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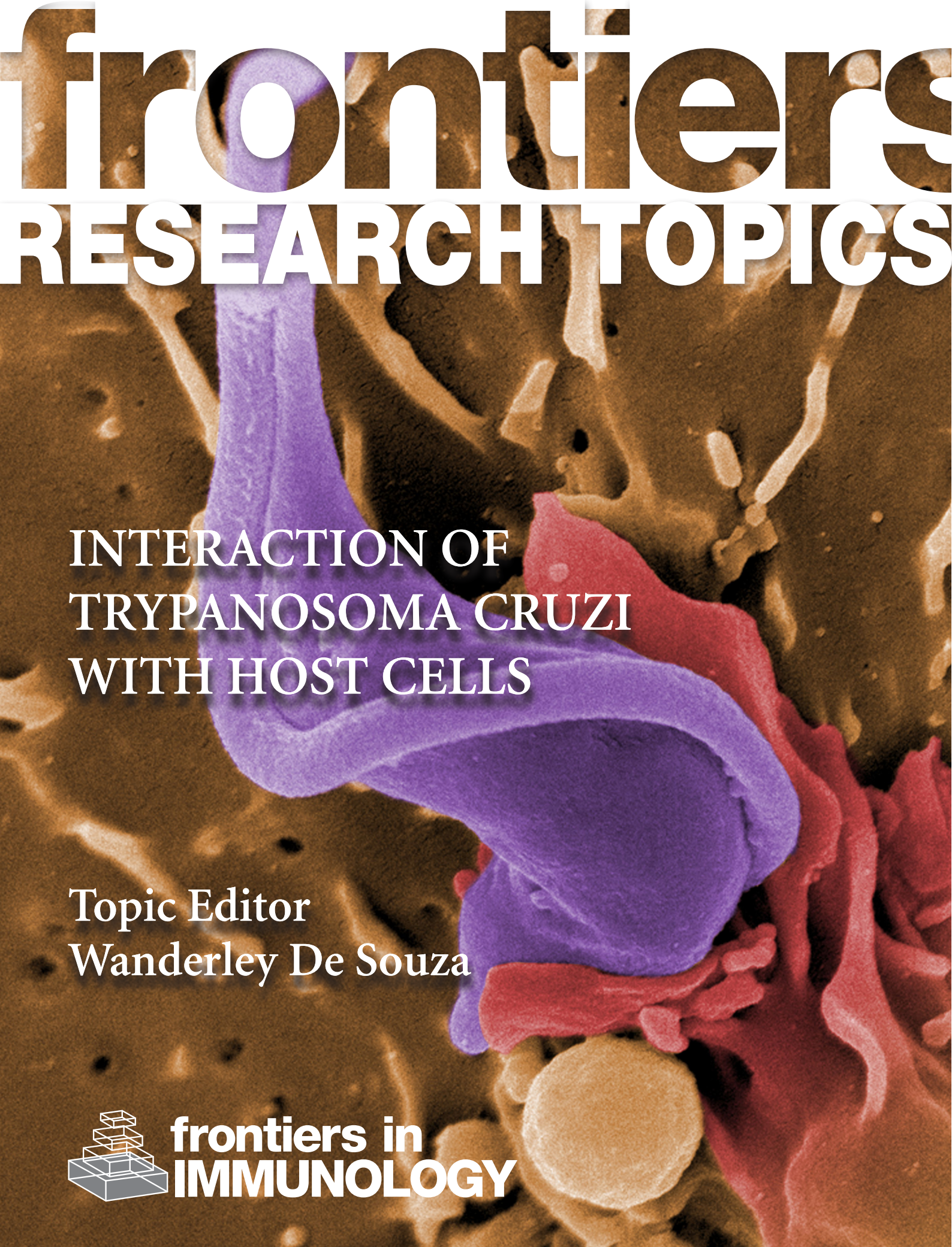

INTERACTION OF TRYPANOSOMA CRUZI WITH HOST CELLS

Topic Editor
Wanderley De Souza

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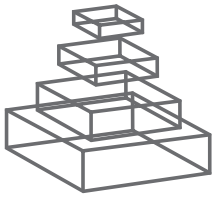

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INTERACTION OF TRYPANOSOMA CRUZI WITH HOST CELLS

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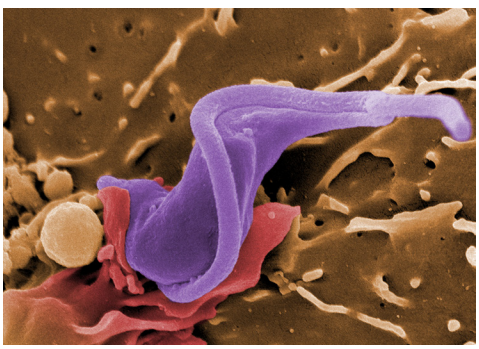

Field emission scanning electron microscopy of an interaction between T. cruzi trypomastigotes and epithelial cells (LLC-MK 2) treated with an macropinocytosis inhibitor. After two hours of interaction a trypomastigote (purple) shows a small portion of itsr body recovered by the macrophage plasma membrane loosely (red region), indicating that the blockage of macropinocytic activity did not impair the membrane extension (Copyright: Emile Barrias, Técia Ulisses de Carvalho and Wanderley de Souza).

Trypanosoma cruzi is a pathogenic protozoan of the Trypanosomatidade Family, which is the etiological agent of Chagas' disease. Chagas' disease stands out for being endemic among countries in Latin America, affecting about 15 million people. Recently, Chagas has become remarkable in European countries as well due to cases of transmission via infected blood transfusion. An important factor that has exacerbated the epidemiological picture in Brazil, Colombia and Venezuela is infection after the oral intake of contaminated foods such as sugar cane, açai and bacaba juices. Trypanosoma cruzi is an intracellular protozoan that exhibits a complex life cycle, involving multiple developmental stages found in both vertebrate and invertebrate hosts. In vertebrate hosts, the trypomastigote form invades a large variety of nucleated cells using multiple mechanisms. The invasion process involves several steps: (a) attraction of the protozoan to interact with the host cell surface; (b) parasite-host cell recognition; (c) adhesion of the parasite to the host cell surface; (d) cell signalling events that culminate in the internalization of the parasite through endocytic processes; (e) biogenesis of a large vacuole where the parasite is initially located, and is also known as parasitophorous vacuole (PV); (f) participation of endocytic pathway components in the internalization process; (g) participation of cytoskeleton components in the internalization process; (h) transformation of the trypomastigote into the amastigote form within the PV; (i) lysis of the membrane of the PV; (j) multiplication of amastigotes within the host cell in direct contact with cell structures and organelles; (k) transformation of amastigotes into trypomastigotes, and (l) rupture of the host cell releasing trypomastigotes into the extracellular space. The kinetics of the interaction process and even the fate of the parasite within the cell vary according to the nature of the host cell and its state of immunological activation.

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EDITORIAL
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Trypanosoma cruzi–host cell interaction

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Keywords: parasitic protozoa, parasite-host cell interaction, cell-to-cell recognition, *Trypanosoma cruzi*, Chagas Disease

Chagas disease was discovered by Carlos Chagas in Brazil in 1909 (1). It is caused by the pathogenic protozoan *Trypanosoma cruzi*, member of Trypanosomatidae family, Kinetoplastida order. Chagas disease is recognized by the World Health Organization as one of the main neglected tropical diseases, affecting about 8–15 million individuals in 18 countries in Central and South America, where it is endemic, as well as countries in North America and Europe (2). At least 30 million people are at risk. Public health programs have significantly reduced transmission of Chagas disease, however, blood and organ transplant transmission in non-endemic countries and a growing number of food borne (especially fruit juices) infections still require special attention. In addition, an increase in the rate of infection in the Amazon region has become a challenge for the control of Chagas disease (3).

Trypanosoma cruzi presents a complex life cycle both in the vertebrate and invertebrate hosts, involving dramatic changes in cell shape (4). Its life cycle involves several developmental stages that are known as amastigotes, epimastigotes, and trypomastigotes. The first two stages are able to divide inside and outside host cells, respectively. The trypomastigote and amastigote stages are also able to infect host cells where they multiply, amplifying the number of parasites, and releasing millions of the infective trypomastigote forms in the intercellular spaces.

This thematic issue deals with the ability of *T. cruzi* to penetrate into host cells. In the first article, de Souza and de Carvalho (5) make a review of the concept of active penetration and suggest that *T. cruzi* always penetrates the host cell through an endocytic process with the formation of a transient parasitophorous vacuole. The second article by Barrias et al. (6) reviews the various mechanisms of endocytosis, which are used by the protozoan to gain access to the intracellular portion of the host cells. These include processes such as classical phagocytosis, participation of membrane rafts, macropinocytosis, and clathrin-mediated endocytosis. In the third article, Calvet et al. (7) analyze in detail the process of interaction of *T. cruzi* with cardiomyocytes, an important host cell, because *in vivo* many of the parasite strains have a tropism for the heart. In the fourth article, Tonelli et al. (8) call the attention to the fact that most probably a large number of molecules are involved in the process of protozoan–host cell interaction and discuss the use of more powerful technologies, such as peptide-based phage display and oligonucleotide-based aptamer techniques. Using these approaches, the results obtained by the group highlight the importance of members of the 85-kDa family on the process of interaction. In the fifth article, Freire-de-Lima et al. (9) point out the relevance of a unique system of sialoglycoproteins and sialyl-binding proteins, which in the case of *T. cruzi* are represented by trans-sialidases. These proteins are involved in parasite–host cell recognition, infectivity, and survival. The sixth and seventh articles by Nde et al. (10) and Ferreira et al. (11) respectively, analyze the role played by components of the extracellular matrix during the interaction of the trypomastigote and amastigote forms of *T. cruzi* with the host cells. Infective trypomastigotes up-regulate the expression of laminin-gamma^{-1} and thrombospondin to facilitate recruitment of parasites to enhance cell infection. The extracellular matrix interactome network seems to be regulated by *T. cruzi* and its gp 83 ligand. The eighth article by Maeda et al. (12) reviews the cell signaling process that takes place during the interaction of metacyclic trypomastigotes, infective forms found in the invertebrate host, with host cells. Special emphasis is given to intracellular calcium mobilization and the triggering the exocytosis of host cell lysosomes during the interaction process mediated by a surface-expressed parasite glycoprotein of 82 kDa. This process leads to the activation of mammalian target of rapamycin (mTor), phosphatidylinositol 3-kinase, and protein kinase C. The last article, by Scharfstein et al. (13) initially analyses the process by which *T. cruzi* trypomastigotes elicit an inflammatory edema that stimulates protective type-1 effector cells through the activation of the kallikrein–kinin system, providing a framework to investigate the intertwined proteolytic circuits that couple the anti-parasite immunity to inflammation and fibrosis.

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Active penetration of *Trypanosoma cruzi* into host cells: historical considerations and current concepts

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In the present short review, we analyze past experiments that addressed the interactions of intracellular pathogenic protozoa (*Trypanosoma cruzi*, *Toxoplasma gondii*, and *Plasmodium*) with host cells and the initial use of the term active penetration to indicate that a protozoan "crossed the host cell membrane, penetrating into the cytoplasm." However, the subsequent use of transmission electron microscopy showed that, for all of the protozoans and cell types examined, endocytosis, classically defined as involving the formation of a membrane-bound vacuole, took place during the interaction process. As a consequence, the recently penetrated parasites are always within a vacuole, designated the parasitophorous vacuole (PV).

Keywords: *Trypanosoma cruzi*, endocytosis, active penetration, phagocytosis, Chagas disease

INTRODUCTION AND EARLY STUDIES

Investigation of the interactions of *Trypanosoma cruzi* with host cells became possible after techniques to cultivate this protozoan in tissue culture were developed. The first approach was described by Kofoid et al. (1935), who showed that the protozoan could survive and multiply in cultures of heart cells from mouse and rat embryos. A subsequent study by Romaña and Meyer (1942) using chick embryo heart cell cultures described the behavior of *T. cruzi* in tissue cultures in detail. Theirs *in vitro* observations of the interaction process in both living and fixed cultures led the authors to describe two mechanisms of cell infection by the protozoan: active penetration and phagocytosis. In this classic paper, it was stated that "in general, the active penetration was more visible with metacyclic forms that, with their great motility, easily crossed the cell surface, penetrating into the protoplasm of fibroblasts and myocytes." To the best of our knowledge, this is the first reference to the active penetration of parasitic protozoans into cells. Six years later, Meyer and Xavier de Oliveira (1948) published a paper and a small book confirming the initial observations and reported that the parasite "can touch the surface of the host cells without penetration. Occasionally, they adhere to the cell surface and suddenly penetrate into the cell." These observations were more clearly demonstrated in a classic film by H. Meyer and A. Barasa. Part of that film which is kept in our laboratory (founded in 1940 by Hertha Meyer) is available at the site of the Brazilian Society of Protozoology (www.sbpz.org.br). The same basic idea initially proposed by Meyer and colleagues was presented in 1973 by Dvorak and Hyde (Dvorak and Hyde, 1973; Hyde and Dvorak, 1973) based on observations of the interaction of *T. cruzi* trypomastigotes of the Ernestina strain with secondary bovine embryo skeletal muscle cells (BESM) and HeLa cells obtained through phase contrast microscopy under controlled conditions. The process of cell invasion was described as an active penetration process in which mechanical activity of the protozoan is prevalent, and the parasites penetrate into the cell through the plasma membrane. According to these studies, the parasites enter cells posterior end first.

It is important to note, although it is not the focus of the present review, that the same basic idea of active penetration has also been applied to other protozoans, such as *Toxoplasma gondii* and *Plasmodium*, which were again analyzed first by Nery Guimarães and Meyer (1942) and Dvorak et al. (1975).

SECOND PHASE

One of the authors of this review (WS) joined the Hertha Meyer's laboratory in 1969 and began the first studies addressing the interaction of *T. cruzi* with chick embryo heart muscle cells and macrophages using electron microscopy. Jim Dvorak visited the laboratory several times between 1972 and 1980, and intense discussions about the concept of active penetration took place at that time. With enthusiasm, Jim defended the idea that *T. cruzi* and *Plasmodium* are able to generate a transient tunnel-like structure in the host cell plasma membrane that is sealed immediately after parasite internalization, and the parasite then establishes intimate contact with the host cell cytoplasm. Up to this point, electron microscopy had not been used to analyze the parasitic protozoan–host cell interaction process. However, a few images obtained in our laboratory showed that recently penetrated trypomastigotes were not in contact with the myofibers of heart muscle cells but were instead located within a membrane-bound vacuole. These observations were made approximately in 1972

when Nadia Nogueira was being trained to work with *T. cruzi* in Hertha Meyer's lab. Subsequently, she moved to Rockefeller University in New York, where she started a project in Zanvil Cohn's laboratory analyzing the interaction of *T. cruzi* with several different cell types, including mouse peritoneal macrophages, HeLa cells, L cells, and calf embryo fibroblasts. During a visit to Dr. Cohn's lab, one of the authors of the present paper (WS) had the opportunity to discuss these electron microscopy images with these investigators, which were subsequently published in a classic paper in 1976 (Nogueira and Cohn, 1976). The images showed clearly that all internalized parasites were initially located in a vacuole, designated the parasitophorous vacuole (PV) following the suggestion of William Trager, who described a vacuole akin to that in Nogueira and Cohn's images in erythrocytes infected by *Plasmodium* (Langreth and Trager, 1973). Similar observations were made in *T. gondii* by Jones and Hirsch (1972). Since that time, it has become clear that in all situations examined to date, *T. cruzi* and certain other intracellular protozoan always penetrate the host cell in a process that is better characterized as an endocytic process, involving the initial formation of aPV. This process takes place independent of the nature of the host cell. Even so-called non-professional phagocytic cells can be penetrated by *T. cruzi* and *T. gondii*. The term induced endocytosis or even induced phagocytosis has been used to refer to a process in which the parasite is able to stimulate endocytic activity in the future host cell.

According to Nogueira and Cohn in the case of *T. cruzi*, subsequent to penetration, the parasites leave the PV in a process they described as "escaping" and then enter into direct contact with the host cell cytoplasm. However, a few years later, it was shown (Carvalho and de Souza, 1989) that there is no escaping, but rather, fragmentation of the PV membrane occurs, most likely due to the activity of enzymes secreted by the parasite, as was subsequently shown by Andrews et al. (1990), associated with the complete disappearance of the PV. Taken together, these observations clearly indicate that an endocytic process is always involved during the process of *T. cruzi* internalization into a host cell. The same basic idea can be extended to other intracellular parasitic protozoa.

A NEW CONCEPT OF "ACTIVE PENETRATION"

We had the opportunity to discuss the data described above as well as the results obtained by several groups in *Plasmodium* and *Toxoplasma* with Hertha Meyer, James Dvorak, Nadia Nogueira, and Zanvil Cohn, and we reached the conclusion that active penetration, as initially defined, does not exist during the process of parasitic protozoa interacting with host cells. A possible exception is observed during infection of host cells by microsporidians, which include a large number of species initially considered to be protozoans but that have more recently been considered to be fungi based on the presence of a large number of genes that, upon phylogenetic analysis, cluster Microsporida with Fungi (see review by Xu and Weiss, 2005). These organisms present a complex life cycle, and their spores contain a structure known as the polar tube. When one of these organisms attaches to the surface of a host cell, signals activate a process that leads to evagination of a structure known as the polar tube, which then penetrates into the host cell, establishing a type of bridge between the cytoplasm of the spore and the cytoplasm of the host cell. The sporoplasm, which may contain one or two nuclei, then flows through the connecting tube and is transferred to the host cell cytoplasm, where it will develop.

After some discussion, it was concluded that the term active penetration could be used to indicate the fact that the parasite plays an important role in the "induction of the host cell invasion process." Indeed, since the first description of the process, it has been clear that the intense movement of the protozoan, especially due to the flagellar beating process, plays some role. This idea was further analyzed by Schenkman et al. (1991), who showed that maintenance of an active energetic metabolism is fundamental for *T. cruzi* to invade cells, as this process is prevented by treatment of the parasites with 2-deoxy-glucose, an inhibitor of glycolysis, as well as sodium azide, antimycin, and oligomycin, which interfere with the mitochondrial metabolism involved in the synthesis of ATP.

The term active penetration has also been employed from another perspective. Kipnis et al. (1979), for example, used it to describe the penetration of bloodstream trypomastigotes into macrophages in a process that was only partially inhibited by cytochalasin B. At present, we know that this compound does not inhibit all forms of endocytosis.

Therefore, we can conclude that the available data, especially those obtained through transmission electron microscopy of thin sections, clearly show that recently penetrated *T. cruzi* of both infective and non-infective forms are always located within a PV that interacts with the organelles of the endosomal–lysosomal system during its short existence. This phenomenon occurs in all the cell types examined to date independent of whether they are professional or non-professional phagocytic cells. The formation of the PV involves the induction of a calcium flux into the host cell via the action of a parasite-derived calcium agonist, which is generated through the action of a parasitic oligopeptidase (Caler et al., 1998), as well as the synaptotagmin VII pathway (Caler et al., 2001), the recruitment of lysosomes (Tardieux et al., 1992), and the participation of microtubules (Tyler et al., 2005) and actin filaments (Rosestolato et al., 2002). Recently, Fernandes et al. (2011) showed that *T cruzi* trypomastigotes mimic the process of wound repair with Ca^{2+}-dependent exocytosis of lysosomes by delivering acid sphingomyelinase to the host plasma membrane, facilitating parasite entry into host cells. These aspects of the invasion process have been extensively reviewed in recent years (Hall, 1993; Burleigh and Andrews, 1995; Yoshida, 2006; Alves and Colli, 2007; de Souza et al., 2010; Caradonna and Burleigh, 2011; Butler and Tyler, 2012; Fernandes and Andrews, 2012; Romano et al., 2012).

ENDOCYTOSIS IS A COMPLEX BIOLOGICAL PROCESS

Our present knowledge of the endocytic process shows that it is more complex than previously thought. Indeed, in addition to the classical phagocytic process, there are several ways a cell can ingest extracellular material of variable dimensions. These mechanisms can be either dependent on dynamin, such as the clathrin- and caveolin-mediated processes, or independent of dynamin, as occurs during processes including macropinocytosis, and lipid

raft-mediated endocytosis. It is likely that other mechanisms will be described in view of the large number of groups attempting to better characterize the endocytic process. As described in another review in this volume (Barrias et al., submitted), *T. cruzi* may use all of these mechanisms to enter host cells. It is possible that the parasite selects the mechanism to be used based on factors such as the nature of the cell and the host cell surface ligand to which binds. Once the parasite binds to and is recognized by the host cell surface it triggers a process that is better described as an induced endocytosis.

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Trypanosoma cruzi: entry into mammalian host cells and parasitophorous vacuole formation

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Trypanosoma cruzi, the causative agent of Chagas disease, is transmitted to vertebrate hosts by blood-sucking insects. This protozoan is an obligate intracellular parasite. The infective forms of the parasite are the metacyclic trypomastigotes, amastigotes, and bloodstream trypomastigotes. The recognition between the parasite and mammalian host cell, involves numerous molecules present in both cell types, and similar to several intracellular pathogens, *T. cruzi* is internalized by host cells via multiple endocytic pathways. Morphological studies demonstrated that after the interaction of the infective forms of *T. cruzi* with phagocytic or non-phagocytic cell types, plasma membrane (PM) protrusions can form, showing similarity with those observed during canonical phagocytosis or macropinocytic events. Additionally, several molecules known to be molecular markers of membrane rafts, macropinocytosis, and phagocytosis have been demonstrated to be present at the invasion site. These events may or may not depend on the host cell lysosomes and cytoskeleton. In addition, after penetration, components of the host endosomal-lysosomal system, such as early endosomes, late endosomes, and lysosomes, participate in the formation of the nascent parasitophorous vacuole (PV). Dynamin, a molecule involved in vesicle formation, has been shown to be involved in the PV release from the host cell PM. This review focuses on the multiple pathways that *T. cruzi* can use to enter the host cells until complete PV formation. We will describe different endocytic processes, such as phagocytosis, macropinocytosis, and endocytosis using membrane microdomains and clathrin-dependent endocytosis and show results that are consistent with their use by this smart parasite. We will also discuss others mechanisms that have been described, such as active penetration and the process that takes advantage of cell membrane wound repair.

Keywords: *Trypanosoma cruzi*, mammalian cell, endocytosis, phagocytosis, active penetration, host cell, interaction

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas disease, is an obligatory intracellular parasite that belongs to the Kinetoplastida order, and it is recognized by the WHO as one of the world's 13 neglected tropical diseases, affecting 16 million people in Latin America. After the initial infection by the parasite, some patients can develop acute signs and symptoms, including fever, hepatosplenomegaly, and inflammatory reactions. These acute symptoms can be spontaneously resolved. However, the majority of patients are asymptomatic. After the acute phase, a symptomatic chronic form can develop 10–20 years after the initial infection, causing irreversible damage to the heart, esophagus, and colon, with severe disorders of nerve conduction in these organs. Therefore, Chagas disease is characterized as a chronic, systemic, and endemic disease affecting approximately 16 million in Latin America (1) and is considered the major parasitic disease burden of the American continent (2). This parasite presents a complex life cycle that occurs in both vertebrate and invertebrate hosts, where three major developmental stages are observed: epimastigotes, trypomastigotes, and amastigotes. The infective forms of *T. cruzi* (amastigotes and trypomastigotes) are able to infect a wide range of nucleated mammalian cells. The intracellular cycle can be divided into several steps and begins when the infective forms attach and are recognized by the host's cell surface (3). Then, cell signaling processes lead to the internalization of the parasite in a process that involves the formation of an endocytic vacuole known as the PV. This review will focus on several processes that have been shown to be involved in the internalization of *T. cruzi*, such as phagocytosis, active entry, endocytosis dependent on membrane microdomains (flotillin- and caveolin-dependent), endocytosis mediated by clathrin and macropinocytosis (**Figure 1**).

RECOGNITION BETWEEN *TRYPANOSOMA CRUZI* AND THE MAMMALIAN HOST CELL: A MECHANISM DEPENDENT ON RECEPTORS AND LIGANDS

Classically, the interaction between host cells and *T. cruzi* has been divided into two different steps: adhesion (which includes recognition and signaling) and internalization (3). The internalization process is described as occurring through several pathways that resemble endocytic mechanisms. These two steps are easily distinguished because interactions performed at 4°C do not allow parasite internalization and the parasites remain attached to the

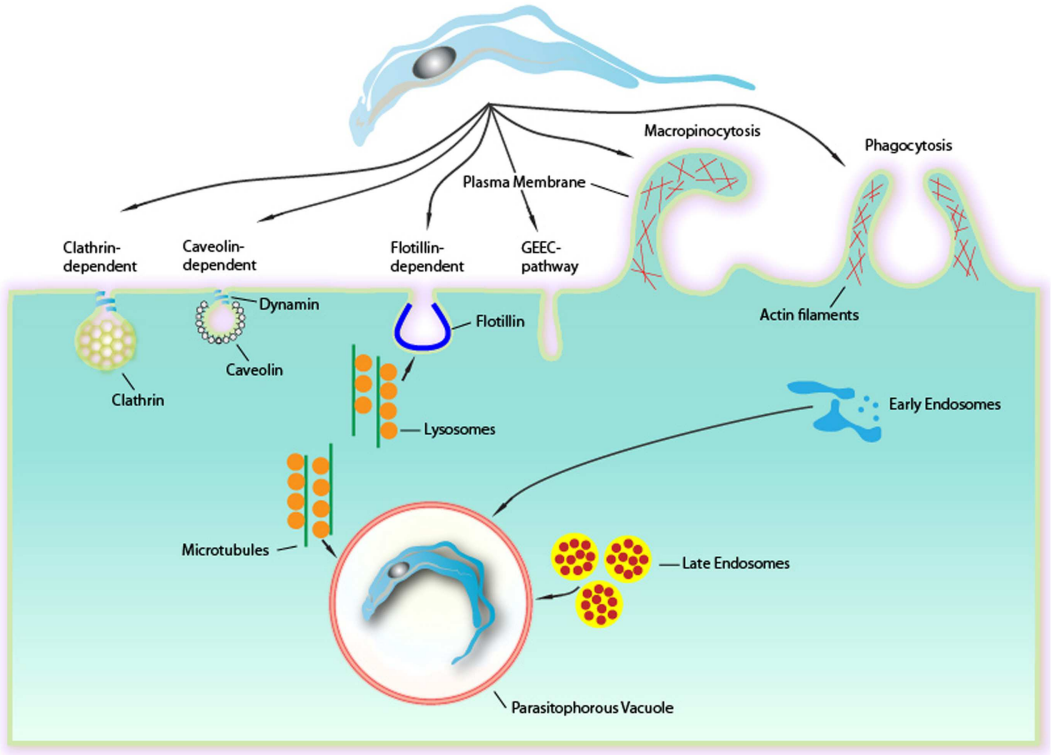

FIGURE 1 | Endocytic mechanisms involved in *Trypanosoma cruzi* entry into mammalian cells can occur via several different mechanisms culminating in a formation of a PV. Although phagocytosis was the first endocytic mechanism described to be used by *T. cruzi*, others mechanisms as clathrin-mediated endocytosis, caveolar-dependent, and lipid raft-dependent endocytosis macropinocytosis seems to be involved. Formation of the PV is always depends on lysosomes. This fusion can occur at the site of entry of the parasite or after entry, with the PV preformed. The fusion of lysosomes in areas of entry-dependent flotillin was recently demonstrated, but it is believed that this can occur in other ways. The targeting of lysosomes to entrance region or the PV occurs via microtubules. Upon entry there is also the fusion of endocytic vesicles (endosomes and late initials) that together with the fusion of lysosomes leads to the maturation of the PV through their acidification. This allowed the destruction of this maturing vacuole of the parasite to escape later.

host cell plasma membrane (PM), suggesting that the internalization process only occurs at higher temperatures (higher than 18°C) (4). Endocytic mechanisms control the lipid and protein composition of the PM, thereby regulating how cells interact with their environments (5). Endocytosis creates an essential interface between eukaryotic cells and their surroundings through the formation, budding, and maturation of PM-derived intermediates. That endocytosis comprises a sophisticated array of different pathways is now widely accepted (6). Mechanisms involved in cellular uptake are important for different processes in a wide variety of cell types. Classically, these mechanisms can be classified into a number of clathrin-independent pathways as well as clathrin-mediated endocytosis (CME), caveolae, phagocytosis, macropinocytosis, and circular dorsal ruffles (5). Additionally, pathogens often exploit endocytic routes to mediate their internalization into cells (7, 8). Although several studies have been conducted in the field of pathogen and host cell interactions, the molecular mechanisms, including the types of endocytic pathways and the proteins involved in cargo recruitment and internalization, are not completely clear (7). Actually, endocytic pathways start with the recognition between the molecules present and exposed on the cell surface and the product that will be internalized (7). Several *T. cruzi* molecules have been described as being involved in the process of invasion. One class of these molecules is the mucins, which are major *T. cruzi* surface glycoproteins (7). Many mucins have been reported as *T. cruzi* ligands because their sugar residues interact with mammalian host cells (9–12). Other *T. cruzi* molecules involved in adhesion are trans-sialidases (active and inactive) and glycoproteins (gp82, gp80, gp35/50, and gp85) (13). With respect to the mammalian host cell, any class of molecules exposed on the host cell surface is believed to have the potential to be a *T. cruzi* receptor ligand. Most of the characterized receptor classes are carbohydrates that contain galactosyl, mannosyl, and sialyl residues (3, 14–19) and lectin-like proteins, such as galectin 3 that bind to carbohydrate residues present on the parasite surface (20–22). Some lectins, as mannose binding lectin, are involved in a humoral pattern-recognition molecule important for host defense. In the case of Chagas' disease this lectin is involved in regulating host resistance and cardiac inflammation during infection (23). Other molecules that function as receptors is possibly involved in the pathogenesis of Chagas' disease are endothelin 1 and bradikinin receptors. They are used by tripomastigotes to

invade cardiovascular cells leading to a chagasic vasculopathy (24). Cytokeratin 18, fibronectin, laminin, and integrins are also receptor molecules because the Tc85 present on the trypomastigote surface has motifs that bind to these molecules, making a bridge between the parasite and the host cell (**Figure 2**) (25–27). We will not describe all the putative molecules involved in *T. cruzi*-host cell interactions because this topic has been discussed in recent reviews (3, 28) and will be covered by other authors in this issue.

PHAGOCYTOSIS

The process known as phagocytosis is a key mechanism of the innate immune response in which macrophages, dendritic cells, and other myeloid phagocytes internalize diverse microorganisms, dead or dying cells, and debris (29). Phagocytosis is an actin-dependent process that can be triggered by several types of ligands and receptors, leading to particle internalization (30). These receptors, called "pattern-recognition receptors" by Janneway (31) because of their capability to recognize pathogens, are present on the entire surface of phagocytic cells and are known as Fc receptors, complement receptors, scavenger receptors, mannose receptors, and receptors for extracellular matrix components (32). Accordingly, a classical zipper type of phagocytosis was described in addition to several unconventional phagocytic routes. In the classical zipper model, after the attachment of a pathogen to the receptor present on the host cell PM, bilateral protrusions extending from the host cell PM engulf the pathogen until a vacuole (completed sealed) is formed. Frequently, this type of phagocytosis occurs after some ligand binds to the Fc receptors or CR receptors (33). Unconventional methods of phagocytosis can be shared among three different groups according to the morphological features. The first is triggered phagocytosis, in which abundant membrane ruffles eventually enclose a spacious vacuole containing the microorganism to be ingested. This mechanism, frequently referred to as triggered macropinocytosis, is commonly driven by entero-invasive bacteria and requires a secretion of a type 3 bacteria protein complex that is responsible for translocating bacterial proteins into the host cells (34). Another unconventional mechanism is overlapping phagocytosis, which is morphologically described as forming pseudopods that do not fuse but slide past each other, resulting in pseudopod stacks to which lateral pseudopods are added. Coiling phagocytosis is characterized by the extension of unilateral pseudopods that rotate around the pathogens. Both overlapping and coiling phagocytosis are predominantly observed in professional phagocytic cells, indicating that this process is driven by the host cell (32). The signaling triggered by the pathogen varies depending on the nature of the receptors used. Basically, exposure to multivalent ligands induces clustering of these receptors in the plane of the membrane, initiating the phosphorylation of some tyrosine kinases. The remodeling of actin is unambiguously required for pseudopod extension, and in the case of FcγR, polymerization is driven by Rac1 and/or Rac2 and Cdc 42. Additionally, phosphoinositides provide an

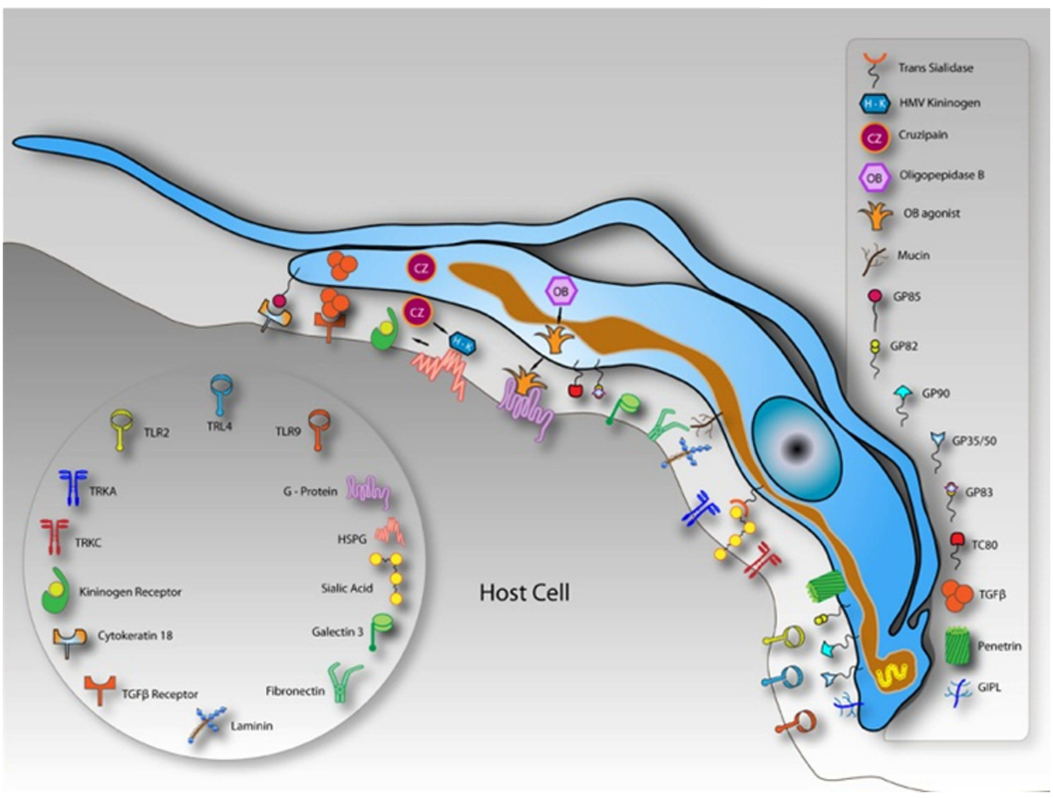

FIGURE 2 | Schematic model demonstrating molecules involved on parasite-host cell interaction process and exposed on the surface of a hypothetical host cell and in trypomastigotes of *Trypanosoma cruzi*. After Ref. (3).

important contribution to actin remodeling during phagocytosis. Phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-3,4,5-participate in actin assembly, driving pseudopod formation. The conversion to phosphatidylinositol-3,4,5-trisphosphate is required for pseudopod extension and phagosomal closure. Phospholipases A and D have been considered essential to phagosome formation (35). With respect to the *T. cruzi* entry process, Nogueira and Cohn (36) were the first to propose that trypomastigotes enter peritoneal macrophages, L929, HeLa cell line and calf embryo fibroblasts by a phagocytic process because the treatment of these host cells with cytochalasin B (a drug that blocks the extension of actin filaments) inhibited the parasite internalization. Using cardiac muscle cells, Barbosa and Meirelles (37) demonstrated by transmission electron microscopy that trypomastigotes bind and induce a typical phagocytic process with host cell pseudopod extensions. These studies suggested the participation of endocytic mechanisms in both professional and non-professional phagocytes. In 1991, Hall et al. (38), using a macrophage cell line, described that the PV containing trypomastigotes presents CR3 receptors, β1 integrin, lysosomal membrane glycoproteins (lgp), and Fc receptors (the last only appears if trypomastigotes were previously opsonized). These results supported the hypothesis that *T. cruzi* can enter the host cell, mainly in macrophages, by phagocytosis. The recognition of Toll-like receptors 2 by trypomastigotes is also capable of inducing a phagocytic process (39) and initiating an inflammatory pathway. Additionally, several groups demonstrated the presence of PM components at the PV membrane, such as galactosyl and glycoconjugate residues (40) and sialoconjugates (41). Several signaling pathways are triggered by phagosome formation and are not different from those involved in the formation of the PV. In professional phagocytes, the activation of tyrosine kinase proteins during the initial contact with trypomastigotes was observed, followed by the recruitment of PI 3-kinase, which culminates in the polymerization of actin microfilaments and pseudopod extension. The participation of tyrosine kinases was demonstrated by Vieira et al. (42) using peritoneal macrophages treated with kinase inhibitors, such as genistein and staurosporine and this group suggested that the main process of trypomastigote entry was by phagocytosis. The participation of Rac1, Rho, and Cdc42 was also observed and will be discussed later. Currently, with new tools to study the endocytic types, the signaling pathways, and cellular components that are involved in different phagocytic mechanisms are being elucidated (macropinocytosis, CME, and participation of membrane microdomains) (5). In relation to amastigote the infection of mammalian cells seems be different when using and comparing different strains. While amastigotes from the *T. cruzi* I lineage (G strain) appears to induce phagocytosis by non-phagocytic cells (43, 44), amastigote from *T. cruzi* II as Y strain is largely phagocytized by macrophages, and occasionally by other cell types (43, 45). The amastigotes' ability to induce phagocytosis was first demonstrated through cytochalasin D host cell's treatment, where Procópio and colleagues (46) observed a drastic reduction of amastigotes penetration after actin polymerization inhibition. The analysis of the interaction type using these new approaches indicates that events initially described as phagocytosis may correspond to other endocytic pathways. The morphological analysis of the initial steps of *T. cruzi* invasion (trypomastigotes or amastigotes) using transmission and scanning electron microscopies revealed that this protozoan uses different mechanisms to invade host cells given that a wide type of morphological events can be observed when they are allowed to interact with the host cells. Using field emission scanning electron microscopy, we showed that even after a short interaction time, trypomastigotes, and amastigotes are ingested by peritoneal macrophages and by non-professional phagocytic cells (LLC-MK_2). The macrophage PM can tightly recover *T. cruzi*, forming a funnel-like structure with bilateral projections of the host cell PM to internalize the parasites in a process described as a classical phagocytosis pathway, forming a long, large protrusion that recovers the parasite body, as characterized in the initial step of trigger phagocytosis (or macropinocytosis), or even forming a structure described as a coiled-coil phagosome in which the host cell PM forms coiled-coil projections (**Figure 3**) (47).

AUTOPHAGY AS AN INDUCTOR OF AUTOPHAGOSOME FORMATION

Phagosomes can also form inside cells in a process described as autophagy. Autophagy is a self-degradative process involved in developmental regulation, the response to nutrient stress, and the clearance of damaged proteins and organelles and plays an important role in balancing sources of energy at critical times in development and in response to nutrient stress (48). Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles, and eliminating intracellular pathogens (48). Indeed, during autophagy, intracellular membranes engulf organelles and cytoplasmic debris, and this process can be used to engulf intracellular microorganisms into a phagosome (called an autophagosome in the case of autophagy). The intracellular machinery involved in this process is complex, involving several classes of proteins, including Atg proteins (proteins related to autophagy) (49). Currently, more than 32 genes for Atg proteins have been described in mammals (49). The formation

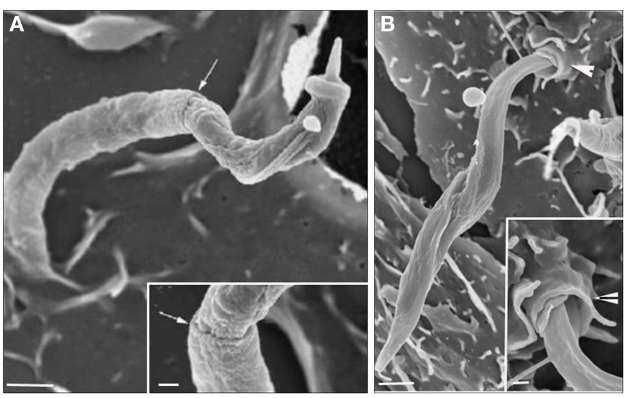

FIGURE 3 | Field emission scanning electron microscopy of the interaction between peritoneal macrophages and *T. cruzi*. (A) *T. cruzi* was partially tightly recovered by the macrophage plasma membrane (PM) in a process described as phagocytosis. (B) *T. cruzi* flagella recovered by host cell PM in a process described as coiled phagocytosis. Bars = 1 μm [After Ref. (47)].

of double membrane autophagosomes also requires the activation of the mTOR protein (mammalian target of rapamycin protein) and recruitment of microtubule-associated protein light chain 3 (LC3B) and lysosome (49). This mechanism can be induced by starvation or by the use of rapamycin (which activates the mTOR pathway). Romano and colleagues (50) demonstrated that both treatments are capable of reducing the internalization of *T. cruzi* into host cells and that the PV is labeled with LC3B, a molecular marker of the autophagy pathway. Martins et al. (51) showed that treating host cells with rapamycin impairs the binding of *T. cruzi* gp82 to the host cell. This surface molecule is required for adhesion and is one molecule described to be responsible for the exocytosis of lysosomes that can lead to trypomastigote internalization (51).

MEMBRANE RAFTS: ENDOCYTOSIS DEPENDENT ON CAVEOLIN OR FLOTILLIN

Due to their characteristic shape, caveolae have long been thought to be dynamic endocytic structures (52). In the case of mammalian cells, basically three different types of caveolin proteins are present: caveolin 1, caveolin 2, and caveolin 3 (52). Caveolin 1 and caveolin 2 are found in almost all cell types (excluding neurons and leukocytes, which do not present caveoles), and caveolin 3 mainly found in muscle cells (52). Each caveolae presents approximately 200 caveolin 1 molecules, and caveolae biogenesis is completely dependent on this protein (52). Based on this information, caveolin 1 is known to be the main caveolae marker. Caveolin 1 is also capable of binding to the GM1 ganglioside and to some GPI-anchored proteins. Cholesterol is another component of caveolae, and its depletion has been shown to promote the disorganization of the caveolar structure (53). The raft-associated proteins, flotillin 1 and flotillin 2, are also reported to play a role in endocytosis. Flotillin proteins show homology with caveolin 1, thus suggesting participation in lipid ordering (5, 54, 55). The domains that contain flotillins are morphologically distinct from caveolae because they display a flattened shape, whereas caveolae are spherical. However, both are enriched in cholesterol, GM1 and GPI-anchored proteins (55).

The host cell PM microdomains have been shown to be involved in *T. cruzi* entry in both non-phagocytic and phagocytic cells (56–58). Fernandes et al. (56) and Barrias et al. (57) showed that cholesterol, the major component of membrane rafts, is involved in the *T. cruzi* entry process because the treatment of the host cells with drugs that remove or immobilize this component, such as beta cyclodextrin and filipin, impairs parasite internalization. We do not know yet if cholesterol is a direct participant in this recognition process or if the alterations caused by its removal or immobilization lead to membrane composition alterations that hide or remove receptors involved in this important process. Previously, Hissa et al. (58) showed that cholesterol depletion reduces *T. cruzi* penetration because lysosome exocytosis became unregulated after this treatment, impairing the release of acid sphingomyelinase from the lysosome, which induces endocytosis. During parasite internalization by the host cell, molecular markers of both types of membrane rafts, such as flotillin 1, caveolin 1, and GM1, were observed at the parasite-host cell PM interface (**Figure 4**) (57). These suggest the participation of microdomains in *T. cruzi* internalization by the host cells.

MACROPINOCYTOSIS AS ANOTHER ROUTE TO *T. CRUZI* PENETRATION

Macropinocytosis represents a regulated form of endocytosis that mediates the non-selective uptake of solute molecules, nutrients, antigens, and some pathogens, such as viruses. This process of endocytosis was originally described as involving the assembly of large extensions of the PM (59). The molecular basis for the formation and maturation of macropinosomes has only recently begun to be defined. Macropinocytic events may begin with external stimuli that trigger the activation of tyrosine kinase receptors, inducing changes in the dynamics of actin filaments, which then leads to PM ruffling. The Ras GTPase superfamily plays an important function in the activation process (60). After activation of the tyrosine kinase receptor, three different signaling pathways are triggered, involving the proteins Rac1, Rabankyrin 5 (an effector of Rab5 protein), Arf6, PI3K, and p21-activated kinase Pak1 (activates Rac1) (61). Rabankyrin 5 has been used as a molecular marker to distinguish macropinosomes from other endocytic compartments (61). In addition, this mechanism is also characterized by the actin-dependent reorganization of the PM to form macropinosomes, which are morphologically heterogenic vesicles that lack coat structures. Na^+/H^+ exchangers have also been described to play an important role in the maintenance of a macropinocytic event. Indeed, drugs that inhibit these exchangers, such as amiloride and EIPA, are widely used to characterize macropinocytosis (60). Although PI3K, Rac, and Cdc 42 have already been described as proteins involved in *T. cruzi* entry into different cell types, Barrias and colleagues (62) recently showed, for the first time, the participation of this pathway in the internalization of trypomastigotes and amastigotes of *T. cruzi* into phagocytic and non-phagocytic cell types. The intense inhibition of the parasite internalization process occurred when the host cells were pre-treated with amiloride (an inhibitor of Na^+/H^+ exchangers) or with rottlerin (an inhibitor of PKC). Host cell treatment with PMA, a stimulator of macropinocytosis caused by PKC stimulation, promotes an increase in parasite internalization. The recruitment of phosphorylated proteins, actin, and Rabankyrin 5 to the site of parasite entry and the characteristic morphology of this process, as shown by fluorescence microscopy, support the view that macropinocytosis is another process used by *T. cruzi* to penetrate host cells (**Figure 5**) (62). Morphologically, the entry of trypomastigotes and amastigotes in peritoneal macrophages closely resembles the process described for macropinocytosis, where there are extensive unilateral extensions of the PM that result in a loose vacuole around the parasite (62).

CLATHRIN-MEDIATED ENDOCYTOSIS

Clathrin-coated vesicles are formed during receptor-mediated endocytosis and organelle biogenesis at the trans-Golgi network (5). The clathrin coat itself is formed by the self-assembly of triskelion-shaped molecules composed of three clathrin heavy chains and associated clathrin light chain subunits (63). The diversity of the cargo and diversity of the adaptor and accessory proteins used to implement vesicle formation reflect the pathways' adaptations to tools suited to the materials being packaged. Some well-known cargoes that use CME are tyrosine receptor kinase, GPCRs, transferrin receptor, LDL receptors, and anthrax toxin

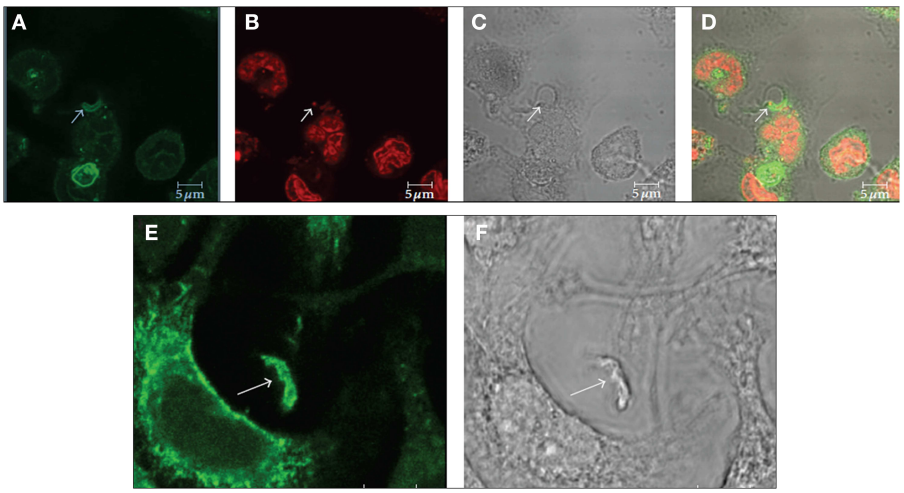

FIGURE 4 | Immunofluorescence microscopy localization of GM1 (A–D) and flotillin 1 (E,F) during internalization of *T. cruzi* by macrophages suggests the participation of membrane microdomains in this process. **(A–D)** Co-localization of GM1, using cholera toxin subunit B **(A)** and an intracellular parasite **(C**: arrow). **(B)** Shows labeling of the nucleus and kinetoplast with propidium iodide. Corresponds to a DIC image; **(D)** is a merge image. **(E,F)** Co-localization of flotillin 1 **(A)**, detected using a specific antibody, and trypomastigotes **(B**: arrows). Bars – 5 μm. After Ref. (57).

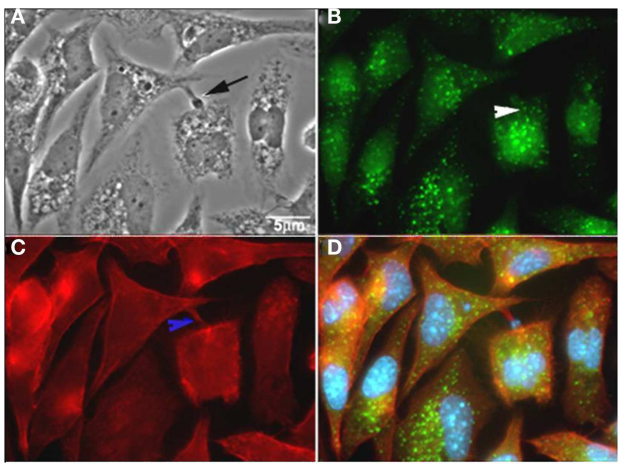

FIGURE 5 | *T. cruzi* co-localizes with rabankyrin 5 and actin. **(A)** Phase contrast; **(B)** rabankyrin 5-Alexa 488; **(C)** phalloidin-Alexa 546; **(D)** merge (rabankyrin 5, phalloidin, and DAPI). Arrow indicate trypomastigotes, white arrowhead indicates rabankirin labeling around parasites and blue arrowhead indicates host cell actin around parasites. After Ref. (62).

(64). Clathrin is also required for the internalization of large structures, such as bacteria (65), fungi hyphae (66), and large viruses (67), in a process that involves cooperation with actin. Recently, Nagajyotic and colleagues (68) demonstrated that the low-density lipoprotein receptor (LDLr) is important in the invasion and subsequent fusion of the PV containing *T.* cruzi with host cell lysosomes, thus suggesting the participation of clathrin-coated pits in parasite internalization because LDL receptors are concentrated in this vesicle. This demonstration was performed using an antibody against the clathrin light chain by immunofluorescence. Although this labeling was clearly observed around the vacuole, further studies should be conducted to demonstrate that the labeling is actually clathrin from the endocytic-coated vesicles and not from another cell site.

ENDO-LYSOSOME PARTICIPATION IN *T. CRUZI* INVASION

After the cargo binds to mammalian cell receptors and its internalization by different endocytic pathways culminating in the activation of many signaling events, the cargo is delivered to heterogeneous organelles known as early endosomes. These organelles are usually complex presenting long thin tubules connected to bulbous or vacuolar elements and pH 6.5–6.0. Early endosomes contain molecular markers, such as the Rab5 and EEA1 proteins ("early endosome antigen"), in their membranes. The tubules are responsible for molecular sorting and vesicle transport to the endoplasmic reticulum, PM, trans-Golgi network, and other destinations (69). This organelle loss tubular elements and matures, transforming in a late endosome. The maturation is marked by the switch of molecular marker Rab5 to Rab7 (70). The late endosome displays a vesicular appearance and moves through cellular microtubules in the minus direction, allowing it to occupy a perinuclear position. In addition to Rab7, late endosomes present Rab9, Cd63, and the mannose-6 phosphate receptor (70). The Lamp1 and Lamp2 proteins, which protect the organelle from acid hydrolases, are acquired during this maturation process through fusion with the lysosome in a coordinating system that culminates in an organelle containing many vesicles inside (multivesicular bodies) and with a low pH range (4.5–5.0). The participation of early and late endosomes in the *T. cruzi*-host cell interaction was first characterized by Wilkowsky and colleagues (71) when they demonstrated the recruitment of Rab5 and Rab7 to the PV containing the protozoan. Woolsey et al. (72), using a short interaction time between *T. cruzi* and non-professional phagocytic cells, showed that 50% or more of the invading *T. cruzi* trypomastigotes use the host cell PM during the PV formation. They suggested that

this process was facilitated by host cell actin depolymerization and showed that this vacuole is enriched in products from PI 3-kinase and that it is negative for lysosomal markers. Approximately 20% of *T. cruzi*-containing vacuoles were positive for EEA1 and Rab 5, and approximately 20% were positive for Lamp1, a lysosome marker. Since 1994 (73), the exocytosis of lysosomes to the parasite site of entry has been described as playing an important role in parasite entry. Lysosomes are placed in the path of the host cell PM along microtubules in a kinesin-dependent method (74). They fuse with the PM in a Ca^{2+}-dependent process (75). This process was described as the unique way the parasite used to enter and be kept inside the host cell. However, this process was subsequently shown to represent only approximately 20% of the parasite entrance, and a lysosome-independent process was described to account for approximately 50% of the parasite internalization process. In addition, in approximately 20% of the internalized parasite, there was participation of the early endosomes, as recognized by EEA1 labeling 10 min post-infection (72). They also described that the PV vacuole containing *T. cruzi* was observed labeled with the lysosome associated protein 1 (LAMP1) as well as with endocytic tracer from pre-labeled lysosomes. These results showed that the lysosome pathway was not the only one that presents fusion with lysosomes. Barr et al. (76) showed that an unusual 120-kDa alkaline peptidase (TSF) from a soluble fraction of *T. cruzi* induces repetitive calcium transients in primary isolated cardiac myocytes from dogs. Using thapsigargin, they also showed that Ca^{2+} depletion from intracellular stores, such as the sarcoplasmic reticulum, is able to inhibit Ca^{2+} transients and trypomastigote invasion. The authors also described that "the Ca^{2+} transients are dependent on release of Ca^{2+} from sarcoplasmic reticulum Ca^{2+} stores, but this release in not dependent on extracellular Ca^{2+} or on the classic model of Ca^{2+}-induced Ca^{2+} release in cardiac myocytes." In 1999, Meirelles et al. (77) also described that the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) participates in trypomastigote invasion into cardiomyocytes because thapsigargin inhibits 75% of this process. Recently, Fernandes et al. (78) showed that the entry of *T. cruzi* trypomastigotes into the host cell wounds the host cell PM by inducing a process of wound repair using Ca^{2+}-dependent exocytosis of lysosomes. The lysosome exocytosis was triggered by an increase in calcium influx, derived from the extracellular space, which enters the host cell as soon as the PM is wounded. The wound repair of the host cell PM was performed with the lysosomal delivery of acid sphingomyelinase to the host PM and formation of endosomes enriched in ceramide, processes that facilitate parasite entry into the host cell (78). Besides, this mechanism may be involved with the tropism of *T. cruzi* for cardic cells since membrane repair is common in muscle cells, explaining part of the Chagas' disease pathology (78).

ACTIN CYTOSKELETON

The participation of the actin cytoskeleton during the initial step of the invasion has, until now, been under debate. The participation of the actin cytoskeleton in *T. cruzi* entry has been suggested since 1976 when Dvorak and colleagues (79) treated different host cells with cytochalasin B and demonstrated unequivocally that the internalization of trypomastigotes was impaired. Rosestolato et al. (80), using different host cells (professional and non-professional phagocytic cells) previously treated with cytochalasin D (CD) and then allowed to interact with the cell culture trypomastigote forms, also showed a drastic reduction of the parasites inside the host cells (81). Additionally, Barbosa and Meirelles (37), using heart muscle cells, clearly showed the evident participation of the actin cytoskeleton during *T. cruzi* invasion. In 2004, Woolsey and Burleigh (72) showed that actin depolymerization by cytochalasin D enhances parasite entry into the host cell at an early step and also blocks lysosome or early endosome fusion at the site of parasite entry. They also described, using NIH-3T3 fibroblasts expressing dominant-negative Rho, that after 15 min of infection, that there were three times more parasites inside than in the control cells but that the number of intracellular parasites drastically decreased until 1 h. They suggested that a cell with continuous actin cytoskeleton alterations was not able to retain the parasites inside the cell, showing the importance of actin polymerization and depolymerization on the interaction process. Our group showed (82) that cells overexpressing Rac 1 exhibited a higher internalization index for *T. cruzi* compared with normal cells. However, after 48 h, a reduced number of parasites were observed. Notably, these different results can be explained by different host cell treatments, whether the cells were washed after the incubation with cytochalasin, the interaction time after the drug treatment, the nature of the parasite strain, and other considerations. We also believe that despite the contradictory results, all these papers contribute to a better understanding of the complex process of the *T. cruzi*-host cell interaction and that it is not good scientific practice to neglect a thorough discussion of all published results, as frequently happens.

During the initial moments of the interaction process with *T. cruzi* trypomastigotes, the host cell transient calcium increase has been reported (73–76). Host cells treated with thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (83) that reduces parasite entry into the host cell (84), showed the participation of the intracellular calcium store in this process and its involvement in lysosome exocytosis.

PARASITOPHOROUS VACUOLE' CLOSURE

In mammalian cells, several molecules that selectively regulate the assembly of an endocytic vacuole have been identified. Among them, dynamin has been shown to play a major role in processes such as CME, synaptic vesicle recycling, phagocytosis, transport from the trans-Golgi network, and ligand uptake through caveolae (85). Dynamin is a GTPase family comprising three isoforms: dynamins 1, 2, and 3 (86). One protein class that interacts with dynamin is phosphatidylinositol-3-kinase (PI3K) (87). Dynamin interacts with the p85 regulatory subunit of PI3K, and this interaction stimulates the GTPase activity of dynamin. Gold and colleagues (88) reported that the inhibition of PI3K prevents the recruitment of dynamin 2 to the site of particle binding, suggesting that in phagocytosis, the activation of PI3K is upstream of dynamin. According to some models, dynamin is a mechanochemical enzyme that is directly responsible for pinching off the vesicle (86). Other authors consider that dynamin is a regulatory protein that recruits the downstream partner, which, in turn, drives the

fission step (87, 89). Using dominant-negative dynamin (K44A) HeLa cells, Wilkowsky and colleagues (71) showed that dynamin is involved in the invasion of *T. cruzi* in non-phagocytic host cells. Subsequently, Barrias et al. (62) showed that the GTPase activity of this protein is important for the fission of PVs in both phagocytic and non-phagocytic cell lines through the use of dynasore, which has the ability to block the GTPase activity of dynamin, acting as a potent inhibitor of endocytic pathways by blocking coated vesicle formation within seconds of its addition.

CONCLUDING REMARKS

More than 100 years after Carlos Chagas' discovery about *T. cruzi* and Chagas' disease, we still have many important gaps in the knowledge of the basic aspects of the protozoan biology and its interaction with host cells. It is now clear that the parasite uses several surface-associated molecules to interact with a not yet completely defined set of macromolecules exposed on the host cell surface. We also now know that several internalization processes are triggered following the parasite ligand-host cell receptor interactions. However, we still do not know which ligand-receptor complex triggers each of the types of internalization. New methods and approaches are necessary to better understand the parasite-host cell interaction process. The use of parasite molecules to recover latex beads and their use to interact with host cells may provide new information as to how different parasite molecules could act in the parasite-host cell junction. Unfortunately, neither gene knock-out nor gene silencing is effective with *T. cruzi*. The use of other methodologies, such as high-throughput technology, gene knock-out of host cell molecules by RNAi and microarray platforms, can provide new insights into this fascinating field of research. Altogether, it is clear that now we have much more information on the process of interaction of *T. cruzi* with host cells, especially the various mechanisms the parasite uses to penetrate into host cells. It is now important to identify the key molecules involved on each process and develop drugs able to inhibit the infection of the cells by the parasite, opening a new approach to the treatment of the acute phase of Chagas disease, where amplification of the infection through successive invasion of the cells plays a fundamental role.

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Current understanding of the *Trypanosoma cruzi*–cardiomyocyte interaction

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Trypanosoma cruzi, the etiological agent of Chagas disease, exhibits multiple strategies to ensure its establishment and persistence in the host. Although this parasite has the ability to infect different organs, heart impairment is the most frequent clinical manifestation of the disease. Advances in knowledge of *T. cruzi*–cardiomyocyte interactions have contributed to a better understanding of the biological events involved in the pathogenesis of Chagas disease. This brief review focuses on the current understanding of molecules involved in *T. cruzi*–cardiomyocyte recognition, the mechanism of invasion, and on the effect of intracellular development of *T. cruzi* on the structural organization and molecular response of the target cell.

Keywords: *Trypanosoma cruzi*, cardiomyocyte, cell recognition, endocytosis, cytoskeleton, cell junction, extracellular matrix, apoptosis

INTRODUCTION

Chagas disease, caused by *Trypanosoma cruzi* infection, has emerged as an important global public health problem due to the many Latin American *T. cruzi*-infected immigrants in non-endemic countries (Pérez-Molina et al., 2012). Although public health programs in the Southern Cone countries have reduced transmission by 70% (Moncayo and Silveira, 2009), blood and organ transplant transmissions in non-endemic countries (Rassi et al., 2009) and outbreaks of foodborne transmission (Pereira et al., 2009; Ríos et al., 2011) have drawn attention to Chagas disease. An estimated 8–15 million individuals in 18 endemic countries in Central and South America are infected, with approximately 30 million people at risk (WHO, 2010; Rassi et al., 2012). Chronic chagasic cardiomyopathy, the most relevant clinical manifestation, is the leading cause of death from heart failure in endemic countries, and accounts for a significant burden of ischemic and inflammatory heart disease in the USA and Europe due to "globalization" of Chagas disease (Moncayo and Silveira, 2009; Moolani et al., 2012). In this review, we summarize current knowledge of the biology of the *T. cruzi*–host cell interaction, highlighting molecular aspects of *T. cruzi*–cardiomyocyte interplay, with a focus on early infection events and the effect of intracellular parasite development on the structure and function of the target cell.

CELL RECOGNITION AND INVASION PROCESS

T. cruzi–CARDIOMYOCYTE RECOGNITION

Interplay between parasite and host cell is essential for *T. cruzi* to successfully adjust to the different microenvironments it occupies in its vertebrate and invertebrate hosts. In the obligatory intracellular phase of its life cycle in the mammalian host, infection is driven by adhesion and internalization events involving a large variety of ligands and/or receptors on the surface of both the parasite and host cell interacting with one another to achieve recognition and invasion. Several different surface molecules in the cardiomyocyte have been implicated in adhesion and internalization by the parasite (**Figure 1**). Carbohydrate residues of membrane glycoconjugates in cardiomyocytes, including galactosyl, mannosyl, and sialyl residues, participate in *T. cruzi* cytoadherence (Barbosa and Meirelles, 1992, 1993), while mannose receptors at the surface of cardiomyocytes modulate parasite entry and are down-regulated by *T. cruzi* infection (Soeiro et al., 1999).

Extracellular matrix (ECM) components are also important in parasite–host cell recognition. Fibronectin, a high molecular weight glycoprotein present at the host cell surface, is recognized by fibronectin receptors of the parasite (Ouaissi et al., 1984), which interact with the RGDS (Arg-Gly-Asp-Ser) sequence of fibronectin and mediate parasite entry (Calvet et al., 2004). Immunization with RGDS peptide induced protection in an experimental murine model of acute *T. cruzi* infection (Ouaissi et al., 1986). Heparan sulfate proteoglycans (HSPG), another class of ECM component widely distributed in mammalian tissues, are also involved in *T. cruzi* attachment and invasion (Ortega-Barria and Pereira, 1991; Calvet et al., 2003). Treatment of trypomastigotes and amastigotes, the infective forms of *T. cruzi*, or cardiomyocytes with soluble heparan sulfate (HS) and heparitinase II, respectively, efficiently inhibited parasite invasion (Calvet et al., 2003; Oliveira Jr. et al., 2008; Bambino-Medeiros et al., 2011). The binding of *T. cruzi* to HSPG involves the recognition of the *N*-acetylated/*N*-sulfated domain of the HS chain by heparin-binding proteins (HBPs) present at the surface of the parasite (Oliveira Jr. et al., 2008). Although *T. cruzi* HBPs are capable of binding HS and chondroitin sulfate (CS), only the HS–HBPs interaction triggers

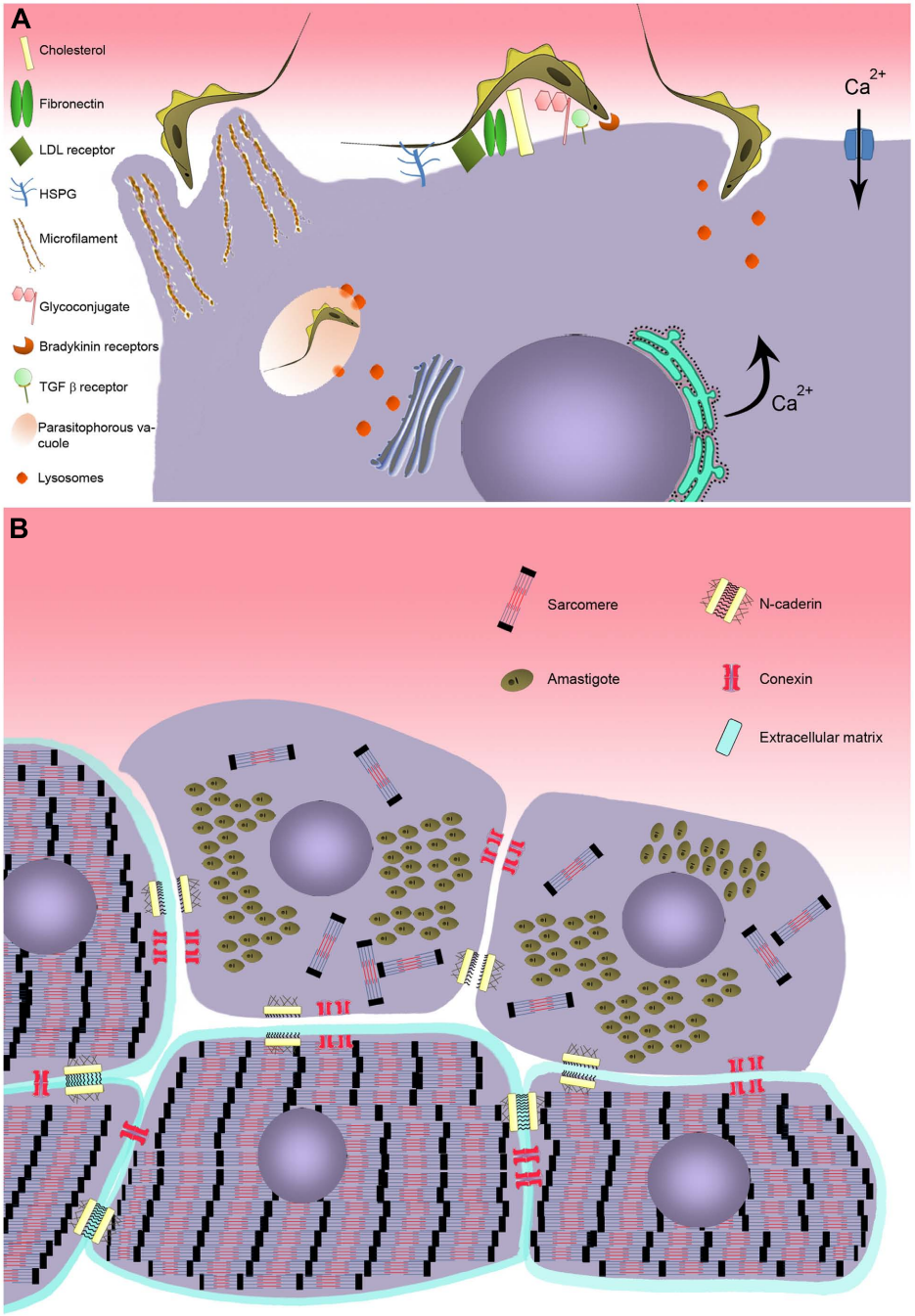

FIGURE 1 | Model of *T. cruzi* invasion in cardiomyocyte. **(A)** Schematic model representing the recognition step involving cell surface molecules of cardiomyocyte during *T. cruzi* invasion. Two distinct mechanism of invasion are represented: actin-dependent and lysosome-dependent mechanisms. **(B)** Effect of *T. cruzi* infection on cardiomyocyte structure. Disturbance on the cardiomyocyte cytoarchitecture is evidenced after *T. cruzi* infection, showing breakdown of myofibrillar and disruption of adherent and gap junctions.

parasite invasion in cardiomyocytes (Calvet et al., 2003; Oliveira Jr. et al., 2008), while HS and CS are involved in vector–*T. cruzi* interactions (Oliveira Jr. et al., 2012).

Lipids also play an important role in *T. cruzi*–host cell interplay. Membrane rafts, enriched in cholesterol and sphingolipids, appear to participate in the invasion process (Barrias et al., 2007; Fernandes et al., 2007; Priotto et al., 2009). Recently, cholesterol has been demonstrated to modulate invasion of cardiomyocytes by *T. cruzi* (Hissa et al., 2012). Depletion of cholesterol from cardiac cell membrane induced an 85–90% reduction of parasite invasion by inhibiting parasites' association with lysosomes. Additionally, the low-density lipoprotein receptor, which is up-regulated in

myocardium of infected mice, also coordinates parasite entry and fusion of the parasitophorous vacuole (PV) with lysosomes (Nagajyothi et al., 2011).

MECHANISMS OF *T.cruzi* INVASION

The large number of molecules involved in recognition of target cells by *T. cruzi* increases the parasite's capacity to explore multiple strategies to ensure propagation in the mammalian host. A number of different mechanisms of *T. cruzi* invasion have been described, involving distinct host cell type, parasite genotype, and developmental stage. At least five models of invasion have been elucidated. (i) An actin-dependent mechanism leads to the rearrangement of microfilaments, inducing the host cell membrane to enclose the parasite (Barbosa and Meirelles, 1995; Procópio et al., 1999; Rosestolato et al., 2002; Ferreira et al., 2006). (ii) Lysosome-dependent mechanisms, involving an increase of transient cytosolic Ca^{2+} levels induced by the parasite, generate cortical actin depolymerization and lysosome recruitment to the parasite binding site (Rodríguez et al., 1999; Hissa et al., 2012). (iii) Activated signaling pathways also participate, including tyrosine kinase receptors (TrKA and TrKC; de Melo-Jorge and PereiraPerrin, 2007; Weinkauf et al., 2011) and phosphatidylinositol 3-kinase (PI3-K; Todorov et al., 2000; Chuenkova et al., 2001; Wilkowsky et al., 2001; Vieira et al., 2002; Woolsey et al., 2003), bradykinin receptors (Scharfstein et al., 2000; Todorov et al., 2003), and transforming growth factor β (TGF-β; Ming et al., 1995; Waghabi et al., 2007). (iv) More recently, sphingomyelinase-mediated plasma membrane repair has been proposed to participate in this process (Fernandes et al., 2011; Fernandes and Andrews, 2012), as has (v) the host cell autophagy pathway (Romano et al., 2009, 2012). Finally, the combination of different mechanisms has been described as coordinating the *T. cruzi* invasion process (Butler and Tyler, 2012).

Elevation of transient intracellular Ca^{2+} levels, an invasion-related effect provoked by *T. cruzi* binding to the host cell membrane (**Figure 1**), has also been demonstrated in cardiac cells (Barr et al., 1996; Garzoni et al., 2003). The increase of cytosolic [Ca^{2+}] has been reported to be brought about in two different ways: (i) by sarcoplasmic reticulum stores, which are sensitive to leupeptin, suggesting a cortical actin depolymerization and lysosome-dependent mechanism of invasion (Barr et al., 1996), and by (ii) extracellular Ca^{2+} influx through membrane Ca^{2+} channels, which are insensitive to leupeptin (Garzoni et al., 2003). Recently, it has been suggested that Ca^{2+} influx may also occur as a result of lesions on the plasma membrane, suggesting that the membrane repair pathway frequently observed in muscle cells may also be involved in cardiac cell invasion by *T. cruzi* (Fernandes and Andrews, 2012).

Transforming growth factor β, a multifunctional family of proteins that controls a range of biological events in most cells, including proliferation and cellular differentiation (Moustakas et al., 2002), has also been shown to participate in *T. cruzi* invasion of cardiomyocytes (Waghabi et al., 2005). *T. cruzi* directly activates latent TGF-β and modulates TGF-β signaling (Waghabi et al., 2005). Inhibition of *T. cruzi* infection in cardiomyocyte was achieved by blockage of the TGF-β receptor type I (TGFβRI)/Smad2 signaling pathway by SB-431542, a TGF-β signaling inhibitor (Waghabi et al., 2007). Besides impairment of parasite invasion, the inhibitor treatment also reduced *T. cruzi* intracellular multiplication and differentiation. Recently, the therapeutic effectiveness of GW788388, an oral inhibitor of TGF-β signaling, has been demonstrated experimentally in acute phase *T. cruzi* infection, leading to a reduction of parasitemia and mortality, and also preventing cardiac fibrosis (de Oliveira et al., 2012).

Bradykinin receptors (B_2R/B_1R) have also been reported to be involved in cardiomyocyte infection by *T. cruzi* (Todorov et al., 2003). This mechanism of invasion is regulated by cooperation between HSPG, kininogen, and cruzipain-1, the major cysteine protease isoform of *T. cruzi*, resulting in the release of kinin. Invasion through the kinin transduction pathway, activated by G protein-coupled bradykinin receptors, induces intracellular Ca^{2+} mobilization from stores in the endoplasmic reticulum (Scharfstein et al., 2000). The B_2R agonist captopril stimulates the invasion of *T. cruzi* while B_2R and B_1R antagonists, present inhibitory effects on cardiomyocytes, suggesting that these receptors interdependently drive invasion of the parasite (Todorov et al., 2003).

As evidenced in other non-professional phagocytic cells (Rosestolato et al., 2002; Ferreira et al., 2006), *T. cruzi* entry is also mediated by an endocytic process in cardiac muscle. A protrusion of cardiomyocyte plasma membrane, orchestrated by cytoskeleton rearrangement, is observed during *T. cruzi*–cardiomyocyte interplay. A dense actin-based membrane skeleton meshwork projects from the sarcolemma and encloses the entering parasite (Barbosa and Meirelles, 1995). This event was drastically inhibited (75%) when cardiac cells were treated with cytochalasin D, an agent that depolymerizes actin filaments, prior to *T. cruzi* infection; no parasite invasion was observed in fixed cardiomyocytes (Barbosa and Meirelles, 1995). Once inside the cells, the parasite is located within a PV that lacks Ca^{2+}–Mg^{2+}-ATPase, adenylate cyclase, and anionic sites (Meirelles et al., 1986) but has carbohydrate residues such as *N*-acetylglucosamine and *N*-acetylgalactosamine (Barbosa and Meirelles, 1992, 1993). Ultrastructural cytochemistry for the lysosomal enzymes aryl sulfatase and acid phosphatase has revealed the fusion of the parasite-containing vacuole with lysosomes (Meirelles et al., 1987). The acidification of the PV by lysosomal fusion, leading to the activation of TC-TOX and disruption of the PV membrane (Andrews et al., 1990; Hall, 1993), is a prerequisite for the trypomastigote to exit the phagosome, also allowing the parasite to be retained intracellularly and complete its life cycle (Andrade and Andrews, 2004, 2005; Mott and Burleigh, 2008).

EFFECT OF *T. cruzi* INFECTION IN CARDIOMYOCYTE PHYSIOLOGY

During the *T. cruzi*–cardiomyocyte interaction the parasite gains control of overall host cell gene expression, including expression of genes related to immune response, inflammation, cytoskeletal organization, cell–cell and cell–matrix interactions, apoptosis, cell cycle, and oxidative stress (Goldenberg et al., 2009; Manque et al., 2011). The intense trypanocidal immune response generated in cardiomyocytes in response to infection by *T. cruzi* results in the production of cytokines, chemokines, and nitric oxide that, while essential elements of the defensive reaction in cardiac tissue

(Machado et al., 2000, 2008; Manque et al., 2011), can also result in cardiac hypertrophy (Petersen and Burleigh, 2003; Waghabi et al., 2009). Several studies report that *T. cruzi* infection stimulates production of nitric oxide synthase 2, matrix metalloproteinase-2 (MMP-2) and MMP-9 in cardiomyocytes, as well as interleukin-6 (IL-6), IL-1β, tumor necrosis factor-alpha and TGF-β (Petersen and Burleigh, 2003; Petersen et al., 2005; Gutierrez et al., 2008; Waghabi et al., 2009; Nogueira de Melo et al., 2010). Peroxisome proliferator-activated receptor γ is also implicated in regulating the inflammatory process (Hovsepian et al., 2011). Moreover, IL-1β-mediated development of cardiomyocyte hypertrophy is orchestrated by Toll-like receptor 2 (Petersen et al., 2005). Proinflammatory cytokines also modulate production of mitochondrial reactive oxygen species, impairing the efficiency of the respiratory chain (Gupta et al., 2009). Mitochondrial disturbance has been identified as an important effect of chagasic cardiomyopathy (Garg et al., 2003; Báez et al., 2011). Inflammatory mediators have also been reported to regulate Rabs expression (Stein et al., 2003) thereby interfering with host cell trafficking. Down-regulation of Rab GTPase proteins, including the effector molecule of Rab5 (EEA1), Rab7, and Rab11, has been demonstrated in *T. cruzi*-infected cardiomyocytes, and it has been proposed that a delayed endocytic pathway may favor microbicidal activity and increase antigen processing (Batista et al., 2006).

Changes in cytoskeletal proteins have also been shown during parasite intracellular development (**Figures 1 and 2**). The complex cytoskeleton organization of cardiomyocytes involved in the contraction–relaxation process of the heart is affected by *T. cruzi* infection (Pereira et al., 1993; Taniwaki et al., 2006). Breakdown of myofibrils has been seen in areas of amastigote nests (Pereira et al., 1993; Taniwaki et al., 2006) and disturbance of intermediate filaments (desmin) and microtubules was also induced by parasite proliferation (Pereira et al., 1993). Interestingly, the actin isoform mRNAs, α-cardiac and β-actin mRNAs, are altered during the parasite intracellular cycle (Pereira et al., 2000). Down-regulation of α-cardiac actin mRNA concomitant with up-regulation of β-actin mRNA suggested the reactivation of non-differentiated cell program. Also within the context of cytoskeletal changes, actin-binding proteins have been demonstrated to be altered in *T. cruzi*-infected cardiomyocytes. Alpha-actinin, an F-actin crosslinker protein that anchors actin to the Z line, and costameres, repeating adhesion structures consisting of vinculin involved in the lateral transmission of contractility force to the sarcolemma, are disrupted and down-regulated in *T. cruzi*-infected cells, reducing strength and force transduction (Melo et al., 2004, 2006). These cytoskeletal disorders are accompanied by deregulation of Ca^{2+} influx, affecting cardiac cell contractility (Taniwaki et al., 2006). One striking feature of trypanocidal drugs is their effect on the recovery of cardiomyocyte cytoskeleton (Garzoni et al., 2004; Silva et al., 2006; Adesse et al., 2011a). Posaconazole, an ergosterol biosynthesis inhibitor with potent trypanocidal activity currently in clinical trials, has been demonstrated to promote the reassembly of the contractile apparatus and microtubule network in *T. cruzi*-infected cardiomyocytes (Silva et al., 2006). The reorganization of myofibrils leads to recovery of cardiomyocyte functionality. Similarly, treatment of *T. cruzi*-infected cardiomyocyte cultures with bisphosphonate risedronate, a farnesyl pyrophosphate synthase inhibitor, and amiodarona, an anti-arrhythmic drug, also fostered the recovery of myofibrils (Garzoni et al., 2004; Adesse et al., 2011a) and may represent interesting alternatives for Chagas therapy.

In addition to disruption of the cytoskeletal architecture by the parasite, cell–cell adhesion (adherens junctions) and intercellular communication (gap junctions), which play important physiological roles in cardiac tissue, are also been disrupted by *T. cruzi* infection (Adesse et al., 2008, 2011b; Melo et al., 2008). Alteration in spatial distribution and down-regulation of the adherence junction proteins N-cadherin and β-catenin in *T. cruzi*-infected cardiomyocytes (Melo et al., 2008) may interfere with tissue integrity and perturb the function of the cardiac conduction system, as has been proposed to be the case in arrhythmogenic cardiomyopathies (Mezzano and Sheikh, 2012). Additionally, electrical conduction disturbance, frequently seen in both acute and chronic phases of Chagas diseases, seems to be related to altered gap junction (connexin-43) coupling of cardiomyocytes induced by *T. cruzi* (de Carvalho et al., 1992, 1994; Adesse et al., 2008, 2011b). Connexin-43 dysregulation has also been attributed to increased levels of TGF-β (Waghabi et al., 2009). Following treatment of *T. cruzi*-infected cardiomyocyte cultures with amiodarone and SB-431542 causes reversal of the disorganization of gap junctions and return to their normal distribution (Waghabi et al., 2009; Adesse et al., 2011a), making these compounds potential therapeutic candidates for treatment of Chagas disease.

Besides their involvement in the early steps of *T. cruzi*–cardiomyocyte recognition, ECM components also present a striking role in chagasic cardiomyopathy pathogenesis since their accumulation leads to fibrosis, disposing patients to heart failure and ventricular arrhythmias (Rassi et al., 2010, 2012). In experimental systems, ECM accumulation begins during the late acute phase of infection (Andrade et al., 1989; Calvet et al., 2004), concomitantly with the onset of inflammatory infiltrates, indicating that the process of fibrogenesis is triggered in the early stages of *T. cruzi* infection. A general increase in ECM transcripts and expression was detected by microarray analysis in acute infection (Garg et al., 2003). Cardiac hypertrophy and ECM remodeling were also seen in a *T. cruzi*-infected 3D cardiomyocyte model (Garzoni et al., 2008; **Figure 2**). Surprisingly, reduction of ECM in *T. cruzi*-infected cardiomyocytes was detected by silver staining in acute infection in mice (Factor et al., 1993). Additionally, *T. cruzi*-mediated down-regulated ECM gene expression in cardiomyocyte cultures (Goldenberg et al., 2009; Manque et al., 2011) and a reduction in the synthesis and spatial distribution of fibronectin were detected in heavily infected cardiomyocytes (Calvet et al., 2004; **Figure 2**) even after TGF-β stimulation (Calvet et al., 2009), suggesting that despite the general enhancement of ECM in the heart, the cells harboring the parasites display low ECM expression. The anti-fibrogenic effect of *T. cruzi* has also been seen in human dermal fibroblasts, with repression of transcription factors that regulate expression of fibroblast genes involved in wound repair and tissue remodeling, including ctgf/ccn2 connective tissue growth factor gene, followed by down-regulation of ECM proteins such as fibronectin and collagen I, suggesting another route of parasite dissemination and infection (Unnikrishnan and Burleigh, 2004; Mott et al., 2011).

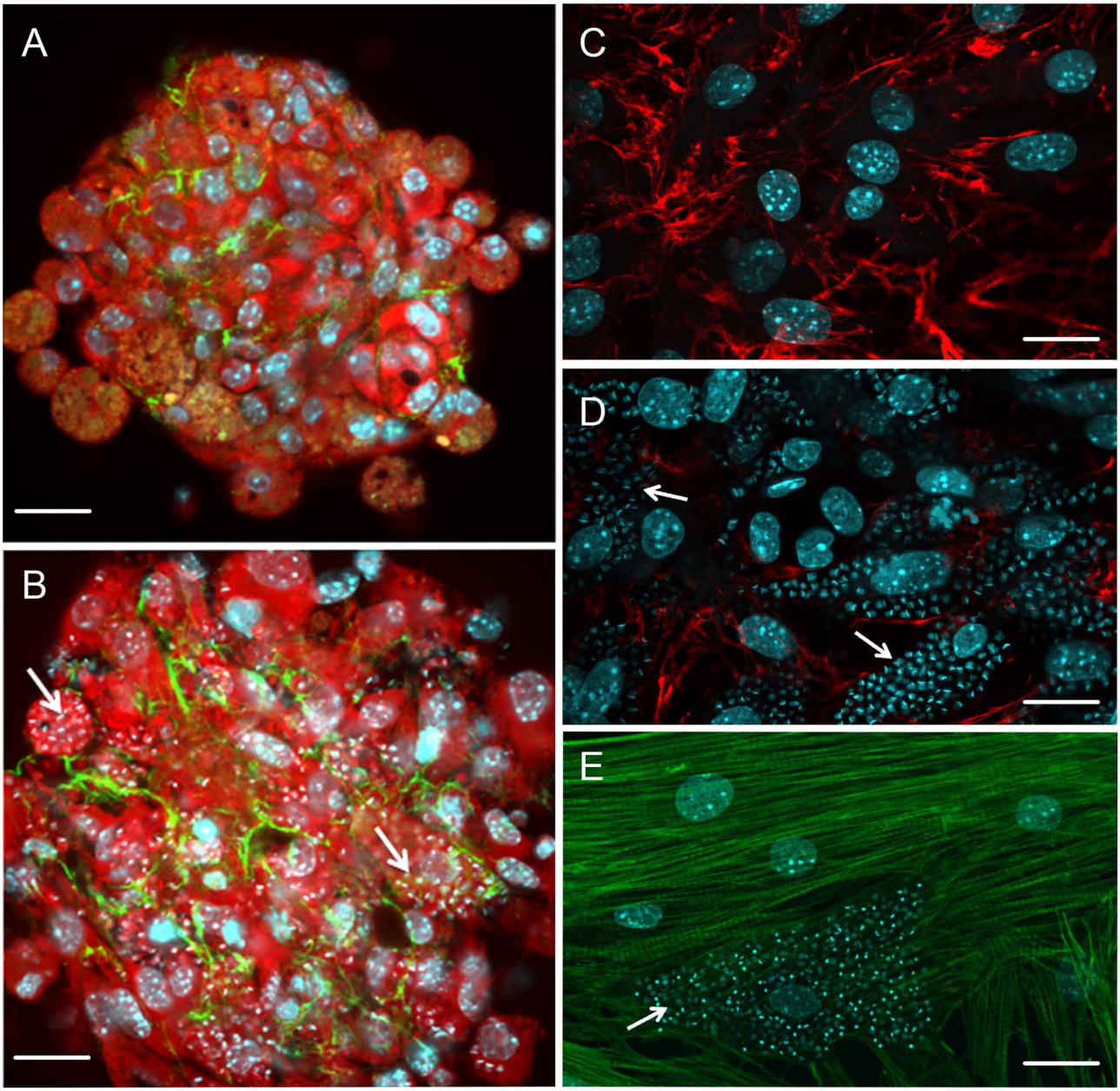

FIGURE 2 | Effect of *T. cruzi* infection on cardiomyocyte cytoarchitecture and extracellular matrix remodeling (A–E). *T. cruzi*-infected cardiomyocyte 3D-culture revealed fibrosis and hypertrophy. Note an increase in spheroid size and the spatial distribution of fibronectin (FN; green) in *T. cruzi*-infected **(B)** compared with the uninfected **(A)** 3D-culture system. However, the FN labeling fainted in highly *T. cruzi*-infected cells. In contrast, the analysis of FN (red) distribution in cardiomyocyte 2D-culture **(C)** demonstrates a reduction of this extracellular matrix component in *T. cruzi*-infected cardiomyocyte culture **(D)**. The low expression of FN in the cells harboring the parasites and the factors involved in the enhancement of FN in 3D-culture are unclear and focus of future investigation. **(E)** Cytoskeletal changes were also evidenced in *T. cruzi*-infected cardiomyocytes, showing complete disorganization of myofibrils. Cardiomyocytes were stained with Evans-blue (red; **A** and **B**) and DAPI (blue; **A–E**), a DNA dye. Arrows indicate intracellular parasites. Bar = 20 μm.

Another point worth discussing relates to the ability of *T. cruzi* to modulate host cell apoptosis, or programed cell death, a physiological process of cell replacement to maintain tissue homeostasis (Mondello and Scovassi, 2010). Pathogens can hijack the host cell apoptotic machinery as an offensive strategy to eliminate the host's immune response (Lamkanfi and Dixit, 2010). Both anti- and pro-apoptotic gene expression are differentially modulated during *T. cruzi*–cardiomyocyte infection, leading to a balance between cell death and survival at different stages of infection (Manque et al., 2011). Induction of apoptosis by *T. cruzi* infection is controversial and seems to be dependent on host cell and parasite genotype (de Souza et al., 2003;

Aoki et al., 2006; Petersen et al., 2006). While fibroblasts are refractory to apoptosis, cardiomyocytes and macrophages differentially undergo apoptosis after *T. cruzi* infection, the latter cell type being highly susceptible. Still, cardiomyocytes infected with *T. cruzi* clone Dm28c have higher levels of apoptosis compared to infection with strains Y and CL (de Souza et al., 2003). Furthermore, the intracellular parasites themselves also undergo apoptosis, hinting at a host attempt to control parasite burden (de Souza et al., 2003, 2010). Interestingly, it has been shown that α2-macroglobulin, a plasma proteinase inhibitor, regulates apoptosis in *T. cruzi*-infected cardiomyocytes and macrophages, impairing the cell death process (de Souza et al., 2008). In contrast, an anti-apoptotic effect has also been demonstrated in cardiac cells (Petersen et al., 2006). The prevention of apoptosis appears to be related to NF-κB activation by inhibiting the signaling of caspases, thus avoiding cell death. Thus, avoidance of apoptosis reduces cardiac damage and may be responsible for the persistence of *T. cruzi* infection.

While our knowledge of *T. cruzi*–host cell interactions has greatly improved, many questions remain open. There are still gaps in our understanding of the molecular interactions involved in cellular recognition and/or signaling pathway in most of the mechanisms of invasion. What are the critical links between these processes? And little is still known about the cooperative role played by the host cell in parasite intracellular growth and differentiation. These questions demand deeper investigation.

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Trypanosoma cruzi extracellular amastigotes and host cell signaling: more pieces to the puzzle

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Among the different infective stages that *Trypanosoma cruzi* employs to invade cells, extracellular amastigotes (EAs) have recently gained attention by our group. This is true primarily because these amastigotes are able to infect cultured cells and animals, establishing a sustainable infective cycle. EAs are thus an excellent means of adaptation and survival for *T. cruzi*, whose different infective stages each utilize unique mechanisms for attachment and penetration. Here we discuss some features of host cell invasion by EAs and the associated host cell signaling events that occur as part of the process.

Keywords: extracellular amastigotes, cell invasion, signaling, mevalonate kinase, protein kinase D

INTRODUCTION

The parasite *Trypanosoma cruzi* employs a variety of distinct strategies to facilitate invasion of mammalian cells. For instance, multiple infective stages [e.g., bloodstream trypomastigotes, metacyclic trypomastigotes, and extracellular amastigotes (EAs)], varying strains and isolates, as well as differing infectivities have been widely acknowledged as significant obstacles to effective treatment. The multitude of invasive strategies employed by *T. cruzi* represents an important survival advantage for the organism and allows for the remarkably wide range of mammalian hosts affected by this parasite. More specifically, *T. cruzi* infects over 100 species from several orders and develops *in vivo* within a variety of cellular niches, including macrophages, muscle tissue, epithelial cells, fibroblasts, and nerve cells. The ability of *T. cruzi* to invade, persist and adapt in both invertebrate and vertebrate hosts is multifactorial, and depends on both the parasite and host fitness. The cellular communication between parasite and its host is constant and has evolved to be relatively benign, as killing the host would not be advantageous to the parasite.

Extracellular amastigotes are either prematurely released from infected cells or generated by the extracellular differentiation of released trypomastigotes (reviewed in Lima et al., 2010). During the acute phase of *T. cruzi* infection within mice, EAs represent 10% of circulating parasite forms and are capable of sustaining an infective cycle in the mammalian host and cells (Andrews et al., 1987).

Among the EA stage, *T. cruzi* I strains (such as the G and Tulahuén strains) are more infective than *T. cruzi* II and VI strains (such as the Y and CL strains; Fernandes and Mortara, 2004; Mortara et al., 2005). In contrast to trypomastigotes, the recruitment of actin is central to the uptake of EA forms in mammalian host cells (**Figure 1**), including HeLa cells, the model cell type used in our studies (Mortara, 1991; Procópio et al., 1998; Mortara et al., 2005).

Here we aim to highlight aspects of host cell invasion by EAs and introduce novel findings including the involvement of molecules from both host cell and the parasite that trigger host cell signaling events.

MAMMALIAN CELL INVASION BY EXTRACELLULAR AMASTIGOTES: THE STORY BEGINS

Prior to the mid 1980s, chronic infection with *T. cruzi* was assumed to be sustained by few trypomastigotes in the bloodstream that had escaped the immune response and invaded new cells. The fate of amastigotes at that time was thought to be restricted to the intracellular growth of the parasite. However, pioneering studies (Behbehani, 1973; Nogueira and Cohn, 1976; Lanar, 1979; Abrahamsohn et al., 1983; McCabe et al., 1984; Carvalho and de Souza, 1986) demonstrated that amastigotes shared some physiological characteristics with trypomastigotes, such as the ability to invade and develop within cells *in vitro* and the ability to infect mice. For instance, McCabe et al. (1984) demonstrated that amastigotes

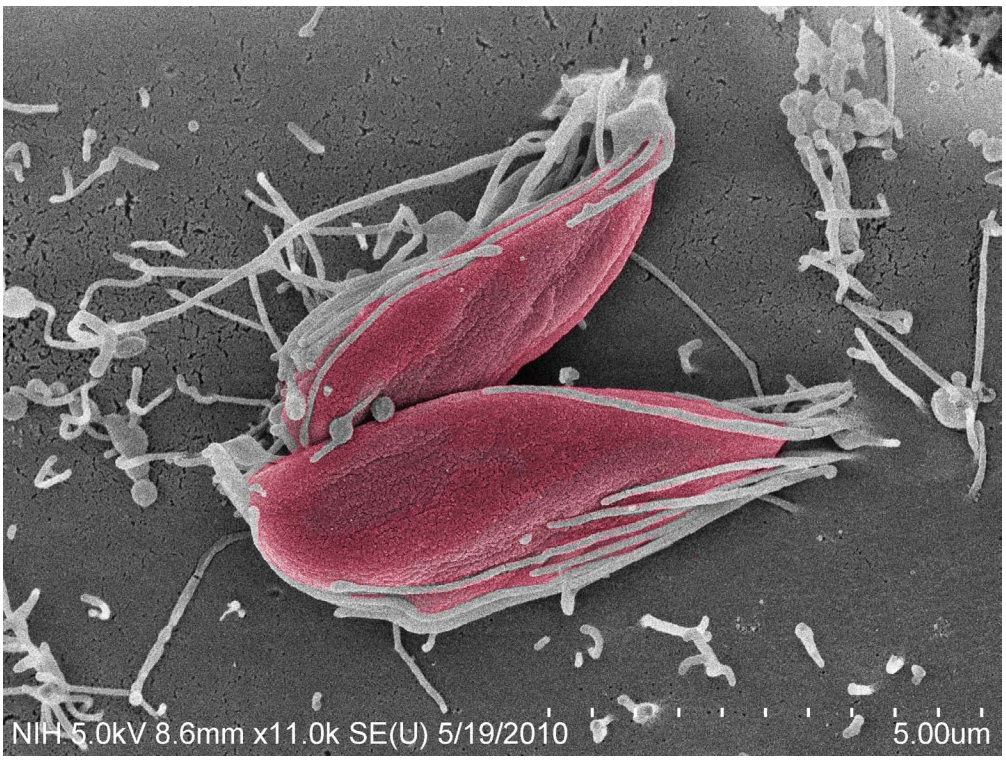

FIGURE 1 | *Trypanosoma cruzi* G strain amastigotes interact with microvilli on the surface of HeLa cells. Parasites (colored in red) imaged by field emission-scanning electron microscopy, attach to HeLa cell surface microvilli. Magnification bar = 5 μm.

isolated from the spleen of mice infected with three different strains of *T. cruzi* (Y, MR, and Tulahuén) were able to infect, survive, and replicate within professional and non-professional (L929 cell line) phagocytes. In a different study, Carvalho and de Souza (1986) obtained highly purified amastigotes (Y strain) from the macrophage-like cell line J774G8. These amastigotes were effectively ingested by macrophages and were able to initiate intracellular replication. Parasite development *in vitro* was measured by means of light microscopy. Moreover, purified amastigotes were highly infective when inoculated into mice, generating high parasitemia and even death (McCabe et al., 1984; Carvalho and de Souza, 1986).

Andrews et al. (1987) found that amastigotes accounted for 10% of circulating forms in the blood of mice during the acute phase of infection. Furthermore, this amastigote form was shown to express a specific surface glycoprotein that was designated Ssp-4. These observations were corroborated when Ley et al. (1988), by using transmission and scanning electron microscopy, demonstrated that extracellular axenically derived amastigotes (i.e., trypomastigote–amastigote differentiation in axenic medium) were able to multiple within cells *in vitro*. When injected intraperitoneally, the amastigote forms were as infective to mice as trypomastigotes. Researchers were also able to measure the infectivity and the multiplication rates of the parasites inside cultured cells (Ley et al., 1988). The process of trypomastigote–amastigote differentiation in axenic medium can be accelerated by lowering the pH of the medium (Tomlinson et al., 1995). EAs can also be obtained from axenic culture media specifically designed for isolating amastigotes (Pan, 1978; Rondinelli et al., 1988). Extracellular (axenically derived) amastigotes resembled intracellular amastigotes with regard to cell body and kinetoplast morphology, and cell surface expression of Ssp-4 (Ley et al., 1988; Barros et al., 1997; Silva et al., 1998).

In vitro, amastigotes are able to infect and complete their life cycle within phagocytic and non-phagocytic host cells, possibly using distinct molecular mechanisms for invasion and escape from the parasitophorous vacuole (Pan, 1978; Carvalho and de Souza, 1986; Ley et al., 1988; Mortara, 1991; Procópio et al., 1998, 1999; Andreoli and Mortara, 2003; Mortara et al., 2005; Florencio-Martínez et al., 2010; Bambino-Medeiros et al., 2011).

In vivo, EAs were intraperitoneally inoculated into groups of five A/J mice and all mice were not only infected, but died after 18–25 days. Two trypomastigote parasitemia peaks were observed on days 7 and 12. Significantly higher numbers of parasites were observed during the second peak in mice that were inoculated with Y strain amastigotes (Ley et al., 1988). EAs were also resistant to complement-mediated lysis (Carvalho and de Souza, 1986; Iida et al., 1989; Fernandes and Mortara, 2004), a feature thought to be required for infectivity. The general conclusion from these studies was that EAs, once prematurely released from dead cells, may persistently infect resident macrophages by initiating an alternative subcycle and/or using an alternative pathway of invasion. It was hypothesized that these events could be crucial for the maintenance of tissue infection and

inflammation (Scharfstein and Morrot, 1999) within the mammalian host.

In 1991, the mechanisms by which amastigotes invade non-phagocytic cells began to be determined. Mortara (1991) observed that amastigotes interact with microvilli on the dorsal surface of HeLa cells leading to microvillus aggregation. This event can be followed by microfilament clustering observable by phalloidin staining and fluorescent microscopy. By a combination of fluorescence and scanning electron microscopy, it was observed that actin aggregates underneath the sites of amastigote adhesion and forms a small clump (Mortara, 1991) that was later called the actin cup-like structure (Procópio et al., 1999). Interestingly, cytochalasin D did not significantly affect parasite attachment but the disruption of cellular microfilaments greatly inhibited amastigote entry (Mortara, 1991; Procópio et al., 1998). In 1998, the actin cup-like structures beneath the amastigotes at HeLa cell entry sites were assessed by confocal microscopy (Procópio et al., 1998). In that study, gelsolin, an actin-binding protein was overexpressed in NIH 3T3 fibroblasts. This resulted in a large increase in the internalization of amastigote forms resulting from enhanced microfilament rearrangement. Components of the HeLa cytoskeleton, integrins, and extracellular matrix, such as α-actinin, ABP_{280}, gelsolin, α5β5 integrin, laminin, and fibronectin, accumulated along with actin at the sites of EA entry (Procópio et al., 1999).

EAs MOLECULES OF ADHESION AND SECRETION: INVASION AND SIGNALING

Adhesion is a crucial initial step for microorganisms to invade any cell. Association of amastigotes with host macrophages was shown to be mediated by the macrophage mannose receptor (MR) and mannose-binding protein (MBP; Kahn et al., 1995). MR and MBP are C-type lectins. MBP strongly and stably binds to amastigotes, and this interaction may contribute to parasite opsonization and cellular invasion. MBP–amastigote interaction is stage specific, can be inhibited by mannan, and requires calcium (Kahn et al., 1996). The amastigote ligands for MBP include SA85-1 and other related surface glycoproteins (Kahn et al., 1996). IFN-γ down-regulates MR expression and thus inhibits parasite invasion and replication within macrophages. It has recently been reported that *in vivo* infection by G strain (TcI) EAs is inhibited by IFN-γ production (Rodrigues et al., 2012). Fibronectin also appears to bridge these initial binding steps (Tulahuén strain; Noisin and Villalta, 1989; Procópio et al., 1999). Our group has previously shown that carbohydrate epitopes expressed on the surface of EAs may also play a role in parasite adhesion to HeLa cells since monoclonal antibodies inhibited the process (Silva et al., 2006).

Cruz et al. (2012) demonstrated that the treatment of HeLa cells with recombinant amastin, a surface glycoprotein abundant in amastigotes, reduced the infectivity of EA forms. Conversely, the ectopic *T. cruzi* expression of amastin accelerated differentiation of amastigotes into trypomastigotes. These results positioned amastin as a potential amastigote molecule able to modulate EA invasion and differentiation within mammalian cells. In addition, amastin might participate in specific host cell signaling culminating in EA invasion.

Trypanosoma cruzi EAs may also secrete proteins in order to aid their attachment and entry into HeLa cells. Silva et al. (2009) identified a hypothetical protein of 21 kDa with no known orthologous in other species. So-called P21 is secreted by the parasites and enhances amastigote invasion, especially when the recombinant protein is added to host cells together with parasites as part of HeLa cell invasion assays. Researchers concluded that P21 triggers unknown host cell signaling events that lead to parasite internalization.

Moonlighting enzymes or protein moonlighting refers to a phenomenon in which a protein can perform more than one function, including unexpected functions (Jeffery, 2009). Many proteins that moonlight are enzymes involved in glycolysis, an ancient universal metabolic pathway. It has been suggested that as many as 7 of 10 proteins in glycolysis and 7 of 8 enzymes of the tricarboxylic acid cycle exhibit moonlighting behavior. Our group has recently characterized a moonlighting protein in *T. cruzi* called mevalonate kinase (MVK), an enzyme involved in isoprenoid synthesis. As part of the *T. cruzi* infection process, this moonlighting protein is involved both in parasite invasion and host cell signaling (Ferreira et al., unpublished). There are numerous intermediates in the mevalonate biosynthetic pathway that play important roles in the post-translational modification of a multitude of proteins involved in intracellular signaling. These intermediates in the mevalonate biosynthetic pathway are essential regulators of cell growth/differentiation, gene expression, protein glycosylation, and cytoskeletal assembly.

Microarray analysis of EA mRNAs has demonstrated that MVK displays higher expression within G (more infective) than in CL (less infective) parasites, suggesting an important role of the MVK pathway in EA infectivity (Ferreira et al., unpublished). We have obtained data indicating that MVK may be a modulator in EA invasion and could become an important target in the development of new drugs to treat Chagas' disease. Moreover, in *T. cruzi*, MVK is secreted and likely participates in the modulation of HeLa cell signaling leading to EA cell invasion. These observations reveal new possibilities for the study of moonlighting protein evolution and function within intracellular parasites.

EAs AND HOST CELL SIGNALING

The first experiments to describe signaling events induced by EAs in non-phagocytic cells were performed in 1998. Procópio et al. (1998) used a protein kinase C inhibitor, staurosporine, and also a tyrosine kinase inhibitor, genistein, to treat HeLa cells before invasion assays with EA. Curiously, staurosporine had no effect whereas genistein moderately inhibited the invasion of EAs in HeLa cells. In Vero cells, genistein had no effect but staurosporine inhibited EA invasion by 82%. The role of RhoA GTPases in host cell invasion by EAs of both G and CL strains of *T. cruzi* was subsequently studied. Rho GTPases regulate three separate signal transduction pathways, linking plasma membrane receptors to the assembly of distinct actin filament structures. Fernandes and Mortara (2004) used non-polarized MDCK cells transfected with different Rho GTPase constructs (RhoA, Rac1, and Cdc42). EA invasion was particularly high in MDCK cells overexpressing either wild type or constitutively active Rac1. Consistently, EA invasion was specifically reduced in the corresponding dominant negative line, suggesting a key role for Rac1-GTPase in the invasion process (Fernandes and Mortara, 2004). On the other hand, in

contrast to a number of bacterial invasion mechanisms (Mounier et al., 2003), amastigote invasion seems to be independent of Cdc42.

Sonicated extracts from G or CL strain of EA induced transient enhancement in HeLa cell intracellular Ca^{2+} levels in a dose-dependent manner. Inhibition of HeLa cell intracellular Ca^{2+} mobilization by thapsigargin or caffeine moderately reduced the infectivity of both G and CL strains (Fernandes et al., 2006). Thus, EAs of both strains trigger calcium signaling in HeLa cells that may be important for the success of EA invasion. Adenylyl cyclase based activation of HeLa cells by exposure to forskolin did not affect infection by either strain. The activation of PI3 kinase in host cells appears to be required for invasion by either the G or CL strain since treatment of HeLa cells with wortmannin reduced EA infectivity (Fernandes et al., 2006). It has also been described that the use of PI3 kinase inhibitors impairs EA (Y strain) internalization into peritoneal macrophages by 60% (Barrias et al., 2010).

We have recently observed that the treatment of non-phagocytic cells with a Src-family tyrosine kinase (SFK) inhibitor, in the absence of serum, reduces EA (G strain) invasion (Bahia et al., unpublished). In addition, EA of the G strain induced time-dependent HeLa cell phosphorylation of SFKs, whose members include Src, Lyn, Fyn, Lck, Yes, and Hck. At this stage, however, the precise Src members involved in this pathway have not been identified. These results suggest that EAs may also exploit the host cell Src pathway in order to invade cells.

EAs AND THE HOST CELL POINT OF VIEW

Host cell plasma membrane microdomains were also shown to be involved in EA *T. cruzi* entry into non-phagocytic cells. Membrane microdomains, also known as lipid rafts, play a unique role in signal transduction by compartmentalizing cell receptors and subsequently boosting downstream signaling to the effectors molecules (Simons and Ikonen, 1997). Cholesterol, the major component of membrane lipid rafts of Vero or HeLa cells was disrupted by methyl β-cyclodextrin (MβCD) and then infected with EAs of G (TcI) and CL (TcVI) strains (Fernandes et al., 2007). Removal of cholesterol from both host cell lines significantly decreased the invasion index of EAs, indicating that host cell cholesterol and/or membrane organization is important for the entry process of both infective forms. After cholesterol repletion, the invasion index of G strain EAs was almost fully recovered. Subunit B of cholera toxin binds to the GM1 ganglioside, a marker for lipid rafts, and the treatment of non-phagocytic cells with CTB (cholera Toxin-B) also decreased EA invasion. In macrophages, the participation of lipid rafts in the internalization of EAs has also been described (Barrias et al., 2007).

Barrias et al. (2010) showed that dynamin, a large GTPase that belongs to a protein superfamily with a critical role in endocytic membrane fission events, plays a role in EA internalization into peritoneal macrophages. Researchers treated macrophages with dynasore, a reagent that has the ability to block the GTPase activity of dynamin, and observed a marked inhibition in the internalization of EAs. However, dynasore did not significantly interfere with parasite adhesion to host cells.

Bambino-Medeiros et al. (2011) showed that amastigote invasion also involves host cell surface heparan sulfate proteoglycans, which localize to signaling membrane microdomains such as lipid rafts. By treating EAs of the Dm28c strain with heparin or heparan sulfate, researchers observed inhibition of EA entry into primary cultures of cardiomyocytes. The authors hypothesized that the binding of amastigote heparin-binding protein could activate different signaling pathways, such as phosphorylation of cortactin by Src activation that would lead to actin polymerization and amastigote entry.

Along these lines, we are currently evaluating the role of protein kinase D (PKD) and cortactin in EA uptake by HeLa cells (Bonfim-Melo, unpublished). PKD is a family of multidomain enzymes (PKD1, 2, and 3). PKD lies downstream of PKCs in a novel signal transduction pathway implicated in the regulation of multiple fundamental biological processes. At the leading edge of migrating cells, active PKD co-localizes with F-actin, Arp2/3, and cortactin. Cortactin has emerged as a key signaling protein in cellular processes such as endocytosis and tumor invasion by interacting with and/or altering the cortical actin network. PKD is an upstream regulator of cortactin. EAs not only recruit PKD (**Figure 2**) and

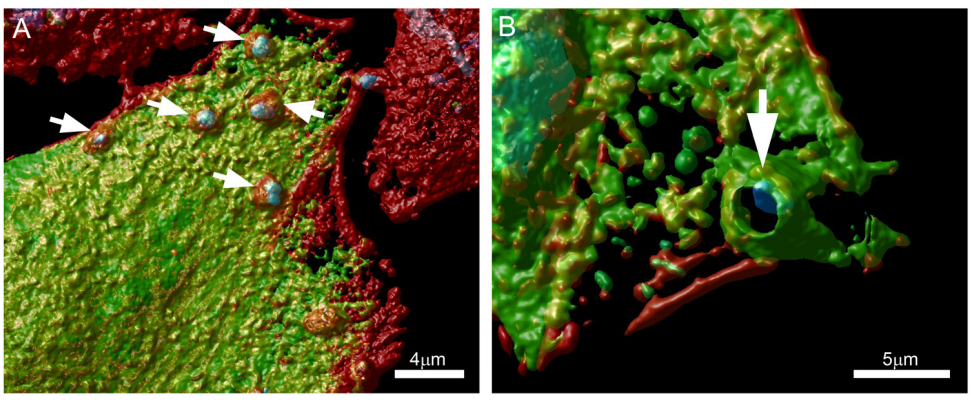

FIGURE 2 | Extracellular amastigotes (EAs) were incubated for 1 h with (A) Vero or (B) CHO cells transfected with a GFP tagged PKD (green) and stained with phalloidin-TRITC (actin) and DAPI (cells nuclei). Arrows show EA recruiting PKD at the invasion sites. Confocal microscopy followed by Huygens Surface Rendering (www.svi.nl). Magnification bars = 4 μm **(A)** and 5 μm **(B)**.

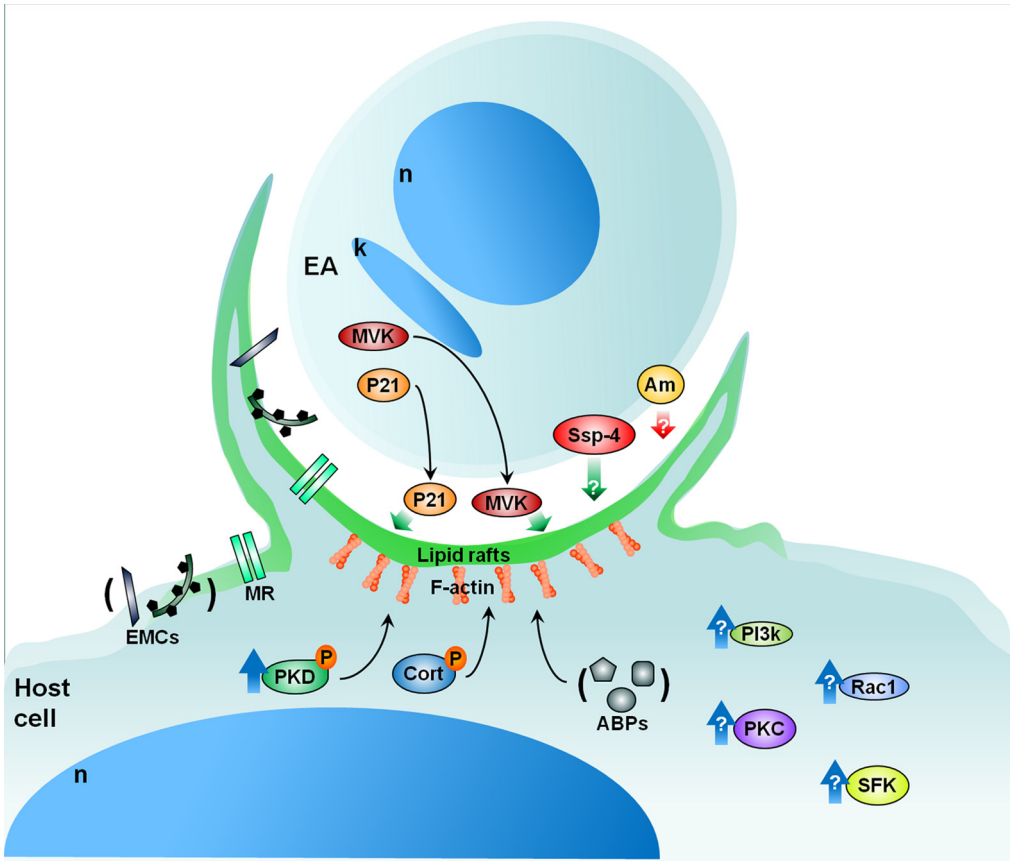

FIGURE 3 | Proposed model for EA signaling. EAs secrete factors such as P21 and MVK that aid in EA uptake by cells in culture. EAs also present specific glycoproteins (i.e., Ssp-4) important for attachment and penetration while other EA glycoproteins (i.e., amastins-Am) may negatively modulate amastigote invasion. From the cellular perspective, lipid rafts are essential to parasite internalization in both macrophages and epithelial cells. Furthermore, the mannose receptor of certain cells may recognize mannose residues expressed by EAs which also participate in EA uptake. Upon contact, EAs recruit PKD, actin (F-actin), and other actin-binding proteins (ABPs) such as cortactin (Cort), gelsolin, and α-actinin. Additionally, EAs likely activate Src-family kinase (SFK), PKC, and Rac1 signaling in host cells. The phosphatidylinositol 3-kinases (PI3k) pathway may also be involved in EA internalization. Cellular heparan sulfate and fibronectin are also important in the process of EA internalization (extracellular matrix components – ECMs). Red and green arrows: negative and positive modulation of the invasion, respectively; blue arrows: host protein activation; black arrows: recruitment. P, phosphorylation; n, nucleus; k, kinetoplast; MVK, mevalonate kinase; MR, mannose receptor. See text for more details.

cortactin to the invasion sites of epithelial non-phagocytic cells, but also induce the phosphorylation of these proteins (Bonfim-Melo, unpublished). These results suggest that unexpected novel roads may also be utilized by *T. cruzi* to invade cells. In order to summarize, the putative cellular molecules and signaling pathways used by EAs are presented in **Figure 3**.

CONCLUDING REMARKS

Extracellular amastigotes of *T. cruzi* are an infective parasite stage that mobilizes molecules and pathways distinct from those engaged by the classical infective trypomastigote forms. This repertoire of molecules may include not only previously characterized carbohydrate epitopes and the P21 secreted component, but also novel components described here such as the secreted form of MVK and the cortactin–PKD pathway.

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Cell signaling during *Trypanosoma cruzi* invasion

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Cell signaling is an essential requirement for mammalian cell invasion by *Trypanosoma cruzi*. Depending on the parasite strain and the parasite developmental form, distinct signaling pathways may be induced. In this short review, we focus on the data coming from studies with metacyclic trypomastigotes (MT) generated *in vitro* and tissue culture-derived trypomastigotes (TCT), used as counterparts of insect-borne and bloodstream parasites, respectively. During invasion of host cells by MT or TCT, intracellular Ca^{2+} mobilization and host cell lysosomal exocytosis are triggered. Invasion mediated by MT surface molecule gp82 requires the activation of mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase (PI3K), and protein kinase C (PKC) in the host cell, associated with Ca^{2+}-dependent disruption of the actin cytoskeleton. In MT, protein tyrosine kinase, PI3K, phospholipase C, and PKC appear to be activated. TCT invasion, on the other hand, does not rely on mTOR activation, rather on target cell PI3K, and may involve the host cell autophagy for parasite internalization. Enzymes, such as oligopeptidase B and the major *T. cruzi* cysteine proteinase cruzipain, have been shown to generate molecules that induce target cell Ca^{2+} signal. In addition, TCT may trigger host cell responses mediated by transforming growth factor β receptor or integrin family member. Further investigations are needed for a more complete and detailed picture of *T. cruzi* invasion.

Keywords: *Trypanosoma cruzi*, cell invasion, cell signaling, Ca^{2+} mobilization, metacyclic rypomastigote, tissue culture trypomastigote

INTRODUCTION

The hallmark of host cell invasion by *Trypanosoma cruzi*, a process that involves diverse parasite and host cell components, is the activation of signal transduction pathways leading to elevation in cytosolic Ca^{2+} concentration in both cells (Docampo and Moreno, 1996; Burleigh and Andrews, 1998; Yoshida, 2006). Ca^{2+}-dependent disruption of host cell actin cytoskeleton that follows interaction with *T. cruzi* facilitates the mobilization of lysosomes to the cell periphery, where the fusion with the plasma membrane contributes for the biogenesis of parasitophorous vacuole, inhibition of this event resulting in impaired parasite internalization (Tardieux et al., 1992; Rodriguez et al., 1995; Martins et al., 2011).

Studies with metacyclic trypomastigotes (MT) generated *in vitro* and tissue culture-derived trypomastigotes (TCT), used as counterparts of insect-borne and bloodstream parasites, respectively, have disclosed that these developmental forms engage distinct sets of molecules and diverse strategies to induce host cell Ca^{2+} signaling and lysosomal exocytosis required for their internalization. Here we summarize the data from experiments performed mostly with non-phagocytic mammalian cells, aiming at understanding the signaling events that lead to *T. cruzi* invasion.

MT SURFACE MOLECULES THAT TRIGGER HOST CELL SIGNALING DURING INVASION

Adhesion to host cells is the first step for *T. cruzi* invasion. Surface glycoproteins with cell adhesion properties expressed in MT, such as gp90, gp82, gp30, and gp35/50, which are differentially expressed in different strains, bind to target cells in a receptor-mediated manner and trigger signaling pathways that may result or not in efficient parasite internalization (Yoshida, 2006).

Gp82, identified by the monoclonal antibody (mAb) 3F6, is a MT-specific surface molecule (Teixeira and Yoshida, 1986). It is a member of a multigene family that belongs to the gp85/*trans*-sialidase superfamily (Araya et al., 1994). Several pieces of evidence indicate that gp82 is engaged by highly infective *T. cruzi* strains to enter host cells (Ramirez et al., 1993; Cortez et al., 2012a). Gp82 is conserved among *T. cruzi* strains from divergent genetic groups, displaying >90% peptide sequence identity (Maeda et al., 2011). MT invasion mediated by gp82 triggers the target cell signaling cascades that result in cytosolic Ca^{2+} mobilization, an event detectable in mammalian cells susceptible to *T. cruzi* infection, such as HeLa and Vero cells, but not in *T. cruzi*-resistant K562 cells (Ruiz et al., 1998). Following gp82 recognition by its still undefined receptor, the available data indicate that Ca^{2+} is released from thapsigargin-sensitive stores, independent of inositol 1,4,5-triphosphate (IP_3), or upon activation of phospholipase C (PLC), generating diacylglycerol (DAG) and IP_3, the former activates protein kinase C (PKC) and the latter promotes Ca^{2+} release from IP_3-sensitive compartments such as endoplasmic reticulum (ER; Ferreira et al., 2006; Maeda et al., 2012; **Figure 1A**). In addition to PKC, two other kinases participate in gp82-mediated MT invasion, namely the mammalian target of rapamycin (mTOR), a conserved Ser/Thr kinase that regulates diverse cell processes, and the lipid kinase phosphatidylinositol 3-kinase (PI3K), as suggests the diminished parasite invasion of cells pretreated with specific inhibitors of these enzymes (Martins et al., 2011; **Figure 1A**). What

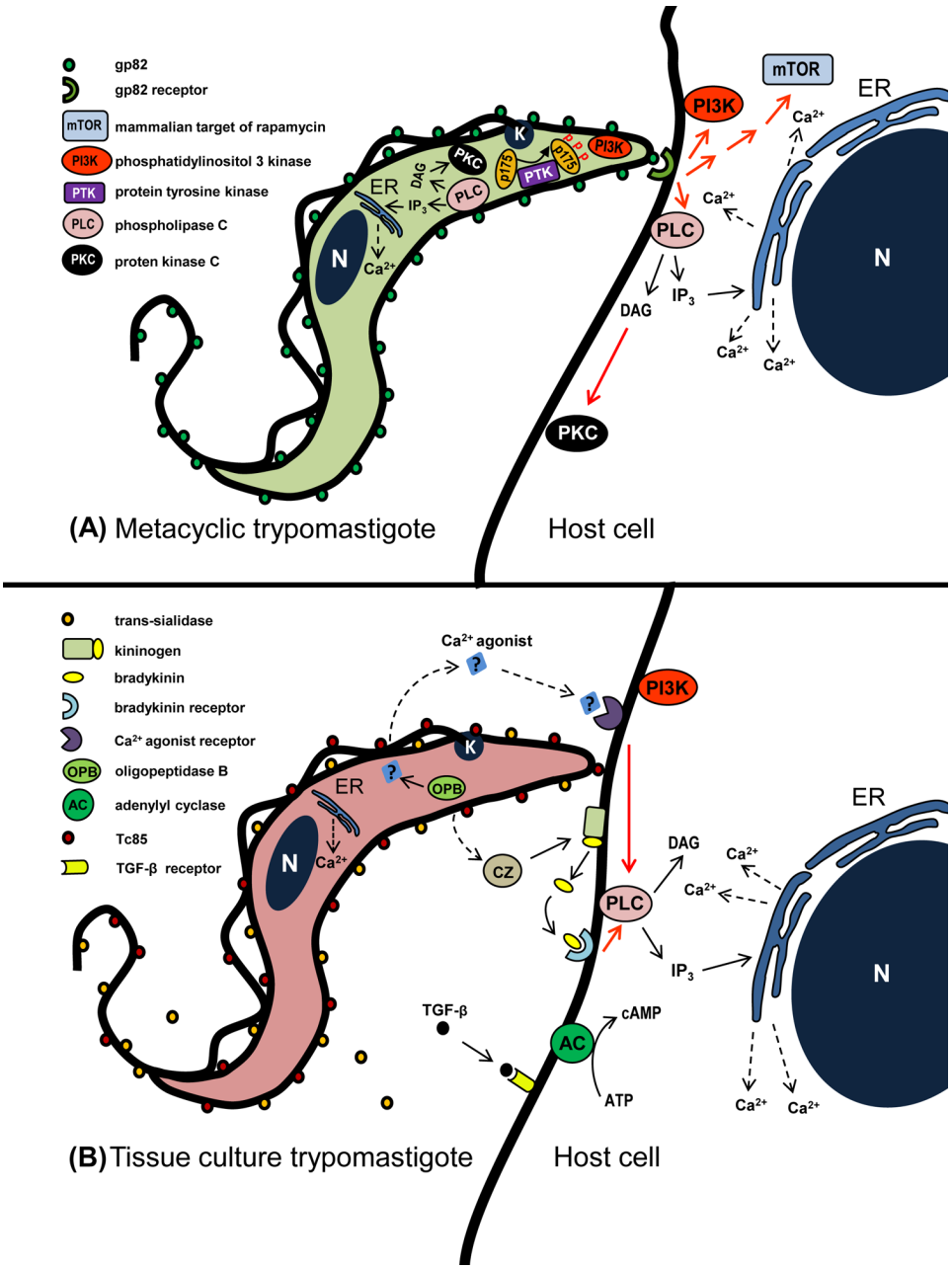

FIGURE 1 | Schematic representation of signaling molecules and pathways that may be activated during *T. cruzi* entry into non-phagocytic mammalian cells. (A) In metacyclic forms that enter host cells in gp82-mediated manner, activation of PLC generates DAG and IP_3. DAG stimulates PKC and IP_3 promotes Ca^{2+} release from IP_3-sensitive compartments. PI3K and PTK are also activated, the latter mediates phosphorylation of p175. In the host cell, the recognition of gp82 by its receptor triggers the activation of PI3K, mTOR, and PLC, the latter generating DAG and IP_3. DAG stimulates PKC and IP_3 promotes Ca^{2+} release from endoplasmic reticulum (ER). **(B)** During TCT interaction with host cells, a Ca^{2+} agonist generated by parasite OPB binds to its receptor and triggers PLC activation. Then IP_3-mediated Ca^{2+} release from ER ensues. Bradykinin, produced from kininogen by the action of TCT cruzipain, binds to bradykinin receptor and triggers PLC activation. Red arrows indicate activation, possibly not directly, but through as one or more as yet undefined elements.

are the connections between these kinases can only be inferred at this point from data available in other systems. PI3K may act on mTOR signaling, provided that the phosphorylation of downstream effectors of mTOR, such as S6K1 and 4E-BP1, is sensitive to rapamycin and also to PI3K inhibitor wortmannin (Chung et al., 1994; Mèndez et al., 1996; Hay and Sonenberg, 2004). Another possible functional association is between PKC and mTOR. A pathway linking epidermal growth factor receptor to mTOR that was critically dependent on PKC has been described in glioma (Fan et al., 2009) and the association of a mTOR homolog with PKC

has been demonstrated in *Saccharomyces cerevisiae* (Kumar et al., 2000). Under some circumstances, PI3K is activated upstream of PKC (Tong et al., 2000). Phosphatidylinositol-3,4,5-P_3, a product of PI3K, appears to directly initiate cellular motility via PKC activation (Derman et al., 1997).

The elevation of cytosolic Ca^{2+} concentration induced by gp82 promotes two associated events that facilitate MT invasion, namely the Ca^{2+}-dependent actin cytoskeleton disruption and lysosome mobilization that culminates in exocytosis (Cortez et al., 2006; Martins et al., 2011; **Figure 2**). During gp82-mediated MT invasion, recently internalized parasites are seen within vacuoles that incorporated lysosome markers (**Figure 2**).

Gp30, a MT-specific surface molecule recognized by mAb 3F6 and expressed in gp82-deficient *T. cruzi* strains, is also implicated in cell invasion (Cortez et al., 2003, 2012a). Like gp82, binding of gp30 to target cells induces Ca^{2+} response and lysosome exocytosis, presumably through activation of signaling pathways involving PI3K, mTOR, and PKC (Cortez et al., 2003, 2012a). Gp30 and gp82 are possibly recognized by the same receptor, as suggests the inhibition of host cell entry of both gp30- and gp82-expressing MT by mAb 3F6, as well as by recombinant proteins based on gp30 or gp82 (Cortez et al., 2003, 2012a).

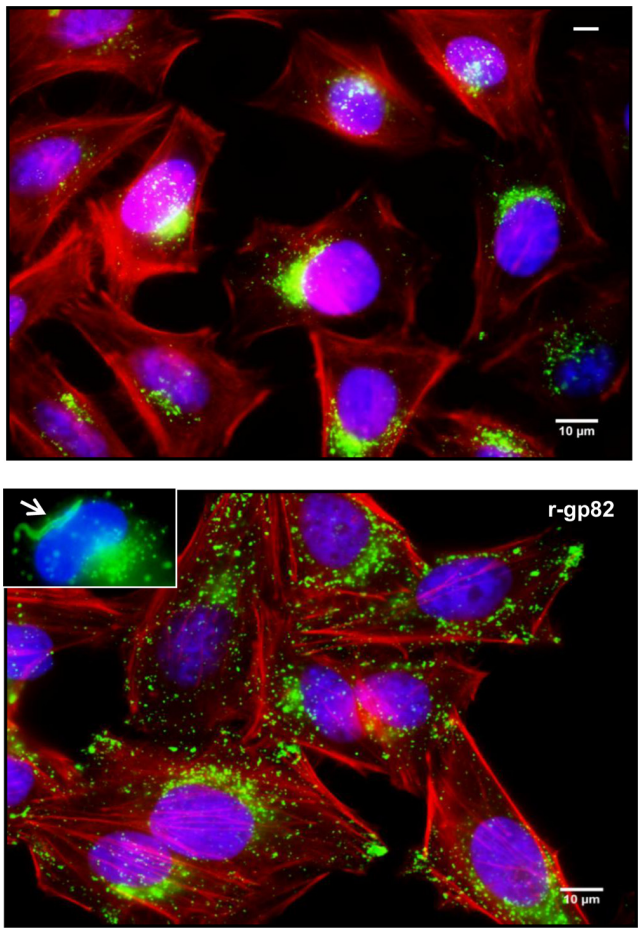

FIGURE 2 | Lysosome mobilization induced by MT gp82. Shown are HeLa cells incubated for 1 h in absence or in the presence of the recombinant protein (r-gp82) containing the complete gp82 sequence. Spread of lysosomes (green) from the perinuclear region to the cell periphery is induced by r-gp82. In the inset, recently internalized MT (arrow) inside the parasitophorous vacuole with lysosome marker is shown.

In addition to gp82 or gp30, MT of different *T. cruzi* strains express variable levels of distinct isoforms of a stage-specific surface molecule gp90, which functions as a negative regulator of parasite infectivity (Málaga and Yoshida, 2001). Expression of gp90 at high levels is invariably associated with reduced capacity to enter target cells (Yoshida, 2006). As opposed to gp82 or gp30, and consistent with its role as down modulator of MT invasion, gp90 does not trigger Ca^{2+} signal upon binding to host cells (Ruiz et al., 1998).

Metacyclic trypomastigotes surface molecules gp35/50 recognized by mAb 10D8, expressed in poorly infective *T. cruzi* strains, are highly glycosylated mucin-like glycoproteins enriched in sialic acid and galactose residues that interact with target cells through their carbohydrate portion (Yoshida et al., 1989; Mortara et al., 1992; Schenkman et al., 1993b). Binding of gp35/50 to target cells triggers intracellular Ca^{2+} elevation, but to a lower degree than gp82 (Ruiz et al., 1998). Removal of sialic acid from gp35/50 increases the capacity to trigger target cell Ca^{2+} response and potentiates MT invasion (Yoshida et al., 1997). It appears therefore that sialyl residues impair parasite–host cell interaction and this is in contrast with the findings with TCT (Schenkman et al., 1991). Gp35/50-mediated invasion apparently requires F-actin recruitment, an event that may be associated with activation of adenylyl cyclase that generates cAMP (Ferreira et al., 2006).

The role played by MT secreted components in parasite internalization remains to be investigated. One such component, SAP (serine-, alanine-, and proline-rich protein), which binds to target cells in a receptor-dependent manner and induces Ca^{2+} signal, participates in the gp82-mediated internalization of MT but plays no role in gp35/50-mediated invasion (Baida et al., 2006). It is possible that SAP acts synergistically with gp82, by triggering Ca^{2+} signal that adds to the response induced by gp82.

SIGNALING PATHWAYS ACTIVATED IN MT DURING INVASION

Gp82-mediated invasion of host cells by MT triggers Ca^{2+} mobilization in the parasite, through signaling cascades involving PLC activation, generation of DAG and IP_3, leading to Ca^{2+} release from IP_3-sensitive reservoirs and PKC stimulation (Yoshida et al., 2000; **Figure 1A**). In addition to involvement of PI3K (Maeda et al., 2012), a protein tyrosine kinase (PTK) activation results in phosphorylation of p175, a protein undetectable in non-infective epimastigotes (Favoreto et al., 1998; **Figure 1A**). PTK activation and Ca^{2+} response are possibly associated events, provided that they are both affected by genistein (Yoshida et al., 2000), a PTK inhibitor that reduces MT infectivity (Neira et al., 2002). MT that invade host cells in a gp35/50-mediated manner may require cAMP and acidocalcisomes, the vacuoles containing a Ca^{2+}/H^{+} exchange system (Docampo et al., 1995), appear to be the main source of Ca^{2+} required for parasite internalization (Neira et al., 2002).

TCT-INDUCED SIGNALING EVENTS IN TARGET CELLS

Diverse *T. cruzi* molecules, either secreted and/or expressed on the cell surface, have been implicated in TCT internalization. Among those known to have cell signal-inducing properties are cruzipain, *trans*-sialidase, trypomastigote small surface antigen (TSSA), and a soluble factor of undefined structure.

Cruzipain, the major *T. cruzi* cysteine proteinase expressed in all developmental forms of different strains (Murta et al., 1990; Paiva et al., 1998), participates in TCT internalization and in intracellular parasite development (Meirelles et al., 1992). From experiments using human umbilical vein endothelial cells or CHO cells overexpressing B_2 type of bradykinin receptor (B_2R), it was postulated that cruzipain acts on cell-bound kininogen and generates bradykinin that, upon recognition by B_2R triggers IP_3-mediated Ca^{2+} influx (Scharfstein et al., 2000; **Figure 1B**), thus promoting parasite invasion, a mechanism that is not ubiquitous, its activation depending on the cell type and the parasite isolate used. Higher expression of functional cruzipain does not correlate with parasite infectivity (Paiva et al., 1998).

Trypanosoma cruzi trans-sialidase (TS), an enzyme that specifically transfers alpha (2-3)-linked sialic acid from host-derived macromolecules to parasite surface molecules, facilitates TCT invasion by sialylating a TCT-specific epitope Ssp-3, which is recognized by target cells through its sialic acid residues and whose signaling properties are unknown (Schenkman et al., 1991). TS may function as a TCT ligand to host cell alpha 2,3-sialyl receptors as a prelude to invasion (Ming et al., 1993). Signaling activities of TS toward mammalian cells include activation of PI3K/Akt pathway that contributes for survival of Schwann cells (Chuenkova et al., 2001), of mitogen-activated protein kinase (MAPK) or extracellular regulated kinase (ERK) pathways that induce neurite outgrowth in PC12 cells (Chuenkova and Pereira, 2001). TCT binds to TrkA, a receptor tyrosine kinase activated primarily by nerve growth factor, in a manner mediated by TS, inducing TrkA autophosphorylation and PI3K/Akt kinase signaling through TrkA-dependent mechanisms (Chuenkova and PereiraPerrin, 2004). Whether these TS-induced signaling mechanisms are associated with TCT invasion is not known. The participation of host cell sialic acid in TCT invasion has been inferred using Chinese hamster ovary cell mutant that is much less susceptible to infection than the parental cell line (Ciavaglia et al., 1993; Ming et al., 1993; Schenkman et al., 1993a). In macrophages, removal of sialic acid with neuraminidase or its blockage with cationized ferritin increased TCT uptake (Araújo Jorge and de Souza, 1984; Meirelles et al., 1984).

Recently, Cánepa et al. (2012) reported that peptides based on TSSA, a mucin-like molecule rich in serine and threonine predicted to be *O*-glycosylated (Di Noia et al., 2002), bind to mammalian cells and induce Ca^{2+} signaling. The question whether the native glycosylated TSSA and synthetic TSSA peptides share the same cell adhesion and signaling properties has not been addressed.

A secreted TCT factor of unknown structure has been claimed to trigger host cell Ca^{2+} mobilization in IP_3-mediated manner (Rodriguez et al., 1995). According to Burleigh et al. (1997), the soluble TCT factor is produced by the action of cytosolic oligopeptidase B (OPB), an enzyme closely related to members of the prolyl oligopeptidase family of serine endopeptidases. The Ca^{2+} agonist, generated from a precursor molecule in TCT cytoplasm, would be exported and its recognition by a target cell receptor, followed by PLC activation and IP_3 production, would release Ca^{2+} from ER (Caler et al., 1998; **Figure 1B**). OPB null TCT had a diminished cell invasion capacity, a Ca^{2+} signal-inducing activity of low intensity and recruited lysosome in a significantly delayed fashion, but preserved the property to induce cAMP elevation in host cells (Caler et al., 2000), which is associated with the ability to potentiate Ca^{2+}-regulated lysosomal exocytosis (Rodriguez et al., 1999). While the ability of Ca^{2+} agonist produced by OPB in disrupting F-actin filaments is associated with increased TCT invasion (Rodriguez et al., 1995), there are reports indicating that actin cytoskeleton disruption results in diminished TCT entry into different cell types, including heart muscle cells (Meirelles et al., 1999; Rosestolato et al., 2002).

Several TCT surface molecules with affinity for extracellular matrix have been implicated in host cell invasion, but little is known about their signal-inducing properties. Among such molecules is a laminin-binding glycoprotein encoded by a multigene Tc85 family belonging to the gp85/*trans*-sialidase superfamily (Giordano et al., 1999). Conserved in all members of gp85/*trans*-sialidase glycoprotein family is the FLY domain (VTVXNVFLYNR). Peptide based on FLY binds to cytokeratin 18 (CK18) on the surface of LLC-MK(2) epithelial cells and promotes dephosphorylation and CK18 reorganization, activating ERK1/2 signaling pathway that leads to increased TCT internalization (Magdesian et al., 2001). This finding with peptide FLY is unlikely to bear any association with TCT entry into host cells because FLY domain is almost completely buried (Cortez et al., 2012b), therefore unavailable for interaction with CK18. In support of this view, transient silencing of CK18 gene in RNAi-treated HeLa cells did not affect binding and invasion of TCT (Claser et al., 2008). Furthermore, a recombinant protein based on amastigote surface protein-2 containing FLY domain failed to bind CK18 (Claser et al., 2008), consistent with the fact that FLY domain is not exposed on the surface.

Cell signaling events during TCT internalization, without association with specific *T. cruzi* molecules, have been reported by many authors. In different cell types, activation of PI3K emerges as a common feature for TCT invasion process. PI3K activated by TCT facilitates lysosome-dependent parasite entry into nonpahgocytic cells (Woolsey et al., 2003). In target cells invaded by a significant fraction of TCT through an lysosome-independent pathway, there is the formation of a host cell plasma membrane-derived vacuole enriched in the lipid products of class I PI3 kinases, initially devoid of lysosomal markers and gradually acquiring lysosome associated membrane protein 1 (Woolsey et al., 2003). This lysosome-independent early event is compatible with the finding that the newly forming TCT compartments first interact with an early endosome and subsequently with other late endosomes, before interaction with lysosomes (Wilkowsky et al., 2002). Using blood trypomastigotes and macrophages, Todorov et al. (2000) found that class I and class III PI3-kinase activities are involved in parasite internalization. PI3K recruitment and assembly of actin filaments were detected at the site of TCT interaction with macrophages (Vieira et al., 2002). In non-phagocytic

Vero, L_6E_9 and NIH 3T3 cells, as well as in human and J774 murine macrophages, PI3K inactivation was ascertained using specific PI3K inhibitors (Wilkowsky et al., 2001). Concomitant with PI3K activation, a strong activation of protein kinase B (PKB/Akt) occurs and, accordingly, transiently transfected cells containing an inactive mutant PKB are more resistant to infection by TCT as compared to the active mutant-transfected cells (Wilkowsky et al., 2001).

Tissue culture-derived trypomastigotes invasion of macrophages also requires PTKs (Vieira et al., 1994). Tyrosine-phosphorylated residues accumulate at the site of TCT association with the cell surface, co-localizing with macrophage F-actin-rich domains (Vieira et al., 2002). Activation of macrophage PKC induced by recombinant gp83, a TCT surface ligand, was also reported (Villalta et al., 1999). Protein phosphatases may also play a role in TCT internalization. Tyrosine dephosphorylation of several proteins is induced by TCT in L_6E_9 myoblasts and the cells, either treated with protein tyrosine phosphatase inhibitors or in the presence of excess phosphotyrosine, become more resistant to invasion by TCT (Zhong et al., 1998). The involvement of alkaline phosphatase has been deduced from experiments with human HEp2 tumor cells that, upon inhibition of the enzyme activity, exhibited a different pattern of actin organization and reduced susceptibility to TCT invasion (Sartori et al., 2003).

Several other host cell components have been implicated in TCT invasion. Ming et al. (1995) found that TCT induce a transforming growth factor β (TGF-β)-responsive reporter gene in TGF-β-sensitive cell lines, and epithelial cells lacking TGF-β receptor I or II, or with dysfunction of the intracellular signaling cascade due to constitutive expression of the cyclin-dependent kinase cdk4 or of the oncogene H-Ras, were more refractory to penetration by TCT. In experiments with human coronary artery smooth muscle cells expressing galectin-3, which increases K-Ras activation and triggers a Ras signal (Elad-Sfadia et al., 2004), there was a decreased TCT adhesion to cells with reduced expression of galectin-3, which was restored by exogenous galectin-3 (Kleshchenko et al., 2004). The β1 subunit of VLA integrin family that links the extracellular matrix to the cortical cytoskeleton was reported to be involved in TCT entry into human macrophages (Fernandez et al., 1993). Recently, acid sphingomyelinase (ASM) was claimed to be required for TCT invasion. Inhibition or depletion of lysosomal ASM markedly reduced the target cell susceptibility to TCT invasion, whereas extracellular addition of ASM stimulated endocytosis, enhanced parasite entry, and restored normal invasion levels in ASM-depleted cells, and ceramide, the product of sphingomyelin hydrolysis, was detected in newly formed parasitophorous vacuoles containing TCT (Fernandes et al., 2011). Triggering of autophagy was also associated with TCT internalization. Romano et al. (2009) have shown that treatment of host cells with mTOR inhibitor rapamycin increased lysosomal-dependent TCT invasion by inducing autophagy. This finding is in sharp contrast with gp82-mediated MT invasion that is inhibited by rapamycin (Martins et al., 2011). Also contrasting with gp82-mediated MT internalization that is impaired in cells prestarved for a short time (Martins et al., 2011), condition that triggers the autophagic pathway, TCT invasion increased upon prestarvation of target cells (Romano et al., 2009). In addition, the absence of Atg5 or the reduced expression of Beclin 1, proteins required at the initial steps of autophagosome formation, reduced the association of parasitophorous vacuole with the lysosomal marker Lamp-1 and diminished TCT entry (Romano et al., 2009).

CONCLUDING REMARKS

What emerges from the data on signaling events during host cell invasion by *T. cruzi* is a still fragmentary picture. Although many parasite as well as host cell components have been identified as playing roles in MT or TCT invasion, these may represent only a small part of the repertoire available for the accomplishment of the critical step for infection. The whole process is beginning to be understood at the molecular level. Furthermore, how the diverse elements are connected and what are the sequences of reactions that culminate in intracellular rearrangements that facilitate parasite internalization have as yet to be clarified. Therefore, a formidable task is still ahead before we can more fully understand the intricate functioning of molecular and cellular machinery involved in *T. cruzi* invasion.

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Sialic acid: a sweet swing between mammalian host and *Trypanosoma cruzi*

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Commonly found at the outermost ends of complex carbohydrates in extracellular medium or on outer cell membranes, sialic acids play important roles in a myriad of biological processes. Mammals synthesize sialic acid through a complex pathway, but *Trypanosoma cruzi*, the agent of Chagas' disease, evolved to obtain sialic acid from its host through a *trans*-sialidase (TcTS) reaction. Studies of the parasite cell surface architecture and biochemistry indicate that a unique system comprising sialoglycoproteins and sialyl-binding proteins assists the parasite in several functions including parasite survival, infectivity, and host–cell recognition. Additionally, TcTS activity is capable of extensively remodeling host cell glycomolecules, playing a role as virulence factor. This review presents the state of the art of parasite sialobiology, highlighting how the interplay between host and parasite sialic acid helps the pathogen to evade host defense mechanisms and ensure lifetime host parasitism.

Keywords: glycoconjugate, sialic acid, sialidase, parasite, immune response

THE SIALIC ACIDS

Sialic acids (Sia) are 9-carbon backbone acidic monosaccharides found at prominent positions of the sugar chains of glycoconjugates present on cell membranes or secreted into the extracellular medium. The most common members of this family are the *N*-acetylneuraminic acid [Neu5Ac] and its derivative the *N*-glycolylneuraminic acid [Neu5Gc] that differ from each other at position 5 (C-5), which is substituted with an acetamido or a hydroxyacetamido moiety respectively (**Figure 1**).

The metabolism of Sia in mammals involves 32 genes that encode enzymes and transporters, distributed among the different compartments of the cell (Wickramasinghe and Medrano, 2011). The final product of this complex biosynthetic pathway, the activated form of Sia (CMP-Sia), is transferred to the non-reducing end of newly synthesized glycan chains by a family of sialyltransferases present in the Golgi lumen. In vertebrates, Sia are commonly linked via an α2–3 linkage to galactopyranose (Gal*p*), via an α2–6 linkage to Gal*p* and *N*-acetylgalactosamine (GalNAc), or via an α2–8 linkage to another Sia (Varki et al., 2009).

Sia-containing glycoconjugates are involved in a myriad of cell functions. Because of the negative charge, these molecules affect the recognition and anti-recognition phenomena. They are targets of Sia-binding lectins and can mask underlying structures, for example impeding the binding of Gal-specific receptors (Sørensen et al., 2009; Rabinovich et al., 2012).

Sia are also important as recognition sites in host–pathogen interactions acting as ligands for parasite adherence, possibly driving natural selection (Varki, 2006). Further, Sia play a major role protecting the infective agent from the host's immune response through molecular mimicry while unsialylated strains are rapidly cleared. The evolutionary advantage of this molecular mimicry is so evident that several parasites exploit cell-surface sialylation to survive within the host environment and establish the infection (Vimr and Lichtensteiger, 2002).

One of the most elegant mechanisms of cell-surface sialylation is exploited by the protozoal parasite *Trypanosoma cruzi*, the etiological agent of Chagas' disease (Coura and Viñas, 2010). *T. cruzi* is not able to synthesize Sia. Instead the parasite scavenges it from host's sialyl glycoconjugates using its *trans*-sialidase activity (TcTS; Previato et al., 1985). TcTS is a modified sialidase, which preferentially transfers α2-3-linked Sia from sialyl β-Gal*p* donor complexes of exogenous origin to acceptor surface mucin-like glycoproteins containing terminal β-galactopyranose residues (**Figure 2**). In the absence of suitable acceptors TcTS transfers Sia to water, then acting as a sialidase (Vandekerckhove et al., 1992).

Once restricted to Latin America, where it affects ∼10 million people, Chaga's disease become a new worldwide challenge. It has now spread to North America, Europe, and the western Pacific region (Chappuis et al., 2010; Clayton, 2010). Molecules of the TcTS family (Freitas et al., 2011) and Sia acceptor glycoproteins (De Pablos and Osuna, 2012) are encoded by hundreds of genes. Combined, the TcTS and the mucin-like glycoproteins are likely to cover most of the parasite surface (**Figure 3B**) creating a parasite–host interface (**Figure 3A**)

In this review we discuss the importance of this unique biological system, comprised by both TcTS and mucin-like Sia acceptor glycoproteins, highlighting how the parasite explores host cell sialylation to establish infection for the lifetime of its host.

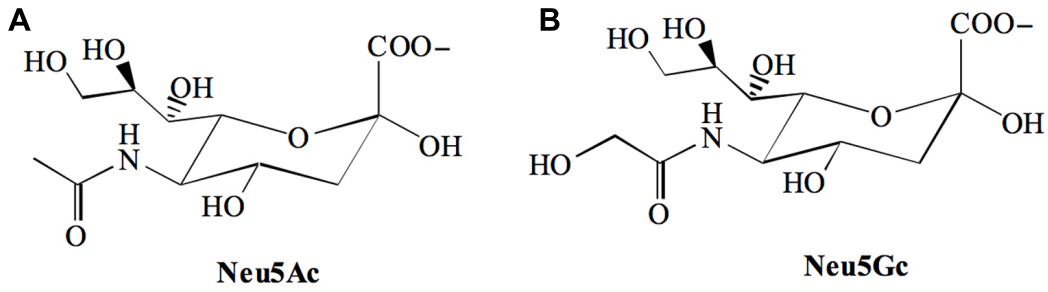

FIGURE 1 | The two major sialic acid found in mammals. (A) *N*-acetylneuraminic acid [Neu5Ac] and, **(B)** *N*-glycolylneuraminic acid [Neu5Gc].

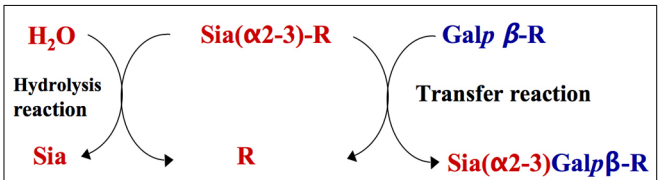

FIGURE 2 | Representation of transfer and hydrolysis activities of TcTS. TcTS preferentially catalyzes the transfer of sialic acid residues from Siaα2–3Galβ1-R containing donors and attaches them in α2–3 linkage to terminal β-Gal*p* containing acceptors (transfer reaction). In the absence of a carbohydrate acceptor, TcTS irreversibly transfers sialic acid to a water molecule, thus functioning as a sialidase (hydrolysis reaction).

THE SIALIC ACID ACCEPTORS ON THE SURFACE OF THE PARASITE AND ITS ROLE IN HOST PARASITE INTERACTION

The Sia acceptors on the surface of *T. cruzi* are mainly a family of highly *O*-glycosylated, threonine-rich mucin like glycoproteins (**Figure 3D**; Buscaglia et al., 2006; Mendonça-Previato et al., 2008) which are glycosylphosphatidylinositol (GPI)-anchored to the parasite membrane (Previato et al., 1995). The Tc-mucins are the major expressed component on the of *T. cruzi* (2×10^6 copies per parasite) and are the third most widely expanded gene family in the genome, comprising more than 1000 genes (Acosta-Serrano et al., 2001; Campo et al., 2004; El-Sayed et al., 2005; De Pablos and Osuna, 2012). Carrying up to 60% of its total mass in carbohydrates, mucins form an elaborate and highly decorated glycocalyx that allows the parasite to interact with and respond to its external environment. Furthermore, glycoproteins expressed on the parasite surface are known to be the major targets of protective immune responses, and this selective pressure presumably drives their expansion and variation among different strains (Minning et al., 2011).

The glycan structure of mucin is complex and heterogeneous among different *T. cruzi* strains. The structure of the oligosaccharides *O*-linked to the mucins of the non-infective epimastigote forms have been described (Previato et al., 1994, 1995; Todeschini et al., 2001, 2009; Agrellos et al., 2003; Jones et al., 2004). These *O*-glycans differ from those found in mammalian systems in three main aspects: (i) they are linked to the peptide backbone through an α-*N*-acetylglucosamine (α-GlcNAc) residue (Previato et al., 1998), rather than an α-*N*-acetylgalactosamine (α-GalNAc; Gill et al., 2011); (ii) they are further substituted by (Gal) on *O*-4 and *O*-6 rather than *O*-3 and *O*-6 as found in mammalian mucins (Varki et al., 2009); (iii) several strains carry a β-galactofuranose (β-Gal*f*) attached to the GlcNAc *O*-4 (Previato et al., 1994; Agrellos et al., 2003; Jones et al., 2004). Two core families were characterized with substitution of the α-GlcNAc *O*-4 by either β-Gal*p* or β-Gal*f*. The Gal*f*β1-4GlcNAc core can be further elaborated by the action of galactopyranosyl or galactofuranosyl transferases. Additionally, substitution at the GlcNAc-ol *O*-6 can occur. Addition of β-Gal*f* to mucins must be verified given that *T. cruzi* presents dozens β-galactofuranosyl transferase genes (El-Sayed et al., 2005; De Pablos and Osuna, 2012) while mammalian hosts glycoconjugates do not present this modification. β-Gal*f* residues might account for host parasite selection since the presence of β-Gal*f*-containing mucins are expressed mainly by strains involved in the sylvatic cycle of *T. cruzi* (Jones et al., 2004).

The major sialylated oligosaccharides (**Figure 3C**) so far characterized comprise a Neu5Acα2-3Gal*p*β1-4GlcNAc (Jones et al., 2004), a Gal*f*β1-4(Neu5Acα2-3Gal*p*β16)GlcNAc (Agrellos et al., 2003), a Gal*p*β1-4(Neu5Acα2-3Gal*p*β1-6)GlcNAc, and a Gal*p*β1-6(Neu5Acα2-3Gal*p*β(1-4)GlcNAc (Todeschini et al., 2001). Characterization of the related monosialylated glycans confirms a previous report showing that incorporation of one Sia residue onto an acceptor hinders entry of a second residue (Previato et al., 1995). Thus, it is possible that concomitant presence of both α2-3-linked Sia and terminal β-Gal*p* residues have biological implications in the *T. cruzi*/host cell interaction.

In addition to the *O*-glycosylation observed in the mucins of epimastigote forms, mucin glycans of infective trypomastigotes derived from infected mammalian cells also contain terminal α-galactosyl residues. α-Gal containing saccharides are epitopes recognized by lytic antibodies found in chronic Chagasic patients (Almeida et al., 1994). Sialylation of parasite glycoconjugates confers significant resistance to killing by the lytic antibodies, which is in agreement with the hypothesis that sialylation must favor parasite survival (Pereira-Chioccola et al., 2000). Further evidence corroborating this hypothesis is that the presence of Sias on the parasite mucins may compromise the activation of the complement pathway (Tomlinson et al., 1994). After sialidase treatment, parasites become more sensitive to complement-induced lysis. Also, parasite uptake by macrophages is increased (Tomlinson et al., 1994).

The importance of Sia on the parasite surface during host cell infection is still controversial. While some studies have shown

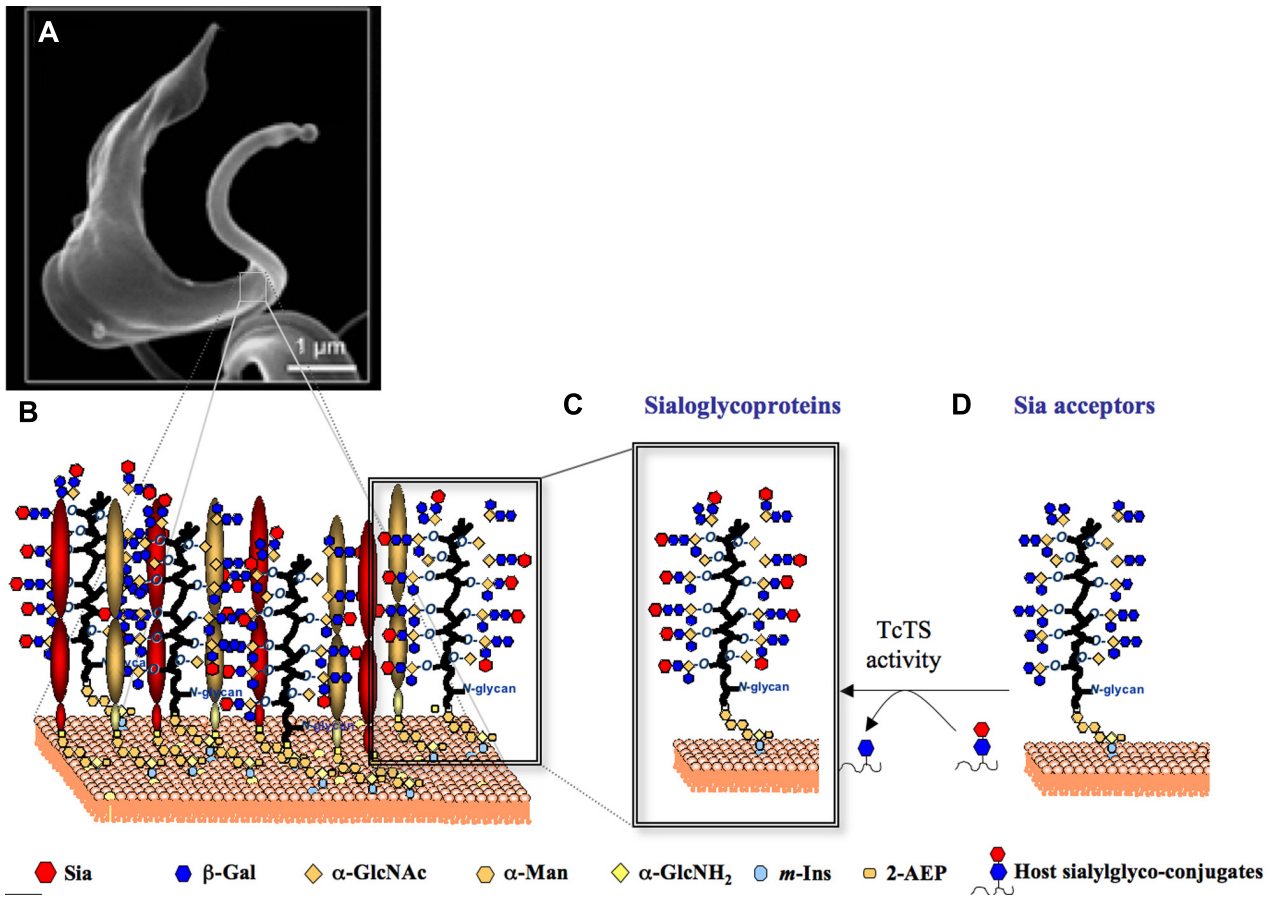

FIGURE 3 | Representation of the TcTS/sialoglycoproteins system on *T. cruzi* trypomastigotes surface. (A) A scanning electron micrograph of a bloodstream form *T. cruzi* trypomastigote (www.fiocruz.br/chagas_eng/cgi/cgilua.exe/sys/start.htm?sid=13) is shown. **(B)** The cartoon represents the GPI-anchored TcTS, $TcTS_{Y342H}$/sialoglycoproteins covering the trypomastigote surface. Sia present on *T. cruzi* sialoglycoproteins **(C)** is transfered from host sialoglycoconjugates to β-Gal*p* containing glycoproteins expressed on parasite surface **(D)** by TcTS activity.

that the presence of Sias in parasite epitopes increases *T. cruzi* infection (Piras et al., 1987; Schenkman et al., 1991), other groups suggest that the presence of Sias is not a requirement and/or may compromise the invasion of host cells (Araújo-Jorge and De Souza, 1988; Yoshida et al., 1997).

Finally, there are increasing evidences to support the Sia-binding Ig-like lectin (Siglecs), on the host cell surface, as the coreceptor for *T. cruzi* mucin (Erdmann et al., 2009; Jacobs et al., 2010). The Siglecs are a family of sialic-acid-binding immunoglobulin-like lectins that promote cell–cell interactions and regulate the functions of cells in the innate and adaptive immune systems through glycan recognition (Varki and Gagneux, 2012). It was demonstrated that *T. cruzi* mucin engagement with the Sia-binding protein Siglec-E promotes immunosuppression of dendritic cells (DC; Erdmann et al., 2009).

TcTS ACTIVITY AND ITS ROLE IN HOST PARASITE INTERACTION

TcTS is part of a protein family known as trans-sialidase/trans-sialidase-like, encoded by more than 1,400 genes (El-Sayed et al., 2005; Freitas et al., 2011). Members of the TcTS gene family can be classified in five groups based on sequence similarity and functional properties (Freitas et al., 2011). Active TS, also namely SAPA (shed acute-phase antigen) expressed by the infective trypomastigote (tTS) and the epimastigote TS (eTS) are grouped into Group I. eTS and tTS have identical enzymatic activities, being highly conserved in their primary sequences (Chaves et al., 1993; Briones et al., 1995; Jager et al., 2008), except for the SAPA domain and their 3′ UTRs, which are completely different in sequence (Jager et al., 2007). Besides, eTS is a trans-membrane protein, while the tTS is associated with the membrane via a GPI linker (Agusti et al., 1997). Group II comprises members of the gp85 surface glycoproteins TSA-1, SA85, gp90, gp82, and ASP-2, which have been implicated in host cell attachment and invasion. FL-160, a representative of group III, is a complementary regulatory protein that inhibits the alternative and classical complement pathways. TsTc13, whose function is unknown, is the representative of group IV and is included in the TcS superfamily because it contains the conserved VTVxNVxL (Alves and Colli, 2008; Yoshida and Cortez, 2008; Souza et al., 2010). Recently a sequence clustering analysis demonstrated that TS family is even more complex and may arbor more groups and subgroups (Freitas et al., 2011).

Several studies suggest that the TcTS can sialylate or desialylate host cells modulating parasite adherence and penetration. Results with Sia-deficient mutants of Chinese hamster ovary (CHO) cells support this hypothesis (Ciavaglia et al., 1993; Ming et al., 1993). Sia deficient cells were less infected than wild-type cells, suggesting that sialylation of glycoconjugates on CHO cells surface is necessary during *T. cruzi* invasion. Moreover, treatment of cells with modified Sia precursors, *N*-propionylmannosamine and other *N*-acylmannosamines, decreased cell invasion by *T. cruzi* (Lieke et al., 2011). Importance of TcTS enzymatic activity in host cell invasion was elegantly proven using an irreversible inhibitor (Carvalho et al., 2010a).

On the other hand, desialylation of sialoglycoproteins found in the membrane of phagolysosomes by TcTS is thought to be important for the escape of the parasite from the cytoplasm of infected cells (Hall et al., 1992; Hall and Joiner, 1993; Rubin-de-Celis et al., 2006).

Glycosylphosphatidylinositol-linked trypomastigote-derived TcTS can be released into the extracellular medium in fairly high amounts during acute *T. cruzi* infection in humans, thus acting distant from the parasite as a soluble factor. Besides its role in mammalian cell invasion, the soluble form of TcTS functions as a virulence determinant molecule, and therefore, could have relevant biological effects on the host immune system. It has been demonstrated (Chuenkova and Pereira, 1995) that *in vivo* injection of tiny amounts of purified native TcTS increases subsequent parasitemia and mortality in *T. cruzi*-infected mice. The effect observed was specific for the transfer activity of TcTS because it did not occur in mice primed with viral or bacterial sialidases. The mechanisms responsible for these effects were not determined but, since TcTS injection into deficient SCID mice did not affect parasitemia or mortality, it was suggested that the enzyme acts on host lymphocytes of the acquired immune system (Chuenkova and Pereira, 1995).

Indeed, multiple effects of TcTS on host T-lymphocyte function were additionally demonstrated. TcTS engagement with α2-3-linked Sia-containing epitopes on CD43 (Todeschini et al., 2002a) from $CD4^+$ T cells triggers costimulatory responses that increase mitogenesis and cytokine secretion, as well as promote rescue from apoptosis (Todeschini et al., 2002b). These results strongly suggest that TcTS could be a key parasite molecule inducing host polyclonal lymphocyte activation, seen as a condition underlying induction of immunopathology and hampering effective vaccination (Minoprio et al., 1989) in the course of *T. cruzi* infection.

Given that surface sialylation might be crucial to decide the final fate of the cells during interaction with thymic lectins (Gillespie et al., 1993; Priatel et al., 2000) alteration of cell sialylation by the soluble TcTS might influence thymocyte development. In fact, alteration of the surface sialylation by TcTS (Mucci et al., 2006) leads to *in vivo* depletion of the $CD4^+CD8^+$ double-positive thymocytes inside the "nurse cell complex" (Leguizamón et al., 1999). Interestingly, thymocyte apoptosis observed after the sialyl residue mobilization requires the presence of androgens (Mucci et al., 2005), suggesting the presence of a dimorphic glycosylation survey in the development of the T cell compartment that can be related to the observed differences in the immune response among sexes (Gui et al., 2012). However, further studies about the molecular mechanism involved in the pro-apoptotic effect of TcTS are necessary. However, we can speculate that TcTS activity can mask or expose β-Gal*p* which is recognized by molecules of the galectin family. Corroborating this hypothesis a role was reported for galectin-3 in death of $CD4^+CD8^+$ immature thymocytes and migration of these cells away from the thymus after *T. cruzi* infection (Silva-Monteiro et al., 2007).

The impact of sialylation mediated by TcTS on $CD8^+$ T cell response of mice infected with *T. cruzi* is an exciting example of how a parasite can manipulate host cell sialylation to favor parasitism. Following infection $CD8^+$ T cell responses are robust and persistent. However, they are significantly delayed (Garg et al., 1997; Tzelepis et al., 2007). This delay contrasts with the rapid appearance of $CD8^+$ T cell responses in other viral, bacterial and even protozoal infections (Kaech et al., 2002), and suggests a operative mechanism of immune evasion. During T cell activation, down-regulation of sialyltransferases (Amado et al., 2004) renders potential Sia acceptors accessible to sialylation through TcTS activity (**Figure 4**). This sialylation may be advantageous to the parasite, since $CD8^+$ T cells resialylated by TcTS present compromised Ag-specific responses and TcTS-treated mice present increased parasitemia (Freire-de-Lima et al., 2010). Cell surface Sia on $CD8^+$ T cells might increase intercellular repulsion and therefore weaken TCR/MHC class I-mediated cell–cell interactions. This would be the opposite of the effect of neuraminidase treatment, which removes Sia residues from various membrane glycoproteins and enhances lymphocyte proliferation (Harrington et al., 2000). In an attempt to establish the nature of the Sia acceptor for TcTS on the $CD8^+$ T cell surface, $CD8^+$ T cells from mice lacking the ST3Gal-I sialyltransferase, an enzyme required for sialylation of core 1 *O*-glycans (Priatel et al., 2000), were infected with *T. cruzi*. Loss of ST3Gal-I sialyltransferase exposes the Galβ1-3GalNAc-Ser/Thr moiety creating an interesting model to establish CD43 as a natural receptor for native TcTS during *T. cruzi* infection. Indeed, infection of mice lacking ST3-Gal-I sialyltransferase restores, at least in part, binding of anti-CD43 S7 mAb, which recognizes Sia-containing epitopes on CD43 of $CD8^+$ T cells. These findings indicated that CD43 is a target receptor for TS on the $CD8^+$ T cell surface. However, resialylation by TcTS was also observed on $CD8^+$ T cells from CD43 KO mice, suggesting that in the absence of CD43 other molecules are substrates for TcTS. Other studies using azido-modified unnatural Sia revealed that CD45 isoforms are Sia acceptors for TcTS activity as well (Muiá et al., 2010).

In infected individuals, alteration of cell surface sialylation by TcTS can also compromise host cell homeostasis. Tribulatti et al. (2005) demonstrated that the administration of TcTS into uninfected mice was able to reduce the Sia content of platelets, exposing terminal galactose residues, which may explain the severe thrombocytopenia observed in *T. cruzi* infected individuals. The recognition of terminal galactose moiety exposed on the platelet surface accelerates platelet clearance by asialoglycoprotein receptor-expressing scavenger cells (Sørensen et al., 2009). The effect of TcTS on the lifetime of other cell types and plasma glycoproteins must be further verified.

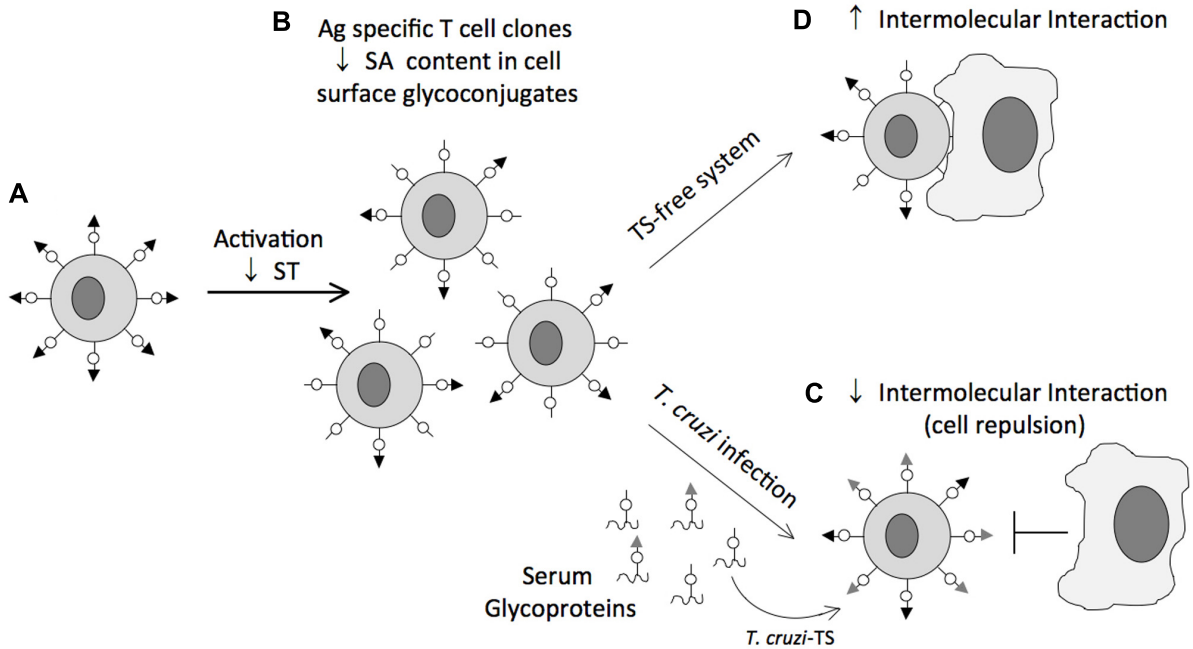

FIGURE 4 | Representative scheme of the glycosylation status of CD8$^+$ T cells during *T. cruzi* infection. Naïve CD8$^+$ T cells are heavily sialylated **(A)**. During T cell activation, down-regulation of sialyltransferases (ST) renders potential sialic acid acceptors accessible to sialylation through TcTS activity **(B)**. This sialylation may be advantageous to the parasite, because CD8$^+$ T cells resialylated by TcTS present compromised Ag-specific responses since sialic acid charge might increases intercellular repulsion and therefore weakens TCR/MHC class I-mediated cell–cell interactions **(C)**. Absence of TS may favor the TCR/MHC class I cell–cell interaction consequently, development of the immune response **(D)**.

Beyond host immune response, it has been observed that TcTS alters the sialylation status of the tyrosine kinase receptor-A (TrkA) in PC12 cells, which leads to receptor internalization, activation, and neuronal differentiation (Woronowicz et al., 2004). Authors demonstrated that the effects observed are triggered by hydrolysis of Sia residues of TrkA by TcTS, as a purified recombinant α2–3-neuraminidase but not a catalytically inactive mutant of TcTS induced the receptor phosphorylation. Such enzymatic activity might be involved in the neural repair and neuroprotection mediated by the TcTS, also called *T. cruzi*-derived neurotrophic factor (Chuenkova and Pereiraperrin, 2011).

The examples described in this section strongly suggest that *T. cruzi* exploits the glycosylation of molecules expressed by the host to evasion of the immune response, thus perpetuating the infection.

INACTIVE TcTS

Inactive TcTS (TcTS$_{Y342H}$) is a parasite adhesin that differs from the active TcTS due to a single mutation of catalytic residue Tyr342, which is mainly changed by a histidine (Cremona et al., 1995). In some *T. cruzi* strains, genes encoding TcTS$_{Y342H}$ members are present in the same copy number as those encoding TcTS (Cremona et al., 1999). However, further studies should be performed in order to address the expression levels and the ratio of the TcTS: TcTS$_{Y342H}$ protein on *T. cruzi* surface.

TcTS$_{Y342H}$ is a unique adhesin containing two sugar binding sites: one for α2,3-Sia and other for β-Gal*p* (Todeschini et al., 2002a, 2004; Oppezzo et al., 2011). Interestingly, the carbohydrate recognition domain for β-Gal*p* residue is formed only after a conformational switch triggered by prior sialoside binding (Todeschini et al., 2004). The bivalent nature of TcTS$_{Y342H}$ might promote glycan cross-linking, which is believed to be essential for cellular signal transduction.

The finding that inactive TS has two carbohydrate binding domains, may explain some apparently contradictory results on the involvement of sialyl and galactosyl epitopes in *T. cruzi*/host cell interaction. While Schenkman et al. (1991) have shown that sialylation of Ssp-3 epitope of mammalian cell-derived trypomastigotes is required for target cell recognition, Yoshida et al. (1997) reported that the removal of Sia from the surface of insect-derived metacyclic trypomastigotes enhances parasite-host interaction. The removal of Sia from *T. cruzi* glycoproteins and the concomitant exposure of cryptic β-Gal*p* residues would favor TcTS$_{Y342H}$ interaction with both host sialoglycoconjugates and terminal β-Gal*p*-containing glycoproteins on the parasite surface, thus enhancing *T. cruzi*/host adhesion. This phenomenon was well characterized for CD22, a mammalian Sia-binding lectin (Varki and Gagneux, 2012). The removal of Sia and concomitant exposure of β-Gal*p* residues from host cell glycans, which occurs as a result of the *T. cruzi* TS reaction may, therefore, be physiologically significant by promoting parasite adherence to, and penetration of host cells.

On the other hand, the parasite might use the active TS to sialylate host cell glycomolecules and generate receptors for TcTS$_{Y342H}$ mediating trypanosome adherence to a target cells. Data showing that sialic acid-deficient cells are less infected than wild-type cells

(Ciavaglia et al., 1993; Ming et al., 1993), suggest that recognition of sialyl residues on CHO cells by $TcTS_{Y342H}$ is necessary during *T. cruzi* invasion.

The hypothesis that $TcTS_{Y342H}$ promotes glycan cross-linking can be corroborated by data showing that both forms, active and $TcTS_{Y342H}$, bind to α2,3-Sia from CD43 on host $CD4^+$ T cells, triggering a co-stimulatory response through mitogen-activated protein kinase ERK1/2 cascade inducing mitogenesis (Todeschini et al., 2002b). It was also shown that both forms of TcTS protect neuronal and glial cells from apoptosis through activation of PI3K/Akt pathway and up-regulate the anti-apoptotic bcl-2 gene (Chuenkova and Pereira, 2000; Chuenkova et al., 2001). Furthermore, we showed that $TcTS_{Y342H}$ binds to α2,3-Sia containing molecules on endothelial cells resulting in NF-κB activation, expression of cell adhesion molecules and rescue from apoptosis. Activation of endothelial cells increases trypomastigotes attachment and invasion, suggesting that $TcTS_{Y342H}$ plays a role in host cell invasion during *T. cruzi* infection (Dias et al., 2008). Further studies are required to establish the overall role of $TcTS_{Y342H}$ in the pathogenesis of Chagas' disease. Data showing that $TcTS_{Y342H}$ competes *in vivo* with the native TcTS for Sia and β-Gal binding sites, inhibiting potential sialylation events from taking place (Freire-de-Lima et al., 2010), might suggest that $TcTS_{Y342H}$ benefits the host during *T. cruzi* infection. Decreased mortality of mice treated with $TcTS_{Y342H}$ suggests a role in prolonging parasite persistence in host tissues and corroborates the role of TcTS-mediated sialylation in the virulence of *T. cruzi*.

Yet the ability of *T. cruzi* to exploit host glycocalyx to attach and establish the infection must be further studied as other inactive members of TcTS might have lectinic properties. Two motifs common to other sialidases, FRIP (xRxP) and Asp box, can be found in various groups of the *trans*-sialidase-like family (Freitas et al., 2011). The FRIP motif, involved in binding the carboxylate group of sialic acid (Gaskell et al., 1995), is found in four out of seven groups of the *trans*-sialidase-like proteins. The Asp box follows the FRIP motif and can be repeated up to five times in the sequences of viral, bacterial, trypanosomatid, and mammalian sialidases. Although its function is unknown, it is worth noting that the Asp box occurs in secreted proteins and in proteins that act on, or interact with, carbohydrates (Copley et al., 2001). Growing evidence has shown that the insect vector-derived metacyclic trypomastigote uses its stage-specific surface molecule gp82, which is member of the gp85/TS superfamily, to bind to gastric mucin and establish *T. cruzi* infection in mice by the oral route (Neira et al., 2003; Staquicini et al., 2010; Cortez et al., 2012).

Findings showing that $TcTS_{Y342H}$ act as a lectin open a new avenue to be explored in the interaction of *T. cruzi* and its hosts. These studies raise the hypothesis that $TcTS_{Y342H}$ helps the parasite to bind to surfaces rich in sialylated glycoconjugates. In addition, $TcTS_{Y342H}$ can act as a lectin triggering cellular signaling, or helping the TcTS from *T. cruzi* surface to transfer and decorate the cellular surfaces with Sia.

TcTS MECHANISM

TcTS is a retaining glycoside hydrolase (Todeschini et al., 2000) member of the family number 33 (GH-33) that comprises the bacterial and eukaryotic exo-alpha sialidases (Withers and Williams, 2012). Unlike it's closely related sialidase, TcTS preferentially transfers sialic acid units to terminal β-Gal*p*-containing molecules and synthesizes α2-3-linkages exclusively. Efforts to decipher the mechanism of TcTS catalysis have been important to the dissection of the mechanism of exo-alpha sialidases (Watts et al., 2006; Newstead et al., 2008) as these enzymes, unlike most of the retaining glycoside hydrolases, do not present a carboxylate correctly placed in the active site to act as a nucleophile. Pioneering studies with fluor-based sugar inactivators (Watts et al., 2003) followed by Lc-MS/MS peptide mapping and then crystallography (Amaya et al., 2004) demonstrated that TcTS operates through a double displacement mechanism involving the transient formation of a covalent sialyl-enzyme intermediate with a Tyrosine (Tyr342), a very conserved residue in exo-sialidases (Vocadlo and Davies, 20083; Davies et al., 2012), while D59 was proposed to act as a general acid/base catalyst.

From such results a classical ping-pong mechanism (Damager et al., 2008) was inferred for TcTS, where the sialosyl aglycone may abandon the active site to allow the entry of acceptor substrate. In this mechanism, as the sialic acid unit approaches the enzyme, it displaces the Tyr119 away from the binding site (Buschiazzo et al., 2002), its carboxylate group interacts with the Arg triad (Arg35, Arg245, Arg314), while its acetamido group interacts with Asp96. Such interactions induce planarization of the sialic acid moiety around the oxygen ring, with C1, C2, and C3 assuming a $^4H_5$ conformation during the transition state. The C2 suffers nucleophilic attack by Tyr342, assisted by Glu230 acting as a general base, and a covalent linkage is formed. The covalent intermediate assumes a $^2C_5$ conformation. The aglycone leaves the catalytic cleft, thus making space for binding of the sialic acid acceptor. Transfer to the acceptor would then occur through attack of the C2 of the sialyl-enzyme intermediate by the 3-OH group of a lactose moiety, or by water (as in other sialidases) which must be deprotonated by the residue acting as acid/base catalyst, Asp59 (Damager et al., 2008). Nevertheless, a mechanism that supports the higher rates for the transfer reaction requires significant conformational changes in the catalytic pocket during substrate binding and catalytic turnover, features not captured in TcTS crystals yet.

Numerous studies that show evidence for the plasticity of the TcTS catalytic cleft have been arisen. For instance, from molecular dynamics simulations it is known that the key hydrophobic residues Y119 and W312 confer flexibility to the catalytic cleft mouth and allow substrates to access the catalytic pocket in a controlled manner (Demir and Roitberg, 2009; Mitchell et al., 2010). Other key residues, which possibly contribute to the plasticity of binding site, were identified by mutagenesis studies (Paris et al., 2001; Carvalho et al., 2010b) or by hybrid quantum mechanics/molecular mechanics simulations (Pierdominici-Sottile and Roitberg, 2011). Crucial evidence for such plasticity rose from the observation that the TS_{Y342H} (Todeschini et al., 2002a) undergoes large conformational changes, upon sialoside binding, leading the overture of a second binding site that accommodates a β-Gal*p* moiety (Todeschini et al., 2004). Active site rearrangement following the sialoside engagement was further proposed for the fully active TcTS (Haselhorst et al., 2004). Nuclear magnetic resonance spectroscopy confirmed that association of the β-Gal*p* within the

TcTS active site succeeding the sialic acid donor is necessary for the transfer reaction to proceed (Haselhorst et al., 2004). Results of TS_{Y342H} incubated with α2-6-sialyllactose in the presence of lacto-*N*-tetraose, showing that incorrect fitting of sialoside into the binding site of TS does not trigger β-Gal*p* binding, corroborate this hypothesis. Furthermore, surface plasmon resonance results showing that lactose binds to an inactive mutant of TS_{D59N} in the presence of α2-3-sialyllactose (Buschiazzo et al., 2002).

This discussion shows that further structural data are needed to shed light into the reaction mechanism that underlies efficient sugar transfer activity rather than simple hydrolysis by TcTS. Given that genomic analysis suggests that TcTS proteins have several point mutations (Freitas et al., 2011), structural and mechanistic works must be persistent, as mutations in key amino acids (Paris et al., 2001; Carvalho et al., 2010a) would produce critical modifications in TcTS catalysis and specificity.

INHIBITION OF SIALIC ACID TRANSFERENCE BY TcTS

Together, the above observations support the hypothesis that TcTS enhances *T. cruzi* virulence by altering host immune responses directed against the parasite. The facts that TcTS presents low homology with mammalian sialidases, and that it is the lonely protagonist for sialic acid acquisition by *T. cruzi*, provide a rationale for a new potential intervention strategy in chemotherapy of Chagas' disease. Beyond the urgency of alternative drugs to treat the illness, to pursuit of TcTS inhibitors has been the target of several research groups that aim to clarify the role of TcTS in the pathogenesis of Chagas' disease. However, compounds that effectively inhibit the catalytic activity of TS have not been described. The advances made in this field were somehow indirect, relying on few strategies like the use of neutralizing antibodies, given that the siRNA mechanism of gene silencing in *T. cruzi* is lacking, and TcTS is coded by multiple genes copies (Freitas et al., 2011) making gene deletion experiments unlikely to be successful (Balaña-Fouce and Reguera, 2007).

Although effective TcTS inhibitors have not yet been reached, efforts made in this area have found interesting lead compounds. The molecules so far tested as TcTS inhibitors are described below as compounds that are analogs to donor or acceptor substrates and compounds that are unrelated to TcTS substrates (**Figure 5**).

DONOR SUBSTRATE ANALOGS (Figure 5)

Unlike the influenza neuraminidase, TcTS activity is barely inhibited by 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA), a transition state analog (Paris et al., 2005), or by its analogous 5,6-dihydro-4H-pyran-2-carboxylic acid derivatives including zanamivir (Neres et al., 2006).

Despite the observed success with the use of S-glycosides as inhibitors of glycosidases including sialidases (Watson et al., 2006) the Neu5Acα2-3-S-Galβ-*O*-octyl was found to show no significant inhibition of TcTS even at millimolar concentrations (Harrison et al., 2001). The low inhibition rates observed by the Neu5Acα2-3-S-Galβ-*O*-octyl suggest that the conformations acquired by this compound in solution might not be recognized by the TcTS. This theory can be supported by NMR studies of the conformational distribution of cellobiose and S-cellobiose linked to the *Streptomyces* sp. β-glucosidase. These data showed that the S-cellobiose presents three conformational families, unlike cellobiose which is only found in two conformations in solution (Montero et al., 1998).

Trapping of the 2,3-difluorosialic acid by the Tyr342 hydroxyl group opened new avenues for the design of irreversible inhibitors for TcTS (Watts et al., 2003). 2,3-Difluorosialic acid temporarily inactivates the TcTS through covalent binding with the hydroxyl group of Tyr342. However, complete inactivation requires very high concentrations of inhibitor (20 mM) and the enzyme spontaneously recovers its full catalytic activity (Watts et al., 2003). Incorporation of aryl groups at C9, like umbelliferyl, benzamide, and 4-(phenyl carbamide) butyramide, led to more than 1000-fold decrease in TcTS reactivation (Buchini et al., 2008). The crystal of the 9-benzoyl-3-fluoro-*N*-acetylneuraminic acid within TcTS showed that the presence of a voluminous group induced a reorientation of the glycerol side chain, which instead interacts with Tyr119, a site occupied by acceptor substrate, explaining low enzyme reactivation by lactose.

An elegant approach for the irreversible inhibition of TcTS was achieved with the 2-difluoromethyl-4-nitrophenyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosid acid (Neu5AcFNP) and 5-acetamido-2-(4-N-5-dimethylaminonaphthalene-1-sulfonyl-2-difluoromethylphenyl)-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosonic acid (dansyl-Neu5AcFP). Characterization of trapped enzyme by mass spectrometry analysis revealed that inactivation of enzyme occurs through a covalent bond formation between the Arg245 and Asp247 residues with the reactive aglycone generated by the hydrolysis of dansyl-Neu5AcFP. Noteworthy is that Neu5AcFNP inhibited infection of mammalian cells by *T. cruzi* trypomastigotes (Carvalho et al., 2010a).

Apart from complex sugar frameworks, some cyclic scaffolds simulating hexose-like moieties, such cyclohexene, benzoic acid, and pyridine-based structures, were tested. A quite simple pyridoxal phosphate structure was reported as a weak non-competitive TcTS inhibitor (Ferrero-García et al., 1993). The same feature was observed for two *N*-acetyl-cyclohexene phosphonate monoalkyl esters (Streicher and Busse, 2006). Trials using a series of piridine-2-carboxylic acid and benzoic acid derivatives described the 4-acetylamino-3-hydroxymethylbenzoic acid as the best compound, with a Ki value of ∼300 μM (Neres et al., 2007). Curiously, this benzoic acid derivative had the same layout as the best cyclohexene phosphonate derivative from the work of Streicher and Busse (2006), evidencing a negative charged group, *N*-acetyl at opposed carbons and a hydroxyl group as pharmacophoric moieties for TcTS inhibition.

ACCEPTOR SUBSTRATE ANALOGS (Figure 5)

In face of fruitless efforts to inhibit TcTS by occupying the donor substrate site, some groups have targeted the acceptor binding site. Lactitol was able to competitively inhibit the TcTS reaction and to interfere with parasite infection in cultured cells (Agusti et al., 2004). Later modifications of the lactitol molecule, by adding Gal*p*, Gal*f*, or benzyl, led to a pentasaccharide with an IC50 of 0.61 mM (Agusti et al., 2007).

The acceptor substrate for the TcTS transfer reaction (Gal*p*) was also used as a source of inspiration for click chemistry synthesis of

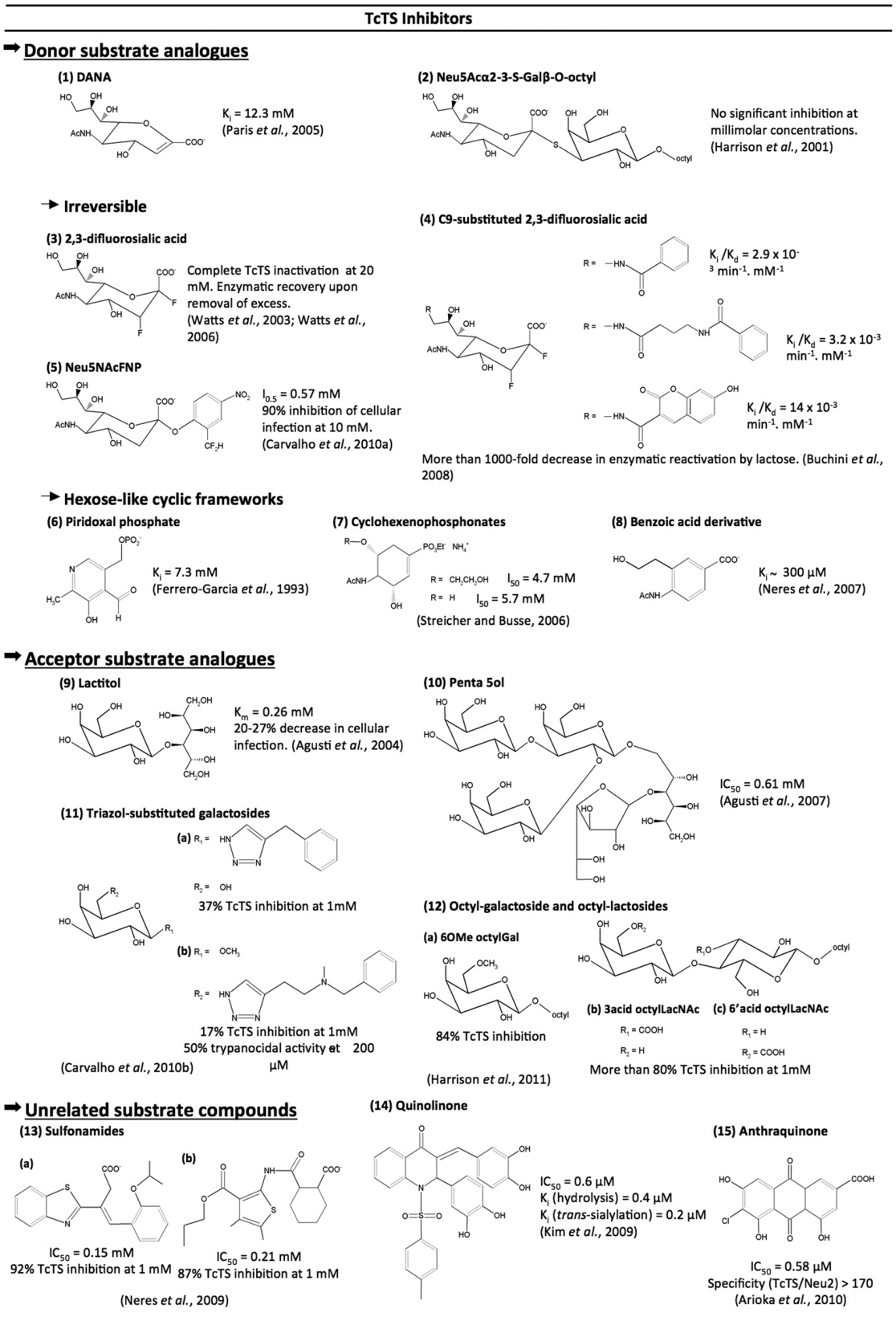

FIGURE 5 | Compounds tested as TcTS inhibitors.

triazoles-substituted saccharides. Starting from galactose derivatives bearing an azide group at C1 or C6, a triazol-substituted saccharide library was made and tested against TcTS. Despite its low inhibitory activity against TcTS, the *N*-methyl benzylamide derivative presented trypanocidal activity (Carvalho et al., 2010b).

Recently, a series of octyl galactosides and octyl *N*-acetyllactosamines were tested against TcTS. Results showed that the TcTS acceptor binding site is intolerant of substitution of β-Gal*p* at positions 2 and 4, whereas substitution at position 6 of the Gal ring is well accepted, highlighting the potential of 6-substituted Gal residues as TcTS acceptor substrates (Harrison et al., 2011).

UNRELATED SUBSTRATE COMPOUNDS (Figure 5)

The 3-benzothiazol-2-yl-4-phenyl-but-3-enoic acid and sulfonamide scaffolds emerged, from virtual screening, as new frameworks for TcTS inhibition (Neres et al., 2009). Sulfonamides figured also as good substituents for chalcones used as TcTS inhibitors by Kim et al. (2009). Of the compounds tested, the tetrahydroxylated quinolinone inhibits both hydrolytic and TcTS activities at millimolar concentration (Kim et al., 2009). Similar to chalcones, various flavonoids and anthraquinones were systematically screened from a large library. A highly hydroxylated anthraquinone was the best inhibitor of TcTS, with an IC_{50} of 0.58 μM (Arioka et al., 2010). Moreover, this compound did not inhibit Neu2, a mammalian neuraminidase, demonstrating that its inhibition is reasonably specific to TcTS (Arioka et al., 2010). Therefore, this last structure represents, to date, the most promising scaffold for a TcTS inhibitor.

Despite great advances made toward TcTS inhibition, works highlighting the enzyme's plasticity (Demir and Roitberg, 2009; Mitchell et al., 2010) showing the existence of an acceptor binding site (Todeschini et al., 2004; Damager et al., 2008) suggest that the structure of TcTS with two cavities would be a better framework for rational drug design aimed to TcTS inhibition.

PERSPECTIVES

Sia participate in a large range of biological processes between *T. cruzi* and its host, and just a few examples are considered here. In fact, the effect of Sia in the pathogenesis of Chagas' disease remains unknown.

Given the role of Sias as "self-associated molecular patterns" recognized by molecules such as Siglecs (Paulson et al., 2012), TcTS activities can perturb natural self-recognition phenomena, perhaps increasing inflammatory responses by exposing desialylated "danger-associated molecular patterns" (Varki, 2011; Rabinovich et al., 2012).

Moreover, from evidence showing that desialylation of Toll-like receptor 4 (Amith et al., 2010; Abdulkhalek et al., 2011) is essential for receptor activation and cellular signaling, we can speculate that TcTS can also modify host response to "pathogen-associated molecular patterns."

Recent advances in the glycobiology field will surely help to understand how a glycosidase evolved to have a highly efficient *trans*-glycosidase activity and to put forward the design of specific and potent *TcTS* inhibitors. These molecules will help to disclose the real roles of TcTS/sialoglycoproteins in *T. cruzi* biology, the pathogenesis of Chagas' disease, and perhaps help the treatment of this illness.

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Regulation and use of the extracellular matrix by *Trypanosoma cruzi* during early infection

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Chagas disease, which was once thought to be confined to endemic regions of Latin America, has now gone global becoming a new worldwide challenge. For more than a century since its discovery, it has remained neglected with no effective drugs or vaccines. The mechanisms by which *Trypanosoma cruzi* regulates and uses the extracellular matrix (ECM) to invade cells and cause disease are just beginning to be understood. Here we critically review and discuss the regulation of the ECM interactome by *T. cruzi*, the use of the ECM by *T. cruzi* and analyze the molecular ECM/*T. cruzi* interphase during the early process of infection. It has been shown that invasive trypomastigote forms of *T. cruzi* use and modulate components of the ECM during the initial process of infection. Infective trypomastigotes up-regulate the expression of laminin γ-1 (LAMC1) and thrombospondin (THBS1) to facilitate the recruitment of trypomastigotes to enhance cellular infection. Silencing the expression of LAMC1 and THBS1 by stable RNAi dramatically reduces trypanosome infection. *T. cruzi* gp83, a ligand that mediates the attachment of trypanosomes to cells to initiate infection, up-regulates LAMC1 expression to enhance cellular infection. Infective trypomastigotes use Tc85 to interact with laminin, p45 mucin to interact with LAMC1 through galectin-3 (LGALS3), a human lectin, and calreticulin (TcCRT) to interact with TSB1 to enhance cellular infection. Silencing the expression of LGALS3 also reduces cellular infection. Despite the role of the ECM in *T. cruzi* infection, almost nothing is known about the ECM interactome networks operating in the process of *T. cruzi* infection and its ligands. Here, we present the first elucidation of the human ECM interactome network regulated by *T. cruzi* and its gp83 ligand that facilitates cellular infection. The elucidation of the human ECM interactome regulated by *T. cruzi* and the dissection of the molecular ECM/*T. cruzi* interphase using systems biology approaches are not only critically important for the understanding of the molecular pathogenesis of *T. cruzi* infection but also for developing novel approaches of intervention in Chagas disease.

Keywords: laminin γ-1, thromospondin-1, calreticulin, gp83, ECM interactome, cellular infection, *Trypanosoma cruzi*, systems biology

LAMC1 AND THBS1 ARE REQUIRED FOR *T. cruzi* INFECTION

As part of our efforts to identify the molecular signatures induced by *Trypanosoma cruzi* in mammalian cells during the early infection process, we analyzed the kinetics of the extracellular matrix (ECM) human transcriptome response. The result of this analysis showed that the only ECM proteins, LAMC1 and THBS1, were important in the early infection process. Accordingly, silencing the expression of LAMC1 and THBS1 by stable RNAi significantly

Abbreviations: 4A4, gp83 monoclonal antibody that neutralizes *T. cruzi* infection; ACHE, acetylcholinesterase (Yt blood group); APCS, amyloid P component, serum; APP, amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer disease); ATF7IP, activating transcription factor 7 interacting protein; ATXN7L2, ataxin 7-like 2; B5, *T. cruzi* p45 mucin monoclonal antibody; BCAM, basal cell adhesion molecule (Lutheran blood group); BCL6, B-cell CLL/lymphoma 6; BIND, Biomolecular Interaction Network Database; BMP1, bone morphogenetic protein 1; bBTBD10, BTB (POZ) domain containing 10; C3, complement component 3; CALR or CRT, calreticulin; CCDC53, coiled-coil domain containing 53; CCSB-HI1, Center for cancer Systems Biology-Human Interactome; CD36, CD36 molecule (thrombospondin receptor); CD44, CD44 molecule (Indian blood group); CD47, CD47 molecule; CFH, complement factor H; cGMP, cyclic guanosine monophosphate; COL1A1, collagen, type I, alpha 1; COL2A1, collagen, type II, alpha 1; COL3A1, collagen, type III, alpha 1 (Ehlers–Danlos syndrome type IV, autosomal dominant); COL5A1, collagen, type V, alpha 1; COL6A1, collagen, type VI, alpha 1; COL7A1, collagen, type VII, alpha 1 (epidermolysis bullosa, dystrophic, dominant and recessive); CORO1A, coronin, actin binding protein, 1A; CSNK1A1, casein kinase 1, alpha 1; CSNK2A1, casein kinase 2, alpha 1 polypeptide; CSNK2A2, casein kinase 2, alpha prime polypeptide; CTGF, connective tissue growth factor; CTSG, cathepsin G; CUBN, cubilin (intrinsic factor-cobalamin receptor); CYHR1, cysteine/histidine-rich 1; DAG1, dystroglycan 1 (dystrophin-associated glycoprotein 1); DCN, decorin; DHFR, dihydrofolate reductase; DIP, Database of Interacting Proteins; E3T3C1, C-terminal domain of TSP-1, containing the last type II repeat domain, the three type III repeat domains and the carboxy-terminal domain; ECM, extracellular matrix; EGF, epidermal growth factor; ELA2, elastase 2, neutrophil; ELN, elastin (supravalvular aortic stenosis, Williams–Beuren syndrome); ERK 1/2, extracellular signal-regulated kinase; F2, coagulation factor II (thrombin); FBLN2, fibulin 2; FCGR2A, Fc fragment of IgG, low affinity IIa, receptor (CD32); FGA, fibrinogen alpha chain; FN1, fibronectin 1; GEMIN4, gem (nuclear organelle) associated protein 4; GFI1B, growth factor independent 1B transcription repressor; gp83 or Tcgp83, *T. cruzi* trypomastigote ligand or trypomastigote surface 83 kDa glycoprotein; HCASM, human coronary artery smooth muscle cell; HPRD, Human Protein Reference database; HRG, histidine-rich glycoprotein; HSPG2, heparan sulfate proteoglycan 2; INF-γ, Interferon gamma; IGFBP5, insulin-like growth factor

reduced *T. cruzi* infection in cells (Nde et al., 2006; Simmons et al., 2006). The cells in which stable LAMC1 RNAi or THBS1 RNAi were performed showed significant reduction in the protein expression level of LAMC1 or THBS1 compared to cells stably transfected with vector alone or scrambled LAMC1 or THBS1 and the kinetics of *T. cruzi* infection were also dramatically reduced (Nde et al., 2006; Simmons et al., 2006). These studies showed that *T. cruzi* requires LAMC1 and THBS1 for early infection and indicated that host LAMC1 and THBS1 play critical roles in the early process of *T. cruzi* infection (Nde et al., 2006; Simmons et al., 2006). The important roles played by LAMC1 and THBS1 encouraged our group to dissect their molecular role in *T. cruzi* infection by looking at the ECM/*T. cruzi* interphase and elucidating the gene-networks and interactomes triggered by *T. cruzi* and its surface molecules involved in the early infection process (Cardenas et al., 2010; Nde et al., 2010). This will be critically reviewed in the next sections.

T. cruzi SURFACE GP83 UP-REGULATES LAMC1 TO RECRUIT PARASITES AT THE ECM TO ENHANCE INFECTION

Gp83 is a ligand expressed in all *T. cruzi* strains and employed by the parasite to attach and enter macrophages as well as non-phagocytic cells (Lima and Villalta, 1988; Villalta et al., 1998, 1999, 2001, 2008). Notably, it is expressed only in invasive trypomastigotes (Villalta et al., 1992) and is more expressed in highly infective trypomastigote clones (Lima and Villalta, 1989). Monovalent Fab fragments of the gp83-specific monoclonal antibody 4A4 inhibit gp83 binding to myoblasts, fibroblasts, and macrophages, block trypanosomes from attaching to and entering these cells, and neutralize *T. cruzi* infection *in vivo* (Villalta et al., 2001). Trypomastigotes release gp83 via parasite glycosylphosphatidylinositol–phospholipase C (PLC) cleavage to activate the host MAPK pathway and PKC in order to promote parasite infection (Villalta et al., 1998, 1999; Nde et al., 2006).

Exposure of gp83 ligand to human cells increases the level of LAMC1 transcripts and its expression in mammalian cells, leading to an increase in cellular infection by *T. cruzi* (Nde et al., 2006). This increase in cellular infection was seen as over-attachment and entry of trypomastigotes into human cells over-expressing LAMC1, under the influence of gp83 ligand, resulting in high parasite multiplication at 72 h. These observations together with the fact that knocking down the expression of LAMC1 by RNAi dramatically reduces *T. cruzi* attachment, entry and multiplication within cells strongly support the hypothesis that host LAMC1, which is regulated by the parasite gp83, plays a crucial role in the early process of cellular infection.

The regulation of infection by the gp83 ligand represents a parasite escape mechanism in which invasive trypomastigotes release gp83 to efficiently gain entry into human coronary artery smooth muscle (HCASM) cells by manipulating LAMC1, which is the most abundant isoform of laminin in humans (Sasaki et al., 2004).

Trypanosoma cruzi must navigate through the basal lamina, which contains LAMC1, and surrounds individual muscle cells such as HCASM cells before infecting these cells. The fact that a *T. cruzi* trypomastigote ligand increases LAMC1 transcript levels in HCASM cells, correlates well with the finding that laminin is deposited in the hearts of patients infected with Chagas' disease (Milei et al., 1993). This suggests that the regulation of LAMC1 in heart cells by gp83 may be involved in heart pathology.

Since the *T. cruzi* gp83 ligand remodels the ECM by up-regulating the expression of LAMC1, together with the report that *T. cruzi* presents laminin receptors on its surface (Giordano et al., 1999), indicates that the parasite exploits LAMC1 to navigate through the ECM, recruit trypanosomes and facilitate infection. Thus, the *T. cruzi* gp83 ligand is a virulence factor that modifies LAMC1 expression in the ECM and contributes to the pathogenesis of *T. cruzi* infection in human heart cells.

T. cruzi MODULATES THE LAMC1 SUB-NETWORK

Recently, the first LAMC1 sub-network modulated by invasive trypomastigotes during the early process of infection was elucidated (Nde et al., 2010). To populate and build the interaction

binding protein 5; IL-12, interleukin 12; IPI, International Protein Index; ITGA1, integrin, alpha 1; ITGA2, integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor); ITGA3, integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor); ITGA4, integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor); ITGA6, integrin, alpha 6 (alpha subunit of VLA-6 receptor); ITGAV, integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51); ITGB1, integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12); ITGB3, integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61); ITGB4, integrin, beta 4; JAG1, jagged 1 (Alagille syndrome); KNG1, kininogen 1; KEGG, Kyoto Encyclopedia of Genes and Genomes; LAMA1, laminin, alpha 1; LAMA2, laminin, alpha 2 (merosin, congenital muscular dystrophy); LAMA3, laminin, alpha 3; LAMA5, laminin, alpha 5; LAMB1, laminin, beta 1; LAMB2, laminin, beta 2 (laminin S); LAMB3, laminin, beta 3; LAMC1, laminin, gamma 1 (formerly LAMB2); LAMC2, laminin, gamma 2; LAMC3, laminin, gamma 3; LGALS3, lectin, galactoside-binding, soluble, 3; LGALS3BP, lectin, galactoside-binding, soluble, 3 binding protein; LIM2, lens intrinsic membrane protein 2, 19kDa; LRP1, low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor); LRP5, low density lipoprotein receptor-related protein 5; LTBP1, latent transforming growth factor beta binding protein 1; MATN2, matrilin 2; MEF, mouse embryo fibroblast; MEK, mitogen-activated protein kinase kinase; MEP1A, meprin A, alpha (PABA peptide hydrolase); MiMI, Michigan Molecular Interactions; MMP-2, matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase); MMP-9, matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase); MUC7, mucin 7, secreted; MYOC, myocilin, trabecular meshwork inducible glucocorticoid response; NID1, nidogen-1; NID2, nidogen-2 (osteonidogen); NO, nitric oxide; NT5E, 5′-nucleotidase, ecto (CD73); NTSP, N-terminal domain of TSP-1; ONECUT1, one cut homeobox 1; PDE1A, phosphodiesterase 1A, calmodulin-dependent; PDEA, phosphodiesterase 6A, cGMP-specific, rod, alpha; PDGFB, platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog); PLAT, plasminogen activator, tissue; PLC, phospholipase C; PLG, plasminogen; PPI, protein–protein interaction; PTPRF, protein tyrosine phosphatase, receptor type, F; RAF, v-raf-1 murine leukemia viral oncogene; RAS, rat sarcoma viral oncogene homolog; RNAi, RNA interference; RPSA, ribosomal protein SA; SAA1, serum amyloid A1; SAA2, serum amyloid A2; SCARB2, scavenger receptor class B, member 2; SCD4, stearoyl-CoA desaturase 5; SDC1, syndecan 1; SDC3, syndecan 3; SIP1, survival of motor neuron protein interacting protein 1; SMAD2, SMAD family member 2; SNAPIN, SNAP-associated protein; SPARC, secreted protein, acidic, cysteine-rich (osteonectin); SPP1, secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1); SUFU, suppressor of fused homolog (Drosophila); SV2A, synaptic vesicle glycoprotein 2A; SV2B, synaptic vesicle glycoprotein 2B; SV2C, synaptic vesicle glycoprotein 2C; Tc45, *T. cruzi* 45 kDA surface mucin; Tc85, *T. cruzi* 85 kDa surface glycoprotein; TcCRT, *T. cruzi* calreticulin; TFPI, tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor); TGF-α, transforming growth factor alpha; TGF-β, transforming growth factor beta; TGFB1, transforming growth factor, beta 1; TSP-1/THBS1, thrombospondin 1; TMPRSS6, transmembrane protease, serine 6; TNFα, tumor necrosis factor alpha; TNFAIP6, tumor necrosis factor, alpha-induced protein 6; TNFRSF11B, tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin); TNFRSF1A, tumor necrosis factor receptor superfamily, member 1A; TRAP; thrombospondin-related anonymous protein; TP53, tumor protein p53; TSC2, tuberous sclerosis 2; ZNF512B, zinc finger protein 512B; ZNF8, zinc finger protein 8.

network, the Michigan Molecular Interactions (MiMI) cytoscape plugin (version 3.2) was used. MiMI retrieves molecular interactions from MiMI database and displays the interaction network with cytoscape. MiMI gathers and merges data from well-known protein interaction databases including BIND, DIP, HPRD, RefSeq, SwissProt, IPI, and CCSB-HI1. The plugin also integrates with other NCBI tools for literature information, document summarization, and pathway matching (Gao et al., 2009). The RT-PCR verified transcripts were used as the initial population nodes. MiMI was used to query for the initial nodes and their respective nearest neighbors to one degree. The networks were then merged for interconnections and the interactome was visualized in cytoscape (version 2.6.1) using the yFiles: organic layout format (Cline et al., 2007).

The first elucidated LAMC1 sub-network interactome mobilized in cells by *T.* cruzi involves the following genes CCDC53, NID1, NID2, SNAPIN, LAMA1, SV2A, CD44, LAMA5, ATF71P, ITGB4, LAMB1, LAMB2, SV2C, SV2B, ITGA6, and BCL6 (Nde et al., 2010). Our group is fully investigating the roles of the members of this sub-network in the infection process. Accordingly, the ITGA6 gene that encodes integrin alpha-6 that combines with beta 4 as a laminin receptor, or with beta 1 in the integrin VLA-6 participates in cell adhesion as well as cell-surface mediated signaling. The integrin, beta 4 (ITGB4) gene that encodes the integrin beta 4 subunit, a receptor for the laminins, tends to associate with alpha 6 subunit and appears to be involved in cell invasion. Entactin, also known as nidogen-1 (NID1) is a component of the basement membrane that connects the networks formed by collagens and laminins to each other and plays a role in cell interactions with the ECM. Furthermore, LAMA1 (Laminin, alpha 1) interacts with Fibulin-2 (FBLN2), an ECM protein that binds various extracellular ligands and calcium and appears to be involved in the infection process. Interestingly, BCL6 is a central hub of the interaction LAMC1 sub-network, which is also a common central hub to five of the seven sub-networks reported by one degree of connection to the initial seed nodes (Nde et al., 2010).

The elucidation of the LAMC1 sub-network interactome mobilized by *T. cruzi* is critically important to understand the molecular pathogenesis of *T. cruzi* infection derived from the ECM/*T. cruzi* gp83 ligand interplay and for the development of approaches for intervention. We expect that continuing to explore systems biology in the early process of *T. cruzi* infection will rapidly bring novel approaches for the treatment and management of Chagas disease.

THBS1 INTERACTS WITH *T. cruzi* SURFACE CALRETICULIN TO ENHANCE CELLULAR INFECTION

Thrombospondins have been described as "matricellular proteins" because they play a role in regulating cellular responses and ECM remodeling in the pericellular microenvironment but they are non-essential components of the mature matrix fibrils (Bornstein, 2002). The role of THBS1 *in vitro* and *in vivo* is complex and context specific, because it interacts with a wide array of cellular proteins. THBS1 is a large homotrimeric glycoprotein containing several domains that can bind to cell surface receptors and extracellular molecules (Chen et al., 2000) such as the N-terminal heparin-binding domain (NTSP), procollagen region, type 1, 2, and 3 repeats and a C-terminal domain (Elzie and Murphy-Ullrich, 2004). The molecule also contains highly conserved epidermal growth factor (EGF) repeats, type 3 repeats and a C-terminal domain, which includes the signature domain (Carlson et al., 2005) that can interact with integrins and CD47 (Lawler and Hynes, 1989; Gao and Frazier, 1994). The C-terminal domain of the thrombospondin family is highly conserved compared to the N-terminal domain, which is different for each thrombospondin isoform.

Calreticulin (CRT) is a major intracellular well-conserved calcium-binding chaperone, which was identified in skeletal muscle (Michalak et al., 1992) and is present in the cells of all higher organisms except erythrocytes (Michalak et al., 1999; Johnson et al., 2001a,b). Numerous reports have implicated CRT in several cellular functions and the molecule has significant non-endoplasmic reticulum functions in normal physiology and human disease status (Gold et al., 2010).

Calreticulin has also been described in some parasite species such as *Schistosoma mansoni*, *Onchocerca volvulus, Necator americanus, Leishmania donovani*, and *Plasmodium falciparum.* However, the role that this protein might play in parasite interactions with the host immediate microenvironment remains unknown (Nakhasi et al., 1998; Ferreira et al., 2004, 2005).

Previous studies from our group showed that invasive *T. cruzi* trypomastigotes up-regulate the expression of THBS1 in HCASM cells (Simmons et al., 2006). Furthermore, knockdown of THBS1 rendered mammalian cells less susceptible to cellular infection by *T. cruzi* indicating that THBS1 also plays an important role in the process of cellular infection by *T. cruzi*. However, the mechanisms by which THBS1 is up-regulated by the parasite to modulate cellular infection are not completely known. The elucidation of *T. cruzi* surface molecules that interact with THBS1 to enhance cellular infection will advance our understanding of the molecular pathogenesis of *T. cruzi* infection. We anticipated that NTSP, which is specific to this isoform of thrombospondin, would be essential in the interaction with the parasite because it is different for all thrombospondin isoforms compared to the conserved C-terminal domain (Carlson et al., 2005).

In *T. cruzi*, it has been suggested that the parasite surface CRT (TcCRT; Ferreira et al., 2004; Aguilar et al., 2005) could play a role in enabling the pathogen to evade the host immune response by interacting with the C1q component of complement (Aguilar et al., 2005). The mechanism by which the parasite CRT interacts with host proteins to enhance the process of cellular invasion remains unknown. We recently hypothesized that *T. cruzi* uses its surface TcCRT to exploit matricellular proteins regulated by the parasite to enhance cellular infection.

We recently reported that *T. cruzi* up-regulates the expression of THBS1 to enhance the process of cellular invasion. Very recently our group characterized a novel THBS1 interaction with *T. cruzi* that enhances cellular infection (Johnson et al., 2012). We showed that labeled THBS1 interacts specifically with the surface of *T. cruzi* trypomastigotes. In **Figure 1** we show the full-length THBS1 and the NTSP domain that interact with TcCRT. Pre-exposure of recombinant NTSP or THBS1 to *T. cruzi* significantly enhanced cellular infection of wild-type mouse embryo fibroblasts (MEF) compared to the C-terminal domain of THBS1, E3T3C1. In addition, blocking TcCRT with antibodies significantly inhibited the

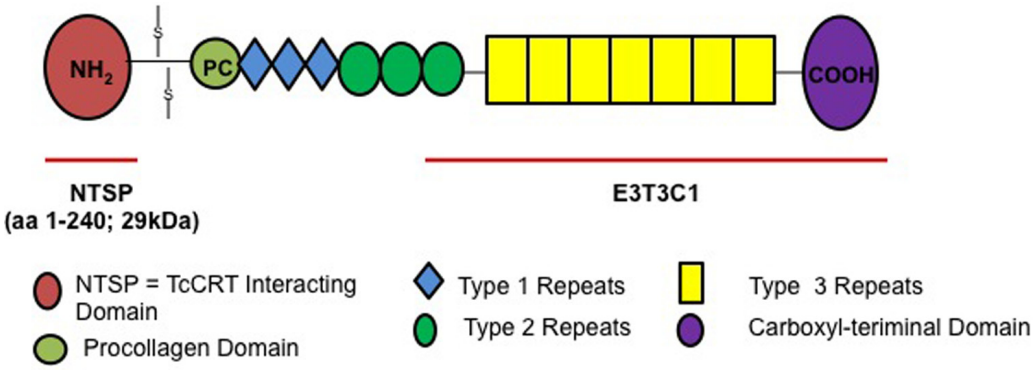

FIGURE 1 | THBS1 structure and NTSP domain that interacts with TcCRT to enhance *T. cruzi* cellular infection.

enhancement of cellular infection mediated by the TcCRT–THBS1 interaction. Taken together, these findings indicate that THBS1 interacts with TcCRT on the surface of *T. cruzi* through the NTSP domain and that this interaction enhances cellular infection. Thus, surface TcCRT is another virulence factor that enhances the pathogenesis of *T. cruzi* infection through THBS1, which is up-regulated by the parasite.

The observation that THBS1 interacts with the parasite membrane TcCRT is also in agreement with the recent discovery that CRT, which was previously thought to be exclusively intracellular, is also expressed on the surface of the parasite (Arosa et al., 1999; Xiao et al., 1999; Yan et al., 2010). The identification of CRT homologs in other parasites such as *Onchocerca*, *Schistosoma*, and *Leishmania* (Michalak et al., 1992; Nash et al., 1994; Joshi et al., 1996) suggests that the protein functions as an intracellular chaperone but its role in the process of infection of those parasites remains unknown.

Our group showed that surface TcCRT is a virulence factor that interacts with host THBS1 to enhance cellular infection by *T. cruzi* (Johnson et al., 2012). In order to explore the significance of host THBS1 and TcCRT in cellular infection by *T. cruzi*, our group used THBS1 KO MEF and WT MEF in infection assays. We observed that host THBS1 and parasite surface TcCRT are important for MEF cellular infection by *T. cruzi*. The significance of surface TcCRT in enhancing cellular infection by *T. cruzi* was supported by the fact that specific antibodies to TcCRT significantly reduced cellular infection. The identification of TcCRT as a virulence factor expressed on the surface of the parasite can be exploited to provide new insights into the molecular pathogenesis of *T. cruzi* infection. TcCRT expression on the parasite surface may modulate the vertebrate complement system as an immune escape mechanism (Ferreira et al., 2005). Taken together, these findings indicate that THBS1 interacts with TcCRT on the surface of *T. cruzi* through the NTSP domain and that this interaction enhances cellular infection by *T. cruzi*.

T. cruzi MODULATES THE THBS1 SUB-NETWORK

The matricellular THBS1 glycoprotein inhibits angiogenesis and modulates endothelial cell adhesion, motility, and growth. THBS1 interacts with several cell adhesion receptors, including CD36, integrins, and integrin-associated proteins and inhibits matrix metalloproteinase enzymes thereby remodeling the cellular microenvironment (Sid et al., 2004). We have shown that *T. cruzi* up-regulates the expression of THBS1 to facilitate the invasion process (Simmons et al., 2006). Knockdown of THBS1 by RNAi significantly inhibited cellular invasion by *T. cruzi*. The increased level of *THBS1* expression significantly modulates the interactome cross-talk between the cells, as we found the largest protein–protein interaction (PPI) sub-network occurred in the THBS1 seed network, with 51 nodes interconnected with 151 edges (Nde et al., 2010). This network topology will potentially favor parasite multiplication and survival. During this cross-talk, higher levels of THBS1 activate TGF-β (Ribeiro et al., 1999), a major pro-fibrotic cytokine causing modification of the ECM milieu. THBS1 then interacts with several host proteins including the COL7A1 gene product, which then interacts with other proteins from the laminin family of genes to make the ECM conducive to parasite mobility and cellular invasion. The modulation of THBS1 gene networking profile is highly critical for trypanosome mobility and cellular invasion. Other parasites (including the human pathogens *Plasmodium*, *Toxoplasma*, and *Cryptosporidium*), which do not regulate human THBS1, use a transmembrane thrombospondin-related anonymous protein (TRAP) for gliding motility and invasion of vertebrate host cells (Kappe et al., 1999; Sibley, 2004; Morahan et al., 2009). TRAP proteins produced by the parasite can also activate TGF-β, an anti-inflammatory cytokine that counteracts the effects of inflammatory cytokines like IL-12, INF-γ, and TNF-α thereby facilitating parasite survival. TSP1, also binds to matrix metalloproteinase-2(MMP-2) and appears to be involved in the infection process.

Modulation of THBS1 expression in HCASM cells by *T. cruzi* is essential because THBS1 blocks the cytoprotective activity of nitric oxide (NO) by antagonizing the NO/cGMP signaling pathway thereby negatively regulating vascular tone, vascular smooth muscle cells adhesion, chemotaxis, and proliferation (Isenberg et al., 2008). Increased THBS1 transcripts in parasitized HCASM cells may suggest that THBS1 also contributes in part to the pathology caused by *T. cruzi*. THBS1 is required for the infection process of *T. cruzi* as evidenced by RNAi of that specific isoform. The up-regulation of host THBS1 expression by *T. cruzi* to facilitate the

infection of human cells represents an additional mechanism that contributes to the pathogenesis of *T. cruzi* infection.

The first elucidated THBS1 sub-network interactome mobilized in cells by *T. cruzi* involves the following genes: TSC2, TNFRSF1A, COL1A1, COL2A1, COL3A1, COL5A1, COL7A1, SDC1, SDC2, SDC3, SCD4, SPARC, LTBP1, HRG, ITGB1, ITGB3, ITGA3, ITGA4, ITGAV, FGA, SPP1, F2, CD47, MMP2, MMP9, CALR, TFPI, ZNF8, LRP1, LRP5, TP53, CTSG, PDGFB, ELA2, CORO1A, LAMB3, CFH, PDEA, JAG1, DCN, TGFB1, CD36, FN1, PLG, TNFAIP6, SCARB2, DHFR, and KNG1 (Nde et al., 2010). The roles of the members of the THBS1 sub-network in the infection process are also being investigated fully by our group as indicated above in this section. The new advances in the area will facilitate the identification of the molecular signature induced by *T. cruzi* in cells via the TcCRT–THBS1 interphase as well as the development of small molecule inhibitors to interrupt the critical initial steps of *T. cruzi* infection.

T. cruzi USES LAMININ AND GALECTIN-3 TO PROMOTE CELLULAR INFECTION

The ECM, human lectins, and parasite mucins have been shown to play an important role in the early process of *T. cruzi* infection. Accordingly, human galectin-3 binds to a trypomastigote surface mucin (Moody et al., 2000; Turner et al., 2002) and to HCASM cells in a lectin-like manner (Kleshchenko et al., 2004) to significantly increase the adhesion of trypomastigotes to these cells. Silencing galectin-3 expression in cells by antisense approach significantly reduces trypomastigote cell adhesion. Galectin-3 molecules interact with a *T. cruzi* 45 kDA mucin surface protein on one hand and with laminin on the other, via their carbohydrate recognition domains and are joined together through the R-domains (Moody et al., 2000; Kleshchenko et al., 2004). In this way, galectin-3 binds to laminin and trypomastigotes to recruit them to the ECM thus facilitating initial infection. Thus, galectin-3 provides a bridge between parasites and laminin in host cell thereby enhancing infection. Prior to cellular infection the infective trypomastigotes bombard host cells with PLC-cleaved gp83 to activate the ERK1/2 pathway to up regulate laminin primed cells to enhanced infection via the laminin-45 mucin-galectin-3 pathway (Moody et al., 2000; Kleshchenko et al., 2004). These studies pointed out a novel *T. cruzi*–host cell interaction mediated by gp83-laminin-45 mucin- galectin-3 that recruits significant number of parasites at the ECM to facilitate cellular infection. Other *T. cruzi* surface antigens bind to laminin (Giordano et al., 1999) and fibronectin (Ouaissi et al., 1986) and have been postulated to participate in the infection process, however these interactions have not been studied using systems biology approaches. Overall, we can conclude that the parasite modulates some ECM components and interacts with them to facilitate infection by exploiting these molecules and human lectins to recruit parasites in the early process of infection.

Galectin-3 binds to the surface of HCASM cells in a granular manner, distributed around the cellular membrane, polarized, and more pronounced at the cellular ends (Kleshchenko et al., 2004). The receptors for human galectin-3 are distributed in patches on the cell surface and are more abundant at the cellular terminal regions. The binding of galectin-3 to trypomastigotes is also seen as granular, restricted to some areas of the trypanosome membrane, and polarized (Kleshchenko et al., 2004).

Galectin-3 is also implicated in the association of *T. cruzi* trypomastigotes with laminin (Moody et al., 2000). Binding of trypomastigotes to laminin is enhanced by galectin-3 and this enhanced binding of trypanosomes is inhibited by lactose. Co-immunoprecipitations indicate that galectin-3 binds to the 45 kDa trypomastigote surface protein and this binding is also inhibited by lactose (Moody et al., 2000). The monoclonal antibody B5 that recognizes the trypomastigote 45 kDa surface mucin blocks trypomastigote attachment to heart myoblasts (Moody et al., 2000; Turner et al., 2002). A working model proposes that galectin-3, released by human cells, forms bridges between *T. cruzi* and laminin. Since nearly all the tissues which *T. cruzi* infects are surrounded by basement membranes of which laminin is a major constituent, its ability to effectively interact with laminin is critically important for passage through the membrane barrier. These studies suggest that this is a trypanosome trapping mechanism, which enables the organisms to accumulate in the basement membrane prior to invasion of heart myoblasts, making galectin-3 a candidate molecule, which enhances the pathogenesis of *T. cruzi* via laminin and a *T. cruzi* surface mucin.

Galectins have long been suspected of modulating cell to ECM interactions in a novel fashion (Liu and Rabinovich, 2005). Data suggest that one mechanism involves the ligation of mammalian cells to ECM proteins, which also interact with galectin-3 such as laminin and elastin (Liu and Rabinovich, 2005). The other mechanism involves the interaction of galectins with the polylactosamine residues of integrins, resulting in the modulation of cellular adhesion to ECM proteins (Rabinovich et al., 2002). Whereas most of these studies were done in mammalian systems, it has been suggested that galectins expressed by *Entamoeba* may be critical in their interactions with host cells (Petri et al., 1990). It is likely that in parasitic organisms, the binding of the organisms to the ECM proteins or cell surface glycoconjugates may be the primary adhesion mechanisms mediated by galectins.

Galectin-3 is also expressed in B cells from *T. cruzi*-infected mice (Acosta-Rodríguez et al., 2004) and is up-regulated by *T. cruzi* infection of mice (Vray et al., 2004). The fact that galectin-3 is secreted by macrophages and by other cells, including HCASM cells, suggests that released galectin-3 modulates infection via laminin. The concentrations of galectin-3 that increase trypanosome adhesion to HCASM cells *in vitro* are similar to the concentrations of galectin-3 present in fluids *in vivo* (Sano et al., 2000). Furthermore, the concentrations of galectin-3 in fluids *in vivo* increase approximately 300-fold during microbial infection (Sato and Hughes, 1994). These observations suggest that the parasite may have adapted to the host and that it takes advantage of a host inflammatory molecule, galectin-3, to bind to host cells via laminin and a *T. cruzi* mucin to enhance infection.

Previous studies have shown that parasite molecules bind to immobilized laminin (Giordano et al., 1999) and that human galectin-3 enhances this interaction (Moody et al., 2000), indicating that the trypanosome interacts with laminin via galectin-3.

T. cruzi SURFACE GP83 REGULATES THE ECM INTERACTOME

The genetic architecture of the early *T. cruzi* infection process of human cells is very limited. To understand this aspect of the infection, we conducted gene transcription microarray analysis followed by gene network construction of the host cell response in primary HCASM cells infected with *T. cruzi* or exposed to *T. cruzi* gp83. Using THBS1, LAMC1, LGALS3, and ERK1/2 as the seed nodes for biological network construction, we built an interactome network of the early *T. cruzi* infection process, which centered on the ECM. ERK1/2 was used as one seed mode, since *T. cruzi* gp83 activates ERK1/2 in cells to up-regulate the LAMC1 expression and infection. After seeding the initial four nodes, the network was expanded to one degree of direct biological interaction, resulting in base interaction networks with the seed node as a center point in each. In order to populate and build our interaction network we used a similar approach to determine the modulation of the LAMC1 sub-network as indicated above. **Figure 2** shows the ECM interactome that is modulated by *T. cruzi* gp83 ligand. The first step in the regulation of the ECM interactome by *T. cruzi* during the initial infection process involves triggering gp83 receptors in the cell via activation of ERK1/2 by PLC-cleaved gp83 which results in the up-regulation of LAMC1 and activation of the LAMC1 sub-network which directly cross-talks with the LGALS3 sub-network to recruit parasites and enhance cellular infection on one side. On the other side, triggering gp83 receptors in the cell via ERK1/2 cross-talk with the THBS1 sub-network, also enhances cellular infection and cross-talk at a distance with LGALS3, which also recruit parasites via Tc45 mucin, and LAMC1 (**Figure 2**). Tc85 interacts with laminin and may mobilize the ECM interactome. It is also possible that *T. cruzi* MMP9 like

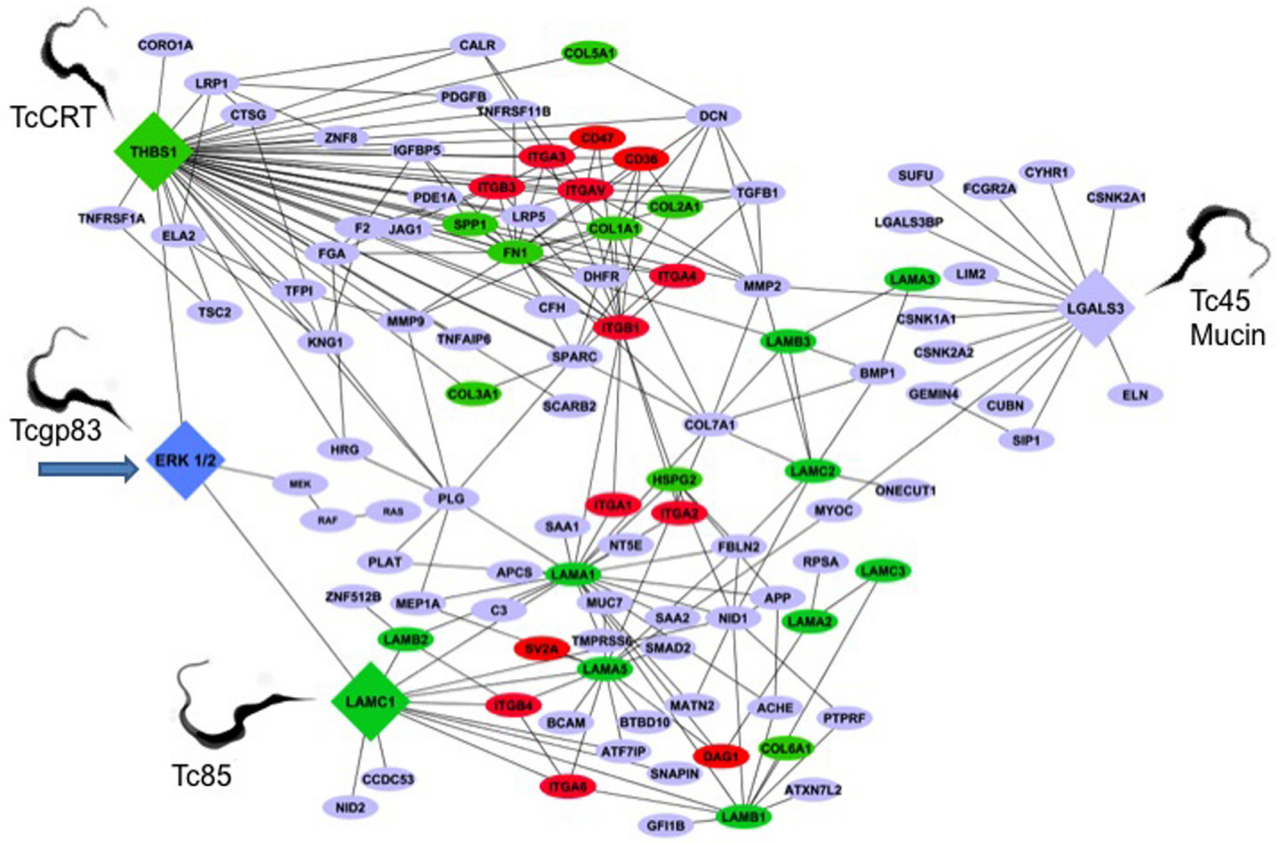

FIGURE 2 | *Trypanosoma cruzi* gp83 regulates the ECM interactome and trypanosome usage of the ECM during early infection to enhance cellular infection. *T. cruzi* gp83 ligand triggers gp83 receptors in the cell via ERK1/2 to up-regulate LAMC1 which cross-talks with LGALS3 and THBS1 to enhance cellular infection using selected parasite surface molecules such as TcCRT, TC45 mucin, and Tc85. Arrow points to the first signal. The interactome was generated using MiMI Cytoscape plugin (version 3.2). The MiMI interface uses a database which is itself constructed from merged data of well-known protein interaction databases as described in the text of the review. The network was connected by using ERK1/2, Laminin gamma-1 (LAMC1), Thrombospondin-1 (THBS1), and Galectin-3 (LGALS3) as initial seed nodes (large DIAMOND nodes). Node expansion was done to one degree of biological protein–protein interaction partners of the seed nodes using the Michigan Molecular Interaction Database. Proteins mapping to the canonical "ECM-Receptor Interaction Pathway" of KEGG (hsa04512) are highlighted as following: GREEN nodes are ECM ligands (laminins, collagens, fibronectin, heparan sulfate, and thrombospondin-1). RED nodes are ECM receptors: (integrins, cd35, cd47, and SV2A). BLUE nodes are not part of the KEGG ECM–ECM Receptor Pathway, but have established biological interactions with ECM–Receptor interaction nodes. The global interactome was visualized in Cytoscape (version 2.6.3), yielding the ECM interactome involved in early *T. cruzi* infection. Trypomastigotes pointed in the figure are culture trypomastigotes released from infected cells expressing surface Tcgp83, TcCRT, and Tc45 mucin. All of these trypomastigote surface molecules are also expressed in blood trypomastigotes.

activity that degrades the ECM may regulate the ECM interactome (Nogueira de Melo et al., 2010). This ECM-focused interactome contains 108 nodes representing protein-coding genes connected by 222 edges representing biological interactions between nodes (**Figure 2**).

Increased THBS1 expression significantly modulates the interactome cross-talk between cells. This change in network topology potentially favors parasite invasion and infection of host cells. As such, THBS1 interacts with several proteins ranging from adhesion receptors (CD36 and CD47) to structural proteins (COL7A1) and zymogens. CD47 is a receptor for the C-terminal domain of THBS1 and this interaction may be important in membrane transport and signal transduction. In addition, CD47 is involved in intracellular calcium increase, which occurs when the cell adheres to the ECM. An increase in cytosolic Ca^{++} in *T. cruzi* trypomastigotes was detected at the single cell level after association of the parasites with myoblasts. Ca^{++} mobilization in host cells was also detected upon contact with trypomastigotes. Confirmatively, pretreatment of the parasites with the Ca^{++} chelators decreased trypomastigote association to myoblasts indicating that calcium mobilization is required for cell invasion (Moreno et al., 1994).

The structural protein COL7A1 is typically found in the basement membrane and also associates with THBS1. Once COL7A1 interacts with THBS1, it makes the ECM conducive to parasite motility and cellular invasion with the help of proteins from the laminin family of genes. Plasminogen (PLG) links LAMC1 and THBS1. PLG (a zymogen) is cleaved into plasmin (a serine protease), and angiostatin (an angiogenesis inhibitor). Plasmin is known to cleave fibronectin, THBS1, and LAMC1, thus taking part in ECM modifications.

Trypanosoma cruzi must navigate through the basal lamina, which contains LAMC1. The *T. cruzi* gp83 ligand modifies LAMC1 expression in the ECM and contributes to the pathogenesis of *T. cruzi* infection in human heart cells. The fact that the LAMC1 network is connected to THBS1 through PLG and COL7A1 and matrix metalloproteinase 2 (MMP2) suggests that this network could facilitate parasite mobilization. MMP2 is a type IV collagenase that participates in the rearrangement of the ECM, which could facilitate parasite mobilization. In the LAMC1 sub-network there is an indirect connection (through LAMA1) to dystroglycan (DAG1). DAG1 is a dystrophin-associated glycol-protein responsible for transmembrane linkage between the ECM and host cell cytoskeleton. The extracellular form of DAG1 can bind to merosin alpha-2-laminin in the ECM (Yamada et al., 1994). If *T. cruzi* alters the dystroglycan complex, it could consequently manipulate or weaken the host cell cytoskeleton prior to gaining entry into the cell. This could yet be another mechanism by which *T. cruzi* invades host cells. In reference to the ECM network reviewed, LAMC1 also has a second-degree interaction (one node in between the genes) with LGALS3 through myocilin (MYOC). MYOC is a secreted protein believed to have a role in cytoskeletal function, specifically vesicular transport and ECM conformation (Caballero et al., 2000). Unlike other intracellular pathogens, which avoid contact with host cell lysosomes, *T. cruzi* requires the low-pH environment of lysosomes to initiate egression from the vacuole and delivery to the host cell cytoplasm where replication takes place (Burleigh, 2005). Therefore, the control of vesicular trafficking by *T. cruzi* improves the rate of trypomastigote entry and amastigote replication in the host cell. The fact that LAMC1 is connected to LGALS3 through MYOC in the ECM interactome suggests the importance of LGALS3 in the manipulation of host ECM by *T. cruzi*.

Increased LGALS3 expression in the ECM promotes the adhesion of *T. cruzi* to host cells and subsequent infection (Kleshchenko et al., 2004). In addition, LGALS3 has numerous ECM interacting partners (Dumic et al., 2006), including collagen IV, hensin, laminins, fibronectin, vitronectin, tenascin, and elastin. LGALS3 regulates adhesion of these ECM proteins to a variety of host cells. Matrix metalloproteases, which are more active in *T. cruzi* infected mice, regulate LGAL3 function (Gutierrez et al., 2008). When metalloproteases are activated in the ECM, they can cleave LGALS3 and negatively regulate its function. Consequently, increased activation of MMP1 and MMP9 is associated with ECM destruction and myocarditis in *T. cruzi* infection.

Multiple types of collagen interact with the three central seed nodes, THBS1, LGALS3, and LAMC1. A glimpse of the importance of collagen in early *T. cruzi* infection was reported (Velge et al., 1988).

SOME POTENTIAL IMPLICATIONS OF THE ECM IN INFECTION

Expression profiling microarray studies of *T. cruzi* infected cells have shown that some ECM genes are regulated (Goldenberg et al., 2009; Mukherjee et al., 2003), however those genes were not confirmed by RT-PCR, nor their function in the process of *T. cruzi* infection was determined. Several reports suggest that some parasite proteases may degrade the ECM. The possible role of trypanosomatid surface proteases in parasite survival and infection has been discussed (Marino et al., 2003; Yao, 2010). It was suggested that the gp85/trans-sialidase interacts with the ECM components (Alves and Colli, 2008) and speculated that the prolyl oligopeptidase Tc 80 may be involved in the infection possibly by acting on ECM components (Grellier et al., 2001). Interestingly, it was found that in a *T. cruzi* infected mouse embryo hepatocyte cell line, there was a reduction of MMP9 (Nogueira de Melo et al., 2004), as well as some matrix components during the late phase of infection (60th to 90th day; Magalhaes-Santos et al., 2002). Pinho et al. (2002) reported that uncharacterized parasite antigens bind to non-infected cells and that ECM components were recognized by antibodies, however the molecular characterization of antigens and the specificity of these possible interactions were not considered. Furthermore, Santana et al. (1997) showed that a Tc 80 proteinase hydrolyzes collagen type in rat mesentery. It is possible that that ECM derived from *T. cruzi* infected endothelial cells directs phenotypic expression (Morris et al., 1990).

It has been suggested that there are fibrotic implications during *T. cruzi* infection involving the ECM. Accordingly, Calvet et al. (2009) suggested that TGF-β and TNF-α stimulate fibronectin expression in uninfected cells of *T. cruzi*-infected cultures, whereas cells harboring the parasites display low or no fibronectin fibrils. It was observed that there are sequential changes of the connective matrix components of the myocardium (fibronectin and laminin) during fibrosis in infected mice (Andrade et al., 1989). The fact that

T. cruzi-mediated down-regulation of CTGF expression requires *de novo* host cell protein synthesis and that *T. cruzi* interferes with the host fibrogenic response suggest that this complex process requires input from multiple host cell signaling pathways (Unnikrishnan and Burleigh, 2004). Although it has been demonstrated that cardiomyocytes are able to synthesize cytokines upon *T. cruzi* infection, Calvet et al. (2004) suggest that matrix remodeling is dependent on cytokines secreted by inflammatory cells recruited in immune response. These reports suggest that *T. cruzi* modulates the ECM during the infection process with potential implications in the pathology of the disease.

CONCLUDING REMARKS

Despite an appreciation of the involvement of the ECM in *T. cruzi* infection, the difficulty in delineating regulatory networks in the process of cellular infection and disease, has until now prevented a comprehensive assessment of *T. cruzi* infection at the molecular level. The use of systems biology approaches to elucidate the ECM interactome network regulated by *T. cruzi* and its gp83 ligand that mediate trypanosome attachment and entry are critically important to understand the molecular pathogenesis of *T. cruzi* infection and to design novel approaches for intervention and disease management. There is an expectation that systems biology approaches applied to understand *T. cruzi* infection would bring new innovative strategies for the treatment and control of Chagas disease. So far the use of systems biology to understand the role of the ECM in *T. cruzi* infection has significantly enhanced the understanding of *T. cruzi*–host cell interaction and pathogenesis of *T. cruzi* infection. Accordingly, here we elucidate the first ECM interactome triggered by the *T. cruzi* gp83 ligand during the first step of cellular infection to recruit trypanosomes at the ECM to enhance cellular infection. In the shot gun used by *T. cruzi* to regulate the ECM interactome to gain cellular entry and evade the host reported here, *T. cruzi* gp83 triggers gp83 receptors in the cell via ERK1/2 to up-regulate LAMC1 which cross-talks with both LGALS3 and THBS1 to enhance cellular infection using selected parasite surface molecules such as TcCRT and TC45 mucin. Based on this new information, current efforts are being pursued by our group to elucidate the molecular signature induced by *T. cruzi* in cells. Since *T. cruzi*–host cell interaction is of a complex nature, the elucidation of the global interactome and sub-interactome networks will help understand the complex *T. cruzi*–host interphase that mediate parasite attachment and entry, and develop new means for the treatment of the highly neglected and complex Chagas disease, which has no current effective drugs or vaccines.

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Selection of binding targets in parasites using phage-display and aptamer libraries *in vivo* and *in vitro*

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Parasite infections are largely dependent on interactions between pathogen and different host cell populations to guarantee a successful infectious process. This is particularly true for obligatory intracellular parasites as *Plasmodium*, *Toxoplasma*, and *Leishmania*, to name a few. Adhesion to and entry into the cell are essential steps requiring specific parasite and host cell molecules. The large amount of possible involved molecules poses additional difficulties for their identification by the classical biochemical approaches. In this respect, the search for alternative techniques should be pursued. Among them two powerful methodologies can be employed, both relying upon the construction of highly diverse combinatorial libraries of peptides or oligonucleotides that randomly bind with high affinity to targets on the cell surface and are selectively displaced by putative ligands. These are, respectively, the peptide-based phage display and the oligonucleotide-based aptamer techniques. The phage display technique has been extensively employed for the identification of novel ligands *in vitro* and *in vivo* in different areas such as cancer, vaccine development, and epitope mapping. Particularly, phage display has been employed in the investigation of pathogen–host interactions. Although this methodology has been used for some parasites with encouraging results, in trypanosomatids its use is, as yet, scanty. RNA and DNA aptamers, developed by the SELEX process (Systematic Evolution of Ligands by Exponential Enrichment), were described over two decades ago and since then contributed to a large number of structured nucleic acids for diagnostic or therapeutic purposes or for the understanding of the cell biology. Similarly to the phage display technique scarce use of the SELEX process has been used in the probing of parasite–host interaction. In this review, an overall survey on the use of both phage display and aptamer technologies in different pathogenic organisms will be discussed. Using these techniques, recent results on the interaction of *Trypanosoma cruzi* with the host will be highlighted focusing on members of the 85 kDa protein family, a subset of the gp85/TS superfamily.

Keywords: combinatorial methods for diagnosis and therapy, phage display, aptamers, SELEX, Kinetoplastidae, apicomplexa

INTRODUCTION

Phage display technology and RNA and DNA aptamers, developed by the SELEX process (Systematic Evolution of Ligands by Exponential enrichment), were described over two decades ago and since then contributed to a large number of small peptides or structured nucleic acids for diagnostic or therapeutic purposes or for the understanding of the cell biology (**Figure 1**). Comparison of normal cells with cancer or infected cells and vascular endothelium of organs are the most visible examples. The small peptides or nucleic acids of interest can be modified to increase their half-life in the organism or to be conjugated to other molecules, such as fluorescence probes, nanoparticles, or immobilized matrices, increasing their potential use.

PHAGE DISPLAY

The use of filamentous bacteriophages (virus that infect bacteria) to express and display foreign protein fragments or peptides started in the mid-1980s when a portion of the gene encoding the *EcoRI* endonuclease was fused to the gene coding for the pIII protein coat from a M13 virus (Smith, 1985). The result of this original experiment was the production of hybrid filamentous bacteriophages, or fusion phages, expressing and displaying the product of the fusion gene into the minor capsid protein pIII on the surface of the phage particle (Smith, 1985). This approach represented a landmark in the field of molecular genetics, because firstly foreign DNA was directly linked to the replicating phage genome and secondly, the phage operated like an "expression vector" with the foreign DNA being expressed as a "protein" associated to the phage protein coat (Smith and Scott, 1993; Smith and Petrenko, 1997).

Most phage-display work has used filamentous phage strains (M13 and its close relatives fd and f1) as vectors. Filamentous bacteriophage consists of a circular single-stranded DNA (ssDNA) genome covered by a few thousand copies of the major coat

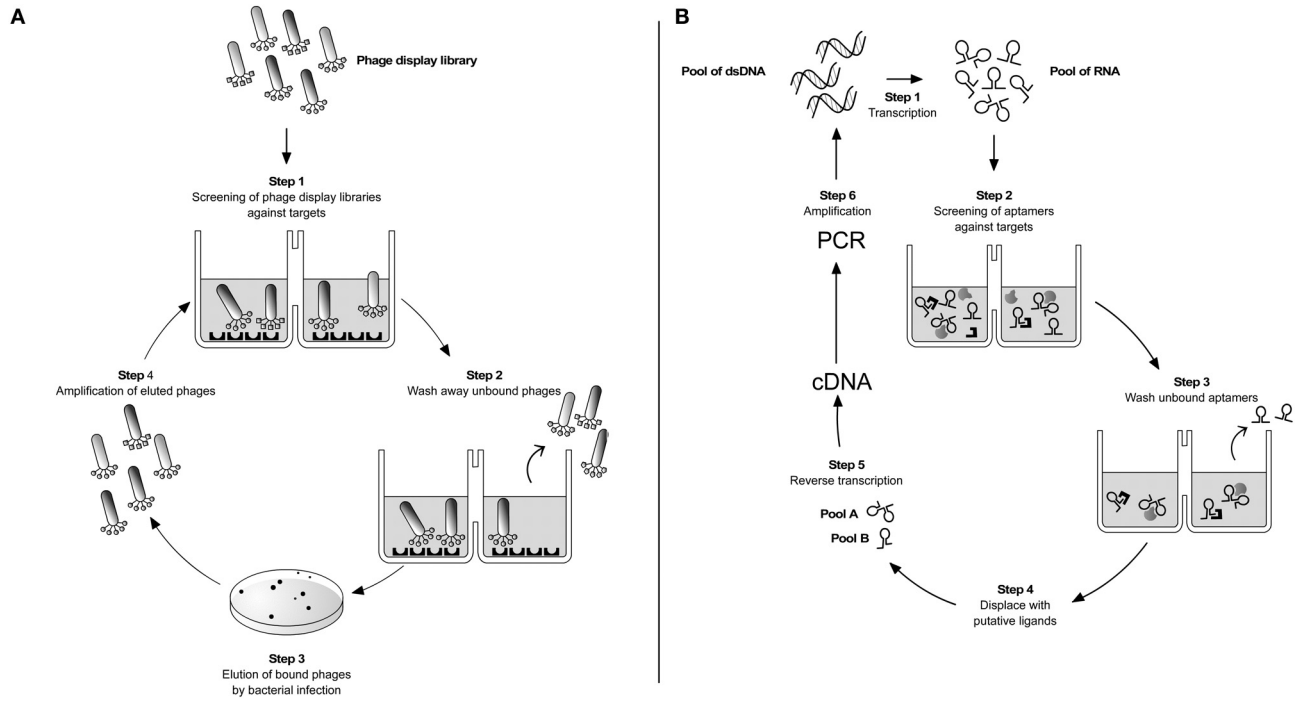

FIGURE 1 | Parasite targetting by combinatorial techniques. (A) Phage display. A phage library displaying potential ligand proteins on their surface is exposed to an immobilized target (step 1). After washing away unbound phages (step 2), binders are eluted by *Escherichia coli* infection and plated on LB-agar (step 3). Clones are then amplified producing a phage mixture that is enriched with relevant (i.e., binding) phage (step 4). The repeated cycling of these steps is referred as "panning." At the end of 3–4 rounds of panning the enriched phage population is recovered by infection of a suitable bacterial host and sequenced to identify the interacting peptides or protein fragments. **(B)** SELEX. It is based on a stretch of single-stranded nucleic acid, which can be RNA or single-stranded DNA (ss-DNA). These are chemically synthesized to have a random stretch usually from 8 to 40 nucleotides, flanked by constant sequences. In the case of RNA SELEX, the synthetic DNA template is transcribed into a pool of 10^{13}–10^{14} different RNA molecules (step 1). The pool is incubated with the desired targets and due to the sample diversity some of the aptamers will bind to their targets (step 2). After washing out unbound RNAs (step 3) the different RNA pools are displaced by incubation with ligands of interest (step 4). By reverse transcription (step 5) and PCR amplification (step 6) selected double-stranded DNAs are reconstructed. The same cycle is repeated over 8–12 times until purified sequences specific for a given ligand are selected. The DNAs are cloned and sequenced. This iterative method follows the same logic when single-stranded DNA sequences are used as aptamers instead of RNA (Ulrich and Wrenger, 2009).

protein pVIII (∼2700 copies in wild type phage) with each end capped by five copies of two different sets of proteins: pIII and pVI at the end that binds to bacteria and inject the DNA into the host cell; pVII and pIX at the other end (Marvin, 1998). Each of the five capsidic proteins has been used to display foreign polypeptides on the surface of the M13 bacteriophage but the minor protein pIII is most commonly used (Greenwood et al., 1991; Smith and Petrenko, 1997). Despite the ease manipulation and extraordinary stability of the phage particle, the display of foreign proteins is not without difficulties and imposes that polypeptides have a limited size, sequence, and folding characteristics, as large molecules may compromise the structure and function of the protein coat (Sidhu, 2000). This restraint has led researchers to cast about for new protein display scaffolds resulting in the development of phagemid display systems (Qi et al., 2012). Phagemid was developed as a hybrid of the filamentous phage M13 and a plasmid to produce a vector that can grow as a plasmid, and also be packaged as single stranded DNA in viral particles. When introduced into a bacterial host together with a "helper-phage," phagemid systems allow the display of both fusion and wild type coat proteins attenuating possible defects on phage function (Sidhu, 2000; Qi et al., 2012).

In recent years display of heterologous proteins on the surface of microorganisms is not restricted to the filamentous phage (Smith, 1985). Alternative display systems that use bacteriophage λ (Sternberg and Hoess, 1995), lytic phages like T4 and T7 (Efimov et al., 1995; Ren et al., 1996), eukaryotic viruses such as baculovirus (for a review see Makela et al., 2010), bacteria (Georgiou et al., 1997), and yeast are also used, each one having advantages and disadvantages with respect to each particular application.

The benefits behind the display systems have also encouraged the development of a new generation of selection technologies. Cell-free systems like ribosome (He and Taussig, 2002), mRNA (Takahashi et al., 2003) and DNA display technologies (Yonezawa et al., 2004) are proven to be more advantageous than phage display, as larger libraries (up to 10^{14}) can be constructed. While in the ribosome display, individual nascent proteins are coupled to their corresponding mRNA through the formation of stable ternary protein–ribosome–mRNA (PRM) complexes,

in the other two systems protein fragments and peptides are covalently coupled to a DNA/RNA template (He and Taussig, 2002; Takahashi et al., 2003; Bertschinger and Neri, 2004). These technologies are becoming more commonplace, but the phage display platform remains the leading technology (Sidhu, 2000).

Phage display libraries are heterogeneous mixtures of fusion phages, each one carrying a different foreign DNA insert and displaying a different protein on filamentous phage surface (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990; Smith and Petrenko, 1997). Such libraries containing billions of phage clones (some libraries are as high as 10^{12} diverse) are extensively used to screen and select for peptides that bind with high-affinity to target molecules. The selection procedure known as "panning" or "biopanning" (depending whether selection is performed *in vitro* or *in vivo*, respectively) is simple and involves four major steps (**Figure 1**) (Parmley and Smith, 1988). After 3–5 rounds of selection against the desired target, individual clones are isolated and the primary structure of the binding peptides is deduced by nucleotide sequencing. Peptide sequences obtained in this manner may then be used in alignment search for known proteins (Koivunen et al., 1999).

APPLICATIONS OF PHAGE DISPLAY

One of the earliest applications of phage display technology was to study antigen–antibody binding for the identification of epitopes and mimotopes (small peptides that mimic linear, discontinuous, and even non-peptide epitopes) (Germaschewski and Murray, 1995). However, it was later shown that larger molecules like antibody fragments (scFV, Fab fragment, VHH domains) could be successfully displayed on phage (McCafferty et al., 1990; Benhar, 2001; Bradbury and Marks, 2004; Petrenko, 2008). The development of phages that display antibodies led to the emergence of a new molecular recognition interface to study protein–protein interactions, structure-function relationships, and protein folding and stability (Clackson et al., 1991; Vaughan et al., 1996). As a result, phage displayed cDNA libraries advanced significantly with a plethora of ligands fused to phage particles such as glycoproteins (Celik et al., 2010), enzymes (Soumillion et al., 1994), protease inhibitors (Markland et al., 1996), cytokines (Gram et al., 1993; Buchli et al., 1997), secreted as well as cytoplasmic, nuclear and membrane proteins (Vithayathil et al., 2011) to cite a few examples.

With the increasing number of phage display collections, numerous new applications have emerged. Selections from various libraries have been used to identify peptide agonists and antagonists for receptors (Sidhu, 2000), determine bind specificity of domains (Sparks et al., 1996; Linn et al., 1997), map carbohydrates and protein functional epitopes (Sidhu, 2000; Fukuda, 2012), select antibodies recognizing post-translational modifications (Kehoe et al., 2006), identify targets for the inhibition of tumor-specific angiogenesis (Koivunen et al., 1999; Arap et al., 2002; Zurita et al., 2003), vaccine development (Lidqvist et al., 2008), and molecular imaging with the use of fluorescently labeled phage (Newton et al., 2006; Petrenko, 2008). More recently, there is a strong trend for the use of phage display in medical science with the production of humanized antibodies and development of new therapeutics. Indeed, a few selected peptides and proteins are in clinical or preclinical stages of development and some are reaching the market (Rothe et al., 2006).

THE USE OF PHAGE DISPLAY IN THE STUDY OF PARASITIC INFECTIONS

Infectious diseases may be caused by various pathogens like bacteria, fungi, virus, multi and unicellular parasites. Independently of the mechanism by which the pathogenic agent elicits infection, the surface components are key determinants for the disease progression. Over the years several laboratories have made great effort to elucidate the mechanisms involved in the establishment of infectious diseases and not surprisingly many of them have focused on the discovery of molecules, from both the pathogen and the host. Considering this, much of the work on pathogen–host interaction relied on classical biochemical and cellular biology approaches (purification, cross-linking, immunoprecipitation, and fractionation) to track for ligands and receptors. Nevertheless, with the rapid expansion of the phage display platform and the development of high throughput selections, pathogenic agents and their hosts are currently being mapped for toxins, ligands and interacting proteins as well for the identification of possible drug candidates.

STUDIES ON APICOMPLEXA USING PHAGE DISPLAY

Plasmodium

The phylum apicomplexa encompasses a vast array of organisms, some of which are of major importance in veterinary and medical areas. Members of the genus *Plasmodium*, *Toxoplasma* and *Cryptosporidium*, for example, are pathogens of humans and cause, respectively, malaria, toxoplasmosis and cryptosporidiosis. The genus *Eimeria* contains important parasites of livestocks, especially poultry, causing coccidiosis. All are unicellular eukaryotes and are obligatory intracellular parasites circulating between an intermediate and a definitive host. The potential of the phage display system in the field of parasitology was first demonstrated in studies on malaria.

Plasmodium protozoa are transmitted to humans by the bite of female mosquitoes from the Anopheles genus. Infection in humans begins when the sporozoites, injected with the saliva of the mosquito vector during blood meal, spreads through the bloodstream reaching the liver. Once inside the hepatocytes (exoerythrocytic cycle), sporozoites divide repeatedly and differentiate into merozoites, which once released in the bloodstream infect the red blood cells (RBCs) beginning the intraerythrocytic cycle. The surface of RBCs is covered by sialoglycoproteins from which glycophorins are the major constituent being implicated in merozoite invasion (Howard et al., 1982). To identify the molecular ligands for the glycophorins, a phage display cDNA library from a *P. falciparum* strain dependent on sialic acid to invade RBCs was used in panning assays against purified human MN-glycophorins or isolated RBCs. After four panning rounds, sequencing of individual positive clones and BLAST searches using the PlasmoDB, several parasite proteins that specifically bind to glycophorins and RBCs were identified. Among them were the erythrocyte-binding antigen (EBA-175) and the erythrocyte-binding ligand-1

or EBL-1, both belonging to the superfamily of the erythrocyte binding-like (EBL) proteins (Li et al., 2012). In addition, the phage cDNA insert bearing the binding site for glycophorin encoded a 69-amino acid peptide located within a subdomain (D2) of the Duffy binding-like domain (DBL) of the EBL-1 gene (Li et al., 2012).

A different strategy to study host–parasite interactions during the intraerythrocytic life cycle of *Plasmodium* was the use of a peptide phage display library to identify peptides with affinity for invading merozoites and infected RBCs (iRBCs). When merozoites are prone to invade the erythrocytes, they translocate and expose on their surface a type I integral membrane protein known as the apical membrane antigen-1 (AMA-1) (Triglia et al., 2000). This protein undergoes proteolytic cleavage at around the point of host invasion and this processing is essential for parasite entry as antibodies that prevent AMA-1 proteolysis inhibit RBC invasion *in vitro* (Dutta et al., 2003). Moreover, during the development of merozoites inside the RBCs, *Plasmodium* proteins are associated or inserted into the host plasma membrane dramatically altering the composition and structure of the erythrocyte membrane. This event is an important mechanism exploited by *Plasmodium* to evade the host immune system since erythrocytes become covered with variant antigens (antigenic variation) protecting the parasite from variant-specific antibodies elicited by earlier infections. This variation is mediated by the differential control of a family of surface molecules termed PfEMP1 and encoded by approximately 60 *var* genes as described by the genome sequencing of *P. falciparum* (Aley et al., 1984; Gardner et al., 2002). Considering these data, panning of random peptide phage display libraries against AMA-1 and the altered surface of infected *Plasmodium* erythrocytes were performed to identify mimotopes for merozoites and iRBCs. One of the peptides that bound to AMA-1 was shown to be a potent inhibitor of the invasion of *P. falciparum* merozoites into human erythrocytes (Li et al., 2002). When the targets were the iRBCs, one selected peptide (P1) that constituted more than 40% of the sequenced phage clones bound specifically to the surface of infected erythrocytes and showed anti-malarial activity (Eda et al., 2004).

Phage display has also been used to investigate the host immunological response against the sporozoites in the pre-erythrocytic cycle. Peptides that represented the circumsporozoite protein (CSP), the major surface antigen of the sporozoites, were inserted into the gene of PVIII of filamentous phage and used to immunize rabbits to test for the antigenicity of CSP epitopes. These experiments have shown that CSP can generate a protective antibody response in addition to provide insights into new antigenic epitopes for vaccine development against malaria parasites (Greenwood et al., 1991).

In an effort to control the development of *Plasmodium* inside the invertebrate host, a work focused on the study of *Anopheles*-malaria parasites interaction was undertaken. Using a phage display library and successive rounds of biopanning with female mosquitoes, a single peptide (SM1) was selected that bound specifically to the luminal side of the midgut epithelium and to the distal lobe of the salivary glands inhibiting *Plasmodium* invasion of the two organs (Ghosh et al., 2001, 2002). Examples on the work of malaria research using phage display technology are listed in **Table 1** (Lanzillotti and Coetzer, 2008).

Toxoplasma gondii

Toxoplasma parasites are another good example of apicomplexa that served as excellent models in studies involving phage display technology. Infection with *T. gondii* occurs by the ingestion of either infectious oocysts through contact with cats (the definitive host) or cat feces or by feeding with meat that contains tissue cysts. Although opportunistic in healthy humans, toxoplasmosis is a lethal disease when progressing in chronically infected individual developing immunodeficiency, particularly in AIDS patients. As a consequence, much of the work on *Toxoplasma* and phage display is focused to cover different aspects of the immunological response against this parasite. To identify antigens implicated in human B-cell responses, a lambda phage-display library of *T. gondii* cDNA fragments was screened with sera of infected individuals. As a result, recombinant phage clones were identified carrying B-cell epitopes present in different *T. gondii* antigens like SAG1, GRA1, GRA7, GRA8, and MIC5 (Beghetto et al., 2003). Using a similar strategy, this group screened a panel of sera of pregnant women infected with *T. gondii* and identified an antigenic peptide within the sequence of the dense granule GRA1 protein (p24) that is immunodominant in *Toxoplasma* infections (Beghetto et al., 2001). Altogether, these data demonstrated the potential of phage-display technology for antigen discovery and for the study of the human antibody response on infectious agents. Data in **Table 2** summarize the work on *Toxoplasma* research using phage display technology.

Cryptosporidium parvum

To date, 20 different species of *Cryptosporidium* have been described based on differences on the host species they infect. *Cryptosporidium* species are enteric coccidia parasites causing cryptosporidiosis (an acute, non-bloody, watery-diarrhea) in mammalian hosts. **Table 3** summarizes work on *Cryptosporidium* using phage display technology. *C. parvum* is of particular concern as it infects humans, especially immune compromised individuals where the disease may become chronic and cause a life-threatening gastroenteritis with a high mortality. Infection with *C. parvum* occurs when ingested oocysts release sporozoites in the intestines of a suitable host. The sporozoites then attach to and invade epithelial cells of the gastrointestinal tract where they undergo intracellular development through asexual as well as sexual cycles. Even though many proteins, such as GP900, CP47, TRAP-C1, GP40, and GP15, have been implicated in the infection, increasing knowledge on the host–parasite interacting molecules is crucial for designing strategies to combat cryptosporidiosis. This has been investigated by panning a cDNA library of the sporozoite and oocyst stages of *C. parvum* expressed on the surface of T7 phage against intestinal epithelial cells (IECs). This study has identified a surface 12 kDa protein (CP12) that localizes especially at the apical region of sporozoites (Yao et al., 2007). Another work has identified the CP2, a known surface molecule of sporozoites involved in the invasion process, when the *C. parvum* T7 phage display library was screened by using Caco-2 (human epithelial colorectal adenocarcinoma) cells (Guo

Table 1 | Summary of phage display applications in malaria research.

Target	Library	Result	References
Mosquito epithelia	Random peptides	Identification of SM1 peptide that inhibits salivary gland invasion by *Plasmodium*	Ghosh et al., 2001, 2002
Ookinete surface	Random peptides	Identification of enolase and actin on the surface of ookinetes	Hernandez-Romano et al., 2011
Parasitized erythrocyte	Random peptides	Identification of a peptide that binds to the surface of *Plasmodium*-infected erythrocytes. Isolation of an antibody against a peptide that causes hemolysis of iRBCs	Eda et al., 2004
Erythrocyte surface proteins	*P. falciparum* cDNA phage library	Identification of *Pf*EBL-1 that bind glycophorin B on the surface of erythrocytes	Li et al., 2012
Purified human erythrocyte protein 4.1	*Plasmodium* cDNA phage library	Identification of EBA-175, EBL-1 and a Ser/Thr kinase as ligands for protein 4.1	Lauterbach et al., 2003
AMA-1	Random peptides	Identification of peptides that bind AMA-1 and inhibit parasite invasion of erythrocytes; structure determination of AMA-1 epitope targets of inhibitory human antibodies	Nair et al., 2002; Keizer et al., 2003
	Mouse antibodies	Identification of four antibodies specific for *Plasmodium* AMA-1	Sabo et al., 2007
Anti-AMA-1 and anti-rhoptry mAbs	Random peptides	Identification and structural elucidation of AMA-1 mimics. Antibodies to mimotopes that inhibit erythrocyte invasion by *Plasmodium*	Narum et al., 2006
CSP	Phage library of CSP peptides	Induction of a strong and specific immune response. Structure determination of a CSP epitope	Greenwood et al., 1991; Monette et al., 2001
MSP	Human and mouse antibodies	Isolation of three antibodies against MSP-3 and MSP-1 that inhibit parasite growth	Sowa et al., 2001; Lundquist et al., 2006; Cheng et al., 2007
	Random peptides	Identification of MSP mimotopes	Demangel et al., 1996
SERA5 enzyme	Random peptides	Identification of a peptide that binds the catalytic domain of the enzyme and affects intraerythrocytic development	Fairlie et al., 2008
Pfs48/45 protein	Human antibodies from malaria-immune patients	Isolation of a scFV that reacts with sexual stages of *P. falciparum* and Pfs48/45 protein	Roeffen et al., 2001
Duffy binding protein (DBP)	Human antibodies from malaria-immune patients	Identification of three scFV that binds (DBP) inhibiting adhesion of RBCs	Kim et al., 2007

et al., 2009). Single-chain variable fragment (scFv) phagemid library has also being used to identify for specific scFvs that bind to *C. parvum*. As a result, panning against the surface protein P23, revealed two scFv sequences that were able to detect both native *C. parvum* proteins and recombinant P23. Importantly, the selected fragments showed no cross-reactivity with *E. coli*, *S. pyogenes*, *L. monocytogenes*, *B. cereus*, *G. lamblia* (cysts or trophozoites), or with S16, another dominant surface antigen on *C. parvum* sporozoites (Boulter-Bitzer et al., 2009).

Eimeria

Infection of chicken with ***Eimeria*,** the causative agents of coccidiosis, is a serious problem for the poultry industry being responsible for varying degrees of morbidity and mortality between

Table 2 | Summary of phage display applications in *T. gondii* research.

Target	Library	Result	References
Sera of *T. gondii*-infected individuals	*T. gondii* cDNA phage library	Identification of epitopes of the *T. gondii* antigens	Beghetto et al., 2003; Di Cristina et al., 2004
Sera of *T. gondii*-infected pregnant women	*T. gondii* cDNA phage library	Identification of an epitope GRA1 protein	Beghetto et al., 2001
Anti-*T. gondii* mAbs	*T. gondii* cDNA phage library	Identification of an epitope of GRA3 located in the dense granules of *T. gondii* tachyzoites	Robben et al., 2002
	Random peptides	Identification of a linear epitope within SAG2A that is expressed in *T. gondii* tachyzoite surface	Cunha-Junior et al., 2010
TgMIC2	Mouse antibodies from *T. gondii* immune animals	Identification of scFV antibodies that recognize TgMIC2	Hoe et al., 2005

Table 3 | Summary of phage display applications in *C. parvum* research.

Target	Library	Result	References
C. parvum glycoproteins	*C. parvum*-specific polyclonal antibody library	Identification of antibodies that recognize *C. parvum* glycoprotein and oocyst/sporozoite preparations	Chen et al., 2003
C. parvum oocysts	Human semi-synthetic phage display antibody libraries	Isolation of scFv antibodies that block infection of HCT-8 cells by *C. parvum*	Pokorny et al., 2008
Sporozoite surface antigen S16 and P23	Human semi-synthetic phage display antibody libraries	Isolation and identification of scFV antibodies that bind to *C. parvum*	Boulter-Bitzer et al., 2009, 2010
Intestinal epithelial cells (IECs)	*C. parvum* cDNA phage library	Identification of a surface adherence protein (CP12) from sporozoites	Yao et al., 2007

infected birds. *Eimeria* parasites invade and develop within the avian IECs causing tissue damage that results in blood loss, dehydration, nutrient malabsorption, and increased susceptibility to other opportunistic pathogens. The parasite is acquired by the ingestion of sporulated oocysts that release sporocysts, which in turn release invasive sporozoites. These invade the avian intestinal tract and once inside the host cells they round up as a trophozoite. At the end of the cycle, *Eimeria* parasites leave the cells as merozoites, which further infect fresh host cells. Control of avian coccidia is being a main challenge in veterinary science and, consistently, studies using phage display are directed toward this end. This is reflected on a work where a phage display library was used to screen for peptides with *in vitro* activity against *E. acervulina* and *E. tenella* sporozoites using living purified *E. acervulina* sporozoites as targets. The selected peptide (PW2) disrupted the sporozoite pellicle, similarly to most natural antimicrobial peptides and showed a very low lytic effect on mammalian and avian cells, suggesting a potential use as a drug against avian coccidiosis. A variation on this theme is the panning of antibody libraries against different development of *Eimeria* parasites. Heavy chain (VH) and light chain (VL) antibody libraries against *E. acervulina* merozoites (Zhao et al., 2010) and scFV antibody libraries against *E. tenella* sporozoites (Abi-Ghanem et al., 2008) were used aiming at the development of new tools for diagnosis and therapy against coccidiosis. Selected examples of these applications are listed in **Table 4**.

STUDIES ON KINETOPLASTIDAE–HOST INTERACTIONS USING PHAGE DISPLAY

The Kinetoplastidae are a class of unicellular and flagellated protozoan parasites responsible for serious diseases in humans and animals. Members of this class include all species of Trypanosomatidae, as *Leishmania* and *Trypanosoma* (*T. cruzi* and *T. brucei*).

Trypanosoma cruzi

T. cruzi, the American trypanosome, is an obligatory intracellular parasite that circulates between an invertebrate host (Triatomine insects) and a vertebrate host. In the latter, development of *T. cruzi* parasite begins with an intracellular amastigote form that reproduce within different cell types by binary fission. After successive rounds of replication, amastigotes differentiate into the infective and non-replicative trypomastigotes, which are released from the infected cell. The surface of the infective trypomastigotes of *T. cruzi* is covered by glycoproteins important for the adhesion and/or internalization of the parasite to host cells and one

Table 4 | Summary of phage display applications in *Eimeria* research.

Target	Library	Result	References
Eimeria sporozoites/live	Random peptides	Identification of PW2 peptide with activity against *E. acervulina* and *E. tenella* sporozoites	da Silva et al., 2002
E. tenella sporozoites/cryopreserved	Chicken scFV antibodies from *E. tenella* immune birds	Isolation of an scFV antibody that binds specifically to *E. tenella* sporozoites	Abi-Ghanem et al., 2008
Cryopreserved *E. acervulina* merozoites	Chicken scFV antibodies from *E. tenella* immune birds	Identification of antibody fragments with high specificity and binding capacity for soluble antigens and intact fixed merozoites	Zhao et al., 2010
Anti-GAM56 protein	*E. tenella* cDNA phage display	Identification of EtGAM22 expressed predominantly at the gametocyte stage	Krucken et al., 2008

example is the superfamily of GPI-anchored glycoproteins named gp85/trans-sialidase. The majority of this superfamily members have at the subterminal carboxyl side the motif VTVxNVFLYNR (known as FLY domain) involved in host cell binding (Magdesian et al., 2001). It has long been known that heart and gastrointestinal tract cells are important targets for *T. cruzi* infection playing significant roles in pathogenesis and maintenance of parasitic reservoirs. The nature of this tropism is not well known and understanding the determinants of this feature can contribute to *T. cruzi* control and drug development. Interestingly, random peptide libraries of bacteriophages injected intravenously pointed out to differences in the blood vessels of different organs (Pasqualini and Ruoslahti, 1996; Arap et al., 2002), raising the notion that the vascular bed expressed specific molecular markers or ZIP codes allowing the delivery of cells or molecules to designated targets (Rajotte et al., 1998). Infectious agents could also exploit these differences in the expression of vascular addresses to gain access to target tissues/organs were they could develop. A combination of immortalized endothelial cells (ECs) and phage display methodologies were employed to investigate whether the FLY domain could interact with the blood vessel and contribute to *T. cruzi* tissue homing. Initially, filamentous bacteriophages were genetically engineered to display the FLY peptide on the pIII minor coat protein (FLY phage). Using that strategy, it was shown that the FLY phage, but not the control phages, binds strongly to ECs derived from the heart and the bladder, two organs in which parasites can be found following infection (Tonelli et al., 2010). Furthermore, hybrid phages injected intravenously into mice demonstrated that FLY binds to the vasculature of different organs but is enriched in the vasculature of the heart, bladder, and esophagus in agreement with the cell binding *in vitro* assay (Tonelli et al., 2010). These results indicated that *T. cruzi* tropism at the molecular level could be explained, at least in part, through the interaction of the FLY motif with receptors present in the vascular bed of different organs. Indeed, this was the first experimental demonstration on how the use of phage display technologies can help to elucidate the mechanisms by which *T. cruzi* interacts with blood vessel receptors *in vivo*.

A phage display library was employed to help the identification of B-cell epitopes on the *T. cruzi* trans-sialidase (TcTS) (Pitcovsky et al., 2001). Trans-sialidase, a GPI-anchored glycoprotein, is shed by *T. cruzi*, being detected in the blood of infected patients during the acute phase of the disease. This enzyme is responsible for the direct transfer of sialyl residues from the host cells to acceptor proteins on the surface of infective trypomastigotes. Both, phage display technology and affinity-purified TcTS antibodies from *T. cruzi* infected rabbits were instrumental to isolate several B-cell epitopes located within or near the catalytic N-terminal domain of TS showing reactivity with sera obtained from *T. cruzi* infections. More importantly, some of the selected peptides were exposed on the surface of the TcTS probably explaining their strong antigenicity (Pitcovsky et al., 2001). Using the same approach the trypomastigote-restricted shed acute-phase antigen (SAPA) from *T. cruzi* was also mapped. The SAPA antigen is a 12-amino acid repetitive unit displayed *in tandem* on the carboxyl-terminus of the TcTS and it is the immunodominant antigen during *T. cruzi* infections. Screening of a phage display library of random peptides against anti-SAPA mAbs and purified immunoglobulin G from SAPA-immunized rabbits resulted in the identification of multiple linear overlapping B-cell epitopes within the repeated unit of SAPA (Alvarez et al., 2001). These data shed light on the molecular structure of the SAPA antigen and on how repetitive antigens are recognized by the immune system. **Table 5** summarizes work on trypanosomes.

African trypanosomes

Sleeping sickness or human African trypanosomiasis (HAT) is caused by subspecies of the protozoan parasite *T. brucei (T. b. gambiense* in West and Central Africa). *T. brucei* is transmitted by tsetse flies of the genus Glossina and once inside the vertebrate it escapes from the host immune system by continuously replacing the major parasite antigens on the plasma membrane, the variant surface glycoproteins or VSG. This successful tactic of the parasite to avoid the defense of the host turned *T. brucei* detection laborious and insensitive, limiting the identification of possible infected individuals. This observation led investigators to search for new ways of accurately identifying HAT-specific VSG

Table 5 | Summary of phage display applications in research with *T. cruzi* and *T. brucei*.

Target	Library	Result	References
T. cruzi			
Mouse vascular bed	Hybrid phage displaying the FLY domain	FLY interacts with the endothelium in an organ-dependent manner with a preference for the heart vasculature	Tonelli et al., 2010
TcTS-specific antibodies and anti-SAPA mAbs	Random peptides	Identification of B-cell epitopes located on TcTS and the SAPA antigen	Pitcovsky et al., 2001
T.b. gambiense			
Sera of *T.b. gambiense*-infected mouse and humans	Random peptides	Identification of epitopes of the *T.b. gambiense* VSG antigens	Van Nieuwenhove et al., 2011, 2012

epitopes (mimotopes) possibly recognized by the host immune system. Two studies illustrate well this idea (Van Nieuwenhove et al., 2011, 2012). In one study, two phage display libraries of random peptides (12-mer and cyclic 7-mer) were screened with mouse MAbs against the two predominant *T.b. gambiense* VSGs proteins (LiTat 1.3 and LiTat 1.5). The result was the isolation of several peptides that mimicked epitopes on the native trypanosomal VSGs LiTat 1.5 and LiTat 1.3 (Van Nieuwenhove et al., 2011). Some of the selected peptides were then confirmed as mimotopes for the parasite VSGs since they were able to inhibit the binding of their homologous monoclonal to the corresponding VSG (Van Nieuwenhove et al., 2011). In a second work focused on the screening of phage display libraries against polyclonal antibodies from sleeping sickness patients sera, two mimotopes of VSG have been found to react positively in indirect ELISA with a panel of 102 HAT positive and 102 endemic negative sera (Van Nieuwenhove et al., 2012). Both studies illustrate how the phage display platform can help to isolate epitopes against infectious agents with potential use in diagnostics. **Table 5** summarizes work on trypanosomes.

APTAMERS

The SELEX technology is an oligonucleotide-based combinatorial library method to select high affinity ligands (aptamers, from aptus, "to fit") to almost any target molecule, including peptides, or cells (Cell-SELEX) (Ulrich and Wrenger, 2009; Ahmadvand et al., 2011; Gold et al., 2012). It is based on a stretch of single-stranded nucleic acid, which can be RNA or ssDNA. These are chemically synthesized to have a segment usually from 8 to 40 nucleotides randomly bound. These sequences tend to assume different conformations, due to the formation of: (1) hairpins, which are helical double stranded structures due to complementarity of nucleotides, like in the classical t-RNA secondary structures; (2) G-tetrads, which is an association of four guanine bases through hydrogen bonding forming a planar squared structure; when these tetrads pile on top of each other and are stabilized by a cation, a G-quadruplex is formed (Johnson et al., 2008); (3) bulges, which occur when a duplex helix is interrupted by a single stranded stretch of nucleotides in only one strand; usually this may form a weak point leading to bending of the helical axis (Lilley, 1995); and (4) pseudo-knots formed by the intercalation of stem-loop structures (Staple and Butcher, 2005). These different topologies with varying loop configurations change the whole geometry of the nucleic acid molecules determining the formation of secondary and tertiary structures that provide a large diversity for the recognition of specific molecules and receptors on cell surfaces. Thus, aptamers are bioactive target-specific compounds that can be used to identify and modulate the activity of surface targets embedded in the membrane of cells relevant for human disease, either for diagnostic or therapeutic purposes.

The SELEX technique uses reiterative *in vitro* selection of combinatorial RNA or DNA pools against a target molecule for the identification of aptamers that are high-affinity oligonucleotide ligands. Aptamers isolated by such iterative method recognize their targets with binding specificities and affinities comparable to those of monoclonal antibodies, with the advantage to be easily chemically modified as to avoid nuclease attack. Thus, due to the fact that aptamers may recognize epitopes exclusively expressed by the target cell, they may be employed even to recognize different physiological states of a given cell, being capable, for example, to distinguish between protein isoforms and different conformations of the same protein.

When RNA aptamers are desired, the RNA pool used in the selections is transcribed from a pool of synthetic DNA templates (**Figure 1**). Each of these may contain, for example, 108 nucleotides with a 40 nucleotide randomized region flanked on both sides by constant sequences. The 5′ nucleotide sequence upstream the randomized region is a promoter for T7 phage RNA polymerase (Famulok et al., 2000). Two-fluoropyrimidine triphosphates can be used as substrates for RNA polymerase to provide stability of the transcribed products against nuclease attack (Ito et al., 1998). Such synthetic DNA pool is transcribed using T7 RNA polymerase in the presence of 2′-OH-ATP and GTP and 2′-F-CTP and UTP (Ruckman et al., 1998).

The obtained RNA molecules can now be incubated with any target, a protozoon for example, to enable RNA:cell surface binding to occur (cell-SELEX). The cell:RNA complexes are separated from free RNA molecules and the bound RNA is eluted by competition with the desired ligand. The liberated RNA is then isolated, reverse transcribed, and amplified by standard PCR procedures

(**Figure 1**). This SELEX procedure will be repeated for 9–12 selection rounds until no further increase in binding affinity can be measured (Irvine et al., 1991). To obtain more specificity, one or more of the iterative rounds can be done against a cell close to the one of interest in order to select out aptamers that are shared by different differentiation forms (negative selection). For example, one may use merozoites of *P. falciparum* to eliminate RNAs bound to targets that are shared with sporozoites. The sequence described above follows the same logic when aptamers carrying ssDNA sequences are used instead of RNA (Ulrich and Wrenger, 2009).

Targets can be isolated either by aptamer immobilization on magnetic beads or by affinity chromatography. Also, receptors on cells can be photocrosslinked with their RNA/DNA aptamers to facilitate target protein isolation. Finally, aptamers can be labeled with fluorescent labels to facilitate separations of cells by FACS (Ulrich and Wrenger, 2009).

SOMAmers (Slow Off-rate Modified Aptamers), a term introduced by Gold et al. (2010), are short, single stranded deoxyribonucleotides, bearing dU-modified residues. Like aptamers, they can be selected against any target molecule. The method for selecting SOMAmers with low dissociation rates ($t_{1/2} > 30$ min) and elimination of non-specific binding (employing an excess of a polyanionic competitor, for example) were developed and reported to show high success rates on selection when compared to the traditional aptamers: using four modified nucleotides (5-benzylaminocarbonyl-dU, 5-naphthylmethylaminocarbonyl-dU, 5-tryptaminocarbonyl-dU, and 5-isobutylaminocarbonyl-dU) into the SELEX experiments, the overall success rate increased by ∼84% in approximately 1200 proteins analyzed (Gold et al., 2012). The initial modifications of the pyrimidines have been expanded and the method applied to solve large scale analysis of biological samples, such as finding biomarkers for drug development and for diagnosis (Gold et al., 2012). As reported, more than 800 SOMAmers were already selected, meaning that 800 human proteins can be detected simultaneously in a high throughput assay using 15 μl of sample (Gold et al., 2012). According to Gold and collaborators, the new aptamers are so important for technological application that they call them SOMAmers, to distinguish them from the prior literature.

Although aptamers are most frequently defined as DNA or RNA molecules, peptide aptamers are important members of the group. Selection of peptide aptamers depends mainly from a random peptide library (often thioredoxin A is the scaffold protein) and on yeast two-hybridization system (Liu et al., 2013). The importance of the peptide aptamers in the parasitology can be exemplified by a recent report showing the inhibition of DNA damage repair and survival in *T. brucei* by a peptide aptamer mimicking RAD51-binding domain of BRCA2 (Hall et al., 2011). BRCA2, a multifunctional scaffolding protein, is implicated in different cellular processes, including its interaction with DNA recombinase proteins of the RAD51 family. BRCA2 from *T. brucei*, also essential for Variant Surface Proteins switching, interacts with RAD51 through BRC motifs, a 44 amino acid long repeat unit. Interestingly, since *T. cruzi* and *Leishmania* have similar machineries, and *T. brucei* BRCA2 is distinct from mammalian BRCA2, the authors predict the development of an optimized peptide aptamer that will work against the tri trypanosomatids.

DNA and RNA-aptamers applied to studies on trypanosomatids and apicomplexa are discussed below.

THE USE OF APTAMERS IN THE STUDY OF PARASITIC INFECTIONS

As pointed out, the number of articles in the literature describing the use of aptamers is scarce: while a database search of PubMed reveals almost 3000 publications making use of the term "aptamers" only less than three dozen publications reported the use of aptamers to study pathogens. Of these, most were related to studies on African and American trypanosomes, *Plasmodium* and *Leishmania*, which will herein be focused and are summarized in **Table 6** (Goringer, 2012).

STUDIES ON KINETOPLASTIDAE USING APTAMERS

Trypanosoma cruzi

The interaction of *T. cruzi* trypomastigotes with extracellular matrix (ECM) components is vital for the establishment of infection in the vertebrate host (Alves and Colli, 2007). Recognizing the importance of this interaction to the success of *T. cruzi* infection, the SELEX approach was used to evolve aptamers with binding affinities for ECM molecules such as laminin, fibronectin, thrombospondin, and heparan sulfate. Therefore, serum stable RNA aptamers that bind to parasite receptors to ECM were obtained after eight selection rounds, each one involving the incubation of trypomastigotes with a RNA pool ($\sim 2 \times 10^{13}$ molecules, consisting of 108 nucleotides with 40-nucleotide randomized region), followed by displacement of the bound molecules with ECM components. One or more iterative rounds were made against *T. cruzi* epimastigotes to eliminate aptamers that bind to targets common to both differentiation forms. Ninety-six clones were sequenced and four classes of RNA aptamers were established based on structural analysis. The selected RNA aptamers bind in the nanomolar range to the parasite receptors for heparan sulfate (40 nM), fibronectin (140 nM), laminin (200 nM), and thrombospondin (400 nM) (Ulrich et al., 2002). Importantly, all four selected aptamers, inhibited *T. cruzi* invasion of epithelial cultured cells by 50–70%, with the best inhibition obtained with the laminin class of aptamers. Although these aptamers confirmed the adhesion of *T. cruzi* to the ECM, the invasion blockage was incomplete, even when all the four classes of aptamers were added together. The existence of other molecules involved in the invasion of host cells by the parasite, an already known complex event (Alves and Colli, 2007) and the binding affinity of the aptamers in the nanomolar range may explain the results. In addition, the possible clearance of the aptamers from the surface of the parasite by shedding has to be considered. Remarkably, GPI-anchored surface glycoproteins from the Tc85 group that bind to laminin (Giordano et al., 1999) are shed as membrane vesicles (Torrecilhas et al., 2012), with a 3.5 h half-life (Gonçalves et al., 1991). The data demonstrate that the SELEX technique can be employed to isolate aptamers against parasite targets, which may be useful in inhibiting parasite invasion. However, aptamers that recognize other molecules may be included, as for example molecules that

Table 6 | Summary of the applications of aptamers on protozoan parasite research.

Target	Library	Result	References
T. cruzi			
T. cruzi ligands for ECM components	2′-F-dU/dc-RNA	50–70% inhibition of epithelial cell invasion by *T. cruzi*. The best inhibition was obtained with aptamers targeted to the laminin receptor	Ulrich et al., 2002
T. cruzi	2′-F-dU/dc-RNA	Development of an aptamer-based concentration method for the detection of *T. cruzi* in blood	Nagarkatti et al., 2012
T. brucei			
T. brucei bloodstream forms	2′-F-dU/dC-RNA	Identification of an aptamer family that binds to a flagellar pocket component. Aptamer internalization through the lysosome pathway	Homann and Goringer, 1999
Preparation of VSG variants	2′-F-dU/dC-RNA	Isolation of aptamers with affinity for many VSGs variants. Binding to live parasites	Lorger et al., 2003
T. brucei	2′-NH_2-dU/dC-RNA	Binding to the flagellar attachment zone	Homann et al., 2006
Leishmania			
L. tarentolae isolated mitochondria	Reporter RNA	RNA-aptamer base methodology for measuring RNA editing activity in the low femtomole range	Liang and Connell, 2009
L. tropica	RNA	Identification of signals for RNA import into the mitochondria	Bhattacharyya et al., 2002
L. infantum	DNA	Binding to the surface protein KMP-11	Berberich et al., 1997
L. infantum	DNA	Binding to histones (H2A and H3)	Ramos et al., 2007, 2010
Plasmodium			
P. falciparum PfEMP1	2′-F-dU/dc-RNA	Binding to PfEMP1 exposed at the surface of infected-erythrocytes. Rosette disruption by aptamers	Ghosh et al., 2001, 2002
P. falciparum parasitized erythrocyte	DNA	Inhibition of hemozoin formation and parasite growth by heme binding aptamers	Niles et al., 2009

are also shed and are known to enhance parasite invasion, as gp85/transialidase (Tonelli et al., 2011; Rubin and Schenkman, 2012) to improve the possibility of finding therapeutic approaches through aptamers.

Due to migration, approximately 390,000 humans infected with *T. cruzi* were detected in the United States (∼75% of the cases), Europe, western Pacific, Canada, Japan and Australia (Coura and Vinas, 2010). In this scenario, since blood transfusion is one way of *T. cruzi* transmission, methods with high sensitivity and specificity for the parasite detection are crucial. Most of the methodologies developed are based on antibodies (ELISA assays) or live parasites (PCR). However, false negative results can be obtained in both cases, due to low amount of antibodies (as in the initial phase of the disease) or to few circulating parasites (as in the asymptomatic cases or chronic phase of the disease). Moreover, detection of antibodies is not a good parameter of cure for Chagas' disease, since the antibodies persist in circulation for a certain period of time, even after the parasite killing, in addition to cross-reactivity problems among the trypanosomatids parasites.

Looking for the improvement of a diagnostic method based on live parasites, an aptamer-based concentration of *T. cruzi* in blood was recently reported (Nagarkatti et al., 2012). Using SELEX strategy, serum stable RNA aptamers that bound to live trypomastigotes with high affinity (8–25 nM ranges) were selected. One aptamer (Apt68) bound specifically to trypomastigotes, but not to epimastigotes or other related trypanosomatids (*L. donovani* and *T. brucei*) with high affinity (K_d ∼7.6 nM). It was also shown that Apt68 immobilized on a solid phase was able to capture and aggregate trypomastigotes from different strains. Moreover, using a magnet, trypomastigotes aggregated to Apt68-coated paramagnetic beads could be purified from the blood and detected by real-time PCR assay, even at concentrations as lower as five trypomastigotes/15 ml. The purification step of the parasite prior to DNA extraction presents technical advantages in relation to the direct extraction from the whole blood, in addition to the volume reduction of the blood sample employed.

African trypanosomes

Aptamers against the infective bloodstream forms of *T. brucei* were selected by incubating the parasite with a RNA library (2×10^{15} unique sequences) (Homann and Goringer, 1999). After 12 cycles of selection, 53 clones were sequenced, resulting in

the identification of three structural families of aptamers, one of which being further characterized. The aptamer 2–16 RNA was specific for the bloodstream stage, since it did not react with the insect stage of the parasite, and bound with high affinity (K_d ~60 nM) to a 42 kDa protein located within the flagellar pocket of the parasite. Moreover, the aptamer also bound to two other *T. brucei* strains. In order to be employed *in vivo*, the aptamer 2–16 was chemically modified, retaining the same properties as the original unmodified molecule, as well as a good stability *in vivo* (half-life in serum of 3.4 days) (Adler et al., 2008). Aptamers that recognized the flagellar attachment zone with high affinity ($K_d = 70 \pm 15$ nM) were also selected against the bloodstream stage of *T. brucei* (Homann et al., 2006). The half-life was determined as more than 30 h, showing a good stability in serum, an important characteristic for using the aptamer as a therapeutic device.

As pointed out before, bloodstream forms of *T. brucei* evade the host immune system by switching temporarily the single VSG that covers the surface of the parasites (antigenic variation). Despite the low identity of the amino acid composition among the VSG repertoire, the presence of similar tertiary structures, hidden from the antibody attack, was the rationale for selecting aptamers against the conserved structures (Lorger et al., 2003). For the selection, a homogeneous preparation of VSG (variant 117) was incubated with a combinatorial library of 2×10^{14} unique RNA sequences. After three rounds of selection, the aptamers were incubated with live parasites expressing the same VSG variant to remove RNAs that did not recognize the native membrane-bound protein. Finally, the same scheme was employed using a different VSG (variant 221) and parasites expressing VSG 221. As a result, RNA aptamers common to both VSG variants were obtained. After round nine, 60 individual clones were sequenced and placed into three groups, according to sequence motifs (76% in group I) in addition to orphan RNAs (6%). Aptamers from the three groups bound to the VSGs in the nanomolar or subnanomolar concentration range ($K_d = 0.16 \pm 0.02$ nM for clone nine), recognized other VSG variants from *T. brucei*, as well as from *T. congolense* and showed good serum stability (15 h). The biotin-labeled aptamers/fluorescent-conjugated streptavidin methodology demonstrated binding of the three groups of aptamers to the whole surface of live *T. brucei* expressing different VSG variants. Also, the biotin-labeled aptamers bound to the surface of the trypanosome and detected by anti-biotin antibodies could be used to drive immunoglobulins to the surface of the parasite.

Leishmania tarentolae

RNA editing, a molecular process found in trypanosomatids, regulates gene expression in their sole mitochondrion. It consists in the insertion and deletion of uridylates in mitochondrial mRNAs guided by small RNAs. The process occurs in a multi-protein complex (editosome), with endonuclease, uridylate transferase, uridylate-specific 3′exoribonuclease and RNA ligase being the four major enzymes involved. Incorrect function of editosome leads to parasite death, as shown for *T. brucei* (Schnaufer et al., 2001; Tarun et al., 2008). Although editosomes are common to other Kinetoplastidae parasites, their protein content may not be identical, as shown for example for two new proteins from *T. brucei* (KREPB9 and KREPB10), which are associated with the editing activity of the endonuclease. Both proteins are absent in *Leishmania* and KREP 10 is absent in *T. vivax* (Lerch et al., 2012). RNA editing is an attractive target for chemotherapy, as pointed out by the abundant literature in the field (Niemann et al., 2011).

The identification and exact function of each component of the editosome is then essential and sensitive assays to detect editing activity have been proposed. Among them, a RNA aptamer-based methodology was developed using a mitochondrial population from *L. tarentolae* as an initial source (Liang and Connell, 2009). The method detects the editing activity by an electrochemiluminescent signal generated by an editing responsive conformational change within a ruthenium labeled RNA reporter. It is claimed that the method detects the edited product in the femtomole range and can be performed in small volumes (12 μl in 384-well microtiter plate) making it suitable for high-throughput screening.

Leishmania tropica

The SELEX method was employed to identify the import signals of cytoplasmatic RNA by mitochondrial receptors. After four rounds of SELEX, the pool of aptamers was further selected by its capacity to be efficiently imported by the mitochondria. Four aptamers were studied in more detail and classified in two types by their efficiency in crossing the inner mitochondrial membrane (type I: A and D arm homologues; type II–V-T homologues, a new putative import signal). Furthermore, considering the efficiencies of aptamers transport into the mitochondria, cooperative and antagonistic interactions among them have been reported, which may be important for the regulation of RNA import, as suggested by the authors (Bhattacharyya et al., 2002).

Leishmania infantum

KMP-11 is a surface protein associated with Kinetoplastidae parasites cytoskeleton, such as *T. cruzi*, *T. brucei*, *L. donovani*, and *L. infantum* and differentially expressed during the parasite life cycle (Tolson et al., 1994; Stebeck et al., 1995; Berberich et al., 1997). A high antibody response against KMP-11 during natural infection of *L. infantum* was reported (Berberich et al., 1997), as well as the stimulation of T-lymphocytes proliferation (Tolson et al., 1994). These characteristics raised the possibility that KMP-11 may be an important tool against infection by *Leishmania*. DNA aptamers were then selected against KMP-11 protein and after a 10-round cycle, aptamers that specifically recognized KMP-11 were selected (Moreno et al., 2003) and are awaiting further characterization.

Although highly conserved among different eukaryotes, histones from trypanosomatids show sequence divergences in the amino- and carboxy-terminal domains. High affinity and specific DNA aptamers were then selected against *L. infantum* H2A (Ramos et al., 2007) and H3 (Ramos et al., 2010).

STUDIES ON APICOMPLEXA USING APTAMERS

Plasmodium

During erythrocyte infection *P. falciparum* secretes proteins to the surface of the RBCs associated with cytoadherence of the

infected erythrocytes to the endothelium of blood vessels or to other non-infected erythrocytes, monocytes, and platelets, leading to rosette formation, a phenotype associated with virulence. The presence of infected erythrocytes in microvessels associated with host responses to the sequestered erythrocytes are centrally involved in the disease pathology. Moreover, 48 cycles of invasion and development inside erythrocytes are made possible by the cytoadherence mechanism while the parasite circulates in the bloodstream. Cytoadherence and rosetting are associated with PfEMP-1 protein (parasite-derived erythrocyte membrane protein) that is exposed on the erythrocyte surface and binds to a different number of human cell receptors, such as heparan and chondroitin sulfates, ICAM-1, CD36 (Fairhurst et al., 2012). A good strategy to minimize malaria mislays is to raise ligands for a more conserved region of PfRMP-1 responsible for adhesion, such as the DBL1α, a rosette forming domain. Using a combinatorial library of 5×10^{14} unique RNA sequences and eight rounds of selection against DBL1α, 85 clones were sequenced and analyzed (Barfod et al., 2009). Two aptamers that bind to PfEMP1 on the surface of infected erythrocytes decrease the rosette formation by 35% at 33 nM concentration and by 100% at 38 nM, pointing out to their potential use as candidates for severe malaria therapy (Barfod et al., 2009).

Hemoglobin digestion in *Plasmodium* occurs in acidic vacuoles, as a result of an extensive uptake of erythrocytic cytoplasm by mid ring and mid trophozoite stages, both early intraerythrocytic stages of the parasite (Bakar et al., 2010). The digestion results in the production of globin, a source of amino acids, and free heme molecules (Fe^{2+}-protoporphyrin IX), which are toxic to the parasite. The oxidation of the heme group results in Fe^{3+}-protoporphyrin IX, which precipitates inside the vacuoles as crystals known as hemozoin (malaria pigment) (Weissbuch and Leiserowitz, 2008).

Anti-malarial drugs extensively used in clinic, as chloroquine or artemisinin act during the degradation of hemoglobin and subsequent hemozoin formation (Weissbuch and Leiserowitz, 2008). Artemisinin, for instance, delays hemoglobin uptake and a product of the hemoglobin hydrolysis potentiates its activity (Klonis et al., 2011). The pathway for hemoglobin degradation is then a suitable target for the development of new drugs and aptamers are good candidates. Heme binding DNA aptamers previously selected *in vitro* by SELEX carried out at pH 7–8 (Li et al., 1996; Okazawa et al., 2000) were employed to interfere with the heme-detoxification in *P. falciparum*. The selected aptamers bind equally to heme at acidic pH, the environmental condition of the vacuolar structures responsible for hemoglobin digestion, and inhibit hemozoin formation *in vitro*. Two of the heme-binding aptamers were used to verify their effect on parasite growth. The erythrocytes were first loaded with the aptamers using hypotonic lysis and resealing conditions, reaching the level of 65–85% of aptamers-loaded cells. Parasite growth in the preloaded-heme binding aptamers cells was significantly reduced after 72 h in culture, when compared to the controls (Niles et al., 2009). The aptamers, as suggested, may be useful tools for elucidation and manipulation of pathways important for the parasite survival.

CONCLUDING REMARKS

Phage display and SELEX are powerful methodologies to be employed in a variety of systems to approach different questions, from the understanding of cell biology to biotechnology. Both techniques share important characteristics, such as the possibility to use large oligonucleotide-based or phage display peptide libraries to screen almost any target, including the whole living cell or animal models. Chemical modification of aptamers and peptides poses an additional advantage over the phage display as it allows more stability in blood for therapeutic use. Particularly, aptamers seem to lack immunogenicity, which, associated with high affinity and specificity to their targets may easily substitute antibodies for therapeutic purposes, although a fast clearance of the aptamers have been shown in the literature. Besides its use directly to neutralize its target, aptamers also can be used as agents to deliver nanoparticles loaded with specific therapeutic drugs or iRNA (Liu et al., 2013). Blood-living or transiently blood-living parasites, as *T. brucei*, *T. cruzi*, or *Plasmodium* are good targets for this kind of approach. The same rational can be easily applied for any parasite-induced modification of the host cell surface, such as the knob-like structures in *Plasmodium*-infected erythrocytes. However, its application will be much more complex when parasites that live into the host cell cytoplasm (such as *T. cruzi*) or inside vacuoles (such as *Leishmania*) are considered. Indeed, as the advantages of target therapy become more apparent, the use of phage display and aptamers to retrieve for disease-related antigens are receiving increased attention. Additionally, aptamers-carrying iRNA may have diverse applications, as helping the understanding of the role of the innumerous proteins involved in the parasite–host cell interaction or inhibiting the expression of proteins from parasites, which possess the iRNA machinery. Besides its use on parasite–host interactions, phage display technology may be engineered for a particular functional activity as, for example, to display human antibodies with high affinity and specificity for particular antigens in disease. In humans, phage antibody libraries made from donors who naturally mount an immune response (with viral infections, bearing tumors, or with autoimmune disease) is being used not only to investigate the humoral response in disease but also with clinical purposes (Hoogenboom and Chames, 2000). These would be an interesting approach as therapy against parasitic diseases.

The application of aptamers for molecular imaging is being used in clinics for the evaluation of several diseases (Cibiel et al., 2012) with the great advantage of not being an invasive methodology and may be helpfully applied also in parasitology. An exciting use of the technique for cell biology is the imaging of cellular metabolites with RNA-based sensors recently described, where the intracellular levels of ADP and S-adenosylmethionine were measured (Paige et al., 2012). Since RNA aptamers to any molecule can be rapidly selected, a rapid expansion of the methodology to other small molecules is envisaged.

As shown by the low number of reports in the literature, it is surprising that these methodologies were not, as yet, employed more often in the study of the complex phenomenon of host–parasite interaction, with the aim to develop much needed

diagnostic and therapeutic tools to combat infections caused by protozoan parasites. Notwithstanding, considering the constant and fast improvement of both technologies and their large applicability to different biological problems, it is expected that phage display and SELEX will make, in a near future, significant contributions to the field of protozoan-borne diseases.

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The kallikrein-kinin system in experimental Chagas disease: a paradigm to investigate the impact of inflammatory edema on GPCR-mediated pathways of host cell invasion by *Trypanosoma cruzi*

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Chronic chagasic myocarditis (CCM) depends on *Trypanosoma cruzi* persistence in the myocardium. Studies of the proteolytic mechanisms governing host/parasite balance in peripheral sites of *T. cruzi* infection revealed that tissue culture trypomastigotes (TCTs) elicit inflammatory edema and stimulate protective type-1 effector T cells through the activation of the kallikrein-kinin system. Molecular studies linked the proinflammatory phenotype of Dm28c TCTs to the synergistic activities of tGPI, a lipid anchor that functions as a Toll-like receptor 2 (TLR2) ligand, and cruzipain, a kinin-releasing cysteine protease. Analysis of the dynamics of inflammation revealed that TCTs activate innate sentinel cells via TLR2, releasing CXC chemokines, which in turn evoke neutrophil/CXCR2-dependent extravasation of plasma proteins, including high molecular weight kininogen (HK), in parasite-laden tissues. Further downstream, TCTs process surface bound HK, liberating lysyl-BK (LBK), which then propagates inflammatory edema via signaling of endothelial G-protein-coupled bradykinin B_2 receptors (BK_2R). Dm28 TCTs take advantage of the transient availability of infection-promoting peptides (e.g., bradykinin and endothelins) in inflamed tissues to invade cardiovascular cells via interdependent signaling of BKRs and endothelin receptors (ETRs). Herein we present a space-filling model whereby ceramide-enriched endocytic vesicles generated by the sphingomyelinase pathway might incorporate BK_2R and ETRs, which then trigger Ca^{2+}-driven responses that optimize the housekeeping mechanism of plasma membrane repair from cell wounding. The hypothesis predicts that the NF-κB-inducible BKR (BK_1R) may integrate the multimolecular signaling platforms forged by ceramide rafts, as the chronic myocarditis progresses. Exploited as gateways for parasite invasion, BK_2R, BK_1R, ET_AR, ET_BR, and other G protein-coupled receptor partners may enable persistent myocardial parasitism in the edematous tissues at expense of adverse cardiac remodeling.

Keywords: bradykinin, cardiomyopathy, cruzipain, endothelins, GPCRs, proteases, kallikrein, *Trypanosoma cruzi*

INTRODUCTION

Afflicting nearly 10 million people in Latin America (Coura and Dias, 2009), Chagas disease is a pleiomorphic clinical entity caused by *Trypanosoma cruzi*, a parasitic protozoan that undergoes obligate intracellular development in the mammalian host. Extremely polymorphic (Macedo and Pena, 1998), the natural populations of *T. cruzi* have been recently subdivided into six discrete taxonomic units (DTUs) named *T. cruzi* I to *T. cruzi* VI (Zingales et al., 2009), of which at least four are known to be involved with human pathology (Miles et al., 2009). Whether transmitted to humans via mucosal wounds inflicted by hematophagous vectors of the reduviid family or, indirectly, by oral ingestion of contaminated juices (Coura and Dias, 2009; Cortez et al., 2012), the insect-derived infective forms (metacyclic trypomastigotes) induce an acute phase that may be asymptomatic, or life-threatening. Characterized by high blood parasitemia, the sequels of severe acute disease may include hepatosplenic pathology, myocarditis, and more rarely, encephalitis. Lasting a few months, the acute symptoms subside with the onset of immunity, but the effector response is not capable of eradicating the intracellular parasites, leading to a chronic infection, characterized by low-grade tissue parasitism and positive serology. Several years later, about 30% of the patients develop a full-blown chronic chagasic myocardiopathy (CCM), characterized by the presence of inflammatory T cell infiltrates, myocardial fibrosis, complex arrhythmias, thromboembolism, and ventricular aneurysms (Marin-Neto et al., 2007). Patients with severe forms of CCM may have heart failure and sudden death, while the remaining chagasic patients (indeterminate stage) remain asymptomatic for decades. In the south cone of America, chagasic patients may also develop digestive system abnormalities (megacolon and/or megaesophagus), albeit in lower frequency than CCM.

CCM: CONVERGING PATHOGENIC MECHANISMS

Nearly a century after the discovery of Chagas disease, we have come to realize that the mechanisms responsible for the variable

clinical manifestations during the chronic phase are still elusive. Cardiac parasympathetic depopulation, microvascular derangement, and low-grade myocardial inflammation directly induced by parasites and T cell-dependent immunopathology seem to converge in the genesis of CCM. After decades of debate, there are persuasive arguments supporting the notion that the primary cause of CCM is a low-grade, persistent parasitism of the myocardium (Tarleton, 2001). A large body of studies in mice and humans indicated that chronic myocarditis is critically dependent on the recruitment of parasite-specific (type 1) effector CD8 T cells to the infected cardiac tissues (Padilla et al., 2009; Silverio et al., 2012).

While not dismissing the relevance of intracardiac infiltrates in the progression of CCM, vascular pathologists argued that low-grade infection could lead to the accumulation of microvascular lesions in the chagasic heart, ultimately resulting in myocardial hypoxia, which in turn may aggravate collateral injury inflicted by pathogenic T cells infiltrating the heart (Morris et al., 1990; Rossi, 1990; Higuchi et al., 1999, 2003). Subsequent studies in experimentally infected animals shed light on the mechanisms by which *T. cruzi* induces microvasculopathy (Andrade et al., 1994; Tanowitz et al., 1999). Initial observations ascribed the formation of vasospasm to the pathogenic activity of endothelins (ETs), a potent class of vasoconstrictor polypeptides (Tanowitz et al., 1999). Of further interest, these workers reported that endothelin-1 (ET-1) expression is up-regulated in parasitized cardiovascular cells (Petkova et al., 2000). Follow-up studies in chronically infected mice demonstrated that cardiac remodeling significantly ameliorated in transgenic lines in which the ET gene was specifically removed from cardiomyocytes, while ablation of this gene in endothelial cells has not significantly reduced heart fibrosis (Tanowitz et al., 2005). Of further interest, the plasma levels of ETs are elevated both in chagasic patients and infected mice (Petkova et al., 2000; Salomone et al., 2001).

While the research linking infection-associated vasculopathy to the function of ETs progressed, our group reported that trypomastigotes generate proinflammatory kinin peptides extravascularly. Follow-up studies indicated that the local activation of the kallikrein-kinin system (KKS) translates into mutual benefits to the host/parasite relationship during the course of chagasic infection (Scharfstein and Andrade, 2011; Scharfstein and Svensjö, 2012). In the present article, we will review the results of these studies and advance the proposition that *T. cruzi* may take advantage of interstitial edema in the inflamed heart to potentiate their infectivity via cooperative signaling of multiple G protein-coupled receptors (GPCRs). The rationale of this hypothesis lies on two fundamental premises: (i) due to the low-grade parasitism observed in chronic infection, there are intermittent "flares" of plasma leakage in the inflamed myocardium (ii) the microvascular edema is temporally linked to the release of parasites from ruptured pseudocysts (Scharfstein and Morrot, 1999).

Studies in various experimental models indicated that tissue culture-derived trypomastigotes (Dm28c) swiftly activate microvascular beds through the activation of the KKS (Todorov et al., 2003; Monteiro et al., 2006; Schmitz et al., 2009; Scharfstein and Andrade, 2011; Andrade et al., 2012). Based on these initial observations, we predicted that the sudden diffusion of plasma-borne constituents (antibodies, complement components, kininogens, ETs) through parasite-laden tissues may affect the delicate host/parasite balance established in the chronically infected myocardium. Although the flagellated trypomastigotes released from pseudocysts may rapidly move away from the primary foci of infection, hence seeking for safer targets elsewhere in the myocardium, we proposed that the transient rise of plasma proteins in the edematous interstitial spaces might favor generation of infection-promoting peptides, such as bradykinin (BK), in the peripheral tissues (Scharfstein et al., 2000; Todorov et al., 2003; Andrade et al., 2012). Before outlining the arguments supporting this working hypothesis, we will present readers with an overview of the essential structural and functional features of the KKS.

MOLECULAR BASIS OF KKS ACTIVATION AND REGULATION

Recently implicated in thrombo-inflammatory processes (Müller et al., 2009; von Brühl et al., 2012), the KKS is a hub-like network of proteolytic enzymes which, among other biological functions, release the proinflammatory "kinin" peptides from an internal segment of their plasma-borne precursors, the kininogens. Generation of kinins may involve multiple processing enzymes: in the bloodstream, plasma kallikrein (PK) releases the nonapeptide BK from high molecular weight kininogen (HK) upon activation of the contact system by negatively charged surfaces, such as platelet-derived polyphosphates (**Figure 1A**). In the extravascular spaces, lysyl-BK (LBK) is excised from low molecular weight kininogen (LK) or HK by tissue kallikrein, a serine protease that is constitutively expressed in multiple tissues. It is also known that kinins can be generated by alternative proteases. For example, in chronic inflammation kininogens may be processed by the concerted action of neutrophil elastase and mast cell tryptase, leading to the release of a slightly larger kinin, Met-LBK (Kozik et al., 1998). In the context of infections, kinins can be directly liberated from the kininogens by the action of microbial cysteine proteases, such as gingipain from *Porphyromonas gingivalis* (Imamura et al., 1994), staphopain A from *Staphylococcus aureus* (Imamura et al., 2005), streptopain from *Streptococcus pyogenes* (Herwald et al., 1996), and cruzipain (Del Nery et al., 1997; Scharfstein et al., 2000; Monteiro et al., 2006).

The biological responses mediated by BK and LBK are mediated by bradykinin B2 receptor (BK_2R), a subtype of heterotrimeric G-protein-coupled BKR (**Figure 1A**) which is constitutively expressed by several cell types, including cardiomyocytes, pain-sensitive neurons, vascular endothelial cells, smooth muscle cells (Leeb-Lundberg et al., 2005). As explained later on in this text, kinins (BK/LBK) also activate BK_2R expressed by sentinel cells of the innate immune system, such as conventional dendritic cells (DCs; Aliberti et al., 2003; Bertram et al., 2007; Kaman et al., 2009).

Once released from HK/LK, the intact kinins (BK/LBK) have a half-life of <15 s in the plasma, hence must swiftly activate BK_2R via the paracrine mode (**Figure 1A**). Since excessive activation of the kinin system may have adverse effects to the vascular system, the kinin/BK_2R pathway is fined-tunned by overlapping regulatory mechanisms, such as (i) down-regulation of surface BK_2R

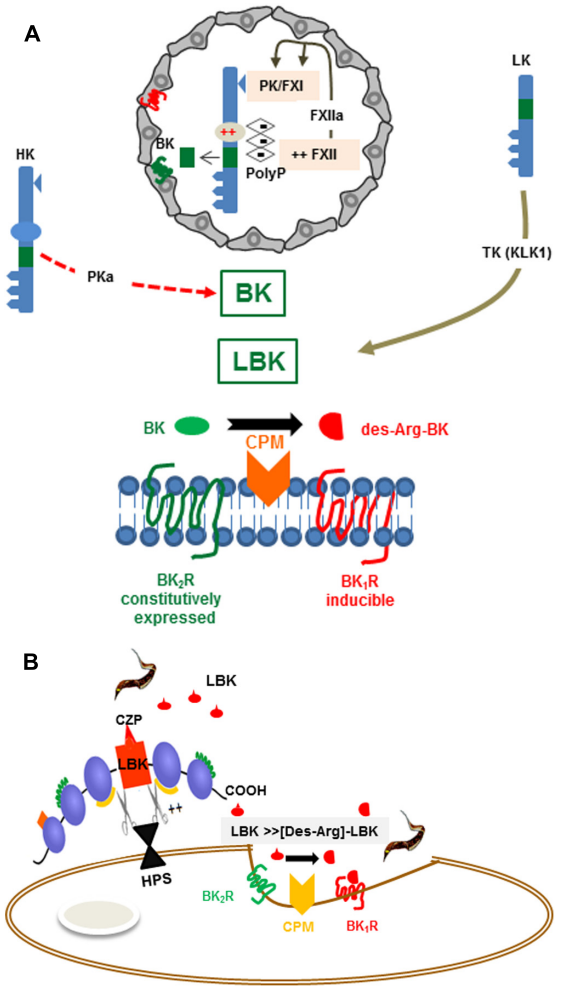


FIGURE 1 | (A) Activation of the kallikrein-kinin system *Top*: The scheme depicts platelet-derived polyphosphates (PolyP) acting (intravascularly) as endogenous activators of the KKS (contact phase of coagulation). The proteolytic cascade is initiated as the negatively charged PolyP (long chains) bind to (i) FXII and (ii) HK, via the histidine-rich (D5) domain. PolyP-induced conformational changes in FXII lead to its autocatalytic cleavage. FXIIa then cleaves the plasma kallikrein (PK) zymogen, generating PKa. After several cycles of reciprocal activation between PKa/FXIIa, FXIIa activates FXI, the downstream effector of the intrinsic coagulation pathway, whereas PKa cleaves HK, releasing the internal nonapeptide bradykinin (BK, green rectangle). In inflammatory conditions, the plasma-borne HK/LK diffuse through post-capillary venules. As the extravascular levels of kininogens rise, these kinin-precursor molecules undergo cleavage by tissue kallikrein (TK), which liberates the decapeptide lysyl-BK. Acting by the paracrine mode, the intact form of kinins (BK or Lysyl-BK) binds at high-affinity to BK_2R, a subtype of BKR that is constitutively expressed in a broad range of cell types. *Bottom*: The scheme depicts the activation requirements of BK_1R, a GPCR subtype whose expression is strongly upregulated in inflamed/damaged tissues. Generation of high-affinity ligands for the inducible BK_1R depends on metabolic processing of the intact kinins (BK or lysyl-BK) by the metallopeptidase kininase I (carboxypeptidase N or membrane-bound carboxypeptidase M). **(B)** BK1R is an inducible gateway for *T. cruzi* entry in cardiovascular cells. *T. cruzi* relies on the enzymatic versatility of the major lysosomal cysteine protease (cruzipain) to release LBK from HK molecules docked to the host cell surfaces via binding to heparan sulfate proteoglycans. Once released, the short-lived lysyl-BK is rapidly processed by membrane-bound carboxypeptidase M, generating [des-Arg]-lysyl-BK, which then enhances parasite infectivity through the signaling of the inducible BK_1R.

(ii) degradation of intact kinins by various metallopeptidases, including angiotensin converting enzyme (ACE, kininase II) – a transmembrane di-peptidyl carboxypeptidase (Skidgel and Erdos, 2004) highly expressed in the endothelium lining and, to a less extent, in other cell types, including innate sentinel cells such as monocytes and DCs (Danilov et al., 2003). Besides degrading intact kinins, thus reducing hypotension, ACE increases blood pressure through the formation of angiotensin II, a potent vasopressor octapeptide. Noteworthy, the transmembrane (somatic) enzyme undergoes cleavage by disintegrin and metalloproteinase (ADAM)-type "sheddase" (Parkin et al., 2004), leading to the accumulation of soluble forms of ACE in the blood and other body fluids.

In contrast to the constitutive BK_2R, the expression of BK_1R is strongly – but transiently – up-regulated during inflammation (Marceau and Bachvarov, 1998; **Figure 1A**). For example, ET-1 and angiotensin II induce BK_1R expression through the signaling of ET_AR and AT_1 (angiotensin 1 receptor) respectively, leading to activation of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) cascade (Medeiros et al., 2004). BK_1R is also induced by IL-1β, TNF-α, and IFN-γ via the NF-κB transcription factor (Marceau and Bachvarov, 1998; Medeiros et al., 2004; Moreau et al., 2007). Differently from BK_2R, the inducible BK_1R is not triggered by "intact" kinins (BK/LBK). Instead, BK_1R is activated by the kinin metabolites [des-Arg]-BK or [des-Arg]-LBK, both of which are generated by carboxypeptidase N (CPN)/carboxypeptidase M (CPM) (kininase I)-mediated cleavage of the C-terminal arginine residue of BK/LBK (**Figure 1A**).

BIOLOGICAL FUNCTIONS OF BKRs

In the cardiovascular system, kinins/BK_2R control the blood flow through nitric oxide (NO)-dependent vasodilation. In contrast to the beneficial role of kinins in cardiovascular homeostasis, there is evidence that dysregulated BK_1R signaling drives myocardial fibrosis and impairs heart function in different experimental models (Spillmann and Tschöpe, 2012).

Beyond inducing detrimental cardiac responses, it is well-established that BK_1R plays a major role in hyperalgesia (Calixto et al., 2004; Gabra et al., 2005; Cunha et al., 2007). Studies in mice subjected to traumatic brain injury revealed that blood-brain leakage and recruitment of inflammatory leukocytes to the CNS is blocked by a specific BK_1R antagonist (Raslan et al., 2010). Although inflammation is usually initiated through the signaling of the constitutive BK_2R, the sustenance of the inflammatory response depends on the signaling of endothelial BK_1R. McLean et al. (2000) were the first to report that the trans-endothelial leukocyte migration is enhanced as result of up-regulated expression/signaling of endothelial BK_1R. Recent progress in studies of experimental autoimmune encephalitis (EAE) demonstrated that BK_1R increases the recruitment of pathogenic effector T cells into the CNS (Dutra et al., 2011; Göbel et al., 2011; **Table 1**).

More recently, a growing number of studies indicated that immune resistance against infection by parasitic protozoan (Monteiro et al., 2006, 2007; Svensjö et al., 2006; Nico et al., 2012; Scharfstein and Svensjö, 2012) and bacterial pathogens (Kaman

Table 1 | Role of BKRs in immunity: literature update.

Experimental System	Bradykinin receptor subtype	Functional Roles of BKRs	Reference
T. cruzi infection (mice)	BK_2R	Induction of CD11c+ DC (splenic) maturation and upregulation of effector (type 1) CD4 and CD8 T cells	Monteiro et al. (2006); Monteiro et al. (2007)
L. donovani and *L. chagasi* (hamsters and mice)	BK_2R	Inflammatory edema induced by promastigotes; stimulation of promastigote uptake and modulation of the intracellular growth of leishmania in macrophages	Svensjö et al. (2006)
Visceral Leishmaniasis (mice)	BK_2R	Immune resistance to acute infection	Nico et al. (2012)
L. monocytogenes infection (mice)	BK_2R	Modulation of innate immunity and infection control	Kaman et al. (2009)
P. gingivalis infection-bucal (mice)	BK_2R	Upregulation of Th1 and Th17 responses (submandibular lymph node)	Monteiro et al. (2009)
In vivo leukocyte trafficking across mesenteric postcapillary venules (mice)	BK_1R	Trans-endothelial leukocyte migration	McLean et al. (2000)
EAE (mice)	BK_1R	Th1 and Th17 responses development and clinical progression of EAE	Dutra et al. (2011)
		Induction of blood brain barrier disruption and T cell transmigration into the CNS	Göbel et al. (2011)
		Limiting infiltration of pathogenic subsets of effector CD4 T cells into the CNS	Schulze-Topphoff et al. (2009)
In vitro DC migration (human)	BK_2R	Stimulation of DC migration	Bertram et al. (2007)
	BK_1R	Inhibition of DC migration	Gulliver et al. (2011)
Allergic inflammation (mice)	BK_1R/BK_2R	Induction/inhibition of migration and activation of eosinophils	Vasquez-Pinto et al. (2010)
	BK_2R	Induction of DC maturation and IL-12-dependent Th1 polarization	Aliberti et al. (2003)

et al., 2009) is critically dependent on activation of innate sentinel cells via the BK_2R pathway (**Table 1**).

BKRs: DOMAIN COMPARTMENTALIZATION, REGULATION, AND SIGNAL TRANSDUCTION PATHWAYS

Stably expressed at the cell surface, the constitutively expressed BK_2R is rapidly desensitized/internalized via GRK4α-mediated receptor phosphorylation upon ligand binding, thus yielding a transient signaling response (Blaukat et al., 2001). Using BK_2R-fusion constructs, Enquist et al. (2007) revealed that upon BK binding, the internalized receptors colocalize with transferring in endosomes, prior to entry in the arrestin-dependent, clathrin-mediated recycling pathway. A second trafficking pathway, described in smooth muscle cells and fibroblasts, indicated that BK also promotes the redistribution of BK_2R and their coupling G proteins to caveolar rafts (de Weerd and Leeb-Lundberg, 1997).

Although the cellular systems vary from one report to another, it is well-established that BK binding to BK_2R promotes the formation of BK_2R homodimers and oligomer complexes of higher order (Leeb-Lundberg et al., 2005). Kang et al. (2004) reported that BK_2R and BK_1R form heterodimers in transfected HEK293 cells. Notably, BK_2R also forms heterodimeric complexes with-type-1 angiotensin receptors (AT_1) in vascular smooth muscle cells and transfected HEK293 (AbdAlla et al., 2001).

As to the signal transduction pathways, it is well established that BK frequently induces BK_2R-dependent $[Ca^{2+}]_i$ transients and PLC-β-dependent hydrolysis of phosphoinositides, both of which commonly coupled to the activation of PKC isoenzymes α, ε, and ξ. Although BK_2R often signals through $G\alpha_q$ and $G\alpha_i$, in some cellular systems BK-driven activation of the constitutively expressed BKR is coupled to Gαs and Gα12/13 (Leeb-Lundberg et al., 2005). Similar to the effects induced by other GPCR agonists, BK sequesters BK_2R along with $G\alpha_q$ and $G\alpha_i$ in caveolar compartments of smooth muscle cells (de Weerd and Leeb-Lundberg, 1997).

In fibroblasts, a cell-type often used in models studies of *T. cruzi* interaction with non-professional phagocytic cells, BK_2R activation is associated with transient tyrosine phosphorylation

and activation of focal adhesion kinase as well as other focal-adhesion substrates (Leeb-Lundberg et al., 1994). In another study with fibroblasts, BK stimulated peripheral actin microspikes and membrane ruffling via activation of Cdc42 and Rac-1 (Kozma et al., 1995). Studies with endothelial cells showed that BK, acting as a potent vasodilator, transiently stimulates endothelial cell production of NO via endothelial nitric oxide synthase (eNOS) through mechanisms involving both calcium-dependent and phosphorylation cascades mediated by PI3K/Akt (Kuhr et al., 2010).

KKS HAS A DUAL ROLE IN EXPERIMENTAL CARDIOMYOPATHIES

All the components of the KKS are present in the heart. In addition to contributing to vascular tone and inflammation in the cardiac tissues, the KKS influences extent of extracellular matrix (ECM) remodeling, angiogenesis, and stem cell mobilization (Spillmann et al., 2006). Of possible relevance to the understanding of KKS function in CCM, experimental studies in mouse models of diabetic cardiomyopathy and myocardial ischemia indicate that specific alterations in kinin homeostasis may either ameliorate or worsen cardiac pathology after cardiac pathology (Spillmann and Tschöpe, 2012).

As mentioned earlier, the beneficial effects of ACE inhibitors are due to their dual biological functions: these potent drugs block ACE-driven conversion of angiotensin I into the vasopressor angiotensin II and inhibit the enzymatic breakdown of kinins (BK_2R agonists), thus increasing the half-life of "intact" kinins in the circulation. Noteworthy, ACE inhibitors reduce myocardial damage (e.g., reduction of infarction size) yet these effects were nullified upon treatment with BK_2R antagonists or in BK_2R-deficient mice (Yang et al., 2001), thus indicating that signaling of BK_2R has a major function in the remarkable protective effects of ACE inhibitors.

Studies in streptozotocin (STZ)-induced models of diabetic cardiomyopathy have shown that early left ventricle (LV) and systolic dysfunction is improved in mice overexpressing tissue kallikrein (KLK1), whereas wild-type heart develop massive fibrosis (interstitial and perivascular) due to focal accumulation of collagen and fibrillar fragmentation. Although KLK1 has a pro-collagenase activity by itself and may activate matrix metalloproteinases (MMPs), the host-protective effects of KLK1 overexpression in diabetic cardiomyopathy were abolished upon treatment with HOE-140 (BK_2R antagonist), thus implying that KLK1-mediated release of kinins improves cardiac function. Additional studies suggested that activation of the KLK1/BK_2R pathway reduces cardiac fibrosis through the activation of the plasminogen activator/MMP2-dependent fibrinolytic cascade (Spillmann et al., 2006). Additional studies revealed that BK_2R signaling that BK_2R signaling improves cardiac function by (i) reducing apoptosis and chamber dilatation in the myocardium (Chao et al., 2008), (ii) optimizing angiotensin II-induced (NO-dependent) neovascularization (Munk et al., 2007), (iii) restoring S2a-mediated sarcoplasmatic Ca^{2+} uptake (Tschöpe et al., 2005), and/or (iv) promoting homing of endothelial progenitor cells to ischemic muscles (Kränkel et al., 2008).

T. cruzi TRYPOMASTIGOTES LIBERATE KININS FROM SURFACE-BOUND KININOGENS VIA CRUZIPAIN

The first clues suggesting that *T. cruzi* is equipped with a kininogenase came from enzymatic analysis of the substrate specificity of cruzipain (Del Nery et al., 1997), a lysosomal-like cysteine protease classified as member of clan A of the C1 peptidase family (Cazzulo et al., 2001; Alvarez et al., 2012). At first sight, the discovery that cruzipain acted as a "kininogenase" seemed paradoxical because kininogens are members of the cystatin family of cysteine protease inhibitors, hence rely on cystatin-like domains to potently inactivate papain-like enzymes, including cruzipain itself (Stoka et al., 1995). Consistent with this, *in vitro* data showed that cruzipain hydrolyzes soluble forms of HK at slower rates as compared to tissue kallikrein. This conundrum was settled by awareness that HK binds to negatively charged sulfated proteoglycans, such as heparan or chondroitin sulfates via the histidine-rich positively charged motif ($D5_H$) localized at the C-terminal end of the BK (D4) sequence (Renné et al., 2000; Renné and Muller-Esterl, 2001). Based on this information, Lima et al. (2002) hypothesized that the spatial orientation of cell-bound HK docked to heparan sulfate proteoglycans was not suitable for cruzipain binding and inactivation by the cystatin-like inhibitory domain (**Figure 1B**). Indeed, model studies performed with cruzipain and HK in the test tube offered circumstantial support to this hypothesis (Lima et al., 2002): the addition of heparan sulfate (at optimal concentrations) drastically reduced the cysteine inhibitory activity of soluble HK on cruzipain while reciprocally increasing the catalytic efficiency of the parasite protease, measured with synthetic peptides flanking the BK sites. Consistent with these results, heparan sulfate potentiated HK processing by cruzipain, generating multiple HK breakdown products and promoting accelerated kinin release (**Figure 1B**). Combined, these biochemical studies suggested that the substrate specificity of the parasite protease was re-directed as result of reciprocal interactions between sulfated proteoglycans with the substrate (HK) and protease (cruzipain) molecules (Lima et al., 2002), hence increasing the efficiency of the kinin-release reaction in peripheral sites of infection.

Previously characterized as a therapeutic target of Chagas disease (McKerrow et al., 2009), cruzipain is a remarkably versatile virulence factor of *T. cruzi*. Beyond activation of the KKS (see below), the proteolytic activity of cruzipain was implicated in mechanisms of parasite virulence/pathogenicity, such as lence/pathogenicity, such as (i) enhancement of trypomastigote invasion and intracellular replication of amastigotes in cardiomyocytes (Meirelles et al., 1992; Scharfstein et al., 2000), (ii) degradation of proinflammatory chemokines (Benítez-Hernández et al., 2010), and (iii) subversion of innate microbicidal responses in parasitized macrophages through interference with the activation of NF-κB (Doyle et al., 2011).

KININ RELEASE IN PERIPHERAL SITES OF *T. cruzi* INFECTION DEPENDS ON THE COOPERATIVE ROLES OF tGPI AND CRUZIPAIN

Vertebrate hosts may control infection by rapidly mobilizing soluble and cellular-based microbicidal systems that destroy pathogen

invaders at the cost of limited self-tissue destruction. Perturbations of steady-stated tissue homeostasis are sensed by sentinel cells of the innate immune system through specialized pattern-recognition receptors (PRRs; Medzhitov and Janeway, 1997). In many infections, the activation of PRRs lead to rapid secretion of pre-formed vasoactive mediators by innate sentinel cells (e.g., eicosanoids, leukotrienes, chemokines, TNF-α), which then activate the endothelium lining, rendering them sticky for circulating neutrophils. Further downstream, vasoactive mediators generated/released at the neutrophil/endothelial interface impair the integrity of the endothelial barrier, hence opening the "flood gates" (DiStasi and Ley, 2009).

Studies in a mouse model of subcutaneous (footpad) infection provided the first evidence that Dm28c TCTs (tissue culture trypomastigotes) induce inflammatory edema through the activation of the kinin system (Todorov et al., 2003; **Figure 2A**). A footpad edema potentiated by the ACE inhibitor captopril was observed 2 h p.i. in wild-type B6 mice, but not in BK_2R-deficient mutants. Using pharmacological tools, we showed that the constitutively expressed BK_2R orchestrated the early phase (2 h) edema, whereas BK_1R (acting in "cross-talk" with BK_2R) sustained the inflammatory response (24 h; Todorov et al., 2003). Intriguingly, Dm28c epimastigotes failed to induce a conspicuous inflammatory edema via the kinin pathway despite the fact that these vector-borne non-infective forms express abundant levels of cruzipain. In a subsequent study, it became clear, for reasons that will be explained further below, that cruzipain was necessary but not sufficient to generate kinins in hamster cheek pouch (HCP) topically exposed to Dm28c epimastigotes.

Insight of the functional interplay between Toll-like receptor (TLR)-driven innate responses and the KKS emerged from analysis of the mechanisms underlying TCT-evoked microvascular leakage responses induced by topically applying the pathogen to the HCP (Monteiro et al., 2006). This unconventional system proved advantageous because it dispensed the use of needle to inject parasites, hence avoiding activation of the KKS by bleeding and traumatic injury. Using intravital microscopy, we found that Dm28c TCTs induced discrete plasma leakage responses in the HCP within a few minutes of parasite application. Noteworthy, the microvascular reactions were attenuated by HOE-140 (BK_2R antagonist) or by pretreating the TCTs with an irreversible cruzipain inhibitor *N*-methyl-piperazine-Phe-homoPhe-vinyl sulfone (K11777), an anti-parasite drug that will be soon tested in clinical trials (Engel et al., 1998; McKerrow et al., 2009).

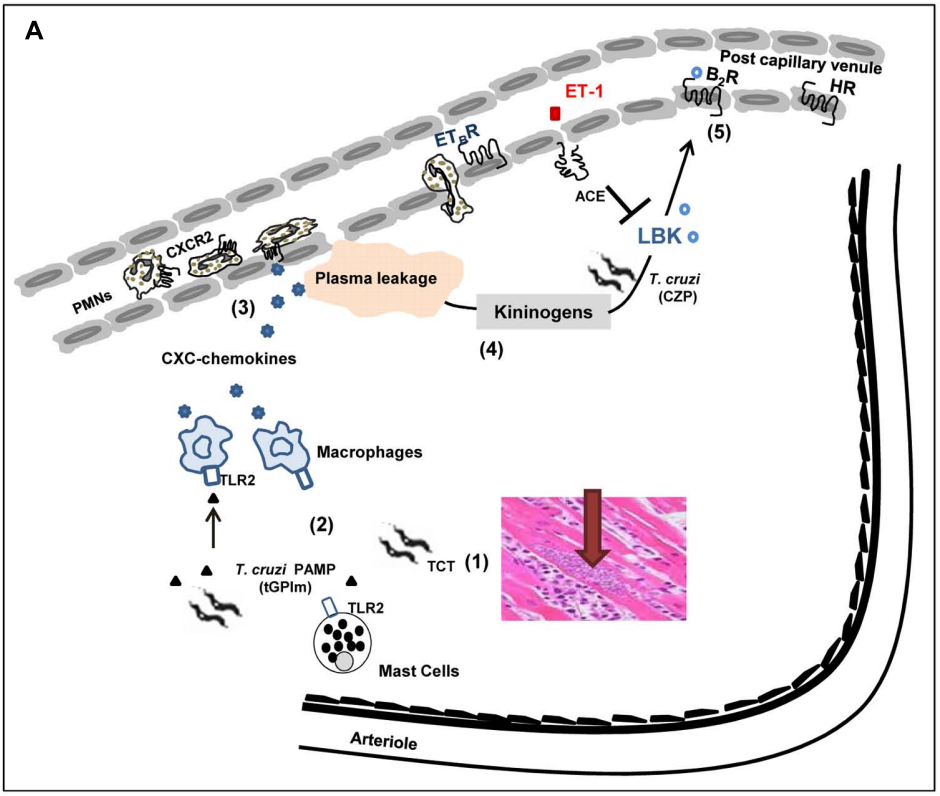
Monteiro et al. (2006) highlighted the fact that purified cruzipain (activated) topically applied to the HCP failed to induce significant plasma leakage even in the presence of ACE inhibitors. However, purified (activated) cruzipain induced a strong BK_2R-driven leakage response when it was applied in combination to purified HK. The dependence on a supply of exogenous HK suggested that, in resting state conditions (i.e., in the absence of inflammation), the levels of endogenous kinin-precursor proteins in the interstitial spaces are insufficient to allow for significant activation of the kinin system. Based on these observations, Monteiro et al. (2006) predicted that Dm28c TCTs might bear developmentally regulated proinflammatory molecules (i.e., absent in Dm28c epimastigotes) which control the rate-limiting step of KKS activation in parasite-laden tissues: the influx of the plasma-borne kininogen (cruzipain substrate) into peripheral sites of *T. cruzi* infection (**Figure 2A**). Seeking to identify these factors, Monteiro et al. (2006) then turned their attention to tGPI, a mucin-linked lipid anchor previously characterized by Almeida and Gazzinelli (2001) as a TLR2 ligand that was expressed at high-levels exclusively in TCTs. Consistent with the working hypothesis, Dm28 TCTs failed to evoke inflammatory edema in $TLR2^{-/-}$ mice or in $BK_2R^{-/-}$ mice (Monteiro et al., 2006). In contrast, these infective parasites evoked a prominent edema both in wild-type mice and TLR4 mutant (C3H/HeJ). These results argued against a role for GIPL, a lipid anchor of epimastigotes previously characterized as TLR4 ligand (Oliveira et al., 2004), in the activation pathway that generates vasoactive kinins in peripheral sites of infection.

Next, Monteiro et al. (2006) studied the functional interplay between TLR2 and the KKS by injecting TLR2-deficient mice with a parasite suspension supplemented (or not) with purified HK. Strikingly, the injection of HK in infected $TLR2^{-/-}$ mice rescued the edema response. Moreover, the edema responses which HK induced in $TLR2^{-/-}$ infected mice were abolished by HOE-140, or alternatively, by pre-incubating the TCTs with the irreversible cruzipain inhibitor K11777. Combined, these results supported the hypothesis that TCTs induce the initial leakage/accumulation of the HK (cruzipain substrate) in peripheral tissues via TLR2, whereas cruzipain amplifies this inflammatory response through the release of BK_2R agonist, at the downstream end of the inflammatory cascade (**Figure 2A**).

In order to further confirm this hypothesis, Monteiro et al. (2006) injected (s.c.) purified tGPI, combined or not to activated cruzipain, in wild-type naïve mice or in $TLR2^{-/-}$ or $BK_2R^{-/-}$ mutants. Edema measurements showed that tGPI/cruzipain potently induced microvascular responses via the TLR2/BK_2R-pathway and these proinflammatory effects were further potentiated by ACE inhibitors. Beyond their effects on the microcirculation, the released kinins link TLR2-driven inflammation to innate immunity by triggering BK_2R expressed on resident/migrating DCs, converting them into T_H1-directing antigen-presenting cells (APCs; Monteiro et al., 2006). Conversely, ACE counter-modulates T_H1-polarization via the trans-cellular the TLR2/BK_2R pathway by degrading BK/LBK, an endogenous signal that drives DC maturation (Aliberti et al., 2003; Monteiro et al., 2006, 2007; Scharfstein et al., 2007).

NEUTROPHIL LINKS TLR2/CXCR2 TO THE PROTEOLYTIC (KKS) PHASE OF INFLAMMATION

A key event at early stage of infection, the interaction of circulating neutrophils with the endothelium has profound effects on the outcome of the inflammatory process. One of the most common consequences of the interactions that take place on the luminal side of post-capillary vessels is the increased vascular permeability, a biological response that leads to the accumulation of protein-rich edema fluid in interstitial spaces. Although the list of endogenous soluble factors that increase vascular permeability is extensive, they usually impair the integrity of the endothelial barrier through the triggering of $[Ca^{2+}]_I$ – the via signaling of heterotrimeric



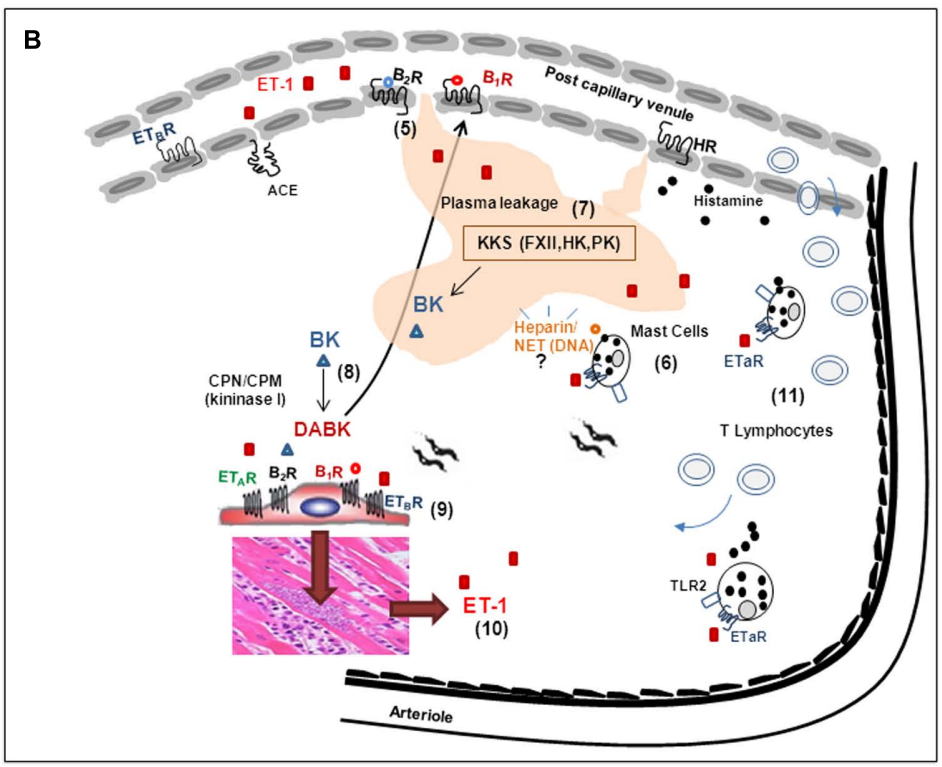


FIGURE 2 | Influence of interstitial edema on host/parasite balance in the myocardium: lessons from studies with the Dm28c strain of *T. cruzi*. **(A)** (1) Despite the scanty parasitism in the myocardium of chronic chagasic patients, parasite-containing pseudocysts occasionally burst, releasing high numbers of pro-inflammatory trypomastigotes into the surrounding interstitial spaces. (2) Innate sentinel cells, such as macrophages and/or mast cells,

(Continued)

FIGURE 2 | Continued
initiate inflammation through the sensing of tGPI, a TLR2 ligands shed by trypomastigotes. (3) In response to TLR2 engagement, tissue macrophages release CXC chemokines, which in turn activate neutrophils/endothelium via CXCR2. (4) Vascular permeability is increased, leading to the diffusion/accumulation of plasma proteins (including kininogens) through parasite-laden interstitial spaces. Once bound to heparan sulfate proteoglycans, HK suffers proteolytic attack by cruzipain (CZP) secreted by trypomastigotes. (5) The released kinins (LBK) amplify plasma leakage through the activation of endothelium BK_2R. The levels of intact kinins keep rising, despite the counter-regulatory role of ACE. **(B)** (6) Whether originating from plasma (following diffusion to sites of infection) or secreted by parasitized cardiomyocytes, ET-1 activates perivascular mast cells via ETAR. Upon degranulation, the mast cell release histamine (which further increases plasma leakage) and heparin, in the peripheral tissues. (7) The plasma "contact" phase system may be activated by mast cell-derived heparin and/or other negatively charged molecules of endogenous origin, such as DNA present in extracellular traps. Once activated by FXIIa, PKa releases BK from HK, thus propagating the proteolytic cascade. (8) Intact kinins are metabolized by kininase I (CPM and/or CPN), generating [des-Arg]-BK, which in turn activate BK_1R (whose expression is up-regulated in a broad range of heart cells, including cardiomyocytes-shown at the center of the illustration). (9) The parasites take advantage of the local availability of infection-promoting peptides (ET1, BK and/or [des-Arg]-kinins) to persistently invade cardiovascular cells through the signaling ("cross-talk") between $BK_2R/BK_1R/ET_AR/ET_BR$. (10) Intracellular parasite outgrowth leads to increased expression of pro-oxidative/pro-fibrotic ET-1 and proinflammatory chemokines. (11) Adverse cardiac remodeling ensues, as result of vicious cycles of ET-1-driven triggering of cardiac mast cells, endothelial activation and BK1R-driven recruitment of pathogenic subsets of effector $CD8^+$ T cells into the heart.

G-proteins, i.e., a pathway required to induce myosin-dependent contraction and junctional disruption of the endothelial cells.

In the HCP studies described in the previous section, Monteiro et al. (2006) observed that circulating leukocytes rapidly adhered to the luminal face of post-capillary venules. Complementary studies performed in neutrophil-depleted wild-type mice revealed that Dm28 TCTs failed to evoke a conspicuous edema in these animals. Similar to the pharmacological maneuvers employed with $TLR2^{-/-}$ mice, already described, the deficient phenotype of neutrophil-depleted mice was rescued upon injection of TCT supplemented with HK (Monteiro et al., 2006). Collectively, these results supported the concept that neutrophils, acting at the early stages of the inflammatory response, link innate immunity (TLR2-driven) to the KKS by driving the diffusion/accumulation of plasma-borne kininogens into parasite-laden tissues (**Figure 2A**).

In a subsequent study, Schmitz et al. (2009) investigated whether macrophages could also play a role in the trans-cellular "cross-talk" mediated by $TLR2/BK_2R$. First, they asked whether macrophages incubated with TCTs or tGPI *in vitro* stimulated the secretion of CXC chemokines (KC/MIP-2). The results revealed that wild-type macrophages robustly responded to Dm28c TCTs (but not to epimastigotes), whereas $TLR2^{-/-}$ macrophages were unresponsive to both stimuli. Turning to the HCP model, intravital microscopy studies showed that the drug repertaxin (CXCR2 antagonist) blocked leukocyte accumulation in the microvascular beds of HCP topically exposed to TCTs and inhibited the paw edema in *T. cruzi*-infected mice (Schmitz et al., 2009). Collectively, these findings supported the concept that Dm28c TCTs evoke inflammatory edema via the $TLR2/CXCR2/BK_2R$ axis, a sequential pathway of activation forged by the trans-cellular "cross-talk" of tissue resident macrophages, neutrophils and the endothelium (**Figure 2A**).

FURTHER EXPANSION OF THE INFLAMMATORY WAVE: THE CONVERGENCE BETWEEN ENDOTHELIN AND KININ PATHWAYS

Encoded by distinct genes, ET-1, 2, and 3 are closely related peptides expressed by endothelial cells, cardiac myocytes, and cardiac fibroblasts (Goto, 2001; Kedzierski and Yanagisawa, 2001). Synthesized as pre-pro-endothelin, these precursor proteins are cleaved by ET-converting enzymes (ECE) forming big-endothelin, which upon further processing yields 21 amino acids ET peptides that activate cardiovascular cells via GPCRs subtypes, ET_AR and ET_BR (Dhaun et al., 2007).

Apart from its powerful vasoconstrictor effects, the pleiotropic ET-1 induces plasma exudation (Filep et al., 1993; Sampaio et al., 2000). Hemodynamic changes such as those provoked by shear stress are sensed by the endothelium, which responds by activating eNOS and early up-regulating ET-1 mRNA. Once released, NO and ET-1 modulate tissue homeostasis extravascularly through the activation of other cell types, such as mast cells (Maurer et al., 2004).

Recently, Andrade et al. (2012) investigated the functional interplay between the kinin and ETs pathways at the early stages of infection by Dm28c TCTs. Remarkably, the application of subtype-specific antagonists of ET_AR or ET_BR, or the BK_2R antagonist (HOE-140) on the HCP prior to TCTs markedly reduced (∼70%) leukocyte accumulation in microvascular beds. In addition, these endothelin receptor (ETR) antagonists blunted plasma leakage in the hamster cheek pouch and blocked the inflammatory edema in *T. cruzi*-infected mice. Collectively, these results indicated that ETRs (both subtypes) and BKRs, may propagate the inflammatory wave intiated via TLR2/CXCR2 through the proteolytic activation of the KKS.

MAST CELLS MAY COUPLE TLR2 TO ETRs/BKRs-DRIVEN PATHWAYS OF INFLAMMATION

Levick et al. (2010) have recently drawn attention to the importance of cardiac mast cells in adverse myocardial remodeling. Given the precedent that mast cells sense *Mycobacterium tuberculosis* via TLR2 (Carlos et al., 2009), Andrade et al. (2012) have speculated that Dm28c TCTs may directly activate ET-positive mast cells (Ehrenreich et al., 1992) via TLR2, perhaps upregulating the extravascular levels of ETs in the chagasic myocardium. Although plausible, the pathogenic role of TLR2-positive cardiac mast cells in CCM was not object of systematic investigations so far. Focusing on other aspects of chagasic pathology, Meuser-Batista et al. (2011) have recently reported that cardiac mast cells die by apoptosis in CBA-infected mice, presumably reflecting reduced production of stem cell factor in chagasic heart.

Studies in other disease settings have shown that ET-1 activates ET_AR-positive mast cells in autocrine manner, inducing secretion of potent vasoactive mediators, such as histamine,

leukotriene C4 (Yamamura et al., 1994), and TNF-α (Coulombe et al., 2002). Of further interest, there is evidence that ET-1 induces metalloproteinase-driven ventricular remodeling in models of chronic heart pressure/volume overload (Murray et al., 2004; Janicki et al., 2006) and modulate cardiac contractility through the induction of mast cell degranulation via ET_AR (Eszlári et al., 2008). Although the role of the ET-1/mast cell axis was not directly investigated in CCM, there is evidence that plasma levels of ET-1 are elevated both in chagasic patients and experimentally infected mice (Petkova et al., 2000; Salomone et al., 2001), presumably reflecting increased shear stress or other infection-associated hemodynamic alterations in these individuals. Furthermore, as highlighted earlier in this text, the expression of ETs is up-regulated in parasitized cardiomyocytes (Tanowitz et al., 2005). Thus, irrespective of the source of ET-1, it is conceivable that the sudden rise in the extravascular levels of this potent pro-oxidative mediator may lead to the activation of perivascular ET_AR-positive mast cells in the chagasic heart. According to this hypothetical scenario, mast cell degranulation via ET_AR may release histamine, chemokines, along with a myriad of vasoactive mediators, in the parasite-ladden cardiac tissues (**Figure 2B**). More recently, Nascimento et al. (2012) have started to investigate this hypothesis by interfering with mast cell function in HCP topically exposed to Dm28 TCTs. Using mast cell stabilizers, we found that plasma leakage (BK_2R-driven) was indeed inhibited, thus suggesting that mast cell degranulation is required for overt activation of the KKS in peripheral sites of chagasic infection (**Figure 2B**).

Thus far, the mechanisms by which mast cells may propagate the KKS cascade in peripheral sites of *T. cruzi* infection remain unknown. In an interesting precedent coming from mouse models of allergic inflammation, Oschatz et al. (2011) have recently proposed that heparin (a mast cell storage product) acts as a typical endogenous "contact" activator, i.e., it activates PKa in FXIIa/HK-dependent manner, thereby releasing BK. Alternatively, parasite-induced activation of cardiac mast cells may activate the KKS through the formation of DNA-containing extracellular traps (von Köckritz-Blickwede et al., 2008). Further studies may determine if the ET/mast cell/KKS pathway plays an important role in the pathogenesis of CCM.

BK_2R IS ESSENTIAL FOR DEVELOPMENT OF ACQUIRED IMMUNE RESISTANCE TO CHAGASIC INFECTION

Comprising a heterogeneous population of professional APCs, DCs are widely but sparsely distributed in peripheral tissues and lymphoid organs (Shortman and Naik, 2007). Strategically positioned in T cell-rich areas of secondary lymphoid tissues, the resident DCs are specialized in antigen-presentation to $CD4^+$ and $CD8^+$ T cells. In steady-state conditions, immature DCs present MHC-restricted antigen peptides to virgin T cells in the absence of co-stimulatory molecules, hence contributing to the maintenance of peripheral tolerance (Sela et al., 2011). However, during infection, the immature DCs sense "danger" motifs expressed by pathogens through distinct families of PRRs, such as TLRs or intracellular NOD2-like receptors (NLR; Akira, 2009). In addition, conventional DCs may sense the threat to tissue integrity via receptors for endogenous proinflammatory mediators, such as ATP, uric acid (Sansonetti, 2006), and BK (Aliberti et al., 2003; Monteiro et al., 2007). Stabilized by cognate interactions with co-stimulatory molecules (CD80/86, CD40, and MHC), the prolonged encounters between antigen-bearing DCs and naïve T cells are essential for TCR activation. During the course of DC/T cell interaction, the "mature" APCs deliver T_H polarizing cytokines, such as IL-12p-70, which is critically required for T_H1 development.

Efforts to characterize the activation pathways controlling DC maturation in the context of *T. cruzi* infection have initially converged to nucleic acid-sensing TLRs (3, 7, and 9). In a key study, Caetano et al. (2011) showed that transgenic mice strains which lack functional UNC93B1 as well as functional endosomal TLRs (TLR3, 7, and 9), were as susceptible to *T. cruzi* infection as mice deficient in TLR3/7/9 (Caetano et al., 2011). In the same study, it was documented that *T. cruzi*-infected macrophages and DCs from 3 day mice displayed low IL-12p40 and INF-γ responses. Based on these results, it was inferred that recognition of intracellular parasites require UNC93B1-driven translocation of the nuclei acid-sensing TLRs from the endoplasmic reticulum to the endolysosomes (Caetano et al., 2011). In spite of this conceptual advance, other studies suggest that DCs might sense *T. cruzi* through TLR-independent pathways. For example, Kayama et al. (2009) showed that *T. cruzi* induces maturation (up-regulation of MHC class II, CD40, and CD86) of $MyD88^{-/-}TRIF^{-/-}$ mice bone-marrow-derived DCs as efficiently as wild-type bone-marrow-derived DCs. Using fetal liver DCs as target cells, these authors linked the *T. cruzi*-induced responses (IFN-γ production and DC maturation) to the activation of the NFATc1 pathway. These results implied that innate immunity is not exclusively controlled by nucleic acid-sensing TLRs.

Several years ago, we reported that that BK, acting as a typical endogenous danger signal (i.e., T_H1-directed endogenous adjuvant) induced DC maturation (IL-12 and up-regulated expression of co-stimulatory molecules) via BK_2R (Aliberti et al., 2003). In a key finding, we subsequently reported that BK_2R-deficient mice succumbed to acute challenge by Dm28c TCTs (i.p. route). Analysis of the immune dysfunctions underlying the susceptible phenotype of $BK_2R^{-/-}$ mice at early stages of infection showed a modest, but significant drop in the frequency of intracardiac type-1 effector T cells. Intriguingly, however, as the acute infection progressed in $BK_2R^{-/-}$ mice, the immune deficiency was intensified and generalized, involving both the extra-lymphoid and lymphoid compartment. Of note, the decayed T_H1 response of $BK_2R^{-/-}$ was accompanied by a corresponding rise in IL-17-producing T cells (T_H17). The premise that the deficient adaptive response of $BK_2R^{-/-}$ mice was a secondary manifestation resulting from impaired $BK_2R^{-/-}$ DC maturation was confirmed by systemically injecting wild-type $BK_2R^{+/+}$ DCs into the susceptible $BK_2R^{-/-}$ mice, prior to pathogen injection. Remarkably, this DC transfer maneuver rendered the recipient $BK_2R^{-/-}$ mice resistant to acute *T. cruzi* challenge, and restored their capability to generate protective IFN-γ-producing $CD4^+$ $CD44^+$ and $CD8^+$ $CD44^+$ effector T cells, while conversely suppressing the potentially detrimental T_H17 ($CD4^+$ subset) anti-parasite responses.

In the same study, Monteiro et al. (2007) further demonstrated that Dm28c TCTs potently activated $BK_2R^{+/+}$ $CD11c^+$ DCs (splenic origin) but not $BK_2R^{-/-}$ DCs, using IL-12 secretion and expression of co-stimulatory molecules (CD86, CD80, CD40) as read-out for DC maturation *in vitro*. Of further interest, K11777-treated trypomastigote failed to robustly activate wild-type DCs, thus linking generation of the BK_2R agonist (DC maturation signal) to the proteolytic activity of cruzipain. Noteworthy, Dm28c TCTs also induced the maturation of (splenic) $TLR2^{-/-}$ $CD11c^+$ DCs and TLR4 mutant (C3H/HeJ) via BK_2R, thus precluding cooperative signaling between this GPCR and either one of these surface PRRs. Admittedly, these results do not exclude the possibility that enhanced parasite uptake via the BK_2R pathway might have indirectly facilitated nucleic acid-sensing by TLRs residing in endolysosomes of immature splenic DCs. In any case, whether acting as a classical sensor receptor, and/or as a upstream pathway that potentiates TLR-signaling by parasite DNA or ssRNA, these results are in line with the concept that kinin "danger" signals proteolytically released by TCTs convert splenic $BK_2R^{+/+}$ DCs into inducers of type 1 immunity (Monteiro et al., 2007). Considering that the splenic parenchyma is continuously exposed to plasma proteins, it is conceivable that flagellated trypomastigotes navigating through the splenic stroma might be faced with abundant levels of blood-borne kininogens, most likely associated to ECM or cell-surface sulfated proteoglycans. Accordingly, we may predict that antigen-bearing $CD11c^+$ DCs (bearing *T. cruzi* antigens) residing in the spleen (and/or liver) stroma are converted into T_H1 inducers following exposure to high-levels of kinin "danger" signals. To this date, studies of BK_2R function in human DCs were limited to lineages derived from monocytes exposed to granulocyte-macrophage colony-stimulating factor (GM-CSF)/IL-4. Using this experimental model, Bertram et al. (2007) reported that BK_2R signaling promotes DC migration although BK, *per se*, did not induce the maturation of human DCs. However, in subsequent studies Kaman et al. (2009) suggested that BK_2R signaling potentiates the maturation of DCs previously "primed" via TLR4. In view of the marked phenotypic heterogeneity of DCs (Shortman and Naik, 2007), studies with a more representative range of human DCs are required to determine whether BK_2R is a key sensor of *T. cruzi*.

In conclusion, the analysis of BK_2R function in different models of acute *T. cruzi* infection strongly suggests that activation of the kinin system fuels anti-parasite immunity. In the next section we will review evidences indicating that generation of kinins in sites of infection may also benefit *T. cruzi*, hence translating into mutual benefits to the host–parasite equilibrium.

TCTs RELY ON CRUZIPAIN ACTIVITY TO INVADE CARDIOVASCULAR CELLS VIA THE BK_2R PATHWAY

As mentioned earlier, studies of *T. cruzi* interaction with mouse cardiomyocytes showed that membrane-permeable irreversible inhibitors of cruzipain impaired trypomastigote invasion and halted intracellular growth of amastigotes (Meirelles et al., 1992). While the studies on cruzipain-mediated pathways of invasion were in progress, Tardieux et al. (1992) reported that *T. cruzi* invades non-phagocytic cells through the induction of calcium-regulated pathway of lysosomal exocytosis. Subsequent studies revealed that the oligopeptidase B-mediated processing of a polypeptide precursor accumulating in the *T. cruzi* cytoplasm generated the calcium-inducing signal (Caler et al., 1998; Morty et al., 1999) that propelled parasite internalization. In a parallel development, Leite et al. (1998) reported evidences that GPCRs were the signal transducers of the $[Ca^{2+}]_i$-inducing signals generated by the oligopeptidase B-dependent peptide. Consistent with their working hypothesis, parasites genetically deficient in oligopeptidase B showed impaired infectivity, suggesting that proteolytic generation of the cytoplasmic Ca^{2+}-inducing signal was indeed required for the development of infective phenotype. In another interesting study, Santana et al. (1997) described the biochemical properties of Tc80, a serine protease displaying collagenase activity. Subsequently characterized as a prolyl oligopeptidase, Tc80 localizes in a vesicular compartment close to the flagellar pocket of trypomastigotes. Synthetic inhibitors of Tc80 potently inhibited *T. cruzi* (Y strain) invasion of non-phagocytic cells without interfering with the $[Ca^{2+}]_i$-inducing activity of parasite extracts (Grellier et al., 2001).

Further exploring the role of cruzipain as a virulent factor, Scharfstein et al. (2000) reported that activation of BK_2R potentiated parasite uptake by non-phagocytic cells. Whether using CHO transfected cells, human umbilical vein endothelial cells (HUVECs), primary cultures of mouse (neonatal) cardiomyocytes (Todorov et al., 2003), or primary culture of human smooth muscle cells (HSMCs; Andrade et al., 2012), these studies indicated that the parasites relied on cruzipain activity to generate a $[Ca^{2+}]_i$-inducing signal (BK_2R agonist) from HK displayed on host cell surfaces. Although the genetic ablation of the multicopy cruzipain genes remained a technical obstacle, experiments performed with membrane-permeable irreversible inhibitors of cruzipain and parasites overexpressing the cruzipain gene strongly suggested that the trypomastigotes critically depended on the enzymatic activity of cruzipain to stimulate parasite uptake by BK_2R-positive target cells (Scharfstein et al., 2000). The authors hypothesized that upon trypomastigote attachment (posterior end) to host cell surfaces, the enzymatically active forms of cruzipain – localized in the flagellar pocket (Murta et al., 1990; Souto-Padrón et al., 1990) – rapidly diffuse into the secluded spaces formed by the juxtaposed host/parasite membranes (Scharfstein et al., 2000). Once confined to "synapses" (Tyler et al., 2005; Butler and Tyler, 2012), the active protease should be protected from targeting by natural inhibitors, such as cystatins, soluble forms of kininogens (Stoka et al., 1998), and α2-macroglobulin (Araújo-Jorge et al., 1994; Morrot et al., 1997) present in interstitial fluids. Considering that BK_2R is sequestered to membrane rafts/caveolae (de Weerd and Leeb-Lundberg, 1997; Haasemann et al., 1998), we hypothesized that surface-bound HK (cruzipain substrate) may translocate in the plasma membrane before being delivered to the site of synapse (**Figure 3**), thus ensuring efficient kinin release/BK_2R-mediated signal transduction (Scharfstein et al., 2000; Santos et al., 2006; Scharfstein and Andrade, 2011; Andrade et al., 2012).

While analyzing the outcome of infection in cultures of HUVECs (resting state), we found that BK_2R did not promote parasite uptake in the absence of ACE inhibitors. This was not unexpected, because expression of ACE, a transmembrane

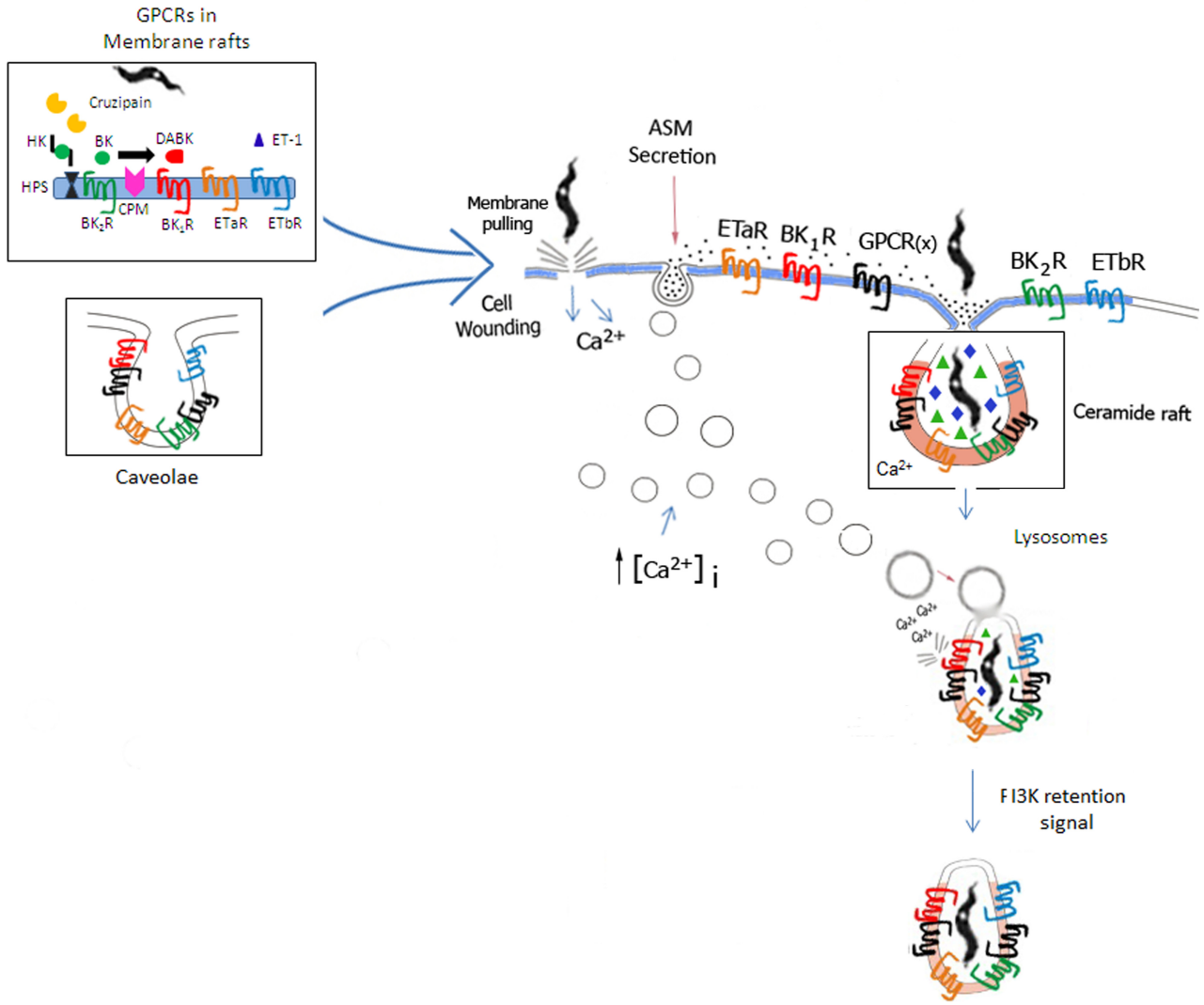


FIGURE 3 | Space filling model of GPCR-mediated invasion of cardiovascular cells. Based on the unified model of *T. cruzi* invasion proposed by Fernandes and Andrews (2012), the scheme illustrates how GPCRs (including BKRs/ETRs) originally confined to lipid rafts or caveolae (left side of panel) might be incorporated into the molecular platforms that coalesce around ceramide-enriched vesicles generated by the housekeeping mechanisms of plasma membrane repair from cellular wounds. According to Fernandes and Andrews, extracellular calcium influx resulting from cell wounds (center of panel) trigger Ca^{2+}-regulated lysosomal exocytosis, which in turn leads to the secretion of acid sphingomyelinase (ASM). Acting pericellularly, ASM generates ceramide-enriched endocytic vesicles. Next, BKRs, ETRs, along with several other GPCRs partners and several accessory signaling molecules translocate from lipid rafts/caveolae into the ceramide-platforms. In the resting state, signal transduction via $BK_2R/ET_AR/ET_BR$ (cross-talk) may likely depend on formation of homo or hetero-oligomers in multimolecular platforms coalescing in ceramide-enriched vesicles. During inflammation, the repertoire of GPCRs in the ceramide-rich endocytic vesicles is modified by the incorporation of inducible molecules, such as BK_1R and CPM. ET-1 (host cell origin/autocrine route) and des-Arg-kinins (released from surface bound HK by cruzipain, then subsequently metabolized by CPM) bind to their respective GPCRs (ET_AR/ET_BR and BK_1Rs). Feedback cycles might enhance the efficiency of the housekeeping plasma-membrane repair mechanisms via BKR/ETR-driven (i) induction of the canonical pathway of Ca^{2+}-regulated lysosomal exocytosis and/or (ii) induction of the calcium-dependent lysosomal fusion with the nascent parasitophorous vacuole (Andrade and Andrews, 2004) and/or PIK3-dependent retention signals (Woolsey and Burleigh, 2004).

metallopeptidase that potently degrades intact kinins, is strongly upregulated by endothelial cells. As predicted, BK_2R-dependent parasite uptake by HUVECs is potentiated by ACE inhibitors (Scharfstein et al., 2000; Todorov et al., 2003; Andrade et al., 2012).

Noteworthy, ACE inhibitors were not essential for BK_2R-driven parasite invasion of cardiomyocytes or smooth muscle cells (Todorov et al., 2003; Andrade et al., 2012). Although the levels of ACE were not measured, it is likely that these muscle cells express lower levels of ACE as compared to HUVECs. Along similar lines, TCTs induced IL-12 responses on splenic $CD11c^+$ DCs via BK_2R irrespective of presence/absence of ACE inhibitors (Monteiro et al., 2007). In summary, the outcome of Dm28c TCT interaction with cells that constitutively express BK_2R is controlled by ACE in cell-specific manner.

THE INDUCIBLE BK_1R: A UBIQUITOUS GATEWAY FOR *T. cruzi* INVASION OF INFLAMED TISSUES?

A major challenge in Chagas disease research is to predict the clinical outcome of the chronic cardiomyopathy. About 30% of these patients develop a progressive form of cardiomyopathy characterized by the presence of diffused inflammation/fibrosis. As mentioned in the introduction, pathologists noticed the presence of microvascular abnormalities and altered ECM patterns in the heart of CCM patients (Higuchi et al., 1999). To our knowledge, BK_1R expression was not systematically investigated in heart autopsy studies of chagasic patients despite the indications that dysregulated BK_1R function might be detrimental to the heart in other disease models. For example, in animal models of STZ-induced diabetic cardiomyopathy (Westermann et al., 2009), BK_1R-deficient mice showed attenuated cardiomyopathy as compared to wild-type mice, as evidenced by the decrease of cardiac inflammation, fibrosis, oxidative stress, and significant improvement of left ventricular function. Pertinent to studies linking heart remodeling to the ET up-regulation by parasitized cardiomyocytes (Tanowitz et al., 2005), there is indication that oxidative stress induced by ET-1 (ET_AR-driven) and angiotensin I (AT_1-driven) up-regulates BK_1R, leading to activation of PI3K and MAPK in smooth muscle (Morand-Contant et al., 2010). Although not directly examined, it is conceivable that NF-κB-induction by proinflammatory cytokines secreted by antigen-specific intracardiac $CD8^+$ T cells (Padilla et al., 2009; Silverio et al., 2012) might also up-regulate BK_1R in the chagasic heart.

Todorov et al. (2003) were the first to demonstrate that Dm28 TCTs evoke interstitial edema via the sequential activation of BK_2R/BK_1R. In the same study, they highlighted the dichotomic nature of kinin signaling pathways: the parasites were able to invade activated target cells via the BK_1R/CPM pathway (**Figure 1B**). Differently from results observed in resting HUVECs (which critically depend on ACE blockade to efficiently internalize TCTs via BK_2R), the trypomastigotes invade lipopolysaccharide (LPS)-treated HUVECs through interdependent signaling ("cross-talk") between BK_1R and BK_2R (Todorov et al., 2003). Notably, the "cross-talk" between BK_2R and BK_1R was also observed in invasion assays performed with primary mouse cardiomyocytes (Todorov et al., 2003), which are cell types that spontaneously express BK_1R in culture systems.

Although studies with non-specific kininase I inhibitors have tentatively linked BK_1R-dependent parasite uptake to the processing activity of these carboxypeptidases, the role of the transmembrane carboxypeptidase CPM was not directly addressed in the above studies. As mentioned in the introduction, BK_2R agonists (BK/LBK) are converted into the BK_1R agonists (i.e., [des-Arg]-BK/LBK) either by surface CPM or by a soluble (plasma-borne) CPM (**Figure 1A** and **Figure 2B**). Since the interaction medium used in our invasion assays was free of serum, it seemed unlikely that CPN was the processing enzyme critically involved in BK_1R-driven parasite invasion of LPS-HUVECs or cardiomyocytes. Instead, we inferred that CPM is likely the enzyme generating the BK_1R agonist that propels parasite invasion in our *in vitro* assays. Interestingly, Sangsree et al. (2003) studied the functional interplay between CPM and BK_1R in great details in human lung microvascular cells activated by IL-1β and IFN-γ. First, they showed that BK_1R signaling is responsible for the sustained NO response induced by intact BK (BK_2R agonist), implying that CPM converted the intact BK into [des-Arg]-kinins. Using cells transfected with genes conding for CPM and BK_1R, the authors disrupted lipid rafts with methyl-beta-cyclodextrin (MβCD). As predicted, this maneuver reduced the BK_1R-dependent increase in $[Ca^{2+}]_i$ in response to stimulation with intact BK_2R agonists, whereas addition of cholesterol rescued this BK_1R-driven response. After showing that CPM and BK_1R co-localized in lipid raft/caveolin-enriched membrane fractions (Zhang et al., 2008), they found that CPM/BK_1R physically interact on the cell membrane, based on co-immunoprecipitation, cross-linking, and fluorescence resonance energy transfer analysis. In an elegant experiment using a novel fusion protein containing CPM at the N-terminus of the BK_1R, Zhang et al. (2008) showed that these transfected cells were $[Ca^{2+}]_i$ responsive upon stimulation with intact kinins, but (as predicted) this response was no longer impaired by MβCD or by CPM antibody.

As already mentioned, in invasion assays performed with LPS-HUVECs or cardiomyocytes, we found that ACE inhibitors were not required to promote parasite uptake via BK_1R, as opposed to the ACE inhibitor-dependent phenotype displayed by resting HUVECs (Todorov et al., 2003; Andrade et al., 2012). This implies that CPM-dependent generation of the BK_1R is prioritized over the ACE-dependent pathway of BK/LBK degradation, hence consistent with the findings reported by Zhang et al. (2008). Since both CPM/BK_1R and BK_1R/BK_2R are sequestered into lipid rafts and/or caveolae, it is conceivable that these GPCRs segregate together with HK (bound to heparan sulfate proteoglycans) into specialized plasma membrane microdomains (**Figure 3**). Of further interest, although BK_2R and BK_1R signal cells through fairly similar intracellular pathways, the regulation of inducible BK_1R differs from BK_2R in that the former is desensitized upon agonist binding only to a limited degree (Enquist et al., 2007).

BKRs AND ETRs AS GATEWAYS FOR INVASION OF CARDIOVASCULAR CELLS: FUNCTIONAL LINK WITH THE SPHINGOMYELINASE-DEPENDENT PLASMA MEMBRANE PATHWAY?

In a previous section, we reviewed results of studies showing that Dm28c TCTs induce inflammatory edema through mechanisms involving cooperation between BKRs and ETRs (Andrade et al., 2012). To evaluate whether the parasites could exploit the ET pathway for invasion purposes, Andrade et al. (2012) studied the interaction of Dm28c TCTs with three types of host cells: HUVECs (which only express ET_BR), primary cultures of HSMCs (which express ET_AR and ET_BR), and mouse neonatal cardiomyocytes (expressing both subtypes of ETRs). Using multiple pharmacological tools (subtype-specific ETR antagonists; neutralizing antibodies for each GPCR) and, in addition, interference RNA, Andrade et al. (2012) demonstrated that TCTs invade "resting" HUVECs via ET_BR, while invasion of the muscle cells involved activation of both subtypes of ETRs. Notably, the combined treatment of the muscle cell cultures with ET_AR and ET_BR subtype-specific antagonists failed to decrease parasite infectivity of HSMC or cardiomyocytes over values induced by the individual drugs, thus recapitulating the "cross-talk" observed between BK_2R/BK_1R in cardiomyocytes

or LPS-HUVECs (Todorov et al., 2003). Importantly, studies with CHO-ET_AR or CHO-ET_BR-transfected cells demonstrated that parasite invasion was efficiently blocked in subtype receptor specific manner, implying that formation of ET_AR/ET_BR heterodimers, "*per se*," is not an absolute requirement for parasite entry. Since G-protein-coupled B_2R and ETR compartmentalize in lipid rafts/caveolae (de Weerd and Leeb-Lundberg, 1997; Bremnes et al., 2000; Okamoto et al., 2000; Harada et al., 2002; Ostrom, 2002), we reasoned that in transfected mammalian cells the density levels of any particular GPCR (in lipid rafts/caveolae) might be well above those found in target host cell types that naturally overexpress these receptors (Andrade et al., 2012). If true, in natural muscle cells, the reduced proportion of any given subtype of GPCR may be compensated by cooperative interactions involving physical association with alternative GPCR "partners." Assuming that ligand generation is not a limiting factor, this "space-filling model" predicts that GPCRs that might be present at higher density in such microdomains should have a better chance to coordinate signaling responses upon ligand binding, as seems to be the case for BK_2R, ET_AR, and ET_BR (Andrade et al., 2012; **Figure 3**).

Although we did not prove that BK_2R, ET_AR, and ET_BR form homo or hetero-oligomers of higher order in host cell attachment sites, our pharmacological studies support this possibility. Whether using subtype-specific GPCR antagonists, neutralizing antibodies, or iRNA interference (ET_AR), our results showed that parasite invasion was inhibited roughly to the same extent. Furthermore, we did not observe additive effects by combining receptor subtype antagonists or iRNA approaches. These results suggested that pharmacological blockade of one GPCR partner is sufficient to dismantle the signaling function of entire unit, consequently reducing parasite internalization in 40–60% (Andrade et al., 2012).

Several years ago, it was demonstrated that cholesterol-depleting drugs reduce host cell susceptibility to *T. cruzi* infection (Fernandes et al., 2007). In a subsequent study, we explored the possibility that BKRs and ETRs compartmentalize in lipid rafts/caveolae, by treating HSMCs with MβCD (Andrade et al., 2012). As predicted, the cholesterol-depleting drug drastically reduced parasite (Dm28c) entry in HSMCs whereas addition of exogenous cholesterol to MβCD-HSMCs restored $ET_AR/ET_BR/BK_2R$-dependent pathways of *T. cruzi* invasion. Although the physical association of these GPCRs was not demonstrated at the molecular level, these results suggest that the cross-talk between ETRs and BK_2R may critically depend on the integrity of lipid rafts/caveolae (**Figure 3**). Interestingly, confocal microscopy studies performed with antibodies to anti-ET_AR or anti-ET_BR showed that parasite–cell interaction sites contained increased clusters of these GPCRs. These results are consistent with the concept that ET-1 and "kinins" activate their cognate GPCRs in lipid rafts/caveolae, perhaps translocated to synaptic sites (Scharfstein et al., 2000). As explained below, it is also possible that ET-1, ET precursors and HK (bound to heparan sulfate) exert their functional roles as agonists and/or precursors of GPCR ligands after their internalization in ceramide-enriched vesicles (**Figure 3**).

In a breakthrough, Fernandes et al. (2011) and Fernandes and Andrews (2012) elaborated a unified mechanistic concept for parasite invasion of non-phagocytic host cells which nicely embraced two seemingly distinct internalization routes (Andrade and Andrews, 2004; Woolsey and Burleigh, 2004), the first one driven by calcium-regulated lysosomal exocytosis, leading to plasma membrane fusion and a lysosomal-independent pathway involving parasite entry though nascent parasitophorous vacuoles. In this unified mechanism of infection, trypomastigotes invade host cells by subverting a housekeeping mechanism of repair from cell wounds which crucially depends on acid sphingomyelinase (ASM)-driven formation of ceramide-enriched endocytic vesicles. Triggered by the formation of stable lesions in the host plasma membrane, the repair response is initiated by the influx of extracellular calcium. This event in turn leads to calcium-regulated lysosomal exocytosis and ASM secretion to the extracellular environment. Further downstream, ASM cleaves the polar head group of sphingomyelin, generating ceramide. The repair process is terminated by the formation of endocytic vesicles, which then reseal the original plasma membrane lesions as they internalize. *T. cruzi* trypomastigotes are thought to inflict plasma membrane injuries upon adherence of the vibrant flagellum/cell body to the cell surface. Invasion "*per se*" occurs after the formation of ceramide-rich endocytic vesicles (Fernandes et al., 2011).

Although we have not provided direct evidence that BK_2R, BK_1R, ET_AR, ET_BR are translocated from lipid and/or caveolar microdomains into ceramide rafts of muscle cells (HSMC or cardiomyocytes) or HUVECs, this possibility deserves to be explored in light of evidence that ceramide-enriched microdomains spontaneously fuse to generate large macrodomains containing receptor clusters in multimolecular platforms (Jin et al., 2008). Using endothelial cells derived from coronary arteries, these authors reported that Fas-ligand driven activation of death receptor mediates the formation of redox signaling platforms in lipid rafts via ceramide production by ASM-driven hydrolysis of sphingomyelin. They also showed that formation of ceramide-enriched signaling platforms was canceled in endothelial cells treated with inhibitors of lysosomal function. In other words, the requirements for the formation of multimolecular signaling platforms in ceramide rafts of endothelial cells seem to recapitulate the requirements for parasite invasion via the house-keeping mechanisms of repair from cell wounds described by Fernandes and Andrews (2012). According our "space-filling model" (**Figure 3**), the signal transduction responses coordinated by these various GPCRs (e.g,., $BK_2R/ET_AR/ET_BR$) may either occur (i) within the synaptic sites formed soon after adhesion and/or (ii) after translocation of these GPCRs into ceramide rafts, hence integrating the above mentioned multimolecular signaling platforms. Sufficiently flexible, this GPCR-dependent model of parasite internalization is compatible with linear or converging activation pathways. For example, the linear activation mode predicts that specific GPCR subtypes are differentially targeted to lipid rafts/caveolae or to ceramide-enriched vesicles and/or to nascent parasitophorous vacuoles (**Figure 3**). Once translocated to nascent parasitophorous vacuoles, some of these GPCR subtypes may generate parasite retention signals through PI3K-dependent remodelling of the actin cytoskeleton (Woolsey and Burleigh, 2004), and/or drive calcium-dependent lysosomal fusion to parasitophorous vacuole (Andrade and Andrews, 2004). Multiple contacts with the pathogen and signals transmitted by soluble agonists available

at high levels in the edematous extracellular environment, such as ETs, BK, DABK, may then fuel parasite uptake *in vivo* through converging signaling pathways.

The possibility that these GPCRs might serve as gateways for *T. cruzi* infection of heart cells is worth exploring. According to Fernandes and Andrews (2012), *T. cruzi* propensity to invade muscle cells is dictated by the need of muscle cells to constantly repair sarcolemma injuries via the Ca^{2+}-regulated pathway of lysosomal exocytosis and ASM-driven ceramide generation (McNeil and Steinhardt, 2003). Future studies may clarify whether the signals that promote parasite uptake by cardiomyocytes via interdependent signaling of ($BK_2R/ET_AR/ET_BR$) may increase the efficiency of the house-keeping mechanisms of muscle cell repair from sarcolemma wounds. If confirmed, the convergence of house-keeping and GPCR-mediated signaling pathways may translate into mutual benefits to the host/parasite relationship, at least so during the indeterminate stage of chronic infection. Interestingly, the concept that BK_2R signaling is protective to the heart tissues is supported by evidences that this pathway restores S2a-mediated sacroplasmatic Ca^{2+} uptake (Tschöpe et al., 2005; Spillmann et al., 2006). As chronic pathology evolves, perhaps the harmonious trade-off between parasite and cardiac muscle is gradually compromised owing to the ubiquitous up-regulation of BK_1R by heart cells, including cardiomyocytes, cardiac fibroblasts, and inflammatory and/or TGF-β (regulatory/pro-fibrotic) macrophages. Flares of plasma leakage elicited by trypomastigotes occasionally released by from ruptured pseudocysts may propitiate the rise of extravascular levels of [des-Arg]-BK/LBK (BK_1R agonists) and ET-1. Furthermore, the extravascular levels of ET-1 are further elevated as result of up-regulated expression of this the pro-oxidative mediator by parasitized cardiomyocytes (Tanowitz et al., 2005). A vicious cycle may be installed, favoring parasite entry in heart cells via the $ET_AR/ET_BR/BK_1R$ pathway at expense of increased inflammation and adverse cardiac remodeling (**Figure 2B**).

HOST/PARASITE BALANCE, KKS AND THE PHENOTYPIC VARIABILITY OF *T. cruzi* SPECIES

The characterization of molecular determinants of pathogenic outcome is a major goal of basic and clinical research on CCM. Although the phenotype of the Dm28c may not be faithfully reproduced by every isolate of the highly polymorphic *T. cruzi* species, currently segregated into six DTUs (I–VI; Zingales et al., 2009), we may anticipate that comparative studies with other isolates should offer clues to understand the molecular determinant of pathogenicity of *T. cruzi*. For example, it will be interesting to know whether parasite strains belonging to particular DTUs share (or not) the ability to induce inflammatory edema through the cooperative activation of the TLR2/CXCR2/$BKR_2R/BK_1R/ET_AR/ET_BR$ pathway. Although the expansion/contraction of this proteolytic cascade must certainly depend on additional parasite and host factors, the few players so far identified may help to investigate the phenotypic variability of natural *T. cruzi* populations. For example, strain-dependent variability on the expression levels of tGPI or, alternatively, in the extent of obscure mechanisms controlling shedding of lipid vesicles enriched with tGPI-linked mucins (Trocoli-Torrecilhas et al., 2009) might influence the trypomastigote ability to activate the KKS (Scharfstein and Andrade, 2011)

Alternative *T. cruzi* ligands are also known to activate innate sentinel cells through TLR4 (Oliveira et al., 2010), TLR9 (Bafica et al., 2006), or NOD1 (Silva et al., 2010), but it is presently unknown whether these pathways may interconnect to the KKS. Interestingly, Petersen et al. (2005) implicated TLR2 as the main upstream regulator of hypertrophy which *T. cruzi* trigger in isolated cardiomyocytes. On the other hand, *T. cruzi* strains that might express/shed higher levels of tGPI might somewhat blunt this potentially adverse phenotype by favoring low-grade release of cardioprotective kinins via cruzipain as described by Monteiro et al., 2006. In the chronic settings, the benefits of anti-apoptotic effects attributed to purified cruzipain (Aoki Mdel et al., 2006) and BK_2R signaling (Chao et al., 2008) may be offset by the up-regulation of BK_1R, a pathway that may synergize with TLR2 and ET_AR, hence fueling inflammatory edema (**Figure 2**) and cardiac hypertrophy in chronically infected patients.

Another mechanism that may underlie the variable phenotype of *T. cruzi* strains is the variable expression profiles of cruzipain isoforms (Lima et al., 2001). For example, it is well established that cruzipain 2 (Dm28c strain) has narrow substrate specificity as compared to the major cruzipain isoform, i.e., the parasite kininogenase (Scharfstein, 2010). Predictably, strain-dependent variability in the ratio of expression between these two cruzipain isoforms may have impact on *T. cruzi* ability to invade host cells expressing BKRs (influence on tissue tropism) as well on its capacity to induce interstitial edema and T_H1 responses via the kinin pathway. For similar reasons, we may predict that variations in the expression levels of chagasin, a tight-binding endogenous inhibitor of papain-like cysteine proteases – originally described in *T. cruzi* (Monteiro et al., 2001), may also influence the phenotype of *T. cruzi* strains. This possibility is supported by evidences (Aparício et al., 2004) indicating that TCTs of the G strain, which are poorly infective, display increased chagasin/cruzipain ratios as compared to Dm28c. Importantly, the infectivity of the G strain was enhanced upon addition of cruzipain-rich culture supernatants from Dm28 TCTs. In the same study, the authors pointed out that that vesicles shed by TCTs might serve as cruzipain substrates, presumably generating hitherto uncharacterized infection-promoting signals (Scharfstein and Lima, 2008). Hence, strain-dependent differences in the expression levels of tGPI and cruzipain isoforms may have impact on host/parasite relationship, either because kinins influence parasite infectivity, DC function and their ability to steer T_H1-type effector development.

CONCLUDING REMARKS

Host defense to infections depend on the mobilization of two distinct strategies in order to minimize infection-associated pathology: the first, innate immunity, is mobilized at the onset of infection with the purpose to eliminate or at least limit the spread of pathogens to the tissues (Medzhitov, 2009). The second strategy, involving products generated by proteolytic cascades and multiple endogenous "danger" signals emanating from injured tissues, has evolved to limit and repair the tissue damage inflicted by the microbial pathogen. The distinction between

the mechanism underlying these overlapping processes in chronic parasitic infections, such as Chagas disease, is important because they profoundly affect the evolutionary dynamics of the of host–pathogen interactions (Rausher, 2001). Here we reviewed data showing that infective forms of *T. cruzi* can evoke interstitial edema through the activation of an inflammatory pathway initiated by one PRR (TLR2) and amplified through the "sequential" activation of the following GPCRs: CXCR2/BK_2R/BK_1R/ET_AR/ET_BR axis. Although presented as a linear cascade process for didactic reasons, this paradigm provides a useful framework to investigate the activation pathways that interconnect innate immunity to the KKS, a hub-like proteolytic network that generates proinflammatory kinin peptides while simultaneously activating three other proteolytic cascades, i.e., complement/coagulation/fibrinolytic systems – none of which were thus far explored in the context of CCM.

The evidence that activation of the kinin/BK_2R pathway benefits the mammalian host emerged from our immunological studies (Monteiro et al., 2007). The analysis of the susceptible phenotype of BK_2R-deficient mice (acute chagasic infection) demonstrated that DC sensing of the kinin "danger" signal via BK_2R was essentially required for optimal generation of type 1 (host protective) effector T cells (Monteiro et al., 2007).

The dual role of the KKS in experimental Chagas disease is so far based on evidences that Dm28c trypomastigotes exploit BKRs/ETRs as (non-exclusive) gateways for cellular invasion of a broad range of non-phagocytic cells, including cardiomyocytes. We have proposed that trypomastigotes sharing the Dm28c phenotype might be able to transiently generate infection-promoting peptidergic ligands for GPCRs, such as BK and ET-1, in inflamed tissues through the induction of intersticial edema.

Interestingly, studies in other models of heart disease have associated KKS/BK_2R signaling with protective cardiac functions, such as reducing apoptosis and chamber dilatation in the myocardium (Chao et al., 2008) and promoting restoration of S2a-mediated sarcoplasmatic Ca^{2+} uptake by cardiac cells (Tschöpe et al., 2005). Thus, it is tempting to speculate that BK_2R signaling may bring about mutual benefits to the host/parasite equilibrium in the chagasic heart, perhaps during the quiescent phase (indeterminate) of chronic disease. Interestingly, there are a few reports describing beneficial effects of continuous ACE inhibitor treatment in animal models of Chagas disease (Leon et al., 2003; de Paula Costa et al., 2010). Although the clinical/pathological benefits of ACE inhibitors in CCM might be partially or totally ascribed to blockade in generation of profibrotic angiotensin II, these results argue against an earlier proposition that ACE inhibitor-dependent up-regulation of the BK_2R pathway might aggravate infection-associated pathology (Scharfstein et al., 2000).

In several models of sterile inflammation, the initial tissue damage is sufficient to initiate self-propagating biochemical and immunological processes with the aim to protect and rescue tissue integrity. Depending on the magnitude of the trauma, metabolic status, and host genetics, the collateral tissue damage associated with the activation of endogenous pathways of inflammation in the parasitized heart (Machado et al., 2000) may be extreme, causing irrevesible leison on cardiomyocytes. Studies *in vitro* have implicated TLR2 signaling in *T. cruzi*-induced pathways leading to cardiac hypertrophy (Petersen et al., 2005). It will be interesting to know whether the infection-associated damage of cardiomyocytes can be attenuated upon kinin release and BK_2R signaling, perhaps reminiscent of some of the BK_2R-dependent protective responses observed in ischemia reperfusion and diabetic cardiomyopathy (Messadi-Laribi et al., 2008; Tschöpe and Westermann, 2008).

Concerning BK_1R, recent work in animal models of focal brain injury highlights the importance of therapeutic intervention on the KKS as a way to limit secondary damage caused by brain-barrier leakage (Raslan et al., 2010). As shown by these authors, pharmacological blockade of the inducible BK_1R (but not BK_2R) had remarkable protective effects on traumatic brain injury. Of interest in this context, we have previously reported that BK_1R antagonist blunts the edematogenic response elicited by Dm28c TCTs in mice pretreated with LPS (Todorov et al., 2003).

Since chagasic patients display higher levels of ET-1 in the bloodstream (Petkova et al., 2000; Salomone et al., 2001), it is plausible that the edema resulting from pseudocyst rupture may promote the diffusion of blood-borne ET-1 through perivascular cardiac tissues. If true, these events may in turn trigger cardiac mast cell degranulation via the ET-1/ET_AR axis, thus linking the ET pathway to the KKS (**Figure 2B**). Furthermore, considering that the expression of BK_1R is up-regulated by the pro-oxidative ETs (Morand-Contant et al., 2010), and that ET-1 expression is robustly increased in parasitized cardiomyocytes (Tanowitz et al., 2005), it is conceivable that *T. cruzi* has evolved strategies to improve its infectivity at expense of increased inflammation, as recently shown in mice infected with the Y strain of *T. cruzi* (Paiva et al., 2012). Following a similar line of reasoning, we may predict that ET-1 and [des-Arg]-kinins, acting cooperatively, may propel parasite entry in cardiovascular cells through the signaling of BK_1R, a subtype of GPCR that is ubiquitously up-regulated in inflamed tissues.

The sporadic formation of an intramyocardial edema in the proximity of an inflammatory lesion generated by pseudocyst rupture may alter tissue homeostasis, consequently modifying the dynamics of host/parasite interaction in this microenvironment. For example, the trypomastigotes navigating through intercellular cardiac spaces might be targeted by high-titered complement fixing/lytic antibodies (Krettli et al., 1982; Almeida et al., 1994) and/or by antibodies that inhibit host cell invasion (Schenkman et al., 1994; Tonelli et al., 2011). Induction of interstitial edema via activation of KKS might favor the rapid diffusion of these host protective antibody specificities into peripheral sites of infection. In addition, the inflammatory edema may allow the diffusion of antibodies against the C-terminal domain of cruzipain (GP25; Scharfstein et al., 1983). Antibody targeting of the immunodominant sulfated epitopes (Duschak and Couto, 2009) displayed on the C-terminal domain of cruzipain may either limit trypomastigote ability to efficiently invade cardiovascular cells (Meirelles et al., 1992; Scharfstein et al., 2000) and/or prevent anti-apoptotic responses which cruzipain otherwise induces in cardiomyocytes (Aoki Mdel et al., 2006).

For similar reasons, the heart conduction system might be targeted by auto-antibodies that are either specific for M2 muscarinic

receptors (Medei et al., 2007) and/or β1-adrenergic receptors (Labovsky et al., 2007). Depending on their titers and fine-specificity, these auto-antibodies may evoke arrhythmia as a secondary consequence of plasma leakage elicited by trypomastigotes. If confirmed, this hypothesis may legitimate attempts to treat chagasic patients with drugs that target the ET/mast cell/KKS axis: by limiting leakage of auto-antibodies into the myocardium, these drugs may also mitigate cardiac arrhythmia.

Considering the long span of chronic infection, the myocardium of patients exhibiting deficient IL-10 production by macrophages or regulatory T cells (Costa et al., 2009) might be particularly prone to up-regulate the BK_1R pathway in response to excessive collateral damage inflicted by pathogenic subsets of T cells. In this hypothetical scenario, the infiltrating type 1 effector $CD8^+$ T cells may relentless fuel the pro-fibrotic pathways coordinated by BK_1R/ETR. Of further interest, recent studies of sterile inflammation in transgenic mice subjected to cardiac pressure overload revealed that mitochondrial DNA (i.e., simulating symbiotic bacteria) leaks into autophagic vesicles of cardiomyocytes (Oka et al., 2012). Strikingly, the authors reported that the down-regulation of DNAse II, a lysosomal enzyme, fuels sterile inflammation in TLR9-dependent manner, provided that the mice are subjected to pressure overload (Oka et al., 2012). Although not explored in the context of CCM, it may not be surprising if chagasic heart debilitated by microvascular lesions, hypoxia, and immunopathology may be hyper-responsive to TLR9 signaling induced by *T. cruzi* mitochondrial DNA and/or self-DNA (from non-infected cardiomyocytes). If confirmed, it will be intriguing to know if BK_1R and ETRs, acting cooperatively in the pro-oxidative environment that prevails in the chagasic heart (Dhiman and Garg, 2011) may up-regulate TLR9 and/or STING, another recently characterized sensor of self-DNA (Ahn et al., 2012). Ongoing studies in chagasic mice treated with BK_1R blockers may clarify whether this therapeutic strategy may reduce parasite tissue load as well as myocardial fibrosis. Moreover, it is conceivable that BK_1R antagonists may reduce chronic inflammation by preventing microvascular leakage and/or blocking parasite-induced recruitment of pathogenic subsets of anti-parasite CD8 T effector cells into the myocardium (Silverio et al., 2012), as reported in EAE (Dutra et al., 2011; Göbel et al., 2011).

In summary, our studies on the KKS have provided a general framework to investigate the intertwined proteolytic circuits that reciprocally couple anti-parasite immunity to inflammation and fibrosis in experimental CCM. Although limited to a single *T. cruzi* strain, Dm28c, the knowledge that emerged from 20 years of studies on this emerging field suggest that plasma leakage into the myocardium may shift the dynamics of host/parasite interaction in the inflamed heart through the activation of BKRs/ETRs. Hopefully, some of the lessons taken from this experimental work may help to elucidate the mechanisms underlying susceptibility to CCM, perhaps assisting efforts to improve the therapeutic management of chagasic patients.

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