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The molecular biology and immune control of chronic *Toxoplasma gondii* infection

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Toxoplasma gondii **is an incredibly successful parasite owing in part to its ability to persist within cells for the life of the host. Remarkably, at least 350 host species of** ***T. gondii*** **have been described to date, and it is estimated that 30% of the global human population is chronically infected. The importance of** ***T. gondii*** **in human health was made clear with the first reports of congenital toxoplasmosis in the 1940s. However, the AIDS crisis in the 1980s revealed the prevalence of chronic infection, as patients presented with reactivated chronic toxoplasmosis, underscoring the importance of an intact immune system for parasite control. In the last 40 years, there has been tremendous progress toward understanding the biology of** ***T. gondii*** **infection using rodent models, human cell experimental systems, and clinical data. However, there are still major holes in our understanding of** ***T. gondii*** **biology, including the genes controlling parasite development, the mechanisms of cell-intrinsic immunity to** ***T. gondii*** **in the brain and muscle, and the long-term effects of infection on host homeostasis. The need to better understand the biology of chronic infection is underscored by the recent rise in ocular disease associated with emerging haplotypes of** ***T. gondii*** **and our lack of effective treatments to sterilize chronic infection. This Review discusses the cell types and molecular mediators, both host and parasite, that facilitate persistent** ***T. gondii*** **infection. We highlight the consequences of chronic infection for tissue-specific pathology and identify open questions in this area of host-*** ***Toxoplasma*** **interactions.**

Introduction

Toxoplasma gondii is a single-cell obligate intracellular protozoan parasite acquired by the eating of contaminated foods. Feline species are *T. gondii*'s definitive hosts, meaning that cats facilitate sexual recombination of the parasite and shed millions of highly infectious, environmentally stable oocysts (1). *T. gondii* is unique in its incredibly broad intermediate host range, which includes humans, livestock (sheep and pigs are particularly important for human transmission), birds, and rodents, among others (2). These intermediate hosts support the asexual tachyzoite and bradyzoite tissue cyst forms of the parasite. Mollusks, which concentrate oocysts by filtering contaminated water, are an additional vector for transmission to humans (3). After consumption of bradyzoite tissue cysts or oocysts, *T. gondii* invades the small intestine of its host (4, 5). Recent work from Laura Knoll's laboratory suggests that the parasite may sense linoleic acid in the feline gut as a critical signal for sexual stage differentiation in these species (6). Passing through the cat confers a tremendous benefit to the parasite in terms of genetic diversity and range expansion, and facilitating transmission to cats appears to be a major pressure driving parasite evolution. Given the importance of the predator-prey cycle between rodents and cats, rodents may be a particularly important host for *T. gondii*. As will be discussed, this conclusion is supported by the observations that *T. gondii* expresses a sophisticated cadre of effectors that intersect mouse immune signaling (7–9) and that infected rodents lose their natural aversion to feline urine (10, 11) and can become severely wasted (12–14), all of which may facilitate transmission via predation of a rodent host.

Rates of human *T. gondii* infection range from 10% in the United States to over 50% in France, Colombia, and Brazil (15–17). Acute infection can cause flu-like symptoms; however, immune-competent individuals clear the majority of parasites during acute infection. Surviving parasites persist as slow-growing bradyzoite tissue cysts, most abundant in tissues with limited immune surveillance, including brain, eye, cardiac, and skeletal muscle (18). Contracting *T. gondii* during pregnancy can be lethal to the fetus, which also has a minimal immune system (19). Tissues that were not classically considered "immune privileged" also harbor parasites, based on the observation that transplant recipients of kidney, liver, heart, or lung have contracted toxoplasmosis from an infected donor (20–24). However, chronic infection in these tissues is almost unstudied, as parasite frequency is incredibly low. The immune response to *T. gondii* is sustained throughout chronic infection, and this is evident in elevated *T. gondii*–specific IgG and IFN-γ in the sera, both of which are essential for parasite restriction (25). If the immune system is suppressed during chemotherapy, organ transplant, or AIDS, for example, *T. gondii* can revert to tachyzoite replication (26, 27). This process, known as recrudescence, can be lethal if parasitemia is not controlled with drugs. The most frequently prescribed regimens are pyrimethamine combined with sulfadiazine or clindamycin; trimethoprim in combination with sulfamethoxazole can be used as an alternative (28). However, these antiparasitic treatments are poorly tolerated, and hypersensitivity to sulfa drugs is particularly common. Currently,

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no treatments have been developed that clear tissue cysts, maybe because of the slow growth of bradyzoites, their sequestration within neurons, and/or the difficulty of developing drugs that cross the blood-brain barrier. This is an area of outstanding need, as new haplotypes of *T. gondii* are emerging that associate with severe ocular disease in immune-competent patients (29, 30).

Population genetics of *T. gondii*

In North America and Europe, environmental isolates of *T. gondii* predominantly belong to three major strains or types: type I, type II, and type III. These types are notable in that virulence, measured by lethal dose (LD), differs by several logs in inbred strains of mice (e.g., C57BL/6, CBA/J, BALB/c). Type I is the most virulent (LD_{100} of 1–10 tachyzoites), compared with type II (LD_{50} of 100–1000) and type III (LD_{50} of ~100,000 to 1 million), which are substantially less aggressive in vivo (31, 32). Human infection is dominated by type II in North America and Europe; however, a fourth type, haplogroup 12, was recently isolated from North American patients and wild animals (33). In Asia and Africa, region-specific clonal lineages have also been isolated (34). Strains that do not fit the pattern of clonal lineage expansion have been identified in South America belonging to haplogroups 4 through 15 (35–37). Whole genome sequencing indicates that genomic admixture and recombination between a limited number of ancestral strains account for *T. gondii*'s genetic diversity. The relationship between strains was determined by comparison of the inheritance pattern of large gene haploblocks. These haploblocks encode virulence-associated, secreted parasite effector proteins, suggesting that the unique assortment of effector alleles may also control pathogenesis and/or transmission rates (38). Importantly, there is evidence of coevolution between virulent haplotypes and mouse IFN-inducible immunity-related GTPase (IRG) genes, which are critical mediators of cell-intrinsic parasite killing in mouse models (39, 40).

Immune determinants of dissemination

Activating a robust immune response is critical to both host and parasite survival. Bradyzoite cysts are resistant to peptic proteases, but tachyzoites are not: if the host dies before chronic infection is established, parasite transmission does not occur. In keeping with this paradigm, the parasite has evolved effectors that selectively activate host immune cell signaling in addition to strategies to avoid sterilizing immunity. In acute and chronic infection, *T. gondii* grows and persists within a parasitophorous vacuole membrane (PVM). The PVM is generated from the host plasma membrane as the parasite ratchets its way into the cell using injected parasite effector proteins. This process avoids the lytic environment of the endo/lysosomal compartments and accounts for the parasite's remarkable capacity to infect almost any nucleated cell type in vitro (41). In vivo, however, the cell types harboring the parasite are more limited. After a parasite cyst is ingested, *T. gondii* invades the distal jejunum of the small intestine in mice (42, 43). The precise host cell types mediating invasion (e.g., M cells, epithelial cells) are not clear; however, *T. gondii* sporozoites and tachyzoites have been observed in intestinal epithelial cells (44). Tachyzoites are also observed within infiltrating immune cells (43–45). The CCL2/CCR2 chemokine axis is a conserved mechanism of monocyte recruitment in mice and humans (46). The parasite effectors *Tg*14-3-3 and *Tg*WIP have been shown to promote hypermotility in infected human and murine dendritic cells (47, 48). Dendritic cell hypermotility has been observed in vivo, suggesting that it may be a stealth mechanism that facilitates parasite dissemination while avoiding detection by circulating immune effectors (49, 50).

Cell-autonomous immunity to *T. gondii*

The long evolutionary relationship between *T. gondii* and mammalian hosts is evident in analysis of the pathways infected cells use to detect and destroy the parasite (39). Three main arms of innate immune sensing have been described in *T. gondii* infection: the Toll-like receptors (TLRs), the IFN-inducible GTPases, and the inflammasomes. TLR and IL-1 receptor (IL-1R) signaling through MyD88 is a central mediator of IL-12 secretion and the protective Th1 response to *T. gondii* (51). In mice that are intraperitoneally infected with *T. gondii*, the parasite protein profilin directly binds and activates TLR11, contributing to IL-12 production and parasite restriction (52). However, profilin is an actin-modifying protein sequestered within parasites and TLR11 signals from endosomal compartments, suggesting that this pathway may be mostly activated by phagocytosed, dead, or dysfunctional parasites (Figure 1). Consistent with this model, after oral infection, TLR11-deficient mice had minimal defects in their Th1 response compared with mice deficient in MyD88 or TLR2, TLR4, and TLR9; however, treating TLR11-deficient mice with antibiotics phenocopied MyD88-deficient mice (53, 54). These data indicate that gut commensal microbiota can prime a protective immune response to *T. gondii* independent of parasite recognition by TLR11. It is notable that human TLR11 is a pseudogene, indicating alternative innate sensing mechanisms to detect and destroy *T. gondii* in human cells.

The inflammasome links detection of microbial components or cell damage associated with infection to the release of IL-1 family cytokines and, often, inflammatory cell death. While the inflammasome response to protozoa is understudied in comparison with bacterial and viral pathogens, what is known about *T. gondii* recognition suggests major differences (55, 56). The inflammasome sensors NLRP1 (in mice) and NLRP3 (in mice and humans) have been shown to process and release IL-1β in response to *T. gondii* infection (Figure 1 and refs. 57–59). Mice deficient in NLRP3, caspase-1, and/or caspase-11 have a higher parasite burden in vivo (57, 58, 60). However, unlike better-studied inflammasome triggers (e.g., the NLRP1 protease anthrax lethal toxin or bacterial pathogens that activate NLRP3), pyroptotic host cell death is not observed in the mouse or human cells (57–59). Unlike murine macrophages, the NLRP3 inflammasome in human monocytes is activated independent of the TLR pathway via Syk and CARD9 signaling; and IL-1β release is independent of the pore-forming gasdermin D (59, 61–63). Open questions in the molecular mechanism of the inflammasome response to *T. gondii* include what parasite signals activate the inflammasome, why proptosis is not engaged, and whether this is the result of active parasite manipulation.

Recent data also suggest crosstalk between the inflammasome and the IFN-inducible GTPases, a pathway that surveys the cell for foreign or damaged membranes and targets them for clearance downstream of IFN-γ. In human cells the dynamin-superfamily guanylate-binding protein 1 (GBP1) localizes to the PVM; this triggers release of parasite DNA into the host cell cytosol, where it is detected

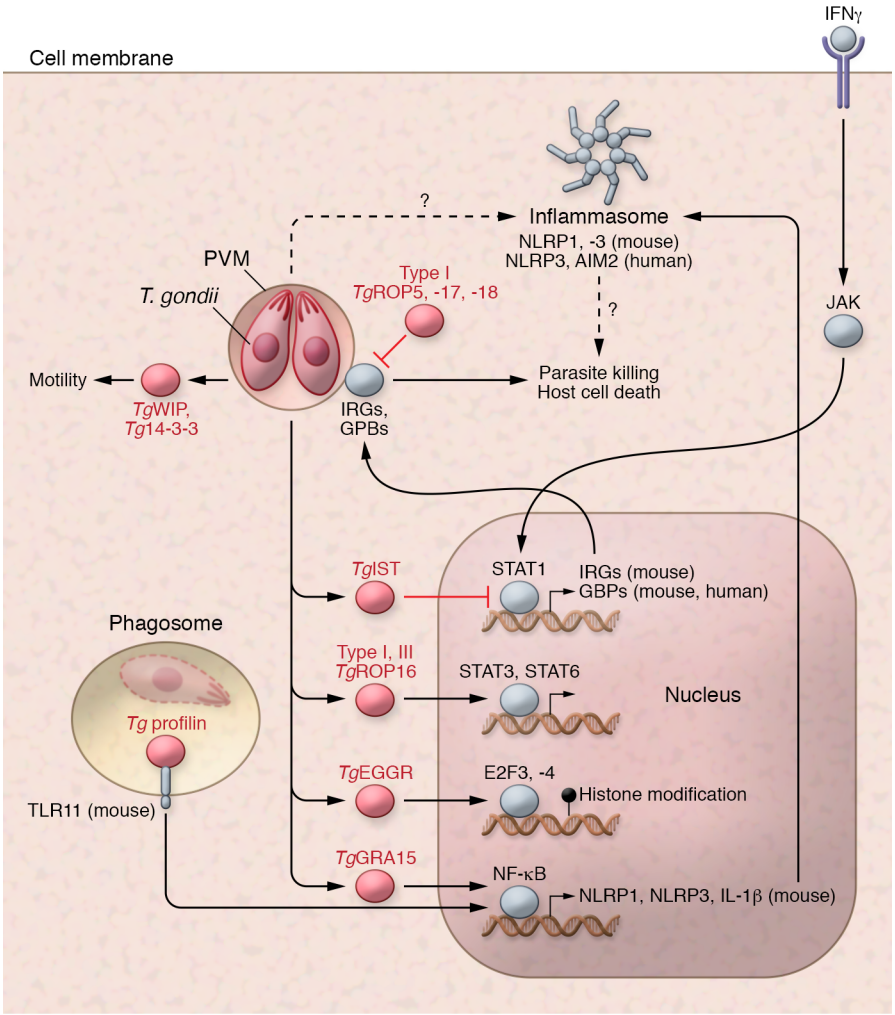

Figure 1. Innate immune signaling and the influence of parasite effectors. *T. gondii* grows within a parasitophorous vacuole membrane (PVM) that protects the parasite from cytosolic immune sensors and avoids fusion with the endolysosomal compartments containing Toll-like receptors (TLRs). In the mouse, TLR11 recognizes *Tg* profilin, an actin-modifying protein that is exposed once dead or damaged parasites are phagocytosed. TLR11 is a pseudogene in humans. *Tg*GRA15 can promote host NF-κB phosphorylation and nuclear translocation. In mice, NF-κB stimulation is necessary for transcriptional regulation of the inflammasome components NLRP1, NLRP3, and IL-1; however, human monocytes can engage an NLRP3 inflammasome independent of NF-κB prestimulation. A detailed mechanism of inflammasome activation, parasite killing, and host cell death remains elusive, particularly in regard to signal integration with IFN-γ. IFN-γ signaling induces STAT1 translocation to the nucleus and upregulation of IFN-responsive genes, including immunity-related GTPases (IRGs, mouse) and guanylate-binding proteins (GBPs, human and mouse), which functions to attack parasite vacuole, leading to parasite killing and host cell death. In human cells, GBP1 is necessary for this process, which leads to AIM2 activation of an alternative apoptosis pathway. The type I parasite rhoptry proteins, *Tg*ROP5, 17, and 18, can dismantle the function of the mouse IRGs IRGa6 and IRGb6 at the PVM, inactivating GBP attack and parasite killing. The parasite dense granule effector *Tg*IST is a nuclear repressor of STAT1 transcription. *Tg*ROP16 is a kinase that phosphorylates and activates host STAT3 and STAT6. *Tg*EGGR affects host gene expression through E2F3- and E2F4-mediated epigenetic modifications. In infected monocytes and dendritic cells (DCs), *Tg*WIP and 14-3-3 proteins promote cell mobility, a putative mechanism of intracellular parasite dissemination in vivo.

by the inflammasome sensor AIM2 (Figure 1 and refs. 63, 64). For reasons that are still unclear, a pyroptotic inflammasome response is not engaged; instead, an alternative apoptotic pathway of host cell death is activated (63, 64). Unlike the human system, mice rely on an expanded family of p47 IRGs to detect the parasite vacuole downstream of IFN-γ. Mouse IRGM1 and IRGM3 regulate the interaction between IRGa6, IRGb6, and phospholipids at the PVM (65). A broader range of mouse GBPs have been implicated in *T. gondii* clearance; however, the mechanism of parasite killing and host cell death in mouse cells is not known (66–68). The importance of the IRG system in parasite clearance is underscored by the observation that type I parasites express a triad of secreted effectors, rhoptry protein 5 (ROP5), ROP17, and ROP18, which bind to and inactivate the GTPase function of IRGa6 and IRGb6 (69, 70). This inactivation is a major mechanism of type-specific virulence, as type II and type III parasites express alleles of *Rop5* or *Rop18*, respectively, that cannot effectively subvert IRG attack. Although there has been tremendous progress toward identifying the classes of cell-autonomous immune signaling in response to *T. gondii*, the field lacks an integrated model of cell-autonomous sensing across these pathways for both mouse and human systems, particularly in cell types other than fibroblast, monocyte, and macrophage.

Innate instruction of adaptive immunity

T. gondii recognition by innate immune sensors triggers a Th1-polarized, $CD8^+$ T cell–dependent immune response that is necessary for host survival. There are many excellent reviews on the immunobiology of infection (7, 8, 71, 72), so we will touch briefly on aspects of the acute immune response that are necessary for the progression to chronic infection. Mice deficient in IL-12, TNF-α, and IFN-γ or their signaling pathways die of parasite overgrowth in acute infection (73–76). IFN-γ and IL-12 deficiency is rare in humans and has not been correlated with increased susceptibility to toxoplasmosis; however, monocyte-derived macrophages from *IFNGR1*-deficient patients fail to restrict *T. gondii* after IFN-γ stimulation compared with healthy-donor macrophages (77, 78). Mice deficient in the IL-6 pathway fail to mount a protective B cell response and die in early chronic infection (79). IL-10 and regulatory T cells play an equally important role in host survival by limiting the magnitude of the inflammatory response and bystander damage (80–85).

A growing number of *T. gondii* effectors have been identified that are secreted into the host cell to control immune signaling. These effectors are released from secretory organelles known as the rhoptries (ROP) and the dense granules (GRA), and many of

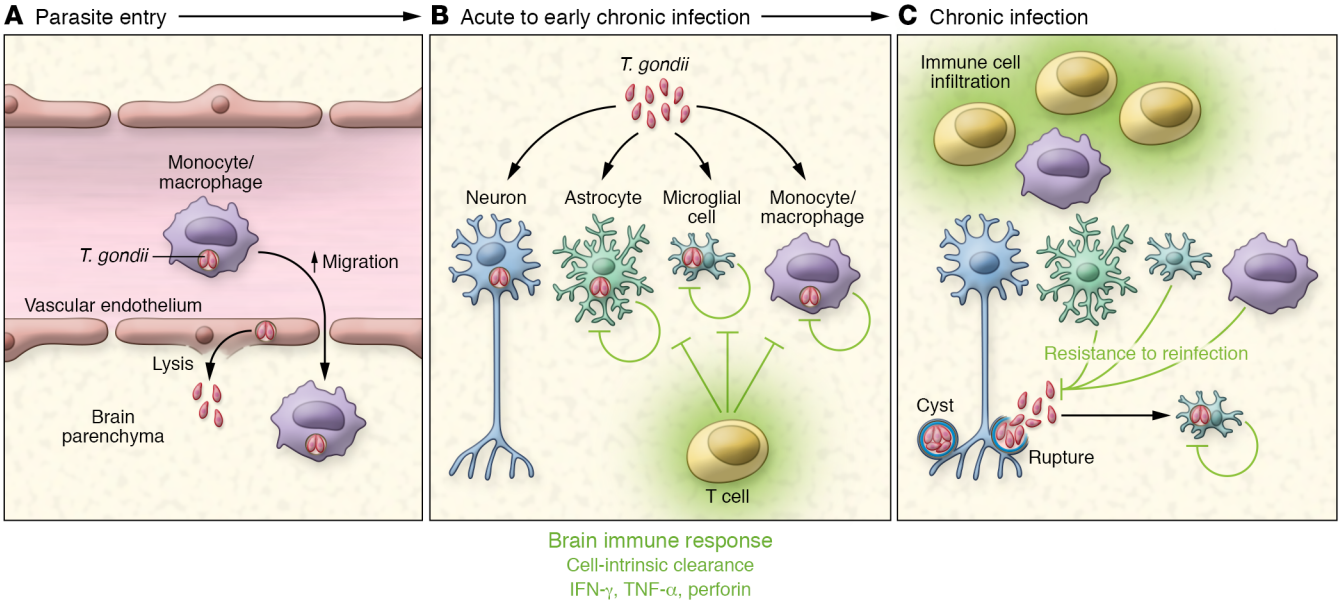

Figure 2. *T. gondii* entry and control of persistent infection in the brain. (**A**) In acute infection, *T. gondii* is frequently observed in immune cells, including monocytes and dendritic cells, with hypermigratory behavior. During infection, blood-brain barrier (BBB) permeability increases and monocytes accumulate in the endothelial lumen, interacting with endothelial cells. These observations have led to the hypothesis that migratory immune cells deliver *T. gondii* to the BBB and, perhaps, smuggle them into the brain. Replicating parasites are also observed in brain endothelial cells, whose subsequent lysis may be a mechanism of *T. gondii* entry into the brain. (**B**) During acute infection parasites are observed infecting neurons, astrocytes, microglia, and infiltrating immune cells. Astrocytes and microglia as well as peripheral monocytes can clear parasites with cell-autonomous immune pathways. (**C**) As chronic infection progresses, infected astrocytes and microglia or the parasites within them are cleared and cysts are primarily observed within neurons. Most parasite cysts are not associated with immune infiltrate; however, individual parasites or parasite debris can be observed colocalizing with immune infiltrate.

the effectors are polymorphic across strains and play a role in virulence (Figure 1). GRA15 activates NF-κB, and GRA24 activates the p38 MAPK pathway to promote expression of IL-12 and IL-18, the upstream regulators of IFN-γ and T cell activation (86, 87). ROP16 is a serine-threonine kinase that directly phosphorylates STAT3 and STAT6 and dampens IL-12 production, which may be consistent with the concept that fine-tuning immune response is necessary for host survival and parasite transmission (88, 89). However, the effector *Tg*IST was recently identified as an inhibitor of IFN receptor signaling. *Tg*IST binds STAT1 and forms an inhibitory complex with the nucleosome remodeling deacetylase (Mi-2/NuRD) complex. This suppresses transcription of IRF1-dependent cytokines, MHC class II expression and antigen presentation, and inducible nitric oxide synthase (iNOS) expression, which kills parasites by producing reactive nitrogen species (90, 91). Similarly, *Tg*TEEGR interacts with E2F3 and E2F4 transcription factors, and forms a nuclear complex with a catalytic subunit of polycomb repressor complex to block NF-κB–mediated expression of proinflammatory cytokines like IL-1β and IL-6 (92). These effectors are among 200–300 predicted secreted effector proteins in the parasite genome, the majority of which have not been characterized, particularly in the context of chronic infection.

Parasite entry to the brain

In chronic infection, the central nervous system contains the highest frequency of parasites per gram of tissue. The potential implications of neural infection for host behavior and homeostasis have led to great interest in understanding the biology of *T. gondii* infection in the brain. Our understanding of chronic central nervous system infection is almost exclusively based on murine models of infection. There are many open questions, beginning with how the parasite traverses the blood-brain barrier (BBB). Using intravital microscopy, *T. gondii* has been imaged replicating within brain endothelial cells and then directly entering the brain (93). Mice infected intravenously with the *T. gondii* RH strain had a higher brain parasite load than mice infected with the CPS strain, which cannot replicate in vivo, suggesting that *T. gondii* growth within vascular endothelial cells may be an important stopover before direct entry into the brain (Figure 2A and ref. 93). Perfusion of Evans blue dye shows increased BBB permeability during chronic *T. gondii* infection, accompanied by reduced blood flow and capillary rarefication which may permit immune cell entry into the brain (94). Using intravital microscopy, $CCR2^+$ monocytes are found to accumulate, exhibiting rolling and cradling behavior at the BBB (95). This observation, coupled with the high frequency of infection of dendritic cells and their hypermotility phenotype, has led to the Trojan horse hypothesis: that parasites traverse the BBB within immune cells (Figure 2A and refs. 49, 96). Although direct evidence for this model is lacking, antibody depletion of $CD11b^+$ leukocytes correlated with reduced brain parasite load; and adoptive transfer of *T. gondii*–infected $CD11c^+$ or $CD11b^+$ cells into naive mice led to neural infection (97).

Chronic infection in the central nervous system

Analysis of mouse brain sections and an extremely limited number of healthy human brain samples indicates that most intracellular

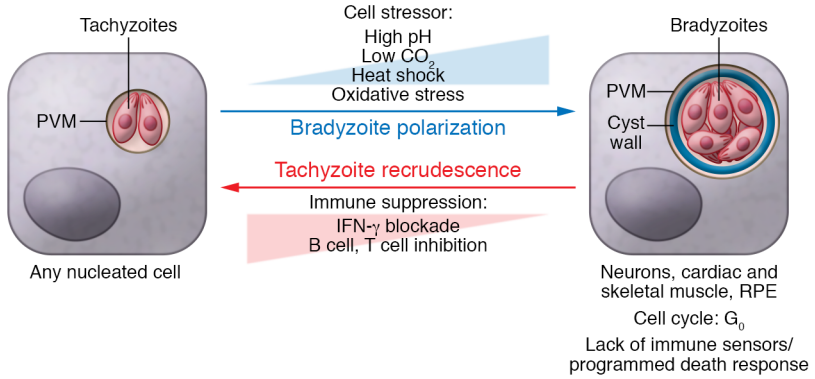

Figure 3. Environmental and host cell–specific pressures driving the *T. gondii* tachyzoite to bradyzoite transition. Left: *T. gondii* tachyzoites can invade almost any nucleated host cell type and grow within the PVM formed from host plasma membrane. In vitro, a range of tissue culture stress conditions can upregulate bradyzoite-specific genes. As parasites polarize to a bradyzoite transcriptional profile, they synthesize a heavily glycosylated cyst wall beneath the PVM. The frequency and rate of bradyzoite differentiation are also influenced by the host cell type, cell cycle status, the host cell lifespan, and inflammatory signals in vitro. In vivo, cysts are most frequently observed in neurons, cardiac muscle, skeletal muscle, and retinal pigment epithelial cells. If the host is immune-suppressed, parasites shift toward a replicative tachyzoite form in a process referred to as recrudescence, which is associated with tissue damage, particularly in the eye.

cysts are not associated with immune infiltration (98). However, within the same brain section, inflammatory foci can be observed containing parasites or parasite debris, activated microglia, macrophages, and T cells (99). Depleting IFN-γ or $CD4^+$ and $CD8^+$ T cells leads to parasite recrudescence (100). Taken together these data suggest that intracellular cysts are relatively immunologically silent; however, cysts that lyse (spontaneously or through recrudescence) are recognized and quickly contained by infiltrating immune cells (Figure 2C).

Experiments using parasites engineered to secrete Cre recombinase in Cre reporter mice have demonstrated that neurons are the major cell type interacting with the parasite in the brain, although T cells, monocytes or macrophages, microglia, and astrocytes are reporter-positive early in brain infection (Figure 2B and refs. 101–103). These data also suggest that rather than having a tropism for neurons, *T. gondii* is cleared from non-neuronal cell types in the brain. Consistent with this model, disabling the IFN-γ signaling in mouse astrocytes by knocking out the transcription factor STAT1 led to greater incidence of cysts within astrocytes (104); and IFN-γ depletion increased the percentage of infected astrocytes (102). Subsequently, the ability of mouse astrocytes to restrict *T. gondii* growth in response to IFN-γ was shown to depend on IRGM3 (IGTP), not iNOS; IRGM3 and IRGa6 disrupted the PVM and, in one study, led to parasite egress (105–107). In human astrocytes, IL-1β in combination with IFN-γ induced iNOS-dependent killing of *T. gondii* (108), whereas TNF- and IFN-γ limited *T. gondii* growth by tryptophan starvation via upregulated indoleamine 2,3-dioxygenase (IDO) (109). The parasite effector *Tg*GRA15 has been shown to limit IDO-mediated parasite restriction in cultured glioblastoma and neuroblastoma cell lines (110). IFN-γ in combination with TNF-α or LPS has also been shown to activate parasite killing functions of human and murine microglia through iNOS-dependent and -independent mechanisms (111–113). Microglia can also produce IFN-γ and TNF-α, which are critical for central nervous system restriction of the infection (114); IFN-γ, in particular, has been shown to induce adhesion molecule expression on vascular endothelial cells and promote the expression of CXCL9, CXCL10, and CCL5, which recruit peripheral immune cells to the brain (115, 116). Most of these data are from in vitro experiments, and better tools to study microglia and astrocyte function in vivo will be important to clarify which pathways control central nervous system infection.

While brain-resident immune cells contribute to *T. gondii* restriction, the role of cell-autonomous immunity in neurons is less clear. A recent study using OVA-expressing parasites and conditional MHC class I–deficient mice demonstrated that neurons can present *T. gondii*–derived antigens to initiate a $CD8^+$ T cell response (117); however, whether endogenous parasite epitopes are efficiently presented on neurons is yet to be examined. Brain-infiltrating $CD8^+$ T cells have been shown to control cyst burden indirectly through IFN-γ secretion, and, to a lesser extent, via perforin-dependent killing of infected cells (118–120). It is notable that perforin has been shown to trigger parasite egress in vitro, suggesting that perforin may limit parasite growth but other cells are responsible for parasite killing, potentially through cell-autonomous immunity (121). A minimal reliance on perforin-mediated *T. gondii* clearance also fits a model wherein cellular cytotoxicity should be limited in the brain to promote survival of neurons, which have an extremely limited regenerative capacity.

Parasite determinants of cyst formation and chronic infection

Currently there are no therapeutic tools that effectively target bradyzoite cysts and sterilize chronic infection. Our understanding of bradyzoite biology is weaker than our understanding of tachyzoite biology. This is linked to long-standing technical challenges associated with genetic manipulation of bradyzoite-specific genes that are required to perform "necessary and sufficient" experiments. However, the recent bloom in CRISPR/Cas9 tools has led to gains in this arena (122, 123).

The transition between tachyzoite and bradyzoite has commonly been referred to as "switching"; however, recent studies suggest that stage conversion is a continuum under epigenetic and transcriptional regulation rather than a finite life stage. Bradyzoite polarization can be induced by cell stressors including alkaline media, heat shock, and oxidative stress (refs. 124–126 and Figure 3). IFN-γ treatment has been shown to induce bradyzoite gene expression in infected macrophages but not fibroblasts, suggesting that cell type–specific differentiation signals may also exist (127). Compared with fibroblasts, infected neuronal or skeletal muscle cells support a stronger expression of bradyzoite markers and a higher frequency of cyst development (128). It is worth

noting that neurons and muscle cell types are historically difficult to culture, suggesting that cell stress signals may be relevant to bradyzoite development in these models. However, terminally differentiated myotubes are reported to support a higher frequency of bradyzoites compared with dividing myoblast progenitor cells, suggesting that cell cycle may provide developmental cues for the parasite development as well (129).

Although the precise signals are unclear, histone methylation and acetylation are important epigenetic regulators of bradyzoite differentiation. Treating tachyzoites with arginine methyltransferase inhibitor, AMI-I, induces a reduction of histone H3R17 methylation and bradyzoite differentiation in vitro (130). The *T. gondii* histone acetyltransferase *Tg*GCN5a is enriched at promoter regions of bradyzoite-specific genes, and *Tg*GCN5a-deficient parasites fail to upregulate the bradyzoite markers *Bag1* and *Ldh2* under stress (131). Treating infected cells with the histone deacetylase inhibitor FR235222 induces bradyzoite differentiation through inhibiting *Tg*HDAC3 (132). Phosphorylation of the *T. gondii* eukaryotic initiation factor 2 α subunit (*Tg*eIF2α) is enhanced under stress conditions and is necessary for bradyzoite differentiation (133). Guanabenz, an eIF2α dephosphorylation inhibitor, has been shown to impair tachyzoite proliferation and promote bradyzoite differentiation in vitro (134).

The ApiAP2 family of transcription factors are emerging as central regulators of bradyzoite differentiation. This family consists of 67 genes, many of which are associated with bradyzoite stage–specific expression. Specifically, AP2XI-4– and AP2IV-3–knockout parasites have reduced expression of bradyzoite-specific genes after in vitro switch; and AP2XI-4–null *T. gondii* forms fewer cysts in mice (135, 136). AP2IV-4 knockouts express some bradyzoite-specific genes under tachyzoite culture, but had fewer brain cysts in mice (137). Using a CRISPR/Cas9 guide RNA library targeting mostly AP2 domain–containing proteins and predicted nucleic acid–binding proteins, bradyzoite formation deficient 1 (BFD1), a Myb-like transcription factor, was recently identified as a key regulator of bradyzoite differentiation in vitro and in vivo in mice. Interestingly, *Bfd1* mRNA is expressed in tachyzoites; however, protein expression is only induced by stress conditions (138). It remains to be seen whether immunosuppression induces any parasite recrudescence in mice infected with BFD1-deficient parasites and how BFD1- and AP2-family proteins coordinate bradyzoite differentiation.

T. gondii bradyzoite cysts are often defined by formation of a cyst wall consisting of heavily glycosylated proteins underneath the PVM (139). The cyst wall is essential for transmission, protecting the parasite from gastric proteases and the low pH of the stomach. Parasites deficient in the cyst wall–localized bradyzoite pseudokinase 1 (BPK1) were more sensitive to pepsin digestion and less orally infectious than WT parasites (140). Parasites that were rendered genetically deficient in cyst glycoproteins, including loss of the nucleotide-sugar transporter *Tg*NST1 or the heavily glycosylated cyst wall protein *Tg*CST1, have defects in cyst number, cyst stability, and infectivity during oral infection (141–143). The cyst wall may also protect bradyzoites from enzymatic attack during chronic infection. The Wilson laboratory demonstrated that chitinase-expressing, alternatively activated (M2) macrophages were able to recognize and degrade chitin-like polysaccharides in the cyst wall (144). Consistent with this observation, a GWAS identified single-nucleotide polymorphisms in the intergenic region of the human CHIA locus, which expresses chitinase, that were significantly associated with *T. gondii* infection (145).

Ocular toxoplasmosis

T. gondii infection is the most frequent cause of posterior uveitis, also referred to as chorioretinitis or inflammation of the retina and choroid (pigmented vascular coat of the eye) (30). This is one area of *T. gondii* infection that has been more extensively studied in patients than in animal models, which have been limited until recently. Type II strains, most frequently associated with infection in Europe and North America, are associated with chorioretinitis (146, 147). Historically, ocular toxoplasmosis was associated with congenital infection; however, rates of disease associated with postnatal infection are rising and associated with new *T. gondii* strains (148). Over 70% of patients presenting with acute ocular toxoplasmosis already have ocular scars, suggesting that disease progression is driven by the inflammatory response to recrudescent *T. gondii* leading to the accumulation of tissue damage over time (149). Immune-competent individuals are able to control ocular infection, but early antiparasitic treatment is critical to limit the extent of retinal damage (150). Human retinal vascular endothelial cells are more sensitive to infection than other endothelial cell types, suggesting a potential mechanism of entry into the eye (151). *T. gondii* cysts have been observed in retinal pigmented epithelial cells (152). In a mouse model of ocular toxoplasmosis, retinal pigment epithelial cells and infiltrating immune cells expressed the T cell inhibitory ligand PD-L1 (153). This may be an important mechanism to limit tissue pathology, although the parasites may exploit this axis for persistence. IFN-γ and IL-6, which are both critical in restricting systematic parasitemia (79, 100), were elevated in the vitreous humor of mice with ocular lesions. However, intraocular injection of an IFN-γ–blocking antibody impaired parasite control and worsened tissue damage, while, perhaps counterintuitively, injection of an IL-6–blocking antibody improved parasite control and minimized ocular damage (154–156). Patients infected with virulent South American haplotypes of *T. gondii*, which have been associated with aggressive chorioretinitis, had less IFN-γ and IL-17 but higher IL-13 and IL-6 levels in the eye compared with European patients infected with virulent type I (157). However, it is currently not clear whether these differences in immune regulation control ocular disease severity.

Behavioral and metabolic changes of chronic *T. gondii* infection

In mice and rats, infection with *T. gondii* leads to a well-established loss of innate aversion behavior to felines, which has been proposed to benefit the parasite by facilitating transmission via predation (11, 158, 159). Whether these behavioral phenotypes are driven by specific changes in neural activity or a more general effect of inflammation is an open question. The observation that *T. gondii* expresses two aromatic amino acid hydrolases that produce L-DOPA, AAH1, and AAH2 led to the hypothesis that the parasite could modulate dopaminergic neuron function. However, deletion of AAH2 failed to alter brain dopamine levels, neuroinflam-

mation, or behavioral alterations in *T. gondii*–infected mice (160, 161), although these genes are necessary for oocyst development in the cat (162). Notably, mice infected with an avirulent mutant of type I *T. gondii* or the related organism *Neospora caninum*, which are cleared before establishing chronic infection, exhibit loss of aversion behavior even though chronic infection is not sustained (163). These data suggest that acute inflammation may be sufficient to trigger sustained behavioral changes, although the molecular bases for behavioral changes in *T. gondii* infection are unclear. Recently, the olfactory GPCR trace amine-associated receptor 4 (TAAR4) was shown to recognize 2-phenylethylamine, a metabolite enriched in urine of predators, including feline species. There is no homolog of TAAR4 in humans, but mice deficient in TAAR4 do not engage in avoidance behavior to bobcat and mountain lion urine (164). That the olfactory neurons expressing TAAR4 are altered or damaged during *T. gondii* infection is a compelling hypothesis that remains to be tested.

Sustained interaction with the immune system is a hallmark of *T. gondii* infection: throughout chronic infection humans and mice have high titers of *T. gondii*–specific IgG and sera cytokines. There is growing evidence that *T. gondii* infection is associated with cachexia in mice, an immune-metabolic disease of sustained muscle wasting. Cachexia positively correlates with parasite load and inflammation severity; however, hypermetabolic weight loss cannot be rescued by diet supplementation (13, 165–167). In oral infection, intestinal barrier inflammation resolves during chronic infection, but commensal dysbiosis does not (14, 168); however, dysbiosis is not sufficient for cachexia, as uninfected cage mates experienced a similar microbial shift but did not develop cachexia (14). Chronically infected mice have sustained changes in splenic and lymph node architecture and are more susceptible to acute viral challenge (169). Moreover, cachectic mice were more susceptible to LPS challenge than mice that recovered weight (170). Recently, mice deficient in the IL-1R axis were shown to recover from acute cachectic weight loss, although chronic parasite burden was similar to that in wild-type mice (171). A study from the Wohlfert laboratory showed that infection-induced myositis could be reversed by depletion of regulatory T cells, which were enriched in skeletal muscle (172). Parasite biology that promotes behavior modification and cachexia in rodent hosts may provide a selective advantage to *T. gondii* by increasing the likelihood of predation and transmission to feline hosts. It is important to note that there is currently no evidence of cachexia in immune-competent humans with chronic *T. gondii* infection. However, cachexia is a predictor of mortality in almost every chronic human disease with limited experimental tools to probe sustained disease. The interaction between *T. gondii* and mice is proving an informative model to understand the pathophysiology of cachexia, which can be applied to understand other disease settings.

Conclusions and future directions

T. gondii's ability to establish a persistent chronic infection is essential for parasite transmission. However, there is much to learn about this stage of infection in animal and human hosts. Deep sequencing has unraveled a far greater diversity in *T. gondii* gene assortment than originally thought, which has opened the door to understanding how parasite genetics influences pathology associated with chronic infection. CRISPR/Cas9 tools are expanding our ability to manipulate the *T. gondii* genome to understand how gene expression in bradyzoites controls differentiation, cyst stability, and oral infectivity of the parasite. Bradyzoite biology is intimately linked to the immune response during chronic infection. The coming decades will likely reveal mechanisms of cell-autonomous immunity to chronic *T. gondii* infection in the brain and other chronically infected tissues, as well as reveal the costs of the chronic inflammatory response for host homeostasis. A better understanding of this biology is needed to develop therapeutic strategies that effectively target bradyzoite cysts. Given the long evolutionary relationship between mammalian hosts and *T. gondii*, such studies are likely to discover important information about the regulation of immune functions during chronic inflammation more broadly.

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