

Video Article

Forward Genetics Screens Using Macrophages to Identify *Toxoplasma gondii* Genes Important for Resistance to IFN- γ -Dependent Cell Autonomous Immunity

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Abstract

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular protozoan pathogen. The parasite invades and replicates within virtually any warm blooded vertebrate cell type. During parasite invasion of a host cell, the parasite creates a parasitophorous vacuole (PV) that originates from the host cell membrane independent of phagocytosis within which the parasite replicates. While IFN-dependent-innate and cell mediated immunity is important for eventual control of infection, innate immune cells, including neutrophils, monocytes and dendritic cells, can also serve as vehicles for systemic dissemination of the parasite early in infection. An approach is described that utilizes the host innate immune response, in this case macrophages, in a forward genetic screen to identify parasite mutants with a fitness defect in infected macrophages following activation but normal invasion and replication in naïve macrophages. Thus, the screen isolates parasite mutants that have a specific defect in their ability to resist the effects of macrophage activation. The paper describes two broad phenotypes of mutant parasites following activation of infected macrophages: parasite stasis versus parasite degradation, often in amorphous vacuoles. The parasite mutants are then analyzed to identify the responsible parasite genes specifically important for resistance to induced mediators of cell autonomous immunity. The paper presents a general approach for the forward genetics screen that, in theory, can be modified to target parasite genes important for resistance to specific antimicrobial mediators. It also describes an approach to evaluate the specific macrophage antimicrobial mediators to which the parasite mutant is susceptible. Activation of infected macrophages can also promote parasite differentiation from the tachyzoite to bradyzoite stage that maintains chronic infection. Therefore, methodology is presented to evaluate the importance of the identified parasite gene to establishment of chronic infection.

Video Link

The video component of this article can be found at <https://www.jove.com/video/52556/>

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular, protozoal pathogen. It is the causative agent of toxoplasmosis, a health hazard in immunocompromised individuals. It is also the model system for other apicomplexan pathogens that infect humans including *Cryptosporidium* and *Cyclospora*. Toxoplasmosis is most commonly acquired through ingestion of food or water contaminated with the bradyzoite or oocyst stage of the parasite. Upon ingestion, these stages convert to the tachyzoite stage of the parasite that replicates within host cells and disseminates systemically. T cells, IFN- γ and, to a lesser extent, nitric oxide¹⁻⁴, are important for control of infection but are not capable of eliminating the disease, as a proportion of tachyzoites convert to the bradyzoite stage that are protected within tissue cysts resulting in a long-lived chronic infection. In fact, there are no therapeutics effective against the chronic cyst stage of the disease. Severe toxoplasmosis is most often due to the reactivation of persistent infection, with the bradyzoite stage of the parasite converting back to the rapidly replicating tachyzoite stage characteristic of primary and acute infection.

Early survival in the face of the innate immune response is important to allow the parasite to reach sufficient parasite numbers, as well as to reach distal sites, to enable establishment of chronic infection. *T. gondii* has evolved strategies to counteract host defense mechanisms that likely contribute to its ability to replicate and disseminate early in infection. First, *T. gondii* forms a unique PV during parasite invasion that is largely segregated from the endocytic and exocytic processes of the host cell compared to other intracellular pathogens⁵⁻⁹. Also, like all successful intracellular pathogens *T. gondii* modifies its host cell to create a permissive environment for growth. This includes reprogramming host cell gene expression by altering host cell transcription factors including those important for regulating cell activation¹⁰⁻¹⁵. ROP16¹⁶⁻¹⁹, GRA15²⁰, GRA16²¹ and GRA24²² have all been shown to be important in regulating the transcriptional response and cell signaling cascades of host cells infected with *T. gondii*. Recent studies using genetic crosses between parasite strains with distinct phenotypes have been highly productive in identifying parasite genes that underlie parasite genotype-dependent traits including evasion of immunity related GTPases (IRGs)^{16,19,23-26}. In mice,

immunity related GTPases (IRGs) are critical for the control of Type II and III genotypes of the parasite while the very virulent Type I genotypes have evolved mechanisms to evade the murine IRGs. However, it is also clear that the parasite has evolved mechanisms to evade antimicrobial mediators in addition to the IRGs and that some of these mechanisms may be conserved across parasite genotypes^{27,28}. In addition, very little is known about the critical mediators of cell autonomous immunity against *T. gondii* during human toxoplasmosis. Parasite genes important for resistance to mediators of cell autonomous immunity may also be important for survival during tachyzoite to bradyzoite conversion which can also be triggered by host immune responses. For example, nitric oxide at high levels can suppress parasite replication in infected macrophages but it can also stimulate tachyzoite to bradyzoite conversion resulting in cyst production³⁰⁻³².

ToxoDB is a functional genomic database for *T. gondii* that functions as a critical resource for the field in terms of providing sequence information for the parasite genome and access to published and unpublished genomic scale data including community annotations, gene expression and proteomics data³³. Similar to many protozoal pathogens, the majority of the genome consists of hypothetical genes with no information available based on gene homology to provide insight into their potential functions. Thus, forward genetics is a powerful tool to identify novel parasite genes important for immune evasion, cyst conversion and other functions critical for parasite pathogenesis as well as for conversion between distinct developmental stages. An additional strength of forward genetics is that it can be used as a relatively non-biased approach to interrogate the parasite as to the genes that are important for specific tasks in pathogenesis, including immune evasion and cyst formation. Recent improvements in next generation sequencing for mutational profiling have made it a method of choice for identifying the responsible parasite genes from forward genetics studies using both chemical and insertional mutagenesis³⁴⁻³⁷.

It is important to identify vulnerabilities in *T. gondii* that can be exploited to enhance the effectiveness of cell autonomous immune mechanisms against the parasite particularly those that may also be active against the resistant cyst stage. Toward this aim, an *in vitro* murine macrophage infection and activation model was developed to identify mutations in the parasite that specifically impair *T. gondii* fitness following activation of infected macrophages but not in naïve macrophages. This macrophage screen was used to interrogate a library of *T. gondii* insertional mutants in order to ultimately identify *T. gondii* genes important for resistance to nitric oxide^{27,28}. The isolation of a panel of *T. gondii* mutants with impaired resistance to activation of infected macrophages, particularly a marked sensitivity to nitric oxide, proved the utility of the screen to identify parasite genes important for resistance to mediators of cell autonomous immunity other than the resistance mechanisms described for the murine IRGs²⁸. Insertional mutagenesis has advantages over chemical mutagenesis in terms of generating a limited number of random mutations in each parasite clone and, in theory, easier identification of the site of mutation. However, identifying the genomic site of plasmid insertion in *T. gondii* insertional mutants, in practice, has been surprisingly difficult in many cases³⁷. Insertion of a plasmid into a gene is also likely to disrupt the function of a gene in contrast to chemical mutagenesis that typically results in single nucleotide changes. However, chemical mutagenesis with either N-ethyl-N-nitrosourea (ENU) or ethylmethane sulfonate (EMS) may offer an increased ability to analyze a larger portion of the parasite genome, compared to insertional mutagenesis, as it creates multiple single nucleotide polymorphisms (estimated at 10 -100) per mutant^{34,38}. Moreover, recent advances in whole genome profiling has made it possible to use next generation sequencing to identify the most likely candidate genes responsible for the identified phenotype of a mutated parasite^{34,38}. Regardless of the mutagenesis approach, confirmation of the role of the parasite gene in resistance to macrophage activation ultimately requires gene deletion and complementation to fulfill molecular Koch's postulates.

The ability to dissect the function of a gene by genetic manipulation of both the parasite and the macrophage is important as many of the genes identified via forward genetics in *T. gondii*, as well as other pathogens, are still characterized as hypothetical genes with little to no sequence homology to other proteins with known functions. The current paper outlines a general approach that can be used to identify whether the disrupted gene in a mutant is important for resistance to a known or unknown mediator of cell autonomous immunity. The initial analysis of host antimicrobial factors is performed by evaluating the survival of wild type and mutant parasites in macrophages from wild type mice versus those with specific gene deletions in inducible nitric oxide synthase (iNOS), gp-91 phox (NADPH oxidase), and specific immunity related GTPases (IRGs). This will determine if the identified parasite genes are important for resistance to nitric oxide, reactive oxygen intermediates or immunity related GTPases²⁸ respectively or if an unknown immune mechanism is involved. Activation of infected macrophages with both IFN- γ and LPS, described in the current protocol, results primarily in the isolation of parasite genes important for resistance to nitric oxide²⁸. The use of pharmacological agents that induce nitric oxide in the absence of macrophage activation (nitric oxide donors) confirmed that the majority of the genes identified were important for resistance to nitric oxide rather than nitric oxide in concert with additional mediators associated with macrophage activation²⁸.

Step one and two describe a forward genetics screen designed to isolate parasite mutants with a fitness defect following activation of infected bone marrow-derived macrophages *in vitro*. Step one describes a dose titration analysis to empirically determine a dose of IFN- γ and LPS to use for macrophage activation that reduces parasite replication but does not fully inhibit replication of the wild type *T. gondii* parental strain that is used for creation of the library of parasite mutants. Step two describes the forward genetic screen of the mutant clones in macrophages in 96-well plates. Step three outlines an approach to confirm the phenotype of each mutant identified in the screen of the 96 well plates and to evaluate whether the defect in each mutant affects parasite survival, replication, or cyst production in response to macrophage activation. Step four describes the use of bone marrow-derived macrophages from mice with deletions in specific antimicrobial pathways to identify the immune mediators to which the parasite mutant is specifically susceptible. Step five outlines an approach to determine if a parasite mutant is also compromised for *in vivo* pathogenesis as evaluated by cyst production in the brains of infected mice.

Protocol

NOTE: All protocols that involve the use of animals were performed in accordance with the guidelines and regulations set forth by the New York Medical College's Animal Care and Use Committee.

NOTE: Detailed protocols for chemical mutagenesis³⁸, isolation of parasites by limiting dilution³⁸, isolation of murine bone marrow derived macrophages³⁹, growth of *T. gondii* in human foreskin fibroblast (HFF) cells and cyst production in macrophages and basic immunofluorescence analysis (IFA)³² are referenced. Carry out all cell culture at 37 °C in 5% CO₂ in D10 media (Dulbecco's Modified High Glucose Eagle Medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin). Keep all reagents sterile throughout cell isolations and cell culture.

1. Dose Titration of IFN- γ and LPS to Determine the Concentrations to Use to Activate Infected Macrophages for the Forward Genetics Screen

- Culture murine bone marrow-derived macrophages O/N in eight chamber glass slides at a concentration of 3×10^5 cells/ml in 250 μ l of D10 media per chamber. Use macrophages one to two-weeks after isolation from bone marrow.
NOTE: Chamber slides are purchased that have a growth enhancing RS wash.
- Use a hemocytometer to count wild type parasites harvested from human foreskin fibroblast (HFF) cells grown in a T25 tissue culture flask. Resuspend parasites at a concentration of 5×10^5 wild type parasites per ml in D10 media. This concentration will result in a multiplicity of infection of approximately 1:1 as the macrophages will have proliferated O/N. Use polystyrene or PET tubes for all manipulations with parasites as the parasite sticks to polypropylene.
- Remove the D10 media in the chamber slides and replace with 250 μ l of the suspension of wild type parasites. Gently add the parasite suspension to each chamber of the slide by allowing it to flow down the inner face of each chamber. Do not allow macrophages to dry out at any point during the protocol.
- Cover chamber slides infected with parasites with the chamber slide cover and place in an incubator at 37 ° C in 5% CO₂ for 4 hr to allow time for the parasite to invade and establish a PV.
- Make dilutions of LPS and IFN- γ in 1 ml of D10 media in sterile tubes for the dose titrations. For dose titrations keep either LPS or IFN- concentration constant and vary the other stimulus⁴⁰.
 - For example, keep LPS constant at 10 ng LPS per ml and vary the concentration of IFN- γ (0, 1 unit/ml, 10 units/ml, 100 units/ml, 1,000 units/ml). Keep IFN- γ constant at 100 units/ml and vary the concentration of LPS (0, 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml). Briefly sonicate LPS stock for 2 min without heating in a bath sonicator (not with a tip sonicator) prior to dilution.
NOTE: Sonication is recommended to disrupt micelle aggregates formed by LPS that may not bind properly to host cells.
- 4 hr after initiation of co-culture between the parasites and adherent macrophages in the chamber slide, discard the D10 media in each chamber and replace it with 300 μ l of the appropriate dilutions of IFN- γ and LPS in D10 media. Include a control with only D10 media to evaluate parasite replication in the absence of macrophage activation.
- Re-cover chamber slides with the chamber slide cover and place in an incubator at 37 ° C in 5% CO₂ for 24 to 30 additional hr.
NOTE: Incubation time depends on the doubling time of the wild type parasite strain that can range from 6-12 hr depending on the parasite strain. A time point is chosen prior to parasite lysis of naïve macrophages but long enough to enable at least 3-4 doubling times.
- Make a 2.5% formaldehyde solution in Dulbecco's phosphate buffered saline (PBS). Discard the media in the chamber slide and replace with 300 μ l of the 2.5% formaldehyde solution. Fix for 20 min at RT.
- Rinse slide(s) twice with 300 μ l of PBS per chamber. For each rinse, discard the PBS in the slide and add new PBS gently down the inner face of each slide chamber to avoid disrupting the macrophages adhered to the slide. Add 300 μ l of PBS per chamber and place cover on chamber slide to prevent slides from drying out prior to staining.
NOTE: Slides can be placed at 4 C for up to 3 days at this point prior to staining.
- Staining protocol for *T. gondii* detection by immunofluorescence microscopy (IFA).
 - Make a 0.2% Triton X-100 solution in PBS (permeabilization buffer). Place the solution in a tube in a 37 ° C water bath and briefly vortex it to ensure the Triton X-100 goes into solution. Discard the PBS in the chamber slides and replace with 300 μ l of the permeabilization buffer. Leave at RT for 30 min.
 - Make a solution of 10% goat serum in PBS (blocking solution). Discard the permeabilization buffer in the slide and replace with 300 μ l of blocking solution per chamber. Leave at RT for 30 min.
NOTE: Fetal calf serum can be substituted for goat serum in all staining protocols.
 - For a one step IFA staining protocol, make a 1:1,000 dilution of a fluorescently-conjugated antibody to *T. gondii* in blocking solution. Remove blocking solution from slide and add 150 μ l of antibody solution per chamber. Replace cover slide on chamber slide to prevent cells from drying out. Incubate for 1 hr at RT.
NOTE: Antibody concentration must be empirically determined depending on the source. Antibody is purchased directly conjugated to a fluorochrome or conjugated to a fluorochrome by the user.
 - For a two-step IFA staining protocol with an unconjugated antibody to *T. gondii* and a fluorescently conjugated secondary antibody, stain slides with 150 μ l of unconjugated primary antibody against *T. gondii* in blocking buffer for 1 hr at RT. Rinse 3x with 300 μ l of PBS plus 1% goat serum (wash buffer).
 - Add 150 μ l of fluorescently-conjugated secondary antibody specific for the species of origin for the primary antibody. Replace cover slide on chamber slide to prevent cells from drying out. Incubate for 1 hr at RT.
 - Rinse slides 3x with PBS plus 1% goat serum (washing buffer). For each rinse, remove the solution in the chamber slides by dumping out its contents and replace with 300 μ l of washing buffer.
 - Rinse slides 2x with 300 μ l of PBS.
 - Remove the chamber from the chamber slide as per manufacturer's instructions.
 - Mount slides with mounting media containing DAPI (4'-6-diamidino-2-phenylindole, dilactate) and a 22 mm x 50 mm coverslip.
- Examine slides using phase contrast and fluorescence microscopy. Determine the average number of parasites per vacuole by examining a minimum of 100 PVs per chamber well. The chosen dose of IFN- γ and LPS for the screen needs to be sufficient to suppress, but not prevent, replication of wild type parasites (see **Figure 1**).

2. Isolation of Parasite Mutants with a Fitness Defect Following Activation of Infected Macrophages

NOTE: A library of random *T. gondii* mutants is required for the forward genetics screen. Random mutagenesis of *T. gondii* can be performed by chemical (ENU/EMS) or insertional mutagenesis^{27,28,38}. Following mutagenesis, clone parasites by limiting dilution and grow individual clones in 96-well plates containing adherent HFF cells in a volume of 200 μ l of D10 media^{32,38}. It is critical that 96-well plates for screening of parasites in

macrophages by microscopy have optical bottoms to enable microscopic screening. Phase contrast and fluorescence microscopy for screening stained 96-well plates requires an inverted fluorescence microscope with phase contrast 4, 10, 20 or 40X objective equipped for long working distances. A 4X objective is useful for seeing the entire well but better resolution of the parasites is achieved with the 20X objective.

1. Transfer 50 μ l of tachyzoite mutants grown in confluent HFF cells in 96-well tissue culture plates into two replicate 96-well plates containing murine bone marrow-derived macrophages (1-2 weeks after isolation) in 100 μ l of D10 media.
NOTE: Perform the initial screen in 96-well plates of the parasite based on volume of parasite culture to avoid having to count the number of parasites transferred per each well. Thus, the microscopy analysis in 2.6 is based qualitatively on whether parasites have generally replicated or not replicated. Step 3 describes the approach to confirm the phenotype of the mutants in chamber slides using equal numbers of wild type or mutant parasites to infect the macrophages.
 1. Directly add parasites to the D10 media already in each well. Infect two wells with wild type tachyzoites as a positive control for parasite replication.
2. Place the infected cells into an incubator (37 ° C and 5% CO₂) for 4 hr to allow parasites time to invade the cells and create their PV.
3. Remove media from one plate by dumping the contents of the plate. Use a single flip of the wrist to dump the media in the plate into a discard basin containing a detergent cidal for the parasites.
 1. Add 100 μ l of "macrophage activation media" using a sterile basin for the media and a multichannel pipette. Use the optimal concentration of LPS and IFN- γ determined from Step 1 for the macrophage activation media. Similarly remove media from the duplicate control plate and replace with 100 μ l of D10 media.
4. Place the infected cells into an incubator (37 ° C and 5% CO₂) for an additional 24-30 hr.
5. Staining protocol for 96-well plates for IFA.
 1. Discard the media in the plate and replace with 100 μ l of 2.5% formaldehyde in PBS. Incubate for 20 min at RT . Use a sterile basin for the formaldehyde and a multichannel pipette.
 2. Discard the formaldehyde solution in the plate and add 100 μ l PBS to rinse the formaldehyde from the plate.
 3. Discard the solution in the plate and add 100 μ l of permeabilization buffer (PBS with 0.2% Triton X-100) to each well. Incubate for 30 min at RT .
 4. Discard the solution in the plate and add 100 μ l of blocking solution (PBS with 10% goat serum) to each well. Incubate for 30 min at RT .
 5. Discard the solution in the plate. Add 50 μ l/well of a fluorescently-conjugated anti-*T. gondii* antibody diluted 1:1,000 in PBS plus 1% goat serum. Incubate for 1 hr at RT with the plate cover on and on a rocker.
 1. Alternatively use an unconjugated primary antibody against *T. gondii* followed by staining with a fluorescently-conjugated secondary antibody as described in 1.10.3.1.
 6. Discard the antibody solution in the plate and replace with 100 μ l/well of PBS plus 1% goat serum. Perform this rinse 3x followed by 2 additional rinses with PBS alone. Leave 200 μ l of PBS in each well and replace the cover on the 96 well plate to prevent the wells from drying.
NOTE: The plate with the cover on can be wrapped in parafilm and placed at 4 ° C for up to 3 days prior to analysis by microscopy.
6. Examine cells under an inverted fluorescent microscope using a 20-40X objective. Select mutants that replicate in the naïve macrophage plate but predominantly fail to replicate beyond one parasite/vacuole in "activated macrophages" or that appear amorphous or degraded in "activated macrophages".
NOTE: The number of wild type parasites per vacuole will depend on the dose of IFN- γ and LPS used but ideally is around 4 parasites per vacuole; a number that reflects growth suppression but not complete inhibition of replication by the activation stimulus.

3. Evaluate the Mutants to Determine if the Defect is at the Level of Parasite Survival or Replication Following Activation of Infected Macrophages

1. Transfer selected parasite mutants from 96-well plates (contents of entire well) to T25s containing HFF cells and allow replication.
2. Culture bone marrow derived macrophages O/N in 8 chamber glass slides at a concentration of 3×10^5 cells/ml in 250 μ l of D10 media. Prepare one slide to compare parasite replication in naïve macrophages and an additional slide to compare parasite replication following activation of infected macrophages.
3. Harvest tachyzoite parasites and count in a hemocytometer. Add 5×10^4 *T. gondii* parasites to a chamber well containing macrophages in D10 media and culture for 4 hr. Include one well with wild type parental parasites as a control. Inoculate an identical replicate slide with the same parasite clone that will not receive activation media in order to monitor replication of parasites in naïve macrophages.
4. Discard D10 media from 1 of the replicate chamber slides and replace with 250 μ l of "activation media". Discard D10 media from the other replicate chamber slide and replace with 250 μ l of D10 media. Incubate both the "activated" and "naïve" macrophage slide with the chamber slide cover on at 37 ° C and 5% CO₂ for an additional 24-30 hr.
5. Fix, permeabilize, and block slides for IFA staining and analysis of parasites as described in Step 1. Perform IFA staining with the chambers intact.
 1. Co-stain cells with an antibody to lysosomal membrane associated 1 (LAMP1) or LysoTracker and antibody to *T. gondii* to evaluate whether activation of infected macrophages is triggering fusion of the mutant PVs with lysosomes (**Figure 4**).
 2. Use antibodies to specific compartments/organelles within the parasite/PV for staining by IFA to identify alterations in the parasite mutant that are evident early following macrophage activation²⁸.
NOTE: Such alterations may provide insight into the mechanism that underlies the deficient survival/replication of the mutant following macrophage activation.
6. Examine slides using a 100X phase oil objective and fluorescence microscopy.

1. Evaluate parasite replication by determining the number of parasites per PV in 100 random vacuoles. Perform at least 2 counts of 100 vacuoles each for statistical analysis.
2. Evaluate general parasite morphology using both fluorescence analysis as well as phase contrast microscopy. View parasites under phase 100x objective. Healthy parasites have parasites tightly enclosed within a close-fitting parasitophorous vacuole. Parasites that have amorphous spacious vacuoles with phase dense rims or amorphous parasites suggest parasite death rather than just a defect in replication (**Figures 1 and 3**).

4. Evaluate whether the Susceptibility of Mutant Parasites to Activation of Infected Macrophages is Associated with Known Anti-microbial Mediators

1. Isolate bone marrow-derived macrophages from wild type C57/BL6 mice, iNOS^{-/-}, gp91-phox^{-/-} or Irgm1/Irgm3^{-/-} mice³².
2. Culture respective bone marrow derived macrophages O/N in 8 chamber glass slides at a concentration of 3×10^5 cells/ml in 250 μ l of D10 media.
3. Proceed with parasite challenge, IFA staining, and analysis as described in Step 3.
4. Determine if the ability of the mutant parasites to survive and replicate following activation of infected macrophages is restored in the absence of reactive oxygen or nitrogen species or specific immunity related GTPases²⁸.
5. Use pharmacological agents, such as nitric oxide donors, to evaluate whether specific anti-microbial mediators are sufficient by themselves to impair mutant parasites in HFF cells or naïve macrophages or if they only act in concert with other mediators of macrophages activation²⁸.

5. Evaluate whether the Defect in the Mutant Parasite Compromises Chronic Infection

1. Isolate tachyzoites from wild type, mutant or targeted gene deleted parasites grown in T25s containing HFF cells. Parasites must be freshly lysed from HFF cultures.
2. Count parasites using a hemocytometer. Resuspend parasites in Hanks balanced salt solution (HBSS) at a concentration per ml appropriate for a sub-lethal dose of the parasite delivered in 200 μ l by intraperitoneal (IP) injection.
3. Use a 1 ml tuberculin syringe and 25 G needle to inject parasites with a 200 μ l volume intraperitoneally (IP). The dose depends on the wild type strain of the parasite and the mouse genotype.
NOTE: Type I genotypes of the parasite are lethal to mice during the acute stage of infection regardless of parasite dose and are not appropriate for studies of chronic infection in the absence of chemotherapy for *T. gondii* to suppress parasite replication.
4. Thirty days after parasite challenge, sacrifice mice by inhalation of CO₂ from a pressurized tank in an uncrowded chamber (a standard size mouse cage may contain no more than 5 mice) followed by cervical dislocation.
5. Isolate the brain from the mouse using established procedures⁴¹⁻⁴³.
 1. Lay the mouse on its front side. Spray the head with 70% ethanol to sterilize the area.
 2. Use sharp scissors to cut through the brain stem. Use dissecting scissors to make a shallow cut laterally around the right and then the left side of the skull starting from the base of the brain stem. Use forceps to gently peel back the skull to expose the brain.
 3. Gently lift the brain and place scissors between the brain and base of the skull to cut the olfactory nerve to free the brain. Lift out the brain with sterile forceps or a spatula and place in a bacteriological Petri plate containing 10 ml of sterile PBS.
6. Cut the brain in half with a sterile scalpel through the center between the right and left hemispheres.
7. Add half of the brain to a small mortar containing 1 ml of PBS. Use the mortar and pestle to make a fine tissue suspension of the brain that can pass through a wide bore 20-200 μ l pipette tip.
NOTE: The other half of the brain should be fixed in 2.5% formaldehyde for 1 hr if tissue sections are needed for histopathology.
8. Place 100 μ l of the brain lysate in a 1.7 ml microcentrifuge tube. Add 1 ml of 2.5% formaldehyde in PBS into the tube containing the brain lysate to fix the brain suspension for staining. Incubate at RT for 30 min.
9. Centrifuge the lysate at 8,000 x g for 5 min in a microcentrifuge and carefully discard the supernatant.
10. Add 1 ml of permeabilization/blocking buffer to the lysate (10% goat serum, 0.2% Triton X-100 in PBS). Incubate at RT for 30 min.
11. Centrifuge lysate at 8,000 x g for 5 min and remove supernatant.
12. Add 200 μ l of FITC-conjugated dolichos biflorus agglutinin (DBA). Use a 1:100 dilution of the lectin in PBS plus 10% goat serum. Gently resuspend the lysate by pipetting. Incubate for 1 hr at RT.
NOTE: DBA is a ligand that binds to CST1 on the parasite cyst wall.
13. Centrifuge lysate at 8,000 x g for 5 min and discard supernatant. Add 1 ml of PBS to the pellet. Incubate at RT for 5 min. Repeat PBS wash 3x. Remove the supernatant following the final wash using a pipette.
14. Use a wide bore 20-200 μ l pipette tip to draw up 5 μ l of the stained brain lysate and place on a microscope slide. Mount the slide with a 25 x 25 mm cover slip to create a wet mount of the brain lysate. Make 3 replicate slides using 5 μ l of brain lysate each.
15. Count the number of cysts per each 5 μ l aliquot by fluorescence microscopy using a 10X objective (**Figure 6**).
NOTE: Some cysts are large (8 or more parasites approximately) while some are very small (1-2 parasites per cyst). Count total number of cysts per each slide but document number of large versus number of small cysts.
16. Add the number of cysts detected in the three different 5 μ l aliquots and multiply by the total dilution factor x 2 (only half of the brain was made into a lysate) to estimate the number of total cysts per brain.

Representative Results

Toxoplasma gondii replicates freely in naïve macrophages and has a doubling time between 6-12 hr depending on the strain of the parasite. **Figure 1** shows representative parasites in naïve versus activated bone marrow-derived macrophages. **Figure 2** shows the general morphology of parasites in HFF host cells at 2, 4, 8, 16 and 32 parasites/PV. In the current protocol, the parasite is allowed to invade naïve macrophages and establish a nascent parasitophorous vacuole (PV) prior to the delivery of a potent activation stimulus, LPS and IFN γ , to the infected

macrophages. In this model, replication of wild type parasites is slowed, but parasite replication still proceeds for approximately 24 hr during which time the macrophages become progressively more adept at inhibiting parasite replication. The screen is designed to function as a modified competition model between the time required for potent macrophage activation versus the time during which the wild type parasite or parasite mutant can continue to replicate within its PV. The first step in the screen is to choose a dose of LPS and IFN- γ to be used for activation of infected macrophages. The dose is empirically determined by evaluating parasite replication in macrophages activated with either a constant dose of IFN- γ in combination with a range of LPS concentrations or a constant dose of LPS with a range of IFN- concentrations (Step 1). The dose of the activation stimuli needs to be evaluated for the parental wild type parasites used for creation of the parasite mutants by chemical or insertional mutagenesis. Ideally the dose of LPS and IFN- γ will permit parasite replication to an average of 2-8 parasites per PV 24-30 hr after activation compared to 8-16 parasites per PV in naïve macrophages (**Figures 1 and 2**).

Once the appropriate concentration of LPS and IFN- γ is determined, the mutants are screened in 96-well optical bottom tissue culture plates. The optical bottom in the plates is important because irregularities in the plastic that is used for traditional tissue culture plates makes it difficult to achieve the appropriate resolution to screen the parasites by phase contrast and fluorescence microscopy. Phase contrast and fluorescence microscopy of the 96-well plates also requires an inverted fluorescence microscope with phase contrast 4, 10, 20 or 40x objective equipped for long working distances. Mutants are screened in replicate plates containing murine bone marrow-derived macrophages. After challenge with parasite mutants, infected macrophages in the wells of one plate are activated with LPS and IFN- γ while the other plate containing infected macrophages is cultured in only D10 media. Plates are incubated at 37 ° C and 5% CO₂ for 24-30 hr after addition of LPS and IFN- γ . After incubation, cells are fixed, stained for parasites and analyzed by IFA using both fluorescence and phase contrast microscopy. Mutants are selected that have normal parasite numbers and replication in naïve macrophages but fewer parasites or smaller vacuoles with fewer parasites in infected macrophages that are activated. Once mutants are selected they should be rescreened in chamber slides (Step 3) in naïve and activated macrophages in two independent experiments to enable analysis of parasite morphology at higher magnifications (100X objective and oil) and to confirm that they have normal replication in naïve macrophages but a defect in replication/survival following macrophage activation.

The phenotypes of the mutants with defects in resistance to activation of infected macrophages typically fall into two broad categories: parasites that appear morphologically intact but are unable to replicate beyond a single parasite per PV; and parasites that appear to be degraded and may also be in amorphous spacious PVs (**Figures 1 and 3**). The morphology and ultrastructure of the parasite and PV is best viewed on slides using a 100X objective and oil and by phase contrast microscopy combined with fluorescence analysis. Staining of parasites for IFA with an antibody to lysosomal associated membrane protein-1 (LAMP1) is useful to determine whether the defect in the mutant is associated with an inability of the PV to prevent fusion with lysosomes. Shown in **Figure 4** is a parasite within a phagolysosome (LAMP1 positive) versus a parasite that is in a PV that is largely segregated from the endocytic system of the host cell (LAMP1 negative). The phagolysosome is evident by a continuous solid rim of LAMP1 staining around the entire parasite. In contrast, a PV often has lysosome organelles in the vicinity but no continuous rim of LAMP1 staining around its circumference. The use of antibodies to defined structures/organelles within the parasite and/or PV are useful in follow up IFA studies to identify anomalies in each mutant associated with activation of infected macrophages. Such interrogations with antibodies may provide insight into the mechanisms that underlie the replication/survival defect in a mutant. For example, **Figure 5** shows the *T. gondii* mitochondrion stained with monoclonal antibody 5F4 anti-F1 ATPase beta subunit (a kind gift from Peter Bradley) 24 hr after activation of macrophages infected with wild type parasites or parasite mutants. *T. gondii* was co-stained with a monoclonal anti-*T. gondii* antibody. Wild type *T. gondii* has a single mitochondrion that extends around the circumference of the parasite. The parasite's sole mitochondrion in wild type parasites following activation of infected macrophages was intact compared to a fragmented mitochondrion in mutant parasites. Mitochondria fragmentation was evident not only in degraded/amorphous parasites but also in parasites that displayed normal morphology as evaluated by phase contrast microscopy. This suggests that the defect in the parasite mutant may increase the susceptibility of the parasite mitochondrion to host cell mediators induced by macrophage activation.

The current model uses a combination of IFN- γ and LPS to activate infected macrophages. IFN- γ stimulates the transcription factor STAT1. LPS, like TNF- α , stimulates the transcription factor NF- κ B. Known IFN- γ -dependent antimicrobial mediators important in cell autonomous immunity against *T. gondii* include an array of IFN- γ -dependent immunity related GTPases (IRGs, GBPs) and reactive nitrogen and oxygen species in addition to other agents. In order to determine if the defect in each mutant is specifically associated with production of known IFN- γ -inducible antimicrobial mediators, bone marrow-derived macrophages are isolated from iNOS^{-/-}, gp 91 phox^{-/-} or specific IRG/GBP gene deleted mice and assayed for activity against the mutant parasites. The combination of IFN- γ and LPS to activate infected macrophages preferentially results in mutants with enhanced susceptibility to nitric oxide compared to wild type parasites²⁸.

Activation of infected macrophages can induce stage differentiation of the parasites from tachyzoites to bradyzoites that are contained in tissue cysts. Such cysts are characteristic of chronic infection. Thus, parasite mutants that are defective for replication/survival following activation of infected macrophages may also be defective for conversion to cysts during *in vivo* infection. In order to evaluate cyst production during chronic infection, mice are challenged intraperitoneally (ip) with a non-lethal dose of the parental parasite strain or the mutant clone. Cysts in the brain range in size from less than 10 μ m in diameter to greater than 50 μ m as shown in **Figure 6**. Count the number of both large and small cysts by IFA but keep the counts separate in order to determine if the parasite mutant is impaired for total cyst production or just for establishment and maintenance of large cysts.

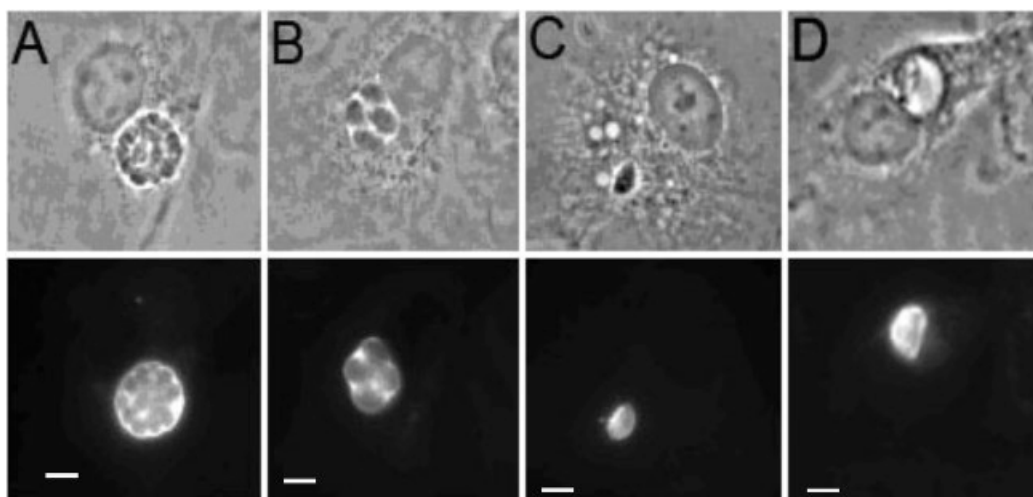


Figure 1. Naïve murine bone marrow-derived macrophages are permissive for replication of *T. gondii* but activation with IFN- γ and LPS substantially inhibits replication. (A) A parasitophorous vacuole (PV) of wild type *T. gondii* 24 hr after invasion of naïve murine bone marrow-derived macrophages. (B) A PV of wild type *T. gondii* parasites consisting of four parasites per vacuole 24 hr after activation of infected macrophages. (C) An example of a mutant parasite unable to replicate and still at one parasite/PV 24 hr after activation of infected macrophages. (D) An example of a mutant parasite that appears degraded within an amorphous PV 24 hr after activation of infected macrophages. The top row shows phase images of the parasites and the bottom row shows fluorescent images using a polyclonal anti-serum against *T. gondii*. The scale bar represents 5 μ m. [Please click here to view a larger version of this figure.](#)

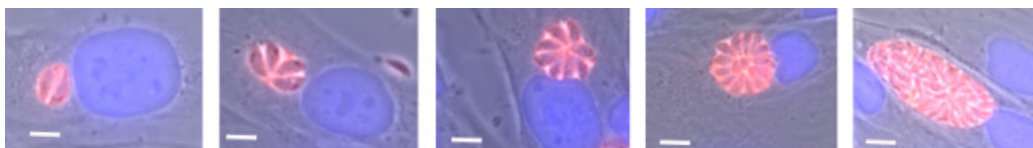


Figure 2. Parasite replication evaluated by the number of parasites per PV. The pictures show 2, 4, 8, 16 and 32 parasites per PV in HFF cells. Each picture is a merged phase image that shows polyclonal antibody staining of the parasite in red and the HFF nucleus in blue (DAPI). The scale bar represents 5 μ m. [Please click here to view a larger version of this figure.](#)

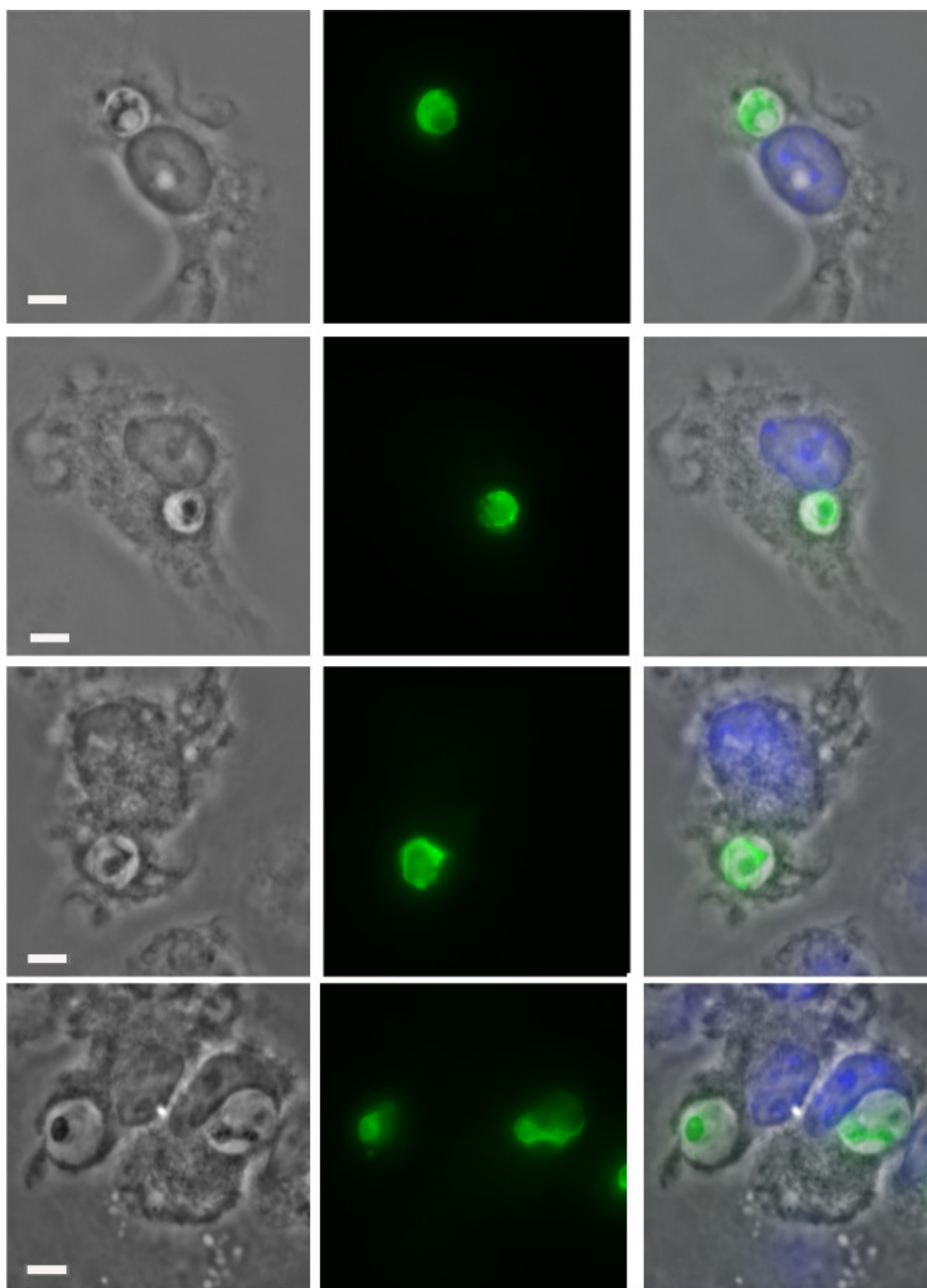


Figure 3. Mutants exhibit a range of phenotypes following activation of infected macrophages. The column on the left is a phase image of the parasite in macrophages, the center column is a fluorescent image using an antibody to *T. gondii*, and the right column is a merge of the phase and fluorescent image. The parasite is shown in green and the macrophage nucleus in blue. The scale bar represents 5 μ m. [Please click here to view a larger version of this figure.](#)

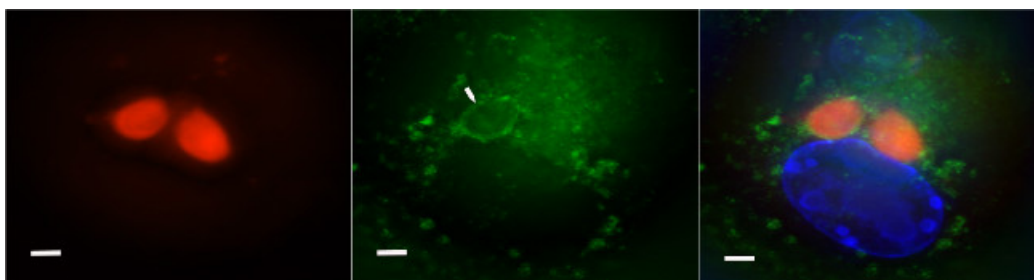


Figure 4. LAMP1 staining distinguishes PVs from phagolysomes-containing parasites. Parasites are stained with a polyclonal anti-*T. gondii* antibody (red) and LAMP1 with the mAb 1D4B (green). An arrow marks a parasite PV that has fused with lysosomes. The parasite without the arrow is in a parasitophorous vacuole (PV) that has not fused with lysosomes. The scale bar represents 5 μ m. [Please click here to view a larger version of this figure.](#)

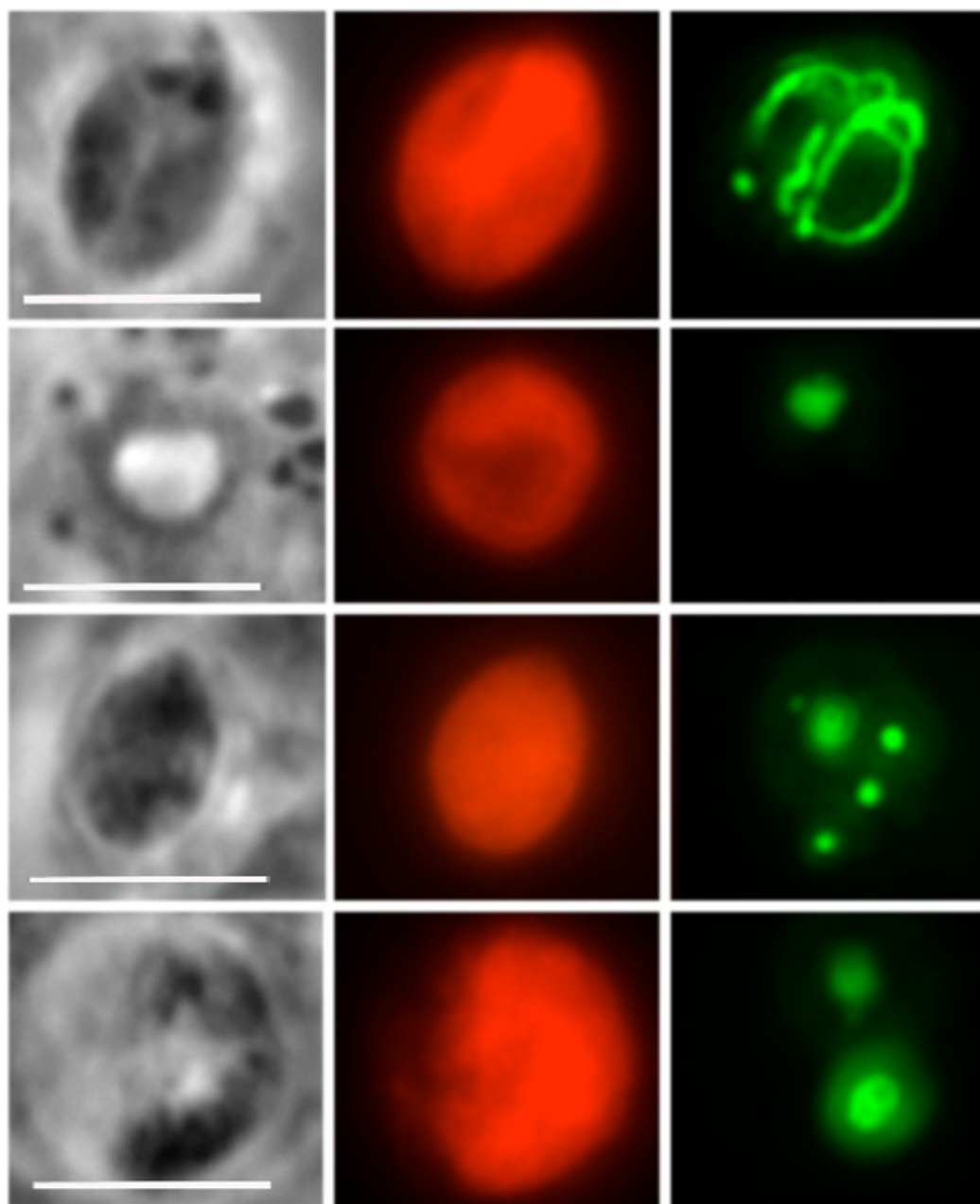


Figure 5. Wild type parasites maintain an intact mitochondrion while the mitochondrion is degraded in parasite mutants following activation of infected macrophages. Mitochondrion (green) in wild type parasites (top panel) compared to three different mutant *T. gondii* parasites at different stages of degradation (bottom three panels) 24 hr after activation of infected macrophages. The scale bar represents 5 µm. [Please click here to view a larger version of this figure.](#)

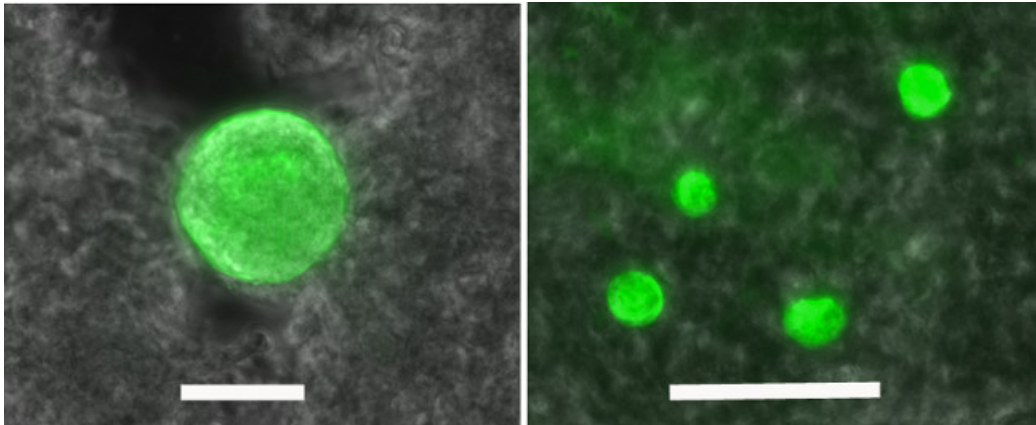


Figure 6. Detection of parasite cysts in the brain using FITC-conjugated dolichos biflorus lectin. The cyst wall labeled with the lectin is shown in green. The scale bar represents 50 μm . [Please click here to view a larger version of this figure.](#)

Discussion

The described protocol provides a non-biased approach that uses activation of murine bone marrow-derived macrophages and forward genetics to isolate *T. gondii* mutants with a defect in their ability to survive activation of infected macrophages. The phenotype of the mutants following macrophage activation typically falls into one of two broad categories: 1) The parasites appear intact but fail to replicate beyond 1 parasite per PV; 2) The parasites appear degraded and may be in spacious, amorphous PVs. The fact that the mutants have a phenotype like wild type parasites in naïve macrophages in the absence of activation proves the protocol can specifically be used to identify mutants that are specifically impaired in their ability to resist macrophage activation. The use of primary bone marrow-derived macrophages is recommended versus the RAW 264.7 macrophage cell line for the screen because the morphology of the parasites and parasite number per vacuole are more easily visualized in bone marrow-derived macrophages.

The described screen combined with forward genetics provides a versatile tool to dissect parasite genes and ultimately genetic pathways important for resistance to specific mediators of cell autonomous immunity. Such forward genetics studies are important to identify parasite genes that can be followed like a string to begin unravelling parasite pathways important for resisting specific antimicrobial mediators both *in vitro* and during pathogenesis. A previous difficulty with forward genetic approaches was identification of the key gene disrupted in each mutant. Cosmid library complementation in *T. gondii* has been quite effective for functional complementation of cell division mutants. However, the fact that replication of wild type parasites are also suppressed by IFN- γ and LPS just to a lesser extent than the mutants, makes functional complementation more difficult than for cell division mutants. Whole genome mutational profiling for both chemical and insertional mutants in *T. gondii* has recently emerged as a productive, and even cost effective, avenue to identify the genes responsible for phenotypes of mutants in forward genetics screens^{34,36,37,44}. Consequently, whole genome mutational profiling using next generation sequencing has enhanced the potential uses of chemical mutagenesis of *T. gondii* in forward genetic studies to identify genes important for specific functions. Such a forward genetics approach as described in the current protocol are important as most of the genome of *T. gondii* remains hypothetical with a majority of predicted genes having no homology to other genes or functional domains that might aid in the identification of candidate parasite genes important for immune evasion.

IFA analysis of 96 well plates is not generally considered a high throughput screening method. In our experience it is reasonable for one person to stain and screen 10 96-well plates in a day or approximately 960 mutants. In the paper by Skariah *et al.*, approximately 8,000 mutants were analyzed and 14 independent mutants were isolated that were significantly impaired for survival/replication in activated but not naïve macrophages²⁸. The impaired survival of the mutants was predominately the result of increased susceptibility to nitric oxide. The limitation in the overall method is primarily at the level of obtaining single clones of the parasite rather than screening by microscopy as the initial screen in 96 well plates is qualitative and reasonably rapid. Confirmation of the phenotype of mutants identified in the screen of the 96 well plate is followed up in Step 3 by quantitative and qualitative analysis using macrophages in chamber slides and by adding equal numbers of wild type or mutant parasites. The use of other analytical screening methods such as fluorometry at the total population level of parasites, rather than the individual parasite level as evaluated by microscopy, are problematic in the current protocol as many of the degraded parasites stain with the polyclonal antibody against *T. gondii* as robustly as wild type parasites with the difference in the mutant being more qualitative than quantitative at the level of fluorescence intensity. Also, the dose of IFN- γ and LPS required to isolate mutants with a defect in resistance to activation of infected macrophages results in suppressed replication of wild type parasites albeit at later time points. Therefore, measurement of total parasite number for the forward genetics screen including the use of luminescent parasites is problematic. However, it is possible the staining protocol could be eliminated in the screening step if the parental parasite clones used for chemical or insertional mutagenesis expressed a constitutive or inducible fluorescent or luciferase marker. The described protocol using IFA and microscopy screening of macrophages in 96 well plates allows for isolation of mutants defective for survival/replication in activated macrophages but it also clearly misses some potential mutants due to the limited microscopic resolution achievable using the 96-well plate format as at this resolution amorphous swollen vacuoles that stain for parasite antigen may be mistaken for healthy replicating parasites within a normal PV. Substitution of 96 well cover glass plates, instead of 96 well plates with an optical surface, is likely to achieve greater resolution during the screen of infected macrophages in 96 well plates.

The vast majority of the mutants identified using IFN- γ and LPS in the described protocol to activate macrophages following parasite invasion have a defect in their ability to protect themselves from nitric oxide²⁸. In this regard, the screen although intended to be non-biased, may enrich for the isolation of parasite mutants with increased susceptibility to specific antimicrobial mediators depending on the activation conditions, the type and species of macrophages and the timing of parasite invasion relative to macrophage activation that are chosen for the screen. Thus the innate immune cell chosen for the screen and the activation stimuli applied are critical parameters that impact the type of antimicrobial mediators

to which the resultant parasite mutants are likely to be susceptible. The genotype of the parasite is also a critical parameter as Type I genotypes of the parasite are relatively resistant to immunity related GTPases (IRGs) induced in response to IFN- γ while the Type II and III genotypes are more susceptible^{26,45,46}. In the described protocol, macrophages are activated after parasite invasion. Although the Type II genotype, Prugnaud strain, used in the described screen is susceptible to the action of immunity related GTPases, allowing the parasite to invade macrophages first enables replication of wild type parasites before full induction of the IRGs. Also, it is not clear that IRGs are effective against the parasite if they are induced in macrophages subsequent to parasite invasion and initiation of replication.

The described protocol uses macrophages to identify parasite genes important for resistance to IFN- γ -dependent mediators of cell autonomous immunity, particularly nitric oxide. The isolation of a pool of parasite mutants that share a marked susceptibility to nitric oxide as well as a shared phenotype of nitric oxide-dependent mitochondrial fragmentation provides a unique set of tools to identify parasite genes and pathways important for resistance to nitric oxide and for understanding the action of nitric oxide more broadly against *T. gondii* and other eukaryotic pathogens. The described protocol with minor modifications can be used for forward genetics studies to identify parasite genes important for resistance to different mediators of cell autonomous immunity. Potential modifications include altering the type of host cell infected, the activation stimuli, or the timing of activation relative to parasite invasion. For example, pre-activation of murine macrophages with IFN- γ before addition of parasites would result in activation of immunity related GTPases. Such a model could be used to identify parasite genes in Type I genotypes of *T. gondii* that may contribute to the resistance mediated by ROP18 and ROP5 to immunity related GTPases^{24,25}. Mediators of cell autonomous immunity against *T. gondii* in human innate immune cells are not as well defined as in rodents. Thus, the use of human peripheral blood monocytes to screen chemical mutants of *T. gondii* might identify both parasite genes important for survival during human infection in innate immune cells as well as mechanisms important in humans for antimicrobial resistance to *T. gondii*. Parasite genes important for resistance to specific antimicrobial mediators can be identified by isolating mutants unable to survive exposure of infected host cells to pharmacological agents such as reactive oxygen or nitrogen species. Similarly the protocol can be modified to isolate parasite mutants with increased susceptibility to inflammasome activation⁴⁷⁻⁴⁹ or ATP stimulation of the macrophage purinergic receptors⁵⁰.

Taken together, the protocols describe an approach using forward genetics and innate immune cells to isolate parasite mutants important for *T. gondii* resistance to IFN- γ -dependent cell autonomous immunity. Importantly, the approach put forth is versatile and can be readily modified to isolate parasite genes important for evading specific antimicrobial mediators, parasite survival in specific types of host cells or resistance to stress conditions encountered during toxoplasmosis. Furthermore, parasite genes important for resisting host cell activation in many cases may also play a role directly or indirectly in cyst production *in vivo* during infection.

Disclosures

The authors have no competing financial interests.

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