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Original article

The regulatory CD4+CD25+ T cells have a limited role on pathogenesis of infection with *Trypanosoma cruzi*

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Abstract

Recent reports have established an important role of CD4+CD25+ T cells in the immune regulation of infectious diseases, autoimmune disorders and cancer. In the present work, we investigated whether these cells had a regulatory role during *Trypanosoma cruzi* infection, using the Colombian strain. Inactivation of CD4+CD25+ cells *in vivo* conferred mice slightly more resistant to infection with the Colombian strain of *T. cruzi*, as evidenced by lower parasitemia and mortality rates. The augmented resistance to infection with Colombian strain did correlate with increased activation of effector CD4 cells. It was antibody-independent, since no difference in levels of IgM, IgG, IgG1 and IgG2a[b] recognizing *T. cruzi* antigens was observed throughout the infection of CD25-inactivated and control mice. Regarding pathogenesis, inflammatory infiltrate and frequency of CD4 and CD8 T cells or macrophages in the cardiac tissue was similar in both groups. Together, our data indicate that CD4+CD25+ cells have a limited role on host resistance during early *T. cruzi* infection. Despite exhaustive investigation, we did not observe any role for these regulatory cells in the pathogenesis of experimental chronic Chagas' disease.
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Keywords: Trypanosoma cruzi; Regulatory T cells; CD4+CD25+

1. Introduction

Chagas' disease, caused by the hemoflagellate protozoan *Trypanosoma cruzi*, afflicts 18 million people in Latin America [1]. *T. cruzi* infection up-regulates the synthesis of pro-inflammatory cytokines, including interleukin-12 (IL-12) which, in turn, induces the production of interferon-gamma (IFN-γ) [2]. IFN-γ and tumor necrosis factor alpha (TNF-α) stimulate macrophages to synthesize nitric oxide that is responsible for the control of protozoan multiplication during the acute phase of infection [3,4]. This immune response also plays a central role in morbidity, which is associated with inflammatory lesions, polyclonal activation of T and B lymphocytes and hypergammaglobulinemia [5,6].

T. cruzi infection directly affects the host immunoregulation followed by intense inflammatory response that can cause deleterious effects on the host [3,7]. The cytokines IL-10 [8] and IL-4 [9] prevent excessive inflammation and pathology, during acute and chronic phases, respectively. Depletion of either T CD4+ cells or IL-12 delays death of IL-10-knockout mice, suggesting that T CD4+ cells and exacerbated production of IL-12 play an important role in the development of *T. cruzi* pathology [8].

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T CD4+CD25+ (Treg) cells comprise a special lymphocyte lineage with regulatory functions directed to infectious and autoimmune processes. This subset of regulatory cells constitutively express CD25, the high affinity IL-2 receptor molecule on their surface [10] and the transcription factor Foxp3 [11–13] as well as other markers such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced TNFR-related protein (GITR) [14]. The regulatory role of CD4+CD25+ cells has been reported in several types of infection [14].

One recent report indicated that depletion of Treg cells did not enhance protective immunity, control of parasite replication and survival in mice infected with *T. cruzi* [15]. However, the role of CD4+CD25+ T cells in the immunopathogenesis of Chagas' disease remains not duly understood. The pathogenesis of *T. cruzi* infection is especially interesting because it has both infectious and autoimmune components. Our main hypothesis was that Treg cells would be involved in both prevention of an initial exacerbated inflammatory process and control of myocarditis resolution. Colombian *T. cruzi* strain was chosen because it has an intense tropism for cardiac tissue, inducing intense cardiomyopathy associated with the high rate of animal survival [16], which enables the study of induction and resolution of cardiomyopathy. Our findings suggest that Treg cells exerts a limited immunoregulatory function during the acute phase of infection, by down-regulating activation of CD4+ T cells, but has no critical role on pathogenesis of chronic infection.

2. Materials and methods

2.1. Animals

C57BL/6, C3H/HeJ and BALB/c female mice 6–8 weeks old were obtained from Centro de Pesquisas René Rachou, Oswaldo Cruz Foundation, and procedures performed following the institutional animal care guidelines.

2.2. Antibodies used in vivo for functional inactivation studies

Affinity-purified PC61, a rat IgG1 anti-CD25 monoclonal antibody (mAb) [17] and GL113 (rat IgG1 anti-*Escherichia coli* β-galactosidase) were used in different experiments. For *in vivo* inactivation of CD25, 1 mg of PC61 was injected intraperitoneally in PBS on days −3 and −1 prior to infection of mice with *T. cruzi*. Control mice were injected with GL113. The inactivation of CD4+CD25+ was confirmed by FACS analysis (B & D FACScan) of splenocytes using anti-CD4 phycoerythrin (PE)- and anti-CD25 fluorescein isothiocyanate (FITC)-labeled antibodies (Pharmingen, San Diego, CA, USA) (Fig. 1).

2.3. Parasites and experimental infection

Mice were infected intraperitoneally with 25, 50 or 1000 blood trypomastigotes of the Colombian *T. cruzi* strain [18] or with 50 or 100 blood trypomastigotes of the Y *T. cruzi* strain [19]. Parasitemia was assessed in the tail blood at different days post-infection [20]. Mouse survival was monitored daily.

2.4. Quantification of serum antibodies against epimastigote antigen (Epi-Ag)

Groups of five mice (injected with either GL113 or PC61) were bled from the retro-orbital plexus on different days after *T. cruzi* infection with 50 parasites. Serum was stored at −20 °C for measurement of levels of IgM, IgG, IgG1 and IgG2a[b] anti-*T. cruzi* antibodies by an ELISA employing, 96-well plates coated with Epi-Ag.

2.5. Immunostaining for flow cytometric analysis

Spleens were harvested on day 11 post-infection (p.i.) with *T. cruzi* and cell suspension prepared in RPMI medium

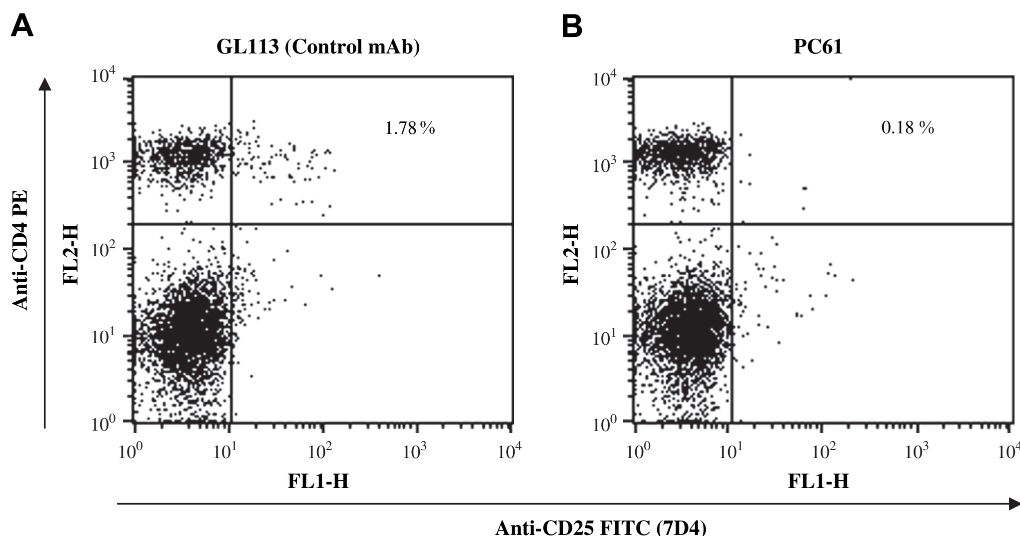

Fig. 1. Representative flow-cytometric analysis of spleen cells from mice treated with control (GL113) (A) or inactivating antibody (PC61) (B) 3 days prior to sacrifice. The cells were stained with anti-CD4-PE and anti-CD25-FITC (7D4 clone). The dot plots shown were gated on small lymphocytes.

containing 5% fetal bovine serum. Five hundred thousand cells were stained with anti-CD69 FITC and PE-labeled antibodies against CD4, CD8 or anti-CD49b (Pan-NK) molecules (Pharmingen). After labeling, the cells were examined by flow cytometry. The software CellQuest (BD Biosciences, San Jose, CA) was used for analysis.

2.6. Ex vivo proliferation analysis and cytokine measurements

Spleen cells were plated at 6×10^6 cells/well in 24-well plates and cultured in the presence or absence of different stimuli for 2 days. Stimuli used in cultures included Epi-Ag (at 10 μg/ml) and concanavalin A (Vector Laboratories) (at 5 μg/ml). The culture supernatants were harvested at 48 h of culture and IFN-γ and IL-10 concentrations measured by sandwich ELISA.

2.7. Heart and serum cytokine measurements in T. cruzi-infected mice

Hearts were harvested at different days p.i. with *T. cruzi* (50 parasites) in 1 ml lysis solution (2.34 g NaCl, 50 μl Tween 20, 500 mg bovine serum albumin (BSA), 1.7 mg phenylmethylsulfonyl fluoride (PMSF), 4.48 mg benzethonium chloride, 37.2 mg EDTA and 2 μl of aprotinin), homogenized, and centrifuged at $10{,}000 \times g$ for 10 min at 4 °C. Supernatants were frozen at −70 °C until use. Mouse Inflammation Kit from BD™ (Cytometric Bead Array—CBA) was used to quantify IL-6, IL-10, MCP-1, IFN-γ, TNF-α and IL-12p70 in sera and heart extracts, according to the manufacturer's instructions. The fluorescence produced by the CBA beads was measured on a FACScan flow cytometer (BD Biosciences) and analyzed with its software.

2.8. Histological evaluation

Hearts were fixed in 4% formaldehyde in PBS, embedded in paraffin, sectioned, stained with hematoxylin–eosin (H&E), examined under light microscopy, and analyzed using specific computer program (KS 300 program; Karl Zeiss, Berlin, Germany) attached to a micro-camera and microscope. The total lesion area of each animal is the mean of lesion areas obtained from ten sections analyzed. Alternatively, removed hearts were embedded in tissue-freezing medium (Tissue-Tek, Miles Laboratories), and stored in liquid nitrogen. Serial 5- to 7-μm-thick sections were fixed in cold acetone and subjected to immunoperoxidase staining [16]. The cells stained for CD4 (clone GK 1.5; Amersham), CD8 (clone 53-6.7; Amersham) and macrophages (clone F4/80; Caltag), and *T. cruzi* amastigote forms were counted under light microscopy.

2.9. RNA and DNA extraction for real-time PCR

Total RNA was isolated from cardiac tissue using Trizol reagent (Invitrogen). RNA was quantified and 6 μg utilized for cDNA synthesis using reverse transcriptase. For DNA extraction, samples were immerged in 150 μl alkaline lysis solution, pH 12 (25 mM NaOH; 0.2 mM Na_2–EDTA · $2H_2O$) and then heated for 1 h at 95 °C (mixing every 5 min). Afterwards, samples were cooled at 4 °C and treated with 150 μl neutralization solution, pH 5.0 (40 mM Tris–HCl). After neutralization, samples were centrifuged at $2000 \times g$ for 2 min and supernatant stored at −70 °C.

2.10. Real-time assay for measuring in vivo expression of Foxp3 and CCL4 mRNA and quantification of tissue parasite loads

Real-time PCR was carried out in an ABI PRISM 7900 Sequence Detection System using fluorogen SYBR Green I (Applied Biosystems). Real-time PCR for parasite quantification was performed as previously described [21]. Host DNA in the samples was confirmed by genomic level measurements of TNF-α PCR products in the same sample. Purified *T. cruzi* DNA (American Type Culture Collection, Manassas, VA) was sequentially diluted for curve generation in aqueous solution containing equivalent amounts of DNA from uninfected mouse tissues. CCL4 and Foxp3 messenger RNA copy numbers were normalized using β-actin copies. Primers (listed as 5′ to 3′) were: β-actin forward (FOR): GGATGCAGAAGGAGAT TACTG, reverse (REV): CGATCCACACAGAGTACTTG; CCL4 FOR: TGGTGCTGAGAACCCTGGA, REV: TCTCTCC TCTTGCTCGTGGC; Foxp3 FOR: CCCAGGAAAGACAG CAACCTT, REV: TTCTCACAACCAGGCCACTTG; TNF-α FOR: CATCTTCTCAAAATTCGAGTGACAA, REV: TGGGAGTAGACAAGGTACAACCC; and "satellite" DNA (*T. cruzi*) FOR: GCTCTTGCCCACAMGGGTGC, where M = A or C, REV: CCAAGCAGCGGATAGTTCAGG.

2.11. Statistical analysis

Differences between groups were compared using Student's *t* or Mann–Whitney test. Differences between days were compared using one-way ANOVA or Kruskal–Wallis test, followed by Tukey and Dunn post hoc tests, respectively. Log-rank test was used for comparison of survival rates. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of inactivation of CD25 on parasitemia and mortality curves of T. cruzi-infected C57BL/6 mice

The first question we asked was if CD25-inactivation would increase resistance of mice to *T. cruzi*. C57BL/6 mice were inoculated intraperitoneally with anti-CD25 (PC61) or an isotype matched control (GL113) on days 3 and 1 prior to infection with different amounts of trypomastigote forms of the Colombian *T. cruzi* strain. At the infection dose of 25 (not shown) and 50 parasites, we observed significantly lower parasitemia and mortality levels (Fig. 2A and B) ($P < 0.05$). By increasing the dose to 1000 parasites, this effect was no longer

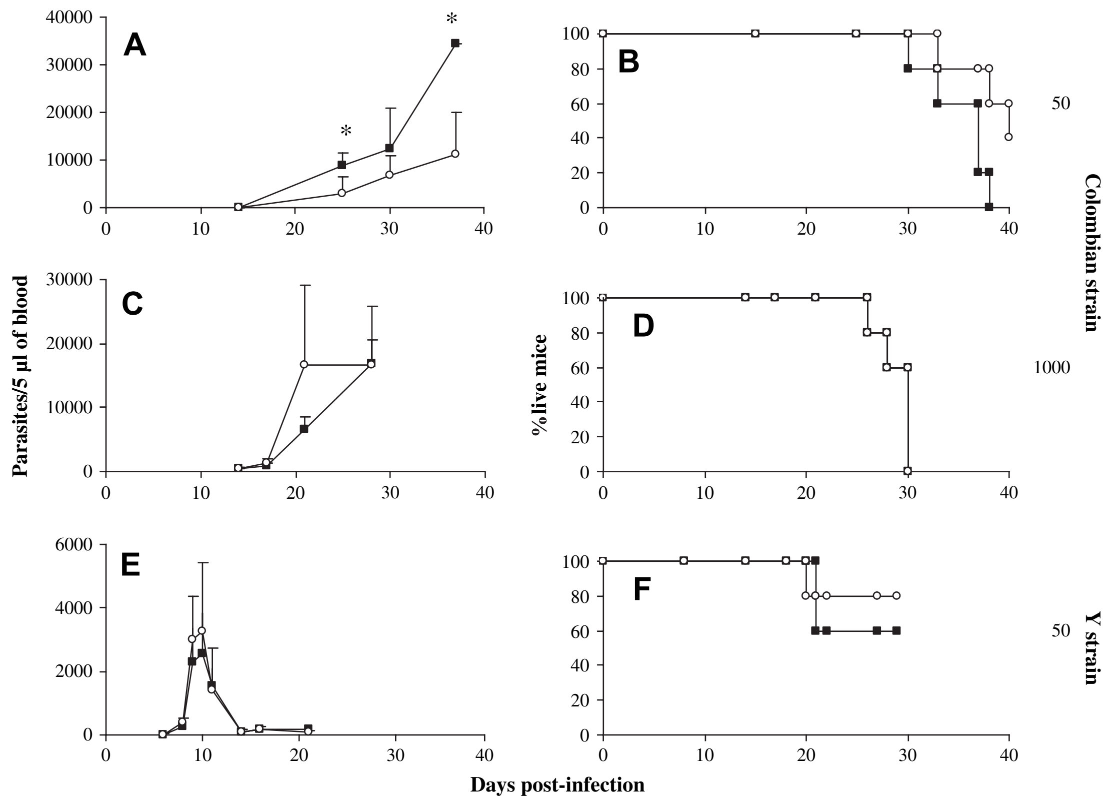

Fig. 2. Inactivation of CD25+ cells induces resistant to *T. cruzi* infection. C57BL/6 mice were injected with anti-CD25 (circles) or with isotype matched control antibody (squares) and were challenged with 50 (A and B) or 1000 (C and D) parasites of the Colombian strain. Number of parasites in 5 µl of blood (A, C) and survival curves are shown (B, D). Experiment with 1000 parasites was performed twice and experiments with 50 parasites were repeated three times. For A, B and D $n = 5$, for C $n = 6$. Asterisk indicates statistically significant differences ($P < 0.05$) calculated by Student's *t*-test. (E, F) Number of parasites in 5 µl of blood and survival curves, respectively, of C57BL/6 mice (4–5 mice per group) injected with anti-CD25 (circles) or with isotype matched control antibody (squares) and challenged with 50 parasites of the Y strain. This experiment was repeated three times.

observed, on parasitemia indices or mortality rates (Fig. 2C and D). Considering the fact that mice with different genetic backgrounds may develop immune responses with varied profiles when infected with *T. cruzi*, we decided to test Treg cells inactivation throughout the course of *T. cruzi* infection in C3H/HeJ and BALB/c mice, using two challenge doses (50 and 1000 parasites). The results were similar to those generated by C57BL/6 mice (data not shown).

In an attempt to provide support to our findings, the same assay was repeated, but using the *T. cruzi* Y strain. C57BL/6 mice were injected with PC61 and GL113 and inoculated with two infective doses of *T. cruzi*, 50 (Fig. 2E and F) and 100 parasites (data not shown). Differently from what was observed with the Colombian strain, no difference was observed between CD25 inactivated and control mice regarding both circulating parasites and mortality rate.

3.2. *Effect of inactivation of CD25 on pro-inflammatory response elicited during* T. cruzi *infection*

In order to investigate if cytokine levels were correlated to the increased resistance in PC61-treated mice to *T. cruzi*, blood samples were analyzed on different days post infection (p.i.). Cytokines IL-6, IL-10, IL-12p70, IFN-γ, TNF-α and MCP-1 were quantified using CBA. Fig. 3 shows the results for IL-6, IFN-γ, TNF-α and MCP-1. A tendency to increased cytokines levels in CD25-inactivated mice at 11 days p.i. was observed. However, the observed difference for IFN-γ ($P = 0{,}148$) and TNF-α ($P = 0{,}254$) levels of PC61-treated mice versus the control group (GL113) was not significant. No difference was observed between the cytokine levels in sera of PC61-treated mice and the control group on later time-points.

3.3. *Effect of inactivation of CD25 on acquired immune responses elicited during* T. cruzi *infection*

We next investigated whether CD25 inactivation would lead to increased activation of CD4, CD8 and NK cells. Spleen cells from CD25-inactivated mice and control mice 11 days p.i. were labeled with antibodies specific to CD69. We verified that CD25-inactivated animals presented a significant increased frequency of CD4+ cells expressing CD69 (29 ± 2.65) ($P < 0.05$) when compared to control mice (GL113) (20 ± 2.8) (Fig 4A). Regarding CD8 (GL113: 14 ± 1.63; PC61: 21 ± 5.11) and NK cells (GL113: 7.4 ± 1.21; PC61: 11.1 ± 2.26), there was no significant difference, although the PC61-treated group exhibited a tendency to increased frequency of CD69-bearing activated cells among these cell subsets (Fig. 4A).

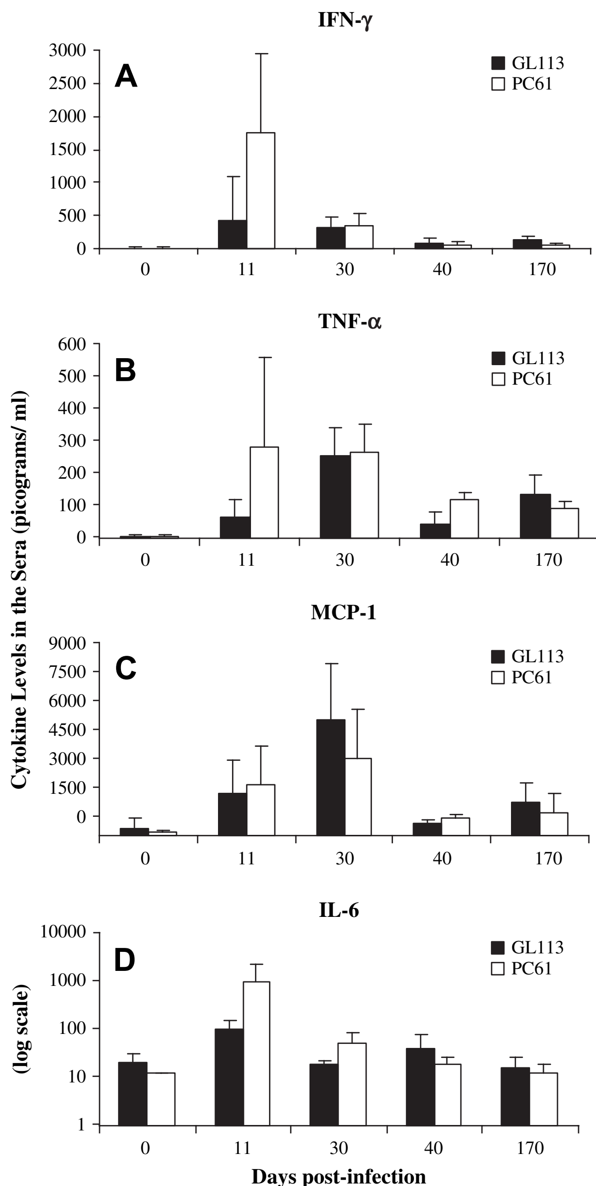

Fig. 3. Effect of CD25-inactivation on serum cytokines levels of mice infected with *T. cruzi*. Quantification by CBA (Cytometric Bead Array) of IFN-γ (A), TNF-α (B), MCP-1 (C) and IL-6 (D) in the serum from infected C57BL/6 mice with 50 parasites/animal (Colombian) at days 0 ($n = 5$), 11 ($n = 3$), 30 ($n = 8$), 40 ($n = 3$) and 170 ($n = 5$) post infection (p.i.). Control mice (GL113): black bars and CD25-inactivated (PC61): white bars. The values shown represent the average plus standard deviation.

We also determined the secretion levels of IFN-γ (effector functions) and IL-10 (modulatory function) by splenocytes of mice infected with *T. cruzi*. As shown in Fig. 4, at 30 days p.i. the levels of IFN-γ were higher ($P < 0.05$) in PC61-treated animals when compared to control group (Fig. 4B). Concerning IL-10 levels (Fig. 4C), no difference between the groups was observed.

Serum immunoglobulin levels (IgG, IgG1, IgG2a^b and IgM) were quantified on different days p.i. (0, 15, 30, 45 and 170). No differences were observed between the two groups. Kinetics of immunoglobulin production was similar in both groups (data not shown).

3.4. Effect of inactivation of CD25 on myocarditis of mice infected with T. cruzi

Hearts from CD25-inactivated mice were harvested on different days p.i. and stained with H&E. Inflammation was similar between CD25-inactivated and the control group (Fig. 5C). Nevertheless we decided to investigate whether the nature of the cells in the inflammatory infiltrate was different between the groups. Immunohistochemistry results did not show any qualitative differences regarding CD4, CD8, macrophage or *T. cruzi* antigens among the groups (data not shown).

We also evaluated whether the cytokines produced in the cardiac tissue were different between groups. Levels of IFN-γ, MCP-1 and IL-12p70 were analyzed using CBA (Fig. 5). On day 30 p.i. (Fig. 5D and E), maximum production of IFN-γ and MCP-1 was observed, in both groups of infected mice when compared with non-infected control mice ($P < 0.05$), but no difference between PC61- and GL113-treated animals was observed. There was no difference for other cytokines analyzed (IL-6, IL-10, and TNF-α) as well (data not shown).

In order to assess the role of CD4+CD25+ T cells in the development of chagasic myocarditis, we quantified the expression of CCL4 (the most potent chemoattractant of CD4+CD25+ cells in mice) [22] and Foxp3 (a transcription factor specifically expressed by Tregs in mice) throughout infection with Colombian strain of *T. cruzi* in mice. Results from the assessment of CCL4 and Foxp3 gene expression as well as quantification of *T. cruzi* DNA in the heart from infected mice at different times post infection (16, 31, 45 and over 90 days p.i.) are depicted in Fig. 6. DNA and RNA quantification was performed through real-time PCR and the results shown refer to increased gene expression or tissue parasitism relative to day zero. At 31 days p.i., the heart tissue showed enhanced production of CCL4 ($P < 0.01$) compared to the other days p.i. (Fig. 6A), which overlapped with maximum tissue parasitism (Fig. 6C). Regarding Foxp3 expression, an increased expression was observed throughout infection, however no statistical difference was observed in the different days post infection (Fig. 6B).

4. Discussion

T CD4+CD25+ cells are known to control autoimmune diseases, down-regulate immune responses against infectious agents and enable the parasite to escape from the host immune system [14]. In the context of *T. cruzi* infection, there is a strong ‘‘non-specific’’ activation of the host immune system during the acute phase and a possible autoimmune disorder during the chronic phase [23]. Importantly, blockade of CTLA-4, a receptor expressed by CD4+CD25+ T cells, results in increased IFN-γ synthesis and host resistance to *T. cruzi* infection [24]. Therefore, investigating the role of CD4+CD25+ in experimental infection could provide further information in the pathophysiology of Chagas’ disease. Here we demonstrated that CD25-inactivated mice infected with *T. cruzi* had slightly augmented IFN-γ and TNF-α production,

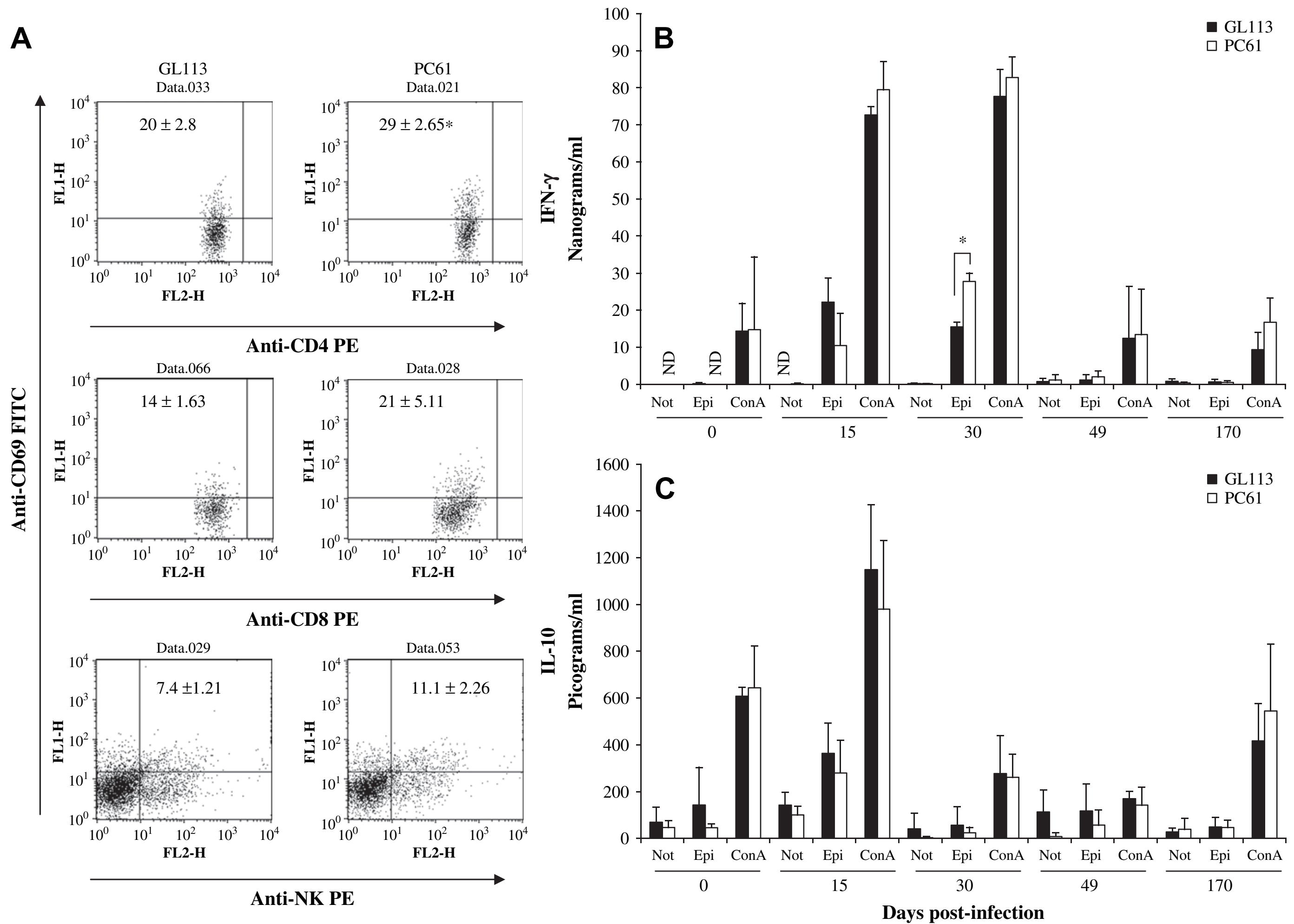

Fig. 4. Mice with inactivation of CD25+ have more CD4+ activated and your splenocytes produce more IFN-γ when stimulated with *T. cruzi* antigen. (A) Representative plots show average percentage plus standard deviation (three samples per group) of CD4, CD8 and NK activated cells (CD69+) collected from mice spleens treated with PC61 (inactivating antibody) or isotype matched control antibody (GL113) at 11 days p.i. with 50 parasites of the Colombian *T. cruzi* strain. The dot plots shown were gated on CD4+ or CD8+ after side-scatter-versus-CD4 or CD8-positive cells; for NK+ the dot plots shown were gated on blast lymphocytes. The asterisk indicates that the difference is statistically significant ($P < 0.05$) when compared CD4+CD69+ from both control and CD25-inactivated animals through Student's *t*-test. In (B) and (C) splenocytes from C57BL/6 mice injected with either control antibody (black bars) or PC61 (white bars) were collected at days zero, 15, 30, 49 and 170 post infection with 50 parasites, stimulated with epimastigote antigens (Epi) or concanavalin A (ConA) and the levels of IFN-γ and IL-10 were measured in culture supernatants 48 h after stimulation. The values shown represent the average plus standard deviation of five animals; the asterisk indicates that the difference is statistically significant ($P < 0.05$) when we compared cytokine levels produced by splenocytes from both control and CD25-inactivated animals, after stimulation at the same day of infection, by Student's *t*-test. ND, not detected.

which correlated with a significant increased activation of CD4 cells (11 days p.i.) and enhanced resistance to acute infection with Colombian strain of *T. cruzi*. In contrast, we found no effect of inactivation of CD25+ T cells in chronic *T. cruzi* infection.

Studies involving human and mice suggest that CD4+ Th1 lymphocytes (CD4 Th1) are important cells for inducing protective immunity in both acute and chronic infection with *T. cruzi* [7]. Accordingly, CD4-depleted mice are more susceptible to infection [25], and transference of CD4 Th1 cells assures protective immunity [26]. CD4 Th1 cells may enhance resistance to *T. cruzi* infection through different forms such as: (i) giving rise to the production of IFN-γ, which up-regulates the generation of nitric oxide by macrophages, an important effector molecule that controls intracellular parasite replication; (ii) leading to cytotoxic CD8 lymphocytes expansion, main effector cells that recognize and destroy infected cells; and (iii) promoting switch and synthesis of immunoglobulin isotypes, also involved in control of parasitemia [7].

Both production of nitric oxide by macrophages and CD8+ T cells have been shown to be critical for host resistance to early infection with *T. cruzi*. We believe the former may explain the higher efficacy observed on parasite control in CD25-inactivated mice. Hence, in addition to the increased production of IFN-γ by spleen cells we also observed a tendency to increased serum level of the pro-inflammatory cytokine TNF-α (11 days p.i.), which contributes for the production of nitric oxide by macrophages and control of *T. cruzi*. Indeed, TNF-α deficiency results in increased parasitemia levels and mortality rate [2], due to an impaired nitric

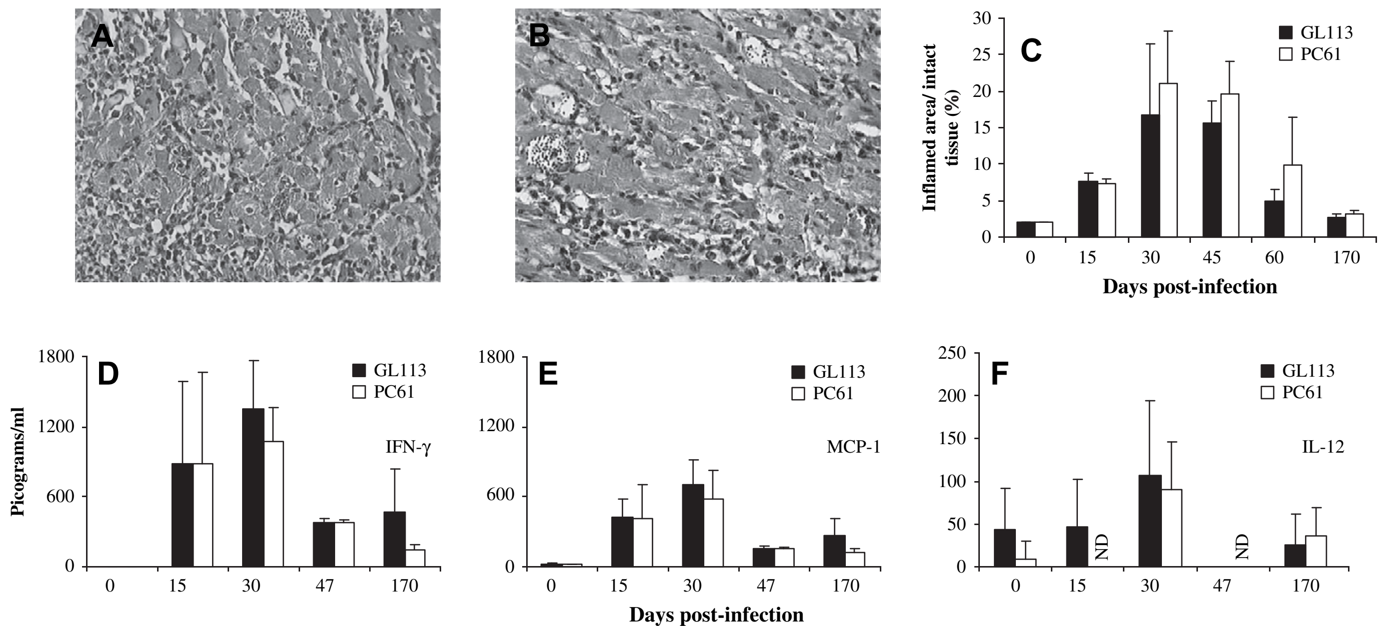

Fig. 5. Inflammatory infiltrate and cytokine levels in the cardiac tissue from both control and CD25-inactivated animals infected with *T. cruzi*. Control C57BL/6 mice (GL113) (black bars) and CD25-inactivated (PC61) (white bars) were euthanized at different days p.i. with 50 parasites of the Colombian strain for quantification of cytokines and histological analysis. Inflammatory infiltrate in the cardiac tissue from control mice (A) and CD25-inactivated mice (B) at 30 days p.i. (HE; magnification 20 ×). In (C), the average and positive standard deviations are shown for each inflammation area in relation to the whole area obtained from cardiac sections of four mice per group. Levels of IFN-γ (D), MCP-1 (E) and IL-12 p70 (F) extracted from the cardiac tissue at days 0 ($n = 3$), 15 ($n = 4$), 30 ($n = 7$), 47 ($n = 3$), and 170 ($n = 4$) p.i. Averages and positive standard deviations are shown.

oxide production by macrophages [27]. Alternatively, enhanced CD8+ T-cell activation and expansion could be responsible for the more efficient control. Although we found an increased number of activated CD8+ T cells, in CD25-inactivated mice, this difference was not significant. In addition, Kotner and Tarleton [15] found no change on specific CD8+ T-cell responses in *T. cruzi*-infected mice depleted of Treg cells. A less appealing hypothesis is the role of Tregs on antibody production, since antibodies have a secondary role on host resistance to infection, and we observed no difference in the *T. cruzi*-specific humoral response profile between CD25 inactivated and control mice.

Importantly, IL-10−/− mice succumb during acute *T. cruzi* infection with controlled parasitism, but an intense pro-inflammatory response and myocarditis [8]. In contrast, we observed comparatively mild changes in terms of parasitism and pro-inflammatory response in the CD25-inactivated mice infected with *T. cruzi*. Thus, our results indicate that Tregs are not likely the main IL-10 source during infection with *T. cruzi*. Considering the direct correlation of IFN-γ and IL-10 responses to parasite antigen, a potential source of this regulatory cytokine during *T. cruzi* infection are the recently described IL-10-producing Th1 cells [28]. Similar results were obtained when we infected C57BL/6 mice (not CD25-inactivated) with the Colombian strain of *T. cruzi* (data not shown).

Our results also indicate that the role of Treg cells in the acute infection with *T. cruzi* appears to be dependent on parasite strain. Thus, no difference was observed between CD25 inactivated and control mice regarding both circulating parasites and mortality rate (Fig. 2E and F) in mice infected with the Y strain of *T. cruzi*. Further, Kotner and Tarleton [15], investigating the *T. cruzi* strains Brazil and Tulahuen, verified that Treg cells showed no evident function in the murine chagasic infection. A possible explanation for the divergence observed in the role of CD4+CD25+ cells in the infections caused by both *T. cruzi* strains, Y and Colombian, is that Tregs may be involved in escape of Colombian strain from the process of initial up-regulation of IL-12 and IFN-γ [29].

Finally, we evaluated the role of Tregs on chronic myocarditis elicited by infection with *T. cruzi*. CCL4 comprise the most chemoattractant cytokine to CD4+CD25+ cells in mice [22]. Infection of macrophages and other antigen-presenting cells with *T. cruzi* up-regulate the production of CCL4 [16]. Thus, we hypothesized that there is a increased migration of Treg cells to the heart throughout the course of infection to modulate cardiac inflammation, diminishing tissue damage and also enabling the parasite to escape immune system mechanisms. Aiming to test such a hypothesis, we carried out quantitative PCR in different stages of infection using primers specific to CCL4 and Foxp3 (a transcription factor specifically expressed in T CD4+CD25+ cells) [11–13]. Despite the increased production of CCL4, there was no corresponding migration of CD4+CD25+ cells to the cardiac tissue. Other investigators [15] have found similar results; they reported little expression of Foxp3 and its product—the scurfin protein—in the muscular tissue, when compared to the spleen expression in mice chronically infected with the *T. cruzi*. Such data, together with histological and immunohistochemical analyses, in which inactivation of CD4+CD25+ cells caused no significant changes in populations of the main cells involved in *T. cruzi* infection control, i.e. CD4 and CD8 cells and

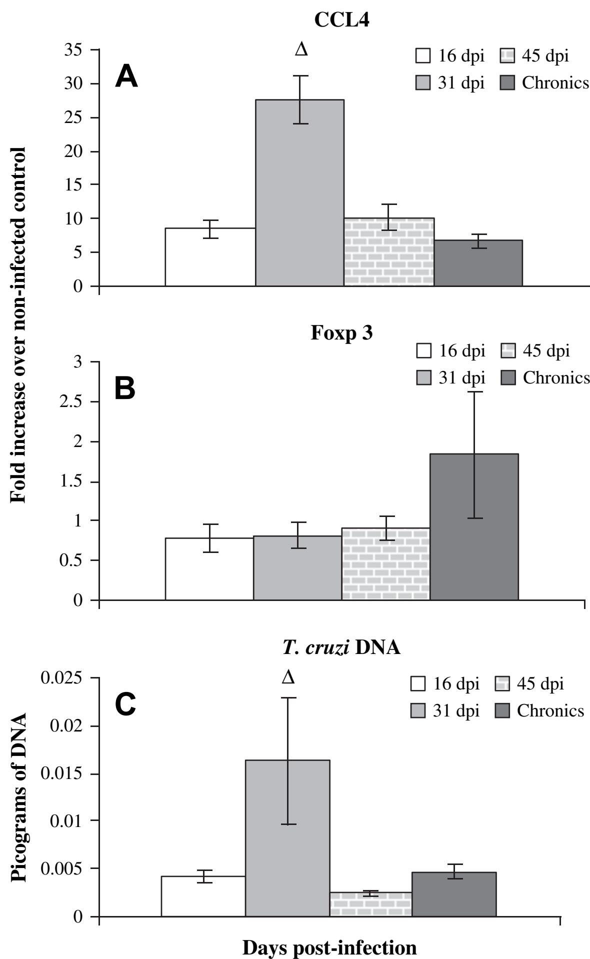

Fig. 6. Levels of cardiac Foxp3 and CCL4 throughout the course of infection with *T. cruzi*. Real-time PCR was carried out for quantification of CCL4 (A), Foxp3 (B) and *T. cruzi* DNA (C) in C57BL/6 mice hearts ($n = 4$) infected with 50 parasites of the Colombian strain. The mice hearts were collected at days 16, 31 and 45 days p.i. and also during chronic phase (>90 days p.i). (A) and (B) show average ± standard error of increased expression of mRNA of CCL4 and Foxp3 normalized by the constitutive gene Beta actin. (C) Measured *T. cruzi* DNA in picograms. In (A), the triangle indicates statistical difference ($P < 0.01$) at day 31 p.i. when compared with other days p.i. by Tukey's test, and (C) shows that *T. cruzi* DNA at 31 days p.i. is statistically superior ($P < 0.05$) to 45 days p.i. by Dunn's test.

macrophages, suggest that Treg cells play no evident role in experimental chagasic myocarditis.

In conclusion, our study demonstrate that, inactivation of regulatory CD4+CD25+ cells (Treg cells) leads to slightly increased resistance to a specific *T. cruzi* strain. Such enhanced resistance was associated with up-regulation of CD4 cells activation and production of IFN-γ in acute phase of infection. However, we observed no significant histological changes and inflammatory cells at the cardiac level. Taken together, our findings indicate that Treg cells have a very limited (or no) role in the pathophysiology of experimental infection with *T. cruzi*.

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