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Administration of anti-CD3 monoclonal antibody during experimental Chagas' disease induces $CD8^+$ cell-dependent lethal shock

F. JACOBS, C. DUBOIS, Y. CARLIER* & M. GOLDMAN *Laboratoire Pluridisciplinaire de Recherche Expérimentale Biomédicale, Hôpital Erasme, and *Department of Parasitology, Université Libre de Bruxelles, Brussels, Belgium*

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SUMMARY

The injection of the 145-2C11 anti-CD3 MoAb in mice induces a polyclonal T cell activation resulting in the release of several cytokines, including interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). As these cytokines are known to be involved in the host defence against *Trypanosoma cruzi*, we measured serum levels of IFN-γ and TNF-α after injection of the 145-2C11 MoAb in the course of experimental murine Chagas' disease. Compared with control mice, *T. cruzi*-infected BALB/c mice were found to be primed to secrete very high levels of IFN-γ and TNF-α from the second and the first week of infection, respectively, up to the chronic phase. *In vivo* cell depletion experiments indicated that $CD8^+$ T cells were responsible for these dramatic hyperproductions of IFN-γ and TNF-α. While all control mice survived anti-CD3 MoAb injection, a high lethality rate was observed in *T. cruzi*-infected mice within 24 h after anti-CD3 MoAb challenge. Pretreatment with neutralizing anti-IFN-γ MoAb or depletion of $CD8^+$ T cell population dramatically decreased the mortality induced by anti-CD3 MoAb in *T. cruzi*-infected mice. Finally, we showed that anti-CD3 MoAb injection in *T. cruzi*-infected mice was followed by a massive release of nitric oxide (NO) metabolites, which was partially reduced by IFN-γ or TNF-α neutralization. The administration of the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) before anti-CD3 MoAb challenge did not prevent and even enhanced lethality in *T. cruzi*-infected mice, suggesting that NO overproduction and lethal shock are not causally related. We conclude that injection of anti-CD3 MoAb in the course of experimental Chagas' disease induces a $CD8^+$ cell-dependent shock mediated by IFN-γ and TNF-α.

Keywords anti-CD3 monoclonal antibody *Trypanosoma cruzi* interferon-gamma tumour necrosis factor-alpha

INTRODUCTION

The acute phase of Chagas' disease in mice is associated with a T cell defect characterized by a decreased production of IL-2 in response to parasite antigens and T cell mitogens [1–3]. In contrast, production of interferon-gamma (IFN-γ) during the second week of infection is increased, as indicated by accumulation of IFN-γ mRNA in spleen and increased *in vitro* secretion of IFN-γ by spleen cells [4]. An *in vitro* study suggested that $CD3^+$, $CD4^-$, $CD8^-$ cells represent an important source of IFN-γ during *Trypanosoma cruzi* infection [4], but the role of these cells in the *in vivo* secretion of IFN-γ is unknown. IFN-γ is clearly involved in resistance against *T. cruzi* infection. Indeed, several *in vitro* studies indicated that IFN-γ enhances the ability of macrophages to clear the parasite [5,6]. Moreover, injection of rIFN-γ in the early phase of murine Chagas' disease prevented the development of morbidity and mortality associated with the infection [7,8]. The dramatic effects of *in vivo* neutralization of IFN-γ by anti-IFN-γ MoAb on the evolution of the infection [9,10] provided definitive evidence for a crucial role of endogenous IFN-γ in host defence against *T. cruzi*. It is likely that this protective effect of IFN-γ involves the induction of nitric oxide (NO) synthase activity, as NO production by infected macrophages was shown to be critical for parasite killing [11–15].

The hamster MoAb 145-2C11 that specifically recognizes the ϵ chain of the mouse CD3 complex [16] induces an acute polyclonal T cell activation resulting in massive secretion of several cytokines. *In vivo*, injection of the 145-2C11 MoAb into normal mice induces a rapid release of IFN-γ and tumour necrosis factor-alpha (TNF-α) in the bloodstream and a transient non-lethal shock syndrome [17–19]. In the present study we compared the *in vivo* production of IFN-γ and TNF-α induced by 145-2C11 MoAb injection at different time points during acute Chagas' disease. The observation that mice were primed to secrete very high levels of IFN-γ from day 14 and TNF-α from day 7 after parasite inoculation prompted us to investigate whether $CD4^+$ and/or $CD8^+$ cells were responsible for IFN-γ and TNF-α production in this model. Moreover, the high lethality rate induced by anti-CD3 MoAb

Correspondence: Michel Goldman MD, Hôpital Erasme, Department of Immunology, Route de Lennik, 808, B-1070 Brussels, Belgium.

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injection in *T. cruzi*-infected mice led us to study the role of IFN-γ and TNF-α in the increased toxicity of the anti-CD3 MoAb during experimental Chagas' disease.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 8 weeks of age, were purchased from Bantin and Kingman Ltd. (Hull, UK) and were maintained in our animal facilities on standard laboratory chow.

Parasites

Mice were infected intraperitoneally with 100 blood-form trypomastigotes of the Tehuantepec strain of *T. cruzi* as previously described [20]. For this purpose, blood taken from an acutely infected mouse was mixed with heparin and diluted in Alsever solution. Control mice received the same quantity of Alsever-diluted blood from an uninfected mouse.

MoAbs and nitric oxide synthase inhibitor injected in vivo

The hamster MoAb 145-2C11 directed against mouse CD3 was produced as supernatant of 145-2C11 hybridoma [16].

The following rat MoAbs were produced in ascitic form: GK1.5, an anti-CD4 IgG2b MoAb [21]; H35-17.2, an anti-CD8 IgG2b MoAb [22]; R46A2, a neutralizing anti-IFN-γ IgG1 MoAb [23]. As control, we used ascites of LO-DNP-2 hybridoma cells (kindly provided by Dr H. Bazin, Experimental Immunology Unit, Université Catholique de Louvain, Belgium), secreting a rat IgG1 MoAb with anti-DNP specificity.

Purified TN3 19-12 hamster anti-mouse TNF-α IgG1 MoAb and its isotype control MOPC21 (CB1) were generously provided by Cell Tech (Slough, UK). The nitric oxide synthase (NOS) inhibitor used in experiments designed to analyse the role of NO *in vivo* was N-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical Co., St Louis, MO).

All the MoAbs used for *in vivo* depletion or stimulation had endotoxin levels <15 pg/ml as determined by a Limulus amoebocyte lysate assay (LAL-QCL-1000; Whittaker MA Bioproducts, Walkersville, MD).

Monitoring of cell depletion

The $CD4^+$ and $CD8^+$ T cell amount was monitored by fluorescent antibody staining of spleen cells and flow cytometric analysis. Splenocytes (5×10^5) were stained at 0°C with optimal dilutions of PE-conjugated L3T4 anti-CD4 MoAb or FITC-labelled Lyt2 anti-CD8 MoAb, both obtained from Becton Dickinson (San Jose, CA). Viable cells (10^4), identified by their ability to exclude propidium iodide, were analysed by flow cytometry (FACStarplus; Becton Dickinson) after gating on lymphocytes according to forward and orthogonal light scatter characteristics.

Determination of IFN-γ and TNF-α serum levels

Serum levels of IFN-γ were quantified, as previously described [24], by two-site ELISA using the F1 and Db-1 rat anti-mouse IFN-γ MoAbs, kindly provided by Dr A. Billiau (Katholieke Universiteit Leuven, Leuven, Belgium) and P. H. Van Der Meide (TNO Health Research, Rijswijk, The Netherlands), respectively. The lower limit of detection of IFN-γ in this test was 1 U/ml.

Serum samples were assayed for TNF-α by two-site ELISA using two rabbit anti-mouse TNF-α polyclonal antibodies kindly provided by Dr W. Buurman (University of Limburg, Maastricht, The Netherlands). The lower limit of detection of TNF-α in this assay was 80 pg/ml.

NO_2^-/NO_3^- assay

Serum samples were assayed for NO_2^- and NO_3^- (stable end products of NO) after reduction of NO_3^- into NO_2^- by copperplated cadmium [25]. Briefly, 50 μl of each sample were first deproteinized by incubation with 200 μl $ZnSO_4$ (75 mM) and 250 μl NaOH (55 mM) for 10 min at room temperature. After centrifugation at 1000 ***g*** for 10 min, 200 μl of supernatant and 200 μg of activated cadmium were mixed together and stirred at room temperature for 1 h. Activated cadmium was prepared as follows: 5 g cadmium powder (100% mesh; Johnson Matthey, Karlsruhe, Germany) were first plated with copper by stirring in 20 ml of 5 mM $CuSO_4$. Excess metallic Cu was removed by extensive washing with glycine–NaOH buffer pH 9·7. Copperplated cadmium was then dried on filter paper and immediately used for the reduction of NO_3^- to NO_2^-. Reduced samples were incubated with an equal volume of Griess reagent and absorbance was measured (A_{450}) on a microplate reader (Multiscan MCC/340; Labsystems, Helsinki, Finland). NO_2^- concentrations were calculated from a reduced $NaNO_3^-$ standard curve ranging from 5 mM to 0·5 μM [26]. The lower limit of detection of NO_3^- in this test was 80 μM.

Statistical analysis

Results are expressed as mean $\pm$ s.e.m. Comparisons between groups were made using either Mann–Whitney test or analysis of variance test using a program for statistics and epidemiology [27].

RESULTS

IFN-γ and TNF-α release induced by anti-CD3 MoAb injection in the course of experimental Chagas' disease

The release of IFN-γ and TNF-α in serum was first studied after i.p. injection of 5 μg of the anti-CD3 MoAb given as a single dose at one of the following time points after parasite inoculation: 0, 7, 14, 21, 28 or 120 days. Preliminary experiments established that serum TNF-α and IFN-γ levels peak at 90 min and 4 h, respectively, after anti-CD3 MoAb injection, both in controls and in *T. cruzi*-infected mice. Therefore, in each group, mice were bled before, 90 min or 4 h after anti-CD3 MoAb injection. IFN-γ serum levels obtained before MoAb injection were <2 U/ml during the whole course of the infection. The peak levels of IFN-γ, measured 4 h after anti-CD3 MoAb injection, remained fairly constant during the first week of the infection, but massively increased (about 100-fold above levels in uninfected mice) on day 14 and remained at such high levels when the anti-CD3 MoAb challenge was given when mice had entered the chronic phase (day 120) (Fig. 1). In absence of anti-CD3 MoAb administration, TNF-α serum levels remained below detection threshold during the course of *T. cruzi* infection. On the other hand, peak TNF-α serum levels obtained after anti-CD3 MoAb injection in *T. cruzi*-infected mice increased after day 7 of injection and reached a peak during the second week (Fig. 1). These high levels were, as for IFN-γ, maintained during the chronic phase of the infection (Fig. 1). The hyperproduction of IFN-γ and TNF-α induced by anti-CD3 MoAb injection in *T. cruzi*-infected mice was also apparent after injection of a higher dose (50 μg) of the MoAb (data not shown and Table 1). As trypomastigotes were detectable in blood after day 10 of infection and peaked between days 21 and 28 (data not shown), the

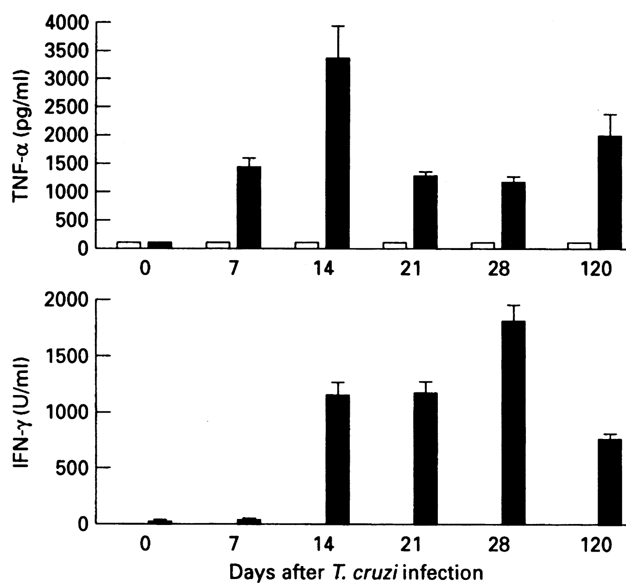

Fig. 1. Release of tumour necrosis factor-alpha (TNF-α) and IFN-γ in serum 90 min and 4 h, respectively, after injection of 5 μg anti-CD3 MoAb in control mice (day 0) and during *Trypanosoma cruzi* infection. Results are presented as mean ± s.e.m. of at least five mice per group. □, Before anti-CD3 MoAb injection; ■, after anti-CD3 MoAb injection.

hyperproduction of IFN-γ and TNF-α in response to anti-CD3 MoAb clearly preceded the peak of parasitaemia.

The major role of CD8$^+$ cells in hyperproduction of IFN-γ and TNF-α in T. cruzi*-infected mice*

To determine which T cell population(s) was responsible for the production of IFN-γ and TNF-α in this model, mice were depleted of CD4$^+$ or CD8$^+$ T cells *in vivo* by administration of either anti-CD4$^+$ or anti-CD8$^+$ MoAb before challenge with a high dose (50 μg) of anti-CD3 MoAb. These experiments were performed 18 days after *T. cruzi* infection after verification that the depleting regimen (1 mg of anti-T cell subset MoAb given on day −1 and day −3 before anti-CD3 MoAb injection) resulted in an efficient elimination of the relevant subset as indicated by flow cytometry of spleen cells: <1% of CD4$^+$ or CD8$^+$ cells were detectable after injection of corresponding MoAb. In mice on day 18 of *T. cruzi* infection, CD8$^+$ cell depletion resulted in a 80% decrease in the release of IFN-γ and a 90% decrease in the release of TNF-α, whereas CD4$^+$ cell depletion had no significant effect (Table 1), indicating that the hyperproduction of IFN-γ and TFN-α after anti-CD3 MoAb injection in *T. cruzi*-infected mice is due to priming of CD8$^+$ cells.

IFN-γ is involved in the lethal shock induced by anti-CD3 MoAb in T. cruzi*-infected mice*

Although control uninfected mice developed prostration, piloerection and diarrhoea after injection of 50 μg anti-CD3 MoAb, all these animals recovered from this shock syndrome within 48 h, as previously described [17–19]. In contrast, during the acute phase of *T. cruzi* infection (on day 18 after parasite inoculation) 13/20 (65%) and 22/38 (58%) of mice died within 24 h after injection of 0·5 or 5 μg of anti-CD3 MoAb, respectively, and the lethality rate reached 100% when a dose of 50 μg was administered. This lethality was related to the MoAb injection, as in the absence of MoAb injection all infected mice were still alive on day 20 after *T. cruzi* inoculation. During the chronic phase of *T. cruzi* infection (day 120), all mice were still alive 24 h after injection of 0·5 or 5 μg of anti-CD3 MoAb, but the lethality rate reached 100% (10/10) when a high dose (50 μg) of the MoAb was injected.

In order to determine the role of IFN-γ and TNF-α in the anti-CD3 MoAb-induced lethality in *T. cruzi*-infected mice, mice were pretreated with 1 mg of a neutralizing anti-IFN-γ and/or 0·5 mg of anti-TNF-α MoAb 2 h before anti-CD3 MoAb challenge.

Table 1. IFN-γ and tumour necrosis factor-alpha (TNF-α) serum levels after *in vivo* injection of anti-CD3 MoAb in CD4$^+$ or CD8$^+$ T cell-depleted mice

	IFN-γ (U/ml)‡		TNF-α (pg/ml)‡	
MoAb treatment†	Control mice	*T. cruzi*-infected mice §	Control mice	*T. cruzi*-infected mice §
—	317 ± 51	2848 ± 365	227 ± 48	6862 ± 516
Anti-CD4$^+$	327 ± 71	2663 ± 306	232 ± 14	6145 ± 807
Anti-CD8$^+$	267 ± 12	557 ± 73*	140 ± 24	665 ± 333**

† Anti-CD4$^+$ or anti-CD8$^+$ MoAbs were injected 3 and 1 days before anti-CD3 MoAb challenge.

‡ IFN-γ and TNF-α serum levels were determined 4 h and 90 min, respectively, after injection of a high dose (50 μg) of anti-CD3 MoAb.

§ Results are expressed as mean ± s.e.m. of at least six mice per group and are representative of three similar experiments. In *T. cruzi*-infected mice, experiments were performed on day 18 after parasite injection.

*$P < 0{\cdot}001$; **$P < 0{\cdot}01$ compared with intact or CD4$^+$-depleted mice.

Table 2. Effects of IFN-γ and/or tumour necrosis factor-alpha (TNF-α) neutralization, and CD4$^+$ or CD8$^+$ cell depletion on anti-CD3 MoAb-induced lethality in *Trypanosoma cruzi*-infected mice

MoAb pretreatment†	Anti-CD3 MoAb-induced lethality (%)‡
None	10/23 (43)
Anti-IFN-γ	2/25 (8)*
LO-DNP	11/25 (44)
Anti-TNF-α	3/20 (15)
CB1	7/20 (35)
Anti-IFN-γ + anti-TNF-α	0/10 (0)*
Anti-CD4	9/14 (64)
Anti-CD8	0/15 (0)**

† Anti-IFN-γ MoAb and anti-TNF-α or their isotype controls (LO-DNP and CB1) were injected 2 h before administration of 5 μg of anti-CD3 MoAb. Anti-CD4 or anti-CD8 MoAbs were given as indicated in Table 1.

‡ Lethality rates were determined 24 h after anti-CD3 MoAb injection.

*$P < 0{\cdot}05$; **$P < 0{\cdot}01$ compared with mice without MoAb pretreatment.

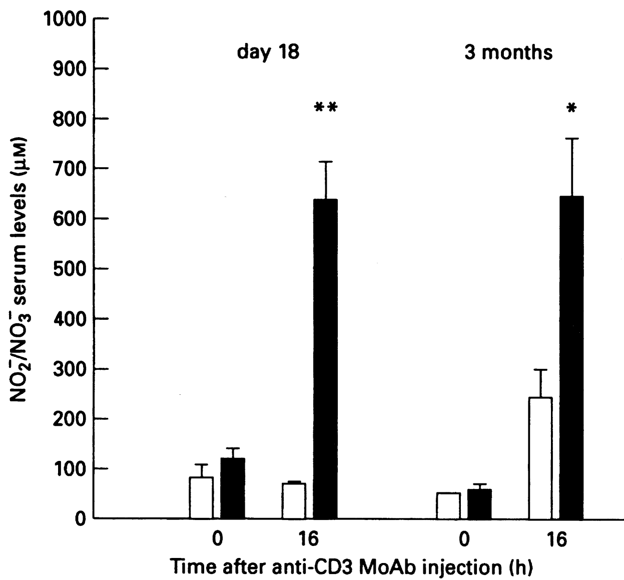

Fig. 2. NO_2^-/NO_3^- serum levels before and 16 h after injection of 5 μg anti-CD3 MoAb in control (□) and *Trypanosoma cruzi*-infected mice (■). Experiments were performed on days 18 and 120 after *T. cruzi* infection. Results were expressed as mean ± s.e.m. of at least five mice in each group. $*P = 0{\cdot}01$; $**P < 0{\cdot}0001$ compared with control mice after anti-CD3 MoAb injection.

In preliminary experiments, we ascertained that the injected amounts of anti-IFN-γ or anti-TNF-α MoAb efficiently neutralized corresponding cytokines in the circulation of anti-CD3 MoAb-injected mice (data not shown). As shown in Table 2, IFN-γ neutralization dramatically decreased the lethality induced by the anti-CD3 MoAb, TNF-α neutralization also decreased the lethality rate, but the effect of anti-TNF-α MoAb did not reach statistical significance. All mice injected with both anti-IFN-γ and

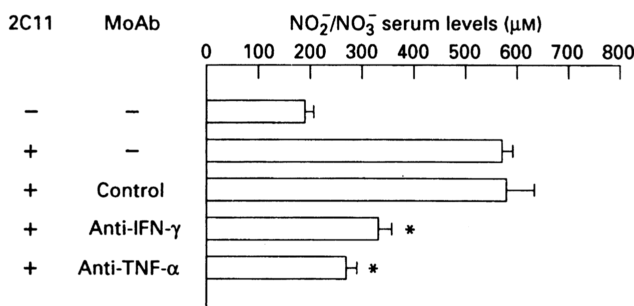

Fig. 3. Effects of IFN-γ and tumour necrosis factor-alpha (TNF-α) neutralization on the release of NO_2^-/NO_3^- induced by anti-CD3 MoAb injection in *Trypanosoma cruzi*-infected mice. NO_2^-/NO_3^- serum levels before and 16 h after injection of 5 μg anti-CD3 MoAb in *T. cruzi*-infected mice. Control (LO-DNP-2 and CB1) or anti-TNF-α and anti-IFN-γ MoAbs were injected as indicated in Table 2. Experiments were performed on day 18 after *T. cruzi* infection. Results are shown as mean ± s.e.m. of at least five mice and are representative of three different experiments. The control MoAb group included five mice injected with CB1 MoAb and five mice injected with LO-DNP-2, as the results were not significantly different. $*P < 0{\cdot}01$ compared with mice injected with control MoAb.

anti-TNF-α MoAbs survived the anti-CD3 MoAb injection. Since $CD8^+$ cells were shown to be responsible for hyperproduction of IFN-γ and TNF-α (see previous section), we reasoned that $CD8^+$ cell depletion should also protect *T. cruzi*-infected mice from anti-CD3 MoAb toxicity. Indeed, as shown in Table 2, all $CD8^+$ cell-depleted mice survived anti-CD3 MoAb injection, while $CD4^+$ cell depletion had no significant effect.

IFN-γ is involved in release of NO metabolites induced by anti-CD3 MoAb injection in control and T. cruzi-*infected mice*

As NO recently emerged as an important mediator of cytokine toxicity [28], we considered its possible involvement in anti-CD3 MoAb-induced shock. Preliminary experiments ascertained that NO_2^-/NO_3^- release observed in serum after *in vivo* administration of anti-CD3 MoAb peaked 16 h after injection in both infected and uninfected mice (data not shown). First, we found that basal NO_2^-/NO_3^- serum levels were not significantly different in *T. cruzi*-infected mice (on day 18 after parasite inoculation) compared with controls (Fig. 2). While injection of 5 μg anti-CD3 MoAb entailed no significant increase in NO_2^-/NO_3^- levels in control mice, a massive release of these NO metabolites was observed in *T. cruzi*-infected mice 16 h after injection of the same MoAb dose (Fig. 2).Moreover, injection of anti-CD3 MoAb during the chronic phase (day 120 after *T. cruzi* infection) was also followed by massive release of NO metabolites.

We then analysed the role of IFN-γ and TNF-α in the *in vivo* induction of NO production by anti-CD3 MoAb. As shown in Fig. 3, both anti-IFN-γ and anti-TNF-α MoAb administration in *T. cruzi*-infected mice partially prevented the release of NO metabolites, indicating that IFN-γ and TNF-α were involved in the hyperproduction of NO metabolites induced by anti-CD3 MoAb injection.

NOS inhibition does not protect T. cruzi-*infected mice from anti-CD3 MoAb-induced lethality*

To approach the role of NO in the shock syndrome induced by anti-CD3 MoAb injection, *T. cruzi*-infected mice received, on day 18 of infection, 2 mg intraperitoneally of L-NAME, a competitive inhibitor of NOS. L-NAME was given 30 min before, simultaneously with, and 2, 4 and 6 h after anti-CD3 MoAb injection. This protocol was previously shown to efficiently inhibit NO release in models of septic shock [26,29]. As previously observed in those models, L-NAME administration in *T. cruzi*-infected mice did not prevent and even appeared to increase anti-CD3 MoAb-induced lethality: lethality rates were 12/14 in L-NAME-injected mice *versus* 9/14 in mice injected with anti-CD3 MoAb alone.

DISCUSSION

The data presented in this study indicate *T. cruzi*-infected mice are primed to produce high amounts of IFN-γ and TNF-α upon *in vivo* polyclonal T cell activation induced by anti-CD3 MoAb injection. This priming occurred early after parasite inoculation—at the moment of exponential increase of the parasitaemia—and persisted during the chronic phase of the disease. By *in vivo* depletion of $CD4^+$ or $CD8^+$ T cells, we demonstrated that $CD8^+$ cells were involved in the massive IFN-γ and TNF-α overproduction induced by anti-CD3 MoAb injection in *T. cruzi*-infected mice. While it is likely that $CD8^+$ cells represent the major source of IFN-γ, the cellular source of TNF-α might be either $CD8^+$ cells or macrophages or both. Indeed, $CD8^+$ cell-derived IFN-γ might activate

parasite-primed macrophages to synthesize high levels of TNF-α. The priming of $CD8^+$ cells for IFN-γ synthesis might be relevant to the protective role of $CD8^+$ cells in the natural course of infection, as both IFN-γ and $CD8^+$ cells were shown to be involved in resistance against the parasite [30,31]. In the present model, the very high levels of IFN-γ induced by the injection of anti-CD3 MoAb are detrimental, as they are involved in the development of a severe shock syndrome. Although our data indicate a major role for $CD8^+$ cells in the systemic release of IFN-γ, they do not exclude the participation of other cell types in the secretion of IFN-γ in certain lymphoid compartments [32,33]. As a matter of fact, $CD3^+$, $CD4^-$, $CD8^-$ cells were shown to be involved in the *ex vivo* secretion of IFN-γ by spleen cells from *T. cruzi*-infected mice [4].

The enhanced toxicity of anti-CD3 MoAb in *T. cruzi*-infected mice did not allow assessment of any possible beneficial effect of this treatment on the course of infection. Indeed, the T cell activation induced by administration of low doses of anti-CD3 MoAb was reported to prevent lethal infections due to *Sendai virus* [34] or *Listeria monocytogenes* [35]. In *T. cruzi*-infected mice, doses as low as 0·5 μg anti-CD3 MoAb still induced significant lethality, indicating that the response of $CD8^+$ cells in terms of IFN-γ production is so intense that the use of this form of immunotherapy can not be applied to Chagas' disease.

IFN-γ as well as TNF-α were previously found to be involved in anti-CD3 MoAb toxicity [19]. Induction of NO synthase by these cytokines is thought to be a major event in the pathogenesis of septic shock [36]. Herein, we reported that anti-CD3 MoAb induces a systemic release of NO metabolites and that the priming for IFN-γ and TNF-α production in the course of *T. cruzi* infection is responsible, at least in part, for this NO overproduction. Although NO was involved in the haemodynamic changes occurring during septic shock [28], the vasoactive properties of NO as well as its ability to inhibit platelet aggregation and adhesion might protect vital organs from the prothrombotic properties of IFN-γ and TNF-α [37,38]. Indeed, inhibition of the constitutive and inducible forms of NOS by agents such as L-NAME was previously shown to increase the toxicity of bacterial toxins [26,29]. It is therefore not surprising that in this model, too, L-NAME administration appeared to enhance lethality.

We conclude that injection of anti-CD3 MoAb in the course of experimental Chagas' disease induces a massive release of IFN-γ and TNF-α. Although IFN-γ appears to play an important role in the host defence against the parasite, the levels of IFN-γ reached after anti-CD3 MoAb injection in *T. cruzi*-infected mice are so high that they result in a lethal shock syndrome. Although Chagas' disease is usually considered as a contraindication to organ transplantation because of reactivation of the parasite infection, some patients infected with *T. cruzi* might still receive heart transplants for end-stage cardiac failure [39]. Our data suggest that the use of OKT3 MoAb as immunosuppressor should be avoided in these patients, as it could result in a more severe form of the cytokine release syndrome which is a common complication of OKT3 administration [40,41].

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