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Trypanosoma cruzi calreticulin: A novel virulence factor that binds complement C1 on the parasite surface and promotes infectivity

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ABSTRACT

In *Trypanosoma cruzi*, calreticulin (TcCRT) translocates from the endoplasmic reticulum (ER) to the area of flagellum emergence. We propose herein that the parasite uses this molecule to capture complement C1, in an infective apoptotic mimicry strategy. Thus, TcCRT/C1 interactions, besides inhibiting the classical pathway of complement activation as previously shown in our laboratories, will also promote infectivity. This fact correlates with significant increases in TcCRT mRNA levels during early infection stages of a VERO cell line. *In vitro*, the collagenous and globular C1q domains simultaneously bind TcCRT and antigen aggregated Igs, respectively. Accordingly, mouse immunizations with TcCRT induced humoral responses that, after challenge, correlated with increased parasitemia. Thus, on the parasite surface, whole Igs anti-TcCRT promote C1 deposits on trypomastigotes while, as expected, $F(ab')_2$ fragments decrease it. Likewise, pretreatment of the parasites with whole anti-TcCRT antibodies augmented parasitemia and mortality in mice. In contrast, pretreatment with $F(ab')_2$ fragments anti-TcCRT, devoid of their capacity to provide additional C1q binding sites, was protective. Most important, while pretreatment of trypomastigotes with C1q increased infectivity in the RAW murine cell line, as well as mice mortality and parasitemia, the $F(ab')_2$ fragments significantly interfered with the C1q-dependent infectivity. Differently from other surface molecules involved in infectivity, TcCRT uses C1 as an adaptor molecule to recognize host cells. As expected, since TcCRT is one of several cell surface parasite molecules participating in infectivity, attempts to interfere with the C1/TcCRT interactions with $F(ab')_2$ fragments, were moderately but significantly effective, both *in vitro* and *in vivo*.

Introduction

Trypanosoma cruzi is the protozoan that causes Chagas' disease (Chagas 1909), an acute and chronic illness affecting 20 million people in Latin America and causing 50,000 deaths per year (Dias et al. 2002; WHO 2002). Several *T. cruzi* surface molecules promote infectivity. Gp82, gp30, gp35/50, trans-sialidase, gp85 and calcineurin B, are all metacyclic and tissue culture-derived trypomastigote surface molecules, with Ca^{2+} signal-inducing activities. They play important variable roles in host cell invasion (Araya et al. 2008; Yoshida and Cortez 2008). Here we propose that *T. cruzi* trypomastigote calreticulin (TcCRT), differently from the other described parasite surface receptors involved in infectivity, uses complement component C1 as an adaptor molecule to recognize host cells. We also propose that attempts to interfere with the C1/TcCRT interactions with whole Igs or their $F(ab')_2$ fragments will have opposite and predictable outcomes, both *in vitro* and *in vivo*. Previously, we have shown that *T. cruzi* calreticulin (TcCRT) translocates from the endoplasmic reticulum (ER) to the area of flagellum emergence on the cell surface (Ferreira et al. 2004). There, TcCRT allows the parasite to recruit C1, thus inhibiting the classical pathway of human

Abbreviations: CRT, calreticulin; TcCRT, *Trypanosoma cruzi* calreticulin; rTcCRT, recombinant TcCRT without its leader and KEDL sequences; pDNA/*TcCRT* and pSecTag/*TcCRT*, plasmids expressing the gene coding for TcCRT without its leader and KEDL sequences; pGM-CSF, plasmid expressing the gene coding for GM-CSF; ER, endoplasmic reticulum.

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complement activation (Ferreira et al. 2004). In 1989, other laboratory (Rimoldi et al. 1989) showed that C1q plays a role in increasing parasite infectivity *in vitro*, although the parasite receptor for the complement component was not identified. Here, we propose that such receptor is parasite surface CRT and that intervention of the natural TcCRT/C1 interaction with laboratory-modified Igs, such as $F(ab')_2$ fragments, will result in decreased parasite infectivity.

Pharmacological treatment of the infection, although effective in some cases, is complicated by the toxicity of the main drugs used (Nifurtimox and Benznidazole) (Castro and Diaz de Toranzo 1988). Therefore, identification and immune intervention of different molecular targets, such as those involved in *T. cruzi* infectivity and in the parasite capacity to inactivate the complement system, together with conventional pharmacological therapies, may result in synergic or even additive effects.

We have cloned, sequenced and expressed the TcCRT gene. It codes for a 45 kDa protein containing the "KDEL"-ER retention sequence and presents more than 50% identity to the human counterpart (Aguillon et al. 1995, 2000a). Most important, natural and experimental infections expose TcCRT to B cells, since the production of anti-TcCRT IgG is readily detected in infected humans (Aguillon et al. 1997; Marcelain et al. 2000), mice (Aguillon et al. 2000b; Ribeiro et al. 2009) and rabbits (Aguilar et al. 2005; Ribeiro et al. 2009). These antibodies, upon binding to cell surface TcCRT, should promote additional C1q capture by the parasite, a reflection of the capacity of the collagenous and globular C1q domains to simultaneously bind TcCRT and the Fc portions of antigen aggregated Igs, respectively (Ferreira et al. 2004; Painter 1984).

Similarly, ligands on the surface of apoptotic cells, such as mammalian CRT among others, can recruit C1, thus promoting the necessary phagocytosis and clearance of these cells (Obeid et al. 2007). Thus, the *in vitro* and *in vivo* evidences presented here strongly support the proposal that translocation of TcCRT from the ER to the membrane, not only will inhibit the classical pathway of complement but, in a parasite apoptotic mimicry effort, it will also promote infectivity, most likely by generating effective C1q-mediated "eat me signals".

Materials and methods

Semiquantitative RT-PCR detection of TcCRT transcription during the T. cruzi cycle

VERO cells (from the anthropomorphic *Cercopithecus aethiops*), cultured for 24 h, were infected with MF trypomastigotes, as previously described (Faundez et al. 2005). Total RNA was extracted (TRIZOL, Invitrogen, Life Technologies, USA) from infected cells and from supernatant trypomastigotes, harvested at different times post-infection. 1 μg total RNA was reverse transcribed (Super Script First-Strand Synthesis System, Invitrogen, Life Technologies, USA). First we measured TcCRT mRNA expression up to 96 h post-infection, using forward 5′-GGAATTCCACGGTGTACTTCCACGAG-3′ and reverse 5′-GGAATTCCATCACG GTCACCCTTTTT-3′ primers that yielded a 1135-bp product. Then, at 48 h (amastigotes) and 144 h (free trypomastigotes) post-infection, we used the same TcCRT forward primer and a reverse one (5′-CTCGAGCCAGTCTTCTTCGAGCTG-3′) to amplify a 542-bp TcCRT message. For PCR amplification of *T. cruzi* GAPDH, an internal control, forward 5′-GACACTTGTGATGGGCGTGAAC-3′ and reverse 5′-CGTGCTCGGAATGATGTTGAC-3′ primers generated a 250-bp product. For *C. aethiops* CRT, the forward 5′-TCTCAGTTCCGGCAAGTTCT-3′ and reverse 5′-TCTGAGTCTCCGTGCATGTC-3′ primers yielded a 231-bp product. Forward 5′-CGTGCGTGACATTAAGGAGA-3′ and reverse 5′-AGCACTGTGTTGGCGTACAG-3′ primers were used for *C. aethiops* β-actin, an internal 273-bp control. Preliminary experiments demonstrated that all PCR reactions were at the exponential amplification phase.

Measurement of C1q binding to solid phase bound TcCRT/anti-TcCRT immune complexes

Microtitration plates sensitized with rTcCRT (1.28 μM in PBS) were blocked for 2 h at 37 °C with 3% (w/v) BSA in PBS (PBS–BSA). After washing, wells were coated with rabbit polyclonal anti-rTcCRT antibodies (Aguilar et al. 2005) in 1% (w/v) PBS-BSA or with media alone. Different amounts of human C1q (Complement Technology, Tyler, Texas, USA), diluted in 1% (w/v) PBS–BSA, were added and incubated for 1 h at 37 °C. C1q specific binding to immobilized rTcCRT, in the presence or absence of anti-rTcCRT, was detected with goat anti-human C1q (Sigma–Aldrich, USA), followed by affinity purified horseradish peroxidase (HRP)-conjugated anti-goat antibodies (Calbiochem, USA).

Assessment of T. cruzi infectivity in actively TcCRT immunized animals

All experiments with animals were approved by the Institutional Bioethics Committee, in agreement with international criteria. Proper veterinary supervision was permanently provided. Bloodstream Tulahuén trypomastigotes were maintained in irradiated BALB/c mice from our Animal Facility.

The PCR-amplified *TcCRT* gene product, without its leader and KEDL sequence, was ligated into pSecTag2B and pcDNA 3.1(−) vectors (Invitrogen, USA), at EcoRI restriction sites (pSecTag/*TcCRT* and pDNA/*TcCRT*, respectively). Reverse and forward primers used were 5′-GGAATCCATCACGGTCACCCTTTTT-3′ and 5′-GGAATTCACCA TGACGGTGTACTTCCA-3′, respectively (Gibco BRL, Life Technologies, USA). pSecTag/*TcCRT* includes, at the 5′-end, the murine immunoglobulin κ chain leader sequence, to allow TcCRT secretion. Endotoxin free plasmids were obtained from pSecTag/*TcCRT*, pDNA/*TcCRT*, pSecTag2B, or pGM-CSF (for murine GM-CSF, kindly donated by Dr. Oscar R. Burrone, Center for Genetic Engineering and Biotechnology, Italy) (Aida and Pabst 1990; Sanbrook 1989).

Two groups (6 animals each) were immunized twice s.c., 7 days apart, with 50 μg rTcCRT, followed by an i.p. injection a week later. At day 46, an i.p. booster was administered. The first injection contained CFA, while the last three contained its incomplete version (IFA). Controls received PBS with CFA or IFA.

Four groups (6 animals each) received four i.m. injections every 15 days and at day 81, containing 50 μg of each of the following constructs: pSecTag/*TcCRT* plus pSecTag2B or plus pGM/CSF (used to stimulate a humoral immune response (Gerloni et al. 1998; Iwasaki et al. 1997; Mahvi et al. 1997; Mellstedt et al. 1999; Pasquini et al. 1997; Warren and Weiner 2000), pSecTag2B plus pDNA/*TcCRT* or plus pGM/CSF (negative control).

For combined genetic and conventional immunizations, a similar protocol was used, except that, after the third DNA injection (day 46), all mice received 50 μg rTcCRT s.c. in CFA. Controls included immunizations with pSecTag2B plus pGM-CSF and PBS. Blood was collected before each immunization and 1 week after the last one.

Detection of specific antibodies in TcCRT immunized animals

Nunc Maxisorb polystyrene plates (Fisher Scientific, USA) were coated with 3 μg/mL rTcCRT per well, diluted in carbonate buffer, pH 9.6, followed by overnight incubation at 4 °C. Control wells received buffer alone and were blocked with 0.5% (w/v) soybean

proteins (PBS–SBP) (Aguillón et al. 1992). Each step was followed by washings with PBS/0.05% (v/v) Tween-20. Sera from immunized mice, diluted in PBS–SBP, were added in triplicate wells, followed by incubation (2 h at 37 °C). Affinity purified HRP-conjugated goat anti-mouse Igs (Sigma–Aldrich, USA) were added, followed by standard detection procedures.

All immunized mice were challenged i.p., at day 54, with 500 bloodstream trypomastigotes. Clinical parameters, including parasitemia, were monitored until 25 days post-infection, when the animals were euthanized.

Flow cytometry analysis for C1q binding to trypomastigotes and epimastigotes in the presence of Igs anti-TcCRT and their F(ab′)$_2$ fragments

3×10^6 trypomastigotes or 2.5×10^6 epimastigotes from the Dm28c clone were incubated for 30 min at 4 °C, after adding 6 μg C1q plus 1 μg polyclonal Igs anti-TcCRT or their F(ab′)$_2$ fragments, in a final volume of 20 μL. After washing 3 times with non-supplemented RPMI, the parasites were incubated with FITC conjugated polyclonal Igs anti-human C1q, for 30 min at 4 °C. After another series of washings, the parasites were fixed with 1% (v/v) paraformaldehyde, for 30 min at 4 °C and analyzed by flow cytometry (BD FACSCanto, BD Biosciences, San Diego, CA, USA), using a WinMDI software.

In vitro T. cruzi infectivity assay

The infection was performed as described (Rimoldi et al. 1989), with some modifications. Briefly, 1×10^6 MF or Dm28c trypomastigotes were alternatively incubated, 30 min at 24 °C, with: (a) 2 μg C1q, (b) 10 μg F(ab′)$_2$ anti-TcCRT S domain (TcS, the TcCRT region responsible for the binding to the collagenous C1q tails (Ferreira et al. 2004)) or anti-TcCRT, and (c) 10 μg anti-TcS or anti-TcCRT, plus 2 μg C1q. The murine macrophage cell line RAW 264.7 (Sigma–Aldrich, USA) was cultured in RPMI supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin, at 37 °C and left to adhere 2 h before trypomastigote infection, at a 1:10 cell:parasite ratio. After incubation at 37 °C for 3 h, non-internalized parasites were washed away with non-supplemented RPMI. Then, the cells were fixed with 90% cold-methanol, for 20 min at 4 °C, washed twice with PBS and incubated 2 min at 24 °C with DAPI. After another series of washings, the cells were mounted for fluorescence analysis. The infected cells or the parasites internalized by 100 cells were counted, analyzing 300 total cells for each experimental condition, in duplicates.

Determination of T. cruzi infectivity in vivo after alternative treatment with anti-TcCRT antibodies, F(ab′)$_2$ fragments or C1q

Six 12-week old, female BALB/c mice were infected i.p. with 10^3 Dm28c trypomastigotes (control group). Other three groups were infected with the trypomastigotes, alternatively pre-incubated, for 30 min at room temperature, with 10 μg human C1q, 30 μg of anti-TcCRT polyclonal IgG or 30 μg of anti-TcCRT F(ab′)$_2$ polyclonal IgG fragments, both reagents generated in our laboratories (Aguilar et al. 2005). Parasitemia was measured every 2 days, starting at day 5 post-infection. Survival was measured during 2 months.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 4 software. Conventional Student's one-tailed *t*-tests, two-way ANOVA, together with Bonferroni's post-test assays were used, as indicated. Exact *p* values, equal or smaller than 0.05, were considered significant. All experiments shown are representative of at least two experiences, performed on different opportunities.

Results

T. cruzi CRT mRNA levels are increased during the early infective stages

Given the TcCRT ability to recruit C1q, in the area of parasite flagellum emergence (Ferreira et al. 2004), and the reported role of this complement component in parasite infectivity (Rimoldi et al. 1989), we asked whether TcCRT expression increases during cell infection in VERO cell cultures. While no differences were observed in the host CRT mRNA expression, at any time post-infection, or between infected and non-infected cells (Fig. 1A), TcCRT mRNA expression levels were three times higher at 24 than at 48, 72 or 96 h post-infection (Fig. 1B). As shown in Fig. 1C, no differences were observed in TcCRT mRNA levels between trypomastigotes, recently released from host cells, and intracellular amastigotes after 48 h of infection.

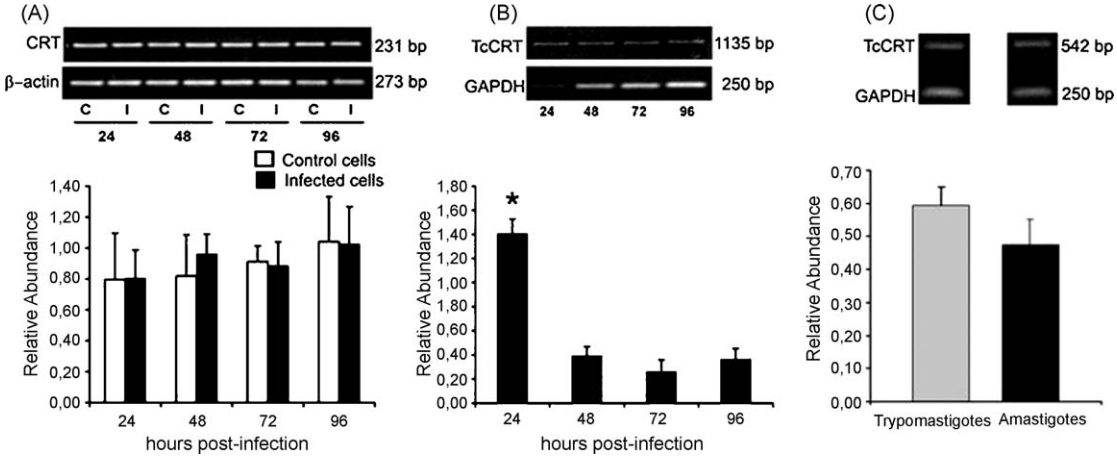

Fig. 1. *T. cruzi* CRT mRNA levels increase during the early *in vitro* infective stages. VERO cells were incubated with trypomastigotes. Total RNA was extracted at different times after infection and semiquantitative RT-PCR was performed. Primers for host CRT/β-actin (A), and TcCRT/GAPDH (B) mRNAs yielded the indicated products. Total RNA from VERO cells was extracted at 48 h (amastigotes) and at 144 h (recently released trypomastigotes) post-infection. TcCRT and GAPDH primers yielded the products shown in (C). Bars represent relative mRNA abundance, expressed as means of 3 or 4 experiments ± SD. In A–C, the top corresponds to electrophoreses and, the bottom, to the respective mRNA corrected densitometries.

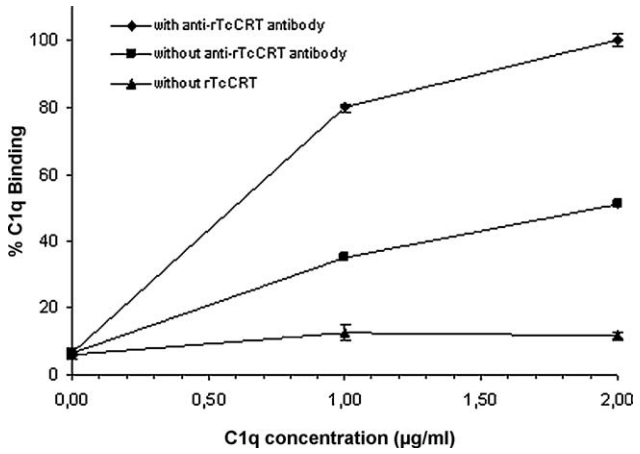

Fig. 2. Whole anti-rTcCRT polyclonal antibodies indirectly increase C1q binding to the solid phase immobilized parasite protein. Wells were sensitized with rTcCRT. Rabbit anti-rTcCRT antibodies were then added, followed by different C1q concentrations. Binding of C1q to rTcCRT was then detected. Results represent percentage of C1q binding (some SDs are too small to be visible).

In vitro C1q binding to immobilized rTcCRT, increases in the presence of anti-rTcCRT antibodies

Given the C1q role in parasite infectivity (Rimoldi et al. 1989), the fact that TcCRT interacts with both C1 and C1q (Ferreira et al. 2004; Valck et al. 2010) and since *T. cruzi* infection elicits the production of specific anti-TcCRT antibodies in both humans and experimental animals (Aguilar et al., 2005; Aguillon et al. 1997, 2000b; Marcelain et al., 2000), we asked whether these antibodies, upon binding TcCRT, could increase parasite infectivity. *In vitro*, the human C1q/rTcCRT interaction is strongly and indirectly enhanced by whole Igs against the parasite molecule (Fig. 2). On the other hand, we have shown that this interaction is directly abrogated *in vitro* by anti-TcCRT F(ab′)$_2$ fragments, from the same Igs (Aguilar et al. 2005).

TcCRT induces strong humoral immune response in BALB/c mice

As shown in Fig. 3, specific anti-TcCRT antibodies were detected after conventional (Fig. 3A), DNA (Fig. 3B) or combined immunization protocols (Fig. 3C). Mainly IgG1, recognizing native TcCRT on the parasite surface (indirect immunofluorescence assay), was detected (results not shown). Administration of a GM-CSF gene resulted in an earlier onset of anti-TcCRT specific antibody production (Fig. 3B). A murine Ig κ chain leader sequence was inserted in the pSecTag/*TcCRT* construct, thus allowing the parasite protein to be secreted. Accordingly, only pSecTag/*TcCRT* immunized animals showed anti-TcCRT specific antibodies, as compared to mice injected with pDNA/*TcCRT* (Fig. 3B). In agreement with earlier studies (Robinson et al. 2003), DNA immunization elicited a lower antibody response, than rTcCRT (Fig. 3A and B).

Immunization with TcCRT increases parasitemia in BALB/c mice

As mentioned, several protocols of active immunization with the recombinant protein, with its coding DNA or combinations thereof, promoted the generation of specific anti-TcCRT antibodies. Notably, the induced anti-TcCRT response was accompanied, in all cases, by increased parasitemia in the *T. cruzi*-challenged mice (Fig. 4). This is particularly evident at days 10 and 14 post-challenge.

In vitro, IgG anti-TcCRT and their F(ab′)$_2$ fragments have different and predictable opposite effects on C1q incorporation onto the parasite surface

As shown in Fig. 5, whole IgG anti-TcCRT recruits C1q on the trypomastigote surface (Fig. 5B). About 70% decrease in C1q recruitment is obtained when the parasites are pretreated with F(ab′)$_2$ fragments anti-TcCRT (Fig. 5C). In epimastigotes, the non-infective parasite form, obtained from axenic cultures, marginal C1q binding was observed, irrespective of the Ig treatment (Fig. 5D–F).

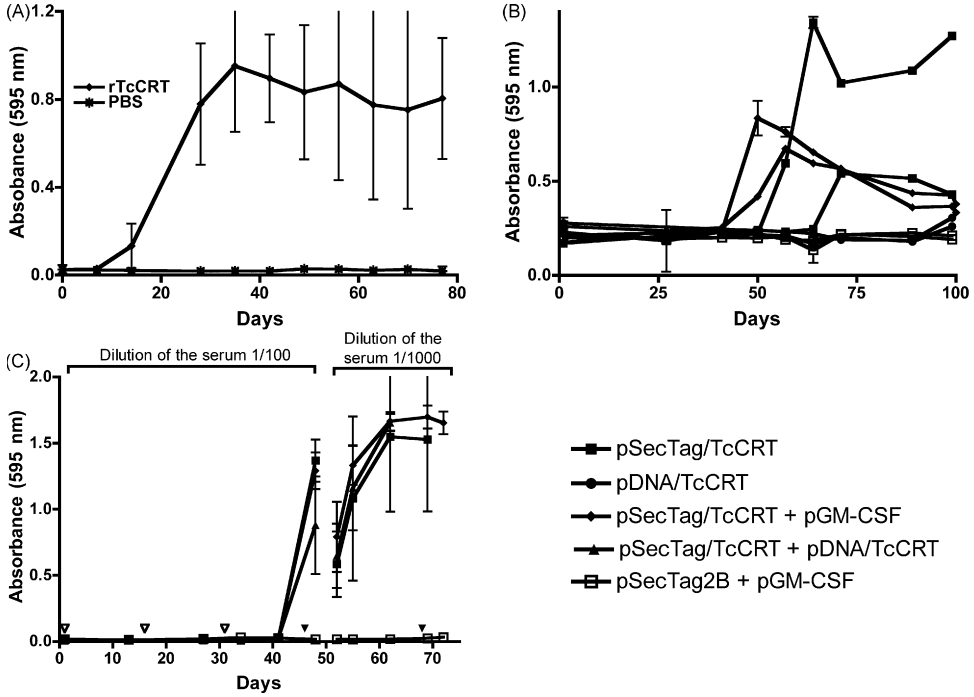

Fig. 3. TcCRT induces humoral immune responses in mice after different conventional and DNA immunization protocols. BALB/c mice were inoculated with rTcCRT (A) and/or with different genetic constructs (B and C), as indicated. Average values ± SD are shown.

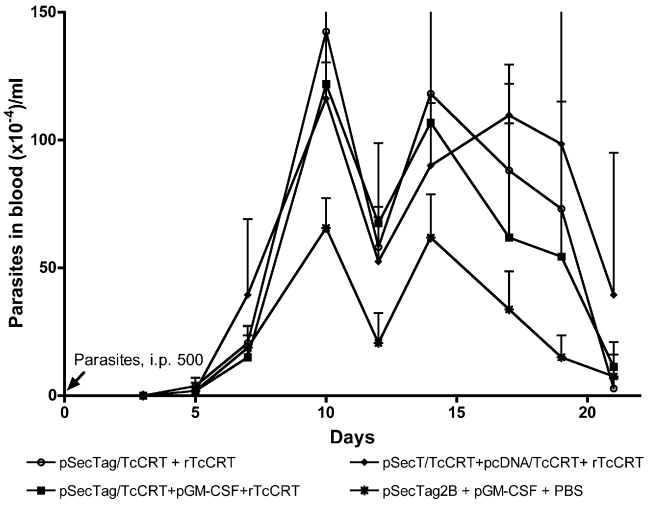

Fig. 4. Parasitemia increases in mice immunized with TcCRT. BALB/c mice were inoculated with different genetic constructs and rTcCRT, as indicated. The animals were challenged and parasitemia was measured. Mean values ± SD are shown. Significant differences in parasitemia, between experimental and control groups were as follows: at days 10, 12 and 17, pSecTag/*TcCRT*, $p = 0.034$, 0.009 and 0.032, respectively, and pSecTag/*TcCRT* plus pDNA/*TcCRT*, $p = 0.004$, 0.043, and 0.008, respectively; at day 12, pSecTag/*TcCRT* plus pGM-CSF, $p = 0.036$; at day 19, pSecTag/*TcCRT* plus pDNA/*TcCRT*, $p = 0.029$.

The C1q capacity to promote parasite infectivity in vitro is reverted by F(ab′)$_2$ anti-TcCRT Ig fragments

The effect of F(ab′)$_2$ Ig fragments anti-TcCRT on MF strain and Dm28c clone trypomastigote infectivity was tested on the RAW cell line (Fig. 6). As expected, pretreatment of parasites with C1q increases the RAW cell infection by MF trypomastigotes, by 35%. This increase in infectivity was fully reverted by pretreatment of the parasites with F(ab′)$_2$ Ig fragments anti-TcCRT (Fig. 6A). Similar results were obtained when the infectivity of different parasites (Dm28c clone) and different counting procedures (number of parasites internalized by the host cells) were used (Fig. 6B).

In vivo, IgG anti-TcCRT and their F(ab′)$_2$ fragments have different and predictable opposite effects on T. cruzi infectivity: a possible role for C1q

In agreement with the previous *in vitro* findings, pretreatment of parasites with whole anti-TcCRT antibodies augmented their infectivity, as reflected by increased parasitemia, mainly 9 days post-infection (Fig. 7A). As predictable, parasite pretreatment with F(ab′)$_2$ fragments, devoid of their capacity to bind C1q and derived from the same antibodies, were protective, as reflected by decreased parasitemia (mainly at 16 days post-infection, Fig. 7B) and increased mice survival (Fig. 7D). Most important, pretreatment of trypomastigotes with C1q, considerably increased both parasitemia (mainly at days 9 and 16) and mice mortality (Fig. 7C and D, respectively).

Discussion

We propose here that *T. cruzi* calreticulin is an additional trypomastigote cell surface receptor and virulence factor participating in trypomastigote infectivity by virtue of its capacity to use C1 as an adaptor molecule to recognize host cells. Altogether, the *in vitro* and *in vivo* results presented here indicate that TcCRT (translocated from the parasite ER to the membrane), by means of recruiting C1 (apoptotic mimicry effort), also promotes infectivity. At present, there is no reported evidence with regard to the mechanisms involved in vertebrate CRT or TcCRT translocation to the membrane and subsequent release to the extracellular milieu, where it fulfills a variety of functions.

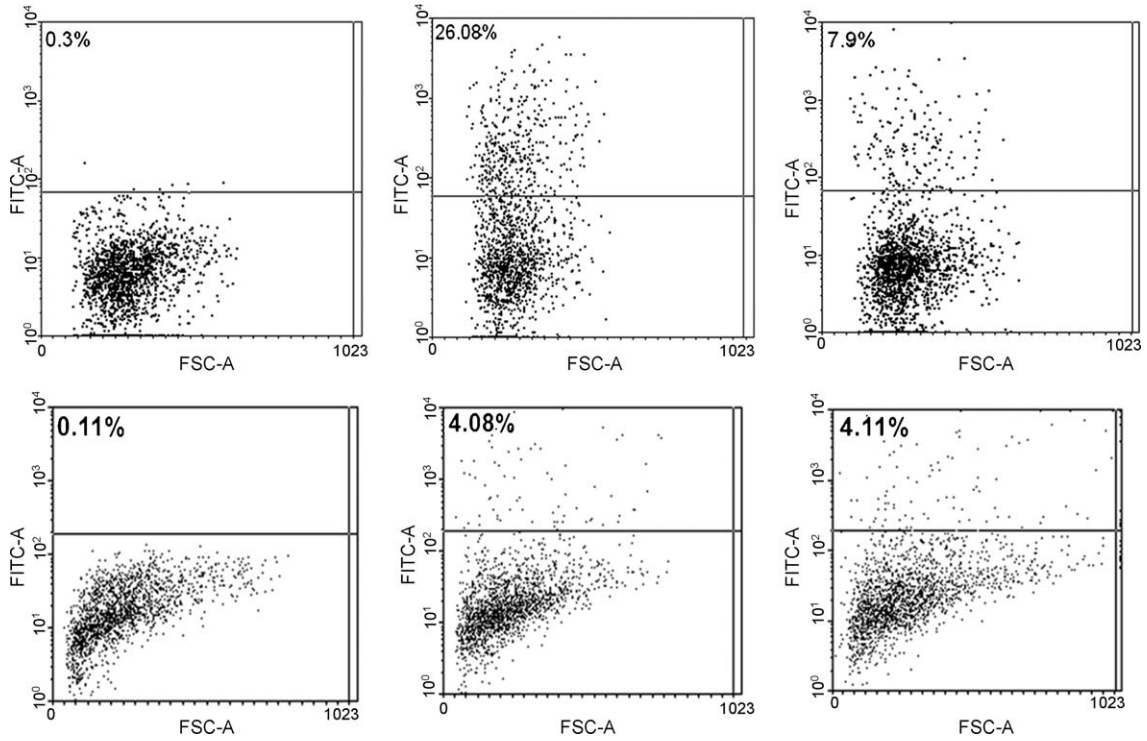

Fig. 5. IgG anti-TcCRT and their F(ab′)$_2$ fragments have different and predictable opposite effects on C1q incorporation onto the parasite surface. 3×10^6 trypomastigotes or 2.5×10^6 epimastigotes from the Dm28c clone were incubated with: Ig anti-human C1q – FITC (A and D), C1q plus Igs anti-TcS (B and E), and C1q plus F(ab′)$_2$ anti-TcS (C and F). C1q incorporation was detected with FITC Ig followed by flow cytometry. Results are representative of two experiments.

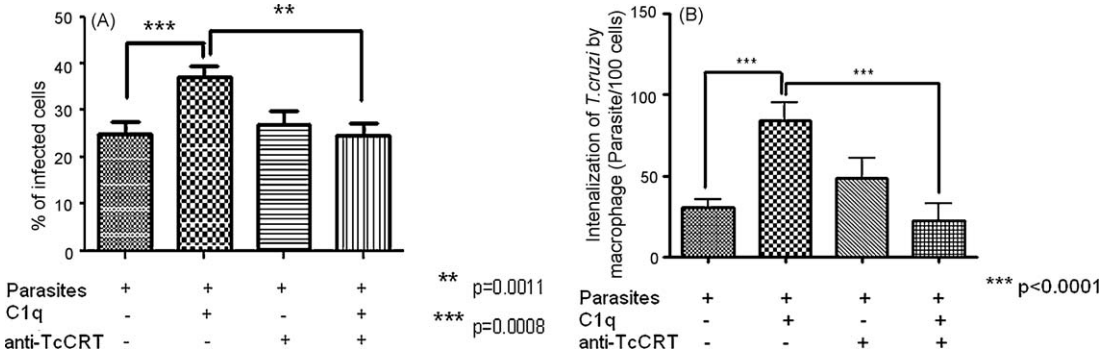

Fig. 6. F(ab′)$_2$ fragments anti-TcCRT inhibit C1q-mediated *T. cruzi* infectivity. 10^5 RAW-264 cells were infected with 10^6 MF strain (A) or Dm28c clone (B) trypomastigotes, pretreated with C1q or with F(ab′)$_2$ fragments anti-TcS or -TcCRT plus C1q. Cells were stained with DAPI and the number of infected cells (A) or parasite internalized (B) by 100 cells were counted. 300 cells were analyzed for each experimental condition. Each experiment was executed in duplicates. Results were statistically validated using non-paired one-tailed *t*-tests.

More recently, we have shown that C1q, after binding TcCRT, loses its capacity to incorporate the serine proteases C1r and C1s and thus to activate the classical pathway of complement. A similar result is obtained when the parasite molecule binds and inactivates C1 (q,r,s) (Valck et al., 2010). Since at present we have no evidences indicating that C1 and C1q behave differently with regard to their capacity to promote infectivity, in this report the acronyms C1 and C1q are frequently used indistinctly.

In agreement with TcCRT importance during the early stages of the parasite/host cell interaction, an significant increase in its mRNA expression was detected at 24 h post-infection (Fig. 1). Afterwards, the TcCRT mRNA levels were decreased by 75% and, in recently released trypomastigotes, were similar to those of intracellular amastigotes, after 48 h of infection. Therefore, increased mRNA TcCRT levels are detected only around pre-infective (cell contact and penetration) stages. The *T. cruzi* life cycle starts when one or few trypomastigotes enter the host cell and, at 24 h post-infection, a low level of mitotic replication into amastigotes is expected. Therefore, the level of a "housekeeping" gene mRNA (such as GAPDH) will be necessarily relatively low, if compared with later stages, when active amastigotes replication has already occurred. Thus, the relatively high levels of TcCRT mRNA, observed at 24 h, should be the product of a relatively small number of intracellular parasites. Conversely, the low relative levels, observed later on, should be the product of a larger number of parasites. We have interpreted this finding as indicative of a higher active TcCRT synthesis during the early infective stages. This phenomenon, paralleled by enhanced TcCRT translocation from the ER to the parasite area of flagellum emergence, is followed by augmented capacity to recruit C1 and improved infectivity. Thus, in spite of its KEDL-ER retention sequence, TcCRT translocates to the parasite surface and binds C1 (Ferreira et al. 2005, 2004), an important "eat me" signal for phagocytic cells (Obeid et al. 2007; Rimoldi et al. 1989).

In spite of its ER location, it is now well accepted that CRT from mammals plays a series of important functions, not only outside the ER, but also in the extracellular milieu (Gold et al. 2009). Although, the mechanisms by which mammal and *T. cruzi* CRT access the extracellular environment are still unknown, the presence of the parasite molecule on the surface of infective trypomastigotes has

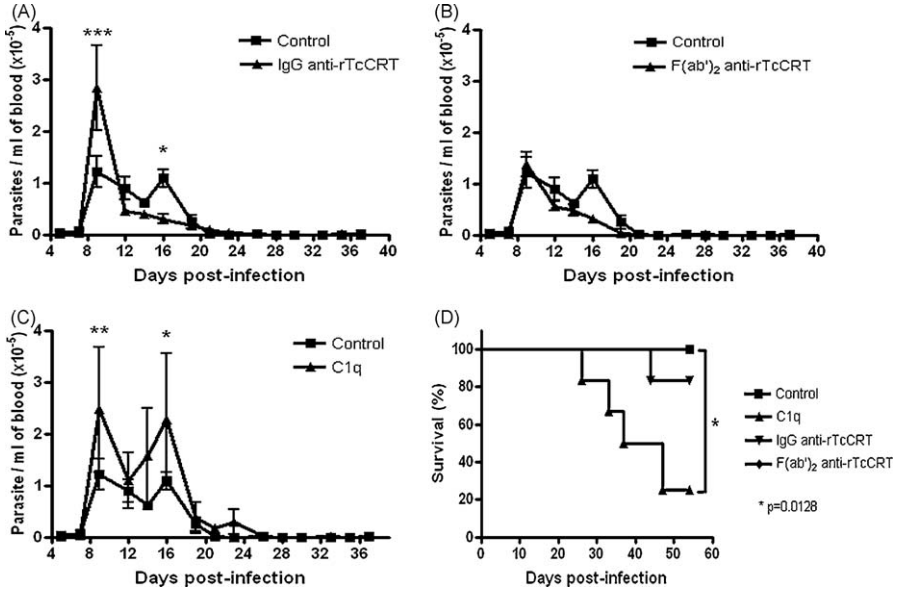

Fig. 7. IgG anti-TcCRT and their F(ab′)$_2$ fragments have predictable opposite effects on *in vivo T. cruzi* infectivity: a role for C1q. BALB/c mice were infected with trypomastigotes (control group). Other three groups were infected with the trypomastigotes alternatively pre-incubated with anti-TcCRT polyclonal IgG (A), anti-TcCRT F(ab′)$_2$ polyclonal fragments (B) and human C1q (C). Parasitemia was measured on indicated days. Survival was measured during 2 months (D) (results of control and F(ab′)$_2$-treated animals are superimposed). Figs. A–D summarize a single experiment. Since Figs. A–C were disaggregated to facilitate visualization, the control group is necessarily the same in all of them). Mean values ± SD are shown. Significant differences in parasitemia, between experimental and control groups were found mainly on days 9 and 16 by ANOVA. $^*p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

been previously established (Ferreira et al. 2004) and further confirmed in this report.

Four experimental evidences allow us to propose important *in vivo* roles for C1q in *T. cruzi* infectivity, all derived from its multiple binding capacities (to parasite surface TcCRT, via its collagenous tails, to the CH2 domains of antigen aggregated Igs, via its globular heads and to mammalian cell receptors): First, C1q increases *T. cruzi* infectivity *in vitro* (Rimoldi et al. 1989); second, we have previously shown that *T. cruzi* infection elicits the production of specific anti-TcCRT antibodies in both humans and experimental animals (Aguilar et al. 2005; Aguillon et al. 1997, 2000b; Marcelain et al. 2000); third, as shown here, the human C1q/rTcCRT *in vitro* interaction is indirectly increased by whole Igs against the parasite molecule (Fig. 2) and, fourth, this *in vitro* interaction is abrogated by anti-TcCRT $F(ab')_2$ fragments, prepared from the same Igs (Aguilar et al. 2005).

TcCRT immunogenicity increases infectivity *in vivo*, most likely through its capacity to generate specific complement fixing IgG1 antibodies (Burton and Woof 1992; Xu et al. 1994). Such were the cases after conventional, genetic and combined immunization protocols (Fig. 3). As expected, co-administration of a GM-CSF gene, by enhancing the antigen-presenting capacity of dendritic cells and facilitating B- and T-cell-dependent immunity (Garg and Tarleton 2002), mediated early production of anti-TcCRT specific antibodies. Insertion of a murine Ig κ chain leader sequence in the pSecTag/*TcCRT* construct, allowed the parasite protein to be secreted *in vivo*. Accordingly, only pSecTag/*TcCRT* immunized animals showed anti-TcCRT specific antibodies, as compared to mice injected with pDNA/*TcCRT*. As expected from earlier studies (Robinson et al. 2003), DNA was less immunogenic than rTcCRT immunizations.

Based on the previous results, it was predictable that the induced anti-TcCRT response should be accompanied by increased infectivity, as reflected by higher parasitemia in the *T. cruzi*-challenged mice. Thus, regardless of the immunization protocol used (Fig. 4), significant increases in the main expected parasitemia peaks were observed. The infection controls, non-immunized with TcCRT, show two lower parasitemia peaks, at 10 and 14 days post-infection (Fig. 4). Most likely, immunization with TcCRT induces C1q binding antibodies, a fact consistent with our proposal that C1q participates in the increased parasite infectivity observed in these immunized animals.

TcCRT-bound C1q locates mainly in the area of trypomastigote flagellum emergence (Ferreira et al. 2004), involved in the initial parasite/target cell contact (Bisaggio et al. 2006; Kollien et al. 1998). However, the possibility that *T. cruzi* trypomastigotes possess other cell surface immunogens that also generate C1q-fixing IgG antibodies, increasing their infectivity, cannot be ruled out at the present time. In addition, simultaneous participation of other infectivity promoting mechanisms, such as Fc and/or CR1-2 complement receptors, is also possible. Moreover, previous reports show that mice immunized with plasmids containing various *T. cruzi* genes produce antibodies, Th1 cytokines, and $CD8^+$ T cell protective immune responses (Boscardin et al. 2003; Garg and Tarleton 2002; Rodrigues et al. 1999). However, the TcCRT concentration in the area of flagellum emergence (Ferreira et al. 2004), may render anti-TcCRT antibodies more suitable to promote infectivity given their capacity to recruit C1q to an area relevant in the parasite/host cell contact and infectivity. Since at least six other molecules present on the parasite surface have been described as relevant in infectivity (Araya et al. 2008; Yoshida and Cortez 2008), interference with the TcCRT/C1 interaction should be partially effective in decreasing parasite infectivity.

IgG anti-TcCRT and their $F(ab')_2$ fragments have different and predictable opposite effects on C1q incorporation onto the parasite surface *in vitro*, as shown in Fig. 5. Thus, the trypomastigote capacity to recruit C1q on its surface when coated with whole IgG anti-TcCRT, decreases to one fourth when the parasite are pretreated with $F(ab')_2$ fragments anti-TcCRT. Probably, this reflects significant blockade of the ongoing TcCRT/C1q interactions and prevention of additional Fc-dependent C1q capture. Most important, in epimastigotes, the non-infective parasite form obtained from axenic cultures, marginal C1q binding was observed, irrespective of the Ig treatment. This result is consistent with the fact that this non-infective parasite form displays only a minor fraction of the TcCRT molecules exposed by infective trypomastigotes, as determined by flow cytometry analysis (Fig. 5). Perhaps, at least part of the epimastigotes incapacity to infect cells, both *in vitro* and *in vivo*, could be explained by limited translocation of the chaperone from the ER to the outer membrane.

The C1q capacity to promote parasite infectivity *in vitro* by MF and Dm28c parasites is reverted by $F(ab')_2$ anti-TcCRT Ig fragments, as summarized in Fig. 6. As expected, C1q promoted increased infection of RAW cells by 35%. In both cases, the infectivity was fully reverted by pretreatment of the parasites with $F(ab')_2$ Ig fragments anti-TcCRT.

As expected, positive or negative modulation of *in vivo* and *in vitro* parasite infectivity by anti-TcCRT antibodies could be predicted based on the structure of the Ig used for parasite pretreatment. While whole anti-TcCRT antibodies did increase infectivity and mortality (Fig. 7), their bivalent $F(ab')_2$ counterpart, devoid of their capacity to provide additional C1q binding sites, displayed inhibitory properties, both *in vitro* and *in vivo* (Fig. 6). Under the experimental conditions used, the parasite interacts only with one pulse of anti-TcCRT antibodies, whole or fragmented. This situation may be different under natural or artificial active immunization conditions, where the presence of specific antibodies should be more persistent in time. Most important, pretreatment of trypomastigotes with C1q considerably increased both parasitemia (mainly around the expected peaks) and mortality (Fig. 7). Probably, preincubation of the parasites with C1q provided a readily available source of this ligand that facilitated swift infection at the very early stages.

Clinical examination of $F(ab')_2$ immunized animals showed improved parameters, as compared to untreated, immunized with whole IgG or C1q-inoculated groups, were hirsutism, diarrhea, dehydration and prostration were more prevalent. These results correlate with the *in vitro* experiments presented in Fig. 6.

About 90% of C1q is present in plasma as a complex with the C1r and C1s serine proteases (Steino et al. 2004). It remains to be determined whether both C1 and C1q are equally effective in promoting infectivity *in vivo*, when compared at equimolar terms, as well as the relevant roles of MBL and ficolins.

Passive *in vivo* administration of polyclonal immunoglobulins (IVIg) has been long reported as an important immune modulation strategy (Galeotti et al. 2009; Vani et al. 2008). At this stage, we cannot rule out the possibilities that part of the observed effects in our whole IgG or $F(ab')_2$-treated experimental animals may be due to such properties (i.e. the inhibitory effect on parasitemia observed on day 16, Fig. 7).

As mentioned, the fact that $F(ab')_2$ fragments anti-TcCRT partially inhibit infectivity, both *in vitro* and *in vivo*, is expected, since at least other six parasite surface molecules are also relevant in the infective process (Araya et al. 2008; Yoshida and Cortez 2008). Interference at all seven levels, combinations of additive or synergic pharmacologic and immunologic strategies may be necessary for a more drastic decrease in infectivity. Given the effective mechanisms that *T. cruzi* displays to escape from the parasitophorous vacuole (Yoshida 2006), a TcCRT/C1q-mediated "eat me signal" may facilitate parasite infection of phagocytic cells, as an initial important infective strategy (Kroemer et al. 2005; Marcelain et al. 2000; Obeid et al. 2007). The fine crystallographic structure of the C1q/TcCRT has not been yet elucidated. However, given the multi-

meric structure of C1q, with 6 A, B, C trimeric chains conforming its collagenous tail, it is conceivable that repeated TcCRT binding sites are available on the complement component.

Apoptotic mimicry, defined by phosphatidylserine exposure, described in another trypanosomatid, *Leishmania amazonensis*, also results in increased infectivity (Wanderley et al. 2006). Attempts to block TcCRT activity or inhibit its expression by RNAi, have not been undertaken in our laboratory, since other investigators have shown that this is not a feasible strategy for other proteins in *T. cruzi* (DaRocha et al. 2004). Since the parasite has multiple copies of this gene (data not shown) and, on the other hand, the CRT gene inactivation is lethal *in uterus* in mammalians (Michalak et al. 2002), our current efforts aimed at knocking out the TcCRT gene may face unpredictable results.

In synthesis, TcCRT is an important parasite virulence factor, most likely participating in apoptotic mimicry strategies. Among the known parasite surface molecules participating in infectivity, TcCRT is the only one that does it by interacting with the host's complement system, thus using C1 as an intermediate adaptor ligand between the parasite and the host cell CRT. By recruiting C1q, surface TcCRT not only inhibits complement activation but also promotes C1-dependent phagocytosis, an initial infective parasite requirement in naïve individuals. In infected individuals, this capacity will be increased by anti-TcCRT Igs. The CRT/C1 interaction may be considered a unifying strategy that mediates, at least partly, not only the *T. cruzi* uptake by mammalian phagocytic cells, but also the ingestion of apoptotic vertebrate cells. Thus, in spite of the evolutionary distance between trypanosomatids and mammals, the phagocytic removal of apoptotic and tumor cells, by macrophages and dendritic cells has also been proposed as strongly dependent on C1 (Bohlson et al. 2007), recruited by membrane-translocated vertebrate CRT (Obeid et al. 2007).

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