmentalization of the response following recruitment of responsive cells to the heart. This latter interpretation is supported by the evidence that a predominance of $CD8^+$ T cells has been observed in the inflammatory infiltrate of mice infected with *T. cruzi* [13] and in human chronic chagasic myocardial lesions, where tumour necrosis factor-alpha (TNF-$\alpha$) and granzyme have also been demonstrated [14].

*Trypanosoma cruzi*-infected $\beta_2$-microglobulin-deficient mice had a reduction in immunosuppression and in their acute inflammatory reaction, but exhibited a higher parasite burden and higher mortality than their $\beta_2$-microglobulin $+/-$ littermates [11]. Cytotoxic cells may contribute to reducing parasite numbers by destroying the infected cells, but can participate in tissue destruction leading to pathology, as has been suggested [13,14]. It is also noteworthy that $\beta_2$-microglobulin $-/-$ mice, although showing normal IFN-$\gamma$ levels, were less able to kill *T. cruzi* amastigotes, indicating that this cytokine may not be of great importance to the reduction of the parasite load. Out results indicate that IFN-$\gamma$ is not involved in the regulation of cytotoxic responses from Chagas' disease patients.

A significant non-antigen-specific and not MHC-restricted cytotoxic response, which probably includes NK and LAK cell activity, was observed in the splenocyte population of *T. cruzi*-sensitized mice [15]. Our data demonstrate that NK cells are the predominant cells in killing K562 target cells in Chagas' disease patients. NK activity has also been detected in the spleens, blood and hearts of acutely *T. cruzi*-infected rats [16].The exact role that these non-specific cytolytic mechanisms play in protective immunity to this parasite remains to be determined. The fact that such activity can be induced by specific *in vitro* antigen challenge suggests that such a mechanism might also operate *in vivo* and could contribute to the development of chagasic tissue lesions. According to this idea, it is interesting to observe that asymptomatic patients showed high cytolytic responses, possibly reflecting some destruction of myocardium. In order to evaluate this possibility, asymptomatic cardiomyopathy patients should be followed up for many years. Unfortunately, such a follow up is very difficult to carry out.

IL-12 is a cytokine with multiple activities on T and NK cells, including the induction of production of IFN-$\gamma$ and increased cytotoxicity. In this study, we demonstrate that IL-12 was able to restore the cytotoxic response of PBMC from symptomatic cardiomyopathy controls. This effect, however, was observed also in normal controls, showing that the effect of IL-12 is directly on the cytotoxic cells (NK cells). In fact, IL-12 is able to induce accumulation of mRNA for perforin in NK cells [17]. The effect of IL-12 on the cytotoxic activity in this system does not seem to be due to increased IFN-$\gamma$ production, since anti-IFN-$\gamma$ MoAb did not abate the effect of IL-12. Absence of blockade of the IL-12 effect by anti-IFN-$\gamma$ MoAb has also been observed in a system evaluating cytotoxic activity in human CTL, where addition of IL-12 resulted in a marked augmentation of cytotoxicity per cell with a small increase in cell number [18].

It is important to point out that the present study deals primarily with cytotoxicity against tumour target cells. Such results cannot be extrapolated to class I-mediated responses against infected cells. Further studies will be done to evaluate patients' cytotoxic responses to autologous *T. cruzi*-infected macrophages.

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