REVISITING THE LIFE CYCLE OF PARASITIC PROTOZOA

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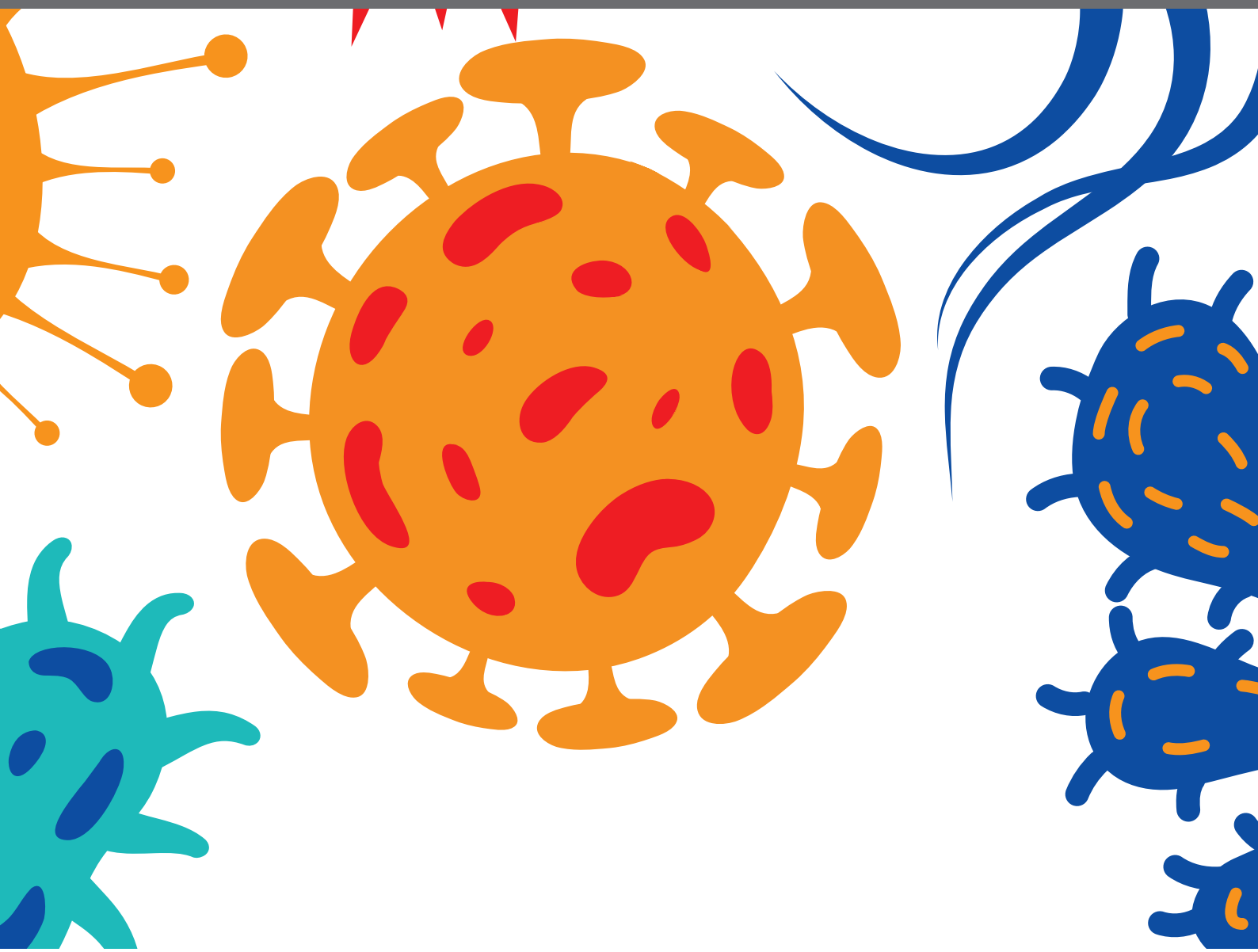

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REVISITING THE LIFE CYCLE OF PARASITIC PROTOZOA

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Table of Contents

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REVIEW
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Calcium in the Backstage of Malaria Parasite Biology

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The calcium ion (Ca^{2+}) is a ubiquitous second messenger involved in key biological processes in prokaryotes and eukaryotes. In *Plasmodium* species, Ca^{2+} signaling plays a central role in the parasite life cycle. It has been associated with parasite development, fertilization, locomotion, and host cell infection. Despite the lack of a canonical inositol-1,4,5-triphosphate receptor gene in the *Plasmodium* genome, pharmacological evidence indicates that inositol-1,4,5-triphosphate triggers Ca^{2+} mobilization from the endoplasmic reticulum. Other structures such as acidocalcisomes, food vacuole and mitochondria are proposed to act as supplementary intracellular Ca^{2+} reservoirs. Several Ca^{2+}-binding proteins (CaBPs) trigger downstream signaling. Other proteins with no EF-hand motifs, but apparently involved with CaBPs, are depicted as playing an important role in the erythrocyte invasion and egress. It is also proposed that a cross-talk among kinases, which are not members of the family of Ca^{2+}-dependent protein kinases, such as protein kinases G, A and B, play additional roles mediated indirectly by Ca^{2+} regulation. This statement may be extended for proteins directly related to invasion or egress, such as SUB1, ERC, IMC1l, IMC1g, GAP45 and EBA175. In this review, we update our understanding of aspects of Ca^{2+}-mediated signaling correlated to the developmental stages of the malaria parasite life cycle.

Keywords: Ca^{2+} signaling, *Plasmodium*, intracellular messenger, homeostasis, invasion, egress

INTRODUCTION

A plethora of cell types employ the calcium ion (Ca^{2+}), mobilized from extracellular and/or intracellular environments, to coordinate different Ca^{2+}-dependent processes. The control of intracellular Ca^{2+} signals is dynamic. Overall, fluctuations in Ca^{2+} concentrations are modulated by an influx and/or efflux promoted by membrane channels, such as store-operated calcium channels (SOCEs), plasma membrane Ca^{2+}-ATPase (PMCA) and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps. Generally, this orchestration in the Ca^{2+} concentration follows a signaling pathway that obeys the order: stimuli, G-protein coupled receptor, phospholipase C (PLC)

activation, mobilization of phosphatidylinositol 4,5-biphosphate (PI(4,5)P2), production of inositol 1,4,5-phosphate (IP3), IP3 recognition by IP3-sensitive receptor channels (IP3Rs) in the endoplasmic reticulum (ER) and downstream Ca^{2+} cascade (Berridge et al., 2003; Clapham, 2007).

In the group of apicomplexan parasites, the protozoan parasites *Toxoplasma gondii* and *Plasmodium* spp. are the most well-established study models. In *T. gondii*, Ca^{2+} signaling is involved in specific parasite processes: motility, conoid extrusion, attachment, invasion and egress from the host cell (Borges-Pereira et al., 2015; Hortua-Triana et al., 2018). Similarly, Ca^{2+} homeostasis and signaling have been extensively studied in *Plasmodium* species. Malaria is still the most life-threatening vector-borne disease globally, with an estimated 409,000 deaths and 229 million cases reported in 2019 (Global Malaria Programme: WHO Global, 2020). The increase and dissemination of antimalarial resistance (Cowman et al., 2016; Phillips et al., 2017; Global Malaria Programme: WHO Global, 2020), together with the augmentation of malaria cases since 2015, point to an urgent need for the discovery of new antimalarial drugs. The *Plasmodium* life cycle is strongly regulated by fluctuations in Ca^{2+} cellular levels, with deficiency causing impairment in parasite growth and invasion rate (Wasserman et al., 1982). This ion also acts as a messenger regulating critical *Plasmodium* biological processes. As such, proteins involved in Ca^{2+} homeostasis and signaling are strong candidates as new antimalarial targets (Gazarini et al., 2007; Vidadala et al., 2014; Mossaad et al., 2015; Bansal et al., 2016; Fang et al., 2017; Iyer et al., 2018). In this review, we present an overview of the mechanisms related to the Ca^{2+} homeostasis in *Plasmodium* species and an update of the main downstream Ca^{2+} signaling pathways and effectors involved in the parasite motility, invasion, development, and egress.

CA^{2+} HOMEOSTASIS IN MALARIA PARASITES

Ca^{2+} signaling is widely conserved in Eukaryotes, with reliance on this ion as a secondary messenger to switch on or off diverse biological process. Given their evolutionary distance from other Eukaryotes, malaria parasites represent a challenging task for the study of Ca^{2+}-mediated mechanisms, with Ca^{2+} uptake by this microorganism presenting several peculiar features. Since *Plasmodium* asexual developmental stages are predominantly intracellular in red blood cells (RBCs), Ca^{2+} has to cross several barriers to reach the parasite, which include the red blood cell membrane (RBCM) and parasitophorous vacuole membrane (PVM) (Kirk, 2001; Kirk, 2004; Kirk and Lehane, 2014).

Ca^{2+} fluctuations in *Plasmodium* species are very complex and demand the support of intracellular Ca^{2+} storage. For example, gametocytes or schizont fractions from *Plasmodium chabaudi* infected RBCs (iRBCs) present 10-20 times more Ca^{2+} than uninfected RBCs. Moreover, it has been observed that this ion concentrates in parasite storage compartments (Tanabe et al., 1982). This pattern of Ca^{2+} concentration was also observed in *Plasmodium falciparum* (Adovelande et al., 1993). To overcome these barriers and promote the observed intracellular Ca^{2+} increase, malaria parasites facilitate RBCM permeability, causing increased Ca^{2+} influx and decreased Ca^{2+} efflux (Tanabe et al., 1982; Desai et al., 1996). A nonselective cation conductance at RBCM, induced by *P. falciparum* growth, has been proposed as a mechanism involved in Ca^{2+} permeability (Brand et al., 2003; Duranton et al., 2003). Furthermore, Na^{+} associated to Ca^{2+} influx is also involved in intracellular parasite growth by this mechanism, potentially involving an ethylisopropyl-amiloride (EIPA)-sensitive channel (Brand et al., 2003).

In addition to the RBCM, PVM is another barrier to Ca^{2+} reaching the *Plasmodium* parasite. Using a cell-attached path clamp method, a 140-pS channel that is permeable to Ca^{2+}, other ions and nutrients was identified and proposed to mediate this transport through the PVM (Desai et al., 1993). Despite such advances, mechanisms involving Ca^{2+} transport into malaria parasites remain poorly understood, with considerable attention now given to this area with regard to potential therapeutic intervention. Blocking the *Plasmodium* translocon for exported proteins machinery (PTEX)-mediated protein export across the PV and out into the RBC cytosol by conditional knockdown approach, significantly reduced Ca^{2+} permeability in iRBCs (Kushwaha et al., 2018), revealing that exported parasite proteins are potentially involved in Ca^{2+} uptake and transport.

Determination of the concentration of intracellular Ca^{2+} in apicomplexan parasites is still controversial, primarily because of the technical limitations due to inhibitors, ionophores and fluorometric measurement assay sensitivities. It is widely accepted that the intracellular concentration of Ca^{2+} is around 0.09-0.1 μM in physiological conditions, similar to those found in other Eukaryotes (Alleva and Kirk, 2001; Moreno et al., 2011; Lourido and Moreno, 2015). Nonetheless, an increase of up to a hundred-fold in Ca^{2+} concentrations was noted in the late stage of the intraerythrocytic cycle forms, ranging from 1-10 μM (Glushakova et al., 2013). Also, a high Ca^{2+} concentration (40 μM) was reported in the parasitophorous vacuole (PV) required for proper parasite development (Gazarini et al., 2003). More recently, by using the Ca^{2+} sensor yellow cameleon (YC)-Nano, dynamic measurement of intracellular Ca^{2+} in different life stages of *P. falciparum* shows significant fluctuations throughout the parasite development: ring (~370 nM), trophozoite (~30 nM), schizont (~310 nM), merozoite (~950 nM), and gametocyte (stage III, ~130 nM, stage IV-V, ~520 nM) stages (Pandey et al., 2016).

Actors modulating such Ca^{2+} fluctuations have now begun to be identified, although it is still a subject under debate. For example, cytoplasmic Ca^{2+} increase may be related to potassium (K^{+}) availability, especially when parasites are faced with an abrupt change from high to low K^{+} concentration. Exposition of *P. falciparum* merozoites to an ionic environment with a low K^{+} concentration (which is the environment usually found by parasites after egress from RBCs) increases the levels of cytosolic Ca^{2+} (Singh et al., 2010). This leads to the production of cyclic-adenosine monophosphate (cAMP) by PfACβ

(adenylyl-cyclase β) upon HCO_3^- (bicarbonate ions) stimulation, followed by activation of protein kinase A (PKA) and microneme secretion (Dawn et al., 2014; Kumar et al., 2017). However, exactly how K^+ acts on signaling for merozoite maturation and invasion is controversial, in contrast to intracellular cationic remodeling in iRBC (Pillai et al., 2013).

In addition, it was also demonstrated that a putative and conserved protein member from the Epac (exchange protein directly activated by cAMP, PF3D7_1417400) pathway in *P. falciparum* is potentially involved in the rise of cytosolic Ca^{2+} levels, facilitating *P. falciparum* merozoite invasion by triggering microneme secretion (Dawn et al., 2014). Nonetheless, this pathway is apparently not required for parasite growth and egress (Patel et al., 2019). Moreover, key elements in this Ca^{2+} mobilization were shown to involve the serpentine GPCR-like receptor *Pf*SR25, a monovalent cation sensor coupled to PLC in triggering the cytoplasmic Ca^{2+} increase. Data also support the involvement of PfSR25 in parasite stress survival (Moraes et al., 2017).

Host molecules can also modulate parasite Ca^{2+} levels. For example, melatonin, which appears as a critical signal controlling synchronous maturation of *Plasmodium in vivo*, triggers an increase in Ca^{2+} cytoplasmic concentration through Ca^{2+} release from intracellular stores by an IP3-dependent pathway activation (Gazarini et al., 2003; Beraldo et al., 2005; Beraldo et al., 2007; Alves et al., 2011; Pecenin et al., 2018). Under melatonin stimulation, Ca^{2+} mobilization is affected by the melatonin receptor antagonist luzindole, the PLC inhibitor U73122 (Hotta et al., 2000) and IP3 receptor blockers (2-APB, 2-aminoethyl diphenylborinate derivatives) (Beraldo et al., 2007; Pecenin et al., 2018). Together, these data support a complex Ca^{2+}-signalling network in high demand for intraerythrocytic parasite development (**Figure 1**).

CA^{2+} STORAGE ORGANELLES

Endoplasmic Reticulum (ER)

The ER is the central organelle for Ca^{2+} storage, with a specific pathway to control calcium influx and efflux in the cell of apicomplexan parasites (Moreno et al., 2011; Lourido and

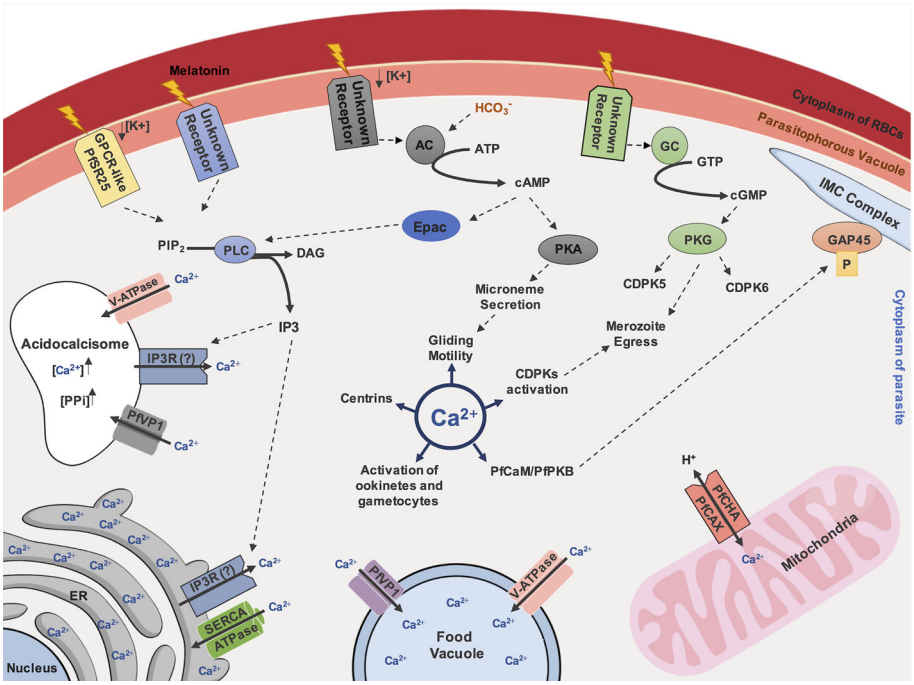

FIGURE 1 | Ca^{2+}-dependent signaling pathway in *Plasmodium* species. Ca^{2+} inside the cytoplasm of parasite controls important processes for parasite survival, such as gliding motility, mediated by activation of PfCaM/PfPKB complex and following phosphorylation of the IMC member protein, GAP45. Additionally, centrins, CDPKs activation, activation of ookinetes and gametocytes are described as Ca^{2+}-regulated. A GPCR-like protein, named as PfSR25, has been described as potential regulator in Ca^{2+} homeostasis in malaria parasites, depending on availability of potassium (K^+) and mediated by IP3 signaling. Melatonin was also described as a trigger for IP3 dependent pathways. Endoplasmic Reticulum (ER) is reported as the major storage of Ca^{2+} and the uptake of this ion possibly depends on SERCA-type Ca^{2+}-ATPases. Ca^{2+} discharge depends on receptors activated by IP3, nonetheless, an IP3R remains to be discovered in *Plasmodium* species. The presence of V-ATPase and VP1 on the food vacuole and acidocalcisome membranes are related to the Ca^{2+} uptake upon an acidic environment maintenance. Acidocalcisome could also have an IP3R that allows exit of Ca^{2+}. Calcium can also enter in mitochondria through Ca^{2+}/H^+ antiporter called PfCHA/PfCAX. Activation of PKA and PKG, by cAMP and cGMP, respectively, generated by adenylyl-cyclase (AC) and guanylyl-cyclase (GC), respectively, could also participate in Ca^{2+} homeostasis, however the membrane receptors that stimulate theses pathways remains to be elucidated. Still, upon HCO_3^- activation, AC can also stimulate Epac activation by cAMP, triggering IP3 signaling through PLC activation. Additionally, a cross-talk among kinases are also proposed to be associated to the merozoite egress mediated by proteolytic cascade events.

Moreno, 2015). Both *P. falciparum* and *T. gondii* have Ca^{2+} pumps in the ER membrane, known as SERCA-type Ca^{2+}-ATPases, that provide Ca^{2+} transport activity (Eckstein-Ludwig et al., 2003; Nagamune et al., 2007; Nagamune et al., 2008). *Pf*ATP6 is the only SERCA-type Ca^{2+}-ATPase found in the *P. falciparum* genome (Gardner et al., 2002). Due to structural similarities to a SERCA inhibitor, known as thapsigargin (Thg), the antimalarial drug, artemisinin, was thought to act against *Pf*ATP6, occasionally inhibiting Ca^{2+} mobilization into ER. Initial evidence for this hypothesis were observed in *Xenopus* oocystes expressing different *P. falciparum* transporters, including *Pf*ATP6 (Eckstein-Ludwig et al., 2003). However, molecular docking and experimental validation assays showed that the interaction between *P. falciparum* SERCA (*Pf*SERCA) and dihydroartemisinin (dART) was ~2.3-fold weaker than those observed between human SERCA and dART, indicating that dART do not inhibit *Pf*SERCA pump activity, refuting the initial conclusion (Pandey et al., 2016).

Generally, Ca^{2+} mobilization from ER storage requires IP3 activation. The production of this molecular signal is provided by PLC (Singh and Chitnis, 2012; Brochet and Billker, 2016). Although IP3-mediated Ca^{2+} release from intracellular stores have been widely reported (Lovett et al., 2002; Alves et al., 2011; Glushakova et al., 2013; Pecenin et al., 2018; Borges-Pereira et al., 2020), no genetic information is known about the presence of IP3R in apicomplexan (Lourido and Moreno, 2015; Garcia et al., 2017). It has been widely accepted that a different IP3-dependent mechanism may exist in apicomplexan to mobilize Ca^{2+} from intracellular stores (Moreno et al., 2011; Lourido and Moreno, 2015). This statement is based on many reports, which have shown that upstream inhibition of the IP3 pathway by using PLC inhibitor (Hotta et al., 2000; Beraldo et al., 2005; Beraldo et al., 2007), and downstream inhibition by using IP3 receptor blocker (Beraldo et al., 2007; Pecenin et al., 2018) and SERCA inhibitor (Alves et al., 2011; Glushakova et al., 2013; Pecenin et al., 2018; Borges-Pereira et al., 2020), all lead to the blockage of Ca^{2+} mobilization in- or outward from the cytosolic environment or IP3-sensitive stores. Since there is no clear evidence that an IP3R exists at the ER in *Plasmodium* species, how Ca^{2+} mobilization occurs into this compartment and how the ER may contribute to Ca^{2+} homeostasis through an IP3-sensible mechanism are still unresolved.

Acidic Organelles

Other Ca^{2+}-storage organelles are described in Apicomplexans, such as acidocalcisomes and food vacuole (FV), which stock Ca^{2+} in an acidic environment (Lourido and Moreno, 2015). The acidocalcisome is a lysosomal-like compartment, rich in pyrophosphate (PPi), polyphosphate (PolyP) complexed with Ca^{2+} and other cations (Moreno and Docampo, 2009; Docampo and Huang, 2016). This organelle was observed in *P. falciparum* by Ruiz et al. (2004) after being described in other parasites, such as *Trypanosoma brucei* (Vercesi et al., 1994), *Trypanosoma cruzi* (Docampo et al., 1995) and *T. gondii* (Moreno and Zhong, 1996).

Two enzymes found in the *P. falciparum* genome, described as vacuolar-H^+-pyrophosphatase (VP1) and vacuolar-H^+-ATPase (V-ATPase), can use PPi and ATP, respectively, to pump protons toward the lumen of acidocalcisomes, providing acidification of the structures, supporting Ca^{2+}-storage maintenance in these organelles (Docampo et al., 2005). VP1 and V-ATPase are also localized in the FV in *Plasmodium* species, suggesting this acidic compartment may also have a role in regulating Ca^{2+}-storage (Saliba et al., 2003). The potential role of these acidic organelles in Ca^{2+} storage is supported by the V-ATPase and VP1 blockage in malaria parasites by their respective inhibitors, bafilomycin A_1 and amino-methylene-diphosphonate (AMDP), causing an increase in cytosolic Ca^{2+} levels (Luo et al., 1999; Biagini et al., 2003).

While the FV in malaria parasites can store around 300-400 nM of Ca^{2+}, this compartment is not considered a major intracellular Ca^{2+} store organelle (Biagini et al., 2003; Rohrbach et al., 2005). Despite the pH-dependency for Ca^{2+} maintenance in the FV, measurement of this ion is challenging, considering the different pH of cellular compartments (Rohrbach et al., 2005). Moreover, the role of FV is associated with hemoglobin degradation (Moura et al., 2009; Tong et al., 2018), chloroquine (CQ) action and CQ-resistance in malaria parasites (Ehlgen et al., 2012; Tong et al., 2018). *P. falciparum* chloroquine resistance transporter (*Pf*CRT), present in the food vacuole membrane (FVM), is apparently very important to balance these processes (Ehlgen et al., 2012; Lee et al., 2018), including its participation in the release of Ca^{2+} from FV (Lee et al., 2018).

The involvement of Ca^{2+} in the functions of FV was initially suggested in *P. chabaudi* by using CQ, where the balance between concentration of intracellular Ca^{2+} and Ca^{2+} in acidic organelles were affected (Gazarini et al., 2007). This could be explained by the parasite's FV permeability to low-micromolar levels of CQ, leading to Ca^{2+} efflux (Ch'ng et al., 2011). Despite FV potentially playing a role in dynamic intracellular Ca^{2+} storage during asexual intraerythrocytic development (Biagini et al., 2003; Lee et al., 2018), the peculiar metabolic features of this organelle shed light on the possibilities for rational drug design against *Plasmodium* species. For example, a recent report showed that from the 400 Pathogen Box compounds, 10 displayed disruption of FV Ca^{2+} levels comparable to those with CQ, suggesting a compromised FV membrane integrity leading to programmed cell death (PCD) in the parasite (Tong et al., 2018).

A number of reports have discussed new perspectives on acidocalcisomes in parasites. In *T. gondii*, the Ca^{2+}/H^+-ATPase (TgA1) and a vacuolar-type H^+-pyrophosphatase (TgVP1) are localized in these organelles (Luo et al., 2001; Drozdowicz et al., 2003). Gene disruption revealed that TgA1 is required for polyphosphate storage, intracellular Ca^{2+} homeostasis, microneme secretion, invasion and virulence (Luo et al., 2005). Moreover, Ca^{2+} uptake occurs in these structures by proton pumping activity (Rohloff et al., 2011). In *Trypanosoma brucei*, an IP3R was found in the acidocalcisomes, suggesting that, besides the usual pathway for ER Ca^{2+} release, IP3 can also provide additional regulation for Ca^{2+} mobilization from acidocalcisomes (Huang et al., 2013). Proteomic analysis of this

structure in *T. brucei* confirmed the presence of IP3R. The presence of VP1, V-ATPase and vacuolar-Ca^{2+}-ATPase (TbPMC1) was also revealed, highlighting evidence of an acidic environment for Ca^{2+} maintenance (Huang et al., 2014). Given the evolutionary evidence of the acidocalcisome (Docampo et al., 2010), it should be not surprising that similar mechanisms may be found in *Plasmodium* species, supporting Ca^{2+} homeostasis in these parasites (**Figure 1**).

Mitochondrion

Besides the primary role of mitochondria in cellular energy metabolism, they can store Ca^{2+} in both human and murine malaria species (Uyemura et al., 2000). Parasite mitochondria can accumulate part of the Ca^{2+} released in the cytoplasm by pharmacological agents, suggesting a role in maintaining Ca^{2+} homeostasis (Gazarini and Garcia, 2004). Interestingly, melatonin modulates transcript levels of three genes potentially related to mitochondria fusion/fission in *P. falciparum*: FIS1, DYN1 and DYN2 (Scarpelli et al., 2018). Considering that melatonin has already been associated with Ca^{2+} mobilization (Gazarini et al., 2003; Beraldo et al., 2005; Beraldo et al., 2007; Alves et al., 2011; Pecenin et al., 2018), *Plasmodium* mitochondrion fusion/fission could potentially be controlled by Ca^{2+} signaling during the asexual life cycle.

In addition, the expression of the mitochondrial Ca^{2+}/H^{+} antiporter gene *pfcha* (or Ca^{2+}/H^{+} exchanger, PfCAX) from *P. falciparum* in the *Xenopus laevis* oocystes has been shown to cause Ca^{2+} uptake after the alkalinization of the intracellular environment, suggesting that the out- or inward-directed Ca^{2+} proton movement is pH-dependent (Rotmann et al., 2010). Regarding this feature in Ca^{2+} transport in *P. berghei*, PbCAX expression has been observed in certain sexual stages (gametocytes, zygotes and ookinetes), essential to ookinete forms and parasite transmission to the mosquito *in vivo*, but not essential to the erythrocytic stages of *P. berghei*. A *pbcax* disrupted strain revealed a stage-specific role of this transporter for *Plasmodium* survival (Guttery et al., 2013).

Ca^{2+} disturbance of the *Plasmodium* FV caused by CQ-treatment directly affects the mitochondrial transmembrane potential (Ch'ng et al., 2011; Tong et al., 2018) and triggers a PCD-like phenotype (Tong et al., 2018), providing evidence for Ca^{2+}-regulating a functional interplay between *Plasmodium* FV and mitochondria. Additional studies are required to understand the roles of the malaria parasite mitochondrion in Ca^{2+} fluctuation and how CQ affects mitochondrial membrane potential in a Ca^{2+}-dependent manner in the FV (**Figure 1**).

CA^{2+} BINDING PROTEINS (CaBPs)

CaBPs are conserved among species and present a helix-loop-helix structural motif, known as an EF-hand motif. This motif is generally pair-structured and exposes its calcium-binding domain where two Ca^{2+} ions connect to it. Analysis of the *P. falciparum* genome databank (PlasmoDB) identified 103 potential proteins with EF-hand motifs. Nonetheless, this number is undoubtedly overestimated due to the divergence of the EF-hand motif and some rifins. Without rifins, 43 proteins containing EF-hands were recorded in *P. falciparum* (Lourido and Moreno, 2015). Some reports have proposed that this number is even lower, with about only 30 putative CaBPs (Brochet and Billker, 2016). Three main families of CaBPs are categorized in the Apicomplexa: the calmodulin (CaM) family (including centrins or caltractrins), the calcineurin B-like (CBL) family and the Ca^{2+}-dependent protein kinases (CDPK) family (Moreno et al., 2011; Lourido and Moreno, 2015; Brochet and Billker, 2016).

Calmodulin in *P. falciparum* (PfCaM) is localized diffusely in the cytoplasm during mature stages of the intraerythrocytic cycle and at the apical pole end of merozoites within the ductule of rhoptries (Scheibel et al., 1987). Furthermore, a protein kinase B (PfPKB) interacts with PfCaM, which is not a member of the CDPK family, in the schizont/merozoite stages of *P. falciparum*. PfPKB is regulated by PfCaM in a Ca^{2+}-dependent manner when the generation of IP3 by PLC mediates Ca^{2+} release. Consequently, PLC is an upstream modulator of PfPKB activity, regulating Ca^{2+} levels inside the parasite and allowing PfCaM-PfPKB interaction (Vaid and Sharma, 2006; Vaid et al., 2008). This protein complex phosphorylates PfGAP45, an anchoring protein of the actin-myosin motor complex from the IMC (inner membrane complex) (Vaid et al., 2008).

Current understanding is limited regarding a group of four *P. falciparum* centrins (PfCENs 1 to 4: PF3D7_0107000, PF3D7_1446600, PF3D7_1027700 and PF3D7_1105500, respectively), that contain four EF-hand motifs. This group of proteins are involved in parasite cell division at centrosome-like structures, probably in a Ca^{2+}-dependent manner (Mahajan et al., 2008). A recent report showed that during mitosis, PbCEN-4 is localized at distinct perinuclear foci, suggesting an association to the putative centrosomal structure, known as the microtubule-organizing center (MTOC) in *P. berghei*. Moreover, *cen-4* gene does not seem to be compensated by increased transcription levels of other centrins and it is dispensable for malaria proliferation (Roques et al., 2019). In contrast, large-scale functional screening of *P. berghei* showed that *cen-1* and *cen-2* genes are essential for parasite survival (Bushell et al., 2017) (**Figure 1**). Other CaBPs and their participation in diverse cellular processes in malaria parasites will be discussed in the next sections (**Figure 2**).

CA^{2+}-DEPENDENT PROTEIN KINASES (CDPKs)

Protein phosphorylation is one of the most studied post-translational modifications in eukaryotic cellular processes. Regarding the importance of Ca^{2+}/CaM in kinase activation, many classes of Ca^{2+}/CaM-dependent kinases (CaMKs) are known in mammals. *P. falciparum* protein kinase 2 (PfPK2) is the unique homolog of human functional CaMK that phosphorylates its substrate in a Ca^{2+}- and CaM-dependent manner, and it is expressed during invasion (Kato et al., 2008).

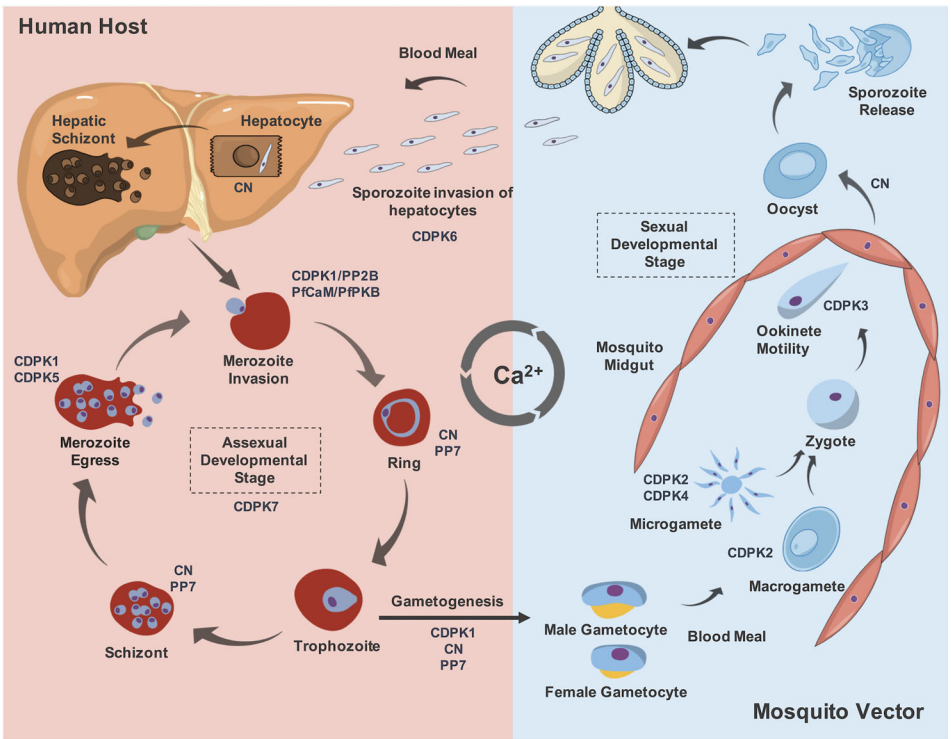

FIGURE 2 | The role of some described Ca^{2+}-binding proteins involved in the development stage differentiation and their expression throughout *Plasmodium* life cycle. During the blood meal, the mosquito vector from genus *Anopheles*, inoculate sporozoites released from its salivary glands that will invade hepatocytes. This process is described to be CDPK6-dependent. Moreover, CN allows sporozoite-to-liver stages development inside hepatocytes. Following formation of merozoites in the liver cells, they are released in the bloodstream to continue asexual development stages, invading new RBCs. This process is mediated by CDPK1, CN and PfCaM/PfPKB complex. The role of CDPK7 to maintain the asexual developmental stages is also reported. In addition, the presence of the phosphatases CN and PP7 are implicated in the ring and schizont stages. After schizont maturation, the merozoites are released into the bloodstream to invade new RBCs, which mechanism that requires the action of CDPK1 and CDPK5. Some parasites pass through a morphological transition to form gametocytes, and this process depends on CDPK1, CN and PP7. After a blood meal of the mosquito vector, these forms maturate into male exflagellated and female gametocytes, named as microgametes and macrogametes, respectively. For this transition, CDPK2 and CDPK4 are required. These forms are fused into a zygote, which maturates to a motile ookinete. The ookinete motility is regulated by CDPK3. The ookinete exits from the lumen of mosquito midgut as an oocyst and this transition is mediated by CN. The maturation of the oocyst will release new sporozoites which will migrate into the salivary glands of the mosquito vector. Thus, in an eventual blood meal, these new sporozoites will infect a new host and complete the parasite life cycle in order to propagate malaria disease.

However, apicomplexan parasites use a group of Ca^{2+}-dependent protein kinases (CDPKs), which are not present in humans. Canonical CDPKs have four EF-hand Ca^{2+}-binding domains attached to the C-terminus of a catalytic kinase domain that shows high homology with CaMK. While CaMKs can self-inhibit through a C-terminal helix, CDPKs are regulated by their Ca^{2+}-binding domains. In these cases, CDPKs undergo structural and conformational changes, promoting the regulation of other proteins by phosphorylation (Wernimont et al., 2010). *P. falciparum* possesses seven CDPKs (PfCDPK1 to PfCDPK7), with correspondent orthologs in plants, but not in animals or fungi (Kadian et al., 2017; Ghartey-Kwansah et al., 2020). It has been proposed that CDPKs could be a novel field for exploration of new antimalarial drugs (Hui et al., 2015).

For instance, transcriptomic data analysis has suggested that PfCDPK1 is primarily expressed in the late schizont stage (Bozdech et al., 2003; Le Roch et al., 2003). In agreement with this, PfCDPK1 is found in the PV and merozoite membrane throughout schizogony and merozoite egress, and performs crucial roles in the invasion process (Azevedo et al., 2013; Bansal et al., 2013). PfCDPK1 is known to phosphorylate both the myosin light chain and an IMC member, PfGAP45, in mature schizonts *in vitro*, when merozoites are formed (Green et al., 2008). As previously reported, PfGAP45 is also phosphorylated by PfPKB (Vaid et al., 2008), but it is proposed that this IMC member is phosphorylated on CDPK1 non-dependent phosphosites (Green et al., 2008). PfCDPK1 knockout mutants showed that this kinase is required for normal growth of *P. falciparum* during asexual proliferation, with critical involvement in gametogenesis, making its transmission to the mosquito unfeasible (Bansal et al., 2018). In contrast, CDPK1 deletion in *P. berghei* showed no difference for the asexual development and host cell invasion, suggesting different functions of the homologs in both species (Jebiwott et al., 2013).

PfCDPK1 mutant parasites on the bulky gatekeeper residue T145M (gatekeeper residue in the wild-type is a Thr, modified to a Met at the position 145 in the mutants) showed prominently

reduced activity compared to wild-type parasites. This lower activity seems to be compensated by PKG, influencing the up-regulation of transcription levels of CDPK5 and CDPK6 in the CDPK1 T145M mutant parasites (Bansal et al., 2016), suggesting that a Ca^{2+}- based signaling may modulate a very collaborative role in the CDPK family and other kinases in malaria parasites (Green et al., 2008; Brochet et al., 2014). Some reports have highlighted the importance of PfCDPK1 in the phosphorylation of members of IMC, such as GAP45 and IMC1g (Green et al., 2008; Kumar et al., 2017). PfCDPK1 knock-down mutants using the FKBP destabilization domain (DD) showed different patterns of phosphorylation in the protein-partners, revealed by iTRAQ-based phosphoproteomic analysis, including the phosphorylation pattern on S149 of PfPKA, which is a kinase also involved in Ca^{2+}-signaling mediated by cAMP (Kumar et al., 2017). Additionally, PfCDPK1 can phosphorylate PfSERA5 (*P. falciparum* serine repeat antigen 5). The PfCDPK1 inhibitor, purfalcamine, blocked SERA5 phosphorylation, leading to the blockage of merozoite egress (Iyer et al., 2018). Despite this evidence on PfCDPK1 as a promising target for therapeutic intervention, a recent chemical genetics approach casts doubt on this suitability for blood stages (Green et al., 2016). Nevertheless, PfCDPK1 continues to represent a good target for a mosquito transmission-blocking strategy, as previously mentioned (Bansal et al., 2018).

In contrast to PfCDPK1, PfCDPK2 function is poorly understood in *Plasmodium*. In all rodent and some other malaria species, the *cdpk2* gene is lacking (Tewari et al., 2010). Initially thought as an essential gene in *P. falciparum*, a recent report has pointed out that in PfCDPK2 knockout mutants obtained by CRISPR-Cas9, it is dispensable in asexual proliferation in *P. falciparum*. Still, CDPK2 seems to play an essential role in male gametocyte exflagellation and possibly in female gametocytes, compromising parasite transmission to mosquitoes (Bansal et al., 2017). Likewise, CDPK4 has been demonstrated to play crucial roles in gametocyte exflagellation (Billker et al., 2004; Ojo et al., 2012). The bumped-kinase inhibitor 1 (BKI-1), which is more than 20-fold more selective for PfCDPK4 over PfCDPK1, inhibited the microgamete exflagellation of *P. falciparum*, but did not block asexual parasite proliferation. A strong correlation between PfCDPK4 activity inhibition and blockage of exflagellation by a series of closely related BKI analogues was observed, supporting that the exflagellation blockage was due to the inhibition of PfCDPK4 rather than other kinases. Furthermore, BKI-1 blocks *P. berghei* transmission to mosquitoes (Ojo et al., 2012).

The apparent role of CDPK4 in the onset of axoneme motility, DNA condensation and cytokinesis during the first 10 min of exflagellation induction has been reported (Fang et al., 2017). An increased interest in the CDPK4 as a new antimalarial target for pyrazolopyrimidine-based inhibitors has also been reported, which could result in new therapeutic strategies for malaria treatment in the near future (Vidadala et al., 2014). Regarding the sexual stages of development, CDPK3 is intimately implicated in regulating the motility of the ookinete in the mosquito vector midgut (Ishino et al., 2006; Siden-Kiamos et al., 2006). *In vitro* migration assays also suggested that this motility is stimulated by Ca^{2+} mobilization from intracellular stores (Ishino et al., 2006).

PfCDPK5 is an important regulator of parasite egress, a highly coordinated event requiring PfSERA5 (Dvorin et al., 2010; Absalon et al., 2018). The egress in CDPK5-deficient merozoites is impaired. PfCDPK5 is localized within micronemes and plays a central role in the microneme protein discharge, correlating a defect in this process to the impaired egress observed in PfCDPK5-deficient parasites. In addition, PKG has been identified as an important protein that cooperates in the egress signaling pathway together with PfCDPK5 (Absalon et al., 2018). This could explain the increased transcriptional expression levels of PKG and PfCDPK5 in the PfCDPK1 mutants as mentioned above, suggesting an integrated cross-talk among kinases in malaria parasites (Bansal et al., 2016), including their role in Ca^{2+} mobilization in gametocyte activation of *P. berghei* and egress of merozoites in *P. falciparum* (Brochet et al., 2014).

Functional studies to understand the roles of CDPK6 and CDKP7 are still lacking. PbCDPK6 has been demonstrated to play a critical role in sporozoite invasion of cells with high expression of heparan sulphate proteoglycans (HSPGs), such as hepatocytes, involving the induction of the circumsporozoite protein (CSP) cleavage upon contact with hepatocytes (Coppi et al., 2007). As previously highlighted, PfCDPK6 could be playing a compensatory role in the asexual blood stages of *P. falciparum* in the absence of a functional PfCDPK1 (Bansal et al., 2016). Still, additional studies need to be performed to address this question adequately. On the other hand, PfCDPK7 is an atypical member of the CDPK family, containing a pleckstrin homology domain adjacent to the kinase domain and two Ca^{2+} -binding EF-hands, present at its N-terminus. PfCDPK7 interacts with PIP_2 through its pleckstrin domain, suggesting that this feature may determine its subcellular localization, possibly at ER exit sites. Moreover, knockout mutants of PfCDPK7 have also shown its importance for the growth of asexual stages of development, presenting abnormal morphology (Kumar et al., 2014). Despite this evidence on the roles of CDPK6 and CDPK7, their downstream signals, which may be implicated in other biological processes, are still largely unknown (**Figure 2**).

CA^{2+}-RELATED PHOSPHATASES

Sixty-seven candidate phosphatases were identified in the *P. falciparum* genome by *in silico* analysis (Pandey et al., 2014). At least three serine/threonine protein phosphatases (STPP) are involved in Ca^{2+} signaling: STPP 2B catalytic subunit A (Wilkes and Doerig, 2008; Singh et al., 2014), STPP 7 (PP7) (Kumar et al., 2004; Wilkes and Doerig, 2008; Singh et al., 2014) and a putative STPP 8 (PPP8), which is inferred as containing a Ca^{2+} binding site EF-hand (Yang and Arrizabalaga, 2017; Mitchell et al., 2019).

Calcineurin (CN), also known as STPP 2B or PP3, is a heterodimeric complex containing a catalytic subunit (CNA) and a regulatory subunit (CNB) (Steinbach et al., 2007). CN is

conserved from yeast to humans (Yang and Arrizabalaga, 2017) and involved in several cellular processes. It has been extensively studied and reviewed (Crabtree, 2001; Wilkins and Molkentin, 2004; Liu et al., 2015; Park et al., 2019). High Ca^{2+} concentration induces the formation of a Ca^{2+}-CaM complex, leading to CN activation, the release of its autoinhibitory domain and exposition of the active site to dephosphorylate its target (Rusnak and Mertz, 2000; Park et al., 2019).

CN in *Plasmodium* spp. is required for host cell attachment and invasion in a receptor-dependent pathway distinct from the AMA1-RON2 (apical membrane antigen-1/rhoptry neck protein 2) system but with some degree of functional overlap (Paul et al., 2015). CN knockdown demonstrated an increase of sensibility to an invasion-inhibitory antibody directed against basigin, an important receptor for RBC invasion, suggesting that CN regulates this process (Duraisingh et al., 2008; Otto et al., 2014; Paul et al., 2015). This might occur regardless of apical organelle proteins involved in invasion (Paul et al., 2015). However, CN has also been described as essential to Ca^{2+}-dependent microneme secretion, and its activity is increased after the exposure of merozoites to a low K^+ environment. The mechanism involving CN and microneme secretion includes regulating apical actin depolymerization (Singh et al., 2014).

Stage-specific conditional degradation of CN in *P. berghei* further demonstrates its role in gametocyte development, fertilization and ookinete-to-oocyst and sporozoite-to-liver stage transitions (Philip and Waters, 2015). CN protein expression and/or activity regulation might provide a regulatory hub during the parasite cell cycle. The protein has been detected at the schizont, ring, sporozoite, merozoite and gametocyte stages, but not in the trophozoite stage (Wilkes and Doerig, 2008; Pandey et al., 2014). Activity inhibition by cyclosporin and FK506 resulted in increased levels of phosphorylated HSP90, phosphoglycerate kinase, actin-1, adenosine deaminase and glyceraldehyde-3-phosphate dehydrogenase. Moreover, actin-1 is potentially a direct substrate of CN in *P. falciparum* (Singh et al., 2014).

Similar to CN, protein phosphatase 7 (PP7) contains EF-hands and IQ (the first two amino acids of the motif: Ile and Gln) calmodulin-binding motifs but, in contrast, is monomeric. The CaM-binding motif was found to inhibit phosphatase activity in *Arabidopsis* PP7 (Dobson et al., 2001; Kutuzov et al., 2001; Yang and Arrizabalaga, 2017). PP7 is not detected at the trophozoite stage but at the schizont, ring, merozoite and gametocyte stages (Dobson et al., 2001; Pandey et al., 2014). These observations indicate that PfPP7 is regulated across all parasite stages and could constitute a potential target to control the parasite cell cycle. PP8 or EFPP is a putative STPP with a long N-terminal domain with EF-hand motifs and is specific to apicomplexans. Mutations were observed in their catalytic domain which put into question their phosphatase activity. Their functions have not yet been investigated (Kutuzov and Andreeva, 2008; Yang and Arrizabalaga, 2017).

Most studies involving the roles of Ca^{2+} signaling and phosphatases are focused on calcineurin. However, other phosphatases without Ca^{2+}-binding motifs could be affected by Ca^{2+} signaling through their protein partners possessing these motifs. According to STRING prediction and Gene Ontology analyses, amongst the 67 candidate phosphatases identified in *P. falciparum*, ten potentially interact with proteins involved in Ca^{2+} signaling: putative acid phosphatase (PF3D7_0918000), putative protein phosphatase 2C (PF3D7_0829100), putative 4-nitrophenylphosphatase (PF3D7_0715000), putative protein phosphatase (PF3D7_0802800), putative RNA lariat debranching enzyme (PF3D7_1340600), putative acid phosphatase (PF3D7_1403900), hypothetical protein (PF3D7_1464600), hypothetical protein (PF3D7_1469200), protein phosphatase 2C (PF3D7_0410300) and putative phosphoesterase (PF3D7_1206000) (Pandey et al., 2014). However, the effects of Ca^{2+} on the phosphatase protein-interaction network remain poorly understood in *Plasmodium* species. Therefore, biochemical assays and phosphatase protein partner screenings are a reasonable approach for discovery of new antimalarials (Khalife and Pierrot, 2016).

OTHER EFFECTORS INVOLVED IN CA^{2+} SIGNALING

P. falciparum reticulocyte binding-like protein 1 (PfRh1) performs a role in initial sensing of Ca^{2+} followed by signal transduction, causing erythrocyte binding antigen-175 (EBA-175) release from microneme and allowing tight junction formation (Gao et al., 2013). The biochemical pathways regarding Ca^{2+} modulation that led to microneme secretion are largely unknown, highlighting the need for further studies in *Plasmodium* species. As previously mentioned, components of the motor complex involved in merozoite invasion are phosphorylated by PfCDPK1 (Green et al., 2008; Vaid et al., 2008). In *P. berghei* sporozoites, this complex is involved in gliding motility and host cell invasion. Living-cell imaging studies demonstrate that while cytoplasmic elevated Ca^{2+} levels are required for gliding, alone this is insufficient, since artificial increases using an ionophore allowed adhesin translocation to the surface but no gliding motion (Carey et al., 2014). Moreover, the *P. falciparum* inner membrane complex 1l (PfIMC1l) has been proposed as a protein to potentially connect this motor complex to the IMC membrane. It is also involved in gliding and invasion processes in a Ca^{2+}-dependent manner. PfIMC1l interacts directly with Ca^{2+} and its interaction with actin is enhanced in the presence of this ion (Kumar et al., 2019). The gliding motility used by ookinete and merozoite invasion is also supported by CDPK4, in a compensatory manner to CDPK1 (and vice versa). Both Ca^{2+}-dependent kinases are involved in IMC stability, phosphorylating the glideosome-associated protein 40 (GAP40) and the CDPK4 substrate SOC6 (PBANKA_070770), involved in IMC biogenesis (Fang et al., 2018).

Following microneme secretion, the interaction of PfEBA-175 and the RBCs receptor glycophorin A (glyA) results in a cytoplasmic lowering of Ca^{2+} levels, which, in turn, stimulates the release of rhoptry proteins such as cytoadherence-linked

asexual protein gene 3.1 (CLAG3.1/RhopH1) and *P. falciparum* reticulocyte binding-like protein 2b (PfRh2b) (Singh et al., 2010). Rhoptry discharge in RBCs contributes to the tight-junction and PV formation, modifying the host cell environment (Boothroyd and Dubremetz, 2008; Santos and Soldati-Favre, 2011). The repression of *P. berghei* rhoptry neck protein 11 (PbRON11) in sporozoites reduced attachment and motility, leading to the impairment of the infection in the mosquito salivary gland and hepatocyte cells. This protein contains putative EF-hand domains and might act by controlling rhoptry protein secretion in a Ca^{2+}-dependent manner (Bantuchai et al., 2019).

Merozoite egress from RBCs is triggered by elevation of cyclic guanosine monophosphate (cGMP) and PKG activation, essential for the protein discharge of secretory organelles, known to support this process (Collins et al., 2013; Alam et al., 2015). Correlation of Ca^{2+} with parasite egress was previously reported (Collins et al., 2013; Glushakova et al., 2013). Events documented in the final hour of the cell cycle include Ca^{2+} release from ER of the schizonts, activation of PfCDPK5 and, in the last 10-20 minutes of the cell cycle, vacuole swelling and red blood cell cytoskeleton destabilization by calpain, a host enzyme activated by Ca^{2+} (Glushakova et al., 2013). More recently, PKG was found to interact with and phosphorylate a multipass membrane protein, termed as important for Ca^{2+} mobilization 1 (ICM1). Conditional knockdown of ICM1 revealed an essential role in Ca^{2+} mobilization to initiate both *Plasmodium* gametogenesis and merozoite egress (Balestra et al., 2021). Additionally, guanylyl-cyclase alpha (GCα)-null mutant parasites were unable to synthesize cGMP for PKG activation in schizonts, leading to a reduction in Ca^{2+} release from internal stores (Nofal et al., 2021).

Conditional gene disruption of the *P. falciparum* phosphodiesterase β (PfPDEβ) leads to a dramatic reduction in schizont cAMP and cGMP hydrolytic activity, resulting in elevated cAMP levels and inappropriate cAMP-induced increased phosphorylation of PKA substrates. In addition, PKA seem to assume a compensatory role with PKG, in order to phosphorylate *P. falciparum* myosin A (*Pf*MyoA), an important component of the so-called glideosome, a parasite complex involved in host cell invasion, in PfPDEβ mutants, bypassing the need for PKG activity by elevated cAMP levels upon Ca^{2+} signaling, possibly by PKA action (Flueck et al., 2019). Together, these findings point towards PfPDEβ regulating cAMP and cGMP production, followed by PKA and PKG activation. Nonetheless, the molecular dynamics of Ca^{2+} signaling associated with these events are still poorly understood.

A family of proteins containing multiple EF-hand motifs, named as the CREC family (calumenin, reticulocalbin 1 and 3, ERC-55, Cab-45), has been remarkably underexplored, considering that proteins from this family are widely found from protozoans to mammals (Honoré and Vorum, 2000). A member of this family is found in the ER of *P. falciparum*, known as PfERC (endoplasmic reticulum-resident calcium-binding protein) (La Greca et al., 1997). This protein is a key regulator of the egress proteolytic cascade of *P. falciparum* merozoites. The use of SERCA inhibitor cyclopiazonic acid (CPA) and an ionophore, ionomycin, did not change the amounts of cytosolic Ca^{2+} in knockdown parasites bearing a glucosamine-inducible ribozyme gene (PfERC-*glmS*) from ER or neutral Ca^{2+} storages, suggesting that the availability of Ca^{2+} from different sources does not change upon knockdown of PfERC. Moreover, PfERC is required for the complete maturation of the aspartic protease plasmepsin X (PMX) in a Ca^{2+}-dependent manner, which is required to cleave the subtilisin-like protease (SUB1) (Fierro et al., 2020).

Additional evidence for Ca^{2+} importance for SUB1 discharge and proteolytic cascade events have been reported. Chelation of intracellular Ca^{2+} in *P. falciparum* schizonts blocks the SUB1 discharge from merozoite exonemes into PV, resulting in a decrease of SERA5 proteolytic cleavage and harming PVM rupture and merozoite egress (Agarwal et al., 2013). Mature SUB1 discharge into PV results in the proteolytic cleavage of protein family members involved in merozoite egress and RBC invasion, such as SERA5 and MSP1 (merozoite surface protein 1) (Nasamu et al., 2017; Pino et al., 2017). Additionally to SERA5, SERA6 has been associated to the parasite egress from RBCs upon SUB1 catalytic processing into the PV (Ruecker et al., 2012). In the absence of SERA6, the rupture of RBCM does not occur, suggesting that SERA6 could be associated to an additional proteolytic cascade event related to the β-spectrin cleavage of host cell cytoskeleton (Thomas et al., 2018). Moreover, autocatalytic maturation of SERA6 needs a PV-located protein cofactor, named merozoite surface antigen 180, which is also a SUB1 substrate. This multi-step proteolytic process is required for dismantling the host RBC cytoskeleton facilitating the parasite egress (Tan et al., 2021). Therefore, it remains to be further described how Ca^{2+} may modulate actors in these proteolytic cascade events.

Ca^{2+} signaling has also been shown to be involved in pre-erythrocytic cycle stages. After hepatocyte invasion, elongated sporozoites transform into a spherical form (exo-erythrocytic form, EEF) in a temperature-dependent process (Shortt and Garnham, 1948; Meis et al., 1983; Kaiser et al., 2003). It has been proposed that the Ca^{2+} signal regulates this morphological transition, with intracellular Ca^{2+} increased at the center of a bulbous structure in *P. berghei*, reinforcing that Ca^{2+} plays central roles in diverse life-cycle stages (Doi et al., 2011). Sporozoite salivary gland proteome analyses revealed several components that could be involved in the Ca^{2+} signaling pathway at this stage, such as G-protein-coupled receptors, adenylyl and guanylyl cyclases and a carbonic anhydrase. Host proteins are also involved in EEF transformations and Ca^{2+} signaling. Protein kinase C–mediated NF-κB activation induces expression of CXCR4 (C-X-C chemokine receptor type 4) in hepatocytes and intracellular Ca^{2+} elevation, essential to EEF development (Bando et al., 2019). The interplay between host and parasite proteins, however, remains highly elusive.

CONCLUDING REMARKS

Plasmodium species contain distinctive features when compared to other eukaryotes. Such characteristics define its phylum or

genus, in which attachment to the host cell, motility, invasion and egress are essential for survival and dissemination. Since Ca^{2+} signaling regulates important and specific *Plasmodium* cellular processes such as microneme secretion, attachment, gliding motility, invasion and egress, actors involved in these pathways, which are regulated by this ion, could be considered potential drug targets. Striking progress to achieve a broader understanding of Ca^{2+} signaling in *Plasmodium* has been made, including the potential involvement of host compounds in Ca^{2+} uptake, such as K^+, Na^+, ionic strength and melatonin (Brand et al., 2003; Gazarini et al., 2003; Singh et al., 2010; Pillai et al., 2013; Pecenin et al., 2018).

However, several gaps in understanding remain in these organisms, covering mechanisms involved in increased Ca^{2+} uptaking by iRBCs, together with transport through PVM and the parasite cellular membrane. Moreover, IP3R or alternative functional protein identification in *Plasmodium* would be an important breakthrough to explore Ca^{2+} mobilization and storage, as well as backstage actors which support those processes as promising therapeutic targets. Actually, a plethora of *Plasmodium* proteins with standard and unusual Ca^{2+} binding motifs, which are known or suspected to be involved in Ca^{2+} signaling, could be explored to this end. This also includes proteins without Ca^{2+}-binding motifs acting as indirect effectors.

The association of classical techniques employed to study permeability, protein channels and pumps, together with more recent high-throughput approaches is a promise to fulfill these gaps. Mass spectrometry-based proteomics (Garcia et al., 2018; Blomqvist et al., 2020; Garcia et al., 2021), including novel proteomic approaches to understand *in vivo* protein-partners, such as BioID and APEX-2 proximity-labelling techniques (Rhee et al., 2013; Kehrer et al., 2016; Boucher et al., 2018; Birnbaum et al., 2020), metabolomics (Beri et al., 2019) and new Ca^{2+} ratiometric techniques coupled to imaging reporters (Brochet et al., 2014; Carey et al., 2014; Pandey et al., 2016; Absalon et al., 2018; Borges-Pereira et al., 2020) are examples of such technologies required for improved understanding of the role of Ca^{2+} in the backstage of malaria parasite biology and drug screening assay design.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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GLOSSARY

IP3R	inositol-1,4,5-triphosphate receptor
IP3	inositol-1,4,5-triphosphate
ER	endoplasmic reticulum
SOCEs	store-operated calcium channels
PMCA	plasma membrane Ca^{2+}-ATPase
SERCA	sarco(endo)plasmic reticulum Ca^{2+}-ATPase
PLC	phospholipase C
$PI(4,5)P_2$	phosphatidylinositol 4,5-biphosphate
RBCs	red blood cells
RBCM	red blood cell membrane
PVM	parasitophorous vacuole membrane
iRBCs	infected red blood cells
uRBCs	uninfected red blood cells
EIPA	ethylisopropyl-amiloride
PTEX	*Plasmodium* translocon of exported proteins
PV	parasitophorous vacuole
cAMP	cyclic-adenosine monophosphate
*Pf*ACβ	*P. falciparum* adenylyl-cyclase β
HCO_3^-	bicarbonate ion
Epac	exchange protein directly activated by cAMP
Rap1	Ras-related protein 1
*Pf*SR25	G-protein-coupled receptor-like
U73122	inhibitor of PLC
2-APB	2-aminoethyl diphenylborinate, inhibitor of IP3 signaling
PfATP6	a *P. falciparum* SERCA-type Ca^{2+}-ATPase
Thg	thapsigargin
FV	food vacuole
PPi	pyrophosphate
PolyP	polyphosphate
VP1	vacuolar-H^+-pyrophosphatase
V-ATPase	vacuolar-H^+-ATPase
CQ	chloroquine
PfCRT	*P. falciparum* chloroquine resistance transporter
FVM	food vacuole membrane
TgA1	*T. gondii* Ca^{2+}/H^+-ATPase
TgVP1	*T. gondii* vacuolar-type H^+-pyrophosphatase
AMDP	amino-methylene-diphosphonate
TbPMC1	*T. brucei* vacuolar-Ca^{2+}-ATPase
*Pf*CHA or *Pf*CAX	*P. falciparum* Ca^{2+}/H^+ antiporter or *P. falciparum* Ca^{2+}/H^+ exchanger
CaBPs	Ca^{2+} binding proteins
EF-hand motif	motif for Ca^{2+} binding
CaM	calmodulin
CBL	calcineurin B-like family
CDPKs	Ca^{2+}-dependent protein kinases family (1-7)
*Pf*PKB	*P. falciparum* protein kinase B
*Pf*GAP45	*P. falciparum* glideosome-associated protein 45
IMC	inner membrane complex
*Pf*CENs	*P. falciparum* centrins (1-4)
MTOC	microtubule-organizing center
CaMKs	Ca^{2+}/CaM-dependent kinases
*Pf*PK2	*P. falciparum* protein kinase 2
PKG	cGMP-dependent protein kinase
PKA	cAMP- dependent protein kinase
IMC1g	inner membrane complex protein 1g
iTRAQ	isobaric tag for relative and absolute quantification
*Pf*SERA5	*P. falciparum* serine repeat antigen 5
HSP90	heat-shock protein
HSPGs	heparan sulphate proteoglycans
CSP	circumsporozoite protein
STPPs	serine/threonine protein phosphatases
CN	calcineurin
CNA	catalytic subunit of the calcineurin
CNB	regulatory subunit of the calcineurin
AMA1-RON2	apical membrane antigen-1/rhoptry neck protein 2
PP7	protein phosphatase 7
IQ	the first two amino acids of the calmodulin binding motif: Ile and Gln
PPP8	serine/threonine protein phosphatase 8
EBA-175	175 kDa erythrocyte binding antigen
*Pf*Rh1	*P. falciparum* reticulocyte binding-like protein 1
*Pf*IMC1l	*P. falciparum* inner membrane complex 1l
GAP40	glideosome-associated protein 40
SOC6	substrate of CDPK4 6
glyA	glycophorin A
CLAG3.1/ RhopH1	cytoadherence-linked asexual protein gene 3.1
*Pf*Rh2b	*P. falciparum* reticulocyte binding-like protein 2b
*Pb*RON11	*P. berghei* rhoptry neck protein 11
cGMP	cyclic guanosine monophosphate
ICM-1	important for calcium mobilization-1 protein
cKO	conditional knockout
GCα	guanylyl cyclase alpha
PfPDEβ	*P. falciparum* phosphodiesterase beta
PfMyoA	*P. falciparum* myosin A
CREC family	Calumenin, Reticulocalbin 1 and 3, ERC-55, Cab-45
*Pf*ERC	*P. falciparum* endoplasmic reticulum-resident calcium-binding protein
CPA	cyclopiazonic acid
SUB1	subtilisin-like protease
PMX	plasmepsin X
MSP1	merozoite surface protein 1
EEF	exo-erythrocytic form
NF-κB	nuclear factor κB
CXCR4	C-X-C chemokine receptor type 4
BioID	proximity-dependent biotin identification
APEX-2	ascorbate peroxidase-2

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BRIEF RESEARCH REPORT
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The Presence of *Leishmania braziliensis* DNA in the Nasal Mucosa of Cutaneous Leishmaniasis Patients and the Search for Possible Clinical and Immunological Patterns of Disease Progression: A Cross Sectional Study

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Leishmania braziliensis is the most important causal agent of American tegumentary leishmaniasis (ATL), and 3 to 5% of patients develop mucosal lesions. The mechanisms related to parasite and host immune interactions and the parasite life cycle that lead to dissemination to the mucosa are poorly understood. We aimed to detect *L. braziliensis* DNA in the nasal mucosa of cutaneous leishmaniasis (CL) patients with early mucous dissemination and to relate those findings to specific inflammatory responses. Nasal swabs were collected from patients with the cutaneous form of ATL. *L. braziliensis* DNA was investigated using TaqMan-based real-time PCR. The levels of serum cytokines (IL-12, IL-6, TNF-α, IL-10, IL-1β and IL-8) were measured by a multiplex cytometric array. A Poisson regression model was used to test prevalence ratios (PRs) and multivariate interactions of clinical and laboratory characteristics. Of the 79 CL patients, 24 (30%) had *L. braziliensis* DNA in the nasal mucosa. In the multivariate model, parasite DNA presence in mucosa was associated with a reduction in IL-12 levels (PR = 0.440; p=0.034), increased IL-6 levels (PR = 1.001; p=0.002) and a higher number of affected body segments (PR = 1.65; p<0.001). In this study, we observed a higher rate of early dissemination to the nasal mucosa than what was previously described. We suggest

that an enhanced Th1 profile characterized by higher IL-12 is important for preventing dissemination of *L. braziliensis* to the mucosa. Further evaluation of parasite-related interactions with the host immunological response is necessary to elucidate the dissemination mechanisms of *Leishmania*.

Keywords: *leishmaniasis*, immunology, multivariate analysis, mucocutaneous, flow cytometry

INTRODUCTION

Tegumentary leishmaniasis is a vector-borne disease with an incidence of approximately 0.7 to 1.2 million cases each year (World Health organization, 2021). The disease burden measured in disability-adjusted life years is higher than that of diseases such as leprosy, dengue fever and Chagas disease (Hotez et al., 2004). One of the main factors related to the negative impact of the disease is the possibility of sequelae related to the compromise of the nasal mucosa (Costa et al., 1987; Strazzulla et al., 2013). The presence of mucosal lesions is characteristic of American tegumentary leishmaniasis (ATL); it is estimated to occur in 3% to 5% of all patients with the disease in the Americas and is primarily caused by *Leishmania braziliensis* (Lessa et al., 2007).

Mucosal leishmaniasis (ML) is an outcome of ATL resulting from parasite dissemination from uncontained cutaneous leishmaniasis (CL) (Marsden et al., 1984; Amato et al., 2009). The presence of the parasite in the mucosa triggers an intense immune response dependent on macrophage activation (Da-Cruz et al., 2002; Carvalho et al., 2007; Conceição-Silva et al., 2018). This intense cellular reaction is responsible for the development of granulomatous lesions in the nasal septum and oropharyngeal structures (Conceição-Silva et al., 2018), reducing the quantity of parasites in late stages and diminishing the sensitivity of parasitological and molecular biology methods for diagnosis (Gomes et al., 2014). The natural history of ML shows that a possible paradox related to immunology exists. Although the presence of *Leishmania* in the mucosa over time stimulates an intense granulomatous response, earlier in disease progression, the parasite escapes from the host immunological response during dissemination periods to the mucosa (de Magalhães et al., 1986; Ávila et al., 2018; Martínez-López et al., 2018).

Early in the disease course, ML can coexist with active cutaneous lesions (Figueroa et al., 2009), but due to delayed access to healthcare, patients are normally diagnosed when the effects of destructive lesions, such as nasal septal perforation or destruction of facial architecture, have already occurred (Sampaio et al., 2019). The process begins with the dissemination of the parasite to the nasal mucosa, leading to microscopic and finally macroscopic changes (Amato et al., 2009). At first, lesions are oligosymptomatic and are not easily observed by the patient, which contributes to the delayed diagnosis (Boaventura et al., 2006). Later steps of disease development have been the focus of research in the field, limiting our understanding of the initial physio-pathogenic stages, including dissemination (Maretti-Mira et al., 2012; Ávila et al., 2018). *L. braziliensis* can be demonstrated in the nasal mucosa of patients with active cutaneous lesions (Figueroa et al., 2009; Romero et al., 2010; Canário et al., 2019), making these patients an interesting model to study early steps of disease development.

Here, we aimed to detect *L. braziliensis* DNA in the mucosa of ATL patients with active CL and to relate those findings with clinical and systemic immunological mediators related to the early dissemination (ED) of the parasite to the nasal mucosa.

METHOD

We performed a cross-sectional study with patients attending from February 2017 to December 2020 the Leishmaniasis Clinic at the University Hospital of Brasilia, a referral service for leishmaniasis diagnosis and treatment in the Brazilian mid-western region. Initially, patients with active cutaneous lesions suggestive of leishmaniasis were screened for inclusion, and clinical and laboratory data were collected. After initial investigation, patients under immunosuppressive therapy or without a confirmed diagnosis of CL were excluded, and only patients with cutaneous lesions confirmed to be ATL were included. All CL cases were defined according to a reference standard based on the results of clinical evaluation, indirect immunofluorescense, direct skin exam, culture of skin aspirates in Novy-MacNeal-Nicolle medium and polymerase chain reactions of skin fragments as described elsewhere (Gomes et al., 2014; Gomes et al., 2014).

Patients with ATL early dissemination (CL-ED) were defined as active CL patients with the presence of *L. braziliensis* DNA in the nasal mucosa. Patients with negative testing were considered controls (CL-ED-Neg). In our study, all CL patients were examined by an assistant dermatologist with anterior rhinoscopy and oroscopy and were referenced for endoscopic examination of the ear, nose, and throat by an expert otorhinolaryngologist. Dermatological clinical examination also included measurement of cutaneous lesions using an adhesive ORC-9752 scale (Orc forensics, Oregon City, USA). In any patient with CL in whom an active mucous lesion was concomitantly identified, we applied Lessa's classification as previously described: I, nodulation without ulcerations; II, superficial ulcerations; III, deep ulcerations; IV, septum perforation; and V, destruction of nasal architecture and altered facial structure (Lessa et al., 2012).

Anterior nasal swabs (Absorve™ sterile Sample collection swab, São Paulo, Brazil) were collected from all included patients. The swabs were rotated five times in each nasal fossa at the

anterior septum and inferior turbinate head. DNA extraction from the nasal swab samples was performed using the PureLink Genomic DNA Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Real-time polymerase chain reactions (RT-PCR) were performed with a TaqMan-specific probe for *L. braziliensis* detection (forward 5'-TGCTATAAAATCGT ACCACCCGACA-3', reverse 5'-GAACGGGGTTTCTGTAT GCCATTT-3'), a probe for FAM (6-carboxyfluorescein; TTGCAGAACGCCCCTACCCAGAGGC), and TAMRA (6-carboxytetramethylrhodamine) on a QuantStudio 1 (Thermo Fisher Scientific, Waltham, USA), as described by Gomes et al. (2017). To evaluate circulating cytokine levels, plasma from each patient was isolated, and tumour necrosis factor (TNF)-α, interleukin (IL)-10, IL-1β, IL-8, IL-6 and IL-12p70 levels were measured using the Human Inflammatory Cytokine Cytometric Bead Array (Becton Dickinson, Franklin Lakes, USA) with a FACSVerse flow cytometer (Becton Dickinson).

For sample size calculation, we aimed at a power of 0.9 and an alpha of 0.05. The calculation was performed based on the expected levels of TNF-α extrapolated from a study by Da-Cruz et al (Da-Cruz et al., 1996), considering that the levels of this cytokine in subjects with ED would be similar to those observed in ML patients (231.4 ± 76.3 pg/ml) and that patients without ED would have a cytokine level comparable to that in CL patients (43.5 ± 8.2 pg/ml). We considered that approximately 11% (Gomes, 2014) of the CL patients would have ED and that 50% of the initially screened subjects would have CL confirmed after investigation. With these assumptions, we included 91 patients to have the desired power and alpha.

Regression imputation using the dependent variable and significant covariates was used to estimate three missing values of the total area of the lesions. The association between ED and clinical variables (sex, age, lesion evolution time, total area of the lesions, number of lesions, number of affected body segments, presence of mucosal symptoms and location of the lesions - hip or legs, arms, face or neck, and chest or abdomen) and cytokine levels was tested using two samples T tests for the parametrical variable "age" and Wilcoxon rank sum tests for all the other variables that were shown to be non-parametrical. All the variables associated with ED having $p<0.25$ were included in the multivariate model. Multivariate analysis was performed with Poisson regression with robust variance (Zou, 2004), and variables were chosen using the stepwise backward selection strategy. At each step, model improvement was evaluated by the Bayesian information criterion. Multicollinearity was also evaluated using the variance inflation factor (VIF). All statistical analyses were performed with Stata Statistical Software Release 16 (StataCorp LLC, College Station, USA). This study was approved by the institutional review board of the University of Brasilia (1.521.691), and subjects signed an informed consent form before inclusion.

RESULTS

We recruited 93 patients, 14 of whom were excluded, nine for not having a leishmaniasis diagnosis and five for having an alternative diagnosis. The remaining 79 patients constituted our sample, with *L. braziliensis* DNA being identified in the nasal mucosa of 24 (30.3%) (**Figure 1**). The typical patient of our population was 41 years old, with a single lesion being approximately two months old on average. After

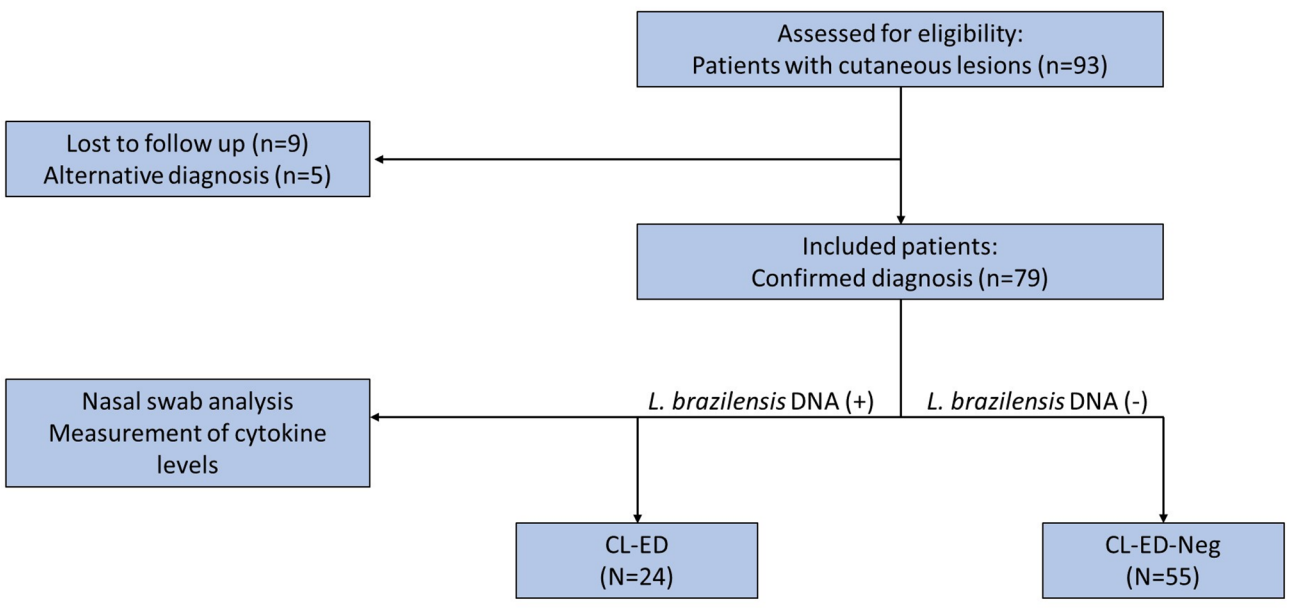

FIGURE 1 | Flow chart showing eligible and included patients as well as the final group configuration. n, number of patients; CL-ED, cutaneous leishmaniasis with early dissemination to mucosa; CL-ED-Neg, cutaneous leishmaniasis without early dissemination to mucosa.

otorhinolaryngological endoscopic examination, as expected, clinically active mucous lesions were found in only five patients in the CL-ED group (**Table 1**). In these patients, the lesions were early, not surpassing grade II in Lessa's classification (Lessa et al., 2012) (**Table 1**). All patients were treated according to the national CL treatment protocol with meglumine antimoniate at a daily dose of 20 mg Sb5+/kg for 20 days.

Cytokine levels are disclosed in **Table 2** and were apparently similar between groups in the univariate analysis. In the univariate model, ED was significantly associated with a higher number of affected body segments (p=0.005) and a higher number of lesions (p= 0.003) (**Table 2**). Important variables in the univariate analysis were initially entered into the model (**Table 2**). High multicollinearity was found, and the TNF-α level was dropped from the model based on its VIF of 14.06 and its higher p value in the univariate model. After the selection of variables, the only variables that remained significant and thus were included in the model were IL-12 level (prevalence ratio (PR) =0.44; 95% confidence interval (CI): 0.21-0.94; p=0.034), IL-6 level (PR=1.001; 95%CI: 1.0005-1.002; p=0.002) and number of affected body segments (PR= 1.65; 95%CI: 1.36-2.01; p<0.001).

DISCUSSION

In this study, we observed that 30.3% of patients with active cutaneous ulcers had molecular evidence of the presence of *L. braziliensis* in the nasal mucosa. In another study from an *L. braziliensis* endemic area, Canário et al. found parasite DNA in the mucosa of 7.8% CL subjects with clinically healthy nasal mucosa (Canário et al., 2019). This difference may be partially explained by the distinct inclusion criteria of the studies, since we did not exclude patients with CL if they were shown to have simultaneous early mucosal lesions.

In addition, all mucosal lesions found were early, not surpassing grade II of V in Lessa's classification (Lessa et al., 2012). Conversely, other studies have shown that by the time of diagnosis, approximately 60% of active ML cases are classified as grade III or greater (Lessa et al., 2012; de Lima et al., 2021). Thus, the early diagnosis of mucosal lesions in this study was associated with shorter disease duration, three months on average, and consequently less severe disease. Our data thus support the proposition by Boaventura et al. that earlier diagnosis may be made if patients with cutaneous lesions are subjected to systematic otorhinolaryngological examination

TABLE 1 | Description of early mucosal lesions of five patients with cutaneous leishmaniasis with concomitant mucous lesions and a positive result for *Leishmania braziliensis* DNA from nasal swabs.

ID	Clinical findings	Grade†
PCT1	Haematic crust on the nasal septum and swollen inferior turbinate mucosa	II
PCT2	Enanthem characterized by small red dot-like areas in the septal mucosa	I
PCT3	Infiltrative lesion at the base of the tongue	I
PCT4	Haematic crust in the left nasal septum region	II
PCT5	Aphthous lesion on the hard palate and nasal septum mucosa	II

†According to Lessa's staging system: I, nodulation without ulcerations; II, superficial ulcerations; III, deep ulcerations; IV, septum perforation; V, destruction of nasal architecture and altered facial structure; ID, patient identification number.

TABLE 2 | Univariate analysis of possible risk factors for early dissemination of *Leishmania braziliensis* to the nasal mucosa.

Variables	CL-ED-Neg	CL-ED	p value
Age years (SD)†	42.6 (17.6)	38.4 (18.4)	0.17
Time since disease onset, median (IQR)	2 (2)	2 (1.5)	0.87
Number of lesions (SD)†	1 (1)	1 (4.5)	0.003
Number of affected body segments, median (IQR)†	1 (0)	1 (1)	0.005
Total area of the lesions (cm^2)†	11.94 (12.22)	17.56 (16.07)	0.097
Sex			
Male (%)	35 (63.6)	15 (62.5)	1
Female (%)	20 (36.6)	9 (37.5)	
Mucosal symptoms (%)	13 (26.3)	7 (29.17)	0.580
Cutaneous lesions on the legs and hip (%)	22 (40)	12 (50)	0.464
Cutaneous lesions on the arms (%)†	16 (29)	11 (45.8)	0.198
Cutaneous lesions on the thorax and abdomen (%)†	3 (5.4)	5 (20.8)	0.051
Cutaneous lesions on the head and neck (%)†	5 (9)	6 (25)	0.080
Cytokine levels (pg/ml)			
IL-12 (SD)†	4.09 (2.8)	3.19 (0.2)	0.170
TNF-α (SD)†	7.93 (11.7)	44.79 (195.9)	0.240
IL-10 (SD)	3.21 (2.1)	2.54 (0.7)	0.390
IL-6 (SD)†	15.98 (41.6)	41.41 (171)	0.170
IL-1β (SD)†	6.12 (1.3)	6.06 (2.2)	0.160
IL-8 (SD)	243.2 (576.6)	421.08 (1,216.3)	0.850

TNF, tumour necrosis factor; IL, interleukin; SD, standard deviation; IQR, interquartile range. †Variables initially entered in the model. CL-ED, cutaneous leishmaniasis with early dissemination to mucosa; CL-ED-Neg, cutaneous leishmaniasis without early dissemination to mucosa.

(Boaventura et al., 2006). On the other hand, the proportion of patients with evidence of ED to the nasal mucosa in our study was lower than those found in Colombia (45%-58%) (Figueroa et al., 2009; Martínez-Valencia et al., 2017). It is possible that this difference between studies is partially explained by the *Leishmania* species responsible, since in Colombia, most of the isolates are from *Leishmania panamensis* (Martínez-Valencia et al., 2017), and most mucosal lesions (61%) due to this species occur simultaneously with the active ulcer (Osorio et al., 1998).

Clinical lesions on the mucosa can be difficult to access, and early lesions can lead to only discolouration of mucosa long before the beginning of mucous-specific symptoms (Nishino et al., 1986; Oliveira et al., 1995; Silveira, 2019). Consequently, these lesions can sometimes only be found by expert examiners with the use of special equipment and magnification. Once ML requires longer courses of treatment, the recognition of early mucous lesions is paramount for achieving satisfactory cure (Ministério da Saúde do Brasil SDVES and Departamento de Vigilância Epidemiológica, 2017).

Dissemination of the parasite from an initial cutaneous lesion is assumed to be the main mechanism of ML development (Marsden et al., 1984; Amato et al., 2009). Although conceivable, multiple infections are unlikely to be the culprit for this form. Parasite similarity occurs between cutaneous and mucosal lesions of the same patient, with the parasite genetic variability in these patient lesions being less prominent than the variability between different patients, as shown in an *L. braziliensis* kDNA signature study (Oliveira et al., 2013). Another possibility is that the parasite disseminates later in the disease process. It has been suggested that amastigotes from ML patients have a decreased ability to be internalized and grow more slowly than those from CL patients (Gomes et al., 2016) and that stationary phase promastigotes from ML are more resistant to nitric oxide (Ávila et al., 2018). These findings suggest that ML parasites are transmitted by vectors as promastigotes that are initially more resistant and more likely to disseminate (Ávila et al., 2018). Additionally, the protective immune responses against *L. braziliensis* are weaker in the early disease process (Unger et al., 2009). Thus, parasite and host immune factors make it more likely that early dissemination is the essential step leading to the development of ML in most cases.

In line with the pool of evidence found in the literature, our univariate analysis showed that the number of lesions and the number of affected body segments were associated with ED of *L. braziliensis* to the nasal mucosa. A greater number of lesions was also found to be associated with ED in another study (Maretti-Mira et al., 2012). Interestingly, the clinical risk factors we found were the same as those found in previous studies (Marsden et al., 1984; Cuentas et al., 1984; Turetz et al., 2002), suggesting a predictive relationship between ED and the possible development of ML. The confirmation of this relationship, however, would require a study with long-term follow-up, since many patients commonly have cutaneous lesions a long time before the development of ML (with 37.1% having lesions more than 5 years earlier and 5.7% having lesions more than 15 years earlier according to one classical study (Marsden et al., 1984).

ML is characterized by a marked inflammatory infiltrate that is responsible for maintaining autoaggression in the presence of few or no parasites (de Magalhães et al., 1986). The inflammatory process is driven by excess proinflammatory mediators such as TNF-α and IFN-γ that are unchecked by regulatory cytokines such as IL-10 and TGF-β (Bacellar et al., 2002; Campos et al., 2018). TNF-α is the most studied mediator (Castes et al., 1993; Cabrera et al., 1995; Da-Cruz et al., 1996; Ribeiro-de-Jesus et al., 1998), but other inflammatory cytokines, such as IL-6 (Castellucci et al., 2006), IL-1β (Moreira et al., 2017) and IL-8 (Vargas-Inchaustegui et al., 2009), may also play a role. In the classical paradigm framework, the development of ML is related to a Th1 phenotype characterized by CD4+ T lymphocytes expressing IFN-γ. In this context, IL-12 is an inducer, and IL-10 is an opposing cytokine (Ribeiro-de-Jesus et al., 1998; Silveira, 2019). However, the same inflammatory mediators linked to protection also play a role in tissue destruction associated with ML (Hartley et al., 2014). Some authors have suggested that this apparent paradox may be associated with the dynamics of inflammatory cytokine expression in relation to disease evolution (Ribeiro-de-Jesus et al., 1998; Unger et al., 2009), with early diminished IFN-γ and TNF-α expression being associated with worse prognosis (Unger et al., 2009). We investigated the association of key cytokines with the ED of *L. braziliensis* to the nasal mucosa to better understand the role of the early immune response in the development of ML. To account for possible confounding clinical variables, we opted to use statistical multivariate modelling to assess the association between cytokine levels and the outcome. Higher IL-6 levels were significantly associated with ED in our final multivariate model (PR=1.001; p=0.002). Although IL-6 has been implicated in the development of mucosal disease (Castellucci et al., 2006), in our study, the effect size (PR=1.001; p=0.002) was too low for the result to be considered of physio-pathological significance, especially considering the effect sizes of the other independent variables included in the model.

IL-12 was a protective mediator against ED of *L. braziliensis* to the nasal mucosa (PR=0.44; p=0.034; 95%CI: 0.21-0.94) in our final multivariate model. IL-12 is a cytokine classically associated with the development of the Th1 cytokine response (O'Garra and Vieira, 2007) and is in the proinflammatory cytokine milieu associated with the development of ML (Ribeiro-de-Jesus et al., 1998; Silveira, 2019). This protective role of IL-12 seems contradictory since most studies have linked the development of ML with the proinflammatory Th1 response (Castes et al., 1993; Da-Cruz et al., 2002; Bacellar et al., 2002). However, this finding may be explained by the scarcity of studies that address the early immunopathological steps leading to ML development. In a transcriptomic study with ten patients with CL, five of whom later developed ML, Maretti-Mira et al. suggested that patients prone to mucosal disease have an insufficient or delayed development of immune response (Maretti-Mira et al., 2012). In another study by Gomes et al, IFN-γ knockout (KO) mice

were infected with amastigotes from patients with ML and CL. Interestingly, in mice infected with ML-derived amastigotes, cutaneous lesions appeared later, but a greater number of parasites were observed in the spleen (Gomes et al., 2016). The same group later showed that the stationary-phase promastigotes derived from ML are more resistant to killing by nitric oxide (NO) and reactive oxygen species than are the CL-derived promastigotes. On the other hand, a lower resistance of ML amastigotes to NO was observed. Altogether, the findings of these two studies suggest that earlier in infection, ML parasites have a weaker capacity to stimulate the immune response at the site of infection but a greater ability to disseminate (Ávila et al., 2018). The delayed development of a protective immune response may facilitate parasite dissemination, and chronic systemic exposure to *Leishmania* antigens could in turn lead to the exacerbated inflammatory immune response associated with mucosal disease. This idea is in accordance with the finding that in cured ML patients, the duration of active disease is positively associated with antigen-induced production of IFN-γ but negatively associated with IL-10 production (Nogueira et al., 2014).

It is important to state that although this study represents the evaluation of a representative population the presence of untested confounders is always a limitation of clinical observational studies. This is especially true for the study of host-parasite immunological interaction. The present results show that future experiments are feasible and paramount for the study of dissemination mechanisms present in ML cases. The use of more complex laboratory methods, the search for a wider panel of mediators, a wider profile of clinical characteristics and the investigation of *Leishmania* RNA virus associations are important factors that must be searched in future protocols and that may significantly influence the clinical presentation of CL and ML.

Our study shows that the Th1 response, characterized by higher IL-12 levels, possibly helps limit the disease to the cutaneous site of infection, preventing dissemination to the nasal mucosa. We hypothesize that during the cutaneous phase of the disease, an insufficient rather than exacerbated immune response is responsible for the dissemination of the parasite and later development of mucous disease. These findings are, however, of exploratory nature and deserve to be more thoroughly investigated in other studies. Our study also highlights the importance of a detailed search for mucous commitment in CL to avoid insufficient treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitê de Ética em Pesquisa da Faculdade de Medicina da Universidade de Brasília - UnB. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

DB: conception, design, data acquisition, analysis, interpretation of data, drafting, final approval. OT: data acquisition, analysis, interpretation of data. CA: data acquisition, analysis, interpretation of data. GF: data acquisition, analysis and interpretation. SS: data acquisition and analysis. BR: data acquisition and analysis. CG: conception, design, data acquisition, analysis, interpretation of data, drafting, final approval, supervision. RS: conception, design, data acquisition, analysis, interpretation of data, drafting, final approval, supervision. All authors contributed to the article and approved the submitted version.

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Trypanin Disruption Affects the Motility and Infectivity of the Protozoan *Trypanosoma cruzi*

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The flagellum of Trypanosomatids is an organelle that contributes to multiple functions, including motility, cell division, and host–pathogen interaction. Trypanin was first described in *Trypanosoma brucei* and is part of the dynein regulatory complex. *Tb*Trypanin knockdown parasites showed motility defects in procyclic forms; however, silencing in bloodstream forms was lethal. Since *Tb*Trypanin mutants show drastic phenotypic changes in mammalian stages, we decided to evaluate if the *Trypanosoma cruzi* ortholog plays a similar role by using the CRISPR-Cas9 system to generate null mutants. A ribonucleoprotein complex of SaCas9 and sgRNA plus donor oligonucleotide were used to edit both alleles of *Tc*Trypanin without any selectable marker. *Tc*Trypanin –/– epimastigotes showed a lower growth rate, partially detached flagella, normal numbers of nuclei and kinetoplasts, and motility defects such as reduced displacement and speed and increased tumbling propensity. The epimastigote mutant also showed decreased efficiency of *in-vitro* metacyclogenesis. Mutant parasites were able to complete the entire life cycle *in vitro*; however, they showed a reduction in their infection capacity compared with WT and addback cultures. Our data show that *T. cruzi* life cycle stages have differing sensitivities to *Tc*Trypanin deletion. In conclusion, additional work is needed to dissect the motility components of *T. cruzi* and to identify essential molecules for mammalian stages.

Keywords: *Trypanosoma cruzi* (*T. cruzi*), trypanin, motility, detached flagellum, metacyclogenesis, CRISPR-Cas9, SaCas9

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INTRODUCTION

Trypanosoma cruzi is a protozoan parasite of the Trypanosomatidae family, which bears a group of early diverging eukaryotes. This parasite is the etiological agent of Chagas disease, a potentially life-threatening illness that affects about 6–8 million people around the world (Mills, 2020). *Trypanosoma cruzi* epimastigotes (a replicative form) differentiate into metacyclic trypomastigotes (MTs), the infective and non-replicative forms in triatomine insects. MTs are released within insect feces and, once introduced into a host, can invade almost all nucleated cells. Once inside the host cells, they differentiate into amastigotes, a replicative form containing a short flagellum. After a period of multiplication intracellularly, the amastigote forms transform into bloodstream trypomastigotes; these cells egress the host cells, cross the extracellular matrix, and swim in a crowded and viscous environment (blood) to be able to reach different cell types of the mammalian organism (Ferri and Edreira, 2021). The most studied *T. cruzi* virulence factors are surface proteins, such as gp82, gp63, trans sialidase, and MASP (Bartholomeu et al., 2009; Belew et al., 2017; de Castro Neto et al., 2021). The contribution of parasite motility in this physiological process remains to be elucidated.

Trypanosoma brucei and *Leishmania mexicana* parasite motility has been explored extensively compared with *T. cruzi*. To understand *T. brucei* parasite motility, 41 genes were selected based on conserved flagellum genes among other motile eukaryotic organisms and silenced using RNAi approaches. This strategy allowed the identification of new components of motile flagella and the characterization of the phenotype of the mutant based on the severity of the motility defects (Baron et al., 2007). A similar approach was used to characterize 20 new proteins associated with the *T. brucei* paraflagellar rod identified through proteomics (Portman et al., 2009). Using CRISPR/Cas9-assisted gene deletion, Beneke et al. dissected the components of *L. mexicana* flagellum proteome (Beneke et al., 2019). The authors obtained 56 mutants to flagellum proteins with altered swimming speed and morphological defect phenotypes. Interesting, some of the mutants were unable to develop in the insect vector, as observed by infection assays (Beneke et al., 2019).

Unlike *T. brucei*, *T. cruzi* lacks RNAi machinery (DaRocha et al., 2004a), but recent advances on CRISPR/Cas9 technology were adapted to dissect the gene function in this organism (Peng et al., 2014; Lander et al., 2015; Soares Medeiros et al., 2017; Burle-Caldas et al., 2018; Chiurillo and Lander, 2021). Despite these advances in the genetic manipulation methodologies in *T. cruzi*, only few flagellar components, gp72, PFR-1, and PFR-2, were characterized (Cooper et al., 1993; Lander et al., 2015). The lack of gp72 caused detached flagella and reduced metacyclogenesis. Though they were able to infect host cells in culture, they were not able to generate bloodstream trypomastigotes; instead, only amastigote forms were released (de Jesus et al., 1993; Cooper et al., 1993). The multicopy genes PFR-1 and PFR-2 were edited using CRISPR/Cas9 technology, and the null mutant also showed a detached flagellum, and they were unable to build the paraflagellar rod structure as shown by transmission microscopy (Lander et al., 2015).

Trypanosoma cruzi and *T. brucei* have conserved flagellar structures, such as an axoneme with 9 + 2 microtubules, and kinetoplastid-specific extra-axonemal structure called paraflagellar rod (PFR). The PFR and axoneme are surrounded by the membrane flagellum and the PFR is linked to microtubules 4 to 7 of the axoneme similar to that in *T. brucei* (De Souza, 2002; Langousis and Hill, 2014). Similar to other Trypanosomatidae, the flagellum beating in *T. cruzi* starts at the tip and propagates to the base. The distance and speed vary cell to cell, with persisting and tumbling modes of motility, with some parasites presenting an intermediate state. This tumbling period seems important for changes in directionality (Ballesteros-Rodea et al., 2012; Walker and Wheeler, 2019). Axoneme structure is necessary for motility in trypanosomatids (Langousis and Hill, 2014). Dynein motors drive the sliding of the microtubules and flagellum beating is produced by the temporal–spatial control of dynein conformations (Lin and Nicastro, 2018). The regulation of dynein conformations requires the nexin–dynein regulator complex (NDRC), where one of the most studied component in trypanosomatids is the protein Trypanin (Ralston and Hill, 2006; Kabututu et al., 2010), the orthologs of which are variously called N-DRC4, GAS8, GAS11, and PF2.

Trypanin was well-characterized in *T. brucei*, an ~54-kDa protein conserved among other trypanosomatids and found mainly in the cytoskeletal fraction (Hill et al., 2000). RNAi targeting of *Tb*Trypanin in procyclic forms causes an uncoordinated flagellar beat and non-directional motility, giving a tumbling motion (Hutchings et al., 2002). *Tb*Trypanin is essential in *T. brucei* bloodstream forms as observed by RNAi experiments, suggesting that perhaps flagellum beating contributes to separating subpellicular microtubules and thus cytokinesis initiation (Ralston and Hill, 2006). Later genetic analysis of other motile components of the flagellum confirmed that normal motility is required for cytokinesis (Broadhead et al., 2006; Ralston et al., 2006).

Later analysis of the *Chlamydomonas* insertional mutagenesis clone pf2 with a defect in motility revealed mutation of *Tb*Trypanin ortholog disrupted N-DRC confirmed by electron microscopy. *pf2* mutants showed defects in the 96-nm repeat of the flagellar axoneme. The N-DRC is a complex of proteins that responds to signals from radial spoke proteins interacting with the central pair complex, coordinating dynein motor activity temporally and spatially and commonly associated with inner arm dyneins (Rupp and Porter, 2003). In mammals, the Trypanin homolog is called GAS11 (growth arrest specific 11) in humans and GAS8 in mice. The finding that GAS11 is expressed in cells without a motile cilium and is associated with the cytoskeleton suggests that, in addition to its suspected role in the regulation of axonemal dynein, it also participates in microtubule/dynein-dependent processes outside the axoneme (Bekker et al., 2007; Colantonio et al., 2006; Evron et al., 2011).

In this work, we characterized *T. cruzi* Trypanin by generating null mutants using CRISPR-Cas9 methodology. We found that *Tc*Trypanin −/− mutants have several phenotypic changes including reduced epimastigote growth and

differentiation into metacyclic trypomastigotes, increased ratio of parasites with detached flagellum, motility defects, and reduced infection capacity.

MATERIAL AND METHODS

Parasite Maintenance and Growth Curve

Trypanosoma cruzi epimastigotes from the Dm28c clone were cultured in a liver infusion tryptose (LIT) medium supplemented with heat-inactivated fetal bovine serum (10%), hemin, and penicillin/streptomycin (Camargo, 1964). MTs were obtained using the protocol described by Contreras et al. (1985). Briefly, epimastigote cultures at the stationary phase were centrifuged at 3,000×*g* and the cell pellets were washed with 1× PBS, then 5×10^8 parasites were resuspended in 1 ml of triatomine artificial urine (TAU, 190 mM NaCl, 17 mM KCl, 8 mM sodium phosphate buffer, 2 mM $MgCl_2$, 2 mM $CaCl_2$, pH 6.0) and incubated for 2 h at 28°C. After that, the culture was diluted 100-fold in TAU3AAG medium (TAU supplemented with 50 mM sodium glutamate, 10 mM L-proline, 2 mM sodium aspartate, and 10 mM glucose) and incubated for 72 h at 28°C. Then, we calculated the MT yield as previously reported (Balcazar et al., 2017) with minor change by using horse serum instead of human serum. For the growth curve analysis of *Tc*Trypanin −/− mutants and wild-type epimastigotes, log-phase cultures were diluted to 1×10^5 parasites/ml and the parasite multiplication was quantified daily using a Neubauer chamber. Parasite cultures reaching the stationary phase were diluted 10-fold for counting.

To do differential counting of nuclei and kinetoplast per cell, logarithmic phase parasites were DAPI stained as follows: 5×10^6 parasites were harvested and cells were pelleted at 3,000×*g* for 5 min. The cells were washed twice with PBS, and the parasites were coated on slides with Fluoromount-G™ Mounting Medium with DAPI (Thermo Fisher). At least 100 random parasites for each cell line were analyzed using fluorescence microscopy.

Cell Cycle Analysis

Epimastigote forms at logarithmic phase ($5–6 \times 10^6$ parasites/ml) were fixed with 70% methanol for 16 h, washed twice with PBS, resuspended in 100 µl of 2× PI solution (3.4 mM Tris–HCl, 10 mM NaCl, propidium iodide 30 µg/ml, and RNAse 100 µg/ml), and added to 100 µl of PBS. These samples were analyzed by flow cytometry using the FACSCanto II equipment, and data were analyzed using the FlowJo 7.6 version software.

Bioinformatics Analysis

For phylogenetic tree analyses, Trypanin ortholog amino acid sequences from representative flagellated organisms were obtained from the TriTrypDB. This included the Trypanin ortholog from *Homo sapiens*, *Mus musculus*, and *Danio rerio*. The accession number of each sequence and multiple alignment data are found in **Supplementary Figure 1**. The sequences were analyzed using the SeaView v. 5.0.4 software (Gouy et al., 2010) setting BioNJ with bootstrap of 1,000 replicates.

Plasmid Construction and Single Guide RNA Transcription

Single guide RNA (sgRNA) design to target *Tc*Trypanin (C4B63_48g99) was performed using EuPaGDT (http://grna.ctegd.uga.edu/), selecting *T. cruzi* Dm28c (TriTryDB-28) as a reference genome and the Cas9 nuclease from *Staphylococcus aureus* (*Sa*Cas9) (Peng and Tarleton, 2015). To ensure that the sgRNA could be used to target other *T. cruzi* strains, a BlastN search was done using 50 nt (25 bases upstream of the SaCas9 cleavage site plus 25 downstream) as query, against all available genomes at TriTrypDB. The BlastN output was manually curated.

For sgRNA *in-vitro* transcription, we constructed a plasmid containing a T7 promoter, the SaCas9 sgRNA scaffold, and Hepatitis Delta Virus ribozyme (HDV), which we named pT7-SaCas9sgRNA-*Bsa*I (**Supplementary Figure 2A**). In this plasmid, the specific region of the sgRNA can be replaced by digestion with *Bsa*I followed by cloning of annealed oligonucleotides. The sgRNA-Trypa442 template was created by oligonucleotide annealing of sgRNA-Trypa442-Plus (ATAGGAGAGTCATGAGATGCGGATT) and sgRNA-Trypa442-Minus (AAACAATCCGCATCTCATGACTCTC) and cloning in the *Bsa*I sites of pT7-sgRNA scaffold-HDV, pT7-SaCas9-sgRNA-Trypa442 (**Supplementary Figure 2B**). pT7-SaCas9-sgRNA-Trypa442 was used as a template for the sgRNA *in-vitro* transcription using MEGAscript™ T7 Transcription Kit (Thermo Scientific), following the instructions of the manufacturer. sgRNA quantity and quality were confirmed by Nanodrop™ quantification and 2% agarose gel electrophoresis.

To restore *Tc*Trypanin expression, the full-length coding sequence without the stop codon of *Tc*Trypanin was PCR amplified with Trypa-For-*Xba*I (5′-AAATCTAGAATGCCACCAAAGGCGGTTCGTG-3′) and Trypa-Rev-*Bam*HI (5′-AAAGGATCCCGACAATTCCCGCCGTCAGAAA-3′) oligonucleotides, using genomic DNA from the Dm28c clone as a template. The PCR product was digested with *Xba*I/*Bam*HI and cloned at the same restriction sites in the plasmids pTREX-Amastin::GFP-Neo (Cruz et al., 2012) and pTREX-Amastin::HA-Hygro (unpublished results) replacing the delta-amastin coding sequence. The resulting vectors allow the expression of *Tc*Trypanin fused to GFP or HA tag. These plasmids were transfected by electroporation using the Amaxa Nucleofector, and the G418-resistant population was obtained as described by DaRocha et al. (2004b) and Pacheco-Lugo et al. (2017).

CRISPR/Cas9 Editing and RFLP Genotyping

Wild-type epimastigotes of Dm28c were chosen to obtain mutant parasites with both alleles of *Tc*Trypanin edited. We performed the protocol described by Soares Medeiros et al. (2017) and Burle-Caldas et al. (2018). Briefly, 5×10^6 log-phase parasites were electroporated with 20 µg of affinity-purified SaCas9 recombinant protein, 10 µg of sgRNA, and 30 µg of single-stranded DNA (ssDNA) donor. We electroporated

epimastigote cultures twice with an interval of 7 days between each electroporation, following the electroporation conditions previously described (Pacheco-Lugo et al., 2017). After 48 h of each electroporation, the genomic DNA (gDNA) was isolated using PureLink™ Genomic DNA Mini Kit (Thermo Fisher) from part of the culture to confirm the gene disruption, by PCR reactions using oligonucleotides that allow the amplification of the entire CDS of *Tc*Trypanin. The PCR product was purified, digested with *Bam*HI enzyme, and electrophoresed in 1% agarose gel. *Tc*Trypanin −/− amplicon with Trypa-For-*Xba*I and Trypa-Rev-*Bam*HI primers (described above) is 1,402 bp long and, after *Bam*HI restriction, generates two bands (926 and 428 bp) detected on agarose gel. The resulting restriction digested fragments (restriction fragment length polymorphism—RFLP) was detected by ethidium bromide staining, and visualization was done with the UVP Bioimaging system.

After confirmation of gene editing, transfected parasites were cloned by limiting dilution in a 96-well ELISA plate to obtain clonal populations. To improve parasite growth during the cloning step, 90 µl of conditioned medium (media from the log-phase culture were recovered by centrifugation followed by supernatant filtering) was added to each well, and the plate was maintained in a wet chamber at 28°C and monitored each week for clone growth. One month later, we collected several clones and tested them with RFLP, as described above, to confirm the *Tc*Trypanin null mutant clones (*Tc*Trypanin −/−).

Video Microscopy

Epimastigotes from the axenic culture of *Tc*Trypanin −/−, *Tc*Trypanin-addback (AB), and WT parasite were collected and counted in a Neubauer chamber. Ten microliters of cell culture at a density of 5×10^6–1×10^7 parasites/ml of the cell was placed on a microscope slide and filmed for at least 30 s at 5 frames per second using a Leica AF6000 Modular System microscope at ×20 magnification using darkfield illumination. Cell swimming was analyzed as previously described (Wheleer, 2017). Briefly, cells were identified in each frame of the darkfield image using a maxima finding algorithm and then cell swimming paths were generated by connecting cell locations based on their movement over the preceding two frames. Mean swimming speed was calculated from these swimming paths.

Immunofluorescence and Localization of GFP-Tagged *Tc*Trypanin

Cell cultures of *Tc*Trypanin −/−, *Tc*Trypanin −/− carrying pTREX *Tc*Trypanin::HA, and wild type were fixed with 4% paraformaldehyde and coated on poly-lysine coverslips for 20 min. Coverslips were washed with PBS and the cells permeabilized with PBS + 0.1% Triton X-100 and then blocked with 3% (m/v) BSA for 1 h at room temperature (RT). The parasites were incubated with a dilution 1:100 of the monoclonal antibody 2F6 (mAb 2F6), which recognizes a flagellar protein of ~70 kDa (Ramos et al., 2011), for 16 h at 4°C, washed three times with PBS + 0.05% Tween-20, and incubated with the secondary antibody anti-mouse conjugated with Alexa-488 (1:500). The coverslips were washed three times, mounted on a microscope slide, and analyzed by confocal microscopy using a Nikon equipment (confocal microscope A1R multiphoton). Parasites *Tc*Trypanin −/− (pTREX-*Tc*Trypanin::GFP), WT (pTREX-*Tc*Trypanin::GFP), and WT (pTREX-GFP) were fixed with paraformaldehyde 4%, coated on poly-lysine coverslips for 20 min, mounted with Fluoromount-G™ Mounting Medium with DAPI (Thermo Fisher), and visualized in a Nikon confocal microscope.

Cytoskeleton Preparation

Epimastigotes (5×10^6) of *Tc*Trypanin −/− (pTREX-*Tc*Trypanin::GFP), WT (pTREX-*Tc*Trypanin::GFP), and WT (pTREX-GFP) were coated on poly-lysine coverslips and incubated for 20 min at RT. To preserve the parasite cytoskeleton, 40 µl of cold PEME (100 mM PIPES, 1 mM $MgSO_4$, 0.1 mM EDTA, 2 mM EGTA, pH 6.9) + Triton X-100 (1% v/v) was added and incubated for 10 s and washed twice with PBS Gadelha et al., 2005. Then, the parasites were fixed with 4% paraformaldehyde for 10 min at RT, washed with PBS, and resuspended in 200 µl of PBS. The coated coverslips with fixed parasites were mounted with Fluoromount-G™ Mounting Medium with DAPI (Thermo Fisher) and visualized in a Nikon confocal microscope.

Western Blot and Cell Fractionation

To extract the epimastigote cytoskeleton in cell suspension, WT Dm28c expressing GFP or *Tc*Trypanin −/− expressing *Tc*Trypanin::GFP cultures was centrifuged at 3,000×g and washed twice with PBS, and the cell pellet was incubated with 50 µl of ice-cold PEME + Triton X-100 (1%) buffer on ice for at least 5 min. The samples were centrifuged at 16,000×*g* for 20 min at 4°C. The pellet (cytoskeleton-enriched fraction) and supernatant fractions were collected in two microtubes. The pellet was washed twice with ice-cold PEME, resuspended in 50 µl of cold PEME, and stored at −20°C until the Western blot assay.

Total cell extracts were obtained by harvesting epimastigotes followed by centrifugation at 3,000×*g*. The cell pellet was washed with PBS and resuspended in SDS-PAGE loading buffer to have 1×10^7 parasites per loading in a polyacrylamide gel. After SDS-PAGE, the proteins were transferred to a PVDF membrane, which was blocked with 5% non-fat milk and incubated with polyclonal anti-GFP (1:1,000) at 4°C for 16 h. After the first antibody incubation, the membrane was washed three times with PBS + Tween 0.05% (v/v) and incubated with anti-rabbit conjugated with peroxidase (1:1,000) at 37°C for 1 h. Finally, the membrane was washed three times with PBS + Tween and antibody recognition was detected using the ECL Chemiluminescence Kit (Thermo Fisher) and X-ray film. Images were acquired exposing X-ray films on the UVP Bioimaging system.

Cell Infection Assays and Tissue-Cultured Derived Trypomastigote Count

Cultures of LLC-MK2 cells were treated with trypsin (0.05%) (Thermo Fisher) and washed twice with PBS. Cells (4×10^4) were

placed in a 24-well plate containing coverslips and cultured in RPMI 1640 (Thermo Fisher) supplemented with 5% fetal bovine serum (FBS) in 5% CO_2. WT, *Tc*Trypanin −/−, and *Tc*Trypanin −/− AB tissue-cultured derived trypomastigotes (TCTs) were harvested from supernatants from previously infected cultures and counted in a Neubauer chamber. Infection assays were performed using an infection ratio of 10 parasites per LLC-MK2 cell (MOI of 10:1) during 2 or 4 h. After the infection period, cells were washed with 1× PBS three times to remove extracellular forms, and fresh RPMI media supplemented with 5% of FBS was added. After 4 days of infection, the TCT release was analyzed by counting in a Neubauer chamber. TCT releasing was determined by harvesting TCTs 4, 5, 6, and 7 days after infection in the same conditions.

Scanning and Transmission Electron Microscopy

Log-phase epimastigotes were centrifugated at 3,000×*g* for 5 min and washed with 1× PBS. Then, the parasites were processed as described by de Almeida et al. (2021). Samples were analyzed using a scanning electron microscope (Jeol JSM-6010 Plus/LA) operating at 20 keV.

RESULTS

Phylogenetic Analysis of *Tc*Trypanin

Trypanin is a highly conserved protein with 37% identity between GAS8 (*Homo sapiens*) sequence and *Tc*Trypanin

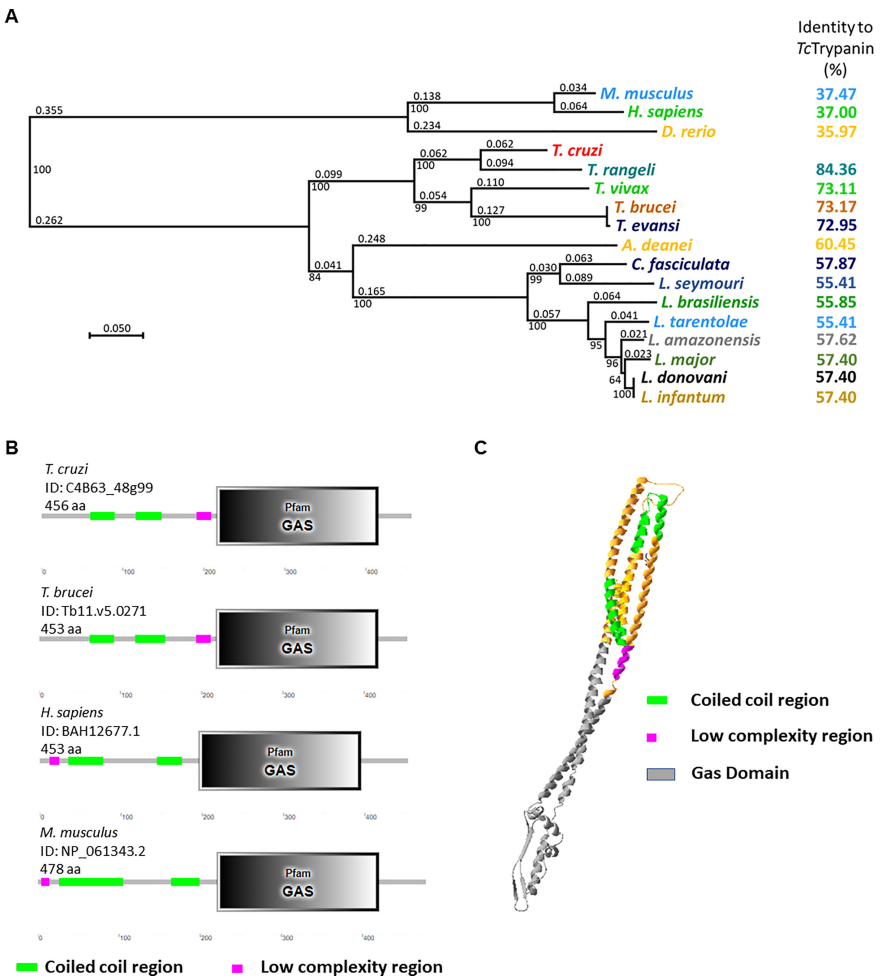

FIGURE 1 | Bioinformatics analysis of *Tc*Trypanin. **(A)** Phylogenetic tree from protein sequences of related tripanosomatids. Amino acid sequences were aligned using SeaView software, and the aligned sequences were used to generate a phylogenetic tree (SeaView 5.4). **(B)** Conserved domains found in *Tc*Trypanin and *Trypanosoma brucei*, human, and mouse orthologs. The bionformatic tool SMART (http://smart.embl-heidelberg.de/) found the conserved GAS domain and coiled coil region at similar regions. **(C)** 3D structure prediction of *Tc*Trypanin protein. The molecular model was generated by the Phyre3 server (Kelley et al., 2015) from the sequence of the *Tc*Trypanin protein from Dm28c (C4B63_48g99). The model shows the predicted tertiary structure that shows higher confidence with colicin IA from *Escherichia coli*. The color scheme corresponds to the features found by SMART analysis.

(*T. cruzi* Dm28c clone—ID: C4B63_48g99). As expected, phylogenetic analysis shows that the *Trypanosoma rangeli* SC58 strain (ID: TRSC58_03641) is the closet related Trypanin among trypanosomatids followed by the *T. brucei* sequence (ID: Tb927.10.6350), sharing 76.32% identity (**Figure 1A**). *Tc*Trypanin presents higher divergence when compared with *Leishmania* species (55.41% to 57.62% identity). For additional evidence of its conservation, we searched for conserved Pfam domains using the SMART software (Letunic, 2004). We found the growth arrest-specific superfamily domain (GAS) (e-value: 6.7×10^{-60}) starting at position 216 to 415, similar to its orthologs (**Figure 1B**). This domain is present in proteins

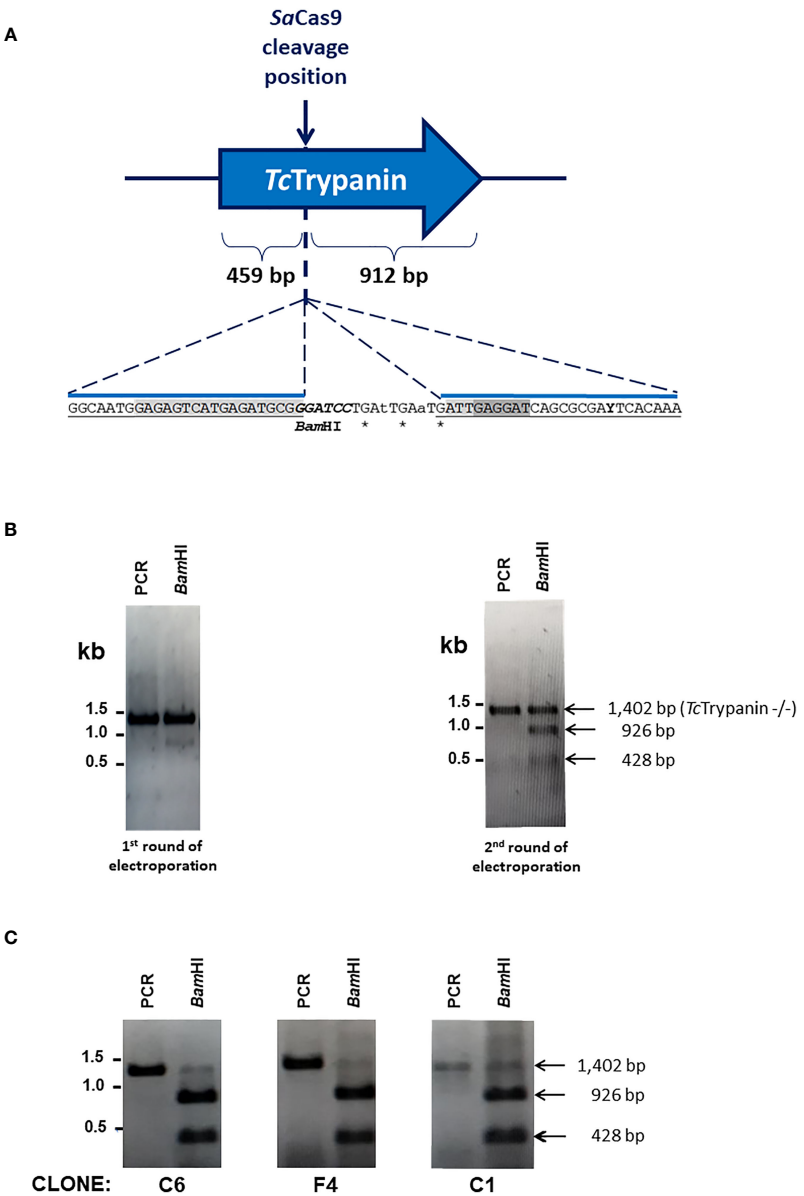

FIGURE 2 | Genome editing of *Tc*Trypanin using the CRISPR/Cas9 RNP complex. **(A)** Schematic representation of the sgRNA targeting site by *Sa*Cas9 RNP complex in the *Tc*Trypanin gene (C4B63_48g99). The RNP complex cleaves right after nucleotide 459 of *Tc*Trypanin coding sequence (CDS length: 1,371 bp). It also represented the insertion point of a *Bam*HI restriction site (italics) to easily track parasite editing through PCR-RFLP and stop codons (asterisks) to ensure coding sequence disruption by homologous recombination using a donor oligonucleotide. The sequence highlighted in light gray in the donor sequence corresponds to the sgRNA target site, and the dark gray sequence is the PAM sequence. **(B)** PCR-RFLP of *Tc*Trypanin showing genome editing of wild-type parasites. The image gels show undigested PCR product (amplicon sizes: WT *Tc*Trypanin = 1,387 bp and *Tc*Trypanin –/– = 1,402 bp) and *Bam*HI-digested (*Bam*HI) PCR product of the full-length ORF of *Tc*Trypanin of two cultures. The left image corresponds to the PCR-RFLP of a mixed population of parasites transfected once with *Sa*Cas9 RNP plus donor sequence. The right gel corresponds to PCR-RFLP of a culture retransfected with RNP complex. **(C)** The PCR-RFLP of individual clones containing both *Tc*Trypanin alleles edited.

required for diverse cellular functions such as microtubule organization and cellular division. Flagellated organisms such as *Chlamydomonas* and *T. brucei* have a homolog of GAS8 named N-DRC and *Tb*Trypanin, respectively, and knockdown, disruption, or null mutants showed an altered swimming phenotype. Despite the conservation of *Tc*Trypanin with GAS-related sequences, when we predicted the *Tc*Trypanin 3D structure, it showed higher alignment coverage with colicin IA (structure confidence 98.1) (**Figure 1C**) and myosin II heavy chain (confidence 97.6) (data not shown) likely as it is a long alpha-helix.

It is likely that *Tc*Trypanin forms a coiled-coil tertiary structure, as can be seen in the *Tc*Trypanin model generated by Phyre3 (**Figure 1C**).

Disruption of *Tc*Trypanin Using the *Sa*Cas9 RNP Complex

To disrupt *Tc*Trypanin, wild-type Dm28c epimastigotes were transfected with a mixture of *Sa*Cas9 recombinant protein preincubated with *in-vitro*-transcribed sgRNA and a donor ssDNA. The donor oligonucleotide has 65 bases corresponding to 3 stop codons at different open reading frames plus a *Bam*HI restriction and 25 bases long homology arms (**Figure 2A**). Since only a small part of the population was edited as detected 3 days after transfection, this population was transfected again, and the new population showed close to 50% of the parasites edited, as detected by RFLP (**Figure 2B**). The *Tc*Trypanin edited population was cloned in a 96-well plate and the clones were checked by RFLP to identify parasites with both *Tc*Trypanin alleles disrupted. Three out of 12 clones were *Tc*Trypanin −/− as shown by RFLP (**Figure 2C**). The use of the ssDNA donor inserting restriction site allowed us to easily identify *Tc*Trypanin −/− clones and occasionally genotype the parasites. Anyhow, the gene disruption was also confirmed by DNA sequencing (**Supplementary Figure 3**).

*Tc*Trypanin −/− Shows Reduced Parasite Growth and Partially Detached Flagellum in Epimastigotes

Based on our experience and previously reported work (Santos et al., 2018), epimastigotes from Dm28c clone show log phase (1st to 4th day) reaching 0.65 to 0.85 × 10^7 parasites/ml, early stationary phase (4th to 7th day) reaching 1.2 × 10^7 parasites/ml, and stationary phase from day 7 to 10. *Tc*Trypanin disruption is not essential for epimastigote survival, which allowed us to select fully edited clones. However, when we assessed the parasite epimastigote growth profile, *Tc*Trypanin disruption interfered with parasite growth compared with WT cells. The reduction on *Tc*Trypanin −/− growth is small, only becoming significant on the 7th day (**Figure 3A**). To better determine if the phenotypic changes are related to *Tc*Trypanin disruption, we generated an addback culture by overexpressing *Tc*Trypanin fused to HA tag (*Tc*Trypanin::HA). The addback culture, named *Tc*Trypanin-AB, was tested for *Tc*Trypanin::HA expression. As shown in **Supplementary Figure 4**, Western blot and immunofluorescence assay using anti-HA confirm *Tc*Trypanin::HA expression. The growth defect was reversed by *Tc*Trypanin::HA overexpression in *Tc*Trypanin −/− addback culture (**Figure 3A**).

To analyze if this impact on the growth rate could be related to cell cycle progression, log-phase epimastigotes were fixed and stained with propidium iodide. *Tc*Trypanin mutants showed a statistically significant increase in the number of cells in G1 (52.80% ± 6.69%) compared with WT cells (34.96% ± 5.16%) and *Tc*Trypanin −/− AB (35.5% ± 6.64%) (**Figures 3B, C**). DAPI staining analysis of the kinetoplast/nucleus content in epimastigotes showed no evidence for cytokinesis impairment; instead, there is a slight decrease in the number of mitotic cells (2N2K cells) in *Tc*Trypanin −/− epimastigotes (6.5% ± 0.7%) compared with the WT cells (10% ± 1.4%), though it was not statistically significant (**Supplementary Figure 5**). These results demonstrate that *Tc*Trypanin is not crucial for *T. cruzi* cell cycle progression and cytokinesis in the epimastigotes forms.

Morphology of the *Tc*Trypanin Mutant and Protein Localization

Parasite cultures were subjected to immunofluorescence and scanning electron microscopy (SEM) analysis. Similar to what was described for the *Tb*Trypanin knockdown (Ralston et al., 2006), *Tc*Trypanin disruption led to epimastigote flagellum detachment (**Figure 4A**). *Tc*Trypanin −/− culture presented up to 8% of the epimastigotes with partially or totally detached flagella compared with WT parasites (**Figure 4B**).

To determine if *Tc*Trypanin disruption can interfere with parasite size (cell body area) and flagellum length, we performed the measurement of the flagellum from images obtained by immunofluorescence using a monoclonal antibody (clone 2F7) previously described as a flagellum marker (Ramos et al., 2011) and SEM (**Figure 5A** and **Supplementary Figure 6**). The measurement of the flagellum length and cell body area revealed that *Tc*Trypanin −/− parasites are smaller and have a shorter flagellum compared with WT and addback epimastigotes (**Figures 5B–D**). WT parasites exhibited a mean flagellum length of 9.51 ± 3.68 μm, while *Tc*Trypanin −/− showed a shorter flagellum (6.89 ± 2.23 μm), and the addback rescued the flagellum length (9.62 ± 3.29 μm) (**Figure 5B**). Body area was calculated to find morphological defects in the cell body. We found that WT cells have a mean area of 13.6 ± 3.43 μm^2, while *Tc*Trypanin −/− cells are smaller (8.97 ± 2.09 μm^2), and addback cells recovered their normal size (12.35 ± 3.15 μm^2) (**Figure 5D**). Similar results were found when SEM images were analyzed (**Supplementary Figure 6**).

*Tc*Trypanin::GFP localization in fixed parasites (**Figure 6A**) or cytoskeleton preparation (**Figure 6B**) by confocal microscopy showed a distribution unlike *Tb*Trypanin, as described using an epitope tag (Hill et al., 1999) or using polyclonal antibodies (Hill et al., 2000; Hutchings et al., 2002) in *T. brucei*. The GFP-tagged protein localizes to patches of the cortical cytoskeleton (**Figure 6B**). Western blot assay of whole-cell extracts of GFP and *Tc*Trypanin::GFP expressing parasites was conducted. As shown in **Supplementary Figure 7A**, epimastigotes expressed GFP (approximately 26 kDa) and *Tc*Trypanin::GFP (approximately 82 kDa) at the expected size. Furthermore, cell

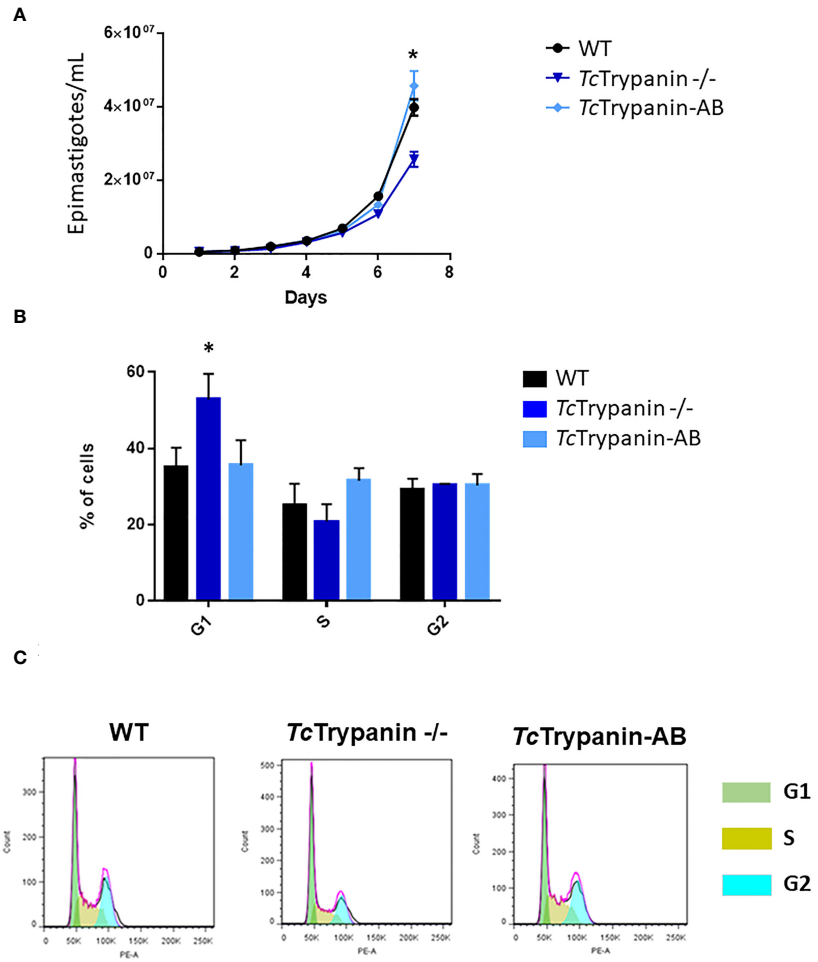

FIGURE 3 | *Tc*Trypanin −/− grows slower than WT and addback cells and presents higher number of cells at the G1 phase. **(A)** Growth curves of WT (Dm28c), *Tc*Trypanin −/−, and *Tc*Trypanin-AB (addback) epimastigotes. The growth curves were performed during 7 days, and the parasites were counted each day (three biological replicates). Asterisk indicates that there is a difference in parasite growth on day 7 (*t*-test, $p < 0.05$). **(B)** Bar graphs show the percentage of cells at each phase of the cell cycle (two biological replicates). The flow cytometry analysis was taken at day 5. Asterisk indicates that G1 cells are more frequent in *Tc*Trypanin −/− cells than in WT cells (*t*-test, $p < 0.05$). **(C)** Flow cytometry chart. Data were analyzed with FlowJo 7.6 version, and analysis of the cell cycle was applied after appropriated gating (this is a representative experiment).

cytoskeleton preparation of parasite suspension followed by Western blot detection of *Tc*Trypanin::GFP confirmed its presence in the cytoskeleton fraction (**Supplementary Figure 7B**), similar to what was described for *Tb*Trypanin in *T. brucei* (Hill et al., 2000). *Tc*Trypanin either has a different subcellular localization to *Tb*Trypanin or N-terminal GFP tagging has disrupted its normal localization. We suspect the latter.

Motility Is Strongly Impaired in the *Tc*Trypanin Mutant

Since *Tb*Trypanin knockdown is required for directional cell motility in *T. brucei* procyclic forms, we decided to determine *Tc*Trypanin −/− epimastigote motility by video microscopy. The images in **Figures 7A–C** represent the motility traces of distinct epimastigotes from WT, *Tc*Trypanin −/−, and addback cultures. Wild-type epimastigote cell behavior is similar to previously reported data (Ballesteros-Rodea et al., 2012; Sosa-Hernández et al., 2015), with cell-to-cell variation, where some cells swim productively and some tumble. In *Tc*Trypanin −/−, the motility is strongly reduced with no productively swimming cells (**Figures 7A–C**), which we quantified using path length, mean swimming speed, and count of tumbling-like behaviors (**Figures 7D–F**). WT epimastigotes showed a higher path length (65 ± 53.09 μm) compared with *Tc*Trypanin −/− (44.27 ± 22 μm), while the *Tc*Trypanin::HA addback culture (62.87 ± 43.79 μm) recovered the path length defect (**Figure 7D**). The mean speed of the *Tc*Trypanin −/− mutant (2.867 ± 1.59 μm/s) was reduced compared with WT (5.793 ± 3.63 μm/s), with the addback partially recovering swimming speed (4.617 ±

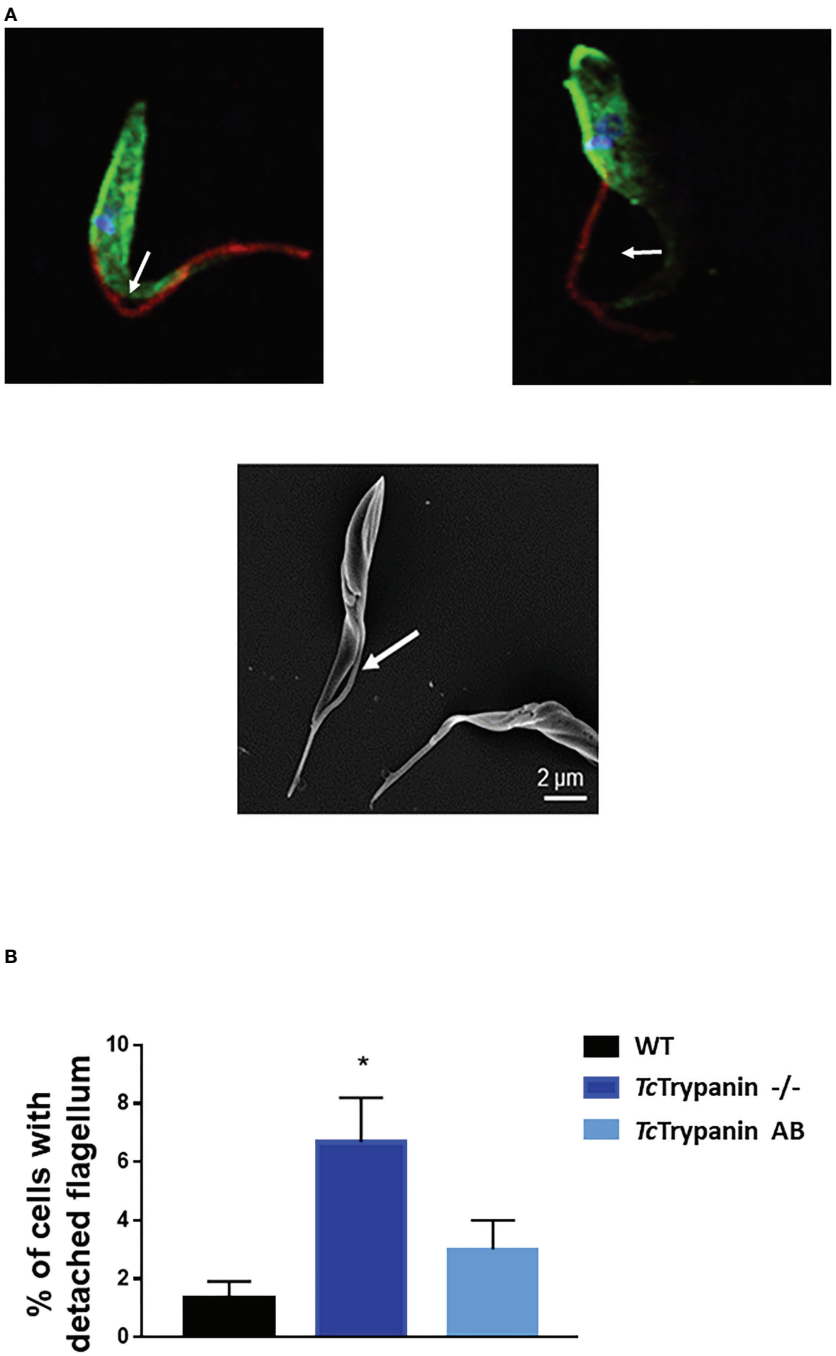

FIGURE 4 | *Tc*Trypanin disruption affects flagellum attachment. **(A)** Immunofluorescence of *Tc*Trypanin disrupted parasites showing detached flagella (white arrows). Mutant parasites (*Tc*Trypanin –/–) were stained with anti-actin (green) and the monoclonal antibody 2F7, a flagellum marker (red) (upper images). The bottom image is a SEM image showing a parasite with a partially detached flagellum. **(B)** Bar graphs plotting the frequency of partially detached flagellum from at least 100 random parasites from immunofluorescence images. The asterisk represents a statistically significant diference between WT and *Tc*Trypanin –/– cells (*t*-test, $p < 0.05$).

2.68 µm/s) cultures (**Figure 7E**). The *Tc*Trypanin –/– mutants still moved, while not swimming productively, in a tumbling-like behavior. The *Tc*Trypanin –/– parasites presented higher tumbling propensity compared with the other cultures (**Figure 7F**). It is important to highlight that these cells are not immotile despite the short distance traveled, and similar to *T. brucei*, *Tc*Trypanin mutants seem to have a tumbling and twitching motion.

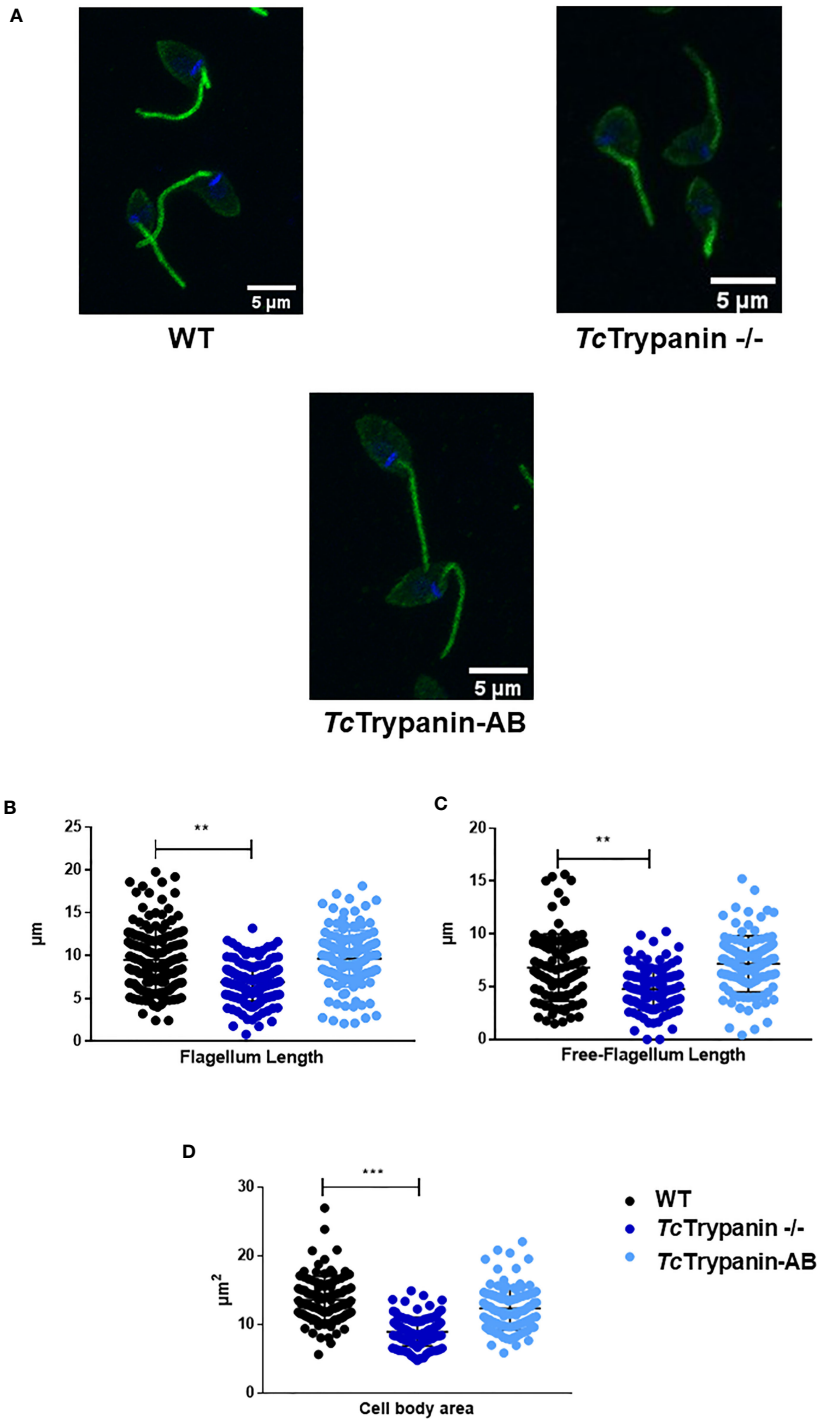

FIGURE 5 | Cell body and flagellum sizes are affected in *Tc*Trypanin –/– mutants. **(A)** Immunofluorescence of epimastigotes with the flagellum marker mAb 2F7. Representative images from parasites immunolabeled with mAb 2F7 (green). **(B–D)** Measurements of flagellum length, free flagellum length, and cell body area. Data of parasite morphology were obtained by measuring the length of the labeled flagellum and cytoplasmic area using ImageJ software. Scatter plot showing data from measures of *N* = 150 parasites stained with monoclonal 2F7 (flagellum marker). The asterisks represent statistically significant difference between WT and *Tc*Trypanin –/–cells ("**" t-test, $p < 0.01$; "***" $p < 0.001$).

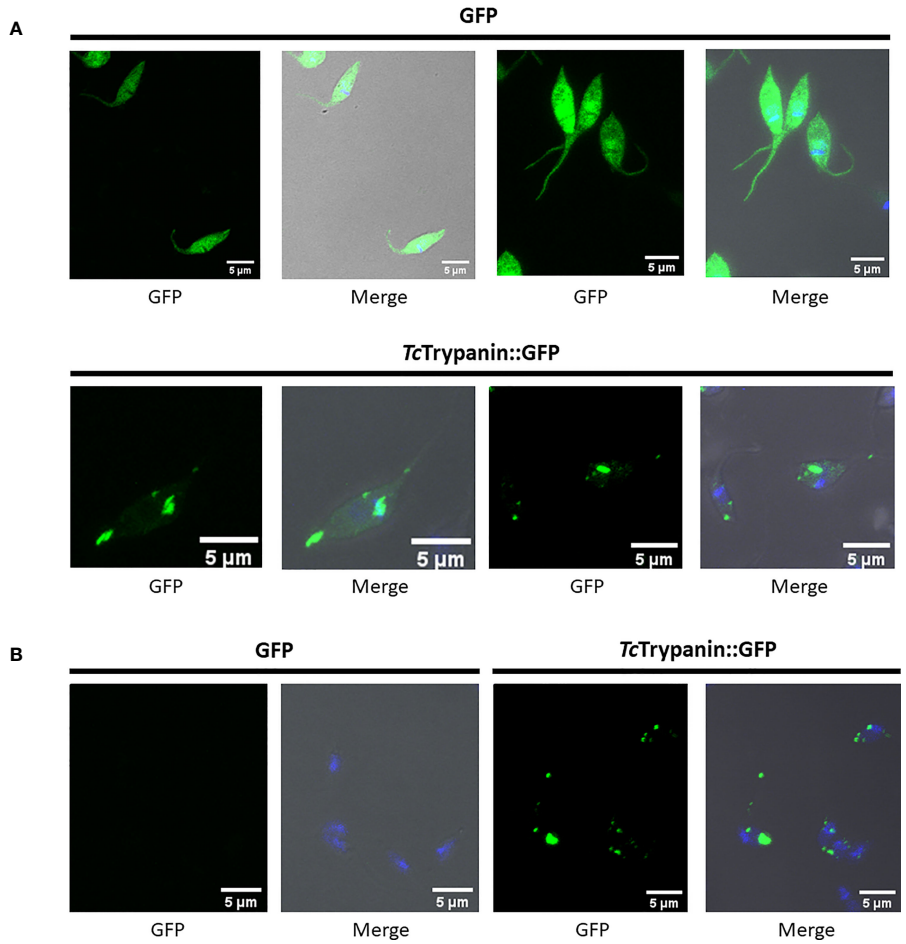

FIGURE 6 | Localization of *Tc*Trypanin::GFP. **(A)** WT parasites were transfected with the plasmid pTREX-*Tc*Trypanin::GFP or pTREX GFP (control plasmid). After G418 resistance selection, the parasites were fixed and analyzed by confocal microscopy. **(B)** The same cultures were processed as described in the *Material and Methods* section to obtain parasite cytoskeleton using PEME + Triton X-100 buffer. For each analyzed field, shown are the GFP channel (left image) and merged images (right image). Merged images correspond to phase-contrast plus GFP (green) and DAPI (blue) channels.

*Tc*Trypanin Mutants Have Metacyclogenesis and *In-Vitro* Infection Rates Affected

To test whether *Tc*Trypanin disruption can somehow affect epimastigote differentiation into metacyclic trypomastigotes *in vitro*, *Tc*Trypanin −/− mutant and wild-type epimastigotes were induced to differentiate for 3 days using TAU + TAU3AAG medium as previously described (Contreras et al., 1985). Wild-type parasites presented 25.76% ± 3.39% of MTs, while *Tc*Trypanin −/− parasites had a much lower number of MTs (1.5% ± 0.14%). The addback of *Tc*Trypanin::HA partially recovered the WT phenotype, by showing 14.28% ± 2.81% (**Figure 8A**). Despite the lower differentiation capacity, the MTs were able to infect LLC-MK2 cells and release TCTs for *in-vitro* infection assays (**Figures 8B**–**D**).

TCTs from a second round of infections were tested on *in-vitro* infection assays as described in the *Material and Methods* section. Mammalian cells were exposed to TCT parasites for 2 or 4 h and washed at least three times. Twelve hours after infection, the cells were stained with Giemsa and counted. After 2 hours of infection, WT showed a higher infection rate of 56.9% ± 6.94% and cells infected with *Tc*Trypanin −/− presented 39.12% ± 9.03%, while addback cultures showed an intermediary rate of 52.08% ± 10.75%. The overall difference between WT and *Tc*Trypanin mutant means was 12.96%, and this difference was statistically significant ($p < 0.05$), while a non-significant difference was detected between WT and addback cultures (**Figure 8B**). When we increased the infection time to 2 h (4 h of infection), WT TCTs were able to invade 70.06% ± 5.56% of cells, while *Tc*Trypanin −/− parasites displayed 59.84% ± 7.55% of infected cells. The overall difference between the means of WT and *Tc*Trypanin −/− was reduced to 10.2% (**Figure 8C**). We suspect this lower infection capacity by the *Tc*Trypanin mutant may be influenced by parasite motility. Performing the same

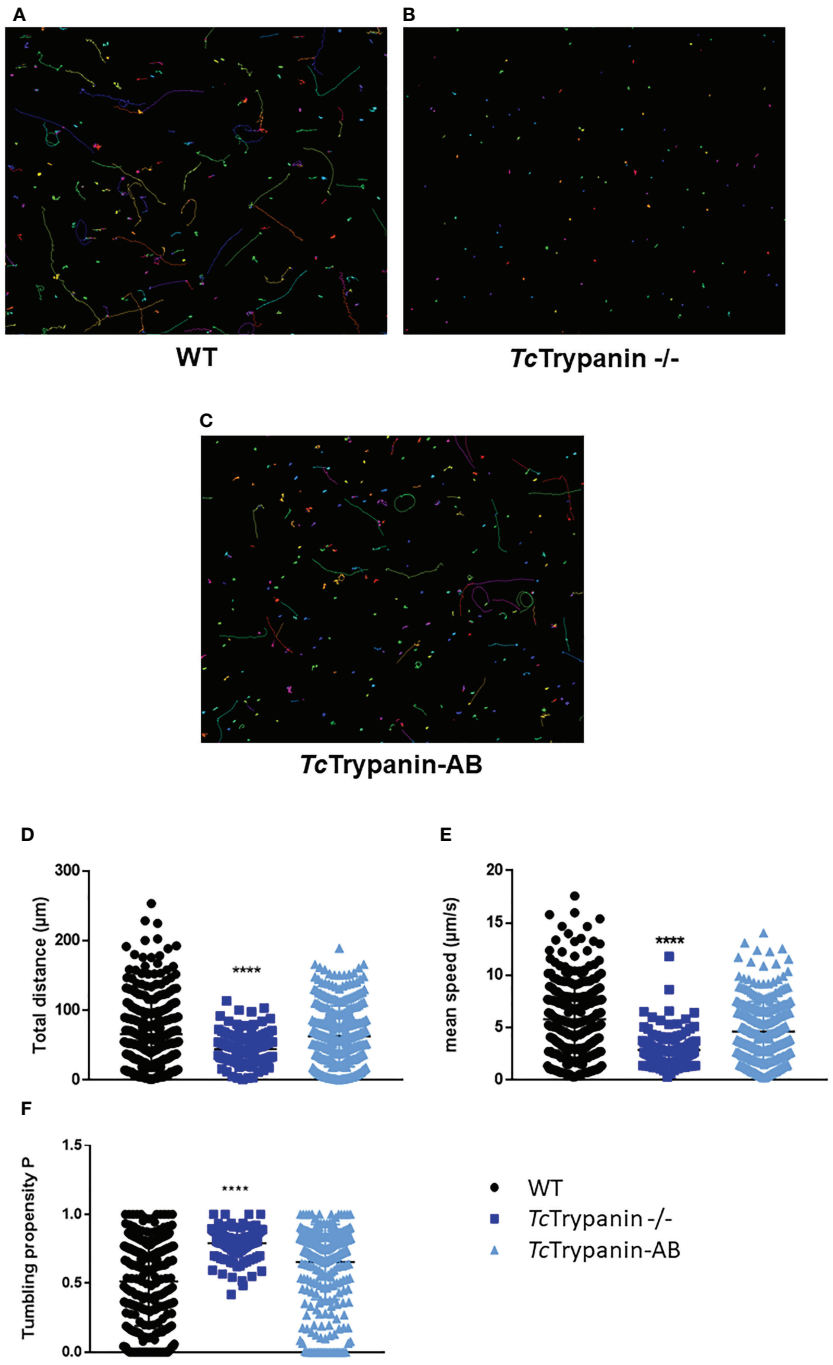

FIGURE 7 | Motility analysis of *Tc*Trypanin –/– by video microscopy. Tracks of WT **(A)**, *Tc*Trypanin –/– **(B)**, and *Tc*Trypanin-AB **(C)** parasites. Epimastigotes were harvested and placed on a microscope slide for video microscopy at ×20 objective. The motility traces of each epimastigote were acquired and converted into images. Different lines or dot colors represent distinct parasites. **(D, E)** Scatter plot of variables from motility analysis. Data from total distance **(D)**, mean speed **(E)**, and tumbling propensity **(F)** were plotted. Two-way ANOVA with multiple comparison and Dunnett test showed a statistical difference with p <0.0001 (four asterisks).

infection conditions, we also evaluated TCT release kinetics after infection per 2 or 4 h (**Figures 8D, E**). All the infected cultures showed a peak of TCT release at day 5, when cells infected with WT parasites during 4 h showed a mean of 1.5 × 10^6 TCTs/ml released, whereas cells infected with *Tc*Trypanin –/– TCTs released 0.85 × 10^6 parasites/ml, while again, the addback parasites rescued the TCT release (1.3 × 10^6 parasites/ml) (**Figure 8E**). In the 2-h cell infection experiment, we detected a

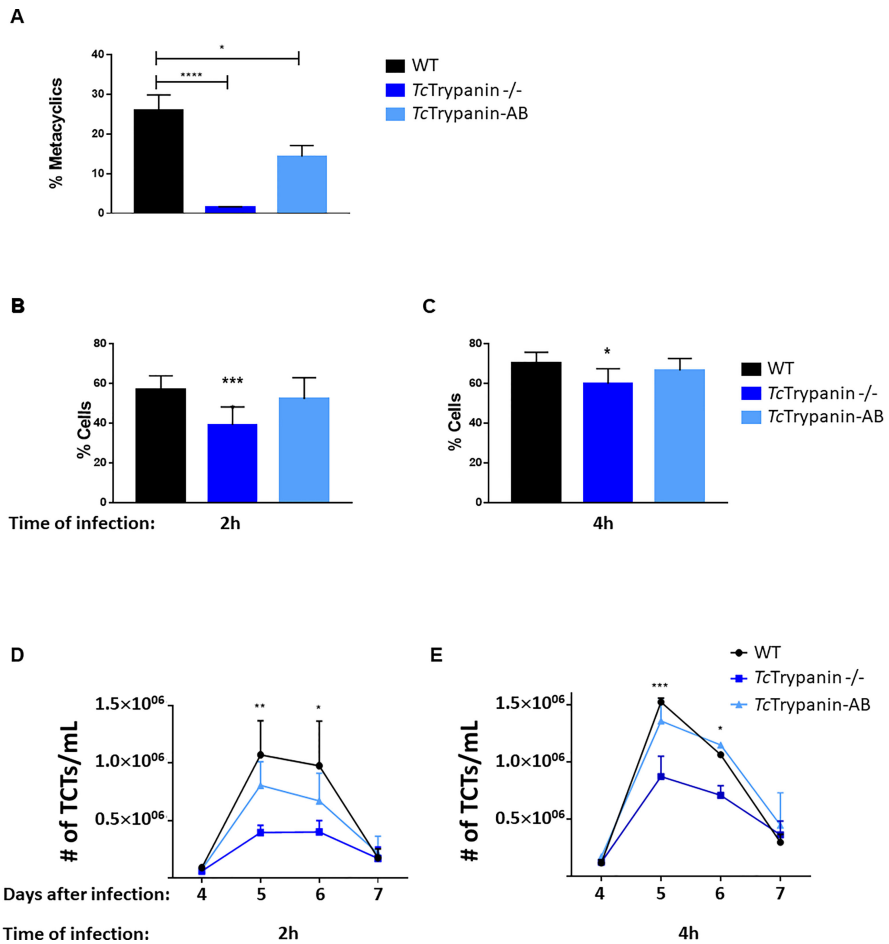

FIGURE 8 | Metacyclogenesis, infection rates, and TCT releasing. **(A)** *Tc*Trypanin disruption affects metacyclogenesis. Epimastigotes from the stationary phase were incubated with TAU medium, and 3 days later, metacyclics were counted. Asterisks indicate statistically significant results (one-way ANOVA, multiple comparison, "*" with $p < 0.05$, "****" with $p < 0.0001$). Values from two biological replicates and each of them was run in two technical replicates. **(B, C)** Infection rates of LLC-MK2 cells. Monolayer cultures were challenged with TCTs from WT, *Tc*Trypanin −/−, addback culture. The infections were performed for 2 **(B)** or 4 h **(C)** and Giemsa stained for counting the number of infected cells. This experiment was performed as in three independent replicates with two technical replicates. Asterisk indicates the difference between WT and *Tc*Trypanin −/− (statistical significance was performed in two-way ANOVA, multiple comparison, "*" with $p < 0.05$, "***" with $p < 0.001$). **(D, E)** TCT cell releasing dynamic from the supernatant of infected cells. After 4 days of infection, the number of released TCT parasites was counted in a Neubauer chamber. Data were compared with WT and *Tc*Trypanin-AB with two-way ANOVA and the difference was statistically significant as indicated by asterisks ("*" with $p < 0.05$,"**" with $p < 0.01$, "***" with $p < 0.001$). Values are derived from three independent replicates.

similar behavior, where the WT TCT-infected culture released 1×10^6 parasites/ml, *Tc*Trypanin −/− 0.39×10^6, and addback 0.8×10^6 parasites/ml (**Figure 8D**). Taken together, the infection rate (2 and 4 h of TCT incubation) of WT and *Tc*Trypanin mutants somehow correlates with the TCT release, which may suggest that the intracellular stage replication or differentiation may not be affected drastically.

DISCUSSION

The flagellum of pathogens is a multifunctional organelle frequently associated with parasite infectivity. In *T. cruzi*, the most studied virulence factors are surface proteins. Only recently, with the advance of new genetic editing tools, the initial investigation of motility and infectivity factors has been conducted in this parasite (Arias-del-Angel et al., 2020; Rodríguez et al., 2020). *Trypanosoma cruzi*, like *T. brucei*, needs to travel through the extracellular matrix (a viscous environment) and cross obstacles during a natural infection (Heddergott et al., 2012). Additionally, *T. cruzi* has to attach to cells for receptor recognition (Campetella et al., 2020) and to trigger events for internalization (Maeda et al., 2012). Besides that, the functional characterization of the flagellar component is poorly explored in *T. cruzi*. In this work, we showed for the first time the functional characterization of one axonemal protein of *T. cruzi* and its involvement in motility and infectivity, suggesting the role of motility as an infectivity factor in *T. cruzi*.

To ablate *Tc*Trypanin expression, we failed to replace this gene by selectable markers (NeoR and HygroR) using conventional knockout strategy (unpublished data). However, to disrupt *Tc*Trypanin in *T. cruzi* Dm28c, we performed genome editing with CRISPR/Cas9. For *T. cruzi*, successful editing can be achieved by different methods such as the stable expression of *Sp*Cas9 followed by the transient transfection with *in-vitro*-transcribed sgRNA (Peng et al., 2014; Burle-Caldas et al., 2018; Romagnoli et al., 2018), the stable expression of both *Sp*Cas9 and sgRNA (Lander et al., 2015), the stable expression of T7 RNA polymerase and *Sp*Cas9 followed by the transient transfection of a DNA template for the sgRNA expression directed by the T7 promoter (Costa et al., 2018), and last but not least, the marker free editing by the transfection of the ribonucleoprotein complex (*Sa*Cas9 + sgRNA) protein *Sa*Cas9 (Soares Medeiros et al., 2017; Burle-Caldas et al., 2018). Recombinant protein electroporation was also applied for CRE recombinase delivery to manipulate *T. cruzi* gene expression (Pacheco-Lugo et al., 2020). By applying the *Sa*Cas9 protein delivery, we easily disrupted *Tc*Trypanin using a donor oligonucleotide to insert stop codons plus a restriction site, when compared with the conventional approach. This approach was so powerful that it allowed us to identify mutant parasites a few days after transfection without the use of selectable markers. This reserves selectable markers for additional genetic manipulation steps such as complementation. Currently, a marker-free approach has been used to attenuate *Leishmania major* for vaccine development (Zhang et al., 2020), a requirement for use in preclinical and clinical studies (Karmakar et al., 2021). The *Tc*Trypanin −/− parasite we have developed here are suitable to be tested as a vaccine candidate, since it showed reduced motility and lower infectivity *in vitro*.

*Tc*Trypanin was not essential in insect or mammalian stages. The null mutant had a lower growth rate and an accumulation of cells in G1 in epimastigotes compared with wild-type and addback cultures. Functional analysis of some motile components, including *Tb*Trypanin by mRNA knockdown, showed moderate to severe defects in the cytokinesis of *T. brucei* parasites (Hutchings et al., 2002; Baron et al., 2007). Cytokinesis defects of *Tb*Trypanin knockdown produce a clump of parasites suggesting cytokinesis failure (Ralston et al., 2006); again, we did not observe this kind of phenotype in the *T. cruzi* life cycle stages. This suggests that a motile flagellum has less contribution on *T. cruzi* division, which may be linked with *T. cruzi* having an amastigote life cycle stage. It is important to highlight that *Tc*Trypanin −/− epimastigotes are not immotile and have an active flagellar beating that could be enough for cytokinesis despite its lack of directionality. Whether or not motility is essential for *T. cruzi* cytokinesis might be further explored by deleting genes such as PF16 or another CMF with a severe motility defect (Baron et al., 2007). A previous work with gp72-KO is also informative (de Jesus et al., 1993). In this report, the authors show that gp72 (FLA-1 in *T. brucei*) mutants have detached flagella, and the epimastigote mutants are able to differentiate into infective metacyclic forms (reduced compared with WT). However, the infected cells do not release tissue culture-derived trypomastigotes, only amastigote-like forms with short flagella.

The reduction of cell size was an unexpected result, though the shape of trypanosomatids depends on subpellicular microtubules (SM) (Sinclair et al., 2021), and *T. brucei* cell motility seems to shape SM (Sun et al., 2018). Sun et al. showed that flagellar detachment caused by RNAi of FLA1BP leads to abnormal SM handedness compared with parasites with a normal flagellar wave. Otherwise, differences in cell body size were not reported. This may be due to the transient nature of the RNAi knockdown compared with the permanent disruption of *Tc*Trypanin −/− by CRISPR-Cas9 or differing morphogenesis in *T. brucei*. Flagellum attachment zone proteins (FAZ) form a complex that maintains flagellum attached to the cell body, but that also regulates the cell cycle and coordinate flagellum length, and FLA1BP silencing in *T. brucei* reduces flagellum length (Sun et al., 2012). Hutchings et al. (2002) suggest that *Tb*Trypanin is necessary for normal flagellum attachment. It is unclear whether this is a direct role or an indirect impact of defective motility, but flagellum detachment may contribute to defective morphogenesis in the *Tc*Trypanin −/− mutant.

Our data show that *Tc*Trypanin disruption has a higher impact on TCT infectivity depending on the time of infection (4 vs. 2 h) compared with wild-type parasites, suggesting that motility might be important for parasites to find host cells. Hence, mutant parasites need more time to infect a cell host and will likely be more exposed to the immune system during an *in-vivo* infection. For *T. brucei*, motility impairment leads to higher targeting by the higher clearance of antibodies (Shimogawa et al., 2018). It is possible that the TCTs from *Tc*Trypanin mutant will be less infective on *in-vivo* infection in an animal model. The reduced TCT releasing in *Tc*Trtypanin −/− correlates with fewer infected cells, suggesting that amastigote replication is not affected in the *Tc*Trypanin −/−.

In summary, the *Tc*Trypanin null mutant affected several features of the *T. cruzi* cell biology and life cycle, including epimastigote growth, cell body and flagellum size, flagellum attachment, metacyclogenesis, infection rates, and cell motility. Some of these phenotypic variations were compatible with its ortholog knockdown in *T. brucei*. Despite many similarities with *T. brucei*, the *Tc*Trypanin −/− mutant can go through an entire life cycle. These data reinforce the need for additional studies to understand the functioning of flagellar components in *T. cruzi*.

CONCLUSIONS

We described that *Tc*Trypanin absence causes some motility defects that do not impair cytokinesis in the epimastigote forms. It is also remarkable that these mutants can complete infection cycles despite the reduction in infectivity and metacyclogenesis. This ability to go through the entire life cycle suggests that parasite motility does not affect parasite viability, as seen by the knocking out of proteins related to motility (e.g., gp72/FLA-1 and Trypanin). These data highlight the need for a large-scale functional genomics approach of flagellar components of *T. cruzi*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JS-G, BB, NS-M, LVM, JSM, LAP-L, LCSM, and RW performed the experiments. JS-G drafted the manuscript. JS-G, NS-M, and WD critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.807236/full#supplementary-material

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The Enhanced Expression of Cruzipain-Like Molecules in the Phytoflagellate *Phytomonas serpens* Recovered From the Invertebrate and Plant Hosts

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Phytomonas serpens is a protozoan parasite that alternates its life cycle between two hosts: an invertebrate vector and the tomato fruit. This phytoflagellate is able to synthesize proteins displaying similarity to the cysteine peptidase named cruzipain, an important virulence factor from *Trypanosoma cruzi*, the etiologic agent of Chagas disease. Herein, the growth of *P. serpens* in complex medium (BHI) supplemented with natural tomato extract (NTE) resulted in the increased expression of cysteine peptidases, as verified by the hydrolysis of the fluorogenic substrate Z-Phe-Arg-AMC and by gelatin-SDS-PAGE. Phytoflagellates showed no changes in morphology, morphometry and viability, but the proliferation was slightly reduced when cultivated in the presence of NTE. The enhanced proteolytic activity was accompanied by a significant increase in the expression of cruzipain-like molecules, as verified by flow cytometry using anti-cruzipain antibodies. In parallel, parasites incubated under chemically defined conditions (PBS supplemented with glucose) and added of different concentration of NTE revealed an augmentation in the production of cruzipain-like molecules in a typically dose-dependent way. Similarly, *P. serpens* recovered from the infection of mature tomatoes showed an increase in the expression of molecules homologous to cruzipain; however, cells showed a smaller size compared to parasites grown in BHI medium. Furthermore, phytoflagellates incubated with dissected salivary glands from *Oncopeltus fasciatus* or recovered from the hemolymph of infected insects also showed a strong enhance in the expression of cruzipain-like molecules that is more relevant in the hemolymph. Collectively, our results

showed that cysteine peptidases displaying similarities to cruzipain are more expressed during the life cycle of the phytoflagellate *P. serpens* both in the invertebrate and plant hosts.

Keywords: *Phytomonas serpens*, tomato, *Oncopeltus fasciatus*, invertebrate vector, interaction, cruzipain

INTRODUCTION

The genus *Phytomonas* (Kinetoplastea: Trypanosomatidae) was first mentioned in the early 1900s when proposed a classification for flagellate trypanosomatids found in plants (Camargo, 1999; Jaskowska et al., 2015). Infection by *Phytomonas* spp. can be associated to pathological syndromes in plants of great economic importance such as coffee, cassava, coconut and oil palm (Dollet, 1984; Camargo, 1999). On the other hand, in many cases flagellates parasitize plants without apparent pathogenicity (Camargo, 1999; Jaskowska et al., 2015). *Phytomonas serpens* is a flagellate isolated for the first time in the sap of tomato fruits (*Solanum lycopersicum*) (Gibbs, 1957; Jankevicius et al., 1989). There is no precise available information about the pathogenicity of trypanosomatids in edible fruits such as tomatoes, oranges and grapes, although flagellates usually remain limited around the point of inoculation. When it comes to *P. serpens*, the presence of these trypanosomatids provoke only yellowish spots on the fruit surface that are not ascertained to be real injuries to tomato; however, a loss in nutritional quality and, above all, a loss in economic value added to the product are undoubtedly documented (Camargo, 1999).

Phytomonas serpens is transmitted to tomatoes through the bite of hemipteran phytophagous insects, such as *Phthia picta* and *Nezara viridula* (Jankevicius et al., 1989; Camargo, 1999). In insects, ingested parasites initially colonize intestinal sites, where the first multiplication of *P. serpens* cells occurs. The parasites then migrate through hemolymph to salivary glands, where they resume proliferation (Mcghee and Hanson, 1964). Transmission occurs when insects with infected salivary glands feed on the fruit (Jankevicius et al., 1989; Camargo and Wallace, 1994).

Among trypanosomatids, *P. serpens* represents an excellent biological study model due to the expression of molecules similar to those described in human pathogenic species (Jaskowska et al., 2015). The parasite expresses a 63-kDa cell surface polypeptide that is similar to the main metallopeptidase present in *Leishmania* spp, called gp63 or leishmanolysin; however, these molecules do not show any proteolytic activity in the phytoflagellate (Breganó et al., 2003; Santos et al., 2007; d'Avila-Levy et al., 2014). Breganó et al. (2003) demonstrated that *P. serpens* displays antigens similar to those of *Trypanosoma cruzi*, which were strongly recognized by the serum of patients with Chagas disease besides being capable of providing protective immunity in susceptible BALB/c mice. Subsequently, our group identified in *P. serpens* the presence of two 38- and 40-kDa cysteine-type peptidases that have similar antigenic properties to the main cysteine peptidase of *T. cruzi*, called cruzipain (Santos et al., 2006; Santos et al., 2007; Elias et al., 2012). This finding is directly related to the strong reactivity of *P. serpens* antigens with sera from patients with Chagas disease (Santos et al., 2007).

The important roles of cysteine peptidases in *P. serpens* are strengthened by the modulation of their expression when the parasite is cultured in different culture media. Elias and collaborators (Elias et al., 2008) reported a strong reduction in the 38- and 40-kDa peptidase activities when parasites were grown in either liver infusion trypticase or yeast extract media, when compared to parasites grown in Warren medium. Additionally, cruzipain-like molecules were differentially modulated according to the proteins present in the culture medium (Elias et al., 2008). Cultivation of *P. serpens* in phosphate-buffered saline (PBS)-glucose supplemented with human and bovine albumins led to a reduction in the expression of these proteins by about 50% and 25%, respectively, while mucin and fetal bovine serum did not alter their production. In contrast, immunoglobulin G (IgG) and hemoglobin drastically enhanced its surface expression by about 7- and 11-fold, respectively, when compared with parasites incubated in PBS-glucose (Elias et al., 2012). Incubation of *P. serpens* in PBS-glucose supplemented with hemoglobin at different concentrations also induced a dose-dependent increase in the activity of both cysteine peptidases; in addition, a significant increase in the secretion of cruzipain-like molecules was also observed after incubation of the parasites with 1% hemoglobin, in comparison with parasites incubated in PBS-glucose (Elias et al., 2012).

With these premises in mind, the present study aims to evaluate and compare the modulation of the expression of cruzipain-like cysteine peptidases in the presence of natural tomato extract (NTE), as tomato is the original plant host of *P. serpens*, as well as during the interaction process with the plant host and with the phytophagous hemipteran *Oncopeltus fasciatus*, usually employed as an invertebrate host model for studies of this parasite (Santos et al., 2006; Dias et al., 2012).

MATERIALS AND METHODS

Parasite and Growth Conditions

Phytomonas serpens (isolate 9T; CT-IOC-189), isolated from tomatoes, was provided by Coleção de Tripanossomatídeos, Instituto Oswaldo Cruz – Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. Promastigote forms (1×10^6 parasites/ml) were grown in 3.7% (w/v) brain–heart infusion (BHI) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 26°C for 48 h. Cellular growth was estimated by counting the parasites in a Neubauer chamber. The cellular

viability was monitored through parasite motility (Elias et al., 2008).

Preparation of NTE Medium

Fresh and mature tomatoes of the species *Solanum lycopersicum* were washed in a solution of distilled water with 0.01% sodium hypochlorite. Then, tomatoes were macerated, weighted and diluted in PBS (150 mM NaCl, 20 mM phosphate buffer, pH 7.2) to a final concentration of 3 g/ml. After dilution, the extract was centrifuged at 3,500×g for 30 min. The supernatant, called NTE, was removed and subjected to sterilization through filtration on a Millipore 0.22-μm membrane.

Cell Morphology and Ultrastructural Analysis

In order to detect morphological alterations, *P. serpens* promastigotes were grown in BHI medium supplemented or not with 25% (BHI-NTE 25%) or 50% NTE (BHI-NTE 50%) for 48 h at 26°C. Then, the parasites were washed three times in PBS, fixed with methanol for 5 min, stained with Giemsa and then observed under a Zeiss microscope (Axioplan, Oberkochen, Germany). In parallel, each experimental population was mapped by flow cytometry (FACSCalibur, BD Bioscience, USA) using a two-parameter histogram of forward scatter (FSC) versus side scatter (SSC) to measure two morphometric parameters: cell size and granularity, respectively. At the same time, aliquots were collected from each culture medium before inoculation (0 h) and after 48 h of growth to measure pH values over time. Ultrastructural analysis was performed by scanning electron microscopy (SEM), in which promastigotes were cultivated under the same conditions and then fixed for 40 min at 25°C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation, cells were washed in cacodylate buffer and postfixed with a solution of 1% OsO_4, 0.8% potassium ferrocyanide and 5 mM $CaCl_2$ in the same buffer for 20 min at 25°C. Cells were dehydrated in graded series of acetone (30–100%) and then dried by the critical point method, mounted on stubs, coated with gold (20–30 nm) and observed in a Jeol JSM 6490LV scanning electron microscope (Massachusets, USA).

Parasite Extracts

Phytomonas serpens promastigotes grown in BHI medium supplemented or not with 25% or 50% NTE for 48 h were harvested by centrifugation for 5 min at 500×g at 4°C and washed three times with cold PBS. Then, parasites were lysed by the addition of 1% Triton X-100 (Sigma Aldrich, St Louis, USA) and homogenized using a vortex by alternating 30-seg shaking and 1-min cooling intervals. Then, the cellular extract was centrifuged at 1,500×g for 15 min at 4°C. Protein concentration was determined by the method described by Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Cysteine Peptidase Activity Assays

Cysteine peptidase activity was determined using *P. serpens* promastigotes grown in the absence (control) or in the presence of 25% or 50% of NTE. Cellular parasite extracts were obtained as described in the previous item. The fluorogenic peptide substrate *N*-benzyloxycarbonyl-L-phenylalanyl-L-arginine-(7-amino-4-methylcoumarin) (Z-Phe-Arg-AMC) (Sigma Aldrich, St Louis, USA) was used as a specific cysteine peptidase substrate. The 5-mM stock solution of the fluorogenic substrate was prepared in DMSO. The reaction was started by the addition of the substrate (20 μM) to the parasite extract (10 μg protein) by diluting in 0.1M sodium phosphate buffer, pH 5.0, containing 2 mM dithiothreitol (DTT). The reaction mixture was incubated at 37°C for 15 min and the clearance of the fluorogenic substrate was monitored continuously by a spectrofluorometer (SpectraMax Gemini XPS, Molecular Devices, CA, USA) using emission and excitation wavelengths of 460 and 380 nm, respectively. Concomitantly, cysteine peptidase activities were assayed in gelatin-containing sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Heussen and Dowdle, 1980). Samples containing 50 μg protein from each system were resuspended in SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol and 0.002% bromophenol blue). Peptidases were assayed and characterized by 10% SDS-PAGE with 0.1% co-polymerized gelatin as substrate. After electrophoresis at a constant voltage of 120 V at 4°C, SDS was removed by incubation with 2.5% Triton X-100 for 1 h at room temperature under constant agitation. Then, gels were incubated at 37°C in sodium phosphate buffer, pH 5.0, supplemented with 2 mM DTT for 48 h in the absence or in the presence of the following cysteine peptidase inhibitors: cystatin (1 μM), leupeptin (1 μM), antipain (1 μM), iodoacetamide (1 μM) and E-64 (1 μM). After incubation, gels were washed twice with distilled water, and stained for 24 h with 0.2% Coomassie brilliant blue R-250 in methanol-acetic acid-water (50:10:40) and destained overnight in a solution containing methanol-acetic acid-water (5:10:85). The molecular masses of the peptidases were estimated by comparison with the mobility of low molecular mass standards (Thermo Fisher Scientific, Massachusetts, USA). The densitometric analysis was performed using the ImageJ program.

DETECTION OF CRUZIPAIN-LIKE MOLECULES

Modulation of Expression by NTE

Promastigotes of *P. serpens* were cultivated in BHI medium supplemented or not with 25% or 50% NTE for 48 h. Alternatively, parasites (8×10^8 cells) were incubated in PBS-glucose 2% supplemented or not with 0.1, 1 or 10% NTE for 3 h at room temperature. After this period, parasites were centrifuged and cells were fixed for 15 min in 2% paraformaldehyde in PBS (pH 7.2) at room temperature, and then washed with the same buffer. Morphological integrity was verified by optical microscopic observation. Cells were incubated for 1 h at room temperature with a 1:250 dilution of rabbit anti-cruzipain polyclonal antibody raised against *T. cruzi* (kindly provided by Dr Juan-Jose Cazzulo, Instituto de Investigaciones Biotecnologicas, Universidad Nacional de General San Martin,

Buenos Aires, Argentina). Then, cells were incubated for an additional hour with a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (IgG) (Sigma Aldrich, St Louis, USA). Finally, cells were washed three times in PBS and analyzed in a flow cytometry (FACSCalibur, BD Bioscience, USA) equipped with a 15-mW argon laser emitting at 488 nm. Non-treated cells and those treated with the secondary antibody alone were run in parallel as controls (d'Avila-Levy et al., 2006). The results were expressed as the percentage of fluorescent cells (%FC) and mean of fluorescence intensity (MFI).

Modulation of Expression by Tomato Fruit (*Solanum lycopersicum*)

Parasites cultivated in BHI medium were collected by centrifugation, washed in PBS and counted in a Neubauer chamber. Concomitantly, tomatoes were initially treated for 15 min in a solution of chlorine and water (20 ppm) and left for 30 min under ultraviolet light. Then, parasites (5×10^4 cells resuspended in 5 µl PBS) were inoculated in 3 sites previously chosen in tomatoes. Fruits were incubated for seven days at 26°C, during which no signs of decomposition were visible. Finally, tomatoes were cut, seeds separated and parasites removed by endocarp filtration. Parasites were then collected and prepared for flow cytometry using anti-cruzipain antibody, as described previously.

Modulation of Expression by the Salivary Glands of the Phytophagous Insect *O. fasciatus*

A milkweed bug (*O. fasciatus*) culture was purchased from Carolina Biological Supply Company, Burlington, North Carolina, USA and originated the colony maintained in our department. Adult *O. fasciatus* (Hemiptera: Lygaeidae) were kept in plastic pitchers under a 12 h light/dark cycle at 28°C with 70-80% relative humidity and fed with peeled and toasted sunflower seeds and distilled water (Romeiro et al., 2000). Salivary glands of *O. fasciatus* were carefully dissected, washed three times in ice-cold PBS and resuspended in PBS-glucose 2%. In parallel, parasites cultivated in BHI for 48 h were collected by centrifugation, washed in PBS and counted in a Neubauer chamber. Then, parasites (8×10^8 cells) were incubated with dissected salivary glands for 3 h (Santos et al., 2006). After incubation, parasites were collected by centrifugation and treated with anti-cruzipain antibody and analyzed by flow cytometry, as described previously. Control systems were used with parasites incubated only in PBS-glucose 2%.

Modulation of Expression by the Hemolymph of *O. fasciatus*

Parasites were grown for 48 h in BHI, washed three times in PBS and counted in a Neubauer chamber. Then, parasites were resuspended in PBS (4×10^5 cells) and injected, with a Hamilton syringe, on the side of the junction between the second and third thoracic segments of *O. fasciatus*. After 72 h of infection, hemolymph was collected by clipping off the first two pairs of insect legs (Alves e Silva et al., 2013). Then, hemolymph was centrifuged at 5,000×g for 10 min to separate parasites, which were washed in PBS and processed for analysis by flow cytometry using the anti-cruzipain antibody. After incubation, the parasites were collected by centrifugation and treated with anti-cruzipain antibody and analyzed by flow cytometry, as described previously.

Statistics

All experiments were performed in triplicate, in three independent experimental sets. Results were analyzed statistically by Student's t-test (in the comparisons between two groups) and by Analysis of Variance One-Way ANOVA followed by a Tukey-Kramer post-test (in comparisons between three or more groups). In all analyses, p values of 0.05 or less were considered statistically significant. All analyzes were performed using the program GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Effects of NTE on the Growth Rate and Morphology of *P. serpens*

The first step of this study was to evaluate the effect of NTE on *P. serpens* morphology and growth. Analysis by flow cytometry showed that parasites grown in BHI-NTE 25% and BHI-NTE 50% for 48 h presented similar cell size and granularity when compared to parasites grown in the standard BHI medium, as revealed by both FSC and SSC flow cytometry parameters (**Figure 1**). Corroborating this result, both light and scanning electron microscopies revealed no morphological differences between these populations, in which *P. serpens* had the typical promastigote form presenting an elongated cell body, an anterior kinetoplast in relation to the nucleus and a single long flagellum (**Figure 1**). The presence of NTE in the culture medium was also able to reduce the proliferation rate of *P. serpens* in a dose-dependent manner: the cultivation in BHI-NTE 25% promoted a reduction of approximately 17%, while the cultivation in BHI-NTE 50% of around 30% (**Figure 1**). Before inoculation, pH values in the different culture media showed that the higher the concentration of NTE, the lower the pH value; after 48 h of culture, there was an acidification of BHI medium and, more prominently, in BHI-NTE 25% medium. The pH of BHI-NTE 50% medium remained practically constant after 48 h of growth (**Figure 1**).

Effects of NTE on the Proteolytic Activities of *P. serpens*

Our next goal was to evaluate the expression of cysteine peptidases in the different populations through chemical dosages using the fluorogenic peptide substrate Z-Phe-Arg-AMC and gelatin-SDS-PAGE. The data indicated an increase of approximately 1.9- and 2.4-times in the cleavage of the fluorogenic substrate when parasites were cultivated in the presence of NTE at 25% and 50%, respectively, as compared to parasites grown in BHI medium (**Figure 2A**). Corroborating these data, the analysis through SDS-PAGE containing gelatin as

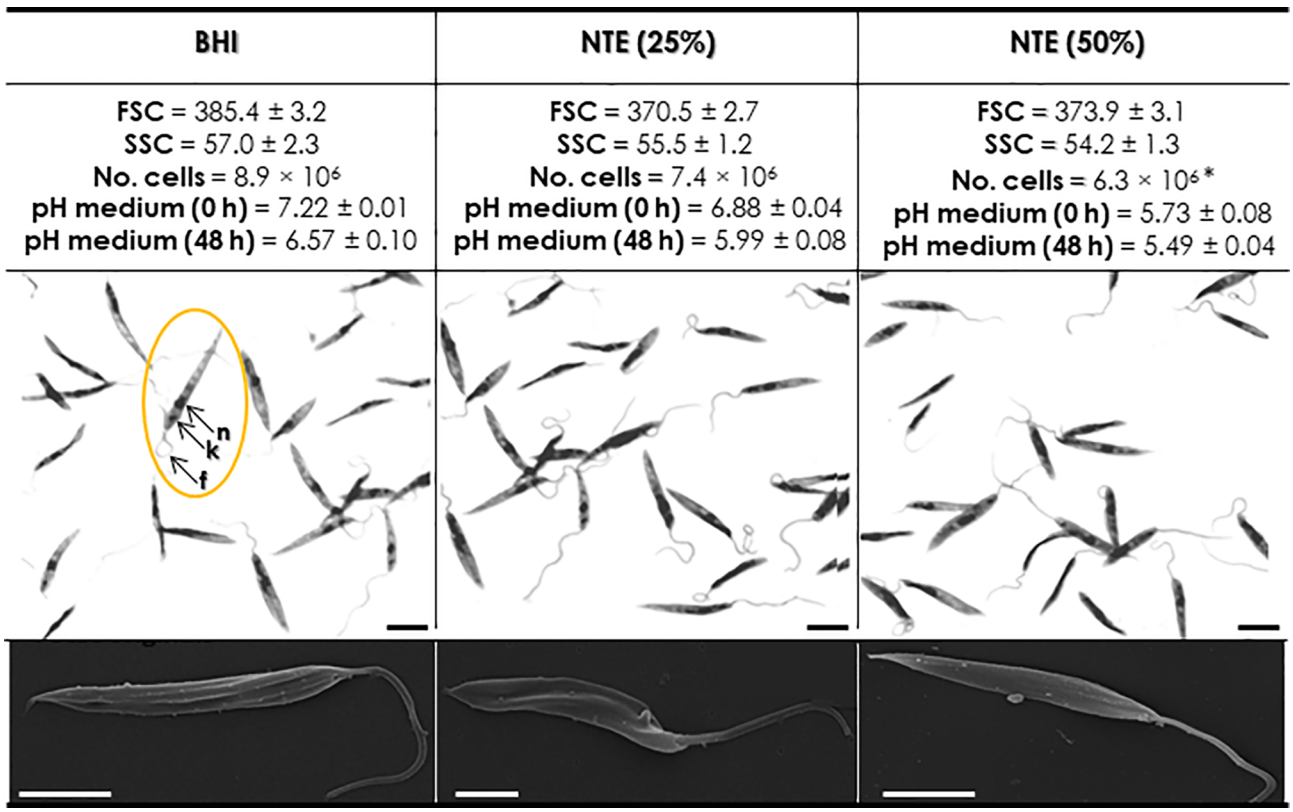


FIGURE 1 | Effects of natural tomato extract (NTE) on *Phytomonas serpens* morphology and growth after cultivation in BHI medium for 48 h in the absence (BHI) or in the presence of different concentrations of NTE (25 and 50%). pH values were also measured in each culture medium before inoculation (0 h) and after 48 h of growth. Cells were analyzed by flow cytometry in order to measure two morphometric parameters, forward scatter (FSC) and side scatter (SSC). The values expressed represent the mean of fluorescence intensity of three independent experiments. The symbol (*) represents the significant difference ($P < 0.05$; Student's *t*-test) between treated and control groups. The influence of NTE was also evaluated during cell proliferation (initial inoculum of 1 x 10^6 parasites/mL) by counting cells in a Neubauer chamber after 48 h. In parallel, Giemsa-stained smears were analyzed under an optical microscope. Promastigote forms present a kinetoplast (k), the central nucleus (n), an elongated cell body and a flagellum (f) attached to the parasite cell body. Bars: 10 μM. In scanning electron microscopy analysis of promastigotes, note that no morphological changes were identified after growth of parasites in these different media. Bars: 5 μm.

substrate revealed the expression of two major bands with molecular masses of approximately 38 and 40 kDa (**Figure 2B**); the densitometric analysis showed that the band intensity was higher in the presence of NTE, indicating a 50% and a 54% increase when parasites were grown in BHI-NTE 25% and 50% versus BHI, respectively. Furthermore, the proteolytic activity bands were inhibited, totally or partially, by different cysteine peptidase inhibitors (cystatin, leupeptin, antipain, iodoacetamide and E-64), allowing to characterize them as cysteine peptidases (**Figure 2C**) as previously described by our group (Santos et al., 2006; Elias et al., 2008; Elias et al., 2009). The proteolytic class was confirmed in cell extracts of parasites grown in BHI medium, but similar results were detected in parasites cultivated in NTE-supplemented media (data not shown).

Effects of NTE on the Cruzipain-Like Molecules Expressed by *P. serpens*

Analysis using flow cytometry showed that parasites cultivated either in BHI-NTE 25 or 50% for 48 h promoted a dose-dependent increase in the expression of cruzipain-like molecules, as can be seen by the increase in both %FC and MFI parameters (**Figure 3**). In this context, the presence of 25% and 50% NTE in the culture medium promoted a 5.2- and 8.8-fold increase in the percentage of positive cruzipain-like cells as well as 2.3- and 4-fold increase in the MFI, respectively, as compared to the growth in BHI medium (**Figure 3**). When parasites were alternatively incubated in PBS-glucose supplemented with different concentrations of NTE for 3 h, a dose-dependent positive modulation in the expression of cruzipain-like molecules was observed (**Figure 4**). When compared to control parasites cultivated in PBS-glucose, *P. serpens* incubated with PBS-glucose supplemented with 0.1, 1 and 10% NTE showed a 3.0-, 5.6- and 8.9-fold increase in the percentage of cells labeled with anti-cruzipain antibody as well as 1.6-, 2.7-, and 8.4-fold increase in MFI, respectively (**Figure 4**).

Effects of *In Vitro* Infection of Tomatoes on the Cruzipain-Like Molecules Expressed by *P. serpens*

Since *P. serpens* promastigotes were able to increase the expression of cruzipain-like molecules when in contact with

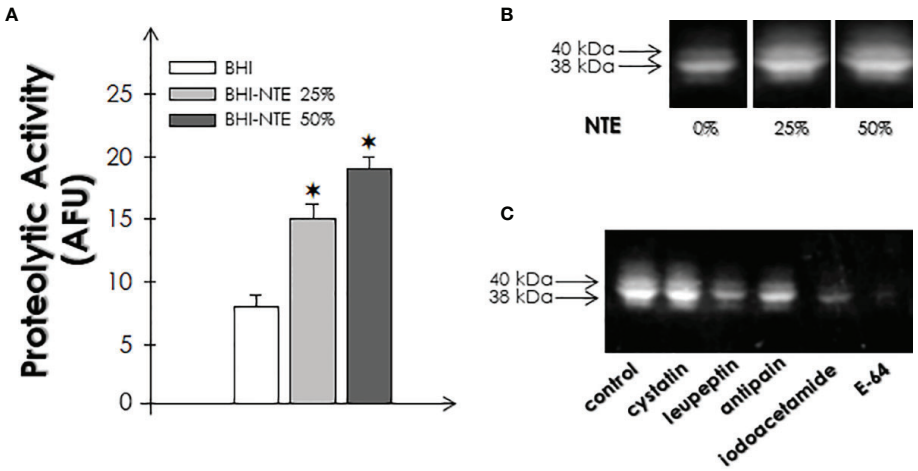
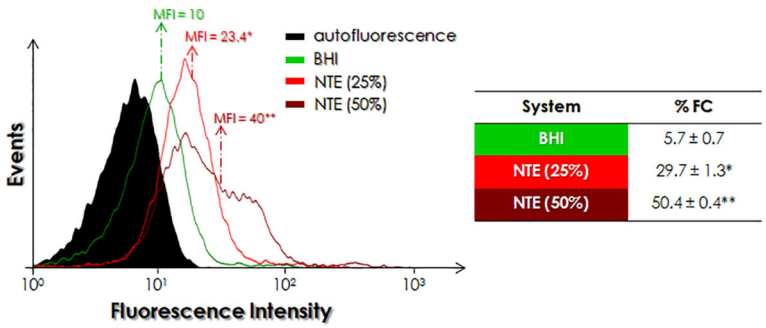


FIGURE 2 | Effects of natural tomato extract (NTE) on cysteine peptidase activity in promastigotes of *P. serpens* cultivated in BHI medium for 48 h in the absence or in the presence of different concentrations of NTE (25 and 50%). **(A)** Cysteine peptidase activity in parasite lysates was assessed by measuring the hydrolysis of Z–Phe–Arg–AMC. Results are expressed as arbitrary fluorescence units (AFU). Data shown are the mean ± standard deviation (SD) of three independent experiments performed in triplicate. Symbols denote statistical differences ($P < 0.05$; Student's *t*-test) between cells grown in BHI medium and cells grown in BHI-NTE medium (25% or 50%). **(B)** The peptidase profiles in cell extracts were analyzed by means of gelatin-SDS-PAGE; gel strips were incubated at 37°C in sodium phosphate buffer, pH 5.0, supplemented with 2 mM DTT. **(C)** Gel strips were also incubated in the absence (control) or presence of cysteine peptidase inhibitors: 1 μM cystatin, 1 μM leupeptin, 1 μM antipain, 1 μM iodoacetamide and 1 μM E-64. Molecular masses, expressed in kDa, are represented on the left. The proteolytic class was determined in cell extracts of parasites grown in BHI medium, but samples from BHI-NTE media were tested in parallel, and similar results were detected.



System	% FC
BHI	5.7 ± 0.7
NTE (25%)	29.7 ± 1.3*
NTE (50%)	50.4 ± 0.4**

FIGURE 3 | Detection of cruzipain-like molecules in *P. serpens* cultivated in BHI medium supplemented or not with either 25% or 50% natural tomato extract (NTE). Promastigotes were cultured for 48 h and then fixed and processed for flow cytometry analysis using anti-cruzipain antibody. The histogram expresses the mean of fluorescence intensity (MFI) levels. Values represented in the table express the percentage of fluorescent cells (%FC). Each experiment was performed at least three independent times. The symbols indicate the experimental systems considered statistically significant from the BHI medium (*, $P < 0.05$ and **, $P < 0.01$; Student's *t*-test).

NTE supplemented in BHI (**Figure 3**) and in PBS-glucose (**Figure 4**) media, we decided to evaluate the modulation of expression of cruzipain-like molecules in parasites recovered from infected tomatoes. In this sense, promastigotes inoculated directly into tomatoes and cultivated for seven days were collected and analyzed using flow cytometry. Initially, we verified whether the cultivation of the parasites in the fruit was able to promote morphological changes. Our data indicated a small reduction in parasite size (FSC = 11.2 ± 0.9) as compared to parasites cultivated in BHI medium (FSC = 16 ± 0.4). Similarly, a small decrease in the granularity parameter was also verified in *P. serpens* cultivated in tomato (SSC = 66 ± 6) as compared to cells cultivated in culture medium (SSC = 76.8 ± 4.7) (**Figure 5A**). Afterward, we evaluated the expression of cruzipain-like molecules on parasites. As verified in the fluorescence intensity histogram, flagellates recovered from the fruit expressed a greater number of molecules similar to cruzipain than control parasites cultivated in BHI culture medium (**Figure 5B**). The data indicate

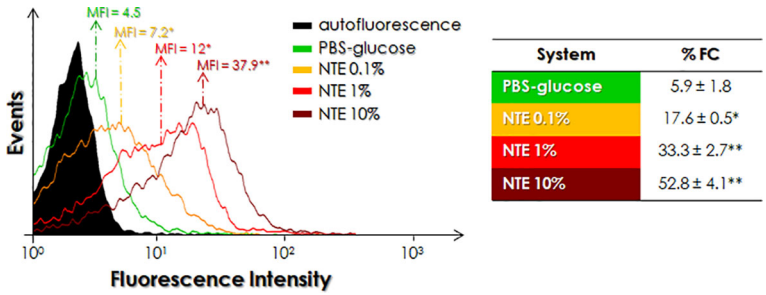


FIGURE 4 | Detection of cruzipain-like molecules in *P. serpens* cultivated in PBS-glucose supplemented or not with 0.1%, 1% or 10% natural tomato extract (NTE). Promastigotes were cultured for 3 h and then fixed and processed for flow cytometry analysis using anti-cruzipain antibody. The histogram expresses the mean of fluorescence intensity (MFI) levels. Values represented in the table express the percentage of fluorescent cells (%FC). Each experiment was performed at least three independent times. The symbols indicate experimental systems considered statistically significant from the PBS-glucose medium (*, $P < 0.05$ and **, $P < 0.01$; Student's *t*-test).

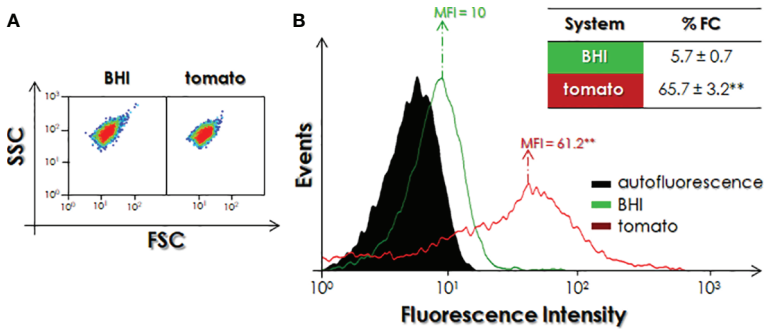


FIGURE 5 | Detection of cruzipain-like molecules in *P. serpens* after infection of tomatoes (*S. lycopersicum*). Promastigotes recovered from infected tomatoes or cultured in BHI medium were washed and analyzed by flow cytometry. **(A)** Morphometric parameters analysis, forward scatter (FSC) and side scatter (SSC); **(B)** Analysis of the expression of cruzipain-like molecules by flow cytometry using anti-cruzipain antibody. The histogram expresses the mean of fluorescence intensity (MFI) levels. Values represented in the table express the percentage of fluorescent cells (%FC). Each experiment was performed at least three independent times. The symbols indicate experimental systems considered statistically significant from the BHI medium (**, $P < 0.01$; Student's *t*-test).

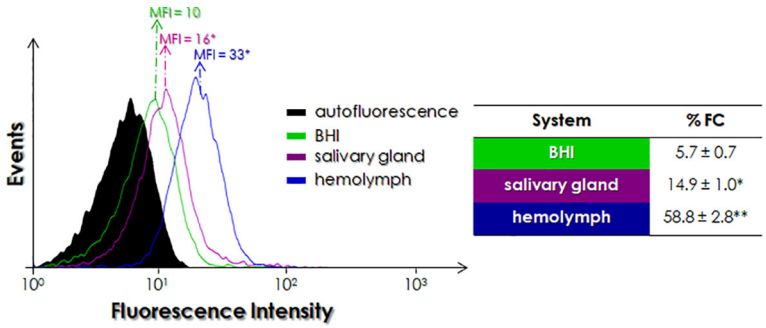
a significant enhancement in both %FC (11.5-fold) and MFI (6.1-fold) parameters from parasites cultivated in tomatoes when compared to control parasites (**Figure 5B**).

Effects of *In Vitro* Infection of *Oncopeltus fasciatus* on the Cruzipain-Like Molecules Expressed by *P. serpens*

The expression of cruzipain-like molecules by *P. serpens* was also modulated by *O. fasciatus*. The data represented in the histogram in **Figure 6** indicate a significant increase in MFI both in parasites incubated with dissected salivary glands and in those recovered from infected insects, when compared to *P. serpens* incubated in BHI medium. This augment was more prominent in parasites isolated from hemolymph than in those incubated with salivary glands (**Figure 6**). The percentage of labeled cells was also higher in the parasites isolated from previously infected *O. fasciatus*, when compared to control parasites grown in BHI. There was a 10.3-fold increase in the %FC for cruzipain-like molecules in parasites isolated from hemolymph, while a 2.6-fold increase in the %FC was seen in parasites incubated with the salivary glands.

DISCUSSION

Phytomonas serpens is a heteroxenic parasite that finds extremely different environments during its life cycle: intestine/hemolymph/salivary glands of insects and endocarp of tomatoes; hence, the parasite depends on its adaptation to all these environments in order to proliferate, colonize and survive. In this regard, peptidases are used by trypanosomatids to hydrolyze proteins, allowing the parasite to acquire necessary amino acids to its proliferation as well as to overcome protective barriers of insects and plants, through cleavage of antimicrobial peptides and degradation of cell wall protein components (Mosolov et al., 2001; Mosolov and Valueva, 2004). Production of proteolytic enzymes to degrade host barriers is a common strategy used by microorganisms to colonize tissues and



System	% FC
BHI	5.7 ± 0.7
salivary gland	14.9 ± 1.0*
hemolymph	58.8 ± 2.8**

FIGURE 6 | Detection of cruzipain-like molecules in *P. serpens* after infection of *Oncopeltus fasciatus*. Parasites recovered after parasite interaction with explanted salivary glands of *O. fasciatus* and recovered from the hemolymph of infected insects were washed, resuspended in PBS and processed for flow cytometry analysis. The histogram expresses the mean of fluorescence intensity (MFI) levels using anti-cruzipain antibody. Values represented in the table express the percentage of fluorescent cells (%FC). Each experiment was performed at least three independent times. The symbols indicate the experimental systems considered statistically significant from the BHI medium (*, $P < 0.05$ and **, $P < 0.01$; Student's *t*-test).

eventually cause diseases; thus, the greater the plasticity of these enzymes, the more effective they will be in host colonization processes.

The first aspect exploited in this work was the parasite ability to survive in BHI culture medium supplemented with natural tomato extract, herein called NTE. We observed that BHI-NTE is favorable for the growth and maintenance of the viability of *P. serpens*, since parasites were able to survive and proliferate without showing any morphological changes. As previously determined by our group, incubation of *P. serpens* in PBS-glucose supplemented with different proteins (BSA, human serum albumin, hemoglobin, mucin, IgG and fetal bovine serum) did not lead to loss of cell viability neither promoted alterations in the measurement of cell size and granularity of the parasites (Elias et al., 2012). In the present study, *P. serpens* was able to grow in a complex medium (BHI) with up to 50% of NTE, although the increase in the concentration of tomato extract resulted in a reduction in the growth rate, probably due to the pH acidification of the culture medium promoted by the addition of NTE and/or due to the dilution of the rich medium (BHI) with the less nutritive NTE.

Jankevicius et al. (1989) had previously infected tomatoes with *P. serpens* and observed that the flagellates were able to multiply actively in the fruits sap; in addition, parasites isolated from the fruit had a smaller body length, body width and flagellum length when compared to parasites cultivated in the culture medium. In this report, similarly, we found that *P. serpens* recovered from infected tomatoes showed a reduction in cell size as compared to parasites grown in BHI medium. In this sense, the great polymorphism of *P. serpens* was previously observed through the presence of two subpopulations of parasites isolated from the digestive tract and salivary glands of the insect vector *P. picta*: one formed by small flagellates and the other one containing large parasites (Jankevicius et al., 1989). In addition, a report showed that 72 h post-infection of *O. fasciatus* with *P. serpens*, 40% of the parasites present in the circulation and almost 100% of the parasites attached to salivary glands of *O. fasciatus* had a long slender cell body (Alves e Silva et al., 2013). Since *P. serpens* has the same evolutionary form throughout its life cycle, the observed size differences according to the host where the parasite is found remains unknown. However, Alves e Silva et al. (2013) proposed that probably the long slender form present in the insect can help the parasite to escape from phagocytosis of hemocytes of invertebrate hosts.

The previous observation by our group that cysteine peptidases from *P. serpens* are able to degrade different proteinaceous substrates reinforces the wide versatility of these enzymes (Elias et al., 2008). In this study, it was possible to observe that expression of cysteine peptidases increases when parasites were cultivated in axenic culture media (BHI or PBS-glucose) containing NTE in a dose-dependent manner. Cysteine peptidases from *P. serpens* have already been biochemically characterized, and identified as homologous molecules to *T. cruzi* cruzipain (Elias et al., 2008; Elias et al., 2009). In *T. cruzi*, this enzyme presents comparable expression levels at all stages of the parasite's life cycle, although it is found at higher levels in the epimastigote forms, present in the invertebrate host, than in amastigotes or trypomastigotes forms (Branquinha et al., 2015). In epimastigote forms, cruzipain is found in reservosomes, organelles similar to lysosomes, where protein degradation occurs for nutritional purposes. In trypomastigote and amastigote forms, expression of cruzipain is located to the flagellar pocket and cell surface, respectively (Branquinha et al., 2015). In *P. serpens*, cruzipain homologues are located in different cellular compartments, including cytoplasm, membrane, flagellum, and inside flagellar pocket (Elias et al., 2009; Elias et al., 2012).

The increased expression of cysteine peptidases promoted by the presence of NTE in the culture medium was accompanied by an increase in the expression of epitopes reactive to anti-cruzipain antibodies, suggesting the importance of these proteins during establishment of the infection in the fruit host. In the present work, the increased expression of cruzipain-like proteins could also be observed in the direct interaction between *P. serpens-S. lycopersicum* (tomato), in parasites incubated with salivary glands of *O. fasciatus* and in parasites recovered from the hemolymph of infected insects. Previously, it was reported that *T. cruzi* isolated from infected *R. prolixus* showed a drastic increase in the expression of surface cruzipain molecules (Uehara et al., 2012). Santos et al. (2006) found that cruzipain-like molecules in *P. serpens*

are essential during the invertebrate host life cycle, since treatment of the parasite with either cysteine peptidase inhibitors or anti-cruzipain antibodies promoted a drastic reduction in the interaction process between *P. serpens* and explanted salivary glands of *O. fasciatus*. Interestingly, another report showed that SDS-PAGE analysis using salivary gland extract of *O. fasciatus* as substrate identified a proteolytic activity of cruzipain-like molecules and that these enzymes act by cleaving a 115-kDa polypeptide located on the surface of salivary glands. These data suggest that cruzipain-like molecules are responsible for the cleavage of proteinaceous components present on salivary glands, promoting exposure of key surface receptors for the interaction between *P. serpens* and the insect (Elias et al., 2008). In *T. cruzi*, cruzipain is also crucial for the interaction of the parasite with the invertebrate host, since treatment of *T. cruzi* epimastigotes with anti-cruzipain antibodies or with cysteine peptidase inhibitors (cystatin, antipain, E-64, leupeptin, iodocetamide) decreased the parasite adhesion to *R. prolixus* midgut. Furthermore, mutant epimastigotes overexpressing the endogenous cruzipain inhibitor (chagasin) displayed low rates of adhesion to insect dissected midguts *ex vivo*; in addition, *in vivo* experiments also revealed low levels of colonization of *R. prolixus* midgut and rectum by mutant parasites in comparison to wild-type cells (Uehara et al., 2012).

The acidification of the culture medium containing NTE raised the question as to whether this environment should be considered more hostile for parasite growth. Since an increase in cysteine peptidase activity was also verified in parasites recovered from infected tomatoes, it is possible to correlate this fact to the adaptation to growth in a more acidic medium. Furthermore, it is well reported in the literature that secondary metabolites produced by plants can play a fundamental role in the resistance of plants to parasitic infections (Friedman, 2002; Evangelista et al., 2018). For instance, the glycoalkaloid α-tomatine has a potent antimicrobial action and it is found mainly in green tomatoes, with decreased concentration during fruit maturation (Medina et al., 2015). α-tomatine and its aglycone structure, tomatidine, display *in vitro* toxicity against *P. serpens*, but the lower levels found in mature tomatoes are not able to act as an effective barrier against *P. serpens* infection (Medina et al., 2015). However, prolonged exposure time to tomatidine may decrease the parasite's growth (Evangelista et al., 2018). Additionally, peptidase inhibitors are also produced by tomatoes in order to protect the plant against pathogens and predators (Friedman, 2002). In this sense, the increased expression of cysteine peptidases may also be acting as a compensatory mechanism due to the production of peptidase inhibitors, ensuring parasite survival.

The knowledge about the metabolism of *P. serpens* is essential to understand its pathological potential for tomato fruits, and the proteomic analysis of the parasite allowed the identification of enzymes of metabolic pathways located in glycosome, mitochondrion and cytosol (Dos Santos Júnior et al., 2018). However, no enzymatic activity involved in the hydrolysis of polysaccharides into monosaccharides, such as amylases, amylomaltases, invertases or carboxymethylcellulases, were identified in *P. serpens*. On the other hand, peptidases present in these parasites seem to be aid the establishment of the infection in the fruit and in the invertebrate host. In this report, we verified that cysteine peptidases can help in different processes, either in the degradation of protein substrates that will provide necessary amino acids for their proliferation and nutrition, or during the interaction with the fruit and insect contributing to the survival of the parasite in these environments. Altogether, our data indicate that cruzipain-like molecules are expressed throughout the life cycle of *P. serpens*, playing essential roles in the parasite's interaction processes with the invertebrate as well as with plant hosts, which must be further explored.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

All authors conceived and designed the experiments. SO, CE, and FD performed the experiments. All authors analyzed the data. AL, CD'A-L, AS, and MB contributed reagents/materials/analysis tools. All authors wrote and revised the paper. All authors contributed to the research and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work. All authors have read and agreed to the published version of the manuscript.

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BRIEF RESEARCH REPORT
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Causative Agents of American Tegumentary Leishmaniasis Are Able to Infect 3T3-L1 Adipocytes *In Vitro*

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Although macrophages have long been considered key players in the course of *Leishmania* infections, other non-professional phagocytes have lately been shown to maintain low levels of the parasite in safe intracellular niches. Recently, it was demonstrated that the adipose tissue is capable of harboring Old World *L.* (*L.*) *infantum* in mice. However, there is no evidence of experimental adipocyte infection with New World *Leishmania* species so far. In addition, it was not known whether adipocytes would be permissive for formation of the unique, large and communal parasitophorous vacuoles that are typical of *L.* (*L.*) *amazonensis* in macrophages. Here we evaluated the ability of *L.* (*L.*) *amazonensis* and *L.* (*V.*) *braziliensis* promastigotes and amastigotes to infect 3T3-L1 fibroblast-derived adipocytes (3T3-Ad) using light and transmission electron microscopy. Our results indicate that amastigotes and promastigotes of both species were capable of infecting and surviving inside pre- and fully differentiated 3T3-Ad for up to 144 h. Importantly, *L.* (*L.*) *amazonensis* amastigotes resided in large communal parasitophorous vacuoles in pre-adipocytes, which appeared to be compressed between large lipid droplets in mature adipocytes. In parallel, individual *L. (V.) braziliensis* amastigotes were detected in single vacuoles 144 h post-infection. We conclude that 3T3-Ad may constitute an environment that supports low loads of viable parasites perhaps contributing to parasite maintenance, since amastigotes of both species recovered from these cells differentiated into replicative promastigotes. Our findings shed light on the potential of a new host cell model that can be relevant to the persistence of New World *Leishmania* species.

Keywords: 3T3-L1, adipocyte, infection, *Leishmania*, microscopy

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INTRODUCTION

At least 20 species belonging to the *Leishmania* genus cause leishmaniasis, a complex disease with different clinical manifestations that leads to 20,000-30,000 annual deaths worldwide (Akhoundi et al., 2016; WHO, 2021). The disease has shown increased incidence and expansion of transmission to new territories in recent years (Pigott et al., 2014; Cotton, 2017).

Two main stages can be recognized throughout the life cycle of *Leishmania*: oval-shaped cells with an interiorized/reduced flagellum (amastigotes) and fusiform cells with a prominent flagellum (promastigotes). Amastigotes are taken up by female sand flies with the blood of an infected vertebrate and, upon reaching the digestive tract of the insect, transform into promastigotes that attach to the gut epithelium. After a few days, the parasites are released from the gut epithelium and accumulate in the insect's stomodeal valve as non-replicative metacyclic promastigotes, prior to inoculation in the vertebrate host dermis where they will subvert innate defense mechanisms and infect mononuclear phagocytic cells, mainly macrophages. Within these cells, metacyclic promastigotes tolerate increase in temperature (~25 to ~34°C), decrease in pH and low iron availability, features directly responsible for triggering promastigote-to-amastigote differentiation inside phagolysosomal compartments (parasitophorous vacuoles, PVs) (Sacks and Kamhawi, 2001; Mittra et al., 2013). After transformation, amastigotes divide by binary fission several times and rupture the host cell. Finally, the mononuclear phagocyte system will internalize these newly released parasites both locally and after dissemination, leading to the classical leishmaniasis symptoms (McCall et al., 2013).

Infected patients can develop the cutaneous form of leishmaniasis that affects skin, lymph nodes and mucous membranes, or the visceral form, with spleen, bone marrow and liver parasitism. Disease treatment is challenging due to high cost, drug toxicity, hospitalization need for parenteral drug administration, and variable efficacy that can lead to disease recurrence. In addition, reports of parasite resistance to antimonials, used as the first drug choice in many regions, have been a matter of concern in the context of antileishmanial chemotherapy (Darcis et al., 2017; Souza et al., 2017; Alcântara et al., 2018).

The cellular and molecular mechanisms underlying the chronicity of leishmaniasis, common in relapsing patients, are not fully understood. However, a few studies revealed that hyperactivation of the inflammatory response mediated by *Leishmania* plays an important role during the establishment of chronic infections (Navas et al., 2014). Furthermore, distinct cell types, such as fibroblasts and hepatocytes, may be involved in disease control and spread of the parasite to different tissues (Bogdan et al., 2000; Morehead et al., 2002; Vianna et al., 2002; Cavalcante-Costa et al., 2019). Adipocytes, for example, are not only storage cells within tissues and organs, but are also associated with inflammatory signaling in metabolic diseases (Cristancho and Lazar, 2011). Interestingly, Allahverdiyev and co-authors described experimental infections of adipocytes derived from mesenchymal stem cells with the following Old World *Leishmania* species: *Leishmania (Leishmania) donovani*, *L. (L.) major*, *L. (L.) tropica*, and *L. (L.) infantum* (Allahverdiyev et al., 2011). Also, a very recent study showed that murine adipose tissue harbors Old World *L. (L.) infantum* (Schwing et al., 2021).

There is no evidence of experimental adipocytic infection with New World *Leishmania* species in the literature so far. However, given the implication of adipocytes in inflammatory signaling and recent evidence of successful *in vitro* and *in vivo* infection by Old World *Leishmania* species, it is reasonable to speculate that the establishment of adipocytic infections with *Leishmania* species that cause American Tegumentary Leishmaniasis (ATL), i.e., *L.* (*L.*) *amazonensis* and *L.* (*Viannia*) *braziliensis*, may not only be feasible, but also play an important role in parasite persistence. In fact, in addition to the pathologies being quite distinct in the regions where both species circulate, there are also differences related to the formation and organization of the intravacuolar environment occupied by species of the *Leishmania mexicana* complex (i.e., *L. (L.) amazonensis*) when compared with other species. Taken together, all aspects above-mentioned highlight the importance of knowing this new model of infection by *Leishmania* spp.

METHODS

Parasite Cultivation

Leishmania (L.) amazonensis (IFLA/BR/67/PH8 WT and GFP strains (Renberg et al., 2015)) and *Leishmania (V.) braziliensis* (MHOM/BR/75/2903 strain) promastigotes were cultivated in medium 199 with 20% heat-inactivated fetal bovine serum (FBS), 10 mM adenine, 5 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich, Merck KGaA, USA) in 25 cm^2 cell culture flasks at 25°C. Cultures of *L. (V.) braziliensis* were supplemented with 10% FBS and 5% sterile male human urine. *L. (L.) amazonensis* and *L. (V.) braziliensis* amastigotes were cultivated axenically as previously described (Miguel et al., 2013) in modified medium 199 supplemented with 20% FBS acidified to pH 4.8 and pH 5.2, respectively. Passages were kept for up to two weeks at 32°C for *L. (L.) amazonensis* and 34°C for *L. (V.) braziliensis*.

Differentiation of 3T3-L1 Fibroblasts

3T3-L1 fibroblasts were grown in DMEM medium (Gibco, Thermo Fisher) supplemented with 10% FBS, 2 mM L-glutamine, 0.1 M sodium pyruvate, 40 mM HEPES and 1% penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich) in 25 cm^2 culture vented flasks at 37°C with 5% CO_2. The culture was split every three days from 10^5 cells/mL. 3T3-L1 fibroblast-derived adipocytes (3T3-Ad) were obtained as previously described (Zebisch et al., 2012), with some modifications. The total period of cell differentiation consisted of 14 days, with medium replacement every 48 h. Initially, 10^4 cells/mL were seeded in 24-well plates (Corning) containing 13 mm coverslips. On day 3, cultures at 70-80% of confluence were exposed to the adipogenic cocktail containing 0.25 mM dexamethasone, 2 µg/mL insulin and 0.5 mM IBMX (Sigma-Aldrich) for 48h at 37°C, 5% CO_2. On day 5, the supernatant was replaced by fresh medium containing insulin at 1 µg/mL for additional 48 h. On days 7-14, medium replacements were performed every 48 h. Pre-adipocytes containing small lipid droplets and a few mature 3T3-Ad were detected from day 8. Mature 3T3-Ad were observed until day 14.

In Vitro Infections and Staining Protocols

Pre- and mature 3T3-Ad were infected with *Leishmania* late stationary phase-promastigotes or axenic amastigotes (multiplicity of infection (MOI) = 20) in 24-well plates containing 13 mm glass coverslips and kept at 34°C with 5% CO_2. After 4 h of incubation, wells were washed three times with warm PBS (1X) and incubated for 1, 24, 48 and 144h at 34°C with 5% CO_2. Next, coverslips were washed twice with warm PBS (1X) and fixed in 4% formaldehyde solution for 1h. Microscopic examination of unstained cells was performed using EVOS imaging systems (ThermoFisher Scientific). In parallel, cultures were sequentially stained as follows: one part Oil-Red O stock solution in ddH_2O Zebisch et al. (2012) (2:1; v:v) for 2h, washed three times with ddH2O and then incubated with Giemsa solution for 15 min. Next, coverslips were gently rinsed in water. Intracellular parasite counts were obtained by quantifying the parasite number for at least 100 3T3-Ad per condition under a light microscope (100x oil immersion objective, Eclipse E200, Nikon).

For BODIPY lipid staining, coverslips were washed twice with warm PBS (1X) and fixed with 4% paraformaldehyde for 30 min at room temperature. Following fixation, samples were washed as describe above and permeabilized with 0.5% Triton for 20 min. Next, coverslips were incubated with 1µg/mL BODIPY 493/503 (Invitrogen) for 20 min. Samples were washed and mounted with Prolong Gold Antifade With Dapi (Invitrogen) and observed with the Zeiss Axio Imager 2 epifluorescence microscope. Images were processed using ImageJ 1.50b software (NIH USA).

For infections with *L.* (*L.*) *amazonensis* expressing GFP, 3T3-Ad were incubated with axenic amastigotes (MOI=20) in 24-well plates containing 13 mm glass coverslips and kept as described above. Coverslips were washed in warm PBS (1X) and carefully mounted onto microscope slides for examination of GFP amastigotes (Ex/Em 488/510 nm) using a Zeiss Axiovert 135 microscope equipped with digital camera (Orca II, Hamamatsu) controlled by Metamorph software (Universal Imaging). The tests were carried out in triplicates and with at least two independent experiments. Statistical analyzes were performed with Origin8 software (OriginLab) using Student's t-test, with differences considered significant when p-value < 0.05.

Transmission Electron Microscopy

Samples of 3T3-Ad infected with *Leishmania* were washed three times with warm PBS (1X), fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer and 3 mM CaCl2 pH 7.4 for 5 min at room temperature and 1h at 4°C. The samples were washed three times with 0.1M sodium cacodylate buffer and 3mM CaCl2 at 4°C and three times with 0.1 M imidazole buffer pH 7.4 at 4°C to produce prominent electron-opaque staining of lipid droplets. Next, the cells were post-fixed with 2% osmium tetroxide in 0.1 M imidazole buffer pH 7.4, for 30 minutes at 4°C. Next, after five washes with ddH2O water at 4°C, samples were treated with filtered 2% aqueous uranyl acetate overnight at 4°C. After this, five washes with ddH2O water at 4°C was performed and sequential dehydration with ethanol was done at 4°C. Next, samples were embedded in Epon resin mixed with ethanol (1:1) under constant agitation for 30 min at room temperature. Embedding with pure resin was repeated five times. Samples were included in pure Epon resin for 72h at 60°C for complete polymerization. Finally, the material was sectioned and contrasted with 2% aqueous uranyl citrate for 20 min and lead citrate for 10 min at room temperature. Transmission electron microscopy analysis was performed using a LEO 906 Microscope (Zeiss) at the Electron Microscopy Laboratory (Institute of Biology, UNICAMP).

Assessment of Amastigote Viability

Amastigotes' viability was assessed by monitoring the transformation of intracellular parasites into promastigotes after 144 h of *in vitro* infection. For this, infected 3T3-Ad cultures were lysed with 0.04% SDS (sodium dodecyl sulfate) in 300 µL PBS (1X) through 10 cycles of resuspension of the content using a 30-G needle in 1 mL sterile syringes. The suspension was collected and washed with alternate cycles of centrifugation. First, suspensions were centrifuged at 100 x g for 10 minutes at 4°C for recovering supernatants containing amastigotes. Next, samples were resuspended in cold PBS (1X) and centrifuged at 800 x g for 10 minutes at 4°C to remove supernatants, while pellets were resuspended in PBS (1X). This last cycle was repeated twice. Pellets containing amastigotes were seeded in 96-well plates (10^5/mL) with M199 medium for promastigote differentiation at 25°C for up to 9 days. Growth curves after differentiation of viable cells were determined by counting the number of promastigotes with a Neubauer chamber every other day.

RESULTS

Adipocyte cultures were established by incubation of 3T3-L1 fibroblasts with the differentiation cocktail for 8 days. With this protocol we reached a differentiation rate of approximately 70-80% and several cells containing Oil-Red O-stained lipid droplets were easily identified by Giemsa staining and light microscopy (**Figure 1A**). Differentiated cultures (referred to as 3T3-Ad) were incubated with *L. (L.) amazonensis* promastigotes for 4 h and various staining protocols were tested to determine the best fixation/dye method that allowed proper visualization of *Leishmania* and 3T3-Ad interactions. Contact of the flagellar tips of promastigotes with 3T3-Ad was detected in methanol-fixed preparations stained with Giemsa, where lipid droplets were visualized as hyaline spaces (**Figure 1B**), and also by live light microscopy imaging (**Video 1; Supplementary Material**). Promastigotes close to 3T3-Ad were also seen by transmission electron microscopy (**Figure 1C**).

Internalized *L. (L.) amazonensis* promastigotes typically differentiated into amastigotes after 48 h, and occupied large PVs surrounded by lipid droplets (**Figures 1D–F**). Clear visualization of PVs interspersed with lipid droplets in infected pre-adipocytes was possible using the Giemsa and Oil-Red O co-staining protocol (**Figure 1G**). However, in fully differentiated 3T3-Ad, that contains larger lipid droplets, observation of the

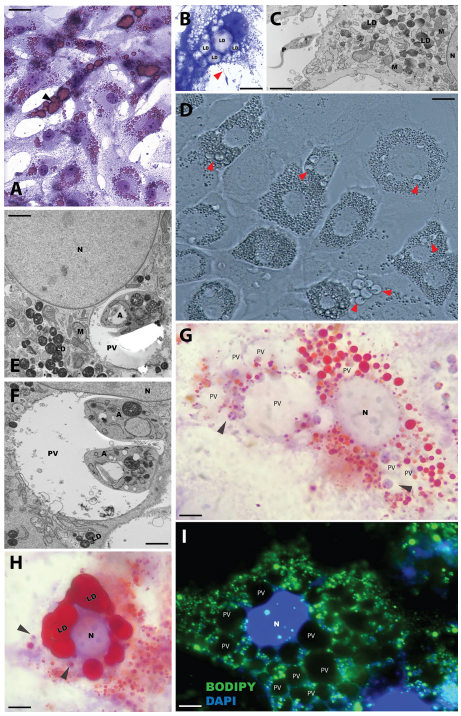

FIGURE 1 | Representative micrographs of 3T3-Ad *in vitro* infected with *L. (L.) amazonensis*. **(A)** 3T3-L1 fibroblasts undergoing adipocyte differentiation after 14 days, in which lipid droplets are stained with Oil-Red O (arrowhead) and are observed in differentiated adipocytes stained simultaneously with Giemsa. Scale bar = 8 µm. **(B)** Differentiated adipocyte stained with Giemsa, in which hyaline lipid droplets (LD) spaces can be seen, were incubated with *L. (L.) amazonensis* stationary phase promastigotes (MOI=20) for 1 h. Note the promastigote attached to the adipocyte by the flagellum (red arrowhead). Scale bar = 12 µm. **(C)** Ultrastructure showed a promastigote ("P") close to the plasma membrane of an 3T3-Ad. "M": mitochondria, "LD": electron-opaque lipid droplets after osmium-imidazole treatment, "N": nucleus. Scale bar = 5 µm. **(D)** Unstained infected 3T3-Ad as described in **(B)**, observed by phase contrast microscopy after 48 h. Red arrows point to PVs containing amastigotes. Scale bar = 6 µm. **(E, F)** Ultrastructure of PV with amastigotes **(A)** infected for 48h "M": mitochondria, "LD": electron-opaque lipid droplets after osmium-imidazole treatment, "N": nucleus. Scale bar = 0.5 µm. Representative 3T3-L1 pre-adipocyte **(G)** and 3T3-Ad **(H)** infected for 48 h and stained with Giemsa and Oil-Red O, where small red droplets (LD) are dispersed throughout the cytosol close to PVs harboring amastigotes (arrowheads) **(G)**. Bulky droplets (LD) stained in red **(H)**, with amastigotes nearby (arrowheads). "N": nucleus. Scale bars **(G)** = 2.5 µm; **(H)** = 3.5 µm. **(I)** Fluorescence microscopy of infected 3T3-Ad for 48 h. Lipid droplets stained with BODIPY 493/503 (green) are closely seen with PV. "N": nucleus of host cell and parasites stained in blue with DAPI. Scale bar = 6 µm.

parasites was challenging and depended on the focal position (**Figure 1H**). *L. (L.) amazonensis*-infected 3T3-Ad were also examined by fluorescence microscopy after staining the lipid content with BODIPY 493/503 (green) (**Figure 1I**). Undoubtedly, these photomicrographs clearly evidenced BODIPY staining in bodies close to amastigote-containing PVs (**Figure 1I**).

To avoid possible deformation of lipid droplets caused by staining protocols and to more precisely determine the intracellular distribution of parasites, assays were conducted in live cells with green fluorescent amastigotes (GFP – *L. amazonensis*). **Figure 2** shows that after 144 h, amastigotes internalized in 3T3-Ad were tightly localized between large and abundant lipid droplets.

Taken together, our results indicate that the expanded and communal PVs typical of *L. (L.) amazonensis*-infected macrophages also form in non-phagocytic cells containing lipid droplets O (**Figures 1E–G**). However, fully differentiated adipocytes imposed visual challenges due to possible compression of the large communal PVs by the large lipid droplets. The typical morphology for *L. (V.) braziliensis* PVs in macrophage infections, i.e., single and tight vacuoles harboring the amastigote stage, was also observed for 3T3-Ad containing reduced or increased number of lipid droplets (**Figure 3**).

Once the presence of the parasite was confirmed in our cell model, our next step was to investigate whether 3T3-Ad were able to sustain viable *L.* (*L.*) *amazonensis* and *L.* (*V.*) *braziliensis* intracellular parasites. Both stages were able to infect and persist in 3T3-Ad for up to 144 h, despite the detection of fewer amastigotes after 48 h (**Figures 4A, B**). Intracellular parasites were recovered and *in vitro* promastigote differentiation assays were performed, followed by assessment of the parasite's ability to replicate by counting the cells over a period of 9 days (**Figures 4C, D**). Our results indicate that amastigotes of both *Leishmania* species remained viable in 3T3-Ad infected for 144 h, being capable of differentiating into promastigotes and replicating in culture.

DISCUSSION

Mononuclear phagocytes are recognized as the main target cells for parasitism by *Leishmania* amastigotes. However, a number of cell types have been suggested as potential additional infection sites by *Leishmania* (Rittig and Bogdan, 2000), including adipocytes (Allahverdiyev et al., 2011; Schwing et al., 2021). Studies concerning the adipose tissue have been limited to morphofunctional aspects for many decades due to its putative stable and immutable nature. However, this paradigm has been revisited with an increasing number of studies correlating this tissue to cardiovascular diseases, diabetes and inflammatory modulation (Cristancho and Lazar, 2011; Groeneveld, 2020). Moreover, evidence has been accumulating indicating that the adipose tissue may play a role in infections caused by bacteria, virus and protozoan parasites, perhaps by providing nutrients to allow the pathogen's proliferation and survival (Shoemaker et al., 1970; Desruisseaux et al., 2007; Tanowitz et al., 2017; Bouzid et al., 2017; Bourgeois et al., 2019).

Concerning the 'trypanosomatid-adipocyte' interaction, it has been reported that *Trypanosoma brucei* and *T. cruzi* can associate with mouse white and brown adipose tissues, respectively (Shoemaker et al., 1970; Trindade et al., 2016). However, replicative forms of *T. brucei* are extracellular and, for *T. cruzi*, nucleated host cells are susceptible to active invasion and colonization by its replicative forms. With regard to the biology of *Leishmania* infection, its preference for phagocytic cells, particularly macrophages, has been very clear and

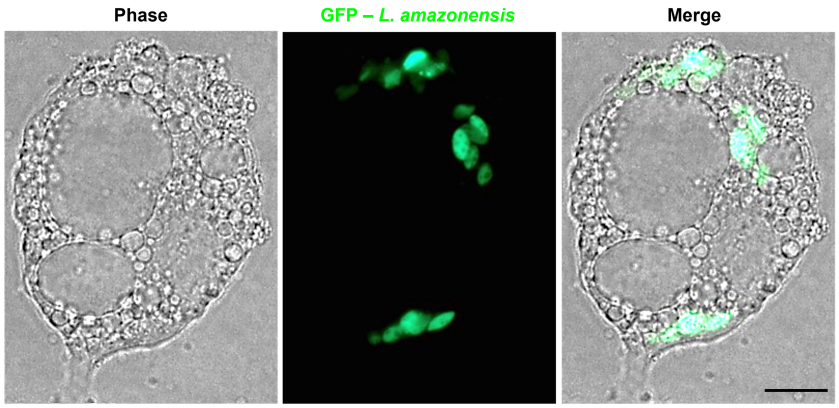

FIGURE 2 | Unfixed 3T3-Ad infected with GFP – *L. (L.) amazonensis* for 144 h. Fully-differentiated 3T3-Ad showing intracellular amastigotes expressing GFP (green) accumulated among lipid droplets. Scale bar = 5 μm.

considered to be directly associated with the development of leishmaniasis (Novais et al., 2014).

Leishmaniasis is classically manifested in its cutaneous or visceral form, affecting skin layers and internal organs, respectively. In both cases, adipocytes should be found occupying interstitial compartments, suggesting that *Leishmania* may interact with adipocytes in the event of both cutaneous and visceral leishmaniasis. Based on this hypothesis and on the fact that there is no evidence in the literature showing that ATL-causing species are capable to infect adipocytes, we investigated the ability of promastigotes and amastigotes of *L. (L.) amazonensis* and *L. (V.) braziliensis* to infect and replicate in 3T3-Ad.

Several specific staining protocols and microscopic techniques were employed in our study to confirm the

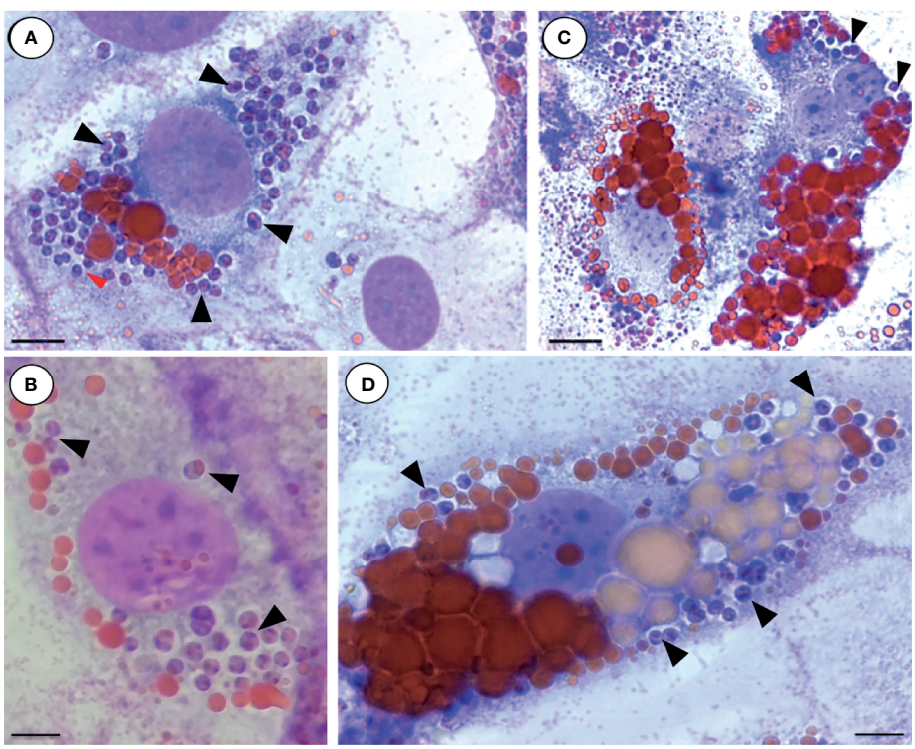

FIGURE 3 | Infection of 3T3-Ad with *L. (V.) braziliensis*. 3T3-Ad presenting reduced **(A, B)** or increased number of lipid droplets **(C, D)** were infected with axenic amastigotes of *L. (V.) braziliensis* for 24 h (MOI=20) and stained with Giemsa (purple nuclei and purple amastigotes) and Oil-Red O (lipid droplets in red). Arrowheads point to single amastigotes and individual PVs. Bars **(A)** = 6 μm; **(B, D)** = 5 μm, **(C)** = 8 μm.

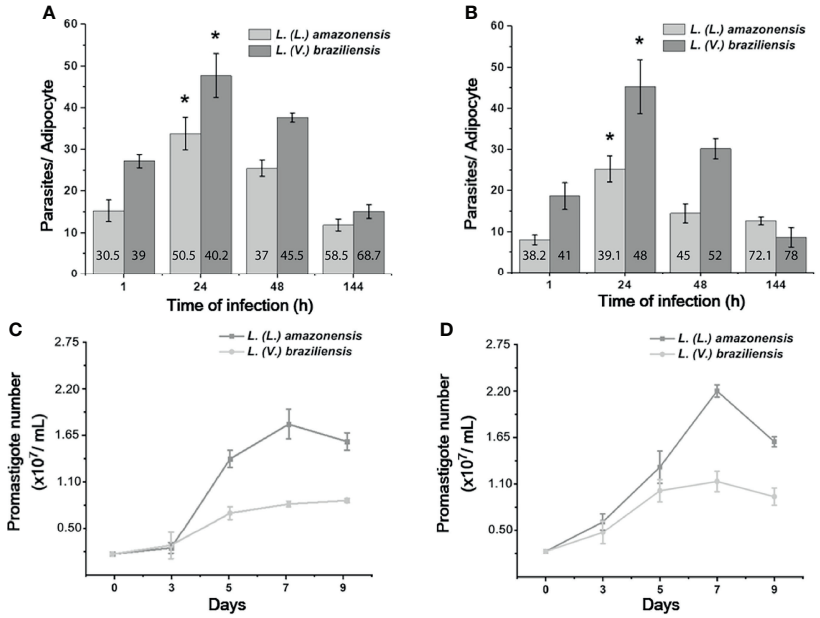

FIGURE 4 | *In vitro* kinetics of *Leishmania* intracellular growth after 3T3-Ad infection, and parasite viability. The mean number of amastigotes per 3T3-Ad ± standard deviation was determined after infection with promastigotes **(A)** or axenic amastigotes **(B)** and incubation for 1, 24, 48 and 144 h, for *L. (L.) amazonensis* and *L. (V.) braziliensis*. At least 100 3T3-Ad cells were counted per condition. Assays were performed in triplicate and the assays were repeated at least twice. Asterisks indicate a statistically significant difference (p-value < 0.05) for each time point *vs*. 1 h (Student's t test). Numbers in the bars indicate the mean of infected 3T3-Ad (%) per condition. **(C, D)**: Growth curves of parasites recovered from 3T3-Ad infections with promastigotes or amastigotes, respectively, after 144 h. Amastigote-promastigote differentiation was carried out as described in the 'Methods Section' and recovered parasites resuspended in M199 were kept at 25°C. On the 3rd day, promastigotes were observed, which grew exponentially until reaching the maximum density peaks around day 7. Experiments represent the mean of triplicates ± standard deviation.

adipocytes' permissiveness to *Leishmania*. The panel in **Figure 1** illustrates the infective process of *L. (L.) amazonensis* in 3T3-Ad by revealing the interaction of promastigotes *via* flagellar tip (**Video 1; Supplementary Material**) and the presence of intracellular amastigotes at longer times of infection. When macrophages are infected by *L. (L.) amazonensis*, unique expanded and communal PVs containing several amastigotes are observed (Real et al., 2010) and, in our assays, large communal PVs were also detected in 3T3-Ad containing *L. (L.) amazonensis*, especially in host cells with low amounts or smaller sizes of lipid droplets (i.e., pre-adipocytes) (**Figure 1G, I**). In fact, the 3T3-L1-differentiation process is considered a dynamic process, as it promotes droplet remodeling during lipolysis and cell growth. Paar et al. showed that the growth of lipid droplets during human adipose-derived stem cells differentiation into adipocytes is equivalent to the process observed in 3T3-L1 cells (Paar et al., 2012). Our 3T3-Ad reached full differentiation as determined by the presence of larger droplets, and although this made it more challenging to observe the limits of PVs, it was clear that these cells contained viable amastigotes (**Figures 1H**, **2**). With regard to *L. (V.) braziliensis* infection, Giemsa and Oil-Red O-stained cells showed intact individualized PVs containing single amastigotes (**Figure 3**), as expected for this species in macrophage infections. Thus, an important finding of our study is that it clearly indicates that the dynamics of generation and maintenance of the adipocytic vacuolar environment, which is very different between *L. (L.) amazonensis* and *L. (V.) braziliensis*, is controlled by the parasite itself, not depending on unique characteristics of phagocytic host cells, as it has been previously shown that fibroblasts are capable of sustaining *Leishmania* infections (Morehead et al., 2002; Wilson et al., 2008).

ATL caused by *L. (L.) amazonensis* and *L. (V.) braziliensis* show different lesion aspects with inflammatory infiltrates that vary in their major cellular components (de Souza et al., 2011; de Oliveira and Brodskyn, 2012). However, phagocytic cells usually reveal the communal PV architecture greatly altered when filled with *L. (L.) amazonensis* amastigotes. This histopathological modulation of the vacuolar environment seems to be a strategy for parasite survival, as it may lead to the dilution of molecules with leishmanicidal action (Sacks and Sher, 2002; Wilson et al., 2008).

Quantification of the parasite burden revealed that promastigotes and axenic amastigotes had a similar capacity to infect 3T3-Ad, with infection levels peaking after 24 h for both species. Moreover, amastigotes of *L. (L.) amazonensis* and *L. (V.) braziliensis* were viable intracellularly up to after 144 h, as parasites were able to differentiate into flagellate promastigote forms, reproduce asexually and reach maximum density in culture growth curves (**Figure 4**). It is interesting to point out that infections with reduced MOI (2 and 5) were not efficient in sustaining *L. (V.) braziliensis* infections in our preliminary assays

(data not shown). During the establishment of our experimental conditions, we observed that the most comparable infection rates were found using MOI=20 for both species. Perhaps differential infectivity potential presented by distinct strains from different species explain the success related to the ability of infecting adipocytes. These parameters should be considered in future studies, especially considering that ATL caused by *L. (L.) amazonensis* usually show substantial parasite burden while *L. (V.) braziliensis* infection leads to infiltrates with scarce amastigotes (Pereira et al., 2017). In this sense, adipocytes could play a distinct role in parasite maintenance that deserves to be investigated in depth in terms of species specificity.

Our findings with an easily accessible cell line open avenues for investigating whether adipose cells represent a suitable environment for *Leishmania* survival, especially related to its dependence on lipid metabolism/metabolites to survive in the host (Saunders and McConville, 2020), and what is the possible impact(s) of residence in adipose tissues for the pathogenesis of the disease. In addition, our results showed for the first time that New World *Leishmania* species that are causative agents of ATL are able to infect adipocytes *in vitro*, and maintain their ability to generate markedly distinct PVs in non-phagocytic cells. This unexplored aspect of the *Leishmania*-host interaction can be of great value for a deeper investigation of chronic cases, which are generally related to ineffective treatment regimens or natural relapses of ATL.

Furthermore, it has been shown that *Leishmania* persistence plays a relevant role in parasite dissemination, especially for New World species (Ramirez and Guevara, 1997; Soliman, 2006). In this sense, one of the most intriguing issues surrounding the pathogenesis of different clinical forms of leishmaniasis concerns the characterization of hideouts that serve as refuges for amastigotes to reproduce slowly. Adipocytes should be further investigated as possible sites for *Leishmania* persistence as they are capable of producing nitric oxide (NO), although at lower rates than macrophages (Pilon et al., 2000). Since the longstanding expression of inducible NO synthase is related to the persistence of *Leishmania* within host macrophages (Bogdan et al., 1996), it is plausible to suggest that another cell type involved in the infection course could allow the maintenance of this pathogen in certain organs where adipocytes are abundant, such as skin and viscera, which are relevant to specific clinical manifestations of different *Leishmania* species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NA and DM: conceptualization of the study. BM, KM, SC, and DM: acquisition, analysis, and interpretation of the data. DM: writing - original draft preparation. BM, KM, SC, NA, and DM: writing - review and editing. SC, NA, and DM: supervision, project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.824494/full#supplementary-material

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Trypanosoma Species in Small Nonflying Mammals in an Area With a Single Previous Chagas Disease Case

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Trypanosomatids are hemoflagellate parasites that even though they have been increasingly studied, many aspects of their biology and taxonomy remain unknown. The aim of this study was to investigate the *Trypanosoma* sp. transmission cycle in nonflying small mammals in an area where a case of acute Chagas disease occurred in Mangaratiba municipality, Rio de Janeiro state. Three expeditions were conducted in the area: the first in 2012, soon after the human case, and two others in 2015. Sylvatic mammals were captured and submitted to blood collection for trypanosomatid parasitological and serological exams. Dogs from the surrounding areas where the sylvatic mammals were captured were also tested for *T. cruzi* infection. DNA samples were extracted from blood clots and positive hemocultures, submitted to polymerase chain reaction targeting SSU rDNA and gGAPDH genes, sequenced and phylogenetic analysed. Twenty-one wild mammals were captured in 2012, mainly rodents, and 17 mammals, mainly marsupials, were captured in the two expeditions conducted in 2015. Only four rodents demonstrated borderline serological *T. cruzi* test (IFAT), two in 2012 and two in 2015. *Trypanosoma janseni* was the main *Trypanosoma* species identified, and isolates were obtained solely from *Didelphis aurita*. In addition to biological differences, molecular differences are suggestive of genetic diversity in this flagellate species. *Trypanosoma* sp. DID was identified in blood clots from *D. aurita* in single and mixed infections with *T. janseni*. Concerning dogs, 12 presented mostly borderline serological titers for *T. cruzi* and no positive hemoculture. In blood clots from 11 dogs, *T. cruzi* DNA was detected and characterized as TcI (n = 9) or TcII (n = 2). Infections by *Trypanosoma rangeli* lineage E (n = 2) and, for the first time, *Trypanosoma caninum*, *Trypanosoma dionisii*, and *Crithidia mellificae* (n = 1 each) were also detected in dogs. We concluded that despite the low mammalian species richness and degraded environment, a high *Trypanosoma* species richness species was being transmitted with the predominance of *T. janseni* and not *T. cruzi*, as would be expected in a locality of an acute case of Chagas disease.

Keywords: Trypanosomatidae, mammalian host, *Trypanosoma cruzi* clade, infection, Atlantic Forest

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INTRODUCTION

Trypanosomatids are obligate parasites capable of infecting invertebrates, vertebrates, and plant hosts (Hoare, 1966; Vickerman, 1976). To date, 25 genera are described within this order and are classified according to the number of hosts involved in the development of their life cycle (d'Avila-Levy et al., 2015; Maslov et al., 2019; Kostygov et al., 2020; Lukeš et al., 2021): a) a group called monoxenic parasites formed by species that classically have only one definitive host, the invertebrate animals; and b) a second group called heteroxenic that have two hosts to complete their life cycle, an invertebrate animal and the other can be a vertebrate animal or a plant. Nineteen genera are recognized as tripanosomatid monoxenous (d'Avila-Levy et al., 2015; Kaufer et al., 2017; Kostygov et al., 2020; Lukeš et al., 2021): *Angomonas*, *Blastocrithidia*, *Blechomonas*, *Crithidia*, *Herpetomonas*, *Kentomonas*, *Jaenimonas*, *Lafontella*, *Leptomonas*, *Lotmaria*, *Novymonas*, *Obscuromonas*, *Paratrypanosoma*, *Rhychoidomonas*, *Sergeia*, *Strigonomonas*, *Vickermania*, *Wallacemonas*, and *Zelonia*. Its definitive hosts are insects of the orders Diptera, Hemiptera, and Siphonaptera, such as mosquitoes, flies, bees, and fleas (Kozminsky et al., 2015). Six genera are classified as heteroxenous trypanosomatids (d'Avila-Levy et al., 2015; Kaufer et al., 2017; Maslov et al., 2019): *Endotrypanum*, *Leishmania*, *Paraleishmania*, *Porcisia*, and *Trypanosoma*, which infect vertebrate animals, and *Phytomonas*, which is capable of infecting plants. The genera *Leishamania* and *Trypanosoma* infect a diversity of species of vertebrate animals and are the most studied genera due to their medical and economic importance (Simpson et al., 2006).

The clade denominated *Trypanosoma cruzi* is composed by, at least, nine different species and other operational taxonomic units that infect a broad range of mammalian species (Lopes et al., 2018; Clément et al., 2019; Rodrigues et al., 2019; Alves et al., 2021) and, from whose the transmission cycle is known, are transmitted by hematophagous insects, such as cimicids and triatomines (Gardner and Molyneux, 1988a; Jansen et al., 2018). The species included in this clade are distributed on five continents (Asia, Africa, Oceania, Europe, and the Americas) (Noyes et al., 1999; Hamilton et al., 2009; Hamilton et al., 2012; Lima et al., 2012; Lima et al., 2013; Lima et al., 2015; Barbosa et al., 2016; Botero et al., 2016; Lopes et al., 2018; Mafie et al., 2018; Wang et al., 2019). According to phylogenetic analysis, the following species are classified into this group: i) *T. livingstonei*, a species described in African bats (Lima et al., 2013); ii) *T. noyesi* species described in Australian marsupials (Botero et al., 2016); iii) *T. janseni*, species first described in marsupials of the species *Didelphis aurita* in the Atlantic Forest of Brazil (Lopes et al., 2018); iv) *T. wauwau* and *Trypanosoma* sp. neobats described in neotropical bat species from Latin America (Lima et al., 2015); v) *T. vespertilionis* described in European and African bats (Hamilton et al., 2012); vi) *T. conorhini* a cosmopolitan species described in rodents (Hamilton et al., 2012); vii) three isolated representatives of a monkey, a civet and an African bat (Hamilton et al., 2009); viii) *T. teixeirae* described in an Australian bat (Barbosa et al., 2016); ix) *T. rangeli*, a mammalian multihost species in Latin America (Stevens et al., 1999); and x) species of the subgenus *Schizotrypanum*: *T. cruzi*, *T. dionisii*, and *T. erneyi* (Lima et al., 2012). *Trypanosoma cruzi*, the etiological agent of Chagas disease (CD) in humans, is a multihost parasite that infects at least eight mammalian orders, and it is transmitted by triatomines (Jansen et al., 2018). It presents a genetic diversity in which seven genotypes, denominated as discrete typing units (DTUs), TcI to TcVI and Tcbat, are recognized (Zingales et al., 2009; Zingales et al., 2012). Indeed, an increasing number of new species and new host–parasite interactions have been recently discovered within the family, especially when studying wild-free-ranging mammals. Even *T. cruzi*, which is a well-studied parasite, still demonstrates gaps in the knowledge of its ecology, transmission, and epidemiology. Exactly this point gave rise to this study.

The aim of this study was to investigate *Trypanosoma* sp. infection in small nonflying mammals in the Atlantic Forest of Mangaratiba municipality, Rio de Janeiro state, southeast Brazil. For this purpose, three excursions were carried out: one in 2012 and two in 2015. Thus, our objective was to monitor possible changes in the enzootic scenario of *Trypanosoma* spp. infection in nonflying small mammals in the area. Our intent was to assess whether there was an alteration in the profile of the enzooty with the increase in the mammalian infection rate by *T. cruzi* or whether the enzooty remained stable.

MATERIAL AND METHODS

Ethical Statement

The capture of small sylvatic mammals was authorized by the Sistema de Autorização e Informação em Biodiversidade—SISBIO of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA)-(permanent license number 3365-1) and by the Instituto Estadual do Meio Ambiente (INEA/RJ) under license number 028/2015. Mammalian blood sample collection and euthanasia were performed according to the Ethical Committee for Animal Use of the Oswaldo Cruz Foundation (license LW-81-12).

Study Area

Mangaratiba municipality is located in the Rio de Janeiro Green Coast, 85 km from Rio de Janeiro municipality. It occupies a 356 km² area, and its estimated population was 41,557 inhabitants in 2016, according to the Instituto Brasileiro de Geografia e Estatística (IBGE). In 2012, an acute case of CD confirmed by parasitological, serological, and molecular tests was reported (Sangenis et al., 2015). The case was acquired through the contaminative route by a man who slept in a hammock. Two areas were chosen to investigate *Trypanosoma* spp. transmission cycle: i) Fazenda Batatal, where in 2012 the case of CD occurred, and ii) Vale do Sahy, where Cunhambebe Park is located approximately 15 km from where the acute CD occurred (**Figure 1**).

Small Sylvatic Mammals' Capture

On the first expedition in 2012 (dry season—August), three linear transects were established in the Fazenda Batatal location: i) two close to the place where the man became infected and ii) one in a forest fragment farther away but still within the same

FIGURE 1 | Mangaratiba municipality study locations in 2012 and 2015. The red star represents the area where acute CD occurred in 2012. The blue dot is where the Cunhambebe Park is located. Data source: Instituto Brasileiro de Geografia e Estatística—IBGE (www.ibge.gov.br); Google Earth (https://www.google.com.br/intl/pt-BR/earth/).

region. Sherman® (H. B. Sherman Traps, Tallahassee, FL, USA) and Tomahawk® (Tomahawk Live Traps, Tomahawk, WI, USA) type traps were placed on the ground and/or understory and baited with universal bait (Dario et al., 2016). Two surveys were performed in 2015 at Fazenda Batatal and Vale do Sahy locations in the wet (March) and dry (September) seasons employing the same capture methodology. The capture effort was 440 traps for 4 nights in 2012 and 480 traps for 4 nights in each of the 2015 expeditions.

For all the animals, morphological characteristics and body measurements were recorded for taxonomic identification. The identification of rodents was confirmed by karyological analyses (Bonvicino et al., 2005). Only rodent specimens that required taxonomical confirmation obtained by karyotyping were sacrificed and the carcasses were deposited as voucher specimens in the collection of the Nacional Museum (Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, Brazil). All captured animals were manipulated according to the safety manual for the use of wild mammals in research (Dario et al., 2016) and were anesthetized (9:1 ketamine chlorhydrate 10% and acepromazine 2%) for blood sample collection (by cardiac puncture) for parasitological and serological exams.

Dog Survey

An active search for dogs was conducted in the residences near the locations where the sylvatic mammals were captured in both years. With the informed consent of their owners, blood samples were collected using Vacutainer® tubes containing EDTA by puncturing the femoral vein of the dog. A questionnaire was used to record the name, age, sex, size, and primary function (hunting, companionship, or protection) and anatomical peculiarities. Dogs from the same house were considered to be a single event.

Trypanosomatid Infection Survey

Parasitological and serological methods were used to identify *Trypanosoma* species infection in sylvatic mammals and dogs. The parasitological methods included (i) fresh blood examination and (ii) hemocultures (300 µl each of animal blood in two tubes containing Novy McNeal-Nicole (NNN) supplemented with Liver Infusion Tryptose (LIT) overlay supplemented with 10% fetal bovine serum and 140 mg/ml antibiotic). Hemocultures were examined fortnightly for five months. Positive cultures, which demonstrated parasite growth, were amplified, cryopreserved, and deposited in the Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores, COLTRYP/Fiocruz. The liquid phase of initially positive hemocultures, in which the flagellate did not thrive successfully, was centrifuged at 4,000*g*, and the resultant sediments were stored at −20°C for molecular identification. In addition, mammalian whole blood was centrifuged at 1,180*g* for 15 min, and the blood clot was used in the molecular analysis (Alves et al., 2021).

Serological analyses were performed by indirect immunofluorescence antibody test (IFAT) to detect IgG antibodies in sera of sylvatic mammals and dogs (Camargo, 1966). Reference strains I00/BR/00F (TcI) and MHOM/BR/1957/Y (TcII) from axenic cultures were mixed in equal (1:1) proportions and used as antigens. The sera from Murinae rodents and plasma from dogs were tested with anti-rat IgG and anti-dog IgG, respectively, conjugated with fluorescein isothiocyanate (Sigma, St. Louis, MO, USA). The marsupial sera were tested as described in (Xavier et al., 2014) using an in-house anti-*Didelphis* spp. IgG and the reactions revealed using an anti-rabbit IgG also conjugated with fluorescein isothiocyanate. The cutoff values for the IFAT were 1:40 for marsupials and dogs and 1:10 for rodents (Herrera et al., 2005). To confirm the serological results of the dogs, an enzyme-linked immunosorbent assay (ELISA-Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil) was performed. The cutoff value for ELISA was an optical absorbance ≥0.200, mean ±3 standard deviations. In addition, two negative and two positive control sera were added to each reaction of the dog. For the IFAT assays in wild mammals, specific positive and negative controls were also added according to each mammalian family.

To exclude cross-reactions between *T. cruzi* and *Leishmania* sp., an IFAT using a mixture of axenic cultures of *L. infantum* (IOC/L579—MHOM/BR/1974/PP75) and *L. braziliensis* (IOC/L566—MHOM/BR/1975/M2903), an ELISA and a rapid test for the diagnosis of canine visceral leishmaniasis (CVL) (TR DPP®, Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil) were performed. Serological positivity criteria for dog samples were obtained according to Xavier et al. (2012): dogs with serological titers higher for *Leishmania* sp. than for *T. cruzi* were considered infected only for *Leishmania* sp. when *T. cruzi* titers were ≤1 : 80 and as mixed infected when titers were >1 : 80.

Trypanosomatid Species Molecular Characterization

Total genomic DNA from positive hemocultures and blood clots was extracted using phenol–chloroform (Sambrook and Russel, 2001) and ammonium acetate precipitation (Rodrigues et al., 2019). For *Trypanosoma* species identification, nested polymerase chain reaction (nested-PCR) for the small subunit ribosomal RNA gene (SSU rDNA) (external primers TRY927F 5'CAGAAACGAAACACGGGAG3' and TRY927R 5'CCTACTGGGCAGCTTGGA3'; internal primers SSU561F 5'TGGGATAACAAAGGAGCA3' and SSU561R 5'CTGAGACTGTAACCTCAAAGC3') (Noyes et al., 1999; Smith et al., 2008) was performed for positive hemocultures and blood clot DNA, while a convencional PCR for glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) gene (GAPTRY-mod F 5'GGBCGCATGGTSTTCCAG3' and GAPTRYr R 5'CCCCACTCGTTRTCRTACC3') (Borghesan et al., 2013) was performed only for hemoculture samples. *Trypanosoma. cruzi* strain SylvioX/10cl1 was used as a positive control, and we included one reaction with distilled water instead of DNA as a negative control.

The PCR products (~650 bp for the SSU rDNA gene and ~800 bp for the gGAPDH gene) were visualized using a 2% agarose gel stained with ethidium bromide and purified according to the manufacturer's instructions (Illustra GFX PCR DNA and gel band purification kit—GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Both strands of DNA were then sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 DNA sequencer available at the PDTIS/Fiocruz sequencing platform. To obtain SSU rDNA and gGAPDH consensus sequences, DNA strands were assembled using SeqMan (DNASTAR Lasergene, Gatc, Konstanz, Germany) and then edited using MegaX (Kumar et al., 2018). The SSU rDNA and gGAPDH sequences from hemoculture isolates were concatenated for phylogenetic analysis to increase the robustness of the results.

Trypanosomatid sequences from this study and others retrieved from GenBank were aligned using the algorithmL-INS-i in MAFFT v.7.0 (Katoh and Standley, 2013). Phylogenetic reconstructions by Bayesian inference (BI) and maximum-likelihood (ML) methods were performed using the Mrbayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and IQ-tree (Nguyen et al., 2015) programs, respectively. The best nucleotide substitution models were chosen according to the corrected Akaike information criterion (cAIC) in ModelFinder (Kalyaanamoorthy et al., 2017) for each phylogenetic analysis. All the programs were available on the Phylosuite v1.2.2 platform (Zhang et al., 2020). For the ML inference branch support, ultrafast bootstrapping (Hoang et al., 2018) with 5,000 replicates with 1,000 maximum interactions and 0.99 minimum correlation coefficients and the SH-aLRT branch test with 5,000 replicates were applied. In the BI, the Bayesian Markov chain Monte Carlo (MCMC) method was used to assign trypansomatids prior to information. Four independent runs were performed for 20 M with sampling every 2,000 generations with 25% burn-in from each run. The final trees were visualized on Figtree v 1.4.3.

RESULTS

The enzootic scenario of *Trypanosoma* spp. transmission in the area remained stable and different from all other areas that reported cases and outbreaks of acute CD. We observed a low richness of sylvatic mammal species and low *T. cruzi* transmission in this region of the Atlantic Forest. However, we observed an important diversity of trypanosomatid species being transmitted in the area, mainly among dogs.

Small Sylvatic and Synanthropic Mammals' Occurrence

In the first expedition in 2012, 21 mammal specimens were captured, mainly rodents, in Fazenda Batatal, which is where the case happened: *Akodon cursor* (n = 13), *Oxymycterus judex* (n = 3), *Oligoryzomys nigripes* (n = 2), *Euryzygomatomyz americanatus* (n = 1), *Nectomys squamipes* (n = 1), and *Didelphis aurita* (n = 1). The capture success was of 4.8%. During the two expeditions performed in 2015, 15 small sylvatic mammals (seven individuals in March and eight individuals in September) were captured and identified into four species: *D. aurita* (n = 10), *Marmosa paraguayana* (n = 1), *A. cursor* (n = 3), and *Euryoryzomys russatus* (n = 1). Two synanthropic rodent species, *Rattus rattus* and *Rattus norvegicus*, were also captured (**Table 1**). The capture success was 3.54%. Comparing the three expeditions, we observed a change in mammalian species captured. In 2012, the predominance of rodent species (n = 20; 90.9%) was observed, and in 2015, *D. aurita* predominance (n = 10; 58.8%) was observed in both locations.

Parasitological and Serological Survey in Small Sylvatic and Synanthropic Mammals and Domestic Dogs for *Trypanosoma* spp. Infection

None of the parasitological exams (fresh blood and hemoculture) performed in the sylvatic mammals captured in 2012 were positive. In contrast, of the 17 hemocultures performed in 2015, six from *D. aurita* were positive (**Table 1**): five of them were successfully amplified, one failed to obtain parasite

TABLE 1 | Small sylvatic and synanthropic mammals captured and *Trypanosoma* sp. infection in Mangaratiba municipality, RJ state in 2015.

Location	LBCE	COLTRYP	Species	Molecular characterization		GenBank accession number		
				Hemoculture	Blood clot	SSU rDNA hemoculture	gGAPDH hemoculture	SSU rDNA blood clot
Fazenda Batatal	17242	–	*Marmosa paraguayana*	–	–	–	–	Not performed
	17245	–	*Akodon cursor*	–	–	–	–	Not performed
	17246	00608	*Didelphis aurita*	*T. janseni*	*Trypanosoma* sp. DID	OL314519	OL314513	OL314521
	17247	00607	*Didelphis aurita*	*T. janseni*	*T. janseni*	OL314518	OL314512	OL314525
	17248	00604	*Didelphis aurita*	*T. janseni*	–	OL314515	OL314509	Not performed
	17250	–	*Akodon cf. cursor*	–	*T. janseni*	–	–	OL314524
	17251	–	*Rattus norvegicus*	–	–	–	–	Not amplified
	17254	–	*Akodon cf. cursor*	–	–	–	–	Not amplified
	18335	–	*Didelphis aurita*	–	*Trypanosoma* sp. DID	–	–	MH404845*
Vale do Sahy	17249	–	*Rattus rattus*	–	–	–	–	Not performed
	17243	00605	*Didelphis aurita*	*T. janseni*	–	OL314516	OL314510	Not performed
	17244	00606	*Didelphis aurita*	*T. janseni*	–	OL314517	OL314511	Not performed
	17252	–	*Didelphis aurita*	–	–	–	–	Not amplified
	17253	–	*Didelphis aurita*	*T. janseni*	*Trypanosoma* sp. DID	OL314520	OL314514	OL314523
	18336	–	*Didelphis aurita*	–	–	–	–	Not amplified
	18337	–	*Didelphis aurita*	–	*Trypanosoma* sp. DID	–	–	OL314522
	18338	–	*Euryoryzomys russatus*	–	*T. janseni*	–	–	OL314526

*Sequence deposited and published by Rodrigues et al. (2019).

amplification, and molecular characterization was performed in the culture sediment (LBCE17253). The DNA sequences obtained from SSU rDNA and gGAPDH were identified as *T. janseni*. In the SSU rDNA sequence alignment, a single nucleotide polymorphism (SNP) was observed at site 975 between COLTRYP00604, KY243025 and the other *T. janseni* sequences. According to the phylogenetic analysis (**Figure 2**), the samples formed two groups of *T. janseni*. Serologically, all marsupials were negative, and four rodents were positive for *T. cruzi* infection: two *O. judex* (LBCE18303 and LBCE18316—serological titer 1:20) in 2012 and two synanthropic rodents (*R. novergicus* LBC17251 and *R. rattus* LBCE17249—serological titer 1:40) in 2015.

The forms with dog data filled before blood collection showed that we were not dealing with the same animal already examined in a previous excursion. Actually, the turnover rate of dogs in the rural areas of the country is significant. In addition, only adult dogs were examined. All fresh blood examinations and hemocultures were negative for *Trypanosoma* infection in the dogs from the Fazenda Batatal (n = 21—2012; n = 26—2015) and Vale do Sahy (n = 32) areas that were examined. Twelve dogs were seropositive for *T. cruzi* infection (**Table 2**).

Of the 17 specimens of small mammals captured in 2015, 11 blood clot samples were obtained for molecular characterization. Seven samples showed infection with trypanosomatids (**Table 1** and **Figure 3**), namely, *T. janseni* in *A. cursor* (n = 1), *E. russatus* (n = 1), *D. aurita* (n = 1), and *Trypanosoma* sp. DID in four *D. aurita* (**Table 1**). Two of the blood clot samples (LBCE17246 and LBCE17253) presented infection by *Trypanosoma* sp. DID, while in the hemoculture, molecular characterization showed that these samples were infected by *T. janseni* (**Figures 2**, **3** and **Table 1**). Regarding dogs, 16 blood clot samples demonstrated DNA of *C. mellificae* (n = 1) (**Figure 4**), *T. cruzi* DTU TcI (n = 9), *T. cruzi* DTU TcII (n = 2), *T. dionisii* (n = 1), and *T. rangeli* subpopulation E (n = 2) (**Figure 3**), and *T. caninum* (n = 1) (**Figure 5**). Comparing the results observed in the serological and blood clot molecular characterization, three samples characterized as *T. cruzi* belonged to dogs that presented serological titers and positive ELISA for *T. cruzi*: LBT6230, LBT6244, and LBT6702 (**Table 2**), but the sample LBT6702 presented infection by *T. caninum* in blood clot molecular characterization.

DISCUSSION

In this study, we demonstrated a peculiar enzootic transmission cycle of trypanosomatid species among sylvatic mammals and dogs from the Mangaratiba Atlantic Forest, where one case of acute CD had been diagnosed 3 years before. The enzootic scenario in relation to *T. cruzi* transmission did not change between 2012 and 2015. No sylvatic mammals presented infectivity competence for *T. cruzi* transmission. The difference observed was that more marsupials were captured in 2015 than in 2012, and some sylvatic mammals were infected by *T. janseni*. There are four aspects to be highlighted in this study that are uncommon in areas of CD: 1) there were very few reports of invasion of triatomines in houses; 2) there were no domiciled triatomines; 3) *T. cruzi* was the least prevalent trypanosomatid species in wild mammals; and 4) dogs were demonstrated to be infected by the higher diversity of Trypanosomatid species.

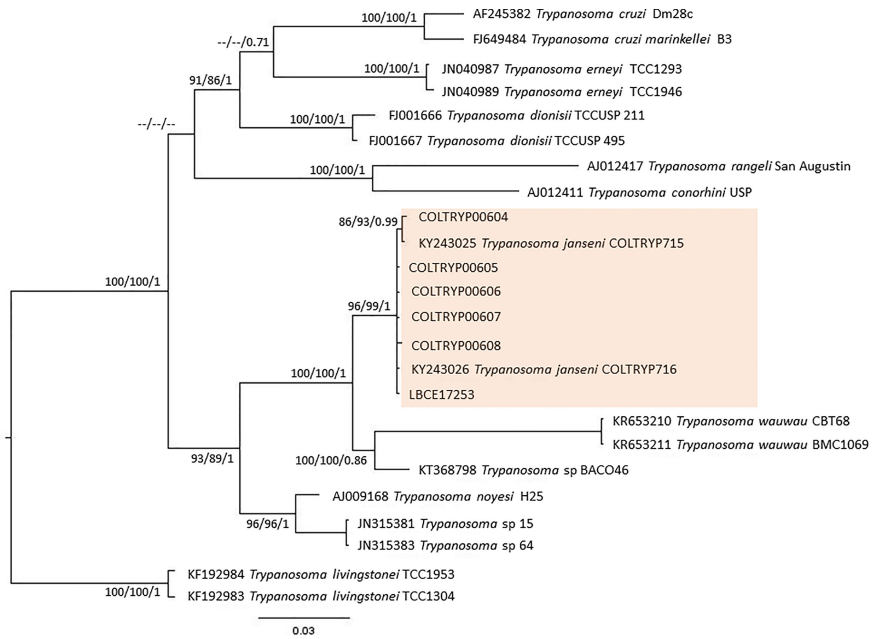

FIGURE 2 | Concatenated (SSU rDNA + gGAPDH) *T. cruzi* clade phylogenetic tree based on 1121 base pair fragment lengths of sylvatic mammal hemocultures. The tree was inferred using transitional3 equal frequencies plus gamma distribution (TIM3e+G) and generalized time reversible with invariant sites and amino acid frequencies plus gamma distribution (GTR+F+I+G) models for ML and BI, respectively. The numbers at nodes correspond to ML (ultrabootstrap, SH-aLRT) and BI (posterior probability). The scale bar shows the number of nucleotide substitutions per site. The red square represents group formed *T. janseni* sequences from different culture isolation.

TABLE 2 | Trypanosomatidae infection in dogs (serological tests for *T. cruzi* and molecular blood clot for trypanosomatids) in Fazenda Batatal and Vale do Shahy locations, Mangaratiba municipality, Rio de Janeiro state.

Year	LBT	Location	IFAT	ELISA	Blood clot	GenBank accession number
2012	3476	Fazenda Batatal	1:80	Positive	Not performed	–
2015	6212	Fazenda Batatal	1:80	Positive	Not amplified	–
2015	6213	Fazenda Batatal	1:40	Bordeline	*T. cruzi* TcI	OL314533
2015	6215	Fazenda Batatal	1:80	Positive	Not amplified	–
2015	6218	Fazenda Batatal	1/20	Positive	*T. cruzi* TcI	OL314534
2015	6219	Fazenda Batatal	1:160	Positive	Not amplified	–
2015	6701	Fazenda Batatal	1:20	Negative	*T. cruzi* TcI	OL314530
2015	6702	Fazenda Batatal	1:80	Positive	*T. caninum*	OL314539
2015	6704	Fazenda Batatal	1:40	Negative	*C. mellificae*	OL314540
2015	6705	Fazenda Batatal	1:20	Negative	*T. rangeli*	MN661344*
2015	6706	Fazenda Batatal	1:20	Negative	*T. rangeli*	MN661345*
2015	6707	Fazenda Batatal	1:20	Negative	*T. cruzi* TcI	OL314529
2015	6226	Vale do Sahy	1:20	Positive	*T. cruzi* TcI	OL314537
2015	6228	Vale do Sahy	1:40	Negative	*T. dionisii*	OL314538
2015	6229	Vale do Sahy	Negative	Negative	*T. cruzi* TcI	OL314532
2015	6230	Vale do Sahy	1:80	Positive	*T. cruzi* TcI	OL314531
2015	6234	Vale do Sahy	1:20	Positive	*T. cruzi* TcII	OL314536
2015	6240	Vale do Sahy	1:80	Positive	Not amplified	–
2015	6241	Vale do Sahy	1:160	Positive	Not amplified	–
2015	6244	Vale do Sahy	1:160	Positive	*T. cruzi* TcII	OL314535
2015	6245	Vale do Sahy	1:80	Positive	Not amplified	–
2015	6709	Vale do Sahy	1:80	Negative	*T. cruzi* TcI	OL314528
2015	6711	Vale do Sahy	Not performed	Not performed	*T. cruzi* TcI	OL314527
2015	6715	Vale do Sahy	1:80	Positive	Not amplified	–
2015	6716	Vale do Sahy	1:80	Positive	Not amplified	–

**Sequence previously published by Dario et al. (2021a).*

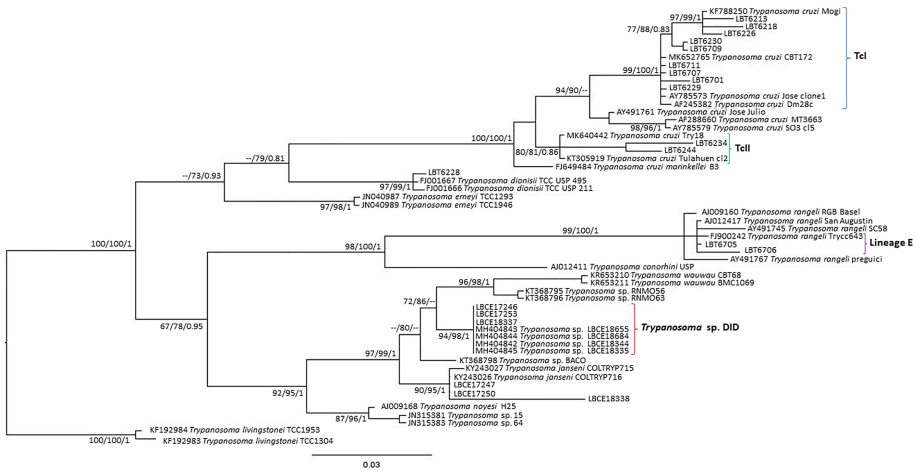

FIGURE 3 | *Trypanosoma cruzi* clade phylogenetic tree based on 692 base pair SSU rDNA fragment lengths from sylvatic mammal and dog blood clots. The tree was inferred using transitional3 with amino acid frequencies and freeRate heterogeneity (TIM3+F+R2) and generalized time reversible with amino acid frequencies plus gamma distribution (GTR+F+G) models for ML and BI, respectively. The numbers at nodes correspond to ML (ultrabootstrap and SH-aLRT) and BI (posterior probability). The scale bar shows the number of nucleotide substitutions per site. The blood clot samples grouped with *T. cruzi, T. rangeli, Trypanosoma* sp. DID and *T. janseni*. The blue curly brackets represent the sequences that grouped with *T. cruzi* TcI sequences. The green curly brackets represent the sequences that grouped with *T. cruzi* TcII/TcVI sequences. The purple curly brackets represent the sequences that grouped with *T. rangeli* lineage E sequences. The red curly brackets represent the sequences that grouped with *Trypanosoma* sp. DID sequences.

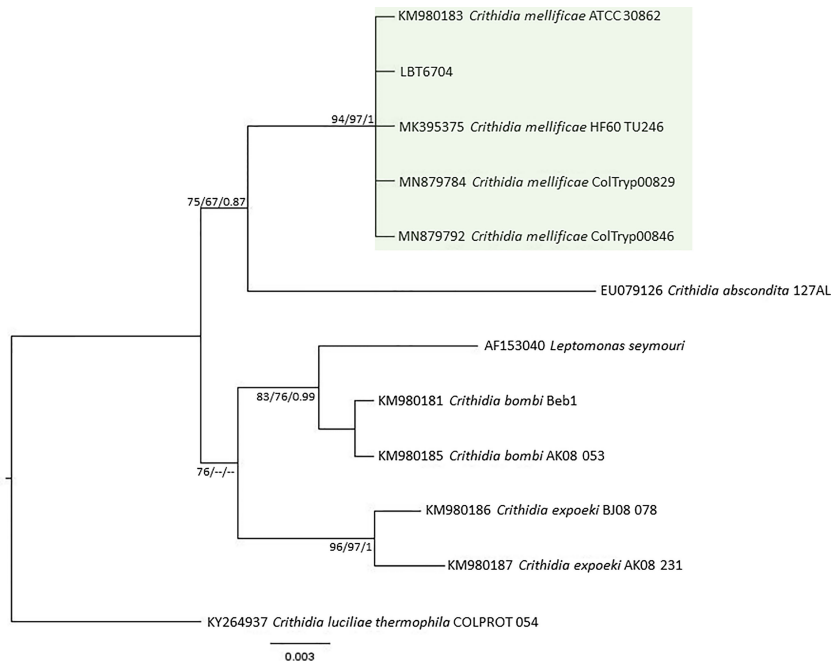

FIGURE 4 | *Crithidia mellificae* phylogenetic tree based on 528 base pairs SSU rDNA fragment length from dog blood clot. The tree was inferred using transitional3 equal frequencies plus gamma distribution (TIM3e+G) and symmetrical plus gamma distribution (SYM+G) models for ML and BI, respectively. The numbers at nodes correspond to ML (ultrabootstrap and SH-aLRT) and BI (posterior probability). The scale bar shows the number of nucleotide substitutions per site. The green square represents the clade formed by LBT6704 with other *C. mellifiace* sequences from insects and mammals.

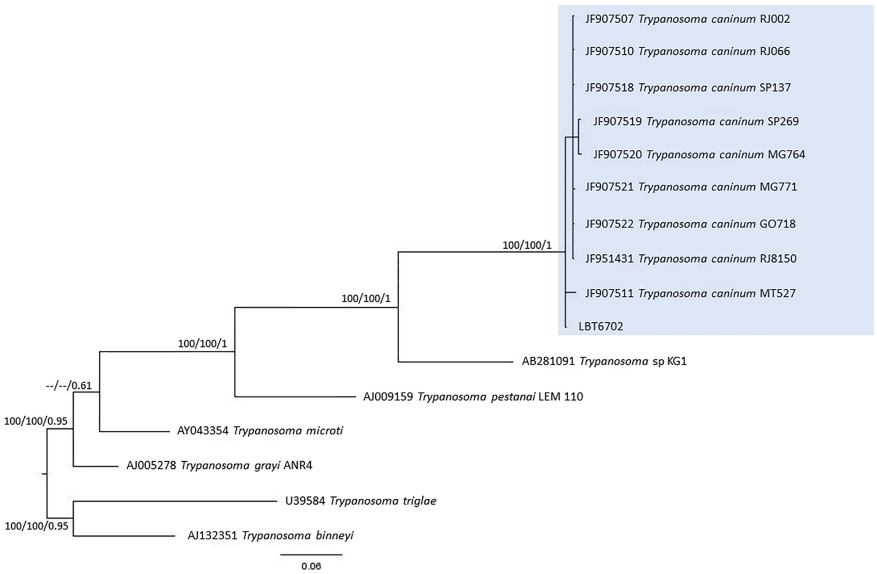

FIGURE 5 | *Trypanosoma caninum* phylogenetic tree based on 605 base pair SSU rDNA fragment lengths from dog blood clots. The tree was inferred using transitional with amino acid frequencies and freeRate heterogeneity (TIM+F+R2) and symmetrical plus gamma distribution (SYM+G) models for ML and BI, respectively. The numbers at nodes correspond to ML (ultrabootstrap, SH-aLRT) and BI (posterior probability). The scale bar shows the number of nucleotide substitutions per site. The blue square represents the clade formed by LBT6702 with other *T. caninum* sequences from different Brazilian states.

One of the species recently described within the *T. cruzi* clade is *T. janseni* (Lopes et al., 2018). This species was described from spleen and liver axenic cultures of a *D. aurita* marsupial of the Atlantic Forest of Rio de Janeiro state. Phylogenetically, this species was demonstrated to group together with *T. wauwau* and trypanosomatids from neotropical bats (Lopes et al., 2018). In this study, *T. janseni* was found to infect *D. aurita* and two rodent species: *A. cursor* and *E. russatus*. Three points suggest a possible biological diversity of *T. janseni*: i) by the ability to grow on NNN + LIT medium of some isolates in contrast to others probably representing a different genetic makeup (Berbigier et al., 2021); ii) sequencing showed a SNP, and the phylogenetic tree separated *T. janseni* into two groups. Obviously, the sequenced gene fragment is very small, and we would need whole 18S rDNA sequencing to evaluate this heterogeneity. Moreover, these data turn tempting to speculate that *T. janseni* also exhibits a certain degree of diversity, as is so common in these organisms. The high rates of marsupials infected by *T. janseni* observed in different Brazilian biomes reinforce that the genus *Didelphis* is one of the oldest hosts of parasite species from the *T. cruzi* clade and may also be the ancient host of *T. janseni* (Lopes et al., 2018; Rodrigues et al., 2019). However, *T. janseni* was demonstrated to be adaptable to other mammalian taxa, since it was identified in two rodent species (*A. cursor* and *E. russatus*) in this study and in Berbigier et al. (2021), and it was found infecting a dog, as demonstrated by PCR of blood clot samples (Malavazi et al., 2020).

Didelphids are known for their capacity to harbor several *Trypanosoma* species (Rodrigues et al., 2019). *Trypanosoma* sp. DID was described by Rodrigues et al. (2019) in *D. aurita* and *D. albiventris* from the Atlantic Forest and Cerrado biomes, respectively, and it is phylogenetically correlated to the clade formed by *T. wauwau*, *Trypanosoma* sp. neobats and *T. janseni*. Apparently, the set of results on *Trypanosoma* sp. DID shows one single taxonomic unit, since there is no genetic difference between the sequences, all grouped together. The occurrence of *Trypanosoma* sp. DID was already reported in mixed infections with *T. cruzi* (Nantes et al., 2021), and we observed this in *D. aurita* in single and mixed infections with *T. janseni*. Since they belong to the same clade, these findings raise the following questions: what is the possible mutual impact of a concomitant infection of *Trypanosoma* sp. DID with *T. janseni* in an opossum? Are these mixed infections with two or more species of *Trypanosoma* stable? These mixed infections demonstrate how important it is to use different diagnostic methodologies mainly if sylvatic mammals are studied and, once again, reinforce that mixed infections are the main infection pattern in nature.

The serological diagnosis for *T. cruzi* in *D. aurita* was reliable because no cross-reaction with *T. janseni* and *Trypanosoma* sp. DID was observed. However, there are some cases that deserve attention: i) the dog samples LBT6218 and LBT6701 were positive and negative in ELISA test, respectively, but displayed IFAT serological titers that were one dilution under the cutoff point (1:40), but their blood clot analyses demonstrated *T. cruzi* TcI. This could be explained by the paucity of parasites and maybe a more recent infection—we detected only DNA of *T. cruzi* and no positive parasitological testes ii) two dogs (LBT6213 borderline and LBT6704 negative ELISA tests), displayed cut off serological titers in IFAT (1:40). The identification of *C. mellificae* suggests cross reaction and shows the importance of always using two diagnostic tests in regard to diagnosing trypanosomatid infection.

Dogs exhibited the highest richness of trypanosomatid species infection, as demonstrated by blood clot molecular characterization: *T. cruzi* (DTUs TcI and TcII), *T. dionisii*, *T. caninum*, *T. rangeli*, and *C. mellificae*. *Crithidia mellificae*, a trypanosomatid classically referred to as a monoxenous insect parasite, is increasingly demonstrating itself as a generalist species capable of infecting an expressive number of mammal species (Rangel et al., 2019; Alves et al., 2021; Dario et al., 2021b). It is interesting to observe that *C. mellificae* is able to infect different species of mammals, and here, we include one more host, the domestic dog. The same for *T. dionisii*, classically described infecting bats (Gardner and Molyneux, 1988b), once again showed a broad mammalian host range since it has also been observed infecting dogs. This trypanosomatid species has also been described to infect marsupials, carnivores and humans (Dario et al., 2016; Rodrigues et al., 2019).

Trypanosoma rangeli is a multihost parasite transmitted by triatomines exclusively from the *Rhodnius* genus (Guhl and Vallejo, 2003) and is a heterogeneous parasite in which five lineages or two groups are recognized according to the molecular marker used: lineages A, B, C, D or E for nuclear markers (Maia da Silva et al., 2004; Maia da Silva et al., 2009) and KP1(+) or KP1 (−) for kDNA (Vallejo et al., 2002). Concerning *T. rangeli*, we question ourselves how this parasite species is transmitted in the southeastern Atlantic Forest, since its transmission is related to *Rhodnius* triatomine species (Guhl and Vallejo, 2003), and we report its occurrence in an area where this genus is not reported, showing that there are many gaps in the knowledge of elementary aspects of the biology even of this species, one of the most studied within the family. Although Gurgel-Gonçalves et al. (2012) mentioned that *R. domesticus* occurs in the southeast Atlantic Forest, it is difficult to find the species. There is only one report about its occurrence in Espírito Santo state (Corrêa-do-Nascimento et al., 2020). Even when performing a search for triatomines in the region, the genera that probably we would have found were *Triatoma* and *Panstrongylus* and not the genus *Rhodnius*.

Trypanosoma caninum, a trypanosomatid species classified outside the *T. cruzi* clade, was described from intact skin samples from domestic dogs, and little is known about its biology. Numerous cases of natural dog infection by this *Trypanosoma* species have already been recorded in different regions of Brazil. Phylogenetic analyses revealed that all *T. caninum* isolates analyzed were grouped in the same cluster, regardless of geographic precedence or genetic marker used. Additionally, electronic and optical microscopy analysis showed the presence of atypical epimastigote forms, without free flagellum, in axenic cultures (Madeira et al., 2009; Barros et al., 2014; Barros et al., 2015). Here, we observed the encounter of *T. caninum* for the first time in a blood sample, which is not surprising since the genus *Trypanosoma* is defined as a hematozoan. The question is why, among so many dogs examined, this species has never been observed in peripheral blood and always in skin tissue (Madeira et al., 2009; Barros et al., 2012). *Trypanosoma caninum* is a trypanosomatid species described to date exclusively by isolation of intact fragments from domestic dogs in different Brazilian biomes (Madeira et al., 2009; Barros et al., 2012). Many aspects of this parasite species remain unknown, namely, their evolutionary forms in vertebrate hosts and possible vectors (Barros et al., 2012; Barros et al., 2014; Fonseca-Oliveira et al., 2018). Although *T. caninum* has never been isolated by hemoculture, we identified the infection in a dog blood clot sample. This was possible because blood clot molecular identification is a sensitive methodology, especially in identifying trypanosomatids that are difficult to isolate in culture media (Rodrigues et al., 2019).

Although showing the greatest richness of trypanosomes, dogs showed only cryptoparasitemias, since no blood culture was positive and the presence of trypanosomatid species in the blood could only be detected by PCR of blood clots. The set of results indicated indirect evidence of low infectivity in dogs for any trypanosomatid species in the three excursions. We have performed two different parasitological tests that demonstrated the infectivity potential of a mammal: i) fresh blood exam (direct test) and ii) hemoculture (indirect test), and both presented negative results for dogs. In fact, dogs rarely demonstrated high parasitemias by *T. cruzi* in Brazil (Jansen et al., 2017). These results showed that in the peripheral circulation, there is no circulating parasite and the chance of a triatomine specimen get infected in the blood meal is low, however, this would only be confirmed by performing vector feeding assays. We observed six dogs with positive IFAT for *T. cruzi* infection. One of these dogs even presented a titer of 1:160. These sera were also positive by ELISA, but these dogs were negative by blood clot PCR for *T. cruzi*. Positive blood clot PCRs for *T. cruzi* were observed in the dogs (n = 11) with lower serological tests, including four negative ELISA testing dogs, which probably reflects recent infections.

The aim of this study was to understand *T. cruzi* transmission in an area where there was a case of acute CD in Mangaratiba. However, to our surprise, infection by *T. cruzi* was uncommon among the captured mammals, as demonstrated by parasitological exams (molecular characterization) and by low serological titers in the three expeditions performed. We attempted to search for triatomines during the fieldwork and in the surrounding area where acute CD occurred; however, no specimens were found. Regarding triatomine occurrence in the area, the dwellers did not recognize triatomine insects or mention its occurrence. Only after our fieldwork, after we informed them about these insects, five triatomine specimens (one in 2012 and four in 2015) were reported and delivered by the residents (Roque ALR, personal communication; Dario MA, personal communication). All the specimens were identified as *Triatoma tibiamaculata*, and only one specimen (2012) was infected by *T. cruzi*, as detected by the intestinal content exam, and it was characterized as *T. cruzi* DTU TcI (Roque ALR, personal communication). We hypothesize that the infected *T. tibiamaculata* (Sangenis et al., 2015) involved in the human case probably became infected and moved further away from the study area. This observation raises the question concerning the displacement capacity of triatomines that, in addition to flying, can also travel by air currents. A similar transmission pattern was observed in the Atlantic Forest of Espírito Santo state (neighboring state in the north of Rio de Janeiro state) (Dario et al., 2017).

We rule out the importance of dogs in the transmission cycle of *T. cruzi* in the area because they presented only cryptic infection. Moreover, they were bred free-range and rural dogs that use a wide range of living areas, i.e., they may also have acquired the infection in a more distant area. In addition, no other mammalian species were infected, and the presence of triatomines was not reported by the locals or by the sanitary authorities. This scenario has not changed after three years.

In conclusion, we demonstrated that even highly degraded areas may maintain a high diversity of trypanosomatid species being transmitted. The *T. cruzi* clade proved to be increasingly diverse, and certainly, in the future, more species and/or genotypes will be revealed within this group. Finally, the acute CD case was an unfortunate coincidence because no other case has been reported.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee for Animal Use of the Oswaldo Cruz Foundation (license LW-81-12). The capture of small sylvatic mammals was authorized by the Sistema de Autorizaçao e Informaçao em Biodiversidade—SISBIO of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (permanent license number 3365-1) and by the Instituto Estadual do Meio Ambiente (INEA/RJ) (license number 028/2015).

AUTHOR CONTRIBUTIONS

AJ and AR contributed to the conception and design of the study. MD, CL, SX, PD'A, AR, and AJ undertook the research and analyses. MD and AJ wrote the draft of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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A Stroll Through the History of Monoxenous Trypanosomatids Infection in Vertebrate Hosts

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The Trypanosomatidae family encompasses unicellular flagellates and obligate parasites of invertebrates, vertebrates, and plants. Trypanosomatids are traditionally divided into heteroxenous, characterized by the alternation of the life cycle between an insect vector and a plant or a vertebrate host, including humans being responsible for severe diseases; and monoxenous, which are presumably unique parasites of invertebrate hosts. Interestingly, studies reporting the occurrence of these monoxenous trypanosomatids in humans have been gradually increasing, either associated with *Leishmania* co-infection, or supposedly alone either in immunocompromised or even more sporadically in immunocompetent hosts. This review summarizes the first reports that raised the hypothesis that monoxenous trypanosomatids could be found in vertebrate hosts till the most current reports on the occurrence of *Crithidia* spp. alone in immunocompetent human patients.

Keywords: heteroxenous, immunocompromised hosts, monoxenic, pathogenicity, trypanosomatidae, immunocompetent host

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INTRODUCTION

The family Trypanosomatidae encompasses eukaryotic flagellates, unicellular and obligatory parasites of invertebrates, vertebrates, and plants. The unique morphology of its single mitochondrion DNA (called kinetoplast) is an apomorphy of the Class Kinetoplastea, and its specific cellular positioning in relation to the nucleus and the point of flagella emersion allows the identification of specific life cycle forms, some of which are genus-specific (reviewed by d'Avila-Levy et al., 2015). Trypanosomatids are traditionally divided into heteroxenous, characterized by alternating the life cycle between an insect vector and a vertebrate host or a plant; and monoxenous, which are parasites presumably exclusive of invertebrate hosts, mainly insects. The heteroxenous trypanosomatids are the causative agents of severe human diseases that are mainly transmitted by an insect vector, such as Chagas disease (caused by *Trypanosoma cruzi*), sleeping sickness (caused by *Trypanosoma brucei sensu lato*), and the various forms of cutaneous and visceral leishmaniasis (caused by *Leishmania* spp.) (Vickerman, 1994). On the contrary to these pathogenic flagellates, the monoxenous (or "lower") trypanosomatids have received considerably less effort and attention by the scientific community since these parasites are found, a priori, only in the digestive

tract of insects, and even its pathogenicity to insects is questionable, with few exceptions. Nevertheless, species typically non-pathogenic to humans are important models for understanding the biological behavior, biochemistry and molecular biology of pathogenic trypanosomatids (d'Avila-Levy et al., 2015). In addition, these organisms are being explored as vaccine candidates; for example, *Phytomonas serpens* confers protective immunity against *T. cruzi* (Pinge-Filho et al., 2005; da Silva et al., 2013); and as a platform to produce folded eukaryotic proteins, such as erythropoietin and insulin produced by *Leishmania tarentolae* (Dortay and Mueller-Roeber, 2010). Finally, monoxenous trypanosomatids are attracting the attention of researchers in the field due to the increasing reports on the occurrence of these presumably non-pathogenic trypanosomatids in humans (Dedet et al., 1995; Jiménez et al., 1996; Pacheco et al., 1998; Miller, 2000; Ferreira and Borges, 2002; Chicharro and Alvar, 2003). Here, we will present a historical review since the first reports that raised the hypothesis that monoxenous trypanosomatids could be found in vertebrate hosts till the recent reports on the occurrence of *Crithidia* spp. in immunocompetent human patients.

FIRST REPORTS ON THE OCCURRENCE OF MONOXENIC TRYPANOSOMATIDS IN HUMANS

A comprehensive bibliographic survey on the biology and physiology of monoxenous trypanosomatids by McGhee and Cosgrove (1980) described a possible human infection attributed to the genus *Herpetomonas*. The authors reported a patient with undefined symptoms (such as fever and moments of unconsciousness) admitted to a hospital in Texas (USA). Several tests were performed, including a liver biopsy, which subsequently was inoculated in culture medium. After seven days, it was possible to observe the proliferation of flagellates. However, neither promastigote nor epimastigote forms were observed. The authors suggested that the flagellate found in the patient's sample was a species of the genus *Herpetomonas* based on the microscopic observations and the culture behavior, but the possibility of culture contamination by the culture medium manufacturer or by sample handling in the hospital was not ruled out (McGhee and Cosgrove, 1980). In addition, this report also mentioned evidence of *Herpetomonas megaseliae* (syn. *Herpetomonas muscarum* (Borghesan et al., 2013) infection in lizards and mice (Daggett et al., 1972; McGhee and Cosgrove, 1980) and species of *Crithidia*, *Leptomonas*, and *Blastocrithidia* that managed to grow in chicken embryos maintained at 37°C (Schmittner and Mcghee, 1970). However, the authors also stated that previous claims of pathogenicity of monoxenic trypanosomatids in infection of vertebrates were made but that none of these cases could be sustained after close examination. To sum up, McGhee & Cosgrove challenged: "Although there is no proof of lower trypanosomatids infecting vertebrates, the possibility exists and should be considered by attending physicians and veterinarians" (McGhee and Cosgrove, 1980).

In 1991, an unusual clinical manifestation was reported in an immunosuppressed child in the Republic of Guinea-Bissau. The case drew physicians' attention, as it had symptoms caused by *Leishmania* species that cause visceral leishmaniasis. However, leishmaniasis had never been reported in the country by that moment. This led the researchers to suppose that the symptoms were caused by some unknown species of *Leishmania* or another opportunistic trypanosomatid present only in reptiles or small mammals, different from the well-known pathogenic species (Sabbatani et al., 1991).

Later, in 1995, a report on the island of Martinique (France) of a human immunodeficiency virus (HIV)-positive patient who developed the diffuse cutaneous nodular syndrome, usually caused by *Leishmania* species, also caught the attention of researchers (Dedet et al., 1995). The parasites isolated from the skin lesions were submitted to isoenzymes characterization and optical and electron microscopy. Interestingly, the parasite differed isoenzymatically from all known *Leishmania* species. The authors assumed that the parasite present in the lesion could be a monoxenous trypanosomatid since both microscopies revealed the presence of the kinetoplast and opisthomastigote stages (Dedet et al., 1995), and the successful infection could be related to the immunodeficiency of the patient. Some years later, Boisseau-Garsaud et al., 2000) reported a second case, but this time in a patient not affected by any immunodeficiency, in the same location (Martinique - France) presenting a localized skin lesion. The isoenzyme analysis revealed that this parasite showed the same isoenzymatic profile of the parasite described previously by Dedet and colleagues. As none of the two patients had left the island in their entire lives, the authors suggested that an insect parasitic monoxenous trypanosomatid occurring in that region could be infecting humans in a diffuse or localized manner depending on the health status of the host (Boisseau-Garsaud et al., 2000). However, two years later, Dedet research group submitted the two clinical isolates to gene sequencing. Following the isoenzymic characterization, both strains were identical to each other and distant from any other known *Leishmania* species, although related to *L. enriettii*. Thus, it was not possible to conclude whether these isolates would be grouped within *Leishmania* (*Leishmania*), *Leishmania* (*Viannia*) or if they should belong to a new clade within the euleishmania group (Noyes et al., 2002). Several years later, these isolates were recognized as *L. martiniquensis*, with a divergent taxonomic position, distinct from any other existing taxa responsible for an endemic focus of cutaneous leishmaniasis in Martinique (Desbois et al., 2014).

In 1996, Jiménez and colleagues reported a case in Madrid of a patient who, in addition to having immunosuppression, was an injecting drug user. This patient was hospitalized with an extensive medical history of infections and, among them, clinical suspicion of visceral leishmaniasis. Although it was not possible to observe amastigote forms in the bone marrow aspirate, some flagellates were isolated in culture, and promastigote forms were observed by optical microscopy. The isoenzymatic characterization revealed a pattern different from

Leishmania reference strains, and the sample did not hybridize with *Leishmania*-specific probes. Moreover, isolated promastigotes could not infect either BALB/c mice or Syrian golden hamsters. Therefore, the authors ruled out the possibility of *Leishmania* infection and suggested that the patient could be infected by a monoxenous trypanosomatid that could cause leishmaniasis-like symptoms in HIV-positive patients. The contamination route could be syringe washing with water contaminated with parasitic insect feces (Jiménez et al., 1996).

In Brazil, Pacheco et al. (1998) identified amastigotes in a bone marrow aspiration from an HIV-positive patient with clinical symptoms of visceral leishmaniasis. The patient's place of residence is endemic for *L. braziliensis*, a species responsible for cutaneous leishmaniasis, which raised the hypothesis of visceralization of a typical cutaneous species. The parasite grew in culture as promastigotes and could not establish infection in mouse peritoneal macrophages, suggesting the non-pathogenicity of the flagellate (Pacheco et al., 1998). The isoenzyme analysis revealed that the isolated parasite did not correspond to all the assessed known genera, including the classical pathogenic ones. Southern blot hybridization technique revealed homology with the monoxenous species *Leptomonas pulexsimulantis* (generally parasitic species of dog fleas) (Pacheco et al., 1998). The authors assumed that the patient's immunosuppressed condition probably facilitated the establishment of opportunistic monoxenous trypanosomatids in the organism already weakened by HIV (Pacheco et al., 1998).

At the beginning of the 2000s, the progressive increase in reports of leishmaniasis symptoms in patients caused by non-leishmania trypanosomatids prompted Chicharro and Alvar (2003) to publish the first review on the topic - Monoxenous trypanosomatids in immunosuppressed patients. Although the pathogenicity of monoxenous trypanosomatids is debatable, the authors argued that these reports are not surprising since individuals severely immunocompromised because of HIV infection may also be more susceptible to other infections, including trypanosomatids, which are generally considered non-pathogenic.

In 2008, an immunosuppressed patient was admitted to a hospital in Montpellier (France), the symptoms being fever and persistent headache. The patient had been HIV-positive since 1991 and had a history of antiretroviral failure with low CD4+ T cell counts, leading to many opportunistic diseases. Among the exams performed, a blood sample was inoculated in an NNN medium. Four weeks later, kinetoplastid flagellates with morphology different from that of typical *Leishmania* promastigotes were isolated. The patient was discharged from the hospital and recovered spontaneously without any anti-leishmanial treatment. Subsequent attempts to re-isolate the parasite either from the blood or from the bone marrow were unsuccessful. Sequencing of the *5S* and *18S* ribosomal DNA region from the isolated parasite revealed genetic proximity to the monoxenous trypanosomatid *Herpetomonas samuelpessoai* (Morio et al., 2008). It seems clear that the patient's immune status can pave the road for opportunistic infections, like the monoxenic trypanosomatids. It is interesting to note that all these reports identified the later after culture isolation, which creates an important bias that will be later addressed.

CO-INFECTIONS LEISHMANIA AND LEPTOMONAS

Besides the possible infections by monoxenous trypanosomatids reported in immunosuppressed patients, another phenomenon has been attracting attention in the scientific community: the co-infection of monoxenous trypanosomatids, mainly of the genus *Leptomonas*, and *Leishmania* species. This co-infection has been reported increasingly, and most of these reports come from the Indian subcontinent, where visceral leishmaniasis or Kala-azar is frequent (Srivastava et al., 2010; Ghosh et al., 2012; Selvapandiyan et al., 2015; Thakur et al., 2020).

By screening 120 patients with kala-azar symptoms in the Indian subcontinent, Srivastava et al. (2010) obtained successful culture isolates from all splenic aspirates. The restriction fragment length polymorphisms (RFLP) of the *Hsp70* gene from the isolates identified nine samples with a restriction pattern that differed from known *Leishmania* species. Furthermore, *Hsp70* and *18S* gene sequencing of two isolates revealed a genetic relatedness to *Leptomonas seymouri* (Srivastava et al., 2010). This report is interesting because it identifies a potential monoxenic trypanosomatid in 9 different kala-azar patients from different regions of India with no history of HIV/AIDS. Although, as expected, BALB/c inoculation with *L. donovani* led to death after 20 days, while the BALB/c group inoculated with one of the atypical isolates survived and 45 days after infection, DNA was extracted, and the product of rDNA-ITS1 PCR analyzed by gel electrophoresis revealed a pattern consistent with *Lept. seymouri* and *L. donovani* (Srivastava et al., 2010). This raised concerns on the original culture: was it mixed with a higher proportion of *Lept. seymouri* that outnumbers but does not eliminate *L. donovani* in culture? Kala-azar causes a significant immunosuppression that could pave the road for *Lept. seymouri* infection; therefore, in mice, the few *L. donovani* cells would increase, but the inoculum would not be enough to establish a deadly infection. Two years later, another research group showed that *Lept. seymouri* was identified in four out of 29 DNA clinical samples (peripheral blood or skin biopsy) from patients with visceral leishmaniasis or post-kala-azar dermal leishmaniasis (PKDL). In addition, two out of seven parasites isolated in culture from blood samples revealed similarity to *Lept. seymouri* (Ghosh et al., 2012), which was first identified due to an atypical ITS1 PCR product, followed by DNA sequencing and phylogenetic analysis (Ghosh et al., 2012). Later, a screening by ITS1-RFLP of cutaneous leishmaniasis biopsies from 57 patients in a new endemic region in India revealed that 38.5% (22/57) of the samples presented two PCR products with a restriction fragment profile consistent with *L. donovani* and *Lept. seymouri*. Subsequently, the amplicons from representative samples (n=9) of the possible co-infection biopsies were sequenced, revealing maximum identity with *L. donovani* and *Lept. seymouri* (Thakur et al., 2020). Considering, that the

PCR was performed directly in the biopsies, this is an important piece of evidence towards the participation of monoxenic trypanosomatids in leishmaniasis physiopathology.

Usually, when patients manifest the typical clinical features of visceral leishmaniasis, PKDL or cutaneous leishmaniasis, the test performed to confirm the diagnosis is either the detection of the parasite in smears on a stained slide or culture positivity; in some cases, ELISA tests are also used. However, DNA sequencing is rarely performed to confirm the identity of the species found. This has led the authors to question whether all the parasites isolated from these patients with leishmaniasis may be infected with *L. donovani* and any potential co-infecting monoxenous trypanosomatid species, since they are not typed (Srivastava et al., 2010; Thakur et al., 2020). Interestingly, the authors stated that the detection of the non-pathogenic species in patients in the Indian sub-continent must be carefully considered: first, a more in-depth study on the clinical relevance and susceptibility of this parasite to infect humans is worth; second, considering that the co-infections were identified in an area where there are a relevant number of patients resistant to the antimonial used for the treatment of visceral leishmaniasis, this strain of *Leptomonas* could be more resistant to antimony or could act synergistically to *L. donovani* leading to treatment resistance (Ghosh et al., 2012).

In this same direction, Singh et al. (2013) using the next generation sequencing of samples collected from patients affected by visceral leishmaniasis in India, identified *Lept. seymouri* in co-infection with *L. donovani*. Although both species were in concomitance in splenic aspirates, it was not possible to attribute the pathogenicity of *Leptomonas* to the clinical cases. Furthermore, the two species have low genetic divergence and are morphologically similar: both have promastigote forms within the host insect. Thus, the researchers also believe that cases like this are underestimated and that the presence of *Leptomonas* or another monoxenous associated with the *Leishmania* species is greater than reported (Singh et al., 2013).

To evaluate the mechanisms that may favor the appearance of monoxenous trypanosomatids in vertebrate hosts, Kraeva et al. (2015) further investigated two of the clinical isolates of *Lept. seymouri* found in Singh's work (Singh et al., 2013). Although the isolates proved to be adapted to grow at high temperatures and they were able to remain in the digestive tract of the insect vector, *Lept. seymouri* was unable to establish an infection in mammalian macrophages *in vitro*. The authors concluded that despite the adaptations undergone by *Lept. seymouri*, its occurrence in a vertebrate host would only be possible under host immunosuppressant conditions. Those authors also point out that some DNA sequences deposited at Genbank as *L. donovani* are in fact *Lept. seymouri* and emphasized the importance of specific markers for identifying monoxenous species (Kraeva et al., 2015).

CRITHIDIA OCCURRENCE IN IMMUNOCOMPETENT PATIENTS

In 2019, Ghobakhloo and colleagues reported the occurrence of *Crithidia* either alone or in co-infection with *L. major* in human patients in Iran with no apparent immunosuppression. As expected, out of 167 patients screened, 92.8% had *L. major* as the only causative agent, while 5.4% accounted for co-infections, and in only 1.8% of the cases (4 patients), *Crithidia* was the sole agent. The study was focused on detecting insect trypanosomatids based on previous reports in Iran that provided preliminary evidence on the occurrence of insect trypanosomatids in immunocompetent patients. Considering the skepticism of the scientists on the occurrence of "monoxenous" trypanosomatids in healthy individuals and the general belief that all reports up to date are due to the culture strain/clone biased selection, the authors collected samples directly from the biopsies and performed microscopy and PCR amplification. Using two sets of gGAPDH primers, one that amplified *Leishmania* and the other *Crithidia*, the authors provided sequences that showed similarity to *C. fasciculata* reference sequences (Ghobakhloo et al., 2019). In addition to the molecular characterization of the clinical samples, the authors also analyzed some aspects of the isolates obtained in culture. The isolates were able to survive, even if less active and rounder, at a higher temperature that mimicked the temperature of the human body, in contrast to a *C. fasciculata* reference strain obtained from the Centre of Research in Infectious Disease, Laval University (Quebec, Canada). In addition, the clinical isolate of *Crithidia* was able to infect two macrophage cell lines (J774 and THP1). However, as expected, the infection index was lower when compared to *L. infantum* and *L. major* infection. Furthermore, the reference strain of *Crithidia* did not show the same behavior, it was unable to establish itself inside the macrophages (Ghobakhloo et al., 2019).

These reports open interesting discussions: for a time, the findings of monoxenous trypanosomatids in humans were associated with immunodeficiencies in the patient. In this work, some isolates of *Crithidia* were recovered from the lesions of immunocompetent patients. In addition, when *Leishmania-Crithidia* co-infection was reported, the clinical cases were more severe when compared to patients who had only *Leishmania* isolated from the lesion. Another important observation is that, as described in the co-infection between *Lept. seymouri* and *L. donovani* (Ghosh et al., 2012), some of these co-infections did not respond to treatment with antimonial, which raised the question of the possible role of monoxenous trypanosomatids in resistance to the therapy (Ghobakhloo et al., 2019).

This article was refuted in the same year by Kostygov et al., 2019) who claimed that the methodologies used in Ghobakhloo's report were not sufficient to state that *Crithidia* was the sole agent causing skin damage in immunocompetent patients. Kostygov and colleagues claimed that the immune status of the patients was not assessed as well as the primer used to amplify gGAPDH from *Leishmania* could not amplify *L. infantum* (Kostygov et al., 2019). Subsequently, the authors replied that the *Crithidia* spp. were identified in patients with cutaneous leishmaniasis resistant to glucantime treatment, which led the researchers to a closer follow up of these patients. To this end, all patients were examined for immunodeficiency diseases, which were excluded, and then biopsies and smears from patients' lesions were collected under

sterile conditions for parasite characterization. One sample was taken for culture, and three smears were prepared for microscopic study and PCR amplification. Regarding the PCR primer specificity, the authors replied that it could discriminate among *L. infantum, L. major*, and *L. tropica* (Motazedian, 2019). Concerning the specificity of the PCR primer designed to amplify *Leishmania*, the primer specific for *Crithidia* did amplify a fragment directly from the patients' smears. The amplicon was sequenced and revealed similarity to *Crithidia*. The same sample revealed only *Crithidia* in the cultures, subsequently identified in Dr. Marc Ouellette's research group (Laval University Research Center for Infectious Diseases, Quebec, Canada). Kostygov hypothesis would be that these four samples would be a co-infection between *L. infantum* and *Crithidia*, and the authors failed to detect the former by the PCR primers used. Then, as expected, in culture, the monoxenic counterpart outgrows the fastidious *Leishmania*.

In the same year, another report drew a lot of attention from the scientific community, also reaching the general press (Maruyama et al., 2019). The authors described one fatal case of a visceral leishmaniasis-like, being more severe and resistant to the treatment. The patient, which had a negative HIV diagnosis, died of disease and surgical complications. The parasite was isolated in culture from skin lesion and bone marrow. The isolated parasite is more closely related to *C. fasciculata* than to any *Leishmania* by several approaches, including whole-genome sequencing. After intravenous infection in female BALB/c mice with both isolates, only the bone marrow isolate was detected again in the liver and spleen; however, at significantly lower levels than the positive control (*L. infantum*), while the skin strain was detected at very low levels only in the liver. Therefore, those authors performed an artificial skin infection on the mice's ear, revealing that the skin strain caused lesions even more extensive than that resulting from the positive control (*L. major*) (Maruyama et al., 2019). Subsequently, Domagalska and Dujardin published a comment letter where they raised concerns to what we can call – "environment biased selection hypothesis". The inoculation in the culture medium from the patient's biopsy can lead to the monoxenic counterpart prevailing in culture, although few *Leishmania* cells can remain. After that, in the mouse, the few *Leishmania* cells can outnumber the *Crithidia*. Therefore, a molecular identification after the experimental infection can help solve this puzzle (Domagalska and Dujardin, 2020). Finally, the last report sequencing the *ITS1* gene directly from buffy coat samples in 14 patients (seven symptomatic and seven asymptomatic) in Iran revealed that one asymptomatic sample presented close similarity (99.75%) to *C. fasciulata* (Rezaei et al., 2020).

OCCURRENCE OF "MONOXENOUS" TRYPANOSOMATIDS IN NON-HUMAN MAMMALIAN HOSTS

Human infection possibly caused by insect trypanosomatids calls the attention of the scientific community from the first report. However, not only humans are reported as possible unusual mammalian hosts for these trypanosomatids (Maslov et al., 2013). The possibility that insect trypanosomatids could be found in mammalian hosts started in the early description of these parasites, already by morphological observations (McGhee and Cosgrove, 1980). The first time, to our knowledge, that a DNA sequence was provided from monoxenic trypanosomatids isolated from mammals was from rodents and dogs in Egypt several years after the original isolation. The original parasite kept in culture for several years was sequenced and reported to be close to *Herpetomonas* (Podlipaev et al., 2004). A long jump in years and a more recent report performed DNA sequencing screening on 593 insectivorous bats from 8 species in the USA, revealing that 5 (0.8%) were positive for *Blastocrithidia* spp. The incidence is not to be neglected since the positivity for *T. cruzi*, and *T. dionisii* were 0.17% and 1.5%, respectively (Hodo et al., 2016). The screening was performed on DNA extracted from blood and heart tissue with no culture manipulation (Hodo et al., 2016). Another bat screening performed in Brazil revealed that out of 181 bat specimens from 18 species, one was found positive for a species belonging to the *Crithidia* genus, most likely *C. mellificae* (Rangel et al., 2019). The DNA amplification and sequencing were performed out of a culture isolate after rich medium cultivation (Rangel et al., 2019). In a following report from the same research group, a screening in 72 sylvatic animals captured in three distinct biomes in Brazil (Atlantic Forest, Cerrado, and Pantanal) revealed choanomastigote forms in 21 fresh hemoculture preparations (from marmosets, coatis, crab-eating fox, and ocelots). Parasites could not be observed in direct blood examination (Dario et al., 2021). DNA extraction from these hemocultures and *18S* and *gGAPDH* genes sequencing revealed a close similarity with *C. mellificae* (Dario et al., 2021).

The description of natural infections of monoxenous trypanosomatids in mammals has a tendency to increase. However, the actual parasite-host relationship is not fully established, not even if it is a parasite as classically defined. There are though several reports demonstrating successful *in vitro* infections by monoxenous trypanosomatids in humans and mouse cellular culture, as well as the successful growth of these parasites at 37 °C (Santos et al., 2004; Barreto-de-Souza et al., 2008; Matteoli et al., 2009; Pereira et al., 2010).

FINAL CONSIDERATIONS

The first report of a monoxenous trypanosomatid causing disease in mammals occurred as early as the 1980's. The reports are increasing in number and consistency. One should consider that for the general physician routine and patient handling and treatment, parasite isolation and identification is not necessary, and it is rarely performed. Therefore, the cases that resulted in a scientific report were either because *Leishmania* was not an occurring pathogen in the country or because the physicians' faced hurdles in the patient treatment, which required an "out of the box" approach. Anyway, the routine leishmaniasis diagnosis can never reveal the occurrence of monoxenic trypanosomatids: direct smears observation and

immunoassays can reveal positivity for a monoxenic trypanosomatid without distinguishing from *Leishmania*. Isoenzymes and DNA sequencing can reveal the identity of the pathogen. However, the former requires parasite isolation in culture, while the latter can be performed directly in the patients' biopsy, but it is not frequently performed. Although it seems unequivocal that monoxenic trypanosomatids are occurring in wild mammals and humans and even in immunocompetent ones, the "environment biased selection hypothesis" is far from ruled out. Therefore, a co-infection monoxenic trypanosomatids/*Leishmania* can be more common than imagined; the subsequent culture inoculation can favor the fast-growing monoxenic counterpart, which outgrows *Leishmania* without completely eliminating it from the culture media. Then, a mice infection can promote the selection in the other direction. Therefore, in order to take steps ahead on this intriguing pathway that could completely change our understanding of the trypanosomatids life cycle, it is critical that DNA amplification and sequencing is performed in the original biopsy, in the cultured sample, and then upon parasite isolated after the experimental infection. Nevertheless, it is time to move forward. The occurrence of monoxenic trypanosomatids in mammals is still viewed with suspicion by the scientific community. Several published reports needed to justify that there was no misidentification or contamination during the handling of the material, and primer specificity is questioned. Extensive screening of clinical samples by direct DNA sequencing would shed some light on the frequency of monoxenic trypanosomatids in humans and, more importantly, provide further evidence on their relevance in treatment resistance. Subsequently, further questions arise: how do they reach the lesion? As a secondary infection by a fly or as direct inoculation by a vector? The questions that arise are endless, but the first and urgent approach is – are they alone or in a co-infection with *Leishmania*? A detailed study using the powerful DNA sequencing tools available today may provide a significant increase in knowledge about the possible role of monoxenous trypanosomatids in the pathogenic process, either alone or in co-infection with traditional pathogenic species.

AUTHOR CONTRIBUTIONS

CMDL and CBM wrote the first version of the manuscript. All the authors discussed, conceived and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Released Parasite-Derived Kinases as Novel Targets for Antiparasitic Therapies

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The efficient manipulation of their host cell is an essential feature of intracellular parasites. Most molecular mechanisms governing the subversion of host cell by protozoan parasites involve the release of parasite-derived molecules into the host cell cytoplasm and direct interaction with host proteins. Among these released proteins, kinases are particularly important as they govern the subversion of important host pathways, such as signalling or metabolic pathways. These enzymes, which catalyse the transfer of a phosphate group from ATP onto serine, threonine, tyrosine or histidine residues to covalently modify proteins, are involved in numerous essential biological processes such as cell cycle or transport. Although little is known about the role of most of the released parasite-derived kinases in the host cell, they are examples of kinases hijacking host cellular pathways such as signal transduction or apoptosis, which are essential for immune response evasion as well as parasite survival and development. Here we present the current knowledge on released protozoan kinases and their involvement in host-pathogen interactions. We also highlight the knowledge gaps remaining before considering those kinases - involved in host signalling subversion - as antiparasitic drug targets.

Keywords: excreted kinase, eukaryote, protozoa, signal transduction, antimicrobial therapy

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INTRODUCTION

The efficient manipulation of their host cell is an essential feature of intracellular parasites, which they achieve by secreting effectors to maintain their replicative niche within the host cell and to hijack important host pathways. Among those effectors, kinases have been shown to regulate a wide range of pathways, such as signalling or metabolic pathways. The potential key role of secreted kinases in the subversion of host cell signalling pathways, make them candidates of choice for the development of new antiparasitic treatments, particularly with the growing concern of drug resistance. Targeting secreted effectors may reduce the risk of drug resistance, as any mutation to bypass the drug effect may prevent their extra-parasite role and thus result in high fitness costs for the parasite. Despite their importance, only few parasite-secreted kinases have been studied and their functions in the host cell characterised. We focused on two phylums:

-The Apicomplexa (Alveolata) with ***Plasmodium* spp.**, ***Toxoplasma gondii*** and ***Eimeria tenella***, the causative agents of malaria - transmitted by female anopheles mosquitoes -, toxoplasmosis and coccidiosis, respectively;

-The kinetoplastids (Euglenozoa) with ***Leishmania* spp.**, the causative agent of leishmaniasis, transmitted by the bite of a female sand fly and ***Trypanosoma cruzi*,** the causative agent of Chagas disease, spread by Triatominae.

In the present mini review, we describe the known mechanisms of parasite effector secretion and compare the secreted kinome from phylogenetically distant intracellular protozoan parasites. We give examples of the host functions of the few studied secreted kinases and highlight the scientific gaps remaining to fully understand host signalling subversion by parasites.

MECHANISMS OF PARASITE EFFECTOR RELEASE/SECRETION

Parasites release virulence factors either as soluble molecules or inside extracellular vesicles (EVs), leading to the modification of the biological and immune functions of their host cell to ensure their survival (Silverman et al., 2010b; Regev-Rudzki et al., 2013). Apicomplexa and kinetoplastids parasites display different mode of host-parasite interactions due to the specificity of their host cell and their mode of cell entry, which might be partly reflected in the mechanism of parasite protein secretion.

Common to Most Apicomplexa and Kinetoplastids

Exocytosis, driven by the active transport of secretory vesicles is the eukaryotic conventional secretion system for proteins containing a hydrophobic domain in N-terminal position [Signal Peptide, SP, see (Beer and Wehman, 2017) for an illustrated review]. These protein-containing vesicles traffic from the Golgi apparatus to the plasma membrane, fuse with the plasma membrane to release the secreted proteins in the extracellular environment (Colombo et al., 2014). This secretion system does not account for all the molecules exported by parasites. In *Leishmania*, 98% of the secreted proteins lack SP, suggesting the presence of other secretion pathways (Silverman et al., 2008). The second mechanism is unconventional protein secretion (UPS) and refers to proteins either exposed on the cell surface or in the extracellular medium (Balmer and Faso, 2021). The third mechanism is through extracellular vesicles (EVs), lipid-bound vesicles which either bud from the plasma membrane (microvesicles) or are derived from multivesicular bodies that fuse with the plasma membrane (exosomes) (Dlugonska and Gatkowska, 2016; Mathieu et al., 2019; Babatunde et al., 2020) and for more details in mechanisms of secretion see (Teng and Fussenegger, 2021).

Specific to Apicomplexa

Apicomplexa have developed specific strategies to release proteins into the host cell, which might be the consequence of cell entry by invasion, contrary to phagocytosis or endocytosis for kinetoplastids. Cell invasion requires the fast discharge of microneme and rhoptry proteins (perforins, lipases, proteases, adhesins and kinases) implicated in gliding motility, parasite attachment, formation of the moving junction and the hijacking of host cell pathways, which might not be compatible with the slower release of proteins by the secretory or exosomal pathways (Tomavo et al., 2013; Bisio and Soldati-Favre, 2019). Proteins are targeted to those compartments by conventional SP and specific motifs. Microneme secretion is triggered by signalling events, involving intracellular cyclic nucleotides, calcium level and phosphatidic acid (Dubois and Soldati-Favre, 2019) and is followed by rhoptry secretion (Aquilini et al., 2021). In addition to micronemes and rhoptries, *Plasmodium* species create, in the host cell cytoplasm, a network of membranous structures of parasite origin, called Maurer's clefts. These structures are attached to the host cytoskeleton and act as extracellular secretory and trafficking organelles for the parasite (Lanzer et al., 2006) but little is known about their biogenesis and functions. Finally, some proteins contain PEXEL motifs in *Plasmodium* (Marti et al., 2004) and TEXEL motifs in *Toxoplasma* (Coffey et al., 2015), which are required for their release *via* exporters located on the parasitophorous vacuole membrane (PVM). The PVM derives from the host cell membrane and is modified by incorporation of parasite proteins, to avoid phagolysosome fusion (de Koning-Ward et al., 2009; Marino et al., 2018). This pathway corresponds to the default constitutive secretion pathway (Venugopal et al., 2020).

EXOPROTEOMES OF APICOMPLEXA AND TRYPANOSOMATIDS

There is a growing body of data on the exo-proteome, whatever the mechanism of secretion used by parasites. It contains a range of protein classes including proteases, kinases, membrane proteins, heat shock proteins or nucleic acids, which induce specific modifications in the host cell (Montaner et al., 2014). Only little is known about the mechanisms involved in cargo selection of these EVs. *Leishmania* HSP100 has a strong impact on protein cargo composition: its deletion affects the immune status of the host cell and parasite survival (Silverman et al., 2011). The EV composition is sensitive to environmental cues (Hassani et al., 2011) and might contribute to the spread of drug resistance (Douanne et al., 2020). EVs have an essential role during infection (Torrecilhas et al., 2020); co-egestion of *Leishmania* and its EVs by the sand fly induces the inflammatory recruitment of neutrophils and macrophages (Atayde et al., 2015). EVs are involved in immune evasion; *T. cruzi* exosomes aggravate the infection due to severe inflammatory response and increase the parasite burden (Trocoli Torrecilhas et al., 2009). Several vesicular virulence factors from *T. cruzi* have been involved in host invasion, intracellular parasite proliferation or immune evasion (Costa et al., 2016). For Apicomplexa, *T. gondii* exosomes has been

shown to activate a pro-inflammatory immune response (Li et al., 2018), and small non-coding RNAs and genomic DNA contained in EVs released from RBC infected by *P. falciparum* are detected by the STING pathway, favouring parasite survival (Sisquella et al., 2017). However, accessing parasite-derived EVs is challenging, as apicomplexans parasites cannot be cultured without their host cell, thus most of the data available on their exo-proteome is in fact from host-derived EVs.

SECRETED/EXCRETED PARASITE KINASES

Phosphorylation, an essential reversible post-translational modification, affects every cellular process (Ardito et al., 2017). It acts as a molecular switch for many biological processes, including signal transduction networks in response to extracellular stimuli. Phosphorylation is catalysed by kinases, which transfers phosphate from ATP onto proteins, sugars or lipids. Upon phosphorylation, the chemical properties, conformation, localisation and/or activity of the molecule change, inducing rapid downstream effects in the cell (Hunter and Sibley, 2012). To survive, intracellular pathogens need to exploit the host pathways either to fulfil their needs for proliferation or to inhibit the host defence responses. Targeting the phospho-proteome of the host is the fastest way to subvert a large repertoire of biological and immune processes (Regev-Rudzki et al., 2013; Carrera-Bravo et al., 2021).

Kinases

Most studies on kinases refers to protein kinases. Protozoan parasite kinomes contain orthologues for 6 of the 8 groups of conventional eukaryotic PKs (ePK): AGC, CAMK, CK1, CMGC, STE and TKL) and some "others" that share ePK folding but cannot be assigned to any major ePK group from humans (Peixoto et al., 2010; Talevich et al., 2011). One additional group Kinetoplastids, NEK family, is involved in cell cycle and cytoskeletal functions. Apicomplexa contains also specific ePK (FIKK, ROPK and WNG), differentially conserved and/or duplicated within Apicomplexa parasite phylum (Ward et al., 2004; Beraki et al., 2019). While only one FIKK gene was identified in coccidia (*Toxoplasma, Eimeria*) and in most *Plasmodium* species, this group is expanded in *P. falciparum* with 20 kinases and in several *Plasmodium* sp. infecting apes (Adderley et al., 2021). Most of the rhoptry proteins are kinases (ROPK), either active, inactive (lacking a complete catalytic triad) or non-canonical (active with differences in conserved residues) (Bradley et al., 2005). Finally, very recently, a new group of four kinases specific to coccidia, and missing the typical glycine loop was identified (WNG1-3 and BPK1) (Beraki et al., 2019). BPK1 is associated with bradyzoite cyst wall, with a crucial role in *in vivo* cyst infectivity (Boothroyd, 2013; Buchholz et al., 2013). In *Eimeria*, two WNGs are predicted, but their functions remain unknown. To date, only TgWNG1 has been functionally characterized: it is involved in the phosphorylation of GRA, a family of effectors stored in dense granule vesicles and secreted to develop the intra-vacuolar network, implicated in survival of parasite. Although as important as protein kinases, there are no comprehensive studies available on carbohydrate, lipid, nucleoside or other kinases, but only individual publications (Pereira et al., 2011).

Host Functions of Secreted Kinases

Proteomic characterisation of parasite exo-proteomes revealed the presence of only few kinases, mostly involved in pathways such as glycolysis, cellular energy homeostasis or cell signalling (**Table 1**). While in Apicomplexa most secreted kinases target proteins, in *Leishmania* and *T. cruzi*, more than half of the kinases target nucleosides, carbohydrates or lipids (**Figure 1**). Eight kinases are released by both kinetoplastids and Apicomplexa (**Table 1**, bold), suggesting that host subversion mediated by those kinases might be conserved between parasites. Five kinases might be kinetoplastid-specific (**Table 1**, italic) and only one might be Apicomplexa-specific (CDPKs, **Table 1**, underlined). This low number of specific kinases may be due to the scarce proteomic data available for these parasites.

Glycolytic Kinases

Glycolytic kinases are located in the glycosomes of kinetoplastids and in the cytosol and the apicoplast of Apicomplexa (Saito et al., 2002; Fleige et al., 2007). They regulate glycolysis but have additional biological functions, as moonlighting proteins. For instance, *Leishmania* hexokinase, a glycolytic enzyme, also acts as a haemoglobin (Hb) receptor, allowing Hb internalisation (Krishnamurthy et al., 2005). *L. donovani* aldolase, another glycolytic enzyme, interacts with and activates the host SHP-1 (protein tyrosine phosphatase). SHP-1 inhibits M1 macrophage polarization, creating a more favourable environment for *Leishmania* (Nandan et al., 2007; Garg et al., 2020). Although important in number, nothing is known about the host functions of glycolytic kinases, but their release by most the parasites suggest important roles in the host cell.

Nucleoside Diphosphate Kinase (Ndk)

Ndk catalyses the transfer of phosphate from nucleoside triphosphate (NTP) to nucleoside diphosphate (NDP) to maintain ATP cellular homeostasis (Kolli et al., 2008). This kinase also plays roles in the regulation of gene transcription, DNA repair, differentiation and apoptosis (Yu et al., 2017). Ndk seems important for drug resistance in *T. cruzi* and in *Leishmania*, its overexpression leads to a decrease sensitivity to antimony (Sb^{III}) (Moreira and Murta, 2016); for DNA damage responses in *T. cruzi* (Miranda et al., 2008); and for parasite replication in *T. gondii* (Lykins et al., 2018). In these parasites, Ndk is released in EVs (Silverman et al., 2008; Silverman et al., 2010a; Bayer-Santos et al., 2013; Lee et al., 2014; Brossas et al., 2017). In *Leishmania*, the release of ndk prevents extracellular ATP (eATP)-mediated cytolysis of infected macrophages (Kolli et al., 2008). eATP, a signal nucleotide, binds to and

TABLE 1 | Kinases secreted by protozoan parasites.

Organism	Kinase Name	Kinase Class	References	Stages
Leishmania	6-phospho-1-fructokinase, putative	carbohydrate	Silverman et al., 2008; Silverman et al., 2010a; Douanne et al., 2020	Stationary-phase promastigotes, logarithmic promastigotes
	adenosine kinase, putative	nucleoside	Silverman et al., 2008	Stationary-phase promastigotes
	adenylate kinase, putative	nucleoside	Silverman et al., 2008; Silverman et al., 2010a; Hassani et al., 2011; Douanne et al., 2020	Stationary-phase promastigotes, logarithmic promastigotes and axenic amastigotes
	casein kinase I, putative CK1.2	protein	Silverman et al., 2008; Silverman et al., 2010a	Stationary-phase promastigotes
	casein kinase II, alpha chain, Putative LmjF.02.0360	protein	Douanne et al., 2020	Logarithmic promastigotes
	mevalonate kinase	lipid	Bamra et al., 2021	Promastigotes and amastigotes
	cdc2-related kinase 9	protein	Silverman et al., 2008	Stationary-phase promastigotes
	glycogen synthase kinase3-putative	protein	Douanne et al., 2020	Logarithmic promastigotes
	glycosomal phosphoenolpyruvate carboxykinase, putative	other	Silverman et al., 2008; Hassani et al., 2011; Douanne et al., 2020	Stationary-phase promastigotes and logarithmic promastigotes
	hexokinase, putative	carbohydrate	Silverman et al., 2008; Hassani et al., 2011	Stationary-phase promastigotes
	mitogen activated protein kinase, putative,map kinase, putative	protein	Silverman et al., 2008	Stationary-phase promastigotes
	mitogen-activated protein kinase 3, putative,map kinase 3, putative	protein	Silverman et al., 2008	Stationary-phase promastigotes
	nucleoside diphosphate kinase b	nucleoside	Silverman et al., 2008; Silverman et al., 2010a; Hassani et al., 2011; Douanne et al., 2020	Stationary-phase promastigotes, logarithmic promastigotes and axenic amastigotes.
	phosphoglycerate kinase	other	Douanne et al., 2020; Mandacaru et al., 2019; Ribeiro et al., 2018; Brossas et al., 2017; Queiroz et al., 2016; Bayer-Santos et al., 2013; Hassani et al., 2011	Logarithmic promastigotes, stationary-phase promastigotes and Epimastigotes, Vero cells infected with trypomastigotes, Trypomastigotes, Tissue culture-derived trypomastigotes and trypomastigotes.
	protein kinase, putative LmjF34.0030	protein	Silverman et al., 2008	Stationary-phase promastigotes
	pyruvate dehydrogenase lipoamide kinase, putative	other	Silverman et al., 2008	Stationary-phase promastigotes
	pyruvate kinase, putative	other	Silverman et al., 2008; Silverman et al., 2010a; Douanne et al., 2020; Ribeiro et al., 2018	Stationary-phase promastigotes and logarithmic promastigotes, Tissue culture-derived trypomastigotes.
	pyruvate phosphate dikinase, putative	other	Silverman et al., 2008; Silverman et al., 2010a; Douanne et al., 2020	Stationary-phase promastigotes and logarithmic promastigotes
	serine/threonine-protein kinase, putative LINF_290033500/ LmjF29.2570 identity to human Nek1	protein	Douanne et al., 2020	Logarithmic promastigotes
	serine/threonine-protein kinase, putative LmjF25.2340 identity to human AKT1	protein	Silverman et al., 2010a	Stationary-phase promastigotes
	tagatose-6-phosphate kinase-like protein	carbohydrate	Silverman et al., 2008	Stationary-phase promastigotes
Trypanosoma cruzi	*adenosine kinase, putative*	nucleoside	Brossas et al., 2017	Trypomastigotes
	mevalonate kinase	lipid	Ferreira et al., 2016	Metacyclic trypomastigote and extracellular amastigote cultures
	adenylate kinase, putative	nucleoside	Queiroz et al., 2016; Ribeiro et al., 2018	Trypomastigotes, Tissue culture-derived trypomastigotes
	arginine kinase	other	Bayer-Santos et al., 2013; Queiroz et al., 2016; Brossas et al., 2017; Ribeiro et al., 2018; Mandacaru et al., 2019	Epimastigotes, Vero cells infected with trypomastigotes, Trypomastigotes, Tissue culture-derived trypomastigotes and trypomastigotes.
	fucose kinase	carbohydrate	Ribeiro et al., 2018	Tissue culture-derived trypomastigotes
	galactokinase	carbohydrate other	Ribeiro et al., 2018	Tissue culture-derived trypomastigotes

(Continued)

TABLE 1 | Continued

Organism	Kinase Name	Kinase Class	References	Stages
	glycosomal phosphoenolpyruvate carboxykinase, putative		Queiroz et al., 2016; Ribeiro et al., 2018; Mandacaru et al., 2019	Trypomastigotes, Tissue culture-derived trypomastigotes and trypomastigotes.
	hexokinase	carbohydrate	Bayer-Santos et al., 2013, Mandacaru et al., 2019	Epimastigotes and trypomastigotes.
	mitogen-activated protein kinase, putative	protein	Bayer-Santos et al., 2013; Brossas et al., 2017; Ribeiro et al., 2018	Epimastigotes, Vero cells infected with trypomastigotes, Tissue culture-derived trypomastigotes
	NIMA-related kinase, putative	protein	Queiroz et al., 2016	Trypomastigotes
	nucleoside diphosphate kinase B	nucleoside	Bayer-Santos et al., 2013; Brossas et al., 2017; Queiroz et al., 2016; Ribeiro et al., 2018; Mandacaru et al., 2019	Epimastigotes, Vero cells infected with trypomastigotes, Trypomastigotes, Tissue culture-derived trypomastigotes and trypomastigotes.
	phosphatidylinositol-3-Kinase	lipid	Bayer-Santos et al., 2013	Epimastigotes
	Protein kinase Tc00.1047053506211.210 MAPKKK identity to human PAK1/PAK3	protein	Ribeiro et al., 2018	Tissue culture-derived trypomastigotes
	Protein kinase, putative TcCLB.508641.170 identity to human PKC theta	protein	Brossas et al., 2017	Vero cells infected with trypomastigotes
	protein kinase-A catalytic subunit	protein	Queiroz et al., 2016	Trypomastigotes
	pyridoxal kinase, putative	other	Queiroz et al., 2016; Ribeiro et al., 2018	Trypomastigotes, Tissue culture-derived trypomastigotes
	pyruvate kinase 2, putative	other	Queiroz et al., 2016	Trypomastigotes
	pyruvate phosphate dikinase 2	other	Queiroz et al., 2016	Trypomastigotes
	pyruvate phosphate dikinase 1	other	Bayer-Santos et al., 2013; Ribeiro et al., 2018	Epimastigotes, Tissue culture-derived trypomastigotes
	serine/threonine protein kinase *TcCLB.508153.400/ TCSYLVIO_004423 identity to human Nek1*	protein	Bayer-Santos et al., 2013; Ribeiro et al., 2018	Epimastigotes, Tissue culture-derived trypomastigotes
	serine/threonine-protein kinase 10, putative TcCLB.506401.110 identity to human STK10	protein	Queiroz et al., 2016	Trypomastigotes
Plasmodium	Camk2d calcium/ calmodulin_dependent protein kinase II_ delta isoform 1	protein	Martin-Jaular et al., 2011	Trophozoite *P. yoelii* infected reticulocyte
	Camk2d Isoform 1 of Calcium/ calmodulin_dependent protein kinase type II delta chain	protein	Martin-Jaular et al., 2011	Trophozoite *P. yoelii* infected reticulocyte
	Camk2d Isoform 2 of Calcium/ calmodulin_dependent protein kinase type II delta chain	protein	Martin-Jaular et al., 2011	Trophozoite *P. yoelii* infected reticulocyte
	Camk2d Isoform 3 of Calcium/ calmodulin_dependent protein kinase type II delta chain	protein	Martin-Jaular et al., 2011	Trophozoite *P. yoelii* infected reticulocyte
	Camk2d Isoform 4 of Calcium/ calmodulin_dependent protein kinase type II delta chain	protein	Martin-Jaular et al., 2011	Trophozoite *P. yoelii* infected reticulocyte
	casein kinase 2, alpha subunit	protein	Abdi et al., 2017	Trophozoite *P. falciparum* infected erythrocyte
	diacyl glycerol kinase	lipid	Gualdron-Lopez et al., 2018	Trophozoite *P. vivax* infected erythrocyte
	FIKK10.1	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK13	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK14	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK1	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK4.1	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK4.2	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK7.1	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK9.1	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte

(Continued)

TABLE 1 | Continued

Organism	Kinase Name	Kinase Class	References	Stages
	FIKK9.3	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK9.6	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK10.2	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK11	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	FIKK12	protein	Hiller et al., 2004	Trophozoite *P. falciparum* infected erythrocyte
	pacsin2 Protein kinase C and casein kinase substrate in neurons protein 2	protein	Martin-Jaular et al., 2011	Trophozoite *P. yoelii* infected reticulocyte
	phosphatidylinositol 4-kinase, putative	lipid	Abdi et al., 2017	Trophozoite *P. falciparum* infected erythrocyte
	phosphoglycerate kinase	other	Mantel et al., 2013; Abdi et al., 2017	Trophozoite *P. falciparum* infected erythrocyte
	pseudo protein kinase 1, putative PF3D7_0321400	protein	Abdi et al., 2017	Trophozoite *P. falciparum* infected erythrocyte
	pyruvate kinase	other	Vincensini et al., 2005; Mantel et al., 2013	Trophozoite *P. falciparum* infected erythrocyte
	serine/threonine protein kinase, putative PF3D7_1441300	protein	Abdi et al., 2017	Trophozoite *P. falciparum* infected erythrocyte
	calcium-dependent protein kinase CDPK1	protein	Lal et al., 2009	Purified microneme organelle
	calcium-dependent protein kinase CDPK4	protein	Lal et al., 2009	Purified microneme organelle
	adenylate kinase	nucleoside	Vincensini et al., 2005	Trophozoite *P. falciparum* infected erythrocyte
Toxoplasma	calcium-dependent protein kinase CDPK1	protein	Wowk et al., 2017; Ramirez-Flores et al., 2019	Tachyzoite *T. gondii* infected human foreskin fibroblast, Acellular tachyzoites
	calcium-dependent protein kinase CDPK2A	protein	Wowk et al., 2017	Tachyzoite *T. gondii* infected human foreskin fibroblast
	calcium-dependent protein kinase CDPK3	protein	Wowk et al., 2017; Ramirez-Flores et al., 2019	Tachyzoite *T. gondii* infected human foreskin fibroblast, Acellular tachyzoites
	casein kinase I	protein	Wowk et al., 2017	Tachyzoite *T. gondii* infected human foreskin fibroblast
	CMGC kinase, CK2 family	protein	Wowk et al., 2017; Ramirez-Flores et al., 2019	Tachyzoite *T. gondii* infected human foreskin fibroblast, Acellular tachyzoites
	hexokinase	carbohydrate	Wowk et al., 2017; Ramirez-Flores et al., 2019	Tachyzoite *T. gondii* infected human foreskin fibroblast, Acellular tachyzoites
	nucleoside diphosphate kinase	nucleoside	Lee et al., 2014	Acellular tachyzoites
	phosphoenolpyruvate-carboxykinase I	other	Wowk et al., 2017	Tachyzoite *T. gondii* infected human foreskin fibroblast
	phosphofructokinase PFKII	carbohydrate	Wowk et al., 2017	Tachyzoite *T. gondii* infected human foreskin fibroblast
	phosphoglycerate kinase	other	Wowk et al., 2017	Tachyzoite *T. gondii* infected human foreskin fibroblast
	pyruvate kinase	other	Wowk et al., 2017	Tachyzoite *T. gondii* infected human foreskin fibroblast
	rhoptry kinase family protein ROP39	protein	Wowk et al., 2017	Tachyzoite *T. gondii* infected human foreskin fibroblast
	selenide, water dikinase	other	Wowk et al., 2017	Tachyzoite *T. gondii* infected human foreskin fibroblast
	ROP2 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	ROP4 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	ROP5 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	ROP8 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	ROP11 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	ROP16 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	ROP17 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	ROP18 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	ROP38 - PK-like	protein	Bradley et al., 2005	Purified rhoptry organelle
	WNG1 (With-No-Gly-Loop)	protein	Beraki et al., 2019	Bradyzoite
	WNG2	protein	Beraki et al., 2019	Bradyzoite
	WNG3	protein	Beraki et al., 2019	Bradyzoite
	BPK1 bradyzoite pseudokinase 1	protein	Buchholz et al., 2013	Bradyzoite
Eimeria[a]	**pyruvate kinase**	other	Labbé et al., 2006	Schizont from *in vitro* infected cells

(Continued)

TABLE 1 | Continued

Organism	Kinase Name	Kinase Class	References	Stages
	hexokinase	carbohydrate	Sun et al., 2016	Sporozoite from *in vitro* infected cells
	calcium-dependent protein kinase CDPK1	protein	Dunn et al., 1996	Sporozoite from *in vitro* infected cells
	calcium-dependent protein kinase CDPK2	protein	Dunn et al., 1996	Sporozoite from *in vitro* infected cells
	calcium-dependent protein kinase CDPK3	protein	Han et al., 2013	Sporozoite and schizont from *in vitro* infected cells
	calcium-dependent protein kinase CDPK4	protein	Wang et al., 2016a	Sporozoite and merozoite from *in vitro* infected cells
	ETH_00000075	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00005190 - EtROP1	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00005400	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00005840	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00005905	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00020620	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00026495	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00027695	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00027700	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00027855	protein	Oakes et al., 2013	Purified rhoptry organelle
	ETH_00028765	protein	Oakes et al., 2013	Purified rhoptry organelle
	WNG1, predicted	protein	Beraki et al., 2019	ND[b]
	WNG4, predicted	protein	Beraki et al., 2019	ND[b]

[a]*Secreted kinases listed for Eimeria are underestimated, due to a lack of datasets. Eimeria pyruvate kinase (Labbé et al., 2006), hexokinase (Sun et al., 2016) and CDPK (Dunn et al., 1996; Han et al., 2013; Wang et al., 2016a) are secreted by an unknown mechanism.*

[b]*Not determined.*

Kinase class refers to the kinase substrate. Kinases common to kinetoplastids and Apicomplexa are indicated in bold, kinases specific to kinetoplastids or to Apicomplexa are indicated in italic or underlined, respectively. Based on experimental procedures of cited references for Apicomplexa, kinases from organelles are secreted in the host cell cytoplasm, not in the extracellular medium. To the author knowledge, only P. falciparum *TKL2 and PfCK1 were detected in extracellular medium and at the erythrocyte membrane (Abdi et al., 2013) and maybe associated to immunomodulatory functions. Most of the secreted proteins of Kinetoplastids are released inside the host cell. Ndk and AK seem to be secreted in the extracellular environment of the host cell due to their role. Limitations concerning secretome preparation and characterization have been reviewed (Severino et al., 2013).*

activates the P2X7 receptor, which is responsible for the pore formation in the membrane of macrophages, resulting in cell death (Kolli et al., 2008; Kulkarni et al., 2019). By transferring phosphate from ATP to NDP, Ndk decreases eATP, thus prevents ATP-induced changes in mitochondrial permeability of macrophages. Furthermore, Ndk participates in the host purine salvage by protozoan parasites by utilizing eATP to produce other NTPs such as GTP (Kolli et al., 2008) These functions might be conserved in *T. cruzi* and *T. gondii*, which also release Ndk.

Casein Kinase 1

Casein kinase 1 (CK1) is a serine/threonine protein kinase that regulates a wide range of biological processes (Xu et al., 2019; Rachidi et al., 2021). In *Leishmania*, three paralogs are released: L-CK1.4 through the classical secretory pathway, L-CK1.5 and L-CK1.2 *via* exosomes (**Table 1**). Nothing is known about the role of these paralogs in the host cell, except for L-CK1.2. This kinase phosphorylates human IFNAR1 receptor, physiological target of human CK1α, to promote its ubiquitination and subsequent degradation, leading to the attenuation of the cellular response to interferon α/β (Liu et al., 2009). Recently additional host proteins phosphorylated by L-CK1.2 were identified (Smirlis et al., 2022). Several pathways, such as apoptosis, actin skeleton organisation or RNA processing were shown to be potentially regulated by L-CK1.2, which corresponds to pathways altered during *Leishmania* infection (Smirlis et al., 2020). These findings suggest that L-CK1.2 might replace human CK1 and phosphorylate host proteins to modify the immune status of the host cell. Among the three CK1 isoforms encoded by *T. gondii*, only CK1α is secreted in EVs (Donald et al., 2005; Wowk et al., 2017; Rachidi et al., 2021). In contrast to its kinetoplastid orthologs, it is still unclear whether TgCK1α is essential for *T. gondii* survival or what are its functions in the host cell. However, TgCK1α is not a candidate drug target, as its deletion increases *T. gondii* virulence (Wang et al., 2016b). Finally, *P. falciparum* expresses only one CK1, which is secreted by potentially hijacking the trafficking system of the host cell (Dorin-Semblat et al., 2015). Ten PfCK1-interacting host proteins were identified and are involved in various pathways, such as post-translational modifications, translation and protein trafficking/export (Batty et al., 2020).

Adenylate Kinase (AK)

AK catalyses the transfer of a phosphate group from ATP to AMP to generate two ADPs. It regulates homeostasis of adenine nucleotides and plays an important role in the regulation of the energy metabolism. AK has been detected in the exo-proteome of *Leishmania* and *T. cruzi* (**Table 1**). Recent data from *L. donovani*, suggests that AK2a prevents ATP-mediated cytolysis of macrophages, similarly to Ndk (Kulkarni et al., 2019).

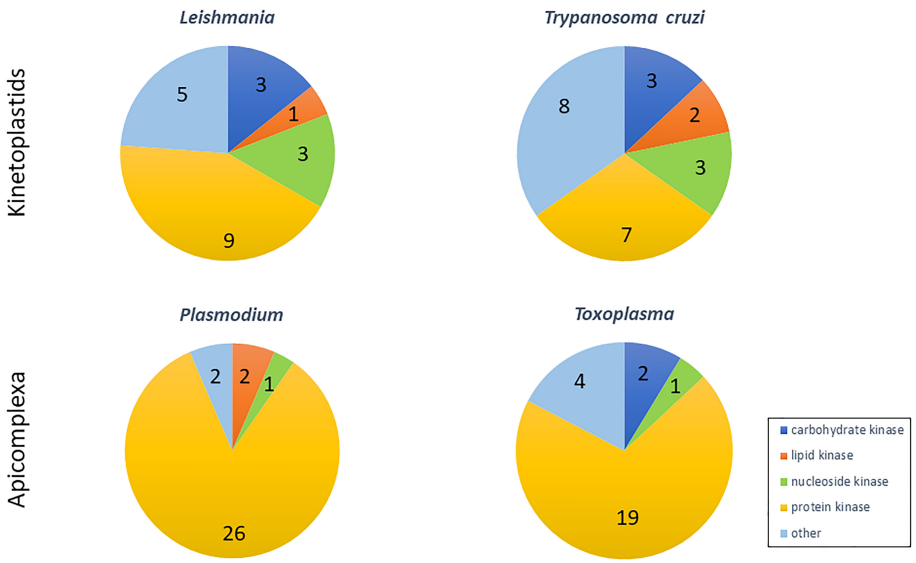

FIGURE 1 | Proportion and number of kinases classes according to their substrates, identified in secretome of Kinetoplastids (*Leishmania* and *Trypanozoma*) and Apicomplexa (*Toxoplasma* and *Plasmodium*).

Mevalonate Kinase

L. donovani Mevalonate kinase (MVK) is a glycosomal enzyme, secreted by the parasite *via* a non-classical secretion pathway (Bamra et al., 2021). MVK catalyses the phosphorylation of mevalonic acid into mevalonate-5-phosphate, which is part of the cholesterol biosynthesis pathway. Macrophage infection with *L. donovani* over-expressing MVK leads to an increase in parasite internalisation. During extracellular amastigotes invasion, *T. cruzi* MVK induces the phosphorylation of host Src/FAK, involved in cytoskeleton remodelling of the host (Ferreira et al., 2016), and the phosphorylation of the host P38 and ERK leading to cytoskeleton and microfilament remodelling, which favour parasite internalisation. Moreover, LdMVK is an immuno-suppressor, which favours anti-inflammatory cytokines through ERK1/2, increasing parasite survival (Bamra et al., 2021).

FIKKs

The 18 FIKKs secreted by *P. falciparum*, display an important non-redundant role in cytoskeletal connections, nutrients permeability and ubiquitination of RBC proteins, as shown by the phosphoproteomic profile of their systematic invalidated mutants (Davies et al., 2020). For instance, FIKK4.1 and FIKK4.2 are involved in cytoadhesion of the RBC to the vascular endothelium, by regulating the number/size of knobs formed on the RBC membrane (Kats et al., 2014). FIKK4.1, FIKK7.1 and FIKK12 phosphorylate host cell cytoskeleton proteins, thus modifying RBC rigidity (Nunes et al., 2010; Brandt and Bailey, 2013). Finally, FIKK9.1, FIKK10.1 and FIKK10.2, exported *via* Maurer's clefts, are essential for parasite survival (Siddiqui et al., 2020).

ROPKs

ROPK, secreted from the rhoptries, are involved in host-pathogen interaction. Although not all ROPK are functionally characterized, a systematic and targeted *T. gondii* ROPK knockout screen (Fox et al., 2016) highlighted the role of 20 ROPKs in the establishment of a chronic infection. After their secretion in the host cell cytoplasm, TgROP5, TgROP17 and TgROP18 form a complex on the cytosolic side of the PVM (Etheridge et al., 2014). TgROP5 binds immune-related GTPases (IRG) to decrease their polymerisation rate (Behnke et al., 2012). IRG are then phosphorylated by TgROP18, to prevent their recruitment to the PVM and preserve it (Fleckenstein et al., 2012). Additionally, TgROP17 is also involved in GRA translocation through the PVM, in association with the MYR complex (Panas et al., 2019). TgROP16 (Saeij et al., 2006), TgROP17 (Drewry et al., 2019), TgROP18 (Fentress and Sibley, 2011) and TgROP38 (Peixoto et al., 2010) are known to interfere with and regulate host pathways such as immune response and apoptosis. TgROP16 is localized to the host cell nucleus after invasion (Ong et al., 2010), phosphorylates signal transducer and activator of transcription STAT6 and STAT3 (Yamamoto et al., 2009; Butcher et al., 2011) to bypass the protective immune-response of the host cell. In *E. tenella*, among 28 ROPKs differentially expressed during the life-cycle (Ribeiro E Silva et al., 2021), only EtROP1 has been functionally characterised (Diallo et al., 2019). It interacts with host p53, to inhibit host cell apoptosis and induce G0/G1 cell cycle arrest. Interestingly, EtROP1 kinase activity is only required for cell cycle arrest, supporting the hypothesis of an additional kinase that would be responsible for p53 phosphorylation. ROPK inhibitors may offer new therapeutic treatments to control coccidiosis (Simpson et al., 2016).

CONCLUDING REMARKS

Released divergent kinases that alter host signalling pathways are interesting as they co-evolve with their host targets to insure their proper function within the host and are thus less prone to mutations that would lead to drug resistance. Some compounds that target those secreted kinases have already been identified. *P. falciparum* Pyruvate Kinase is efficiently targeted by antimalarial drugs, such as LZ1 (Fang et al., 2019) or suramin, which also targets trypanosomatids Pyruvate Kinase (Zhong et al., 2020). L-CK1.2 has been validated as a drug target and several compounds with anti-leishmanial activity have been identified, for review see (Rachidi et al., 2021). The similarity between PfCK1 and L-CK1.2 suggests that it might also be a good antimalarial drug target. Given the expanse of their effects on the host cell, understanding the roles that kinases secreted by parasites play in the subversion of host cell signalling will help uncover crucial drug targets.

AUTHOR CONTRIBUTIONS

AS and NR wrote the first draft of the manuscript. SS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Role of Virulence Factors of Trypanosomatids in the Insect Vector and Putative Genetic Events Involved in Surface Protein Diversity

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Trypanosomatids are flagellate protozoans that can infect several invertebrate and vertebrate hosts, including insects and humans. The three most studied species are the human pathogens *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp. which are the causative agents of Human African Trypanosomiasis (HAT), Chagas disease and different clinical forms of leishmaniasis, respectively. These parasites possess complex dixenous life cycles, with zoonotic and anthroponotic stages, and are transmitted by hematophagous insects. To colonize this myriad of hosts, they developed mechanisms, mediated by virulence factors, to infect, propagate and survive in different environments. In insects, surface proteins play roles in parasite attachment and survival in the insect gut, whilst in the mammalian host, the parasites have a whole group of proteins and mechanisms that aid them invading the host cells and evading its immune system components. Many studies have been done on the impact of these molecules in the vertebrate host, however it is also essential to notice the importance of these virulence factors in the insect vector during the parasite life cycle. When inside the insect, the parasites, like in humans, also need to survive defense mechanisms components that can inhibit parasite colonization or survival, e.g., midgut peritrophic membrane barrier, digestive enzymes, evasion of excretion alongside the digested blood meal, anatomic structures and physiological mechanisms of the anterior gut. This protection inside the insect is often implemented by the same group of virulence factors that perform roles of immune evasion in the mammalian host with just a few exceptions, in which a specific protein is expressed specifically for the insect vector form of the parasite. This review aims to discuss the roles of the virulence molecules in the insect vectors, showing the differences and similarities of modes of action of the same group of molecules in insect and humans, exclusive insect molecules and discuss possible genetic events that may have generated this protein diversity.

Keywords: Trypanosomatids, virulence factors, insect vectors, host parasite interaction, parasite genetic variability

INTRODUCTION

The three most studied species of trypanosomatids are the human pathogens *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp. which are the causative agents of Human African Trypanosomiasis (HAT), Chagas disease and different clinical forms of leishmaniasis, respectively. These parasites are flagellate protozoans that possess complex dixenous life cycles, infecting several invertebrate and vertebrate hosts, and are transmitted by hematophagous insects (Kaufer et al., 2017; Sylvia and Aldo, 2019). The transmission from the mammal host to the insect vector occurs when tsetse flies, triatomine bugs or sand flies ingest a blood meal, taking parasites, which may be inside infected cells or free-living in the bloodstream. Following ingestion, the parasites differentiate into their insect form and colonize several areas of the vector digestive system. To complete the life cycle, these parasites are transmitted back to the vertebrate hosts *via* different mechanisms: *T. brucei* is inoculated in the host by tsetse flies, while *T. cruzi* is eliminated with feces or urine by triatomine bugs and *Leishmania* is regurgitated by sand flies (Cholewiński et al., 2015; Geiger et al., 2016).

To persist in this variety of hosts, they developed mechanisms, mediated by their virulence factors, to infect, propagate and survive in these different environments. Many studies have reported the impact of these molecules in the vertebrate host. They are mostly composed of parasites' surface proteins that perform roles of evading the immune system by inhibition of cytokines production, protection against complement-mediated lysis, adhesion, invasion and survival inside host cells (Geiger et al., 2016; Stijlemans et al., 2016; de Castro Neto et al., 2021). However, it is also essential to discuss the importance of the virulence factors in the insect vector during the parasite life cycle. In a similar way to the human infection, once the parasites are ingested by insects, they need to endure defense mechanisms and structures that are in place to inhibit their colonization and survival. The anatomy and physiology of the anterior gut, the midgut peritrophic membrane barrier, digestive enzymes activity and excretion alongside the digested blood meal are a few examples of these mechanisms (Franco et al., 2012).

The parasite's protection in this situation is often performed by the same group of virulence factors used in the mammalian host with just a few exceptions, in which a certain protein is expressed specifically by the insect vector form of the parasite (Schlein et al., 1990; Aksoy, 2019; Herreros-Cabello et al., 2020). The presence of these molecules with function in both, vertebrate and invertebrate hosts, may be a result of trypanosomatid evolution, since it has been proposed that they may have been first originated as an insect-borne parasite, which later became digenetic (Hamilton et al., 2004).

This review aims to display the role of these virulence factors in the insect vector, showing how the same group of molecules have different modes of action in insect, when compared to their roles during the human infection, and discuss possible genetic events that may have generated host-specific protein subsets.

VIRULENCE FACTORS IN INSECTS

T. brucei

During the invertebrate host life cycle, once ingested by the insect, *T. brucei* differentiates from bloodstream to procyclic forms. The onset of the differentiation process may be associated with the parasite exposure to the insect's proteases and the higher pH in the tsetse fly midgut (Ponte-Sucre, 2016). At this point of the life cycle, the parasite's membrane is still covered by the variant surface glycoprotein (VSG), anchored by glycosylphosphatidylinositol (GPI), that is the most abundant surface protein on bloodstream forms, responsible for evading the host's adaptative immune response (Horn, 2014; Pays et al., 2014). Following parasite ingestion by the insect, a change in the trypanosome surface coat occurs and the VSGs are quickly removed by proteolysis and GPI hydrolysis, catalyzed by major surface proteases (MSP) and phospholipase C (PLC), respectively (Moreno et al., 2019) and released into the midgut of the tsetse fly (Ponte-Sucre, 2016). Once in the midgut, cells responsible for producing the peritrophic matrix (PM), a semipermeable, chitinous barrier that lines the midgut, incorporate the released VSGs. The incorporation leads to a decrease in expression of the tsetse fly microRNA (mir-275), interferes with the Wnt-signaling pathway and the activity of Iroquois/IRX transcription factor family (Aksoy et al., 2016). These events impair the role of the PM that functions as a barrier to infection by pathogens, and promote parasite proliferation in the gut during the early stages of the infection process (Aksoy et al., 2016; Ponte-Sucre, 2016; Aksoy, 2019).

At the same time, while in the insect midgut, the VSGs of trypanosome surface coat are replaced by procyclins, a stage-specific group of proteins which is the main acceptor of sialic acid in *T. brucei* (Engstler et al., 1993; Pontes de Carvalho et al., 1993; Montagna et al., 2002; Jackson, 2015). They comprise a small multigene family, anchored by GPI that is differentially expressed during infection (Urwyler et al., 2005). Procyclic trypomastigotes use a GPI-anchored trans-sialidase to transfer sialic acids from the host glycoconjugates to the procyclins and glycosylphosphatidylinositols (GPIs) expressed on their surface (Engstler et al., 1993; Pontes de Carvalho et al., 1993; Montagna et al., 2002; Nagamune et al., 2004). Surface sialic acids protect the trypanosomes from the hostile environment of the midgut of tsetse flies (Nagamune et al., 2004). Procyclins may also be involved in the protection of the parasite surface against the activities from digestive enzymes mediated by their C-terminus proteases resistance domain (Acosta-Serrano et al., 2001b), hydrolases in the tsetse fly midgut and could be essential for the differentiation of procyclic trypomastigote to the metacyclic form (McConville et al., 2002).

T. cruzi

Relative to *T. cruzi*, by the time these parasites enter the gut of the insect vector through the blood meal, they are challenged by the invertebrate host defense mechanisms and components aimed to digest the ingested blood. These components comprise hemolytic factors, proteases, oxygen and nitrogen radicals and components of the vector humoral immune system (Garcia et al., 2007). To

assist the parasite survival during the life cycle in the insect, *T. cruzi* Type-1 glycoinositolphospholipids (GIPLs) molecules (Colli and Alves, 1999), similar to the GIPLs) present in *Leishmania* and widely present on the surface of *T. cruzi* epimastigotes, are responsible for parasite attachment on the insect's gut (Garcia et al., 2007; Nogueira et al., 2007).

Mucins are also a group of proteins with great importance during the establishment of infection. In vertebrates, they are responsible for cell adhesion, immunomodulation of host defense and complement system evasion (Almeida et al., 1999; Colli and Alves, 1999; Acosta-Serrano et al., 2001a). In the insect vector, they are, perhaps, one of the best characterized group of proteins (TcSMUG) involved in the protection of *T. cruzi* expressed during this stage of the parasite life cycle. TcSMUG are present in the epimastigote form and are categorized in two groups: L (large) and S (small), based on their mRNA size and structure (Di Noia et al., 2000). The TcSMUG S found in the epimastigote and metacyclic trypomastigote stages are major acceptors of sialic acid transferred by trans-sialidases (Schenkman et al., 1993; Pech-Canul et al., 2017) and were identified as the backbone for the GP35/50 mucins (Herreros-Cabello et al., 2020). They were shown to be resistant to proteases, which may protect the parasite in the insect vector intestinal tract (Mortara et al., 1992). On the other hand, the TcSMUG L, exclusive to epimastigotes, are glycoconjugates that do not accept sialic acids (Urban et al., 2011), and might be implicated in adhesion to the vector midgut surface (Gonzalez et al., 2013).

In *T. cruzi*, the glycan structures on these mucins may vary depending on the host. They have also shown to possess stage-specific variations, observed throughout the parasite life cycle, with strain specific-modifications present in the oligosaccharides branching from the peptide backbone (Previato et al., 2004; Mendonça-Previato et al., 2005; Giorgi and de Lederkremer, 2020). The role of these glycan structures has also been studied in GIPLs and Gp35/50 kDa mucins relative to parasite attachment to the insect digestive tract *via* assays using native molecules purified from the epimastigotes, recombinant Gp35/50 kDa mucin-like proteins, transgenic epimastigotes over-expressing Gp35/50 kDa mucins and chemically synthesized oligosaccharides (Mendoza et al., 2006; Nogueira et al., 2007; Cámara et al., 2019; Giorgi and de Lederkremer, 2020).

Adhesion of epimatigotes to the insect midgut was inhibited by GIPLs purified from epimastigotes of Y strain (*T. cruzi* lineage TcII), while mucins had no such inhibitory effect on the parasite binding to the midgut (Nogueira et al., 2007). On the other hand, several lines of evidence indicated that Gp35/50 kDa mucins play a role in the parasite attachment towards triatomine hindgut rather than midgut tissues (Cámara et al., 2019; Giorgi and de Lederkremer, 2020). Namely, a) native and recombinant Gp35/50 kDa mucins bind to the *Triatoma infestans* rectal ampoules; b) ex *in vivo* attachment assays showed that the binding of transgenic epimastigotes over-expressing Gp35/50 kDa mucins to the insect hindgut was 2-3 fold higher than those of wild type epimastigotes; c) synthetic oligosacharides based on Gp35/50 kDa mucins specifically inhibited the epimastigote attachment to *T. infestans* rectal ampoule.

The 63 kDa glycoprotein (gp63), another widely studied *Leishmania* virulence factor, encoded by a multigene family highly expanded in *T. cruzi* genome (El-Sayed, 2005), may contribute to the parasite's virulence in the insect stage *via* its metalloprotease activity (Cuevas et al., 2003). A study has also shown the participation of gp63 in the parasite's adhesion to *Rhodnius prolixus* midgut *via* assays using divalent metal chelators or anti-Tcgp63-I antibodies caused an impaired *T. cruzi* adhesion to the vector midgut (Rebello et al., 2019).

As for others specific *T. cruzi* virulence factors, cruzipains, which are cysteine proteases responsible for cell invasion and immune evasion during the vertebrate host infection (McKerrow et al., 2006), were also reported to contribute to parasite adhesion to the insect's midgut (Uehara et al., 2012). Trans-sialidases (TS) comprise a multigene family that plays an important role by transferring sialic acid to mucin-like proteins on the cell surface and facilitate invasion in the vertebrate host (Schenkman et al., 1994). TS family also has a subset of genes expressed exclusively on epimastigotes (Schenkman et al., 1994; Briones et al., 1995; Retana Moreira et al., 2021) and they seem to possess roles of protection from glycolytic enzymes and adhesion to the insect's gut (Schenkman et al., 1994).

Leishmania spp.

Upon insect ingestion, *Leishmania* spp., tend to use the same classes of molecules that also help the parasite to evade the human's immune system, with their roles summarized on **Table 1**. Generally during the vertebrate host life cycle, glycoconjugates like lipophosphoglycans (LPG), proteophosphoglycans (PPG) and gp63 perform roles of attachment to the host's cells, modulation, and evasion of the immune system components (de Castro Neto et al., 2021). To survive in the insect, following blood meal ingestion, these parasites, still in the intracellular amastigote stage, start their differentiation to promastigotes, exit the macrophages and are exposed to midgut hydrolytic enzymes. However, a dense glycocalyx barrier formed by LPG and PPG provides protection against the action of these enzymes and also inhibit the release of midgut proteases (Schlein et al., 1990). LPG also plays a significant role by mediating promastigotes adhesion to midgut epithelial cells, which prevents the parasite elimination with the whole blood meal and ensures the completion of its life cycle (Sacks et al., 2000; Ilg, 2001).

Besides the PPGs actions in interacting and modulating the mammalian immune system (Piani et al., 1999), it seems that this group of proteins main role is the maintenance of the digenetic life cycle, especially during the transmission process from the vector to the vertebrate host (Rogers et al., 2004). To that end, *Leishmania ssp*. produces a gel-like obstruction in the sand fly anterior midgut, called Promastigote Secretory Gel (PSG), composed mainly by secretion of filamentous proteophosphoglycan (FPPG) and other related molecules. This obstruction may stimulate to the regurgitation of the parasites by the sand flies during the blood feeding (Franco et al., 2012; Giraud et al., 2019). The PSG was also reported to be released with the *Leishmania* during transmission and showed to positively influence the development of the disease (Rogers et al., 2004). Furthermore, PSG extracted from the insect

vector, produced by *L. major* and *L. tropica*, was able to intensify infections in mice (Giraud et al., 2019).

Gp63, considered one of the main surface proteins in *Leishmania*, has been widely studied specially for its immune evasion roles during the vertebrate host infection, briefly summarized on **Table 1**. Furthermore, this group of proteins also play significant roles in the insect vector, mainly associated with parasite adhesion to the insect's intestinal epithelium and degradation of its protein components (D'Avila-Levy et al., 2006a; Pereira et al., 2009). This degradation may be involved with the parasite's actions against the insect's defense molecules and may have significant roles in the parasite's nutrition pathway, due to its function as an endopeptidase, broad substrates specificity and optimum pH (D'Avila-Levy et al., 2014). Despite it has been hard to obtain direct evidence of the protein's nutritional role in insects, the adhesion events have been widely demonstrated both in monoxenous trypanosomatids, e.g., *Crithidia guilhermei* (D'Avila-Levy et al., 2006a), *Leptomonas* spp. (Pereira et al., 2009), *Angomonas deanei* (D'Avila-Levy et al., 2008) and *Herpetomonas muscarum* (Nogueira de Melo et al., 2006); and

TABLE 1 | Groups of virulence factors from *T. brucei*, *T. cruzi* and *Leishmania spp*., with different functions on vertebrate and invertebrate hosts.

Virulence factor	Host	Function	Reference
T. brucei			
Variant surface glycoprotein (VSG)	Vertebrate	Adaptive immune response evasion	(Horn, 2014; Pays et al., 2014)
	Insect	Reduce the peritrophic matrix barrier function and help the parasite establish infection of the gut	(Aksoy et al., 2016)
Procyclin	Vertebrate	Not present	
	Insect	Protection of the parasite surface against digestive enzymes and metacyclicogenesis	(Acosta-Serrano et al., 2001b; McConville et al., 2002)
T. cruzi			
Glycoinositolphospholipids (GIPLs)	Vertebrate	Downregulation of immunomodulatory components on macrophages and dendritic cells	(Brodskyn et al., 2002)
	Insect	Adhesion to the insect's gut	(Garcia et al., 2007; Nogueira et al., 2007)
Glycoprotein gp63	Vertebrate	No clear function detected on vertebrate host cell stages (trypomastigote and amastigote)	(Cuevas et al., 2003)
	Insect	Metalloprotease activity in the insect stage	(Cuevas et al., 2003)
		Adhesion to the insect's gut	(Rebello et al., 2019)
Cruzipain	Vertebrate	Cell invasion and immune evasion	(McKerrow et al., 2006)
	Insect	Adhesion to the insect's gut	(Uehara et al., 2012)
Trans-sialidases (TS)	Vertebrate	Transfer of sialic acid residues	(Schenkman et al., 1994)
		Host cell adhesion and evasion	
	Insect	Protection from the gut glycolytic enzymes	(Schenkman et al., 1994)
		Adhesion to the insect's gut	
Mucins	Vertebrate	Cell adhesion	(Almeida et al., 1999; Acosta-Serrano et al., 2001a)
		Immunomodulation of host defense	
		Complement system evasion	
	Insect	TcSMUG S	(Mortara et al., 1992; Herreros-Cabello et al., 2020)
		Protection against proteases in the insect's intestines	
		TcSMUG L Adhesion to the insect's gut	(Gonzalez et al., 2013)
Leishmania spp.			
Lipophosphoglycans (LPG)	Vertebrate	Complement system evasion	(Forestier et al., 2015)
		Adhesion to macrophages	(Cunningham, 2002)
		Impairment of phagosome maturation and acidification	(Lodge et al., 2006; Borges et al., 2021)
	Insect	Protection from insect's digestive enzymes	(Schlein et al., 1990)
		Adhesion to the insect's gut	(Sacks et al., 2000; Ilg, 2001)
Proteophosphoglycans (PPG)	Vertebrate	Adhesion to macrophages	(Piani et al., 1999)
		Modulation of macrophages at the early stage of infection	
	Insect	Protection from insect's digestive enzymes	(Schlein et al., 1990)
		Involved with the production of the Promastigote Secretory Gel (PSG), that may be responsible for the regurgitation of the parasites by the sand flies during the blood feeding	(Franco et al., 2012; Giraud et al., 2019)
Glycoprotein gp63	Vertebrate	Complement system evasion	(Isnard et al., 2012; Shao et al., 2019)
		Macrophage binding and entry	(Olivier et al., 2012)
		Downregulation of cellular compounds aimed to parasite elimination	(Podinovskaia and Descoteaux, 2015)
	Insect	Adhesion to the insect's gut	(D'Avila-Levy et al., 2006a; Pereira et al., 2009; Soares et al., 2017)
		Parasite protection against the insect's defense mechanisms	(D'Avila-Levy et al., 2014)

dixenous trypanosomatids, e.g., *Phytomonas serpens* (D'Avila-Levy et al., 2006b), *T. cruzi* (Rebello et al., 2019) and *Leishmania* spp. (Soares et al., 2017). In Leishmania, *in vitro* and *in vivo* studies in different species showed parasites that had this protein blocked by anti-gp63 antibodies, chemical chelators (Soares et al., 2017) or when down-regulated (Jecna et al., 2013) substantially inhibited attachment in the midgut of insects from the *Lutzomyia* genus. A similar down-regulation study also showed that gp63 seemed to be important for *L. amazonensis* early stage development in *Lutzomyia longipalpis* (Hajmová et al., 2004), while a complete gene deletion in *L. major* showed the opposite result in the sand fly vector (Joshi et al., 2002). This may show that gp63 may present divergent roles in different *Leishmania* species relative to interactions with their respective insect vector. These events may also be a consequence of gp63 divergent gene copy numbers and sequence variability across the *Leishmania* species, which will be further discussed in this review, that may allow the parasite to infect a variety of insect hosts. Another hypothesis that should also be considered is the redundant roles of the other cited virulence factors in insects. In case of total absence or decreased amount of gp63, it would be compensated by other molecules like LPG and PPG that perform similar adhesion and protection roles, as displayed in **Table 1**.

Notably, most of the virulence factors molecules groups involved in the human immune system evasion are also responsible to assure parasite's survival in their insect vector (**Figure 1**), despite being whole different hosts and environments. A few of the genes coding for some of these molecules, e.g., gp63, PPG and mucin may be traced back to precursor genes present in the non-parasitic near relative of trypanosomatids, *Bodo saltans*. Based on that fact, it is reasonable to speculate that the probable origin of parasitism and what would become the first systems of cell defense were originated by random genetic events of gene losses and gains in the trypanosomatid ancestor (Jackson et al., 2016). They allowed (1) the pre-existing molecules to change and adapt that protozoa to infect other hosts and (2) the creation/expansion of unique multigene families present in each parasite (Jackson et al., 2016). These events initially would allow the survival of these organisms inside insect's intestines, followed by future adhesion roles that would permit them to attach, multiply and be transmitted to other hosts, establishing a monoxenous life cycle. Evidence of this gradual evolutionary step can be observed by the presence of the gp63 orthologs in monoxenous trypanosomatids and even in the free living kinetoplastid *B. saltans*, as previously mentioned in this review. Another example of that, could be the presence of precursor coding sequences in genomes of even more early monoxenous trypanosomatids, e.g., *Paratrypanosoma confusum* and *Leptomonas pyrrhocoris*. *P. confusum* was discovered infecting mosquitoes and is considered of great importance to the study of parasitism origins, due to its phylogenetic classification as an intermediate kinetoplastid, that lies between the free-living bodonids and parasitic trypanosomatids (Flegontov et al., 2013). Whereas *L. pyrrhocoris* is phylogenetically closer to *Leishmania* genus and insights from its genome may elucidate how *Leishmania* acquired its dixenous life cycle (Flegontov et al., 2016). Their annotated genomes [*Paratrypanosoma confusum* (Skalický et al., 2017) and *Leptomonas pyrrhocoris* (Flegontov et al., 2016)] available in the TriTryp database show coding sequences for LPG, gp63, PPG, cruzipain, TS and mucin, which are important virulence factors for both humans and insects, as mentioned previously in this review. Since these organisms only infect one host, the evolutionary event that led to the dixenous life cycle happened

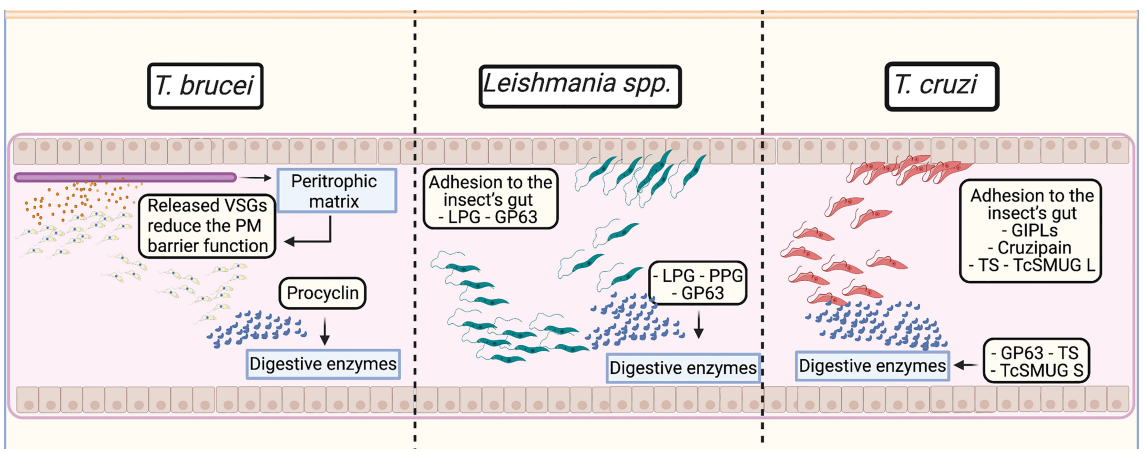

FIGURE 1 | Virulence factors roles in their insect vectors intestine tract. ***T. brucei*:** Variant surface glycoprotein (VSGs) released from the membrane are incorporated by the peritrophic matrix, reducing its protective function by interfering with the host cell internal pathways. Following VSG release, the surface coat is replaced by procyclins that may protect the parasite against digestive enzymes and hydrolases in the tsetse fly midgut. ***Leishmania* spp.:** Inside the invertebrate vector intestines, lipophosphoglycans (LPGs) and gp63 ensure the parasite adhesion to the midgut epithelial cells and alongside proteophosphoglycans (PPGs), protect the parasite against the action of host digestive enzymes. ***T. cruzi*:** The parasite adhesion is performed by glycoinositolphospholipids (GIPLs), cruzipain, trans-sialidases (TS) and the mucin group TcSMUG L. The protection against the digestive enzymes may be performed by gp63 *via* its metalloprotease activity; TS may be involved in protection from glycolytic enzymes; and the mucin group TcSMUG S may perform roles of protease resistance.

in separate occasions for the *Trypanosoma* and *Leishmania* genus (Jackson, 2015).

GENETIC MECHANISMS CONTRIBUTION TO GENE VARIABILITY AND VIRULENCE IN TRYPANOSOMATIDS

Most of the proteins involved in the virulence in invertebrate and vertebrate hosts of *T. brucei, T. cruzi* and *Leishmania* spp., are part of multigene families generated by genome remodeling in these parasites. Among other roles during the infection, these genes are the basis for the specific cell surface content displayed by each parasite genus and may be associated with the disease in humans and survival inside insects (Jackson, 2015). This diversity is possible due to their genomic plasticity and evolutionary need to move through different hosts and be exposed to different environments during the life cycle. The evolutionary events that originated these genes could vary from a mutation by a single amino acid substitution to a whole chromosomal duplication (with their coding and non-coding regions), however, the gene duplication events are considered to be the main mechanism responsible for producing new material for evolutionary innovation (Andersson et al., 2015; Jackson, 2015).

These duplications can be achieved by different genomic processes. For instance, subtelomeric regions of *T. cruzi* are enriched with members of multigene families encoding (glyco) proteins (TS, RHS, MASP, mucins, DGF-1 and GP63) that are subjected to intense genomic rearrangements, typical for that chromosomal area (Kim et al., 2005; Moraes Barros et al., 2012). Moreover, other non-syntenic regions (disruptive compartments) in *T. cruzi* genome, composed of TS, MASP and mucins multigene families, have also a role in the homologous recombination, serving as an homology site (Berná et al., 2018). These regions are susceptible to double-strand breaks of DNA, by retrotransposon nucleases, that tend to be repaired by homologous recombination. However, if during the repair, non-homologous chromatids are used, new gene variations may be created (Herreros-Cabello et al., 2020). If these variants are proved to be functional, it can promote new host-parasite interactions that would lead to parasite evasion in their hosts (Herreros-Cabello et al., 2020). It is suggested that part of gene expansion of virulence factors comprised of multigene families located in the subtelomeric regions, like VSG in *T. brucei* (El-Sayed et al., 2000), trans-sialidases (Freitas et al., 2011), mucins and MASPs (Mucin-Associated Surface Proteins) in *T. cruzi*, are due to their chromosome location (Herreros-Cabello et al., 2020).

Another process that can also increase gene diversity is the gene conversion mechanism that promotes the duplication of genes in tandem arrays from one common gene, but with sequence divergence (Jackson, 2007). Regardless of the gene duplication mechanism that generates the paralogs, these events are responsible to produce the materials for the evolutionary improvement that allows organisms to adapt and increase in complexity (Andersson et al., 2015; Jackson, 2015). Following the gene duplication event, the new copy may (1) present a novel function, (2) lose the function due to a mutation and be silenced as a pseudogene or (3) keep the function of the original gene. However, the latter case may be accompanied by accumulative mutations that would reduce their total capacity to the level of the single-copy ancestral gene (Lynch and Conery, 2000).

The presence of this genomic duplication is clearly seen in the abundant tandem arrays in trypanosomatids, especially in proteins involved with virulence, such as VSG in *T. brucei* (Jackson, 2007), gp63 in *Leishmania* spp. (Castro Neto et al., 2019) and mucins (Buscaglia et al., 2006) and TS in *T. cruzi* (Kim et al., 2005; Moraes Barros et al., 2012; Berná et al., 2018). These events are highly common in trypanosomatids, probably as a mechanism of increasing polycistronic transcription abundance for genes which are in need for a high expression rate (Jackson, 2007). These processes that lead to the genetic diversity of these parasites proteins may directly affect the outcome of host-parasite interactions, since they may produce different strains, which cause diverse responses in their hosts (Ponte-Sucre, 2016).

Genetic Events Associated With VSG Variation in *T. brucei*

For *T. brucei*, the high genetic variation of its main antigenic protein (VSG) is a result of homologous recombination or transcriptional switching among approximately 15 different VSG expression sites with a repertoire of approximately 1,000-2,000 different genes. They possess long transcription units with other expression site-associated genes (ESAGs) apart from VSG, which also plays a role in *T. brucei* antigenic variation (Reis-Cunha et al., 2017; Sima et al., 2019). To do that, silent VSG genes are copied and placed into expression sites, replacing the old gene (Rudenko, 1999). This event creates several categories of VSG genes, which are composed in its majority by pseudogenes, that are used by the parasite to produce chimeric genes by gene conversion and increase VSG variability (Roth et al., 1989). Despite the gene conversion method, silent VSG genes may also switch, through telomere exchange, from a silent to an active VSG expression site (Rudenko, 1999), by a DNA repair process (Horn, 2014; Sima et al., 2019).

Impact of Genetic Polymorphisms in Subsets of Proteins From *T. cruzi* Multigene Families Involved in the Parasite Virulence

The trypanosomatids (*T. brucei* and *Leishmania* spp.) have most of their virulence surface proteins as part of multigene families. In *T. cruzi* the main representatives of this group are the TS and the mucins. The TS and TS-like genes comprise a number around 1,500 genes depending on the strain, that can be separated by sequence similarity into 8 groups (Freitas et al., 2011). This provided the parasite an array of different functions which are present in many different stages of the life cycle (Herreros-Cabello et al., 2020).

Relative to the mucins, despite having groups of genes being differentially expressed in the insect and mammalian hosts, they tend to present wide sequence polymorphisms (Buscaglia et al., 2006). These variations are more common in mucins expressed

in the mammalian host than in the insect host, which may be attributed to their localization by the subtelomeric regions of the chromosome and immune system pressure during infections. Besides being used to divert recognition by the immune system, these variations also generated differences in the genome to the point to differentiate these proteins in distinctive function groups (De Pablos and Osuna, 2012). The genes expressed in mammals also are divided into two groups: TcMUC I (predominant in amastigotes) and TcMUC II (present in trypomastigotes). In their genomic structure they possess a central region with high variability sequence, which is long in TcMUC II and short TcMUC I. This region is known for having binding sites for glucose and sialic acid and may be responsible for the high glycosylation rates of these proteins, when compared to the ones expressed in the epimastigote stage (De Pablos and Osuna, 2012; Herreros-Cabello et al., 2020).

Another group of proteins that also have a hypervariable central region in their sequence are the MASPs. In terms of gene numbers, they are considered the second largest protein family in *T. cruzi*. Despite having a highly conserved N- and C- terminal regions, they are cleaved during the protein maturation, leaving only the hypervariable region exposed at the parasites' membrane (El-Sayed, 2005; Bartholomeu et al., 2009). This case of high variation can also be attributed to the subtelomeric regions of the genes, but also to probable cases of homologous recombination due to the highly conserved UTR sequences (Souza et al., 2007).

Surface Proteins Variability and Their Role in *Leishmania* spp. Virulence

Leishmania does not possess the same expression mechanisms as the VSGs of *T. brucei*. They rely in other genetic events, like gene sequence variation and expansion throughout the genome, to contribute to host immune evasion. The carbohydrate moieties of molecules like PPGs (McConville et al., 2002) and LPGs, for instance, present high variation among themselves, which increases their chances of interacting with a myriad of components in the insect and vertebrate hosts. These variations in LPGs are so remarkable that they can be stage and species specific, besides presenting different functionalities (Franco et al., 2012; Bifeld and Clos, 2015; Valente et al., 2019). Studies in *L. major* metacyclic form show that this stage is resistant to complement mediated lysis, whereas the promastigote form present in the insect is highly susceptible. This difference is conferred by changes in the molecule PG moiety that is approximately twice as long in the metacyclic form, when compared to the ones present on the promastigote. Despite this modification impairs the promastigote survival of the complement attack, it is what allows the parasite to detach from the insect gut prior transmission and migrate to their mouthparts (Franco et al., 2012; Bifeld and Clos, 2015). In addition, variations to the LPG side chains carbohydrates have also been implicated in species specific parasite-vector attachment. It has been shown that the vector *Phlebotomus papatasi* ability to only transmit *L. major* is linked to the parasite's specific LPG structure, which comprises multiple terminally exposed β-linked galactose residues, responsible for binding to the vector's gut. *In vitro* assays with parasites that do not possess this carbohydrate side chain patterns have failed to bind to *P. papatasi* mid guts (Sacks, 2001).

The same principle for LPGs can be applied for gp63 proteins, which are expressed by a variety of species-specific copy number and coding sequences, as shown in **Table 2**. Notably, there is a substantial increase in the number of coding sequences for these genes in *L. braziliensis*, which is a species belonging to subgenus *Viannia*, when compared to species of the *Leishmania* subgenus (*L. major, L. infantum and L. mexicana*), as observed in TritrypDB database and early genomic studies (Victoir et al., 1995; Victoir et al., 2005).

These genes are organized in tandem and are generally distributed in three chromosomes (10, 28 and 30/31), depending on the species. Most of the genes are located on chromosome 10, as shown in **Table 2**, varying in number from 4 to 33 and with fewer genes (1 or 2) on chromosomes 28 and 30/31. A phylogenetic study showed that the genes present on chromosomes 28, 30 and 31 are more similar to genes present on monoxenous trypanosomatids, like *Leptomonas* and *Chrithidia* (Castro Neto et al., 2019). This may imply that these genes are probably the ones with a functional role during the invertebrate host stage of the life cycle, but more studies are needed to confirm this hypothesis.

Overall, these genes seem to have expanded forming paralogs by recombination events mainly on chromosome 10 of *L. braziliensis*, which is proposed to be more involved with the mammal host infection (Castro Neto et al., 2019). These events, besides increasing the numbers, produce sequence variation that may also contribute to parasite's virulence. Despite maintaining a conserved core domain, studies performed in *L. braziliensis* showed that regions encoding surface peptides possibly involved in host–parasite interaction, presented high variability (Victoir et al., 2005; Castro Neto et al., 2019). However, due to current sequencing methods and the high similarity rate among these genes, more studies are needed to point out the actual number, gene variation and functional role of these different gp63 variants in their hosts. We speculate that this would be an adaptive strategy of the parasite to improve virulence during the

TABLE 2 | Gp63 gene repertoire among the main medical interest species of *Leishmania* distributed by subgenus and chromosome.

Species	Subgenus	Chromosome 10 genes	Chromosome 28 genes	Chromosome 30/31 genes	Total genes
L. major	*Leishmania*	4	1	1	6
L. infantum	*Leishmania*	13	2	1	16
L. mexicana	*Leishmania*	5	1	1	7
L. braziliensis	*Viannia*	33	–	6	39

mammal host infection and also allow them to infect a myriad of different insect vectors.

CONCLUDING REMARKS

The proteins directly or indirectly involved in host parasite mechanisms and immune system evasion today, in invertebrate and vertebrate hosts, may have been originated by genetic events that made this possible during millennia of evolution. We can speculate that these variations, mainly on surface proteins involved with host-parasite interaction traced back to early kinetoplastids, allowed the once free-living protozoans to start infecting insects producing new species, that later could infect vertebrate hosts. This was probably made possible due to the genomic events described on this review (gene conversion, reciprocal recombination events and mutations, associated with duplications that led to the creation of multigene families that proved to be essential during the parasite's evolution). These non-stopping genetic variations are constantly producing gene variants that may improve the host-parasite interaction in their hosts, facilitating parasite survival and proliferation.

There is no question on the importance of virulence factors studies for the host parasite relationship in vertebrate hosts. However, the roles of these molecules in the insect vector requires further studies, to better characterize them or find novel factors involved in this stage of the life cycle. Understanding the genomic events that led to their variations and allowed them to have groups of molecules with specific roles on different hosts may contribute to the analyses of these proteins' functions and features, which may open new possibilities to the development of new translational studies relative to the control of insect vectors in endemic areas. In addition, these comprehension of genetic mechanisms or events that contributed to the virulence of these parasites can give an important contribution to the improvement in the search of novel targets in drug, vaccine, and diagnostic research. It is important to not label these groups as a whole and be aware of their sequence variation and subsets of molecules with actual and specific roles in their respective stage in the parasite's life cycle.

AUTHOR CONTRIBUTIONS

ACN wrote the manuscript and prepared the table and figure. JS and RM reviewed and edited the manuscript, table, and figure. All authors contributed to the article and approved the submitted version.

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Nucleospora hippocampi n. sp., an Intranuclear Microsporidian Infecting the Seahorse *Hippocampus erectus* From China

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The life cycle, ultrastructure, and molecular phylogeny of a new intranuclear microsporidian, *Nucleospora hippocampi* n. sp., infecting the intestine of the *Hippocampus erectus*, were described. The histopathology revealed an extensive infection, mainly in the columnar epithelium of the intestinal mucosa layer. The enterocytes were the important target cell for *Nucleospora hippocampi* n. sp. infection. Transmission electron microscopy results showed that this microsporidian developed directly within the host cell nucleoplasm. In the intranuclear life cycle, the transformation from meront to sporogonial plasmodium was recognized by forming electron-dense disc structures, which were considered the polar tube precursors. The microsporidian showed the typical morphological characteristics of the family Enterocytozoonidae in the formation and development of spore organelles prior to the division of the sporogonial plasmodium. According to wet smear observation, eight spores were generally formed in a single host nucleus. Mature spores were elongated ovoids that were slightly bent and measured 1.93 × 0.97 μm. The isofilar polar tube was arranged in 7~8 coils in one row. Phylogenetic analysis of its small subunit ribosomal DNA sequences demonstrated that the parasite belonged to the *Nucleospora* group clade. The histological, ultrastructural, and molecular data support the emergence of a new species in the genus *Nucleospora*. This is the first report of *Nucleospora* species in Asia and threatened syngnathid fishes.

Keywords: seahorse, *Nucleospora*, transmission electron microscopy, intranuclear parasitism, intestinal disease

INTRODUCTION

Seahorses are highly modified pipefish and are commonly traded for traditional medicine, ornamental display, and aquarium fish (Koning and Hoeksema, 2021). As a threatened fish species, seahorses are being commercially cultured to solve overexploitation and the increasing demand in global trade. The high-density pressure in the culture makes them more vulnerable to many infectious diseases, including vibriosis, mycobacteriosis, fusariumsis, parasitosis, and

microsporidiosis (Koldewey and Martin-Smith, 2010; LePage et al., 2015). Few microsporidians have been reported in the seahorse. Only *Glugea heraldi* has been formally described by Blasiola Jr. (1979) as infecting the wild-caught *Hippocampus erectus* (Teleostei: Syngnathidae).

Microsporidia are obligate intracellular eukaryotic parasites with a close fungal relationship in evolutionary origin (Corsaro et al., 2016; Bass et al., 2018). Most microsporidian species live in the cytoplasm of their host cells directly or indirectly, but few microsporidians exhibit unique intranuclear infections, such as *Enterospora* spp. (Stentiford et al., 2007; Palenzuela et al., 2014; Yan et al., 2018), *Nucleospora* spp. (Chilmonczyk et al., 1991; Freeman and Kristmundsson, 2013), and *Desmozoon lepeophtherii* (synonym *Paranucleospora theridion*) (Freeman and Sommerville, 2009; Freeman and Sommerville, 2011). To date, no intranuclear microsporidians have been described as infecting syngnathid fishes.

In March 2019, a disease of the cultured lined seahorse *Hippocampus erectus* broke out in Hainan province, China, characterized by a severe intestinal lesion. By microscopic examination, numerous microsporidian spores were discovered in the intestinal mucus and white feces of diseased fishes. In this paper, we applied both ultrastructural observation and molecular analyses to identify the microsporidian found in the *Hippocampus erectus* intestine. The morphological, ultrastructural, and molecular data support the idea that it is a new intranuclear species of the genus *Nucleospora*.

MATERIALS AND METHODS

Sample Collection

Sick seahorses, *Hippocampus erectus*, were collected from recirculating tank systems in the Qionghai Research Center of the East China Sea Fisheries Research Institute, Hainan province, China (19°22′24″N, 110°40′10″E) in March and August of 2019. The temperature of the water ranged from 18°C to 28°C, and the salinity ranged from 28 to 30 ppt. A total of 153 samples (body length: 2~10 cm) were necropsied after anesthetization with MS222 for euthanasia. All fish experiments were conducted according to the national standard guidelines for ethical review of animal welfare (GB/T 35892-2018).

Histology

The gastrointestinal tracts were partially cut along the longitudinal axis. The inner gastrointestinal contents and some epithelial cells were scraped off, smeared on microscopic slides, and observed under a Leica DM4B light microscope. After light microscopic examination, the tissues containing microsporidian spores were preserved in different fixative solutions for processing for histology, electron microscopy, and molecular biology analyses, respectively.

Portions of the gills, body musculature, heart, kidney, liver, spleen, intestine, and gall bladders were fixed in Davidson's fixative for 24 h. The tissues were then processed using routine histological techniques. Tissue sections (5 μm) were stained using Masson's trichrome method (Chang et al., 2019). Images were captured using a Leica camera DMC6200 with the Leica Application Suite X (version 3.4.2) software.

Transmission Electron Microscopy

Small pieces of affected intestine tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h. The fixed tissues were rinsed three times in 0.1 M phosphate buffer and then postfixed in 1% osmium tetroxide (OsO_4) for 1 h at 4°C. After washing three times with the same buffer, specimens were dehydrated through a graded acetone series, embedded in epoxy resin 812, and then polymerized at 60°C for 16 h. Semithin sections (1 μm) were stained with Toluidine Blue to target infected areas. Ultrathin sections (60–80 nm) were mounted on uncoated copper grids and stained with uranyl acetate and lead citrate. Samples were examined using a Tecnai G2 Spirit Biotwin transmission electron microscope at 80 kV.

Polymerase Chain Reaction Amplification

The counterpart tissues were preserved in 100% ethanol at the time of dissection. After ethanol evaporation, total genomic DNA was extracted from three fish using the TIANamp Marine Animals DNA Kit (Tiangen, China), following the manufacturer's instructions. Isolated DNA was eluted in 50 μl of TE buffer and stored at −20°C for polymerase chain reactions (PCR).

The previously published primer set, 18F (5′-CACCAGGT TGATTCTGCC-3′) and 580R (5′-GGTCCGTGTTT CAAGACGG) (Vossbrinck et al., 1993; Vossbrinck et al., 2004), was used to amplify the microsporidian rDNA sequence, including the whole small subunit rRNA gene (SSU rRNA), the internal transcribed spacer (ITS), and the partial large subunit rRNA gene (LSU rRNA).

Amplifications were performed in a total volume of 50 μl containing 25 μl of PrimeSTAR® Max premix (Takara, China), 2 μl of 10 μM for each pair of primers, 19 μl of sterile water, and 2 μl of genomic DNA template. PCR conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 52°C for 35 s, and 72°C for 90 s, with a final extension at 72°C for 5 min.

Cloning and Sequencing Analysis

The amplified PCR products were detected by 1% agarose gel electrophoresis and purified using an Agarose Gel DNA Purification Kit (Takara, China). The purified fragments were cloned into the pMD19 T-vector, transformed into competent *Escherichia coli* DH5α cells, and plated on Luria–Bertani agar plates containing ampicillin (Amp-LB). After overnight culture, the positive clones verified by colony PCR were amplified in liquid Amp-LB medium. PCR fragments from bacterial clones containing the correct insert size were sequenced with primers M13F/M13R using an Applied Biosystems 3730xl DNA analyzer by Sangon Biotech (Shanghai) company. The internal primers

870F (5′-TGCGGCTTAATTTGACTCAAC-3′) and 870R (5′-GTTGAGTCAAATTAAGCCGCA-3′) were used for the complete overlap in DNA sequencing (Freeman et al., 2013). The obtained sequences were carefully examined and assembled using BioEdit software (version 7.2.5).

Phylogenetic Analysis

The sequences were evaluated using the BLASTn programs in NCBI to compare them with those available in the microsporidian SSU rDNA databases. To assess the phylogenetic position of this species with other microsporidians, 28 microsporidian SSU rDNA sequences were selected from the NCBI GenBank database based on the BLAST search results and literatures. Sequences were aligned using the ClustalW algorithm in MEGA software version 10.0 with default settings (Kumar et al., 2018). The model of "General Time Reversible Model + gamma-distributed (GTR+G)" was chosen based on estimated results of "Find Best DNA/Protein Models (ML)" to construct the Maximum likelihood (ML) tree in MEGA X. The robustness of the resulting tree was assessed using 1,000 bootstrap replicates. The Bayesian phylogenetic inference was performed using MrBayes version 3.2.7 (Ronquist et al., 2012). The GTR substitution model with gamma-distributed rate variation across sites was set for tree reconstruction. Bayesian searches were run for 1,000,000 generations and sampled every 1,000 generations. The convergence of Markov chain Monte Carlo (MCMC) analyses was diagnosed in the Tracer version 1.7.2.

RESULTS

Clinical Signs and Epidemiology

Infected lined seahorses exhibited anorexia, spiritlessness, occasional white feces, and chronic mortality. After necropsy, there were no obvious tissue damage and other infection symptoms. The gut of the severely infected fishes had a slight whitish appearance. No other organ observed was found infected. Microsporidian infections were found in both juvenile and adult stages of the lined seahorses.

Light Microscopy

Microscopic examination revealed that microsporidian spores were commonly present in the intestinal tract and feces of infected *Hippocampus erectus*. In a wet mount, the infected host enterocyte cell generally contained eight spores (**Figure 1A**). The spores had an ellipsoid shape (**Figure 1B**). Occasionally, empty spores were seen in the feces (**Figure 1C**). Fresh spores averaged (2.66 ± 0.21) × (1.44 ± 0.19) μm [n = 50; range (2.04–3.12) × (0.99–1.85) μm]. The prevalence of microsporidian infection among individuals was 22.2% (34 of 153).

Histopathology

The varying numbers of cells in the epithelium layer were infected compared with the normal intestinal tract tissue (**Figures 2A, B**). Affected cells include the enterocytes and goblet cells (**Figures 2B**). Based on the overwhelming infection observed, the former were the preferred target cells for the parasite. Parasite stages appeared as eosinophilic granular and red staining (**Figures 2B, C**). The microsporidians were seemingly surrounded by the membrane structures (**Figures 2B, C**), which were subsequently confirmed to be the host's nuclear envelope by transmission electron microscopy (TEM) (**Figure 3**). No infection was observed in other host organs.

Ultrastructure of Microsporidian Development

Different proliferative stages of the parasite directly develop in the nucleoplasm of enterocytes. The earliest stage observed was rounded uninucleate meronts, 1.3~2.0 μm in diameter, characterized by a large single nucleus and a simple plasmalemma (**Figure 4A**). The outer layer of the meront plasmalemma deposited

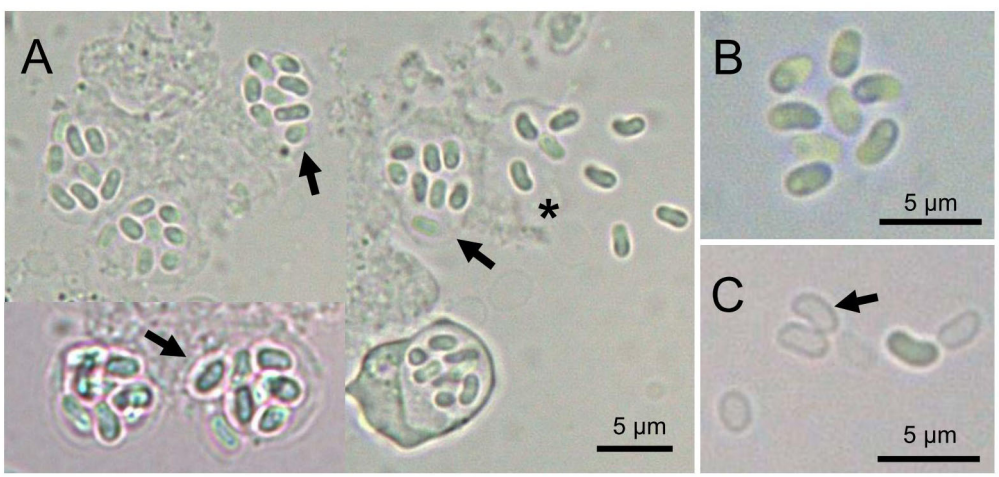

FIGURE 1 | Light microscopy of *Nucleospora hippocampi* n. sp. **(A)** The infected enterocytes contain eight spores (arrows). Eight spores are released from the ruptured enterocyte (asterisk). **(B)** Higher magnification of eight fresh spores. **(C)** The arrow shows the empty spore shell.

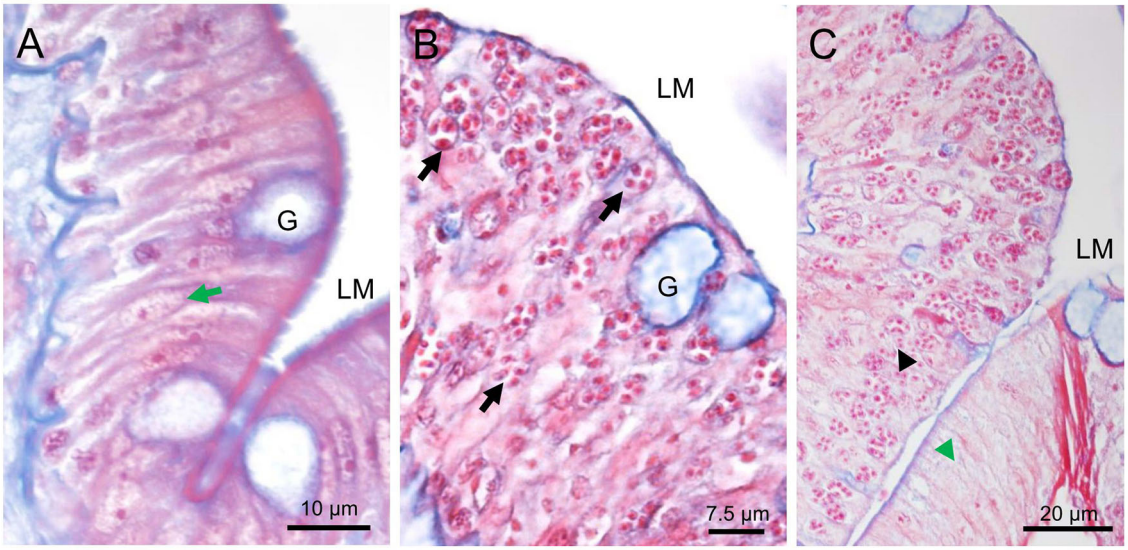

FIGURE 2 | Histopathology of *Nucleospora hippocampi* n. sp. infection in the intestinal epithelia of the *Hippocampus erectus*. Masson's stain. **(A)** Normal columnar epithelium of the intestine: intestinal lumen (LM), host nucleus (green arrow), and goblet cell (G). **(B)** The intestinal epithelium with microsporidian infection (black arrows). **(C)** The infected region (black arrowhead) and uninfected region (green arrowhead) of the intestinal epithelium layer.

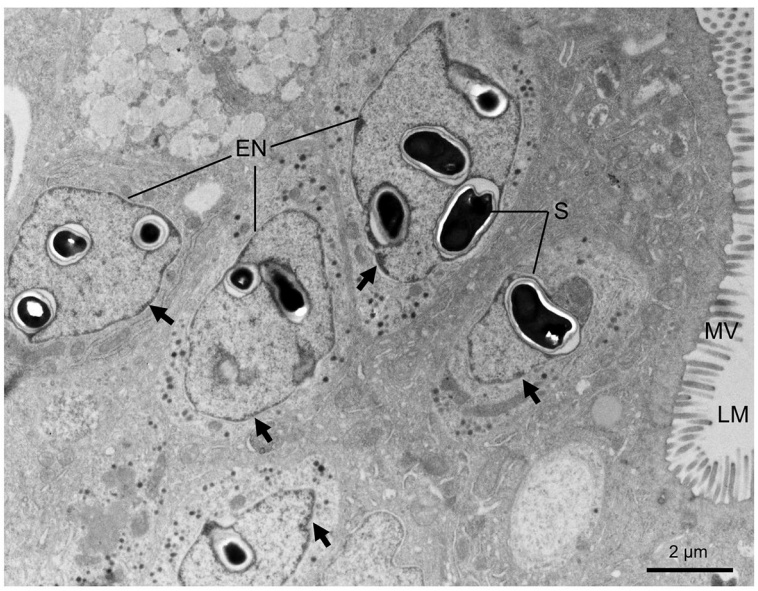

FIGURE 3 | Transmission electron micrographs showing the microsporidian *Nucleospora hippocampi* n. sp. infecting the intestine cells of the *Hippocampus erectus*. Intranuclear infections occur in five enterocytes. Affected nuclei with marginalized chromatin (black arrows). LM, intestinal lumen; MV, microvilli; S, spore; EN, enterocyte nucleus.

electron-dense materials (**Figures 4A, D**). The uninucleate meront underwent mitosis to form binucleate meront (**Figures 4B, C**). During mitosis, the spindle plaques and microtubules appeared; the meront nuclear envelope preserved; the claviform cytoplasm, which might be endoplasmic reticulum, was increased. The binucleate meront continued to divide without cytokinesis, giving rise to a multinucleate meront with numerous claviform cytoplasm (**Figure 4D**). The transformation from multinucleate meront to multinucleate sporogonial plasmodium was easily recognized by the formation of the electron-dense disc (EDD) structures

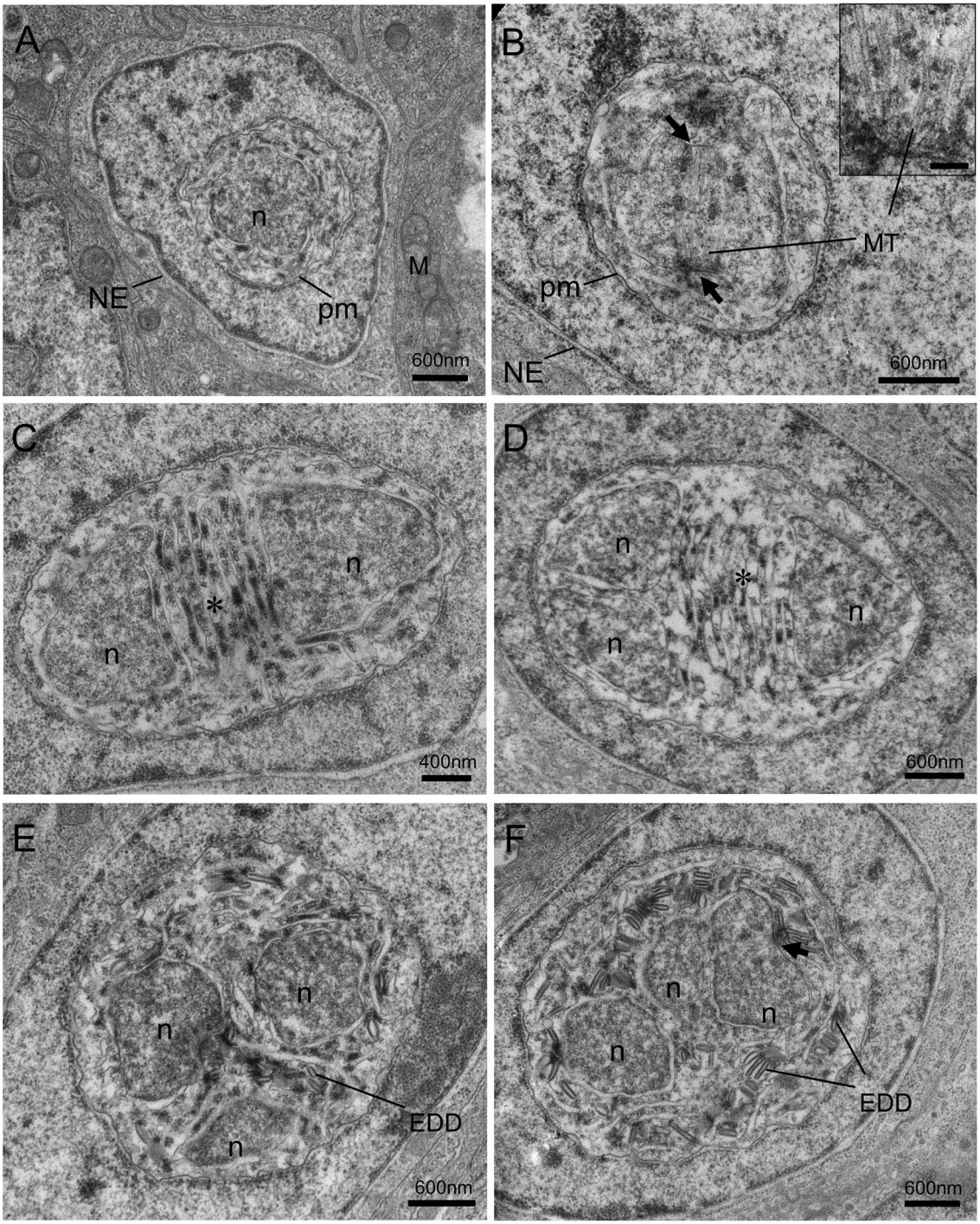

FIGURE 4 | Transmission electron micrographs of the merogonic and early sporogonic stages of *Nucleospora hippocampi* n. sp. in the nuclei of intestinal epithelial cells of the *Hippocampus erectus*. **(A)** Early uninucleate meront stage within the nucleoplasm of the host nucleus. Host nuclear envelope (NE), host mitochondria (M), the monokaryon (n), and plasma membrane (pm) of the meront. **(B)** The uninucleate meront undergoing karyokinesis. Long microtubules (MT) radiate from two spindle plaques (black arrows). Inset showing the details of the microtubules and spindle plaque. Insert scale bar = 0.1 μm. **(C)** The binucleate meront contains two nuclei (n) separated by claviform cytoplasm (asterisk). **(D)** The trinucleate meront with three nuclei and claviform cytoplasm (asterisk). **(E)** The electron-dense discs (EDD) appear in the cytoplasm of late trinucleate meront (or early sporogonial plasmodium). **(F)** An early sporogonial plasmodium with increasing EDD around three nuclei. The spindle plaque (black arrow) forming on one nucleus indicates nucleus division again.

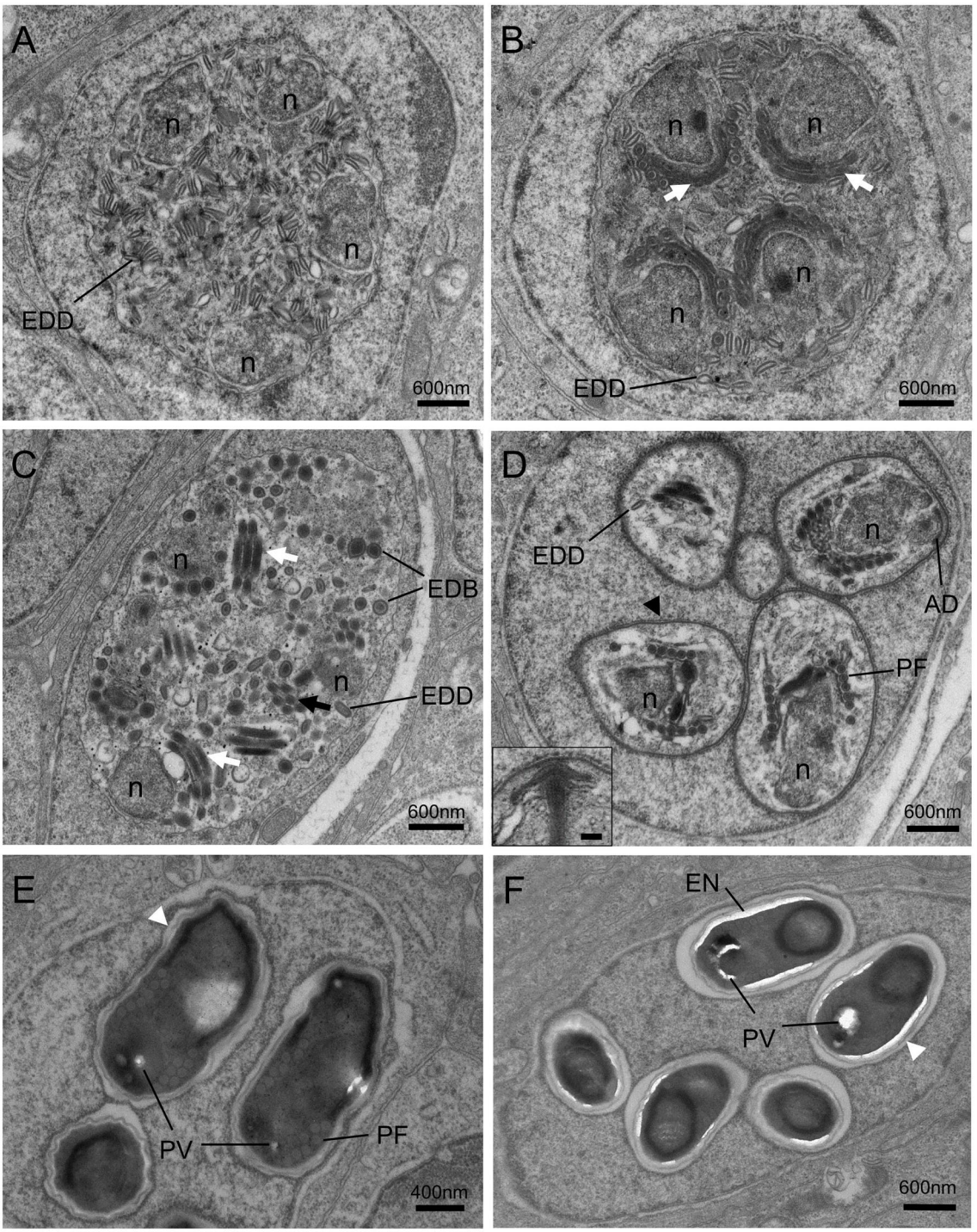

FIGURE 5 | Transmission electron micrographs of the sporogonic stages of *Nucleospora hippocampi* n. sp. in nuclei of intestinal epithelial cells of *Hippocampus erectus*. **(A)** The sporogonial plasmodium contains numerous electron-dense discs (EDD) and four nuclei (n) distributed close to the inner edge of the cytomembrane. **(B)** Multinucleate sporogonial plasmodium contains four sets of the primordial polar tube (white arrows). EDD is associated with primordial polar tube formation. **(C)** Late-stage sporogonial plasmodium with increased precursors of the polar tube (white arrows) and reduced EDD. The EDD transform into rounded electron-dense bodies (EDB), then the EDB coalesce into primordial turns of the polar tube (black arrow). **(D)** The sporoblasts (five are visible) develop in direct contact with the host nucleoplasm. The sporoblasts showing parasite nucleus (n), coiled polar tube with 6–7 turns (PF), and thickened plasmalemma (arrowhead). Insert showing the magnification of anchoring disk (AD). Insert scale bar = 0.1 μm. **(E)** Late immature spore with preformed winding spore wall (white arrowhead), posterior vacuole (PV), and polar tube (PF). **(F)** Mature spore with smooth spore wall (white arrowhead) and thickening electron lucent endospore (EN).

(**Figure 4E**). As development progressed, more electron-dense discs were synthesized in the early sporogonial plasmodium cytoplasm. The nuclei underwent further division (**Figure 4F**) and migrated to the inner edge of its cytomembrane (**Figures 5A, B**).

The electron-dense discs of multinucleated sporogonial plasmodium gradually decreased and turned into rounded electron-dense bodies (EDB) (**Figures 5A–C**), which were the raw materials for the manufacture of the primordial polar tube. At this stage, several units of polar tube precursors, each closing to the individual nuclei respectively, were assembled in the late sporogonial plasmodium (**Figures 5B, C**). The sporogonial plasmodium was separated into several uninucleate sporoblasts by cytokinesis. Individual sporoblasts could not divide further. During the development, the precursor of an anchoring disk formed in each sporoblast (**Figure 5D**), and the number of regularly arranged polar filament coils increased at this developing stage. Maturation of liberated sporoblasts into immature spores was characterized by the transformation of a moderately thickened membrane into a winding spore wall (**Figure 5E**). The electron-lucent endospore became visible and progressively thicker as the spore matured (**Figures 5E, F**). Considering that the host nucleus was spherical and the ultrathin section only showed a cut surface of the host nucleus, we hypothesized that eight sporoblasts were generated from the multinucleated sporogonial plasmodium (**Figure 5D**) and that these eight sporoblasts eventually developed into eight spores in one host nucleus.

Mature spores were ellipsoidal and slightly bent (**Figure 6A**). The spores were measured at 1.93 ± 0.17 (1.68–2.49; n = 50) μm in length and 0.97 ± 0.08 (0.74–1.24, n = 50) μm in width. The spore wall was composed of a thick electron-transparent endospore and an electron-dense exospore. A layer of electron-dense materials was deposited outside the spore wall (**Figure 6D**). The thickness of the spore wall was about 70 nm. Inside the spores, the anchoring disc was umbrella-shaped (**Figure 6E**); the polaroplast consisted of two distinct lamellar parts: a tightly packed lamellae part and a loose tubular internal part composed of 5 layers (**Figures 6B, F**); the single nucleus was located in the midregion of the spore (**Figure 6G**); the posterior vacuole was observed at the posterior end of spore (**Figures 6A, C, G**); and the isofilar polar tube usually formed 7-8 coils in a single layer close to the spore wall, with a diameter of approximately 80 nm (**Figure 6H**).

Molecular Analyses

The PCR fragments amplified using the primer pair 18F/580R were 1,922 bp in length, containing complete SSU rDNA (1,269 bp), a complete ITS region (269 bp), and partial LSU rDNA (384 bp). Three rDNA sequences of *Nucleospora hippocampi* n. sp. isolated from three infected *Hippocampus erectus* were deposited in GenBank with accession numbers MW229242 to MW229244, respectively. These rDNA sequences showed low intraspecific variations (>99.4% similarity).

The BLASTn search results revealed no known microsporidian sequences available in GenBank that matched exactly to the obtained sequences. Comparison of the nucleotide sequences showed that the *Nucleospora hippocampi* n. sp. SSU rDNA data (1,269 bp of Genbank MW229243) showed a 96% sequence identity (1,202/1,251 bp) to *Nucleospora salmonis* (Genbank U78176), a 95% sequence identity (1,200/1,263 bp) to *Nucleospora cyclopteri* (Genbank KC203457), and a 96% sequence identity (391/406 bp) to *Nucleospora braziliensis* (Genbank KT777455). The SSU rDNA sequences of other microsporidians, including *Obruspora papernae* (Genbank HG005137), *Paranucleospora theridion* (Genbank KR187185), *Enterocytozoon hepatopenaei* (Genbank KX981865), *Enterospora canceri* (Genbank HE584634), and *Enterocytozoon bieneusi* (Genbank L07123), showed 90%, 89%, 87%, 86%, and 83% identity with *Nucleospora hippocampi* n. sp., respectively.

The Maximum likelihood and Bayesian phylogenetic analyses produced similar tree topology (**Figure 7**), placing three *Nucleospora hippocampi* n. sp. isolates within a highly supported clade (bootstrap percentages (BP) = 99; posterior probabilities values (PP) = 1). Furthermore, the new microsporidian was strongly supported to cluster with *Nucleospora salmonis*, *Nucleospora cyclopteri*, and *Nucleospora braziliensis* in the large *Nucleospora* clade (BP = 98; PP = 1). *Nucleospora* spp. was classified in group II as a sister taxon to group I, which included *Desmozoon* (=*Paranucleospora*), *Obruspora*, and several undescribed genera. The family Enterocytozoonidae, including the genera: *Enterocytozoon*, *Enterospora*, *Nucleospora*, *Obruspora*, and *Desmozoon*, was closely related to the family Hepatosporidae (group IV) (Stentiford et al., 2011). Based on the new classification view of Microsporidia (Vossbrinck and Debrunner-Vossbrinck, 2005), this parasite belonged to clade IV, known as class Terresporidia.

Taxonomic Description

Taxonomic rankings

Super-group: Opisthokonta Cavalier-Smith (1987)

Super-phylum: Opisthosporidia Karpov et al. (2014)

Phylum: Microsporidia Balbiani (1882)

Class: Terresporidia Vossbrinck and Debrunner-Vossbrinck (2005)

Family: Enterocytozoonidae Cali and Owen (1990)

Genus: *Nucleospora* Hedrick, Groff and Baxa (1991)

Nucleospora hippocampi n. sp.

Diagnosis of the species: Monokaryotic spores are elongated ovoids that are slightly bent. Eight spores are frequently observed in one host nucleus without forming the interfacial membrane. Fresh spores measure approximately 2.66 × 1.44 μm. Fixed spores are 1.93 × 0.97 μm in size. The spore wall is about 70 nm of thickness. The single nucleus is positioned in the posterior half of the spore. The isofilar polar tube coils have 7–8 turns arranged in a single layer, about 80 nm in diameter. The posterior vacuole is situated at the posterior end of the spore and is surrounded by the polar filaments. Polaroplast with two regions: densely arranged lamellae and loose tubular lamellae. The intranuclear life cycle of *Nucleospora hippocampi* n. sp. is inferred in **Figure 8**.

Type-host: Lined seahorse *Hippocampus erectus* (Teleostei: Syngnathidae).

Type-locality: The Hainan province, China (19°22′24″N, 110° 40′10″E).

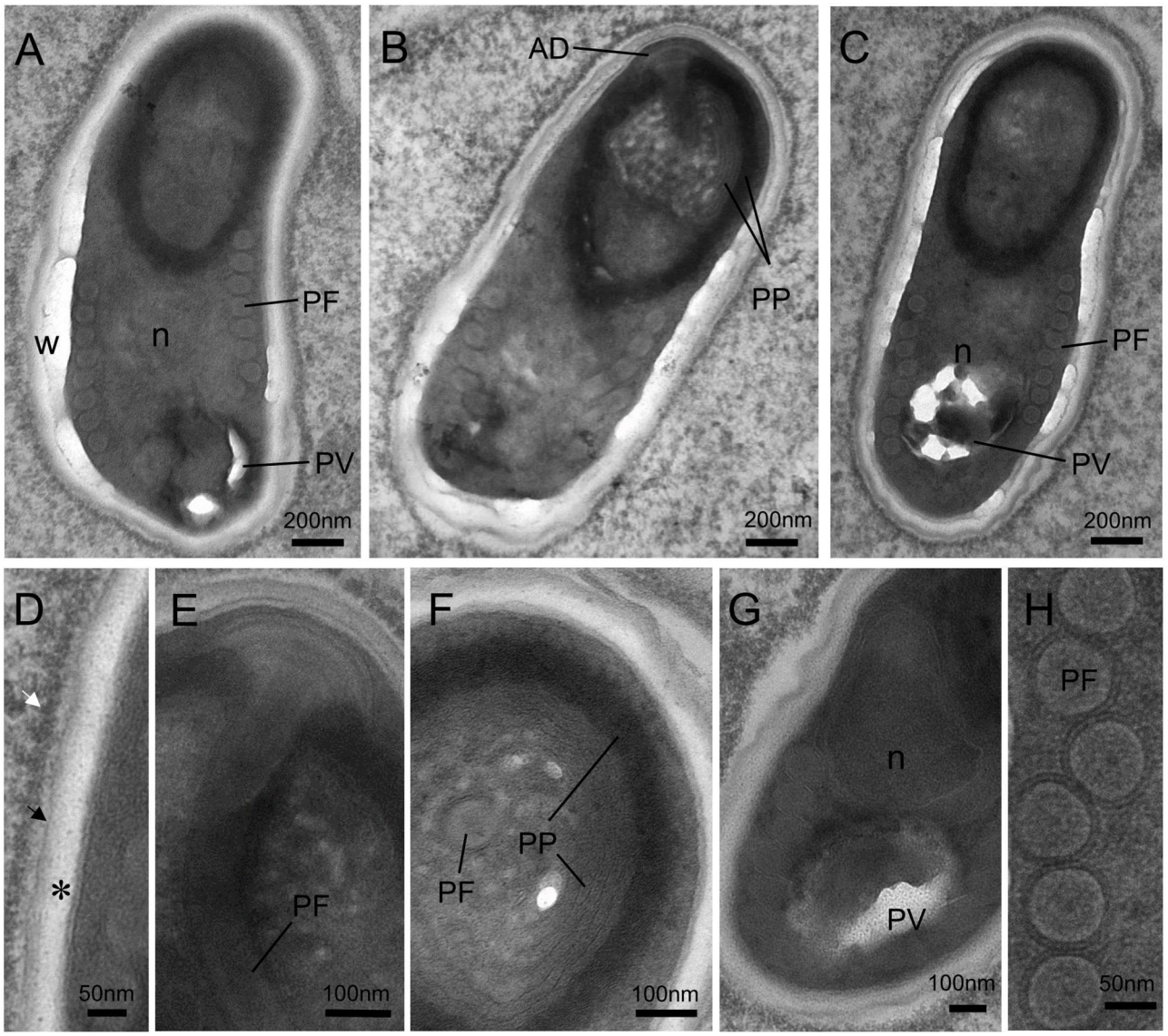

FIGURE 6 | Transmission electron micrographs showing the ultrastructure of mature spores of *Nucleospora hippocampi* n. sp. in the *Hippocampus erectus*. **(A–C)** Three spores showing the typical microsporidian structures and organelles. The spore wall (W), anchoring disk (AD), polaroplast (PP), posterior vacuole (PV), polar filament (PF), and the single nucleus (n) are indicated. **(D)** Ultrastructural detail of spore wall showing the exospore (black arrow), the endospore (asterisk), and the plasmalemma (arrowhead). Notice the electron-dense materials deposited on the outer surface of the exospore (white arrow). **(E)** Detail of terminal anchoring disk and associated polar tube (PF). **(F)** High magnification of a transverse section of a spore showing the lamellar region of the polaroplast (PP). **(G)** The single nucleus (n) is situated beside the posterior vacuole (PV). **(H)** Detail of the isofilar polar filament (PF).

Site of infection: Intestinal epithelial cells.

Prevalence: 34 of 153 (22.2%) lined seahorses examined by light microscopy.

Type-material: Paraffin sections and resin-embedded tissues are deposited in the East China Sea Fisheries Research Institute, China Academy of Fishery Sciences, Shanghai.

Type SSU rDNA sequence: GenBank accession No. MW229243.

Etymology: The specific epithet "hippocampi" derives from the generic name of the host.

DISCUSSION

Nucleospora hippocampi n. sp. Is a New Microsporidian

The ultrastructural morphology of this microsporidian development shows one of the key features of the family Enterocytozoonidae: the spore organelles are almost fully developed before the sporogony plasmodium divides into sporoblasts (Sprague et al., 1992). Moreover, molecular data

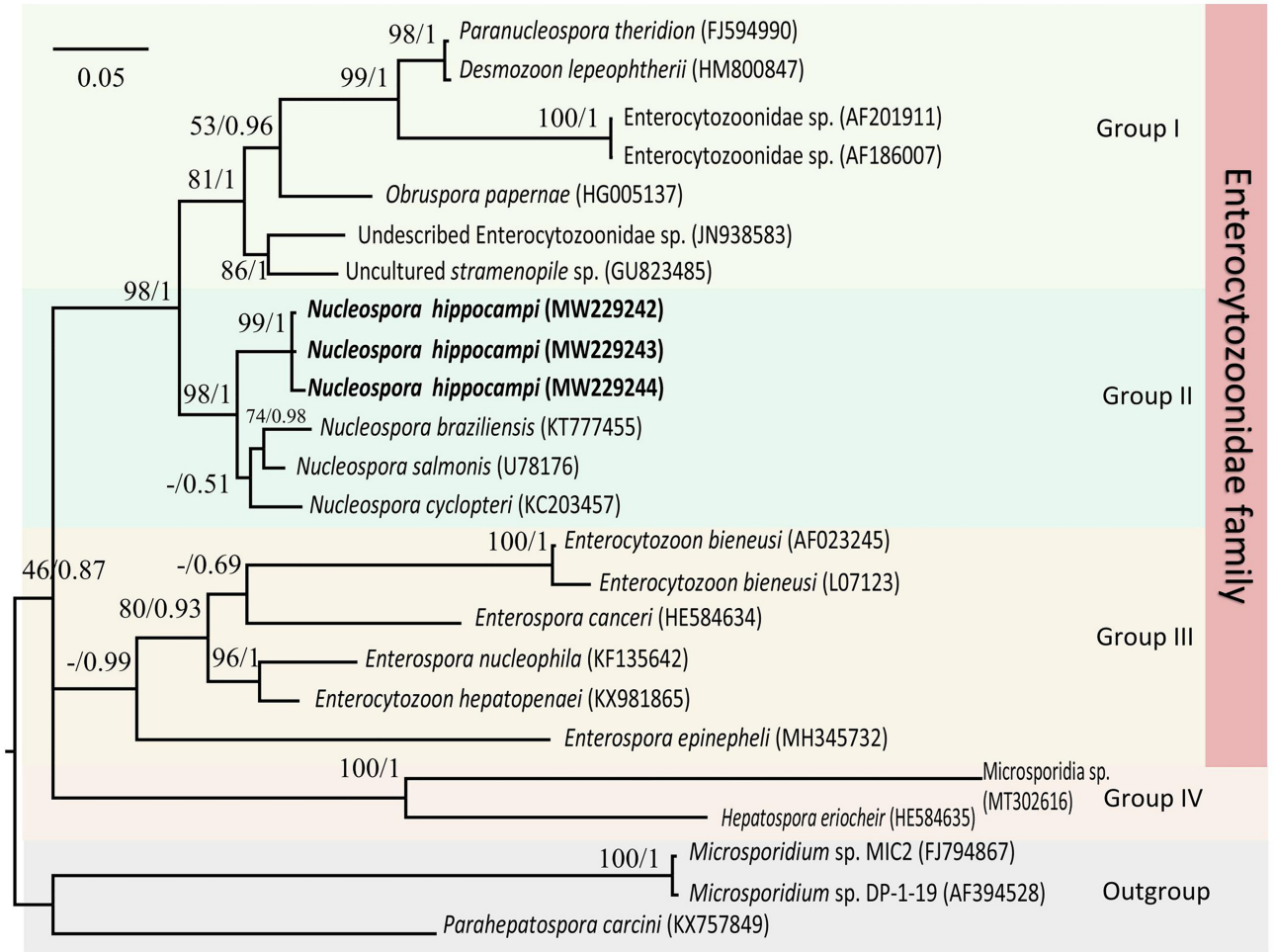

FIGURE 7 | The Maximum likelihood and Bayesian analyses based on SSU rDNA sequences showing the relationship of *Nucleospora hippocampi* n. sp. and other selected microsporidian species. *Parahepatospora carcini* (KX757849), *Microsporidium* sp. MIC2 (FJ794867), and *Microsporidium* sp. DP-1-19 (AF394528) are used as the out-group. Values at nodes represent Maximum-likelihood bootstrap support percentages (BP)/Bayesian posterior probabilities (PP). The scale bar represents the estimated number of substitutions per nucleotide site.

further reveals its phylogenetic position within the *Nucleospora* group on the Enterocytozoonidae family.

To date, four *Nucleospora* species have been described: *Nucleospora salmonis* (previously called *Enterocytozoon salmonis*), *Nucleospora secunda*, *Nucleospora cyclopteri*, and *Nucleospora braziliensis*. The biological features of all members are compared in **Table 1**. The presented *Nucleospora hippocampi* n. sp. is distinguished from other *Nucleospora* species by differences in shape and size, the number of turns of the polar tube, host cell type and tissue tropism, and the number of spores in a host cell section. It is worth mentioning that the SSU rDNA sequence divergences between *Nucleospora hippocampi* n. sp. and other *Nucleospora* species also support the morphological differences. Taken together, a new species of the *Nucleospora* genus is proposed for establishment. It is the first report of *Nucleospora* species in the seahorse.

Awareness of the Potential Threat of *Nucleospora hippocampi* n. sp. to the Seahorse

This parasite belongs to the emergent *Enterocytozoon* group Microsporidia (EGM) (Stentiford et al., 2019). EGM has been responsible for many emergent diseases in varied hosts over the past 50 years. For instance, *Enterocytozoon bieneusi* is the most prevalent human microsporidian, especially infecting the intestinal epithelial cells of severely immune-suppressed humans, and is associated with self-limiting diarrhea, malabsorption, and wasting (Matos et al., 2012). *Enterocytozoon hepatopenaei* has recently caused substantial economic losses in global shrimp aquaculture and is associated with slow growth and white-face syndrome (Chaijarasphong et al., 2020). *Enterospora nucleophila* infects the farmed gilthead sea bream (*Sparus aurata*), causing an emaciation syndrome and significant mortality (Palenzuela et al., 2014). In

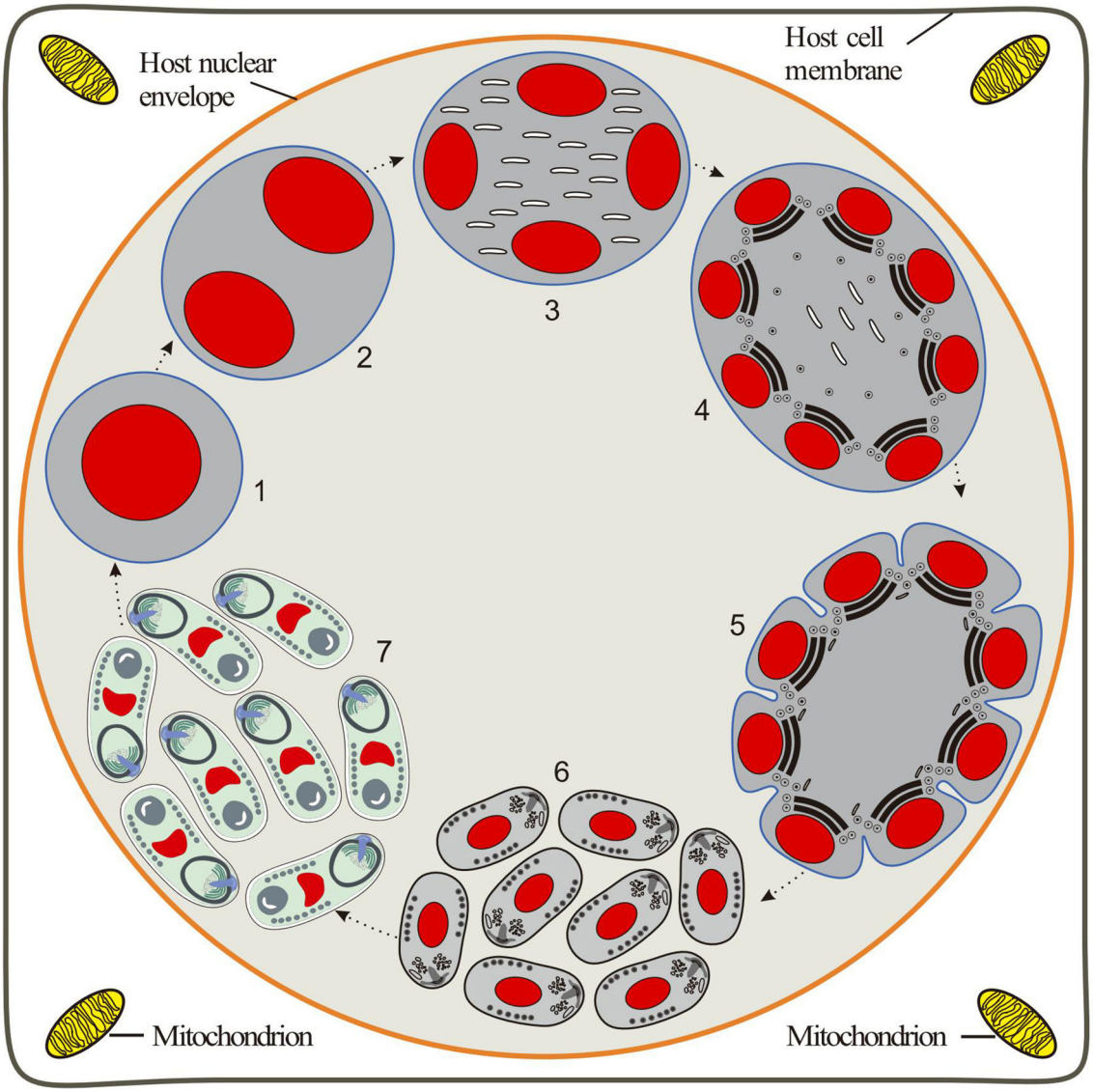

FIGURE 8 | Diagrammatic representation of the proposed intranuclear life cycle of *Nucleospora hippocampi* n. sp. (1) Uninucleate meront free in the host nucleus. (2) Binucleate meront formed by nucleus mitosis without cytokinesis. (3) The nuclear divides again to produce a tetra-nucleate meront with numerous claviform cytoplasm. (4) The proliferative sporogonial plasmodium shows electron-dense disks and eight units of polar tube precursors around the inner edge of its plasmalemma. (5) The syncytial sporogonial plasmodium produces eight uninucleate sporoblasts by rosette-like budding. The invagination of plasmalemma segregates each nucleus with polar tube precursors into each sporoblast. (6) The individual sporoblasts develop into immature spores. (7) Eight mature spores are formed in direct contact with the host nucleoplasm.

TABLE 1 | Biological and molecular data of the microsporidians in the genus *Nucleospora*.

Species	Spore shape	Spore size (μm)	No. of spores in per nucleoplasm	PF coils	Infection cell type	Tissue tropism	Host	Habitat	SSR DNA (GenBank No.)	References
Nucleospora salmonis	Ovoid	2 × 1 (TEM)	1–8	8–12	Hematopoietic cell, blood leukocyte	Spleen, kidney	*Oncorhynchus tshawytscha*	Euryhaline	U78176	Chilmonczyk et al. (1991); Docker et al. (1997)

(Continued)

TABLE 1 | Continued

Species	Spore shape	Spore size (μm)	No. of spores in per nucleoplasm	PF coils	Infection cell type	Tissue tropism	Host	Habitat	SSR DNA (GenBank No.)	References
Nucleospora secunda	Ellipsoid	1.65 × 0.82 (TEM)	7–18	4–5	Enterocyte	Intestine	*Nothobranchius rubripinnis*	Freshwater	Not available (na)	Lom and Dyková (2002)
Nucleospora braziliensis	Ovoid	1.34 × 0.61 (fresh)	na	na	na	Gill, gut, heart, kidney, liver, muscle, spleen, and stomach	*Oreochromis niloticus*	Euryhaline	MW491352	Rodrigues et al. (2017)
Nucleospora cyclopteri	Elongate ovoid	2.53 × 1.04 (TEM), 3.12 × 1.30 (fresh)	1–14	10–12	Lymphocyte	Kidney	*Cyclopterus lumpus*	Marine	KC203457	Freeman et al. (2013); Freeman and Kristmundsson (2013)
Nucleospora hippocampi n. sp.	Elongate ovoid, slightly bent	2.66 × 1.44 (fresh), 1.93 × 0.97 (TEM)	1–8	7–8	Enterocyte	Intestine	*Hippocampus erectus*	Marine	MW229243	This study

particular, other species in the *Nucleospora* genus, such as *Nucleospora salmonis* and *Nucleospora cyclopteri*, have caused economic damage in fish aquaculture (Sakai et al., 2009; Naung et al., 2021). Hence, as one member of this highly pathogenic EGM, the potential threat of *Nucleospora hippocampi* n. sp. should be taken seriously in cultured seahorse.

For many years, the theory of host–parasite population dynamics has long held the interest of ecologists. In some hosts, the role of microsporidia in population regulation has been reported (Kohler and Hoiland, 2001; Stentiford et al., 2013), such as daphnia, locust, honeybee, and mosquito (Han et al., 2020). Seahorses are fascinating sea creatures, but at present, the global seahorse populations are in severe decline. Furthermore, all seahorse species are on the International Union for Conservation of Nature (IUCN) Red List. Because infection data for wild seahorses are not available, it is unclear whether *Nucleospora hippocampi* n. sp. plays a role in exacerbating the decline of seahorse resources. Further research on epidemiology is needed to evaluate its effect on host richness.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MW229242.

ETHICS STATEMENT

The animal study was reviewed and approved by East China Sea Fisheries Research Institute. All fish experiments were carried out in accordance with the laboratory animal guidelines for ethical review of animal welfare (China national standard GB/T 35892-2018).

AUTHOR CONTRIBUTIONS

WF and HT designed the experiments. YW performed the experiments and prepared the manuscript. NY, YH, and JZ participated in pathology experiment. LL constructed the figures. XZ and XL assisted in sample collection. SZ, RM, and XCL assisted in molecular analyses. All authors revised and approved the submitted version of the manuscript.

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