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Parasite-host glycan interactions during *Trypanosoma cruzi* infection: *trans*-Sialidase rides the show

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Abstract

Many important pathogen-host interactions rely on highly specific carbohydrate binding events. In the case of the protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease, glycointeractions involving sialic acid (SA) residues are pivotal for parasite infectivity, escape from immune surveillance and pathogenesis. Though unable to synthesize SA *de novo*, *T. cruzi* displays a unique *trans*-Sialidase (TS) enzyme, which is able to cleave terminal SA residues from host donor glycoconjugates and transfer them onto parasite surface mucins, thus generating protective/adhesive structures. In addition, this parasite sheds TS into the bloodstream, as a way of modifying the surface SA signature, and thereby the signaling/functional properties of mammalian host target cells on its own advantage. Here, we discuss the pathogenic aspects of *T. cruzi* TS: its molecular adaptations, the multiplicity of interactions in which it is involved during infections, and the array of novel and appealing targets for intervention in Chagas disease provided by TS-remodeled sialoglycophenotypes.

Keywords

Sialic acids; *trans*-Sialidase; pathogenesis; glycobiology of infection; Chagas disease; *Trypanosoma cruzi*

INTRODUCTION

Trypanosoma cruzi (Kinetoplastida, Trypanosomatidae) is a parasitic protozoan that alternates between haematophagous triatomine vectors and a variety of mammalian hosts, including humans. Chagas disease, caused by infection with *T. cruzi*, is the highest impact parasitic disease and leading cause of infectious cardiomyopathy throughout Latin America [1]. Due to migratory trends of infected populations to non-endemic regions, this illness is

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now also recognized as an emergent threat to global public health [1]. Despite this alarming epidemiological situation, vaccines are not yet available, and the current arsenal of trypanocidal drugs is restricted to two rather old compounds: Benznidazole and Nifurtimox. Both require prolonged regimes, have unclear therapeutic efficacy, and display serious secondary effects [1]. In this context, there is an urgent need to develop innovative therapies, particularly those exploiting novel mechanisms of action.

In order to establish a persistent infection, *T. cruzi* must strike a balance between causing disease and staying below the radar of the mammalian immune system. This parasite has accordingly evolved a broad range of self-protective mechanisms and elusion devices, including the establishment of an intracellular replicative niche and the formation of quiescent or dormant forms [2]. Prominent among *T. cruzi* evasion strategies, is the manipulation of the sialic acid (SA) signature of the infection. SAs are a family of acidic, nine-carbon monosaccharides, usually found at terminal positions of oligosaccharide chains of membrane-anchored and/or secreted glycoconjugates [3]. Mainly two SA species, *N*-Acetylneuraminic acid and *N*-Glycolylneuraminic acid, can be found in mammals, where they are involved in a broad range of biological processes, including the regulation of cell-to-cell adhesion and signaling phenomena [3]. The mechanistic basis for SA-modulated phenotypes is quite variable, but the central theme is that their strong negative charge tends to alter the range of binding partners of scaffolding glycoconjugates and, hence the overall landscape of cell glycointeractions. Of note, SAs are major forces underlying intercellular recognition events required for the elicitation and resolution of effective immune responses, and therefore it should not come as any surprise that sialylation constitutes the glycosylation step most widely manipulated by pathogens [4]. It was shown, for instance, that a variety of bacteria, protozoa and fungi develop ‘protective shields’, instrumental to the colonization of the host and/or to inhibit immune recognition and clearance, upon surface sialoglycoconjugates [5, 6]. In this context, it is worth noting that surface sialylation is a well-recognized microbial determinant of complement resistance [7]. Other pathogens express lectins that enable their initial attachment to SA-containing structures on target cells or tissues [8–10]. Finally, certain microorganisms induce sialylation changes in the host as an indirect way to undermine the immune system and/or the integrity of endothelial barriers [11, 12]. Outside of hijacking the glycosylation machinery, usually through the expression of sialyltransferases [13, 14], a stronger case can be made for direct alteration of the host cell surface SA signature by microbial sialidases [15, 16].

Despite being unable to synthesize SA *de novo*, *T. cruzi* incorporates this carbohydrate through the action of a unique enzyme: the *trans*-Sialidase (TS). This molecule combines many of the SA-based parasitic traits mentioned above, *i.e.* SA lectin, sialidase and sialyltransferase activities, all in a single, multitasking polypeptide. TS binds to and subsequently cleaves terminal SA residues linked α2,3 to β-galactopyranoses (ßGal*p*) from host donor macromolecules. However, instead of just hydrolyzing SA residues as for classical sialidases, TS is able to catalyze the formation of an equivalent SAα2–3βGal*p* glycotope in the glycans of mucin-type proteins displayed on the parasite membrane [18]. The generation of such surface SA-containing structures is crucial for protection and infectivity of trypomastigotes, the parasite forms present in the mammalian bloodstream [19]. In addition, the regulated shedding of TS into the bloodstream allows *T. cruzi*

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trypomastigotes to manipulate the surface sialylation pattern, and thereby the biological properties of different host cell types including thymocytes, lymphocytes, erythrocytes, platelets and endothelial cells. TS-induced alterations in the host sialylglycophenotype correlate with immune dysfunction, hematological alterations, susceptibility to *T. cruzi* infection and disease. These molecular features, together with the absence of a functional counterpart in the mammalian host, enthroned TS as a very promising target for the development of new treatments and vaccines against Chagas disease [20].

Recent and comprehensive reviews about the structural, and biochemical properties of *T. cruzi* TS prompt us to cover these aspects only briefly [17, 21]. Here, we will rather aim to assess the role(s) played by this molecule in the interaction with the mammalian host: from the molecular adaptations underlying its pathogenic effects to the multiplicity of parasite and host TS-altered sialoglycophenotypes that take place during *T. cruzi* infections.

2- Biological adaptations of *T. cruzi* TS, or how to make a virulence factor out of an enzyme.

2.1- Genomic features

The most parsimonious hypothesis proposes that TS emerged in an ancestor of the trypanosome lineage, most likely as an upgraded sialidase [22]. This event predated the splitting of salivarian and non-salivarian trypanosomes, which occurred ~250–500 million years ago [23]. The 'primal' sialidase may have evolved spontaneously in this ancestral organism or, most likely, may have been acquired from a microbe sharing the same niche (*i.e*. the insect gut) (Fig. 1). Whatever the case, TS activity was readily adopted by different clades of the *Trypanosoma* genus, including the African trypanosomes *Trypanosoma brucei* [22] and *T. congolense*, in which a small family of TS molecules was found [24]. However, it was in *T. cruzi* where this evolutionary 'spin off' was actually pushed up to its very limits. Successive events of gene duplication followed by diversification and recombination led to a tremendous increase in the genetic dosage and functional complexity associated to this enzyme. Indeed, *T. cruzi* evolved a huge repository of >1,400 TS and TS-like genes and pseudogenes, collectively known as the gp85/*trans*-Sialidase gene family, in which eight rather robust groups can be delineated [25–27]. Members of this family are very polymorphic, bearing as low as 30% sequence identity between each other, though they are unified by certain structural features. These include a sequence associated to tissue tropism known as FLY [28] as well as typical bacterial/viral sialidase motifs such as Asp-boxes and the FR(I/D)P tetrapeptide (Fig. 2) [25–27]. In addition, all of gp85/*trans*-Sialidase family members display an *N*-terminal signal peptide and a *C*-terminal sequence compatible with the post-translational anchoring to the plasma membrane through a glycosylphosphatidylinositol (GPI) moiety, both of which ensure their localization at the parasite surface (Fig. 2) [29]. Throughout this review, we will focus our analysis on molecules displaying *trans*-sialidase/sialidase activity, which are restricted to the gp85/*trans*-Sialidase Group I. It is worth mentioning, however, that other family members are likely involved in several aspects of *T. cruzi* biology, particularly in the interaction with the mammalian host (reviewed in [28]). Importantly, and besides molecule- or group-specific features/functions, it should be stressed that the redundancy and sheer diversity of the Gp85/

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trans-Sialidase family may have been selected for as an additional strategy to cope with the mammal immune system [30, 31].

Group I of gp85/*trans*-Sialidases family includes genes expressed by insect-dwelling, epimastigote forms (*eTS*) and those expressed by trypomastigotes, characterized by the presence of an antigenic and repetitive *C*-terminal region (Fig. 2). This region is known as SAPA (for Shed Acute Phase Antigen), as TS is actively secreted by *T. cruzi* trypomastigotes (see below), and recognized mainly by sera from patients coursing the acute phase of Chagas disease [32, 33]. The *N*-terminal and catalytic region of TS (and likely also of eTS) folds into two distinct domains: a β-propeller, which contains the catalytic pocket, and a lectin-like domain (named as such due to its structural similarity to plant lectins) [34]. These domains are connected by a long α-helix (see inset, Fig. 2). Both TS and eTS display *trans*-Sialidase/sialidase activity with very similar kinetic properties and SA acceptor-donor specificity [35]. However, eTS is a rather simple molecule, streamlined for the transference of SA residues between glycoconjugates whereas TS display additional sequences (such as SAPA) involved in moonlighting, *i.e*. non-catalytic, activities. These structural features, together with the acknowledged idea that TS activity was originally involved in the interaction between trypanosomes and arthropod vectors [22, 36], put forward the notion that *eTS* correspond to the 'ancestral' *T. cruzi TS* genes, upon which the trypomastigote-expressed counterparts were crafted [37]. Moreover, it is likely that acquisition of such molecular gadgets was instrumental to the pathogenic repurposing of TS.

T. cruzi is better defined as a taxon composed of multiple 'clonal' strains showing remarkable genetic and phenotypic diversity that were grouped into six evolutionary lineages or DTUs (Discrete Typing Units) [38]. The differential genomic make-up of *T. cruzi* strains is patently reflected in the dosage/diversification of gene families coding for molecules involved in the interaction with the host(s) [39]. In the case of gp85/*trans*-Sialidases, all of the strains analyzed so far were shown to contain *TS* genes, with dosages ranging from 1 to 40 copies per haplotype [40, 41]. Despite previous studies claiming the existence of phenotypic TS^{null} parasites [42], strict conservation and expression of *TS* genes across the *T. cruz*i taxon was also supported by comprehensive phenotypic [43] and immunological surveys [44, 45]. Intriguingly, these studies also revealed that the activity of enzyme displayed by different strains does not correlate with *TS* gene dosage [40, 43].

Along with *TS* and *eTS* genes, a third kind of closely related genes can be found in the *T. cruzi* genome. These code for molecules lacking *trans*-Sialidase/sialidase activity and were hence called 'inactive TS members' or iTS. Notably, all *iTS* genes analyzed so far present a T/C transition on codon 342, which determines a Tyr_{342}His replacement on the deduced polypeptides (Fig. 2). This sole mutation accounts for their lack of *trans*-sialylation capacity, as supported by biochemical and structural data [34, 46, 47]. iTS molecules bear SAPA repeats and display extremely high conservation in sequence (>95% identity at the amino acid level) and structure to enzymatically active TS [34, 40, 48]. This structural conservation includes the *N*-terminal domain, and even the 'inactivated' catalytic pocket [48]. Indeed, iTS molecules are able to bind substrates of the TS reaction (αSA2–3βGal*p* and/or glycoproteins bearing this glycotope) and even retain residual hydrolytic activity [41, 48, 49], suggesting they function as parasite surface adhesins *in vivo* ([50], see also Section 3). The structural

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conservation of iTS molecules is further stressed by their recognition by a TS-neutralizing monoclonal antibody (13G9) specific to a conformation-dependent epitope located in the enzymatic pocket [51].

Genotypic analyses showed that *iTS* genes, instead of presenting taxon-wide distribution such as *TS* genes, were restricted to parasite strains clustering to DTUs TcII, TcV and TcVI [40]. This peculiar phylogenetic distribution, along with the fact that *iTS* genes always show SAPA repeats and the same catalytic inactivating T/C transition, strongly suggest that they originated from an enzymatically active *TS* gene by a single mutation event. In the framework of currently accepted *T. cruzi* evolutionary history [38], this primal mutation most likely occurred early after the stemming of the TcII branch from the main *T. cruzi* clade, which is dated at 3–88 million years ago, depending on the analyzed genes [38]. The resultant *iTS* gene was strictly conserved in TcII strains and transmitted during relatively recent (~100,000 years ago) TcII recombination events that led to the emergence of hybrid TcV and TcVI lineages [38, 40]. As in the case of *TS* genes, distinct strains of *T. cruzi* DTU TcII, TcV and TcVI seem to have undergone differential expansion of the *iTS* dosage [40]. The absence of mutation accumulation on *iTS* genes from independently evolving strains strongly suggests that their diversification is under strong negative selection. Based on available data [41, 48–52], it is tempting to speculate that this selective pressure was driven by structural, and hence functional constraints (see below). In this framework, development, upholding and expression of iTS molecules may be envisaged as an additional, genomic-based trick devised to optimize TS pathogenic function. A schematic representation of the proposed evolution of *TS* genes in *T. cruzi* is presented in Fig. 1.

2.2- Biological features of SAPA repeats

The facts that eTS molecules do not bear SAPA repeats and, more importantly, that TS proteins devoid of SAPA conserved full enzymatic activity provided the first hint that this *C*-terminal extension was involved in non-catalytic activities [53, 54]. Because of its repetitive nature and the robust humoral responses that this sequence elicits early upon *T. cruzi* infection [32, 33], it was initially considered that SAPA operated as an antigenic decoy, drawing the attention of the immune system and thereby delaying and/or preventing the elicitation of protective responses. Indeed, the appearance of antibodies able to neutralize TS activity (TS-neutralizing antibodies, TS-NtAbs) is a much later event in the course of infection, which coincides with the decline in parasitemia at the end of the acute phase [33, 55, 56]. In contrast with short-lived anti-SAPA humoral responses, TS-NtAbs can be found in ~100% of chronically infected individuals [33, 55, 56], and even in treated patients that met the parasitological cure criterion (*i.e.* negative results in conventional serological and parasitological assays) [57]. This switch in anti-TS antibody specificity (from anti-SAPA to TS-NtAbs) allowed serologic discrimination of patients in the acute *vs.* chronic phases of the disease, and thereby the improvement of Chagas disease diagnostic applications [32, 57–59]. Moreover, the fact that TS-NtAbs are also widely detected in sera from *T. cruzi*-infected mammals support their epidemiologic value [44, 45].

Subsequent work from our labs unveiled a complex antigenic arrangement on TS, apparently engineered to skew humoral responses in the infected host. This antigenic structure is

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centered in SAPA, which bears multiple and partially overlapping B cell epitopes in each repeat [60], though it also relies on the presence of a mesh of SAPA cross-reacting B cell epitopes localized to the *N*-terminal region of TS [61, 62]. In this regard, it is most notable the case of the so-called epitope 1443 [62]. This epitope, restricted to gp85/*trans*-Sialidases Group I molecules, is flanked by two disulfide bond-forming Cys residues and thereby folds into an exposed and immunogenic loop that protrudes from the protein surface [34]. Antibodies to epitope 1443, however, do not neutralize TS activity. Moreover, mutant molecules lacking 1443 elicit an improved TS neutralizing response as compared to wild-type molecules, strongly suggesting that this epitope evolved to delay the elicitation of TS-NtAbs [62].

From a structural standpoint, the rigid, 'stalk-like' conformation of SAPA was proposed to assist TS activity on the trypomastigote surface by projecting the *N*-terminal catalytic region above the parasite glycocalix and thereby easing its access to SA donors and acceptors. Alternatively, the identification of high molecular mass TS species on the supernatant of cultured trypomastigote, which were sensitive to mild proteolysis, led to the hypothesis that SAPA repeats may promote enzyme multimerization [54]. This proposal, however, needs to be reevaluated in view of our recent studies on the biogenesis and physiology of the trypomastigote membrane [63, 64]. As shown, TS is not secreted as a GPI-less, 'soluble' protein upon the action of phospholipases on the trypomastigote membrane. On the contrary, this molecule is largely, if not exclusively, shed into the milieu as part of membrane-derived micro-vesicles [64, 65], which may account for the above mentioned high molecular mass TS species [54].

Interestingly, SAPA improves the pharmacokinetics of TS in the bloodstream [66–68], although this was shown using recombinant TS instead of native, micro-vesicle-associated molecules. Most importantly, prolonged persistence in the bloodstream is a likely determinant underlying the effective reach of immune organs or cells far from the infection site, and hence the pathogenic effects of TS (and by extension also of iTS, see below). The ability of SAPA to increase the bloodstream half-life of TS/iTS could be conveyed to other molecules of biotechnological interest and, most interestingly, it could be dissected from its strong immunogenicity [68].

Besides the antigenic and structural aspects, SAPA repeats, independently of TS/iTS activities, may also contribute to *T. cruzi* pathogenesis. As shown, SAPA interacts with endothelial cells or Peripheral Blood Mononuclear Cells (PBMCs), thereby inducing the secretion of the proinflammatory cytokine IL-6 [69]. The consequences in terms of the infection are not clear, but IL-6 is required for parasite specific response and host resistance to *T. cruzi* [70]; and may also facilitate endothelium activation and parasite extravasation, as shown for African trypanosomes [71]. On the other hand, SAPA was shown to stimulate immunoglobulin secretion by inducing B cell proliferation in a T cell independent manner, suggesting it may have a role in the polyclonal lymphocyte activation and hypergammaglobulinemia observed during the acute phase of *T. cruzi* infection [70, 72].

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Overall, SAPA repeats display a multiplicity of antigenic, structural and biological features (summarized in Fig. 2), which seem ideally suited to optimize the *in vivo* functioning of bloodstream trypomastigote *trans*-sialidases (TS and iTS).

3- Pathogenic effects of *T. cruzi* trypomastigote *trans*-Sialidases (TS and iTS)

Trypomastigote *trans*-Sialidases (TS and iTS) display a variety of pathogenic effects that lead to hematological disorders and to the dampening of the immune surveillance on the infected host (summarized in Fig. 2 and Table 1). Since these effects may be mediated by *trans*-sialylation/sialylation events (*i.e.* restricted to TS molecules) and/or by SAα2–3βGal*p* lectin activity (*i.e.* achieved by both TS and iTS molecules), they will be discussed separately in this section. It is worth mentioning that *T. cruzi* TS has also been proposed to moonlight as a parasite-derived neurotrophic factor, involved in the modulation of the balance between neuronal degeneration and regeneration [73]. However, and since these effects do not apparently involve TS catalytic and/or lectin activities, they will not be covered here.

3.1 TS activity-associated effects

In the absence of genotypic *TS*^{null} parasites, a key evidence supporting TS as a major *T. cruzi* virulence factor is provided by the fact that the amount of expressed (surface-associated and/or shed) TS activity correlates with the extent of pathogenesis induced by different strains in experimental infections [43]. In agreement, passive transfer of TS-NtAbs into *T. cruzi*-infected mice ameliorates histological alterations [74, 75] and thrombocytopenia [76].

Pathogenic effects of TS are mainly related to *trans*-sialylation events on the surface of distinct host cell types/tissues. However, some effects can be associated with desialylation events. For instance, bloodstream TS activity detected during the early steps of the infection is responsible for the reduced amount of SA on the surface of platelets and red blood cells, and hence for inducing thrombocytopenia and erythropenia in the acutely infected host [76, 77]. In this regard, it is worth noting that shedding of sialidase activity by bloodstream forms of African trypanosomes also correlates with erythrocyte desialylation and anemia in the infected host [16].

TS activity modifies the sialylation pattern of many cell types [74, 78, 79], being of particular relevance on the immune system. As shown, parasite-borne TS (or injection of the recombinant enzyme), induces several histologic abnormalities such as apoptosis in central and peripheral immune organs [78, 80, 81]. For instance, TS triggers apoptosis of immature $CD4^+CD8^+$ thymocytes inside the nurse cell complexes [74, 80]. This, together with the leakage of this cell population to the periphery leads to transient thymic aplasia, a signature of early *T. cruzi* infection [82]. On the other hand, in *in vitro* experiments, TS is able to co-stimulate the proliferation induced by Concanavalin-A lectin or anti-CD3 antibody in a mixed culture of T cell lymphocytes and Antigen Presenting Cells (APC). There are some controversies in the literature on this issue. Gao and Pereira described that this effect is

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independent of CD43 or CD40 molecules and mediated by Bruton's tyrosine kinase and IL-6 while Todeschini et al have reported that the CD43 pathway is clearly involved [83, 84]. Therefore, there is a lot of work ahead to understand the molecular pathways that govern the T cell activation by the TS. Moreover, TS may also alter the surface sialylation code of $CD8^+$ effector T cells, thereby impairing the cytotoxic response and favoring parasite persistence [85].

In addition to the actions on T cells it was recently described that TS induces the secretion of the pro-inflammatory cytokine IL-17 by B cells. At variance with T helper (Th) 17 cells, the molecular pathway that trigger the secretion of IL-17 in B cells involves the sialylation of the membrane glycoprotein CD45 and is independent of the transcription factors RORγt and Ahr [86]. In *T. cruzi*-infected mice, B cells are the major source of IL-17 at the early stage, and contributes to the control of the parasite, possibly through the upregulation of specific $CD8^+$ responses [86, 87]. Interestingly, a decline in IL-17 serum level has been recently proposed as a biomarker for assessing anti-*T. cruzi* therapeutic efficacy [88].

All the described pathogenic effects/immune alterations that depend on SA mobilization are observed during the acute phase of *T. cruzi* infection. They are associated to the TS activity shed into the bloodstream, which cannot be detected later on in the infection due to i) a dramatic drop in parasitemia and ii) the elicitation of TS-NtAbs [55, 57]. SA incorporation into the trypomastigote surface, on the contrary, is most likely mediated by 'local' TS, which may have been previously accumulated into the cytoplasm of the infected cell [21]. Thus, it seems to be two discrete pools of TS activity in the infected host: i) a systemic one, that is mainly relevant during the acute phase and that contributes to host colonization, pathogenesis and immune disorders, and ii) a locally-relevant one, which is mainly involved in trypomastigote surface coat sialylation and hence in parasite protection and infectivity (see next section). Based on our findings, it may be hypothesized that at variance with systemic TS activity, this latter pool is not majorly affected by NtAbs, probably due to the absence of edema at the sites of parasite replication.

3.2 Lectin activity-associated effects

Different studies using recombinant iTS molecules, showed that this isoform can recapitulate several of the alterations induced by TS in the host immune system physiology [50, 52, 84]. Therefore, these effects may be in principle attributed to the lectin activity towards SAα2–3βGal*p*-containing structures, which is common to iTS and TS molecules [74, 78].

Although it is known that the protective response against *T. cruzi* infection is mediated by the Th1 phenotype, eliciting an adequately balanced Th1/Th2 response is critical for host survival [89, 90]. Tipping off this balance towards a Th2 phenotype is conducted *via* the hijacking of the IL-10 pathway in APC, which translates into a decrease in IL-2 and IFNγ secretion with concomitant increment in IL-4 production by $CD4^+$ T cells [52]. It is important to note that iTS and TS are involved in the induction of IL-2 suppression *in vitro* and *in vivo*, a hallmark of the acute phase of *T. cruzi* infection [52]. In addition, *in vitro* experiments have shown that iTS and TS induce $CD4^+$ T cell co-stimulation, with concomitant secretion of IL-4 and, hence a Th2 bias [84]. The same authors have also shown

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that TS and/or iTS rescue $CD4^+$ T cells from programmed cell death via CD43 interaction [84]. These findings provide a context where a harnessing system of the potential extreme damage that an uncontrolled Th1 response might provoke is induced. In further support, the co-administration of iTS and TS to infected mice, showed that the presence of iTS reduce the pro-inflammatory capacity of TS [91]. In the same line, iTS administration to infected mice reduced the number of cells infiltrates in heart [91]. Indeed, it is worth mentioning that stimulation of the NF-kB pathway on endothelial cells by iTS induces the up-regulation of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1, with a concomitant increase in parasite infectivity [92].

Due to their extreme similarity to TS molecules and the co-occurrence of iTS and TS on the trypomastigote surface, the precise sub-cellular distribution and biological meaning of iTS molecules is a quite difficult task to assess. However, recent studies using parasite lines expressing iTS (developed upon natural *iTS*^{null} parental background) indicate that iTS molecules are effectively displayed on the trypomastigote surface [50], and that their SAα2–3βGal*p* lectin activity play significant roles in the physiologic scenario of parasite infection. As shown, *iTS*-transfected parasites display exacerbated adhesion/invasion to non-phagocytic cells *in vitro* and increased virulence *in vivo* [50]. Altogether, these findings strongly support that iTS proteins exert alternative and/or complementary roles to TS in *T. cruzi* virulence and pathogenesis.

4.- TS and the parasite sialoglycophenotype

Establishment of specific glycointeractions with the host are needed throughout the *T. cruzi* life-cycle, including for those parasite forms dwelling in the insect (epimastigotes and metacyclic trypomastigotes). For instance, glycointeractions with endothelia of the triatomine digestive tract is critical for the survival and proliferation of epimastigotes [93]. In this phase of the life-cycle, however, the parasite relies on non-sialylated rather than sialylated surface glycoconjugates to engage in such contacts [93–95]. This is consistent with the fact that epimastigote mucins (known as Gp35/50) are poorly sialylated, which may be attributed to the low concentration of suitable SA donors in the gut lumen of triatomines [96], and to the very low expression of TS activity in this stage [35].

Differentiation into infective metacyclic trypomastigotes, correlates with an increase in TS expression [35]. This leads to massive sialylation of metacyclic surface Gp35/50 mucins, which contribute to the first steps of infection of the mammal host, particularly in cases of oral transmission [97]. Once in the mammal, Gp35/50 may also play supporting roles in the recognition and/or signaling of the target cell. Interestingly, these effects are dependent on the parasite genotype and, at least for some parasite strains, modulated by the sialylation status of Gp35/50 [98].

The scaffolding polypeptides and the *O*-glycans from bloodstream trypomastigote mucins (known as tGPI-mucins) are different than those of Gp35/50 [18, 99]. Of note, tGPI-mucins bear non-reducing, terminal αGalactopyranosyl (αGal*p*) residues, which are not SA acceptors in the TS reaction [100]. These αGal*p*-containing structures are foreign to humans and therefore, highly immunogenic during *T. cruzi* infections [100]. Anti-αGal antibodies

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have the capability to lyse bloodstream trypomastigotes in a complement-dependent or independent manner, strongly suggesting that they are involved in controlling parasitemia [19]. As mentioned, incorporation of SA residues onto tGPI-mucins by means of TS occurs immediately upon trypomastigote egress from the infected cell, and is critical to prevent parasite lysis by yet undefined mechanisms. On one hand, it may be considered that the negative charges in SA residues somehow stabilize the surface coat. Experiments showing that neutralization of negative charges by addition of $MgCl_2$ reverses the protective effect of sialylation support this hypothesis [19]. Alternatively, it may be proposed that sialylation of nearby βGal*p* residues imposes steric hindrance to the binding of anti-αGal antibodies and/or to the subsequent capping of tGPI-mucins on the trypomastigote surface. This idea is however not supported by recent data showing that tGPI-mucins bearing terminal αGal*p* and βGal*p* residues are sorted to mutually exclusive membrane domains on the trypomastigote surface [64, 65]. Whatever the case, sialylation of tGPI-mucins protects trypomastigotes from lytic antibodies and, most likely, also from the action of complement [21].

In addition to their protective effects, formation of SAα2–3βGal*p* glycomarkers on the parasite surface was shown to be involved in trypomastigote recognition and invasion of host non-phagocytic cells [101]. Other groups, however, described a minor effect for the trypomastigote sialoglycophenotype on target cell recognition [102, 103]. Whether these differences are related to the experimental set-up and/or the genetic background of the parasite strains analyzed remains to be addressed. More recently, *in vitro* studies indicated that both the SAα2–3βGal*p* lectin activity of TS/iTS molecules and/or the generation of such sialoglycomarkers on the target cell surface by parasite-anchored TS contribute to trypomastigote internalization [50, 104]. Pioneer studies described that following invasion, TS-mediated mobilization of SA residues from host cell sialoglycoconjugates (and particularly from LAMP 2) was critical for the timely escape of trypomastigotes from the parasitophorous vacuole, a critical step in the progression of parasite intracellular development [21]. These findings were recently corroborated using genetically modified cell lines and/or parasites, as well as elegant co-infection models [105, 106]. Upon differentiation into replicative amastigotes, the parasite shuts down TS expression, which is compatible with its intracellular life style and hence, absence of suitable SA donors. Moreover, these parasite forms are completely refractory to TS activity as they do not express appropriate SA acceptors on their surface [107]. Intriguingly, amastigotes do express mucin-like products [108, 109], and bear huge amounts of αGal epitopes on their surface [19].

On a final note, it is worth noting that tGPI-mucins may also contribute to *T. cruzi* pathogenesis. Vesicles containing tGPI-mucin-positive are shed profusely by trypomastigotes and, independently of antibody opsonization, they are internalized by epithelial cells and macrophages. This, in turn, triggers pro-inflammatory responses and enhances parasite virulence by yet undefined mechanisms [110]. Most interestingly, tGPI-mucins were shown to bind to specific Siglecs (SA-binding Ig-like lectins), and subsequently modulate their downstream functions in the innate and/or adaptive host immune response [111–115]. Since Siglec manipulation is strictly dependent on the sialylation status of tGPI-mucins [114, 115], this phenomenon may be therefore considered

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as an indirect TS effect on the host. Table 1 summarizes the molecular/cellular targets of TS (both on the parasite and the insect/mammal host), and their associated effects.

5. Conclusions

Cleavage and release of SA residues from host glycoconjugates by classical sialidases is a widely used pathogen strategy with crucial roles in nutrient acquisition and pathogenesis. Decoration of microbial surface glycoconjugates with SA, on the other hand, usually results in a dampening of the host immune response. Therefore, development of a molecule with *trans*-sialidase activity should be considered a major achievement in the evolution of trypanosomes, as it allowed these parasites to couple SA scavenging activity with the formation of protective and/or adhesive structures on their own surface. As discussed here, TS plays multiple key roles on *T. cruzi* basic biology and virulence (summarized in Box 1). On one hand, TS enables SA incorporation on the parasite surface, which is critical for the insect-to-mammal host switching, a major bottleneck in the *T. cruzi* life cycle. Most importantly, once in the mammalian host, parasite multiplication and persistence in tissues is strictly dependent on the timely sialylation of its surface glycoconjugates. On the other hand, the controlled shedding of this molecule into the bloodstream provides the trypomastigotes with the opportunity to manipulate the surface sialylation pattern, and hence the signaling/functional properties, of different target cells. Different genomic and molecular adaptations such as a repetitive extension (SAPA) that improves protein pharmacokinetics while, at the same time, delays the elicitation of neutralizing responses in the infected mammal and/or the co-expression of structurally conserved though enzymatically inactive molecules (iTS) seem ideally suited to optimize TS pathogenic function *in vivo*. Though still fragmentary, collective evidence from *in vitro* and *in vivo* studies indicate that changes in the host sialoglycophenotype strictly correlate with hematological alterations, immune dysfunction and susceptibility to *T. cruzi* infection. Moreover, certain pathological alterations associated to acute Chagas disease such as thrombocytopenia, polyclonal B cell activation and thymus atrophy may be directly associated to the effect of secreted TS.

Development of novel genetic, molecular and cellular tools aimed at deepening our knowledge of these multitasking molecules and of their targets/effects during *T. cruzi* infections will provide a significant impact in the parasitology/glycobiology/immunology areas. As a first step towards filling this gap, we developed a method allowing the high throughput identification of TS targets *in vivo*. This method is based on the use of a synthetic 'SA donor' analog, *i.e.* a SA residue with an azide group on its carbon 5, which is α2–3-linked to a ßGal*p* group [107]. When added exogenously, such compound is recognized as a suitable SA donor by *T. cruzi* TS, hence leading to the formation of a Neu5Azα2–3Galβ signature on SA acceptor glycoconjugates. So far, this method allowed the identification of TS targets on the surface of different cell types [79], as well as the undertaking of structural/functional studies on *T. cruzi* membrane composition and dynamics [29, 64, 116–119]. This method, together with other tools able to shed new structural/functional light into TS biology will pave the way to the identification of novel and appealing targets for intervention in Chagas disease

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BOX 1:

Experimental evidence supporting trypomastigote *trans*-Sialidases (TS and/or iTS) as major virulence factors during *Trypanosoma cruzi* infections.

- There is a strict correlation between strain virulence and TS expression
- TS/iTS are involved in the adhesion/invasion of the target cells
- TS is involved in trypomastigote protection from immune mechanisms and in the escape from the parasitophorous vacuole into the infected cell cytoplasm, where it differentiates and replicates.
- TS/iTS (and also SAPA repeats) induce major alterations in the host immune system.
- Administration of recombinant TS into naïve animals recapitulates several physiopathologies associated to acute *T. cruzi* infection such as thrombocytopenia and alterations of the histoarchitecture of the spleen, thymus and ganglia.
- Appearance of TS-neutralizing antibodies (TS-NtAbs) in *T. cruzi* infected mammals correlate with a decrease in parasitemia; and passive transfer of TS-NtAbs into mice ameliorates histological findings upon challenge.
- Ectopic-expression of TS/iTS molecules enhances parasite virulence.

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Highlights

- *T. cruzi* strain virulence correlates with *trans*-sialidase (TS) expression
- TS is involved in *T. cruzi* adhesion/invasion of mammalian target cells
- TS induces major alterations in mammalian host immune system
- In naïve animals, TS recapitulates physiopathologies induced during infection
- TS-neutralizing antibodies ameliorate histological damages upon *T. cruzi* infection

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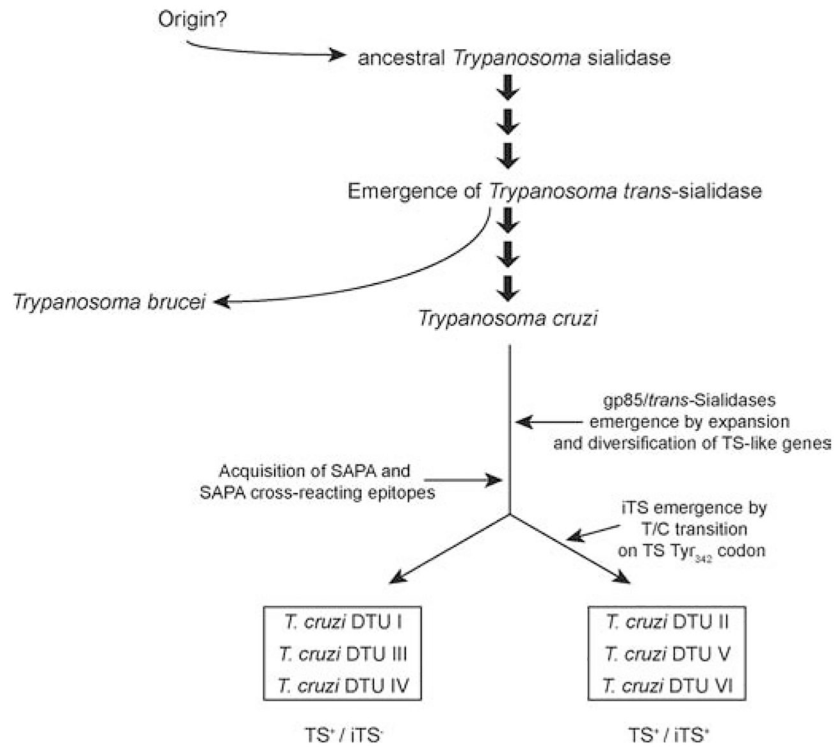

FIGURE 1:
Possible evolution of *trans*-Sialidase genes in *T. cruzi*.

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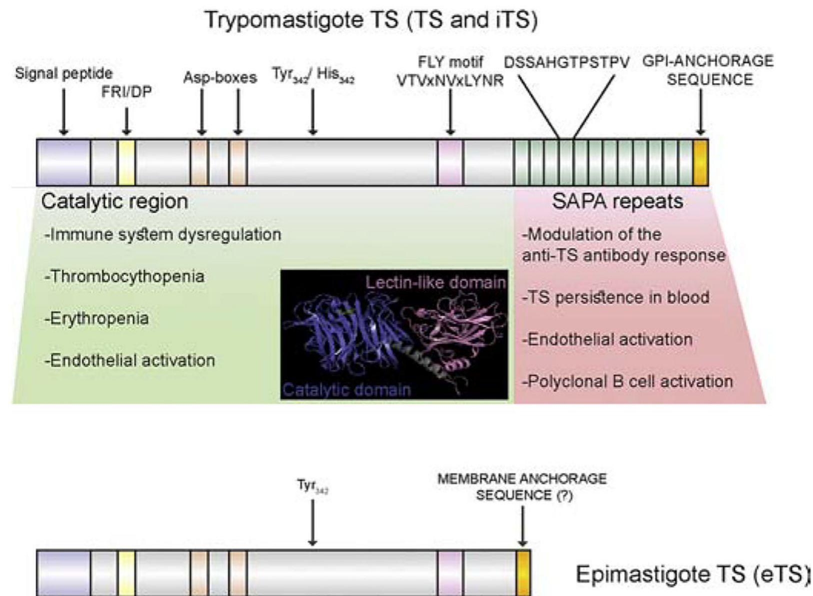

FIGURE 2: Structural and biological features of molecules belonging to the Group I of *T. cruzi* Gp85/*trans*-Sialidases.

Schematic representation of predicted TS molecules expressed by trypomastigote (TS and iTS, above) and epimastigote (eTS, below) forms of *T. cruzi*. For trypomastigote TS, identity of residue 342 discriminates between enzymatically active TS (Tyr) and inactive iTS (His) molecules. The position of predicted signal peptide, membrane-anchoring sequence, and typical bacterial sialidase motifs (Asp-boxes, FR[I/D]P tetrapeptide) is indicated. The sequences of the adhesive FLY motif (VTVxNVxLYNR, where x means any amino acid) and of a typical SAPA repeat (DSSAHGTPSTPV) are also shown. (?) means that the nature of the eTS membrane anchor has not been yet experimentally addressed. For trypomastigote TS molecules, the major biological effects of the *N*-terminal, catalytic region and of the SAPA repeats are indicated below. The structure of the catalytic region of TS, showing the presence of two independent domains (the catalytic one and a lectin-like one) connected by a α-helix is shown in the inset. GPI, glycosylphosphatidylinositol.

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Table 1:

Sialoglycophenotypes associated to TS activity along the *T. cruzi* life-cycle.

Developmental Stage	TS expression	Targets	On Parasite: Phenotype	On Host: Targets in host	On Host: Phenotype	References
Epimastigote	−/+ (eTS)		• Parasite protection?	?	?	[93, 95]
Metacyclic Trypomastigotes	++	Gp35/50 mucins	• Parasite Protection • Improved infectivity	Lamp 2 (on non-phagocytic cells)[1]	• Parasitophorous vacuole loosening	[21, 97, 98, 106]
				Lamp 2 (on non-phagocytic cells)[1]	• Parasitophorous vacuole loosening	[19, 21, 101][104]
Trypomastigote	+++	tGPI-mucins	• Protection against immune mechanisms • Improved infectivity	• $CD4^+CD8^+$ Thymocytes[2] • $CD4^+$T-cell[2] • $CD8^+$T-cell[2] • B-cells[2] • Platelets[2] • Erythrocytes[2] • Endothelial cells[2]	• Immune system modulation • Hematological disorders	[43, 50, 52, 74–78, 80, 81, 84–87,91,92]
Amastigote	-	-	-	-	-	

[1] Molecular target of parasite-associated TS activity.

[2] Cellular targets of secreted TS activity.