Immunobiology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Exogenous Calreticulin, incorporated onto non-infective *Trypanosoma cruzi* epimastigotes, promotes their internalization into mammal host cells

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ARTICLE INFO

Article history:
Received 3 May 2016
Accepted 27 October 2016
Available online xxx

Keywords:
Chagas
Trypanosoma cruzi
Calreticulin
Complement
Infection

ABSTRACT

Chagas disease is an endemic pathology in Latin America, now emerging in developed countries, caused by the intracellular protozoan *Trypanosoma cruzi*, whose life cycle involves three stages: amastigotes, epimastigotes, and trypomastigotes. *T. cruzi* Calreticulin (TcCRT), an endoplasmic reticulum resident chaperone, translocates to the external cellular membrane, where it captures complement component C1, ficolins and MBL, thus inactivating the classical and lectin pathways. Trypomastigote-bound C1 is detected as an "eat me" signal by macrophages and promotes the infective process. Unlike infective trypomastigotes, non-infective epimastigotes either do not express or express only marginal levels of TcCRT on their external membrane. We show that epimastigotes bind exogenous rTcCRT to their cellular membrane and, in the presence of C1q, this parasite form is internalized into normal fibroblasts. On the other hand, Calreticulin (CRT)-deficient fibroblasts show impaired parasite internalization. In synthesis, CRT from both parasite and host cell origin is important in the establishment of C1q-dependent first contacts between parasites and host cells.

1. Introduction

Chagas disease is a chronic ailment, described by Carlos Chagas in 1909 (Chagas, 1909). It is endemic in Latin America, with approximately 8 to 9 million seropositive humans and an undetermined number of domestic and wild animal reservoirs. Available treatments are modestly efficacious and with serious side effects. Due to migratory processes, this disease is now increasingly being reported outside the endemic zone. Thus, it is estimated that there are near to one million infected patients in the USA, and approximately one hundred thousand in the rest of the world (Coura and Vinas, 2010). This disease has acute and chronic stages (Chagas, 1911). The acute stage starts a few days after the infection and fever, headache, muscle pain and shivers are common symptoms. This stage lasts nearly 30 days, where high parasitemia and cellular parasitism are evident. The chronic stage appears in about 30% of the infected patients, having variable symptomatology and duration.

The etiologic agent is the hemiflagellated intracellular protozoan *Trypanosoma cruzi*, transmitted to mammal hosts through the feces of haematophagous arthropods from the *Triatominae* family, such as "kissing bugs" (Canals et al., 1998). *T. cruzi* is a unicellular organism, with characteristic structures of eukaryotic cells. The parasite has a life cycle consisting of 3 stages. Infective, non-replicative trypomastigotes are present in the feces of the arthropod vector, and penetrate mammal cells near the bite site (Coura and Vinas, 2010; Brener, 1973). There, trypomastigotes transform into amastigotes that, after several replication cycles, transform back into infective trypomastigotes that disseminate *via* lymph and blood. In the arthropod vector, infective trypomastigotes and non-infective replicative epimastigotes are present. Epimastigotes, such as those used in this study, were obtained *in vitro*, under special culture conditions (Maya et al., 1997).

Several *T. cruzi* surface molecules have been shown to promote infectivity. Among them gp30, gp35/50, gp82, gp85, trans-sialidase, and calcineurin B, are present in both metacyclic and tissue

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http://dx.doi.org/10.1016/j.imbio.2016.10.020
0171-2985/

culture-derived trypomastigotes, with Ca^{2+} signal-inducing activities, playing important variable roles in the parasite attachment to host cells and invasion (Yoshida and Cortez, 2008; Araya et al., 2008).

T. cruzi Calreticulin (TcCRT) is also an important *T. cruzi* virulence factor. It is a 45-kDa protein (Ramos et al., 1991), isolated, cloned and characterized in our laboratory and others (Ramos et al., 1991; Aguillon et al., 1995, 2000, 1997; Labriola et al., 1999). TcCRT has the same domains as in other species (N, P, C and S), with similar functions to human CRT such a potent antiangiogenic effect (Molina et al., 2005), participation in the ER quality control for glycoprotein folding and interaction with Cruzipain (Caramelo and Parodi, 2008), and among its infection-promotion properties, it can bind Thrombospondin 1 (TSP-1), thus enhancing the invasion of mouse embryonic fibroblasts (Johnson et al., 2012). *In vitro*, TcCRT is translocated from the endoplasmic reticulum (ER) to the trypomastigote flagellum emergence zone where, through its S domain, it captures C1 (Ferreira et al., 2004), the first component of the classical pathway of the complement system. The interaction of TcCRT with the collagenous tails of C1q inhibits the activation of C1, followed by downregulation of the whole classical pathway (Ferreira et al., 2004; Valck et al., 2010). This mechanism is used by trypomastigotes to resist complement-mediated lysis. Additionally, C1q binding to TcCRT on trypomastigotes promotes increased infectivity (Ramirez et al., 2011a,b), since it mimics a physiological apoptotic cell removal signal. Apoptotic cells translocate CRT to the external membrane. When this molecule binds C1q, an "eat me" signal is generated which, among other possibilities, is recognized by macrophages through their own CRT (cC1qR, also present on the cellular macrophage membrane) (Eggleton et al., 2000; Coppolino and Dedhar, 1998; Basu et al., 2001; Ogden et al., 2001). As a consequence, C1q binding to TcCRT on trypomastigotes allows the parasite to evade complement-mediated lysis and acts as a potent virulence factor (Ferreira et al., 2004). Once the parasite is phagocytized, it escapes the parasitophorous vacuole and continues its infective cycle. TcCRT virulence is confirmed by blocking its capacity to bind C1q with specific anti – rTcCRT $F(ab')_2$ antibody fragments (devoid of their C1-binding capacity, located in the Fc portion) leading to a considerable decrease in parasite internalization by macrophages (Ogden et al., 2001; Ramirez et al., 2011a,b). A second confirmation of TcCRT as a potent virulence factor emerges from our recent obtainment of epimastigotes where one of the two alleles coding for TcCRT was inactivated. These parasites show decreased resistance to complement lysis, impaired replication levels and marginal capacity to transform into the trypomastigote stage (metacyclogenesis), using conventional culture media (Sanchez Valdez et al., 2013; Sanchez-Valdez et al., 2014).

Not much is described about TcCRT in the epimastigote context. Lack of TcCRT on their surface (Sosoniuk et al., 2014) may explain, at least partially, why epimastigotes are non infective, both *in vivo* and *in vitro*. Moreover, *in vivo*, epimastigotes should have two related disadvantages (*i.e.*: incapacity to inactivate, *via* TcCRT, the classical pathway of the complement system, with consequent susceptibility to lysis, and inability to bind C1q, thus resulting in impaired infectivity). Epimastigotes lack TcCRT expression on their surface, however, intracellular expression of the protein, seems to be normal. In this context, TcCRT participates in ER quality control of glycoprotein folding (Caramelo and Parodi, 2008), and is translocated to the cytosol, in an ER Calcium-dependent manner (Labriola et al., 2010).

Hence, the *T. cruzi* infective process is largely C1–mediated, but TcCRT dependent. Specifically, TcCRT is responsible for at least 40% of the trypomastigote infectivity *in vitro* (the parasite capacity to contact and subsequently to infect host mammal cells) (Ramirez et al., 2011a,b), and about 80% in an *ex vivo* system (Castillo et al., 2013). For these reasons, we hypothesize that the binding of recombinant TcCRT (rTcCRT) to the epimastigote cellular membrane, promotes its internalization into host cells. Here we show that epimastigotes did bind exogenous rTcCRT to their cellular membrane and, in the presence of C1q, these parasite forms were internalized into CRT-sufficient (normal) fibroblasts and only marginally into their hemiallelic CRT-deficient counterpart.

2. Materials and methods

2.1. Epimastigotes

T. cruzi Dm28c epimastigotes were obtained from an axenic culture (Laboratory of Drug Biochemistry, Metabolism and Resistance, ICBM, Faculty of Medicine, University of Chile). The parasites were grown at 28 °C in modified Diamond's medium as previously described (Maya et al., 1997).

2.2. Quantification of exogenously bound rTcCRT to epimastigotes' cellular membrane

2.2.1. Functional evaluation of rTcCRT

Microtitration plates (Nunc MaxiSorp, USA) were coated with 100 μl/well of rTcCRT (5 μg/ml). Nonspecific binding sites were blocked with 3% w/v Bovine Serum Albumine (BSA) in Phosphate Buffer Saline (PBS). Then, 0–4 μg/ml of pure human C1q were added in a final volume of 100 μl in Veronal Buffer (5,5-diethylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione). Bound C1q, as an evaluation of rTcCRT function, was detected with an affinity-purified rabbit anti-human C1q antiserum (DAKO, Carpinteria, CA), followed by affinity-purified Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit Ig antibody (DAKO). HRP (405 nm) activity was assessed by addition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) with H_2O_2. Washing with PBS/0.05% Tween 20 was performed following each step. As controls, microtitration wells were coated as follows: (i) without rTcCRT, (ii) with rTcCRT detected with a rabbit anti-rTcCRT antibody followed by a goat anti-rabbit IgG (DAKO), (iii) with C1q detected with an affinity-purified rabbit anti-human C1q antiserum (DAKO, Carpinteria, CA), followed by an affinity-purified HRP-conjugated goat anti-rabbit Ig antibody (DAKO).

2.2.2. Functional evaluation of fluorescein isothiocyanate (FITC) labeled rTcCRT (F-rTcCRT)

rTcCRT was labeled with FITC using a commercial kit (FluoReporter® FITC Protein labeling kit, Invitrogen, USA). Microtitration plates were coated with 100 μl/well of F-rTcCRT (5 μg/ml). Nonspecific binding sites were blocked with 3% w/v BSA in PBS. Then, 0–4 μg/ml pure human C1q was added in a final volume of 100 μl in Veronal Buffer. Bound C1q was detected with an affinity-purified rabbit anti-human C1q antiserum (DAKO, Carpinteria, CA), followed by affinity-purified HRP-conjugated goat anti-rabbit Ig antibody (DAKO). HRP (405 nm) activity was assessed by addition of ABTS with H_2O_2. Washing with PBS/0.05% Tween 20 was performed following each step. Controls were designed as described above.

2.2.3. Binding of F-rTcCRT to Dm28c epimastigotes

These parasites were obtained from axenic cultures. Binding of F-rTcCRT to the cellular membrane of epimastigotes was detected using flow cytometry (FACScan, BD Biosciences). The parasites were washed twice in 2% w/v BSA in PBS by centrifugation at 450*g*, 5 m at 4 °C, suspended in 2% w/v BSA in PBS and treated with 3–12 μg of F-rTcCRT per 5×10^5 parasites for 30 m at room temperature (final volume 50 μl), followed by 3 additional washes. As control, the capacity of 3–12 μg of unlabeled rTcCRT per 5×10^5 parasites to inhibit the binding of 3 μg F-rTcCRT to parasites was tested. After

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the last washing step, the parasites were suspended in 100 μl of FACS buffer and a total of 10.000 events were observed.

2.3. Quantification of rTcCRT, C1q and host's murine CRT (MmCRT) effect over epimastigotes penetration

Wild type ($crt^{+/+}$, K41) or CRT-deficient ($crt^{-/-}$, K42) murine embryonic fibroblasts (MEF) were obtained in one of our laboratories (MM), by generating a Calreticulin knockout vector (Mesaeli et al., 1999).

2.3.1. Infection of murine embryonic fibroblasts (MEF) K41 with epimastigotes treated with rTcCRT and C1q

Infection was performed by co-incubating cells with parasites, as previously described (Ramirez et al., 2011a,b). MEF K41 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (SIGMA, USA), supplemented with 10% fetal bovine serum (SFB), 2 mM L-Glutamine and penicillin/streptomycin, at 37 °C, in 5% CO_2. 10^6 epimastigotes were tested as follows: (i) without treatment, (ii) with 6 μg of rTcCRT, (iii) with 10 μg of C1q, (iv) with 6 μg of rTcCRT followed by 10 μg of C1q, (v) with 6 μg of rTcCRT followed by 15 μg of a polyclonal rabbit anti-rTcS F(ab')$_2$ antibody fragment and 10 μg of C1q, (vi) with 6 μg of rTcCRT followed by 15 μg of a polyclonal rabbit anti-rTcS F(ab')$_2$ antibody fragment, (vii) with15 μg of a rabbit polyclonal anti-rTcS F(ab')$_2$ antibody fragment, (viii) with15 μg of a rabbit polyclonal anti-rTcS F(ab')$_2$ antibody fragment followed by 10 μg of C1q. They were all co-cultured with the fibroblasts. All treatments were incubated 30 m at room temperature. After each incubation the parasites were washed by centrifugation with 1× PBS/BSA 2% and co-cultured with MEF K41 $crt^{+/+}$, in a 10:1 ratio (10^6 parasites per 10^5 cells), for 4 h at 37 °C in 5% CO_2. Non – internalized parasites were removed by exhaustive washing with 1X PBS and then, the samples were stained with 2-(4-amidinophenyl)-1H-indole-5-carboxamidine (DAPI) as described in 3.1.1. The samples were analyzed by fluorescence microscopy (Nikon Eclipse E400), and the internalized parasites, every 100 cells for each condition, were counted.

2.3.2. DAPI nuclei staining

Samples were fixed with cold 90% methanol for 20 m at 4 °C, washed twice with 1X PBS, and incubated with DAPI (1 mg/ml) for 2 m at room temperature. After the first washing step with 1X PBS, the cells were mounted in fluorescence slides. Samples were analyzed by fluorescence microscopy.

2.3.3. Infection of MEF K42 with rTcCRT and C1q treated epimastigotes

The infection assay was performed as described in 3.1, except that MEF K42 cells ($crt^{-/-}$) were cultured in DMEM media, in a similar experimental design as described in 3.1.

Fig. 1 shows typically internalized (A) and externally attached (B) parasites. A parasite was considered as internalized, by judging the nucleus location and fluorescence intensity

2.4. Statistical analysis

Data were statistically validated using one tail unpaired *t*-test and GraphPad Prism 5 software. FCS Express 4 Flow Research Edition was used to analyze the flow cytometry data.

3. Results

3.1. Exogenous rTcCRT binds to epimastigotes' cellular membrane

3.1.1. F-rTcCRT retains its capacity to bind C1q

Since rTcCRT binds to the collagenous portion of C1q (Ferreira et al., 2004), we used this property as a criterion to evaluate whether the conjugation with the fluorophore (FITC) modifies this protein function. By ELISA we evaluated the C1q binding capacity of both rTcCRT and F-rTcCRT. Microtitration plates were coated with either rTcCRT (Fig. 2A) or F-rTcCRT (Fig. 2B) and treated with increasing C1q concentrations. According to a previously described function, in Fig. 2A we show that under an rTcCRT excess, C1q binds in a dose-dependent manner. Labeling of rTcCRT with FITC does not change its C1q binding capacity. In Fig. 2C, a highly significant correlation between both assays was obtained ($p < 0.0001$), indicating that both soluble receptors are equivalent in their C1q binding capacity.

3.1.2. F-rTcCRT binds to the cellular membrane of Dm28c epimastigotes

F-rTcCRT binds to the epimastigote cellular membrane in a dose-dependent manner (Fig. 3A). On the other hand, F-rTcCRT and its unlabeled counterpart compete for a membrane receptor on the epimastigote cellular membrane (Fig. 3B), as determined by flow cytometry. Fig. 3C–D show a representative histogram from each assay.

3.2. Quantification of rTcCRT, C1q and MmCRT effect over epimastigotes internalization

3.2.1. Different treatments to epimastigotes, in the presence or absence of C1q, increase the parasite internalization ratio into MEF K41 CRT+/+ cells

The internalization of epimastigotes by MEFs was observed by fluorescence microscopy. Cells with at least one internalized parasite were quantified. The presence of C1q and exogenous TcCRT promotes an increase in the internalization of epimastigotes by CRT-sufficient fibroblasts (Fig. 4A). This increase is reversed by anti-TcS domain F(ab')$_2$ antibody fragments, since they block the rTcCRT/C1q binding.

3.2.2. Epimastigotes treated with rTcCRT and C1q are not internalized in MEF K42 crt-/–

We have proposed that in the *T. cruzi* infective process, C1q acts as a bridge between parasite and host CRTs. Since MEF K42 $crt^{-/-}$ cells lack CRT, using the same treatment groups as in 2.1, an important decrease in the parasite internalization ratio was observed (Fig. 4B). Moreover, the different treatments did not induce modifications in the internalization ratio.

A comparison of both assays is shown in Fig. 4. Parasite internalization was evaluated by fluorescence microscopy.

4. Discussion

T. cruzi infection is highly complex, mediated by a variety of different molecules, both at the membrane and cytoplasmic levels. We have shown that TcCRT, by virtue of its capacity to bind C1q, importantly mediates trypomastigote infectivity (Ramirez et al., 2011a,b). While in infective trypomastigotes TcCRT is found in both intra and extracellular locations, especially in the flagellum emergence zone, in non-infective epimastigotes, expression of TcCRT on the membrane is marginal or absent (Ferreira et al., 2004; Ramirez et al., 2011a,b; Sosoniuk et al., 2014). For these reasons, we explored whether the decreased expression of TcCRT or lack thereof on *T. cruzi* Dm28c epimastigotes could explain, at least partly, its incapacity to infect mammal cells.

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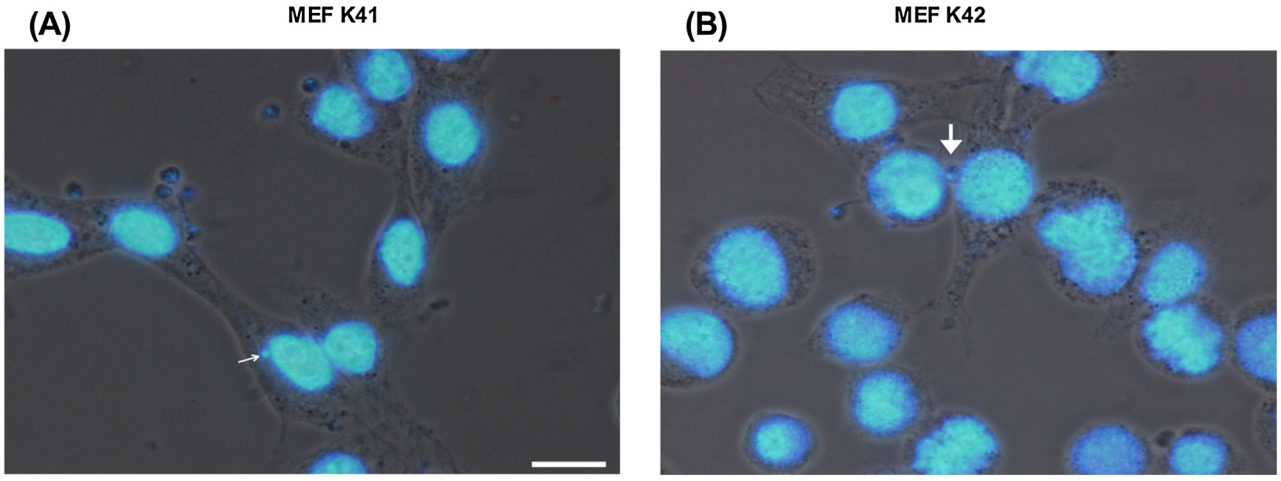

Fig. 1. Typically internalized (A) and externally attached (B) parasites. To consider a parasite as internalized, both nucleus proximity and fluorescence intensity were considered. If in the contrast phase the parasite was seen outside the cell, it was excluded from the quantification. White arrows indicate parasites. Magnification bar represents 10 μm, and both images are a superposition of the contrast phase with an immunofluorescence photograph.

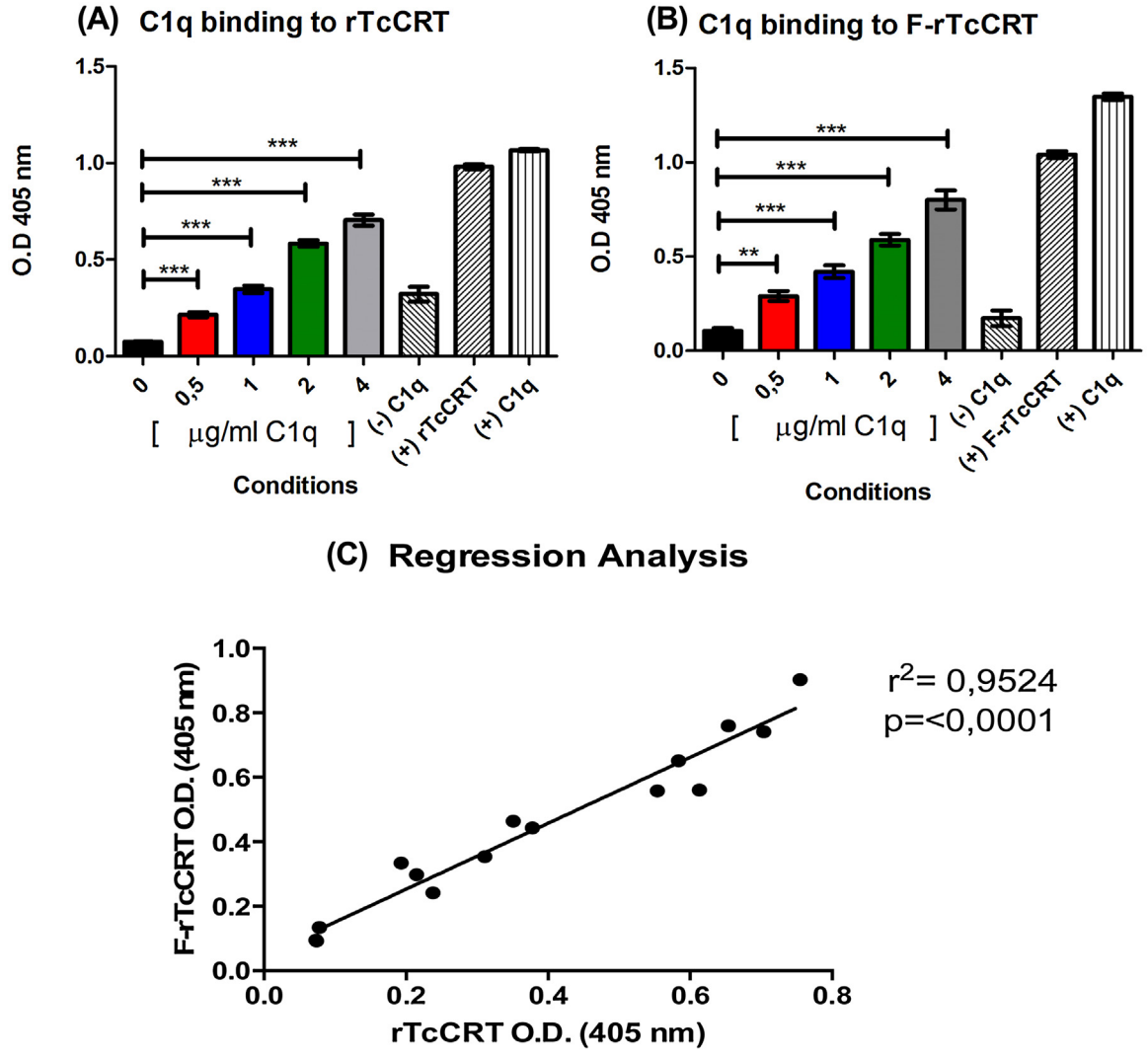

Fig. 2. F-rTcCRT preserves the C1q-binding function of its unlabeled counterpart. rTcCRT and F-rTcCRT binding capacity was measured by ELISA. Microtitration plates were sensitized with rTcCRT (A) or F-rTcCRT (B) and incubated with increasing concentrations of C1q. C1q binding to rTcCRT was detected with a goat anti-rabbit IgG HRP-conjugated antibody. As negative control, wells were blocked with 1X PBS/BSA 3% w/v, and as positive controls the wells were sensitized with rTcCRT, F-rTcCRT or C1q, as indicated. Results are shown as averages of triplicate observations and their SEMs. Data were analyzed using unpaired one tail *t*-test. In (C) a regression analysis between both assays is shown.

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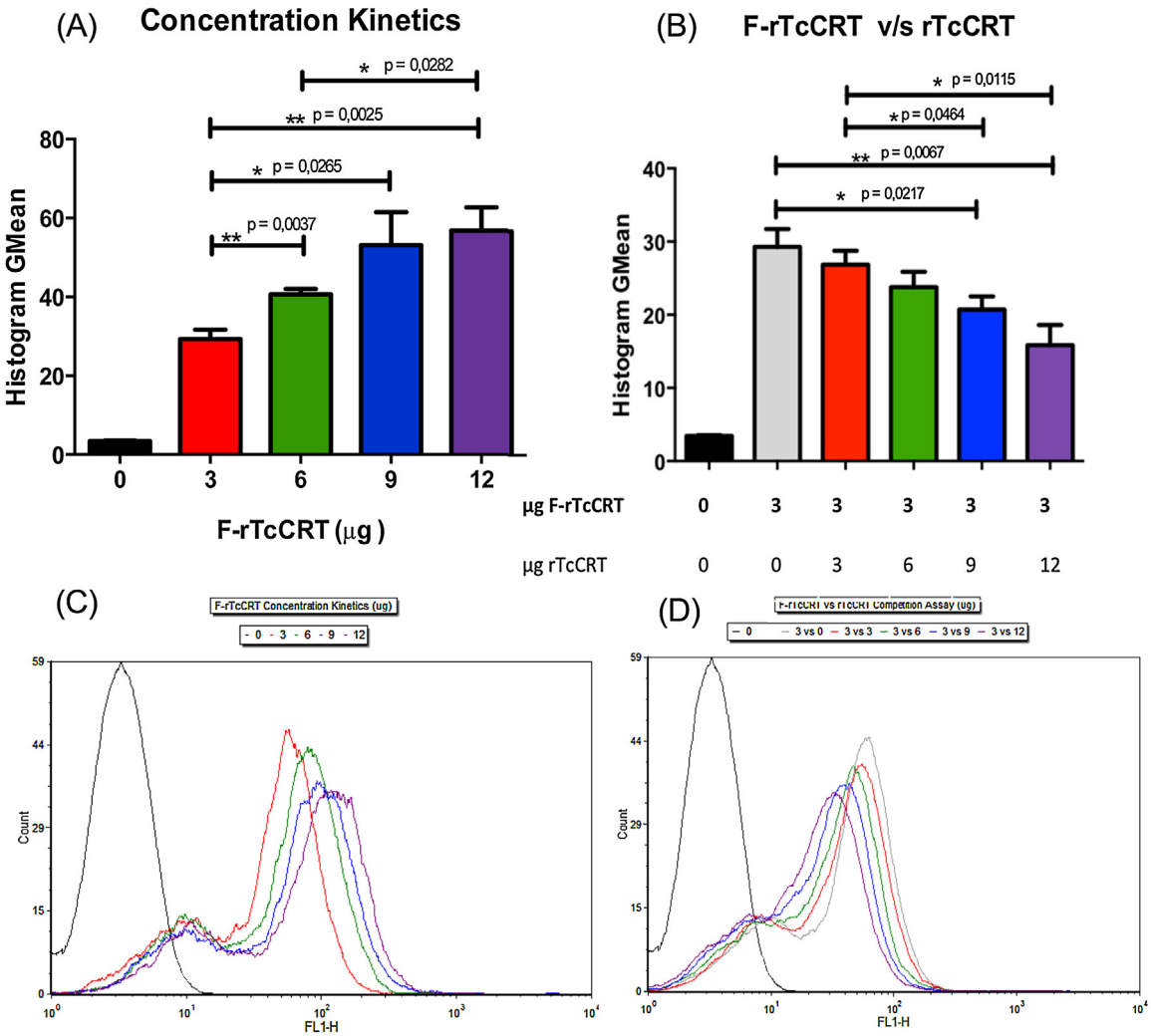

Fig. 3. rTcCRT binds in a specific and concentration-dependent manner to the epimastigote membrane: Direct binding of rTcCRT to the epimastigote external cellular membrane was detected by flow cytometry. (A) Concentration kinetics of F-rTcCRT binding. (B) A basal F-rTcCRT concentration (3 µg/500.000 parasites) was mixed with increasing rTcCRT concentrations, as indicated. These mixtures were then incubated with the parasites. (C) and (D) Representative flow cytometry graphics of the respective A and B panels. Bar graphics show the Geometric Mean of the readings obtained and are representative of triplicate experiments. Data were analyzed using unpaired one tail *t*-test showing the SEMs.

Since the TcCRT aminoacidic sequence does not incorporate a transmembrane domain, the lack of TcCRT on the parasite could be caused by a defect somewhere in the translocation pathway and/or a lack of anchorage molecules needed to complete the binding process of the protein to the cellular membrane. Perhaps, in epimastigotes, similar to mammals, CD91- and ERp57-like molecules (Gold et al., 2010) are also involved. To evaluate whether CRT binds to the cellular membrane of epimastigotes, rTcCRT was labeled with FITC (F-rTcCRT). Since the labeling process can modify the protein's functionality, we first determined whether F-rTcCRT could maintain its capacity to bind C1q (Fig. 2A–B). Moreover, a highly significant correlation between both assays was also obtained (Fig. 2C) and the binding efficiency of both molecules was similar, as indicated by the curve slope.

We then evaluated the capacity of epimastigotes to bind TcCRT, by measuring the binding of F-rTcCRT (Fig. 3). The protein binds to the parasite surface, so the existence of CRT anchoring molecules on these parasite forms is conceivable. Moreover, the binding of rTcCRT to epimastigotes is dose dependent (Fig. 3A) and specific, as shown in a competition assay between F-rTcCRT and rTcCRT (Fig. 3B). Even though unlabeled rTcCRT does displace the binding of F-rTcCRT, this binding-inhibition does not occur in a 1:1 ratio. We believe that this is because the labeling process increases significantly the molecular weight of each rTcCRT molecule, giving a physical advantage over the cold protein to bind to the parasite surface. Alternatively or concomitantly, since we did not use F-rTcCRT under saturating conditions, free binding sites for unlabeled competing rTcCRT may be available. Besides the possibility that TcCRT binds to CD91 and ERp57-like cell surface molecules, there is the possibility that membrane proteins such as $\alpha_2\beta_1$ Integrins (Gold et al., 2010) may also participate.

As seen in Fig. 4, baseline epimastigote penetration seems to be restricted to *in vitro* cultures, since *in vivo* they are easily cleared out by complement action. The high internalization ratio observed when epimastigotes are treated only with rTcCRT (Fig. 4) may be explained, at least partly, by remnants of functional bovine C1q present in the heat inactivated FBS.

Previously, we have shown that the S domain of TcCRT is involved in its binding to C1q (Ferreira et al., 2004). Accordingly, as shown in Fig. 4A, when parasites were pre-treated with anti-rTcS $F(ab')_2$ antibody fragments (group 5), the binding to C1q was blocked, and parasite internalization returned to control levels. This inhibitory effect was not evident when parasites were treated with rTcCRT and anti-rTcS $F(ab')_2$ antibody fragments (group 6). Perhaps,

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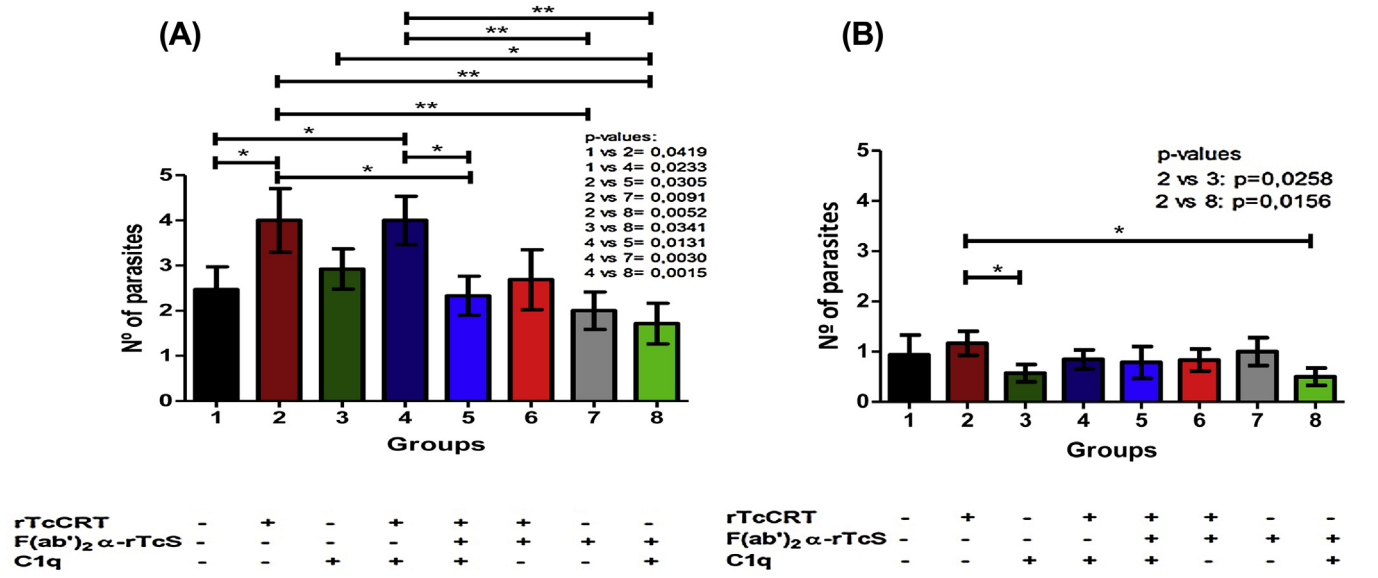

Fig. 4. rTcCRT treatment, in the presence or absence of C1q, increases the internalization of parasites in (A) K41 MEF cells (wild-type) but not in (B) K42 MEF cells (CRT-deficient): The parasite internalization was evaluated by DAPI staining, in fluorescence microscopy. Bars indicate the number of internalized parasites every 100 cells, and represent a triplicate. Data was analyzed using unpaired one tail *t*-test, showing the SEMs. Using ANOVA, K41 and K42 fibroblasts were analyzed in their capacity to internalize epimastigotes, in the presence or absence of C1q and/or rTcCRT. This capacity was highly superior in wild type fibroblasts ($p < 0.0001$).

not all rTcCRT molecules on the parasite cellular membrane were blocked.

On the other hand, in the MEF K42 *crt*$^{-/-}$ cells, basal internalization of epimastigotes notoriously decreased as compared to the internalization on K41 wild-type cells (Fig. 4B). Additionally, different treatments did not produce a difference in the parasite internalization ratio in this cellular line, most likely due to the absence of endogenous CRT.

In this study we describe that epimastigote treatment with both exogenous rTcCRT and C1q promotes internalization of this parasite form into fibroblasts expressing the mammal chaperone. The fact that CRT-deficient fibroblasts are much less prone to be infected with TcCRT/C1q sensitized epimastigotes (Fig. 4) indicates that CRT, from both parasite and host cell origin, is necessary to execute the complement-mediated infective process. Further studies are needed to define whether internalized epimastigotes are able to continue its differentiation process within the host cell.

In synthesis: (i) Epimastigotes bind rTcCRT onto their external membrane. (ii) Epimastigotes increase their penetration into CRT-sufficient cells, mediated by an interaction with rTcCRT and C1q, and (iii) In CRT-deficient cells, epimastigotes penetration is absent or marginal, and independent of the TcCRT and/or C1q treatment provided.

The mechanism used by *T. cruzi* to translocate CRT to the cellular membrane is still not clear. Whether the molecular mechanisms described by Wiersma et al. (Wiersma et al., 2015), to explain failures in CRT translocation in mouse and human models have predictive value for *T. cruzi* epimastigotes, remains to be determined. These notions are at least partially backed up by the fact that epimastigotes hemiallelic K.O. for TcCRT (*TcCRT+/–*) or overexpressing the gene for TcCRT (*TcCRT+/++*), show a notoriously decreased or increased resistance to complement and infectivity, respectively, as compared to their wild type counterparts (Sanchez Valdez et al., 2013; Sanchez-Valdez et al., 2014).

Conflict of interest

The authors declare that there are no conflict of interest.

Acknowledgments

This work was executed in the Laboratory of Immunology of the Microbial Aggression, Program of Immunology, Biomedical Sciences Institute, University of Chile, Chile, with financial support from the Associative Investigation Project ACT-112 and FONDECYT 1095095. Supported by a Grant from the Canadian Institutes of Health Research (CIHR) (to M.M. MOP15291). We thank Mr. Enrique Moraga and Mr. Álvaro Rojas for their fundamental technical support.

References

Aguillon, J.C., Bustos, C., Vallejos, P., Hermosilla, T., Morello, A., Repetto, Y., Hellman, U., Orn, A., Ferreira, A., 1995. Purification and preliminary sequencing of Tc-45, an immunodominant Trypanosoma cruzi antigen: absence of homology with cruzipain, cruzain, and a 46-kilodalton protein. Am. J. Trop. Med. Hyg. 53, 211.
Aguillon, J.C., Harris, R., Molina, M.C., Colombo, A., Cortes, C., Hermosilla, T., Carreno, P., Orn, A., Ferreira, A., 1997. Recognition of an immunogenetically selected Trypanosoma cruzi antigen by seropositive chagasic human sera. Acta Trop. 63, 159.
Aguillon, J.C., Ferreira, L., Perez, C., Colombo, A., Molina, M.C., Wallace, A., Solari, A., Carvallo, P., Galindo, M., Galanti, N., Orn, A., Billetta, R., Ferreira, A., 2000. Tc45, a dimorphic Trypanosoma cruzi immunogen with variable chromosomal localization, is calreticulin. Am. J. Trop. Med. Hyg. 63, 306.
Araya, J.E., Cornejo, A., Orrego, P.R., Cordero, E.M., Cortez, M., Olivares, H., Neira, I., Sagua, H., da Silveira, J.F., Yoshida, N., Gonzalez, J., 2008. Calcineurin B of the human protozoan parasite Trypanosoma cruzi is involved in cell invasion. Microbes Infect. 10, 892.
Basu, S., Binder, R.J., Ramalingam, T., Srivastava, P.K., 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity 14, 303.
Brener, Z., 1973. Biology of Trypanosoma cruzi. Annu. Rev. Microbiol. 27, 347.
Canals, M., Bustamante, R.O., Ehrenfeld, M.H., Cattan, P.E., 1998. Assessing the impact of disease vectors on animal populations. Acta Biotheor. 46, 337.
Caramelo, J.J., Parodi, A.J., 2008. Getting in and out from calnexin/calreticulin cycles. J. Biol. Chem. 283, 10221.
Castillo, C., Ramirez, G., Valck, C., Aguilar, L., Maldonado, I., Rosas, C., Galanti, N., Kemmerling, U., Ferreira, A., 2013. The interaction of classical complement component C1 with parasite and host calreticulin mediates trypanosoma cruzi infection of human placenta. PLoS Negl. Trop. Dis. 7, e2376.
Chagas, C., 1909. Nova tripanozomiase humana: estudos sobre a morfolojia e o ciclo evolutivo do Schizotrypanum cruzi n. gen., n. sp., ajente etiolojico de nova entidade morbida do homem. Mem. Inst. Oswaldo Cruz, 159.
Chagas, C., 1911. Nova entidade morbida do homem: rezumo geral de estudos etiolojicos e clinicos. Mem. Inst. Oswaldo Cruz 219.
Coppolino, M.G., Dedhar, S., 1998. Calreticulin. Int. J. Biochem. Cell Biol. 30, 553.

Please cite this article in press as: Sosoniuk-Roche, E., et al., Exogenous Calreticulin, incorporated onto non-infective *Trypanosoma cruzi* epimastigotes, promotes their internalization into mammal host cells. Immunobiology (2016), http://dx.doi.org/10.1016/j.imbio.2016.10.020

Coura, J.R., Vinas, P.A., 2010. Chagas disease: a new worldwide challenge. Nature 465, S6.
Eggleton, P., Tenner, A.J., Reid, K.B., 2000. C1q receptors. Clin. Exp. Immunol. 120, 406.
Ferreira, V., Valck, C., Sanchez, G., Gingras, A., Tzima, S., Molina, M.C., Sim, R., Schwaeble, W., Ferreira, A., 2004. The classical activation pathway of the human complement system is specifically inhibited by calreticulin from Trypanosoma cruzi. J. Immunol. 172, 3042.
Gold, L.I., Eggleton, P., Sweetwyne, M.T., Van Duyn, L.B., Greives, M.R., Naylor, S.M., Michalak, M., Murphy-Ullrich, J.E., 2010. Calreticulin: non-endoplasmic reticulum functions in physiology and disease. FASEB J. 24, 665.
Johnson, C.A., Kleshchenko, Y.Y., Ikejiani, A.O., Udoko, A.N., Cardenas, T.C., Pratap, S., Duquette, M.A., Lima, M.F., Lawler, J., Villalta, F., Nde, P.N., 2012. Thrombospondin-1 interacts with Trypanosoma cruzi surface calreticulin to enhance cellular infection. PLoS One 7, e40614.
Labriola, C., Cazzulo, J.J., Parodi, A.J., 1999. Trypanosoma cruzi calreticulin is a lectin that binds monoglucosylated oligosaccharides but not protein moieties of glycoproteins. Mol. Biol. Cell 10, 1381.
Labriola, C.A., Conte, I.L., Lopez Medus, M., Parodi, A.J., Caramelo, J.J., 2010. Endoplasmic reticulum calcium regulates the retrotranslocation of Trypanosoma cruzi calreticulin to the cytosol. PLoS One 5.
Maya, J.D., Repetto, Y., Agosin, M., Ojeda, J.M., Tellez, R., Gaule, C., Morello, A., 1997. Effects of nifurtimox and benznidazole upon glutathione and trypanothione content in epimastigote, trypomastigote and amastigote forms of Trypanosoma cruzi. Mol. Biochem. Parasitol. 86, 101.
Mesaeli, N., Nakamura, K., Zvaritch, E., Dickie, P., Dziak, E., Krause, K.H., Opas, M., MacLennan, D.H., Michalak, M., 1999. Calreticulin is essential for cardiac development. J. Cell Biol. 144, 857.
Molina, M.C., Ferreira, V., Valck, C., Aguilar, L., Orellana, J., Rojas, A., Ramirez, G., Billetta, R., Schwaeble, W., Lemus, D., Ferreira, A., 2005. An in vivo role for Trypanosoma cruzi calreticulin in antiangiogenesis. Mol. Biochem. Parasitol. 140, 133.
Ogden, C.A., deCathelineau, A., Hoffmann, P.R., Bratton, D., Ghebrehiwet, B., Fadok, V.A., 2001. C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J. Exp. Med. 194, 781.
Ramirez, G., Valck, C., Ferreira, V.P., Lopez, N., Ferreira, A., 2011a. Extracellular Trypanosoma cruzi calreticulin in the host-parasite interplay. Trends Parasitol. 27, 115.
Ramirez, G., Valck, C., Molina, M.C., Ribeiro, C.H., Lopez, N., Sanchez, G., Ferreira, V.P., Billetta, R., Aguilar, L., Maldonado, I., Cattan, P., Schwaeble, W., Ferreira, A., 2011b. Trypanosoma cruzi calreticulin: a novel virulence factor that binds complement C1 on the parasite surface and promotes infectivity. Immunobiology 216, 265.
Ramos, R., Juri, M., Ramos, A., Hoecker, G., Lavandero, S., Pena, P., Morello, A., Repetto, Y., Aguillon, J.C., Ferreira, A., 1991. An immunogenetically defined and immunodominant Trypanosoma cruzi antigen. Am. J. Trop. Med. Hyg. 44, 314.
Sanchez Valdez, F.J., Perez Brandan, C., Zago, M.P., Labriola, C., Ferreira, A., Basombrio, M.A., 2013. Trypanosoma cruzi carrying a monoallelic deletion of the calreticulin (TcCRT) gene are susceptible to complement mediated killing and defective in their metacyclogenesis. Mol. Immunol. 53, 198.
Sanchez-Valdez, F.J., Perez Brandan, C., Ramirez, G., Uncos, A.D., Zago, M.P., Cimino, R.O., Cardozo, R.M., Marco, J.D., Ferreira, A., Basombrio, M.A., 2014. A monoallelic deletion of the TcCRT gene increases the attenuation of a cultured Trypanosoma cruzi strain, protecting against an in vivo virulent challenge. PLoS Negl. Trop. Dis. 8, e2696.
Sosoniuk, E., Vallejos, G., Kenawy, H., Gaboriaud, C., Thielens, N., Fujita, T., Schwaeble, W., Ferreira, A., Valck, C., 2014. Trypanosoma cruzi calreticulin inhibits the complement lectin pathway activation by direct interaction with L-Ficolin. Mol. Immunol. 60, 80.
Valck, C., Ramirez, G., Lopez, N., Ribeiro, C.H., Maldonado, I., Sanchez, G., Ferreira, V.P., Schwaeble, W., Ferreira, A., 2010. Molecular mechanisms involved in the inactivation of the first component of human complement by Trypanosoma cruzi calreticulin. Mol. Immunol. 47, 1516.
Yoshida, N., Cortez, M., 2008. Trypanosoma cruzi: parasite and host cell signaling during the invasion process. Subcell. Biochem. 47, 82.
Wiersma, V.R., Michalak, M., Abdullah, T.M., Bremer, E., Eggleton, P., 2015. Mechanisms of translocation of ER chaperones to the cell surface and immunomodulatory roles in cancer and autoimmunity. Front. Oncol. 5, 7.

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