


Dissecting Amyloid Beta Deposition Using Distinct Strains of the Neurotropic Parasite *Toxoplasma gondii* as a Novel Tool

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Abstract

Genetic and pathologic data suggest that amyloid beta (A β), produced by processing of the amyloid precursor protein, is a major initiator of Alzheimer's disease (AD). To gain new insights into A β modulation, we sought to harness the power of the coevolution between the neurotropic parasite *Toxoplasma gondii* and the mammalian brain. Two prior studies attributed *Toxoplasma*-associated protection against A β to increases in anti-inflammatory cytokines (TGF- β and IL-10) and infiltrating phagocytic monocytes. These studies only used one *Toxoplasma* strain making it difficult to determine if the noted changes were associated with A β protection or simply infection. To address this limitation, we infected a third human amyloid precursor protein AD mouse model (J20) with each of the genetically distinct, canonical strains of *Toxoplasma* (Type I, Type II, or Type III). We then evaluated the central nervous system (CNS) for A β deposition, immune cell responses, global cytokine environment, and parasite burden. We found that only Type II infection was protective against A β deposition despite both Type II and Type III strains establishing a chronic CNS infection and inflammatory response. Compared with uninfected and Type I-infected mice, both Type II- and Type III-infected mice showed increased numbers of CNS T cells and microglia and elevated pro-inflammatory cytokines, but neither group showed a >2-fold elevation of TGF- β or IL-10. These data suggest that we can now use our identification of protective (Type II) and nonprotective (Type III) *Toxoplasma* strains to determine what parasite and host factors are linked to decreased A β burden rather than simply with infection.

Keywords

Alzheimer's disease, amyloid beta, neurodegeneration, neuroprotection, *Toxoplasma*

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder marked by memory loss, behavior changes, and ultimately, death. An estimated 5.4 million Americans and 46.8 million people worldwide are afflicted (Alzheimer Association, 2016). The costs of caring for AD patients is estimated at \$236 billion/year but are projected to rise to over \$1 trillion in 2050 (Alzheimer Association, 2016). These data, combined with the lack of effective treatments despite decades of research, highlight the importance of taking innovative strategies to find new therapies.

Although uncertainty remains about the mechanisms behind disease initiation and progression, both the

amyloid cascade hypothesis and oligomeric amyloid hypothesis bring together genetic and pathologic data. They suggest that cleavage of the amyloid precursor protein (APP) into amyloid beta (A β) oligomers that form

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A β plaques initiates the pathological process of AD (Hardy and Higgins, 1992; Haass and Selkoe, 2007; Ferreira and Klein, 2011).

For over two decades, researchers have been targeting many molecular pathways in search of therapeutic drug targets, including the A β pathway (Graham et al., 2017). Several attempts have been made to target A β by administration of monoclonal antibodies, but only recently has one of these antibody treatments shown promise in removing A β in AD patients (Bohrmann et al., 2012; Farlow et al., 2012; Garber, 2012; Sperling et al., 2012; Moreth et al., 2013; Sevigny et al., 2016). However, treatment is lengthy and expensive, highlighting the necessity for novel and broad approaches to understanding APP-A β dynamics that may lead to alternative treatment options. Interestingly, the neurotropic parasite *Toxoplasma gondii* has shown particular promise in prevention of A β in the CNS (Jung et al., 2012; Möhle et al., 2016). *Toxoplasma* is an intracellular parasite that naturally, persistently, and asymptotically infects the CNS of both humans and rodents. Three studies have shown that mice chronically infected with one particular strain of *Toxoplasma* are protected against a second CNS insult (Arsenijevic et al., 2007; Jung et al., 2012; Möhle et al., 2016). Two of these studies found a >60% reduction in A β deposition in infected mice compared with uninfected AD mice (Jung et al., 2012; Möhle et al., 2016). One study suggested that this protection was secondary to *Toxoplasma* inducing the anti-inflammatory cytokines TGF- β and IL-10 (Jung et al., 2012) while the other suggested it was due to increases in infiltrating monocytes capable of phagocytosing A β (Möhle et al., 2016). Despite these exciting findings, both studies left a number of unanswered questions: Is protection specific to the *Toxoplasma* strain used? Is a persistent CNS infection required for A β protection? Finally, given the vast number of changes between the CNS of an infected brain and an uninfected brain (Hermes et al., 2008; Pittman et al., 2014), were the previously noted “protective” differences simply due to infection?

To answer these questions, we sought to leverage the genetic diversity of *Toxoplasma*. *Toxoplasma* has over 15 different strain types, of which, the best studied are the original three canonical strains (Type I, Type II, and Type III; Howe and Sibley, 1995; Su et al., 2012). These strains were defined by their phenotypic differences in acute virulence in mice with RFLP mapping confirming their genetic divergence (Sibley and Boothroyd, 1992). Comparing these strains has led to a greater understanding of the host and parasite genes responsible for strain-specific effects (Saeij et al., 2006; Taylor et al., 2006; Saeij et al., 2007; Laliberté and Carruthers, 2008; Reese et al., 2011; Behnke et al., 2012). Thus, we

hypothesized that if the *Toxoplasma* effect against A β was strain specific, we would have a model that would allow us to identify the *Toxoplasma*-induced CNS changes linked solely to A β protection. To test this hypothesis, we infected a third human amyloid precursor mouse model (J20; Mucke et al., 2000), with each of the three canonical strains of *Toxoplasma* and compared them to uninfected mice. At 6 months postinfection (mpi), we evaluated hippocampal A β plaque load, CNS T cell and macrophage/microglial responses, and the overall CNS cytokine milieu. We found that only mice infected with the Type II strain had a decrease in A β plaque burden, despite both persistent strains inducing an overall pro-inflammatory CNS environment, equivalent T cell infiltration, and a doubling of the number of CNS macrophages/microglia. Although CNS levels of TGF- β and IL-10 were increased in Type II-infected mice, they were also increased in Type III infection implying these cytokines are not the driving factors for Type II-mediated protection. Collectively, these data suggest that the effect of *Toxoplasma* infection on A β deposition is specific to Type II strains and confirms the effect is consistent across multiple AD mouse models. Our data also suggest that we have now established a model by which we can leverage strain-specific A β phenotypes to distinguish CNS changes and parasite genes specifically associated with protection rather than simply with infection.

Materials and Methods

Mice

Thirty female hemizygous B6.Cg-Tg(PDGFB-APP^{SwInd})20Lms/2J/Mmjax (J20) mice (Mucke et al., 2000) were purchased from MMRRC (<https://www.jax.org/strain/006293>). These transgenic mice express a mutant form of the human APP that contains both the Swedish (K670N/M671L) and the Indiana (V717F) mutations (*APP^{SwInd}*). Expression of the transgenic insert is directed by the human platelet-derived growth factor beta polypeptide promoter. Mice were housed in microfiltration cages in University of Arizona specific-pathogen-free Animal Care Facility on a 12 hr light/dark cycle and were provided with food and water ad libitum. All experiments were carried out in strict accordance with the Public Health Service Policy on Human Care and Use of Laboratory Animals. The protocol was approved by the University of Arizona's Institutional Animal Care and Use Committee (#12-391).

Toxoplasma gondii Infection

At 3 months of age, mice were intraperitoneally injected with one of three distinct strains of *Toxoplasma*

gondii: attenuated Type I (RH Δ ku80 Δ ROP5; Reese et al., 2011); Type II (Pru Δ hpt); or Type III (CEP Δ hpt; Saeij et al., 2006). Parasites were maintained in human foreskin fibroblasts, (gift of John Boothroyd, Stanford University, Stanford, CA) using DMEM, supplemented with 10% fetal bovine serum, 2 mM glutagro, 100 I.U./ml penicillin and 100 μ g/ml streptomycin before being syringe-released, counted, and diluted to an inoculum of 100 K Type I, 10 K Type II, or 50 K Type III in 200 μ l USP Grade sterile PBS. To monitor the course of infection and track the health of the mice, they were weighed weekly for 6 months.

Tissue Processing

At 9 months of age, 24 mice (6 of 8 Type I, 1 died 2 weeks postinfection [wpi], 1 died 3 mpi; 5 of 8 Type II, 1 died 2.5 wpi, 1 died 15 wpi, 1 died 6 mpi; 8 of 8 Type III, no deaths; and 4 of 6 uninfected, 1 died prior to infection, 1 died 6.5 wpi) were anesthetized with ketamine (24 mg/ml) and xylazine (4.8 mg/ml) then transcardially perfused with 0.9% saline. Brains were harvested as follows: The left hemisphere of the brain was fixed in 4% paraformaldehyde in phosphate buffer and kept at 4°C overnight, rinsed in PBS then transferred to 30% sucrose in PBS, and stored at 4°C; the right half of the brain was cut coronally into two quarters, placed in separate tubes, flash frozen then stored at -80°C. Postfixed brains were sagittally sectioned to 40 μ m thickness on a freezing sliding microtome (Microm HM 430) into 12 sequential tubes so that each tube contained every 12th section spaced 480 μ m apart. Sections were stored free-floating in cryoprotective media (0.05 M sodium phosphate buffer containing 30% glycerol and 30% ethylene glycol) until stained and mounted on slides.

Immunohistochemistry

Free-floating tissue sections were stained using standard techniques. In brief, for immunohistochemical staining, sections were washed, endogenous peroxidases quenched with 0.6% H₂O₂ in 0.3% Triton-X/TBS for 40 min, then blocked with 3% Goat Serum in 0.3% Triton-X/TBS for 1 hr and incubated with the following primary antibodies: polyclonal rabbit anti-beta amyloid (ab2539, abcam, 1:1000); polyclonal rabbit anti-Iba1 (019-19741, Wako Pure Chemical Industries, Ltd., 1:3000); monoclonal hamster anti-mouse CD3 ϵ 500A2 (550277, BD PharmingenTM, 1:500). The following secondary antibodies were used: biotinylated goat anti-rabbit (BA-1000, Vector Laboratories, 1:500) and biotinylated goat anti-hamster (BA-9100, Vector Laboratories, 1:500). ABC (32020, Thermo Fisher)

followed by 3,3'-Diaminobenzidine (SK-4100, Vector Laboratories) was used to detect biotinylated antibodies. For immunofluorescent staining, sections were washed, blocked with 3% Goat Serum in 0.3% Triton-X/TBS for 1 hr and incubated with the following primary antibodies: monoclonal rabbit anti-TMEM119 (ab209064, abcam, 1:200) and polyclonal chicken anti-Iba1 (ab139590, abcam, 1:1000). The following secondary antibodies were used: polyclonal goat anti-rabbit 488 (A11008, Thermo Fisher Scientific, 1:200) and polyclonal goat anti-chicken 568 (A11041, Thermo Fisher Scientific, 1:200). Sections were washed then incubated in DAPI (4',6-Diamidino-2-Phenylindole, Dilactate; D3571, Thermo Fisher Scientific, 300 μ M @ 1:1000).

Image Analysis

All images were obtained on either a Leica DMI6000 motorized inverted microscope with a Leica DCF450 camera and 20 \times /0.60 objective using Leica Application Suite X software or an Olympus IMT-2 inverted light microscope with a Hamamatsu ORCA-100 grayscale CCD camera and 20 \times /0.70 objective. To analyze the number of CD3⁺ and Iba1⁺ cells and percent area of Iba1⁺ cells in the cortex, 12 fields of view from three sections/mouse were thresholded in Simple PCI software (Hamamatsu, Sewickley, PA). All other analyses were performed by thresholding or manually counting cells with FIJI software (Schindelin et al., 2012) from three sections per mouse except for A β plaque quantitation where two sections/mouse were analyzed. For all hippocampal quantification, a standard area that encompassed the whole hippocampus was used. For immunofluorescently stained sections, for each section, 100 cells from across the cortex and 100 cells from the hippocampus were analyzed using Zeiss Zen black software for Iba1 and TMEM119 colocalization. For each mouse, 3 sections were analyzed.

Real-Time PCR for Toxoplasma Parasite Burden

DNA was isolated from the rostral quarter of frozen brain tissue samples using a DNeasy Blood & Tissue Kit (69504, Qiagen) following manufacturer instructions. Amplification of the highly conserved, 35-fold repeat B1 gene was performed using SYBR[®]Green fluorescence detection with the Eppendorf Mastercycler[®] ep realplex 2.2 system using forward primer 5'-AGG TCG GTG TGA ACG GAT TTG-3' and reverse primer 5'-AGC GTT CGT GGT CAA CTA TCG ATT G-3'. Each reaction contained a final volume of 20 μ l comprising 10 μ l SYBR[®]Green PCR Master Mix (4472908, Applied Biosystems), 0.5 μ l of each primer at a concentration of 10 μ M/ μ l, 4 μ l of DNA at a concentration of 10 ng/ μ l,

and 5 μ l of sterile nuclease free water. The reaction was carried out as follows: 50°C for 2 min, 95°C for 2 min then 40 cycles of 15 s at 95°C and 1 min at 60°C followed by a melting curve analysis. Standards, samples, and negative controls were analyzed in triplicate for each run. In uninfected mice, the lowest average cycle threshold (CT) value was 37; therefore, values ≥ 37 were considered below the level of detection. The comparative CT method was used to evaluate levels of parasite burden relative to Type III-infected mice. The equation used was $2^{-\Delta\Delta CT} = [(CT \text{ B1 gene} - CT \text{ of GAPDH}) \text{ for each mouse} - \text{Avg} (CT \text{ B1 gene} - CT \text{ of GAPDH}) \text{ for Type III mice}]$.

Protein Extraction, Multiplex Cytokine Assay, and ELISAs

Caudal quarter of flash-frozen brain tissue was homogenized in radioimmunoprecipitation assay buffer (1% Triton-X, 0.1% SDS, 1 \times PBS) plus 1% phosphatase inhibitor cocktail (P5726-1ML, Sigma-Aldrich) and 1% protease inhibitor cocktail (P8340-1ML, Sigma-Aldrich). Samples were sonicated on ice in 3-s bursts until homogeneous with buffer, then centrifuged at 4°C for 3 min at 13,000 r/min. Supernatant was collected, respun, and the final supernatant placed on ice. The concentration of each sample was measured using a Direct Detect[®] Infrared Spectrometer before being stored at -80°C. Cytokines were assessed using the MILLIPLEX-MAP-Mouse-Cytokine/Chemokine-Magnetic-Bead-Panel (MCYTOMAG-70K, EMD Millipore; Dinel et al., 2014; de Theije et al., 2014; Lubitz et al., 2014). This panel allows for detection of 25 cytokines and includes quality controls for each cytokine/chemokine in the kit. After placing duplicate samples on the plate, the plate was analyzed with a Luminex[®] MAGPIX xPONENT 4.2 System which uses Milliplex Analyst software and Luminex[®] technology to detect individual cytokine quantities. TGF- β was analyzed using the Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit (MB100B, R&D Systems) and read using a VERSAmax tunable microplate reader (Molecular Devices). Both kits were performed following manufacturer's instructions with each sample measured in duplicate.

Statistical Analysis

Statistical analyses were performed using Prism 7.0 software. For comparisons between groups of four, except for quantitative PCR analysis, all raw data were square root transformed to improve distributional characteristics prior to analysis. Multiplex cytokine data were analyzed using multiple comparisons after repeated measures

analysis of variance. All other data were analyzed by one-way analysis of variance with Fisher's least significant difference post hoc test.

Results

Only Type II Infection Significantly Decreases Hippocampal A β Plaque Burden

Previous studies demonstrated that one specific Type II *Toxoplasma* strain protected against A β deposition (Jung et al., 2012; Möhle et al., 2016). To determine if this A β protective effect was *Toxoplasma* strain specific, we infected 3-month old J20 human amyloid precursor protein (hAPP) model mice (J20; Mucke et al., 2000), with each of the three genetically distinct, canonical strains of *Toxoplasma* (Type I, II, and III), including using a different but genetically similar Type II strain than the prior work (Su et al., 2012; Lorenzi et al., 2016). As Type I strains are universally lethal during acute infection (Sibley and Boothroyd, 1992), here we used an attenuated Type I strain that lacks a major acute virulence locus (Reese et al., 2011; Behnke et al., 2012). This attenuated strain has been shown to cause only an acute infection with no CNS persistence (Reese et al., 2011; Ingram et al., 2013). For simplicity, from here forward, we refer to this strain simply as Type I. J20 mice are known to show A β deposition at ~5 to 10 months of age. During this time period, and consistent with human pathology, plaques form in the hippocampus first before spreading to the cortex (Mucke et al., 2000). Consistent with Jung et al. (2012), we sacrificed the mice at 6 mpi (9 months of age) and assessed extracellular A β deposition by immunohistochemistry (Figure 1(a)). Plaque deposition within the hippocampus was quantified and compared between groups in three different ways: percent area of A β staining (Figure 1(b)), number of plaques (Figure 1(c)), and average size of plaques (Figure 1(d)). There was no significant difference in percent area of positive staining, number, or size of plaques between Type I and Type III-infected mice as compared with uninfected (saline-injected mice; Figure 1(a)–(d)). Only Type II-infected mice showed a significant decrease in all three parameters compared with uninfected (Figure 1(a)–(d)). Together, these data indicate that the protective effect of *Toxoplasma* infection on A β deposition is unique to Type II strains.

Macrophage/Microglia Response Significantly Increased in Type III Infection

Given the findings by Möhle et al. (2016) and others (Lai and McLaurin, 2012; Hohsfield and Humpel, 2015) that infiltrating macrophages can act as the major phagocytic

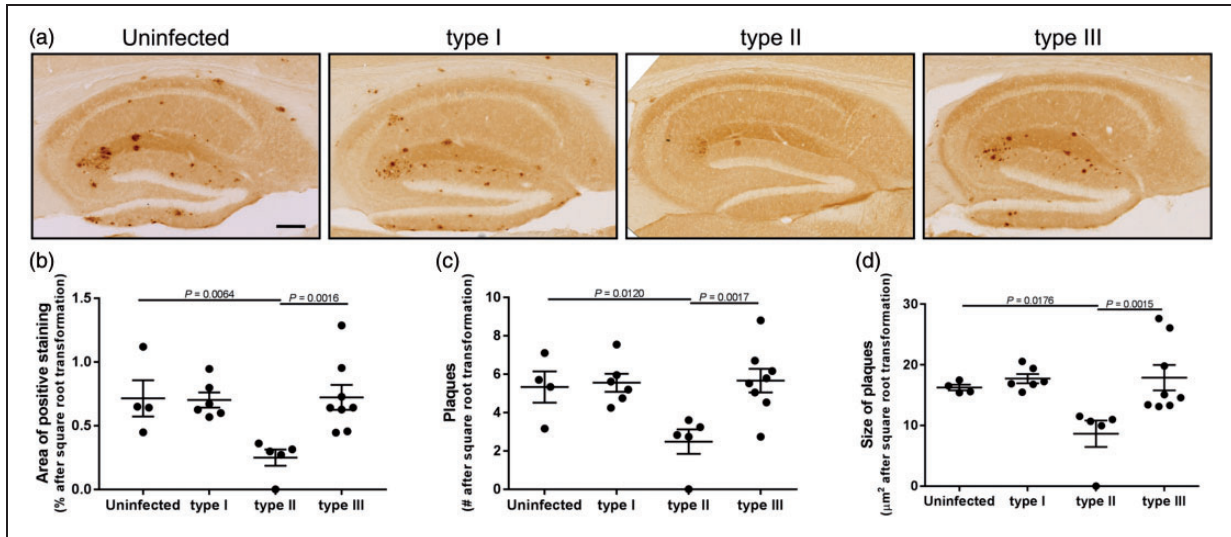


Figure 1. Hippocampal amyloid- β plaque burden is significantly reduced in Type II-infected J20 mice. Brain tissue sections from J20 uninfected or infected mice (as indicated) were stained for amyloid- β ($A\beta$) and imaged by light microscopy. Resulting hippocampal images were evaluated with Fiji for noted parameters. (a) Representative images immunohistochemically stained for $A\beta$, scale bar = 200 μm . (b) Quantification of the percent area of positive $A\beta$ staining in uninfected, Type I-, Type II-, and Type III-infected J20 mice. (c) As in (b) except now quantification is of the average number of $A\beta$ plaques per mouse hippocampus. (d) As in (b) except now quantification is of the average size of hippocampal plaques per mouse. For (b) to (d), each dot represents one mouse. Two sections/mouse, four to eight mice/group, bars represent mean \pm SEM.

population against $A\beta$ deposition, we sought to determine if infection with different strains of *Toxoplasma* led to differences in macrophage/microglial activation. We evaluated the macrophage/microglial immune response across our groups at 6 mpi by immunohistochemically staining for Iba1, a marker that stains both macrophages and microglia (Kanazawa et al., 2002; Tanaka et al., 2003; Le Blon et al., 2014). As we cannot distinguish between macrophages or microglia using this marker, for accuracy, we refer to these cells as macrophage/microglia. We then evaluated the stained cells for morphology, as well as quantifying the percentage of positively stained tissue and the overall numbers of Iba1⁺ cells in both the cortex and the hippocampus. In the cortex, uninfected and Type I-infected mice had macrophage/microglia with a ramified, unactivated morphology, while Type II-infected mice showed a slight increase in macrophage/microglia ramification and Type III-infected mice showed a hyper-ramified/bushy morphology with processes that appear thicker and darker compared with all other groups (Figure 2(a), insets). Interestingly, only in Type III-infected mice did these changes result in a significant increase in staining compared with uninfected mice (Figure 2(b)). Conversely, quantification of the number of Iba1⁺ cells revealed that both Type II- and Type III-infected mice had almost double the number of Iba1⁺ cells compared with uninfected mice (Figure 2(c)). Similar results were found in the hippocampus, with only Type III-infected mice showing a significant increase

in Iba1⁺ staining and both Type II- and Type III-infected mice showing double the number of Iba1⁺ cells compared with uninfected and Type I-infected mice (Figure 2(d)–(f)). Interestingly, in the hippocampi of uninfected and Type I-infected mice, clusters of Iba1⁺ cells were found (Figure 2(d)) in patterns similar to that of plaque deposition in mice of this age in this brain region (Figure 1(a)). Little to no cell clustering was observed in hippocampi from Type II-infected mice, and minimal clustering was observed in hippocampi from Type III-infected mice (Figure 2(d)). These data demonstrate that both the protective (Type II) and nonprotective (Type III) strains show an equivalent increase in the number of Iba1⁺ cells in the brain, suggesting that an increase in macrophage infiltration and/or microglial proliferation is not sufficient to offer protection against $A\beta$. However, the differences in morphology between Iba1⁺ cells in Type II and Type III infection suggest that these macrophage/microglia may be differentially activated. Finally, our analyses also suggest that percentage of Iba1⁺ staining does not necessarily correlate with the number of macrophage/microglia present in the cortex.

Increased Numbers of *TMEM119*[−]/Iba1⁺ Cells in the Hippocampus of Uninfected and Type I-Infected Mice

As both macrophages and microglia are Iba1⁺, and *Toxoplasma* infection is known to result in

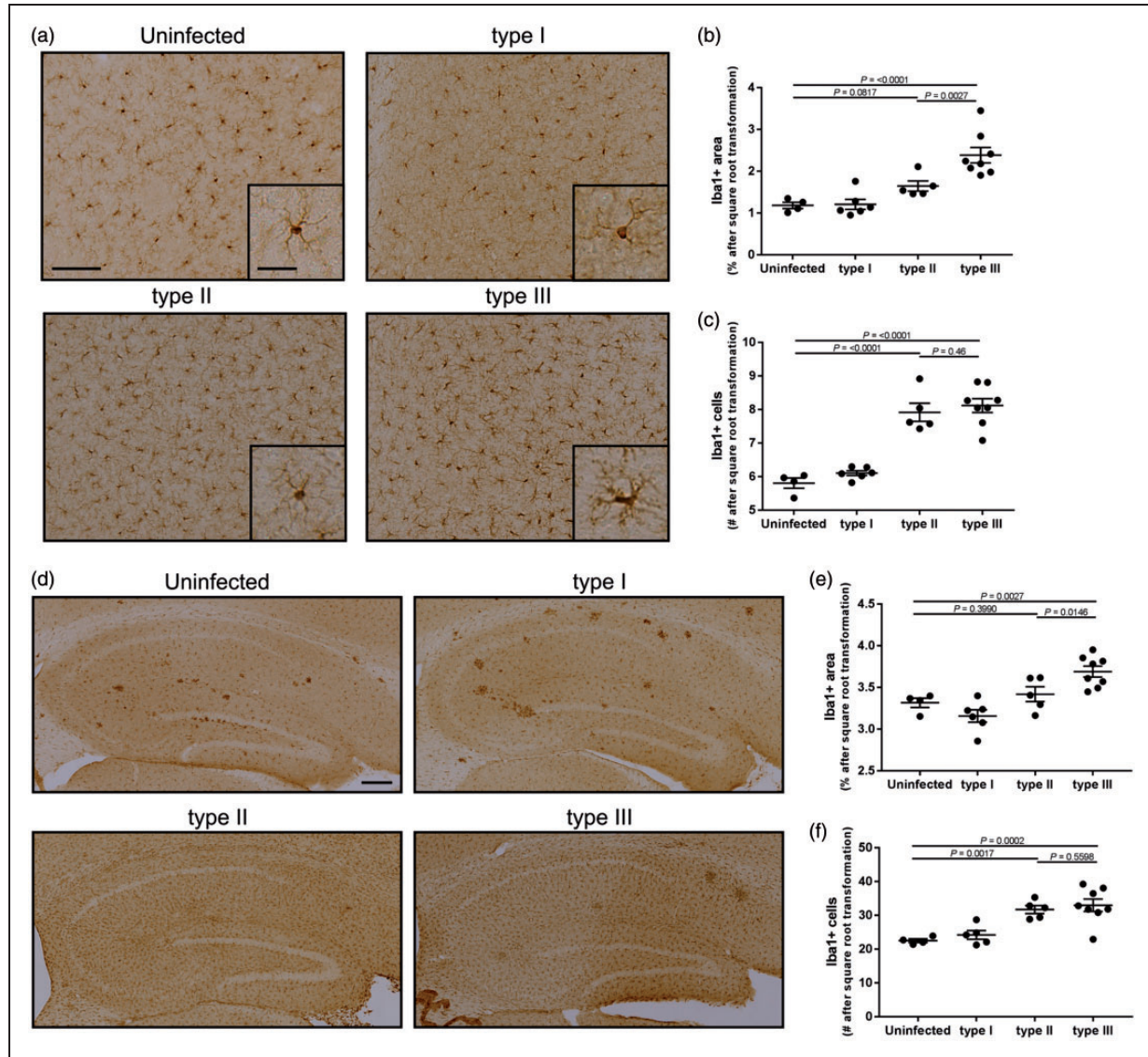


Figure 2. Both type II and type III infection cause an increase in CNS macrophage/microglia number, but only type III infection produces a more activated phenotype. Brain tissue sections from J20 uninfected or infected mice (as indicated) were stained for Iba1, a macrophage/microglial marker, and evaluated by light microscopy. (a) Representative cortical images of Iba1 staining in uninfected, type I, type II, and type III infected mice, scale bar = 100 μ m. Insets show a magnification of an individual Iba1+ cell, scale bar = 25 μ m. (b) Quantification of percent area of Iba1+ staining in each group as noted. Quantification was done using Simple PCI software. (c) Quantification of the number of Iba1+ cells in each group. Quantification was done using Fiji. (D, E, F) As in (a-c) for hippocampus, except all quantification was done using Fiji, scale bar = 200 μ m. For (b), (c), (e), (f), each dot represents one mouse; 3 sections/mouse, 4-8 mice/group, bars represent mean \pm SEM.

monocyte-derived macrophages in the brain (Nance et al., 2012; Biswas et al., 2015; Möhle et al., 2016), we sought to determine the contribution of infiltrating macrophages to the increase in Iba1+ cell numbers. To do this, we fluorescently stained tissue sections with an anti-Iba1 antibody and the newly described anti-TMEM119 antibody that has been reported to specifically label resident microglia (Bennett et al., 2016). Iba1+ cells from across the hippocampus and cortex were identified as either TMEM119+/Iba1+ (microglia) or

TMEM119-/Iba1+ (macrophage) by colocalization analysis (Figure 3(a)). Uninfected and Type I-infected mice were found to have increased percentages of TMEM119-/Iba1+ cells (macrophages) as compared with Type II- and Type III-infected mice (Figure 3(b)). These TMEM119-/Iba1+ cells were most consistently identified in clusters in the uninfected and Type I-infected mice (Figure 3(c)). This observation is consistent with the prior report(s) that infiltrating macrophages are recruited to clear A β plaques (Lai and McLaurin, 2012; Hohsfield

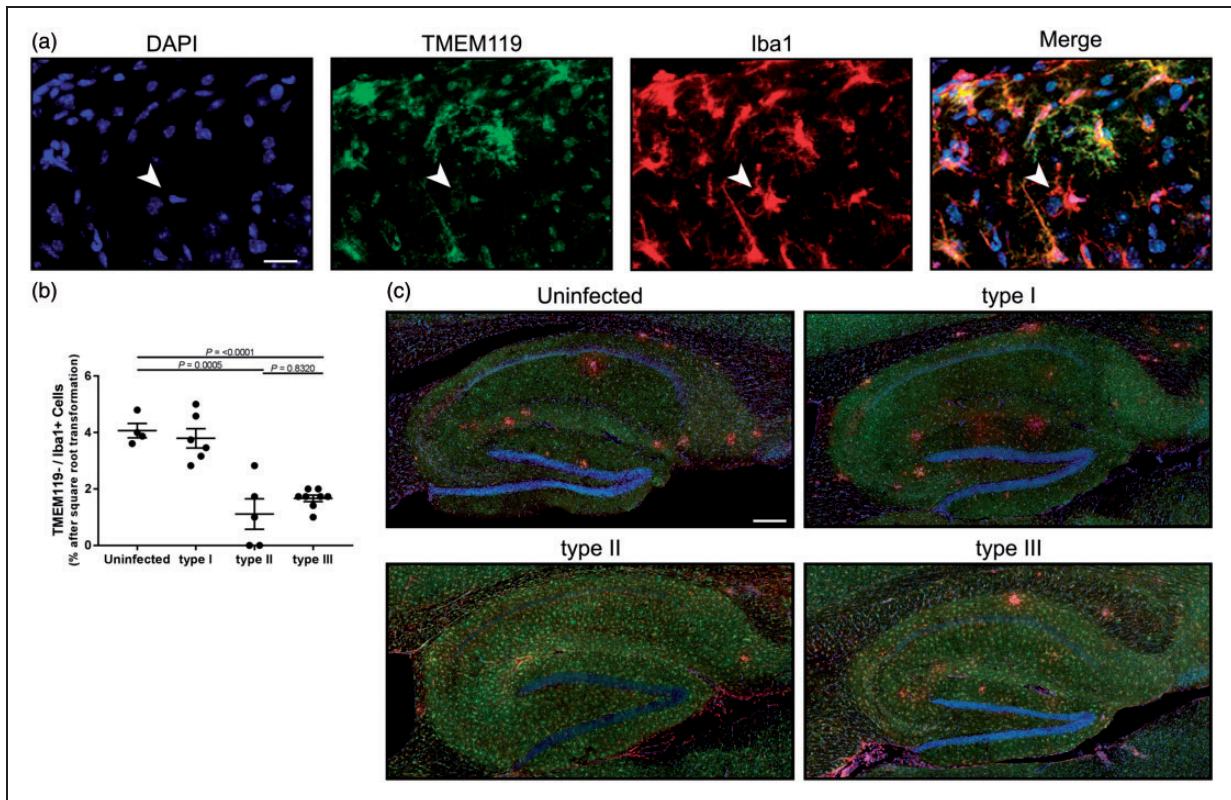


Figure 3. Both Type II and Type III infected mice show decreased percentages of infiltrating macrophages. Brain tissue sections from J20 uninfected or infected mice were stained with DAPI and anti-Iba1 and anti-TMEM119 antibodies then imaged by confocal microscopy. Resulting hippocampal images were evaluated with Zeiss Zen Black software for noted parameters. (a) A representative image from a type II infected mouse showing the different channels (as labeled) and the final merged image. White arrowhead denotes a single Iba1+ cell that does not co-localize with TMEM119 staining. Scale bar = 20 μm . (b) Quantification of the percent of TMEM119-/Iba1+ stained cells in the hippocampi and cortex of uninfected, type I, type II, and type III infected J20 mice. Each dot represents one mouse. 200 Iba1+ cells were evaluated/section (100 from the cortex; 100 from the hippocampus), 3 sections /mouse, 4-8 mice/group, bars represent mean \pm SEM. (c) Representative stitched-grid hippocampal images with merged DAPI, Iba1, and TMEM119 staining, scale bar = 200 μm .

and Humpel, 2015; Möhle et al., 2016). However, as we found few infiltrating macrophages in either Type II- or Type III-infected mice, these data suggest that macrophage infiltration is not the main mechanism responsible for the decrease in plaques found only in Type II-infected mice at 6 mpi.

Chronic Infection With Type II and Type III Parasites Results in Increased T Cell Numbers

While macrophages are the major innate immune cell responsible for control of CNS toxoplasmosis (Suzuki et al., 1988; Mordue and Sibley, 2003), T cells are the adaptive immune cell essential for controlling CNS toxoplasmosis (Parker et al., 1991; Gazzinelli et al., 1992; Denkers and Gazzinelli, 1998; Harris et al., 2010). Additionally, T cells have recently been implicated in determining macrophage/microglial recruitment, activation, and phagocytic capabilities in hAPP mouse models (Baruch et al., 2015, Marsh et al.,

2016). To test whether differences in T cell infiltration might explain the Type II *Toxoplasma* protective effect, tissue sections were stained with a marker for T cells, CD3 (Figure 4(a) and (b)), after which CD3⁺ cells were counted in both the cortex and the hippocampus. As expected, both the uninfected and Type I-infected (acute infection only) J20 mice had virtually no infiltrating T cells in the cortex (Figure 4(c)) or the hippocampus (Figure 4(d)). Consistent with the establishment of a chronic *Toxoplasma* infection, the Type II- and Type III-infected mice showed significantly increased numbers of infiltrating T cells as compared with both the uninfected or Type I-infected mice (Figure 4(c) and (d)). Of note, there was no difference between the numbers of infiltrating T cells in Type II- versus Type III-infected mice in either region (Figure 4(c) and (d)). Therefore, like the Iba1⁺ cell data, these findings suggest that increased T cell infiltration alone is not the key factor responsible for the decrease in plaque burden in the Type II-infected J20 mice.

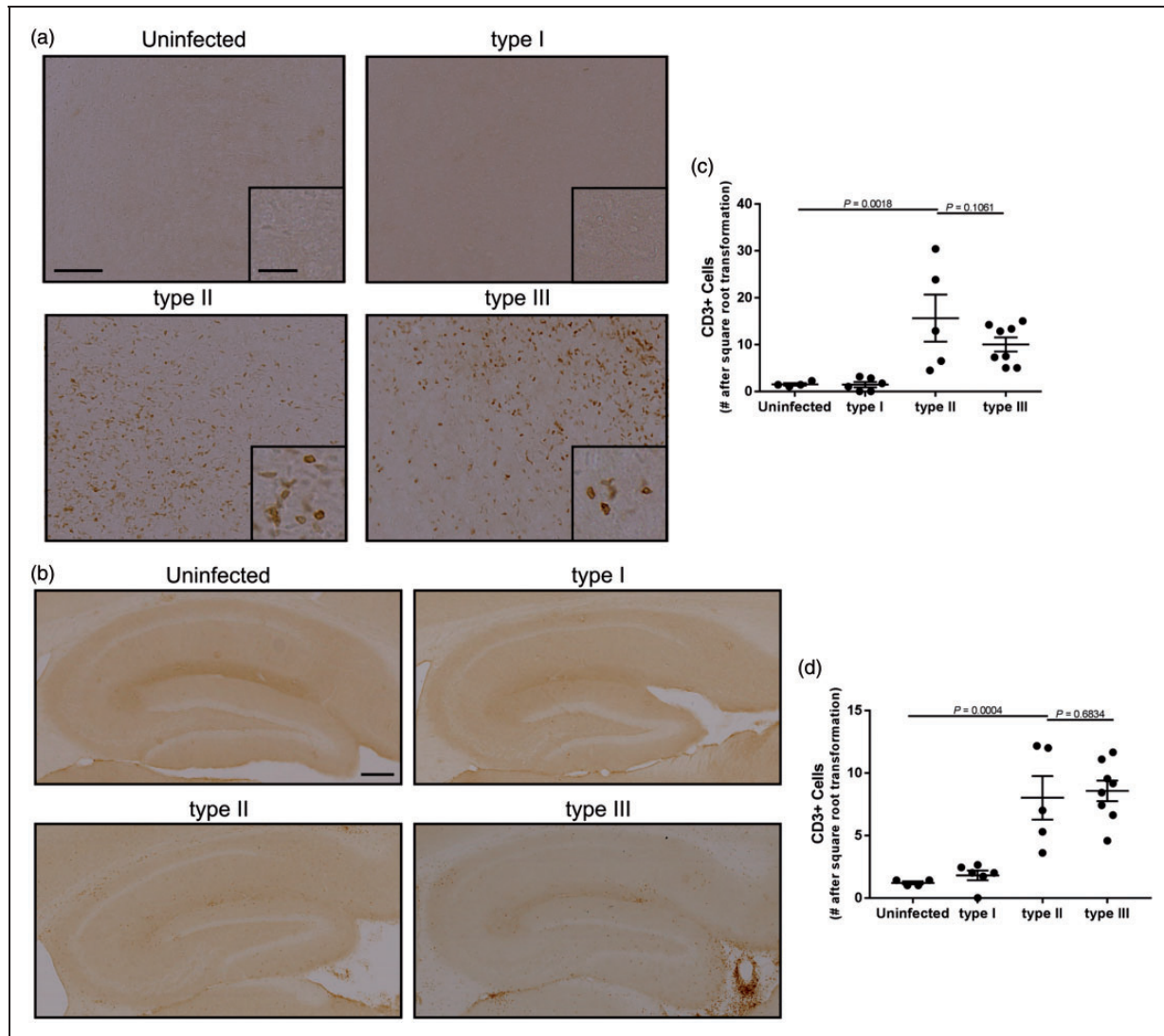


Figure 4. Infection with type II or type III parasites produces chronic CNS T cell infiltration. Brain tissue sections from J20 uninfected or infected mice (as indicated) were stained for the pan T cell marker CD3 and evaluated by light microscopy. (a) Representative cortical images of CD3⁺ staining in uninfected, type I, type II, and type III infected mice, scale bar = 100 μ m. Insets show a magnification of a representative area for CD3⁺ cell identification, scale bar = 25 μ m. (b) As in (a) for hippocampus, scale bar = 200 μ m. (c) Quantification of the number of cortical CD3⁺ cells in each group. Quantification was done using Simple PCI software. (d) As in (c) for hippocampus, analysis was done using Fiji. For (c), (d), each dot represents one mouse; 3 sections/mouse, 4-8 mice/group, bars represent mean \pm SEM.

Pro- and Anti-Inflammatory Cytokines Are Elevated in Type II- and Type III-Infected Mice

Given that we did not find clear differences in the number of infiltrating immune cells that would explain the Type II-only protective effect, we sought to determine if, as suggested by Jung et al. (2012), the Type II-protective effect was secondary to promoting a global anti-inflammatory environment with significant increases in TGF- β and IL-10. To evaluate the CNS cytokine environment, we isolated and analyzed protein from brain homogenates using a multiplex 25-cytokine/chemokine panel. As expected from previous work (Hunter et al.,

1994; Sarciron and Gherardi, 2000; Wen et al., 2010), when compared with uninfected mice, Type II- and Type III-infected mice showed a > 2-fold increase in the following pro-inflammatory cytokines and chemokines: CCL5, CXCL10, interferon gamma (IFN γ), IL-6, and MCP-1 (Figure 5(a)). Of these five cytokines, only IFN γ was significantly different between type II and type III (Supplementary Table 1). Also as expected, the acutely resolved Type I infection did not result in any significant changes in cytokine or chemokine levels when compared with uninfected mice (Figure 5, Supplementary Table 1). Contrary to Jung et al. (2012), we did not find a statistically significant difference in IL-10 levels between

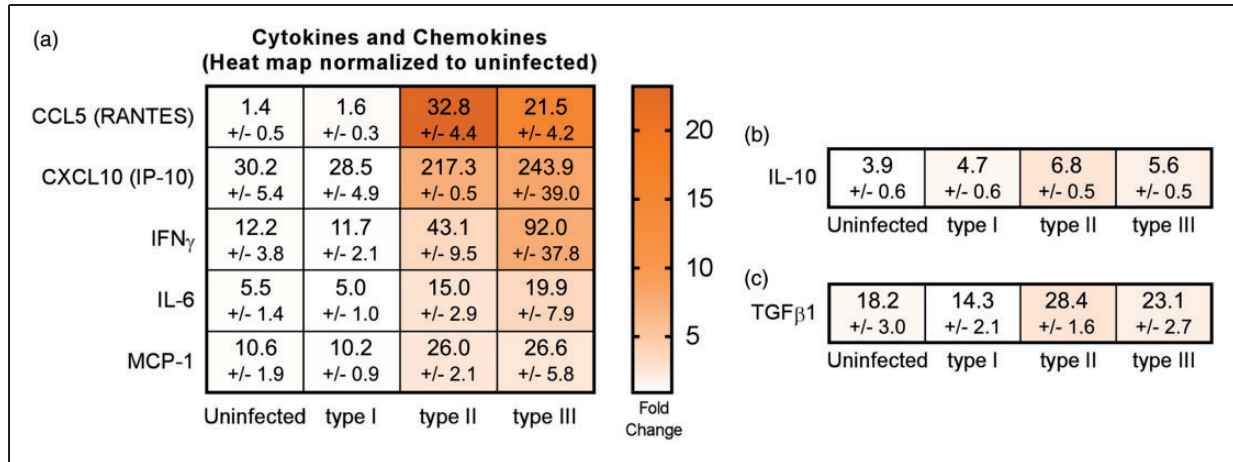


Figure 5. Both Type II- and Type III-infected mice show increased CNS levels of inflammatory cytokines. Protein extracted from brain homogenates from uninfected and infected mice (as noted) was used to quantify multiple cytokines and chemokines via a multiplex cytokine assay (a) and (b) or specific ELISA (c). (a) The table shows the cytokines or chemokines from the 25-plex assay with a > 2-fold change over uninfected. Heat map indicates fold change relative to uninfected and inside each box is the mean pg/mg concentration \pm SEM. Only these five cytokines showed any statistically significant differences between any of the groups (Supplementary Table 1). Cytokines/chemokines with a < 2-fold change over uninfected were: KC, IL-10, IL-5, IL-1A, IL-17, TNF α , MIP-2, MIP-1B, MIP-1a, IL-1B, IL-12p70, IL-9, IL-4, IL-2, IL-13, GM-CSF, IL-7, IL-12p40, G-CSF, and IL-15. (b) As in (a) but for IL-10. (c) Quantification of TGF- β 1 was done by specific ELISA as TGF- β 1 is not part of the multiplex assay. Bars represent mean \pm SEM. For all assays, n = 4–8 mice/group.

uninfected and Type II-infected mice (Figure 5(b)). As TGF- β was not part of the multiplex assay, we used an individual ELISA to assess brain TGF- β levels. While we did find a statistically significant increase in the Type II-infected mice versus uninfected mice ($p = .0233$), this difference was < 2-fold (1.6-fold increase, consistent with the findings by Jung et al. (2012)) and importantly, there was no significant difference between Type II- versus Type III-infected mice (Figure 5(c)). In summary, consistent with the establishment of a chronic CNS infection, Type II- and Type III-infected mice showed a markedly pro-inflammatory environment. Although discrepancies between cytokine and chemokine levels in previously published articles may be explained by choice of infecting parasite or mouse model, the data presented here indicate that a few key cytokines like TGF- β and IL-10 are not the major mediators of Type II-induced A β protection.

Type II-Infected Mice Have Higher Chronic Parasite Burden Than Type III-Infected Mice

In addition to evaluating the neuroinflammatory response, we sought to determine if parasite burden differed between the Type II and Type III strains, as such a difference might represent another factor that would drive changes essential to strain-specific protection versus nonprotection against A β . To quantify parasite burden, we isolated DNA from homogenized brain and used quantitative PCR to measure the level of a *Toxoplasma* gene (B1; Burg et al., 1989; Courret et al., 2005; Harris et al., 2010; Wilson et al., 2005; Nance et al.,

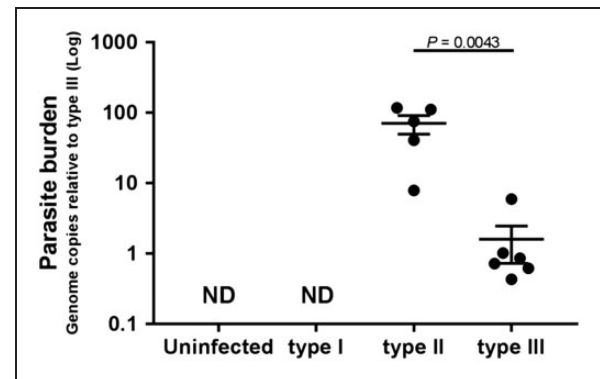


Figure 6. Parasite burden is 75-fold greater in Type II-infected mice than Type III-infected mice. At 6 mpi, brain tissue was harvested and DNA was extracted. Quantification of relative parasite burden was done using qPCR to detect the *Toxoplasma*-specific gene B1. Two Type III-infected mice had undetectable B1 levels and are not shown. Each dot represents one mouse; 4–8 mice/group, bars represent mean \pm SEM. ND = not detectable.

2012; Cekanaviciute et al., 2014). As expected, in mice infected with the attenuated Type I parasites (acute infection only; Behnke et al., 2012; Ingram et al., 2013), we found no detectable levels of B1. All the Type II (5) and all but two Type III-infected mice (six of eight) had detectable levels of B1. Unexpectedly, the Type II-infected mice had a 75-fold greater parasite burden compared with Type III-infected mice (Figure 6). This result was especially remarkable given that the Type

III-infected mice were inoculated with a higher number of parasites (10 K for Type II and 50 K for Type III). These data suggest that the Type II-associated protection against A β deposition could be linked to long-term parasite persistence.

Discussion

Here, we have shown in a third hAPP mouse model that infection with a Type II *Toxoplasma* strain decreases A β deposition by > 60%. Thus, our data reproduce and confirm prior hAPP-*Toxoplasma* studies (Jung et al., 2012; Möhle et al., 2016). Additionally, we have extended this prior work by showing that this A β protection is conferred by a second Type II strain of *Toxoplasma*. We have also shown that this protection does not occur when the infecting strain produces an acute infection only (attenuated Type I strain), nor is a chronic CNS infection with a non-Type II strain (Type III strain) sufficient to induce this protection. Finally, by comparing the CNS cytokine/chemokine environment and the infiltrating immune response, we show that the Type II protection against A β is not simply secondary to inducing TGF- β and IL-10 or increasing the number of infiltrating T cells or macrophages. There are several important implications of these findings. The ability of a second Type II *Toxoplasma* strain to protect against A β deposition shows that this previously observed effect is not dependent upon the specific Type II *Toxoplasma* strain used in the prior studies (both of which used the same Type II strain). In addition, our work further substantiates the prior studies (Jung et al., 2012; Mohle et al., 2016) and highlights the reproducible nature of the Type II protection against A β (three labs, three different hAPP mouse models). The lack of A β protection by the attenuated Type I strain suggests that *Toxoplasma*-induced changes in A β deposition are distinct from previously observed behavioral changes, where acute infection was enough to induce a decrease in fear aversion to cat urine (Ingram et al., 2013). Importantly, as infection with a Type III strain, which also produces a chronic CNS infection, does not confer protection against A β , we have established a robust tool with which we can use comparative analyses to identify changes associated with A β protection rather than simply CNS infection.

Our cytokine data emphasizes the utility of comparing protective (Type II) and nonprotective (Type III) infections. The original hAPP-*Toxoplasma* study suggested that TGF- β and IL-10 drove the protective effect of the Type II strain (Jung et al., 2012). Yet, we found very similar levels of these cytokines in the CNS of mice infected with either the protective Type II strain or the nonprotective Type III strain (Figure 5(b) and (c)), suggesting that elevated levels of TGF- β and IL-10 are not sufficient to drive *Toxoplasma*-associated protection.

Similarly, different reports have implicated the cytokine IFN γ as either decreasing A β plaque deposition or exacerbating plaque deposition (Mastrangelo et al., 2009; Browne et al., 2013; Baruch et al., 2016). In our model, even at 6 mpi, Type II- and Type III-infected J20 mice had high levels of CNS IFN γ , consistent with the central role of IFN γ in the control of toxoplasmosis (Suzuki et al., 1988; Halonen et al., 2001). Yet, these levels of IFN γ were neither positively nor negatively correlated with A β plaque deposition. Collectively, these data suggest that there is a balance of pro- and anti-inflammatory cytokines/chemokines which are important for immune cell infiltration and control of the infection but not specific to a Type II infection and therefore not responsible for decreased A β plaque deposition.

Our immune cell analysis also highlights how *Toxoplasma* strain comparisons might aid in determining the mechanisms by which Type II infection protects against A β deposition. Möhle et al. (2016) recently suggested that the decrease in A β plaque deposition in Type II *Toxoplasma* infected 5xFAD mice was secondary to a profound increase in infiltrating phagocytic mononuclear cells. We found equal numbers of CNS Iba1⁺ cells in both the protective (Type II) and nonprotective (Type III) infection, suggesting that a pure increase in CNS macrophages/microglia was not sufficient to drive protection against A β deposition. However, we did observe morphologic differences in Iba1⁺ cells in Type II- versus Type III-infected mice, suggesting that the Iba1⁺ cells may be differentially polarized (Von Bernhardi et al., 2015; Hellwig et al., 2016; Morganti et al., 2016). Analysis of TMEM119/Iba1 stained sections revealed relatively few infiltrating macrophages in our Type II and Type III mice. This finding was surprising because it is inconsistent with prior reports of the importance of infiltrating monocytes/macrophages in controlling CNS toxoplasmosis, though this prior work was done much earlier in CNS infection (3–8 weeks postinfection) and primarily used flow cytometry to distinguish between microglia and macrophages (Schlüter et al., 1995; Suzuki et al., 2005; Nance et al., 2012; Biswas et al., 2015; Möhle et al., 2016). Of course, it is also possible that in the setting of *Toxoplasma* infection, and/or expression of hAPP, TMEM119 expression occurs in both microglia and infiltrating macrophages. Future studies, perhaps with *Cer2^{+/RFP}/Cx3cr1^{+/GFP}* mice (Mizutani et al., 2012) will need to be done to more definitively identify infiltrating cells versus proliferating resident microglial cells. As we only looked at 6 mpi, it is also possible that at earlier time points Type II infection leads to the accumulation of distinctly polarized macrophages/microglia that are more efficient at degrading A β but less efficient at controlling parasite burden (Figure 6). Such a possibility would be consistent with the Möhle et al. (2016) study and will require follow-up studies to understand the possibility of

microglia versus macrophage A β plaque clearance over time. Additionally, consistent with our Iba1 results, we showed that equivalent numbers of T cells infiltrate the brain in Type II and Type III infection (Figure 4(c),(d)). However, given the recent interest in the influence of T cells in mitigating AD pathology (Baruch et al., 2015; Baruch et al., 2016), more work is needed to establish if Type II and Type III strains drive different T cell populations to the brain, and how these different T cell populations might affect A β plaque burden in the setting of *Toxoplasma* infection.

Finally, we extended the prior work by evaluating the CNS for parasite burden, an analysis that had not been done in the prior two studies. Our finding that Type II infection leads to much higher levels of parasite persistence compared with Type III infection suggests that CNS parasite persistence may lead to distinct changes in the CNS environment that altered A β processing and therefore, deposition. Alternatively, it may be a consequence of prolonged exposure to a cytokine milieu that is further skewed toward a pro-inflammatory profile during infection with Type III parasites, leading to better control of parasite burden when compared with Type II infection. Future studies will use modified Type II strains that have a decreased ability to establish a chronic CNS infection to determine if parasite persistence plays a role in A β protection.

In conclusion, the findings presented here suggest that only Type II infection leads to protection against A β deposition. This finding suggests that Type II infection evokes alterations in CNS cells and/or the environment that are distinct from the vast number of changes induced simply by CNS infection with any strain of *Toxoplasma*. Future work will focus on understanding how different cytokine environments might affect APP processing which could result in either decreased production or increased degradation of A β . In addition, given the findings of Möhle et al. (2016) and Baruch et al. (2015), it will be imperative to characterize differences in the leukocyte populations observed in Type II- versus Type III-infected mice over time. Importantly, comparing infection with these two strains can now be used as a tool to tease out changes in the brain that are linked to protection rather than simply to CNS infection. Ultimately, we hope that determining how Type II *Toxoplasma* infection leads to protection against A β deposition will identify new targets to combat AD.

Summary

In this study, we infected Alzheimer's disease model mice with three strains of *Toxoplasma gondii*. Only one strain showed protection from amyloid beta deposition providing a novel tool to investigate distinct changes associated with protection rather than simply infection.

Author Contributions

C. M. C. and A. A. K. designed experiments; C. M. C., K. E. M., and A. A. K. analyzed data and wrote the paper; C. M. C., W. R. M., and J. F. performed the experiments.

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Declaration of Conflicting Interests

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Supplementary Material

Supplementary material is available for this article online.

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