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The hitchhiker's guide to parasite dissemination

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Abstract

Toxoplasma gondii is a parasitic protist that can infect nearly all nucleated cell types and tissues of warm-blooded vertebrate hosts. *T. gondii* utilizes a unique form of gliding motility to cross cellular barriers, enter tissues, and penetrate host cells, thus enhancing spread within an infected host. However, *T. gondii* also disseminates by hijacking the migratory abilities of infected leukocytes. Traditionally this process has been viewed as a route to cross biological barriers such as the blood-brain barrier. Here we review recent findings that challenge this view by showing that infection of monocytes down-regulates the program of transendothelial migration. Instead, infection by *T. gondii* enhances Rho-dependent interstitial migration of monocytes and macrophages, which enhances dissemination within tissues. Collectively, the available evidence indicates that *T. gondii* parasites use multiple means to disseminate within the host, including enhanced motility in tissues and translocation across biological barriers.

Introduction

Intracellular pathogens exploit host cells as a protected niche that supports growth while avoiding immune detection. Some intracellular pathogens further co-opt host cells as vectors that enable dissemination. This strategy can allow the pathogen to gain access to tissue by invading and translocating through the cells that form epithelial and endothelial barriers. For example, enteric bacterial pathogens such as *Shigella* and *Salmonella* invade the intestine by translocating through epithelial M cells (Vazquez-Torres and Fang, 2000). Alternatively, intracellular pathogens can spread to more distal sites by infecting migratory leukocytes that routinely traffic throughout the host using lymphatic and circulatory vasculature as conduits to reach deep tissues. Dissemination via migrating infected leukocytes often functions through a Trojan horse mechanism in which infected leukocytes ferry intracellular pathogens across biological barriers. Such Trojan horse style spread can lead to devastating consequences, such as when infected blood phagocytes deliver pathogens across the blood-brain barrier (BBB) and into the immune-specialized central nervous system (CNS) compartment (Santiago-Tirado and Doering, 2017; Ueno and Lodoen, 2015). Leukocytes also migrate through interstitial tissue spaces; however, the role of this pathway in pathogen dissemination has not been extensively explored. Infected leukocytes may promote pathogen dissemination by carrying pathogens harbored as intracellular cargo as they migrate into tissues, enter lymphatic and circulatory vessels, or traverse vulnerable biological barriers.

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Toxoplasma gondii is a parasitic protist that causes acute and chronic toxoplasmosis in many warm-blooded vertebrates (Dubey, 2007). In immunocompetent humans, acute toxoplasmosis is typically not life-threatening but leads to systemic parasite dissemination that results in a lifelong chronic infection focused in the CNS (Montoya and Liesenfeld, 2004). *T. gondii* infects a variety of migratory leukocytes and has been theorized to exploit infected leukocytes as Trojan horses to enable BBB penetration. However, recent work suggests that *T. gondii* exploits infected monocytes as shuttles to efficiently disseminate through tissues, rather than using leukocytes to cross endothelial barriers. Here we review the literature surrounding *T. gondii* dissemination to illustrate the diverse mechanisms by which intracellular pathogens can exploit host cells to promote dissemination into a variety of host niches.

Progression of toxoplasmosis

Toxoplasmosis is typically acquired when hosts ingest encysted parasites in the form of either bradyzoite tissue cysts or feline-shed oocysts (Hill and Dubey, 2002) (Figure 1A). Parasites emerge from either oocysts or tissue cysts within the stomach (Dubey et al., 1998). The freed parasites then travel into the small intestine and access the intestinal lamina propria by invading and replicating within or traversing through epithelial enterocytes, events that primarily occur in the ileum (Dubey et al., 2012; Dubey et al., 1997). Upon entering the intestinal lamina propria, parasites infect and replicate within a wide variety of cells, being most abundantly found within monocytes, macrophages and neutrophils (Dubey, 1997; Dubey et al., 1997; Gregg et al., 2013).

T. gondii rapidly spreads from the lamina propria, first into secondary lymphoid tissues and then into distal organs. In murine infection models, Peyer's patches and mesenteric lymph nodes are infected by two to three days post inoculation (Courret et al., 2006; Dubey, 1997; Dubey et al., 2012; Dubey et al., 1997; Gregg et al., 2013). After about one week, parasites will infect all distal organs, including the lungs, heart, spleen, and brain (Dubey, 1997; Dubey et al., 2012; Dubey et al., 1997). *T. gondii* rapidly appears within secondary lymphoid organs and the blood and spleens of infected mice (Courret et al., 2006; Dubey, 1997; Dubey et al., 2012; Dubey et al., 1997; Konradt et al., 2016), suggesting that hematogenous and lymphatic spread are both plausible routes for systemic dissemination. The intrinsic gliding motility of extracellular *T. gondii* (Sibley, 2010) and shuttling of intracellular parasites within migratory host cells likely both contribute to systemic dissemination.

Leukocyte migratory modes

Leukocytes are migratory immune cells and they respond both to environmental signals and the substratum to navigate their journeys. The migration of leukocytes was initially believed to universally depend upon adhesion mediated by transmembrane integrin proteins. However, more recent studies have conclusively shown that leukocytes can also migrate using a lowly-adhesive, entirely integrin-independent mode (Lammermann et al., 2008; Lämmermann and Germain, 2014). Both integrin-dependent and -independent migration are well-suited to promote *T. gondii* dissemination at various stages of infection.

Integrin-dependent leukocyte migration was elucidated by studies that extensively used two-dimensional cell culture systems to model the extravasation of blood leukocytes through endothelium and into tissues (Figure 2). During this process, circulating leukocytes are captured onto endothelial surfaces when glycosylated transmembrane leukocyte adhesins including PSGL-1 and CD44 interact with the endothelial ligands P- and E-selectin (Kansas, 1996; Ley et al., 2007). This interaction is strengthened by shear stress imposed by blood flow (Marshall et al., 2003) and leads to leukocyte rolling over the endothelium. Leukocyte rolling is slowed and eventually arrested when activated leukocyte integrins engage with endothelial ligands such as ICAM-1 and VCAM-1 (Berlin et al., 1995; Chan et al., 2001; Ley et al., 2007). Arrested leukocytes then crawl over endothelial vasculature. Endothelial crawling presumably functions to identify preferred sites for endothelial traversal, which is termed diapedesis. Diapedesis occurs via both transcellular and paracellular routes (Ley et al., 2007) (Figure 2).

During the transendothelial migration (TEM) cascade, integrins fulfill two critical functions: 1) mediating the firm adherence required for leukocytes to maintain endothelial engagement in the presence of blood flow, and 2) serving as essential force transducers during crawling and endothelial traversal (Calderwood and Ginsberg, 2003) (Figure 2). The force that produces leukocyte crawling and endothelial traversal is primarily generated by the concerted action of actin polymerization at the leading edge of migrating leukocytes and myosin II contractility. Actin polymerization drives forward protrusion of the cell membrane, leading to rearward treadmilling of actin filaments. Myosin acts further back in the cell, pulling the cell cortex rearwards (Renkawitz and Sixt, 2010). The cytoskeletal protein talin serves as a universal adaptor that connects the actin cytoskeleton to transmembrane integrins engaged with external ligands (Calderwood and Ginsberg, 2003). This linkage of the leukocyte cytoskeleton to external ligands via integrins and talin is critical for converting the retrograde cytoskeletal forces into forward movement (Renkawitz and Sixt, 2010).

Contrasting the absolute requirement for integrin-mediated adhesion and force transduction for successful migration over and through endothelial vasculature, leukocytes can migrate independently of integrin functionality in confined three-dimensional spaces such as tissue interstitium. The idea that leukocytes could migrate independently of integrins was initially very controversial. However, the feasibility of integrin-independent leukocyte migration was elegantly shown by murine studies that demonstrated that genetic ablation of either the universal integrin adaptor talin or all relevant integrins heterodimers in dendritic cells (DCs) did not cause any defects in DC interstitial migration *in vivo* (Lammermann et al., 2008). Integrin-independent migration is not restricted to DCs, as further work has provided similar evidence for neutrophils (Lammermann et al., 2013) and T cells (Woolf et al., 2007).

The mechanistic basis of force generation during integrin-independent migration remains an area of active investigation. Several models have been proposed, including deformation-based movement, membrane flow, and polarized blebbing (Fackler and Grosse, 2008; Paluch et al., 2016; Renkawitz and Sixt, 2010). These models are generally unified in positing that integrin-independent migration can occur when the environment of a migrating cell eliminates the need for stable adhesion and traction to convert forces into movement. The

interstitium meets this requirement by confining leukocytes in extracellular matrices so that passive disengagement is not possible (Renkawitz and Sixt, 2010). Lowly-adhesive interstitial migration can be driven solely by actin polymerization or the concerted action of actin polymerization and Rho/ROCK-activated myosin II contractility which assists the cell in squeezing through small pores (Lammermann et al., 2008; Lämmermann and Germain, 2014) (Figure 2).

Integrins are expressed by all mammalian cells except erythrocytes (Hynes, 2002), and integrin ligands are ubiquitously found in essentially every body tissue (Humphries et al., 2006). Accordingly, leukocytes likely deploy both integrin-dependent and -independent migratory strategies during interstitial migration. Intracellular pathogens disseminating via a strategy that relies on the hijacking of migratory host cells could thus target integrin-dependent or - independent strategies to spread through tissues. Conversely, a Trojan horse strategy would require the migrating host cell to perform integrin-dependent TEM. Specifically targeting integrin-independent migration might benefit pathogens by targeting spread to deep tissue or enhancing rates of infected leukocyte entry into collecting lymphatics.

Leukocytes as Trojan horses

Because of the severe clinical consequences of toxoplasmic encephalitis (Montoya and Liesenfeld, 2004), much attention has been paid to the potential for migratory blood leukocytes to deliver *T. gondii* across the BBB and into the CNS.

Murine models indicate that following oral *T. gondii* infection, the vast majority of blood leukocytes that harbor parasites are $CD11b^{+}$ cells (Courret et al., 2006). In mice, $CD11b^{+}$ blood leukocytes include inflammatory $CCR2^{+}$ $Ly6C^{hi}$ monocytes and patrolling $CCR2^{-}$ $Ly6C^{lo}$ CX_3CR1^{hi} monocytes (Geissmann et al., 2003). Whether *T. gondii* preferentially infects either subset remains unknown. However, the predominance of $CD11b^{+}$ cells among parasitized blood leukocytes positions monocytes as the most appealing candidate Trojan horse for delivering *T. gondii* into the CNS. The best *in vivo* evidence supporting a role for infected monocytes in promoting *T. gondii* dissemination is that adoptive transfer of *T. gondii*-infected monocytes into mice hastens dissemination to the brain, when compared to transfer of extracellular parasites. Notably, this result may simply reflect that intracellular parasites enjoy enhanced protection from immune defenses such as complement attack while in the bloodstream. Alternatively, *T. gondii* infection might reprogram monocyte motility to promote dissemination. This idea is suggested by a collection of *in vitro* studies that showed that infection alters the rolling and crawling of monocytes interacting with endothelial monolayers or fibronectin-coated substrates (Cook et al., 2018; Harker et al., 2013; Ueno et al., 2014).

Somewhat perplexingly, these studies reported that infection inhibited integrin-mediated adherence *in vitro*, yet enhanced endothelial crawling in a manner sensitive to antibody blockade of integrin-ligand pairings (Cook et al., 2018; Harker et al., 2013). A separate study reported that *T. gondii* infection transiently increases de-adherence of peritoneal macrophages and J774 macrophages to the integrin ligands fibronectin, laminin, and

collagen IV (Da Gama et al., 2004). Parasite traversal of the BBB within a monocyte Trojan horse would require completion of integrin-dependent TEM. Accordingly, perturbing integrin-mediated adherence in infected monocytes seems an inefficient strategy to advance dissemination across the BBB. One study reported that *T. gondii* infection did not significantly decrease monocyte TEM across a human umbilical vein endothelial cell barrier (Ueno et al., 2014), which suggests that inhibited integrin function is eventually overcome. Another study reported increased prevalence of infected $CD45^+/CD11bc^+$ cells in a pool of rat PBMCs following TEM across a model BBB, indicating that infection might enhance TEM in this system (Lachenmaier et al., 2011). However, we recently demonstrated that *T. gondii* infection profoundly decreased monocyte TEM across several *in vitro* models of peripheral and BBB endothelium (Drewry et al., 2019). Moreover, an *in vivo* analysis showed that *T. gondii*-infected monocytes adoptively transferred into mice were abundantly found in brain vasculature but failed to transmigrate across the BBB and into the brain parenchyma (Konradt et al., 2016). The parasitized monocytes found in the blood of *T. gondii*-infected mice thus do not seem optimally poised to ferry parasites across the BBB.

Hitchhiking with migrating leukocytes

Within hours of ingestion by a host, *T. gondii* encounters and infects a variety of migratory leukocytes in the intestinal lamina propria. The rapid spread of *T. gondii* through and out of the lamina propria compartment may be aided by infected leukocytes acting inadvertently as shuttles that carry intracellular parasites throughout the tissue interstitium, and possibly into lymphatic vessels. Travel of infected leukocytes through lymphatics would then deliver intracellular parasites to secondary lymphoid organs (Figure 1B). Although leukocytes are normally thought of as defenders for the host, by inadvertently promoting pathogen dissemination leukocytes are transformed into unwilling accomplices to parasite infection that enable access to new host niches and shield intracellular parasites from engulfment by tissue resident or recruited phagocytes.

The intestinal lamina propria is heavily populated with CX_3CR1^+ macrophages that are continuously replenished by circulating inflammatory monocytes (Zigmond and Jung, 2013). Hence, following oral infection, *T. gondii* could target these incoming monocytes or the monocyte-derived CX_3CR1^+ macrophages to aid spread through and out of the lamina propria. Supporting this model, we recently showed that *T. gondii* infection enhanced monocyte migration through *in vitro* collagen matrices that model interstitium, and *in vivo* interstitial migration of splenic CX_3CR1^+ monocytes (Drewry et al., 2019). This result contrasts another model where infected neutrophils were theorized to passively promote local parasite spread through the intestinal lamina propria, with infection having no discernable impact on neutrophil motility (Coombes et al., 2013). *T. gondii* enhancement of monocyte and macrophage migration required the secreted parasite kinase ROP17 and was inhibited by blockade of Rho/ROCK signaling (Drewry et al., 2019). The Rho/ROCK-dependence of infected monocyte migration and corresponding inhibition of TEM suggest that *T. gondii* specifically activates integrin-independent migration in monocytes and macrophages (Drewry et al., 2019). Infected monocytes carrying hitchhiking parasites could efficiently navigate the confined spaces of tissue interstitium using integrin-independent

migration, an appealing model given the disrupted integrin functionality observed in infected monocytes.

Extensive evidence suggests that *T. gondii* could also exploit infected DCs to promote tissue spread (Bhandage and Barragan, 2019). DCs are antigen presenting cells that primarily reside in peripheral tissues, where they acquire antigens that they subsequently travel to secondary lymphoid tissues to present to T cells (Randolph et al., 2005). As with monocytes, the best evidence for the ability of DCs to promote *in vivo* dissemination comes from experiments showing that adoptive transfer of *T. gondii*-infected DCs results in unusually rapid parasite dissemination to the brain (Lambert et al., 2006). *T. gondii* infection induces an *in vitro* hypermotility phenotype in DCs that includes enhanced migration across plastic transwell membranes and endothelial monolayers, increased crawling velocity and displacement on two-dimensional surfaces (Lambert et al., 2006), and enhanced migration through three-dimensional collagen matrix (Kanatani et al., 2015). The hypermotility of infected DCs correlates to rapid cytoskeletal changes including integrin redistribution (Weidner et al., 2013), requires GABA receptor signaling to a Cav1.3 voltage-dependent calcium channel (Bhandage and Barragan, 2019; Fuks et al., 2012; Kanatani et al., 2017), and can be induced by heterologous expression of a 14–3-3 protein (Weidner et al., 2016). DCs are not typically found in the blood in large numbers, and thus not surprisingly do not account for a meaningful portion of parasitized leukocytes in the blood during murine *T. gondii* infections (Courret et al., 2006). Accordingly, the DC hypermotility phenotype is most likely to be relevant to promoting parasite tissue dissemination, rather than direct traversal of endothelial barriers.

DCs do not only travel from tissues to lymph nodes via afferent lymphatics, but also exit lymph nodes via efferent lymphatics at low rates (Randolph et al., 2005). This route leads to the bloodstream via the lymphovenous valve (Randolph et al., 2017) (Figure 1B). As such, an infected leukocyte with robustly activated tissue migration could potentially deliver an intracellular parasite through tissues, into lymphatics, through a lymph node, and eventually into the blood stream (Figure 1). The plausibility of this model is supported by observations that *T. gondii*-infected DCs traffic to both mesenteric lymph nodes and the spleen in greater abundance than uninfected or LPS-activated DCs following adoptive transfer via intraperitoneal injection (Lambert et al., 2006). Upon entering the blood circulation, *T. gondii* could then penetrate endothelial barriers such as the BBB by using infected leukocytes as Trojan horses, or egress and directly engage a new niche as an extracellular parasite (Figure 1B).

Translocation through host cell portals

T. gondii traverses multiple biological barriers during natural infections, including the intestinal epithelium, BBB, and potentially the maternal-fetal barrier. *T. gondii* could penetrate these barriers as extracellular parasites taking a paracellular route between epithelial or endothelial cells, or as intracellular parasites carried within leukocyte Trojan horses trafficking across the barrier (Arora et al., 2017; Barragan and Sibley, 2002; Barragan and Sibley, 2003) (Figure 1B). However, recent studies suggest that direct invasion of BBB endothelial cells followed by expansive growth within this cellular compartment may be

critical for successful CNS invasion by *T. gondii*. In this context, it is important to point out that prior studies that concluded that adoptive transfer of monocytes (Courret et al., 2006) or DCs (Lambert et al., 2006) enhanced infection of the brain did not distinguish between parasites present within leukocytes lodged in the vasculature *vs*. parasites that had migrated into the parenchyma (either extracellularly or within a host cell).

Notably, *T. gondii* actively invades into and egresses out of host cells in a process powered by a parasite actin-myosin motor (Drewry and Sibley, 2015; Sibley, 2010). Accordingly, *T. gondii* translocation into and through BBB endothelial cells would probably not be mediated by internalization driven by the host cell cytoskeleton as is observed with bacterial translocation through intestinal M cells (Vazquez-Torres and Fang, 2000). Instead, *T. gondii* may actively invade a barrier cell, replicate within that cell, and subsequently egress out into the brain parenchyma. A BBB invasion and replication model is supported by a study reporting that oral infection of mice with tissue cysts led to detection of replicated parasites within brain vasculature endothelial cells, which was followed by detection of parasite cysts in parenchyma regions near vasculature (Konradt et al., 2016). Extracellular parasites were detected in the blood in roughly equivalent quantity as cell-associated parasites (Konradt et al., 2016), which further supports the idea that extracellular *T. gondii* in the blood could seed CNS infections by directly invading BBB cells. A recent study assessing mice intraperitoneally challenged with *T. gondii* reported that parasite invasion of the brain, but not lung or spleen, was restricted by endothelial expression of a dominant negative EGFR expected to protect parasites from autophagy-mediated killing (Corcino et al., 2019). Intraperitoneal infection is not a physiological infection route for *T. gondii* and may prompt dissemination to progress in a different manner than in a natural infection initiated by oral ingestion of parasite cysts. However, if EGFR-mediated protection of parasites in BBB endothelial cells and enhanced brain burden also occurs following oral cyst inoculation, this would further support the idea that parasite development within BBB endothelial cells is a critical stage in successful parasite invasion of the CNS.

T. gondii may reach portals into new tissues such as lymphatic vessels or the BBB endothelium by migrating while extracellular using a substrate-dependent behavior termed gliding (Sibley, 2010). Alternatively, *T. gondii* could also use infected leukocytes to rapidly travel along standard leukocyte trafficking routes. This model would allow *T. gondii* to exploit hijacked migrating leukocytes as both shuttles for dissemination and shields against other host defenses, such as engulfment by activated phagocytes or complement attack in the blood. Upon reaching a target tissue, intracellular parasites could then egress from their host leukocyte and directly invade target endothelial cells (Figure 1B). It is unclear how parasites would optimize the timing of egress from migrating host leukocytes to occur in desirable locations. However, a recent study did report that adherence of infected leukocytes to endothelium triggered parasite egress from the host leukocytes (Baba et al., 2017).

Conclusions and future directions

The impressive ability of *T. gondii* to spread beyond its initial infection nidus in the gut and reach all host tissues including the CNS poses substantial risk to infected hosts. As part of this process, *T. gondii* infection clearly alters the migration of monocytes and DCs *in vitro*,

which could function to promote tissue dissemination and barrier traversal by co-opting leukocyte migratory capacity. Early investigations into the potential for migrating leukocytes to promote *T. gondii* dissemination speculated that the parasite would exploit leukocytes as Trojan horses across biological barriers such as the BBB (Courret et al., 2006). DCs are not optimal candidates to fill this role, as infected DCs are exceedingly rare in the blood during murine infections (Courret et al., 2006). Blood monocytes are more frequently infected and hence better positioned to ferry parasites across the BBB. However, *T. gondii* infection profoundly impairs the ability of monocytes to transmigrate through *in vitro* endothelial barriers (Drewry et al., 2019). The *in vivo* relevance of this inhibited TEM phenotype is supported by reports that infected monocytes adoptively transferred into mice failed to cross into the brain parenchyma (Konradt et al., 2016). Accordingly, we consider *T. gondii* unlikely to exploit infected leukocytes as true Trojan horses across the BBB. Instead, we suggest that *T. gondii* is more likely to act as a hitchhiker that rides through tissues within infected leukocytes. Migrating infected leukocytes could carry parasites through the lymphatic system and eventually into the blood circulation, where parasites likely egress out of the leukocyte and confront barriers such as the BBB as extracellular parasites.

Future studies should establish more precisely how and when infected leukocytes promote *T. gondii* dissemination during natural infections initiated by oral ingestion of parasite cysts. Monocytes and DCs can clearly promote parasite dissemination when introduced into naïve mice via adoptive transfer (Courret et al., 2006; Lambert et al., 2006), but more work is needed to establish the steps where leukocyte migration aids dissemination during natural oral infections. Discriminating between parasites entering the vascular circulation of the CNS *vs.* crossing into the parenchyma will also be crucial. Recent studies reveal that *T. gondii* infection specifically upregulates interstitial migration of monocytes; however, the mechanism by which this occurs is only partially understood (Drewry et al., 2019). This pathway depends on the secreted parasite kinase ROP17 and host Rho signaling. Further studies are needed to define the substrates of ROP17 that drive this response. Intriguingly, very recent data indicate that ROP17 mediates translocation of several dense granule effector proteins across the parasitophorous vacuole membrane (Panas et al., 2019). Accordingly, ROP17 may promote enhanced migration by directly phosphorylating a host target, such as several known phospho-activated RhoGEFs (Hodge and Ridley, 2016), or by mediating the secretion of another parasite effector. In mice subcutaneously challenged with a hypervirulent type I *T. gondii* strain, *ROP17*-deficient parasites exhibited delayed dissemination kinetics that corresponded with a moderate enhancement of mouse survival (Drewry et al., 2019). Intriguingly, *ROP17* deletion in the related type II *T. gondii* strain *Pru* has been shown to almost completely abrogate formation of brain cysts (Fox et al., 2016). Type II *T. gondii* also exhibit less robust extracellular motility than type I parasites (Barragan and Sibley, 2002). Accordingly, it is appealing to speculate that ROP17-dependent enhanced interstitial migration in infected monocytes and macrophages may be especially pivotal for advancing the dissemination of lowly-motile type II parasites. Monocytes and DCs may also be differentially exploited by *T. gondii* for dissemination purposes, as infection enhances integrin-dependent TEM in DCs (Lambert et al., 2006) but potently blocks TEM in monocytes (Drewry et al., 2019). Determining whether monocytes or DCs advance parasite dissemination at specific stages, or if particular subsets of monocytes or

DCs are especially targeted by disseminating *T. gondii* remain key questions for future study.

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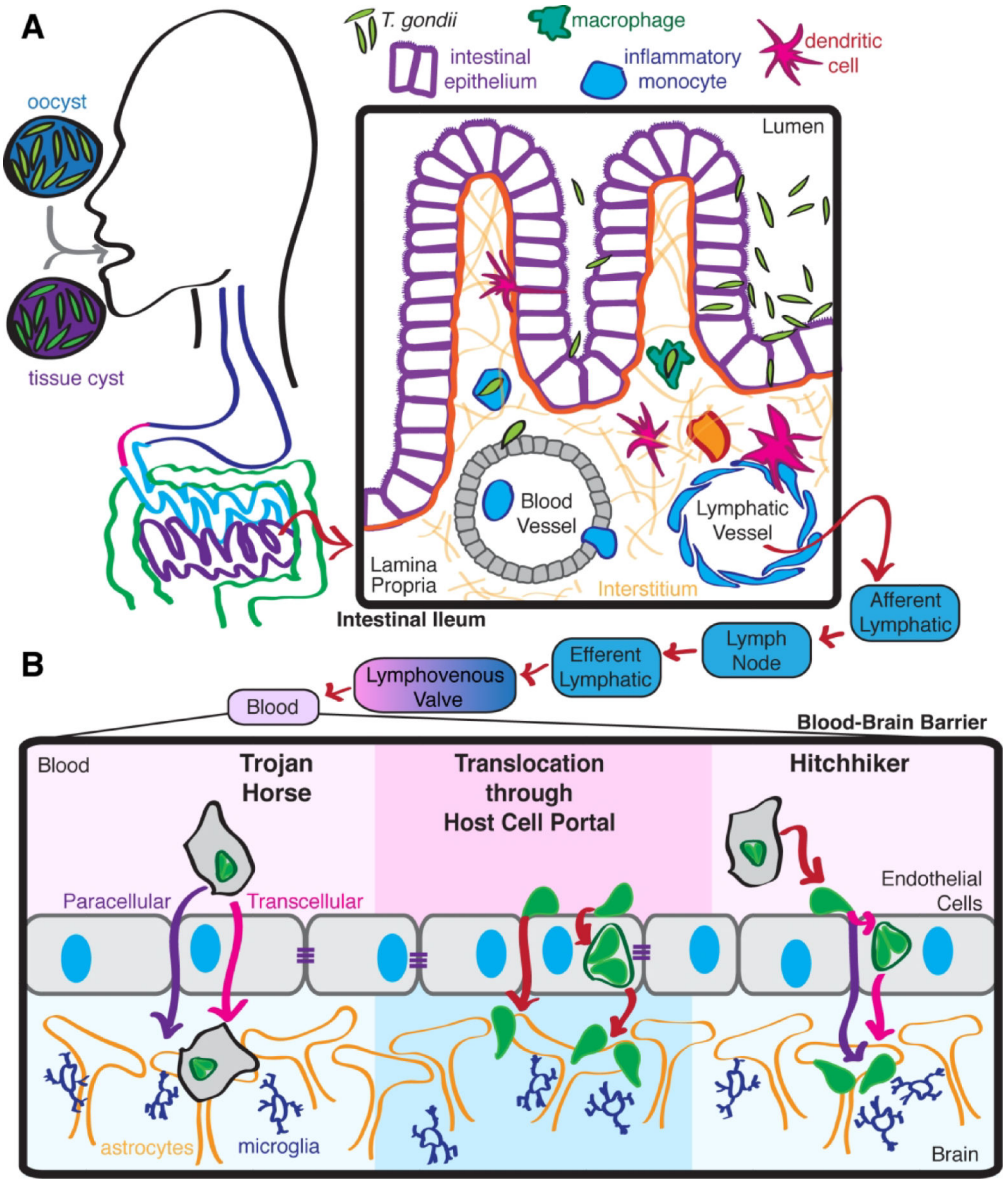

Figure 1. Progression of toxoplasmosis.

(A) *T. gondii* infections are acquired when hosts ingest parasite cysts. Upon reaching the ileum, parasites released from the cysts traverse invade through intestinal epithelium and gain access to the lamina propria. (B) *T. gondii* then spreads systemically. Parasites may invade the CNS when extracellular *T. gondii* directly invade BBB endothelial cells or with the assistance of migratory infected leukocytes using Trojan horse or hitchhiker mechanisms.

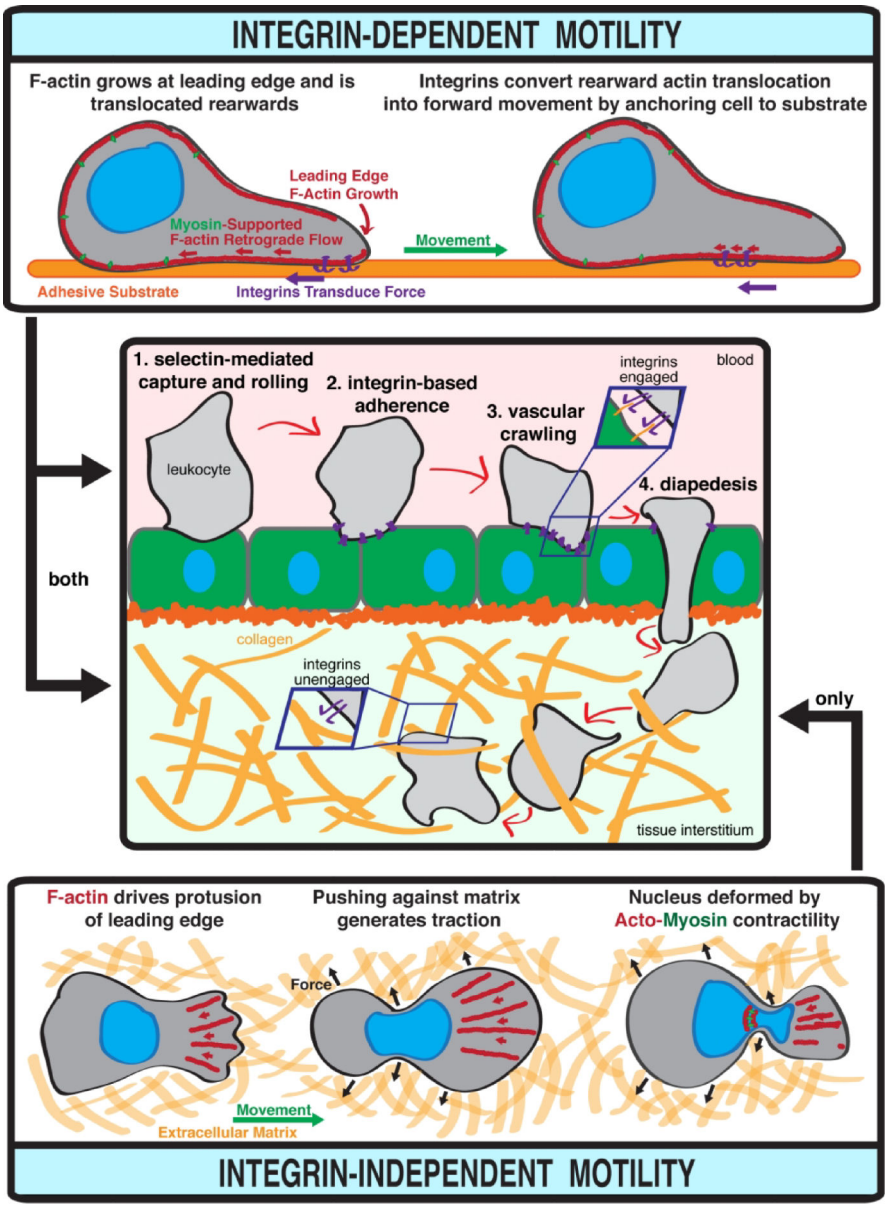

Figure 2. Leukocyte migration.

Integrin-dependent motility enables transendothelial migration of leukocytes from the blood into tissues, and interstitial migration through tissues. Integrin-independent motility only functions in confined three-dimensional spaces such as tissue interstitium.