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Article

Trypanosoma cruzi Infection Activates Transgenic DO11.10 CD4+ T Cells

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Abstract

The acute *Trypanosoma cruzi* infection induces an exuberant immune response; however, despite this, the host is unable to clear the parasite, and the infection progresses to a chronic phase. T and B cells play a crucial role in controlling infections. Although the parasite constitutes a myriad of antigenic determinants capable of activating many T and B cell clones, some antigens trigger a large proportion of CD8 T cells, implying TCR cross-reactivity targeting these determinants. Polyclonal activation may result in an inefficient immune response against the parasite, diverting it to less critical antigenic determinants, allowing for infection persistence, and opening up the possibility of autoimmunity. Cross-reactivity is being demonstrated for CD8 T cells, but not for CD4 T cells. Herein, we demonstrate, by cytometry, that CD4+ T cells, carrying high levels of DO11.10 transgenic TCR, which are responsive to OVA, are activated during the *T. cruzi* acute infection, becoming effector memory T cells that produce cytokines such as IFN- γ and TNF- α , and prior exposure to OVA via the oral route modified cytokine production of these transgenic T cells upon infection. We also demonstrate that *T. cruzi* induces the expression of Foxp3 in a sizable pool of transgenic T cells.

Keywords: chagas disease; *Trypanosoma cruzi*; DO11.10 T cells; ovalbumin; interferon- γ , TNF; interleukin-10; cross-reactivity

INTRODUCTION

Trypanosoma cruzi is the etiological agent of a neglected condition named Chagas disease ([1]). The acute infection is followed by a potent immune response that controls the growth of the parasite ([2]). However, the host can not clear the parasite, and a chronic infection is established ([2]). A very wide range of immune responses can be detected during the acute phase of the disease, keeping parasite growth in check ([2]). Yet, most of the lymphocyte response is polyclonal and may occur through bystander activation ([3]). However, it has been proposed that T cell receptors are cross-reactive to a large extent ([4]). Many molecular mechanisms are proposed to explain TCR cross-reactivity in different models ([5]). Therefore, it is reasonable to argue that T cell cross-reactivity may be one of the reasons to justify the polyclonal T cell activation during *T. cruzi* infection. Cross-reactivity may have important immunological implications. For instance, T cells are positively selected in the thymus by self-peptides and MHC. They may yet interact with and produce immune responses to non-self peptides in the peripheral lymphoid organs ([5,6]). Importantly, infections could trigger and expand clones with cross-reactivity to self-antigens, and therefore, cause autoimmune diseases ([7,8]).

On the other hand, cross-reactivity may help to develop vaccines to prevent or ameliorate autoimmune diseases. For instance, it was described that non-self heat shock protein-derived peptides may induce activation of cross-reactive regulatory T cells, avoiding autoimmune diseases ([9–11]). Moreover, previous contact with pathogens or even non-pathogenic microflora could modify the immune response to an unrelated microorganism, as documented before ([8,12]). For example, we have previously described that aged or thymectomized mice were completely resistant to the infection with *T. cruzi*, whereas young mice are susceptible ([13,14]). Resistance, in that case, correlated with the increase of naturally occurring memory T cells in these animals at the moment of infection due to lack of regulation caused by aging or thymectomy, suggesting that environmental antigen-reactive T cells may help control the parasite load, responding to *T. cruzi* antigens by cross-reactivity ([2]). Interestingly, a great degree of cross-reactivity between different clones of CD8 T cells and *T. cruzi* antigen-derived peptides has recently been demonstrated ([15]). Together, these findings might contribute to explaining important aspects of Chagas disease pathogenicity. Yet, there is no clear information concerning the cross-reactivity of CD4⁺ T cells in the context of class II MHC during *T. cruzi* infection.

In this study, we have described the phenotypes and functional changes of the DO11.10 transgenic CD4⁺ T cell, bearing the TCR recognized by the clonotypic monoclonal antibody KJ1-26, upon acute *T. cruzi* infection. Originally, the DO11.10 transgenic TCR is specific for an Ovalbumin-derived peptide (323-339) in the context of IAd class II MHC ([16]). Therefore, the activation of DO11.10 transgenic CD4⁺ T cells to an unrelated multitude of antigens, such as *T. cruzi*, is rather a surprise. We demonstrate that DO11.10 transgenic CD4 T cells are largely activated to an effector memory phenotype during the acute *T. cruzi* infection in BALB/c mice. Additionally, part of the DO11.10 CD4 T cells begin to express Foxp3. The percentage of cells expressing IFN- γ and TNF also increases in the acute phase. Previous oral immunization with OVA altered the pattern of cytokine production by DO11.10 CD4 T cells, increasing the percentage of IL-10-expressing cells. These results suggest that cross-reactivity between CD4 T cells and class II MHC peptides may be similar to the CD8 T cell compartment and peptides related to class I MHC. These findings might have profound implications for the quality of the immune response during the infection.

MATERIALS AND METHODS

Animals

DO11.10 BALB/c mice (4-6 weeks old) were from the Centro de Pesquisas Gonalo Moniz animal house. The animals were housed in micro-isolators under conventional conditions and were handled under institutional ethical guidelines. The Committee approved all the protocols used in this study for ethics at the Oswaldo Cruz Foundation, under protocol number CpQGM 015-09.

Ovalbumin Immunization

Ovalbumin (A5253, Sigma Aldrich) was dissolved in drinking water at a concentration of 5 mg/mL, filtered, and administered ad libitum for 5 days. A fresh solution was used every day. Mice were rested for 7 days and immunized intravenously in the retroorbital sinus with 300 μ g/0.1 mL of OVA (A5503, Sigma-Aldrich) diluted in PBS. This protocol contains slight modifications from a previously published study ([17]).

Infection

Groups of 5 to 30 mice were infected intraperitoneally with 10³ blood-form trypomastigotes of the Tulahuen strain of *T. cruzi* in 0.2 ml of 0.15 M phosphate-buffered saline (PBS). Control mice received the same volume of PBS. Mice were sacrificed between 22 and 25 days after infection, depending on the experiment. Results did not show significant differences during this brief period.

In Vitro Cell Culture

Splenocytes were cultured in triplicate at a density of 10^7 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-ME, and one mM HEPES (complete medium). Cells were cultured at 37°C and 5% CO₂ for 24 hours in complete medium alone or with a combination of PMA plus Ionomycin (Cell Stimulation Cocktail, Tonbo Bioscience, San Diego, CA, USA), as previously described ([18]). Additionally, Brefeldin-A was added 6 hours before cell harvest to facilitate staining for flow cytometric analysis.

Flow Cytometric Analysis

Spleen cells were isolated as described ([19]) and placed in ice-cold PBS supplemented with 5% FBS and 0.01% sodium azide. Stainings were done as previously described ([19]). In some experiments, CD4 T cells were magnetically sorted using surface staining with APC anti-CD4 (Clone RM4-4), followed by anti-APC microbeads (Miltenyi Biotec, Germany), according to the manufacturer's instructions. Purity was consistently above 95%, as confirmed by cross-checking with a PE-CY5.5 anti-CD4 antibody (Clone GK1.5, a non-epitope-overlapping antibody). The fluorochrome-conjugated monoclonal antibodies used were anti-CD4 (Clones GK1.5 and RM4-4), anti-DO11.10 clonotype (Clone KJ1-26), anti-CD44 (Clone IM7), anti-CD62L (Clone MEL-14), anti-Foxp3 (clone 150D), anti-IL-4 (clone 11B11), anti-IL-10 (Clone JES5-16E3), anti-IFN- γ (Clone XMG1.2), and anti-TNF- α (Clone MP6-XT22), which were purchased from eBioscience, BioLegend, or Caltag. Streptavidin-PE-Cy5.5 from Caltag revealed biotin-conjugated antibodies. Intracellular staining for IL-4, IL-10, IFN- γ , and TNF- α was performed as described ([20]). Buffer kits for intracellular and intranuclear stainings were from eBioscience and used as indicated by the manufacturer. Isotype controls (clones RTK2071 and RTK4530) were used to establish the background for cytokine intracellular assays. After surface staining, the cells were fixed with 1% paraformaldehyde in PBS and analyzed using a FACScan (Becton&Dickinson). Results were analyzed using FlowJo software.

Statistical Analysis

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

RESULTS

Percentage of CD4+DO11.10+ T Cells in Experimental Groups Submitted to Different Treatments

Figure 1 shows that transgenic DO11.10 CD4+ T cells comprise the majority of splenic CD4 T cells in control mice (81.8 ± 3.9). The same situation was observed for mice previously immunized with OVA (83.8 ± 5.1). However, *T. cruzi*-infected mice showed smaller proportions of transgenic T cells in the CD4+ T cell population (41.2 ± 4.4) within 25 days after infection. OVA immunization significantly prevents the diminution of DO11.10 transgenic T cells in the overall CD4+ T cell population (61.9 ± 6.5), indicating that antigen-experienced transgenic T cells may be better preserved during acute infection. Please note that the fluorescence intensity of KJ1-26 clonotype expression was also lower in infected mice, indicating some degree of activation. These results also suggest that DO11.10 transgenic CD4+ T cells may interact with *T. cruzi* antigens in some way.

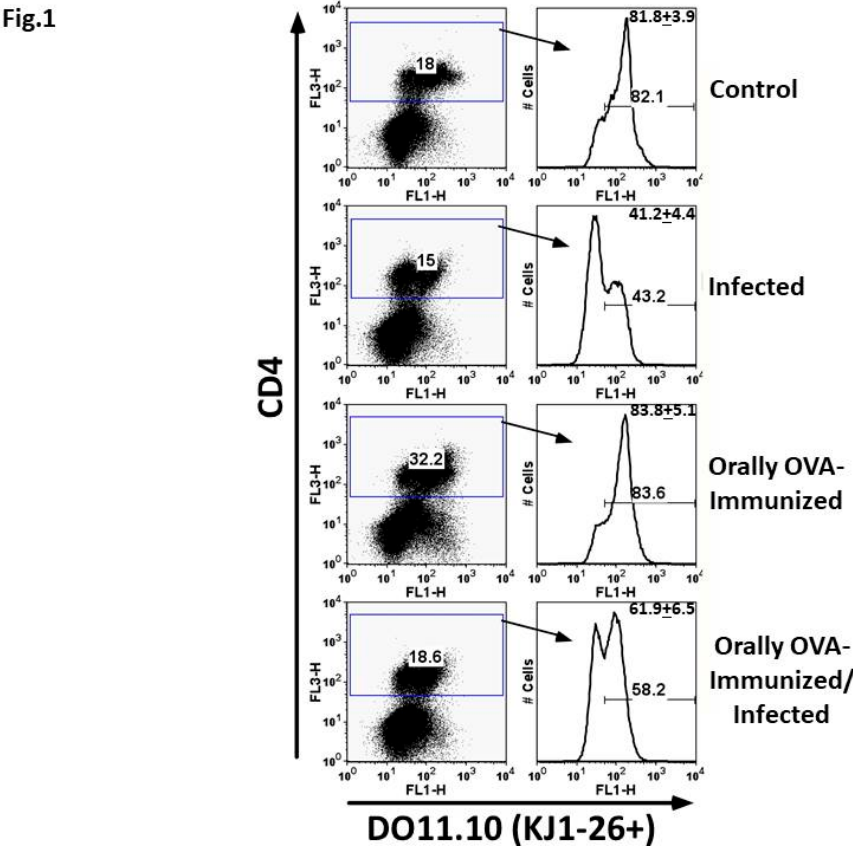


Figure 1. Quantitation of splenic DO11.10 transgenic CD4+ T cells bearing the TCR clonotype, identified by the KJ1-26 mAb. Spleen cells from uninfected control, infected, OVA-immunized, and OVA-immunized/infected transgenic mice obtained on day 25th of infection were studied. A representative histogram from one animal per group is depicted. Numbers in the upper right corner represent the mean ± SD of the percentage of KJ1-26+ T cells inside a CD4 electronic gate. Groups were of five mice each. Experiments were repeated three times, on different occasions, with similar results. The Mann-Whitney test was used to compare two distinct groups of mice ($P < 0.05$ was considered significant).

DO11.10 Transgenic CD4+ T Cells are Activated to Effector Memory Lymphocytes During the Acute T. cruzi Infection

To better understand the activation of DO11.10 transgenic T cells during acute *T. cruzi* infection, the formation of memory T cells was examined 22 days post-infection. In these experiments, CD4+ T cells were previously labeled with an APC anti-CD4 monoclonal antibody (mAb) and then sorted using anti-APC magnetic beads. Figure 2A,B show that DO11.10+CD4+ T cells are predominantly in the resting stage, exhibiting low percentages of markers associated with memory T cells. On the other hand, DO11.10 transgenic mice increased the rate of effector memory transgenic T cells, expressing high levels of CD44 and low levels of CD62L. Immunization with OVA also increases the percentage of effector memory transgenic CD4+ T cells. Spleen cells from OVA-immunized mice contained higher percentages of central memory transgenic T cells compared to the other groups, as well as higher percentages of effector memory transgenic CD4+ T cells compared to the control group.

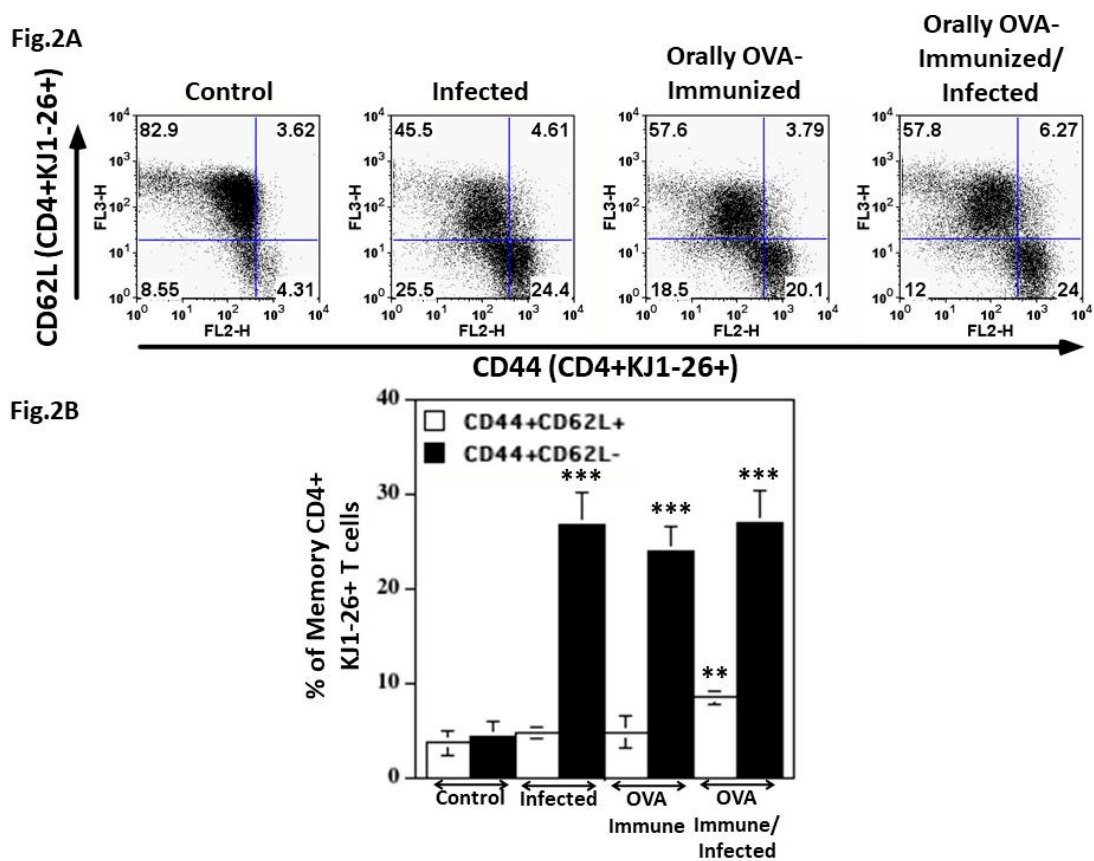


Figure 2. Quantitation of central and effector memory splenic DO11.10 transgenic CD4⁺ T cells. Spleen cells from uninfected control, infected, OVA-immunized, and OVA-immunized/infected transgenic mice obtained on day 22nd of infection were studied. Each dot plot represents one mouse from the same experiment. Central memory transgenic KJ1-26⁺ T cells were defined by the expression of high levels of CD44 and CD62L, whereas effector memory T cells were characterized by high CD44 expression and were negative for CD62L (Figure 2A). In Figure 2B, the mean $\pm$ SD of the percentages of central (white bars) and effector (black bars) memory KJ1-26⁺CD4⁺ T cells are shown. In these experiments, CD4 spleen cells from individual mice were magnetically sorted after staining with APC-labelled CD4 monoclonal antibody (mAb) and microbeads conjugated to anti-APC. Groups were of five mice each. The Mann-Whitney test was used to compare two different groups of mice. (**P < 0.01, ***P < 0.005).

T. cruzi Infection Induces Foxp3 Expression in DO11.10⁺ CD4⁺ Splenic T Cells

We have also studied the Foxp3 expression in DO11.10 transgenic CD4⁺ T cells during the infection in normal or OVA-immunized transgenic mice. Figure 3 shows a representative contour plot from different experimental groups. As previously published, the expression of Foxp3 in CD4⁺ T cells from DO11.10 transgenic mice is very low, if at all. However, we detected a significantly higher number of CD4⁺ KJ1-26⁺ T cells expressing Foxp3 after 25 days of infection (Fig. 3, second contour plot). The protocol used for OVA immunization also resulted in similar numbers of transgenic Foxp3-expressing cells, as shown in Figure 3. Infection in OVA pre-immunized mice did not result in different numbers of Foxp3 expression in transgenic T cells, compared to only infected or OVA-immunized mice.

Fig.3

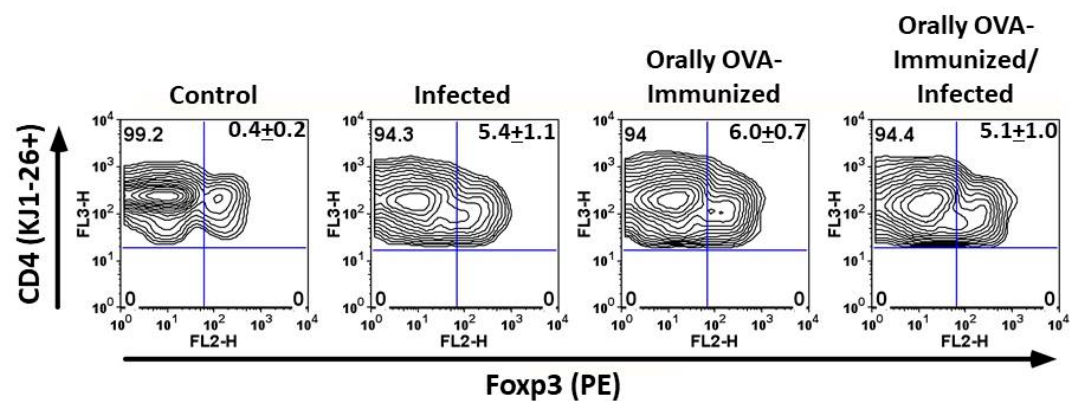


Figure 3. The number of splenic CD4⁺KJ1-26⁺Foxp3⁺ T cells increased after *T. cruzi* infection. In Figure 3, the percentages of splenic CD4⁺KJ1-26⁺Foxp3⁺ T cells are shown. Mice were used on day 25 after *T. cruzi* inoculation. Numbers in the upper right quadrant represent mean ± SD of the percentage of Foxp3⁺ T cells in the CD4⁺KJ1-26⁺ splenic T cells in each experimental group. No significant differences were found when experimental groups were compared among themselves. However, infected, OVA-immunized, and OVA-immunized/infected transgenic mice showed higher percentages of Foxp3⁺ transgenic T cells compared to uninfected, unimmunized controls. Animals were analyzed individually (n = 5 per group). Experiments were repeated on three occasions (days 22 and 25, after infection) with similar results (P < 0.005, Mann-Whitney test).

*DO11.10 Transgenic CD4⁺ T Cells Spontaneously Produce IL-4 and IL-10 upon *T. cruzi* Infection*

Figure 4 shows representative histograms on the production of IL-4 (upper histograms) by spleen cells from control, infected, OVA-immunized, and OVA-immunized-infected DO11.10 transgenic mice after 24 h in culture with medium alone or PMA/Ionomycin. The results show that transgenic CD4⁺ T splenocytes from infected mice presented higher frequencies of IL-4-expressing cells compared to unstimulated controls. In addition, previous OVA immunization significantly reduced the frequencies of IL-4⁺ cells, both with and without infection. Stimulation with PMA/Ionomycin repeated the pattern described above for unstimulated cells. We have also studied the frequencies of IL-10 expression on transgenic CD4⁺ T lymphocytes. The lower histograms in Figure 5 show that infection increased the frequencies of splenic IL-10 transgenic producers compared to control, OVA-immunized, or OVA-immunized-infected DO11.10 mice when spleen cells were cultured for 24 h in medium alone. Again, further stimulation with PMA/Ionomycin just amplified the pattern observed with medium alone. Interestingly, pre-immunization with OVA significantly decreased the frequencies of IL-10-producing transgenic T cells compared to non-immunized mice.

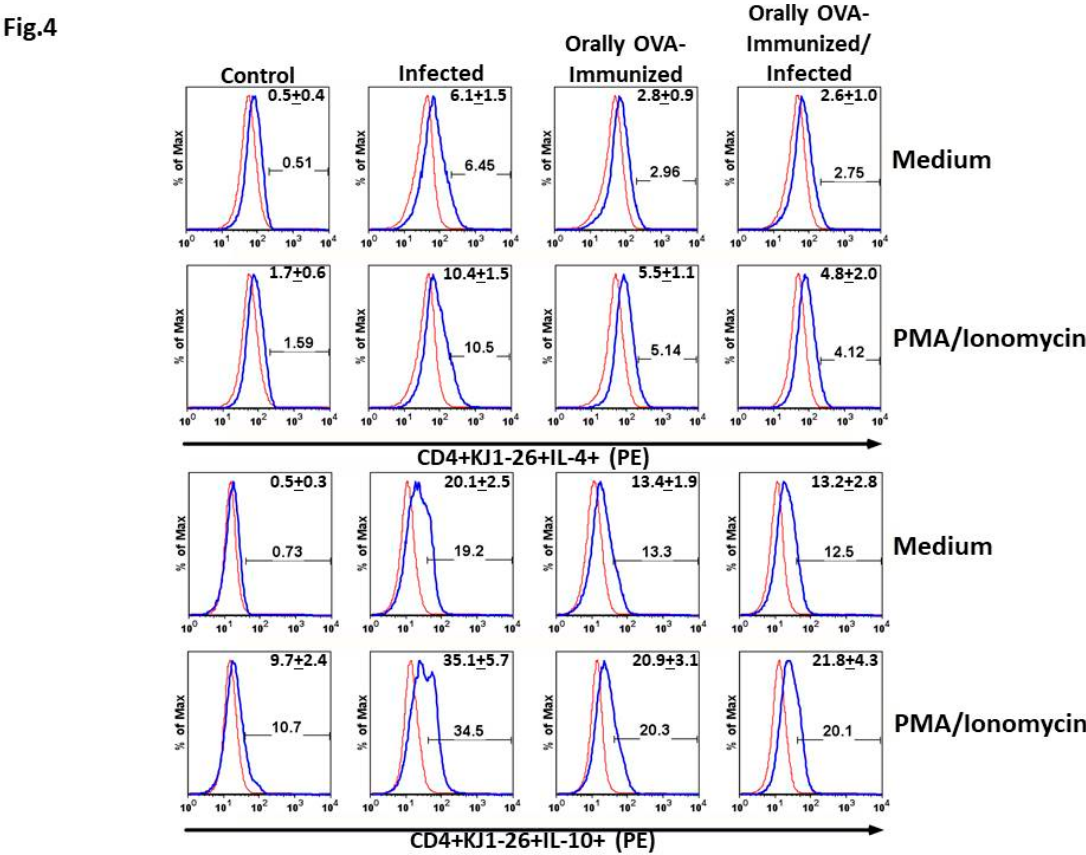


Figure 4. Production of IL-4 and IL-10 by DO11.10 transgenic T cells upon *T. cruzi* infection. The upper and lower sets of histograms show the percentage of splenic IL-4 or IL-10-producing CD4+ KJ1-26+ T cells from the different experimental groups, respectively. To establish the lower limits for IL-4 and IL-10 expression, a negative control was established using isotype mAb controls (red line histogram). Numbers in the right upper corner of each histogram represent the mean of the percentage of IL-4 or IL-10 producer cells $\pm$ SD. Illustrative histograms from one animal for each experimental group are shown. Spleen cells from control (uninfected, unimmunized), infected, OVA-immunized, and OVA-immunized/infected transgenic mice were cultured in complete medium or stimulated with PMA/Ionomycin for 24 hours. Brefeldin A was added for the last 6 hours before cell harvesting. Experiments were conducted 23 days after initial infection. Mice were analyzed individually (n = 5 per group). A total of three independent experiments were performed with similar results. Two groups were compared, using the Mann-Whitney test (P < 0.05 was considered statistically significant).

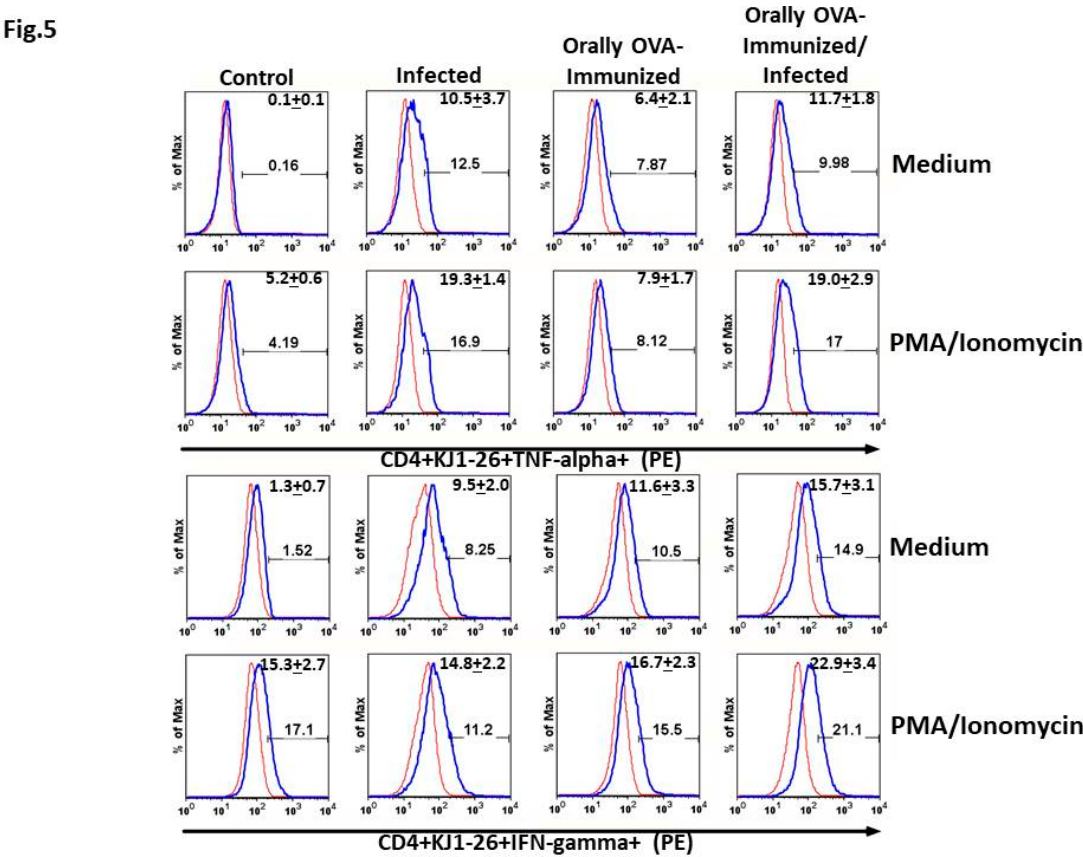


Figure 5. The frequencies of DO11.10 transgenic T cells expressing TNF- α and IFN- γ increased during acute *T. cruzi* infection. The upper set of histograms shows the percentage of splenic TNF-producer CD4+ KJ1-26+ T cells from the different experimental groups. The lower set of histograms represents the percentage of DO11.0 transgenic T cells producing IFN- γ . To establish the lower limits for TNF or IFN- γ -expression, a negative control was established using isotype mAb controls (red line histogram). Numbers in the right upper corner of each histogram represent the mean of the percentage of TNF or IFN- γ -producer cells $\pm$ SD. Illustrative histograms from one animal for each experimental group are shown. Spleen cells from control (uninfected, unimmunized), infected, OVA-immunized, and OVA-immunized/infected transgenic mice were cultured in complete medium or stimulated with PMA/Ionomycin for 24 h. Brefeldin A was added for the last 6 hours before cell harvesting. Experiments were conducted 23 days after initial infection. Mice were analyzed individually (n = 5 per group). A total of three independent experiments were performed with similar results. Two groups were compared, using the Mann-Whitney test (P < 0.05 was considered statistically significant).

T. cruzi Infection Increases the Frequencies of Splenic TNF- α and IFN- γ DO11.10 Transgenic T Cells

Infection with *T. cruzi* increased the frequencies of CD4+KJ1-26+ cells that spontaneously expressed TNF-alpha after 24 hours of culture in complete medium, as shown in Figure 5 (upper histogram set). Further activation with PMA/Ionomycin potentiates the production of TNF-alpha in controls, especially in infected animals. OVA-immunization also increased the spontaneous production of TNF-alpha compared to non-immunized controls. However, neither infection nor PMA/Ionomycin stimulation increases the frequencies of transgenic splenic T cells expressing TNF-alpha in OVA-immunized animals, suggesting that previous immunization has set a limit to the production of TNF as well as IL-4 and IL-10, as shown above.

The frequencies of transgenic splenic T cells that show spontaneous production of IFN-gamma increased upon infection compared to uninfected mice and are similar to those of mice previously immunized with OVA. In addition, OVA-immunized mice exhibited very high percentages of transgenic T cells that produced IFN-gamma spontaneously after 24 h in complete medium during the acute infection. In vitro stimulation with PMA/Ionomycin further increases the frequencies of IFN- γ -producing T cells in all experimental groups compared to unstimulated cells.

DISCUSSION

Signs of a robust T and B cell activation, as well as innate immune cells, are well documented during the *T. cruzi* infection ([2,3]). However, due to the large extent of lymphocyte activation, this response is usually considered polyclonal, where most of the clones would not be cognitively engaged to *T. cruzi* antigen recognition, but rather would be the result of bystander activation by non-specific signals involving PAMPs, DAMPs, and cytokines ([21]). Memory T cells would be favoured in such an activation as they are less dependent on TCR-mediated signals to be activated ([22]). A bystander polyclonal lymphocyte activation would disfavour a parasite-focused immune response by recruiting many non-specific T cells, competing with parasite-specific T lymphocytes, contributing to the establishment of the chronic infection, and, at the same time, opening up the conditions to generate autoimmune diseases upon recruitment and activation of potential autoreactive T and B cells ([5,7,8]). It has been hypothesized for a long time that T cells might embody a large proportion of cross-reactive TCRs so that they could be positively selected by low-affinity interactions with MHC/self peptides in the thymus and still respond to non-self antigens in the peripheral lymphoid organs ([5]). The aforementioned DO11.10 transgenic mice are a notable example of this. New data concerning *T. cruzi* infection suggest that a large proportion of this polyclonal activation may indeed correspond to a highly cross-reactive TCR-dependent response to a few *T. cruzi* antigens. Tarleton's group showed that a large proportion of CD8 T cells in mice infected with *T. cruzi* recognize and respond to a few peptides from two major groups of trans-sialidase peptides ([23]). Recently, the same group has better characterized the polyclonal CD8 T cell response, demonstrating that more than 50% of the total CD8 lymphocytes recognize two peptides from trans-sialidases and one peptide from mucin family proteins. Interestingly, these authors have found that a large degree of cross-reactivity exists among different CD8 T cell clones and peptides derived from distinct *T. cruzi* antigens, such as the trans-sialidases, mucins, and paraflagellar (PAR) proteins ([15]). In addition, they also described that a well-known CD8 T cell epitope, SIINFEKL, derived from Ovalbumin, is also recognized by CD8 T cells engaged in the response to *T. cruzi* antigens ([15]). Although the studies mentioned above support this concept of polyclonal activation due to cross-reactivity for CD8 T cells and class I MHC, no data are available for CD4 T lymphocytes and class II MHC.

In this study, we have shown that upon *T. cruzi* infection, the CD4+DO11.10+ transgenic T cells were diminished among total CD4+ T lymphocytes, as shown in Figure 1. At first glance, this result may suggest that other T cells are expanding at the expense of transgenic T cells. However, the expression levels of the KJ1-16 clonotype fell, indicating that transgenic T cells were being activated. Therefore, some of these cells might escape detection. Another possible explanation is that transgenic T cells underwent apoptosis upon stimulation caused by the infection, as previously described during the acute infection ([24]). Alternatively, one may hypothesize that mature transgenic T cells start to reexpress RAG, and therefore have a second rearranged alpha-chain, becoming a double TCR expressor, due to some infection-induced signal from the lymphoid microenvironment. Although the TCR-revision was described for some peripheral T cell populations in mice and humans ([25–27]), there is no description of that during *T. cruzi* infection. Oral immunization with OVA did not increase or decrease the percentage of DO11.10 transgenic T cells among CD4 T lymphocytes, compared to controls. Still, the frequency of total CD4+ T cells, among total spleen cells, increased substantially. In addition, antigen-experienced transgenic T cells from infected, orally immunized mice were also increased, compared to non-immunized transgenic infected mice. These later results indicate that immunization may modify the functional activities of transgenic DO11.10 T cells during the acute infection. Because of the above-discussed data, we have decided to study the expression of memory T cell markers, namely CD44 and CD62L. These markers divide memory T cells into central (CD44+CD62L+) and effectors (CD44+CD62L-) ([28,29]). Figures 2A and 2B demonstrated that OVA and *T. cruzi* infection have induced memory differentiation in transgenic splenic T cells, and *T. cruzi* infection induced a further augmentation of central memory transgenic T cells in previously immunized mice, compared to the other groups. Taken together, these results confirm our previous

observations, strongly arguing in favour of cross-reactivity between ova and *T. cruzi* antigens being recognized by the same transgenic TCR. It has been described that the DO11.10 transgenic CD4⁺ T cells do not differentiate into Foxp-3⁺ regulatory T cells (Tregs), unless mice were immunized with the cognate antigen in some conditions ([17]). Figure 3 confirms that finding in normal DO11.10 transgenic mice, where the percentage of clonotype-positive CD4 T cells express a very low frequency of Foxp-3. However, *T. cruzi* infection led to an increased expression of Foxp-3 in KJ1-26⁺ CD4 T cells, indicating once more that DO11.10 transgenic T cells were activated by TCR cross-reactivity to an unknown peptide antigen derived from *T. cruzi* antigens. Previous immunization did not improve the Treg frequencies beyond a certain level, a very interesting point as it suggests that the frequency of Tregs is somehow tightly controlled by unknown peripheral factors.

We have further studied the functional activities of DO11.10 transgenic T cells either in control uninfected mice or upon *T. cruzi* infection. We also primed transgenic T cells with OVA *in vivo* to study their cytokine patterns after infection. Figure 4 shows that upon *T. cruzi* infection, the frequencies of IL-4 and IL-10 producers among DO11.10 transgenic CD4⁺ T cells increased significantly compared to uninfected control mice spleen cells; also, the differences were potentiated by non-specific stimulation with PMA and calcium ionophore. Interestingly, previous oral OVA-immunization increased the frequencies of IL-4 and IL-10 producers compared to control non-immunized mice, but failed to further augment the frequencies of IL-4 and IL-10 producers upon *T. cruzi* infection, indicating that memory cells were not more responsive to *T. cruzi* infection, arguing against the activity of non-specific signals acting on transgenic T cells. A situation similar to the one described for IL-4 and IL-10 was found for the frequencies of TNF-producers among DO11.10 transgenic CD4⁺ T cells, as depicted in Figure 5, upper histogram set. However, previous oral OVA-immunization increased the frequencies of IFN- γ -expressing transgenic CD4⁺ T cells. Different from the other cytokines discussed before, the immunization scheme already induced more IFN- γ -expressing transgenic T cells compared to infection in naïve animals, so the immunization protocol we have used skewed the functional activity of transgenic T cells to a TH1 pathway that was further reinforced by the infection in this combination of parasite/mouse strains, as previously demonstrated ([30]).

In conclusion, we have demonstrated that DO11.10 transgenic CD4⁺ T cells were activated during the acute *T. cruzi* infection. We have shown that transgenic T cells acquire markers related to memory T cells, including a reasonable frequency of Tregs. Further, we showed that these cells produced important cytokines during the infection and that the cytokine pattern could be modified by previous exposure to the original antigen. We did not identify the antigens/peptides recognized by the transgenic TCR in this study. Yet, this is the first demonstration that a CD4⁺ transgenic cell, specific for a non-related *T. cruzi*-derived peptide, was indeed activated during this infection by TCR cross-reactivity. The model described may allow further studies on the development of different functional activities during *T. cruzi* infection, memory T cell generation, and the dynamics of memory cells during and after the acute infection or immunization/vaccination. Therefore, this model seems to be highly attractive for new studies.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of FIOCRUZ-BA (CpQGM 015-09).

Data Availability Statement: All the data is presented in the study. Crude data may be obtained from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflicts of interest

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