Mixed signals – how *Trypanosoma cruzi* exploits host-cell communication and signaling to establish infection

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**ABSTRACT**

Chagas disease (American trypanosomiasis) is a ‘neglected’ pathology that affects millions of people worldwide, mainly in Latin America. *Trypanosoma cruzi*, the causative agent, is an obligate intracellular parasite with a complex and diverse biology that infects several mammalian species, including humans. Because of genetic variability among strains and the presence of four biochemically and morphologically distinct parasite forms, the outcome of *T. cruzi* infection varies considerably depending on host cell type and parasite strain. During the initial contact, cellular communication is established by host-recognition-mediated responses, followed by parasite adherence and penetration. For this purpose, *T. cruzi* expresses a variety of proteins that modify the host cell, enabling it to safely reach the cytoplasm. After entry into the host cell, *T. cruzi* forms a transitory structure termed ‘parasitophorous vacuole’ (PV), followed by its cytoplasmic replication and differentiation after PV rupture, and subsequent invasion of other cells. The success of infection, maintenance and survival inside host cells is facilitated by the ability of *T. cruzi* to subvert various host signaling mechanisms. We focus in this Review on the various mechanisms that induce host cytoskeletal rearrangements, activation of autophagy-related proteins and crosstalk among major immune response regulators, as well as recent studies on the JAK-STAT pathway.

**KEY WORDS:** *Trypanosoma cruzi*, Cellular infection, Host cell signaling, Protein kinases, Immune response

**Introduction**

*Trypanosoma cruzi* is an obligatory intracellular pathogenic parasite with a complex heteroxenic life cycle that includes invasion of a mammalian host (Chagas, 1909). For the invasion process to be successful, the parasite needs to establish interaction with the host and evade its defense mechanisms. *T. cruzi* forms an intracellular structure termed the ‘parasitophorous vacuole’ (PV), which plays a crucial role in evading host immune responses and in survival following internalization (Batista et al., 2020a). *T. cruzi* is capable of invading a wide variety of phagocytic and non-phagocytic cell types (Behbehani, 1973; Tardieux et al., 1992; Woolsey et al., 2003; Mortara et al., 2005; Fernandes et al., 2011). This ability depends on a genetic plasticity that enables the parasite to adapt effectively to various conditions that arise during its interaction with the host (Vago et al., 2000; Reis-Cunha et al., 2015, 2018), making it difficult to clarify the physiological processes involved in parasite-host interactions. Another challenge for researchers is the diversity of strains [discrete typing units (DTUs), which are genetic subdivisions within *T. cruzi*] studied (see Glossary), which differ in their infectivity (de Souza et al., 2010).

This variety of mechanisms is associated with its morpho-evolutionary forms, which have biochemical differences, termed epimastigote (EPI), intracellular amastigote (IA), extracellular amastigote (EA), metacyclic trypomastigote (MT), and bloodstream trypomastigote (BT or TCT) (see Glossary). EPI and IA are replicative forms of the parasite found in the insect vector and mammalian host, respectively, while MT is the invasive form arising from the insect. TCT is the circulating form in the mammalian host that is capable of invading cells. EA is another invading form in the mammalian host (De Souza, 1984; Mortara, 1991). The variety of parasite characteristics (i.e. biochemical and morphological differences), in combination with the various types of cells and conditions used in different studies, have led to a large volume of experimental data, some of which seem contradictory.

Regardless of these difficulties, studies of *T. cruzi* are important because it is the etiological agent of Chagas disease (American trypanosomiasis). According to the World Health Organization (https://www.who.int/health-topics/chagas-disease), 6 to 7 million people worldwide have this disease, and another 75 million are potentially at risk of infection. Chagas disease is endemic in South America, and for a long time affected mainly rural regions. It has received increasing attention in recent decades as it has appeared in numerous urban areas around the world as a result of activities associated with human migration (Zingales et al., 2012). A worldwide ‘Chagas disease day’ (April 14) was declared during the 72nd World Health Assembly (2019, in Geneva), reflecting the importance of the disease.

Studies of the biology of the disease are focused mainly on finding effective treatments. Chagas disease, in its chronic phase, presents serious problems with heart function or (in a small percentage of patients) the digestive system, and is sometimes life threatening (Zingales et al., 2012; Pérez-Molina and Molina, 2018). Investigations into parasite communication with its mammalian host during the invasion process have identified signaling pathways and protein complexes, as well as specific proteins, that could serve as pharmacological targets for the interruption or modification of the life cycle of the parasite or the infection process (for a review, see Fernandes and Andrews, 2012; Ferreira et al., 2012; Soares-Silva et al., 2016). The goal is to find a definitive cure for the disease, or at least treatments that are more effective than those currently available.

Pathogenic microorganisms, during evolution, have developed a variety of mechanisms for subverting important signal transduction pathways, including the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways (Bonfim-Melo et al., 2015; Wex et al., 2016; Mazza et al., 2017; Bahia et al., 2018; Batista et al., 2020b). Pathogens also secrete effector molecules that can modulate or alter gene expression and function, protein phosphorylation, the actin cytoskeleton and its regulators, or intracellular trafficking (Song et al., 2015; Ferreira et al., 2016;
**Glossary**

*Metacyclic trypomastigote (MT):* infective form derived from invertebrate host (insects; distal intestine), transmitted by blood meal lesion.

*Bloodstream trypomastigote (BT or TCT):* infective form released from mammalian infected cells.

*"In both MT and BT forms, the kinetoplast is in the basal body posterior region, which is also the location of the flagellar pocket from which the flagellum emerges (left image in illustration)."

*Epimastigote (EPI):* non-infective replicative form found in invertebrate hosts (insects; medium intestine). The kinetoplast is in the anterior part of the basal body, which is also the location of the flagellar pocket from which the flagellum emerges. The EPI form is quickly eliminated by the mammalian complement system (middle image in illustration).

*Intracellular amastigote (IA):* round-shaped cytoplasmic replicative form found in vertebrate hosts (mammals).

*Extracellular amastigote (EA):* infective round-shaped form released prematurely from infected cells (prior to differentiation into trypanastigotes), or from differentiation of BT into EA in blood.

*"In both IA and EA forms, the kinetoplast is located between the nucleus and flagellar pocket; it does not have a notable flagellum (interiorized in the flagellar pocket) (right-hand image in illustration)."

**Box 1. The Trypanosoma cruzi life cycle**

*T. cruzi* alternates between two hosts during its life cycle: a vertebrate (mammal) and an invertebrate (hematophagous insects of the family Reduviidae; genera *Triatoma*, *Rodnius* and *Panstrongylus*) (Brener, 1997; de Souza, 2002; Bern, 2015). Metacyclic trypomastigote (MT)-containing fecal material is eliminated during the insect’s blood meal and infects the vertebrate through scratching that brings the parasite to the meal-induced tissue lesion (Lima et al., 2010; Bern, 2015). In the vertebrate host, MTs are able to invade a wide range of nucleated cells and form a transitory structure termed ‘parasitophorous vacuole’ (PV) (Batista et al., 2020a). After a number of events, such as PV development (i.e. organelles membrane donation) and maturation, which is defined by the acidic characteristic of the lysosomes that fuse with the PV, the parasite disintegrates the PV upon production of the pore-forming TcTox protein (de Souza et al., 2010) and is released into the host cytoplasm, where it differentiates into replicative intracellular amastigote (IA) forms (de Souza, 2002; Fernandes and Andrews, 2012). IA generation leads to blood trypomastigote (BT or TCT) differentiation, followed by cell lysis. In the blood, BTs can either invade new cells to maintain the infection, or be transferred to new invertebrate hosts. In the latter case, BTs differentiate into epimastigote (EPI) replicative forms (middle intestine), which migrate to the distal intestine where they transform back into BT forms, ready to infect another vertebrate host (de Souza, 2002; Bern, 2015). Within the vertebrate host, BTs can differentiate into extracellular amastigote (EA) forms that can infect other cells, providing an alternative cycle of infection. EA can also be generated by early IA-containing cell lysis (Ferreira et al., 2012).

(Watanabe Costa et al., 2016; Lin et al., 2017; Meneghelli et al., 2020) in both phagocytic and non-phagocytic host cells (Stradal and Schelhaas, 2018).

We review here our current knowledge of the mechanisms used by *T. cruzi* to enter and survive in host cells and to maintain its life cycle (see Box 1 for a brief overview). These mechanisms include interactions with host surface proteins to facilitate entry, subversion of host autophagic processes and alterations of signaling pathways in order to impair host immune response.

**Actin integrity and parasite entry**

Intracellular pathogens have developed a variety of strategies to subvert the host cell actin cytoskeleton for their own benefit (for a review, see Stradal and Schelhaas, 2018). Depending on the *T. cruzi* strain and/or developmental form, disruption of host actin polymerization may either inhibit or promote invasiveness (Fig. 1A). Actin recruitment is essential for uptake of EA into mammalian host cells (for a review, see Ferreira et al., 2012; Bonfim-Melo et al., 2018) and disruption of actin filaments by cytochalasin D impairs EA entry. Our group elucidated an actin-mediated pathway for EA entry into HeLa cells (Bonfim-Melo et al., 2015). Cortactin is an F-actin-binding protein, and Src family kinase (SFK)-dephosphorylated cortactin facilitates actin polymerization through a complex of neural Wiskott–Aldrich syndrome family protein (N-WASP, encoded by *WASL*) and the actin-related protein 2/3 complex (N-WASP–Arp2/3), with protein kinase D1 (PKD1; also known as PRKD1) acting as an upstream regulator of cortactin (Eiseler et al., 2010). Interaction of EA with HeLa cells recruits cortactin and PKD1, in addition to actin (Bonfim-Melo et al., 2015), and EAs that enter cells colocalize at the invasion site with actin, cortactin and PKD1 (Fig. 1B). Accordingly, EA invasion is inhibited by depletion of cortactin and/or PKD1, demonstrating that these factors (in addition to actin) play essential roles in EA invasion (Bonfim-Melo et al., 2015). Other actin-regulating molecules have been implicated in EA invasion in recent years. For example, Rac1 and Cdc42 modulate EA-mediated actin recruitment and enhance EA invasiveness (see Fig. 2, labeled 1) (Bonfim-Melo et al., 2018). The ezrin, radixin and moesin proteins (collectively ERM proteins) link actin filaments to the plasma membrane and are present in a variety of actin-rich structures (Bonfim-Melo et al., 2018). Of these, ezrin and radixin have been shown to be essential for EA invasion into HeLa cells (Bonfim-Melo et al., 2018).

Moreover, some studies have shown that actin disruption promotes entry of TCT (Y strain, DTU TcII) (see Glossary) into and multiplication inside macrophages (de Araújo et al., 2016), of TCT G strain (DTU TcII) into B cells (dos Santos et al., 2020) (Fig. 1C,D), and invasion of MT of CL strain (DTU TcVI) into HeLa cells (Onofre et al., 2019). It is generally agreed that TCT actively invades cells, and that cortical actin filaments inhibit cell invasion, as their disruption enhances the invasion process in cell models (dos Santos et al., 2020).
Host autophagy, an essential pathway for *T. cruzi* invasion

The involvement of host autophagy in the *T. cruzi* invasion process has been the subject of numerous studies. Autophagy, the regulated degradation and recycling of surplus or dysfunctional cellular components, is a basic cellular process often observed in responses to intracellular stress (Parzych and Klionsky, 2014; McEwan, 2017). In brief, a double-membrane structure (autophagosome) is generated and then fuses with a lysosome to form an autolysosome, which helps maintain cellular homeostasis by degrading or recycling unneeded components, such as proteins and organelles. Maintaining appropriate autophagic flux depends on a series of proteins that coordinate the different stages of autolysosome formation. mTOR inhibition triggers autophagy initiation; the class III phosphoinositide 3-kinase (PI3K) complex (VPS34–Beclin1–VPS15; VPS34 and VPS15 are also known as PIK3C3 and PIK3R4, respectively, in mammals) is essential in the initial phase of formation of the double-membrane structure, and the Atg12–Atg5–Atg16L complex is involved in processing of microtubule-associated protein 1 light chain 3 family proteins (LC3 or MAP1LC3) from LC3-I (cytosolic) to LC3-II, the phosphatidylethanolamine (PE)-ligated membrane-bound form, during elongation of the initial autophagic structure (Kabeya et al., 2000; Wang et al., 2009; Yang and Klionsky, 2010; Murrow and Debnath, 2013; Ohsumi, 2014; Cao et al., 2019).
Autophagy also plays a role in immune system responses to intracellular pathogens, termed xenophagy (Sharma et al., 2018). Autophagy is necessarily tightly controlled, because excessive induction can lead to cell death, whereas insufficient autophagy can cause infectious disease. A number of intracellular pathogens have adapted to autophagy and are able to exploit it to their advantage, for instance to facilitate their internalization. Furthermore, T. cruzi appears to utilize some of the host autophagy processes to evade the host immune system as it travels to the cytoplasm to complete its life cycle (Box 1) (Duszenko et al., 2011; Mendes et al., 2020).

There are several reasons why exploiting the autophagic pathway is important for T. cruzi: (1) facilitating interactions with intracellular structures formed from host membranes, such as during PV formation (Reignault et al., 2019), (2) obtaining nutrients, and (3) the necessity of an acidic environment for breakdown of its transient PV by activation of TcTOX (a protein with membrane pore-forming activity at acidic pH), and its differentiation (see Box 1) (De Souza et al., 2010).

The relationship between T. cruzi infection and host autophagic pathway has been investigated using phagocytic and non-phagocytic cells, during infection with various parasite strains (e.g. CL-Brener, Y) and morphological forms (mainly trypomastigotes, because they are an infectious form), and employing various strategies, including treatment with autophagy-stimulating drugs (mainly rapamycin) or nutrient-deficient media (to induce starvation). Infection has also been studied in the context of impaired autophagy, for example, upon knockdown of essential autophagic genes (ATG5 or Beclin1), inhibition of autophagy-related proteins (e.g. wortmannin), as well as owing to the presence of phagocyte-specific markers (e.g. LC3) or lysosomal markers [e.g. LBPA (also known as PDCD6IP), Lamp-1 and cathepsin D]. The results obtained from these studies vary greatly, and some appear contradictory, presumably because of differing experimental conditions, and some of these findings are discussed below.

A study by Romano and collaborators on CHO cells expressing GFP–LC3 and infected with TCT (CL Brener and RA strains) revealed, for the first time, a transient interaction between the host autophagosome marker LC3 and the forming PV (Romano et al., 2009). This group also observed a movement of autophagosomes toward the site of parasite contact with the host cell membrane, and a wrapping of parasites in LC3-marked structures (with the

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**Fig. 2. T. cruzi invasion triggers autophagy and modification of the host cytoskeleton.** (1) Extracellular amastigote (EA) invasion requires the recruitment of cortactin (cort), protein kinase D1 (PKD1) and actin to the entry site. Rac1 and Cdc42 are involved in EA-mediated actin recruitment and thus promote effective invasion. SFK dephosphorylates cortactin, thereby inducing actin polymerization via the N-WASP–Arp2/3 complex, which facilitates parasite entry. (2) Under conditions that stimulate autophagy, T. cruzi invasion promotes the recruitment of autophagy structures to the parasite entry site. (3) PV formation involves autolysosomes as determined by co-localisation of autolysosomal markers (autophagosome markers indicated by blue circles and lysosome markers by red ones), which persists until PV breakdown. (4) T. cruzi cruzipain (black cross) enhances survival by upregulating Beclin 2 (Bcl-2) expression, which may be mediated by either the Akt-PI3K or MEK1-ERK1/2 pathway in cardiomyocytes.
autophagosomes participating in PV formation). This interaction was observed during invasion and initial infection (1 to 6 h) and persisted until parasites escape from the PV after fusion to lysosomes that provide the acid environment necessary for the rupture of PV (maturation) but did not affect other stages of infection (see Box 1). PV formation was affected upon use of PI3K inhibitors and knockout of proteins that play key roles in autophagosome formation, such as Atg5 and Beclin1, confirming the contribution of autophagosome membranes to the PV. Overall, induction of host autophagy resulted in a significant increase in the number of infected cells, suggesting that autophagy facilitates parasite entry into the cell. Comparable results were obtained for several different strains, indicating a common mechanism for T. cruzi invasion (Romano et al., 2009).

The same group later showed that induction of host autophagy also increased the amount of the lysosomal markers Lamp-1 and cathepsin D in PVs, indicating that they are derived from autolysosomes (Romano et al., 2012). During periods of starvation, they showed that autolysosomes were transported to sites of membrane where parasite invasion occurred and that this process depends on the level of host autophagy. On the basis of these findings, these authors proposed a model for the generation of new vesicles with autolysosomal features at the infection site that facilitate invasion (Fig. 2, labeled 1 and 2) (Romano et al., 2012).

In another study, infection by T. cruzi (Tulahuen strain) trypomastigotes were shown to induce autophagosome formation in the host (fibrosarcoma cell line HT1080), but their maturation to autolysosomes was inhibited, suggesting that parasite-induced autophagy was incomplete (Onizuka et al., 2017). Here, colocalization of LC3 and Atg16L was observed, indicating that the parasite was able to induce initial phagophore formation during the early stage of infection, but not formation of autolysosomes (Onizuka et al., 2017).

Studies based on in vitro infection of different types of host cells with various pathogen strains suggest that host autophagy is one of the main regulators of the T. cruzi invasion process, and is involved in PV maturation (Salassa and Romano, 2019). However, in vivo (mouse) studies might give rise to different results, considering that one purpose of autophagy is the elimination of pathogenic microorganisms. A recent study addressed this point by examining the autophagic response of peritoneal cells from heterozygous Beclin1-knockdown (Beclin1<sup>−/−</sup>) mice to TCT Y-GFP infection (Casassa et al., 2019). The mice displayed reduced autophagic response (as determined by a reduction in LC3 levels), higher parasitemia level and lower percentage of survival compared to control mice (Beclin1<sup>+/+)</sup>). Treatment of wild-type mice with the autophagic inhibitor (DFMO) had the same effects, indicating that autophagy helps to control acute T. cruzi infection in vivo, in contrast to in vitro observations. The authors concluded that macrophages (in this case peritoneal macrophages) are able to eliminate intracellular parasites during progression of infection, and that autophagy is involved in this process (Casassa et al., 2019).

Another study similarly found that T. cruzi induced the formation of both autophagosomes and of autolysosomes (amastigote-containing vesicles) in macrophages at 4 h after infection, suggesting that the entire autophagic cycle is induced by T. cruzi in macrophages during early stages of infection (Matteucci et al., 2019). A previous study by this group had shown that the NLR family pyrin domain containing 3 (NLRC3) inflammasome plays a role in controlling T. cruzi infection by inducing the production of nitric oxide (NO) through a caspase-1-mediated pathway (González-López et al., 2013). The NLRC3 inflammasome can also inhibit autophagy through cleavage of the signaling molecule Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF; also known as TICAM1) that is induced by caspase-1 activation. Autophagy also regulates activation of the inflammasome, thereby inhibiting host inflammatory response and reducing inflammatory tissue injury (Cao et al., 2019). In this case, autophagy acted as an autonomous effector mechanism against infection, independently of NO production. NLRC3 was evidently important for the process because induction or inhibition of autophagy in NLRC3<sup>−/−</sup> macrophages did not notably affect the anti-parasite response; that is, in the absence of NLRC3, autophagic flow was specifically blocked during infection. Thus, NLRC3 may be necessary for the induction of autophagy, as well as autolysosome assembly in response to infection. The authors concluded that autophagy is mediated by NLRC3 for control of T. cruzi infection. The role of NLRC3 in autolysosome formation may reflect a cellular response against the modulation of autophagy in macrophages that is mediated by T. cruzi (Matteucci et al., 2019).

The degree to which host autophagy is involved in T. cruzi infection varies depending on the type of host cell. In a recent in vitro study of macrophages and cardiac cells infected with T. cruzi, a significant reduction of infection (60%) was observed in macrophages that were subjected to starvation in the initial stages of infection (6 h) (Duque et al., 2020). In the starved cardiac cells, a 42% reduction of infection was observed, but only after 48 h. Similar results were obtained for the number of parasites per cell in both cell types. Induction of host autophagy by starvation may therefore negatively affect parasite internalization, as a negative modulation of infection was observed during autophagy induction in macrophages (Duque et al., 2020). In studies of long-term infection (after 6 h) during the differentiation phase to the amastigote form, neither infection rate nor number of parasites per cell were notably changed in macrophages, whereas both parameters were significantly reduced in cardiac cells, pointing to a response of macrophages specifically to early infection (Duque et al., 2020).

The relationship between host autophagy and the T. cruzi infection process is still a controversial topic. Several previous studies by various groups have demonstrated that certain factors must be considered during experimental design (e.g. cell type, parasite strain, in vitro versus in vivo conditions) because the combination of these factors can affect the obtained results. The studies reviewed here indicate that host autophagy plays a dual role in responses to T. cruzi infection, with it either promoting parasite internalization, by participating in PV formation, or promoting parasite elimination during the initial stages of infection as part of innate immune response to pathogens (xenophagy). The seemingly contradictory results thus may provide a clue to the different cellular mechanisms that are activated during parasite–host interactions under particular experimental conditions. Although the intrinsic mechanisms underlying these processes require further studies, it is clear that parasites have the capability of modulating host autophagy to promote their invasive activity.

The MAPK pathway – a brief introduction

Mitogen-activated protein kinases (MAPKs) are a family of highly conserved Ser/Thr kinases in eukaryotes, particularly mammals (Manning et al., 2002; Mody et al., 2009). Two subgroups of MAPKs can be distinguished – conventional (cMAPK) and atypical (aMAPK). cMAPKs include the well-established group of kinases discussed below: extracellular signal-regulated kinase 1 and 2 (ERK1/2; also known as MAPK3 and MAPK1, respectively), p38 MAPKS (α, β, γ and δ; MAPK14, MAPK11, MAPK12 and MAPK13, respectively), and c-Jun N-terminal kinases 1, 2 and 3 (JNK1, JNK2 and JNK3; MAPK8, MAPK9 and MAPK10, respectively) (Turjanski et al., 2007). Several external signaling
processes (e.g., those triggered by mitogens, cytokines, stress or growth factors) can lead to phosphorylation and consequent activation of MAPKs (Raman et al., 2007; Huang et al., 2009). The MAPK signaling cascade is a highly specialized pathway that integrates distinct signaling routes both upstream [e.g. Tyr kinase receptor, interleukin receptor, Toll-like receptors (TLRs), G-coupled proteins and surface adhesion proteins] and downstream, through their transduction factors and interactions with other cytoplasmic proteins (e.g., adaptors, phospholipases, GTPases and other kinases) (Ihle, 1995; Lemmon and Schlessinger, 2010; Das et al., 2018; Grimsey et al., 2018). The MAPK signaling cascade is involved in many important cellular regulatory pathways, including proliferation, gene expression, apoptosis, stress responses and immune responses (Tjurjanski et al., 2007; Raman et al., 2007; Cargnello and Roux, 2011).

**T. cruzi and MAPK-related immune responses**

*T. cruzi* have evolutionarily developed into an intracellular obligatory parasite in order to survive inside a variety of mammalian hosts (Batista et al., 2020a). Accordingly, the parasite has developed several strategies for entering cells without triggering host defenses (Lima et al., 2010). Parasite interaction with host cells involves various stimuli that lead to not only activation of a single pathway cascade but also orchestrating diverse signaling routes, including those mediated by MAPKs. To date, only a few studies have focused on these pathways, because the MAPK pathways that are induced by *T. cruzi* are not yet well characterized, and, moreover, only few research groups specialize in studying signaling pathways in protozoan parasites. The most studied MAPKs participating in interactions with *T. cruzi* are those that involve the ERK1/2 and p38 (α, β, γ and δ) MAPK pathways, as these play roles in both parasite internalization and immune responses (Soares-Silva et al., 2016).

*T. cruzi* is clearly able to induce inflammation in host cells by stimulating various TLRs (for a review, see Rodrigues et al., 2012), although no specific single surface receptor for invasion of mamalian cells by *T. cruzi* has yet been identified. TLR stimulation is a fundamental process in host recognition of parasites through antigen processing and presentation (Yatim and Lakkos, 2015). Two pathogen-associated molecular patterns (PAMPs) of *T. cruzi*, that is, glycosylphosphatidylinositol (GPI) mucin-like anchors for trypomastigotes and glycoinositolphospholipids (GPIs) for epimastigotes, are recognized by TLR2 and TLR4, respectively (for a review, see Rodrigues et al., 2012). Other parasite factors are recognized by endosomal TLRs, for instance CpG-rich DNA by TLR9 and RNA by TLR6 (for a review, see Rodrigues et al., 2012). The following sections summarize our knowledge to date of *T. cruzi*-mediated induction of MAPK signaling, in relation to both innate and adaptive immune responses.

**MAPK-related innate immune responses to *T. cruzi* infection**

Innate immune responses are fundamental to both elimination of pathogens and maintenance of homeostasis (Blander and Sander, 2012). Numerous *T. cruzi* factors have been shown to modulate cellular responses to entry and survival within the host by modifying immune responses (Maeda et al., 2012; Soares-Silva et al., 2016). Forms of AgC10 mucin, a GPI-anchored surface protein in MT and EPI (see Glossary) interact with the CD62L (L-selectin) receptor, thereby dysregulating the production of interleukin 12 (IL-12) and tumor necrosis factor (TNF) in human macrophages, which promotes parasite survival through an impairment of the immune response (de Diego et al., 1997). TNF and another inflammatory mediator, cyclooxygenase-2 (COX; also known as PTGS2), are regulated by post-transcriptional destabilization of mRNA that is promoted by the suppression of the activity of p38 MAPKs and their substrate, MAPK-activated protein kinase-2 (MAPKAP-K2), which is mediated by AgC10 (Alcaide and Fresno, 2004). AgC10 directly inhibits cytokine production by acting on the p38 pathway, because it also impairs the synthesis of IL-10, an interleukin involved in TNF production (Fig. 3, labeled 3) (Alcaide and Fresno, 2004). Interestingly, AgC10 does not inhibit the production of IL-1β (a COX-2 stimulator) or of interferon-γ (IFN-γ) (de Diego et al., 1997; Alcaide and Fresno, 2004). GPI-mucin purified from TCT (see Glossary) induces a host immune response, whereas EPI GPI-mucins and GPIs have no such effect (Almeida et al., 2000). TCT GPI-mucin and GPI (Fig. 4, labeled 1) induce ERK1/2 activation, resulting in a downregulation of IL-12 expression (Ropert et al., 2001). IL-1β and IFN-γ production are not sufficient for the establishment of an efficient immune response (because of lack of IL-12 and TNF), indicating that the parasite is able to subvert immune responses as they begin (de Diego et al., 1997; Alcaide and Fresno, 2004). Dysregulation of IL-12 synthesis may be essential for subversion of immune response, as this protein is linked to important cellular processes, including cytotoxicity, natural killer (NK) cell cytokine production and regulation of T helper and T CD8⁺ cell proliferation (Borish and Steinke, 2003). In contrast, recognition of glycosylated *T. cruzi* by the macrophage galactose-C type lectin 1 (MGL1; also known as CLEC10A) receptor acts synergistically with TLR2 and TLR4 (Fig. 4, labeled 1) to induce ERK1/2 and NF-κB, resulting in the production of pro-inflammatory factors IL-12 and TNF, and of the anti-inflammatory cytokine IL-10 (Rodriguez et al., 2020).

*T. cruzi* trans-sialidase (TS) enzymes also act as MAPK inducers (Villalta et al., 1998; Chuenkova and Pereina, 2001; Magdesian et al., 2001, 2007). Recombinant surface ganglioside glycoprotein Gp63 from TCT (Gp63-TS) has been shown to modulate TCT cell uptake by inducing ERK1/2 activation in macrophages (Villalta et al., 1998). *T. cruzi*-secreted TSs (or neuraminidases) in cells of the nervous system induce neuroregeneration through the ERK1/2 pathway, and thereby play a protective role by reducing nerve damage in a similar manner to the effects of mammalian neurotrophic growth factors (NGFs) (Chuenkova and Pereina, 2001). Members of the gp85 TS superfamily, which are involved in *T. cruzi* invasion, present a C-terminal FLY domain with a conserved sequence motif (VTVXNVFLYNR) that binds to the cytoskeleton 18 (CK18; also known as KRT18) receptor (Fig. 4, labeled 1) in Rhesus monkey kidney epithelial cells (LLC-MK2) (Magdesian et al., 2001). This interaction results in rapid (within the first minute) ERK1/2 activation and subsequent disassembly of CK18, which promotes *T. cruzi* internalization into these cells (Magdesian et al., 2001, 2007). Other pathogen antigens (e.g. cruzipain) (Fig. 2, labeled 4) promote the survival of cardiomyocytes by upregulating the expression of anti-apoptotic Bcl-2, which may occur via the PI3K/Akt or MEK1–ERK1/2 pathways (Aoki et al., 2006). Indeed, ERK1/2 is activated by TCT invasion of human placental chorionic explants, even under low parasite loads (Castillo et al., 2013).

**MAPK-related adaptive immune responses due to *T. cruzi* infection**

*T. cruzi* is able to enter cells by interfering with host primary defenses, as discussed above. Efficient coordination of innate and specialized immune responses is fundamental for the host resistance against pathogens (Yatim and Lakkis, 2015) and dendritic cells play a key role in this (Eberl, 2016). The presentation of *T. cruzi* antigens through MHC class II of dendritic cells induces their early maturation
and so contributes to resistance to *T. cruzi* infection. These antigens act synergistically with macrophage migration inhibitory factor (MIF) for an efficient production of IL-12, which is dependent on p38 phosphorylation (Fig. 3, labeled 5) (Terrazas et al., 2011). Accordingly, MIF−/− mice exhibit impaired IL-12 production (Terrazas et al., 2011). Efficient IL-12 production is essential for the recruitment of type 1 T helper cells (Th1), which eliminate intracellular protozoa (Soares-Silva et al., 2016). TS secretion by *T. cruzi* plays an important role in the development of Chagas disease. TS proteins are capable of downregulating the Th1 response by inducing IL-10 production in antigen-presenting cells (APC) and CD4+ interacting cells (Ruiz Díaz et al., 2015). This provides a useful strategy for the parasite to avoid immune system recognition by converting an intracellular-related response (Th1) into an
Fig. 4. Activation of ERK1/2, JNK and NF-κB signaling during *T. cruzi* infection. (1) ERK1/2 activation. Trans-sialidase (TS) enzymes (neuroaminidases) directly induce the ERK1/2 pathway. In addition, gp85, a member of the TS superfamily, binds to the cytokeratin 18 (CK18) receptor, which results in its disassembly and reorganization, and rapid ERK1/2 activation, thereby promoting *T. cruzi* internalization. Macrophage galactose-C type lectin 1 (MGL1) receptor acts synergistically with TLR2 and TLR4; this promotes the activation of ERK1/2-mediated pro-inflammatory factors (IL-12 and TNF). *T. cruzi* GPI–mucin induces ERK1/2 activation, which results in the downregulation of IL-12 and CREB synthesis. IL-1β and IFN-γ production is not affected. Furthermore, *T. cruzi*-mediated cardiac pathology requires activation of the Ras–Raf–MEK1–ERK1/2–AP-1 axis, which promotes the synthesis of endothelin-1 (ET-1) and cyclin D1 (Cd1). (2) In contrast, JNK activation of Jun (AP-1 complex) and CREB expression downregulates ERK1/2 during early stage of *T. cruzi* infection. (3) Activation of NF-κB signaling by TNF or by TLR2 and TLR4, leads to transcription of pro-inflammatory genes and so facilitates parasite entry. Alternatively, cruzipain may impair NF-κB signaling during the early stage of infection, thereby promoting IL-10 production and parasite survival. NF-κB also plays a role in the production of pro-inflammatory factors (IL-12 and TNF).
extracellular-related response (Th2), which is directed to combat pathogens outside of the host cell, allowing intracellular parasite to become camouflaged from specialized cell responses. Atrophy and a reduced capacity of thymus typically also occur during pathogenesis (Morrot et al., 2011). In this context, TS can enhance thymocyte migration by mediating actin rearrangements and activating the JNK pathway activation, leading to premature release of double-positive T cells (CD4+ CD8+) (Nardy et al., 2013). This is another useful strategy for the parasite because it promotes the release of heterogeneous thymocytes rather than highly specialized ones (Nardy et al., 2013).

MAPK activation has also been linked to T. cruzi-related cardiac pathology (Huang et al., 2003; Mukherjee et al., 2004), and, in this context, ERK1/2 induction is essential for acute inflammation. Following T. cruzi invasion of endothelial and interstitial cells, adjacent smooth muscle cells undergo proliferation, resulting in cardiomyopathy. This process is mediated by activation of the Ras–Raf–MEK1–ERK1/2–AP-1 axis (Fig. 4, labeled 1), which induces the synthesis of endothelin-1 (ET-1; also known as EDN1) and cyclin D1 (Cd1 or CCND1), which are fundamental for T. cruzi infection and proliferation. IL-1 participates in ERK1/2 activation by regulating ET-1 (Huang et al., 2003; Mukherjee et al., 2004).

Fibrosis development has a severe effect on chagasic cardiopathy (Pérez-Molina and Molina, 2018). This requires transforming growth factor-β (TGF-β) stimulation, followed by p38 and Jun activation, which induces the redistribution of fibronectin in skeletal myoblasts and cardiac fibroblasts, with consequent hypertrophy of cardiomyocytes (Fig. 3, labeled 4) (Silva et al., 2019). Although fibrosis is necessary for repair of tissue damage, it can have adverse effects by disturbing cardiac contractile function.

Because T. cruzi presents as a diversity of strains and pathology-associated outcomes (Zingales et al., 2012), the elucidation of host signaling pathways affected is difficult because different strains display differing behaviors during host interaction. Findings from the various studies described above indicate that activation of MAPK pathways is often strain dependent and/or cell type specific. T. cruzi-induced MAPK signaling, once activated, integrates various pathways, resulting in the expression of both pro-inflammatory and anti-inflammatory cytokines that together orchestrate immune responses in mammalian hosts. T. cruzi, as an intracellular obligatory protozoan, has thus developed strategies (i.e. parasite-derived molecules) to subvert various MAPK-related immune responses within host cytoplasm and sustain infection. As discussed above, there have been different attempts to elucidate T. cruzi-mediated MAPK activation, but in most cases, these have focused on the effects of a single parasite strain or molecule. This approach is related in part to the complexity and difficulty of knocking out T. cruzi genes. Recent advances in technologies such as CRISPR-Cas that facilitate gene editing will improve our ability to fully elucidate complex parasite-host signaling relationships.

MAPK stimulation by EA

Interactions of T. cruzi forms such as EA with the host have received increased attention, but remain poorly understood. EA (see Glossary) are capable of inducing ERK1/2 activation in HeLa cells during invasion (Bonfim-Melo et al., 2018; Ferreira et al., 2019), but the molecular mechanism during immune responses is unknown. A biphasic ERK1/2 activation profile has been correlated to host epithelial cell differentiation capacity in commensal vs pathogenic forms of Candida albicans (Moyes et al., 2010). The first phase of ERK1/2 activation during the host cell interaction process appeared to result from fungus recognition (commensal), whereas during fungal filament surge (pathogenic) the cell response of the host changed from that of a homeostatic state to a danger signal, resulting in a second wave of ERK1/2 activation (Moyes et al., 2010). In preliminary studies, our group obtained similar results with a highly-infective EA strain in non-phagocytic cells, suggesting that ERK1/2 may play a role in EA infection (M.F.B., C.A.N., I.M., D.B. and Marlu Alves Dos Santos, Claudio Vieira Silva and Daniella Castanheira Bartholomeu, unpublished observations). However, another study indicated that epithelial cells of the colon activated JNK, c-Jun, and CREB, and downregulated ERK1/2 (Fig. 4, labeled 2), during early stages of T. cruzi infection (Suman et al., 2018). This may have a role in controlling extracellular matrix composition, whose dysregulation may lead to fibrosis.

Activation of NF-κB in Trypanosoma cruzi infection

The NF-κB signaling pathway plays a key role in inflammatory immune responses and is activated by various TLR stimuli, including membrane surface molecules of trypanosomatids (Dos-Santos et al., 2016; Cerbán et al., 2020). In mammals, this pathway involves the proteins RelA (p65 subunit), RelB, cRel, and p50 and p52 (and its precursor p100). These proteins form dimers in cytoplasm and are bound by inhibitory IκB proteins. The NF-κB pathway can activate gene expression by two means: canonical and non-canonical. In the canonical pathway, activation of an IκB kinase (IKK) complex – composed of IKKα (also known as CHUK), IKκB (IKKβ), and IKκγ (IKBKG or NEMO) – leads to IκB protein phosphorylation and subsequent degradation; this releases RelA or Rel complexes, which translocate into nucleus where they activate their target genes. In the non-canonical pathway, formation of the NF-κB-inducing kinase (NIK) complex leads to IKKα activation and phosphorylation, as well as processing of p100, resulting in release of the RelB–p52 complex and its translocation into nucleus (Baud and Collares, 2016; Khongthong et al., 2019).

In view of its crucial role in innate pro-inflammatory immune responses, NF-κB signaling has long been thought to be involved in responses to T. cruzi infection, particularly in non-immune cells (Huang et al., 1999, 2003; Hall et al., 2000). However, the NF-κB signaling pathway is clearly modulated by multiple factors, because of its role in protection of the host and the ability of the parasite to subvert host immune responses. Recent new insights into the regulation of NF-κB signaling in Chagas disease progression have emerged as follows.

First, activation of NF-κB by TNF treatment has been shown to facilitate T. cruzi invasion into non-professional phagocytic cells, such as the epithelial lines HEK293T and LLC-MK2 (Pinto et al., 2011). A similar effect was obtained with the application of supernatant from infected monocytes, which contains TNF and other cytokines. Here, NF-κB activation evidently played a role in parasite proliferation, as NF-κB inhibition reduced the number of IA (see Glossary) in infected cells (Pinto et al., 2011) (Fig. 4, labeled 3). A second study reported that T. cruzi activated NF-κB signaling in cardiomyocytes via the ERK-MAPK pathway to induce expression of pro-inflammatory genes (Hovsepian et al., 2013). In the later stages of infection, pro-inflammatory responses were modulated by inhibition of NF-κB translocation into nucleus. However, in the early infection stages of mouse macrophages, T. cruzi instead suppressed, rather than activated, NF-κB signaling through the action of cruzipain (Hovsepian et al., 2013). Treatment with cruzipain is known to increase IL-10 levels and enhance parasite survival (Stempin et al., 2002) (Fig. 4, labeled 3).

Furthermore, with regard to other T. cruzi surface molecules, the lipid extracts of strain RA and K98 amastigotes were found to present as a diversity of strains and pathology-
activate NF-κB signaling in HEK cells through TLR2- and TLR6-dependent pathways (Bott et al., 2018). Finally, a recent study indicated that extracellular vesicles of T. cruzi induced NF-κB activation in macrophages and caused severe problems in cases of chronic inflammation (Choudhuri and Garg, 2020).

Taken together, these findings reveal that depending on cell type (non-professional phagocytic versus professional phagocytic) and stage of infection (early versus later), activation or inhibition of NF-κB signaling pathway may either favor the parasite or prevent its establishment therefore, T. cruzi modulates this pathway differently depending on these factors.

**JAK-STAT – a possible protective pathway against T. cruzi infection**

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway has been linked to cell survival and proliferation (Bousoik and Montazeri Aliabadi, 2018) and to antimicrobial innate immune response when activated by IFN (Majoros et al., 2017). Its persistent activation has been correlated with certain inflammatory and immune diseases (Xin et al., 2020).

JAK-STAT signaling begins with binding of JAK proteins to various types of receptors, including cytokine receptors and growth factor receptors. STAT proteins are then activated by phosphorylation, initiating a signaling cascade that leads to STAT translocation to nucleus and transcription of target genes. Besides directly activating transcription factors, the JAK-STAT pathway also communicates with other major pathways, including MAPK-ERK and PI3K-AKT-mTOR, to activate them (Bousoik and Montazeri Aliabadi, 2018).

The role of the JAK-STAT pathway in Chagas disease remain unclear. The importance of canonical activation of JAK-STAT signaling for resistance to intracellular pathogens (Listeria monocytogenes, Toxoplasma gondii and Leishmania spp.) has been demonstrated by several studies (for review see Majoros et al., 2017). However, only a few recent studies have addressed the role of this pathway in T. cruzi infection.

One of these examined the effects of IFN-γ pretreatment in human cell lines (Stahl et al., 2014). Here, treatment prior to T. cruzi exposure created a so-called anti-parasitic state in cells, which resulted from STAT1 activation and enhanced transcription of the indoleamine 2,3-dioxygenase (Ido, herein referring to Ido1) gene. Infection with parasites only (without IFN-γ pretreatment) gave rise to sustained serine and tyrosine phosphorylation of STAT1 and consequent sustained pathway activation. Furthermore, specific STAT1 dephosphorylation at Ser727 occurred in the presence of IIA, which reduced the protective effect of STAT1 owing to a decrease in Ido transcription (Stahl et al., 2014). In STAT1-knockdown cells, IFN-γ had no effect and levels of blood and tissue parasites were higher, reflecting the importance of STAT1 in protecting the host from an imbalance in inflammatory response (Fig. 3, labeled 1,2) (Stahl et al., 2014; Kulkarni et al., 2015).

In addition, phospho-proteomic analysis of T. cruzi-infected colonic epithelial cells revealed increasing levels of p-JAK2 (peak at 180 min) and p-STAT1 (peak at 120 min) (Suman et al., 2018). Here, JAK2-STAT1 upregulation could be correlated with the activation of interferon pathway (Suman et al., 2018), supporting suggestions from previous studies that the JAK-STAT pathway is activated during T. cruzi infection and is linked to inflammatory responses (Tarleton et al., 2000; Bergeron and Olivier, 2006).

Other STAT proteins have also been shown to be involved in Chagas disease development (reviewed in Stahl et al., 2015). For instance, STAT4 and STAT6 have crucial roles in cytokine-mediated immune responses and affect the ability of these responses to limit tissue parasite load. STAT4-deficient mice are highly susceptible to parasite infection, whereas STAT6-deficient mice show enhanced resistance, with fewer parasites detected and low inflammation levels in heart and skeletal muscles (Tarleton et al., 2000).

A recent bioinformatics analysis of transcriptomic profiles of rat myoblasts infected with various T. cruzi strains (Brazil, CL, Y, Tulahuen) aimed to clarify any differential effects of these strains on the JAK-STAT pathway (Nisimura et al., 2020). The genes that were upregulated in all strains included cardiotorphin-like cytokine factor 1 (Clef1), which was thus implicated as a causative factor for cardiac hypertrophy mediated by activation of this pathway. The CL strain had a strong effect on JAK-STAT signaling and gave rise to a higher number of negative correlations between JAK-STAT genes and genes of interest. Although the same JAK-STAT pathway genes were activated in each of the strains, owing to distinct network interactions with different transcripts, the degree of activation of JAK-STAT and other pathways varied between them, thereby affecting Chagas disease development. Strategies for Chagas disease therapy therefore need to take into account the specific T. cruzi genetic background (Nisimura et al., 2020).

The above studies indicate that JAK-STAT pathway exerts a protective effect against infection, which is mediated through STAT1, and that T. cruzi can affect this pathway. Further studies are required to elucidate the mechanism whereby JAK-STAT modulates other pathways during T. cruzi infection, and whether Chagas disease development is affected by crosstalk among these pathways. A better understanding of the JAK-STAT pathway is thus likely to help improve Chagas disease treatments, as it has been shown to promote resistance to infection by other intracellular parasites.

**Concluding remarks**

The relationship between T. cruzi and its host cells is complex and intricate, involving numerous molecular factors from both host and parasite. As an obligate intracellular pathogen, T. cruzi is able to manipulate several host pathways to promote infection and to persist inside the host by remodeling host immune response to its advantage (summarized in Fig. 3). The full elucidation of T. cruzi interactions with mammalian host cell pathways is important to be able to improve therapeutic approaches, but remains challenging because T. cruzi biology is highly complex (similar to the case for other eukaryotic intracellular pathogens), and, moreover, host interactions vary considerably depending on parasite strain and stimulus. Owing to its ability to invade a wide range of cell types (phagocytic and non-phagocytic) and to infect a variety of host organs, T. cruzi is able to utilize numerous strategies to persist inside the host. Furthermore, crosstalk between multiple host signaling pathways makes it difficult to identify specific host proteins or other factors targeted by T. cruzi that could be utilized to suppress parasite invasion and/or infection. Many extensive studies in recent decades have focused on elucidating T. cruzi–host relationships, but improved strategies for a more effective treatment of T. cruzi infection and Chagas disease are still needed. Major advances during the past decade in molecular biological, genomic, bioinformatics and data mining techniques will greatly improve our ability to collect, analyze and integrate research data, and provide us a comprehensive overview of cell communication events during T. cruzi infection, thereby paving the way for effective new Chagas disease treatments in the near future.

**Acknowledgements**

The authors are grateful to Dr S. Anderson for English editing of the manuscript.
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