Immunoparasitology series

Does *Trypanosoma cruzi* calreticulin modulate the complement system and angiogenesis?

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Calreticulin, a calcium-binding protein that is highly conserved in its multiple functions, is present in a wide spectrum of subcellular compartments in virtually every cell of higher organisms. In this article, we propose a dual role for parasite calreticulin, with emphasis on the *Trypanosoma cruzi* model. By modulating the vertebrate complement system, calreticulin might provide the parasite with an effective immune-escape mechanism. Alternatively, by inhibiting angiogenesis, the parasite molecule might protect the host from ongoing neoplasic aggressions. Many questions are still unanswered, particularly those regarding the consequences that these interactions could have *in vivo* for both the parasite and the host.

Introduction

Because parasites must react swiftly to the defensive strategies of their hosts, highly specific molecular interactions occur at the host–parasite interface that normally lead to an equilibrium in the relationship. An important array of host- and parasite-derived molecules participates in these interactions.

Considering the remarkable conservation of both the genomic organization and the amino acid sequence of calreticulin (CRT) throughout evolution, several functional aspects of this molecule are operative in a variety of vertebrate and invertebrate species. Thus, recent studies indicate that, in some parasites at least, CRT participates in modulation of the complement system of the host [1,2]. Moreover, the pleiotropic properties of CRT are reinforced by recent studies involving this parasite molecule in antiangiogenic mechanisms in the host.

CRT, a calcium-binding protein present in virtually every cell of higher organisms, is located primarily in the endoplasmic reticulum (ER) [3,4] and regulates key cellular functions [5,6]. Prominent among these functions, described mainly in vertebrates, are CRT lectin-like chaperone capacity, modulation of gene expression, induction of phagocytosis of apoptotic cells, mediating autoimmunity, antiangiogenesis, inhibition of tumoral growth, and participation in the lytic activity of perforins from T cells and natural killer cells [7].

The consensus features of all CRT proteins are an acidic C-terminal domain, a proline-rich P domain and a globular N-terminal domain [5,8,9]. Importantly: (i) an S domain, which is included in the N and P domains, is involved in the binding of complement components [10,11]; and (ii) the N domain includes 60 C-terminal amino acids that concentrate the antiangiogenic properties, by virtue of their capacity to inhibit endothelial cell proliferation [12].

The primary sequences of CRT, from most of the species studied, initiate with a signal peptide and terminate with KDEL or related ER-retention sequences [7]. Interestingly, CRT has non-ER locations and can also be released from the cell by either active secretory processes or cell death to mediate various functions [13].

CRT from parasites such as *Onchocerca volvulus*, *Schistosoma mansoni, Leishmania donovani* and *Trypanosoma cruzi* is ~50% identical to its human counterpart (HuCRT). Interestingly, the tick *Amblyomma americanum*, while feeding on its host, secretes CRT [14], 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 [2]. Also, seropositive humans produce antibodies against *T. cruzi* calreticulin (TcCRT) [15–17], which strongly suggests that the molecule is available not only to immunocompetent B cells but also to complement components such as C1q, with possible implications for the classical complement pathway, as discussed later.

Thus, by modulating the vertebrate complement system, CRT might provide the parasite with an effective immune-escape mechanism. Concomitantly, by inhibiting angiogenesis, the parasite molecule might protect the host from ongoing neoplasic aggressions. These two emerging features of parasite CRT, with emphasis on the *T. cruzi* model, are reviewed.

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Trypanosoma cruzi calreticulin: a functional homolog of its human counterpart?

Native, Tulahuen strain TcCRT was isolated [16–19] and its gene was cloned, sequenced and expressed [15]. TcCRT from another *T. cruzi* strain was also characterized [20,21]. TcCRT is a 45-kDa immunodominant molecule [18] that has multiple gene copies located in a variable number of chromosomes [15,16]. Because a TcCRT region (TcS: amino acids 159–281) is 50–80% identical to certain functional regions of the HuCRT S domain, it was determined that fluid-phase or trypomastigote-bound TcCRT interacts with host C1q and inhibits the classical pathway of complement activation [1].

The sharing of several functional domains by vertebrate and parasite CRT encourages further investigation of the contributions made by this molecule to the biology of parasites and the interactions with their hosts.

Parasite CRT and the complement system

HuCRT binds to the collagenous region of C1q [22]. A cell-membrane-associated form of CRT might function as a receptor for C1q and collectins [10,11,22–24]. The binding site on HuCRT for C1q and collectins was defined by its 12-kDa S-domain–N-terminal portion [10,11], with functional consequences such as inhibition of the classical complement pathway [10] and of C1q-mediated immune-complex processing [25].

The classical and lectin pathways of complement activation are initiated by related but distinct recognition molecules, with C1q being the only recognition component of the classical pathway. The collagen-like portions of these recognition molecules interact with serine proteases [26,27], which are responsible for the cleavage of C4 and C2 [26,28,29], thus initiating the activation of these pathways.

One important aspect of the host–parasite interaction that has been reviewed recently [30] involves the complement system of the host. Information about a possible role for CRT, from protozoan parasites, in the modulation of human complement is beginning to emerge in the literature [1]. Recombinant hookworm (*Necator americanus*) CRT also binds to and inhibits the biological function of human C1q [2].

Complement-mediated lysis of trypomastigotes *in vitro* requires an intact alternative pathway because serum depletion of factors B and P completely abrogates the lysis of trypomastigotes precoated with immunoglobulin (Ig)G [31,32]. The classical pathway, although unable to lyse trypomastigotes efficiently on its own, provides an enhancing effect on the amplifying properties of the alternative pathway [33]. This impairment of the classical pathway suggests the importance of exploring the existence of other parasite complement-regulatory proteins. A role for TcCRT in these functions has been proposed [1]. In Figure 1, an overview of the classical pathway is shown. Because TcCRT, by virtue of its capacity to bind to the collagenous tails of C1q, inhibits the generation of C4b, impairment of the

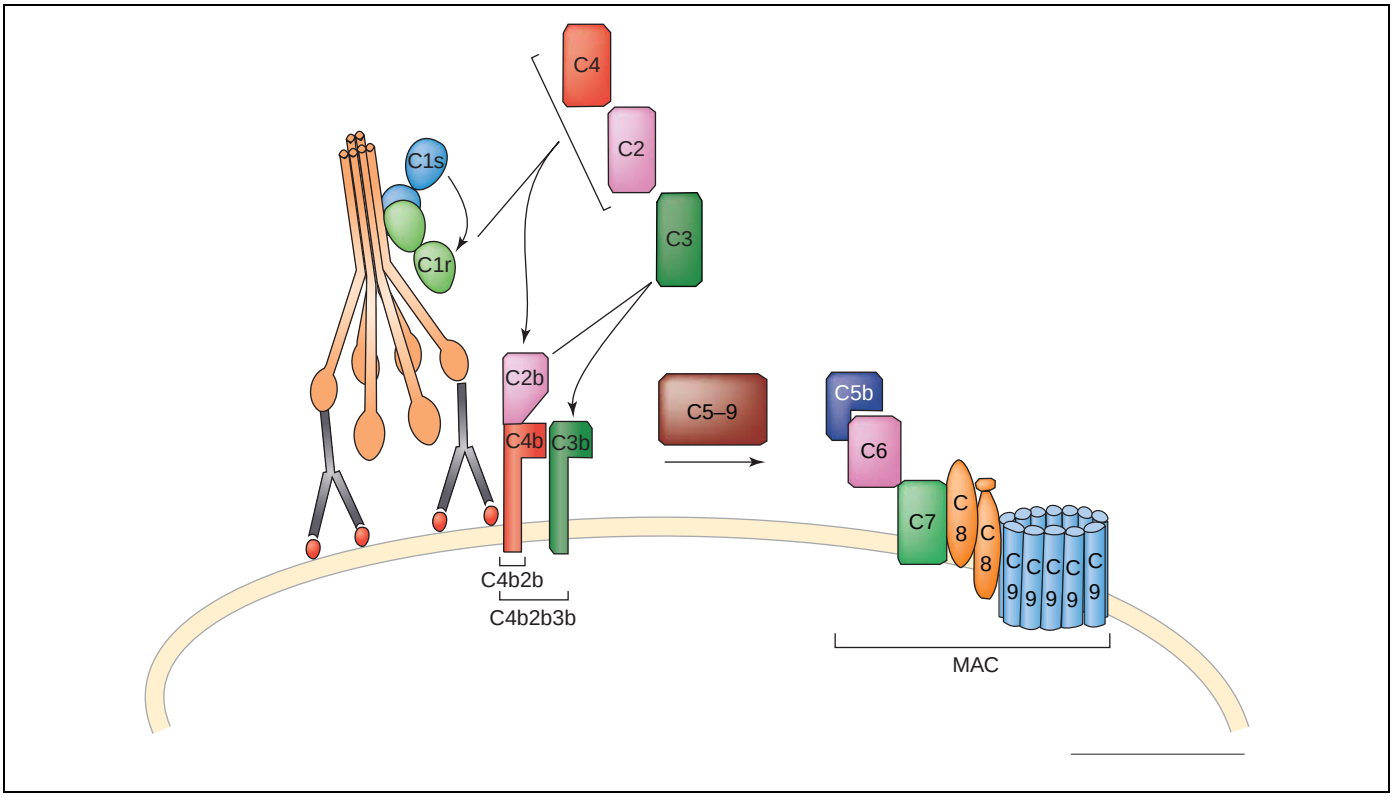

Figure 1. Activation of the classical pathway. C1(q,r,s) recognizes Fc domains of antibodies reacting with antigens on the surface of foreign aggressors (i.e. *Trypanosoma cruzi*). The C1r serine protease activates C1s, which digests C4 and C2 to form C4b2b, which is a C3 convertase that digests C3. Many C3b fragments are generated; most of them will pass to the fluid phase and some will bind covalently to the surface of the foreign aggressor. C3b bound to C4b2b then generates the C5 convertases of the classical pathway (C4b2b3b). C5 binds noncovalently to a site in C3b in the respective convertases and is digested by C2b. The digestion of C5 is the last enzymatic step of the cascade. The formation of the membrane attack complex (MAC) involves the noncovalent binding of C5b to the four terminal components of the cascade (C6–9). The C5b–9 complex acquires amphiphilic properties; the MACs form functional pores in the membrane, mediating the osmotic lysis of the cell.

generation of C3 convertase (C4b2b), C5 convertase (C4b2b3b) and the membrane attack complex (C5b–9) is easily predicted.

Because native TcCRT, in the context of *T. cruzi* trypomastigote infection, is immunogenic in humans [16,17] and mice [18], TcCRT should be available for interaction with complement component C1q, with possible functional consequences. Specific *in vitro* binding of TcCRT to human C1q collagenous tails and a consequent strong inhibition of the classical pathway were demonstrated [1]. Because TcCRT is expressed on the surface of infective trypomastigotes and colocalizes with human C1q, its *in vivo* interaction with C1q might mediate complement inhibition in the microenvironment surrounding the parasite.

In *T. cruzi*, complement-regulatory proteins such as CRP, which is a decay-accelerating factor (DAF)-like protein, participate directly in the stage-specific inhibition of the alternative pathway [33–35]. Thus, $F(ab')_2$ and Fab fragments directed against these proteins make the parasite susceptible to the action of the alternative pathway [36,37]. Apparently, the classical pathway of complement activation would have an amplifying role but it would not be able to elicit an efficient antiparasite lytic response on its own.

Because certain regions within the TcS domain of TcCRT are up to 80% identical to regions within the HuCRT S domain, C1q binds to recombinant TcS in a dose-dependent, specific and saturable manner. Moreover, because C4 activation is impaired, this binding inhibits C1q-dependent complement-mediated hemolysis of Ig-sensitized erythrocytes *in vitro* [1]. Unlike other *T. cruzi* complement regulators described, by strongly inhibiting C4 activation, TcCRT impairs complement activation at its earliest stage. Thus, by virtue of its capacity to bind to and inhibit the function of C1q, TcCRT might contribute to the well-known [33] inability of the classical pathway to have a preponderant role in the defense against *T. cruzi* (Figure 2).

The possibility that TcCRT interacts *in vivo* with the complement system of the host supports our findings that TcCRT is found on the surface of infective trypomastigotes and that it colocalizes with C1q, which is a strong indication that both molecules interact on the infective parasite surface. This correlates with *in vitro* results showing that TcCRT strongly inhibits activation of the classical human complement pathway [1].

The mechanism by which TcCRT inhibits C4 activation remains to be determined. Perhaps TcCRT, by binding to sites occupied by C1s and C1r on the collagenous tails of C1q, mediates C1s and C1r displacement from the C1(q,r,s) module, as hypothesized for HuCRT [38]. Alternatively or concomitantly, TcCRT, through its calcium-binding properties (A. Ferreira *et al.*, unpublished), could mediate the removal of calcium from C1, resulting in the release of both serine proteases.

Perspectives and unanswered questions about complement and TcCRT

A role for TcCRT in parasite cell invasion could be proposed because of the interaction of the parasite molecule, located at the parasite surface, with C1q of the host.

By inhibiting complement activation, extracellular human or parasitic CRT might inhibit immune-complex solubilization, with consequences for the pathogenesis of diseases such as systemic lupus erythematosus and Chagas' disease [39,40]. Also, if complement function is impaired, immune complexes could escape clearance by the mononuclear phagocytic system and end up in tissues in which they would trigger an inflammatory response, including the release of autoantigens [39].

C1q binds to apoptotic cells [23] and stimulates their ingestion by ligating CRT on the phagocyte surface (on which CRT is known as cC1qR) [41]. cC1qR is bound to the endocytic receptor protein CD91. Ingestion of apoptotic cells through CRT–CD91 involves macropinocytosis, which is a primitive and relatively nonselective uptake mechanism for C1q-enhanced engulfment of intact apoptotic cells, cell debris and foreign organisms [23,27,42,43]. Both noninfective *T. cruzi* epimastigotes and vertebrate-stage tissue-culture infective trypomastigotes (TCTs) bind to C1q in a saturable fashion, and internalization by mononuclear phagocytes and fibroblasts of C1q-coated TCT is enhanced compared with untreated parasites. Purified C1q alone potentiates the internalization of TCT, without an additional requirement for C3 fragments or IgG on the target particle [44]. A role for TcCRT in this interaction could be envisaged.

Parasite calreticulin: a role in angiogenesis?

Angiogenesis is a complex multistep process that leads to neovascularization generated from pre-existing blood vessels. It is associated with inflammation, wound healing, tumor growth and metastasis. The generation of new blood vessels is regulated by proangiogenic and antiangiogenic molecules, some of which are currently under clinical and preclinical trials for cancer treatment [45–48].

During the past six years, the role of vertebrate CRT in angiogenesis and tumor growth has been studied extensively. A CRT peptide (amino acids 120–180), a larger molecule (amino acids 1–180) named vasostatin and the whole CRT molecule are potent angiogenesis inhibitors, both *in vitro* and *in vivo* [12,49,50]. Vasostatin inhibits angiogenesis and the *in vivo* proliferation of vascular endothelial growth factor (VEGF)-stimulated endothelial cells by acting directly on these cells; this does not affect the vascularization of established tumors [12,49,51]. The binding of endothelial cells to extracellular-matrix components is impaired by vasostatin [52].

For five decades, there has been speculation about possible mechanisms involved in the *in vivo* experimental growth-inhibitory effect that several *T. cruzi* strains have on a variety of transplanted and spontaneous tumors in animals and humans [53–55]. The induction of a specific antitumoral immune response [56] and the secretion of a 'toxic substance' [54] by the parasite have been proposed to explain the antineoplasic effect of *T. cruzi* infection, but they have not been demonstrated experimentally. (Both explanations are compatible with an evolutionary speculation that this antineoplasic effect would protect the host from prevalent neoplasic aggressions, with evident benefits for the parasite.)

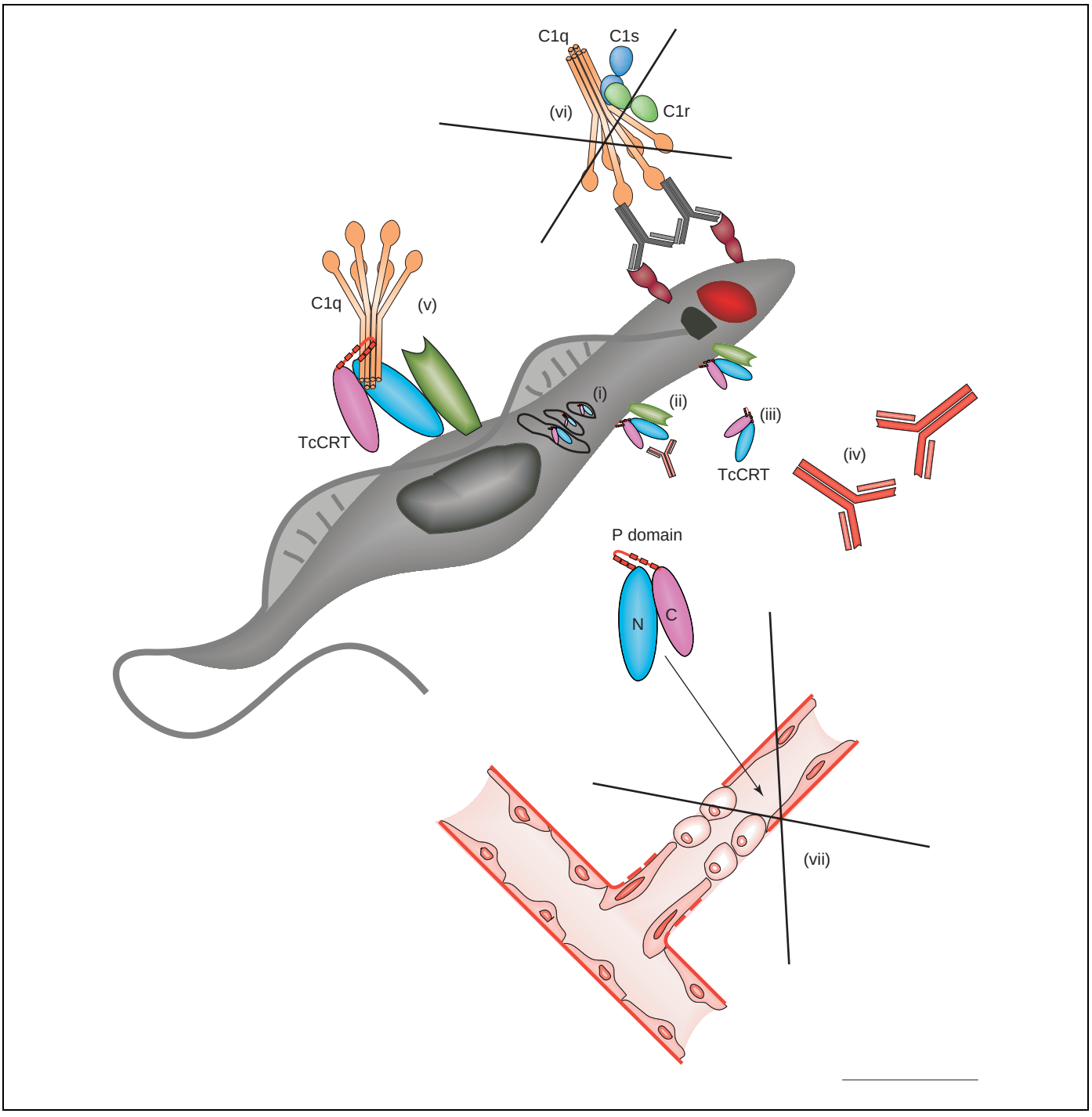

Figure 2. *Trypanosoma cruzi* calreticulin mediates anticomplement and antiangiogenic effects in vertebrate hosts. A trypomastigote produces calreticulin (TcCRT), which is found in the ER **(i)** and on the parasite surface **(ii)**. Released TcCRT **(iii)**, shown here with only three domains (N, light blue; P, red; and C, pink), is immunogenic and generates specific antibodies **(iv)**. TcCRT, bound to the parasite surface by an unknown receptor (green), interacts with the collagenous tails of C1q **(v)** and prevents further activation of the classical pathway of human complement **(vi)**. Released TcCRT also interacts with endothelial cells, mediating antiangiogenic effects **(vii)**, presumably by interfering with the extracellular-matrix binding of these cells.

Neoplasic growth and metastasis are intimately related to neoangiogenesis [45,47,48], and HuCRT has important antiangiogenic properties [12,49,50,52]. Because there is a segment in TcCRT with 46% identity and 60% positivity to a functional antiangiogenic fragment from HuCRT (amino acids 120–180), the issue of whether the parasite molecule shares this property has been addressed recently. Studies performed with recombinant and native TcCRT [57] show a highly significant ($p < 6 \times 10^{-7}$) and specific inhibition of angiogenesis in the chorioallantoic membrane (CAM) of chick embryos (CAM assay) [58]. These results are further substantiated by results obtained with TcCRT synthesized *in situ* by the genetic pSecTag2B–TcCRT construct, versus the empty vector, in the same assay [57]. In summary, similar to HuCRT [12,49,50,52,59,60], both native and recombinant TcCRT have antiangiogenic effects in the *in vivo* CAM assay, even at low concentrations. It remains to be determined whether TcCRT shares with its

vertebrate counterpart the property of binding to laminin, thus interfering with endothelial growth [52]. It could be speculated that these properties, together with the accessibility of this molecule on the trypomastigote surface, explain, at least in part, the reported [55,56] antineoplasic effect of experimental *T. cruzi* infection.

Finally, Figure 2 illustrates the integration of the roles that TcCRT might have in complement and angiogenesis modulation. These are probably just two of the many complex functions of a potentially pleiotropic parasite calreticulin. These functions, exemplified mainly in *T. cruzi*, probably participate in the equilibrium that is frequently reached in the host–parasite relationship.

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