Trends in Parasitology

CellPress
REVIEWS

Review

Trypanosoma cruzi Calreticulin: Immune Evasion, Infectivity, and Tumorigenesis

Galia Ramírez-Toloza,[1,*] Eduardo Sosoniuk-Roche,[2] Carolina Valck,[2] Lorena Aguilar-Guzmán,[1] Viviana P. Ferreira,[3] and Arturo Ferreira[2,*]

To successfully infect, *Trypanosoma cruzi* evades and modulates the host immune response. *T. cruzi* calreticulin (TcCalr) is a multifunctional, endoplasmic reticulum (ER)-resident chaperone that, translocated to the external microenvironment, mediates crucial host–parasite interactions. TcCalr binds and inactivates C1 and mannose-binding lectin (MBL)/ficolins, important pattern- recognition receptors (PRRs) of the complement system. Using an apoptotic mimicry strategy, the C1–TcCalr association facilitates the infection of target cells. *T. cruzi* infection also seems to confer protection against tumorigenesis. Thus, recombinant TcCalr has important antiangiogenic properties, detected *in vitro*, *ex vivo*, and *in ovum*, most likely contributing at least in part, to its antitumor properties. Consequently, TcCalr is useful for investigating key issues of host–parasite interactions and possible new immunological/pharmacological interventions in the areas of Chagas' disease and experimental cancer.

Highlights

TcCalr has essential roles in host–parasite interaction in Chagas' disease.

TcCalr inhibits the classical and lectin pathways of the complement system by binding of their soluble PRRs such as C1, MBLs (mannose binding lectins), and ficolins.

TcCalr binds C1, and this interaction is used as a strategy to infect the mammalian cell.

TcCalr inhibits angiogenesis and interacts with tumor cells, modulating the expression of molecules that increase tumor immunogenicity.

The combined experimental evidence reviewed herein is compatible with the possibility that Chagas' disease protects against concomitant tumors.

TcCalr Is a Key Molecule in Host–Parasite Interaction

Chagas' disease is a chronic zoonosis, originally located in America. However, due to migration of infected people, Chagas is a worldwide disease. Many challenges in diagnosis, therapy, and control of chronic Chagas' disease remain unresolved [1]. *T. cruzi*, its causal agent, has a complex life cycle involving mammalian hosts and insect vectors (Box 1). To understand the host–parasite interactions it is crucial to resolve challenges such as the identification of parasite components that mediate its infectivity and resistance to host immunity.

This review focusses on *T. cruzi* calreticulin (TcCalr, formerly known as TcCRT), a multifunctional, ER-resident chaperone, that the parasite translocates to the exterior. We discuss experimental evidence which indicates that TcCalr contributes to the circumvention of the host's immunity, to the promotion of infectivity, and to the inhibition of **tumorigenesis** (see Glossary) in experimental animals and, possibly, in infected people [2].

TcCalr Is Another Main Virulence Factor That Promotes Infectivity

T. cruzi exhibits a variety of mechanisms to evade specific components of the host's immunity, thus enabling the protozoan to initially establish an infection and then veer towards a chronic stage. Thus, **epimastigotes** are exposed to oxygen-reactive species (ROS), due to UV light and/or the heme groups, present in the blood meal [3,4]. Likewise, infective **trypomastigotes** interact with phagocytic cells, activating NADPH oxidase and producing large amounts of ROS and reactive nitrogen species (RNS) [5]. Consequently, in both *T. cruzi* stages, ROS and RNS damage DNA, but *T. cruzi* has different enzymes to repair and resist these genomic alterations [6–8].

Once *T. cruzi* metacyclic trypomastigotes are released in the feces or urine, they penetrate through discontinuous regions on the epidermis or mucosa and infecting host cells [9]. In this process,

[1]Faculty of Veterinary Medicine and Livestock Sciences, University of Chile, Santiago, Chile
[2]Department of Immunology, Faculty of Medicine, University of Chile, Santiago, Chile
[3]Department of Medical Microbiology and Immunology, University of Toledo College of Medicine and Life Sciences, OH, USA

*Correspondence:
galiaram@uchile.cl (G. Ramírez-Toloza) and aferreir@med.uchile.cl (A. Ferreira).

Box 1. Chagas' Disease, an Important Illness Worldwide

T. cruzi, the causal agent of Chagas' disease, a worldwide neglected tropical disease affecting about 6–7 million people, undergoes a complex life cycle involving an invertebrate hematophagous triatomine vector and an extensive range of mammalian hosts, including humans. Birds are resistant, mainly due to their complement system that can effectively clear the parasite in a sterile way. The vector, a poikilotherm triatomid hematophage arthropod, develops in the intestine as a replicative and noninfective form, the epimastigote, that differentiates to the nonreplicative infective stage, the metacyclic trypomastigote, present in the rectum of the insect vector. During the differentiation process from noninfective epimastigotes to infective metacyclic trypomastigotes (metacyclogenesis), the parasite undergoes complex morphological and biochemical changes, to effectively infect and survive in the hostile homeothermic environment of the vertebrate host.

The primary transmission form in mammalian hosts involves contact with dejections (a mixture of urine and feces) of hematophagous triatomine vectors, which contain metacyclic trypomastigotes, an infective form of the parasite that, somehow, finds its way through the skin or mucosa. The infection has different steps, leading to a chronic illness in 30–40% of infected people who may develop fatal cardiac and intestinal mega syndromes. Early-stage acute infection can also be fatal, especially among infants. However, most of the infected people (60–70%) will remain asymptomatic for the rest of their lives. Other forms of transmission are organ transplantation, blood transfusion, congenital transmission, laboratory accidents, and, a recently described form, oral transmission by consumption of food or drinks contaminated with metacyclic trypomastigotes. This transmission form seems to cause a more severe disease, with higher mortality and severity than the vector-borne disease. Because the causal agent of Chagas' disease can be maintained as reservoirs in a variety of triatomine insects, and in more than 150 mammalian species, it is considered an important zoonosis [1].

trypomastigotes use a variety of virulence factors that interact with nonspecific host components and receptors.

Although *T. cruzi* reproduction is mainly clonal, the parasite exhibits a wide genetic, biochemical, and behavioral diversity. *T. cruzi* has seven discrete typing units (DTUs) (TcI–TcVI and Tc bat) [10]. This diversity is broadly distributed in America [11]. Recently, genetic exchange in the *T. cruzi* life cycle has been proposed, thus identifying determinants of parasite virulence and redefining long-standing theories on clonality in trypanosomatids [12].

Previous reports indicated a tissue tropism by specific lineages of *T. cruzi* [13]. However, current associations among *T. cruzi* genetic heterogeneity, host immune response and genetic factors, and clinical manifestations have been described [11]. Proteomic analysis, comparing strains with different pathogenicity, indicates that strains inducing chronic infection have enriched antioxidant defenses, while those inducing acute infection produce nucleotides and proteins involved in parasite replication and lethality [14]. In *T. cruzi*, gp90, proteins from the *trans*-sialidase (TS) family (gp85, gp82, and TSA-1), gp35/50, gp83, penetrin, cruzipain, oligopeptidase B, Tc80, and TcCalr [15], participate in infectivity. Expression of some of these proteins is variable in different parasite stages and strains [2].

TcCalr is a multifunctional and pleiotropic protein, described in our laboratory, that resides in the ER but, surprisingly, it transits from the ER to the Golgi, reservosomes, nucleus, kinetoplast, flagellar pocket, and cell surface [16–18]. In genetically modified infective trypomastigotes [19], TcCalr is located mainly in the kinetoplast and nucleus, suggesting a secretory pathway in which the kinetoplast accumulates TcCalr to translocate it to the cell membrane [17,20]. In epimastigotes, TcCalr is located mainly in the nucleus [17] and is marginally translocated [20].

Calreticulin (Calr) is a pleiotropic protein present in nucleated cells and highly conserved across species (Box 2) [21]. Calr is translocated and exposed on the cellular membrane as an 'eat me' signal, promoting efferocytosis (uptake and removal of apoptotic cells by **phagocytosis**) and cancer cell removal, with opposite outcomes. While efferocytosis results in a silenced immune response, removal of dying cells results in immunogenic cell death, with activation of innate and adaptive immunity [21]. Calr on apoptotic cells is recognized by two pattern-recognition components of the **complement system** (**C**), C1q and **mannose-binding lectin** (**MBL**). These

Glossary

Angiogenesis: endothelial cell proliferation, resulting in new blood vessels, from pre-existing ones.
Antigen-presenting cell (APC): APCs display peptides in association with MHC molecules.
Arthropods: insects, arachnids, myriapods, and crustaceans. They have a segmented exoskeleton.
B lymphocytes: cells generated in the bone marrow; they produce antibodies and function as professional APCs.
C1: a serum C protein, with 18 polypeptide chains, associated in six heterotrimers and holding two serine protease dimers. It initiates the classical pathway C by attaching to specific domains present in IgG or IgM.
Complement system (C): about 45 serum and cell-surface proteins that interact with one another in a cascade of proteolytic and nonproteolytic interactions with lytic, opsonic, inflammatory, and immune-stimulating consequences.
Cross-antigen presentation: exogenous antigens are taken up by an APC and presented via MHC-I to cytotoxic T cells.
Endothelial cells: cells that line the interior surface of blood and lymphatic vessels.
Epimastigotes: a replicative, noninfective form in the life cycle of trypanosomatid protozoa, present in the middle gut of the insect vector.
Ficolins: homopentameric innate immunity plasma proteins, containing collagen- and fibrinogen-like carbohydrate-recognizing domains.
Helminths: parasite worms that elicit Th2-dependent immunity with eosinophil-rich inflammatory infiltrates and IgE production.
MHC: (major histocompatibility complex) a complex present in chromosome 6 (in humans) and 17 (in mice). It contains genes coding for proteins highly relevant in the main functions of immunity and in other biological areas. MHC class I and II present peptides to $CD8^+$ and $CD4^+$ T cells, respectively.
Mannose-binding lectin (MBL): a plasma-soluble PRR that binds mannose residues present on bacterial cell walls, acting as an opsonin for macrophages.
Natural killer (NK) cells: bone-marrow-derived lymphocytes, different

Box 2. Calreticulins: Structure and Function

Calreticulin (Calr) is a pleiotropic 46 kDa protein, highly conserved in all species. Its main functions are Ca^{2+} sequestration and glycoprotein assembly in the ER. First described in mammals, the Calr structure involved three domains: the N-terminal globular, a flexible proline-rich P intermediate arm-like, and C carboxyl terminal domains [21]. Extracellularly, Calr participates in thrombospondin 1-mediated focal adhesion of T cells, C1q-mediated clearance of apoptotic cells by macrophages, immunogenic dendritic cell-mediated uptake of apoptotic cancer cells leading to curative T cell immunity in mouse models, promotion of antiangiogenesis, and wound-healing [80]. *T. cruzi* calreticulin (TcCalr) is approximately 50% identical to its human counterpart (HuCALR). In *T. cruzi*, TcCalr participates in the inhibition of the classical and lectin C pathways [16], mediates infectivity via TcCalr–C1q interaction on the parasite [15], inhibits angiogenesis in a lower equimolar concentration than HuCALR [86,89,96], reduces tumor growth [87,88,90], and promotes wound healing more efficiently than HuCALR [99,100], a difference that may be explained by the structural bases of TcCalr. In general, Calrs have lectin-site features, being able to bind both carbohydrates and/or proteins. A synthetic peptide (VC-TcCalr), from the TcCalr N-domain, derived from *in silico* models, and synthesized, behaves as a strong dipole, able to interact with charged proteins such as collagen-like tails and scavenger receptors. This fact may explain, at least in part, the dual capacity of TcCalr to bind C1q and to inhibit angiogenesis. HuCALR is less polar and spatially stable, probably due to substitutions of Gln for Gly, Arg for Lys, Arg for Asp, and Ser for Arg that hinder protein–protein interactions [77]. Additionally, small-angle X-ray scattering (SAXS) and crystallography provide information about the flexibility of TcCalr in solution and its open conformation. Structure analysis may be exploited to identify parasite Calr determinants as therapeutic targets, able to be inhibited without affecting the functions of host HuCALR [76].

components attach to apoptotic cells by their globular domains to facilitate ingestion by macrophages. Consequently, Calr, also known as C1q receptor (cC1qR) [22], binds to the collagenous tails of C1q and MBL, and to CD91 on the macrophage cell surface. Then, micropinocytosis of apoptotic cells begins [21,23].

In *T. cruzi* trypomastigotes, TcCalr is exposed mainly in the area of flagellum emergence, where it recruits C1q or **C1** (C1q, containing the C1s and C1r dimeric serine proteases) in a molecular mimicry strategy. Like apoptotic cells, the TcCalr/C1q complex is recognized as an 'eat me' signal by cC1qR/Calr on phagocytes and other cellular types, thus promoting infectivity (Figure 1). As expected, and most important, anti-TcCalr $F(ab')_2$ antibodies inhibit the TcCalr/C1q interaction and, therefore, decrease infectivity *in vitro* and *in vivo* [15]. The TcCalr/C1q-mediated infectivity correlates with an increase in TcCalr mRNA levels in the early infection steps [15].

Noninfective epimastigotes bind exogenously added TcCalr and are internalized by fibroblasts in a C1q-dependent manner. Likewise, Calr-deficient fibroblasts have impaired capacity to internalize TcCalr, indicating again that the TcCalr/C1/Calr complex participates in the invasion process [24]. Moreover, mice inoculated with genetically modified trypomastigotes, carrying a single *TcCalr* allele (*TcCalr*$^{+/-}$) and, therefore with lower TcCalr expression, have undetectable parasitemias and anti-*T. cruzi* IgG antibodies. This fact may be explained by the negative modulation of two properties conferred by the TcCalr: evasion of C and infectivity [25].

The TcCalr-mediated infection strategy is important in *T. cruzi* congenital transmission, relevant in Chagas' disease epidemiology and its emergence in developed countries. The human placenta expresses higher Calr levels during pregnancy [26]. Translocated parasite TcCalr binds and inactivates C1 that will, in turn, bind the parasite to cC1qR present on the placental syncytiotrophoblast (ST). This newly formed TcCalr/C1q/Calr synapsis is important for infection of the human placenta, as measured in an *ex vivo* model [27]. Thus, a molecular basis explaining at least an important part of the parasite contact with the ST has been provided.

Later on, similar Calr functions have been described in other parasites (Table 1). In general, the interactions with C are well conserved among other parasite species, such as **protozoa** [28–30], **helminths** [31–38], and **arthropods** [39,40], inhibiting, in some cases, the classical

from B and T cells; they lyse infected cells as an important arm of the innate immune system.

Opsonization: the process of attaching opsonins, such as IgG or complement fragments, or both, onto microbial surfaces to mediate phagocytosis.

Pattern-recognition receptors (PRRs): receptors in the innate immune system that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).

Phagocytosis: a process executed mainly by macrophages and neutrophils; in this process the membranes of these cells surround foreign particles in an energy-consuming and cytoskeleton-dependent way.

Protozoa: single-celled eukaryotic organisms.

Scavenger receptors (SRs): cell-surface receptors on macrophages, originally defined by their capacity to mediate endocytosis of oxidized or acetylated, low-density lipoprotein particles.

T lymphocytes: $CD4^+$ helper cells and $CD8^+$ cytotoxic cells, participating in cell-mediated adaptive immunity. They mature in the thymus, circulate in the blood, and colonize secondary lymph nodes where their T cell receptors (TCRs) recognize peptides bound to self MHC molecules on APCs.

Trypomastigotes: the infective stage of *Trypanosoma*, present in the mammalian host's bloodstream and, in the vector, as infective metacyclic trypomastigotes.

Tumorigenesis: the generation of new tumors or growths, mainly malignant.

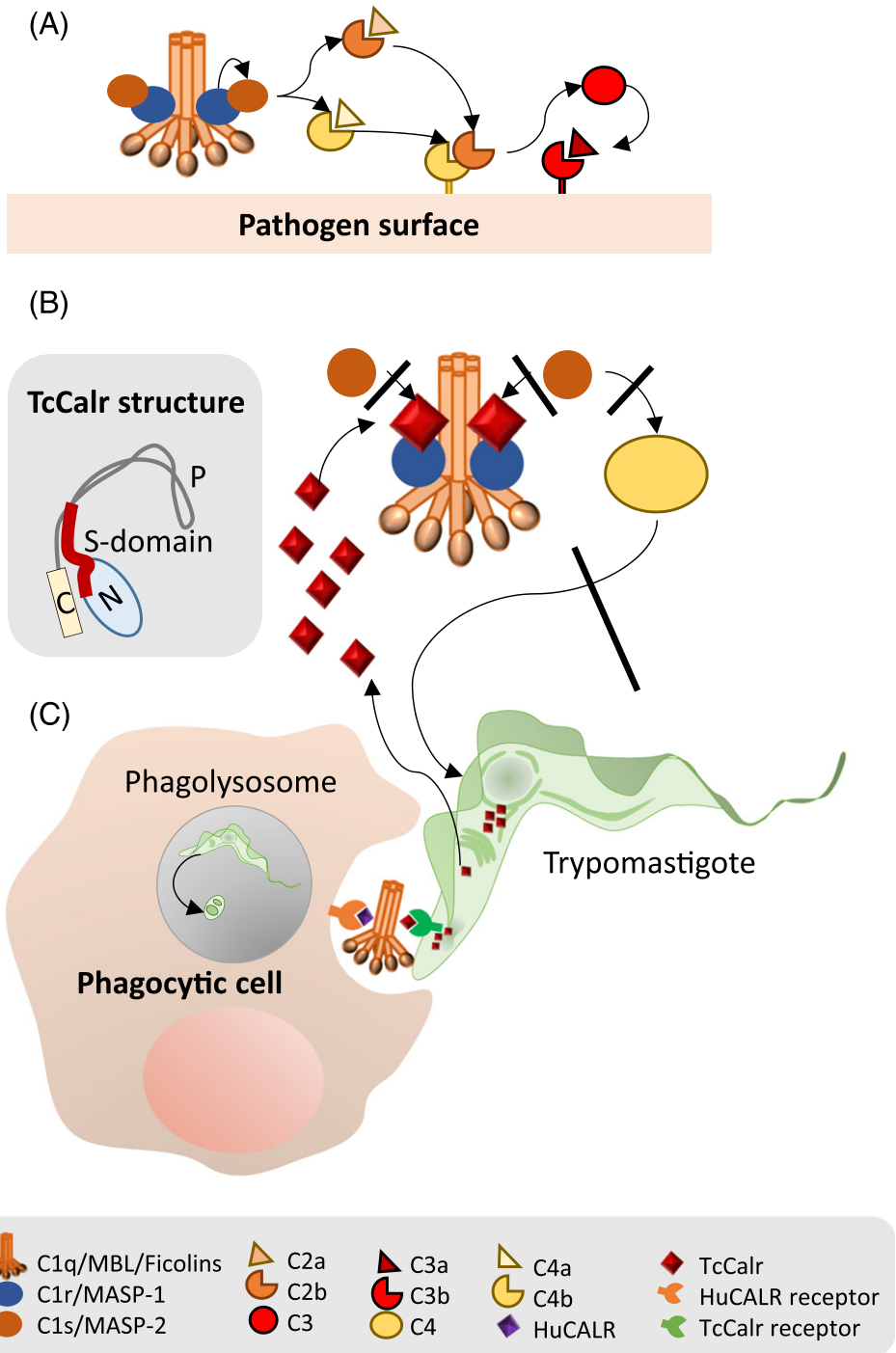

Figure 1. *Trypanosoma cruzi* Calreticulin (TcCalr) Binds C1q, Inhibiting the Complement System and Mediating Infectivity. (A) C1q in the classical pathway, or mannose-binding lectin (MBL) or ficolins in the lectin pathway, are activated by the recognition of danger signals on microorganisms and the binding of serine proteases, C1r and C1s, in the classical pathway, and MASP-1 or 2 in the lectin pathway. Serine-protease C1s activates C4 and C2, generating C4b

(Figure legend continued at the bottom of the next page.)

pathway. In *T. cruzi* [15] and *Trypanosoma congolense* [41], Calr is a virulence and pathogenic factor. Other relevant Calr functions, in a variety of parasitic infections, are the modulation of cellular and humoral responses [42–51] and, in proteomic and genetic analysis, the chaperone is relevant in various aspects of the host–parasite interactions [52–62].

TcCalr Mediates Mechanisms for Evading the Host's Immune System

In the host's bloodstream, trypomastigotes must cope with a highly destructive innate and adaptive immunity in order to swiftly infect a variety of the host's nucleated cells. In this review, we focus on the interaction of TcCalr with the initial stages of C activation. Most likely, these interactions occur amidst an array of immune reactions, including phagocytosis by macrophages and other cells, natural killer cell activation, participation of **B lymphocytes** and $CD4^+/CD8^+$ **T lymphocytes**, as well as the secretion of proinflammatory cytokines such as interferon-γ, tumor necrosis factor, interleukin-12, and interleukin-17 [63]. However, *T. cruzi* specifically evades host immunity at different stages of activation, and successful infection occurs in the presence of an otherwise competent immune system.

In general, noninfective epimastigotes are extremely susceptible to C, while trypomastigotes are highly resistant. The magnitude of both variables is strain-dependent [64]. To evade C activation, trypomastigotes utilize C regulatory proteins. They may be membrane-bound and/or secreted, or captured from the blood, thus acquiring resistance to C actions [2]. Some of the described proteins are: plasma-membrane-derived vesicles (PMVs), *T. cruzi* trypomastigote-decay-accelerating factor (T-DAF), *T. cruzi* C regulatory protein (TcCRP), factor H (FH), gp58/68, and C2 receptor inhibitor trispanning (CRIT) [2]. Inhibition of host C components may act at different levels, interfering with: (i) C-mediated lysis, (ii) generation of C3a and C5a (anaphylatoxins; small C fragments, essential in the recruitment of blood cells to the infection site), and (iii) the **opsonization** process, which mediates phagocytosis of pathogens during acute infections [65].

TcCalr is involved in several key aspects of the host–parasite interplay, such as inhibition of both the classical and lectin pathways of C activation. TcCalr inhibits the classical pathway, interacting with C1 (Figure 1). This interaction inactivates C at the earliest stages of activation. Thus, TcCalr binds to the C1q collagenous tails and interferes with the activation of C1s and subsequent C1s-mediated cleavage of C4 and, consequently, inhibits the entire cascade, including the generation of membrane attack complexes (MACs) [16]. This capacity resides in the central S-domain of TcCalr (aa 159–281), first described in human Calr (HuCALR) with similar functions [66]. TcCalr also interferes with activation of C1s by competing with the $(C1r–C1s)_2$ tetrameric complex capacity to bind C1q, decreasing the C4b generation and membrane deposition and, therefore, the levels of the classical pathway C3 and C5 convertases [16]. This TcCalr capacity to bind C1q is calcium-independent [67]. *In vitro*, both classical pathway serine proteases, C1s and C1r, bind TcCalr, but TcCalr does not inhibit the C4-activating function of solid-phase-bound C1s. Perhaps, C1s inactivation occurs only when the serine protease is part of C1, the complex [C1q, $(C1r, C1s)_2$] [67]. Additionally, we

and C2b, which will form the C3 convertase. This molecule activates C3. (B) TcCalr has different locations inside the parasite and is secreted. TcCalr in the external milieu interacts with C1q, through the S-domain, a short domain located between the N and P domains. This domain competes with C1s to bind collagenous tails of C1q, blocking C1s binding and the activation of C4. This mechanism inhibits the classical and lectin pathways, formation of the membrane attack complex, and lysis of the pathogen. Additionally, (C) trypomastigotes express TcCalr mainly in the area of flagellum emergence. Unlike trypomastigotes, epimastigotes, the noninfective form of the parasite, express a significantly smaller amount of TcCalr on the parasite's surface. TcCalr on the parasite's membrane interacts with C1 complex or C1q. The C1q/TcCalr complex on the parasite is identified by human calreticulin (HuCALR) present on mammalian cells. The TcCalr/C1q/HuCALR interaction allows invasion of host cells. There are many proteins involved in this infectivity process. Inside the phagolysosome, trypomastigotes resist oxidative stress and transform into amastigotes.

Table 1. Some Functions of Calreticulin in the Host–Parasite Interplay[a]

Parasite	Function	Refs
Calr interferes with the C1q binding and/or complement system (C) activation		
Protozoans		
Entamoeba histolytica	EhCalr plays a role in early stages of host–parasite interaction and binds C1q, inhibiting the C classical pathway.	[28,29]
Trypanosoma carassii	TcaCalr binds C1q and inhibits the host classical pathway of C.	[30]
Trypanosoma cruzi	TcCalr binds C1q (and MBL and ficolins), inhibiting the classical and lectin pathway of C.	[16,20,67]
Helminths		
Brugia malayi	BmCalr blocks C1q-mediated host immune response and binds calcium and zinc.	[31,32,38]
Haemonchus contortus	HcCalr binds C1q and prevents blood clotting by binding to Ca^{2+} and clotting factors.	[33]
Opisthorchis viverrini	OvCalr interferes with C activation by C1q binding.	[34,35]
Trichinella spiralis	TsCalr binds C1q, inhibiting the classical pathway of C and C1q-induced macrophage activities.	[36,37]
Arthropods		
Triatoma infestans	TiCalr interacts with C1, inhibiting the classical pathway of C.	[39]
Amblyomma americanum	AaCalr binds C1q, but does not inhibit C.	[40]
Calr is a virulence factor, participating in pathogenicity		
Protozoans		
Trypanosoma congolense	*TcoCalr* is related to virulence and pathogenicity. Anti-Calr antibodies seem to block the immunomodulating action of TcoCalr against the host.	[41]
Trypanosoma cruzi	TcCalr binds C1q on the parasite surface, promoting infectivity. Additionally, TcCalr is secreted, interfering with other processes, such as angiogenesis and tumor growth.	[15,86,88–90,96,98]
Calr modulates the cellular or humoral host immune response		
Protozoans		
Trypanosoma cruzi	TcCalr is immunogenic in immunized animals and infected humans. Additionally, TcCalr seems to improve the immunogenicity against some specific canine tumors.	[16,90]
Helminths		
Dirofilaria immitis	DiCalr is a Ca^{2+}-binding protein, present in excretory–secretory products derived from larvae and adult worms. DiCal is immunogenic in chronically infected microfilaremic dogs.	[42]
Heligmosomoides polygyrus	nHpCalr is expressed and secreted by tissue- invasive larvae (L4); it interacts with Scavenger receptor-A and induces a Th2 response.	[43]
Necator americanus	NaCalr was described as an allergen.	[44]
Onchocerca volvulus	RAL-1, shares a 64.4% identity with calreticulin, inducing cross-reactivity with host Calr.	[45]
Schistosoma japonicum	SjCalr induces maturation of dendritic cells and a Th1 polarized immune response in mice.	[51]
Schistosoma mansoni	SmCalr is a Ca^{2+}-binding protein, immunogenic in mice. SmCalr plays a role during cell proliferation and participates as an antigen to T and B cells.	[46] [47]
Taenia solium	rTsCalr favors a Th2-biased immune response, inducing IL-10 in mucosal and systemic lymphoid organs *in vivo*.	[48]

(continued on next page)

Table 1. (continued)

Parasite	Function	Refs
Arthropods		
Boophilus micropus	BmCRT is present in saliva and it is immunogenic in tick-infested bovines.	[49]
Haemaphysalis qinghaiensis	HqCalr is secreted in their host during blood sucking, promoting humoral response.	[50]

[a]Abbreviations: C, complement system; Calr, calreticulin.

have demonstrated *in vitro* that TcCalr competes with the serine proteases C1r and C1s, but does not displace them from the preformed C1 complex [67].

TcCalr also binds MBL and **ficolins**, thus inhibiting the lectin pathway of C activation (Figure 1) [20]. In addition to MBL, L-, H-, and M-ficolins are **pattern-recognition receptors (PRRs)** in the lectin pathway [68–72]. L- and H-ficolins bind to lipoteichoic acid (LTA) [73] and acetylated sialic acids, both components found in Gram-positive and Gram-negative bacteria, respectively, with subsequent activation of serine proteases (MASPs) [74]. However, L-ficolin (but not H-ficolin) binds to TcCalr. The TcCalr/L-ficolin interaction does not interfere with LTA binding to L-ficolin but does interfere with its activation via LTA. Moreover, L-ficolin binds preferentially to trypomastigotes, as compared to noninfective epimastigotes, which translocate significantly lower amounts of TcCalr to their surfaces [20]. Whether M-ficolin binds TcCalr has not been investigated. Although TcCalr binds to MBL and L-ficolin [16,67], the role of TcCalr/MBL or TcCalr/L-ficolin complexes in *T. cruzi* infectivity has not been addressed. However, a study using two *T. cruzi* strains, susceptible and resistant, suggested that MBL participates in the infectivity process while the parasite deactivates the lectin pathway [75], but the ligand for MBL on the parasite surface is still unknown.

Recently, the crystallographic structure of Calr has been described, identifying interesting conformational rearrangements for future therapeutic investigations about parasite Calr [76]. We have generated several *in silico* predictive TcCalr models to delimit a peptide (VC-TcCalr) at the TcCalr N-domain. Synthetic VC-TcCalr did bind to C1q and was antiangiogenic in chick chorioallantoic membrane (CAM) *in ovum* assay. VC-TcCalr, a strong dipole, interacts with charged proteins such as collagen-like tails and **scavenger receptors** (**SRs**). Comparatively, HuCALR has less polarity and spatial stability, probably due to at least substitutions of Gln for Gly, Arg for Lys, Arg for Asp, and Ser for Arg that hinder protein–protein interactions. These differences may explain, at least in part, how TcCalr inhibits C and has higher efficiency as an antiangiogenic and antitumor agent than HuCALR [77].

Variability in the capacity of parasite strains to express and secrete TcCalr may explain differences in their ability to cope with C. Accordingly, parasites monoallelic for the *TcCalr* gene are significantly more susceptible to C-mediated lysis by classical and lectin pathways, as compared to the wild-type counterparts and, as expected, those carrying an extra copy of this gene overexpress TcCalr and are significantly more resistant [25,78].

As mentioned, several functions are shared and conserved, to differing degrees, by Calr from different species [79,80]. Calr is a surprisingly pleiotropic protein present in all nucleated cells in different organisms, including parasites. During recent years, Calr has also been characterized in parasites such as *Entamoeba histolytica* [28,29] and *Trypanosoma carassii* [30], and in the helminths *Brugia malayi* [32], *Haemonchus contortus* [33], *Opisthorchis viverrini* [34,35] and

Trichinella spiralis [36] (Table 1). As expected, all of these parasitic Calrs interact with host C1q to inhibit the C classical pathway. Additionally, *T. spiralis* Calr–C1q interaction induces macrophage activation [36,37]. Moreover, this mechanism of evading C activation may be important in hematophagous arthropods. We have described and characterized Calr from *Triatoma infestans* (TiCalr), the principal vector of Chagas' disease [39]. TiCalr from the salivary glands of *T. infestans* was cloned and expressed. TiCalr also binds C1 and inhibits the classical pathway. The presence of TiCalr in hematophagous triatomine saliva may control potential activation of C, present in the blood meal, in the digestive tract of these vectors, thus preventing subsequent tissue damage [39]. Curiously, the tick *Amblyomma americanum,* while feeding on its host, also secretes Calr [40], although Calr and C1q interaction does not seem to inhibit C activation [40]. In the nematode *H. contortus*, Calr–C1q interaction prevents blood clotting by binding to Ca^{2+} and clotting factors [33], facilitating the feeding process. This property should be explored in other hematophagous parasites.

TcCalr as a Molecular Link between Chagas' Disease and Cancer

Breast, prostate, cervix uterine, lung, and colorectal, among many other cancers, are a major cause of mortality. Intriguingly, a lower incidence of cancer has been observed in those individuals infected with *T. cruzi*, and cancer prevalence is lower in those areas where the parasite is endemic [81].

Seven decades ago, Roskin [82] and Kliuyeva [81], from the former Soviet Union, proposed that infection with *T. cruzi*, or treatment with parasite extracts, induced specific antitumor immunity, both in experimental animals and humans. These studies were greatly delayed by the 'cold war' conflict [83]. Reports in prominent journals supported these findings [84,85]. However, no molecular basis underlying this phenomenon was proposed. We have determined that TcCalr significantly inhibits **angiogenesis** [86] and tumor development in animal models [87–90], proposing that TcCalr may be responsible, at least in part, for this phenomenon.

T. cruzi Infections, and Treatments with *T. cruzi* Extracts, Significantly Induce Tumor Resistance

Some microorganisms may confer resistance to some types of tumor [91]. Thus, *T. cruzi* infection seems to enhance cancer resistance, as supported by diverse types of experience. In a chronically *T. cruzi*-infected rat model, colon cancer was induced by 1,2-dimethylhydrazine (DMH). In this experimental set up, chronic infection was protective, as compared to non-infected animals [91]. Furthermore, different strains of *T. cruzi* have a tropism for tumor cells, and virulent parasites successfully developed in cancer cells. It was proposed that the anti-cancer activity of *T. cruzi* may be due to a combination of surface cellular antigens and an inhibiting or lysing factor [92].

Additionally, *T. cruzi* generates a chronic infection, induces a highly polarized T helper (Th)1 profile, and replicates in the cytoplasm, leading to $CD8^+$ T cell antigen presentation. For this reason, a recombinant nonpathogenic *T. cruzi* clone was used as a vector for a testis tumor antigen to induce long-term T cell-mediated immunity. The parasite expressed the tumor antigen, activating T cells and delaying tumor growth [93].

In rat models for colon cancer induced by DMH, and mammary cancer induced by *N*-nitroso-*N*-methylurea (NMU), *T. cruzi* antigens induced strong $CD4^+$ and $CD8^+$ antitumor responses. Unknown *T. cruzi* antigens were proposed as responsible [94].

In the studies reviewed above, no parasite native molecule was singled out as responsible for these antitumor effects. Our laboratory has generated a variety of *in vitro*, *ex vivo*, *in ovum*, and

in vivo data, identifying TcCalr as a parasite ER-resident chaperone that, upon translocation to the exterior, experimentally promotes interesting antiangiogenic and antitumor effects. Perhaps these mechanisms may be operative, at least in part, in Chagas' disease patients.

Despite the above evidence, *T. cruzi* infection has also been associated with increased susceptibility to certain kinds of cancer. A comprehensive recent review [95], based mainly on individual case reports, proposes a paradoxical parallelism between Chagas' disease and the presence of cancer. Thus, chronic *T. cruzi* infection has been found to be associated with gastrointestinal cancer, esophageal leiomyosarcoma, uterine leiomyoma, and colon cancer. It is suggested that *T. cruzi*-related carcinogenesis is probably due to host genetic predisposing factors. In these cases, the parasite–host interaction possibly results in chronic inflammation in particular tissues [95].

TcCalr Seems to Mediate an Important Part of *T. cruzi* Antitumor Effects

As mentioned, infective trypomastigotes translocate TcCalr from the ER to the zone of flagellum emergence, an area that first contacts with the susceptible host cell [16,78]. Thus, TcCalr is available to participate in different host–parasite interactions. The inability of *T. cruzi* epimastigotes to translocate TcCalr correlates with their lack of infectivity [20,24]. Furthermore, the number of *TcCalr* genes (one to three) present in *T. cruzi* directly correlates with TcCalr expression, with *in vitro* resistance to the deleterious action of human C and with infectivity [25,78].

The N-terminal domain of recombinant TcCalr (rTcCalr) is antiangiogenic as determined by: (i) inhibition of capillary growth *ex vivo* in rat aortic rings, (ii) morphogenesis, proliferation, and chemotaxis *in vitro* in human umbilical vein **endothelial cells** [86], and iii) *in vivo* angiogenesis in the CAM assay [89]. In equimolar terms, TcCalr is a twofold more efficient antiangiogenic molecule than HuCALR [96]. The effect of TcCalr is specific since it can be reversed by anti-rTcCalr antibodies [89]. In addition, TcCalr is internalized by endothelial cells. However, this internalization is inhibited by fucoidan, a ligand for SR [86], indicating SR as an additional possible receptor for TcCalr on endothelial cells.

The experimental antitumor effect of *T. cruzi* infection can be fully reproduced by exogenously administrated rTcCalr [86]. Most important, polyclonal anti-rTcCalr $F(ab')_2$ fragments, but not their preimmune counterparts, reverse the capacity of rTcCalr to inhibit EAhy926 endothelial cell proliferation and the growth of an aggressive mammary adenocarcinoma cell line (TA3-MTXR) in mice [87]. Thus, the question emerges whether native endogenous TcCalr, in the context of the parasite, may also be responsible for at least an important part of the antitumor effect of *T. cruzi* infection [87,96]. To address this question, mice were inoculated with TA3-MTXR tumor cells and infected with *T. cruzi* trypomastigotes. In parallel, these animals were treated with anti-TcCalr antibodies. As expected, these antibodies, but not their preimmune counterparts, neutralized the antitumor effect of the infection, indicating that native endogenous TcCalr, now produced *in vivo* by the parasite, is responsible for at least an important part of the antitumor effect of *T. cruzi* infection [87].

Based on all the previous experimental considerations, where native TcCalr (secreted or on the parasite) seems to mediate fundamental alterations in the tumor cell microenvironment, we propose a model where these changes may lead to an adaptive immune response, with significant antitumor consequences (Figure 2, Key Figure).

Thus, considering our own results, and those provided by other investigators, once in the bloodstream, trypomastigotes infect endothelial cells (ECs) by recruiting C1 via externalized TcCalr.

Key Figure

Trypanosoma cruzi Calreticulin (TcCalr) Has Antiangiogenic and Antitumor Effects – a Possible Model

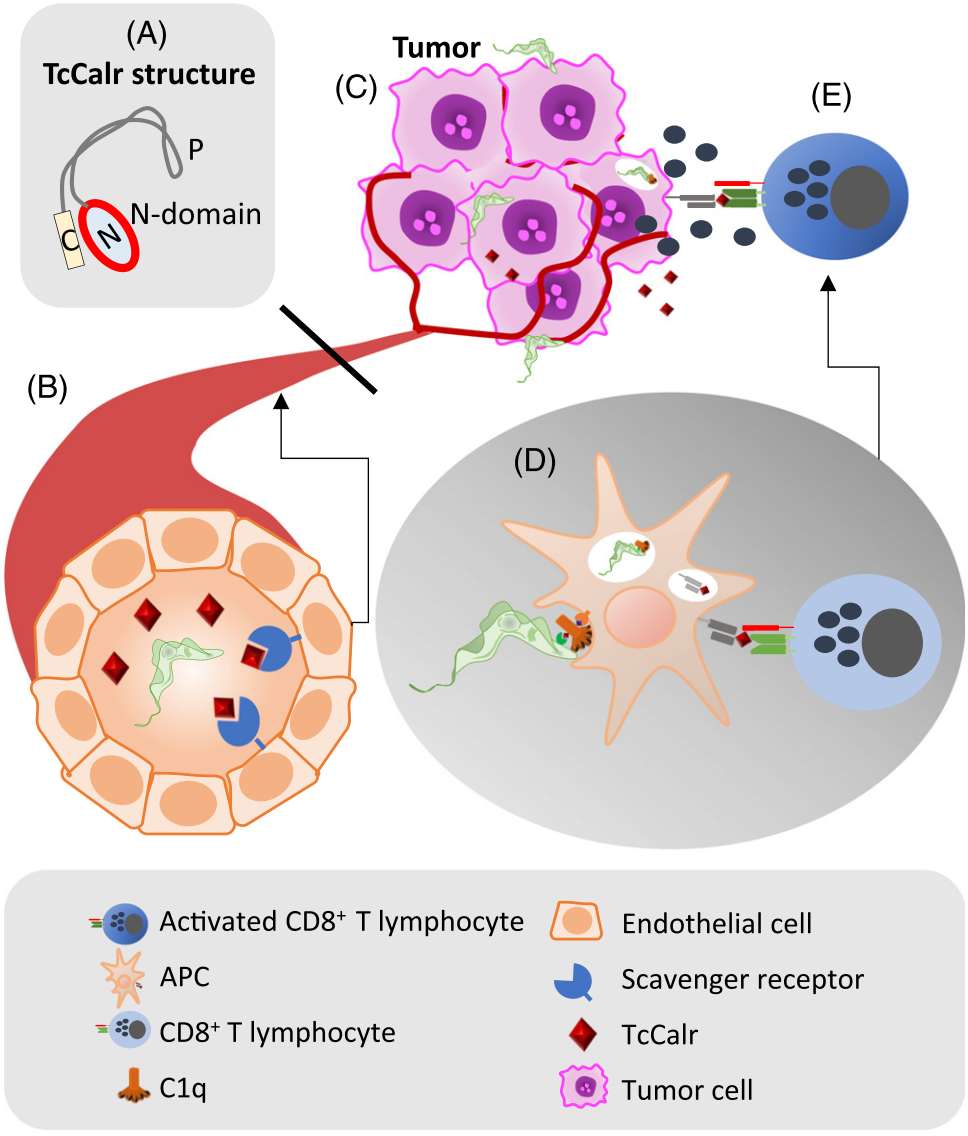

Figure 2. A variety of combined evidence indicates that *T. cruzi* infection may protect against tumor formation. TcCalr is the first described molecule that may be responsible, at least in part, for this protective effect. (A) The N-domain of TcCalr has an antiangiogenic effect, more potent than human calreticulin (HuCALR). (B) This domain, and the whole TcCalr molecule, can inhibit capillary growth, morphogenesis, proliferation, and chemotaxis *in vitro*, *ex vivo*, and *in vivo*. TcCalr is internalized by endothelial cells, and this process is reversed by fucoidan, a ligand for scavenger receptors, that may also bind TcCalr. (C) Additionally, *T. cruzi* infection, *T. cruzi* extracts, or recombinant (r)TcCalr have inhibitory effects on tumor growth. These data may indicate that the antineoplastic effect of *T. cruzi* infection is mediated by TcCalr. Based on our data and other findings, we propose that (D) TcCalr/C1q/HuCALR synapsis mediates cell invasion, and that secreted TcCalr promotes antiangiogenesis, contributing to the generation of a stressful environment for the tumor. Thus, antigen-presenting cells

(Figure legend continued at the bottom of the next page.)

This TcCalr also inhibits angiogenesis and allows contact with ECs via HuCALR (also known as cC1qR [97]) or SRs. Tumor cell phagocytosis by dendritic cells (DCs) is stimulated through C1q recruitment ('eat me' signal). An evoked adaptive immune response could be stimulated by TcCalr (or mammalian Calrs). The possibility exists that DCs expose peptides derived from TcCalr via **cross-antigen presentation** (and/or from still unidentified tumor-specific antigens – TSAs) to cytotoxic T cells in the draining regional lymph nodes. It is also feasible that these putative activated T lymphocytes return to the tumor site and act against tumor cells [98]. A simultaneous role for Th, **natural killer** (**NK**) and NKT cells are matters of current research in our and other laboratories.

Mammalian Calrs are about 95% identical, while TcCalr differs by 50%, in its primary sequence, from these counterparts. Therefore, rTcCalr – upon binding to tumor cells – is structurally more suitable than HuCALR to force their immunogenicity [39]. Recently, we demonstrated that TcCalr binds to canine transmissible venereal tumor (CTVT) cells and to a canine mammary carcinoma cell line, improving the immunogenicity of both tumors. These cells can be engulfed by macrophages and DCs cocultured with rTcCalr, accelerating its maturation and activating T cells. Paradoxically, MHC Class I expression decreases in these cells, which may be related to a down-regulation signaling promotion of the rescue of MHC I [90].

How Does *T. cruzi* Infection Elicit an Antitumor Status in the Mammalian Host? A Proposal for an Integrative Model

The following are possibilities that may explain, at least partly, how TcCalr mediates antitumor effects. (i) The parasite translocates TcCalr to the area of flagellum emergence. (ii) There, the chaperone recruits C1. (iii) TcCalr blocks the C1 capacity to initiate the C classical pathway. (iv) The TcCalr/C1q/HuCALR synapsis mediates cell invasion. (v) On the tumor cell, exogenous TcCalr, together with recruited C1q, acts as 'eat me' signals (apoptotic mimicry) after C1q has been recognized by HuCALR (cC1qR) on DCs. (vi) Secreted TcCalr is antiangiogenic; thus, *T. cruzi* infection may contribute to the generation of a stressful environment for the tumor. (vii) Stressed tumor cells translocate cC1qR, followed by additional C1q recruitment. (viii) **Antigen-presenting cells** (**APCs**), through their cC1qRs, internalize and process TcCalr-bound tumor cells on their way to regional lymph nodes. (ix) APCs may cross-present immunogenic TcCalr-specific peptides (and perhaps from other TSAs), loaded onto **MHC**-I molecules, to $CD8^+$ cytotoxic T lymphocytes. (x) These lymphocytes may attack tumor cells presenting TcCalr-derived peptides and/or from still unidentified TSAs (Figure 2).

So far there is published experimental evidence sustaining proposals (i)–(vii) [15,16,20,24,67,77,86,87,89,96]. Proposals (viii)–(x) are under investigation.

Finally, because mammalian Calrs are about 95% identical in their amino acidic sequence, their immunogenicity across these species is rather restricted. Possibly, the relevant uniqueness of TcCalr resides in extensive amino acid sequence differences (about 50%) from the mammalian counterparts, thus explaining why these antitumor effects are better performed by TcCalr. In fact, TcCalr is phylogenetically closer to plant Calrs (e.g., *Arabidopsis thaliana*) than to mammalian Calrs [39]. Thus, the parasite molecule, upon tagging tumor cells, should

(APCs) internalize and process TcCalr-bound tumor cells on their way to regional lymph nodes. APCs may cross-present immunogenic TcCalr-specific peptides [and perhaps other tumor-specific antigens (TSAs)], loaded onto MHC-I molecules, to $CD8^+$ cytotoxic T lymphocytes, and (E) contingent on the occurrence of this activation, these activated lymphocytes may attack tumor cells that present TcCalr-derived peptides and/or those from still unidentified TSAs.

be able to force their poor immunogenicity in the presence of an apparently otherwise competent immune system.

It should also be considered that parasite and host genetic variability may cause different clinical manifestations, immunopathogenic behavior, protein expression and secretion [14]. Thus, additional studies are necessary to elucidate the roles of parasite and host genetic backgrounds in the outcomes of these complex interactions.

Concluding Remarks

TcCalr, a multifunctional protein with several key roles in the host–*T. cruzi* interaction (Figures 1 and 2), is vital for parasite development since knockout *TcCalr* parasites are unviable [78]. Additionally, trypomastigotes, the infective stage of *T. cruzi*, translocate TcCalr to the cellular membrane where the chaperone will participate in two crucial mechanisms: host immune evasion and mammalian cell invasion. To evade the host immune response, TcCalr binds C1 and MBL/ficolins, soluble PRRs (sPRRs), of the C classical and lectin pathways, respectively [16,67]. The TcCalr/sPRR interaction is an expression of resistance to C-mediated functions (lysis, opsonization, immune stimulation, and inflammation). On the other hand, the TcCalr interactions with C1, and perhaps with MBL or ficolins, facilitate the infectivity process, using a similar mechanism previously described in apoptotic cell removal. This prophagocytic mechanism operates when TcCalr, on the parasite surface, is identified by C1q, and the formed complex TcCalr/C1q is recognized by the HuCALR present on the phagocytic cell surface [15]. Moreover, in *T. cruzi* experimentally infected animals, resistance to tumor development has been substantiated, and it is feasible that similar mechanisms may operate in Chagas' disease patients. TcCalr is a likely important mediator of these mechanisms, mainly due to its antiangiogenic and immunogenic properties [86,87,89,96]. Its capacity to modulate molecules that may participate in tumor immunogenicity may also be operative (see Outstanding Questions). In synthesis, the combined experimental evidence reviewed herein is compatible with the possibility that Chagas' disease protects against concomitant tumors.

Finally, understanding how the parasite modulates the host–parasite interaction and the identification of responsible molecules will not only lead the search for new therapeutic targets or therapies to face Chagas' disease, but may also contribute to the design of additional experimental approaches to other prevalent diseases, such as cancer.

Outstanding Questions

What are the consequences of C1 and MBL/ficolins–TcCalr interaction in cell signaling and in phagolysosome formation?

What is the ligand for TcCalr on the trypomastigote surface?

Are APCs able to present TcCalr-derived peptides in an MHC class I context to $CD8^+$ T lymphocytes?

Do these $CD8^+$ T lymphocytes have a specific antitumor effect in *T. cruzi*-infected individuals?

Since rTcCalr is immunogenic in mammals, is it worth searching for a smaller, less immunogenic peptide? Will the peptide keep the main relevant functions of the whole chaperone molecule?

Does TcCalr, upon tagging tumor cells, force their immunogenicity by acting as a 'tumor-specific antigen'?

Acknowledgments

This work was supported by Chilean FONDECYT/CONICYT Public Grants 1050133, 1095095, 1130099 (A.F.) and 11110251 (G.R.-T.), VID-Universidad de Chile (A.F.); URC-024/16 (G.R.-T.) and FIV-FAVET 12101701-9102-181 (G.R.-T.).

References

1. Pérez-Molina, J.A. and Molina, I. (2018) Chagas disease. *Lancet* 391, 82–94
2. Ramirez-Toloza, G. and Ferreira, A. (2017) *Trypanosoma cruzi* evades the complement system as an efficient strategy to survive in the mammalian host: the specific roles of host/parasite molecules and *Trypanosoma cruzi* calreticulin. *Front. Microbiol.* 8, 1667
3. Vija-Suarez, C.A. *et al.* (2017) Behavioral evidence of oxidative stress by hematophagy in the ocellar system of *Rhodnius prolixus* Stål, 1859 red-eyed mutants. *J. Insect Physiol.* 101, 31–38
4. Graça-Souza, A.V. *et al.* (2006) Adaptations against heme toxicity in blood-feeding arthropods. *Insect Biochem. Mol. Biol.* 36, 322–335
5. Piacenza, L. *et al.* (2009) Fighting the oxidative assault: the *Trypanosoma cruzi* journey to infection. *Curr. Opin. Microbiol.* 12, 415–421
6. Ponce, I. *et al.* (2017) A flap endonuclease (TcFEN1) is involved in *Trypanosoma cruzi* cell proliferation, DNA repair, and parasite survival. *J. Cell. Biochem.* 118, 1722–1732
7. Valenzuela, L. *et al.* (2019) Further *in vivo* evidence implying DNA apurinic/apyrimidinic endonuclease activity in *Trypanosoma cruzi* oxidative stress survival. *J. Cell. Biochem.* 120, 7
8. Machado-Silva, A. *et al.* (2016) How *Trypanosoma cruzi* deals with oxidative stress: Antioxidant defence and DNA repair pathways. *Mutat. Res. Rev.* 767, 8–22
9. Cardoso, M.S. *et al.* (2015) Evasion of the immune response by *Trypanosoma cruzi* during acute infection. *Front. Immunol.* 6, 659
10. De Fuentes-Vicente, J.A. *et al.* (2019) *Trypanosoma cruzi*: A review of biological and methodological factors in Mexican strains. *Acta Trop.* 195, 7
11. Jiménez, P. *et al.* (2019) A systematic review of the *Trypanosoma cruzi* genetic heterogeneity, host immune response and genetic factors as plausible drivers of chronic chagasic cardiomyopathy. *Parasitology* 146, 269–283
12. Schwabl, P. *et al.* (2019) Meiotic sex in Chagas disease parasite *Trypanosoma cruzi*. *Nature Commun.* 10, 1–14
13. Macedo, A.M. *et al.* (2002) Chagas disease: role of parasite genetic variation in pathogenesis. *Exp. Rev. Mol. Med.* 4, 1–16

14. Herreros-Cabello, A. *et al.* (2019) Comparative proteomic analysis of trypomastigotes from *Trypanosoma cruzi* strains with different pathogenicity. *Infect. Genet. Evol.* 76, 104041
15. Ramirez, G. *et al.* (2011) *Trypanosoma cruzi* calreticulin: a novel virulence factor that binds complement C1 on the parasite surface and promotes infectivity. *Immunobiology* 216, 265–273
16. Ferreira, V. *et al.* (2004) The classical activation pathway of the human complement system is specifically inhibited by calreticulin from *Trypanosoma cruzi*. *J. Immunol.* 172, 3042–3050
17. Gonzalez, A. *et al.* (2015) *Trypanosoma cruzi* calreticulin topographical variations in parasites infecting murine macrophages. *Am. J. Trop. Med. Hyg.* 92, 887–897
18. Souto-Padrón, T. *et al.* (2004) Immunocytochemical localisation of calreticulin in *Trypanosoma cruzi*. *Histochem. Cell Biol.* 122, 563–569
19. Gonzalez, A. *et al.* (2018) Variable numbers of calreticulin genes in *Trypanosoma cruzi* correlate with atypical morphology and protein expression. *Immunobiology* 223, 802–806
20. Sosoniuk, E. *et al.* (2014) *Trypanosoma cruzi* calreticulin inhibits the complement lectin pathway activation by direct interaction with L-Ficolin. *Mol. Immunol.* 60, 80–85
21. Schcolnik-Cabrera, A. *et al.* (2019) Calreticulin in phagocytosis and cancer: opposite roles in immune response outcomes. *Apoptosis* 24, 245–255
22. Eggleton, P. *et al.* (2000) C1q receptors. *Clin. Exp. Immunol.* 120, 406–412
23. Ogden, C.A. *et al.* (2001) C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J. Exp. Med.* 194, 781–796
24. Sosoniuk-Roche, E. *et al.* (2017) Exogenous calreticulin, incorporated onto non-infective *Trypanosoma cruzi* epimastigotes, promotes their internalization into mammal host cells. *Immunobiology* 222, 529–535
25. Sanchez-Valdez, F.J. *et al.* (2014) A monoallelic deletion of the TcCRT gene increases the attenuation of a cultured *Trypanosoma cruzi* strain, protecting against an *in vivo* virulent challenge. *PLoS Negl. Trop. Dis.* 8, e2696
26. Crawford, K.E. *et al.* (2012) Calreticulin has opposing effects on the migration of human trophoblast and myometrial endothelial cells. *Placenta* 33, 416–423
27. Castillo, C. *et al.* (2013) The interaction of classical complement component C1 with parasite and host calreticulin mediates *Trypanosoma cruzi* infection of human placenta. *PLoS Negl. Trop. Dis.* 7, e2376
28. Ximénez, C. *et al.* (2014) *Entamoeba histolytica* and *E. dispar* calreticulin: inhibition of classical complement pathway and differences in the level of expression in amoebic liver abscess. *Biomed. Res. Int.* 2014, 127453
29. González, E. *et al.* (2011) *Entamoeba histolytica* calreticulin: an endoplasmic reticulum protein expressed by trophozoites into experimentally induced amoebic liver abscesses. *Parasitol. Res.* 108, 439–449
30. Oladiran, A. and Belosevic, M. (2010) *Trypanosoma carassii* calreticulin binds host complement component C1q and inhibits classical complement pathway-mediated lysis. *Dev. Comp. Immunol.* 34, 396–405
31. Verma, P. *et al.* (2017) Molecular cloning and characterization of protein disulfide isomerase of *Brugia malayi*, a human lymphatic filarial parasite. *EXCLI J.* 16, 824
32. Yadav, S. *et al.* (2018) Metal binding study of calreticulin: An immunomodulatory protein of human filarial parasite *Brugia malayi*. *Int. J. Biol. Macromol.* 117, 1157–1168
33. Suchitra, S. and Joshi, P. (2005) Characterization of *Haemonchus contortus* calreticulin suggests its role in feeding and immune evasion by the parasite. *Biochim. Biophy. Acta* 1722, 293–303
34. Chaibangyang, W. *et al.* (2018) Evaluation of *Opisthorchis viverrini* calreticulin for potential host modulation. *Acta Trop.* 187, 175–181
35. Chaibangyang, W. *et al.* (2017) Molecular and biochemical characterization of *Opisthorchis viverrini* calreticulin. *Korean J. Parasitol.* 55, 643
36. Zhao, L. *et al.* (2017) *Trichinella spiralis* calreticulin binds human complement C1q as an immune evasion strategy. *Front. Immunol.* 8, 636
37. Wang, Z. *et al.* (2018) Mapping of the complement C1q binding site on *Trichinella spiralis* paramyosin. *Parasit. Vectors* 11, 666
38. Yadav, S. *et al.* (2017) Monitoring thermal and chemical unfolding of *Brugia malayi* calreticulin using fluorescence and circular dichroism spectroscopy. *Int. J. Biol. Macromol.* 102, 986–995
39. Weinberger, K. *et al.* (2017) *Triatoma infestans* calreticulin: gene cloning and expression of a main domain that interacts with the host complement system. *Am. J. Trop. Med. Hyg.* 96, 295–303
40. Kim, T.K. *et al.* (2015) *Amblyomma americanum* tick calreticulin binds C1q but does not inhibit activation of the classical complement cascade. *Ticks Tick-borne Dis.* 6, 91–101
41. Bossard, G. *et al.* (2016) Cloning, expression, molecular characterization and preliminary studies on immunomodulating properties of recombinant *Trypanosoma congolense* calreticulin. *Infect. Genet. Evol.* 45, 320–331
42. Tsuji, N. *et al.* (1998) Molecular characterization of a calcium-binding protein from the filarial parasite *Dirofilaria immitis*. *Mol. Biochem. Parasitol.* 97, 69–79
43. Rzepecka, J. *et al.* (2009) Calreticulin from the intestinal nematode *Heligmosomoides polygyrus* is a Th2-skewing protein and interacts with murine scavenger receptor-A. *Mol. Immunol.* 46, 1109–1119
44. Brown, A. *et al.* (1999) A hookworm allergen which strongly resembles calreticulin. *Parasite Immunol.* 21, 439–450
45. Rokeach, L.A. *et al.* (1994) Epitopes of the *Onchocerca volvulus* RAL1 antigen, a member of the calreticulin family of proteins, recognized by sera from patients with onchocerciasis. *Infect. Immun.* 62, 3696–3704
46. Khalife, J. *et al.* (1994) Characterization and localization of *Schistosoma mansoni* calreticulin Sm58. *Parasitology* 108, 527–532
47. El Gengehi, N. *et al.* (2000) A *Schistosoma mansoni* 62-kDa band is identified as an irradiated vaccine T-cell antigen and characterized as calreticulin. *J. Parasitol.* 86, 993–1000
48. Mendlovic, F. *et al.* (2015) Cytokine, antibody and proliferative cellular responses elicited by *Taenia solium* calreticulin upon experimental infection in hamsters. *PLoS One* 10, e0121321
49. Ferreira, C.A.S. *et al.* (2002) Cloning and partial characterization of a *Boophilus microplus* (Acari: Ixodidae) calreticulin. *Exp. Parasitol.* 101, 25–34
50. Gao, J. *et al.* (2008) Cloning and characterization of a cDNA clone encoding calreticulin from *Haemaphysalis qinghaiensis* (Acari: Ixodidae). *Parasitol. Res.* 102, 737–746
51. Ma, L. *et al.* (2017) SjCRT, a recombinant *Schistosoma japonicum* calreticulin, induces maturation of dendritic cells and a Th1-polarized immune response in mice. *Parasit. Vectors* 10, 570
52. Alvarado-Delgado, A. *et al.* (2016) Infection with *Plasmodium berghei* ookinetes alters protein expression in the brain of Anopheles albimanus mosquitoes. *Parasit. Vectors* 9, 542
53. Chen, K. *et al.* (2019) Proteomic analysis of excretory-secretory products from young adults of *Angiostrongylus cantonensis*. *Mem. Instit. Oswaldo Cruz* 114, e180556-e180556
54. Hamilton, S. *et al.* (2018) Characterisation of proteins in excretory/secretory products collected from salmon lice, *Lepeophtheirus salmonis*. *Parasit. Vectors* 11, 294
55. Antunes, S. *et al.* (2012) Functional genomics studies of *Rhipicephalus* (*Boophilus*) *annulatus* ticks in response to infection with the cattle protozoan parasite, *Babesia bigemina*. *Int. J. Parasitol.* 42, 187–195
56. Morgan, M.S. *et al.* (2016) A proteomic analysis of *Sarcoptes scabiei* (Acari: Sarcoptidae). *J. Med. Entomol.* 53, 553–561
57. Kim, Y.-H. *et al.* (2015) Proteomic screening of antigenic proteins from the hard tick, *Haemaphysalis longicornis* (Acari: Ixodidae). *Korean J. Parasitol.* 53, 85–93
58. Wang, Y. *et al.* (2009) *Echinococcus multilocularis*: proteomic analysis of the protoscoleces by two-dimensional electrophoresis and mass spectrometry. *Exp. Parasitol.* 123, 162–167
59. Kumari, S. *et al.* (2008) Th1-stimulatory polyproteins of soluble *Leishmania donovani* promastigotes ranging from 89.9 to 97.1 kDa offers long-lasting protection against experimental visceral leishmaniasis. *Vaccine* 26, 5700–5711

60. Guillou, F. *et al.* (2007) Excretory–secretory proteome of larval *Schistosoma mansoni* and *Echinostoma caproni*, two parasites of *Biomphalaria glabrata*. *Mol. Biochem. Parasitol.* 155, 45–56
61. Gupta, S.K. *et al.* (2007) Proteomic approach for identification and characterization of novel immunostimulatory proteins from soluble antigens of *Leishmania donovani* promastigotes. *Proteomics* 7, 816–823
62. Xu, G. *et al.* (2004) Cloning and sequencing of putative calreticulin complementary DNAs from four hard tick species. *J. Parasitol.* 90, 73–78
63. Cutrullis, R.A. *et al.* (2017) MIF-driven activation of macrophages induces killing of intracellular *Trypanosoma cruzi* dependent on endogenous production of tumor necrosis factor, nitric oxide and reactive oxygen species. *Immunobiology* 222, 423–431
64. Cestari, I. and Ramirez, M.I. (2010) Inefficient complement system clearance of *Trypanosoma cruzi* metacyclic trypomastigotes enables resistant strains to invade eukaryotic cells. *PLoS One* 5, e9721
65. Cestari, I. *et al.* (2012) *Trypanosoma cruzi* immune evasion mediated by host cell-derived microvesicles. *J. Immunol.* 188, 1942–1952
66. Lynch, N.J. *et al.* (2002) *In vivo* pharmacokinetics of calreticulin S-domain, an inhibitor of the classical complement pathway. *Int. Immunopharmacol.* 2, 415–422
67. Valck, C. *et al.* (2010) Molecular mechanisms involved in the inactivation of the first component of human complement by *Trypanosoma cruzi* calreticulin. *Mol. Immunol.* 47, 1516–1521
68. Endo, Y. *et al.* (1996) Cloning and characterization of the human lectin P35 gene and its related gene. *Genomics* 36, 515–521
69. Lu, J. *et al.* (1996) Biosynthesis of human ficolin, an *Escherichia coli*-binding protein, by monocytes: comparison with the synthesis of two macrophage-specific proteins, C1q and the mannose receptor. *Immunology* 89, 289–294
70. Sugimoto, R. *et al.* (1998) Cloning and characterization of the Hakata antigen, a member of the ficolin/opsonin p35 lectin family. *J. Biol. Chem.* 273, 20721–20727
71. Teh, C. *et al.* (2000) M-ficolin is expressed on monocytes and is a lectin binding to N-acetyl-d-glucosamine and mediates monocyte adhesion and phagocytosis of *Escherichia coli*. *Immunology* 101, 225–232
72. Wittenborn, T. *et al.* (2009) Characteristics and biological variations of M-ficolin, a pattern recognition molecule, in plasma. *J. Innate Immun.* 2, 167–180
73. Lynch, N.J. *et al.* (2004) L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of Gram-positive bacteria, and activates the lectin pathway of complement. *J. Immunol.* 172, 1198–1202
74. Gout, E. *et al.* (2010) Carbohydrate recognition properties of human ficolins Glycan array screening reveals the sialic acid binding specificity of M-ficolin. *J. Biol. Chem.* 285, 6612–6622
75. Evans-Osses, I. *et al.* (2014) Differential ability to resist to complement lysis and invade host cells mediated by MBL in R4 and 860 strains of *Trypanosoma cruzi*. *FEBS Lett.* 588, 956–961
76. Moreau, C. *et al.* (2016) Structures of parasite calreticulins provide insights into their flexibility and dual carbohydrate/peptide-binding properties. *IUCrJ* 3, 408–419
77. Peña Álvarez, J. *et al.* (2019) Structural bases that underline *Trypanosoma cruzi* calreticulin proinfective, antiangiogenic and antitumor properties. *Immunobiology*. Published online November 5, 2019. https://doi.org/10.1016/j.imbio.2019.10.012
78. Sanchez Valdez, F.J. *et al.* (2013) *Trypanosoma cruzi* carrying a monoallelic deletion of the calreticulin (TcCRT) gene are susceptible to complement mediated killing and defective in their metacyclogenesis. *Mol. Immunol.* 53, 198–205
79. Ramirez, G. *et al.* (2011) Extracellular *Trypanosoma cruzi* calreticulin in the host–parasite interplay. *Trends Parasitol.* 27, 115–122
80. Eggleton, P. *et al.* (2016) Calreticulin, a therapeutic target? *Exp. Opin. Ther. Targets* 20, 1137–1147
81. Klyuyeva, N.G. and Roskin, G.I. (1963) *Biotherapy of Malignant Tumors*, Pergamon
82. Roskin, G. (1946) Toxin therapy of experimental cancer. The influence of protozoan infections upon transplanted cancer. *Cancer Res.* 6, 363
83. Krementsov, N. (2009) *Trypanosoma cruzi*, cancer and the Cold War. *Hist. Ciênc. Saúde-Manguinhos* 16, 75–94
84. Malisoff, W.M. (1947) The action of the endotoxin of *Trypanosoma cruzi* (KR) on malignant mouse tumors. *Science* 106, 591–594
85. Hauschka, T.S. and Goodwin, M.B. (1948) *Trypanosoma cruzi* endotoxin (KR) in the treatment of malignant mouse tumors. *Science* 107, 600–602
86. Lopez, N.C. *et al.* (2010) Antiangiogenic and antitumor effects of *Trypanosoma cruzi* Calreticulin. *PLoS Negl. Trop. Dis.* 4, e730
87. Abello-Caceres, P. *et al.* (2016) Does native *Trypanosoma cruzi* calreticulin mediate growth inhibition of a mammary tumor during infection? *BMC Cancer* 16, 731
88. Aguilar-Guzman, L. *et al.* (2014) Human survivin and *Trypanosoma cruzi* calreticulin act in synergy against a murine melanoma *in vivo*. *PLoS One* 9, e95457
89. Molina, M.C. *et al.* (2005) An *in vivo* role for *Trypanosoma cruzi* calreticulin in antiangiogenesis. *Mol. Biochem. Parasitol.* 140, 133–140
90. Cruz, P. *et al.* (2019) *Trypanosoma cruzi* calreticulin: *in vitro* modulation of key immunogenic markers of both canine tumors and relevant immune competent cells. *Immunobiology*. Published online December 5, 2019. https://doi.org/10.1016/j.imbio.2019.12.001
91. Oliveira, E. *et al.* (2001) Chronic *Trypanosoma cruzi* infection associated with low incidence of 1,2-dimethylhydrazine-induced colon cancer in rats. *Carcinogenesis* 22, 737–740
92. Kallinikova, V.D. *et al.* (2001) Anticancer properties of flagellate protozoan *Trypanosoma cruzi* Chagas, 1909. *Biol. Bull. Russian Acad. Sci.* 28, 244–255
93. Junqueira, C. *et al.* (2011) *Trypanosoma cruzi* as an effective cancer antigen delivery vector. *Proc. Natl. Acad. Sci. U. S. A.* 108, 19695–19700
94. Ubillos, L. *et al.* (2016) *Trypanosoma cruzi* extracts elicit protective immune response against chemically induced colon and mammary cancers. *Int. J. Cancer* 138, 1719–1731
95. van Tong, H. *et al.* (2017) Parasite infection, carcinogenesis and human malignancy. *EBioMedicine* 15, 12–23
96. Toledo, V. *et al.* (2010) Comparative *in vivo* antiangiogenic effects of calreticulin from *Trypanosoma cruzi* and *Homo sapiens*. *Biol. Res.* 43, 287–289
97. Stuart, G.R. *et al.* (1997) The C1q and collectin binding site within C1q receptor (cell surface calreticulin). *Immunopharmacology* 38, 73–80
98. Ramirez-Toloza, G. *et al.* (2016) Is the antitumor property of *Trypanosoma cruzi* infection mediated by its calreticulin? *Front. Immunol.* 7, 268
99. Ignacio Arias, J. *et al.* (2015) Comparative effect of human and *Trypanosoma cruzi* calreticulin in wound healing. *J. Tissue Eng. Regen. Med.* 9, 41–54
100. Arias, J.I. *et al.* (2018) Different *Trypanosoma cruzi* calreticulin domains mediate migration and proliferation of fibroblasts *in vitro* and skin wound healing *in vivo*. *Arch. Dermatol. Res.* 310, 639–650