DETERMINING IF NEURONS CAN CLEAR *T. GONDII* PARASITES THROUGH A CRE REPORTER SYSTEM

By

SAKTHI KUMAR

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A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

Neuroscience and Cognitive Science

THE UNIVERSITY OF ARIZONA

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Approved by:

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Dr. Anita Koshy
Department of Neurology, Immunology

Abstract

Toxoplasma gondii (T. gondii) is an intracellular parasite that infects the central nervous system (CNS) in up to one third of the human population. *T. gondii* persistence in the CNS is thought to be due in part to an inability of neurons to clear intracellular parasites. In this project, I have studied the mechanisms by which neurons are able to clear *T. gondii* and whether they are able to do so *in vivo.* Using a novel reporter system, we have determined that neurons are capable of clearing parasites in an interferon-γ (IFN-γ) dependent manner. Furthermore, our results suggest that neurons can clear parasites *in vitro* through the activation of a set of interferon-γ (IFN-γ) stimulated immunity-related GTPases (IRGs) known to be involved in the clearance of the parasite in other cell types. Additionally, we have tested this reporter system in a model of infection allowing us to establish whether neurons clear parasites *in vivo*.

1. Introduction

Toxoplasma gondii (T. gondii) is an obligate intracellular parasite that infects the central nervous system (CNS) in up to one third of the world's human population. *T. gondii* can infect a variety of warm-blooded animals. Infection can occur from eating or drinking contaminated food or water (Sibley et al., 2009). Although *T. gondii* infection is prevalent in humans, in immunocompetent individuals it generally causes a life-long, asymptomatic infection. However, in immunocompromised individuals (e.g. AIDs patients), the *T gondii* infection can be fatal (Dubley et al., 1998; Remington & Cavanaugh, 1965).

***T. gondii* life cycle and host cell invasion**

There are two distinct types of *T. gondii* infections—feline and nonfeline infections. Feline infections are associated with sexual replication of *T. gondii* while nonfeline infections are associated with asexual replication. In regard to the asexual part of the *T. gondii* life cycle, there are two main stages of growth—the tachyzoite stage and the bradyzoite stage. Tachyzoites are found during acute *T. gondii* infection and divide rapidly, disseminating throughout the host. In the chronic stage of *T. gondii* infection the tachyzoites differentiate into bradyzoites which divide slowly and form cysts. The cysts are able to persist in the CNS of hosts for life by evading the host cell immune response. If a cyst is ruptured this can lead to the bradyzoites differentiating into tachyzoites, resulting in the reactivation of the infection (Black & Boothroyd, 2000).

As *T. gondii* is an obligate intracellular parasite, to survive it must successfully enter a host cell. To do so *T. gondii* produces a parasitophorous vacuole (PV) derived from

the host cells plasma membrane (Mordue et al., 1999). The ability of the parasite to invade its host cell and form and maintain its PV is driven by the release of two sets of secreted effector proteins. The first set of proteins is housed in the rhoptries, a specialized secretory organelle, and are most commonly identified as ROPs. These rhoptry proteins, or ROPs, are injected into the host cell before *T. gondii* enters the cell. The rhoptries secrete RON proteins that are inserted into the host membrane and form an anchoring point that allows for the formation of a complex called the moving junction. The moving junction allows the parasite to enter its host cell by sequestering the host cell plasma membrane (i.e. simultaneously forming the PV and entering the host cell). Soon after secretion of the RONs, the contents of the rhoptry bulbs are secreted into the host cell, releasing proteins into the host cytosol and the PV lumen. Rhoptry proteins can be secreted into host cells by parasites that do not successfully complete the invasion process, meaning that the effector proteins delivered may alter host cell function even if invasion is aborted (Rastogi et. al., 2019). Once the PV has formed and *T. gondii* is inside of the cell, the parasite releases a second set of proteins from another set of secretory organelles, the dense granules. The proteins are mostly known as dense granule proteins (GRAs). Formation and maintenance of the PV allows the parasite to exist and undergo endodyogeny within a cell while protecting the parasite from host cell-intrinsic defense mechanisms (Mordue et al., 1999).

CNS-*T. gondii* interactions

Our current understanding of *T. gondii*-CNS host cell interactions comes mainly from *in vitro* studies. Previous research has demonstrated that *T. gondii* has the ability to infect two parenchymal CNS cell types (neurons and astrocytes) resulting in cysts

(Halonen et al., 1996; Jones et al., 1986; Lüder et al., 1999). However, *in vivo, T. gondii* almost exclusively persists in neurons as *T. gondii* cysts—the persistent form of the parasite (Ferguson & Hutchison, 1987; Melzer et al., 2010). There are two mechanisms that most easily explain these *in vitro* versus *in vivo* differences. One possibility is that, for unknown reasons, *T. gondii* primarily interacts and infects neurons *in vivo*. Alternatively, another possibility is that *in vivo* all CNS cell types are infected, but only neurons lack the ability to kill the parasite. Until recently, limited data suggested the latter model, that neurons lack cell-intrinsic immune responses that are found in other cells, best-explained *T. gondii* persistence in neurons (Schlüter et al., 2001). However, recent work in the Koshy Lab has questioned this model. (Cabral et al., 2016).

Tracking neuron-*T. gondii* interactions in vivo

To track neurons that have been injected by *T. gondii,* our lab developed a Cre reporter system (TCre) where Cre-recombinase is fused to toxofilin, a rhoptry protein, which is injected into host cells prior to invasion (Koshy et al., 2012; Cabral et al., 2016). To confirm Cre-mediated recombination, we use a Cre-reporter cell line that carries a green fluorescent protein (GFP) transgene that is preceded by a premature stop codon which is flanked on both sides by *loxP* sites. Upon injection of Cre by our transgenic parasites,

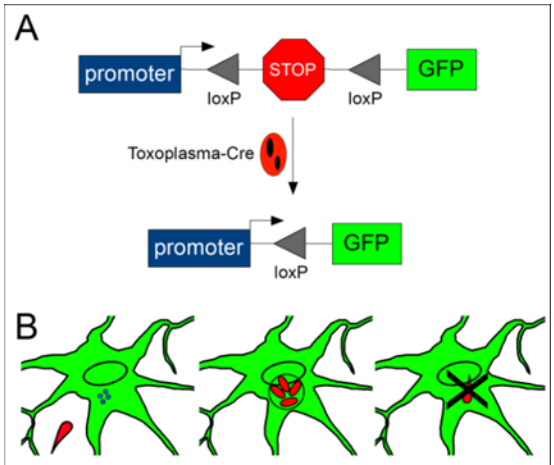

Figure 1. *Toxoplasma*-Cre allows permanent tracking of neurons injected with *Toxoplasma* proteins.
(**A**), Schematic of transgene carried by Cre reporter mice and the effect of injected Cre on recombination. (**B**), Options for how *Toxoplasma*-injected neurons arise. From left-to-right: failed invasion; active infection with cyst development; and active invasion followed by neuron killing of intracellular parasite (unknown mechanisms).

the stop codon is excised and GFP is expressed (Fig. 1). This GFP expression in Cre-reporter mice allows us to track host cells that have been injected with parasite proteins after Cre-mediated recombination *in vivo* (Madisen et al., 2010). Additionally, GFP expression leads to the ability to visualize the whole neuron, including the location of the cyst and the soma (Koshy & Cabral, 2014).

Through our Cre-reporter system, the Koshy Lab has been able to determine that in the CNS parasites almost exclusively interact with neurons. Additionally, up to 95% of these *T. gondii*-injected neurons (TINs) are not invaded, suggesting that neurons may be able to clear parasites. However, as TCre is injected prior to invasion, these uninfected GFP^+ neurons could arise from injection without invasion. There are two distinct phenotypes in neurons: TINs that harbor parasites and TINs without an active infection. Therefore, for TINs without an active infection there are two possibilities that can explain this phenotype: parasites either successfully invade the host cell and are then cleared by cell intrinsic mechanisms or parasites inject the neuron but fail to complete host cell invasion (Koshy et al., 2012). This re-raises the question of whether neurons are able to clear parasites.

Previous research has shown that mouse non-neuronal cells clear intracellular parasites primarily by a set of interferon-γ (IFN-γ) stimulated GTPases: immunity-related GTPases (IRGs) and guanylate binding proteins (GBPs) (Halonen et al., 2001; Schülter et al., 2001). IFN-γ is the major cytokine that is required for both acute and chronic toxoplasmosis partly due to its ability to upregulate the IRGs and GBPs (Steinfeldt et al., 2010; Suzuki & Remington, 1989). Early after infection, the accumulation of IRG on the PV destroys it and results in parasite death. All the proteins in the IRG family function

such that when one of them is disrupted it causes early mortality following infection from *T. gondii* (Steinfeldt et al., 2010).

In order to investigate if and through what mechanisms neurons are able to clear *T. gondii*, we had to use a system that allows us to track and identify TINs. To remove the possibility that parasites could be injecting the neuron but failing to complete invasion, we fused Cre to Gra16 (GCre), a secreted effector protein that is only released into the host cells after invasion is completed. This GCre system solves the problem posed by the TCre system by eliminating the possibility of injection of Cre-recombinase without invasion. Therefore, using this system, if we observe neurons that are GFP^+ and do not harbor parasites this finding would suggest that the neurons cleared themselves of *T. gondii*.

In this project we determined that our GCre system only caused Cre-mediated recombination after the parasite had invaded. Then, we found that neurons can clear parasites through IFN-γ-dependent mechanisms. Finally, we show that clearance in neurons requires an intact IRG system indicating that neurons clear parasites through similar mechanisms found in other cell types. Together, this work indicates that neurons can clear parasites. Current efforts are directed towards leveraging our novel GCre system to identify if neurons clear parasites *in vivo*.

2. Methods

2.1 Parasite presence assay:

Primary neuronal cell cultures were obtained from Cre-reporter mice using previously described methods (Parker et al., 2018). At 12 days *in vitro*, neuronal cultures were

infected with syringe lysed TCre or GCre parasites at a multiplicity of infection (MOI) of 0.5. At 72 hours post infection (hpi), neuronal cultures were fixed with 2 percent PFA and stained with DAPI and an antibody cocktail specific to both tachyzoites and bradyzoites (anti-SAG1 and anti-SRS9 antibodies). The total number of GFP^+ neurons harboring a parasite were counted and divided by the total number of GFP^+ neurons to calculate parasite presence in each of the strains (TCre or GCre).

2.2 IFN-γ clearance assay:

Primary neuronal cell cultures were obtained from Cre-reporter or IRGM1/3 knockout Cre-reporter mice (Collazo et al., 2001; Madisen et al., 2010). These mice were generated in our lab by crossing IRGM1/3 knockout mice with our Cre-reporter mice. Neurons were then stimulated with 100 units of IFN-γ or mock treated with PBS for 24 hours, after which they were infected with syringe-lysed GCre parasites at a multiplicity of infection (MOI) of 0.5. At 72 hpi neuronal cultures were then fixed with 2 percent PFA and stained as above. The total number of GFP^+ neurons harboring a parasite was counted and divided by the total number of GFP^+ neurons to calculate the percentage of clearance.

2.3 Mouse Infection:

Cre-reporter mice, that express tdTomato, were infected with 10K syringe-lysed GCre parasites. At 3 weeks post infection, mice were euthanized, perfused with cold PBS, and their brains harvested. Each brain was halved to separate the left and right hemisphere and each hemisphere was cut into 200-micron thick sections using a vibratome for subsequent downstream imaging of neurons.

2.4 PACT Clearing:

The 200 µm thick sections underwent PACT clearing, which removes lipids from the sections, allowing imaging of the full section. Brain sections were washed in PBS to remove cryoprotectant media before being submerged in a hydrogel monomer solution (2,2'-Azobis[2-(2imidazolin-2-yl) propane] dihydrochloride in 4% acrylamide in PBS), on a rotator in 4ºC over night to ensure saturation of the solution within brain sections. The subsequent day, oxygen was displaced from the samples by bubbling nitrogen into the vials containing hydrogel monomer solution and brain sections for 3 minutes. Samples were then placed in a 42ºC rotating incubator for 2 hours to allow for crosslinking of proteins in brain section with hydrogel monomer solution. After transferring sections to phosphate buffered saline with Tween-20 (PBST) and washing to remove remaining hydrogel solution, 8% sodium dodecyl sulfate (SDS) was added to the samples and placed into the rotating incubator at 45ºC for 30 minutes. Finally, section samples were washed in PBST to remove the SDS (Cabral et al., 2020; Yang et al., 2014).

2.5 Cyst Staining:

In keeping with a previously described protocol we PACT cleared sections, and *T. gondii* cysts were then stained by using Dolichos conjugated to FITC (Dolichos-FITC). Sections were incubated 3% BSA with Dolichos-FITC (1:500) for 24 hours and then washed 2 times in 2%TX/20%DMSO/0.04%NaN_3/PBS. Finally, sections were then washed and placed in sRIMS solution. Samples stayed in sRIMS for 24 hours prior to mounting and imaging.

2.6 Imaging:

PACT cleared sections were imaged on a Zeiss 880 NLO Upright confocal microscope. Images were obtained using 20x lens. The entire neuronal process and soma were captured for analysis.

2.7 IMARIS:

3D rendering of the entirety of neurons with its projections were obtained using the Filaments tool in Bitplane IMARIS (9.2.1). Criteria for neurons rendered was that the entirety of the neuronal projections must be encompassed in the image. Soma that had projections that went out of the field of view were not taken into consideration for analysis. Seed points were modified to identify and connect processes with the respective soma. The Clipping Plane tool was used to scan through the image to correctly trace processes to the cell body. To determine if the selected neuron actively harbored encysted parasites, the image was analyzed for Dolichos staining. Cells without a Dolichos-positive stain do not contain cysts and therefore were inferred to have been infected and cleared by cell intrinsic mechanisms.

3. Results

Previous work in our lab indicated that only about 5% of interacted neurons *in vivo* harbor an active infection indicating that neurons may be capable of clearing parasites (Koshy et al., 2012). However, since our TCre system is unable to differentiate between neurons that have only been injected versus neurons that were injected and invaded we sought to refine the Cre system by fusing Cre-recombinase to Gra16 (GCre), a secreted effector protein that is only released into host cells after full invasion.

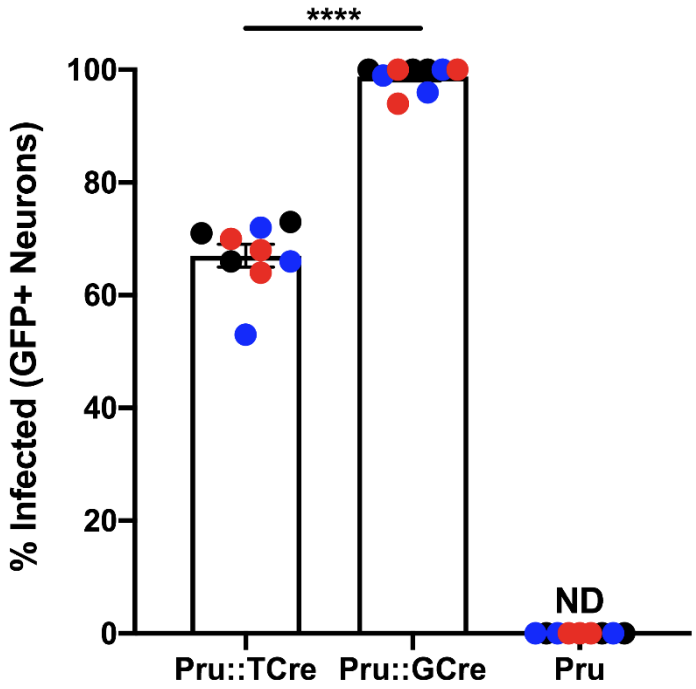

Figure 2. Parasite presence assay. The Type II strain, Prugniaud (Pru), was used for infection. Bars, ± mean SEM. Each dot = 1 coverslip. n ≥ 100 cells/coverslip. 3 coverslips/experiment. ns = not significant. ND = Not Detected. A Welch's t-test was run on the results: *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005, ****p ≤ 0.0001.

Figure courtesy of Joshua Kochanowsky.

Since GCre is only released into host cells after parasite invasion, all GFP-expressing Cre reporter cells should be infected. To test that the GCre system functioned

as expected, we infected primary pure neuron cultures from Cre reporter mice with either Pru::TCre, Pru::GCre or parental (Pru) parasites that do not express Cre-recombinase. We then used epifluorescent microscopy to determine what percentage of GFP^+ neurons harbored parasites. We found that on average 70% of the GFP^+ neurons infected with Pru::TCre had a parasite in them, while on average 30% did not, suggesting that the 30% arose because the parasite injected its protein into the neuron but did not invade it. However, on average 96% of the GFP^+ neurons infected with Pru::GCre had a parasite in them because in the GCre system Cre gets released after invasion of the cell. Therefore, our results suggest that GCre is capable of Cre-mediated recombination and on average 96% of GFP^+ neurons harbor an active infection, indicating that GCre is released after invasion.

Using our GCre reporter system, we can track neurons that are successfully invaded, eliminating neurons that are GFP^+ due to merely injection. Therefore, to determine if neurons are able to clear parasites, we infected primary neuronal cell cultures in the presence or absence of IFN- γ (Fig. 3). We infected with Pru::GCre in the presence of IFN- γ, Pru::GCre in the absence of IFN- γ, and treated neurons with IFN- γ alone. We then used epifluorescent microscopy to determine what percentage of the GFP^+ neurons harbored parasites.

We found that on average 96% of the GFP$^+$ neurons infected with Pru::GCre in the absence of IFN-γ actively harbored a parasite (Fig. 3). These results are similar to the results from the parasite presence assay shown above (Fig. 2) and are expected as in the GCre system Cre is only released after complete invasion of the cell. Meanwhile, on average 78% of the GFP$^+$ neurons infected with Pru::GCre in the presence of IFN-γ actively harbored a parasite, likely because cells are able to clear parasites in the presence of IFN- γ (Fig. 3). Therefore, our results propose that neurons are able to clear parasites during invasion with the presence of IFN-γ.

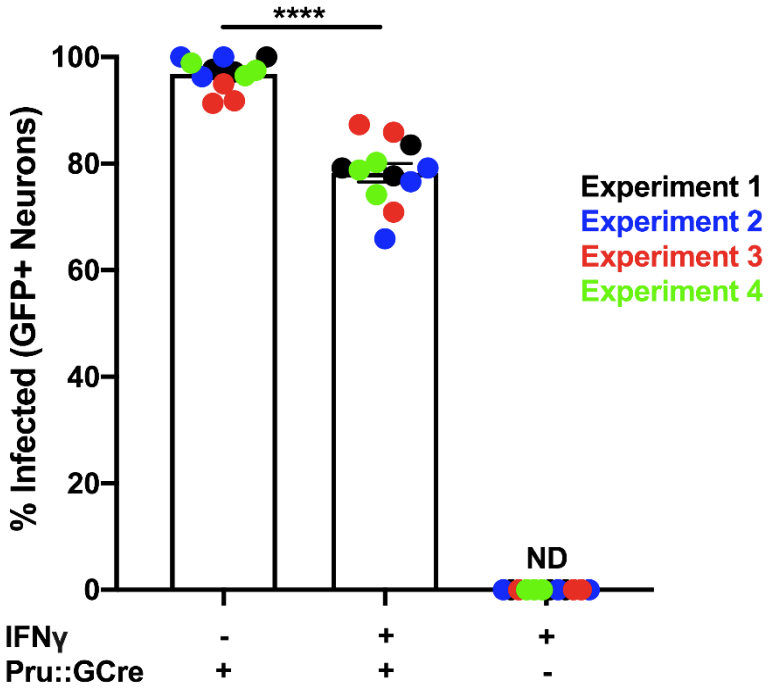

Figure 3. IFN-γ clearance assay. The Type II strain, Prugniaud (Pru), was used for infection. Bars, ± mean SEM. Each dot = 1 coverslip. n ≥ 200 cells/coverslip. 3 coverslips/experiment. ns = not significant. ND = Not Detected. A Welch's t-test was run on the results: *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005, ****p ≤ 0.0001.

Figure courtesy of Joshua Kochanowsky.

Previous research has shown that mouse non-neuronal cell types clear intracellular parasites by a set of IFN-γ-regulated GTPases (IRGs) (Halonen et al., 2001; Schülter et al., 2001). To determine if neurons also use this as the mechanism to clear parasites, we infected primary neuronal cell cultures derived from IRGM1/3 knockout Cre-reporter mice with Pru::GCre. We infected with Pru::GCre in the absence of IFN-γ, Pru::GCre in the presence of IFN-γ, and treated neurons in the presence of IFN- γ alone (Fig. 4).

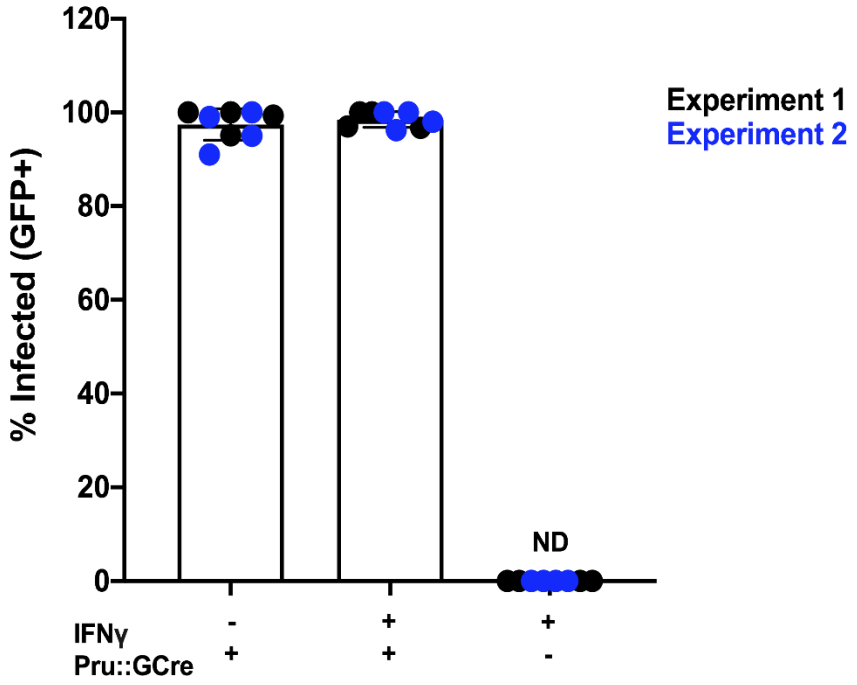

Figure 4. IRGM1-3/zsGreen neuron clearance assay. The Type II strain, Prugniaud (Pru), was used for infection. Bars, ± mean SEM. Each dot = 1 coverslip. n ≥ 100 cells/coverslip. 3 coverslips/experiment. ns = not significant. ND = Not Detected. A Welch's t-test was run on the results: *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005, ****p ≤ 0.0001.

Figure courtesy of Joshua Kochanowsky.

We saw in the IRGM1-3/ GFP$^+$ Neuron Clearance Assay (Fig. 4) that Pru::GCre in IRGM1-3 KO mice stimulated with IFN-γ had roughly the same % of infected GFP$^+$ neurons as Pru::GCre not stimulated with IFN-γ (on average 95%). These results suggest that when IRGM1 and -3 are absent, that neurons are unable to clear parasites suggesting a role for the IRG system in clearance of the parasite in neurons.

Having established that *in vitro* neurons stimulated with IFN-γ are capable of clearing parasites likely through the IRG system, we next sought to determine if clearance occurs *in vivo*. To address whether clearance happens during *in vivo* infections we infected Cre-reporter mice with Pru::GCre. After 3 weeks post infection the brains were harvested, cut into 200-micron thick sections, and cleared using PACT clearing. Then the

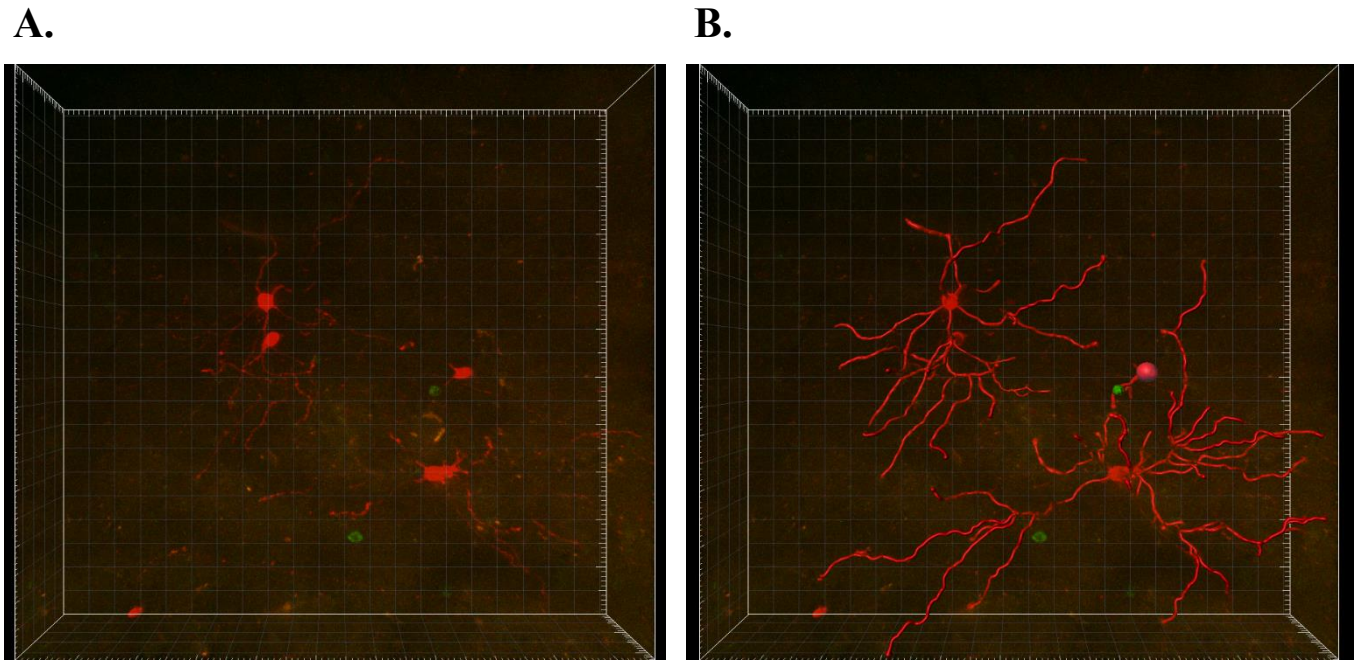

Figure 5. A. PACT cleared sections were imaged on a Zeiss 880 NLO Upright confocal microscope. Images were obtained using 20x lens. The entire neuronal process and soma were captured for analysis. **B.** 3D rendering of the entirety of the neurons with its projections were obtained using the Filaments tool in Bitplane IMARIS (9.2.1). Dolichos stain was used to identify cysts within the image to determine if the cell actively harbored cysts. Cells deplete of Dolichos stain (cysts; green) are inferred to have been infected and cleared by cell-intrinsic mechanisms.

PACT cleared sections were stained with Dolichos (which stains the cyst wall) and imaged by 20x (Fig. 5-A). The results in the image shows that GCre can cause Cre-mediated recombination *in vivo.* As the mice were infected using our GCre system, we know that the fluorescent neurons were injected and invaded by the parasites.

Using IMARIS (9.2.1), 3D renderings of the entirety of neurons with their projections were obtained (Fig. 5-B). Dolichos stains for *T. gondii* cysts. A number of neurons did not contain Dolichos-positive cysts and were concluded to have been infected and cleared by cell intrinsic mechanisms. The 3D neuron rendering (Fig. 5-B) shows that there is no cyst in multiple neurons, suggesting that there is no active infection in the cell, though we might have missed visualizing single parasites as the entire cyst wall was stained.

4. Discussion

The first phase of my project was to determine if our GCre system was functioning as expected. We found that on average 70% of the neurons infected with Pru::TCre had a parasite in them, suggesting that on average 30% do not because the parasite injected the neuron but did not invade it. Meanwhile, on average 96% of the neurons infected with Pru::GCre had a parasite in them because in the GCre system Cre gets released after invasion of the cell. These results suggest that GCre is capable of Cre-mediated recombination and on average 96% of GFP^+ neurons harbor an active infection indicating that GCre is released after invasion. The next part of my project was to use our established GCre system to see if neurons can clear parasites. Our data demonstrated that on average 95% of the neurons infected with Pru::GCre in the absence of IFN- γ had

a parasite in them. We also saw that on average 80% of the neurons infected with Pru::GCre in the presence of IFN- γ had a parasite in them, likely because cells require the presence of IFN- γ to clear parasites. Our results propose that neurons can clear parasites when stimulated with IFN- γ.

After determining that IFN- γ stimulation is necessary for neurons to clear parasites during invasion, we sought to determine if a set of IFN-γ-regulated GTPases (IRGs) is the mechanism by which neurons clear parasites. Our results show that Pru::GCre infected in IRGM-3 KO mice that are stimulated with IFN-γ had a similar % of infected GFP^+ neurons than Pru::GCre not stimulated with IFN-γ (on average 95%). These results suggest that when IRGM1 and -3 are absent, neurons are unable to clear parasites, suggesting an important role for the IRG system in clearance of the parasite in neurons. After determining that neurons can clear parasites *in vitro*, we sought to address whether clearance happens during *in vivo* infections. The 3D neuron rendering demonstrated that there is no cyst in multiple neurons, suggesting that there is no active infection in the cell. However, an alternative explanation is that the neurons could be harboring parasites that have not encysted yet.

The significance of our results is that they suggest that: 1) neurons can clear parasites *in vitro* by a set of IFN-γ-regulated GTPases (IRGs) and 2) neurons may be able to clear parasites *in vivo.* These results are promising as they further validate the theory that neurons can clear parasites and provide an explanation of the mechanism in which they are able to do so. This data brings us one step closer to understanding how the CNS is able to fight *T. gondii* infection.

A future project will be to create a fluorescent version of our Pru::GCre parasite. A problem we came across in our 3D rendered neuron images is that although Dolichos stains *T. gondii* cysts, we cannot exclude the possibility of parasites that have not encysted residing in the neuron. Additionally, it is difficult to visualize small vacuoles or vacuoles that contain only a single parasite. However, using Hoescht staining could make it easier to visualize single parasites as it stains host cell nuclei and parasite nuclei. Also creating and using a fluorescent parasite would allow us to visually determine if a neuron that has been infected and invaded by *T. gondii* is actively harboring parasites or has cleared them. Additionally, *T. gondii* turns into cysts in neurons. Another future project would be to determine if cysts can be cleared or if clearance only occurs at the tachyzoite stages. The more we are able to understand the biological mechanisms underlying *T. gondii,* the closer we will be able to create an effective medical treatment.

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