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In vitro Treatment of a Murine Mammary Adenocarcinoma Cell Line with Recombinant *Trypanosoma cruzi* Calreticulin Promotes Immunogenicity and Phagocytosis

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ABSTRACT

American Trypanosomiasis, a parasitic disease produced by *Trypanosoma cruzi* (*T. cruzi*), endemic in Latin America, infects about 6 million people. During the chronic stage of the infection, approximately 30% of infected people will develop Chagas Disease, the clinical manifestation. Few decades ago it was reported that, during the chronic stage, the parasite interferes with the development of solid tumors. However, the identification of parasite molecules responsible for such effects remained elusive. Years later, we described *T.cruzi* Calreticulin (TcCalr), an endoplasmic reticulum resident chaperone that infective trypomastigotes translocate to the parasite exterior, where it displays anticomplement activities. Most likely, at least some of these activities are related with the antitumor properties of TcCalr, as shown in *in vitro*, *ex vivo*, *in ovum*, and *in vivo* models. In this context we, we have seen that in *vivo* subcutaneous peritumoral inoculation of rTcCalr enhances local infiltration of T cells and slows tumor development. Based on these precedents, we propose that *in vitro* treatment of a mammary adenocarcinoma (TA3 cell line) with rTcCalr, will enhance tumor immunogenicity. In agreement with this proposal, we have shown that: i). rTcCalr binds to TA3 cells in a concentration-dependent fashion, ii). C1q binds to TA3 cells in an rTcCalr-dependent fashion, confirmed by the reversion attained using anti-TcS (a central TcCalr domain that binds C1) F(ab')$_2$ antibody fragments, iii). incubation of TA3 cells with rTcCalr, promotes cell phagocytosis by murine macrophages and, iv). rTcCalr decreases the membrane expression of MHC class II, m-Dectin-1, Galectin-9 and PD-L1, while increasing the expression of Rae-1γ. In synthesis, herein we show that *in vitro* treatment of a murine mammary adenocarcinoma with rTcCalr enhances phagocytosis and modulates the expression of a variety of membrane molecules that correlates with increased tumor immunogenicity.

1. Introduction

Herein we propose that *in vitro* treatment of a murine mammary adenocarcinoma with Calreticulin, a *Trypanosoma cruzi* endoplasmic reticulum chaperone, enhances phagocytosis and modulates the expression of a variety of membrane molecules that, *in vivo*, should correlate with increased tumor immunogenicity.

The term Cancer involves more than 200 pathologies that attack all body tissues. Even though cancer is not commonly infectious, when considering the types with more prevalence in humans, it can be said that it behaves as a global pandemic (World Health Organization, 2015). It is a pathology with a quick development and a varied degree of lethality, that starts with changes in a unique cell. Once these cells start proliferating without control, they can form a primary tumor, and eventually metastasize (Klein, 2008) in the same or to other tissues. There is a series of changes that cells must undergo to transform into tumor cells. In this context, Hanahan and Weinberg described, in 2011, that the cells must: i). maintain a sustained proliferative signaling, ii). evade cellular growth suppressors, iii). activate invasive and metastatic processes, iv). enable replicative immortality, v). induce angiogenesis,

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vi). resist cellular death, vii). deregulate its own energetic capabilities, viii). evade an immune response, ix). generate genomic instability and mutations and, x). generate a pro-tumor inflammation (Hanahan and Weinberg, 2011).

Up to 2013, female breast cancer has shown the greatest global incidence, with a steady increase in the number of cases through the years (Cancer Research UK, 2012). Risk factors for this type of cancer are genetic, biologic, environmental, behavioral or social in nature. Some of the main risk factors are the mutation of the BRCA gene (Njiaju and Olopade, 2012), alcohol or tobacco consumption (Hamajima et al., 2002), or a long term use of birth control pills (Collaborative Group on Hormonal Factors in Breast, 1996), among many others. In general, breast tumors are classified as sarcomas or adenocarcinomas, being the latter the most prevalent one (American Cancer Society, 2015). This tumor starts with the transformation of cells from the epithelium of the mammary gland tissue, that may invade near normal tissues. Given the characteristics of this pathology, in a high number of cases, the patient gets diagnosed in an advanced stage of the disease, one of the main reasons for the high lethality seen in pathology. However, the death rate has decreased over time (Ferlay et al., 2015), since the general population is more informed, there is more accessibility and better tools for early diagnosis and better therapies are available. In the last few years, the morpho-histologic characteristics of the tumor have been proposed as possible bio-markers. This has led to a sub-classification of breast cancer, depending on molecules that are either overexpressed or reduced on their membrane, including ER+ (positive to Estrogen Receptor), HER+ (Positive to HER2) or TNBC (Triple Negative Breast Cancer) (Malhotra et al., 2010).

Conventional anti-tumor therapies often carry a big load, both economical and psychological, together with a wide range of adverse effects, including irreversible systemic damage or even death. Therefore, it is of the utmost importance to keep searching for treatments with a better access and less adverse secondary effects.

Chagas Disease is a chronic ailment, practically incurable, first identified in 1909 by Dr. Carlos Chagas (Chagas, 1909). It is endemic in the region comprehended between Mexico's northern border, and approximately Chile's central region. Around 6 to 7 million infected patients have been detected in Latin America and the Caribbean with about 30% presenting different stages of the disease (Hotez et al., 2012). Given the recent increase in migratory phenomena, among other processes, the infection has gone global, with about a million infected in the US and several hundred thousand in other non-endemic regions (Coura and Vinas, 2010).

The etiological agent is the hemoflagellate, intracellular protozoa *T. cruzi*, transmitted to mammalian hosts through the bite of "kissing bugs", hematophagous arthropods from the *Triatominae* family. In Chile, there have been identified four main species: *Triatoma infestans* (domiciliary), *Mepraia spinolai* (wild environment), *Mepraia gajardoi* and *Mepraia parapatrica* (both in the coastal environments) (Canals et al., 1998; Canals et al., 2000; Frias-Lasserre et al., 2010; Ordenes et al., 1996).

The disease has two clinical stages: acute and chronic (Chagas, 1911). The acute phase starts a few days after the bite, where the host develops clinical signs like fever, head and muscular pain, and shivers (Rassi et al., 2010). This phase lasts approximately 30 days, when high parasitemia and cellular parasitism are evident. The chronic phase presents variable symptomatology and duration. The main symptoms are cardiomegaly, megacolon and megaesophagus and, in some cases, a certain degree of peripheral nervous lesions (Apt et al., 1980; Arribada et al., 1990; Dias, 1989).

In 1946, the Russian couple of scientists, Grigorii Roskin and Nina Klyuyeva, described the usage of a *T. cruzi* preparation that decreased in tumor volume and, in occasions, it mediated complete remission, in both murine and human models (Klieva and Roskin, 1963). This formulation, known as KR preparation, was prescribed as an anti-cancer treatment (Kremen͡tsov, 2002). Years later, Mérieux Laboratory in France commercialized a form known as Cruzin Antibiotic, later discontinued because the mechanism of action was still unknown. Several recent studies have confirmed the antitumor effects described for *T. cruzi* infection, observing that: i. Rats infected with *T. cruzi* resist chemically induced carcinomas (Oliveira et al., 2001), ii. *T. cruzi* has tropism for tumor cells (Kallinikova et al., 2001). iii. A non-pathogenic *T. cruzi* clone used as a vector for a testis tumor antigen activated T cells (Junqueira et al., 2010), and iv. Rats treated with a *T. cruzi* extract show a strong CD4+ and CD8+ antitumor response (Ubillos et al., 2016). However, in all these papers, there was no identification of a parasite molecule as, at least partially, responsible for such antitumor effects.

In 1991 we described *T. cruzi* Calreticulin (TcCalr) (Aguillon et al., 1995; Aguillon et al., 2000; Aguillon et al., 1997; Ramos et al., 1991) (formerly known as TcCRT), a 45 kDa protein, functional and structurally homologous to human CALR (HuCALR) (Ferreira et al., 2004). It has an N-terminal domain, with antiangiogenic and antitumor properties; a P domain, rich in proline and a C-terminal domain. Both P and C domains actively participate in the calcium homeostasis process (Michalak et al., 1999). A central S domain, spanning part of the N and P domains, inhibits the mammal Complement System by binding the q sub-unit of the first component (C1q) of the Classical Pathway (Ferreira et al., 2004; Valck et al., 2010), as well as MBL (Cestari Idos et al., 2009) and Ficolins (37) from the Lectin pathway (Sosoniuk et al., 2014). As HuCALR, rTcCalr is antiangiogenic *in vitro*, *in vivo*, *ex vivo* and *in ovum* (Lopez et al., 2010; Molina et al., 2005; Toledo et al., 2010), with higher activity, when compared with its human counterpart. It is also an important parasite virulence factor (Castillo et al., 2013; Ramirez et al., 2011) and promotes healing (Arias et al., 2018; Ignacio Arias et al., 2015).

We have described that the peritumoral inoculation of rTcCalr, either in mammary adenocarcinoma or melanoma models, decreases the proliferative level of tumor cells *in vitro*, and the tumor volume *in vivo* (Abello-Caceres et al., 2016; Ramirez-Toloza et al., 2014). The *in vivo* antitumor effects of the recombinant protein are practically identical to those obtained with the parasite infection and, in both cases, this effect is reverted with anti-rTcCalr antibodies, but not by their pre-immune counterparts (Abello-Caceres et al., 2016).

We have also proposed that TcCalr binding to Scavenger receptors diminishes the neoangiogenic process, decreasing nutrient and oxygen levels, otherwise available and necessary for tumor growth. This could lower the level of tumor metastasis, and also promote endoplasmic reticulum (ER) stress and the consequent translocation of chaperones in mammalian tumor cells. Furthermore, TcCalr is phylogenetically closer to Calr from vegetables, such as *Arabidopsis thaliana*, than to mammalian Calr, both the human and murine versions included (Weinberger et al., 2017). This is why, among other possibilities, TcCalr could act as a tumor-specific antigen upon interacting with tumors, thus contributing to force tumor immunogenicity *in vivo*.

Even though the molecular mechanism by which TcCalr could be exerting its antitumoral activity is partially described (Abello-Caceres et al., 2016), there is consensus in the fact that a primary action of the parasite chaperone inoculated peritumorally would be one of anti-angiogenic nature. This could mediate stress in tumor cells, stimulating an immune response (*i.e.* immunogenic cell death). On the other hand, the antitumoral action of rTcCalr could be related to stimulation of macrophages, NK cells, or even Cytotoxic T cells. In the antitumor response, TcCalr may act as a DAMP, similarly to its human counterpart. In this case, HuCALR, translocated to the cell membrane, promotes cellular phagocytosis. Moreover, CALR binds to molecules such as CD40 L, FasL or even TRAIL (Duus et al., 2007).

Based on the previous considerations, we propose that *in vitro* treatment of a murine mammary adenocarcinoma with rTcCalr will modulate the expression of a variety of membrane bound molecules that correlate with increased phagocytosis and tumor immunogenicity.

2. Materials and Methods

2.1. TA3 cells

TA3 cells were kindly donated by Dr. Jorge Ferreira (Department of Pharmacology, Faculty of Medicine, Universidad de Chile, Chile). This is a mammary adenocarcinoma cell line, non-adherent, obtained directly from tumors by Hauschka (Hauschka, 1953). The cells were maintained in 25 ml of DMEM, supplemented with 20% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine, and passed every other day to a new bottle. Then, the cells were centrifuged at 142 g for 5 m, and resuspended in 5 ml of DMEM. 1 ml of that suspension was then transferred to a new bottle and 24 ml of fresh medium were added.

2.2. K41 cells

K41 cells are a murine embryonic fibroblast (MEF) cell line, adherent, obtained directly from mice (Mesaeli et al., 1999). The cells were maintained in 10 ml of DMEM, supplemented with 10% FBS, 2% penicillin/streptomycin, and 2% L-glutamine, and passed to a new bottle when 80% confluence was reached. Then, the cells were released with trypsin and centrifuged at 142 g for 5 m, resuspended in 5 ml of the described medium. Then 1 ml of that suspension was then transferred to a new bottle and 9 ml of fresh medium were added.

2.3. Raw 264.7 cells

These cells correspond to a commercial, adherent, murine macrophage cell line (ATCC©, TIB-71™). The cells were processed as described above, except that RPMI supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine was used, instead of DMEM.

2.4. Labeling of rTcCalr with Alexa-660 and FITC

rTcCalr was labeled with the Alexa Fluor™ 660 Protein Labeling Kit or FluoReporter FITC Protein Labeling kit (both from Invitrogen, California, USA), following manufacturer's instructions. Briefly, rTcCalr at 2 mg/ml was mixed with the dye, stirred for 1 h in the darkness, passed through a resin column (provided by the manufacturer) and collected.

2.5. Binding of rTcCalr to TA3 cells

10^5 TA3 cells were treated with 0-4 μM of rTcCalr labeled with Alexa-660 (A-rTcCalr) or FITC (F-rTcCalr) for 30 m at room temperature. Then, the cells were washed three times with PBS 1X by centrifugation at 142 g for 5 m and analyzed by Flow Cytometry, using a BD LSRFortessa Cytometer (BD Bioscience, New Jersey, USA).

2.6. Evaluation of rTcCalr-mediated C1q binding

10^5 TA3 cells were treated with 16 μM rTcCalr, as described, and then incubated with 0.05-0.4 μM C1q (Complement Technologies, Tyler, Texas, USA), for 30 m at room temperature. Afterwards, the cells were washed three times by centrifugation at 142 g. As negative controls, C1q binding was blocked with F(ab')$_2$ anti-TcS antibody fragments (Aguilar et al., 2005) and, alternatively, the cells were incubated with C1q, in the absence of rTcCalr. C1q was detected with an anti-C1q antibody labeled with FITC (Dako, California, USA) and measured by Flow Cytometry.

2.7. TA3 cells rTcCalr- or C1q-dependent phagocytosis

10^5 TA3 cells were stained with a 5 μM CFSE solution for 10 m at 37 °C. Then, the cells were incubated with rTcCalr, C1q, or both subsequently for 30 m at room temperature. Simultaneously, Raw 264.7 cells were starved in un-supplemented RPMI for 2 h. Afterwards, they were stained with Calcein Violet AM (BioLegend, California, USA), following manufacturer's instructions. Later, the cells were co-cultured (1:2, TA3 : Raw 264.7) for 3 h with gentle orbital shaking, at 37 °C. As a negative control, TA3 cells treated with rTcCalr and C1q were co-cultured with Raw 264.7 cells at 4 °C. Cells were then analyzed by Flow Cytometry, and the percentage of phagocytosis was measured by double staining.

2.8. rTcCalr preferential binding to tumor cells and to fibroblasts

TA3 cells were stained with CFSE as described, while K41 cells were stained with Calcein Violet AM. Then, 10^5 TA3 cells were mixed with 10^5 K41 cells and incubated with 1 μM A-rTcCalr for 0.5 and 2 h at room temperature, in the darkness. The cells were then washed three times with PBS 1X at 142 g for 5 m, and analyzed by Flow Cytometry and double staining (Alexa-660 + /CFSE + *vs* Alexa-660 + /Calcein Violet AM +) percentage was compared.

2.9. Evaluation of an immunomodulatory role for rTcCalr

3×10^5 TA3 cells were stimulated for 48 h with 25 and 50 μg of rTcCalr. As controls, cells were stimulated with 25 and 50 μg of Bovine Serum Albumin (BSA) or with 25 IU/ml of IFNγ. Given that rTcCalr is purified using an *E. coli* expression model, we also evaluated the effect of LPS present in the protein solution. After the stimulation, the cells were washed three times with PBS 1x, by centrifugation at 142 g for 5 m, at room temperature, followed by staining with the following monoclonal antibodies: i). Anti H2K^k (mouse, 36-7-5), ii) Anti I-A^k (mouse, 10-3.6), iii) Anti CD16/32 (rat, 93), all from BioLegend, California, USA, iv) Anti m-Dectin-1 (rat, R1-8g7) (InvivoGen, California, USA), v) Anti B7.H3 (rat, MIH35) (BioLegend), vi) Anti B7.H4 (rat, 188) (eBioscience, California, USA), vii) Anti Galectin 9 (rat, 108A-2) and viii) Anti PD-L1 (rat, 10 F.9G2) (both from BioLegend).The cells were then washed, and incubated with an anti-mouse IgG or anti-rat IgG labeled with FITC, an Anti-Rae-1γ-PE (Biolegend), and the respective isotype control, for 30 m, at 4 °C in the darkness. Then, the cells were washed and analyzed by Flow Cytometry.

2.10. Bioethical Considerations

Given the nature of the biological reagents and cells used in this investigation, no bioethical certifications were required.

2.11. Statistical Validations

rTcCalr, C1q and rTcCalr binding to murine tumor cells and fibroblasts, and to both cell types simultaneously present, was analyzed with a two-way ANOVA. To compare the fluorophores' influence in rTcCalr binding, a correlation assay and a Wilcoxon's Rank Sum Tests were performed. When C1q binding was blocked with F(ab')$_2$ fragments, the phagocytosis assay or the expression changes in immunomodulatory molecules were evaluated using Student's t-Tests.

3. Results

3.1. rTcCalr binding to TA3 cells is concentration-dependent and fluorophore independent

Even though we have previously shown the antitumor effects of rTcCalr, evaluation of its binding to the cellular membrane is necessary. rTcCalr was labeled with FITC (F-rTcCalr) or with Alexa-660 (A-rTcCalr), as described in methods. Then, TA3 cells were incubated with increasing concentrations of F-rTcCalr or A-rTcCalr and later analyzed

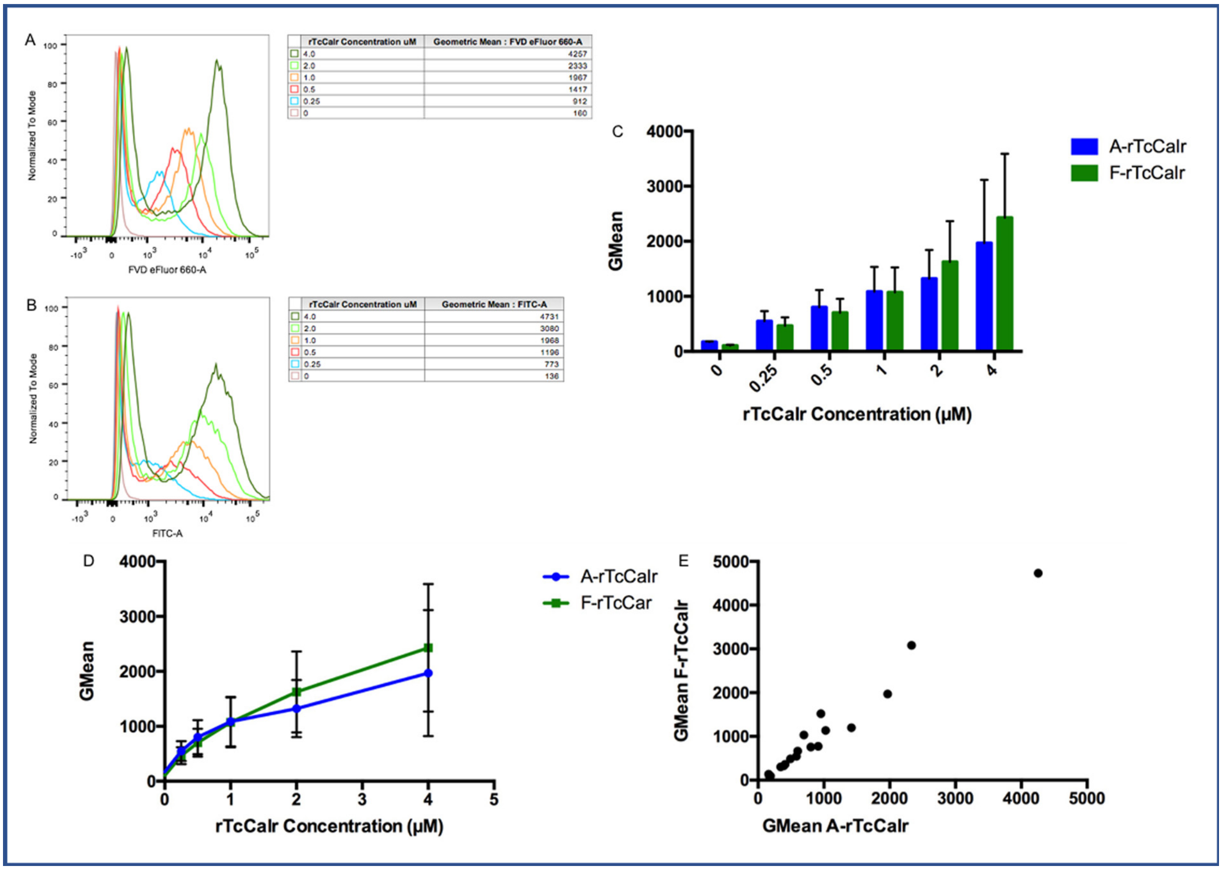

Fig. 1. rTcCalr binding to TA3 cells is concentration-dependent and it is not influenced by the fluorophore used. rTcCalr labeled with either FITC or Alexa-660 behaves similarly in their binding to TA3 cells. TA3 cells where incubated with increasing concentrations of rTcCalr labeled with either Alexa-660 (**A**) or FITC (**B**). When comparing both Calrs (**C**), binding of the protein is similar, irrespective of the fluorophore used ($p = 0.8087$) and the binding is concentration-dependent ($p = 0.0246$). Similar curves were obtained for the concentration-dependent binding of rTcCalr, regardless of the labeling (**D**) ($p = 0.9538$). A similar result is obtained when the data generated with both fluorophores are correlated (**E**) ($r = 0.9814$, $p < 0.0001$). Data are representative of three repetitions. Bars show SEM.

by Flow Cytometry. We observed an increase in the fluorescence with A-rTcCalr (Fig. 1A) and with F-rTcCalr (Fig. 1B). The GMeans obtained for each probe were similar ($p = 0.8087$) independently of the fluorophore used for labeling, and the increase observed in the GMeans was concentration-dependent ($p = 0.0246$) (Fig. 1C). Moreover, both curve slopes were also similar ($p = 0.9538$) (Fig. 1D). On the other hand, a strong correlation between the binding of both reagents ($r = 0.9814$, $p < 0.0001$) is evident, indicating that both fluorophores do not influence rTcCalr binding to the cellular membrane (Fig. 1E).

3.2. C1q binds to TA3 cells, both in a rTcCalr dependent and independent ways

TA3 cells were treated with rTcCalr, as previously described. Afterwards, the cells were incubated with increasing concentrations of C1q. As a negative control, inhibition of C1q binding to rTcCalr was attempted, using F(ab')$_2$ antibody fragments anti-rTcCalr S domain (anti-TcS) and incubated with C1q in the absence of rTcCalr. Even though we detected C1q binding to the cells, we did not observe differences at the C1q concentrations used. However, the binding was rTcCalr dependent (Fig. 2C). In Figs. 2**A** and **2B** we show representative C1q binding histograms in rTcCalr treated (**2A**) or untreated (**2B**) cells. Perhaps, at the concentrations used, the system was saturated. The rTcCalr dependency is also confirmed when using F(ab')$_2$ anti-TcS antibody fragments at twice the rTcCalr concentration, that partially reverted the C1q binding (Fig. 3A). In **Fig 3B** we show a representative histogram.

3.3. rTcCalr binding to tumor cell promotes phagocytosis by murine macrophages

TA3 cells were stained with CFSE and pre-incubated with rTcCalr, C1q or both, and then co-cultured with starved, Calcein Violet AM-stained Raw 264.7 murine macrophages, for 3 h, at 37 °C. As shown in Fig. 4, when treated only with rTcCalr, the number of phagocytosed cells (as seen by the double positive percentage) increases, both over the basal phagocytosis or the negative control performed at 4 °C.

3.4. rTcCalr binds equally to TA3 tumor cells or K41 MEF cells

TA3 and K41 MEF cells were intracellularly stained with CFSE and Calcein Violet AM, respectively. Afterwards, they were mixed in equal parts and the mixture or a sample of each individual cell, were incubated with A-rTcCalr for 0.5 and 2 h. When comparing the binding to each cell type individually, a higher level of binding to K41 MEFs cells was observed (Fig. 5A). However, when the two cell types were mixed no preferential binding was detected (Fig. 5B).

Up until now, the mechanisms we have described for the antitumor effect of rTcCalr are mainly mediated by inhibiting tumor angiogenesis and promoting phagocytosis by binding to the tumor cell, recruiting C1q and thus starting this process. However, given the nature of CALRs in general, it may as well modulate the expression of certain surface molecules that could enhance an antitumor immune response. These possibilities indicated the need to measure the cellular surface expression of the following molecular groups:

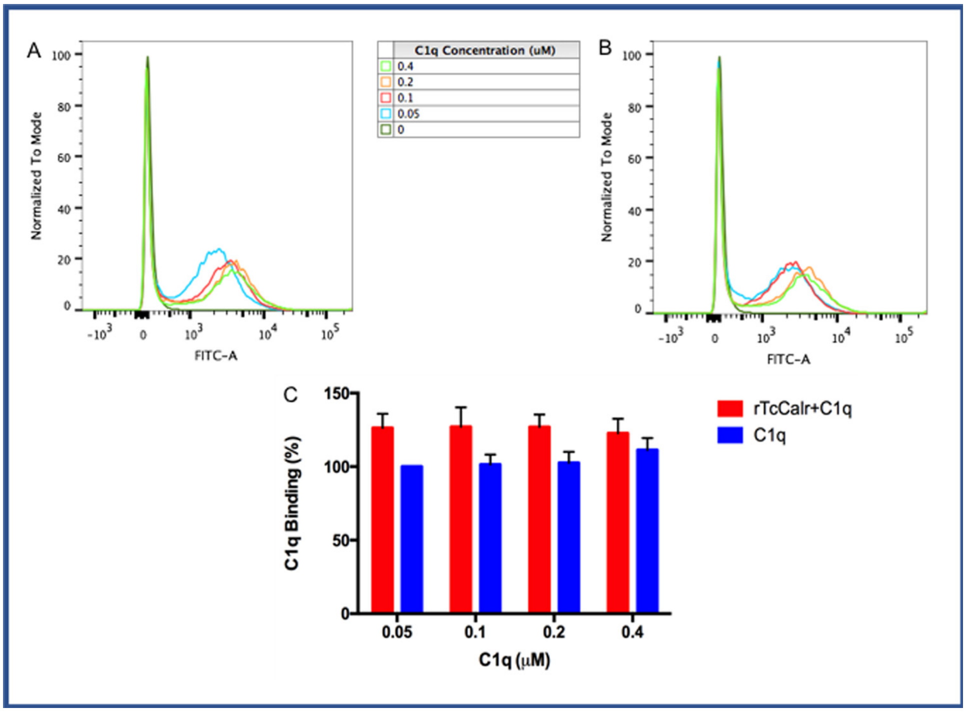

Fig. 2. C1q binds to TA3 cells in an rTcCalr-dependent way. TA3 cells were incubated (**A**) or not (**B**) with one concentration of rTcCalr and afterwards with increasing concentrations of C1q. Data were normalized and then both curves were compared (different concentrations of C1q in the presence or absence of rTcCalr) (**C**). There is an rTcCalr-dependent C1q binding (p = 0.0008), but no differences were observed with the variable C1q concentrations used (p = 0.9757). Data are representative of four repetitions. Bars show SEM.

3.5. rTcCalr decreases MHC class II membrane expression

TA3 cells were incubated for 48 h with rTcCalr, BSA, IFNγ and LPS. After the treatment, cells were washed, and incubated with monoclonal antibodies anti-H2K^{k}, anti-I-A^{k} and the respective isotype control. Finally, the samples were analyzed by Flow Cytometry. Under these conditions, treatment with rTcCalr leads to a decrease in MHC II (Fig. 6B) and an increase in MHC I (Fig. 6A). Even though the decrease in MHC II is achieved only with rTcCalr stimulation, the increase in MHC I is not different from that achieved with LPS. Figs. 6C and 6D show representative histograms.

3.6. rTcCalr decreases m-Dectin-1 membrane expression

The procedure was the same as described, but in this case the cells were incubated with a rat monoclonal antibody anti-CD16/32, anti-m-Dectin-1 and the respective isotype control. Finally, the samples were analyzed by Flow Cytometry. The treatment with rTcCalr leads to a decrease both in CD16/32 (Fig. 7A) and in m-Dectin-1 (Fig. 7B). In the case of CD16/32, the decrease achieved is not different from that of LPS. Figs. 7**C** and **7D** show representative histograms. Bars show SEM.

3.7. rTcCalr decreases the membrane expression of Galectin-9 and PD-L1, while increases Rae-1γ

The procedure used was previously described, but in this case the tumor cells were incubated with rat monoclonal antibodies anti-B7.H3, B7.H4, Galectin-9, PD-L1 and Rae-1γ. The treatment with rTcCalr increases the expression of Rae-1γ (Fig. 8E), decreases the expression of PD-L1 (Fig. 8D) and Galectin-9 (Fig. 8C), while it does not modify the expression of B7.H4 (Fig. 8B) and, specifically, of B7.H3 (Fig. 8A). Figs. 8**F-J** show representative histograms.

4. Discussion

The proposal of Roskin and Klyuyeva, that *in vivo* parenteral *T.cruzi* cell extracts had anti-tumor effects both in mice and humans, was followed by a series of reports corroborating such observations. (Kliueva and Roskin, 1963). More recently, our laboratory has provided evidences that TcCalr, translocated from the ER to the parasite exterior mediates, at least in important part, this effect (van Tong et al., 2017, Ferreira et al., 2004). Here, we propose that cells of a murine mammary adenocarcinoma, TA3, treated with rTcCalr, will display increased *in vitro* tumor immunogenicity, as judged by the chaperone capacity to modulate both phagocytosis (in the presence or absence of C1q) and the expression of membrane molecules known to be involved in promoting an effective immune response.

We first determined that both F-rTcCalr and A-rTcCalr versions bind equally to the TA3 murine mammary adenocarcinoma, as determined by Flow Cytometry (Fig. 1**A-C**). We also showed that this binding increased in parallel with increasing concentrations of the protein and that the kinetics of both curves are similar (Fig. 1**D-E**). In both of the

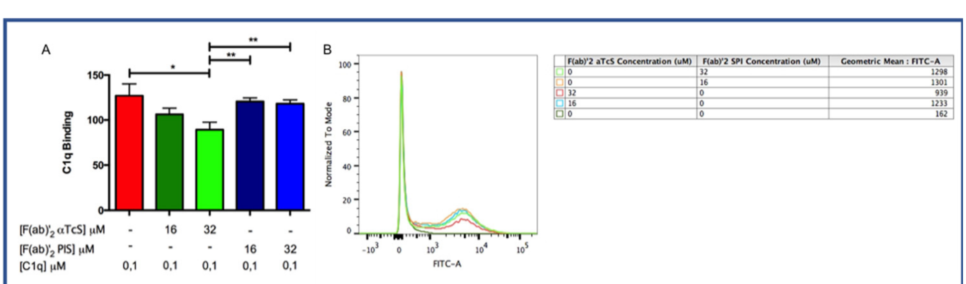

Fig. 3. C1q binding to TA3 cells can be partially reverted by anti-TcS F(ab')$_2$ antibody fragments: After treating TA3 cells with rTcCalr, they were incubated with different concentrations of F(ab')$_2$ antibody fragments, derived from immune or pre-immune sera. At 32 μM, the anti-TcS antibody fragments (**A**) were effective. **B** shows a representative histogram. A decrease in C1q binding is evident in the lower peak (red line). Data are representative of four repetitions. Bars show SEM.

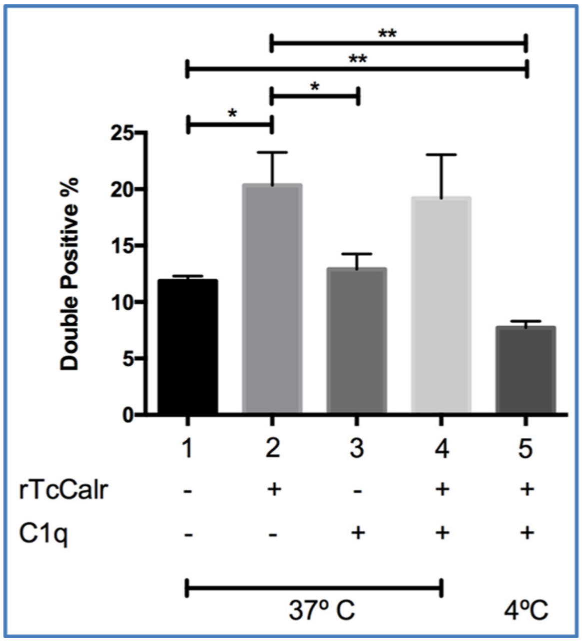

Fig. 4. rTcCalr enhances tumor cell phagocytosis. CFSE pre-stained TA3 cells were incubated with rTcCalr, C1q or both, and then co-cultured with Calcein Violet AM pre-stained and starved Raw 264.7 murine macrophages, for 3 hrs. As a negative control, stained TA3 cells incubated with both rTcCalr and C1q were co-cultured with macrophages at 4 °C. Data shown are representative of three repetitions. Bars show SEM.

representative histograms presented (Fig. 1**A and 1B**), the system did not reach saturation with the rTcCalr concentrations used, shown by the 2 peaks present in all the treatments. Moreover, the distribution observed at the highest TcCalr concentration used (4 μM) (independently of the fluorophore used), correlates with this notion, leaving open the possibility of additional protein binding.

We then evaluated whether TcCalr treatment increases the tumor cell capacity to bind C1q. We observed that there is an increase in C1q binding to rTcCalr treated, as compared to untreated cells (Fig. 2C). Figs. 2.A and 2.B show that there are no differences between the histograms at increasing C1q concentrations. The rTcCalr dependency of C1q is further confirmed when using F(ab)'$_2$ anti-TcS antibody fragments, which diminish C1q binding when using twice as much concentration than the one used for rTcCalr (Fig. 3A). As discussed below, C1q binding in the absence of rTcCalr is feasible, probably given its capacity to bind phosphatidyl serine (Paidassi et al., 2008).

Once both rTcCalr, and rTcCalr-mediated C1q bindings were confirmed, we evaluated whether this treatment influences the phagocytic activity of murine macrophages co-cultured with the treated tumor cells. Treatment with rTcCalr enhances phagocytosis by the murine macrophage cell line (Raw 264.7) (Fig. 4), at a higher level than that achieved only with C1q, and even higher than the negative control. Unexpectedly, the basal phagocytic levels and that observed in the TcCalr + C1q treated group, are similar. Perhaps, the heat inactivated FBS supplementing the RPMI used in the Raw 264.7 culture still carried some active C1q that could remain attached to the macrophages ("mC1q"). This could, in turn, engage with the rTcCalr bound to the tumor cells, starting the phagocytosis process. In cells that were pre-treated with both rTcCalr and C1q, the "mC1q binding sites" could be already occupied with bovine C1q, with no increase in phagocytosis. This pro-phagocytic role of C1q has been also shown in different models. It has been shown that the interaction between TcCalr and C1q is essential in the phagocytosis-like process that *T.cruzi* uses for cell invasion in the infective process, as shown by Ramirez et al (Ramirez et al., 2011). In mammals, the binding of C1q to Calr is a known "eat-me signal", since Calr can translocate in response to apoptotic signals, bind C1q and recruit macrophages to start the apoptotic process (Park and Kim, 2017).

If the parasite chaperone displays a specific binding affinity for tumors, a possible translation into a clinical model could be envisaged. To evaluate this possibility, we designed an *in vitro* approach where we mixed TA3 tumor cells with K41 MEFs and then incubated the mixture with a single concentration of A-rTcCalr, for different periods (Fig. 5B). rTcCalr binding to both cell types was not different. Noteworthy, when incubating with each cell individually, rTcCalr did bind more to MEFs than to TA3 cells (Fig. 5A). Even though the receptor for Calreticulin on the cellular membrane has not been identified, it has been shown that the chaperone binds to CD91 (low density lipoprotein-related protein) (Basu et al., 2001) or phosphatidylserine (PS) (Tarr et al., 2010). This lipid is usually in the inner leaflet of the cellular membrane and it can be translocated to the external leaflet during apoptosis processes or injuries, with consequent activation of the complement system (Fadok et al., 1992). This translocation has also been described in tumor cells, where PS behaves as a cancer biomarker (Sharma and Kanwar, 2018). Maybe, TA3 cells express high levels of PS on the cellular surface, while K41 cells express normal levels of CD91 or other normal ligands for Calreticulin, in a relative absence of PS. Thus, the larger percentage of positive K41 cells could be explained by a smaller number of molecules available for rTcCalr binding. In this context, PS translocation could mean more molecules per cell available, thus yielding a lower percentage of positive cells when analyzing the TA3 lineage.

We then tested the possibility that rTcCalr has an effect on the expression of some tumor proteins with known immune modulatory effects. Thus, we measured the expression of MHC class I and II molecules, to preliminary evaluate the potential capacity of this tumor cell line to cross present rTcCalr-derived peptides to T CD8$^+$ and CD4$^+$ cells. Treatment with rTcCalr increases the MHC I expression in the cellular membrane (Fig. 6A). However, the change achieved was not different than that seen with the treatment with LPS. For MHC II, we observed an rTcCalr concentration-dependent decrease in the

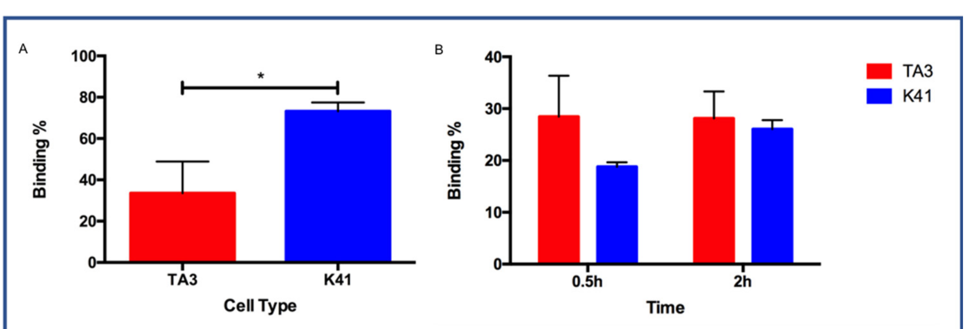

Fig. 5. rTcCRT does not bind preferentially to tumor cells in an *in vitro* model. Pre-stained TA3 cells or K41 MEFs were incubated alone with A-rTcCALR (**A**) or mixed in equal numbers prior to incubating for 0.5 or 2 h (**B**). Then, double positive fluorescence percentages were compared. When incubating A-rTcCALR with each cell type individually, the chaperone bound preferentially to K41 MEFs (p = 0.0124). When comparing binding in a cell-type and time dependent fashion using a two-way ANOVA, no differences were observed (p = 0.2426 for the cell type variable and p = 0.4964 for the time variable). Data shown are representative of three repetitions. Error bars show SEM.

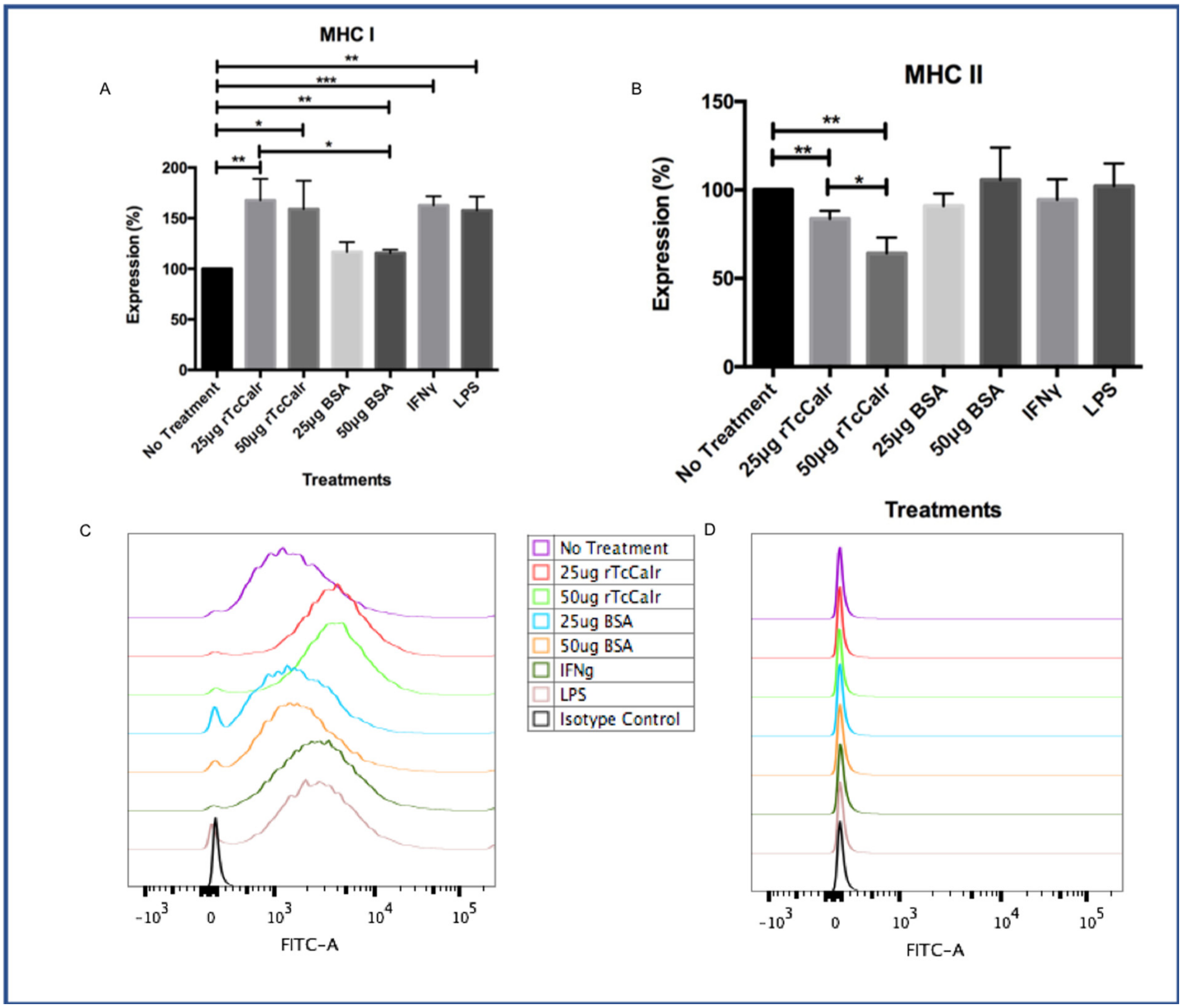

Fig. 6. rTcCalr decreases the membrane expression of MHC class II in TA3 cells. TA3 cells were stimulated with rTcCalr. After 48 h, membrane expression of MHC class I (**A**) and II (**B**) was quantified using monoclonal antibodies. This treatment decreases MHC II and increases MHC class I membrane expression, but the latter is not different from the effect achieved with LPS. (**C**, **D**) Representative histograms of each protein detection. The different groups were analyzed using Student's t Test, and data shown are representative of four repetitions. Bars show SEM.

membrane expression (Fig. 6B). The role of helper T cells in tumor immunity is not completely clear. However, an increase in the activation of these cells could dampen the activity of cytotoxic T cells (Donia et al., 2015). In this context, the decrease of MHC II could benefit the antitumor response.

Next, we tested the presence of receptors relevant in the endocytosis process, a requisite for peptide cross-presentation, specifically CD16/32 (Fc immunoglobulin fraction receptors) and m-Dectin-1 (murine Dectin 1, C Type Lectin receptor). CD16/32 levels decrease approximately by 50% (Fig. 7A), as well as the levels of m-Dectin-1 (Fig. 7B). However, similarly to MHC I, CD16/32 levels also decrease with LPS. Thus, in these two cases, the possibilities to dissect the chaperone effect from that mediated by the endotoxin is reduced. It could be speculated, however, that the decrease in the expression of CD16/32, together with the increase in the expression of MHC I, could reflect a capacity of this tumor cell line to cross-present rTcCalr-derived peptides, to $CD8^+$ T cells.

Given the antiangiogenic effects described for rTcCalr and also the interaction between CALR with molecules such as CD40 or TRAIL (Duus et al., 2007), or receptors such as TNFR (de Bruyn et al., 2015), we next asked whether rTcCalr modifies the expression of immunodulatory molecules present in the tumor-immune synapse. Among these molecules, we selected some of the most relevant ones, such as B7.H3, B7.H4, Galectin-9, PD-L1 and Rae-1γ. We observed a decrease in the levels of Galectin-9 and PD-L1, and an increase in the levels of Rae-1γ (Fig. 8**C-E**), while no changes were registered in the expression of B7.H4 (Fig. 8B). In the case of B7.H3 the expression of this protien decreased with every variable tested (Fig. 8A). An increase in the expression of Rae-1γ could imply, *in vivo*, an enhanced activation of NK cells by involvement of the NKG2D receptor, allowing a stronger lytic activity on tumor cells. Galectin-9 is a pro-apoptotic protein, that also participates in processes of immune modulation. In this context, it can induce Treg differentiation (Seki et al., 2008), as well as $CD4^+$ (Zhu et al., 2005) and $CD8^+$ (Wang et al., 2007) T cell apoptosis.

The fact that rTcCalr decreases the level of Galectin-9 could imply a decrease in Tregs in the tumor microenvironment, together with enhanced helper and cytotoxic responses. On the other hand, PD-L1 inhibits T cell activity by inducing apoptosis when binding to PD-1 on the target cell. PD-L1 is one of two immune targets approved for use in immunotherapy in a clinical setup, along with CTLA-4. B7.H4 reduces the activity of CD4+ and CD8 + T cells by binding to so far unknown receptors. B7.H4 is not usually expressed in a constitutive way in healthy tissues, while its levels increase in different types of cancer (Smith et al., 2014).

B7.H3 is a molecule usually present in APCs, which intervenes on immune checkpoints, similar to PD-L1 or CTLA-4 (Castellanos et al.,

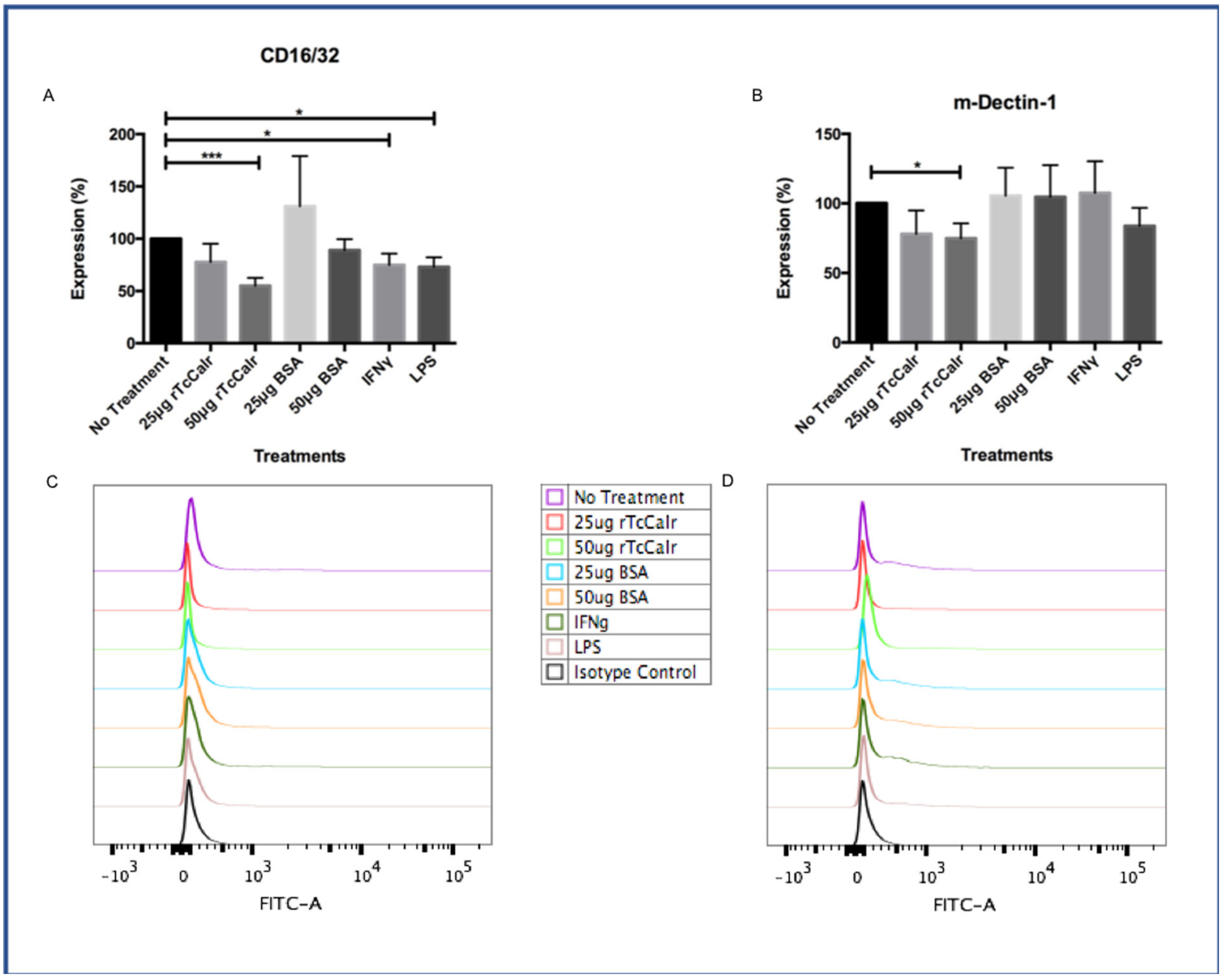

Fig. 7. Treatment with rTcCalr decreases the membrane expression of m-Dectin-1 and CD16/32. TA3 cells were stimulated with rTcCalr. After 48 h, membrane expression of CD16/32 (**A**) and m-Dectin-1 (**B**) was quantified using monoclonal antibodies. The treatment leads to a decline in the expression of m-Dectin-1 and CD16/32, however, this last effect is no different than the effect mediated by LPS. (**C**, **D**) Representative histograms of each protein detection. The different groups were analyzed using Student's t Test, and data shown are representative of four repetitions. Bars show SEM.

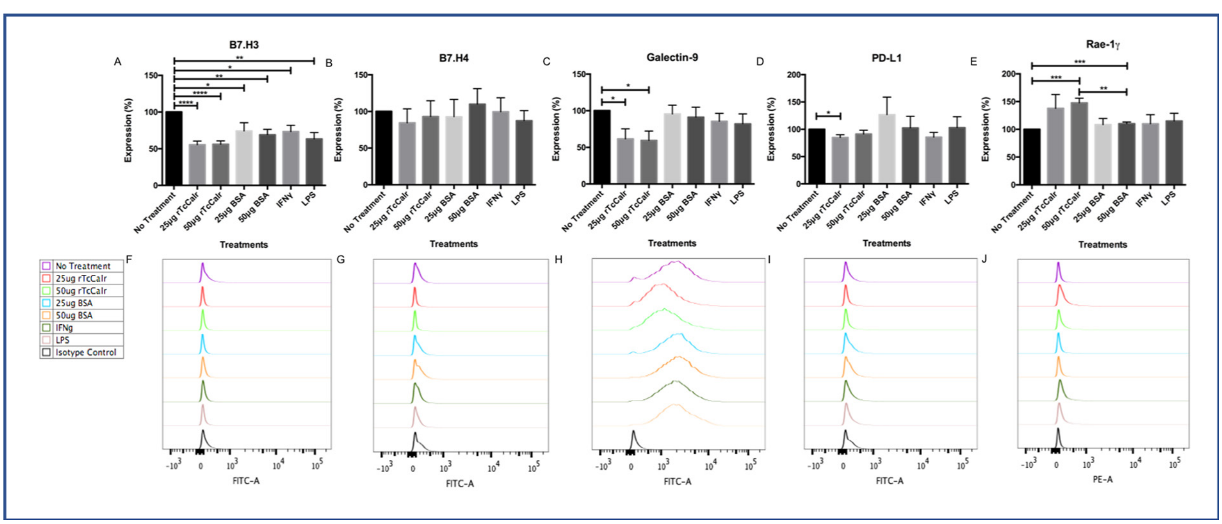

Fig. 8. Treatment with rTcCalr decreases membrane levels of Galectin-9 and PD-L1 and increases the expression of Rae-1γ. TA3 cells were stimulated with rTcCalr. After 48 h, membrane expression of B7.H3 (**A**), B7.H4 (**B**), Galectin-9 (**C**), PD-L1 (**D**) and Rae-1γ (**E**) was quantified using monoclonal antibodies. (**F-J**) Representative histograms of each protein detection. The different groups were analyzed using Student's t Test, and data shown are representative of four repetitions. Bars show SEM.

2017). However, in the last few years it has been proposed that B7.H3 has important functions in the tumor development, such as migration and invasion (Castellanos et al., 2017).

This modulatory effect on cell surface proteins by Calr has also been reported in other proteins by Harada et al (Harada et al., 2006) and later reviewed by Jian et al (Jiang et al., 2014). Harada showed that the presence of Calr in the cell surface inhibits the expression of the cystic fibrosis transmembrane conductance regulator (CFTR), a cell surface cAMP-dependent Cl- channel. However, the ways in which this regulation are achieved are not yet clear, and need further studying.

In synthesis, we have shown that rTcCalr binds to the tumor cell, enhancing C1q binding and the phagocytosis process. In an *in vivo* model, this could result in a slower tumor development and bigger options of recognition of the tumor by the immune system. The lack of rTcCalr binding specificity for the tumor cells used is somewhat unexpected. However, TcCalr interaction with tumor cells may unleash different reactions from those elicited in normal cells. However, this finding should be further analyzed in an *in vivo* model to validate the viability of a clinical use of the protein (*i.e.* via intravenous inoculation). The immune modulation achieved is something that also has to be further explored in an *in vivo* model. The modulations observed allow us to propose a unique role for rTcCalr on tumor cells, where it could exert an antitumor response by inhibiting T cell apoptosis (via Galectin-9 and PD-L1), activating NK cells (via Rae-1γ) and inhibiting a tumor regulatory environment by decreasing differentiation of T cells into Tregs. Thus, in our model, the modulations observed could concur in the antitumor effects displayed by rTcCalr and, likely, those mediated by the experimental or natural *T. cruzi* infection.

CRediT authorship contribution statement

Eduardo Sosoniuk-Roche: Conceptualization, Methodology, Investigation, Writing - original draft. **Pamela Cruz:** Methodology. **Ismael MaldonadoI:** Methodology, Conceptualization. **Bárbara Pesce:** Methodology, Validation. **Marek Michalak:** Writing - review & editing. **Carolina Valck:** Project administration, Validation. **Arturo Ferreira:** Writing - review & editing, Funding acquisition, Supervision.

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