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Review

From the double helix to the post-genomic era: Modern molecular cell biology contributions to disclose *Trypanosoma cruzi* host interactions

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ABSTRACT

Trypanosoma cruzi, the causative agent of Chagas' disease, which affects a large number of individuals in Central and South America, is transmitted to vertebrate hosts by blood-sucking insects. This protozoan is an obligate intracellular parasite. The infective forms of the parasite are metacyclic and bloodstream trypomastigote and amastigote. Metacyclic trypomastigotes are released with the feces of the insect while amastigotes and blood stream trypomastigotes are released from the infected host cells of the vertebrate host after a complex intracellular life cycle. The recognition between parasite and mammalian host cell involves numerous molecules present in both cell types. Here we review the new advances in the understanding of this pathogen interaction with host provided by the rapid progress in Molecular Biology, as the commemoration of the 60 years of the double-helix discovery is imminent.

KEYWORDS: molecular biology, *Trypanosoma cruzi*, host interaction, cell invasion

INTRODUCTION

Sometime, about 150,000 years ago, *Homo sapiens* emerged in eastern Africa and spread throughout the world, possibly in several waves. By the end of the Ice Age, humans had migrated to and inhabited virtually the whole of the face of the Earth, bringing some parasites with them and collecting others on the way. During our relatively short history on Earth, humans have acquired an amazing number of parasites, about 300 species of helminth worms and over 70 species of protozoa. Many of these are rare and accidental parasites, but we still harbor about 90 relatively common species, of which a small proportion cause some of the most important diseases in the world, inevitably, these are the ones that have received the most attention (For complete review) [1].

The first written records of what are almost certainly parasitic infections come from a period of Egyptian medicine from 3000 to 400 BC, particularly the Ebers papyrus of 1500 BC discovered at Thebes. Later, there were many detailed descriptions of various diseases that might or might not be caused by parasites, specifically fevers, in the writings of Greek physicians between 800 to 300 BC, such as the collected works of Hippocrates, known as the Corpus Hippocratorum, and from physicians from other civilizations including China from 3000 to 300 BC, India from 2500 to 200 BC, Rome from 700 BC to 400 AD, and the Arab Empire in the latter part of the first millennium. As time passed, the descriptions of infections became more accurate

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and Arabic physicians, particularly Rhazes (AD 850 to 923) and Avicenna (AD 980 to 1037), wrote important medical works that contain a great deal of information about diseases clearly caused by parasites. In Europe, the Dark and Middle Ages, characterized by religious and superstitious beliefs, held back medical progress until the Renaissance, which released a flurry of activity that eventually led to the great discoveries that characterized the end of the 19th century and the beginning of the 20th. These discoveries included the demolition of the theory of spontaneous generation and the evolution of the germ theory by Louis Pasteur, the demonstration by Pasteur that diseases could be caused by bacteria, the discovery of viruses by Pierre-Paul Emile Roux, the introduction by Robert Koch of methods of preventing diseases caused by microorganisms, and the incrimination by Patrick Manson of vectors in the transmission of parasites. The great personalities of this period made discoveries in a number of fields, and their findings and ideas fed off one another [1].

The discovery in 1953 of the double helix[2], the twisted-ladder structure of deoxyribonucleic acid (DNA), by James Watson and Francis Crick marked a milestone in the history of science and gave rise to modern molecular biology, which is largely concerned with understanding how genes control the chemical processes within cells. In short order, their discovery yielded ground-breaking insights into the genetic code and protein synthesis. During the 1970s and 1980s, it helped to produce new and powerful scientific techniques, specifically recombinant DNA research, genetic engineering, rapid gene sequencing, and monoclonal antibodies. Major current advances in science, namely genetic fingerprinting and modern forensics, the mapping of the human genome, and the promise, yet unfulfilled, of gene therapy, all have their origins in Watson and Crick's inspired work. Also, the mapping of pathogens genomes [3-5], along with new molecular technologies has improved researches by giving rise to new methodologies that have accelerated the understanding of parasite-host interactions. For the purpose of this review, we will discuss the benefits of molecular cell biology in the understanding of *Trypanosoma cruzi* - host cell interactions.

T. cruzi proteins related to host cell invasion

Since the pioneering studies by Herta Meyer and co-workers that initiated *in vitro* studies of *T. cruzi* development within cultured cells [6] followed by the detailed description provided by James Dvorak and Thomas Hyde on how cells become infected by *T. cruzi* trypomastigotes [7], numerous studies have been performed in order to understand the molecular mechanisms that underlie the rather complex process of parasite entry into mammalian host cells. Until the 80's, most of studies developed to characterize *T. cruzi* antigens that interact with host cells were performed by means of labeling parasites with radioactive isotopes, immunopreciptation with polyclonal antibodies, identification with monoclonal antibodies, treatment of parasite lysates with the GPI-specific phospholipase C of *Trypanosoma brucei* and pulse-chase experiments with [^{35}S]methionine-labeled parasites [8-10].

Whole-genome sequencing of *T. cruzi* was published only in 2005 [3]. However, before this progress, authors had already cloned and sequenced a cDNA for a metacyclic trypomastigote- specific glycoprotein with a molecular mass of 90 kDa, termed MTS-gp90. The insert of the MTS-gp90 cDNA clone strongly hybridized with a single 3.0-kb mRNA of metacyclic forms, whereas the hybridization signal with epimastigote mRNA was weak and those with RNAs from other developmental stages were negative, indicating that transcription of the MTS-gp90 gene is developmentally regulated. A series of experiments showed that the MTS-gp90 gene is present in multiple copies in the *T. cruzi* genome, arranged in a nontandem manner, and that there are at least 40 copies of the gene per haploid genome. Sequence analysis of recombinant MTS-gp90 revealed 40 to 60% identity at the amino acid level with members of a family of mammalian stage-specific, 85-kDa surface antigens of *T. cruzi*. However, there are considerable differences in the amino acid compositions outside the homology region [11]. By using antisense oligonucleotides complementary to a region of the gp90 gene implicated in host cell adhesion, authors investigated whether the selective inhibition of gp90 synthesis affected the capacity of metacyclic forms to enter target cells. Parasites were incubated for 24 h with 20 µM PS1, a

phosphorothioate oligonucleotide based on a sequence of the gp90 coding strand; PS2, the antisense counterpart of PS1; or PO2, the unmodified version of PS2 containing phosphodiester linkages. PS2 but not PS1 or PO2 inhibited the expression of gp90. Inhibition by PS2 was dose dependent. Northern blot analysis revealed that steady-state gp90 mRNA levels were diminished in PS2-treated parasites compared to untreated controls. Expression of gp90 was also inhibited by other phosphorothioate oligonucleotides targeted to the gp90 gene (PS4, PS5, PS6 and PS7) but not by PS3, with the same base composition as PS2 but a mismatched sequence. Parasites treated with PS2, PS4 or PS5 entered HeLa cells in significantly higher numbers than untreated controls, whereas the invasive capacity of PS1- and PS3-treated parasites was unchanged, confirming the inverse association between infectivity and gp90 expression [12].

Furthermore, the same group have cloned and sequenced a cDNA clone coding for a metacyclic trypomastigote-specific surface glycoprotein with a molecular mass of 82 kDa (MTS-gp82). The insert of the MTS-gp82 cDNA clone strongly hybridized with a single 2.2-kb metacyclic trypomastigote mRNA, suggesting that the steady-state levels of mRNAs for MTS-gp82 are developmentally regulated. MTS-gp82 is encoded by a multigene family whose members are distributed in several chromosomes. Sequence analysis revealed 40-56% identity at amino acid level between MTS-gp82 and members of *T. cruzi* gp85/sialidase family (TSA-1, Tt34c1, SA85-1.1). MTS-gp82 showed several amino acid motifs that are characteristic of gp85/sialidase family, such as the Asp box (SxDxGxTW), the subterminal (VTVxNVFLYNR) motif and the putative GPI-anchor sequence. On the basis of its structural features, the MTS-gp82 gene could be included in the *T. cruzi* gp85/sialidase family, but constituting a distinct group which is preferentially expressed in metacyclic trypomastigotes [13]. Recombinant proteins and synthetic peptides representing various sequences of gp82, a surface glycoprotein of *T. cruzi* metacyclic trypomastigotes implicated in mammalian cell invasion, were used aiming at the identification of the domain(s) of this molecule required for interaction with target cells. Invasion of cultured HeLa cells by metacyclic trypomastigotes was inhibited by about 80% in the presence of native gp82 or the corresponding recombinant construct J18. Inhibition by recombinant proteins J18a and J18b, containing respectively the N-terminal and the C-terminal portions of gp82, was on the order of 30% and 65%. As compared to J18b (amino acids 224-516), the truncated gp82 fragments J18b1 (amino acids 303-516) and J18b2 (amino acids 357-516) displayed lower inhibitory effect. Compatible with these observations, they found that the recombinant protein J18b, but not J18a or J18b2, binds to HeLa cells in a dose-dependent and saturable fashion. Experiments with ten overlapping synthetic peptides, representing the gp82 portion spanning amino acids 224-333, showed that peptides 4 (amino acids 254-273) and 8 (amino acids 294-313) have significant inhibitory activity on HeLa cell invasion by metacyclic forms. All these results indicate that the portion of gp82 required for mammalian cell attachment and invasion is located in the central domain of the molecule [14]. Additional studies showed that the recombinant protein Del-4/8, lacking 65 amino acids of gp82 central domain (at positions 257 to 321), was virtually devoid of cell-binding activity and lacked the ability to inhibit parasite invasion, in contrast to J18. Constructs with shorter deletions, i.e., Del-4 (deleted from 257 to 271) and Del-8 (deleted from 293 to 321), bound to target cells to a significantly lesser degree than did J18. The sites deleted in recombinant proteins Del-4 and Del-8 contained acidic amino acids critical for cell adhesion. Of a set of synthetic peptides spanning the gp82 central domain, a 22-mer hybrid peptide, p4/8, formed by two noncontiguous sequences (at positions 257 to 273 and 302 to 306) and containing the four acidic residues, competed with the binding of J18 protein to target cells and significantly inhibited (~60%) the penetration of parasites. This peptide, generated by the juxtaposition of sequences that are separated by a hydrophobic stretch in the linear molecule, appears to be mimicking a conformation-dependent cell-binding site of gp82 [15].

More recently, this group used J18, a construct lacking the gp82 central domain (J18*), and 20-mer synthetic peptides based on the gp82 central

domain for gastric mucin binding and HeLa cell invasion assays, or for *in vivo* experiments. Metacyclic trypomastigotes and J18 bound to gastric mucin whereas J18* failed to bind. Parasite or J18 binding to submaxillary mucin was negligible. HeLa cell invasion by metacyclic forms was not affected by gastric mucin but was inhibited in the presence of submaxillary mucin. Of peptides tested for inhibition of J18 binding to gastric mucin, the inhibitory peptide p7 markedly reduced parasite invasion of HeLa cells in the presence of gastric mucin. Peptide p7*, with the same composition as p7 but with a scrambled sequence, had no effect. Mice fed with peptide p7 before oral infection with metacyclic forms developed lower parasitemias than mice fed with peptide p7*. Thus, the results indicated that selective binding of gp82 to gastric mucin may direct *T. cruzi* metacyclic trypomastigotes to stomach mucosal epithelium in oral infection [16].

cDNA clones encoding a Serine-, Alanine-, and Proline-rich protein (SAP) of T. cruzi metacyclic trypomastigotes were isolated and characterized . The deduced peptides translated from these clones were characterised by a high content of residues of alanine, proline, serine, glycine, valine, and threonine distributed in several repeats. The repeats are partially homologous to the serine-, alanine-, and proline-containing motifs of *Leishmania major* and *Leishmania mexicana* proteophospho-glycans. Genes coding for SAP are part of a polymorphic family whose members are linked to members of gp85/sialidase and mucin-like gene families. This is consistent with the hypothesis that this genetic organisation could be a means by which *T. cruzi* co-ordinates the expression of major surface proteins [17]. Aided by the availability of the completed genome sequence of *T. cruzi*, we have now identified 39 full-length sequences of SAP, six pseudogenes and four partial genes. SAPs share a central domain of about 55 amino acids and can be divided into four groups based on their amino (N)- and carboxy (C)-terminal sequences. Some SAPs have conserved N- and C-terminal domains encoding a signal peptide and a glycosylphosphatidylinositol anchor addition site, respectively. The recombinant SAP exhibited an adhesive capacity toward mammalian cells, where binding was dose dependent and saturable, indicating a possible ligand-receptor interaction. SAP triggered the host cell Ca^{2+} response required for parasite internalization. A cell invasion assay performed in the presence of SAP showed inhibition of internalization of the metacyclic forms of the CL strain. These results showed that SAP is involved in the invasion of mammalian cells by metacyclic trypomastigotes [18].

The 80 kDa POP Tc80 (prolyl oligopeptidase of *T. cruzi*) is involved in the process of cell invasion, since specific inhibitors block parasite entry into non-phagocytic mammalian host cells. In contrast with other POPs, POP Tc80 is capable of hydrolysing large substrates, such as fibronectin and native collagen [19]. In order to perform a molecular characterization of this enzyme, the group of researchers presented the cloning of the *POPTc80* gene, whose deduced amino acid sequence shares considerable identity with other members of the POP family, mainly within its C-terminal portion that forms the catalytic domain. Southern-blot analysis indicated that *POPTc80* is present as a single copy in the genome of the parasite. These results are consistent with mapping of *POPTc80* to a single chromosome. The active recombinant protein (rPOP Tc80) displayed kinetic properties comparable with those of the native enzyme. Infective parasites treated with these specific POP Tc80 inhibitors attached to the surface of mammalian host cells, but were incapable of infecting them. Structural modelling of POP Tc80, based on the crystallized porcine POP, suggested that POP Tc80 is composed of an α/β-hydrolase domain containing the catalytic triad Ser^{548}–Asp^{631}–His^{667} and a seven-bladed β-propeller non-catalytic domain. Docking analysis suggests that triple-helical collagen access to the catalytic site of POP Tc80 occurs in the vicinity of the interface between the two domains [20].

Another elegant study was the cloning and characterization of the first cell surface casein kinase II (CKII) substrate (Tc-1) of *T. cruzi*. Analysis of the gene sequence revealed a 1,653-bp open reading frame coding for 550 amino acid residues. Northern blot analysis showed a 4.5-kb transcript that is expressed in invasive trypomastigotes but not in noninvasive epimastigote forms of *T. cruzi*. Southern blot analysis indicates that Tc-1

is a single-copy gene. At the amino acid level, Tc-1 displayed 95% and 99% identity to two hypothetical proteins recently reported by the *T. cruzi* genome project. Analysis of the translated amino acid sequence indicates that the Tc-1 gene has a putative transmembrane domain with multiple cytoplasmic and extracellular CKII phosphosites. Exogenous human CKII was able to phosphorylate serine residues on both recombinant Tc-1 and Tc-1 of intact trypomastigotes. This phosphorylation was inhibited by the CKII inhibitors heparin and 4,5,6,7,-tetrabromo-2-azabenzimidazole. Antibodies to Tc-1 effectively blocked trypomastigote invasion of host cells and consequently reduced parasite load [21].

Trans-sialidase (TS), a parasite enzyme that is used to obtain sialic acid from host glycoconjugates, has been implicated in cell invasion and parasitophorous vacuole (PV) exit. In this context, a recent study showed that trypomastigotes derived from infected mammalian cells express and release 20 times more TS activity than axenic metacyclic trypomastigotes, which correspond to the infective forms derived from the insect vector. Both forms have the same capacity to invade mammalian cells, but cell derived trypomastigotes exit earlier from the vacuole. To test whether high TS expression is responsible for this increased exit from the PV, trypomastigote TS was expressed on the surface of metacyclic forms. Transfected and non-transfected metacyclics attached to and invaded HeLa or CHO cells equally. In contrast, metacyclics expressing TS on the surface escaped earlier from the vacuole than non-transfected metacyclics, or metacyclics expressing TS in their cytoplasm. In addition, trypomastigotes and metacyclic forms expressing TS differentiated earlier into amastigotes. These results indicate that the increased expression of TS in cell-derived trypomastigotes is responsible for the earlier exit from the PV to the cytoplasm and their subsequent differentiation into amastigotes [22].

For the first time in the literature, our group characterized a protein involved in amastigotes cell invasion besides its importance during metacyclic trypomastigotes cell entry. First, we screened *T. cruzi* genomic database in order to find hypothetical proteins that showed high probability of being secreted or membrane anchored and thus, likely to be involved in host-cell invasion. We have cloned and characterized this novel *T. cruzi* protein (P21) that is ubiquitous, secreted, and its recombinant form (P21-His_6) adhered to mammalian cells in dose-dependent manner and upregulated a phagocytosis-like activity [23]. We are currently addressing the pro-phagocytic acitivity of P21-His_6, the mammalian cell surface receptor and the signaling pathway triggered by the recombinant protein. Our results show that P21-His_6 triggers unspecific phagocytosis (inducing macrophage phagocytosis of different intracellular protozoa and zimosan) by binding to CXCR4 receptor, which leads to a cascade of intracellular signaling dependent on PI3-kinase that in turn promote actin polymerization. Also, the recombinant protein inhibits CXCR4 tropic HIV integration into host genome (data not published).

Recently, authors have reported the purification and biochemical characterization of TcPTP1, a protein tyrosine phosphatase from *T. cruzi*. The group demonstrated inhibition of the enzyme by the PTP1b inhibitor BZ3, which on its turn was able to accelerate the differentiation of epimastigotes into metacyclic forms of *T. cruzi* induced by nutritional stress. Additionally, this compound was able to inhibit by 50% the infectivity of *T. cruzi* trypomastigotes [24].

Among the molecular factors that contribute to virulence is a large multigene family of proteins known as gp85/trans-sialidase, which participates in cell attachment and invasion. But whether these proteins also contribute to tissue homing had not yet been investigated. By using a well-designed approach, Bacteriophage expressing an important peptide motif (denominated FLY) common to all gp85/trans-sialidase proteins was used as a surrogate to investigate the interaction of this motif with the endothelium compartment. For that purpose phage particles were incubated with endothelial cells obtained from different organs or injected into mice intravenously and the number of phage particles bound to cells or tissues was determined. Binding of phages to intermediate filament proteins has also been studied. These results indicated that FLY interacts with the endothelium in an organ-dependent manner with significantly higher avidity for the heart vasculature.

Phage display results also show that FLY interaction with intermediate filament proteins is not limited to cytokeratin 18, which may explain the wide variety of cells infected by the parasite. This is the first time that members of the intermediate filaments in general, constituted by a large group of ubiquitously expressed proteins, have been implicated in *T. cruzi* cell invasion and tissue homing [25].

A new scenario for *T. cruzi* protein discovery was opened by the fascinating study where 1161 open-reading frame expressed sequence tags (ORESTES) from the mammalian stages of the VL10 human strain were generated. Sequence clustering resulted in 435 clusters, consisting of 339 singletons and 96 contigs. Significant matches to the *T. cruzi* predicted gene database were found for ~94% contigs and ~69% singletons. These included genes encoding surface proteins, known to be intensely expressed in the parasite mammalian stages and implicated in host cell invasion and/or immune evasion mechanisms. Among 151 contigs and singletons with similarity to predicted hypothetical protein-coding genes and conserved hypothetical protein-coding genes, 83% showed no match with *T. cruzi* EST and/or proteome databases. These ORESTES are the first experimental evidence that the corresponding genes are in fact transcribed. Sequences with no significant match were searched against several *T. cruzi* and National Center for Biotechnology Information non-redundant sequence databases. The ORESTES analysis indicated that 124 predicted conserved hypothetical protein-coding genes and 27 predicted hypothetical protein-coding genes annotated in the CL Brener genome are transcribed in the VL10 mammalian stages. Six ORESTES annotated as hypothetical protein-coding genes showing no match to EST and/or proteome databases were confirmed by Northern blot in VL10. The generation of this set of ORESTES complements the *T. cruzi* genome annotation and suggests new stage-regulated genes encoding hypothetical proteins [26].

Host cell receptors for *T. cruzi* invasion

T. cruzi gp83 ligand, a cell surface trans-sialidase-like molecule that the parasite uses to attach to host cells, increases the level of laminin gamma-1 transcript and its expression in mammalian cells, leading to an increase in cellular infection. Stable RNA interference (RNAi) with host cell laminin gamma-1 knocks down the levels of laminin gamma-1 transcript and protein expression in mammalian cells, causing a dramatic reduction in cellular infection by *T. cruzi*. Thus, host laminin gamma-1, which is regulated by the parasite, plays a crucial role in the early process of infection. This is the first report showing that knocking down the expression of a human gene by RNAi inhibits the infection of an intracellular parasite [27].

Human galectin-3 specifically binds to *T. cruzi* trypomastigote surface proteins to enhance trypomastigote adhesion to the extracellular matrix component laminin [28]. Also, the expression of galectin-3 is required for and mediates *T. cruzi* adhesion to human coronary artery smooth muscle cells [29]. Our group have addressed if galectin-3 would have any impact during amastigote cell invasion. Our results showed that recombinant galectin-3 did not affect *T. cruzi* amastigote invasion of fibroblasts; however, endogenous galectin-3 was found to play an important role during amastigote intracellular trafficking. Galectin-3 was recruited to amastigote PV at different time points after parasite-host cell interaction and accumulated near the lysed PV. To determine if the appearance of galectin-3-enriched arrangements coincided with amastigote escape from the PV, SKBR3, SKBR3 GFP-galectin-3, and SKBR3 GFP-galectin-3 R186S mutant cells were used in invasion kinetics assays (15 min-48 h) and stained with an anti-Lamp-2 (lysosomal membrane glycoprotein-2) antibody for indirect immuno-fluorescence. The presence of galectin-3-containing structures at 6 h post infection occurred mainly in the vicinity of amastigotes that were not Lamp-2 positive. Thus, galectin-3 might be a marker for *T. cruzi* vacuole lysis. In SKBR3 GFP-galectin-3 R186S mutant cells the galectin-3-enriched arrangements were not visualized, showing that this phenotype depends on the lectin activity (data not published).

Authors have addressed the hypothesis that *T. cruzi* pathogenic outcome is influenced by functional interplay between endothelin-1 (ET-1) and bradykinin (BK) receptors. Intravital microscopy

was used to determine whether ETR/B(2) R drives the accumulation of rhodamine-labeled leukocytes in the hamster cheek pouch (HCP). Inflammatory edema was measured in the infected BALB/c paw. Parasite invasion was assessed in CHO overexpressing ETRs, mouse cardiomyocytes, endothelium (HUVECs) or smooth muscle cells (HSMCs), in the presence/absence of antagonists of B(2) R (HOE-140), ET(A) R (BQ-123) and ET(B) R (BQ-788); specific IgG antibodies to each GPCRs; cholesterol or calcium-depleting drugs. RNA interference (ET(A) R or ET(B) R genes) in parasite infectivity was investigated in HSMCs. BQ-123, BQ-788 and HOE-140 reduced leukocyte accumulation in HCP topically exposed to trypomastigotes and blocked edematogenic inflammation in infected mice. Acting synergistically, ET(A) R and ET(B) R antagonists reduced parasite invasion of HSMCs to the same extent as HOE-140. Exogenous ET-1 potentiated *T. cruzi* uptake by HSMCs via ETRs/B(2) R whereas RNA interference of ET(A) R and ET(B) R genes conversely reduced parasite internalization. ETRs/B(2) R-driven infection in HSMCs was reduced in HSMC pretreated with MβCD, a cholesterol depleting drug, or in thapsigargin or verapamil treated target cells. Their findings suggest that plasma leakage, a neutrophil-driven inflammatory response evoked by trypomastigotes via the kinin/endothelin pathways, may offer a window of opportunity for enhanced parasitism of cardiovascular cells [30].

Using a remarkable approach, authors showed that a neuronal cell line (PC12-NNR5) relatively resistant to *T. cruzi* became highly susceptible to infection when overexpressing human neurotrophin receptor TrkC but not human TrkB. Furthermore, trkC transfection conferred a 3.0-fold intracellular growth advantage. Sialylation-deficient Chinese hamster ovarian (CHO) epithelial cells Lec1 and Lec2 also became much more permissive to *T. cruzi* after transfection with trkC. Additionally, neurotrophin-3 (NT-3) specifically blocked *T. cruzi* infection of the TrkC-NNR5 transfectants and of naturally permissive TrkC-bearing Schwann cells and astrocytes, as did recombinant parasite-derived neurotrophic factor (PDNF). Two specific inhibitors of Trk autophosphorylation (K252a and AG879) and inhibitors of Trk-induced MAPK/Erk (U0126) and Akt kinase (LY294002) signaling, but not an inhibitor of insulin-like growth factor-1 receptor, abrogated TrkC-mediated cell invasion. Antibody to TrkC blocked *T. cruzi* infection of the TrkC-NNR5 transfectants and of cells that naturally express TrkC. The TrkC antibody also significantly and specifically reduced cutaneous infection in a mouse model of acute Chagas' disease. TrkC is ubiquitously expressed in the peripheral and central nervous systems, and in non-neural cells infected by *T. cruzi*, including cardiac and gastrointestinal muscle cells. Thus, TrkC is implicated as a functional PDNF receptor in cell entry, independently of sialic acid recognition, mediating broad *T. cruzi* infection both *in vitro* and *in vivo* [31].

In order to explore the role of the Low Density Lipoprotein receptor (LDLr) in *T. cruzi* invasion, authors evaluated LDLr parasite interactions using immunoblot and immunofluorescence (IFA) techniques. These experiments demonstrated that *T. cruzi* infection increases LDLr levels in infected host cells, inhibition or disruption of LDLr reduces parasite load in infected cells, *T. cruzi* directly binds recombinant LDLr, and LDLr-dependent *T. cruzi* invasion requires PIP2/3. qPCR analysis demonstrated a massive increase in LDLr mRNA (8000 fold) in the heart of *T. cruzi* infected mice, which is observed as early as 15 days after infection. IFA shows a co-localization of both LDL and LDLr with parasites in infected heart [32].

An innovative technique, SELEX technique (systematic evolution of ligands by exponential enrichment) was used to evolve nuclease-resistant RNA ligands (aptamer) that bind with affinities of 40-400 nm to parasite receptors for the host cell matrix molecules laminin, fibronectin, thrombospondin, and heparan sulfate. After eight consecutive rounds of *in vitro* selection four classes of RNA aptamers based on structural similarities were isolated and sequenced. All members of each class shared a common sequence motif and competed with the respective host cell matrix molecule that was used for displacement during the selection procedure. RNA pools following seven and eight selection rounds as well as individual aptamers sharing consensus motifs were active in inhibiting invasion of LLC-MK(2) monkey kidney cells by *T. cruzi in vitro* [33].

Lysosomes and PI-3 kinase

Among the paradigmatic studies that laid new insights into the invasion mechanism is the description by the group of Norma Andrews that calcium-dependent lysosomal recruitment takes place during trypomastigote invasion [34, 35]. According to this model, trypomastogotes engage signaling processes that culminate with the formation of parasitophorous vacuole [36, 37].

Other evidences on the participation of components of the early endocytic traffic such as dynamin and Rab5 have indicated that the lysosomal process might be more elaborate and downstream of earlier events [38]. Using a more quantitative approach and for the first time trasfected cell with plasmid encoding AKT and PLC PH domains to identify the role of phosphatidyl-inositol 3-kinase (PI3-K) on the lysosomal pathway, Woolsey *et al*. (2003) [39] were able to firmly confirm previous observations by Wilkowsky *et al*. (2001) [40] that this cellular key component could be involved in a lysosome-independent *T. cruzi* internalization pathway by trypomastigotes. Trypomastigotes that use this route mobilize phosphorylated inositides during the formation of the PV that then matures to become enriched in lysosomal marker Lamp-1. One important input of this work was that for the first time the relative contributions of each mode of entry, namely PI3-K (50%), lysosome (20%), and endosomal route (20%) were estimated [39].

In a brilliant approach Norma Andrews group recently showed that host cell entry by *T. cruzi* mimics a process of plasma membrane injury and repair that involves Ca(2+)-dependent exocytosis of lysosomes, delivery of acid sphingomyelinase (ASM) to the outer leaflet of the plasma membrane, and a rapid form of endocytosis that internalizes membrane lesions [41].

Also, it was recently observed that vacuole containing *T. cruzi* is decorated by the host cell autophagic protein LC3. In addition, the absence of Atg5 or the reduced expression of Beclin 1, two proteins required at the initial steps of autophagosome formation, limited parasite entry and reduced the association between PV and the classical lysosomal membrane glycoprotein-1 (Lamp-1). These results indicate that mammalian autophagy is a key process that favors the colonization of *T. cruzi* in the host cell [42].

A pioneer study on the invasion of *T. cruzi* amastigotes in single Lamp-1 or Lamp-2 knockouts, respectively, or in two independent Lamp-1/2 double-knockout cell lines showed that amastigotes were higher infective in Lamp-2 knockouts. Similarly, amastigotes invasion rate was higher for one of the double knockout clones but not for the other. Higher lysosomal exocytosis correlated with a higher invasion rate and early lysosomal marker acquisition. These findings suggest that lysosomal exocytosis is important to amastigote cell invasion, as well as for trypomastigotes [43].

The intriguing role of lysosome during trypomastigote cell invasion and intracellular traffic raised the question to define if Lamp would have any specific role during *T. cruzi* internalization. It was showed that increased expression of the lysosomal membrane glycoprotein Lamp-1 at the cell surface renders CHO cells more susceptible to trypomastigote invasion in a microtubule-dependent fashion. Mutation of critical residues in the lysosome-targeting motif of Lamp-1 abolished the enhancement of *T. cruzi* invasion. This suggests that interactions dependent on Lamp-1 cytoplasmic tail motifs, and not the surface-exposed luminal domain, modulate *T. cruzi* entry. Measurements of Ca^{2+}-triggered exocytosis of lysosomes in these cell lines revealed an enhancement of beta-hexosaminidase release in cells expressing wild-type Lamp-1 on the plasma membrane; this effect was not observed in cell lines transfected with Lamp-1 cytoplasmic tail mutants [44].

Host gene network expression during *T. cruzi* infection

To understand the genetic architectures of the early invasion process of *T. cruzi*, gene transcription microarray analysis was conducted, followed by gene network construction of the host cell response in primary human coronary artery smooth muscle (HCASM) cells infected with *T. cruzi* or exposed to *T. cruzi* gp83. Using seven RT-PCR verified up-regulated genes (FOSB, ATF5, INPP1, CCND2, THBS1, LAMC1, and APLP2) as the seed for network construction, authors built an interaction network of the early

T. cruzi infection process containing 165 genes, connected by 598 biological interactions. This interactome network is centered on the BCL6 gene as a hub. Silencing the expression of two seed genes (THBS1 and LAMC1) by RNAi reduced *T. cruzi* infection. Overall, their results elucidated the significant and complex process involved in *T. cruzi* infection of HCASM cells at the transcriptome level. This is the first elucidation into the interactome network in human cells caused by *T. cruzi* and its gp83 ligand [45].

To further identify human host factors required for *T. cruzi* infection, authors performed a genome-wide RNAi screen using cellular microarrays of a printed siRNA library that spanned the whole human genome. The screening was reproduced 6 times and a customized algorithm was used to select as hits those genes whose silencing visually impaired parasite infection. The 162 strongest hits were subjected to a secondary screening and subsequently validated in two different cell lines. Among the fourteen hits confirmed, they recognized some cellular membrane proteins that might function as cell receptors for parasite entry and others that may be related to calcium release triggered by parasites during cell invasion. In addition, two of the hits are related to the TGF-beta signaling pathway, whose inhibition is already known to diminish levels of *T. cruzi* infection. This study represented a significant step toward unveiling the key molecular requirements for host cell invasion and revealing new potential targets for antiparasitic therapy [46].

Using a murine microarray to investigate the cellular response caused by invasion of primary murine cardiomyocytes by *T. cruzi* trypomastigotes, authors identified 353 murine genes that were differentially expressed during the early stages of invasion and infection of these cells. Genes associated with the immune response, inflammation, cytoskeleton organization, cell-cell and cell-matrix interactions, apoptosis, cell cycle, and oxidative stress are among those affected during the infection. Their data indicate that *T. cruzi* induces broad modulations of the host cell machinery in ways that provide insight into how the parasite survives, replicates, and persists in the infected host and ultimately defines the clinical outcome of the infection [47]. Also, an extensive microarray analysis of hearts from a mouse model of *T. cruzi* infection identified significant alterations in expression of approximately 12% of the sampled genes. Extensive up-regulations were associated with immune-inflammatory responses (chemokines, adhesion molecules, cathepsins, and major histo-compatibility complex molecules) and fibrosis (extracellular matrix components, lysyl oxidase, and tissue inhibitor of metalloproteinase 1). Their results indicate potentially relevant factors involved in the pathogenesis of the disease that may provide new therapeutic targets in chronic Chagas disease [48].

CONCLUDING REMARKS

Modern Molecular Cell Biology played a significant role in determining the function of gp82 and gp90 during metacyclic trypomastigote cell invasion, also collaborating to uncover the epitopes involved in cell adhesion. It is worth mentioning, the number of new proteins from the parasite associated to cell invasion that were and are being characterized by also using molecular biology tools. Indeed, now we have a complete database to search for more components of the parasite involved in different aspects of its biology, Moreover, these advances will contribute significantly in the establishment of new therapeutic chemotherapy. The use of cells transfected with plasmids encoding the target gene in fusion with fluorescent proteins made possible the observation that earlier events occur before parasite fusion with lysosomes. Moreover, the amazing techniques are gradually making possible to get a panoramic host response to the parasite infection. Probably without the technology behind Watson and Crick discovery most of the knowledge we have today would not have been possible. All these inspiring results seem only to be the beginning of a broad advance science will face in the post-genomic era with the transcriptome, metabolome, diverse high-throughput analysis and systems biology that will provide even more information in a network way, which are premise tools for the near future science.

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