Trypanosoma cruzi calreticulin inhibits the complement lectin pathway activation by direct interaction with L-Ficolin

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ABSTRACT

Trypanosoma cruzi, the agent of Chagas' disease, the sixth neglected tropical disease worldwide, infects 10–12 million people in Latin America. Differently from T. cruzi epimastigotes, trypomastigotes are complement-resistant and infective. CRPs, T-DAF, sialic acid and lipases explain at least part of this resistance. In vitro, T. cruzi calreticulin (TcCRT), a chaperone molecule that translocates from the ER to the parasite surface: (a) Inhibits the human classical complement activation, by interacting with C1, (b) As a consequence, an increase in infectivity is evident and, (c) It inhibits angiogenesis and tumor growth. We report here that TcCRT also binds to the L-Ficolin collagenous portion, thus inhibiting approximately between 35 and 64% of the human complement lectin pathway activation, initiated by L-Ficolin, a property not shared by H-Ficolin. While L-Ficolin binds to 60% of trypomastigotes and to 24% of epimastigotes, 50% of the former and 4% of the latter display TcCRT on their surfaces. Altogether, these data indicate that TcCRT is a parasite inhibitory receptor for Ficolins. The resulting evasive activities, together with the TcCRT capacity to inhibit C1, with a concomitant increase in infectivity, may represent T. cruzi strategies to inhibit important arms of the innate immune response.

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1. Introduction

Trypanosoma cruzi (T. cruzi), the agent of Chagas' disease (Chagas, 1909), an acute and chronic illness affecting 10–12 million people in Latin America (Moncayo, 2003), causes 50,000 deaths per year (Dias et al., 2002; WHO, 2002) and it is the sixth neglected tropical disease worldwide. It is responsible for 0.7 million disability-adjusted life-years (Hotez et al., 2006). Infected individuals can travel to non-endemic countries, where transplacental, organ transplants, blood transfusions, among other possible

means, represent alternative ways for parasite dissemination. Thus, there are now over 300,000 infected people in the United States, more than 5500 in Canada and about 90,000 in Europe, Japan and Australia, altogether (Rodrigues and Albajar, 2010). Consequently, new research, epidemiological, economic, social and political challenges are now emerging (Clayton, 2010; Rodrigues and Albajar, 2010). Moreover, the zoonotic nature of the disease poses new concerns with regard to its impact on wild animals or those with productive or affective values, and their roles as possible parasite reservoirs.

The parasite cycles between arthropod vectors and mammals, Homo sapiens sapiens included. Briefly, haematophagous Triatominae insect vectors (at least six species in America, (Petherick, 2010)), are infected by trypomastigotes during their blood meals. In the insect' digestive tract the parasite cycles to epimastigotes and later into infective trypomastigotes that are deposited onto the host' skin together with the feces. By still controversial means, the parasite penetrates the skin and invades virtually any kind of nucleated cell,

where it transforms into amastigotes, the multiplicative form. After several cycles of mitotic divisions, they transform into infective trypomastigotes and are liberated into the circulation, where they may infect virtually any nucleated cell or new vectors (Burleigh and Andrews, 1998). The disease is acquired mainly *via* the arthropod vectors but increasing importance is recognized to transplacental infection, organ transplants, as well as to the role of blood transfusions and contaminated foods (Coura, 2006; Flores-Chavez et al., 2008; Freilij and Altcheh, 1995; Kun et al., 2009; Yoshida, 2009).

In the human complement system, activated by three pathways (Morley and Walport, 2000), similar to C1 and MBL, Ficolins are danger signal recognition molecules (Fujita et al., 2004). They resemble C1q in electron micrographs and trigger complement activation through binding to the MBL-associated serine proteases (MASPs), functional homologues of C1r and C1s (Dahl et al., 2001; Iwaki et al., 2006; Matsushita and Fujita, 1992; Stover et al., 1999; Thiel et al., 1997). L-, H- and M-Ficolins have been described (Endo et al., 1996; Lu et al., 1996; Sugimoto et al., 1998; Teh et al., 2000; Wittenborn et al., 2010). L and M-Ficolins bind to lipoteichoic acid (LTA) (Lynch et al., 2004) and sialic acid, both cell components found in Gram-positive and negative bacteria, respectively, with subsequent MASPs activation (Gout et al., 2010). Thus, Ficolins significantly contribute to innate immunity.

T. cruzi interacts with the complement system utilizing a variety of defensive and infective strategies (Krettli et al., 1979). Interestingly, infective trypomastigotes are highly resistant to complement activity, while non-infective epimastigotes from axenic cultures are susceptible (Krettli et al., 1979). Several molecules, mainly expressed by trypomastigotes, participate in this resistance; among others, the *T. cruzi* complement regulatory protein (CRP) (Norris et al., 1991; Norris and Schrimpf, 1994), the human decay accelerating factor *T. cruzi* homologue (T-DAF) (Norris et al., 1991), sialic acid (Meri and Pangburn, 1990; Tomlinson and Raper, 1998) and specific lipases with capacity to release complement proteins bound to parasite surface components (Tomlinson and Raper, 1998), have been described.

We have cloned, sequenced and expressed a *T. cruzi* calreticulin gene (*TcCRT*), which codes for a 45 kDa protein, that displays 60% homology and 42% identity with the human counterpart (HuCRT) (Aguillon et al., 2000; Ferreira et al., 2002, 2004a). Similar to HuCRT (Stuart et al., 1996, 1997), *in vitro*, TcCRT inhibits the classical pathway of human complement activation, upon interacting with C1 (Ferreira et al., 2004a,b, 2005) and competing with the $(C1r–C1s)_2$ complex for binding to the collagenous C1q tails, thus interfering in C1 formation (Valck et al., 2010). Also, TcCRT operationally interferes with C1s activity, in a calcium-independent manner, without involving the release of the protease from the C1q recognition molecule (Valck et al., 2010). Moreover, TcCRT, translocated from the ER to the trypomastigote area of flagellum emergence, recruits C1 and C1q, as a likely immune evasion strategy. Complement inhibition and increased infectivity, two central events in the host/parasite interactions, are thus evident (Ferreira et al., 2004b; Rimoldi et al., 1989). (The extraordinary TcCRT pleiotropism is even reflected in its capacity to interact with endothelial cells and to inhibit angiogenesis and tumor growth, a fact that may also influence the host-parasite interactions (López et al., 2010; Ramírez et al., 2011a)).

Knowledge on how the parasite interferes with the activation of the complement system, a main effector arm of innate immunity, may facilitate the development of strategies to interfere with the aggressor infectivity.

Herein, we propose that TcCRT actions extend beyond its interactions with C1–C1q by also inhibiting the human lectin pathway activation, initiated by L-Ficolin binding to LTA. Similar to HuCRT (Lacroix et al., 2009), TcCRT binds to the L-Ficolin collagenous portion but, differently from the human chaperone, it does not bind to H-Ficolin. We also propose that trypomastigotes display significant amounts of TcCRT on their surfaces, as compared to epimastigotes. As a possible consequence, human serum L-Ficolin binds preferentially to trypomastigotes and significantly impairs the L-Ficolin capacity to activate C4. It is then possible that the parasite uses its CRT to inhibit the lectin pathway activation, as a survival strategy. Most likely, this functional parallelism between Ficolins and C1q results from the physical and functional homologies that exist between both molecules, mainly at their collagenous domains.

2. Materials and methods

2.1. Serum Ficolin binding to TcCRT

Serum Ficolin binding to TcCRT was determined by ELISA (Lynch et al., 2004), with some modifications. Microtitration wells (Nunc MaxiSorp, USA) were sensitized with 100 µl/well of recombinant TcCRT (0.01 mg/ml), in carbonate buffer (15 mM Na_3CO_3, 35 mM $NaHCO_3$, pH 9.6), overnight at 4 °C. The remaining solid phase active sites were blocked with 250 µl/well TBS/0.1% w/v BSA for 2 h at room temperature (RT). Then, the wells were washed 4 times with TBS/Tween/Ca^{2+} (TBS/0.05% Tween-20, 5 mM $CaCl_2$). Different dilutions of C1q-depleted serum (Calbiochem, USA), diluted in binding buffer (20 mM Tris–HCl, 10 mM $CaCl_2$, 140 mM NaCl, 0.05% Triton X-100, 0.1% BSA, pH 7.4) were added as a Ficolin source, and incubated 1 h at RT. After washing, L- and H-Ficolin binding to TcCRT was measured using rabbit polyclonal anti-L or H-Ficolin antibodies, generated in one of our laboratories (T.F.), diluted in TBS/Tween/Ca^{2+}. Both reactions were measured with anti-rabbit IgG-alkaline phosphatase (1/10,000 in TBS/Tween/Ca^{2+}), followed by *p*-nitrophenylphosphate (*p*NPP) and detection at 405 nm. As positive and negative controls, some wells were sensitized with anti-human L- or H-Ficolin monoclonal antibodies (GN4 and 4H5 clones, respectively, from HyCult, The Netherlands), or BSA.

2.2. TcCRT effects on L-Ficolin activation

Microtitration wells were sensitized with LTA (1 µg/well in carbonate buffer) during 4 h at RT. The remnant active sites were blocked with 250 µl/well of buffer TBS/0.1% BSA for 2 h at RT. Then, the wells were washed 4 times with TBS/Tween/Ca^{2+}. Human C1q-depleted serum was incubated with TcCRT (0, 1.2, 4.8 µM in binding buffer) during 30 min at 37 °C, before adding to the LTA coated wells. The plate was then incubated for 1 h at RT, washed, and L-Ficolin activation was assessed by adding human C4 (Calbiochem, USA), at 4 µg/ml in BBS^{2+} buffer (4 mM Barbital, 145 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4) to the wells and incubated for 1.5 h at RT. C4b deposition was detected with a chicken anti-human C4-alkaline phosphatase conjugated antibody (ImmunSystem AB, Sweden) (1/1000 in TBS/Tween/Ca^{2+}). The reaction was revealed with *p*-nitrophenylphosphate (*p*NPP) and the colored product was detected at 405 nm.

2.3. LTA binding to L-Ficolin in the presence of TcCRT

Microtitration wells were sensitized with LTA (Sigma–Aldrich, USA) (1 µg/well in carbonate buffer) overnight at 4 °C. Blocking of the remnant active sites and subsequent washings were performed as described in the previous section. Human C1q-depleted serum, as a source of L-Ficolin, diluted 1/15 in binding buffer, was incubated with TcCRT (0, 0.3, 0.6, 1.2, 2.4, 4.8 µM) for 30 min at 37 °C, before addition to the wells, and then incubated for 1 h at RT. After washing, the L-Ficolin binding to LTA, in the presence of TcCRT, was detected using rabbit polyclonal anti-L Ficolin antibodies (1/1000 in TBS/Tween/Ca^{2+}) and a sheep anti-rabbit IgG-alkaline

phosphatase conjugated (1/10,000 in TBS/Tween/Ca^{2+}). The reaction was revealed and read as described above. As a negative control, the assay was performed in the presence of equimolar BSA concentrations.

2.4. L-Ficolin binding to T. cruzi

Four microgram of recombinant human L-Ficolin (Lacroix et al., 2009) were incubated with 2×10^6 epimastigotes or trypomastigotes for 30 min at RT. Then the parasites were washed with PBS, incubated with a polyclonal rabbit anti-human L-Ficolin antibody (1/50 in PBS/0.4% BSA) for 30 min at RT. After a new series of washings, the parasites were incubated with a sheep anti-rabbit IgG-FITC antibody (1/50 in PBS/0.4% BSA) (Sigma-Aldrich, USA), washed, fixed with 1% *p*-formaldehyde and analyzed by flow cytometry (FACS Scan, Becton Dickinson).

2.5. Presence of TcCRT on T. cruzi surface

2×10^6 Epimastigotes or trypomastigotes were incubated with polyclonal rabbit anti-TcS and TcCRT antibodies (1/50 in PBS/0.4% BSA) for 30 min at RT. Then, the parasites were washed with PBS, incubated with sheep anti-rabbit IgG-FITC (1/50 in PBS/0.4%BSA) (Sigma–Aldrich, USA) for 30 min at RT, washed, fixed with 1% *p*-formaldehyde and analyzed by flow cytometry (FACS Scan, Becton Dickinson).

2.6. Statistical validation of experimental data

When pertinent, data were analyzed by two tailed *t*-tests. Experiments shown are representative of at least two trials.

3. Results

3.1. L-Ficolin, but not H-Ficolin, binds TcCRT

Since we previously demonstrated that TcCRT binds C1 and C1q (Ferreira et al., 2004b), we used C1q depleted serum as an L- and H-Ficolin source. L- or H-Ficolin binding to TcCRT was detected with specific polyclonal antibodies, as shown in Fig. 1. L- but not H- Ficolin specifically binds to immobilized TcCRT, as compared to the positive controls represented by binding of L- and H-Ficolin to the respective solid-phase bound antibodies (Fig. 1A and B, respectively).

3.2. TcCRT inhibits L-Ficolin activation but it does not interfere with LTA binding to L-Ficolin

Given the structural homologies between C1q and Ficolins, and since TcCRT interferes with the classical pathway activation (Ferreira et al., 2004b; Valck et al., 2010), we asked whether this inhibitory capacity is also exerted on L-Ficolin. Microtitration wells were sensitized with lipoteichoic acid (LTA). C1q-depleted serum, as a source of L-Ficolin, was incubated with TcCRT before its addition to the wells. To detect L-Ficolin activation, purified C4 was added and C4b deposition was then measured. As shown in Fig. 2 A, under these conditions, TcCRT inhibits L-Ficolin dependent lectin pathway activation. Since neither MBL nor H-Ficolin bind LTA (Lynch et al., 2004), the activation initiated by LTA is mediated by L-Ficolin.

Physiologically, L-Ficolin binds through its globular heads to LTA, present in Gram positive bacteria (Lynch et al., 2004). For this reason, we tested whether TcCRT binding to L-Ficolin affects this important physiologic function. C1q-depleted serum, as a source of L-Ficolin, was incubated with increasing TcCRT concentrations
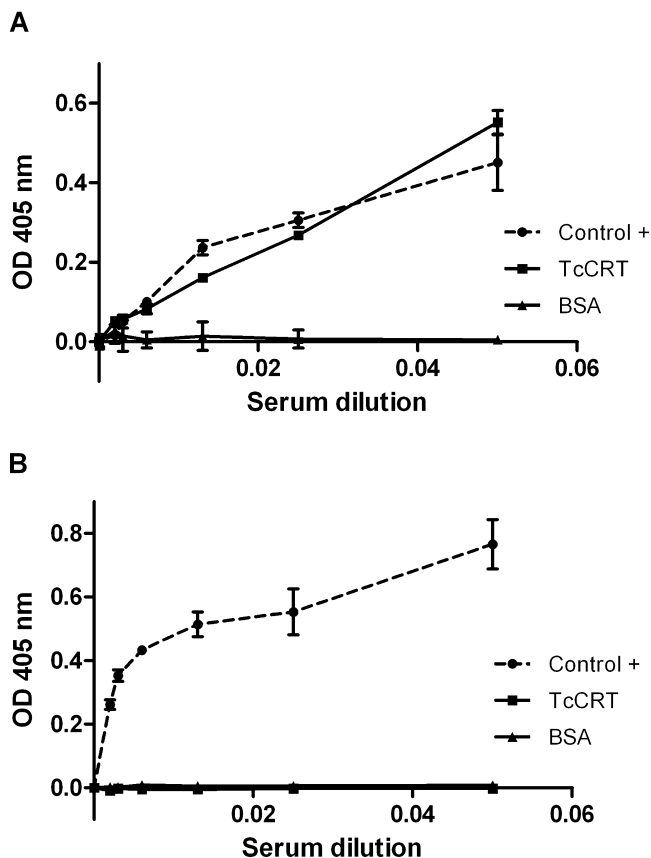

Fig. 1. L- but not H-Ficolin binds to TcCRT. The solid phase was alternatively sensitized with TcCRT, or, as positive control, with anti-human L-Ficolin antibody (A) or anti-human H-Ficolin antibody (B). BSA was used as negative control. After incubation with human C1q depleted serum, the L-Ficolin (A) or H-Ficolin (B) binding to TcCRT was detected with specific polyclonal antibodies. Bars represent standard deviations.

before its addition to microtitration wells sensitized with LTA. L-Ficolin binding to LTA, in the presence of TcCRT, was then detected with a rabbit polyclonal anti-human L-Ficolin antibody. Under these conditions, TcCRT did not affect the L-Ficolin capacity to bind LTA (Fig. 2B).

3.3. Human L-Ficolin binding to T. cruzi epimastigotes and trypomastigotes is quantitatively different

Considering that TcCRT is translocated from the endoplasmic reticulum (ER) to the parasite surface (Ferreira et al., 2004b), and that it binds to L-Ficolin *in vitro* inhibiting its activation, we tested whether L-Ficolin binds to the surface of both *T. cruzi* epimastigotes and trypomastigotes. Non-infective epimastigotes, from axenic cultures, and infective trypomastigotes were incubated with purified recombinant human L-Ficolin. Its binding to the parasites was detected with polyclonal antibodies, followed by flow cytometry analysis. By comparing the density plots shown in Fig. 3A–C(epimastigotes) and D–F (trypomastigotes), and their respective histograms (panels G and H), it can be concluded that human L-Ficolin binds to 24% of epimastigotes and to 49% of trypomastigotes, as compared to the secondary FITC-labeled antibody binding to 12% of trypomastigotes without L-Ficolin. (At present, we do not know the reason for the binding of the anti-Ficolin polyclonal antibodies to untreated trypomastigotes. However, the possibility that trypomastigotes, but not epimastigotes, express an unknown protein that is recognized by these antibodies cannot be ruled out). These results were also confirmed by fluorescence microscopy (not shown).
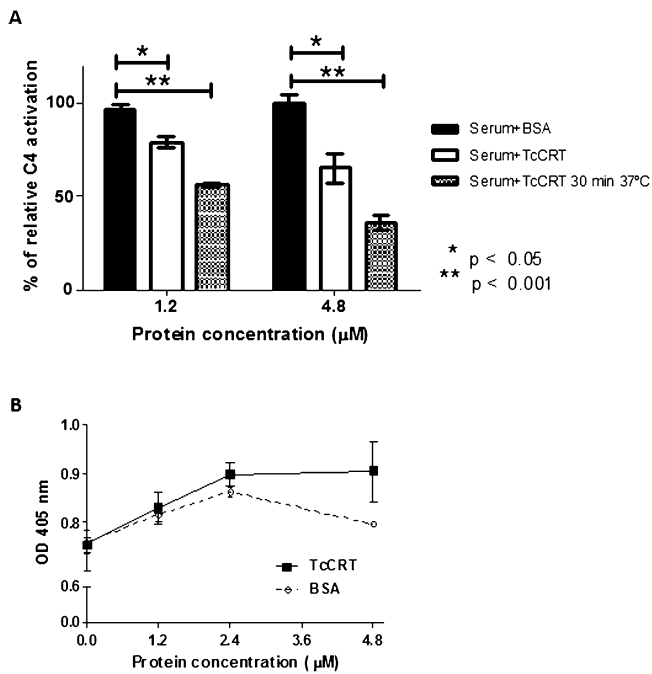
Fig. 2. TcCRT inhibits L-Ficolin activation but it does not interfere with its LTA binding. Microtitration wells were sensitized with LTA. C1q-depleted serum was incubated with increasing concentrations of TcCRT before its addition to the wells. (A) L-Ficolin activation was detected as a function of C4b deposition onto the solid phase. Bars represent standard deviations. (B) L-Ficolin binding to LTA, was determined in the presence of increasing TcCRT concentrations. Bars represent standard deviations. Results were analyzed by two tailed *t*-tests.

3.4. TcCRT is extensively expressed on the surface of trypomastigotes, as compared to epimastigotes

By flow cytometric analysis, 50% of live infective trypomastigotes display TcCRT on their surfaces (Fig. 4D), as compared to 4%

of non-infective epimastigotes (Fig. 4J). This low TcCRT detection in epimastigotes is obtained with both polyclonal antibodies anti TcCRT or against its S-domain (Fig. 4I–J, respectively). In both cases, the preimmune antibodies did generate non-specific lower signals (Fig. 4C and H). (At present, we do not know the reason for the binding of preimmune antibodies to both trypomastigotes and epimastigotes. Since these preimmune sera were generated in adult rabbits, the presence of cross-reactive antibodies is likely. In any case, the immune sera, obtained in the same animals, does discriminate the TcCRT presence in the two parasite forms). Derived histograms are shown in Figs. 4E and K.

4. Discussion

Trypanosoma cruzi calreticulin displays important functions in the vertebrate host-parasite interactions including inhibition of the classical pathway of complement activation (López et al., 2010; Ramírez et al., 2011a,b; Valck et al., 2010). Given the structural and functional homologies between C1q from the classical pathway and Ficolins from the lectin pathway, we tested whether TcCRT interacts with Ficolins, with resulting functional impairment of this pathway. Using human C1q-depleted serum as a Ficolin source, we observed that L-Ficolin (Fig. 1A), but not H-Ficolin (Fig. 1B), bind TcCRT. These results could be explained by the relatively low amino acid homology between H- and L- Ficolins (48%) (Garred et al., 2009). Since HuCRT binds to both L- and H-Ficolins (Lacroix et al., 2009), the preferential TcCRT binding to L-Ficolin could also be explained by the differences (58%) in amino acid sequence between HuCRT and TcCRT. These molecules display 60% homology and 42% identity.

In vitro, activation of the lectin pathway can be monitored in an ELISA, where Ficolins interacting with solid phase bound LTA will activate C4 (Lynch et al., 2004). Normally, in order to prevent C1 interference, lectin pathway activation is studied under high ionic strength. As an alternative, in our experiments we used a human C1q-depleted serum, as a Ficolin source. Specific dose-dependent
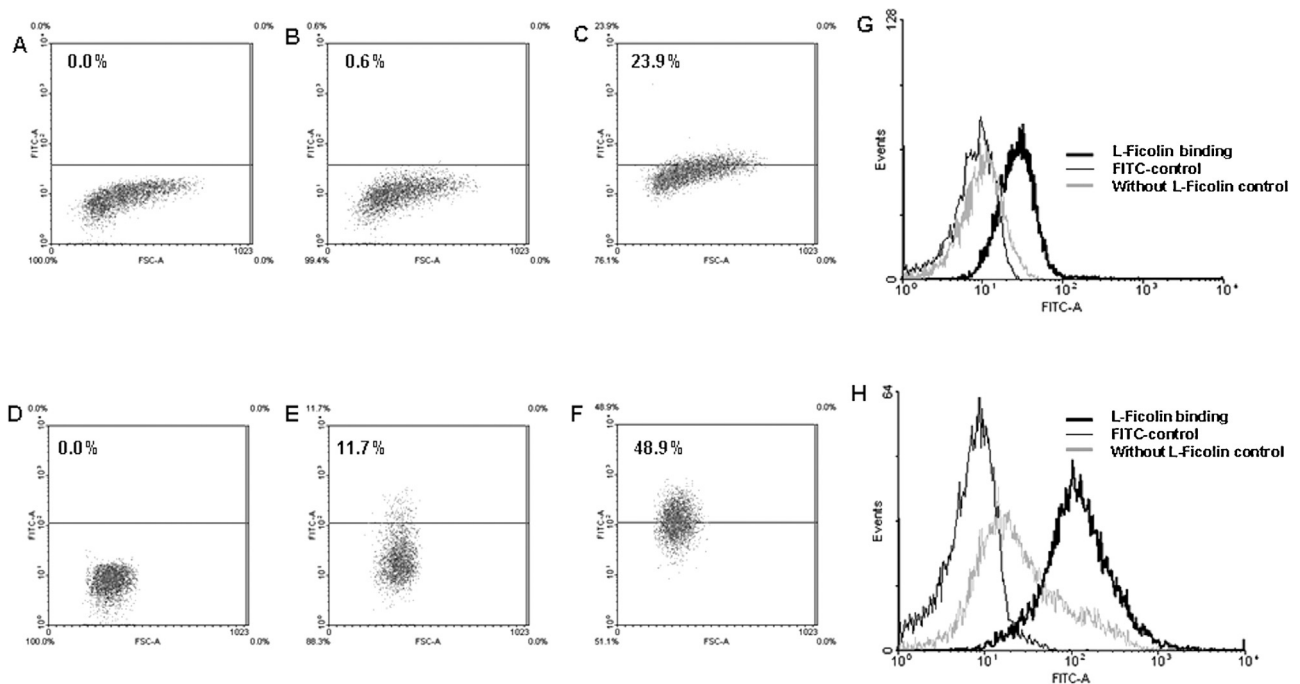

Fig. 3. Human L-Ficolin binds preferentially to trypomastigotes. Epimastigotes or trypomastigotes were sequentially incubated with recombinant L-Ficolin, polyclonal rabbit anti-human L-Ficolin and sheep anti-rabbit IgG-FITC antibodies. After fixing with formaldehyde they were analyzed by FACS Scan. (A–C) and (D–F) Density plots of epimastigotes and trypomastigotes, respectively. (A and D) FITC controls, (B and E) controls without L-Ficolin. (C and F) binding of L-Ficolin. (G and H) Histograms of L-Ficolin binding to epimastigotes and trypomastigotes, respectively.
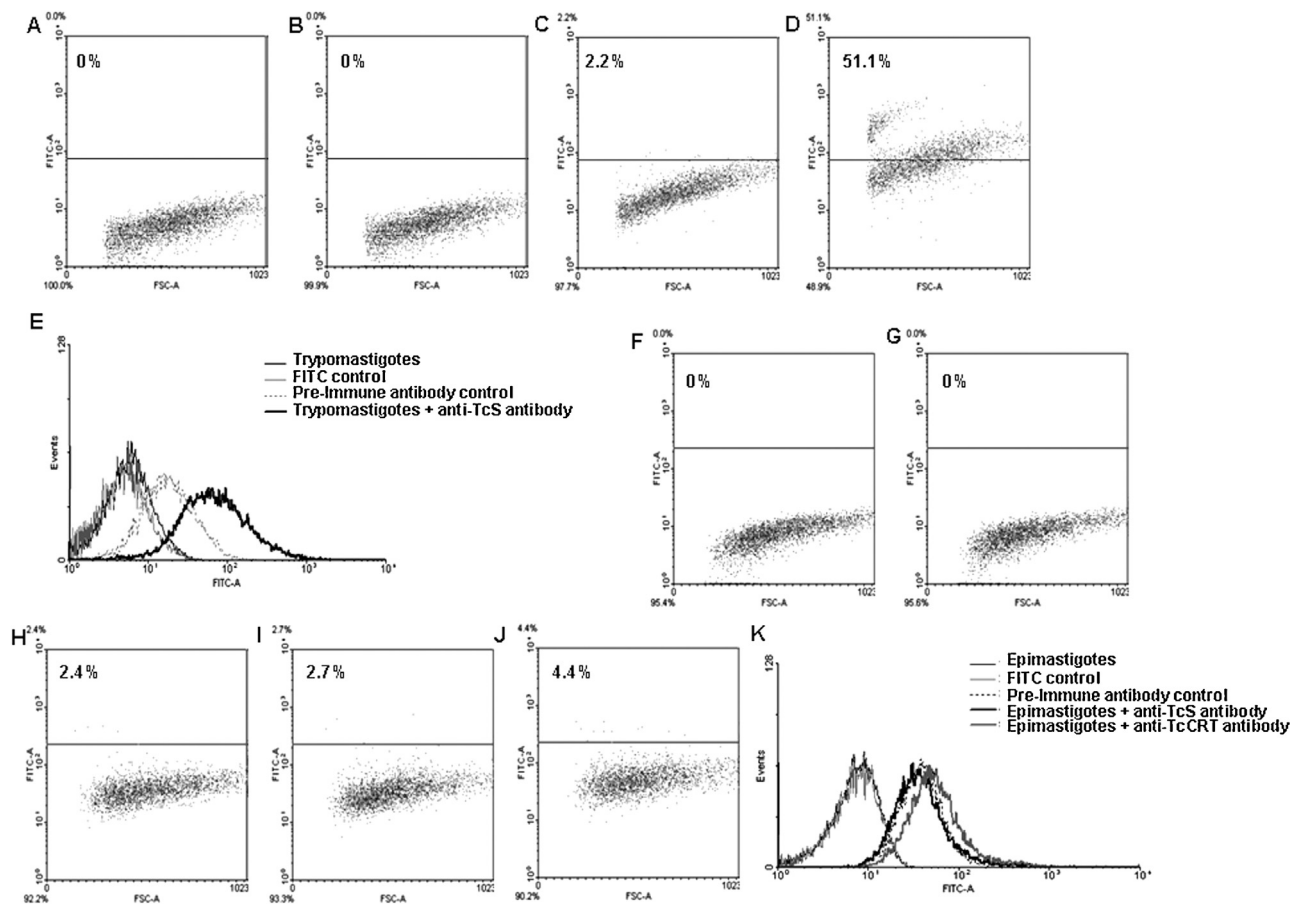
Fig. 4. TcCRT is present in the surface of trypomastigotes and marginally on epimastigotes. Epimastigotes or trypomastigotes were sequentially incubated with recombinant polyclonal rabbit anti-TcS or TcCRT and sheep anti-rabbit IgG-FITC antibodies. After fixing with formaldehyde they were analyzed by FACS Scan. (A–D) and (F–J) Density plots of trypomastigotes and epimastigotes, respectively. (A and F) parasites only. (B and G) FITC controls. (C and H) controls with pre-immune antibodies. (D and I) binding of anti-TcS antibodies. (J) binding of anti-TcCRT antibodies. (E and K) Histograms of detection of TcCRT in trypomastigotes and epimastigotes, respectively.

TcCRT inhibition of L-Ficolin activation was achieved using solid-phase bound LTA (Fig. 2A) (a molecule that does not bind H- or M-Ficolins).

Physiologically, L-Ficolin binds to lipoteichoic acid (LTA), present in Gram positive bacteria (Lynch et al., 2004). As shown here, TcCRT binds to human L-Ficolin and inhibits its activity. This interaction does not take place through the globular fibrinogen-like portion since, pre incubation of L-Ficolin (present in a human C1q-depleted serum) with TcCRT, did not interfere with its binding to LTA (Fig. 2B). This behavior is similar to that described for HuCRT, that binds to the MASP binding site on L-Ficolin (Lacroix et al., 2009).

Recombinant L-Ficolin binds to 60% of trypomastigotes and to 24% of epimastigotes (Fig. 3). These observations agree with those showing that L-Ficolin and H-Ficolin bind to both cultured epimastigotes and trypomastigotes from the Silvio X10/6 strain (Cestari Idos et al., 2009), although no differential quantification was provided.

We have shown that TcCRT specifically binds and inactivates L-Ficolin in its capacity to mediate C4b generation. In other words, in the presence of TcCRT, L-Ficolin cannot be activated by LTA. However, this binding does not impair the L-Ficolin capacity to bind to its natural ligand LTA (Fig. 2B). Whether a direct TcCRT- MASP interaction takes place, with or without release of the Ficolin-associated serine proteases, is unknown. Since most trypomastigotes expose TcCRT on their membrane (Fig. 4), it is highly possible that L-Ficolin binding to the parasite occurs mainly through this protein. Inactivation of L-Ficolin capacity to mediate C4 (and presumably C2) activation, will follow. Whether, similar to C1q (Castillo et al., 2013; Ramírez et al., 2011b), L-Ficolin bound to parasite surface TcCRT plays a preferential role in trypomastigote infectivity, as compared to epimastigotes, is currently under investigation in our laboratory.

Since epimastigotes either do not expose or expose marginally TcCRT on their surface (Fig. 4), L-Ficolin binding to this parasite stage may be also explained by the presence of a different, still unidentified receptor or cell-surface ligand.

In synthesis, we propose here that L-Ficolin binding to TcCRT, with consequent functional inhibition of the lectin pathway may represent a *T. cruzi* strategy to inhibit an important arm of the innate immune response. Given the preferential TcCRT exposure in trypomastigotes, as compared to epimastigotes, this evasive strategy is more relevant for the trypomastigote stage. Thus, the greater epimastigote susceptibility to complement action may be, at least partly due to their lower amount of membrane bound TcCRT. Implicit in this proposal is the necessity that both trypomastigotes and epimastigotes should have surface molecules (different from TcCRT), still unidentified, but clearly detectable as danger signals by the L-Ficolin globular domains. Alternatively, in the absence of Ficolin-binding danger signals, the TcCRT/L-Ficolin interaction may serve the parasite as "eat me" signals, similar to those determined by C1q (Castillo et al., 2013; Ramírez et al., 2011b). This issue is under investigation in our laboratory.

Conflicts of interest statement

Acknowledgments

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