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Original Article

Immunostimulation by *Trypanosoma cruzi* has little effect on immune response against experimental melanoma

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ABSTRACT

In vivo presence of *Trypanosoma cruzi* triggers a persistent innate and adaptive immune response. We aimed to evaluate the immunological activity generated by introducing *Trypanosoma cruzi* in mice with an experimental melanoma. We investigated whether immunity to epimastigote forms of *T. cruzi* could induce anti-tumor activity using an experimental model of melanoma. C57Bl/6 mice received: 1. saline injection (Control). 2. only *Trypanosoma cruzi* (*Tc*). 3. only melanoma cells (B16F0, 100 μL with $1x10^5$ cells); 4. *Trypanosoma cruzi* plus melanoma cells (*Tc*+B16F0). *Trypanosoma cruzi* administration to B16F0 resulted in a low number of splenic NK cells but not NKT cells. Myeloid suppressor cells were decreased in the *Tc*+B16F0 (day 5) and the *Tc* group (day 20), both compared to the B16F0 animals. The *Tc*+B16F0 group presented lower CD4 (but not CD8) splenic T cells that produced IL-10, compared to B16F0. About TNF-α production, no differences between groups were found, but $CD4^+TNF\text{-}\alpha^+$ were higher than CD8 T cells in all studied experimental conditions. After anti-CD3 stimulation, splenic T cells of the *Tc*+B16F0 group produced higher levels of IFN-γ compared to the other experimental groups. Finally, lower $CD4^+CD69^+$ T cells may indicate decreased non-specific activity in the *Tc*+B16F0 compared to the B16F0 group. The association of *Trypanosoma cruzi* with B16F0 resulted in NKT cells' maintenance up to twenty days after tumor injection, probably favoring longevity and early tumor restrainment. The group *Tc*+B16F0 had reduced myeloid suppressor cells involved on the immunoregulatory axis at the beginning of tumor development, which possibly is related to an anti-tumor response as an early immune response.

KEYWORDS: melanoma B16F0, *Trypanosoma cruzi*, natural killer, NKT lymphocytes, tumor cell-regulation, myeloid suppressor cells.

ABBREVIATIONS

FACs	-	Flow Cytometry
FITC	-	Fluorescein
PE	-	phycoerythrin
IE.	-	intra-ear injection
IP.	-	intraperitoneal
IFN-γ	-	interferon-gamma
IL	-	interleukin
MDSCs	-	Myeloid suppressor cells
MHC	-	major histocompatibility complex
MV	-	microvesicles
NK	-	natural killer cells
NKT	-	natural T lymphocytes
T. cruzi	-	*Trypanosoma cruzi*
TNF-α	-	tumor necrosis fator alpha

INTRODUCTION

T. cruzi [1], *Toxoplasma gondii* [2], and *Leishmania* spp. [3] are known to promote different forms of disease and immune responses. Chagas disease is

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related to immune system deregulation and is associated with chronic digestive and heart disorders due to damage to enteric motor innervations. Chagasic mega-syndromes, e.g., megaesophagus and megacolon, are related to dilated cardiomyopathy and gastric alterations [4]. Many different cell populations and cytokines are involved in Chagasic lesions, and inflammatory infiltrates are investigated during this disease [5]. Initial IFN-γ production before the generation of T-cell-mediated immunity is already known to occur during many infections. It may be necessary for the development of resistance to many intracellular pathogens [1]. Natural killer (NK) cells contribute as the primary cell type responsible for IFN-γ production in the early stages of *T. cruzi* infection, and *T. cruzi*-induced IFN-γ biosynthesis requires the presence of live parasites, as described during *T. cruzi* experimental infection [1].

Among the cutaneous neoplasms, melanoma-type lesions are highly malignant at advanced stages and carry a worsening prognosis due to the high probability of metastasis. The tumor microenvironment presents immunosuppressive characteristics, which inhibit the activation and effector function of lymphocytes infiltrated in tumor cells, where they would otherwise mount an adaptive response [reviewed in 6-7]. This inhibition may lead to the exhaustion and senescence of effector immune cells against the tumor [6-7].

William Coley first reported immunotherapy involving the introduction of pathogens capable of stimulating the immune system in the late 1800s. Coley injected into surgically incurable tumors a mixture of bacterial toxins derived from erysipelas and *Bacillus prodigious* [reviewed in 8-9]. However, the substantial toxicity resulting from this regimen, together with inconsistent responses, made this therapy unviable. Live trypomastigote forms of *T. cruzi* can induce microvesicles (MV) by monocytes and lymphocytes [10]. The consequent release of membrane-bound TGF-beta promoted rapid cellular invasion by *T. cruzi* and contributes to parasite avoidance of the complement attack. Infection occurring in the presence of MV or exosomes results in increased parasitemia, contributing to parasite evasion of innate immunity [10]. Non-infective parasites used herein or the use of *T. cruzi* antigens can induce a protective immune response against different types of neoplasia [11] and can modulate the cellular and humoral axis against solid tumors.

Despite the study of mechanisms involved in the anti-tumor response, since tumors contain decreased MHC molecules from the host, the immune system frequently allows and tolerates tumor cell expansion. The present work evaluated whether the presence of *T. cruzi* could influence the development of experimental melanoma. B16F0 tumor cells were used in combination with *T. cruzi* parasites to evaluate tumor cell-development and immune response in C57Bl/6 mice. This study provides new tools to understand whether parasites influence tumor growth and protective immunity against melanoma.

MATERIALS AND METHODS

Animals

Female C57Bl/6 mice between 4-6 weeks of age were raised and maintained at the animal care facility of IGM-Fiocruz-Bahia. These experiments adhere to ethical standards, accordingly, license guidelines (IGM-Fiocruz: CEUA 008-2017, and CEUA 017-2014 project ID 1302). To evaluate the survival and the tumor growth, 10 mice were injected with B16F0 and used in each experiment (three individual experiments, meaning a cumulative number of 30 mice). The animals were anesthetized intraperitoneally with 0.1 mL of a 2 mg Ketamine (Agener, Brasil) with 0.4 mg Xylazine (Dorcipec, MG, Brasil), diluted into 0.1 mL of 0.15M NaCl (saline), each. At the end of the experiment, mice were euthanized with 0.2 mL of a 3 mg Ketamine (Agener, SP, Brasil) with 0.6 mg Xylazine (Dorcipec, Brasil), diluted into 0.1 mL of saline. For the total splenic cell-numbers and flow cytometry, each experiment involved 04 animals per group (n=4 used for the 5^{th} day, and n=4 mice for day twenty of the experimental analysis (two experiments, the total number was n=16)).

Tumor cell inoculation

Melanoma B16F0 cells were cultured in tissue culture flasks containing RPMI medium with 10% of fetal calf serum. Following anesthesia, the mice received melanoma cells using an inoculum of $0.5x10^5$ in 50 μL by intra-ear injection (IE.), or $1x10^5$ in 100 μL of saline dorsal injection.

Trypanosoma cruzi parasites

Non-infective epimastigote forms of *T. cruzi* were derived from the Y strain of *T. cruzi* (*Tc*). Parasites were then washed four times in saline solution and injected *via* intraperitoneal (IP) or intra-ear injection (IE). For *T. cruzi* IP inoculation, $3x10^5$ forms of *T. cruzi* in 0.2 mL of saline were injected one week before B16F0 tumor cell injection. For *T. cruzi* IE, inoculation of $3x10^4$ forms of *Tc* was used ten days after IE tumor cell-injection.

Splenic intracellular cytokine determination

Splenic cells were removed, and after washing, aliquots of $2x10^6$ spleen cells were used for staining. The following monoclonal antibodies were used for staining: anti- CD11c-FITC; anti-CD11b-PE; anti-Gr1-FITC; anti-CD8-FITC; anti-CD4-FITC; anti-αβ-FITC; NK 1.1-PE (Becton and Dickinson, BD Biosciences). Cells were washed in FACs buffer, and reading was done in a flow cytometer (FACS) by collecting 200.000 events on a BDFacsSorter. For intracellular staining, cells were obtained in the same manner as above and were cultured at 37 °C with 5% CO_2 in RPMI 10% FBS in the presence of 2.5 ug/ml brefeldin-A and stimulated with soluble anti-CD3. Cells were then fixed in Fix /Perm BD, washed, permeabilized in Perm /Wash BD Biosciences, and then incubated for another 30 minutes with the following anti-cytokine antibodies: anti-INFγ phycoerythrin (PE), anti-TNFα PE, anti-IL10 PE. After the addition of antibodies, the cells were washed and then read as described above. All the cells were previously incubated with Fc block, and a rat anti-mouse isotype monoclonal antibody was used as a control of each staining. All results are representative of one in two experiments unless indicated in the figures. A (BDFacsort) Flow Cytometer acquired 200.000 events.

Statistical analysis

The following experimental groups were used and compared: saline injection (Control), injected only with melanoma cells (B16F0), or *T. cruzi* injected (*Tc* or *Tc*+B16F0). Log Rank test was used to compare longevity, and Mann Whitney was used to measure tumor size between groups. Splenic cell numbers and cytokine production were compared using a Mann Whitney non-parametric test, as indicated in the figures, and $p = 0.0286$ was considered significant. The results are shown in column graphs as the mean ± standard error of the mean (SEM).

RESULTS

In vivo stimulation with *T. cruzi* avirulent strain slightly prolongs the survival of animals that received a dorsal injection of B16F0 melanoma cells

In Fig. 1, survival analysis demonstrated that *Tc*+B16F0 presented a trend towards increasing the survival rate than the B16F0 group. The group of mice injected with B16F0 started to die on day

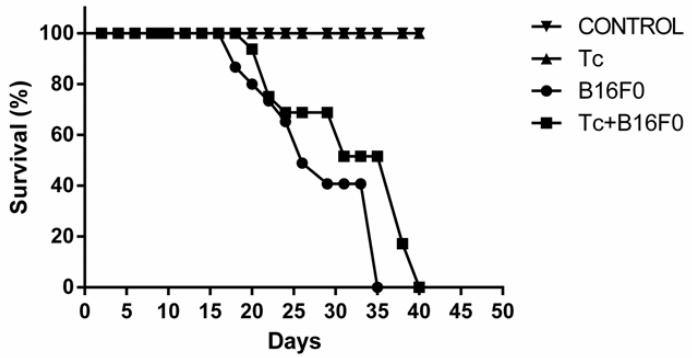

Fig. 1. Survival of C57Bl/6 following dorsal injection of B16F0 tumor cells. Groups of mice were: 1-either dorsally injected with B16F0 tumor cells alone (B16F0). 2- Seven days before B16F0 injection (*Tc*+B16F0), received an intraperitoneal inoculation of *T. cruzi*. 3-Received only the avirulent *T. cruzi* Y strain (*Tc*) or 4-Saline (control). Log Rank test, $p = 0.1253$ in *Tc*+B16F0 compared to B16F0. Results are representative of one in three experiments. n = 10.

17 after tumor inoculation. In the *Tc*+B16F0 group, death began to occur on day twenty after tumor cell inoculation. The B16F0 group reached 100% mortality at 35 days of kinetics, while it was observed in the *Tc*+B16F0 group only after 40 days. These data indicate that *Tc* stimulation induces immune stimulation that could slightly improve survival by five days in C57Bl/6 mice, although there was no significant increased longevity in the *Tc*+B16F0 group.

T. cruzi injection in the pinna of the ear of C57Bl/6 (IE.) and its adjuvant potential to modulate B16F0 development

The following experimental groups were used for longevity and tumor size results (Fig. 2): those that only received tumor cells injected IE. (B16F0); those injected with B16F0 and *T. cruzi* in the 10th day of tumor development (*Tc*+B16F0). When compared to the B16F0, the survival rate of the *Tc*+B16F0 group was not different. Although there was no statistically significant difference between animals that received tumor cells and mice previously inoculated with *Tc*, there was a trend to delay mortality in the *Tc* group (Fig. 2A). Administration of *T. cruzi* did not change the initial tumor cell-expansion *Tc*+B16F0 compared to the group that had received only tumor cells (B16F0) up to the first fifteen days (Fig. 2B). After 17 days, the group that received *T. cruzi* (*Tc*+B16F0) presented a trend toward a mild decrease in tumor size.

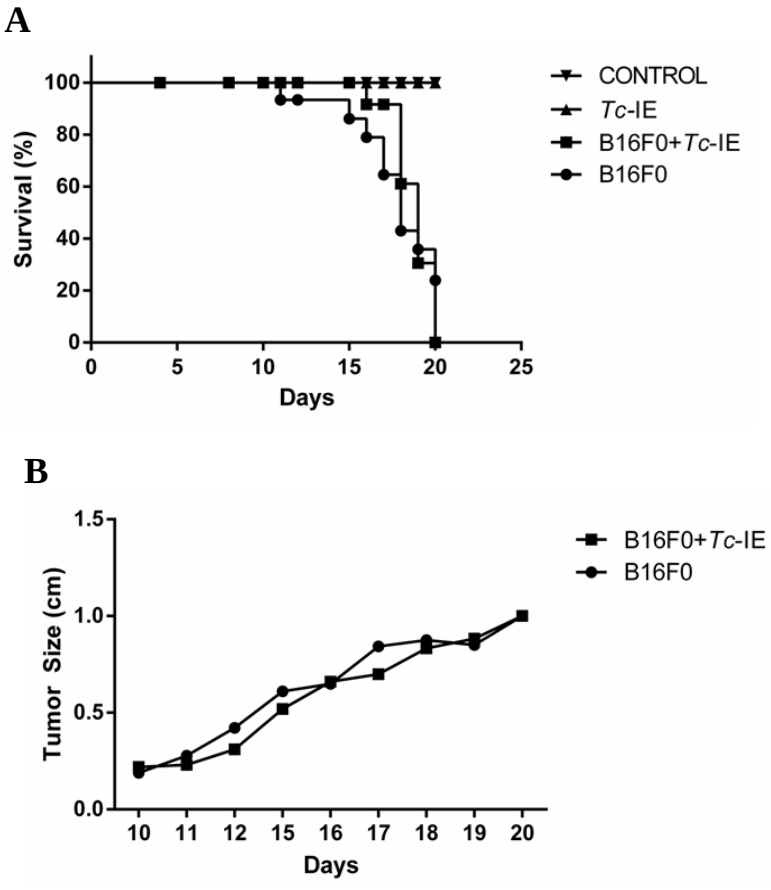

Fig. 2. B16F0 implanted in the pinna of the ear of C57Bl/6 after *Tc* injection. Different experimental groups: 1- Control group (naïve mice) 2 –Injected with *T. cruzi* (*Tc*) 3- Injected with tumor cells IE. (B16F0) 4- Injected with B16F0 and the avirulent strain of *T. cruzi* intraperitoneally (*Tc* +B16F0). In (A), *Tc*+B16F0 and the B16F0 group (Log Rank test, $p = 0.5584$). Fig. 2 (B) shows tumor size measured (in centimeters) where *Tc*+B16F0 and B16F0 groups are shown. $p = 0.8060$. Mann Whitney. n = 10.

Animals that received *Tc* followed by B16F0 tumor cells maintained similar numbers of splenic NKT cells compared to mice that only received tumor cells

NK and NKT cells were evaluated among the different experimental groups, where a decrease in the total number of splenic NK cells was found in the *Tc*+B16F0 group compared to the B16F0 group at five days post tumor inoculation (Fig. 3A). The number of splenic NKT cells at this time point was decreased in the *Tc* compared to the B16F0 group but was not significantly different between the *Tc*+B16F0 and B16F0 groups (Fig. 3B).

***Tc*+B16F0 presented decreased myeloid suppressor cells (MDSC) and reduced numbers of splenic $CD4^+CD69^+$ T cells compared to the B16F0 group**

$CD11b^+Gr1^+$ splenic cells indicate the numbers of MDSCs (Fig. 4). Graphs illustrate $CD11b^+Gr1^+$ gated splenic cells (Fig. 4A). Fig. 4B shows a decrease in $CD11b^+Gr1^+$ cells in the *Tc*+B16F0 group compared to the B16F0 group five days after tumor cell injection. Twenty days after tumor cell injection, MDSCs are lower only in the *Tc* group compared to B16F0 (Fig. 4C). A decrease in the number of these cells in the *Tc*+B16F0 group only five days after tumor injection indicated a possibility to regulate an anti-tumor response, slightly inhibiting tumor progression.

Five days after the dorsal injection of B16F0, a higher number of $CD4^+$ splenic T cells expressing $CD69^+$ was observed in the B16F0 group when individually compared to all the other experimental conditions. The *Tc* group also had an increased number of $CD4^+CD69^+$ T cells compared to the *Tc*+B16F0 group. This reduction in cells can indicate a decrease in non-specific activity in *Tc*-injected groups during early tumor development due to reduced numbers of recently activated CD4 T cells compared to B16F0 injected mice (Fig. 5).

Evaluation of splenic T-cell cytokine profile with IFN-γ, TNF-α, and IL-10 production

Twenty days after B16F0 inoculation, the total splenic cell number was analyzed (Fig. 6), and cytokines were evaluated after *ex vivo* splenic cell stimulation (Fig. 7). The evaluation of the total number of CD4 (Fig. 6A) and CD8 (Fig. 6B) splenic T lymphocytes showed that, among the groups, these cell populations were similar twenty days after tumor inoculation, showing maintenance of the number of T cells. We found that IFN-γ was produced by CD4 and CD8 splenic T cells in all groups after anti-CD3 stimulation (Fig. 7A-B). $CD4^+IFN\text{-}\gamma^+$ is higher in the *Tc* groups (*Tc* and *Tc*+B16F0) than the B16F0 group of mice after anti-CD3 stimulation (Figs. 7A). In the *Tc*+B16F0, $CD8^+IFN\text{-}\gamma^+$ T cells are higher than the B16F0 experimental group

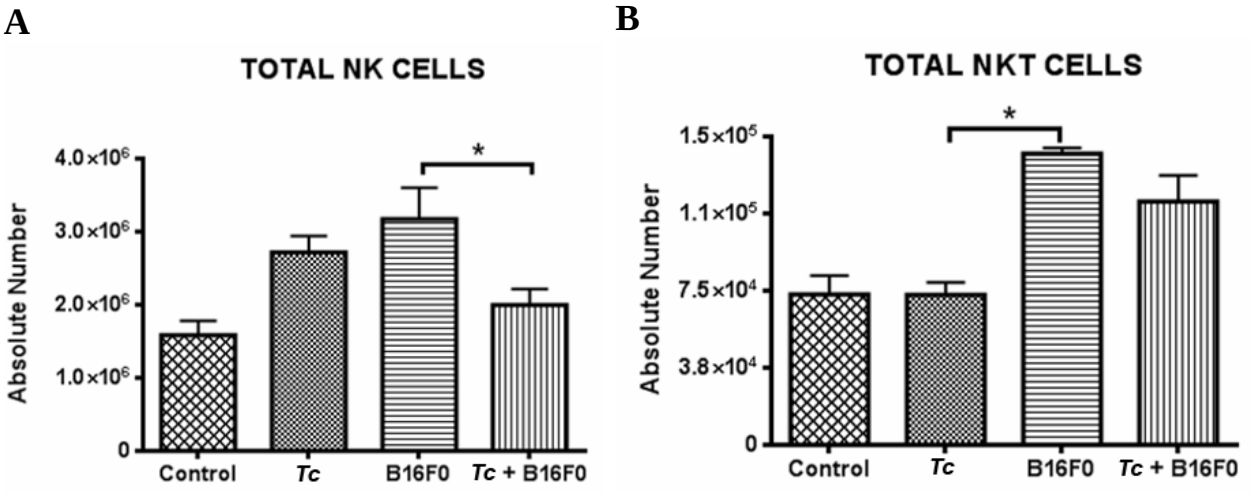

Fig. 3. Absolute numbers of NK and NKT splenocytes (five days after dorsal B16F0 injection). Absolute numbers of NK (A) and NKT (B) cells between *Tc* and B16F0 animals are shown. B16F0 and *Tc*+B16F0 groups (in A) and B16F0 and *Tc* groups (in B) were compared. $p = 0.0286$ (*) indicates statistical significance. Mann- Whitney test. n = 4.

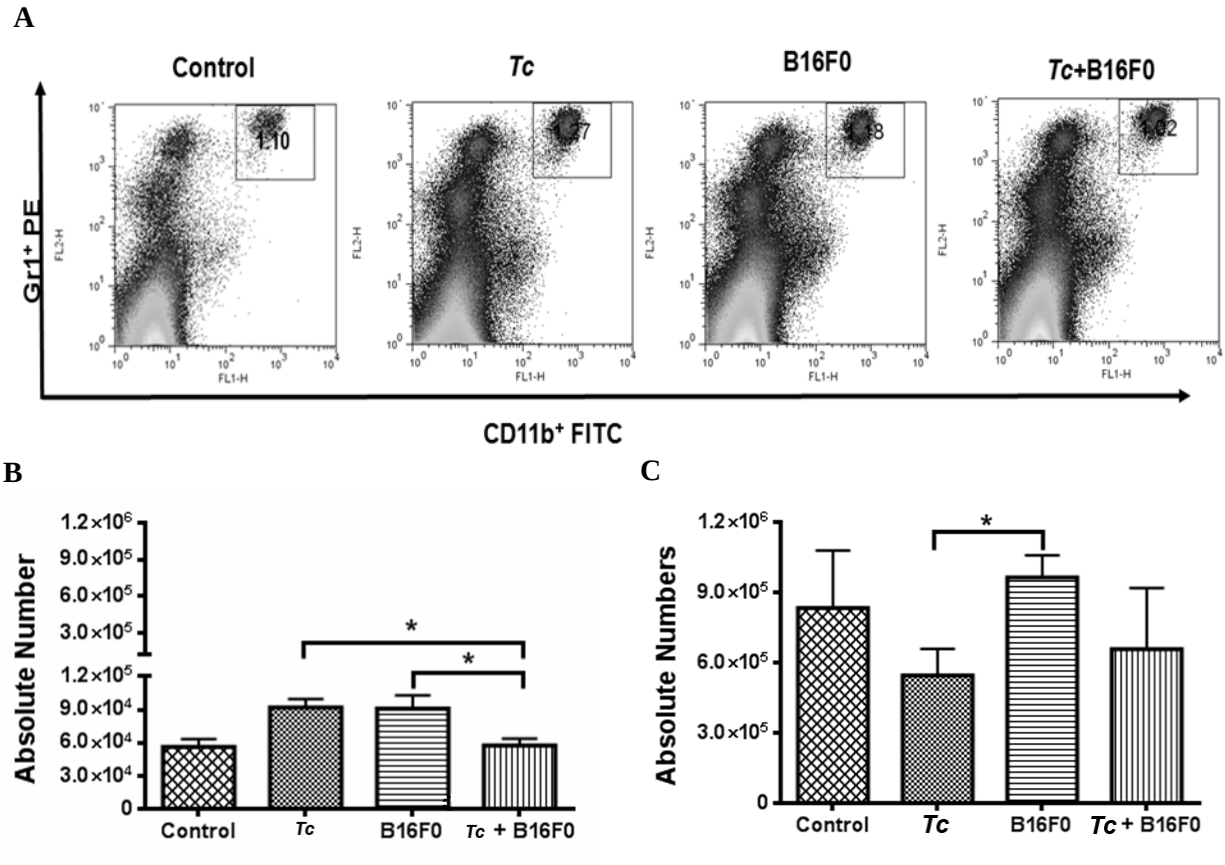

Fig. 4. $CD11b^+Gr1^+$ myeloid-derived suppressor cells found in mouse spleens after dorsal B16F0 injection. (A), Plots represent relative numbers of $CD11b^+Gr1^+$ myeloid-derived suppressor cells (MDSC) in the spleen five days after injecting tumor cells. Relative numbers of $CD11b^+Gr1^+$ MDSCs are depicted, where 1.10, 1.27, 1.18, and 1.02% relative numbers are shown for the control, *Tc*, B16F0, and B16F0+*Tc* respectively. Figs. 4 (B-C) - absolute numbers of MSDCs in *Tc*+B16F0 compared to B16F0 five days after B16F0 injection (B) or twenty days (C) after tumor cells injection. In B-C, $p = 0.0286$ (*) is the statistical significance. n = 4, Mann-Whitney test.

(Fig 7B). The production of splenic TNF-α by CD4 and CD8 T cells stimulated (or not) with anti-CD3 was also measured. No differences were found in groups that were stimulated (or not) with anti-CD3 (Figs. 7C-D), but CD4 T cells were higher than CD8 T cells producing TNF-α. Lower IL-10 was produced by CD4 splenic T cells in the *Tc*+B16F0 group following anti-CD3 stimulation when individually compared to the other groups (Fig. 7E, $p = 0.0286$. Mann Whitney). The B16F0 group presented a lower number of $CD8^+IL\text{-}10^+$ compared to each experimental condition of anti-CD3 stimulation. Thus, increased modulation of the regulatory cytokine IL-10 produced by CD4 T cells and the increased IFN-γ production in *Tc*+B16F0 could indicate an anti-tumor response due to the administration of *Tc* twenty days after the injection of tumor cells.

DISCUSSION

Melanoma prognosis depends on the detection stage, and generally, this disease presents a poor prognosis. Despite additional treatment options besides radiotherapy and chemotherapy, the most effective treatment for this cancer is early diagnosis, followed by the complete surgical removal of the tumor [12, 13]. Different immune mechanisms may likely be acting at various stages of tumor cell growth, which inhibit the mounting of an anti-tumor response. An insufficient number of inflammatory signs at the onset of tumor growth or cells'

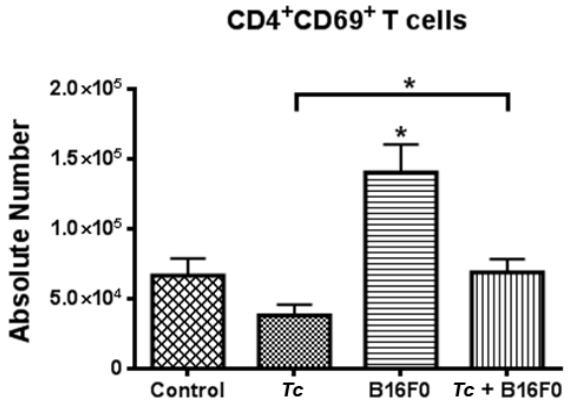

Fig. 5. Decrease in early activation marker expression in peripheral $CD4^+$ T cell subpopulations in both *Tc* groups, compared to B16F0. Spleen cells from control (naïve group), *Tc*, B16F0, or *Tc*+B16F0 mice were stained with anti-CD4 plus anti-CD69 antibodies (a marker for recently activated T cells). FACS analyzed gated $CD4^+$ T cell subpopulations. All experimental groups were individually compared to the B16F0 group. Statistical significance $p = 0.0286$ is represented by (*), Mann Whitney test. n = 4.

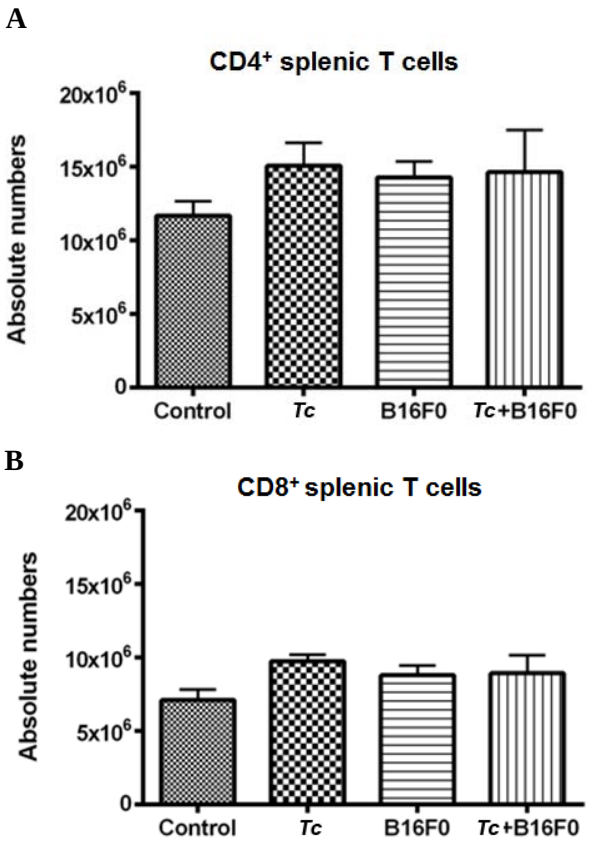

Fig. 6. Relative numbers of splenic CD4 and CD8 splenic T cells evaluation in C57Bl/6 mice twenty days after B16F0 injection. (A), absolute numbers of $CD4^+$ T cells. (B), the total numbers of $CD8^+$ T cells are shown. No significant differences were found between the studied groups. n = 4.

accumulation also causes inhibitory factors favoring tumor development [14, 15, 16].

The *T. cruzi* administration induces immune stimulation that could mildly increase survival by five days in C57Bl/6 mice (Fig. 1). B16F0 group of mice began to die first, but no survival advantage was observed between groups (Fig. 2). Despite the observed differences in time to death in these animals, we have found no statistical significance in the experimental conditions studied. In another study, survivin and *T. cruzi* calreticulin induced a synergistic inhibitory effect on tumor development in a murine melanoma model [17]. In our study, *T. cruzi* only promoted a mild but not significant decrease in the tumor size when both *T. cruzi* and B16F0 were injected IE. (Fig. 2B).

Similarly, *T. cruzi* in B16F0 dorsally injected presented the same tumor size pattern seen with *T. cruzi* IE. injection (not shown). A mildly delayed mortality and containment of tumor progression seen herein in the *Tc*+B16F0 group could be associated with the initial maintenance of T cells present in mouse spleens (Fig. 3), especially NKT cells have potent anti-tumor capabilities and function as the second line of defense against tumors [reviewed in 6]. It is suggestive that distinct from $NK1.1^{negative}$ cells, $NK1.1^{positive}$ T cells can also act as the second line of defense against tumors, especially against B16 melanoma cells [14]. $NK1.1^{positive}$ CD8 T cells favor antigen recognition like conventional T cells and induce killing activity against MDSCs in an antigen-specific manner [14].

Fig. 4 shows that mice injected with B16F0 alone increased the number of MDSCs at the early stages of tumor development five days after tumor cell injection. Thus, MDSCs could interfere with the anti-tumor activity of effector cells in B16F0 alone compared to *Tc*+B16F0. The lower MDSCs in the *Tc*+B16F0 group could also indicate the presence of a pro-inflammatory environment in the spleens of *Tc*+B16F0 mice just at the early stages of tumor injection, but not afterward. Many

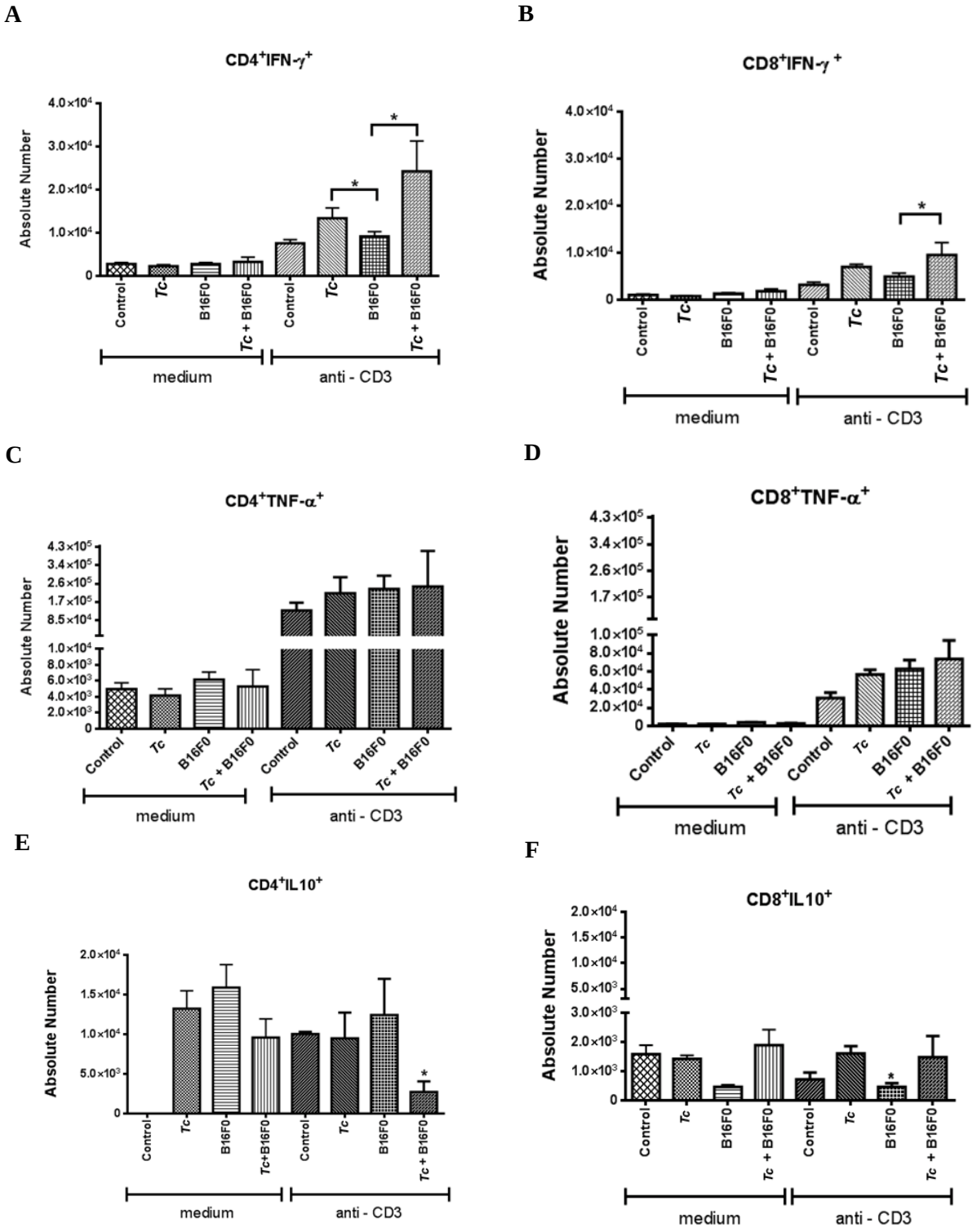

Fig. 7. IFN-γ, TNF-α, and IL-10 production by splenic cells following *ex vivo* stimulation. IFN-γ produced by $CD4^+$ (A) and $CD8^+$ (B) splenic T cells is shown. TNF-α production was measured for CD4 and $CD8^+$ splenic T cells (Fig.7 (C-D)). Fig. 7 (E-F) shows IL-10 produced by CD4 and CD8 T cells, and the *Tc*+B16F0 group was individually compared to B16F0 and *Tc* after anti-CD3 stimulation. Staining was done individually, in triplicates. Differences between groups are shown (*); $p = 0.0286$. Mann Whitney test. n = 4.

tumors may escape the immune response attacks by accumulating myeloid suppressor cells (MDSC), which can suppress the anti-tumor response and are considered suppressors in pathogenic processes [18].

The decreased CD4+CD69+ splenic T cells possibly indicate that the *Tc*+B16F0 group could be generating increased activated/effector T cells (Fig. 5). The depletion of $NK1.1^+$ cells in the *T. cruzi* experimental model is related to a lower frequency of activated/memory T cells in mouse spleens [5, 19]. Antigen-presenting cells such as macrophages, dendritic cells, and B cells are also relevant in the generation and maintenance of effector T-cells [20, 21, and reviewed in 22].

During an immune response, resting T cells are activated and develop effector functions [23]. Effector activated/memory T cells may migrate to inflammatory sites and are of fundamental importance in the defense against tumor growth in other tissues than in the lymphoid tissue [14]. Qualitative systemic cell activation is fundamental in therapeutic approaches improving anti-cancer strategies, leading to better prognosis [14, 24].

In Fig. 7A-B, it is shown that IFN-γ produced following *T. cruzi* contact (right before B16F0 injection) can promote a mildly prolonged survival which correlated to a trend of decrease in tumor size. However, the systemic administration of *T. cruzi* did not prevent tumor expansion (Fig. 2B). Besides, following *in vitro* stimulation at 20 days after the dorsal injection of *Tc*+B16F0, IFN-γ was produced by CD4 and CD8 splenic T cells in all groups, after anti-CD3 stimulation (Fig. 7). IFN-γ production was higher in the *Tc* groups (*Tc* and *Tc*+B16F0) than the other groups of mice after anti-CD3 stimulation (Figs. 7A-B). The induction of NK-mediated IFN-γ biosynthesis by *T. cruzi* requires live and infective parasites [1], but NK cell-number was not increased herein by the presence of a *T. cruzi* avirulent strain. Another study has shown that another avirulent strain of *T. cruzi* induced T cell-mediated immunity and protection against cancer cells, leading to a higher number of IFN-γ secreting splenocytes [25]. Furthermore, the authors argue that this parasite presents high immunomodulatory characteristics due to the expression of Toll-like receptors [26-27].

TNF-α was produced by splenic CD4 and CD8 T cells, and no differences were found in groups that were stimulated (or not) with anti-CD3 (Figs. 7C-D). Thus, increased modulation of the regulatory cytokine IL-10 could indicate an anti-tumor response due to the administration of *T. cruzi* twenty days after the injection of tumor cells. However, the *Tc* group presented higher CD4 T cells producing IL-10 than the control (without stimulation), but a lower number was observed in the *Tc*+B16F0 than the B16F0 group after anti-CD3 stimulation. On the other hand, IL-10 produced by $CD8^+$ splenic T cells was improved in the *Tc*+B16F0 group compared to B16F0, following anti-CD3 stimulation (Fig. 7F, $p = 0.0286$. Mann Whitney). High levels of interleukin (IL-10) can also suppress cells with anti-tumor activity [18]. As such, it is suggested that *T. cruzi* suppresses anti-tumor activity at later stages of *in vivo* tumor development, with a higher number of cells producing IL-10.

The introduction of pathogens such as *T. cruzi*, Leishmania sp., and *T. gondii* is not clarified, and long-term infection can result in either tolerogenic or priming signals [15]. Since both adaptive and innate responses are activated together *in vivo*, pathogens can easily reactivate infection under various conditions. Accumulating evidence indicates that immune system activation is crucial for the efficacy of radiotherapy and chemotherapy interventions during anti-tumor treatment [16]. However, the combination of *T. cruzi* during melanoma development does not increase immune system activity. Accordingly, it is crucial to comprehensively evaluate whether *T. cruzi* can positively modulate melanoma responses to the melanoma control mechanisms. It seems that NKT-like cells exert cytotoxicity against tumor cells and MDSCs through a granzyme B-mediated granule exocytosis pathway [14].

Finally, *T. cruzi* effects, combined with B16F0, were prone to indicate a suppressive immune response despite having more CD4 T cells producing IFN-γ and fewer producing IL-10. Although a lower number of MDSC in group *Tc*+B16F0 only on day 5, MDSCs are not decreased after twenty days of B16F0 injection in the *Tc*+B16F0 compared to the B16F0 experimental group of mice.

CONCLUSION

In future studies, innate immunity's participation should be addressed where the pathogen could shape the immune system to result in trained

immunity [reviewed in 28], enabling an immunological memory through an immune-modulation and a heterologous effect against melanoma. Further, it is reasonable to investigate novel strategies to treat melanoma towards the long-term adaptation of innate immune cells through the presence of *T. cruzi.*

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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