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One Minute Ultraviolet Exposure Inhibits *Toxoplasma gondii* Tachyzoite Replication and Cyst Conversion without Diminishing Host Humoral-Mediated Immune Response

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Abstract

We developed a protocol to inactivate *Toxoplasma gondii* (*T. gondii*) tachyzoites employing 1 minute of ultraviolet (UV) exposure. We show that this treatment completely inhibited parasite replication and cyst formation *in vitro* and *in vivo* but did not affect the induction of a robust IgG response in mice. We propose that our protocol can be used to study the contribution of the humoral immune response to rodent behavioral alterations following *T. gondii* infection.

Keywords

Toxoplasma; inactivation; cysts; immune response; animal models; behavior

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1. Introduction

Toxoplasma gondii (*T. gondii*) infection is known to affect rodent behavior (Kannan, et al., 2010, Lamberton, et al., 2008, Vyas, et al., 2007, Webster, 2001). While some behaviors, such as aversion to cat odor, are changed by all 3 *T. gondii* clonal subtypes (Ingram, et al., 2013), other behavioral alterations, such as novelty-induced activity and learning and memory, are to some extent dependent upon the parasite strain (Kannan, et al., 2010, Kannan and Pletnikov, 2012). It is unclear whether brain and behavioral alterations are due to a direct consequence of *T. gondii* cysts residing in the brain, or an indirect effect of the immune response to *T. gondii* infection. To discriminate the contributions of direct and indirect effects of *T. gondii* infection, experimental tools are needed to evoke an immune response to *T. gondii* proteins in the absence of active infection.

Tools to induce a *T. gondii* specific immune response without administration of a fully functioning live parasite already exist (Kur, et al., 2009). For example, Toxovax®, a vaccine used to protect sheep, is made from a live attenuated Type I strain (Buxton and Innes, 1995). However, since each clonal lineage of *T. gondii* up-regulates cytokines in a strain-specific manner (Saeij, et al., 2005), Toxovax® may not be suitable to study behavioral alterations specific to other *T. gondii* strains. Other vaccines made from single or combined purified *T. gondii* proteins (e.g. SAG1, SAG1 + GRA4) are able to stimulate an anti-*T. gondii* adaptive immune response (Kur, et al., 2009), but the host immune response to single and/or denatured proteins may be different from the one induced by structurally intact tachyzoites. Consequently, we sought to develop a method to prevent *T. gondii* tachyzoite replication in the host without significantly altering the host's immune response to the parasite.

Ultraviolet (UV) irradiation has been shown to inactivate *T. gondii* tachyzoites. Current published protocols are based on a rather prolonged exposure to UV irradiation (i.e. up to 60 minutes) (Lu, et al., 1999, Zhao, et al., 2013), without clear demonstration that the inactivated tachyzoites are able to stimulate the host immune system in the absence of parasite replication or cyst production in rodent brains (Endo, et al., 1981, Grimwood, 1980, Lu, et al., 1999, Yang, et al., 2010, Zhao, et al., 2013). Here we describe a new rapid and effective method for UV inactivation of *T. gondii* tachyzoites in just 1 minute. An advantage of our method is the use of standard equipment (Stratalinker®), thereby increasing the potential for reproducibility of the protocol. We demonstrate that 1 minute of UV inactivation is sufficient to completely inhibit *in vitro* parasite replication and cyst formation in the mouse brain, while still leading to a robust humoral immune response in mice.

2. Materials and Methods

Serology and *in vivo* parasite detection by PCR and cyst staining were performed using coded samples with the observer being unaware of their origin.

2.1 Parasite purification

Prugnnaud (PRU) tachyzoites (passage 3 *in vitro*) were cultured and purified as previously described (Kannan, et al., 2010).

2.2 UV-Inactivation of Parasites

Purified parasites (30 μ L) resuspended in Dulbecco's Phosphate Buffered Saline (DPBS) (1 $\times$) were added to an ultraviolet (UV)-transparent cuvette (Sarstedt AG & Co) and sealed with Parafilm®. The cuvette was placed horizontally in a UV Stratlinker® (STRATAGENE, UV Stratlinker 2400) and exposed to UV light for 1, 5, or 15 minutes at a power of 3,689.04 μ J/ cm^2 /sec for a total energy exposure of 221342.4, 1106712, or 3320136 μ J/ cm^2 , respectively. This was accomplished by setting the timer on the Stratlinker® for 60 minutes, which keeps the emitted power constant, and stopping the countdown after 1, 5, or 15 minutes. Control 0 minute UV exposed parasites were added to a cuvette but not placed in the Stratlinker®. A schematic of the apparatus has been provided (Figure 1).

2.3 Trypan blue Exclusion

Live and UV-inactivated parasites were checked for damage to the cell membrane via the trypan blue dye test. Trypan blue solution (0.4% in PBS, Cellgro) was mixed with purified tachyzoites (1:1) and immediately visualized under brightfield using a Nikon Eclipse E400 microscope. Images were taken with the program EOS Utility 2.8.1.0 (Canon, EOS Rebel T1i camera).

2.4 Parasite Replication Assay

Purified live and UV-inactivated tachyzoites were examined for *in vitro* replication competency using a modification of an established immunofluorescence procedure (D'Angelo, et al., 2009). Tachyzoites were added to HFF cells growing in 8-chamber slides (Millipore). At 2–9 days post-infection (37°C, 5% CO₂), cell monolayers were rinsed with DPBS, fixed, permeabilized and then immunolabeled with rabbit (Rb) anti-SAG-1 (AbD Serotec, UK) followed by Alexa Fluor 594 goat anti-Rb (red, Life Technologies). DAPI (Invitrogen) for visualizing host cell nuclei was added to the secondary antibody. Stained cells were examined by epifluorescence using a Nikon eclipse E400 microscope with the program MetaVue version 6.2r6. Prior to staining, cells were examined by phase contrast using Axiovert 100 (Carl Zeiss) and images taken with Axiovision Rel 4.8.

2.5 Parasite Red/Green Invasion Assay

Purified live and UV-inactivated tachyzoites were examined for *in vitro* invasion competency as previously described (D'Angelo, et al., 2009). Briefly, purified parasites were added to HFF cells growing in 8-chamber slides (Millipore). At 1 hour post-infection, cells were rinsed, fixed and then immunolabeled with Rb anti-SAG-1. Cells were then permeabilized and immunolabeled with MAb 9e11 anti-SAG1 (Argene Inc., NY, USA) followed by a mixture of Alexa Fluor 594 goat anti-Rb (red, Life Technologies) to detect attached/extracellular tachyzoites and Alexa Fluor 488 (green, Invitrogen). DAPI (Invitrogen) was added to secondary. Cells were visualized via epifluorescence as described above.

2.6 Animals

Male BALB/c mice, 5 weeks old (The Jackson Laboratory, Bar Harbor, ME) were used in this study. Animal protocols were approved by the Animal Care and Use Committee of Johns Hopkins University (JHU). Mice were housed 3–5 per cage with 14.5/ 9.5 hours of light/dark cycle and free access to food and water.

2.7 Mouse Inoculations

Parasites were purified and UV-inactivated as described above. Alum adjuvant (Thermo Scientific) was added drop-wise to inactivated parasite (1:1) with mixing after each drop. Positive control inocula consisted of live parasites only. Negative control inocula consisted of DPBS and alum adjuvant (1:1). All solutions were placed on a rotating shaker (Barnstead Lab-Line) at medium-high speed for 40 min. Mice were injected intraperitoneally with 200 μ L of either 400 UV-inactivated tachyzoites: alum adjuvant (n=5; 2 tachy/ μ L; 1:1), 40,000 UV inactivated tachyzoites with and without alum adjuvant (n=5; 200 tachy/ μ L; 1:1); 300,000 UV-inactivated tachyzoites with and without alum adjuvant (n=5; 1500 tachy/ μ L; 1:1), 400 live tachyzoites (n=4; 2 tachy/ μ L), or mock-infected with DPBS: alum adjuvant (n=3; 1:1).

Boosters were administered 12 days post-infection. Mice first administered UV-inactivated tachyzoites with and without alum adjuvant or mock-infected with DPBS: alum adjuvant were given the same preparations as a booster. Mice first given live parasite were administered DPBS only as a booster.

2.8 Serology

Blood was collected via tail bleed and then centrifuged at $10,000 \times g$ for 10 minutes on a table top centrifuge to separate cells from serum. Anti-*T. gondii* IgG was measured in sera using ELISA kits from IBL America (Minneapolis, MN, U.S.A.) modified as previously described (Xiao, et al., 2009). The primary antibody consisted of diluted serum (1:100) and secondary antibody was enzyme labeled anti-mouse IgG.

2.9 Parasite Detection in Mouse Brain

Mice were sacrificed 4 weeks after initial infection, whole brains were excised, and then washed with cold $1 \times$ PBS. Brains were homogenized by serial passage through 18, 20, and 23 gauge needles in sequential order, and then centrifuged at 3,300 RPM for 5 minutes at room temperature. The pellet was resuspended in 1 mL PBS and divided for 1) parasite cyst staining, 2) parasite DNA detection by real-time polymerase chain reaction (RT-PCR) and 3) parasite viability.

2.9.1 Cyst Staining—Twenty percent of brain homogenate was stained with Fluorescein labeled Dolichos Biflorus Agglutinin (DBA) (1:200; Vector Laboratories) to visualize parasite cysts. Stained brain homogenates were examined using Nikon eclipse E400.

2.9.2 Real-time Polymerase Chain Reaction—Thirty percent of brain homogenate was used for TaqMan real-time PCR analysis. DNA was extracted from 3 samples of brain homogenate per mouse (10% homogenate/ mouse) using a standard kit (MOBio Tissue Kit,

Carlsbad, CA, U.S.A.). Real-time PCR was performed for the unique *T. gondii* 5S rRNA gene (Fazaeli, et al., 2000) using the following primers and probe: 5'-TGC TGC GTT CTT CAT CGT-3' forward, 5'-TGC TTT CAG ATT GCT TCC TAA A-3' reverse, 56-FAM/CGC GAG CCA AGA CAT CCA TTG C/36-TAMSp. Mouse beta-actin (Mm00607939 s1, Life Technologies) was run as a housekeeping gene/ loading control. Each sample was run in triplicate with 100 ng DNA. The PCRs were performed on an ABI7900HT real-time PCR system (Applied Biosystems, Foster City, CA). Real-time results were analyzed using SDS 2.4 software (version 2.4; Applied Biosystems).

2.9.3 Viable Parasite Recovery—Fifty percent of brain homogenate was used to purify brain cysts. To separate parasite cysts from brain cell debris, isotonic percoll was added to brain homogenate and centrifuged at 2,400 RPM for 15 minutes at room temperature. The pellet was resuspended in DPBS (1×) and centrifuged at 3,300 RPM for 10 minutes at room temperature. The remaining pellet was resuspended in HFF medium and plated in duplicate on HFF cells cultured on 8-chamber microscope slides (Millipore). After 2 weeks, cells were washed, fixed, permeablized, stained, and images taken as described under the replication assay section.

2.10 Tissue Cytokine Expression

For cytokine analysis, a separate cohort of mice was generated as described above, with an additional control group (DPBS). Mice were sacrificed 5–8 hrs after the 12 day post-infection booster administration (DPBS, n=3; Live, n=5; Adjuvant, n=3; Inactivated, n=4). Brain and spleen were harvested and immediately frozen on dry ice.

Total RNA was extracted from 50 mg of tissue using the Direct-zol RNA Miniprep kit (Zymo) with on-column DNase treatment per the manufacturer protocol. cDNA was prepared from 1 µg total RNA using the SuperScript III first strand system for reverse transcriptase (RT)-PCR (Life Technologies). No addition of SuperScript III (–RT) was used as a negative control for each sample.

Cytokine expression was measured via PCR using the following cycling conditions: [95° 2'; (94° 20"; Annealing Temp 30"; 72° 30") × 35 cycles; 72° 7']. Primer sequences, annealing temperatures, amplicon size, and RefSeq numbers are listed in Table 1. All cytokine reactions were run with 1.5 mM Mg²⁺, while the β-actin housekeeping gene/loading control was run with 2.0 mM Mg²⁺. Each sample was run in triplicate using 150 ng template cDNA per reaction. PCR products were separated and visualized via electrophoresis on precast gels. Images were taken using a Molecular Imager® Gel Doc™ System (BioRad) and bands quantified using ImageJ (Schneider, et al., 2012). After normalization to β-actin, cytokine expression for mice administered live or inactivated tachyzoites were then normalized to infection controls (DPBS and adjuvant, respectively).

2.11 Statistical Analysis

Data were first tested for normality using IBM SPSS Statistics 21. If passed, independent samples t-Test was run to compare two groups. If failed, data were subjected to a Kruskal-Wallis one-way analysis of variance by ranks with infection as the independent variable.

Mann-Whitney U-test was then run to compare specific infection groups at individual time points; $p < 0.05$ was considered significant.

3. Results

We first sought to determine whether UV exposure affects the integrity of the tachyzoite cell membrane via the trypan blue dye test. We found that, similar to live parasites, purified *T. gondii* tachyzoites exposed to UV for 1 minute were impermeable to vital dye trypan blue whereas longer exposures, i.e. 5 and 15 minutes, resulted in immediate intracellular accumulation of the dye (Figure 2). We then checked whether the replication of 1 minute UV-exposed tachyzoites was compromised. While live tachyzoites successfully replicated and eventually lysed host HFF cells, 1 minute UV-exposed tachyzoites were unable to replicate (Figure 3). As replication may have been affected by the inability to invade HFF cells, we performed an invasion assay with 1 minute UV-exposed tachyzoites. We found that 1 minute UV treatment did not affect the ability of tachyzoites to invade HFF cells (Figure 3, inset). Based on these results, we selected 1 minute UV-exposed tachyzoites to study *in vivo*.

We first optimized the administration condition and dose of inactivated tachyzoites to elicit a robust anti-*T. gondii* IgG response in mice. As expected, injection of 400 live tachyzoites produced a significant increase in anti-*T. gondii* IgG levels compared to injection with adjuvant. We then tested several doses of inactivated parasites with or without adjuvant to determine the optimal dose to elicit a robust anti-*T. gondii* IgG response. Neither 400 nor 40,000 inactivated tachyzoites with or without adjuvant significantly elevated levels of anti-*T. gondii* IgG compared to adjuvant only ($p > 0.05$). However, injections of 300,000 inactivated tachyzoites with or without adjuvant led to a significant rise of anti-*T. gondii* antibodies as compared to adjuvant ($p < 0.05$) (Figure 4). The greatest anti-*T. gondii* IgG response was produced by administration of 300,000 inactivated parasites with adjuvant compared to that following injection of 400 live tachyzoites or 300,000 inactivated parasites without adjuvant (all $p < 0.05$). We therefore selected the dose of 300,000 inactivated tachyzoites with adjuvant to be given to mice (inactivated group) to compare with mice given live parasite (live group) or adjuvant only (adjuvant group).

In order to better characterize the response to different treatment conditions, we looked at the kinetics of anti-*Toxoplasma* IgG levels as a measure of humoral-mediated immunity and mouse body weight as a clinical symptom of sickness. We found that at 20 dpi anti-*Toxoplasma* IgG levels were significantly higher in the inactivated group compared to the live or adjuvant group ($p < 0.05$). However, this difference between the inactivated and live group was resolved by 30 dpi ($p > 0.05$) (Figure 5). Despite the presence of relatively high anti-*Toxoplasma* IgG levels in mice of the inactivated group, these animals did not significantly lose weight as measured at 0, 10, 20 or 30 dpi (all $p > 0.05$). In contrast, mice of the live group had significantly lower body weights compared to mice of the adjuvant or inactivated group at 10, 20, and 30 dpi ($p < 0.05$) (Figure 6).

We also evaluated the expression of cytokines in the brain and spleen as a measure of cell-mediated immunity. After booster administration at 12 dpi, we found an elevation in relative

cytokine expression in the spleen of mice administered live but not inactivated *T. gondii* tachyzoites (Figure 7). IL-12 β expression was significantly higher in the live group compared to the DPBS or adjuvant group ($p < 0.05$). In addition, IL-1 β expression was elevated in the live group compared with the DPBS group ($p = 0.053$) (Figure 7A). We found no significant changes in the expression of spleen TNF α or IFN γ due to live or inactivated *T. gondii* infection (Figure 7B). In addition, we looked for expression of these cytokines in the basal region of the brain. However, none of these cytokines were detected in this brain region of mice administered live or inactivated tachyzoites (data not shown).

We then determined whether the IgG response in mice of the inactivated group was associated with viable *T. gondii* presence in the brain 30 dpi. Viable parasites were found in the brain homogenates from mice of the live group but were absent in the homogenates from mice of the inactivated group (Figure 8). We also looked for the existence of *T. gondii* cysts. Parasite cysts were found in the brains of mice of the live group ($n = 3$) but not in the brains of mice of the inactivated group ($n = 5$) (Figure 8A). To confirm the absence of viable parasites in mice of the inactivated group, their brain homogenates were added to HFF cultures. No *T. gondii* tachyzoite growth was observed from the brains of mice given inactivated parasites. In contrast, there was a robust growth of the parasite from the brains of mice given live tachyzoites (Figure 8B). To further verify that inactivated tachyzoites were not present in the mouse brains, we checked for *T. gondii* DNA via real-time PCR. *T. gondii* specific 5S rRNA genomic DNA was detected in brain homogenates of mice given live parasites, but not in animals given inactivated parasites or adjuvant only. Regardless of treatment conditions, the host specific housekeeping gene/ loading control Beta-actin was observed in all samples and the levels did not differ between groups ($p > 0.05$) (Figure 9).

4. Discussion

The present study indicates that our method of inactivating tachyzoites with 1 minute UV-exposure results in a complete inhibition of tachyzoite replication *in vitro* and absence of viable parasites *in vivo*. Nonetheless, intraperitoneal injection of UV-inactivated parasites triggered a robust IgG immune response in mice. However, inactivated tachyzoites were unable to elicit a significant cytokine response *in vivo*. These results suggest that our method of inactivating *T. gondii* tachyzoites with UV could aid in investigating the role of *T. gondii* humoral-mediated immune activation on rodent behavior.

Our data are largely consistent with prior studies that utilized UV inactivation of *T. gondii* tachyzoites (Endo, et al., 1981, Grimwood, 1980, Lu, et al., 1999, Yang, et al., 2010, Zhao, et al., 2013). For example, we also showed that tachyzoites exposed to UV were able to exclude Trypan blue, suggesting irradiation did not damage the tachyzoite membrane (Grimwood, 1980). We found that 1 minute UV-exposed tachyzoites did not replicate *in vitro*, as has been seen by others using UV inactivation of tachyzoites (Endo, et al., 1981, Grimwood, 1980). In addition, we report that mice administered inactivated parasites had a robust serological IgG response without a decrease in body weight, as has been previously published (Yang, et al., 2010, Zhao, et al., 2013).

In contrast to Grimwood and associates who reported that irradiated tachyzoites can infect BHK cells at a rate similar to live tachyzoites (Grimwood, 1980), we found only a few UV inactivated tachyzoites were capable of binding and invading HFF cells. It is possible that similar rates of infection between live and UV inactivated parasites were not observed in our studies due to differences in the cell type used. It is also possible that we did not see many inactivated parasites at 9 dpi due to parasite degradation in culture. Indeed, it has been shown that inactivated parasites are degraded in macrophage cultures within 48 hrs of infection (Endo, et al., 1981).

In contrast to prior studies that used UV inactivation, we performed a more thorough validation of our method. We confirmed that inactivated parasites were not present in the mouse brain 30 dpi as tested with parasite cyst staining, culturing, and real-time PCR. However, even if we did not detect any live parasites in the brains of mice from the inactivated group at 30 dpi, one cannot completely rule out that a few parasites might have entered the brain but were subsequently cleared. Notably, a transient presence of *T. gondii* in the brain has been suggested to contribute to behavioral abnormalities (Ingram, et al., 2013).

In addition, while prior studies using UV inactivation have only measured anti-Toxoplasma IgG levels (Yang, et al., 2010, Zhao, et al., 2013), we also evaluated cytokine expression in the brain and spleen. Consistent with prior reports, we found that infection with live tachyzoites can up-regulate expression of the cytokines IL-12 β and IL-1 β (Hunter, et al., 1995, Yarovinsky and Sher, 2006). However, UV-inactivated tachyzoites were unable to significantly increase expression levels of these cytokines. This suggests that UV-inactivated tachyzoites induce only humoral-mediated immunity (IgG) *in vivo*. The exact mechanism for the inability of UV-inactivated *T. gondii* to evoke cytokine expression in the spleen has not been determined. Yet, it is tempting to speculate that the impaired invasion and/or replication of inactivated tachyzoites may be a reason for the lack of cytokine production.

There are several caveats to our study. While the anti-Toxoplasma IgG response to the inactivated parasites lasted up to 1 month post infection *in vivo*, the complete time course of this response has not been evaluated. In addition, the specific *T. gondii* antigens that the IgG antibodies are produced against have not been determined. Also, whether the peripheral IgG response is associated with or followed by immune activation (e.g. microglia and astrocyte activation) in the brain has not been assessed. Importantly, such a phenomenon has been seen in other models. For example, peripheral administration of BCG vaccine to rodents leads to immune activation (e.g. up-regulation of cytokines) in the brain (O'Connor, et al., 2009).

There are a number of potential benefits to using the UV inactivation protocol described here to study the role of the host immune response to *T. gondii* infection in behavioral abnormalities. First, in contrast to synthetic immunostimulators such as poly I:C and LPS (TLR3 and 4 ligands, respectively), our protocol permits determining the humoral immune response specific to *T. gondii* or even a particular *T. gondii* strain as the protocol employs structurally intact parasites. In this way, there would be greater construct validity to animal models of behavioral alterations following *T. gondii* infection (Kannan and Pletnikov, 2012, Kannan, et al., 2013). Second, our method is faster than previously reported ones that take

up to 60 minutes of UV exposure (Endo, et al., 1981, Grimwood, 1980, Lu, et al., 1999, Yang, et al., 2010, Zhao, et al., 2013). Third, our protocol uses a standard piece of laboratory equipment, which has the potential to increase reproducibility of the data between research groups.

5. Conclusion

Here we describe a rapid and straightforward method to reliably inactivate *T. gondii* tachyzoites and stimulate a humoral immune response *in vivo*. The inactivated tachyzoites are incapable of replicating *in vitro* and *in vivo*, or forming cysts *in vivo*, but elicit a strong serological anti-*Toxoplasma* IgG response in mice. This methodology can be implemented to study the role of immune activation following *T. gondii* infection in rodent behavioral alterations. In particular, since this method is applicable to the inactivation of any *T. gondii* strain, differences in parasite-strain immune activation can be studied.

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Highlights

- *T. gondii* tachyzoites exposed to UV for 1 minute still have intact membranes.
- With 1 minute UV exposure, tachyzoites are unable to replicate *in vitro* or produce parasite cysts *in vivo*.
- UV-inactivated tachyzoites can stimulate an IgG response but not cytokine response *in vivo*.

Figure 1. Inactivation Apparatus

(A) Parafilm covered cuvette containing 30 uL purified parasite. (B) Placement of cuvette (arrow) in stratalinker.

Figure 2. TrypanBlue Exclusion of UV inactivated tachyzoites

Purified tachyzoites exposed to UV for 0 or 1 minute remain clear in the presence of TrypanBlue. Tachyzoites exposed to UV for 5 or 15 minutes are immediately stained blue.

Inset: tachyzoites stained with TrypanBlue

Scale bar: 50 μ M.

Figure 3. UV exposure prevents *in vitro* tachyzoite replication

HFF cells were infected with either live tachyzoites or tachyzoites exposed to UV for 1 minute and then examined microscopically after nine days. As shown by both phase contrast and immunofluorescence, live tachyzoites replicated normally, resulting in cell lysis. In contrast, UV-exposed tachyzoites, although able to penetrate the host cells (inset), were unable to replicate, leaving only sparse singlet tachyzoites.

Inset: Red/green Invasion Assay. See Methods section for details.

Red and green: Immunofluorescent staining of *T. gondii* tachyzoite surface antigen 1 (SAG1).

Blue: Nuclear staining with DAPI.

Scale bars: 50 μ M.

Figure 4. Anti-Toxoplasma IgG Levels at 20 dpi

Live and 300,000 inactivated tachyzoites with and without adjuvant significantly elevated anti-Toxoplasma IgG levels in mice 20 dpi. There was a significant group effect [$H=23.715$, 6 d.f., $p=0.0001$]. Post-hoc comparisons showed that the antibody levels were significantly higher in live, 300,000 inactivated, 300,000 inactivated with adjuvant groups vs. adjuvant control and 300,000 inactivated with adjuvant vs. live. * denotes $p < 0.05$ relative to adjuvant control. # denotes $p < 0.05$ relative to live parasites. Error bars denote SEM. Adjuvant, $n=3$; live, $n=4$; 400 inactivated + adjuvant, $n=5$; 40,000 inactivated, $n=5$; 40,000 inactivated + adjuvant, $n=5$; 300,000 inactivated, $n=5$; 300,000 inactivated + adjuvant, $n=5$.

Figure 5. Kinetics of Anti-Toxoplasma IgG Levels

Live and inactivated tachyzoites elicit increased IgG levels in mice 20 and 30 dpi. There was a significant group effect at 20 and 30 dpi [$H=9.726$ and 7.477 , 2 d.f., $p=0.008$ and 0.024 , respectively]. Post-hoc comparisons showed antibody levels were significantly lower in the adjuvant group vs. live and inactivated groups at 20 and 30 dpi; inactivated IgG levels were higher at 20 dpi vs. live. * denotes $p < 0.05$ relative to live and inactivated groups, # denotes $p < 0.05$ relative to live. Error bars denote SEM. Adjuvant, $n=3$; Live, $n=4$; 300,000 inactivated + adjuvant, $n=5$.

Figure 6. Kinetics of mouse weight

Live-infected mice weighed significantly less than adjuvant controls and 300,000 inactivated parasite with adjuvant group at 10, 20, and 30 dpi. Adjuvant, n=3; live, n=4; inactivated, n=5, * denotes $p < 0.05$ as compared to adjuvant and inactivated administered mice. Error bars denote SEM.

Figure 7. Cytokine expression in mouse spleen

(A) Increased expression of IL-12 β and IL-1 β in mice administered live *T. gondii* tachyzoites. (B) No change in expression of TNF α and IFN γ . DPBS, n=3; live n=5; adjuvant, n=3; inactivated, n=4; * denotes p = 0.05 as compared to DPBS control; ‡ denotes p = 0.053 as compared to DPBS control. Error bars denote SEM.

Figure 8. Parasite cyst presence and parasite viability from mouse brain

(A) Parasite cyst DBA lectin (green) seen in brain homogenates of live parasite administered mice only. (B) Parasite tachyzoites grew on HFF cells from brain homogenate of mice administered live parasite but not inactivated parasite. Red is immunofluorescent staining of tachyzoite SAG1, blue is DAPI staining of nuclei. Scale bars denote 50 μ M.

Figure 9. Real-time PCR of *Toxoplasma* 5S rRNA gene

Brain homogenates only from mice given live *T. gondii* had detectable levels of parasite DNA 30 dpi. No parasite DNA was detected in the brains of adjuvant controls or inactivated parasite administered mice. Beta-actin loading control was detected in all samples. There was no significant group effect in Beta-actin levels [$F(2,9) = 0.206$, $p = 0.817$]. Adjuvant, $n = 3$; live, $n = 4$; inactivated, $n = 5$.

Table 1

PCR primers for Cytokine Expression

F denotes forward primer orientation, R denotes reverse primer orientation.

Gene Name	Primer Orientation	Sequence (5'-3')	Annealing Temperature (°C)	Product Size (bp)	RefSeq#
Interleukin 1 β (IL-1 β)	F	TGTCTGAAGCAGCTATGGCAAC	55	200	(Gibb, et al., 2008) NM_008361.3
	R	CTGCCCTGAA GCTCTTGTGTGATG			
Interleukin 12 β (IL-12 β)	F	CATCGTTTTGCTGGTGCTC	51	148	NM_008352
	R	GGTCCAGGTGATGTCATCTTC			
Tumor Necrosis Factor α (TNF α)	F	AGACCCCTCACACTCAGATCA	51	147	NM_013693
	R	TGTCTTTTGAGATCCCATGCCG			
Interferon γ (IFN γ)	F	ACTCCTTTTCCGCTTCTG	51	128	NM_008337
	R	AACGCTACACACTGCATC			
β -actin	F	GTGGGAATTTCGTCAGAAAGGACTCCTATGTG	55	537	(Sisto, et al., 2003)
	R	GAAGTCTAGAGCAACATAGCACAGCTTCTC			