Review

Extracellular *Trypanosoma cruzi* calreticulin in the host–parasite interplay

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Calreticulin (CRT) from vertebrates is a calcium-binding protein present mainly in the endoplasmic reticulum (ER). There, it directs the conformation of proteins and controls calcium levels. This review will focus on several extracellular roles of *Trypanosoma cruzi* CRT (TcCRT) in relation to its capacity to inhibit the complement system, mediate parasite infectivity, interfere with angiogenesis and, as a possible consequence, with tumor growth. The TcCRT antiangiogenic effect parallels with the capacity of *T. cruzi* infection to inhibit tumor development *in vivo*. Thus, the TcCRT, complement, and endothelial cell interactions seem to be an evolutionary adaptation to promote prolonged parasite–host relationships.

Why is Chagas disease important?

Trypanosoma cruzi is the agent of Chagas disease. It is an acute and chronic illness affecting 12 million people in America, causing 15,000 deaths per year, and is considered to be one of the most important neglected tropical diseases worldwide [1]. It causes 0.7 million disability-adjusted life-years. There are >100 species of triatomine bugs that can transmit *T. cruzi* [1]. The parasite infects virtually all mammals through altered skin and mucous membranes, and causes acute, but also various mild, symptoms. It can infect all nucleated mammalian cells, where it multiplies and then enters the bloodstream and circulates to reach other tissues. People and animals can also be infected by ingesting food contaminated with feces from an infected arthropod vector [2]. Humans can then transmit infection through donation of blood or organs, as well as across the placenta. The immune system controls the acute infection in most patients, even without treatment. However, in ~30% of chronically asymptomatic infected humans, heart arrhythmia, failure and even sudden cardiac death, as well as a pathologically dilated colon and esophagus may occur [2]. The parasite is also carried by several hundred thousand people (mainly South American immigrants) in Spain, Portugal, USA, Canada, Japan and Australia [1]. Unlike other trypanosomes such as blood-borne *Leishman-*

Glossary

Angiogenesis: Complex physiological process involving the organization of proliferating endothelial cells into new blood vessels from pre-existing vessels. It is also known as 'neovascularization'. This is a normal process in growth and development (as well as in wound healing). However, this is also a fundamental step in the transition of tumors from being dormant to becoming malignant. It is also a fundamental step in various inflammatory processes.
C1: First component of the classical complement activation pathway. It is composed of C1q and the C1r and C1s serine proteases. C1 binds to antigen-bound IgG or IgM via the C1q globular heads.
C2, C3 and C4: Complement proteins that, after activation, can form C3 (C4b,2b) and C5 (C4b,2b,3b) convertases. These are protein enzymatic complexes that can activate C3 and C5, respectively.
C5b-9: Membrane attack complex (MAC) composed of complement components C5b, C6, C7, C8 and C9.
MBL: Mannose-binding lectin. It binds to mannan-derived molecules present in various microbial aggressors. It participates in the lectin pathway of complement activation.
Ficolins: bind to the lipotheichoic acid and related molecules present in various 'microbial aggressors'. It participates in the lectin pathway of complement activation.
C3b and C4b: Opsonic (fagocitosis-stimulating) protein fragments generated during classical activation or lectin complement activation. C3b is also generated via the alternative pathway.
C3a, C4a, C5a: Anaphylatoxic (proinflammatory) protein fragments generated during the classical, lectin or alternative (C5a) pathways of complement activation.
Chicken embryo chorioallantoid membrane (CAM) assay: Experimental *in vivo* model to evaluate the pro- or anti-angiogenic drug effects in fertilized hens' eggs through histological quantification of the vascular reaction in the chorioallantoid membrane (site of exchange of respiratory gases, calcium transport from the eggshell, acid–base homeostasis in the embryo, and reabsorption of ions and H_2O from allantoic fluid).
Epimastigotes: Non-infective and replicative form of *Trypanosoma cruzi* present in the intestine of the vector and in axenic *in vitro* cultures. In this form, the kinetoplast and the flagellar pocket are located between the nucleus and the anterior end. The flagellum emerges at the side of the trypanosome and runs along the body, building a short undulating membrane.
Trypomastigotes: Infective and non-replicative form of *Trypanosoma cruzi*. This form is located in the blood of mammalian hosts or in the rectum of the arthropod vector (metacyclic trypomastigote). This form is released with the feces after the blood-meal. Trypomastigotes have the kinetoplast and the flagellar pocket at the posterior end of the body. The flagellum runs to the anterior end along an undulating membrane.
GM-CSF: Granulocyte-macrophage colony stimulating factor. It is used as a potent cytokine adjuvant based on its activity of inducing the activation and migration of dendritic cells.
Matrigel™: Solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm mouse sarcoma (a tumor rich in extracellular matrix proteins and pro-angiogenic factors). Its major component is laminin, followed by collagen IV, heparin sulfate proteoglycans, and entactin/nidogen. This matrix provides the substrate necessary for the study of angiogenesis *in vitro* and *in vivo*.
Scavenger receptors: Receptors involved in the endocytosis of 'altered' proteins. Thus, the classical class-A scavenger receptors are well recognized for their role in the uptake of acetylated low-density lipoprotein (LDL), whereas oxidized LDL can be taken up by class-B receptors, such as members of the CD36 family and a different scavenger receptor on resident murine peritoneal macrophages.

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ia and *Trypanosoma brucei*, *T. cruzi* cycles between extracellular and intracellular stages. Probably, the intracellular parasite is less susceptible to drugs and to humoral and important arms of cellular and innate immunity. For over 40 years, the only existing treatments have been benznidazole and nifurtimox, which are effective in ≤80% of acute cases after a 60-day course. Their side effects can be severe, and their use can lead to resistance [3].

The identification of *T. cruzi* molecules involved in immune and non-immune modulation of long-term host–parasite interactions is important not only to understand the basic biological aspects of these relationships, but also to develop possible interventions aimed at shifting the balance towards the benefit of the host. Prominent among the array of 'defensive' molecules displayed by the host is the complement system, an essential effector arm of the innate immune response (Box 1).

Among *T. cruzi* regulators, complement regulatory proteins (CRPs), *T. cruzi*-decay accelerating factor (T-DAF) and sialic acid [4–7], inhibit the complement system at diverse stages of its activation, thereby contributing to chronic and equilibrated host–parasite interactions.

Another protein involved in several aspects of the host–parasite interactions is *T. cruzi* calreticulin (TcCRT). The molecular and cellular features of these interactions at the extracellular level are reviewed herein.

Box 1. The mammalian complement system

In mammals, the complement system is composed of ~40 plasma or membrane-bound proteins. It is an essential effector arm of innate immunity, and provides critical links with the adaptive immune system [58]. The system has at least three structurally related, macromolecular recognition modules able to directly interact with a large variety of 'danger signals' on the membranes of live pathogenic aggressors. First, in the lectin activation pathway, these soluble pathogen recognition molecules are mannan-binding lectin (MBL) and ficolins. Second, in the classical activation pathway, recognition of danger is carried out by C1 in a direct or indirect (via antibodies) manner. Lastly, in the alternative pathway, the system is activated by default on any cellular surface that lacks the ability to control complement activation (i.e. absence of regulatory proteins on the surface or inability to efficiently bind soluble regulatory proteins), as occurs on many pathogen surfaces. C1q, together with C1r and C1s, is the recognition and initiating molecule of the classical pathway. It has a molecular weight of 460 kDa with three polypeptide chains (A, B, and C, which are coded for by three different genes) linked together to form a triple helix structure repeated six times [59]. C1q is formed by N-terminal collagen-like regions and C-terminal globular head regions. Activation of the classical complement pathway starts when the C1 globular heads recognize danger signals represented by the antigen-aggregated immunoglobulin CH_2 (IgG) or CH_3 (IgM) Fc domains. Alternatively, C1q can also directly recognize certain pathogen-associated molecular patterns or apoptotic cells. Autoactivation of C1q-bound C1r follows, and C1r activates C1s by limited proteolysis [59]. Activated C1s then activates C4, an essential step in the generation of C3 and C5 convertases [60]. During this process, activated C4b and C3b will be covalently deposited on the recognized membranes and, as such, will constitute potent opsonins or participate in cell-bound convertases. Finally, the C5 convertase will mediate generation of the membrane attack complex [60]. During complement activation, important proinflammatory mediators are also generated, composed of complement component-soluble cleavage fragments such as C3a and C5a. Thus, during the activated cascade, peptides with opsonic, anaphylatoxic, T cell and B cell immune-stimulating and membrane-destructive capacities are generated.

Why is TcCRT central to the host–parasite interaction?

TcCRT has recently been recognized as a significant molecule that, by virtue of its capacity to interact with the first complement component C1, inhibits the system at the earliest stages of its activation [8]. Thus, since cleavage of C4 into C4b and C4a is one of the earliest events in the activation of the classical pathway, interference with this step will inhibit the entire cascade. On the other hand, TcCRT interacts directly with endothelial cells (probably through their scavenger I receptor) and inhibits angiogenesis [9]. These two apparently unrelated properties may have prevailed within this molecule to favor prolonged host–parasite interactions.

Several of the functional features described so far for TcCRT are conserved to differing degrees in CRT from vertebrates (Box 2), including the human molecule (HuCRT).

CRTs from parasites such as *Onchocerca volvulus*, *Schistosoma mansoni*, *Leishmania donovani* and *T. cruzi* are 50% identical in amino acid sequence to HuCRT. The tick *Amblyomma americanum*, while feeding on its host, secretes CRT [10], presumably as a mechanism to divert host defensive responses. The presence of CRT in penetration gland cells of schistosome cercariae suggests a regulatory influence on calcium-dependent proteases in skin penetration and parasite migration [11]. Other functions of TcCRT include its ability to bind monoglucosylated glycans and its involvement in the retention of immature protein species in the endoplasmic reticulum (ER) [12]. Thus, TcCRT participates in the maturation of the lysosomal protease cruzipain [13].

One of the hallmarks of chronic Chagas disease is the development of autoimmune reactivity, which can contribute to tissue damage. The fact that TcCRT triggers an immune response in mice against the host ortholog protein

Box 2. Calreticulin (CRT) from vertebrates

Calreticulin (CRT from vertebrates is a 46-kDa endoplasmic reticulum (ER)-resident and highly pleiotropic calcium-binding protein present in virtually every cell of higher organisms [61]. As a central component of the glycoprotein folding quality-control system, CRT binds monoglucosylated high mannose glycans. Although CRT is a typical ER-resident protein, it has also been found in the cytosol, nucleus, secretory granules, on the plasma membrane, and free in the extracellular space [61]. At present, there is no reported evidence with regard to the mechanisms involved in the translocation of vertebrate CRT or TcCRT to the cell membrane and subsequent release to the extracellular *milieu*, where it fulfills various functions.

Extracellular CRT modulates important phases of the immune response against apoptotic cancer cells [62]. In addition, if applied topically, CRT accelerates wound healing [63,64]. CRT regulates cell adhesion by interacting with the cytosolic tail of the integrin alpha subunit [61], mediates the nuclear export of some steroid hormone receptors [65–67], and regulates the stability or translation of various RNAs [68–72]. CRT can reach the cytosol and nucleus to fulfill these roles by retro-translocation, a process dependent upon its C-terminal domain and following calcium depletion in the ER [73].

All CRT proteins have three domains: globular N-terminus, proline-rich (P), and acidic C-terminus [61]. An S-domain (amino acids 160 to 289) binds to the collagenous tails of C1 [16,17]. The N-domain (60 amino acids) possesses anti-angiogenic properties [35]. From most of the species studied, the primary CRT sequences initiate with a signal peptide and terminate with a KDEL-ER retention sequence [74].

raises the possibility that chagasic cardiomyopathy may be due to an autoimmune response [14]. Although TcCRT is located mainly in the ER, histochemical studies have found it also in the Golgi complex, reservosomes, flagellar pocket, cell surface, cytosol, nucleus and kinetoplast [8,15]. As it occurs with HuCRT, the mechanisms involved in these heterogeneous TcCRT localizations are unknown.

This review focuses on how TcCRT, exteriorized from the ER, targets the first classical complement component C1 and negatively regulates the cascade, promoting parasite infectivity. We will discuss how extracellular TcCRT, by directly interacting with venous and arterial endothelial cells, inhibits angiogenesis and tumor growth. Thus, these TcCRT functions not only illustrate *T. cruzi* reactions to the immune defensive strategies of the host, but also perhaps illustrate an evolutionary adaptation to favor a prolonged interaction with its host.

TcCRT inhibits the C1q-associated C1s and C1r serine proteases

In vitro, TcCRT, similar to HuCRT [16,17], inhibits the human classical pathway, as a consequence of its capacity to interact with the first component C1 [8,18–20]. TcCRT (most probably in a *T. cruzi* immune-evasion strategy) is translocated from the parasite ER to the area of flagellum emergence [8]. There, complement component C1 is swiftly recruited by parasite-bound TcCRT, and the complement system is inhibited at the level of C4b generation, a key initial event in the activation of the entire cascade.

TcCRT not only competes with the $(C1r\text{-}C1s)_2$ tetrameric complex for binding to the collagenous C1q tails, but also interferes with the capacity of C1s to activate C4, in a calcium-independent manner and without involving the release of the serine protease from the C1q recognition module [20]. These complement-evasion strategies mediate an important decrease in C4b generation so, as well as in the resulting C3 and C5 convertases, the classical pathway and derived activities are extensively inhibited. Important consequences in the host–parasite relationships are therefore predictable (Figure 1).

In vitro, human C1r and C1s bind to TcCRT [20]. This fact is consistent with their important homology, not only in overall amino acid sequence, but also in several functional domains [21]. In agreement with these results, TcCRT interaction with solid phase-bound C1 resulted in inhibition of its capacity to activate C4. However, TcCRT does not inhibit the C4-activating function of solid phase-bound C1s [20]. Perhaps the parasite protein inactivates the C1s enzyme only when the serine protease is within a structural context provided by its interaction with the collagenous C1q tails. Recent data on the crystallographic structure of the C1 complex indicate that the serine proteases locate inside the dome generated by the six collagenous tails. This interaction is strong due to the six binding sites that are

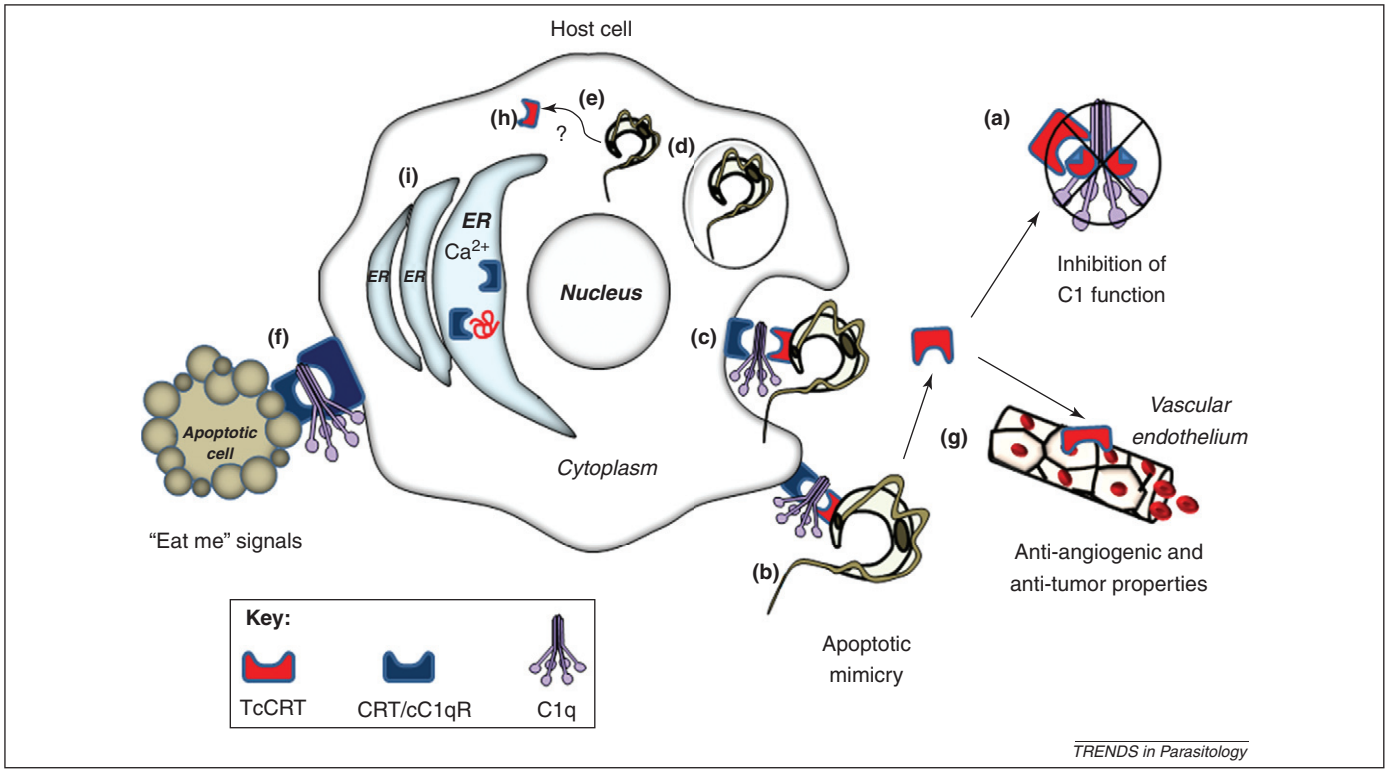

Figure 1. Roles of *T. cruzi* calreticulin in immune evasion, infectivity and angiogenesis.TcCRT is translocated from the ER of the parasite to the area of flagellum emergence, with important consequences in the host–parasite interplay: **(a)** TcCRT interacts with fluid-phase first complement component C1, inhibiting the classical pathway at the earliest activation stages. **(b)** TcCRT recruits C1, thus promoting infectivity. Probably, C1 acts as a bridge between the CRTs of the parasite and host. **(c)** This mechanism is used by the parasite to invade mammalian cells and **(d)** to continue its life cycle (including its retention in the parasitophorous vacuole). **(e)** Perhaps, TcCRT is subsequently released to the cytoplasm of the host cell. **(f)** The CRT-C1q interactions have been described in apoptotic cells as promoting factors for their removal. Thus, 'eat me' signals will promote the parasite uptake by macrophages, **(b)** in an apoptotic mimicry strategy. **(g)** In addition, TcCRT binds to endothelial cells, inhibiting their morphogenesis, migration and proliferation. As a result, venous and arterial capillary growth is inhibited in various experimental arrangements involving several mammal and bird species [9,56,78]. **(h)** Whether, within the infected cell, TcCRT fulfills other roles relevant to the host–parasite interaction is unknown. **(i)** In mammals, CRT has important roles in the ER derived from its ability to bind Ca^{2+} and from its participation as a chaperone in the protein-folding process. Perhaps TcCRT, released by the parasite during its intracellular stages, intervenes in these processes.

generated between the $(C1r\text{-}C1s)_2$ tetramer and C1q [22]. Most probably, to exert its inhibitory effect, the parasite molecule accesses this dome to interact with the proteases or, alternatively, TcCRT interacts with the enzyme catalytic sites when they protrude between the collagenous tails, after C1 interacts with activating danger signals. Although HuCRT and TcCRT prevent binding of the serine proteases to C1q, they do not displace the serine proteases from the preformed stabilized C1 complex [20]. On the other hand, the fact that TcCRT inactivates C1s only in the C1 context may be explained by its dual capacity to interact with the collagenous tails and C1s [20]. A high-resolution crystal structure of the C1q/TcCRT has not been elucidated.

TcCRT is a parasite virulence factor

Why is TcCRT important in parasite 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 and having important variable roles in the invasion of host cells [23,24]. Host C1, upon binding to the trypomastigote surface, also promotes parasite infectivity (there is no evidence indicating that C1 and C1q behave differently with regard to their capacity to promote infectivity so the acronyms C1 and C1q are frequently used interchangeably in this review) [25]. The parasite molecule responsible for recruiting this complement component has been identified as TcCRT [8,26]. Increased parasite infectivity correlates with significant increases in TcCRT mRNA levels during early (cell contact and cell penetration) infection stages of the VERO cell line. Despite its lysine-aspartic acid-glutamic acid-leucine (KDEL)-ER retrieval sequence, TcCRT is translocated from the ER to the parasite area of flagellum emergence. This is followed by an augmented capacity to recruit C1, an important 'eat me' signal for phagocytic cells, leading to increased infectivity (Figure 1) [8,18,25,27].

The TcCRT-C1 interaction can be inhibited with a consequent decrease in parasite infectivity

Immunoglobulin (Ig) molecules generated during *T. cruzi* infection include specific anti-TcCRT antibodies in humans and experimental animals [28,29]. Given the affinity that C1 displays for the CH_2 domain of the Fc portion of IgG, the human C1q-TcCRT interaction, *in vitro*, is indirectly increased by whole anti-TcCRT Ig molecules. In other words, whole IgG molecules interacting with TcCRT on the parasite surface should generate additional eat-me signals with consequent increased infectivity. As expected, these TcCRT-C1q interactions are decreased by anti-TcCRT $F(ab')_2$ portions (which are devoid of the C1-binding Fc fragment), prepared from the same Ig molecules [28]. In agreement with these results, passive immunization of mice with anti-TcCRT $F(ab')_2$ fragments resulted in decreased infectivity, and examination of the animals showed improved parameters as compared with controls where hirsutism, diarrhea, dehydration and prostration were more prevalent [26].

TcCRT is immunogenic and increases parasite infectivity

The presence of TcCRT on the surface of the parasite promotes C1q capture by the parasite and, as a consequence, enhances infectivity [26]. Similarly, ligands on the surface of apoptotic cells, such as mammalian CRT among others, can recruit C1, promoting the necessary phagocytosis and clearance of these cells [27]. The *in vitro* and *in vivo* evidence reviewed here strongly supports the proposal that translocation of TcCRT from the ER to the membrane, mainly in the area of flagellar emergence, not only inhibits the classical pathway of complement but, by mimicry of the apoptotic process, also promotes infectivity (probably by generating effective C1q-mediated eat-me signals). In epimastigotes obtained from axenic cultures, only marginal C1q binding and TcCRT external levels were observed as compared with infective trypomastigotes [26]. Perhaps one of the reasons for the incapacity of epimastigotes to infect cells *in vitro* and *in vivo* could be explained by limited translocation of TcCRT to the outer membrane.

Experimental vaccination with recombinant TcCRT increases T. cruzi infectivity in vivo

The capacity of TcCRT to generate specific complement-fixing IgG1 antibodies may contribute to its increased immunogenicity observed *in vivo* [30,31]. Such was the case after conventional, genetic and combined immunization protocols. Immunogenicity was favored by the co-administration of a granulocyte-macrophage colony stimulating factor (GM-CSF) gene [32] or by insertion of a murine Ig κ chain leader sequence in an immunogenic pSecTag/*TcCRT* construct. As expected, the induced anti-TcCRT response was accompanied by increased infectivity that was reflected by higher parasitemia in the *T. cruzi*-challenged mice [26].

TcCRT inhibits angiogenesis and, as a probable consequence, tumor growth

Angiogenesis is a complex process aimed at providing nutrition, oxygenation and waste removal to various tissues (Box 3). Based on their capacity to bind laminin

Box 3. Angiogenesis and cancer therapy

The function of normal tissue is dependent upon, among several factors, the formation of new vessels, the supply of oxygen and nutrients, as well as the removal of waste products. The process by which new capillaries are formed from the pre-existing ones is called 'angiogenesis', and it occurs in physiological as well as pathological conditions.

Angiogenesis is a complex, multi-step process regulated by the equilibrium of pro- and anti-angiogenic factors which determine the 'angiogenic switch'. To form new blood vessels, endothelial cells must proliferate and migrate to reach remote targets. Also, the host microenvironment must convey signals for cells to multiply and avoid apoptosis [75,76].

The inhibition of angiogenesis was proposed as a cancer therapy almost 40 years ago based on the idea that tumors are highly vascularized and thereby vulnerable at the level of their blood supply. Thus, this therapy is applicable to a wide variety of tumors. Another advantage of anti-angiogenic therapy is that, because of the low mutagenic potential of endotheliocytes, those participating in tumor irrigation should not develop drug resistance. Moreover, a single vessel provides nutrition for thousands of tumor cells and has to be inhibited at only one point to block blood flow to a large area of the tumor. Therefore, targeting the arterial and venous endothelial cells with anti-angiogenic compounds may have consequences for the supply of oxygen and nutrients to the tumor, as well as waste removal. Tumor development is thus significantly delayed [77].

[33] and to inhibit proliferation of endothelial cells, HuCRT and its N-terminal fragment vasostatin display anti-angiogenic properties *in vitro* and *in vivo* [34,35]. These properties of HuCRT are paralleled by inhibitory activities in several tumor models [36–38].

Soviet investigators [39–41], based on experiments performed almost 80 years ago, found that *T. cruzi* infection is a potent inhibitor of tumor growth (a phenomenon recently reproduced in our research team using an *in vivo* mammary tumor model in mice (Ferreira *et al*., unpublished data)). Although anti-tumor effects have been reported for several decades, for various infections with other microbial agents [42], pathogen molecules mediating those statistically based tumor resistances have been poorly defined. The induction of specific immune anti-tumor responses [43] and/or the secretion of 'toxic substances' by the parasite [39,44] were invoked to explain these effects, but experimental evidence has not been provided in this respect.

TcCRT could have an anti-tumor role given its potent anti-angiogenic properties. Since angiogenesis modulators behave differently across species [45], TcCRT was studied with respect to its anti-angiogenic capacity in different experimental arrangements in mammals (including *Homo sapiens sapiens*). TcCRT and its N-terminal vasostatin-like domain (N-TcCRT) display several functions with regard to angiogenesis and tumor growth (Figure 1) [46].

TcCRT inhibits capillary growth in the ex vivo aortic ring assay in rats

In this *ex vivo* angiogenesis assay [22], rat thoracic aortas were dissected into 1-mm thick rings that were embedded in Matrigel™ (an extracellular matrix from a murine tumor), in the presence of endothelial cell growth factor (ECG) and TcCRT.

In this assay, in which the endothelial cells are within the histological context of an artery, (in a physiological quiescent state), TcCRT completely abrogated capillary growth in a dose-dependent manner [46]. Most probably, this *ex vivo* aortic ring assay in rats is a closer correlate to *in vivo* angiogenesis than the *in vitro* capillary morphogenesis carried out with isolated human umbilical cord endothelial vein cells (HUVECs) [9].

TcCRT inhibits capillary morphogenesis in the HUVEC assay

HUVECs were suspended in the presence of TcCRT, N-TcCRT, lipopolysaccharide (LPS), HuCRT or R-TcCRT (amino acids 136–281), and layered on polymerized Matrigel. After incubation, tubular capillary-like structures were quantified [23]. This is also an *in vitro* correlate of important features of *in vivo* angiogenesis in which only the parasite-derived molecules significantly interfered with this process. The relevant TcCRT amino acid sequence spans residues 20 to 191, corresponding to the N-terminal domain. R-TcCRT (an adequate negative control lacking the vasostatin-like domain) did not affect capillary morphogenesis despite its overlapping with N-TcCRT in amino acids 136–191 [46].

Inhibition of migration, proliferation and chemotaxis by TcCRT

In migration, proliferation and chemotaxis of HUVECs, TcCRT was more effective, in molar terms, than HuCRT. Chemotaxis is an essential step in capillary morphogenesis and angiogenesis. In this assay, cells with or without TcCRT are placed in the upper compartment of a chamber [24], separated by a filter from a lower one, containing chemoattractants. The cells on the lower surface of the filter are counted. In HUVECs and Eahy926 cells, migration (the ability of cells to cross the filter of the chemotaxis chamber) was inhibited in a dose-dependent manner by TcCRT. Inhibition of cell migration by TcCRT may explain (at least in part) its potent effects on *in vitro* capillary morphogenesis and *ex vivo* capillary formation [46]. These results are in accordance with those describing the capacity of HuCRT to increase cell binding to the extracellular matrix, with consequent inhibition of cell migration [47,48]. TcCRT and N-TcCRT share the capacity of HuCRT to specifically inhibit proliferation of endothelial cells, a key initial event in angiogenesis [35]. These effects were not observed in fibroblasts when used as negative controls. In HuCRT, the smallest anti-proliferative fragment spans amino acids 120 to 180 [35]. Because, as observed in the morphogenesis assay described above, R-TcCRT had no significant effect on HUVEC proliferation, relevant residues also map between amino acids 20 to 135. TcCRT interferes with pro-angiogenic basic fibroblast growth factor (bFGF, a member of the fibroblast growth factor family) by mechanisms that are incompletely understood. HuCRT also inhibits the proliferation of endothelial cells from diverse origins (such as fetal bovine heart (FBHE) [35], bovine aorta (BAEC) [49], HUVECs [50] and the human umbilical vein endothelial cell line (ECV304) [51]) in response to bFGF and vascular endothelial growth factor (VEGF; a signal protein that stimulates the growth of new blood vessels). R-TcCRT control protein did not affect HUVEC proliferation, or morphogenesis. The inhibition of HUVEC proliferation by TcCRT may imply its involvement in the cell cycle or, alternatively, in the induction of cell death. TcCRT did not induce apoptosis [46]. Therefore, in the TcCRT-mediated inhibition of cell proliferation, a cytostatic effect (rather than the induction of apoptosis) may be mediated by the parasite molecule (Figure 1).

Live HUVEC cells and EAhy926 cells recognize and internalize FITC-TcCRT

HuCRT and TcCRT bind laminin, but only the former interferes with the adhesion of endothelial cells, thereby explaining the anti-angiogenic effects of HuCRT. Thus, the anti-angiogenic effects of TcCRT could be explained by other mechanisms. TcCRT binds to endothelial cells and is then internalized (Figure 1) [46]. The transduction pathways involved are unknown. Scavenger receptor expressed by endothelial cell-I (SREC-I) could be involved in these phenomena. HuCRT binds SREC-I, is endocytosed, and delivers associated peptides for cross-presentation via molecules coded for by major histocompatibility class-I genes (MHC-I) [52]. This fact is compatible with the ability of fucoidin (a specific SREC-I ligand [53,54]) to competitively inhibit the internalization of TcCRT by HUVECs.

Besides being an endocytic receptor, SREC-I is an interesting candidate for signal transduction. Its intracellular domain comprises almost half of the molecule, a surprisingly large proportion among known scavenger receptors. It also contains several potential phosphorylation consensus sites [41,55]. These results are compatible with the possibility that TcCRT internalization is required to mediate its anti-angiogenic effects on endothelial cells. Consistent with these findings, TcCRT also inhibits angiogenesis in the chorioallantoid membrane of the egg of chickens (*Gallus gallus*) [56].

Growth inhibition of a murine mammary tumor cell line

TcCRT significantly inhibited the growth of a murine mammary tumor (TA3 MTXR) and displayed stronger inhibitory effects than HuCRT [46]. The prevalence of tumor development in wild and domestic *T. cruzi* hosts has not been assessed, but many forms of cancer are common in humans (i.e. mammary, prostate, ovarian and cervix-uterine cancers). Thus, the ability of TcCRT to delay tumor growth, together with its anti-inflammatory properties (derived from its capacity to inhibit complement), may represent an evolutionary adaptation of the parasite. This results in increased infectivity, inhibition of concomitant tumors and, as a probable consequence, prolonged survival of the host (Figure 1).

Concluding remarks

TcCRT is a parasite virulence factor that participates in important aspects of the interactions of *T. cruzi* with its vertebrate host (Figure 1). Among the known surface molecules of the parasite participating in infectivity, TcCRT, after being translocated from the ER to the area of flagellar emergence, recruits complement C1 and interferes with the ability of the associated C1r and C1s serine proteases to activate the central component C4. By recruiting C1, surface TcCRT of the parasite also promotes early C1-dependent phagocytosis (reminiscent of the actions of human apoptotic cells). Despite the evolutionary distance between trypanosomatids and mammals, the phagocytic removal of apoptotic cells and tumor cells by macrophages and dendritic cells has also been proposed to be strongly dependent upon C1 [57], which is recruited by membrane-translocated vertebrate CRT [27]. C1 affinity for antigen-aggregated Ig molecules and TcCRT immunogenicity will also contribute to a state of self-sustained *in vivo* parasite infectivity. The interaction between TcCRT and C1 can be partially reversed by $F(ab')_2$ fragments (bivalent antigen-specific Ig fragments devoid of their Fc portions and thus unable to bind the first complement component C1) prepared from anti-TcCRT Ig molecules, thus reversing the TcCRT-promoted infectivity.

Conversely, externalized TcCRT by itself binds to endotheliocytes from arterial and venous emerging capillaries, as tested in various assays in four vertebrate species. Inhibition of angiogenesis follows, which could explain the capacity of this molecule to inhibit tumor growth *in vivo* and, perhaps the anti-tumor effects reported for the parasite infection in experimental *in vivo* arrangements. All these properties map to the TcCRT N-terminal domain.

The combined anti-angiogenic and anti-complement effects of TcCRT may be anti-inflammatory, thus inhibiting the immune response against the parasite. Thus, the anti-angiogenic effect of TcCRT will result in reduction of blood flow, with consequent decreased recruitment of inflammatory cells. Conversely, the anti-complement effect of the parasite molecule will mediate decreases in chemotaxis, phagocytosis and activation of inflammatory cells.

Considering these results together, it could be speculated that the interactions among TcCRT, complement, and endothelial cells are calibrated evolutionary adaptations aimed at protecting the parasite and host, thus promoting long-term interplays. These findings open interesting possibilities for the development of new experimental anti-tumor strategies, particularly if we consider that TcCRT displays stronger anti-angiogenic and anti-tumor effects than its human counterpart. The biotechnological implications of these findings may be envisaged. Whether the anti-angiogenic properties were consolidated first in the parasite chaperone molecule and HuCRT conserved some of these properties as an evolutionary relic or, alternatively, the parasite 'hijacked' this activity from its vertebrate host remains an open question. Although *in vivo T. cruzi* infection and TcCRT treatment inhibit tumor development, it is not possible to causally associate both phenomena (i.e. that *T. cruzi* infection inhibits tumor growth through the anti-angiogenic capacity of TcCRT).

Finally, it could be speculated that, perhaps not all consequences of host–parasite interactions are deleterious to the host. Some cellular and molecular terms of these interactions have been reviewed herein.

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