Targeted Disruption of TgPhIL1 in Toxoplasma gondii Results in Altered Parasite Morphology and Fitness

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Abstract

The inner membrane complex (IMC), a series of flattened vesicles at the periphery of apicomplexan parasites, is thought to be important for parasite shape, motility and replication, but few of the IMC proteins that function in these processes have been identified. TgPhIL1, a Toxoplasma gondii protein that was previously identified through photosensitized labeling with 5-[125I] iodonapthaline-1-azide, associates with the IMC and/or underlying cytoskeleton and is concentrated at the apical end of the parasite. Orthologs of TgPhIL1 are found in other apicomplexans, but the function of this conserved protein family is unknown. As a first step towards determining the function of TgPhIL1 and its orthologs, we generated a T. gondii parasite line in which the single copy of TgPhIL1 was disrupted by homologous recombination. The TgPhIL1 knockout parasites have a distinctly different morphology than wild-type parasites, and normal shape is restored in the knockout background after genetic supplementation with the wild-type allele. The knockout parasites are outcompeted in culture by parasites expressing functional TgPhIL1, and they generate a reduced parasite load in the spleen and liver of infected mice. These findings demonstrate a role for TgPhIL1 in the morphology, growth and fitness of T. gondii tachyzoites.

Introduction

The Phylum Apicomplexa contains a number of medically important parasites including Cryptosporidium spp., which cause diarrheal illness in children and immunocompromised patients, Plasmodium spp., which are the causative agents of malaria, and Toxoplasma gondii, which causes life-threatening disease in immunocompromised people and the developing fetus. Other apicomplexan organisms, including Neospora caninum and Eimeria spp., cause severe disease in domestic animals. Despite the significant morbidity, mortality and economic loss caused by these pathogens, information is lacking regarding many aspects of their basic biology. T. gondii is a powerful model system for studying conserved aspects of apicomplexan biology, due to the ease with which T. gondii can be cultured and genetically manipulated [1,2].

Apicomplexan parasites contain a number of unusual subcellular structures and organelles [3,4], including the inner membrane complex (IMC), a series of flattened vesicles that are tightly apposed to the cytosolic face of the plasma membrane [5,6]. Together, the plasma membrane and IMC are referred to as the pellicle. Underlying the IMC is the subpellicular network, a meshwork of intermediate filament-like proteins [3,7,8]. Additional cytoskeletal structures associated with the pellicle include the conoid, which is a cone-shaped structure composed of a novel polymeric form of tubulin [9], a pair of polar rings located at either end of the conoid [9,10], and 22 microtubules radiating posteriorly from the lower polar ring [10].

Various functions for the IMC, subpellicular network, and subpellicular microtubules have been proposed [3,4,8,11]. During parasite replication, a process known as endodyogeny, the IMC forms early and may provide a scaffold upon which daughter parasites are assembled, as well as mechanical stability for the mature tachyzoites [3,7,12]. Additionally, some components of the myosin motor complex, the proteins that provide the mechanical force for parasite motility and host cell invasion, are anchored in the IMC. Localization of the motor complex between the outer membrane of the IMC and the plasma membrane is thought to be critical for motility and invasion [13–15]. While one member of the myosin motor complex, GAP43, appears to physically connect the IMC to the plasma membrane [16], and the recently identified GAPM proteins may function to anchor the subpellicular network to the flattened IMC vesicles [17], the detailed mechanism(s) by which the IMC, subpellicular network and subpellicular microtubules associate with each other and are organized at the parasite periphery have yet to be elucidated.

TgPhIL1 (Photosensitized INA-Labeled protein 1) was previously identified through photosensitized labeling with 5-[125I] iodonapthaline-1-azide (INA) and localized to the parasite periphery, concentrated at the apical end directly posterior to the conoid [18]. TgPhIL1 is highly conserved among members of the Phylum Apicomplexa but shows little homology to proteins outside of the phylum. When parasites were treated with Clostridium septicum alpha-toxin, which causes the plasma membrane to swell away from the IMC [19], TgPhIL1 remained

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associated with the IMC and/or associated cytoskeletal structures [18]. Although TgPhIL1 labeling with INA suggests this protein is membrane associated, TgPhIL1 is also highly insoluble and extracts much like a cytoskeletal protein [18].

In order to learn more about the function of TgPhIL1, we generated parasites containing a TgPhIL1 deletion. Phenotypic analysis of the knockout parasites revealed that TgPhIL1 is necessary for maintaining the distinctive shape of T. gondii tachyzoites, and parasites lacking TgPhIL1 show both a growth defect in culture and reduced fitness in a mouse model of infection.

Materials and Methods

Ethics statement
All mouse experiments were performed with approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania (Protocol # 801490; "Regulation of the early response to Toxoplasma gondii").

Culture of parasites
Wild-type RH strain T. gondii was maintained by serial passage in confluent primary human foreskin fibroblast (HFF) cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% (vol/vol) heat inactivated fetal bovine serum (FBS), 10 units/ml penicillin G, 10 μg/ml streptomycin sulfate, and 10 mM HEPES buffer, as previously described [20].

Generation of the TgPhIL1 knockout parasite line (RH/TgPhIL1)
All PCR reactions were performed with Pfx polymerase (Invitrogen, Carlsbad CA) unless otherwise indicated. All primers were synthesized by Sigma Genosys (The Woodlands TX) and restriction enzymes were purchased from either New England BioLabs (Ipswich MA) or Invitrogen.

In order to generate the TgPhIL1 knockout construct, PCR was performed on T. gondii genomic DNA using primers PhIL15′KOhis5′ (5′GCTGAGGAGGGAAGATCGAG3′) and PhIL13′KOrevXbaI (5′TGTCCTAGATATCGCAATGCTCGCTGC3′) to generate a 6.5 kb amplicon containing 2.9 kb of TgPhIL1 5′ flanking sequence, the 1.3 kb TgPhIL1 open reading frame (ORF), and 3.1 kb of TgPhIL1 3′ flanking sequence. The amplified product was cloned into pCR-Blunt TOPO (Invitrogen) and inverse PCR was performed on the sequence, the 1.3 kb TgPhIL1 ORF, and PhIL1-Endorev-CATTTCG-3′. Cloning TgPhIL1 under its endogenous promoter for expression were identified by immunofluorescence with anti-TgPhIL1 [18].

Cloning TgPhIL1 and BglII, the product was ligated into pubIMC1-YPFP/sagCAT [22] which had also been digested with XbaI and BglII in order to remove the tubulin promoter and TgIMC1 ORF, generating pTgPhIL1Endo/sagCAT. Complemented parasites were maintained in 20 μM chloramphenicol.

Western blot analysis
Western blot analysis of parasite lysates was performed as described previously [23], using either a rabbit polyclonal anti-TgActin antibody generously provided by Dr. David Sibley [24] or rabbit polyclonal anti-TgPhIL1 [18], at dilutions of 1:10,000 and 1:5,000 respectively.

Immunofluorescence
Parasites were attached to glass coverslips with BD-CellTak (BD Biosciences, San Jose CA) as previously described [23], fixed in phosphate-buffered saline (PBS) containing 4% (vol/vol) paraformaldehyde for 10 minutes at 23°C and permeabilized for 30 minutes at 23°C in PBS containing either 10 mM deoxycholylic acid (for anti-α-tubulin and anti-TgPhIL1) or 0.25% (vol/vol) Triton X-100 (for anti-ISP1 and anti-T. gondii polyclonal serum). The permeabilized parasites were incubated for 15 minutes in PBS containing 2% (wt/vol) BSA (PBS-2% BSA) and one or more of the following primary antibodies: anti-TgPhIL1, diluted 1:500; mouse monoclonal anti-α-tubulin (Sigma-Aldrich, St. Louis MO), diluted 1:400; mouse monoclonal anti-ISP1 (monoclonal 7E8; [25]), diluted 1:2000; and rabbit polyclonal anti-T. gondii (catalog #90700556; AbD Serotec, Raleigh, NC), diluted 1:2000. Immunofluorescence with anti-TgMORN1 [26], 1:500, and anti-TgIMC1 [23], 1:100 was performed after fixation of infected HFF monolayers on glass coverslips in 100% MeOH on ice for 5 minutes. After incubation with primary antibodies, the samples were incubated with a 1:1000 dilution of Alexa488- and/or Alexa546-conjugated secondary antibody (Invitrogen).

Morphometric analysis
Parasites were resuspended in Hanks Buffered Salt Solution (HBSS; Invitrogen) containing 10 mM HEPES, pH 7.2, were attached to either glass coverslips or 8-well chambered coverglasses (Nunc, Rochester NY) using BD-CellTak, incubated for 15 minutes at 23°C with or without various concentrations of mercuric chloride (HgCl2), and visualized by differential interference contrast (DIC) microscopy. The widest point along both the long and short axes of the parasite was determined using ImageJ (http://rsbweb.nih.gov/ij/).

Electron microscopy
Parasites were pelleted at 1,000 x g, resuspended in Karnovsky’s reagent (1% [wt/vol] paraformaldehyde, 2.5% [vol/vol] glutaraldehyde) and incubated for 60 minutes at 4°C. The fixed cells were rinsed 3 times (4 minutes each) in Millonig’s phosphate buffer [27] and embedded in 2% (wt/vol) SeaPrep Agarose (Cambrex BioScience Rockland, Rockland ME), for 15 minutes at 4°C. The samples were then incubated in Karnovsky’s reagent for 15 minutes at 4°C, followed by washing in Millonig’s buffer and trimming of the agarose blocks. 1% (wt/vol) osmium tetroxide (OsO4) was added and the blocks were stored in Millonig’s buffer overnight. The following day, the blocks were serially dehydrated in 35%, 50%, 70%, 85%, 95%, and 100% (vol/vol) ethanol. They were further dehydrated in propylene oxide, infiltrated with and embedded in Spurr’s resin, and allowed to polymerize for 12 hours. Ultrathin sections were cut, placed on nickel grids and contrasted with 2% (wt/vol) uranyl acetate in 50%
Group; RH signals and these were averaged among the 5 mice per cycle numbers were normalized relative to their individual actin.

Quantitect primers for the mouse housekeeping gene counting in the analysis.

Infection to ensure that parasites that failed to invade were not counted in the analysis.

Mouse infections and analysis

Female 6 to 8 week old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained under specific-pathogen-free conditions in accordance with institutional guidelines. 5 mice per group were infected intraperitoneally with 200 μl of PBS containing 1 x 10^6 RH or RHΔTgPhIL1 tachyzoites. Peritoneal cells were harvested on day 7 by lavage with cold PBS, spun onto coverslips and stained using the HEMA-3 kit (Fisher Scientific) as directed by the manufacturer. Parasite DNA levels were measured by real time PCR on DNA isolated from liver and spleen samples 7 days post-infection using the High Pure PCR Template Purification Kit (Roche, Indianapolis IN). The tandemly arrayed, 35-fold-repetitive T. gondii B1 gene [28] was amplified by PCR using primers from the TgPhIL1 upstream genomic sequence, the rhTgPhIL1 ORF, and a chloramphenicol acetyltransferase cassette for selection. The construct was transfected into RHΔTgPhIL1 parasites, which were then subjected to three rounds of selection with chloramphenicol. Immunofluorescence with anti-TgPhIL1 antibody showed that stable transfectants had been generated and that the expressed TgPhIL1 localized properly (data not shown). Three clones positive for TgPhIL1 by immunofluorescence were isolated and analyzed by Western blot with the anti-TgPhIL1 antibody. Each of the complemented clones showed levels of TgPhIL1 expression comparable to that of wild-type parasites (Fig. 1C). Two clones, RHΔTgPhIL1/PhIL1-C5 and RHΔTgPhIL1/PhIL1-C7, were chosen arbitrarily for subsequent experiments.

Generation of a TgPhIL1 knockout parasite line

TgPhIL1 was targeted for disruption by a construct encoding hyg, which confers resistance to the drug phleomycin [21], flanked by 5′ and 3′ sequences from the TgPhIL1 genomic locus (Fig. 1A). Following transfection and three rounds of selection, 6% of the population lacked TgPhIL1 staining by immunofluorescence (Fig. 1B). Parasites in this population were cloned by limiting dilution, and individual clones lacking TgPhIL1 were identified by immunofluorescence. Immunoblotting with anti-TgPhIL1 antibody confirmed the lack of TgPhIL1 expression in several of the clones, one of which (RHΔTgPhIL1; Fig. 1C) was chosen arbitrarily for all subsequent experiments.

For complementation studies, we reintroduced TgPhIL1 into the RHΔTgPhIL1 parasites using a construct containing 2 kb of TgPhIL1 upstream genomic sequence, the TgPhIL1 ORF, and a chloramphenicol acetyltransferase cassette for selection. The construct was transfected into RHΔTgPhIL1 parasites, which were then subjected to three rounds of selection with chloramphenicol. Immunofluorescence with anti-TgPhIL1 antibody showed that stable transfectants had been generated and that the expressed TgPhIL1 localized properly (data not shown). Three clones positive for TgPhIL1 by immunofluorescence were isolated and analyzed by Western blot with the anti-TgPhIL1 antibody. Each of the complemented clones showed levels of TgPhIL1 expression comparable to that of wild-type parasites (Fig. 1C). Two clones, RHΔTgPhIL1/PhIL1-C5 and RHΔTgPhIL1/PhIL1-C7, were chosen arbitrarily for subsequent experiments.

Morphology of the TgPhIL1 knockout parasites

In the course of generating the RHΔTgPhIL1 parasites, it was obvious by light microscopy that these parasites had an altered morphology compared to wild-type (RH) parasites (Fig. 2, compare left and middle panels). To quantify this difference, parasites were measured along their long and short axes at their widest point, confirming that the RHΔTgPhIL1 parasites are indeed shorter and wider than the RH parasites from which they were derived (Fig. 2). Importantly, the shape of the complemented clones appeared similar to that of wild-type parasites, and morphometric analysis confirmed that complementation of TgPhIL1 had indeed restored the length and width of the mutant to wild-type values (Fig. 2, compare left and right panels).

To determine whether the morphological effects of the knockout are further accentuated under conditions of osmotic stress, the parasites were treated with various concentrations of HgCl₂, which disrupts water balance across the plasma membrane by inhibition of aquaporin channels [31,32]. Both the RHΔTgPhIL1 and wild-type parasites swell following treatment with 0.5 μM HgCl₂.
resulting in each case in a decrease in the length to width ratio of \( \sim 15\% \) (Fig. S1). Thus, the already squat-shaped RH\(_D\)TgPhIL1 parasites become even shorter and wider under conditions of osmotic stress.

Ultrastructure of the TgPhIL1 knockout parasites

Given their striking difference in shape, we examined the RH\(_D\)TgPhIL1 parasites by transmission electron microscopy for ultrastructural changes, particularly in the IMC and/or the region of the pellicle just posterior to the conoid, where TgPhIL1 is known to be concentrated ([18]; see also Fig. S3B). No morphological differences in the IMC, the conoid, or the spacing between the IMC and the plasma membrane were observed (Fig. 3).

Conoid extension, motility and invasion of the TgPhIL1 knockout parasites

Since TgPhIL1 localizes in part to a ring just posterior to the conoid, its absence might have an effect on conoid extension. To test this hypothesis, wild-type and RH\(_D\)TgPhIL1 parasites were treated with 1 \( \mu M \) ionomycin, which induces conoid extension [30]. In response to ionomycin treatment, 86\( \pm 6.3\% \) of wild-type parasites extended their conoid compared to 14\( \pm 0.35\% \) of untreated parasites. Similarly, 83\( \pm 1.8\% \) of the RH\(_D\)TgPhIL1 parasites extended their conoids in response to ionomycin treatment, compared to 17\( \pm 2.5\% \) of untreated parasites (Fig. S2), demonstrating that PhIL1 does not play a significant role in conoid extension.

Since the shape of T. gondii is thought to be important for gliding motility [33,34], the alteration in morphology caused by PhIL1 disruption might be expected to affect parasite motility. However, analysis of trails deposited by gliding parasites revealed that the RH\(_D\)TgPhIL1 parasites were able to undergo both circular and helical gliding, and the number and shape of trails deposited by the knockout parasites was qualitatively indistinguishable from the number and shape deposited by wild-type parasites (Fig. 4A). To determine whether RH\(_D\)TgPhIL1 parasites were defective in their ability to invade host cells, quantitative LSC-based invasion assays [29] were performed. Wild-type, RH\(_D\)TgPhIL1, and RH\(_D\)TgPhIL1/PhIL1-C5 parasites were again indistinguishable in their ability to invade host cells (Fig. 4B). Taken together, these
data indicate that PhIL1 plays no detectable role in conoid extension, motility, or invasion.

Other IMC-associated antigens

To determine whether the absence of TgPhIL1 affects the subpellicular microtubules, RHATgPhIL1 parasites were stained with anti-tubulin and visualized by immunofluorescence microscopy. As shown in Fig. S3A (panel a), the subpellicular microtubules in RHATgPhIL1 parasites are of similar length and show a similar distribution to those of wild-type parasites.

Similarly, no differences were observed in the distribution of TgIMC1, a major component of the subpellicular network (Fig. S3A, panel b); TgMORN1, which localizes in part to ring-shaped structures associated with the growing ends of the IMC cisternae during daughter cell formation ([26]; Fig. S3A, panel c); and ISP1, a protein associated with the most apical plate of the IMC ([25]; Figs. S3A, panel d). These data, together with the ultrastructural analyses, suggest the IMC, subpellicular network, and microtubules are not significantly altered in the knockout parasites.

Growth competition assays

To assess how well the knockout parasites grow in vitro, wild-type and RHATgPhIL1 parasites were added to confluent monolayers of HFF cells and the number of parasites per vacuole was counted at various times (0–36 hours) post infection. No difference in growth rate between the wild-type and knockout parasites was detected (data not shown). To look for a more subtle growth defect over a longer time period, equal numbers of wild-type and RHATgPhIL1 parasites were mixed and used to infect the same flask of HFF cells. Each time the parasites lysed the host cell monolayer, culture supernatant containing the released parasites was added to a fresh host cell monolayer. After every third passage, anti-TgPhIL1 immunofluorescence was performed on the released parasites to determine the relative number of wild-type and knockout parasites present. As shown in Fig. 5, wild-type parasites start to outgrow the RHATgPhIL1 parasites by passage 4 and continue to do so at passages 7 and 10. This same experiment was performed with pairwise cultures of the knockout parasites and each of the complemented lines (i.e., RHATgPhIL1 vs. RHATgPhIL1/TgPhIL1-C5 and RHATgPhIL1 vs. RHATgPhIL1/TgPhIL1-C7); like the wild-type parasites, the complemented lines outgrow the PhIL1 knockout parasites at all time points (Fig. 5).

Decreased survival of TgPhIL1 knockout parasites in vivo

Since the RHATgPhIL1 parasites were outgrown by wild-type parasites in vitro, we tested for a growth difference in vivo. Mice were infected intraperitoneally with $1 \times 10^4$ wild-type or RHATgPhIL1 tachyzoites. Seven days post-infection, large numbers of extracellular and intracellular wild-type parasites were observed in the peritoneal fluid. Significantly fewer free and intracellular parasites were detected in the peritoneal fluid of mice infected with the RHATgPhIL1 parasites (Fig. 6A-C). The spleen and liver were also harvested seven days after infection, and quantitative PCR (qPCR) was performed to determine the amount of parasite DNA present. As shown in Fig. 6D, less parasite DNA was present in the spleen...
and of PhIL1 itself may become apparent.

If one of the primary functions of TgPhIL1 is to provide structural stability to the parasite, as suggested by the data reported here, a reduced ability of the knockout parasites to tolerate osmotic or mechanical stresses encountered during in vivo infection might be an underlying cause of the parasite’s reduced fitness. Intriguingly, disruption of the IMC-associated proteins IMC1a and IMC1b in Plasmodium berghei also alters the shape and reduces the mechanical stability of sporozoites and ookinetes, respectively [36,37]. The rate of parasite proliferation early during infection may affect parasite loads later, as the parasite has a limited time window in which to proliferate before the immune system becomes fully activated. The development of drugs that target components of the IMC and cytoskeleton may therefore be a potentially useful approach to disease management.

In the context of a population of parasites, even relatively subtle growth defects such as the one reported here are likely to present a significant selective disadvantage over time, since parasites exhibiting such a defect will be outcompeted during infection by parasites whose growth is not impaired. Several other T. gondii proteins have recently been identified that are non-essential, but make a clear contribution to parasite growth and fitness (e.g., [38–40] and GEW, unpublished data). With the recent development of highly efficient methods for gene replacement in T. gondii [41,42], many more such genes are likely to be identified. In these cases, it may be that we simply do not know the relevant assay, host, or environmental conditions. A broader experimental context is likely to be required for elucidating the biological function of genes such as TgPhIL1, which are beneficial to the parasite but not strictly essential.
Supporting Information

Figure S1 Osmotic stress causes an equivalent amount of swelling in RH and TgPhIL1 knockout parasites. (A-C) Peritoneal exudate cells (PECs) were harvested seven days post-infection, spun onto coverslips and examined for the presence of parasites. Panel A shows that PECs from RH-infected mice carry a significantly higher parasite load than those from RH-TgPhIL1-infected mice (unpaired student t-test, p<0.05). Results shown are the average from five mice, plus or minus standard error. Panels B and C show representative examples of the coverslips from which the data in panel A were derived (Panel B, RH-infected PECs; Panel C, RH-TgPhIL1-infected PECs). Black arrows indicate extracellular parasites and white arrows intracellular parasites. Scale bars = 20 μm. (D) DNA was harvested from spleen and liver samples seven days post-infection, and qPCR was performed to determine the amount of parasite DNA present. Results shown are the average parasite loads from five mice, plus or minus standard error. The liver and spleen each contained a higher load of RH than RH-TgPhIL1 parasites, although only the differences observed in the spleen were statistically significant (unpaired student t-test, p<0.05).
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Figure S2 Conoid extension in RH and TgPhIL1 knockout parasites. Wild-type (RH) and RHΔTgPhIL1 parasites were incubated for 5 minutes in the presence (+) or absence (-) of 1 μM ionomycin, and the percentage of parasites with extended conoids was scored by phase microscopy. The results shown are the average of two experiments ± standard error. The two parasite lines showed no significant difference in ionomycin-induced conoid extension (unpaired student’s t-test, p>0.05).
(TIF)

Figure S3 Localization of α-tubulin, TgIMC1, TgMORN1 and ISP1 in TgPhIL1 knockout parasites. (A) The distributions of α-tubulin, TgIMC1, TgMORN1 and ISP1 were examined in wild-type (RH) and RHΔTgPhIL1 parasites by immunofluorescence microscopy. (a) The splayed subpellicular microtubules of the parasite are indistinguishable in the wild-type and RHΔTgPhIL1 parasites. (b) TgIMC1 localizes to the periphery of the mother cell and the growing daughters during endodyogeny in both parasite lines. (c) TgMORN1 localizes to the basal end of the parasite, the centrocone, and a pair of rings around the dividing nucleus during endodyogeny in both parasite lines (red = TgMORN1; green = TgSAG1, a plasma membrane marker). (d) ISP1 is found in an indistinguishable apical cap-like distribution in the two parasite lines (red = ISP1; green = polyclonal serum directed against total tachyzoite antigens). Scale bars = 5 μm. (B) The localization of PhIL1 is shown for reference: as previously described [18], PhIL1-YFP localizes to the parasite periphery and is concentrated at both the basal end and the apical end just posterior to the conoid (YFP fluorescence, middle panel). The corresponding DIC and merged fluorescence/DIC images are shown in the upper and lower panels, respectively. Scale bar = 5 μm.
(TIF)

Figure 6. Survival and dissemination of TgPhIL1 knockout parasite in a mouse model of infection. Mice were infected intraperitoneally with 1×10⁶ wild-type (RH) or RHΔTgPhIL1 tachyzoites. (A-C) Peritoneal exudate cells (PECs) were harvested seven days post-infection, spun onto coverslips and examined for the presence of parasites. Panel A shows that PECs from RH-infected mice carry a significantly higher parasite load than those from RH-TgPhIL1-infected mice (unpaired student t-test, p<0.05). Results shown are the average from five mice, plus or minus standard error. Panels B and C show representative examples of the coverslips from which the data in panel A were derived (Panel B, RH-infected PECs; Panel C, RH-TgPhIL1-infected PECs). Black arrows indicate extracellular parasites and white arrows intracellular parasites. Scale bars = 20 μm. (D) DNA was harvested from spleen and liver samples seven days post-infection, and qPCR was performed to determine the amount of parasite DNA present. Results shown are the average parasite loads from five mice, plus or minus standard error. The liver and spleen each contained a higher load of RH than RH-TgPhIL1 parasites, although only the differences observed in the spleen were statistically significant (unpaired student t-test, p<0.05).
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Author Contributions

Conceived and designed the experiments: WDB SDG RW LDT CH GEW. Performed the experiments: WDB SDG RW LDT. Analyzed the data: WDB SDG RW LDT CH GEW. Wrote the paper: WDB SDG RW LDT CH GEW.