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Trypanosoma cruzi calreticulin: *In vitro* modulation of key immunogenic markers of both canine tumors and relevant immune competent cells

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ABSTRACT

Recombinant calreticulin from *Trypanosoma cruzi* (rTcCalr), the parasite responsible for Chagas' disease, binds to Canine Transmissible Venereal Tumor (CTVT) cells from primary cultures and to a canine mammary carcinoma cell line. A Complement-binding assay indicated that interaction of the first component C1q with these tumor cells operated independently of the rTcCalr-presence. This apparent independence could be explained by the important structural similarities that exist among rTcCarl, endogenous normal canine and/or mutated calreticulins present in several types of cancer. In phagocytosis assays, tumor cells treated with rTcCalr were readily engulfed by macrophages and, co-cultured with DCs, accelerated their maturation. In addition, DCs maturation, induced by tumor cells co-cultured with rTcCalr, activated T cells more efficiently than DCs, treated or not with LPS. In an apparent paradox, a decrease in MHC Class I expression was observed when these tumor cells were co-cultivated with rTcCalr. This decrease may be related to a down regulation signaling promoting the rescue of MHC I. Possibly, these *in vitro* assays may be valid correlates of *in vivo* sceneries. Based on these results, we propose that rTcCalr improves *in vitro* the immunogenicity of two widely different tumor cell lines, thus suggesting that the interesting properties of rTcCalr to boost immune responses warrant future studies.

1. Introduction

Cancer is one of the most important causes of mortality in humans and domestic animals. Tumors in domestic and wild animal models present advantages in research because they share similar genetic cancer-associated molecular alterations, risk factors and biological and histopathological features, with a broad variety of human cancers. On the other hand, animal and human tumors are susceptible to similar therapies (Di Cerbo et al., 2014; Porello et al., 2006). This is the case of a canine mammary cancer, which exhibits extensive similarity with its human counterpart, thus serving as a possible translational model (Fish et al., 2018). Transmissible cancers are also important cancer study models, and, in addition, these cancers have an important impact on endangered wildlife, either altering population dynamics or producing a population decline or risk of extinction (McAloose and Newton, 2009). Three kinds of transmissible cancers have been detected in nature. Canine transmissible venereal tumor (CTVT), affecting dogs (*Canis lupus familiaris*) and wild canines, the devil facial tumor disease (DFTD) affecting Tasmanian devils (*Sarcophilus harrisii*) and leukemia-like cancer in marine bivalves, affecting mainly soft-shell clams (Metzger et al., 2015). These tumors can be transferred between the recipient and donor animals by a malignant cell, generating a tissue that surprisingly grows despite being a *bona fide* allograft. CTVTs are transmitted mainly during mating and DFTDs through bites (Siddle and Kaufman, 2013). CTVTs and other malignancies avoid destruction by the host immune system by downregulating the expression of tumor class I and/or class II MHC molecules (Yang et al., 1987; Lollini et al., 2005). Simultaneously, these tumors secrete TGF-β1 that negatively modulates cell-mediated cytotoxicity by impairing IFN-γ secretion in T cells and natural killer (NK) cells (Hsiao et al., 2004). DFTD does not increase immunosuppressive cytokine secretion (Morris and Belov, 2013). Instead, this tumor downregulates the expression of MHC class I molecules (Loh et al., 2006). Alternatively, or concomitantly, tumors are known by their capacity to use tolerance and autoimmunity preventing mechanisms to its own benefit, as occurs with the expression of CTLA-4 and PD-1 (Seidel et al., 2018). Therefore, in order to promote tumor rejection, new therapeutic or prophylactic alternatives are needed for the treatment of transmissible tumors (especially in Tasmanian devils which are under severe conservation threat due to DFTD) and domestic animal cancers, in general.

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Trypanosoma cruzi is the protozoan agent of Chagas' disease. The first reports on the anti-tumor effects of *T. cruzi* infection can be traced down to the former Soviet Union. The researchers Grigorii Roskin and Nina Klyuyeva noted certain antitumor activities in mice infected with this parasite (Klyuyeva and Roskin, 1946, 1963). Moreover, an extensive series of experiments in humans, treated with parasite extracts were reported at that time (in 'Biotherapy of Malignant Tumors', N.G. Klyuyeva and G.I. Roskin, Pergamon Press Ltd, Oxford, England). Later, other laboratories have confirmed the original observations of Roskin and Klyuyeva, in several experimental sceneries (Hauschka et al., 1947; Malisoff, 1947; Junqueira et al., 2011; Ubillos et al., 2016). In all these investigations, no parasite molecule has been implied in the antitumor effect of this infection.

In our laboratory, we have reported that *Trypanosoma cruzi* calreticulin (TcCalr, formerly known as TcCRT), an ER-resident chaperone, is largely responsible for the antitumor effect of *T. cruzi* infection (López et al., 2010; Abello-Cáceres et al., 2016; Ramírez-Toloza et al., 2016; Ramírez-Toloza and Ferreira, 2017). TcCalr is a pleiotropic protein with properties that are, to some extent, conserved by its mammal counterpart (Ferreira et al., 2004). In addition, we have previously shown that this ER-resident chaperone is translocated to the parasite exterior. There, the chaperone inactivates the complement classical pathway, thus acting as an important parasite virulence factor (Ramírez et al., 2011), and may be largely responsible for the reported antitumor effect of this parasite infection. Highly relevant in the rejection of a variety of tumors is their capacity to bind the classical first complement component C1 (reviewed in Ramirez-Toloza and Ferreira, 2017). C1 opsonizes cells, thus increasing phagocytosis by immature dendritic cell, macrophages and B cells, followed by processing of tumor antigens and stimulation of cytotoxic T cells in the regional lymph nodes (Mueller et al., 2013). Herein, we propose that the *in vitro* immunogenicity of two canine tumors, a transmissible venereal one (CTVT) and a mammary carcinoma cell line, is increased by cell bound TcCalr. Our results indicate that tumor rejection is mediated, at least partly, by the interaction of TcCalr with the first complement component C1. These *in vitro* assays may represent correlates of the *in vivo* capacity of this parasite chaperone to mediate tumor rejection.

2. Materials and methods

2.1. Dogs

Two- to seven-years-old, female and male dogs with genital CTVT, were recruited from the Veterinary Teaching Hospital, Faculty of Veterinary and Livestock Sciences, University of Chile. Nine dogs were used to extract tumor biopsies for the generation of primary cell cultures and blood samples to obtain PBMCs.

2.2. Bioethical considerations

The study protocol was approved by the Bioethical Committee (CICUA), University of Chile, certificate number 17034-FCS-UCH. All procedures with these animals, performed by trained veterinarians, required a previous written owner consent.

2.3. Proteins

The TcCalr gene was amplified by PCR using platinum Taq polymerase (Invitrogen, San Diego, CA). The primers used were reverse (5′-ggaattccatcacggtcacccttttt-3′) and forward (5′-ggaattccacggtgtacttccacgag-3′) (Invitrogen). The amplified TcCalrDNA was purified and ligated into the EcoRI restriction enzyme site of the pET-28b$^+$plasmid (Novagen, Madison, WI). Competent *Escherichia coli* TOP10F′ bacteria were transformed with the plasmids, plated (Luria-Bertani medium), and selected with 50 μg/ml kanamycin. For protein expression, E. coli BL21(DE3)pLysS was transformed with the corresponding plasmids and grown in the presence of 34 μg/ml chloramphenicol with 50 μg/ml kanamycin. After incubation with isopropyl β-D-thiogalactoside for 3 h, the cells were harvested, sonicated on ice, centrifuged, and the supernatants were filtered. The recombinant TcCalr (rTcCalr) was purified using His Bind resin (Novagen), eluted with buffer containing 1 M imidazole, and dialyzed against 2 mM Tris-HCl and 150 mM NaCl, pH 7.4 (Ferreira et al., 2004). Purity was assessed by conventional SDS-PAGE (Appendix 1). Human C1q was obtained from Complement Technology, Tyler, TX, USA.

2.4. CTVT cells

Biopsies were extracted with a 3 − 4 mm Biopsy Punch (Miltex GmbH, Rietheim-Weilheim, Germany). The specimens were mechanically disaggregated and filtrated with cell strainers (pore size 100 μm) (Corning Falcon, USA). Then, 3 ml of the cell suspension was centrifuged at 100 x g for 30 m at room temperature on a 3 ml Histopaque-1077 gradient (Sigma-Aldrich, USA). The interface cells were cultured with RPMI 1640 (Gibco Laboratories, USA), supplemented with 10 % Fetal Bovine Serum (FBS) (Gibco Laboratories, USA) at 37 °C in a 5 % CO_2 atmosphere.

2.5. CF41.Mg Cells

They are immortalized cells obtained from a mammary tumor from a 10-year-old female beagle (ATCC® code CRL-6232™, Manassas, USA), which were grown in DMEM-high glucose (4.5 g/L) containing 10 % FBS, 2 mM L-glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin sulfate.

2.6. TcCalr and C1q Binding Assays

CTVT and CF41.Mg cells were incubated with variable concentrations of rTcCalr conjugated with FITC or Alexa 660 for 30 m at room temperature and analyzed by flow cytometry (BD LSRFortessa, BD Biosciences). CTVT and CF41.Mg cells exposed to rTcCalr were subsequently incubated with human C1q for 30 m at room temperature. A FITC-conjugated anti-C1q antibody (Dako, Agilent Technologies Inc., USA) was used and the cells were also analyzed by flow cytometry.

2.7. Peripheral blood monocytes cells (PBMCs) isolation

Heparinized blood was collected and fractioned on a Histopaque-1077 gradient (Sigma-Aldrich, USA). The interface cells were cultured in RPMI 1640 (Gibco Laboratories, USA) supplemented with 10 % FBS (Gibco Laboratories, USA) at 37 °C in a 5 % CO_2 atmosphere. To generate macrophages, adherent cells were incubated in conditioning medium with M-CSF. Alternatively, in order to generate immature DCs, adherent cells were incubated in conditioning medium for 6 days with human GM-CSF, Flt3L and canine IL-4. Both, macrophages and immature DCs were characterized by flow cytometry (BD LSRFortessa, BD Biosciences, USA) according to the expression of surface antigens CD14, F-4/80 and MHC-II in macrophages and CD11c and MHC-II in immature DCs. To obtain T cells, the non-adherent cells were cultured in RPMI 1640 supplemented with 10 % FBS at 37 °C in a 5 % CO_2 atmosphere.

2.8. Phagocytosis assay

CTVT and CF41.Mg cells were stained with 1 μM CFSE (Sigma-Aldrich, USA) for 10 m at 37 °C, incubated with rTcCalr and/or C1q and co-cultured with macrophages or immature DCs for 2 h at 37 °C. A negative control at 4 °C was included. To analyze by flow cytometry (BD LSRFortessa, BD Biosciences), macrophages and immature DCs were stained with conjugated antibodies against CD14 and CD11c, respectively. Phagocytosis was evident through the double mark (CD14/CFSE and CD11c/CFSE).

2.9. Mixed lymphocyte reaction (MLR)

Immature DCs were co-cultured with CTVT and CF41.Mg cells

Table 1
Antibodies used in canine tumor cells.

Antibody against:	Reactivity	Conjugate	Clone	Company
CD1a	Human	Alexa Fluor 700	NA1/34-HLK	Novus Biologicals
CD3	Mouse	Brilliant Violet 421	17A2	Biolegend
CD4	Mouse	Brilliant Violet 711	RM4-5	Biolegend
CD8	Mouse	Brilliant Violet 650	53-6.7	Biolegend
CD11c	Dog	APC	Bu15	eBioscience
CD11c	Mouse	APC/Cy7	N418	Biolegend
CD14	Human	PE/Cy7	M5E2	Biolegend
CD25	Human	PE	24212	R&D Systems
CD40	Human	Alexa Fluor 647	LOB7/6	Novus Biologicals
CD45	Mouse	PerCP/Cy5.5	30-F11	Biolegend
CD69	Human	APC	FN50	Biolegend
CD71	Human	PE/Cy7	OKT9	eBioscience
CD80	Mouse	Brilliant Violet 605	16-10A1	Biolegend
CD83	Human	PE	HB15e	Biolegend
CD86	Mouse	Brilliant Violet 510	GL-1	Biolegend
F4/80	Mouse	PerCP	521204	R&D Systems
MHC I	Human	PE	WE/32	Biolegend
MHC II	Dog	FITC	YKIX334.2	eBioscience

(previously incubated with rTcCalr and/or C1q) and then, the expression of MHC-II, CD1a, CD11c, CD40, CD80 (B7.1), CD83 and CD86 (B7.2). Negative and positive controls (LPS) were included.

Once mature DCs were obtained, they were co-cultured with T cells previously stained with CFSE. After 3 days, the presence of CD3, CD4, CD8, CD25, CD69 and CD71 was analyzed by flow cytometry. Negative control, identified in Fig. 8A, B and C as CF41.Mg, refers to dendritic cells exposed only to the culture medium.

Table 1 summarizes the antibody array used in these assays.

2.10. MHC class I and II expression

CTVT and CF41.Mg cells were exposed to rTcCalr and/or C1q for 30 m at room temperature. Then, the expression of MHC class I and II was analyzed by flow cytometry with anti-MHC I and anti-MHC II fluorophore-conjugated antibodies.

2.11. Statistical validations

The data obtained from rTcCalr and C1q binding, phagocytosis, MLR and MHC expression experiments were statistically validated through One-Way ANOVA with Tukey's multiple comparisons by software GraphPad Prism v6.0 (GraphPad software Inc., CA, USA). FlowJo v10 (TreeStar, ORE, USA) was used to analyze flow cytometry results.

3. Results

3.1. Viable CTVT cells are obtained after flow cytometry and sorting

Isolation of CTVT cells, was achieved as proposed in the methods section. About 98 % purity was obtained after sorting and culturing (Fig. 1). The resulting population exhibited similar morphological characteristics as compared with CTVT cells obtained in another laboratory (Liao et al., 2003). The purified CTVT cells displayed high expression of CD45, an indication of their histiocytic origin (Fig. 2). To make sure we worked with CTVT cells, we also determined the karyotype of these cells (Appendix 2).

3.2. rTcCalr binds to CTVT and CF41.Mg Cells

As proposed, rTcCalr binding to CTVT cells was concentration-dependent (0.23–5.5 μM), with saturation obtained at 3.7 μM. This phenomenon was observed in both, rTcCalr conjugated with FITC (F-rTcCalr), or with Alexa 660 (A-rTcCalr), both used at 0.25–8 μM.

The same experiment, with similar results, was performed with A-rTcCalr and the immortalized canine mammary cancer cell line, CF41.Mg, reaching saturation with the labeled chaperone at 4 μM (Fig. 3).

3.3. C1q binds to CTVT and CF41.Mg Cells in an rTcCalr-independent fashion

In both, CTVT and CF41.Mg cells, C1q bound to the tumor cell surface in an rTcCalr-independent manner. Thus, C1q (2 nM) binding to CTVT and CF41.Mg cells, in the presence of rTcCalr at twice the C1q concentration, was similar, a situation also observed in the absence of the chaperone (Fig. 4).

3.4. Tumor cells, co-cultured with macrophages, are readily phagocytosed

CTVT and CF41.Mg cells, treated with or not to rTcCalr and/or C1q, were co-cultured with macrophages. In an apparent paradox, only macrophages exposed to tumor cells showed an important increase in phagocytosis (Fig. 5).

3.5. CTVT and CF41.Mg cells treated with rTcCalr induce DCs maturation

As expected, levels of CD1a and CD40 were increased in DCs in response to the CTVT various treatments (rTcCalr, rTcCalr + C1q and C1q), as compared to the negative controls. All the experiments were performed at 24 h and 48 h. In the case of CTVT cells, the increases were evident at 24 h and 48 h, while in CF41.Mg cells the increases were detected only at 24 h (Fig. 6). Other maturation markers like MHC II, CD11c, CD80 (B7.1), CD83 and CD86 (B7.2) were evaluated. Significant differences were observed between the control and treatment groups, but not among treatments (data not shown).

3.6. $CD4^+$ T cells predominate in peripheral blood samples and have a phenotype slightly different from $CD8^+$ T cells

$CD4^+$ T cells were the predominant phenotype isolated from peripheral blood samples, with a $CD4^+$/ $CD8^+$ ratio close to 2:1. The surface marker that was observed in a larger number of cells was CD25, followed by CD71 and, to a lower extent, CD69. On the other hand, in $CD8^+$ T cells, CD71 was the predominant surface marker, closely followed by CD25 and, by far, the lowest was CD69.

3.7. DCs co-cultured with CF41.Mg cells, treated with rTcCalr, activate T cells more efficiently than LPS and tumor cells alone

As proposed, CD69 and CD71 activation markers were induced

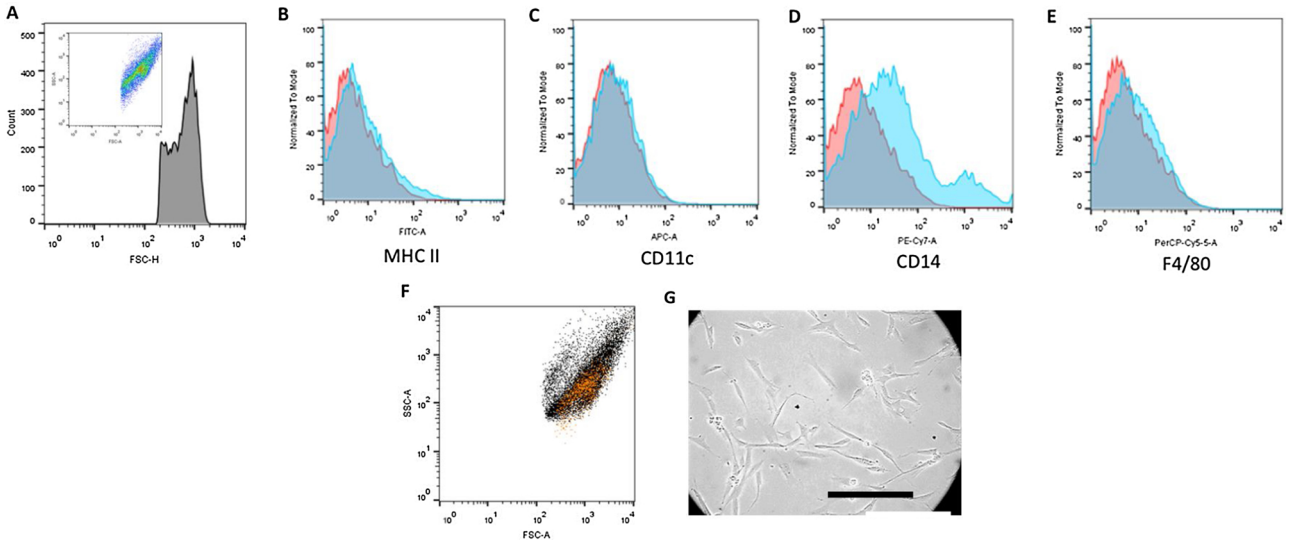

Fig. 1. Sorting of CTVT cells yields a viable population. A) Histogram and dot plot of the cellular mixture. B–E) In red: autofluorescence, in calypso: fluorophore. F) In orange: selected negative cells. G) Microphotography of the selected negative cell cultures, corresponding to CTVT. Scale bar = 100 μm. MHC II, CD11c, CD14 and F4/80 were used as cell markers do not present in CTVT cells.

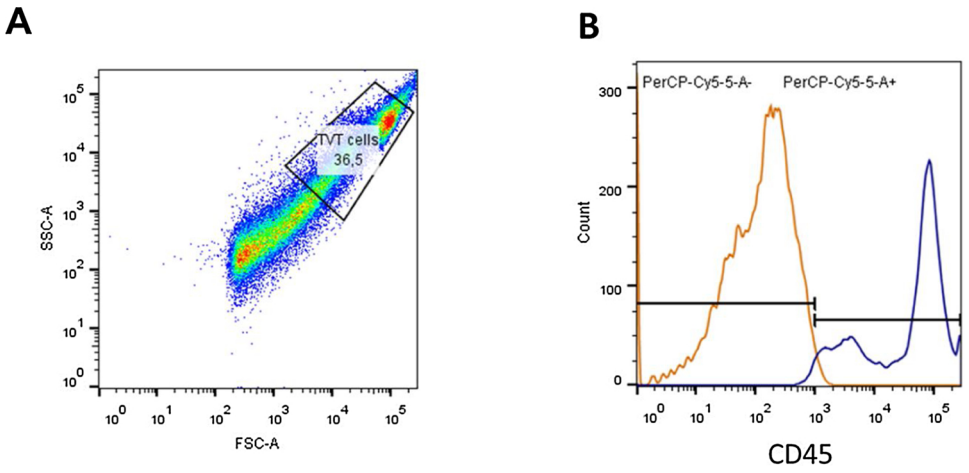

Fig. 2. CTVT cells display CD45, a histiocytic cell surface marker. A) Gate. B) In orange: Autofluorescence. In blue: CD45+ cells.

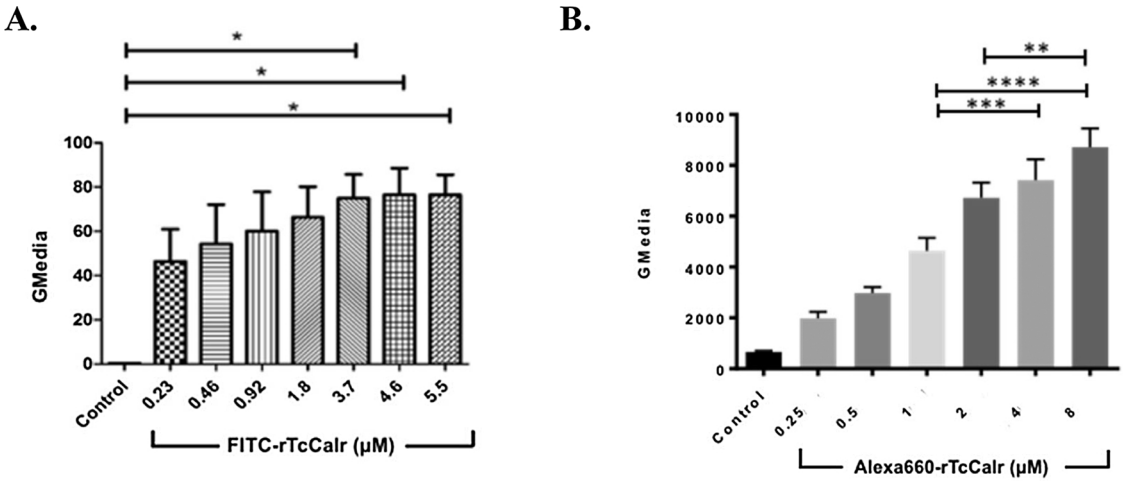

Fig. 3. F-rTcCalr and A-rTcCalr bind to CTVT and CF41.Mg cells respectively, in a concentration-dependent fashion. A and B) Binding of F-rTcCalr and A-rTcCalr, in increasing concentrations, to CTVT and CF41.Mg cells, respectively. Control = 0 μM. While differences were observed in most combinations ($p < 0.0001$), when 4 and 8 μM A-rTcCalr were used, no differences were obtained ($p < 1.00$), a possible indication of saturation.

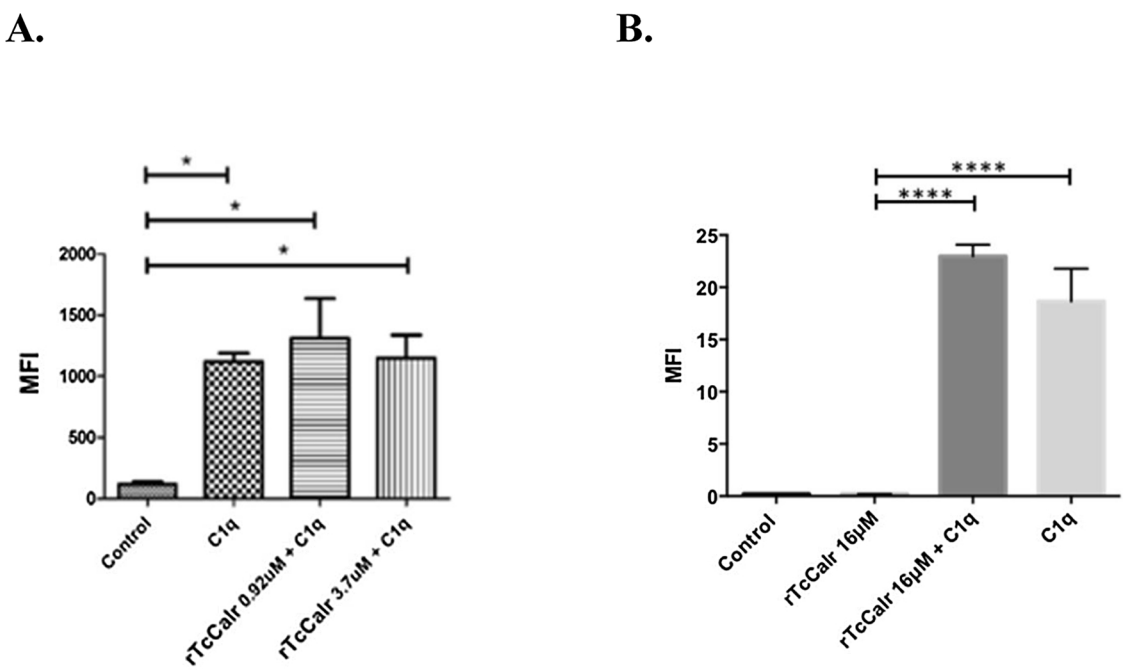

Fig. 4. C1q binds to CTVT and CF41.Mg cells, independently from rTcCalr. A) Binding of C1q (2 nM) to CTVT cells in the presence of rTcCalr. No differences in C1q binding were observed in the presence of rTcCalr at twice the C1q concentration, nor in the absence of the chaperone. B) Binding of C1q (2 nM) to CF41.Mg cells in the presence of rTcCalr. Similar results to those described in A were obtained.

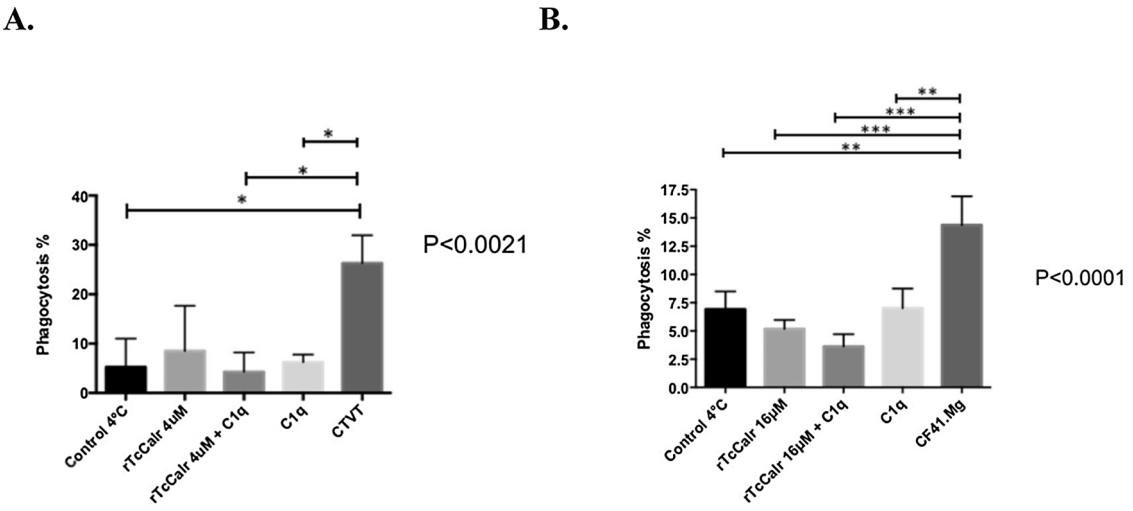

Fig. 5. Tumor cells stimulate phagocytosis. CTVT (A) and CF41.Mg (B) cells stained with CFSE and exposed or not to rTcCalr and/or C1q were co-cultured with macrophages for 2 h. Significant differences were seen only in the cases of macrophages exposed to untreated tumor cells.

earlier in T cells co-cultured for 72 h with DCs, previously exposed to CF41.Mg cells treated with 16 μM rTcCalr (Fig. 8, A and B). In the cases of CD3, CD4, CD8 and CD25, the differences between each group and its control were significant, but no differences were observed among the treatments (data not shown). Notoriously, CFSE expressions in T cells exposed to DCs, co-cultured with CF41.Mg cells treated with rTcCalr and rTcCalr plus C1q, were rather modest, indicating a higher proliferation rate (Fig. 8, C). The negative control, identified in Fig. 8A-C as CF41.Mg, refers to dendritic cells exposed only to the culture medium.

3.8. TcCalr decreased the expression of MHC Class I in CF41.Mg cells

Interestingly, TcCalr and TcCalr with C1q, but not C1q alone, downregulated MHC I expression on CF41.Mg cells after 30 min of exposure at room temperature (Fig. 9A). In Fig. 9B, we observed an increased expression of MHC II in those cells treated with C1q, while no differences between the other groups were observed (control and CF41.Mg cells treated with rTcCalr in presence or not of C1q).

4. Discussion

T. cruzi infection, both in humans and experimental animals, has been long correlated with lower incidences of a large variety of tumors (Klyuyeva and Roskin, 1946; Hauschka et al., 1947; Malisoff, 1947; Junqueira et al., 2011; Ubillos et al., 2016). In these investigations, no parasite molecule was signaled out as responsible for these anti-tumor effects. More recently, work from our laboratory determined that the *T. cruzi* ER-resident chaperone TcCalr, after translocation from the ER to the parasite exterior, exerted, if not all, most of the observed anti-tumor effects (Abello-Cáceres et al., 2016; reviewed in Ramírez-Toloza and Ferreira, 2017). Thus, these observations identify TcCalr as an interesting potential adjuvant in experimental oncologic immunogenic

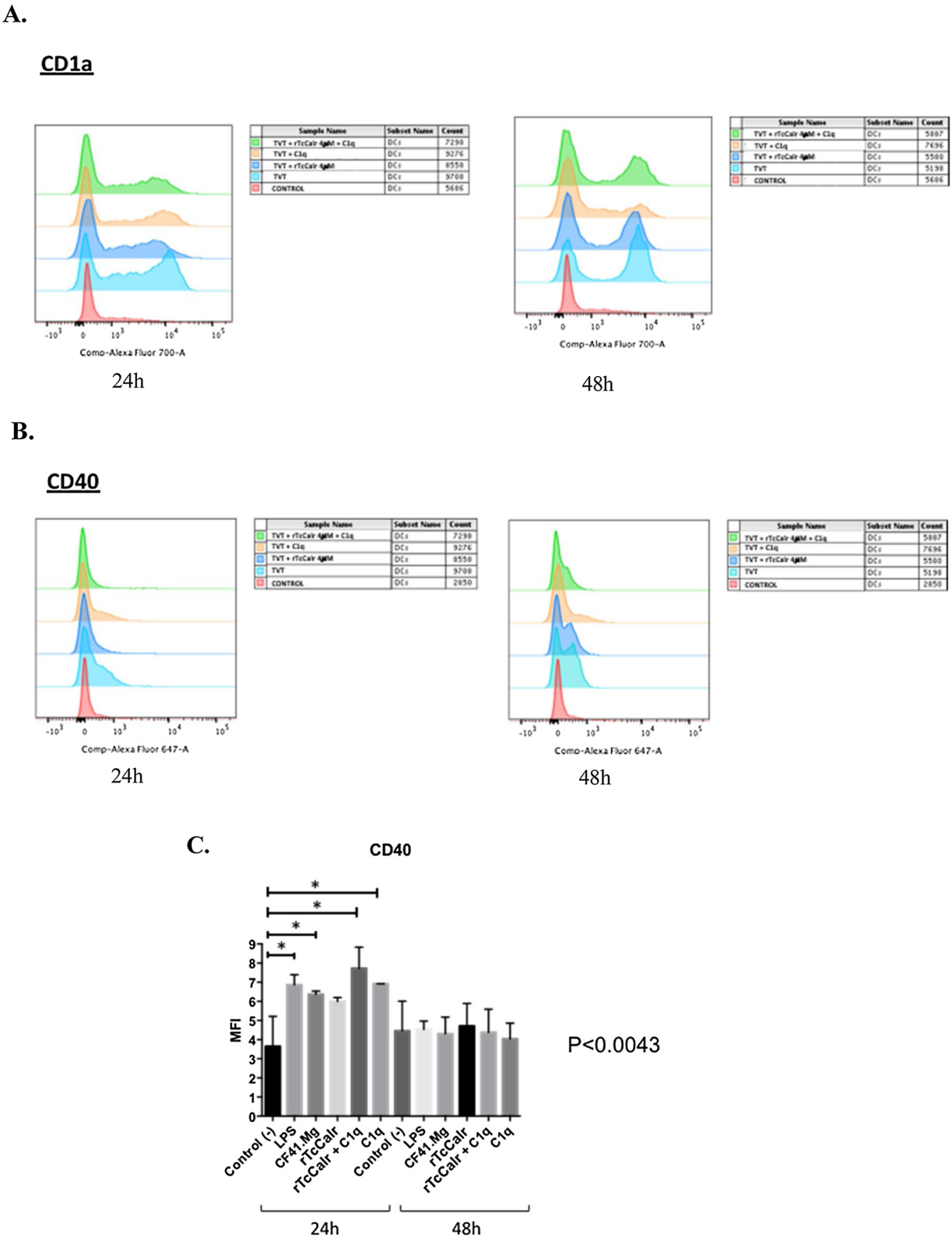

Fig. 6. CTVT and CF41.Mg cells, treated with rTcCalr and C1q, induce the expression of DCs maturation markers in co-cultures. A) and B) Increased levels of CD1a and CD40 compared to the control and the different treatments in CTVT cells, respectively. C) CD40 expression in CF41.Mg cells. All the experiments were performed at 24 h and 48 h. In A and B, rTcCalr was used at 4 μM, while in C it was used at 16 μM.

therapies, with improved activity compared to that described for human calreticulin (CALR) (Korbelik et al., 2015). Both, human and parasite calreticulins seem to act as damage-associated molecular patterns (DAMPs), but after their professional phagocytic cells (APCs) processing, TcCalr seems to be more immunogenic, given its more extensive structural differences with its mammal counterparts (Weinberger et al., 2017).

In this report, using the CTVT and a canine mammary cancer cell

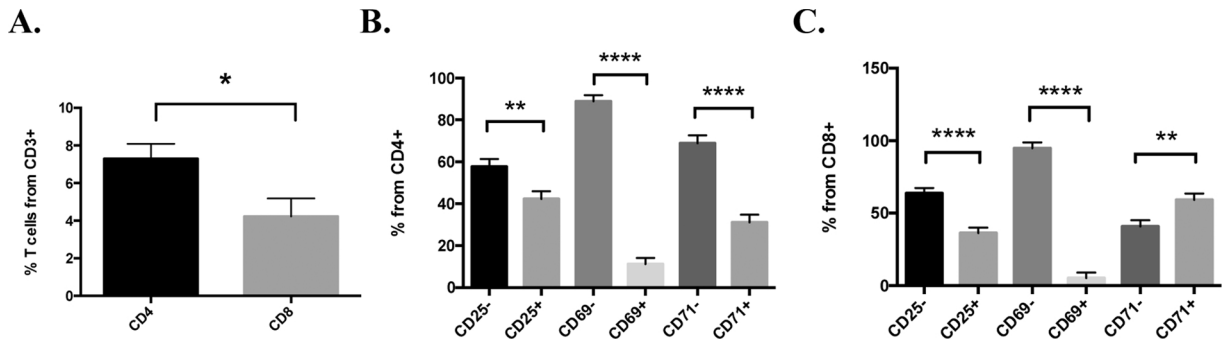

Fig. 7. $CD4^+$ T cells predominated in peripheral blood samples and had a slightly different phenotype from $CD8^+$ T cells. A) Percentage of $CD4^+$ and $CD8^+$ cells extracted from $CD3^+$ cells. B) Percentage of negative and positive cells corresponding to each surface marker; CD25, CD69 and CD71, obtained from the $CD4^+$ T cells. C) Percentage of negative and positive cells corresponding to each surface marker; CD25, CD69 and CD71, obtained from $CD8^+$ T cells.

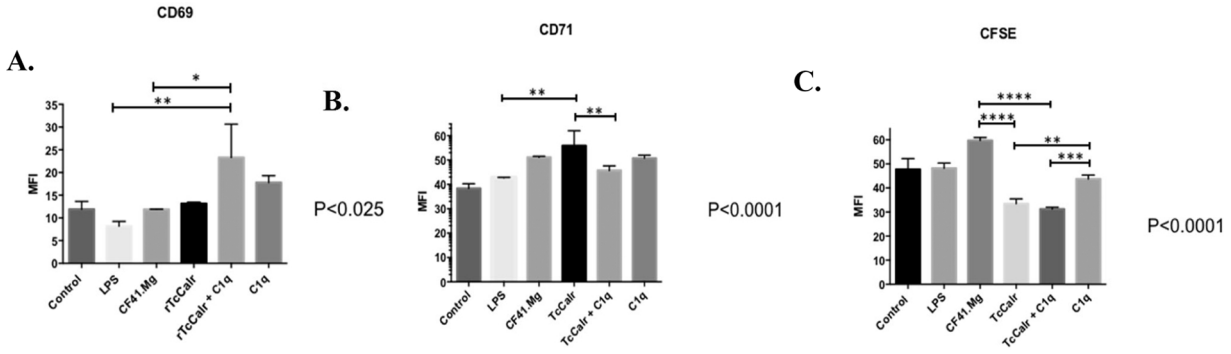

Fig. 8. DCs co-cultured with tumor cells previously treated with rTcCalr induced the expression of T cell activation markers more readily than the other treatments. A) and B) CD69 and CD71 activation markers were induced in T cells co-cultured with DCs, previously exposed to CF41.Mg cells treated with rTcCalr. C) The reduction of CFSE relates with higher proliferation rate in T cells induced by DCs co-cultured with CF41.Mg cells treated with TcCalr.

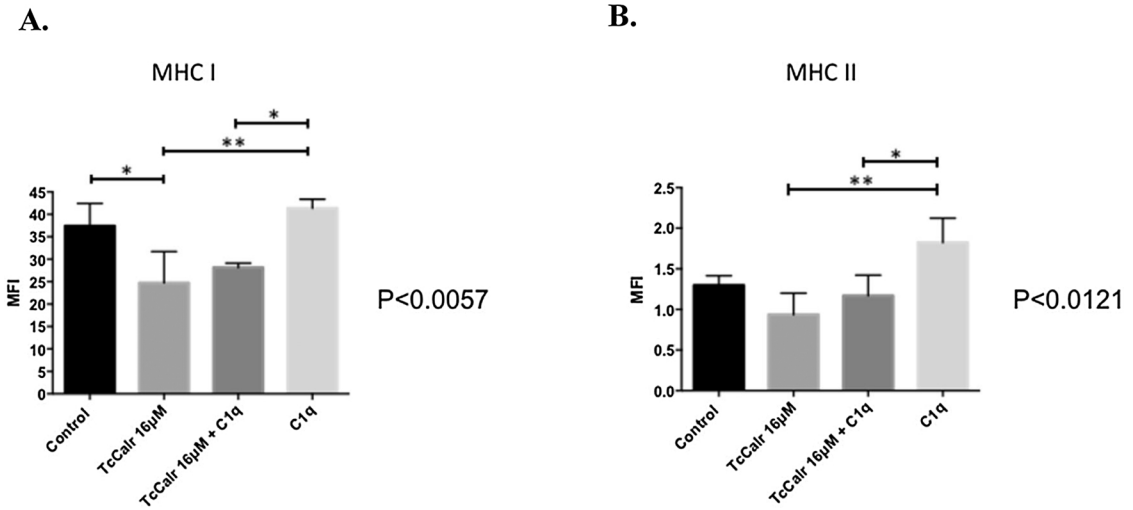

Fig. 9. rTcCalr decreased the expression of MHC Class I in CF41.Mg cells. A) MHC class I expression in CF41.Mg cells treated with rTcCalr and/or C1q for 30 min at room temperature. B) MHC class II expression in CF41.Mg cells treated with rTcCalr and/or C1q for 30 min at room temperature. A) and B) were analyzed by flow cytometry with conjugated antibodies, PE and FITC, respectively.

line, we evaluated whether TcCalr, promoted tumor rejection by increasing the *in vitro* immunogenicity of these tumors. The *in vitro* studies presented herein are possible correlates of the *in vivo* rejection of two widely different canine tumors. These rejections may be mediated, at least in important part, by the interaction of cancer cells with TcCalr and with the first complement component C1. Perhaps, C1-opsonized tumor cells, taken up by APCs, such as dendritic cells, macrophages and B cells, is followed by antigen processing and promotion of cytotoxic and helper T cells responses in the local lymph nodes, our next goal in future *in vivo and ex vivo* assays.

Viability of CTVT cells, obtained through cell sorting, was adequate for the studies performed (Fig. 1). The sorted cells expressed CD45 (Fig. 2), a marker present on histiocytes, thus indicating the origin of these cells and reinforcing the observations obtained by previous studies (Mozos et al., 1996). Furthermore, these cells indeed corresponded to CTVTs, since their chromosome numbers were reduced, as reported by other laboratories (Adams et al., 1968) (Appendix 2).

TcCalr binds to the surface of a broad variety of tumor cells. In our

study rTcCalr binds to the surface of CTVT and CF41.Mg cells (Fig. 3) and to a human mammary cancer cell line (MCF7) (Appendix 3) in a concentration-dependent fashion. C1q and CD91/LRP1 are the best known human calreticulin (CALR) receptors (Duus et al., 2010) and probably both are implicated in the binding of TcCalr to the CTVT cells. CF41.Mg cells have a different histological origin since, differently from CTVT cells, they do not express CD45. It remains to be determined whether C1q and CD91/LRP1 are present in both cell types.

We then observed that C1q binding to CTVT and CF41.Mg cells was in a rTcCalr-independent fashion (Fig. 4). Since calreticulin has been described as a receptor protein for C1q (cC1qR) (Eggleton et al., 1994), our results could be explained by the presence of endogenous canine calreticulin interacting with phosphatidylserine at the external membrane layer of the cell. Tumor cells are under constant stress and calreticulin is externalized under those conditions (Varricchio et al., 2017). In addition, mutations in the calreticulin gene have been described in cancer cells with the consequent expression of the abnormal protein on the cell surface (Panaretakis et al., 2008; Kroemer et al., 2013; Wiersma et al., 2015).

With regard to the phagocytosis assay (Fig. 5), a similar result with a lack of significant differences between rTcCalr and C1q was seen in another study performed by our laboratory (Aguilar-Guzmán et al., 2014), probably explained by the above-mentioned presence of self-endogenous native calreticulin on the tumor cell surface. In the present work, the results obtained may be influenced by an inhibitory effect mediated by CD47, a surface protein also known as integrin-associated protein (IAP). This protein, present in all cells from mammals, acts mainly as a 'don't eat me' signal (Tsai et al., 2010) and currently studied as a potential therapeutic target because it is usually overexpressed in tumors (Chao et al., 2010; Liu et al., 2017). Our results show that cells alone, from both tumors studied, are more potent in inducing phagocytosis by macrophages. The possibility of a positive correlation between exogenous recombinant calreticulin concentration and expression of CD47 should be ascertained. There is evidence that, in the presence of greater calreticulin externalization, tumor cells must compensate by increasing the expression of CD47 to avoid being phagocytosed. Therefore, when exogenous recombinant parasitic calreticulin is added to this equation, the tumor cell, saturated with the chaperone, probably recognizes the signal and quickly begins to express CD47, thus preventing phagocytosis through its binding to the SIRPα (signal regulatory protein α) receptor (Chao et al., 2010; Feng et al., 2018). Therefore, it would be interesting to test rTcCalr in the presence of a CD47 receptor antagonist.

On the other hand, our results in dendritic cells maturation assay (Fig. 6) were similar to those obtained by Li et al., 2015, where DCs increased their CD40 expression in the presence of exogenous calreticulin, polarizing a rather pro-inflammatory phenotype (Bajor et al., 2011). Indeed, probably the secreted cytokines would include type I interferons and acute phase cytokines such as TNFα, IL-1, IL-6 and IL-12 (Blanco et al., 2008). In this scenario, in the context of CTVT, IL-6 produced by T cells would counteract TGF-β1 and would restore NK activity with consequent incremented secretion of IFN-γ and augmented expression of MHC (Hsiao et al., 2004). Thus, an immune response initiated by TcCalr, would be triggered. Also, *in vivo* studies are necessary to test whether this approach would be of benefit for veterinary canine patients affected by CTVT, mammary cancer or other tumors.

A preliminary T cell characterization (Fig. 7), showed a phenotype mostly consisting in $CD4^+$ cells, a fact already known. Additional markers should be tested in future studies. Recently, an increased circulating $CD4^+$, $C25^+$, $Foxp3^+$, T cell population has been described in an experimental model of atopic dermatitis (Rostaher et al., 2018). Since this disease widely affects dogs and humans, it is important to study Tregs in different canine pathological processes, including cancer. On the other hand, similar to humans (Motamedi et al., 2016), canine $CD8^+$ T cells, obtained from peripheral blood, expressed more CD71 than $CD4^+$, in agreement with our observations. However, additional studies are necessary regarding this matter and its implications in the anti-tumor immune response.

Mature DCs obtained by exposing them to tumor cells treated with TcCalr displayed improved antigen presentation, thus activating T cells more efficiently. This was reflected in the increased expression of surface markers CD69 and CD71, and in the increased T cells expansion determined by the loss of the CFSE fluorescent signal (Fig. 8). CD69 and CD71 are early activation markers, so the DCs co-cultured with T cells, probably will trigger a faster response when confronted with tumor cells exhibiting TcCalr on its surface. CD69 is involved in signal transcription leading to the production of IFN-γ and IL-2 (Starska et al., 2011) with the consequent cell-mediated response and possibly a greater T cell priming, resulting in the generation, mainly, of effector CD8 T cells (Bhat et al., 2017). In addition, the parasitic protein would allow the presentation, in the MHC context, of peptide sequences different from the tumor cells ones, thus increasing or forcing the immunogenicity of those tumor cells tagged by the parasite chaperone.

Calreticulin is also part of the peptide loading complex (PLC) that allows the binding of appropriate peptides to MHC class I. Therefore, cells deficient in calreticulin and cells with the mutated version of this protein, such as tumor cells, will have a diminished MHC I expression (Arshad and Cresswell, 2018). In our results (Fig. 9), in CTVT cells, the augmented MHC I and II surface expression would be more related with the regressive stage of the disease, so no differences were observed between groups (Appendix 4). Instead, and rather paradoxically, CF41.Mg cells treated with TcCalr showed a significant decrease in MHC I expression. Possibly rTcCalr internalization or saturating concentration triggers its endocytosis with the consequent non-formation of the PLC, which should be considered in order to approach to the chaperone functional concentration to be used in future experimental treatments *in vivo*. Moreover, rTcCalr has an ATP or calcium binding site with major affinity, competing with canine calreticulin and finally affecting the expression of the MHC I on the cell surface, something similar to what happens with the calreticulin mutants (Wijeyesakere et al., 2015). Conversely, we observed an increase in MHC II expression in the group of cells treated with C1q, but this result should be taken with caution because the numbers on the Y axis were small and could be only indicating a background effect (Fig. 9B).

Recently, we have determined that although a small rTcCalr-derived peptide maintains some antiangiogenic and anti-tumor properties, in equimolar terms, the whole recombinant chaperone is more effective. This could be explained, at least partly, because the TcCalr C1q-binding and antiangiogenic domains are separated in the primary sequence (Peña et al., 2019, in press, available on line).

Current, effective public strategies to control stray dogs, where CTVT prevails, have made very difficult to obtain these specimens. For this reason and, in order to test the validity of our observations in other canine tumor model, we decided to use the CF41.Mg cell line, generated from a canine mammary carcinoma. As expected, TcCalr seems to function similarly, regardless the type of tumor cell, thus providing a more broad value to our findings.

On the other hand, cancer besides being one of the deadliest diseases, is also one with the highest economic burden in the world and, mainly in underdeveloped countries, where many people die waiting for a treatment. Access to cutting-edge treatment is beyond the economic capacity of most patients and, for average people, the only option is to participate in clinical trials. Specifically, immunotherapies like antibodies against HER2/*neu*, PD-1/PD-L1, CTLA-4, among others, are therapies highly expensive and that still do not warrant a cure.

In conclusion, using as models CTVT and CF41.Mg cells, we showed that *T. cruzi* calreticulin improves the immunogenicity of tumor cells. Thus: i) the chaperone binds to the surface of CTVT and CF41.Mg cells in a concentration-dependent manner; ii) in co-cultures, tumor cells treated with TcCalr induce the expression of several surface markers that promote maturation in dendritic cells; iii) in co-cultures, these mature DCs efficiently induce T cell activation, reflected on the expression of surface markers, related with early activation and proliferation. Hence, we experimentally improved the potential

immunogenicity of the treated tumor cells. Our data, taken altogether with previous results from our and other laboratories (reviewed in: Ramírez-Toloza et al., 2016), allow us to propose an alternative adjuvant immunotherapy, still in preclinical experimental stages, with interesting perspectives in veterinary and human oncology.

Author contributions

PC carried out all experiments, analysed the data and wrote the manuscript. SRE and MI contributed with analysis of data and, flow cytometry assays and synthesis of TcCalr respectively. CGT contributed with design of study, data analysis and revision of the manuscript. AF designed the study, performed interpretation of results and reviewed the manuscript. All authors have read and approved the final version of the manuscript.

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Appendix 1 rTcCalr SDS-PAGE

See Fig. A1

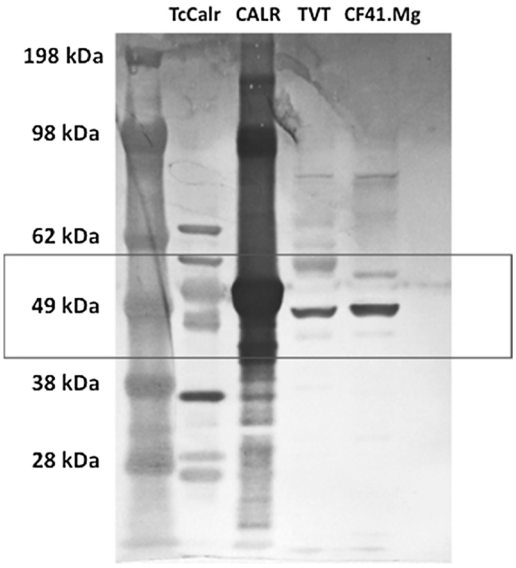

Fig. A1. Calreticulin is present in CTVT and CF41.Mg cells lysates, and is recognized by a rabbit immune serum against CALR. Immunoblot of two purified recombinant proteins (rTcCalr and rCALR) and two cell lysates (CTVT and CF41.Mg) where the presence of endogenous native calreticulins was detected by cross-reactive rabbit anti-CALR antibodies. Calreticulin is near the 49 kDa band of the molecular weight marker. (As usual, the recombinant proteins, unlike the native counterpart, show a higher tendency to degrade).

Appendix 2 CTVT Karyotype

See Fig. A2

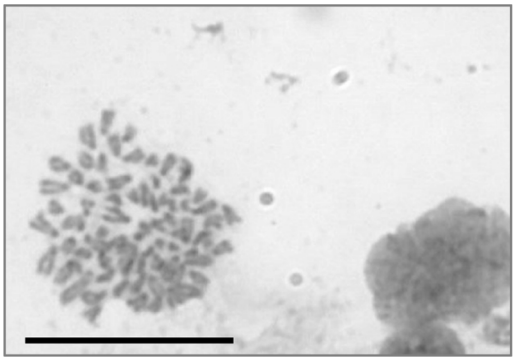

Fig. A2. CTVT cells and normal canine cells have different karyotypes. CTVT contains 68–72 chromosomes, while the normal karyotype in dogs contains 78 chromosomes (100x). Scale bar = 100 μm.

Appendix 3 Binding of rTcCalr to MCF-7 cells

See Fig. A3

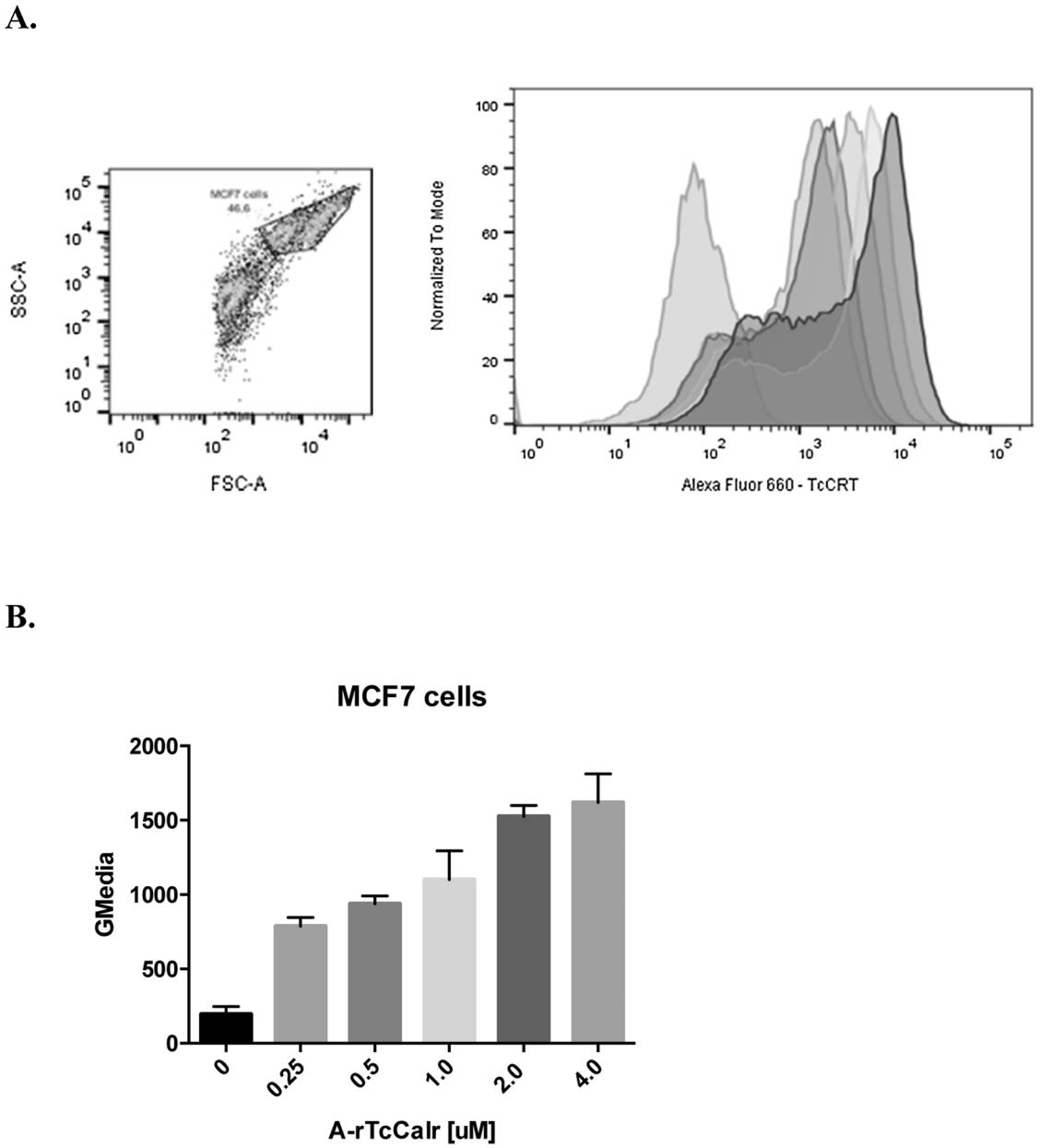

Fig. A3. A-rTcCalr binds to MCF-7 cells, in a concentration-dependent fashion. A) Gate and histogram with the different concentrations of A-rTcCalr. B) Binding of A-rTcCalr, in increasing concentrations, to MCF-7 cells. Control = 0 μM. $p < 0.0002$.

Appendix 4 Increase in MHC I and II in CTVT cells

See Fig. A4

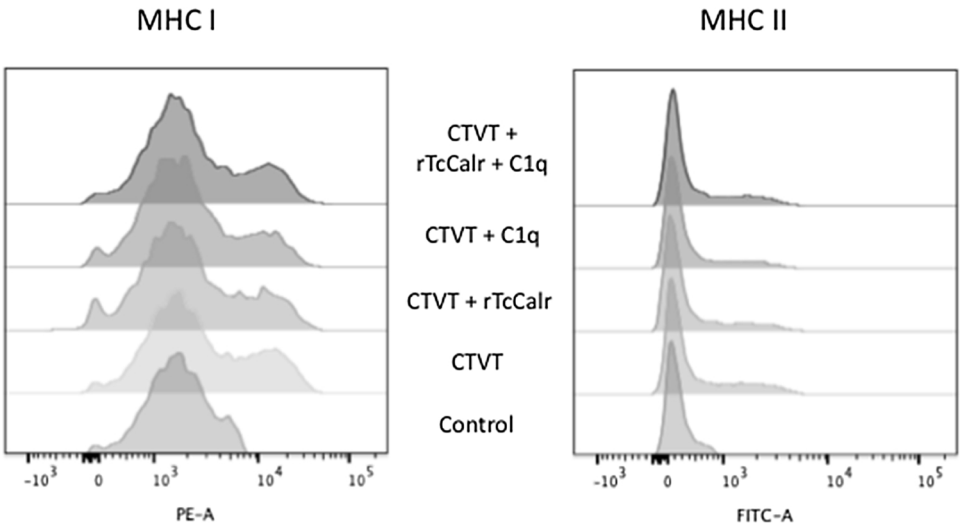

Fig. A4. rTcCalr increased the expression of MHC I and MHC II in CTVT cells, as compared to controls. No differences were observed among the other groups. MHC I and MHC II expression in CTVT cells treated with rTcCalr and/or C1q for 30 min at room temperature. MHC I and MHC II were analyzed by flow cytometry with conjugated antibodies, PE: MHC I and FITC: MHC II. rTcCalr: 4 μM.

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