CHARACTERIZING THE CNS IMMUNE RESPONSE PROVOKED BY TWO GENETICALLY DIVERGENT STRAINS OF *TOXOPLASMA GONDII*

By

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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite that infects a wide range of intermediate hosts, including humans and rodents. For both humans and rodents, once infected *Toxoplasma* establishes a lifelong infection in the central nervous system (CNS). In humans, this tropism for the brain underlies *Toxoplasma*'s ability to cause a wide range of neurological consequences in the immunocompromised, in the developing fetus and occasionally in immunocompetent individuals. The outcomes of symptomatic *Toxoplasma* infection can range from vision-affecting chorioretinitis to encephalitis to death. Recent human data suggest that the genotype of the infecting *Toxoplasma* strain may influence disease severity; however, how these strains differ in their ability to cause disease is not well understood. Previous *in vitro* and acute infection studies in mice have shown that strain-specific polymorphic effector proteins that are secreted and injected into host cells can differentially modulate signaling pathways, affecting cellular functions such as cytokine production. It is unknown if these differences or others lead to strain-specific CNS immune responses. To address this gap in knowledge, we used the mouse model of toxoplasmosis to characterize the neuroinflammatory response generated by two genetically divergent *Toxoplasma* strains (type II and type III). Using immunohistochemistry and an unbiased stereotyped computer program, Simple PCI, we quantified T cell and macrophage responses in the CNS. We also performed a 25-plex cytokine assay to characterize the CNS cytokine milieu in mice infected with either type II or type III parasites. Remarkably, despite having similar parasite burdens at 3 weeks post infection, we found that type III infection causes a significantly higher number of infiltrating T cells and macrophages/microglia and elicits a more robust pro-inflammatory cytokine milieu compared to type II infection. On-going work is

focused on creating transgenic *Toxoplasma* strains that will enable us to dissect out the role of known effector proteins in these strain-specific neuroinflammatory responses.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a wide range of intermediate hosts- from humans to birds (1). In humans and mice, *Toxoplasma* shows a tropism for the central nervous system (CNS) where it establishes a life-long persistent infection. Up to one third of the world's human population is believed to have a chronic, latent CNS infection with *Toxoplasma*. Fortunately, in most individuals with an intact immune system this brain infection appears to be asymptomatic except in the rare cases where immunocompetent individuals develop chrorioretinitis. However, when infected individuals do become immunocompromised, *Toxoplasma* can reactivate and become symptomatic causing devastating outcomes including neurologic disability or in some severe cases death (2). In addition, given that the fetus has an underdeveloped immune response, congenital infection with *Toxoplasma* can also cause symptomatic disease ranging from mild to neurologically devastating.

Toxoplasma has both a sexual and asexual life cycle. The sexual cycle occurs only in felines, which are the definitive hosts for the parasite. The asexual life cycle of *Toxoplasma* can occur in any intermediate hosts, which includes most warm-blooded animals. Hosts acquire *Toxoplasma* infection by ingesting contaminated food or water. *Toxoplasma* has 3 stages: tachyzoites, bradyzoites, and sporozites. Each of these zoites contributes to the life cycle of the parasite. In the asexual life cycle, tachyzoites rapidly divide by endodyogeny, which is the process of producing two daughter cells within the mother cell, and disseminate throughout the host.

Tachyzoites are responsible for causing the host inflammatory response which is thought to be able to clear most tissues of tachyzoites. Bradyzoites, "brady" for slow, are the stage of the parasite that is encysted and is slowly dividing by endodyogeny. It is generally thought that the host immune response puts stress on the parasite causing it to encyst. In turn, the cyst is less immunogenic, allowing it to "hide" from the host immune response permitting these cysts to persist for life. This life-long persistence can become problematic if a patient becomes immunocompromised, increasing the risk of reactivation. Reactivation of latent infection in individuals who are immunocompromised is due to cyst rupture and the conversion of bradyzoites into tachyzoites (3).

In humans, *Toxoplasma* infection causes a wide array of clinical outcomes from chorioretinitis to toxoplasmic encephalitis. Emerging human data suggests that some of the variability in clinical outcomes may be related to the strain of *Toxoplasma* causing the infection (4). A recent study in congenital infected children determined the infecting strain by serology and thus could only identify type II vs. non-type II strains. When comparing the strain type to the clinical outcomes, the data suggested that non-type II infected children had a higher incidence and severity of congenital toxoplasmosis (5)(6)(4). Another small study that was actually able to isolate parasite DNA and genotype the infecting strain suggested that type I strains were associated with aggressive ocular disease in immunocompetent patients while type II strains were associated with more typical reactivation disease in the immunocompromised (7)(8)(9).

Consistent with these data linking the *Toxoplasma* genotype to clinical outcomes, it has long been known that different strains of *Toxoplasma* have differing levels of acute virulence in mice.

In fact, *Toxoplasma*'s 3 canonical strains–type I, type II and type III–were first defined by their acute virulence in mice; these strains were then later confirmed to be genetically distinct (8). Type I is categorized as hypervirulent meaning that one parasite is enough to kill 100% of inoculated mice. Type II is intermediate in virulence with $LD_{50} = 10^3$-10^4 parasites and type III is avirulent with $LD_{50} = 10^4$-10^5 parasites (10). While type I infection is lethal, both type II and type III strains are able to establish a chronic CNS infection in mice. Recent studies have determined that polymorphic genes that affect host cell responses in a strain-specific manner account for the difference in acute virulence in mice (11).

In summary, both human and mouse data suggest that the genotype of the infecting strain can affect disease outcomes. While the parasite genes that underlie the differences in acute virulence in mice have been identified, how different *Toxoplasma* strains might influence human CNS outcome remains unknown. In order to understand how different strains might cause distinct clinical outcomes we need to further understand the strain-specific differences observed in the CNS and define the underlying molecular and cellular mechanisms during *Toxoplasma* infection.

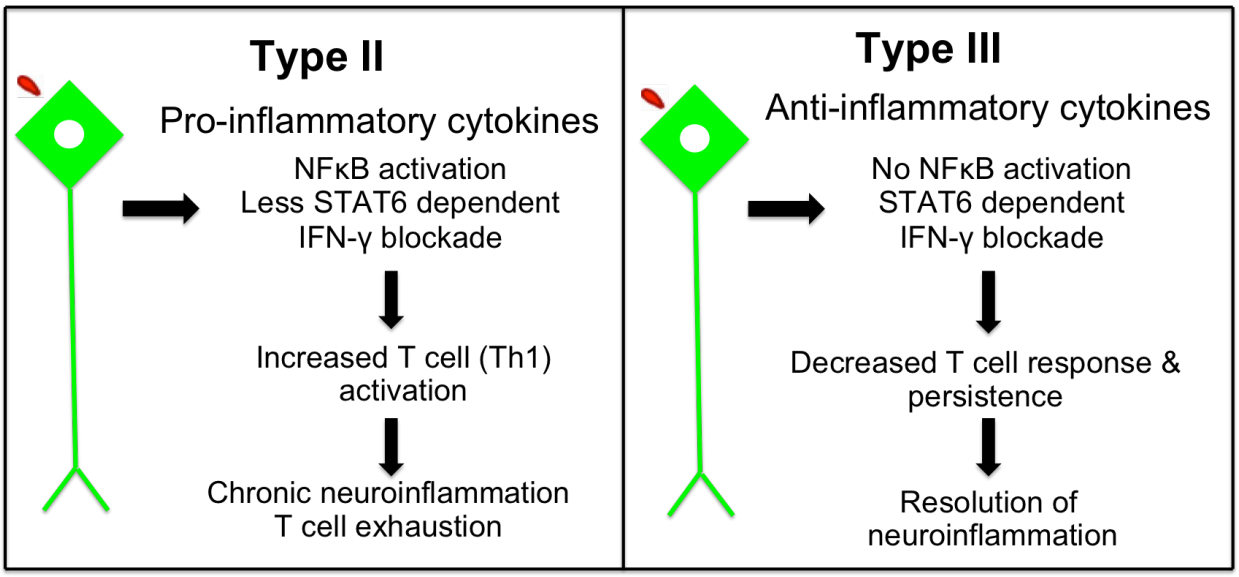
A simple mechanism to cause differences in clinical outcomes due to distinct strains would be if genetically divergent strains of *Toxoplasma* provoked different CNS immune responses. Consistent with this possibility, in the 1980's, Suzuki et al observed that though type II and non-type II strains both encyst in the CNS, they evoke different neuroinflammatory responses. They found that at 4 weeks post infection (wpi), both type II and non-type II strains evoked a similar neuroinflammatory response, but by 16 wpi non-type II infected mice were able to resolve the inflammation whereas the type II infected mice still had neuroinflammation (12). This insight is

significant because it raised the question of what allows both strains to persist in the CNS but cause very different neuroinflammatory responses.

Though strain-specific CNS immune responses are not well studied or understood, a number of *in vitro* studies or *in vivo* studies modeling acute infection have elucidated mechanisms by which different *Toxoplasma* strains can provoke distinct host cell responses acutely. Recent studies have shown that *Toxoplasma* modulates the host cell that it interacts with through the injection and secretion of effector proteins. During invasion, different effector proteins such as ROP16 and GRA15 are injected into the host cell to manipulate host pathways and responses in ways that are favorable for parasite growth and persistence. Polymorphisms in these effector proteins lead to the differential modulation of signaling pathways such as the STAT3/STAT6 and NFκB pathways, which alter the immune response by changes in cytokine production. More specifically, activation of the STAT3/STAT6 transcription factors evoke an increase in anti-inflammatory cytokines while also triggering a decrease in pro-inflammatory cytokines. On the other hand, activation of the NFκB transcription factor results in an increase of pro-inflammatory cytokines. The type I and type III ROP16 allele, but not the type II allele, causes prolonged activation of STAT3/STAT6 transcription factors resulting in an induction of anti-inflammatory cytokines. Conversely, only the type II GRA15 allele activates NFκB transcription factor resulting in activation of a pro-inflammatory cytokine pathway (13).

The ability of these strain-specific host cell changes that might influence cytokine production offered a mechanism by which different *Toxoplasma* strains might ultimately cause different CNS immune responses. Based upon the prior studies detailed above, we hypothesized that type

II infection would drive a greater pro-inflammatory CNS immune response, which would lead to an increase in T cell exhaustion, which is when T cells have been over-stimulated and are no longer able to perform effector function. In turn, this would cause a less effective CNS immune response, resulting in chronic neuroinflammation. Conversely, we hypothesized that type III infection would drive a less-inflammatory CNS immune response, which would lead to quicker resolution of neuroinflammation.



To address this hypothesis, we first needed to establish a reliable mouse model in which we could compare the CNS immune response provoked by type II or type III infection. After establishing this model, we asked three questions. First, is the CNS immune response different between type II and type III infection? We addressed this question by using immunohistochemistry (IHC) to evaluate the T cell and macrophage response in mice infected with either type II or type III parasites. Second, does the quality of the immune response in the CNS differ between type II and type III infection? We used multiplex cytokine assays and flow cytometry to answer this question. Understanding the differences in the quality of an immune response means identifying the different subtypes of immune cells present during infection.

Finally, what are the parasite genes driving these differences in CNS immune response? In order to address this final question, we will be utilizing the CRISPR-Cas9 system to engineer parasite strains that express different alleles of various effector proteins.

METHODS

Mouse infections. We intraperitoneally infected C57BL/6 mice with either type II or type III parasites. Mice used in the study are Cre-reporter mice that express GFP in presence of Cre recombinase (14). Mice were originally obtained from Jackson Laboratories and then bred in the University of Arizona, BIO5 Animal Facility. The parasite strains used in this work have been previously described and express both mCherry and a Toxoplasma:Cre fusion protein that is injected into the host cells during invasion (15)(16).

Parasite strains used and Parasite care. Type II and type III strains were propagated in human foreskin fibroblasts (HFFs) before using it for infecting mice (15).

Perfusion and brain processing for IHC. At appropriate times, brains were harvested, fixed, and sectioned into 40 micron (μm) thick sagittal sections with a microtome as previously described (15). In brief, mice were anesthetized with a 24 mg/ml ketamine and 4.8 mg/ml xylazine mixture, and perfused with 0.9% NaCl containing 10 U/ml heparin after the mice were no longer responding to painful stimulation. Brains were then harvested, drop-fixed in 4% paraformaldehyde (PFA) in phosphate buffer for 24 h, after which the brains were sucrose-embedded using 30% sucrose in PBS. Brains were stored in 30% sucrose/PBS until sectioning.

Sections were stored in cryoprotective media (sodium phosphate buffer, NaH_2PO_4 anhydrous, glycerol, ethylene glycol) until stained and mounted as detailed below.

Parasite Inoculums. The inoculating dose used during each study is described in the appropriate sections. In general, mice were infected with either 10,000 or 50,000 parasites of either type II or type III (four experimental groups: 10K type II, 50K type II, 10K type III and 50K type III) in our first experiments. After analyzing these experiments, we established that 10,000 parasites of either strain gave us the most reliable results and thus subsequent experiments were done using this inoculating dose for each strain.

Quantitative Immunohistochemistry. Standard techniques for DAB immunohistochemistry were used to stain brain sections for T cells and macrophages/microglia. The following antibodies were used: hamster anti-mouse CD3 (1:300) (T cells) and rabbit anti-mouse Iba-1 (1:3000) (macrophages/microglia). We blocked with H_2O_2 to eliminate endogenous peroxidase activity, as well as blocking with goat serum. After applying the appropriate secondary biotinylated antibody (for T cells: goat anti-hamster, and for macrophages/microglia: goat anti-rabbit), we added a 3, 3 –diaminobenzidine (DAB) to detect biotinylated antibodies.
Also performed on the brain sections was a fluorescent stain for cysts. This was done through the use of biotinylated dolichos, which is a lectin that binds to the sugars present on the cyst wall. The secondary antibody, strepavidin 405, is applied and fluoresces in channel 405. After staining, the brain sections are mounted on slides using Vectashield mounting media and then coverslipped.

Microscopy and Statistical Analysis. The DAB-stained brain sections were then imaged on an Olympus IMT-2 inverted light microscope at 20x magnification. Twelve fields of view from each brain section were quantified, and 3 brain sections per mouse as seen in Figure 1. The images

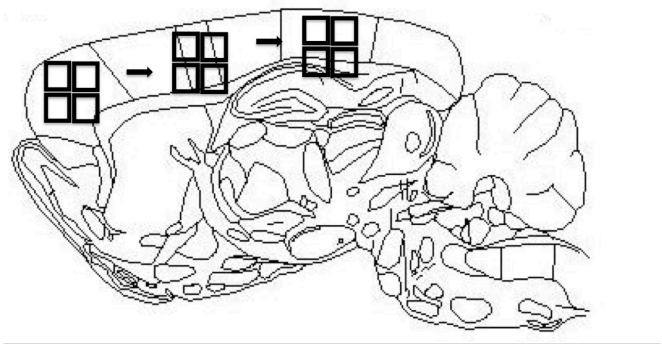


Figure 1. **Quantitative immunohistochemistry was used to count $CD3^+$ T cells and Iba-1^+ macrophages/microglia in the CNS.** For quantification, 3 representative brain sections were analyzed. After appropriate staining, a stereotyped method for quantification of the staining was used. Twelve fields of view along the cortex were evaluated. CD3+ cells were counted individually, whereas Iba-1+ was a collection of percent area in each field of view in order to account for processes.

were then analyzed using an unbiased computer program, Simple PCI, which used a computerized threshold to detect only DAB staining for T cells or macrophages/microglia. The brain sections stained for cysts are analyzed on an EVOS Fluorescence microscope at 20x magnification. In order to confirm the presence of a cyst there must be fluorescence on channel 405 as well as channel 555/568 to indicate the presence of the parasite, which express mCherry.

DNA Extraction and PCR. At 3 wpi, the right frontal lobe of brain was flash frozen and stored at -80 °C until total DNA was extracted. To isolate total DNA we used the DNeasy blood and tissue kit (QIAGEN) and followed the manufacturer's protocol. After DNA was extracted from the samples, the concentration was measured using a NanoDrop spectrophotometer. Parasite

burden was measured by using the same amount of total DNA template and amplifying the *Toxoplasma* B1 gene by quantitative PCR as previously described (17)(18).

Protein Purification and Multiplex Luminex Assay. At 3 wpi, brain tissue was homogenized in cell lysis buffer with protease and phosphatase inhibitor cocktail. Protein concentration for each sample was measured using Direct Detect Spectrometer. The same amount of protein/sample was used in the subsequent assay. A multiplex Luminex bead-based assay using 25-plex mouse inflammatory cytokine and chemokine panel was used to quantify cytokine and chemokines in the protein extraction (Millipore Magpix, Luminex MCYTOMAG-70K-PMX (25-plex cytokine assay)).

Flow cytometry. At 3 wpi, the mice were sacrificed and brains were harvested for flow cytometry. For FACS experiments, mice were perfused with ice cold PBS and harvested brain and spleen were kept in cRPMI media until further processed. First, the cells must be prepared; this included mechanically dissociated tissue, passing it

T cell surface markers	Intracellular T cell marker	APC markers	Cytokines
CD3	FoxP3	F4/80	IFN-γ
CD4		CD11b	TNF-α
CD8		CD11c	Granzyme B
CD25		CD19	IL-10
CD44			
PD-1			

Macrophage markers	Dendritic Cell markers	Neutrophil Markers	B cell marker	T cell marker
F4/80	CD11b	CD11b	CD19	CD3
CD45	CD11c	Gr-1		
CD11b	IA^b			
CD11c	$H2K^b$			
CD16				
CD206 (Macrophage mannose receptor)				
CXCR3				
IA^b				
$H2K^b$				

Table 1. (Top) T cell analysis panel (Bottom) Analysis panel for identifying non-T cell immune cells

Table courtesy of Shraddha Tuladhar.

through syringes, 18 gauge and then 22 gauge. Tissue was then strained through a filter, and cRPMI media was added before the sample was spun down at 937 rcf for 5 mins. Pellet was resuspended and put through Percoll gradient and spun down at 2000 rpm for 25 mins. Layer of leukocytes was collected and media added before sample was spun down again at 937 rcf for 5 mins. Cells were then incubated with antibodies against T cell and macrophage markers as shown in Table 1. Data was then analyzed by FlowJo, immune cell populations were gated by forward scatter and side scatter profile, followed by gating on Live/Dead cells.

RESULTS

Establishment of working mouse model for comparing type II and type III Toxoplasma *infection*

In order to compare the immune response observed in the CNS of the infected mice, we first had to establish an *in vivo* model of *Toxoplasma* infection. *Toxoplasma* virulence has previously been defined in mice with type II being intermediate in virulence and type III being avirulent (10). Thus, in our preliminary studies, the mice were infected with different doses of type II (10,000) or type III (100,000) parasites. However, we were concerned that the immune response difference we found was secondary to differences in the initial parasite inoculums. In order to address this concern, we infected mice with 10,000 or 50,000 parasites of either strain therefore having four experimental groups: 10,000 type II, 10,000 type III, 50,000 type II, and 50,000 type III. We found that infecting mice with 10K of either parasite allows for the most reliable results (Figure 2). For $CD3^+$ cells there was not a significant dose-dependent response when the dose was increased from 10,000 to 50,000. For Iba-1^+ cells we saw that the dose-dependent response is contradictory to what we expected, there was no significant increase in the Iba-1 response with 50 K infection and this increase in parasite inoculum potentially even decreased the response

compared to 10K type III infection. This result could be due to a threshold of response to the infection, meaning that at a certain level of parasite burden the amount of macrophage/microglia response is maxed out.

Difference in the neuroinflammatory response between mice infected with either type II or type III parasites

With the establishment of a consistent model of toxoplasmosis in mice, we began our characterization of the CNS immune response by quantifying the T cell and macrophages/microglia response at 3 wpi. To evaluate these responses, brain sections from mice infected with type II or type III parasites were stained for T cells (anti-CD3) or macrophages/ microglia (anti-Iba-1). Stained sections were then analyzed as described in the methods section, which allows us to quantify the number of T cells infiltrating into the CNS. For macrophages/ microglia, it is not possible to quantify the number of cells, so instead we use the area of the brain that is covered with Iba-1-staining as an estimate of macrophage/microglial infiltration/activation. Using these analyses, we found that there is a greater T cell response and greater macrophage/microglia response in the CNS of mice infected with type III parasites as compared to type II infected mice (Figure 3).

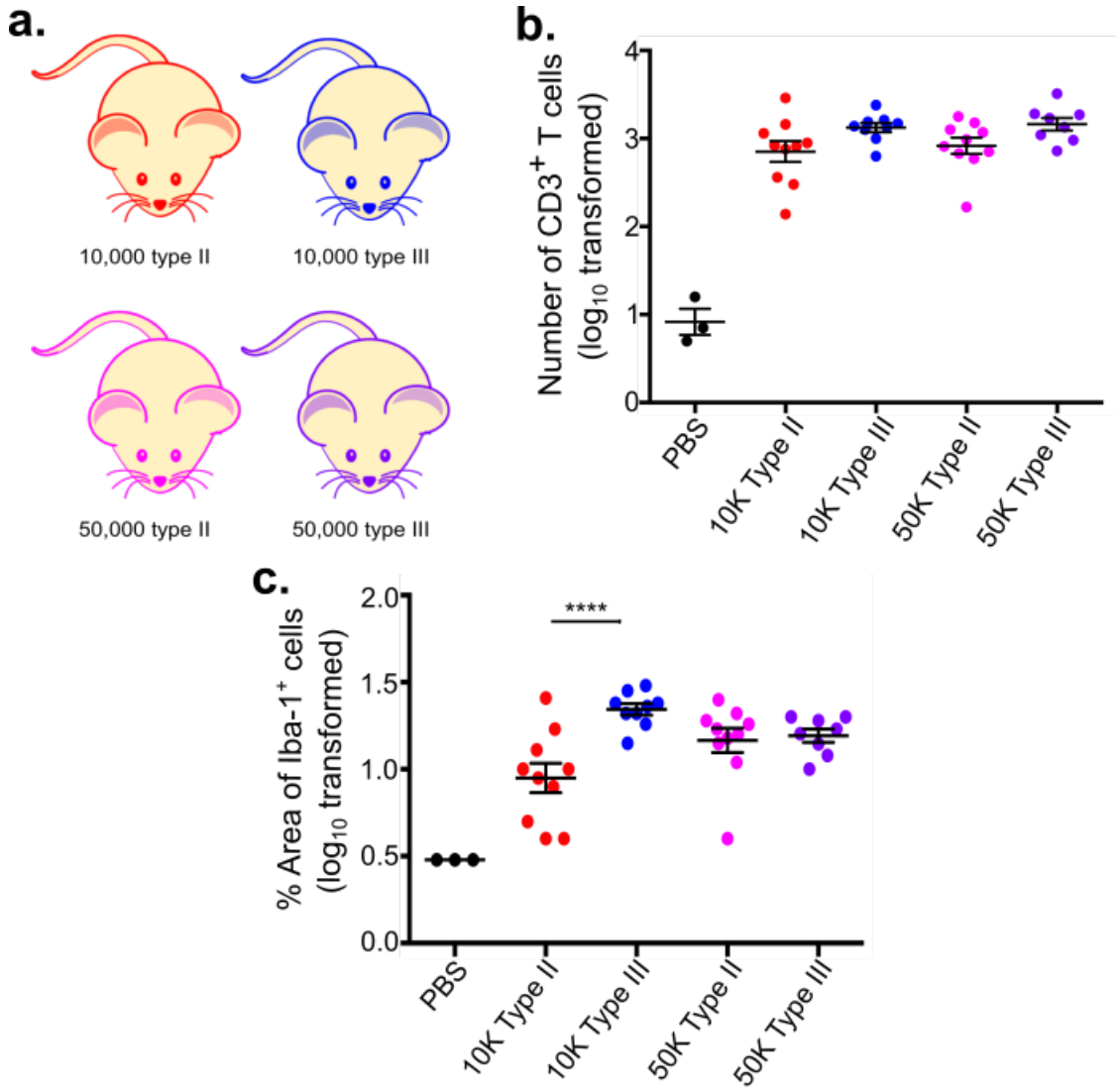


Figure 2**. Establishing a reliable model of parasite infection**. (A) Mice were infected with varying doses of type II and type III parasites. Brains were harvested and sections were stained for T cells (anti-CD3) and macrophage/microglia response (anti-Iba-1). (B) Quantification of T cells infiltrating into the CNS after infection. (C) Quantification of percent area of Iba-1^{+} cells. N= 9-11 mice /strain. N=12 fields of view/section, 3 sections/mouse. ****p<0.0001, non-parametric t-test.

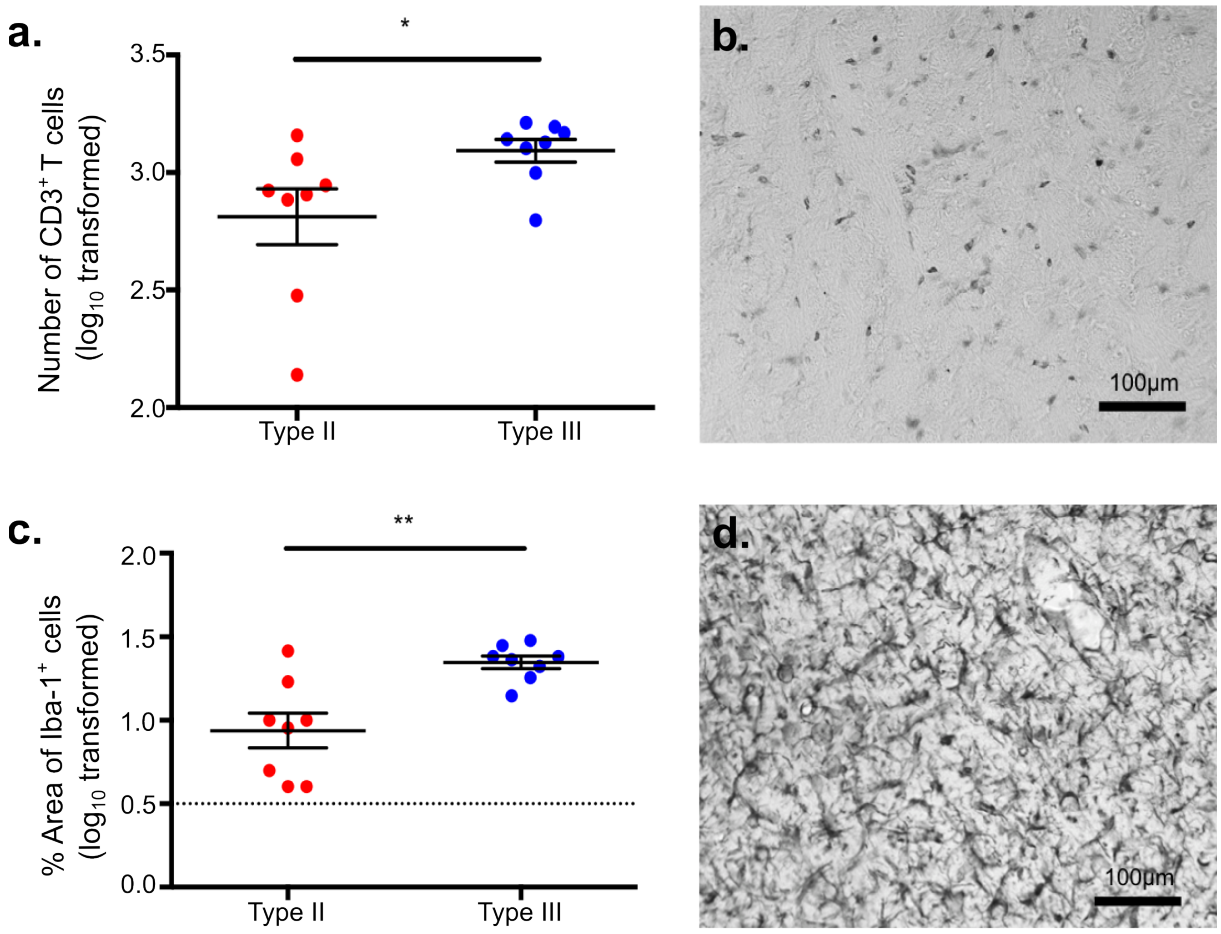


Figure 3. **Difference observed in CNS immune response between mice infected with either Type II or Type III parasites.** Brain sections at 3 wpi were stained for T cells (anti-CD3) or macrophage/microglia (anti-Iba-1) after which the staining was quantified by stereology. (A) Quantification of the number of T cells infiltrating the CNS in type II or type III infected mice. (B) Representative image of T cell staining (anti-CD3). (C) Quantification of the percent area covered by Iba-1+ staining. The dotted line signifies the level of Iba-1+ staining in saline-inoculated mice. (D) Representative image of Iba-1+ staining. N=9-11 mice/strain. N=12 fields of view/section, 3 sections/mouse. Prior to graphing and statistical analysis number were log transformed to improve the distributional characteristics. **p<0.01, non-parametric t-test.

Strain-specific difference observed in the CNS immune response despite similar parasite burden

One of the easiest explanations to understand the difference in immune response between the two strains would be a difference in the parasite burden. To ensure that the difference in the neuroinflammatory response observed between type II and type III was not secondary to CNS parasite burden, we quantified the burden in two ways (Figure 4). First, we quantified the number of cysts in seven sections of brain/mouse. This quantification was done with use of fluorescence in two channels, ensuring that the parasites (mCherry) were housed within the cyst (dolichos). We found no differences in the number of cysts. Given that our cyst counting was done on a very limited sample size (7 sections out of 300 total sections) and the locations of infection vary across mice, we also quantified the amount of *Toxoplasma* DNA present in each sample through quantitative PCR (Q-PCR) of *Toxoplasma'*s B1 gene (17). We used this method to quantify the *Toxoplasma* DNA in 25-30 mg from 25% of brain per mouse. We used the frontal lobes for this quantification because cysts are consistently found in the frontal lobe (19). By Q-PCR, we again found that there is no significant difference between type II and type III parasite burden in the brains of infected mice. Therefore, by two measures the parasite burden between the type II and III infected mice, they did not differ at 3 wpi, suggesting that the differences in immune response were not simply secondary to differences in parasite burden.

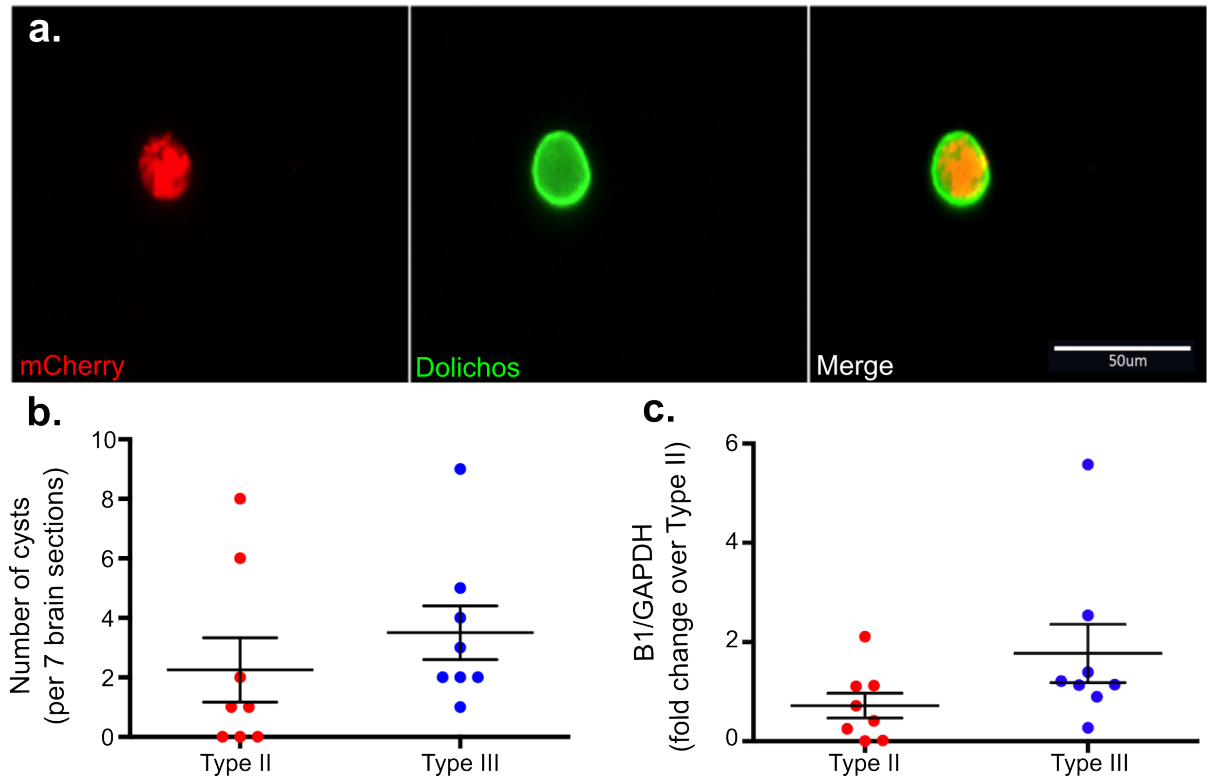


Figure 4. **Difference in the neuroinflammatory response is not driven by CNS parasite burden.** Total DNA was harvested from 10 g of brain at 3 wpi. (A) Representative image of capturing a cyst on the fluorescent microscope. (B) Number of cysts are quantified in 7 equivalent sections per mouse, the difference in cyst number is not significant, p=0.3907, student's t-test. (C) Quantitative PCR on the Toxoplasma B1 gene was used to assess parasite burden. p=0.12, student's t-test

Type III infected mice have a more pro-inflammatory cytokine CNS environment

After characterizing the immune response with IHC, and identifying that the difference observed between the two strains is not due to parasite burden we wanted to further characterize the global changes in the cytokine environment after infection with type II or type III parasites. Therefore, we ran a 25-plex-cytokine assay in order to quantify the cytokine milieu that arises due to infection with either strain of *Toxoplasma*. We found that the CNS of mice infected with type III showed a 2-fold increase across many pro-inflammatory cytokines than when compared to the CNS of type II infected mice (Table 2).

Cytokine	Type II	Type III
IFN-g	22.15	58.47**
IL-6	11.68	25.99*
IP-10	36.59	52.02*
KC	6.62	13.39**
MCP-1	15.87	44.48**
RANTES	161.39	240.98*
TNF-a	7.16	12.38**
G-CSF		
IL-1a		
IL-1B		
IL-12p40		
IL-12p70		
IL-17		
MIP2		

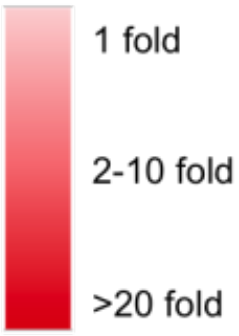


Table 2. **Brain homogenates from type III infected mice show higher levels of pro- inflammatory cytokines compared to the type II infected mice.** At 3 wpi, brains were harvested and proteins isolated from brain homogenates. We performed a 25-plex cytokine assay to analyze the cytokine milieu in the CNS. Data is represented as a fold difference over uninfected brain (saline) and is represented as a heat map. *p<0.05, **p<0.01 for type II vs. type III CNS cytokines, student's t test. Cytokines not listed: G-MCSF, IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-13, IL-15, IL-17, MIP1a, MIP1b.

Difference in the quality of immune response between type II and type III infection

While it was possible that the type III associated increase in CNS pro-inflammatory cytokines was simply secondary to the increase in T cells and macrophages/microglia observed in type III infection, it was also possible that the cytokine differences reflected strain-specific differences in the quality of the immune response. With IHC we are able to quantify T cell and macrophages

and microglial response, however, we were not able to determine different types of T cells and macrophages that were infiltrating to the CNS after *Toxoplasma* infection. Thus, we used a flow cytometry assay, as it allows us to characterize different types of T cells and macrophages that are infiltrating into the CNS after infection. Infected brain tissue was made into a single cell suspension and leukocytes were stained for several different immune cell markers (Table 3), allowing us to stain for specific types of T cells and macrophages (Figure 5). Remarkably, type II infected mice showed an increase in the regulatory T cell (T regs) response. Regulatory T cells are known to dampen the immune response. In addition, some evidence suggests that the immune suppression by T regs creates a more anti-inflammatory environment, which leads to macrophages being more likely to be polarized from M1 (classically activated) to M2 (alternatively activated) macrophages. This could explain the trend we see with macrophage activation where type II infected brains have higher levels of alternatively activated macrophages than when compared to type III infected brains (Figure 6b). Also, type III infected brains have higher levels of classically activated macrophages (Table 4), which might explain the more robust pro-inflammatory response seen in the cytokine milieu. Table 4 summarizes the differences found by flow cytometry in immune cell populations isolated from the CNS of type II vs. type III infected mice.

Cell types	Markers[a]
CD3 T lymphocytes	$CD3^{hi}$
B lymphocytes	$CD19^{hi}$
CD8 T lymphocytes	$CD3^{hi}$, $CD8^{hi}$, $CD4^{lo}$
CD4 T lymphocytes	$CD3^{hi}$, $CD8^{lo}$, $CD4^{hi}$
T regs	$CD3^{hi}$, $CD8^{lo}$, $CD4^{hi}$, $CD25^{hi}$, $FoxP3^{hi}$
T cell exhaustion	$CD3^{hi}$, $CD8^{hi}$, $CD4^{lo}$, PD-1^{hi}, $CD44^{lo}$
Macrophages	$CD45^{hi}$, F4-80^{hi}, $CD11b^{hi/int/lo}$, $CD11c^{int/lo}$
Microglia	$CD45^{int}$, F4-80^{hi}, $CD11b^{int/lo}$, $CD11c^{int/lo}$
Classically Active Macrophages	F4-80^{lo}, $CD11b^{lo}$, $CD11c^{hi/int}$, $CD80^{hi}$, CD86 ^{hi}
Alternatively Active Macrophages	F4-80^{lo}, $CD11b^{hi/int}$, $CD16^{hi}$, 206^{hi}, $CXCR3^{hi}$
Dendritic cells	F4-80^{lo}, $CD11b^{lo}$, $CD11c^{hi/int}$
Neutrophils	F4-80^{lo}, $CD11b^{hi/int}$, Gr-1^{hi}

[a] hi, high level; lo, low level; int, intermediate level

Table 3. Defining various cell types in the brain.

Table courtesy of Shraddha Tuladhar.

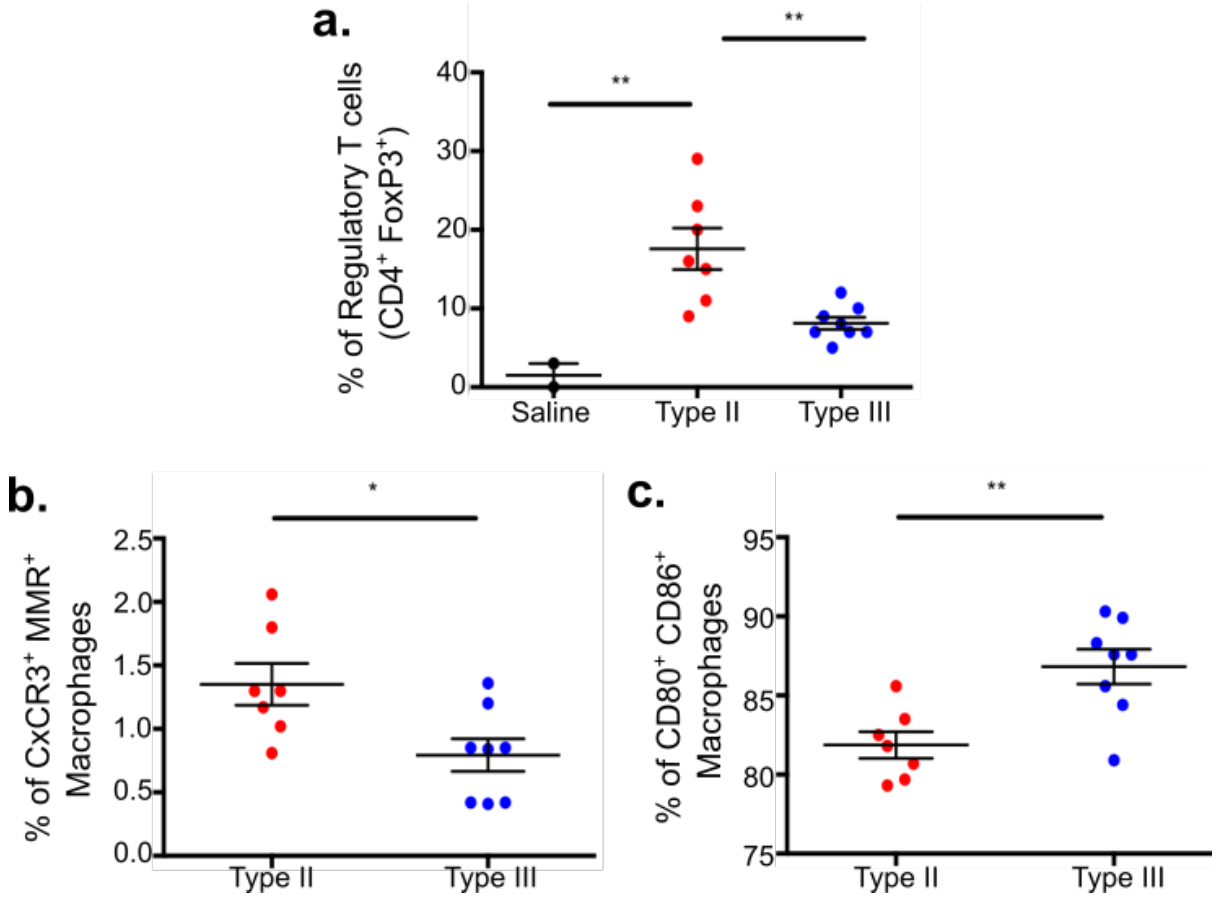


Figure 5. **Qualitative differences between strain-specific CNS immune response.** (A) Difference in regulatory T cell levels between type II and type III. **p<0.01, ANOVA test. (B-C) CxCR3+ MMR+ macrophages represent the M2 phenotype, whereas CD80+ CD86+ macrophages represent the M1 phenotype. Type II has greater levels of M2 macrophages, and Type III has greater levels of M1 macrophages at 3 wpi. *p<0.05, **p<0.01, student's t-test.

Figure courtesy of Shraddha Tuladhar.

Cell Types	Type II	Type III
$CD3^+$ T cells	No difference	
$CD4^+$ T cells	No difference	
$CD8^+$ T cells	No difference	
Exhausted T cells ($CD8^+$ $PD\text{-}1^+$)	No difference	
Regulatory T cells ($CD4^+$ $FoxP3^+$)	↑↑	↑

Cell Types	Type II	Type III
Macrophages ($F4/80^+$ $CD11b^+$)	No difference	
$CD80^+$ $CD86^+$Macrophages	↑	↑↑
$CxCR3^+$ MMR^+ Macrophages	↑↑	↑

Table 4. Summary of strain-specific qualitative CNS immune response differences. Table courtesy of Shraddha Tuladhar.

DISCUSSION

In this study, we were able to compare the CNS immune response that is provoked by two genetically divergent strains of *Toxoplasma.* In the late 1980's, it was shown that mice infected with either type II or non-type II caused distinct neuroinflammatory responses (12). Although these studies provided a histological difference between the two strains, they did not do any quantitative or qualitative analysis. Emerging data suggest that part of the clinical variability found in CNS outcomes may be attributed to different strains that individuals are infected with but how these strains vary in producing different disease outcomes is not well understood (4). Therefore, further understanding the differences in the immune response due to different strains might aid in explaining how *Toxoplasma* strain genotype influences the severity of disease. We

first established a reliable mouse model of CNS toxoplasmosis. Using our newly established model, we found that at 3 wpi the type III *Toxoplasma* strain provoked a more robust macrophage/microglia and T cell response than when compared to type II. Next, we wanted to ensure that the parasite burdens were not the underlying cause for the differences in neuroinflammatory response that we observed. Through Q-PCR analysis of the *Toxoplasma* B1 gene and cyst count we showed that there was no significant difference in parasite burden at 3 wpi between type II and type III infection. As the quantitative immune response between type II and type III infection were different we next wanted to know if there was any difference in the global cytokine response in the CNS of these mice that are infected with either type II or type III. Using a 25-plex Luminex assay, we found that type III infected mice had more pro-inflammatory cytokine milieu than seen in type II infection. Finally, through use of flow cytometry we showed that there are also qualitative differences in the immune response between the two strains.

As summarized above, our studies strongly suggest that type III infection provokes a more pro-inflammatory CNS immune response than type II infection. These data are particularly interesting because they contradict what has been predicted from previous *in vitro* work on the effect of *Toxoplasma* polymorphic effector proteins that differentially modulate host cell signaling pathways depending on the allelic version of the secretory protein (13). As noted previously, *in vitro* $GRA15_{II}$ elicits a more pro-inflammatory cytokine milieu by activation of the NFκB pathway whereas $ROP16_{I/III}$ elicits a more anti-inflammatory cytokine milieu through activation of the STAT 3/STAT 6 pathway (20)(21). Thus, our data potentially suggest that our ability to predict complex phenotypes from *in vitro* assays is limited.

This study establishes that there is a strain-specific CNS immune response provoked by *Toxoplasma* infection, therefore our future directions will address what parasite factors underlie these differences. To accomplish this goal, we will utilize the CRISPR-Cas9 system to engineer transgenic parasite strains. Due to the signaling pathway modulation in response to the polymorphic effector proteins mentioned above, ROP16 and GRA15, these are prime candidates for genetic editing in our transgenic strains. We will be knocking out the endogenous gene while expressing the allelic version of the protein of the other strain. For example, in order to understand the effects of $ROP16_{I/III}$ we will express it in the type II strain but first having knocked out the gene for $ROP16_{II.}$ We will be comparing the following strains: type II, type II ΔROP16, type II ΔROP16 + $ROP16_{I/III}$ and type III, type III ΔGRA15, type III ΔGRA15 + $GRA15_{II}$.

Another future project will be exploring parasite kinetics, or the rate of dissemination. As mentioned previously, we use Cre-reporter mice that express GFP only after Cre-mediated recombination. Since our parasites are engineered to inject a Toxoplasma:Cre fusion protein into the host cells, we are able to quantify the number of cells in the CNS that have interacted with the parasite by counting the CNS cells that express GFP. Interestingly, throughout our work we have seen that at 3 wpi there are significantly more GFP^+ cells in type III infected brains. Since at this time point the parasite burden is not significantly different (Figure 4), another possible explanation is a difference in parasite kinetics. Therefore by quantifying the B1 gene at earlier time points, such as 5 days post infection (dpi) and 10 dpi, we can determine if type III parasites have a kinetic advantage that drives the strain-specific differences in CNS immune response that we observed. Finally, to determine if the CNS immune responses are specific to the CNS or

simply reflect the systemic immune response, we are performing flow cytometry analyses on immune cells isolated from the spleen.

It is clear that at 3 wpi there is a significant quantitative and qualitative difference in the neuroinflammatory response between type II and type III strains. Since we are seeing an overall greater immune response provoked by type III perhaps this more robust response is able to more effectively clear parasites and resolve the inflammation at later time points versus type II that is unable to resolve the inflammation.

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