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REVIEW

Mechanisms of cellular invasion by intracellular parasites

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Abstract Numerous disease-causing parasites must invade host cells in order to prosper. Collectively, such pathogens are responsible for a staggering amount of human sickness and death throughout the world. Leishmaniasis, Chagas disease, toxoplasmosis, and malaria are neglected diseases and therefore are linked to socio-economical and geographical factors, affecting well-over half the world's population. Such obligate intracellular parasites have co-evolved with humans to establish a complexity of specific molecular parasite–host cell interactions, forming the basis of the parasite's cellular tropism. They make use of such interactions to invade host cells as a means to migrate through various tissues, to evade the host immune system, and to undergo intracellular replication. These cellular migration and invasion events are absolutely essential for the completion of the lifecycles of these parasites and lead to their for disease pathogenesis. This review is an overview of the molecular mechanisms of protozoan parasite invasion of host cells and discussion of therapeutic strategies, which could be developed by targeting these invasion pathways. Specifically, we focus on four species of protozoan parasites *Leishmania*, *Trypanosoma cruzi*, *Plasmodium*, and *Toxoplasma*, which are responsible for significant morbidity and mortality.

Keywords Leishmaniasis · American trypanosomiasis · Chagas disease · Toxoplasmosis · Malaria · *Leishmania* · *Trypanosoma cruzi* · *Toxoplasma gondii* · *Plasmodium* · Invasion

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Mechanisms of host-cell invasion in *Leishmania*

Introduction

Leishmania are obligate intracellular protozoa that infect mononuclear phagocytes. The parasites exist in two morphological forms, as an intracellular aflagellated amastigote in the vertebrate hosts, such as humans, dogs, lizards or rodents, and as a motile flagellated promastigote in the invertebrate sand fly vector. Six major *Leishmania* species (*L. tropica*, *L. major*, *L. donovani*, *L. infantum, L. braziliensis*, and *L. mexicana*) cause the three main forms of the disease in humans, dermal cutaneous leishmaniasis, visceral leishmaniasis (VL), and muco-cutaneous leishmaniasis. The form and severity of the disease greatly depend on the infecting *Leishmania* species and the immune status of the host [1].

Life cycle

Leishmania amastigotes are taken up by a female sand fly from an infected vertebrate host during a blood meal. Within the gut of the sand fly, *Leishmania* amastigotes transform via intermediate non-infective procyclic promastigotes into infective metacyclic promastigotes [2]. Infective metacyclic promastigotes are introduced intradermally into the pool of blood of the vertebrate host by the feeding-infected sand fly [3]. Within a short span of time, neutrophils are recruited to site of sand fly bite, where they engulf the promastigotes providing them with temporary shelter [4]. Infected neutrophils or free parasites are then taken up by professional phagocytes (dendritic cells and macrophages), which migrate away from the site of the sand fly inoculation site [5]. *Leishmania* promastigotes survive in the parasitophorous vacuoles where they transform into amastigotes and replicate, eventually overburdening the infected cell leading to rupture. Extracellular amastigotes re-infect local phagocytes leading to subsequent cycles of infection. The transmission cycle is complete when infected phagocytes or extracellular parasites are taken up by feeding sandflies. Amastigotes then transform into promastigotes in the sand fly midgut and then further differentiate to become infective metacyclic promastigotes, which are able to infect a new vertebrate host [6]. *Leishmania* can also be transmitted through blood transfusion from previously infected to naive human hosts [7, 8].

Mediated uptake by host cells at the site of sand fly inoculation

When an infected sand fly releases *Leishmania* promastigotes into the skin, they evade immediate host immune response and destruction by seeking refuge in different types of host cells, which includes dermal macrophages (Fig. 1), fibroblasts, keratinocytes, and dendritic cells (DCs). Metacyclic promastigotes evade immediate destruction by deactivating the host's complement system [9, 10]. In particular, lipophosphoglycan (LPG), which is highly expressed on the surface of metacyclic promastigotes, interferes with the insertion of membrane attack complex [11] and promastigote specific kinases deactivate the classical and alternative complement pathway by phosphorylating complement proteins [12]. Furthermore, the surface-membrane metalloprotease, glycoprotein 63 (known as gp63, leishmanolysin, or major surface protease), cleaves C3b attached to its surface, to an inactive form, C3bi, which

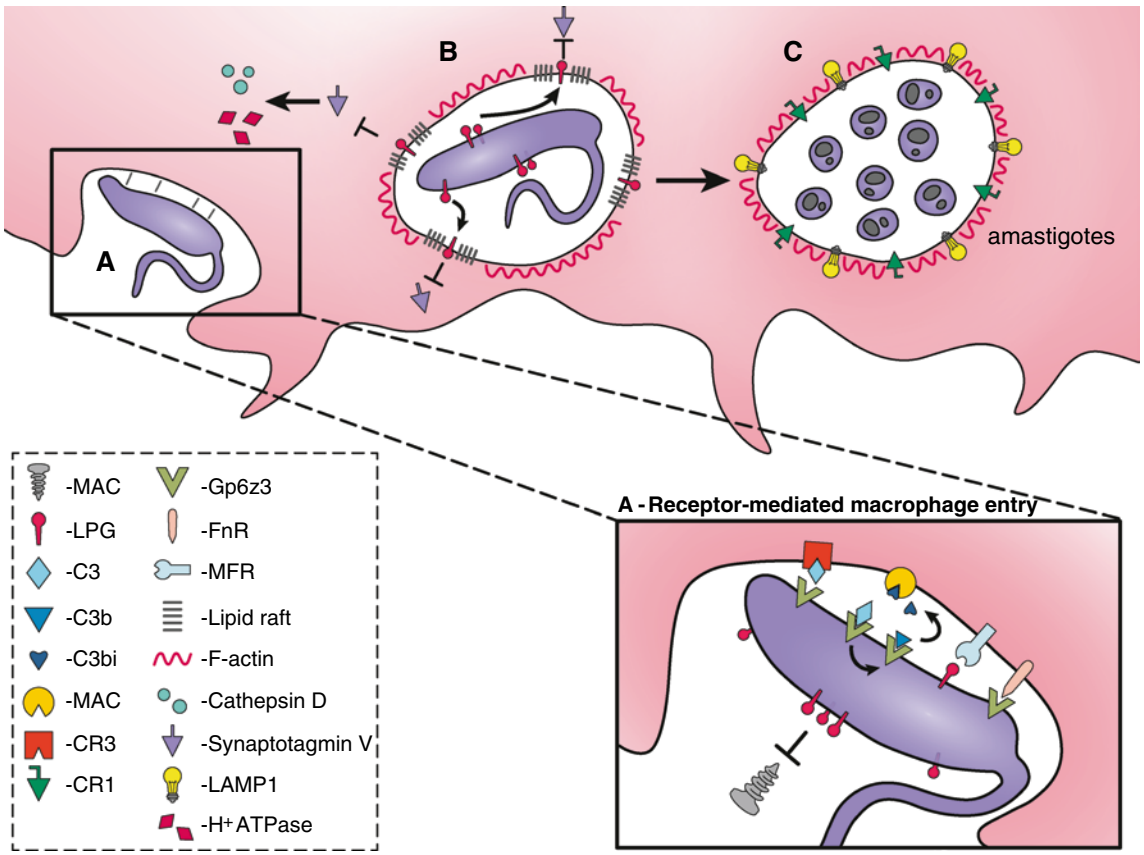

Fig. 1 Mechanisms of host-cell invasion in *Leishmania*

facilitates the complement receptor 3 (CR3)-mediated entry into the host cells [13]. *Leishmania* promastigotes gain entry into the dermal macrophages by utilizing the host CR3, which prevents the parasites from being killed by circulating phagocytes and non-immune serum. Additionally, in dermal macrophages, which lack the respiratory burst machinery, promastigotes have enhanced ability to transform into and multiply as amastigotes compared to other macrophage populations [14]. Similarly, both *Leishmania* promastigotes and amastigotes are actively ingested by the skin fibroblasts [15]. These cells provide a safe environment for *Leishmania* for up to 7 days post-infection, as they produce low levels of nitric oxide even in the presence of lipopolysaccharide and interferon-γ, compared to macrophages [15]. The saliva of sand flies has been shown to have hemostatic and immunomodulatory effects in the host, which affect the infectivity and pathogenesis of *Leishmania* [16]. While many of these effects are common to the saliva from Old (*Phlebotomus* spp.) and New (*Lutzomyia* spp.) World sand flies, there also some effects that are different. Sand fly saliva contains a multitude of components, the most well described is the vasodilator protein called Maxadilan [17, 18]. Sand fly saliva increases the migration of inflammatory cells to the site of deposition, enhancing the interaction of *Leishmania* with potential host cells [19]. The saliva from *Lutzomyia longipalpis* and Maxadilan alone decrease TNF-α, IL-10, and increase IL-6, IL-8, and IL-12 production in LPS stimulated human macrophages [20]. Maxadilan diminishes DTH responses in mice and inhibits T cell activation [20–22], and *Phlebotomus* saliva up-regulates Th2 responses and down-regulates Th1 responses in *L. major* infection [23]. The saliva of *Phlebotomus papatasi* down-regulates NO production in LPS- or IFN-g-activated macrophages [23, 24]. Repeat exposure of hosts to sand fly saliva induced antibody formation and cell-mediated responses to saliva proteins. Interestingly, pre-exposure to sand fly saliva has been shown to be protective to subsequent *Leishmania* infection [25, 26], bringing forth the possibility is using saliva components as a vaccine [27–31]. Work is ongoing in order to understand how the responses to saliva protect against leishmaniasis or if the results of these studies in mice are applicable to humans [32].

Keratinocytes are epithelial cells in the skin that form an important initial barrier between the host and the environment. The cellular interaction between keratinocytes and *Leishmania* is critical in delivering the initial triggers for Th-cell differentiation, although these are not target cells for parasite entry. Moreover, early cytokine expression of IL-1β, osteopontin, IL-12, IL-4, and IL-6 occurs in the keratinocytes of parasite-resistant mice strains following inoculation of *Leishmania* promastigotes. Among these cytokines, IL-6 and IL-4 from epidermal keratinocytes are essential for resistance and generation of protective Th1 immunity [33]. On the other hand, DCs present in the epidermis are highly motile and capable of crawling between interstitial tissue spaces serving as efficient pathogen sensors [34]. These cells internalize *Leishmania* and transport them to lymph nodes for priming of *Leishmania* specific T cells [35]. Interestingly, mice depleted of epidermal DCs had smaller lesions and reduced number of disease promoting T regulatory cells highlighting the role of DCs in promoting host susceptibility [36].

Mediated uptake by neutrophils

Neutrophils are the first cells to arrive at the site of infection within a few minutes of a sand fly bite [37]. This early recruitment of neutrophils might depend on specific cues provided by the release of alarmins (signal for tissue damage), chemokines and cytokines [38, 39]. Neutrophils are crucial cells for early defense against infections as they can kill obligate intracellular pathogens via reactive oxygen species (ROS) [40, 41], neutrophil elastase (NE), or neutrophil extracellular traps (NET) [42]. However, once phagocytic killing has been evaded or silenced, neutrophils can serve as host cells for *Leishmania* promastigotes [37]. Human neutrophils can readily phagocytose *L. major* promastigotes but amastigote recognition or uptake has not been detected by extracellular neutrophils [43]. Lipophoshoglycan and tartrate-resistant acid phosphatase present on the cell surface of *Leishmania* promastigotes inhibits lysosome fusion and the respiratory burst and superoxide anion production in neutrophils [44–46]. *Leishmania* promastigotes release chemotactic lipid *Leishmania* chemotactic factor (LCF), which attracts neutrophils [47] and interacts with Lipoxin A4 receptors (ALX), which deactivates the neutrophil oxidative burst [48]. Neutral elastase, a serine peptidase released by neutrophils, is deactivated by an inhibitor of serine peptidase present in *Leishmania*, which is crucial for intracellular parasite survival [49]. Moreover, virulent inoculum of *Leishmania* promastigotes contains a large subpopulation of apoptotic parasites, which bind to host annexin A5, which induces TGF-β and consequently mediates the silencing of phagocytes and survival of the viable subpopulation [50, 51].

Promastigotes can effectively modulate the short lifespan of neutrophils by delaying or accelerating apoptosis to their advantage. Neutrophils from human blood or murine peritoneal cavity have delayed apoptosis upon *Leishmania* infection [50–52]. On the contrary, neutrophils from infected dermis exhibit a marked increase in apoptosis, indicated by the enhanced expression of phosphatidylserine (PS). Interestingly, this increase in PS expression marks the infected neutrophils to be phagocytosed by dermal DCs [53]. Similarly, infected neutrophils are also phagocytosed

by macrophages indirectly delivering promastigotes forming the basis for the Trojan horse model of macrophage infection [51]. However, recent in vivo studies have shown that neutrophils do not directly transfer their parasites to the macrophages. Instead, the apoptotic neutrophils lyse releasing the apoptotic and viable promastigotes, which are then engulfed by the macrophages [37, 53].

Mediated uptake by macrophages

Macrophages serve as one of the critical host cells for *Leishmania* parasites and it is therein where parasites undergo amastigotes transformation and replicate and deactivate the microbicidal machinery of the host cell. The C3bi (generated by C3b proteolysis by gp63) opsonized metacyclic promastigotes bind to macrophage receptor CR3 facilitating silent entry into the macrophages [13, 54]. Additionally, gp63 binds directly to fibronectin receptors through at least two domains [55, 56] and may also utilize this receptor by forming a bridge between gp63-bound fibronectin [57]. High-mannose type glycans on the surface of promastigotes also facilitate parasite binding through mannose-fucose receptors [58]. Gp63 may also bind directly to CR3 to promote invasion [59]. After initial attachment, *Leishmania* promastigotes initiate caveolae-dependent phagocytosis for entering host macrophages [43]. *Leishmania* LPG is inserted into phagosome cholesterol-rich lipid microdomains, which causes exclusion of the exocytosis regulator, synaptotagmin V, which prevents the acquisition of cathepsin D and the vesicular proton-ATPase, in the newly formed *Leishmania*-containing vacuoles (Fig. 1). This creates an intracellular phagosomal niche that fails to acidify and trigger microbicidal machinery, providing sufficient time for promastigotes to differentiate into amastigotes [60]. Furthermore, vacuoles containing promastigotes interact poorly with late endosomes and lysosomes, as observed by the delayed recruitment of LAMP-1 and Rab7. These events result in the delay of phagosome maturation, which interferes with the recruitment of signal transducers and vesicle trafficking to developing phagosomes [61, 62]. This is characterized by the formation of F-actin coat around the phagosome due to LPG-mediated deactivation of protein kinase C (PKC)α [63] and abnormal retention of actin polymerization machinery (Arp2/3,Wiskott Aldrich Syndrome Protein, myosin and α-actinin) and Cdc42 [64]. This also leads to the LPG-dependent exclusion of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase cytosolic components, $p47^{phox}$and $p67^{phox}$, from the promastigote containing phagosome resulting in deactivation of ROS generation [65]. As promastigotes transform into amastigotes, the parasitophorous vacuoles become acidic (pH 4.7–5.2) [66, 67] and acquire lysosomal hydrolases and late endosomal/lysosomal proteins such as Rab7 and LAMP-1 [67]. Amastigotes multiply within these phagolysosomes until being released from ruptured macrophages and then enter neighboring macrophages through a non-caveolae pathway after ligating Fc-γ and PS receptors [1]. Amastigotes survive in phagolysosomes using several strategies that include: (1) deactivation of the ROS generation machinery [68, 69]; (2) attenuation of the PKC activity [70]; (3) suppression of STAT1 signaling [71]; (4) dysregulation of JAK/STAT signaling by depleting membrane cholesterol [72]; (5) activation of *Leishmania* specific ecto-protein phosphatases, cysteine proteinases or host cellular phosphotyrosine phosphatases or by inducing ceramide generation [73–77] (6) interference with the strength of CD40 cross-linking [78, 79]; (7) inhibition of mTOR complex 1 along with concomitant activation of translational repressor 4E-BP1, which promotes *Leishmania* proliferation [80]. In addition, the phagolysosomes containing *Leishmania* are enlarged due to increased expression of lysosomal trafficking regulator gene (*LYST/Beige*) that protects the parasites from oxidative damage by diluting the microbicidal effects of nitric oxide [81].

Furthermore, *Leishmania* also interferes with the strength of CD40 cross-linking or reciprocally activates MAPK phosphatases (MKP), MKP-1, and MKP-3 resulting in antagonistic regulation of ERK1/2 and p38MAPK, which governs the production of IL-10 and IL-12 by *Leishmania*-infected macrophages [78, 79].

Therapeutic considerations

The current anti-leishmanial drugs antimonials, amphotericin B, pentamidine, and miltefosine have limited therapeutic application due to toxicity and increasing multiple drug resistance. Novel therapeutic approaches are needed and those based on thwarting the mechanisms crucial for cellular invasion by *Leishmania* may hold promising results. Targeting MAPK signaling pathway using the specific MKP-1 inhibitor, triptolide, could be an effective therapeutic option for controlling *L. major infection* [79]. Similarly, AS-605240, a PI3Kγ inhibitor, is another promising therapeutic candidate as it confers resistance against *L. mexicana* infection and showed similar protection as sodium stibogluconate [82]. Furthermore, cysteine proteases, important for intracellular survival of *Leishmania* amastigotes, are targeted by cystatin, a natural cysteine protease inhibitor, for curing experimental VL and could be developed as potential therapeutic candidate [83, 84]. Since *Leishmania* depletes membrane cholesterol and disrupts lipid rafts in host macrophages, therapies based on restoration of cholesterol levels [72, 85, 86] and raft-associated proteins [87, 88] are other promising strategies.

Mechanisms of host-cell invasion by *Trypanosoma cruzi*

Introduction

The lifecycle of *Trypanosoma cruzi* was first described in the early 1900s by the Brazilian physician Carlos Chagas, who while working on malaria, suspected that insects infesting local rural dwellings transmitted human disease. Upon dissection he found motile flagellated parasites within the gastrointestinal tracts of these triatomine insects (family *Reduviidae*). He named the parasite after his mentor Prof. Oswaldo Cruz and determined that these were infective to mammals through experimental infection of monkeys, and further established they caused human disease by finding the parasite in the blood and tissues of acutely and chronically infected patients. In doing so, Chagas has been the only scientist that has completely described the epidemiology, clinical syndrome, etiology, vector and complete lifecycle of a microbial agent. Named after him, Chagas disease (also known as American trypanosomiasis), describes the clinical syndromes caused by infection with *T. cruzi* [89].

Life cycle of *Trypanosoma cruzi*

The life cycle of *T. cruzi* is complex and interesting. Transmission of *T. cruzi* occurs mainly by deposition of infective metacyclic trypomastigotes (MT) present in the urine and feces of hematophagous bugs from the family Reduviidae, of the genera *Triatoma*, *Rhodnius*, and *Panstrongylus*. The development of replicative non-infective epimastigote forms into infective MTs during their transit in the insect gastrointestinal tract is essential to the transmission to human hosts. Once MTs are deposited in the feeding wound they can invade nucleated hosts cells or be engulfed by professional phagocytes (macrophages and DCs) [90]. Human infection by the oral ingestion also occurs through the stomach epithelial invasion of MTs present in insect contaminated preparations of fruit juice [91–95]. After MT host-cell infection, parasites are initially housed in parasitophorous vacuoles but then escape into the host-cell cytoplasm and differentiate into aflagellated amastigotes. Amastigotes replicate in the cytoplasm until they are triggered to differentiate into motile flagellated trypomastigotes, which escape host cells and disseminate in the blood and lymph. The triggers for intracellular differentiation of amastigotes into trypomastigotes are unknown. Bloodstream trypomastigotes lead to further infection by binding to and invading nucleated non-phagocytic cells and are also phagocytized by macrophages, both similar to the initiation of infection that occurs by insect-derived MTs. Amastigotes are also infective and can disseminate in infected hosts. The life cycle is complete when trypomastigotes or amastigotes are ingested by reduviid bugs feeding on mammalian hosts, wherein they differentiate back into replicative epimastigotes. The mechanisms by which *T. cruzi* life cycle forms invade human cells are discussed in more detail below. While insect transmission is the primary mode of parasite acquisition, oral and transplacental routes can also occur and infection through transplanted blood and tissue from chronically infected individuals is increasing worldwide and has prompted expansion of screening practices of donated products [96, 97].

Mechanisms of host-cell invasion

T. cruzi can actively invade multiple non-phagocytic cells. The MTs and bloodstream trypomastigotes (also termed tissue-culture derived trypomastigotes, or TCT, produced in vitro by cultivation of parasites in tissue culture and provide a model of bloodstream forms) are the main infective forms of the parasite and will be referred to as trypomastigotes collectively throughout the rest of this review unless otherwise specified. Infection by amastigotes can also lead to productive infection although the mode of infection by these forms is not entirely understood. Epimastigotes are also infective in vitro but are not widely thought to contribute significantly to natural infection. The process of invasion by trypomastigotes is complex and dynamic and involves the interplay of multiple parasite and host factors. The bulk of studies looking at invasion have been done in vitro with a variety of different host-cell systems. This is complicated by the fact that different parasite strains infect cells by different mechanisms. Parasites can also infect macrophages by phagocytosis or through parasite invasion, as they do in non-phagocytic cells. There is controversy as to which of these is the main mode of parasite entry, however both are operative simultaneously. The information below provides a general overview of the main concepts concerning *T. cruzi* host-cell invasion. The readers may want to refer to several excellent and comprehensive reviews by pioneers in this field for further information [98–101].

Non-phagocytic cell infection

In vitro analysis of parasite invasion of non-phagocytic cells has resulted in the definition of several distinct steps (Fig. 2). Trypomastigotes initiate infection by interacting with the host-cell surface membrane by way of several parasite surface-membrane protein ligands. The host-cell receptors for many of these ligands have not yet been identified. Many members (gp82, gp90, gp85/TS, gp30) of the gp85/*trans*-sialidase (TS) super-family are implicated in the initiation of infection by trypomastigotes [102, 103]. Gp82, and the related gp30, are MT-specific surface

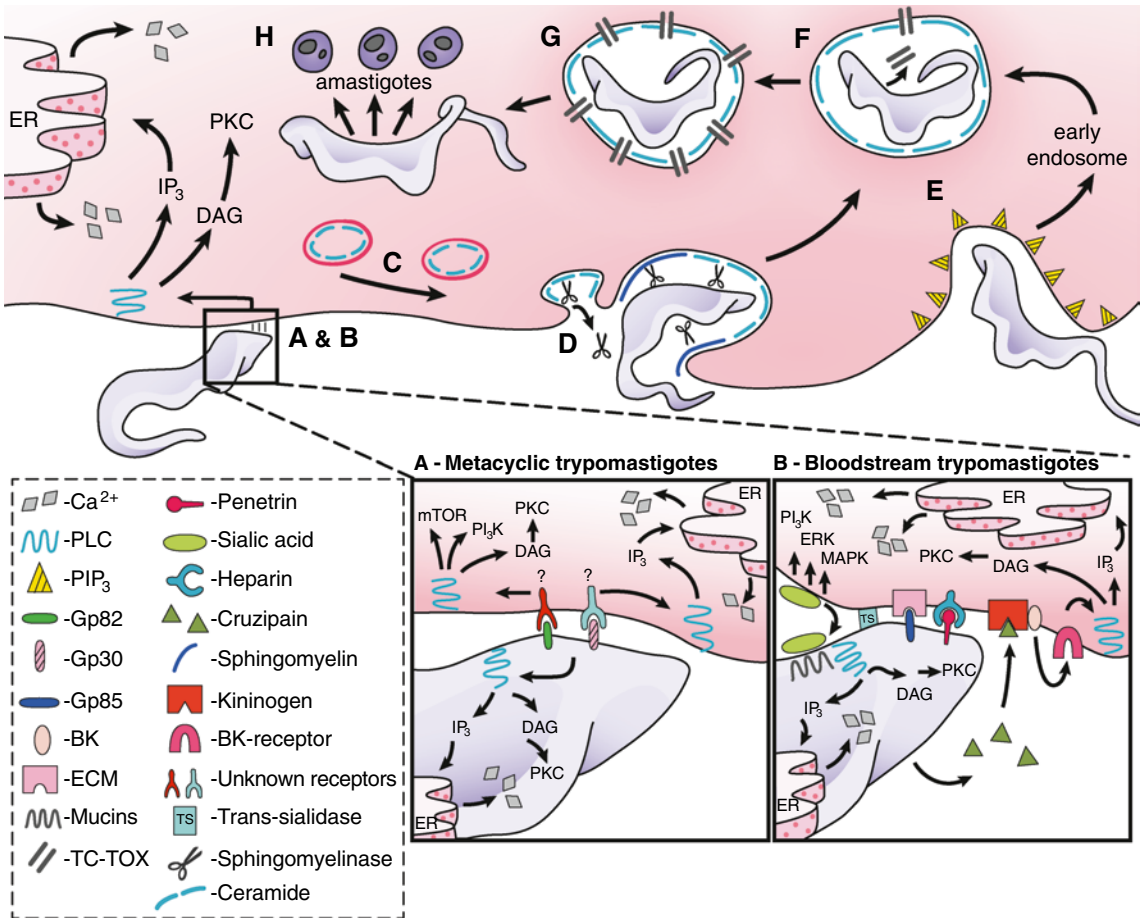

Fig. 2 Mechanisms of host-cell invasion by *Trypanosoma cruzi*

proteins that upon engagement of an unknown receptor(s) mediate bidirectional Ca^{2+} release in both the parasite and host cell [104–106]. On the parasite side, this interaction triggers phospholipase C (PLC) activation generating diacylglycerol (DAG) and inositol triphosphate (IP3), which further activates protein kinase C and release of Ca^{2+} from the endoplasmic reticulum. In host-cell targets, PLC is activated, which ultimately release Ca^{2+} from intracellular stores via IP3-signaling. Recent data indicates that phosphatidylinositol 3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) are also triggered during this process, however the downstream events beyond this are not well elucidated [106]. In some parasite strains, which express gp33/50, a mucin-related protein, host-cell Ca^{2+} release is triggered leading to invasion similar to but not as robustly as that mediated by gp82 [107]. Gp90 expressed in MTs of some parasite strains is a negative regulator of cell invasion and does not trigger Ca^{2+} mobilization or intracellular signaling [107, 108]. Gp82, −30, −90, and −35/50 are expressed to different degrees in various *T. cruzi* strains, which underscores the complex heterogeneity of the mechanisms of parasite invasion [109]. Bloodstream trypomastigotes appear to invade cells in a similar fashion but through a different set of interactions. Host-cell PLC activation occurs through triggering of the host bradykinin receptor, in an interesting mechanism in which parasite secrete cruzipain, a cysteine protease, which cleaves host kininogen to liberate bradykinin [110, 111]. Similar to MT invasion, PLC activation generates IP3, which triggers downstream release of host Ca^{2+}. Ca^{2+} release is also mediated by action of a proteolytic cleavage product generated by oligopeptidase B activity on an unknown parasite protein substrate [112–114]. Parasite surface-membrane *trans*-sialidase (TS) promotes invasion of bloodstream forms through transfer of host surface sialic acid residues onto various parasite mucins-proteins, which are a large family of heavily glycosylated GPI-linked surface proteins [115–117]. Engagement of these sialylated parasite proteins trigger host-cell signaling via activation in a number of different ways including PI3K/Akt-, MAP-kinase and ERK- pathways [118–120].

Parasite binding of extracellular matrix proteins (ECM) (fibronectin, laminin and collagen) and proteoglycans (heparin, heparin sulfate) is also implicated in invasion

[121–124]. The gp85/TS superfamily of proteins been shown to bind multiple ECM components [123]. Also notable is a 60-kDa surface protein, termed Penetrin, which binds heparin and heparin sulfate in a sialic acid-independent manner that promotes invasion [125, 126], and may also associate with ECM components. Recently, homologs of the leishmanial surface protease, gp63, have been described in *T. cruzi*, and it appears as they may be involved in parasite–host cell binding [127, 128], however is it unclear if they are molecularly distinct from Penetrin.

Binding and invasion of host cells by parasites trigger several different, yet overlapping parasite entry pathways. Exploitation each of these is how by *T. cruzi* is such a successful pathogen at infecting multiple cell types. Each of these pathways, which culminate in the encasement of parasites within lysosomal-membrane-based vacuoles, which are necessary for subsequent events leading to productive infection [129].

In the "lysosomal-dependent pathway", lysosomes are recruited to the site of parasite binding in a Ca^{2+}-dependent manner. The exocytosis of lysosomes occurs by their fusion with the surface membrane abutting the parasite, which causes release of acid sphingomyelinase that generates ceramide through cleavage of membrane sphingomyelin [130]. The formation of ceramide in the outer leaflet of the membrane induces endocytosis of the wounded membrane. Parasites take advantage of this by becoming internalized in these lysosomal-based endosomes. This process may require the Ca^{2+}-binding protein synaptotagmin VI and is probably powered by kinesin movement on microtubules that requires Ca^{2+}-calmodulin [131, 132], both correlating with the necessary role of Ca^{2+} in this process. In the "lysosomal-independent pathway" parasites are initially housed in surface-membrane containing phosphoinositides, which eventually fuse with the lysosomes at later times post-infection [133, 134]. The protein dynamin, a GTPase, which associates with PI3K, may be involved in formation and dynamics of vacuole maturation [135, 136]. Prior to lysosomal fusion parasite entry into cells is reversible and parasites can escape the host cell. Thus fusion of these plasma membrane-derived vacuoles with lysosomes is a key step that leads to productive infection [137].

A third pathway has been proposed in which parasites exploit the autophagocytic pathway, which is operative under starvation conditions [138–140]. In this pathway, parasites invade cells and are initially housed in autophagocytic vacuoles, which eventually fuse with lysosomes to create an acidic autophagolysosomal compartment. These vacuoles are thought to be rich in nutrients for parasite survival and polyamines, which promote differentiation of trypomastigotes into amastigotes [141]. Overall, this last model is probably a modified version of the lysosomal-independent pathway specialized for conditions of starvation. The functional significance of actin and whether it is required or deleterious to parasite invasion in these pathways is likely a function of the ligands utilized for entry by different parasite forms [142].

Extracellular amastigotes are also infective to both phagocytic and non-phagocytic cells. Amastigotes of different strains appear to actively invade non-phagocytic host cells through recruitment of actin at the site of parasite attachment [142–144]. *T. cruzi* infection also occurs through the oral route where is has been responsible for several outbreaks [91, 95]. This occurs through parasite contamination of fruit juice (e.g., acai and guava fruit) from crushing of parasite-laden triatomines present on berries during processing. Consumption of contaminated juice delivers MTs into the stomach and duodenum where they invade the gastric mucosal lining. Parasite gp82 is a chief ligand for invasion and the proteolytic degradation of the anti-invasive ligand gp90 further facilitates parasite entry [145–147]. Parasite strains not expressing gp82 invade via the related gp30, which also triggers Ca^{2+} signaling. Parasite strains expressing gastric protease-susceptible gp90 are relatively more infective via the oral route than those expressing protease-resistant gp90 [148].

Phagocytic cell infection

Macrophages are also targets for parasite infection, especially at the initiation of infection when MTs are deposited in the tissue by insects. In addition, amastigotes released from infected cells also infect macrophages. Both stages are taken up by phagocytosis, initially housed in parasitophorous vacuoles, where they can be killed by generation of nitric oxide and peroxynitrite [149–151]. The maturation of parasitophorous vacuoles into phagolysosomes is important for parasite survival, which is required for entry into the host cytoplasm where they avoid exposure from microbicidal radicals. In the cytoplasm parasite differentiate and replicate as they do in non-phagocytic cells. The initial entry of parasites into macrophages is probably by way of both an actin-dependent phagocytic process and an active invasion process similar as those described above in non-phagocytic cells. Recent studies indicate that the integrity of membrane rafts composed of cholesterol, ganglioside GM, flotillin and caveolins are important in trypomastigote and amastigote infection of macrophages [152–154]. It is unclear whether, in the case of macrophages, this affects only active-invasion and/or the phagocytic parasite pathway. However, lipid membrane raft disruption diminishes infection of non-phagocytic cell invasion by trypomastigotes probably by disaggregation of proteins important for signaling pathways that initiate the invasion process.

Therapeutic considerations

The drugs currently used to treat Chagas disease include the nitroheterocyclics compounds benznidazole and nifurtimox, which are indicated in acute and intermediate stage *T. cruzi* infection. The mechanism of action of nifurtimox is not completely understood but is probably due to the induction of oxidative radical formation within the parasite and/or lowering of thiol levels through the production of nitroso-intermediate due to reduction of the nitro-group of the compound by the parasite [155–157]. The action of benznidazole is thought to act by reduction of macromolecule production through interactions of its nitro-group [158, 159]. The efficacy of these drugs in chronic Chagas disease is somewhat controversial and trials are underway to determine if there is any benefit of treatment at this late stage [160–162]. These drugs are significantly toxic, yet are the mainstay of anti-parasitic therapy because there are no other alternatives approved for human use at present. Inhibitors of cruzipain, an important cysteine protease of *T. cruzi*, are promising candidates for future therapy [163–165]. This protease is essential for viability of all stages of *T. cruzi* and is important as several key steps in the life-cycle, including parasite invasion (see above), cell division and differentiation [166]. Preliminary studies indicate that the vinyl sulfone inhibitor, K11777, is a therapeutic candidate and that other compounds that inhibit this enzyme may find there way into the pipeline of new investigational drugs.

Mechanisms of host-cell invasion by apicomplexan parasites

Introduction

Apicomplexan parasites are a unique group of protists united by the presence of an organelle called the apicoplast as well as the apical complex, a structure specifically involved in host-cell invasion. Although there are many organisms that fall under the phylum Apicomplexa, this section of the review will focus primarily on *Plasmodium falciparum*, the causative agent of cerebral malaria, with additional discussion of *Toxoplasma gondii* invasion, the causative agent of Toxoplasmosis.

Malaria is an infectious disease that has plagued man for millennia. Despite enormous struggles at prevention and treatment, currently over 250 million cases occur annually, resulting in an estimated 700,000 deaths, mostly African children [167]. Humans get infected with *P. falciparum* when bit by an infected mosquito, as this is the definitive host. The impact of this disease on the at-risk population is staggering and new, targeted approaches to prevent and cure it are desperately needed. *T. gondii* can infect any mammal, with humans being an accidental host. *T. gondii* can only sexually reproduce in the intestines of cats, the parasite's definitive host, making every other host an intermediate host. In the United States over 60 million people carry the parasite but generally only those with compromised immune systems and pregnant women develop symptoms of disease [168]. Humans get infected by ingesting parasite-contaminated food or water or by ingesting infected and undercooked meat.

Although we understand a great deal regarding the biology of the parasites responsible for malaria and toxoplasmosis, it is only relatively recently that many of the molecular mechanisms by which the parasite recognizes and invades or traverses through host cells have been elucidated. There is a striking complexity of cellular traversal and invasion mechanisms utilized by Apicomplexa parasites for entry into the host cell. For *Plasmodium*, we focus on the two mammalian cell types into which the parasites invade, namely the liver hepatocyte and the erythrocyte. *Toxoplasma* is known to invade all nucleated cells, both non-phagocytic and phagocytic cells. For both species, we discuss notable parasite proteins and organelles involved in each of these invasion processes and highlight the host molecules known to be involved. As these processes represent critical events in the parasite's life cycle, we finish with a brief section describing a number of ongoing efforts to target invasion in an attempt to combat these menacing diseases.

As this is meant to be a general review of Apicomplexan invasion, we regrettably have had to limit the depth of our description. Many excellent reviews exist that are more focused on various aspects of this topic and we direct the reader to these [169–175].

Plasmodium life cycle

Five species of *Apicomplexan* parasites of the genus *Plasmodium* infect humans to cause malaria and, like many parasites, they develop through discrete stages, each particularly adapted to one of its two hosts (the human and the female anopheline mosquito). These include *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and, shown recently to infect humans, *P. knowlesi* [176]. A striking feature of these parasites is their ability to invade a wide variety of cell types during this complex life cycle. This cycle can be broken down into eight steps: (1) In the midgut of an infected female mosquito, the definitive host, diploid zygotes migrate through the chitinous, cell-free peritrophic matrix and penetrate the midgut epithelium, taking up residence between the epithelium and the midgut basal lamina; (2) The zygotes then develops into oocysts that mature and release motile sporozoites that travel

through the mosquito's hemocoel, attaching to and traversing through salivary gland cells to access the interior of the gland; (3) Upon the female mosquito taking a blood meal from the mammalian host, sporozoites are deposited in dermal tissue and use gliding motility to reach dermal vessels, crossing the endothelium into the bloodstream; (4) Sporozoites then rapidly (within minutes) migrate to and cross the sinusoids of the liver by traversal through resident macrophages, called Kupffer cells. Once in the liver parenchyma, sporozoites trans-migrate through a number of hepatocytes by a membrane wounding process, after which they eventually invade a hepatocyte through invagination of the hepatocyte membrane; (5) Within the hepatocyte, sporozoites develop into exoerythrocytic forms that differentiate and replicate to produce haploid merozoites, which are then released into the peripheral bloodstream; (6) Merozoites invade erythrocytes, initiating the erythrocytic cycle of infection. During the erythrocytic stages, parasites replicate and progress through the following developmental stages within erythrocytes: ring, trophozoite and schizont. Merozoites egress from the erythrocyte and re-invade uninfected erythrocytes in a 48–72-h cycle, dependent on the species; (7) During this cycle of development and re-invasion of erythrocytes, some parasites transform into sexual gametes; (8) Gametocytes are taken up by a mosquito, and fertilize to become zygotes, thus completing the life cycle [176].

Plasmodium invasion of the hepatocyte

Invasion of a liver hepatocyte is perhaps best thought of as an ordered process of both extracellular and intracellular steps with the inhibition of any of these resulting in a block in infection. After traversing the dermal epithelium and entry to the bloodstream, sporozoites are carried to the liver where they arrest and undergo gliding motility on the surface of the sinusoids that form the reticuloendothelial system. This is proposed to be mediated through a multivalent interaction of the parasite's surface-expressed circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) with sulfated heparin sulfate proteoglycans (HSPGs), secreted by liver stellate cells, which protrude through the endothelial fenestrae into the sinusoidal lumen. This interaction and arrest in the liver sinusoid explains why circulating sporozoites are virtually undetectable within minutes of infection, discovered by intravenously infecting rats with *P. berghei* sporozoites [177]. Traversal of the liver endothelium in rats does not appear to be through the liver endothelial cells, rather it involves a membrane invagination process (with formation of a parasitophorous vacuole) of the Kupffer cells present in the endothelial lining by *P. berghei* and *P. yoelii* [178]. Functional studies to block the activity of these phagocytic cells, either by heat killing or by specific inhibitors, suggested their involvement in sporozoite traversal, with a smaller percentage of traversal explained by a biologically active process [179]. Stronger evidence is from mouse experiments in which macrophage-colony-stimulating factor 1 (CSF-1) was knocked out, leading to greatly reduced numbers of Kupffer cells. These mice were significantly less susceptible to *P. yoelii* infection, indicating the necessary role of Kupffer in access to the liver parenchyma [180]. Once in the parenchyma of the liver, sporozoites traverse a number of hepatocytes in a non-invasive, membrane-wounding manner before finally invading a host hepatocyte through invagination and formation of a parasitophorous vacuole.

Plasmodium invasion of the erythrocyte

The merozoites released into the bloodstream from the liver exoerythrocytic cycle are small (~1 μm) and somewhat pear-shaped. At their apical end is located a specialized structure known as the apical complex in which reside specialized secretory organelles called rhoptries and micronemes that contain proteins necessary for invasion (Fig. 3). The limited tropism of merozoites is due to specific ligand-receptor interactions between the parasites and the erythrocytes. Initial interactions occur through proteins covering the surface of the merozoite, followed by a reorientation and formation of a tight junction between the erythrocyte and the apical end of the parasite. One of the best described proteins important in this initial interaction is the GPI-anchored protein, merozoite surface protein-1 (MSP-1), which has been suggested to interact with two nonglycosylated extracellular regions of Band 3 protein on the surface of the erythrocyte [181]. Orientation of the parasite to its apical end approximating the erythrocyte membrane then occurs followed by tight junction formation through high-affinity interactions between parasite ligands and erythrocyte receptors. Two families of proteins, the erythrocyte-binding-like (EBLs) protein and the reticulocyte-binding-like (RBLs or *P. falciparum* Rhs) proteins, have been well recognized as major parasite ligands responsible for binding and invasion and have been shown to play a cooperative role in invasion, as functional loss of the former leads to the increased transcription of the latter [182]. Both are localized to apical organelles and are released onto the parasite surface during invasion. EBLs and RBLs both appear to have specific receptors on the surface of erythrocytes, such as EBL-175 binding predominantly to glycophorin A [183]. In *P. falciparum* five EBLs and six RBLs have been described. With the exception of PfRh1, all the PfRhs bind in a sialic acid-independent manner and only PfRh4 has an identified receptor, complement

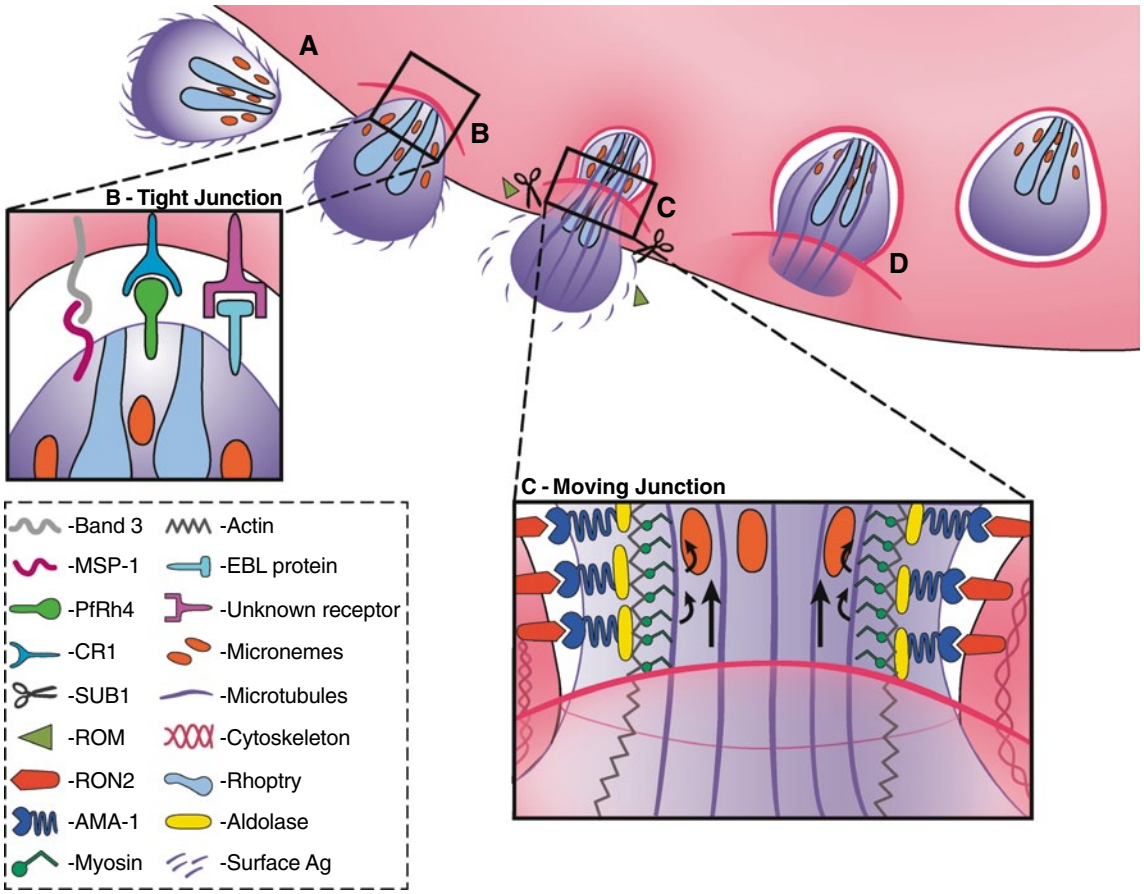

Fig. 3 Mechanisms of host-cell invasion by apicomplexan parasites

receptor 1. Both families are present throughout *Plasmodium* spp. It is notable that *P. falciparum* appears to use multiple and alternative ligand-receptor interactions and is capable of switching invasion mechanisms between sialic acid dependent and sialic acid independent pathways, demonstrated using neuraminidase and trypsin treatments on erythrocytes [184, 185]. In different regions of India and Gambia, field isolates were tested and it was found that the majority of parasites invade by the EBL-175/glycophorin A pathway [186, 187]. However, in Brazil only a minority of field isolates used this pathway [188]. This is evidence that there is variation of invasion mechanisms in filed isolates from different regions of the world.

Another well known binding interaction necessary for invasion occurs between the Duffy antigen and the Duffy Binding Protein (DBP) of *P. vivax*. The Duffy antigen, expressed on human erythrocytes, is the binding receptor for a cysteine rich domain of DBP [189]. Duffy negative [Fy(a-b-)] individuals, most commonly found in Western Africa, are naturally resistant to *P. vivax* infection [190]. The Duffy antigen has also been shown vital for *P. knowlesi* binding in vitro [191, 192]. The cysteine rich domain in *P. knowlesi* DBP is found to bind to Duffy antigen but with different specificity than that of *P. vivax* [189].

Toxoplasma gondii Life cycle

T. gondii invades a wide variety of cell types in a life cycle that can be described in four steps: (1) Tachyzoites are the fast-growing form that causing acute infection in humans and other animals. These infect nucleated host cells, replicating into a large number of progeny and egress by lysing the host cell; (2) Under immune pressure from the host, the parasite can differentiate into a slow-growing form, termed bradyzoites. This occurs in long-living tissue types such as muscle or neuronal cells; (3) Ingestion of animal tissue bradyzoites, by cats or humans releases parasites in the intestinal tract. In cats this results in sexual development and subsequent shedding of oocysts into the environment and in humans it can lead to disseminated infection; (4) Shed oocysts from cats develop into haploid sporozoites in the environment. The oral ingestion of these sporozoites leads to differentiation into tachyzoites and acute infection by humans or other mammals [193].

Toxoplasma gondii invasion of host cells

T. gondii tachyzoites use gliding motility, a mechanism not expanded upon in this review, to facilitate movement and interaction with host cells. Like a *Plasmodium* merozoite, surface antigens aid in the interaction between the tachyzoite and the host cell. One antigen known to have membrane-binding activity is Perforin-like Protein 1 (PLP-1), forming pores in the host-cell membrane after binding and as well as playing a role in egress [194]. The parasite then re-orients its position such that the apical end has contact with the host cell. *T. gondii* secretes proteins needed for invasion from micronemes, rhoptries and dense granules. The contents of the micronemes are secreted first during the initial contact with the host cell, including the full-length cellular form of microneme protein 2 (MIC2), which binds specifically to the host cell [195]. MIC2, along with MIC1, MIC4, MIC6 and MIC8, is involved the interaction between the parasite and the host cell [196, 197]. As the parasite invades the target cell these microneme proteins are proteolytically cleaved from the surface of the parasite. The rhoptries then secrete rhoptry neck proteins (RON). RON2 is inserted into the host-cell membrane of the host cell being invaded, while RON4, 5, 8, 9, and 10 form a complex at membrane surface of the membrane [198–201]. Tachyzoites then actively invade similar to *Plasmodium* merozoites, with the formation of the moving junction and a motor complex described below.

Process of apicomplexan invasion

The process of active invasion is so similar in *Plasmodium* and *T. gondii* that this section summarizes this invasion process used by both species of parasites with differences or unique mechanisms specifically noted.

Following tight junction formation between the parasite and the host-cell surface, parasites forcibly enter through invagination of the host-cell membrane, eventually forming and residing within parasitophorous vacuoles. The process involves a tight junction "motor" termed a moving junction (MJ) (Fig. 3). This junction can be thought of a "ring of contact" between the invading merozoite and the erythrocyte and is used by the parasite to "pull" itself into the cell. Once the parasite is completely inside the host cell, the MJ disappears as the parasitophorous vacuole resolves. No host proteins have been identified in the MJ although studies suggest host cytoskeleton components are involved [202, 203]. The most widely studied parasite protein involved in MJ formation is the apical membrane antigen-1 (AMA1), which is highly conserved across *Plasmodium* sp. [204–207] and *T. gondii* [200, 208]. AMA1 is secreted by micronemes and interacts with RON2, RON4 and RON5 [198, 209] as well as aldolase, an F-actin binding protein. In *T. gondii*, MIC6 also interacts directly with aldolase [196]. The RON proteins are part of a parasite derived protein complex termed the RON complex, which is secreted into the host cell upon MJ formation. The interaction between AMA1 and the RON complex is essential for parasite invasion [209]. In fact, antibodies to AMA1 can block invasion [210] and a 20-residue AMA1 binding peptide also specifically blocks invasion [211, 212], providing evidence that AMA-1 could be a potential vaccine target. As the MJ progresses around the parasite during invasion many proteins are shed from the surface of the parasite [213–215], including AMA-1 [216] and PTRAMP [217], a process required for invasion [214, 218, 219]. Recent work in *Plasmodium* has identified a subtilisin-like serine protease, PfSUB2, as the "sheddase" responsible for this indispensable event [220]. In addition to the role of SUB2 as a sheddase, a number of intramembrane proteases called rhomboids (ROM) are also implicated [215, 221–223]. In *T. gondii*, ROM4 is implicated in cleaving surface proteins to produce a gradient of adhesins, these including MIC2, AMA1, and MIC3 [224].

Therapeutic considerations for malaria

There are many drugs that treat malaria, the most recently developed and effective being multi-drug artemisinin therapies. However, to date all current antimalarials have encountered resistant parasites [225], highlighting the great need for continued drug development. Theoretically, a drug or vaccine that targets invasion of either hepatocytes or erythrocytes can be a useful preventative measure or treatment for malaria. Recent vaccine strategies are focusing on antigens crucial to invasion of hepatocytes, such as CSP, or of erythrocytes, such as EBLs and RBLs. Due to functional redundancy and antigen switching, multi-antigen vaccine strategies are more likely to act synergistically, reducing the chance of resistance and showing a more effective immune response.

Targeting hepatocytes invasion could be a great tool for prophylaxis drugs, vaccines or treatments to prevent recurrent malaria, as in the case of a *P. vivax* infection. The vaccine RTS,S, which is based on CSP from *P. vivax*, was recently reported in phase 3 clinical trials in Africa to be effective at decreasing the malaria rate in young children by nearly 50 % [226, 227]. Serum from rabbits treated with this *P. vivax* CSP-based vaccine has shown cross-species recognition of *P. falciparum* and *P. berghei* sporozoites, which is promising for the use of RTS,S against malaria caused by the various species of *Plasmodium* or even mixed infections [228].

Targeting erythrocytic invasion proteins could also serve as a useful vaccine strategy. Studies of polymorphisms of invasion-related ligands PfEBL and PfRh in field isolates from Colombia, Peru, and Brazil highlight the importance

of understanding the variety of invasion pathways being utilized by parasites in different geographical regions [229]. This needs to be taken into account by developing multi-targeted vaccines, which might overcome "vaccine resistance" and be effective against parasites from different regions, different species of parasites, or mixed infections. As an additional example of this, antibodies against PfRh5 used in combination with seven other merozoite antigens, most notably PfRh4, have been shown to act synergistically in inhibiting growth of *P. falciparum* in culture [230].

Drug-targets include the proteases involved in invasion due to the essentiality of these proteases in egress and re-invasion of erythrocytes. Two peptidyl alpha-ketoamides based on the *P. falciparum* subtilisin-like protease 1 (SUB1) inhibited orthologues in *P. berghei*, *P. vivax* and *P. knowlesi*, suggesting the possibility of "pan-reactive" drugs based on this protease [231]. *P. falciparum* signal peptide peptidase, an intramembrane aspartyl protease associated with micronemes, is shown to interact with a transmembrane receptor on host erythrocytes and antibodies against it block invasion by *P. falciparum* in vitro [232]. Rhomboid proteases may also provide an effective drug target as they are essential to invasion [233, 234]. And as mentioned previously, the AMA1 and RON complex interaction is also of interest in vaccine development as antibodies and binding peptides to AMA-1 block invasion [210, 211, 220].

Therapeutic considerations for toxoplasmosis

Toxoplasmosis is also a treatable Apicomplexan disease, although careful consideration is needed for pregnant women and immune-compromised patients like those infected with HIV. Most medications target only the tachyzoite, leaving the bradyzoite unharmed. Current medications include pyrimethamine, which is also a malaria medication, and sulfadiazine. Vaccine studies using these invasion proteins have been relatively successful. A recent study has shown that a ROP8 DNA-based vaccine, encoding one of the rhoptry proteins secreted and essential for invasion, has induced a protective immune response in mice [235]. A DNA vaccine using a fusion protein of PLP-1 and MIC6 induced protective immunity in mice [236], as did MIC1 and MIC4 [237], indicating that these are promising candidates for vaccines. It is likely that other targets of invasion, as mentioned above for malaria, could also make good drug targets for toxoplasmosis.

Overall, invasion and traversal are crucial mechanisms for Apicomplexan parasite survival and infectivity. With multiple invasion events in multiple cell types, the molecular components of invasion are attractive targets for multi-component drugs and/or vaccines, which have the potential to both increase protective immune responses in humans and decrease chance of parasite resistance.

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