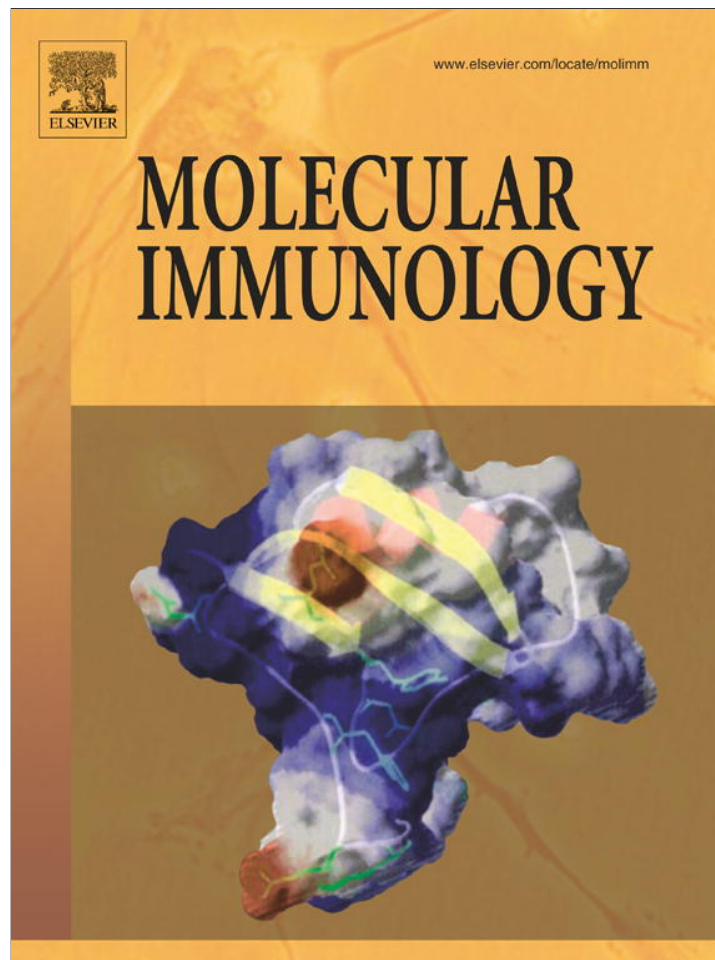


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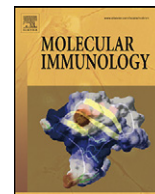
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Trypanosoma cruzi carrying a monoallelic deletion of the calreticulin (TcCRT) gene are susceptible to complement mediated killing and defective in their metacyclogenesis

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ABSTRACT

Trypanosoma cruzi calreticulin (TcCRT) can hijack complement C1, mannan-binding lectin and ficolins from serum thus inhibiting the classical and lectin complement pathway activation respectively. To understand the *in vivo* biological functions of TcCRT in *T. cruzi* we generated a clonal cell line lacking one TcCRT allele (TcCRT+/-) and another clone overexpressing it (TcCRT+). Both clones were derived from the TCC *T. cruzi* strain. As expected, TcCRT+/- epimastigotes showed impairment on TcCRT synthesis, whereas TcCRT+ ones showed increased protein levels. In correlation to this, monoallelic mutant parasites were significantly susceptible to killing by the complement machinery. On the contrary, TcCRT+ parasites showed higher levels of resistance to killing mediated by the classical and lectin but not the alternative pathway. The involvement of surface TcCRT in depleting C1 was demonstrated through restoration of serum killing activity by addition of exogenous C1. In axenic cultures, a reduced propagation rate of TcCRT+/- parasites was observed. Moreover, TcCRT+/- parasites presented a reduced rate of differentiation in *in vitro* assays. As shown by down- or upregulation of TcCRT expression this gene seems to play a major role in providing *T. cruzi* with the ability to resist complement system.

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1. Introduction

Chagas' disease, caused by the hemoflagellate protozoan parasite *Trypanosoma cruzi*, affects about 10 million people in America causing 50,000 deaths per year (WHO, 2002). Currently, the available treatment is based on the use of two drugs, Benznidazole and Nifurtimox, which are effective in 80% of acute cases; however, their side effects can be severe and their use can lead to resistance (Clayton, 2010).

Abbreviations: ER, endoplasmic reticulum; CMK, complement-mediated killing; TcCRT, *Trypanosoma cruzi* calreticulin; TcCRT+, calreticulin overexpressing parasites; TcCRT+/-, calreticulin monoallelic mutant; UGGT-/-, UDP-Glc:glycoprotein glucosyltransferase biallelic null mutant; HYG, hygromycin phosphotransferase gene; NEO, neomycin phosphotransferase gene; NHS, normal human serum; GDH, glutamate dehydrogenase polypeptide.

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During *T. cruzi* life cycle, different forms of the parasite can be recognized. Blood trypomastigotes and amastigotes are the vertebrate-stage parasites and metacyclic trypomastigotes are the infective vector-stage parasites. Epimastigotes are not infectious and are present in the triatomine vector. Host complement system can be activated by classical, alternative and lectin pathways and depends on an activation cascade of several serum proteins culminating in the formation of a membrane attack complex causing death by cell lysis (Krautz and Kretti, 2000). However, amastigotes and blood and metacyclic trypomastigotes typically resist complement lysis in non-immune host serum. In contrast, epimastigotes are susceptible to complement lysis because under natural conditions they never interact with components of the complement system (Iida et al., 1989; Kipnis et al., 1985; Nogueira et al., 1975). Several specific molecules have been reported as complement inhibitors; mainly, they are glycoproteins that destabilize complexes formed by complement proteins. So far, the *T. cruzi* complement regulators characterized are: Gp 58/68 (Fischer et al., 1988); *Trypanosoma cruzi*-Decay Accelerating Factor (T-DAF) (Norris, 1991), Complement Regulator Protein (CRP) (Norris, 1998), Complement C2 Receptor Inhibitor Trispanning (CRIT) (Inal et al., 2005) and *Trypanosoma cruzi* calreticulin (TcCRT) (Ferreira et al., 2004b)

TcCRT is a lectin chaperone polypeptide of 47 kDa, mainly present on endoplasmic reticulum (ER) where it controls calcium levels (Michalak et al., 2009; Souto-Padrón et al., 2004). TcCRT is translocated from the ER to the area of flagellar emergence, where it interacts with serum complement C1 component and interferes with the ability of the associated C1r and C1s serine proteases to activate C4 and C2, inhibiting the generation of C3 and C5 convertases. Thus, the classical complement pathway is inhibited at the earliest stage of its activation. TcCRT also interacts with mannan-binding lectin (MBL) and ficolins inhibiting complement lectin pathway activation (Ferreira et al., 2004b, 2005). In *T. cruzi*, the ER quality control system for glycoproteins is integrated by UDP-Glc:glycoprotein glucosyltransferase (UGGT), glucosidase II and TcCRT (Caramelo and Parodi, 2008). UGGT monoglucosylates proteins during their folding process. Thus, TcCRT, by virtue of its lectin capacity, retains monoglucosylated intermediates. TcCRT interaction with these intermediates not only allows correct retention of unfolded proteins in the ER, but also favors their folding, inhibiting premature oligomerization as well as incorrect disulfide bridging (Conte et al., 2003; Trombetta and Parodi, 2001). In the present work, we applied genetic manipulation techniques to understand the biological role played by TcCRT in *T. cruzi*. TcCRT-defective and overexpressing parasites were obtained through targeted monoallelic deletion and episomal expression, respectively.

2. Materials and methods

2.1. Trypanosome cultures

A *T. cruzi* clone derived from the naturally attenuated TCC strain of *T. cruzi* was used (Basombrio et al., 1982). Epimastigote forms were grown at 28 °C in liver infusion-tryptose medium (LIT) supplemented with 10% fetal bovine serum decomplexed at 56 °C for 60 min, 20 µg hemin (Sigma), 100 IU of penicillin and 100 µg streptomycin per ml. The UGGT null mutant epimastigotes (UGGT^{-/-}) were obtained from the *T. cruzi* CL Brener strain as previously described (Conte et al., 2003).

2.2. TcCRT gene deletion constructs

In order to delete copies of the TcCRT gene in the TCC strain of *T. cruzi*, DNA replacement vectors were constructed via the MultiSite Gateway Three-Fragment Vector Construction kit (Invitrogen) as previously reported (Xu et al., 2009). Briefly, the final DNA constructions for homologous recombination carried either the selectable marker hygromycin phosphotransferase gene (HYG) or neomycin phosphotransferase gene (NEO) flanked by 0.49 kb of the 5' UTR and 1.4 kb of the 3' UTR sequences of the TcCRT gene (Genebank accession number AAHK01000598.1). The final plasmids were denominated pDest.CRT/HYG and pDest.CRT/NEO and the correct distribution of the recombination fragments was corroborated by sequencing. The recombination fragments were amplified by polymerase chain reaction (PCR) using as template the plasmids described above. Amplification fragments of 3.4 kb and 3.2 kb corresponding to the constructions with the HYG and NEO marker were obtained and denominated, respectively, CRT/HYG and CRT/NEO. The fragments were gel purified (PureLink, Invitrogen) and quantified for further electroporation.

2.3. TcCRT overexpression constructs

In order to overexpress the TcCRT gene on the TCC strain we PCR-amplified 1.2 kb corresponding to the complete TcCRT coding sequence (CDS) using genomic DNA from TCC *T. cruzi* strain as template. Complete TcCRT CDS was obtained from Genebank

(accession number AAHK01000598.1) and amplified using primers 5'-GCCAGATATCATGAGGAGAAATGACATAAAA-3' (5' end carrying an EcoRV site) and 5'-TCCTCTCGAGTCAAAACTTCCACCACCGAA-3' (5' end carrying an XhoI site). The purified amplification product was digested with enzymes EcoRV and XhoI, purified again and then ligated into the EcoRV/XhoI digested pTRET expression vector (Vazquez and Levin, 1999). The final construction was denominated pTRET-TcCRT. Correct cloning was checked by PCR, restriction mapping analysis and sequencing using an ABI 3100 automated DNA sequencer.

2.4. Parasites transfection

For deletion of one allele of the TcCRT gene, a total of 50 µg of the purified CRT/HYG or CRT/NEO recombination fragments were used. For the generation of overexpressing parasites a total of 20 µg of circularized pTRET-TcCRT plasmid was used. In both cases transfection was carried out using TCC log phase epimastigotes. A BTX electroporator was used to transfect 1×10^8 cells in 0.4 cm cuvettes, applying two 450 V/500 µF pulses. After keeping the parasites at 4 °C for 10 min, they were seeded into LIT medium. After 48 h, 300 µg/ml of antibiotic hygromycin B or 200 µg/ml of G418 (Sigma, St. Louis, MO, USA) was added. Viable cultures were expanded and tested for resistance. Resistant parasites were cloned by limiting dilution in 96-well plastic plates.

2.5. Molecular characterization

Genomic DNA from transfected clones and from TCC wild type parasites was purified using the phenol-chloroform method. Diagnostic PCR analysis was done to confirm the correct replacement of TcCRT gene. Three pairs of primers were used (Fig. 1A). Pairs 1 and 2 allowed us to check that the recombination fragment was integrated into the TcCRT locus. Pair 1 consists of primer CRT7 (5'-CCTTCCGATGGCATTAGC-3') which anneals upstream of TcCRT gene plus primer H2 (5'-GAAGTACTCGCCGATAGT-3') specific for the HYG sequence. Pair 2 consists of primer CRT93 (5'-ATTCCAAACAACATTGCCGT-3') which anneals downstream of TcCRT gene plus primer H1 (5'-CGTCTGTGAGAAGTTTCTG-3') specific for the HYG sequence. Pair 3 allowed us to detect the recombination fragment integrated into the other TcCRT allele. This pair consists of primer CRT8 (5'-CTGAACCGTTTCTGCCACT-3'), which anneals upstream of TcCRT, plus primer H2. For Southern blot analysis, genomic DNA of mutant and wild type TCC parasites was digested, separated by 0.7% agarose gel electrophoresis and the gels were blotted onto nylon membranes (Hybond-N 0.45 µm pore size filters; Amersham Life Science) using standard methods. Probes were generated by amplification of 0.49 kb and 0.96 kb DNA segments corresponding to the 5' UTR TcCRT and HYG gene respectively. Labeling of the probe and DNA hybridization was performed with the PCR-DIG DNA-labeling and detection kit (Roche Applied Science) according to the manufacturer's protocol.

2.6. Immunodetection of TcCRT

Parasite lysis, SDS-PAGE and Western blotting were performed as described elsewhere (Labriola et al., 1999). Briefly, 1.5×10^5 epimastigotes were frozen overnight and resuspended with 1% NP-40/HEPES 50 mM/NaCl₂ 200 mM and a protease inhibitor transepoxy succinyl-L-leucylamido (4-guanidino) butane (E-64) from Sigma. Total extracts were separated by SDS-PAGE using 10% polyacrylamide gels and the protein bands transferred onto a nitrocellulose membrane (Immobilon-P Millipore). Non-specific binding sites were blocked by incubating the membrane for 30 min in 3% nonfat milk powder. The membrane was then incubated for 1 h with the primary antibody raised against TcCRT

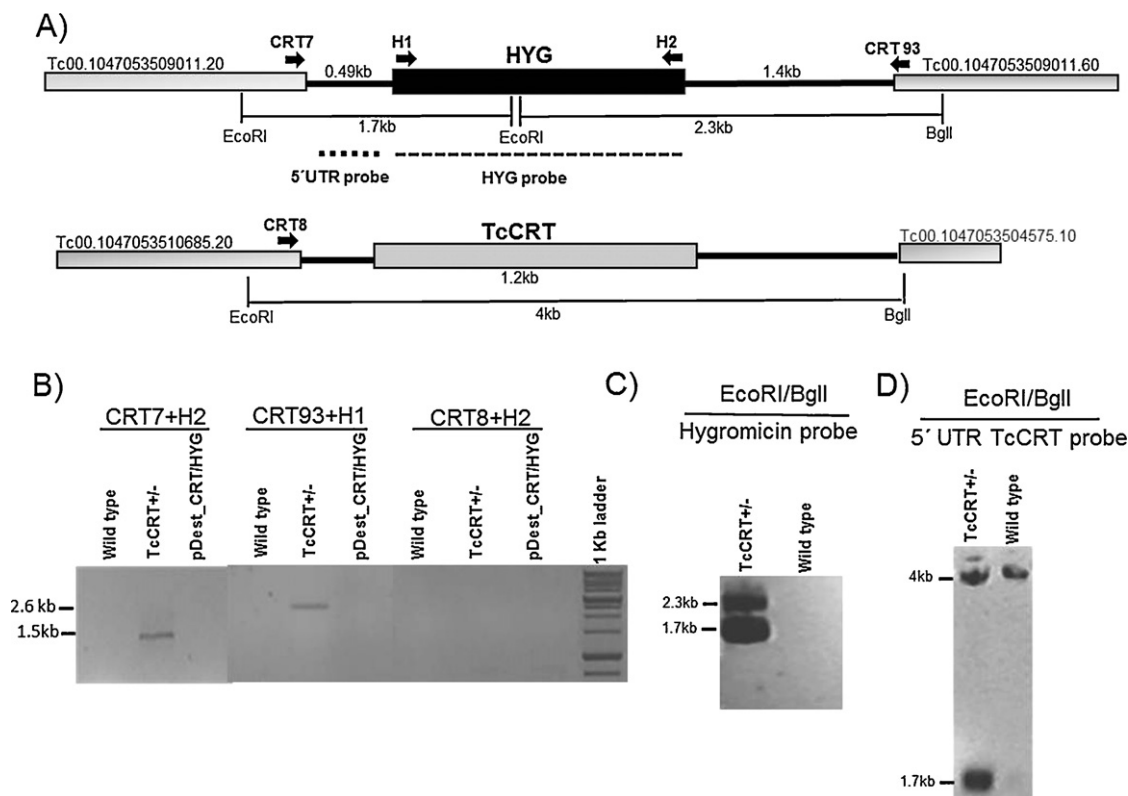


Fig. 1. Deletion of one TcCRT allele in *Trypanosoma cruzi* TCC strain parasites. (A) Schematic representation of the expected genomic loci of TcCRT in TcCRT+/- parasites. (B) PCR analysis using primers annealing on the hygromycin resistance gene and the coding sequences of the up and downstream gene of TcCRT. (C) Genomic Southern blot of TcCRT+/- and wild type parasites. Genomic DNA of TCC wild type and TcCRT+/- clones were digested by a combination of EcoRI/BglI and hybridized with a DNA probe complementary to the hygromycin gene or the 5' UTR of TcCRT gene.

(Labriola et al., 1999; Souto-Padrón et al., 2004) (1:2000 dilution), diluted in blocking solution. Then, the membrane was washed three times in TBS and then incubated for 45 min with anti-rabbit horseradish peroxidase-conjugated IgG (Sigma–Aldrich, St. Louis, MO, USA) diluted 1:30,000 in blocking solution. Antiserum anti-glutamate dehydrogenase (GDH) (Labriola et al., 2010) of *T. cruzi* was used as a control.

2.7. Epimastigote growth assays

4 × 10⁴ epimastigotes from TcCRT+/-, TcCRT+ and wild type parasites were seeded in 24-well plates containing 1 ml of complete LIT medium per well. The number of growing parasites was quantified during 18 days in a Neubauer chamber.

2.8. Complement mediated killing assay

Complement mediated killing (CMK) was assayed using epimastigote forms. We used serial dilutions of normal human serum (NHS) from non-infected individuals to determine the concentration that causes parasite death. Logarithmic phase epimastigote forms of *T. cruzi* (5 × 10⁵) were resuspended in 100 μl of non-supplemented LIT medium (without fetal bovine serum) to avoid any interference with the results. Parasites were incubated with 100 μl of non-supplemented LIT-diluted NHS. The final NHS concentrations used were 0%, 7.5%, 10%, 15%, 20%, 30%, 40%, and 50%. Incubation was done in 1.5 ml tubes at 37 °C. Parasites were then washed twice by centrifugation (11,000 × g, 1 min) in cold PBS. The reaction was terminated by adding 800 μL of cold non-supplemented LIT and incubating on ice. Heat-inactivated NHS (56 °C for 30 min) was used as a negative control. Surviving

parasites were quantified in a Neubauer chamber under a light microscope. In order to determine the kinetics of complement mediated killing, the incubation reactions were stopped at different time points (5, 10, 20, 30, 40, 50 and 60 min). In this experiment we use the serum dilution 10%.

Complement activation by the classical pathway is Ca²⁺ and Mg²⁺ ion-dependent, whereas alternative pathway activation requires only Mg²⁺. Thus, inhibition of the classical pathway was achieved through Ca²⁺ chelation by incubating NHS with 10 mmol/L ethylene glycol tetraacetic acid (EGTA) and 7 mmol/L MgCl₂. Under this condition, killing was attributed to the alternative pathway (Fine et al., 1972). Some CMK assays aimed to restore C1 activity were performed by adding 0.5 μg (0.02 μmol/L) of exogenous C1 (Sigma–Aldrich, St. Louis, MO, USA) to NHS.

2.9. *Triatoma infestans* infection

Laboratory-bred *T. infestans* were used. The degree of colonization of these bugs by *T. cruzi* was tested using metacyclic forms of TcCRT+/-, TcCRT+ and wild type parasites. Parasites were suspended in 1 ml of human blood at a concentration of 3 × 10⁴ parasites/ml, placed inside a tightly closed latex condom, and heated to 37 °C. Groups of 10, second or third-instars bugs, previously fasted for 1 month, were allowed to feed across the latex membrane for 30 min in the dark. Non-engorged insects were discarded, and the remaining ones (engorged) were kept in a chamber at 29 °C with 75% humidity and examined after 30 days. A drop of feces was obtained on a slide by abdominal compression and diluted 1:50 in LIT medium. The concentration of parasites was determined in a Neubauer chamber.

2.10. Metacyclogenesis *in vitro*

The morphologic transformation of TcCRT+/-, TcCRT+ and wild type epimastigotes to the mammalian infective metacyclic trypomastigotes (metacyclogenesis) was studied by incubating epimastigotes with media supplemented with triatome gut homogenate (Isola et al., 1986). Parasites were centrifuged at 3000 rpm for 15 min and resuspended at a final concentration of 5×10^6 parasites/ml. Triatome gut homogenate (10%) was added and quantification of metacyclic trypomastigotes, epimastigotes and intermediate forms was determined daily during 7 consecutive days in Neubauer chambers. Samples were done in triplicate.

2.11. Statistical analysis

Continuous variables were analyzed with the two-tailed Wilcoxon signed-rank test for time course plots and with the Mann–Whitney or Kruskal–Wallis test for single-day measurements using Prism software (GraphPad version 5.0). Values are expressed as mean \pm standard errors of the mean from at least three separate experiments.

3. Results

3.1. Generation of TcCRT single targeted mutant and overexpressing parasites

Hygromycin B resistant parasites could be selected after transfection of the CRT/HYG recombination fragment derived from plasmid pDest_CRT/HYG. PCR and Southern blot analysis of a cloned population confirmed the deletion of one allele of the TcCRT gene. In order to detect if the CRT/HYG recombination fragment was integrated into the correct locus we used different pairs of primers. One primer was specific for a sequence up or downstream of the TcCRT gene, not present in the recombination cassette used for transfection, and the other primer specific for the HYG sequence of the CRT/HYG recombination fragment (see Section 2). The expected genomic loci of TcCRT in single knock-out parasites are shown in Fig. 1A. When using primer CRT7 in combination with primer H2, a band of 1.5 kb was obtained only for mutant parasites as expected. When using primers CRT93 + H1, a band of 2.6 kb was evident also for mutant parasites (Fig. 1B). These results confirmed the correct replacement of one allele of the TcCRT gene in mutant parasites. The replacement was apparently restricted to one allele, since the primer pair specific for the second allele (CRT8 + H2) did not trigger a PCR (Fig. 1B).

Southern blot results also corroborated the correct replacement. Genomic DNAs of TCC wild type and TcCRT+/- parasites were digested with a combination of enzymes EcoRI and BglI, blotted and hybridized with HYG probe. As expected, bands of 2.3 kb and 1.7 kb were evident in mutant parasites while no signal was observed in TCC wild type parasites (Fig. 1C). Moreover, when using the 5' UTR of the TcCRT gene as a probe, bands of 4 kb and 1.7 kb were present in the mutant parasites and only a 4 kb band was observed in wild type parasites confirming the correct replacement of one TcCRT allele (Fig. 1D).

After obtaining parasite clones with the monoallelic TcCRT deletion, attempts were made to generate biallelic TcCRT-/- null mutant clones by retransfecting TcCRT+/- parasites with the CRT/NEO recombination fragment. After several attempts of transfection, no double resistant parasites were obtained. This failure may reflect the essential character of the TcCRT protein for parasite survival.

To accomplish the generation of TcCRT overexpressing parasites, *T. cruzi* TCC epimastigotes were transfected with circularized

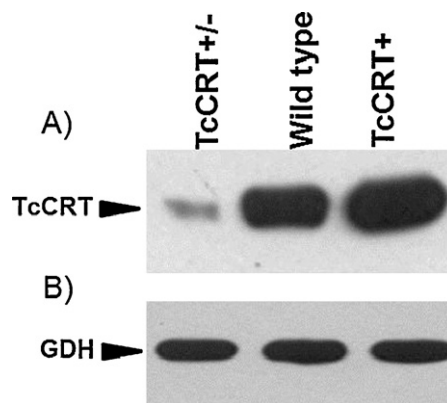


Fig. 2. TcCRT expression progressively increases in TcCRT+/- single targeted mutant, wild type and TcCRT+ epimastigotes. Equal numbers of epimastigotes (1.5×10^5) were analyzed. Cell lysates were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with anti-TcCRT (A) or anti-GDH (B) rabbit serum.

pTREX-TcCRT plasmid and submitted to antibiotic selection. G418 resistant parasites were obtained and cloned. This parasite population was named TcCRT+.

Thus, we generated two transgenic cell lines from TCC wild type strain: TcCRT+/- and TcCRT+ for their use in *in vitro* and *in vivo* experiments.

3.2. TcCRT expression progressively increases in TcCRT+/-, wild type and TcCRT+ parasites

We carried out Western blot analysis using total extracts from TcCRT+/-, TcCRT+ and wild type epimastigotes. Equal numbers of epimastigotes from each parasite populations were analyzed (1.5×10^5). Experiments were performed to examine whether the TcCRT gene alterations were associated with a modification in TcCRT protein synthesis. The primary antibody used as probe was previously characterized and shown to recognize the 47 kDa TcCRT polypeptide of *T. cruzi* (Labriola et al., 1999; Souto-Pradón et al., 2004). TcCRT monoallelic mutants present a significant reduction in TcCRT production, whereas overexpression of the TcCRT gene induced higher levels of TcCRT protein synthesis compared to wild type parasites (Fig. 2A). Densitometric estimation of the amount of TcCRT polypeptide using serial dilutions of each parasite lysate indicated that TcCRT+/- mutant contained ≈ 6 -fold less TcCRT polypeptide than wild type parasites. Moreover, parasites overexpressing TcCRT contained ≈ 2 -fold more TcCRT polypeptide than wild type parasites. In addition, we obtained similar signal intensities when using as control *T. cruzi* anti-GDH antiserum demonstrating that equal quantities of total proteins were analyzed from each parasite population (Fig. 2B).

3.3. Deletion of one allele of the TcCRT gene inhibits the replication capacity of mutant epimastigotes

To assess if deletion of one allele of the TcCRT gene affects replication capacity of mutant epimastigotes, we evaluated the growth behavior of such parasites *in vitro*. We also determined the growth rate of TcCRT+ overexpressing parasites compared to wild type. The number of parasites was recorded daily until stationary phase was reached.

On days 10 and 12 differences in the propagation rate of TcCRT+/- versus TcCRT+ and wild type epimastigotes were observed ($p < 0.001$), suggesting that TcCRT may be implicated in cell proliferation (Fig. 3). However, no differences were observed between TcCRT+ and wild type parasites ($p = 0.9$).

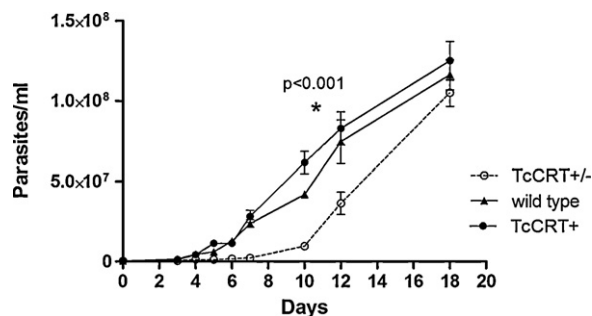


Fig. 3. TcCRT^{+/-} epimastigotes are inhibited in their proliferative capacity *in vitro*. *In vitro* growth curve of TCC TcCRT^{+/-}, TcCRT⁺ and wild-type epimastigote parasites. Each point indicates the average parasite concentrations of four independent wells. These results are representative of three independent experiments. At days 10 and 12 proliferation is different ($p < 0.001$) between TcCRT^{+/-} versus TcCRT⁺ or wild type groups. No differences were observed between TcCRT⁺ and wild type parasites ($p = 0.9$).

3.4. Greater susceptibility and resistance to complement mediated-killing is conferred by TcCRT deletion or overexpression

To determine the kinetics of CMK in TcCRT^{+/-}, TcCRT⁺ and wild type epimastigote forms we performed assays where the reaction was stopped at different incubation times over a time course of 5–60 min at 37 °C. After 10 min, the survival levels of the three populations under study were different from each other ($p = 0.02$) (Fig. 4A). This time point was thus selected for further analyzing the relationship between serum concentrations and killing. CMK assays were performed using serial dilutions of NHS at 37 °C during 10 min incubation. A divergence in killing was found in the three groups analyzed ($p = 0.004$) (Fig. 4B). When NHS concentration

exceeded 10%, the number of epimastigotes TcCRT^{+/-} decreases dramatically to values close to zero, while the other two groups remained resistant. In contrast, TcCRT⁺ parasites were resistant to CMK as compared with the wild type despite the serum concentration used ($p = 0.01$) (Fig. 4B). The global survival rates were 22.12%, 71.66% and 93.04% for TcCRT^{+/-}, wild type and TcCRT⁺ parasites respectively. These divergences disappeared when parasites were incubated with NHS treated with EGTA and MgCl₂, thereby inhibiting the classical and lectin complement pathway (Fig. 4C). All parasites were lysed to a similar extent with no differences ($p = 0.97$) in all concentrations tested.

To test if the C1-TcCRT interaction was responsible for divergences on mutant parasites we restored C1 activity in CMK assays by adding 0.5 μg (0.02 μmol/L) of exogenous C1 to NHS (Fig. 4D). By adding excess of C1, we could reduce the resistance to CMK in TcCRT⁺ parasites ($p = 0.03$), obtaining values comparable with the survival levels of wild type parasites. These results indicated that the susceptibility or resistance observed in these parasites was a consequence of C1 binding on TcCRT polypeptide in the parasite surface. A similar result was obtained using increasing serum concentrations (0–50%, v/v), as a C1 source (results not shown).

Then, we determined if the susceptibilities to CMK are due to TcCRT direct effects or to alterations on the TcCRT-dependent ER quality control system functions. In the ER quality control system UGGT monoglucosylates proteins during their folding process. These monoglucosylate intermediates are substrates for the TcCRT lectin activity on ER (see Section 1). Thus, we conducted CMK assays using CL Brener UGGT^{-/-} parasites. In these parasites, TcCRT cannot fulfill its function as a lectin chaperone. We show that UGGT^{-/-} parasites behave similar to CL Brener wild type, with regard to their susceptibility to CMK ($p = 0.9$) (Fig. 5). Thus, involvement of TcCRT in complement susceptibility and protein folding are independent phenomena.

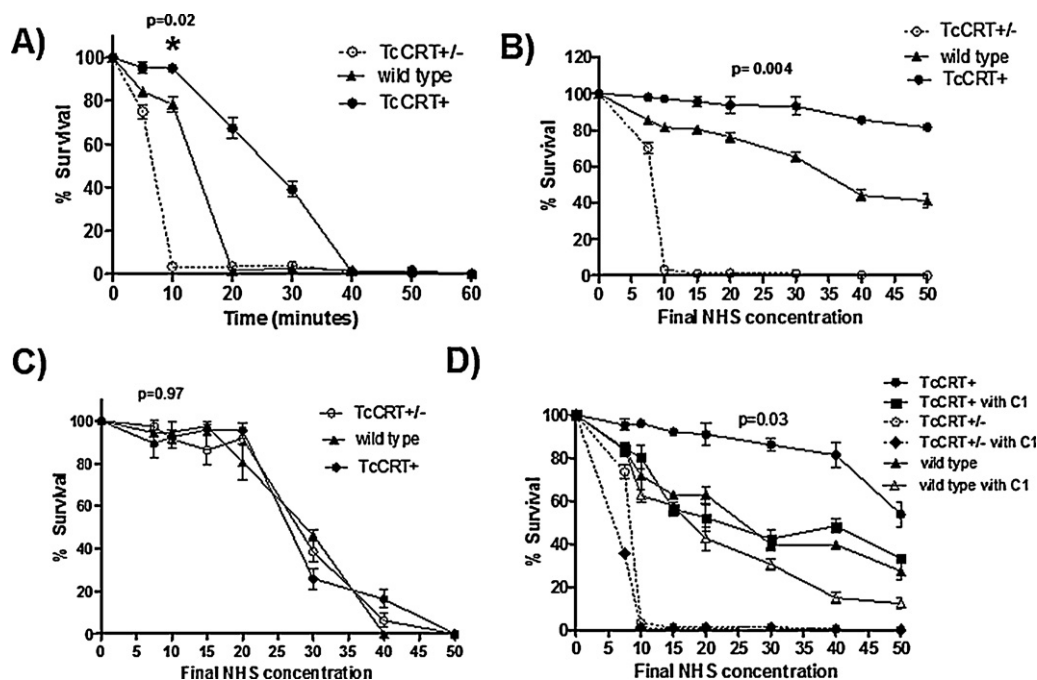


Fig. 4. TcCRT expression directly correlates with increased resistance to classical pathway complement mediated killing, in a C1-dependent fashion. Complement mediated-killing (CMK) assays of *Trypanosoma cruzi* TCC strain TcCRT^{+/-}, TcCRT⁺ and wild type epimastigotes. (A) Kinetics of complement activation. Kinetics are different ($p = 0.02$) when comparing the three curves at 10 min of incubation. (B) CMK dynamics using serial dilutions of NHS and 10 min of incubation at 37 °C. Results are different ($p = 0.004$) in all NHS concentrations when comparing the three curves. (C) CMK assays using 10 mM EGTA/7 mM MgCl₂-treated NHS (alternative pathway activation only). CMKs are not different ($p = 0.97$) at all concentrations when the three curves are compared. (D) CMK assays adding 0.5 μg (0.02 μM) of exogenous C1 to NHS. CMKs are different ($p = 0.03$) between TcCRT⁺ with C1 and TcCRT⁺ at all tested concentrations. Results are shown as means ± s.d. of 3 independent experiments.

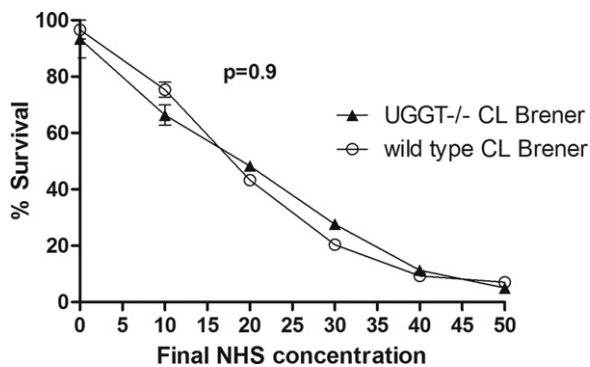


Fig. 5. Inhibition of TcCRT lectin activity does not affect the parasite complement killing properties. Complement Mediated-Killing (CMK) assays of *Trypanosoma cruzi* CL Brener strain UGGT^{-/-} and wild type epimastigotes. Assays were performed using serial dilutions of NHS and 10 min of incubation at 37 °C. UGGT^{-/-} and wild type epimastigotes are not different ($p=0.9$) at all serum concentrations used.

3.5. TcCRT^{+/-} parasites display a reduced metacyclogenesis ability

To evaluate whether a monoallelic deletion of the TcCRT gene affects parasite replication in the vector insect and metacyclogenesis, TcCRT^{+/-}, TcCRT⁺ and wild type parasite development was compared in *T. infestans* vectors. Groups of 10 second- or third-instar bugs were allowed to feed across a latex membrane with human blood artificially infected with 3×10^4 parasites/ml of each of the lines under study. Trypomastigote concentrations were recorded in the feces of the bugs on days 30 after feeding. At this time point, a clear reduction in the ability to multiply inside the vector was detected in TcCRT^{+/-} parasites. The proportion of infected bugs was significantly lower in the group of insects fed on blood infected with TcCRT^{+/-} parasites in comparison to insects fed on wild type and TcCRT⁺ parasites (Fig. 6A). Moreover, the concentration of parasites in feces of each group were different ($p=0.04$). Averages of 1×10^5 , 1.24×10^7 and 9.5×10^6 parasites/ml were respectively detected in bugs fed on TcCRT^{+/-}, wild type and TcCRT⁺ *T. cruzi*-infected blood (Fig. 6A).

In addition, the capacity to develop metacyclogenesis of these mutant and wild type parasites was studied *in vitro* by incubating epimastigotes with media supplemented with 10% triatomine gut homogenate (Isola et al., 1986). Quantification of metacyclic trypomastigotes in the supernatant revealed an inhibition of differentiation in TcCRT^{+/-} parasites ($p=0.03$) compared to the wild type (Fig. 6B).

4. Discussion

In this work, we developed mutants of *T. cruzi* engineered to hamper the ability of *T. cruzi* to evade host defenses and infect insect vectors. Using the Multisite-Gateway System®, we developed plasmid vectors able to eliminate one allele of the TcCRT gene by homologous recombination on the flanking sequences. Thus, we generated a clonal cell line lacking one TcCRT allele on the naturally attenuated TCC *T. cruzi* strain (TcCRT^{+/-}) (Fig. 1). As a positive control, a recombinant *T. cruzi* clone that overexpresses the TcCRT polypeptide (TcCRT⁺) was obtained by transfecting pTREC-TcCRT plasmid. Western blot analyses indicated the under- and overexpression of the TcCRT polypeptide in the respective clones (Fig. 2).

Attempts were made to generate TcCRT^{-/-} null mutant clones but no double resistant parasites could be obtained. This failure may reflect the essential character of the TcCRT protein and the possibility that the *in vitro* growth and infectivity of the mutant is probably dependent on the remaining TcCRT allelic copy. Indeed

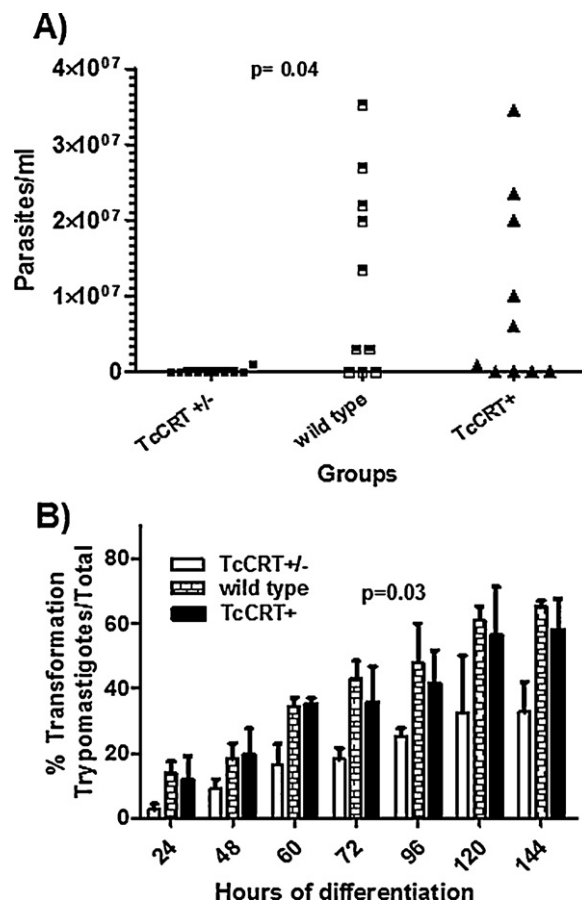


Fig. 6. Metacyclogenesis is strongly inhibited, both *in vivo* and *in vitro* in TcCRT^{+/-} *T. cruzi*. (A) Number of infected insects at day 30 post-feeding with blood contaminated with TcCRT^{+/-}, TcCRT⁺ and wild type parasites. Each dot represents a bug. Metacyclogenesis is different ($p=0.04$) between TcCRT^{+/-} and wild type groups. (B) Kinetics of metacyclogenesis induced by triatomine gut homogenate. The results shown are the means \pm s.d. of 3 independent experiments. Kinetics of metacyclogenesis are different ($p=0.03$) between wild type and TcCRT^{+/-} parasites in all tested time points.

this negative finding could corroborate previous observations indicating that TcCRT, translocated from the ER to the parasite surface is important in infectivity, *via* C1q (Ramírez et al., 2010). TcCRT is a multifunctional *T. cruzi* molecule. It is an important reservoir of Ca²⁺ in the ER and participates in the glycoprotein folding quality control system (Conte et al., 2003; Labriola et al., 1999; Michalak et al., 2009). Besides parasite contact and penetration into the host cell (Ramírez et al., 2010), complete deletion of the TcCRT gene could affect some of these important functions so that a null mutant would not be viable. We were able to detect a significant impairment in the *in vitro* growth rate of TcCRT^{+/-} epimastigotes with respect to its parental, TCC wild type line. These data indicate that deletion of a single allele of TcCRT is sufficient to slow down their growth in axenic cultures (Fig. 3).

To infect its host, *T. cruzi* must evade the cytolytic effects of the complement system. To do this, it uses the so-called complement regulatory proteins (CRPs), which bind key complement proteins thus interrupting the cascade that leads to parasite lysis. The functions of these molecules as complement inhibitors were inferred mainly through the use of overexpressing parasites using pTEX or pTREC vectors (Cestari et al., 2008; Norris, 1998; Tambourgi et al., 1993) but it was never carried out using parasites with gene targeted deletions. Here we used both approaches to study the TcCRT general functions and particularly their role in complement inactivation.

The kinetics of complement mediated killing (CMK) at near physiological conditions indicated that only 10 min of NHS incubation were enough to observe a divergence in the susceptibility of TcCRT+/- and TcCRT+ epimastigotes in contrast to wild type parasites (Fig. 4A).

In CMK assays, where the 3 complement pathways are active, we demonstrated that TcCRT+/- parasites displayed an enhanced susceptibility to killing, especially when serum concentration exceeded 10%. Conversely, TcCRT+ parasites showed levels of complement resistance of approximately 80–98% under the assay conditions. Wild type parasites remained sensitive, displaying an intermediate level (Fig. 4B). Most likely, the results obtained were a consequence of the TcCRT polypeptide's ability to bind and hijack C1 from serum interrupting the classical complement pathway (Ferreira et al., 2004a,b, 2005; Valck et al., 2010). Thus, susceptibility to CMK observed in TcCRT+/- parasites is probably due to decreased C1q hijacking from serum produced by the lower expression of TcCRT on parasite surface. On the other hand, TcCRT+ resistance to CMK is consistent with an increased C1q depletion from serum originated by TcCRT overexpression. According to these observations, when CMK assays were performed adding exogenous C1, a significantly reduction of TcCRT+ parasites resistance were observed (Fig. 4D). A similar result is obtained when increasing concentrations of NHS are added to the system, as a source of C1. When the CMK assays were performed using EGTA-treated NHS, which block the classical and lectin pathway, killing divergences disappeared and parasites were killed to the same extent (Fig. 4C). This result supports TcCRT involvement in the inhibition of the classical and lectin pathway as determined by *in vitro* assays (Ferreira et al., 2004b). Similar results using EGTA-treated NHS were reported for the C2 receptor inhibitor trispanning gene (CRIT) of *T. cruzi*, where the capture of C2 complement component from serum inhibited the activation of the classical pathway (Cestari et al., 2008).

UGGT-/- parasites behave similar to wild type, with regard to their susceptibility to CMK (Fig. 5). Thus, although TcCRT does not play a role in protein folding in UGGT-/- parasites, the translocated parasite protein capacity to down regulate the classical complement pathway, does not seem to be affected in these mutants. Although available UGGT-/- parasites are from CL Brener strain, it is likely that this behavior is also evidenced in the TCC strain parasites. In addition, parasite growth does not seem to be affected when UGGT function is inhibited (Conte et al., 2003). Therefore, it could be proposed that the growth decrease of our TcCRT+/- parasites (Fig. 3) is not due to the lectin TcCRT properties.

In all performed CMK assays, a difference between the results obtained with epimastigotes, as compared to trypomastigotes, is expected since these last forms are naturally resistant to NHS. Probably derived from the genetic modifications, it was not possible to us recover trypomastigotes to perform the assays.

When feeding *T. infestans* with TcCRT+/- parasites the proportion of infected bugs, as well as the concentration of parasites in feces of infected insects was lower than in the wild type group (Fig. 6A). Considering the reduced growth rate of epimastigotes TcCRT+/- (Fig. 3) a possible explanation could be that when TcCRT+/- blood trypomastigotes access the insect's digestive system, they transform into epimastigotes, with possible decrease in their growth, as compared to non-mutant counterparts. Thus, a lower metacyclic trypomastigote number should be expected in the hematophagous feces. These results do not show a decrease in metacyclogenesis in TcCRT+/- parasites, but they support in an *in vivo* assay the TcCRT+/- proliferation reduction summarized in Fig. 3.

TcCRT monoallelic mutation affect the ability of the parasite to multiply and complete the transition to infective stages in *in vitro* experiments as shown by the impairment in metacyclic differentiation by triatome gut homogenate addition to the culture media

(Fig. 6B). Thus, the existence of a direct or indirect relationship between the TcCRT gene product and metacyclogenesis control mechanisms, as suggested by these results, should be analyzed in more detail. Furthermore, the increased production of TcCRT (pTRES-transformed parasites) had no effect on the rate of metacyclogenesis and these parasites behave as wild type clones.

In conclusion, our findings using mutant parasites corroborate the *in vitro* conclusions obtained by Ferreira et al.: TcCRT, on parasite surface, can bind C1 and probably MBL and ficolins from serum inhibiting the classical and lectin complement pathway activation respectively. Thus, parasites with diminished TcCRT expression were susceptible to CMK, the opposite occurs when overexpressed. The evidence presented here strongly argues for the role of TcCRT as a complement evasion strategy and as a virulence factor in *T. cruzi*.

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