ADMINISTRATION OF A NONDEPLETING ANTI-CD25 MONOCLONAL ANTIBODY REDUCES DISEASE SEVERITY IN MICE INFECTED WITH TRYPANOSOMA CRUZI

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The role of CD25+ regulatory T cells during the course of Trypanosoma cruzi infection has been previously analyzed, and the bulk of results have shown a limited role for this T cell subpopulation. In this study, we have used an IgM, nondepleting monoclonal antibody (mAb) aiming at blocking interleukin (IL)-2 activity on CD25+ T cells. The administration of this antibody 10 days before infection increased the resistance of outbred Swiss mice to the Colombian strain of T. cruzi. Anti-CD25-treated mice had lower parasitemia and augmented numbers of effector memory T cells. In addition, these animals showed higher numbers of splenic T cells secreting IFN-γ and TNF-α, both cytokines described to be involved in the resistance to T. cruzi infection. The same treatment also increased the numbers of splenic T cells that produced homeostatic and regulatory cytokines, such as IL-2 and IL-10, and CD4+CD25+ T cells. The administration of nondepleting anti-CD25 mAb at the beginning of the chronic phase, when parasites were cleared from the blood, halted the inflammatory process in the heart, without any signs of infection reactivation. These results indicate that nondepleting anti-CD25 monoclonal antibodies may be useful to treat chronic Chagas’ disease.

Keywords: Trypanosoma cruzi, interleukin 10, regulatory T cells, anti-CD25, monoclonal antibody, mice

Introduction

Immune responses to self or nonself antigens are controlled, among other cells, by regulatory T cells that are CD4+ and express the CD25 molecule. CD25 is the chain that confers high affinity properties to the interleukin (IL)-2 receptor molecular complex [1]. Accordingly, this subtype of regulatory T cells is highly dependent on the presence of IL-2 to proliferate and for the maintenance of its competitive fitness [2]. IL-2 is the main factor controlling the homeostasis of regulatory T cells, since its absence greatly diminishes the numbers of this type of regulatory T cells, generating autoimmune disease [3]. Additionally, it has been shown that some monoclonal antibodies to IL-2, when coupled to IL-2 as a complex, may induce proliferation of regulatory T cells, augmenting their numbers and suppressive function [4]. IL-2 is also important for the development of the effector T cell response [4]. It has been proposed that one of the regulatory T cells activities relates to their capacity to absorb the IL-2 produced by effector T cells in a way that limits the amount of this cytokine, reducing activation of other effector T cells [5].

In addition to the high affinity IL-2 receptor that contains the α-chain (CD25), the IL-2 receptor has also a low affinity molecular complex composed by the β-chain (CD122) and the common γ-chain (CD132) [1]. The CD122 is expressed in some activated T cells, including CD4+CD25+ regulatory T cells, NK cells, and macrophages [6]. Functional inactivation of this molecule (CD122) results in very low numbers of regulatory T cells and in autoimmunity [6]. This suggests that the CD122 receptor is even more important than its high affinity counterpart, as gene inactivation of CD25 does not result in major disturbance of regulatory T cell homeostasis in mice [7]. These studies indicate that IL-2 and its receptors are essential for the homeostatic control of regulatory and effector T cells [6].

Some monoclonal antibodies to the murine high affinity IL-2 receptor chain (CD25) may deplete regulatory T cells and effector T cells. This is the case for the antibody (rat IgG1) produced by the PC-61 hybridoma [8].

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However, there is another monoclonal antibody that was described to bind to the CD25 molecule, blocking IL-2 activity, without depleting regulatory T cells in vivo [9]. This antibody is a rat IgM and is produced by the 7D4 hybridoma [9]. Interestingly, both monoclonal antibodies have been shown to modify the development of immunological responses, suggesting that depletion may not be the only mechanism underlying their biological activity. Humanized monoclonal antibodies to human CD25 are being utilized in clinical practice, and their usefulness is expanding from avoiding transplant rejection to the treatment of some autoimmune diseases [10]. The antibodies used in medical practice are of the nondepleting class, inducing strong down-modulation of the CD25 molecule but, at the same time, preserving the numbers of Foxp3+CD4+ regulatory T cells [11]. Indeed, they are effective at reducing the activation of effector T cells and, therefore, are being used to block immune responses [11].

Trypanosoma cruzi produces a strong immune response to its antigens during the acute phase of the infection. This host immune response controls the parasite load but does not eliminate the infection, which evolves to a chronic phase, and the host remains infected for the rest of its life [12]. During the acute phase of the infection, tissue lesion is induced by the presence of parasites in the tissues and the associated immune response [13, 14]. However, in the chronic phase of the infection, the tissue lesions persist and autoimmune mechanisms are likely to play a role in their perpetuation [15–17].

Previous studies have shown a limited role for CD25+ regulatory T cells, upon depletion of CD25+ cells in T. cruzi-infected mice, using monoclonal antibodies produced by the PC61 hybridoma [18–20]. In addition, the administration of the depleting antibody in the chronic phase of the infection has produced no biological effects [18, 19]. However, the antibody used in those studies eliminated regulatory and effector T cells, at the same time, and, therefore, could be responsible for their conclusions. In this study, we have used a nondepleting antibody produced by the hybridoma 7D4 in the acute and chronic phases of T. cruzi infection. The biological activity of this monoclonal antibody is rather related to a strong immunomodulatory function. Our results show that the administration of a single dose of this monoclonal antibody 10 days before infection results in lower parasitemia, increased conversion of CD4 and CD8 T cells to effector memory cells and increased production of IFN-γ and TNF-α, during the acute phase of the infection. In addition, the numbers of T cells able to produce IL-2 and IL-10 were also increased along with the numbers of splenic CD4+CD25+ regulatory T cells. Administration of the same antibody in the early chronic phase of the infection does not produce any signs of infection reactivation but, instead, strongly reduces the numbers of inflammatory cells in heart tissues within a month of a single administration. These results indicate that manipulation of the effector/regulatory immune response axis by nondepleting anti-CD25 monoclonal antibodies may be useful in the treatment of chronic T. cruzi infection in humans.

Materials and methods

Animals

Swiss Webster mice (1–2 month old) were from the Centro de Pesquisas Gonçalo Moniz animal house. The animals were kept in microisolators under conventional conditions and were manipulated according to institutional ethical guidelines. All the protocols used in this study were approved by the Committee for Ethics of the Oswaldo Cruz Foundation.

Infection and treatment with nondepleting anti-CD25 monoclonal antibody

Groups of five to 20 mice were infected intraperitoneally with 10⁷ blood-form trypomastigotes of the Colombian strain of T. cruzi in 0.2 ml of 0.15 M phosphate-buffered saline (PBS). Control mice received the same volume of PBS. The numbers of parasites were evaluated in 5 μl volumes of blood. PC61 mAb, 7D4 mAb, and rat Ig were semipurified from ascitic fluid or sera, as previously described [21]. One milligram of the antibody preparations was injected intravenously 10 days before infection or in the early chronic phase (60 days after infection).

In vitro cell culture

Splenocytes were cultured in triplicates at a density of 10⁵ cells/well in 24-well plates (Nunc) in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone), 50 mM 2-mercaptoethanol (2-ME) and 1 mM hydroxyethyl-piperazine ethanesulfonic acid (HEPES) (complete medium). Cells were cultured at 37 °C and 5% of CO₂ for 24 h in complete medium alone or in the presence of 2 μg/mL of anti-CD3 monoclonal antibody (clone 2C11). Brefeldin-A was added 8 h before the cells were harvested to stain them for flow cytometric analysis.

Flow cytometric analysis

Spleen cells were isolated as described [22] and placed in ice-cold PBS supplemented with 5% FBS and 0.01% sodium azide. Staining was done as previously described [23]. The fluorochrome-conjugated monoclonal antibodies used were anti-CD4, anti-CD8, anti-CD44, anti-CD62L, anti-CD25 (clone PC61), anti-foxp3, anti-IL2, anti-IL10, anti-IFN-γ, and anti-TNF-α, and were purchased from eBioscience or CALTAG. Biotin-conjugated antibodies were revealed by streptavidin-PE-Cy5.5 from CALTAG. Intracellular staining for IL-2, IL-10, IFN-γ, and TNF-α were performed as described previously [23]. After surface staining, the cells were fixed with 1% para-

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formaldehyde in PBS and analyzed using a FACScan (Becton and Dickinson). Results were analyzed using Flowjo software.

**Histological and quantitative morphological studies**

Heart and skeletal muscle tissues were removed from infected mice, fixed in buffered 10% formalin, and par-

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**Fig. 1.** *In vivo* administration of the monoclonal antibody secreted by the 7D4 hybridoma down-modulates the expression of CD25, sparing most of the foxp3+ T cells. In (A), Swiss mice were treated with a control rat Ig or with PC61 monoclonal antibody (mAb) or with the 7D4 mAb. Splenic or lymph node cells were stained for CD4 and foxp3 as previously described. *Figure 1A* shows gated CD4+foxp3+ splenic (upper panel A) or lymph node (lower panel A) T cells from the different experimental groups, 10 days after antibody administration. Dot plots represent one animal out of four that gave similar results. Numbers in the quadrants represent percentages of the specified subpopulation inside a lymphocyte gate. Numbers in the small square in the upper right quadrants represent the percentage of foxp3 high CD4+ T cells. *Figure 1B* shows lymph node cells from mice treated with rat Ig (upper left dot plots) or 7D4 mAb (middle and lower left dot plots), stained for CD4 and CD25 (surface staining showed in upper and middle left dot plots and intracellular staining showed in lower left dot plots). Right dot plots show the expression of foxp3 and the expression of surface (upper and middle right dot plots) or intracellular (lower right dot plots) CD25 molecule in electronically gated CD4+CD25+ lymph node T cells.
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affin embedded, and sections were used for histological studies. The number of mononuclear cells or intact parasite nests was counted in 30 nonsuccessive microscopic fields, using a 10× ocular and a 40× objective. Counting was performed on paraffin sections of heart muscle tissues from infected mice during acute infection. The slides were coded, and the studies were done double blind.

**Fig. 2.** *In vivo* treatment with nondepleting antibody to CD25 molecules in Swiss mice infected with the Colombian strain of *T. cruzi* resulted in lower parasitemia. Swiss mice were treated with 1 mg of semipurified rat Ig (open circles) or 7D4 mAb (closed circles), 10 days before infection with 103 trypomastigote forms. Data shown are from one representative out of two similar, independent experiments, with 9 to 10 mice/group. Each point represents the mean. *P* < 0.05 (Mann–Whitney test)

**Fig. 3.** *In vivo* treatment with nondepleting anti-CD25 mAb led to an increased number of effector/memory T cells during the acute phase of *T. cruzi* infection in Swiss mice. The numbers of effector memory lymphocytes in mice treated with anti-CD25 (7D4 mAb) or rat Ig as a control group were determined after staining CD4 or CD8 splenic T cells with CD44 and CD62L mAbs. *Figure 3* shows the total numbers of CD4+CD44highCD62Llow (open bars) or CD8+CD44highCD62Llow (closed bars) splenic T cells. Each point (mean ± SD) corresponds to results with 4 to 5 mice stained individually. These studies were performed 20 days after initial infection. The experiment above represents one out of three similar experiments with equivalent results. *P* < 0.05, **P** < 0.01 (Mann–Whitney test)
Statistical analysis

The results are presented as means ±SD. The significance of differences between the experimental and control groups was determined as described for each figure legend. P values below 0.05 were considered significant.

Results

In vivo administration of the monoclonal antibody secreted by the 7D4 hybridoma down-modulates the expression of CD25, sparing most of the foxp3+ T cells

In Fig. 1A, we have compared the in vivo activity of the product of two hybridomas (PC-61 and 7D4). Figure 1A shows that 1 mg of the antibody produced by the PC-61 clone was able to deplete the foxp3+ T cell population. Depletion lasted for at least 10 days, since mice remained partially depleted in the spleen and lymph nodes, particularly for the populations expressing high levels of foxp3. On the other hand, the antibody produced by the 7D4 clone did not induce any depletion in foxp3 populations in the lymph nodes. However, a small population of splenic ultra bright foxp3+ T cells was depleted by this antibody (Fig. 1A). Treatment with the monoclonal antibody produced by the 7D4 clone down-modulated the expression of CD25 molecules on lymph node CD4+ T cells, as shown in Figure 1B. Yet, no depletion of foxp3+ cells could be observed. In addition, when the 7D4 mAb was used in vivo, intracellular staining to detect CD25 molecules, using a monoclonal antibody that recognizes a different epitope (clone PC61), showed that CD4+CD25+ T cells were not depleted, but instead they have internalized the CD25 molecule (Fig. 1B).

In vivo treatment with nondepleting antibody to CD25 molecules in Swiss mice infected with the Colombian strain of T. cruzi resulted in lower parasitemia

Outbred Swiss mice treated with nondepleting anti-CD25 mAb 10 days before infection presented lower parasitemia when compared to mice treated with rat Ig as a control (Fig. 2). This mouse strain infected with the Colombian strain of T. cruzi was extremely resistant, and only 10% of the mice died in the group treated with rat Ig up to day 60 of infection. Mortality, in the group treated with 7D4 mAb, was not observed during the same period of observation (data not shown).

In vivo treatment with nondepleting anti-CD25 mAb led to an increased number of effector/memory T cells during the acute phase of T. cruzi infection in Swiss mice

As the numbers of activated/memory T cells were previously related to resistance in another experimental model of T. cruzi infection [22], we asked if the administration of a nondepleting monoclonal antibody to CD25 molecule could somehow induce higher numbers of memory T cells. In fact, in vivo treatment with nondepleting anti-CD25 resulted in increased numbers of both splenic CD4+ and CD8+ effector/memory T cells (CD44highCD62Llow) when these mice were compared to control Ig-treated mice. These results are shown in Fig. 3.

The production of cytokines such as IFN-γ, TNF-α, IL-2, and IL-10 by T cells and the numbers of splenic CD4+CD25+ T cells were increased in 7D4-treated mice, during the acute phase of the infection

The production of IFN-γ and TNF-α during the acute phase of the infection was previously related to resistance to T. cruzi infection [24–26]. For this reason, we asked if the treatment with nondepleting anti-CD25 was modulating the overall production of these cytokines. Figure 4 shows that the in vivo treatment with 7D4 monoclonal antibody increased the number of splenic CD4+ (Fig. 4A) and CD8+ (Fig. 4B) T cells that produced TNF-α and IFN-γ, respectively, upon anti-CD3 stimulation. However, neither the number of splenic CD8+ T cells that produce TNF-α nor the number of splenic CD4+ that produced IFN-γ was further increased by the 7D4 mAb treatment in relation to infected control groups treated with rat Ig (data not shown). The production of IL-2 and IL-10 by T cells and also the numbers of CD4+CD25+ regulatory T cells, which depend on the presence of IL-2 to expand, were also evaluated. As shown in Fig. 4C, there was a significant increase in the numbers of splenic CD4+IL-2+ T cells from mice treated with nondepleting anti-CD25 mAb compared to controls, when these cells were cultured in complete medium alone. This difference was amplified by stimulation with anti-CD3 mAb. In addition, the total number of splenic CD4+CD25+ T cells increased in infected mice previously treated with nondepleting anti-CD25 mAb (Fig. 4D). The number of CD4+ T cells able to produce IL-10, upon anti-CD3 stimulation, was also increased in mice treated with 7D4 mAb in relation to Ig treated controls (Fig. 4E).

In vivo administration of a single dose of nondepleting anti-CD25 mAb halts heart tissue inflammation without infection reactivation

After the resolution of the acute infection and by day 60 after initial infective challenge, mice received one single injection of rat Ig or nondepleting anti-CD25 mAb. After 1 month, mice were sacrificed and hearts were used for histological analysis. In Fig. 5A, the picture illustrates the typical inflammatory pattern that can be found in these mice at this stage of the infection with focal mononuclear cell inflammatory infiltrate without visible tissue parasites. Figure 5B shows the heart tissue of a mouse previously treated with nondepleting anti-CD25 where the inflam-
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Inflammatory cells were very diminished or absent. Figure 5C shows a quantitative study, confirming that the number of inflammatory cells infiltrating the heart muscle was much lower than in control mice.
Discussion

In this work, we have investigated the role of cells expressing CD25 molecules, including regulatory T cells, in the control of the parasite-induced immune response during the acute phase of the disease and in the early chronic infection.

Administration of a single dose of the nondepleting anti-CD25 mAb (1 mg/mouse) induced a great reduction of the splenic foxp3+ high CD4+ T cells. However, this subpopulation was not depleted in the lymph nodes. This could indicate that the amount of this antibody penetrating the lymph nodes was not enough to induce depletion of this particular ultra bright foxp3+ regulatory T cell population. However, the 7D4 mAb penetrates the lymph nodes, as it induced a strong down-modulation of the CD25 molecule on T cells (Fig. 1B). Intracellular staining aiming at the CD25 detection indicated that this population (CD25+) was not depleted, but instead was internalizing the CD25 molecule, upon antibody binding. Therefore, the administration of the 7D4 mAb induced depletion of splenic regulatory cells expressing high levels of foxp3 and a strong down-modulation of the CD25 molecule in splenic and lymph node CD4+ T cells. The levels of foxp3 expression have been correlated to the potency of the suppressor activity exerted by T regulatory cells [27]. Cells expressing high levels of foxp3 are the most potent regulatory T cells, whereas cells expressing low foxp3 levels are less potent. These findings might help to explain the increased numbers of effector memory splenic T cells found in day 20 after infection in animals previously treated with the 7D4 mAb when compared to infected mice injected with control rat Ig. In addition, the administration of the 7D4 mAb induced higher numbers of splenic CD4+TNF-α+ and CD8+IFN-γ+ T cells and both cells and cytokines are described to function in protective immune responses against T. cruzi infection, thus, helping to explain the lower parasitemia in this model [28].

It should be noted that injection of this antibody 1 day before infection induces slightly higher parasitemia but no
increased mortality (data not shown). This indicates that the antibody used in this particular condition may induce a delay in the formation of the specific immune response, leading to higher parasitemia. This might be related to the presence of high amounts of the antibody in a period where the immune system is mounting a protective immune response. In this case, it is likely that the effect of the antibody is more pronounced on hindering the generation of effector T cells rather than of regulatory cells. In fact, it is being described that IL-2 and CD25 are required in the early phases of T cell activation to generate memory/effector T cells [6]. However, in the model described here, the antibody was administered 10 days before the infection, in a time that, in healthy animals, there should be no stimulus to generate CD25+ effector T cells that the anti-CD25 mAb could act upon. However, the thymus-derived natural CD25+Foxp3+ Treg cells, which are ubiquitous in lymphoid organs even in the absence of infection, would be affected by the presence of the mAb. At the time of the infection, when most of the mAb had been cleared from the circulation, CD25+ effector T cells could then be generated in the absence of effective controlling influences of natural Tregs.

As already pointed out above, one of the proposed mechanisms to explain the biological activity of the regulatory T cells is their capacity to absorb IL-2 and produce a less favorable environment for the development of effector T cells [5]. As a consequence of the immune response against the pathogen, the levels of IL-2 would progressively build up in the microenvironment. At this point, one could argue that the IL-2, even being present in high levels, could not be used by Tregs because the high affinity receptors of these cells would be blocked or their levels would have been down-modulated by the mAb injected one week earlier. However, the high affinity receptor α-chain seems to be dispensable to keep functional regulatory T cells [2]. This is not the case for the β-chain of the low affinity complex (CD122), which, as mentioned above, is being conclusively shown to be of crucial importance for the homeostasis of the regulatory T cell population [4]. In this particular situation, therefore, with the high IL-2 levels accompanying the immune response against the parasite, the CD122 molecule would be competent to transduce an IL-2-conveyed stimulatory signal to the Tregs. Moreover, we have detected augmented numbers of splenic CD4+ T cells able to produce IL-2 (Fig. 4C), which would further increase the numbers of regulatory T cells at a given time point after the infection, as observed in the present study (Fig. 4D).

On the other hand, blocking of CD25 molecules seems to facilitate the proliferation of a non-energetic regulatory T cell subpopulation, namely, the IL-10-producing Tr1, as we have detected a significant increase in splenic CD4+IL-10+ T cells from animals previously treated with 7D4 mAb when compared to infected controls treated with rat Ig (Fig. 4E). Interestingly, by means of still unclear mechanisms, IL-10 has been implicated in the resistance to T. cruzi [29] [30]. This phenomenon might be related to the IL-10 primary anti-inflammatory function, dampening the activity of antigen-presenting cells, their associated T-cell immune responses, and, consequently, avoiding excessive tissue damage during the acute infection [30, 31]. In addition, it might work by increasing the threshold of tissue resistance to bystander lesions, thus, making noninfected tissue less prone to destruction and able to withstand higher levels of inflammatory cytokines such as TNF-α and IFN-γ and, therefore, stronger tissue immune responses. A protective role of this kind from TNF-α-induced apoptosis has already been suggested for IL-10 in heart and striated muscle cells [32–35].

The biological activities described above have motivated us to test this antibody during the early chronic infection where the number of parasites has been controlled but inflammatory cells in previously heavily infected tissues remain. The administration of this antibody in the early chronic phase has not induced any increase in parasitemia or in the amount of tissue parasitism, evaluated in histological sections (data not shown). However, the number of inflammatory cells in the hearts was much lower in mice that were injected with 7D4 mAb when compared to infected controls to which rat Ig was given (Fig. 5A, B, and C). The reduction of the inflammatory reaction in the heart tissue could be due to the increased levels of IL-10 production by Tr1 cells or by IL-10-secreting Th1 cell [36–38]. This latter has been shown to expand during infections and is believed to result from cytokine synthesis switching in effector T cells [37]. They would, therefore, curb the development of the heart disease after effector T cells had controlled the infection. In fact, a proportion of the cells that infiltrate muscle tissue in T. cruzi-infected mice synthesizes both IL-10 and interferon (IFN)-γ (Mengel J. et al., unpublished data) and could be either Tr1 or IL-10-secreting Th1 cells.

It is important to note that we could not find, by flow cytometry, large numbers of CD4+Foxp3+ in different muscle tissues along the infection period. Usually, these cells represent less than 1% of the total CD4+ T cells in infected tissues in different models of T. cruzi infection, using different strains of the parasite and of mice (Nihei, et al., manuscript in preparation). Yet, we were able to detect relatively large numbers of CD4+CD25+Foxp3− effector T cells in the heart and skeletal muscle. Therefore, the regulatory T cells might be suppressing the formation of pathogenic immune responses in the secondary lymphoid organs, and not in the nonlymphoid tissues, since the majority of CD4+CD25+ T cells in lymphoid organs are Foxp3+.

We would favor the interpretation that the injection of the anti-CD25 mAb during the early chronic phase of the T. cruzi infection would affect mainly the CD25+ Tregs, and not memory/effector T cells, which have been described to be less dependent on IL-2 for their maintenance or reactivation [39]. The absence of effects related to parasite load would thus depend on the continuous functionality of these effector cells even in the presence of the anti-CD25 mAb, and perhaps on an augmentation of
the numbers of IL-10 producing Tr1 cells, also resulting from a decrease in CD25+ Tregs. As mentioned above, the presence of IL-10 has been associated with the control of the murine infection by Colombian-strain T. cruzi. After 1 or 2 weeks, when the mAb concentration would be significantly diminished, there would be a transient increase in the immune response against the parasite at the lymphoid tissues, with the generation of IL-2-producing Th1 cells due to the absence of functional CD25+ Tregs. The release of larger amounts of IL-2 by these Th1 cells would then further reinforce the regulatory arm of the immune response by causing a rebound in the numbers of the regulatory Foxp3+ T cells. This, together with the increased numbers of Tr1 cells and perhaps with an increased differentiation of IL-10-secreting Th1 cells from the population of Th1 cells that would have expanded in the first days after the mAb administration, would account for the observed amelioration in heart inflammation.

The infection is a dynamic and an ongoing process, and it has been described that most T cells that have been engaged in a previous immune response usually undergo apoptosis and/or become less able to perform their full functional activity [40]. Therefore, one could propose that this antibody applied in the chronic phase of the infection could mimic the same biological effect it provoked in the acute disease, increasing the process of recruitment of naïve cells to the effector pathway, renewing and potentiating the ongoing immune response, eliminating more effectively residual parasite nests, and, at the same time, controlling the inflammatory response and tissue lesion in a more effective way. Of note, this could be dependent on the dose and time schedule of the antibody administration, as discussed above.

In conclusion, we have provided evidence that nondepleting anti-CD25 monoclonal antibodies may be useful tools in the management of chronic T. cruzi infection, justifying the carrying out of further studies aiming at obtaining supporting data for its clinical use in Chagas’ disease.

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