

Research Article

Host Cell Preference of *Toxoplasma gondii* Cysts in Murine Brain: A Confocal Study

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Abstract *Toxoplasma gondii* is a protozoan parasite that is widely prevalent in humans and typically results in a chronic infection characterized by cysts located predominantly in the central nervous system. In immunosuppressed hosts, such as patients with HIV infection, the infection can be reactivated from the cysts in the brain resulting in a severe and potentially fatal encephalitis. Studies suggest that the chronic infection may also have neuropathological and behavioral effects in immune competent hosts. An improved understanding of tissue cyst behavior is of importance for understanding both the reactivation as well as the neurophysiological consequences of chronic infection. *In vivo* studies have identified neurons as host cells for cysts but *in vitro* studies have found that astrocytes can also foster development of the cysts. In this study we have addressed the question of which neural cell tissue cysts of *T. gondii* reside during chronic infection using a mouse model. Mice were infected with Me49 Strain *T. gondii* and the intracellular localization of the cysts analyzed during the development and establishment of a chronic infection at 1, 2, and 6 months post infection. Brains were fixed, cryosectioned, and stained with FITC-*Dolichos biflorans* to identify the *Toxoplasma* cysts and they were labeled with cell specific antibodies to neurons or astrocytes and then analyzed using confocal fluorescence microscopy. Cysts were found to occur almost exclusively in neurons throughout chronic infection. No cysts were identified in astrocytes, using the astrocyte marker, GFAP. Astrocyte interactions with neuronal-cysts, however, were frequently observed.

Keywords astrocytes, “Toxoplasmic encephalitis,” chronic infection

1 Introduction

Toxoplasma gondii is a protozoan parasite that is widely prevalent in humans and animals worldwide [21,27]. In

humans, infection usually results in a chronic infection characterized by cysts containing bradyzoites located predominantly in muscles and the central nervous system [27]. Evidence indicates that in most individuals, the infection persists for the lifetime of the host as an asymptomatic infection [21]. However, in immune suppressed hosts, such as patients with AIDS, infections can be reactivated from these cysts with transformation of bradyzoites to tachyzoites resulting in severe and potentially fatal encephalitis [25, 26]. Additionally, recent studies suggest that chronic infections can be a cause of cryptogenic epilepsy and that these chronic infections are associated with an increased incidence of schizophrenia and/or psychosis [21, 30, 33–36]. This suggests that chronic infection has a neuropathological and neurophysiological effect on immune competent hosts. Improvements in our understanding of the tissue cyst stage of *T. gondii* during chronic infection should provide important information on the mechanisms of reactivation, neuropathology, and neurophysiological changes associated with chronic infection.

Latent infection of *T. gondii* in the brain is characterized by cysts that contain the bradyzoite stage of the parasite and that are located intracellularly within host cells in the central nervous system [12, 14, 31]. Intact intracellular cysts normally illicit no host inflammatory reaction around infected cells or in the immediate vicinity of infected cysts [11], and because of this it has been suggested that tissue cysts are isolated from the immune response by their host cells. Cysts are surrounded by a thick cyst wall containing branched tubular structures, composed of an electron-dense material and containing the glycoprotein, CST1 [7, 30, 37]. Studies in mice indicate tissue cyst rupture occurs intermittently in immune competent hosts, releasing parasites into the brain, and eliciting an inflammatory reaction that serves to contain the infection [13]. *In vivo* studies have identified neurons as host cells for the cysts based largely on ultra-structural studies in which synapses could be identified [11,

12,31]. However, numerous *in vitro* studies have found that astrocytes can also foster development of the cysts [14,18,20,24,37]. The issue of whether both astrocyte and neuronal host cell type(s) harbor the cyst stage in chronic *T. gondii* infections *in vivo* has not been formerly addressed.

In this study we have addressed the question of whether neurons and astrocytes both serve as the host cells for the cysts of *T. gondii* in a chronic infection murine model. Mice were infected with *T. gondii* Me49 strain, and the intracellular localization of the cysts analyzed during the development and establishment of a chronic infection. Brains were stained with FITC-*Dolichos biflorans* to identify the *Toxoplasma* cysts and were labeled with cell specific antibodies to neurons or astrocytes, and they were then analyzed using confocal fluorescence microscopy. Cysts were found to occur almost exclusively in neurons throughout chronic infection. Astrocyte interactions with neuronal cysts were, however, frequently observed suggesting a mechanism for cysts to affect glial cell physiology in chronic infection.

2 Materials and methods

Antibodies and other reagents. Antibodies used in this study were mouse anti-MAP2, monoclonal antibody (Millipore, MAB378), chicken anti-neurofilament heavy chain polyclonal antibody (Abcam), and rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (Dako-Cytomation). The lectin, *Dolichos biflorans* conjugated to fluorescein, FITC-*Dolichos biflorans* (Sigma), was used to label the *T. gondii* cysts. Secondary antibodies used were goat anti-rabbit Alexa Fluor 647, goat anti-mouse Alexa Fluor 647, or goat anti-chicken Alexa Fluor 647 (Invitrogen).

***Toxoplasma gondii* culture.** *T. gondii*, strain Me49, was maintained in Human Fetal Fibroblasts (HFFs) cells as previously described [19]. Tachyzoites were harvested from HFFs, resuspended in PBS, and used to inoculate mice.

Infection of mice with *Toxoplasma*, fixation of infected brains, and cryosectioning. Male Balb/c mice, 6 weeks old, were inoculated subcutaneously with 200 tachyzoites of *T. gondii* strain Me49. Mice were sacrificed and brains were harvested at 1 month ($n = 2$), 2 months ($n = 2$), and 6 months ($n = 1$) post infection. In addition, brains from age-matched uninfected mice ($n = 3$) were used as controls. The brains of uninfected and infected mice were harvested from the cranium of the mice immediately after they were sacrificed, placed in 4% formalin in phosphate buffered saline (PBS), pH 7.3, and fixed overnight at 4°C with agitation. The brains were then washed 3 times in PBS and incubated in a graded (5%-15%-30%) sucrose solution overnight at 4°C. Brains were then frozen at -70°C, mounted in OCT solution, cut into 20 µm cryosections and serial sections collected on Superfrost slides.

Immunohistochemistry staining and confocal microscopy.

Sections were blocked in PBS with 10% goat serum, 10% fetal bovine serum, and 0.1% Triton X-100, for 1 hour. The sections were incubated with FITC-*Dolichos biflorans* lectin (1 µg/ml) and either anti-MAP2 (1:400), anti-neurofilament heavy chain (1:2000), or anti-GFAP (1:200), respectively, overnight at 4°C with agitation. Sections were washed 3 times with PBS and then incubated with the secondary antibodies for 2 hours in the dark. Secondary antibodies used were anti-mouse Alexa Fluor 647, anti-chicken Alexa Fluor 647, or anti-rabbit Alexa Fluor 647. After incubation in the secondary antibodies, sections were washed 3 times in PBS, allowed to dry, mounted with Prolong Gold (Invitrogen), and covered with a #1.5 glass coverslip. Cryosections were imaged on a Leica Confocal Scanning Laser Microscope SP2 AOBS with the argon laser at 488 nm for the FITC and the krypton laser at 647 for the Alexa Fluor 647 label. The PMT settings were adjusted so that no overlap between the fluorescence in the 488 and 647 channels occurred. Brains were imaged using 100x oil objective and optical sections of 0.2 µm were collected. Confocal images were subsequently analyzed using Imaris software (Version 6.1.0, Bitplane, AG).

3 Results

3.1 Description of the chronic infection with *T. gondii*, strain Me49 in mice

Mice inoculated with tachyzoites of *T. gondii* strain, Me49, appeared normal with no signs of illness at any time throughout the infection period (up to 6 months post infection). Infected mice were sacrificed at 1, 2, and 6 months post infection (p.i.); the brains were fixed and then analyzed for cysts. Cysts were identified by staining with fluorescently labeled lectin, FITC-*Dolichos biflorans*, which stains the cyst wall with low background staining of neural tissue [10,37]. Cysts were observed within the brains of infected mice at all timepoints and were of varying sizes, with the largest cysts, 25–30 µm in diameter (Table 1). Cysts were mostly found as individual cysts, with well-defined cyst walls (Figure 1A). In addition, clusters of cysts were commonly observed with a large cyst (> 50 bradyzoites)

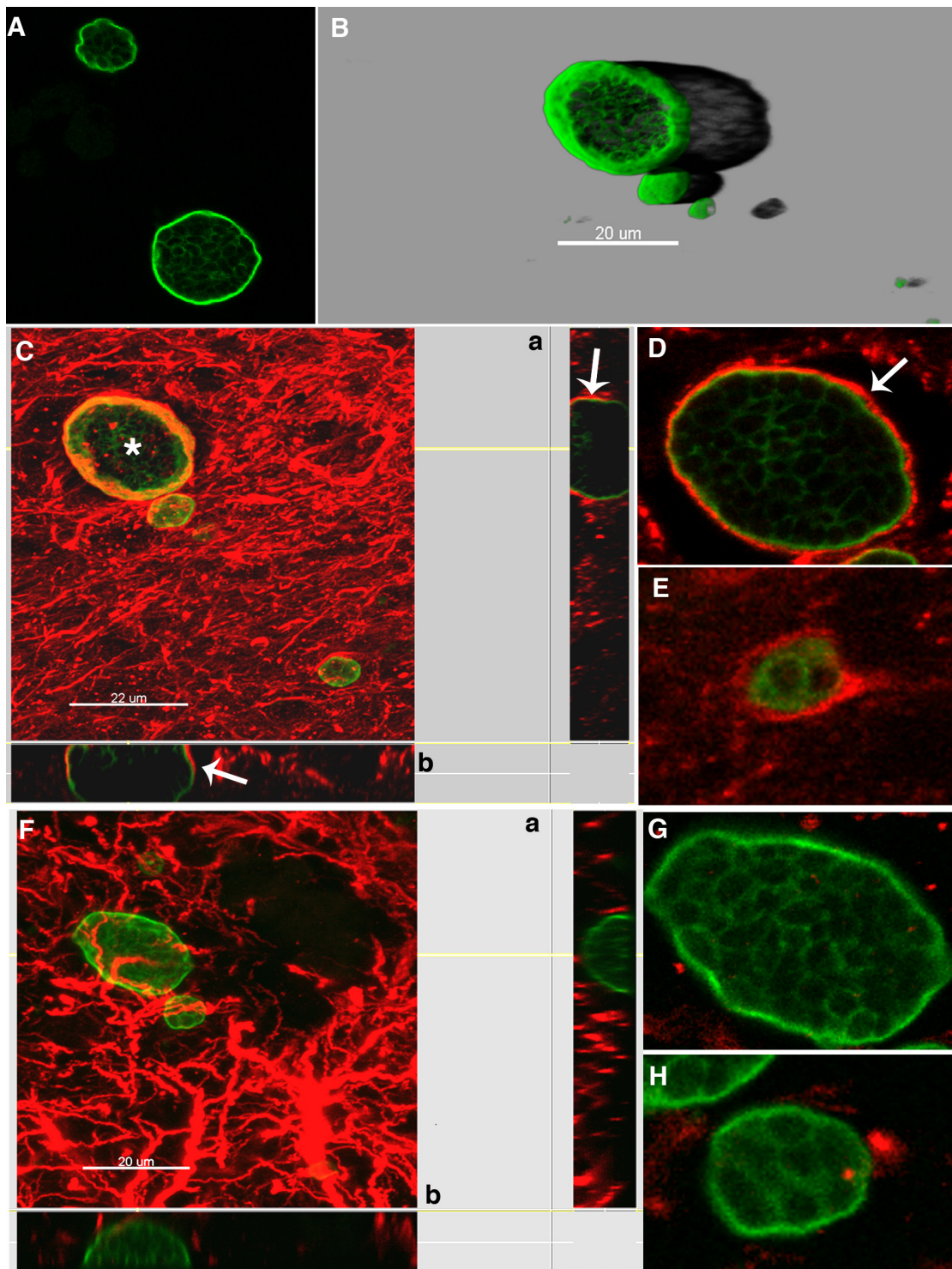
Time post-infection	Range of cyst diameter ¹	Host cell	
		Neurons ²	Astrocytes ³
1 month	7 to 20 µm	Yes	No
2 month	3 to 30 µm	Yes	No
6 month	5 to 25 µm	Yes	No

¹ based on the measurement of a minimum of 10 cysts for each interval

² based on the examination of 57 cysts; 33 positively identified as neurons

³ based on the analysis of 25 cysts; none positively identified as astrocytes

Table 1: Cyst size and host cells harboring cysts in a chronic infection.



(A) Cryosection of murine brain labeled with *Dolichos biflorans*-FITC (DB-FITC) showing the well-defined staining of the cyst wall; (B) field of 5 cysts showing a typical cyst cluster consisting of a large cyst ($> 20 \mu\text{m}$ in diameter) surrounded by 2 smaller cysts (note the 2 small cysts in the close vicinity); (C) Maximal Image Projection of field with 4 cysts double-labeled with stained with DB-FITC (green) and anti-neurofilament heavy chain (NF-H; red); note the close association of the neurofilaments with the cyst wall as shown in the side views of cysts (a and b, arrows; note that a and b are side views of area indicated at the yellow line); (D) Single slice of cyst in C (starred) showing the close opposition of the NF-H with the cyst wall; (E) single slice of a small cyst in a neuron; (F) Maximal Image Projection of field with 2 cysts double-labeled with DB-FITC (green) and anti-GFAP (red); (G and H) single slices of 2 cysts in F showing the absence of GFAP filaments around the cyst wall; note that the GFAP filaments (red) are present outside the cyst wall, but not closely opposed to the cyst wall.

Figure 1: Confocal microscopy of cysts in murine brain.

in close proximity to a slightly smaller cyst, and several small cysts, typically containing 2 to 6 bradyzoites, in the immediate vicinity (Figure 1B). This pattern of cyst distribution was common at 2 and 6 months post infection and may be indicative of reactivation with formation of new cysts, cyst dispersal, and/or cyst replication.

3.2 Immunofluorescence/confocal microscopy analysis of host cells harboring *T. gondii* cysts

T. gondii infected brain sections were stained with FITC-*Dolichos biflorans* (FITC-DB), and either neuron or astrocyte specific antibodies. To identify neurons, an antibody to microtubule associated protein, MAP2, which is a stringent marker for neurons, confined to neuronal cell bodies and dendrites, or an antibody to neurofilament heavy chain (NF-H), which stains intermediate filaments in neuronal cell bodies and axons, was used. To label astrocytes, cryosections were stained with an antibody to the astrocyte specific intermediate type cytoskeletal protein, GFAP. Cryosections were double-labeled with the cyst marker, FITC-DBA, and either MAP2, NF-H, or GFAP, and they were analyzed by confocal microscopy. Optical sections of 0.2 μm were taken and 3D reconstructions, maximum projection images, and single slices were analyzed to assess the host cell harboring the cysts.

Cysts were found within neurons at 1, 2, and 6 months post infection (Table 1). Cysts were identified within neurons by the presence of neurofilaments surrounding the cyst, as illustrated in Figure 1C, and by the close opposition of neurofilaments to the cyst wall as evident from the side views (Figures 1Ca and 1Cb, arrows). Additionally, analysis of single slice images (1 plane of a confocal Z series) verified the neuronal cytoskeletal element (neurofilament) was adjacent (Figures 1D and 1E). Cysts of all sizes, from small cysts, containing 1–2 bradyzoites, to large cysts, > 20 μm in diameter containing more than 50 bradyzoites, were identified to be located in neurons. Cysts were similarly identified to be within neurons using the neuronal marker, MAP2.

In contrast, by a similar analysis, staining with the astrocyte cytoskeletal protein, GFAP, no cysts at either 1, 2, or 6 months post infection could be definitely identified to be present in astrocytes. Astrocytic processes could be identified closely associating with cysts as illustrated in Figure 1F, but the astrocyte marker, GFAP, was always found to be at short distance versus closely opposed to the cyst wall, indicating that the cyst was not located within the astrocyte (Figures 1G and H). Although cysts were not found within astrocytes, numerous astrocyte processes were commonly found to associate around the large cysts, as illustrated in Figure 1F. Cysts with predominant astrocytic processes surrounding them usually had well-defined cyst walls with no indication of cyst rupture, indicating that the cysts were intact.

3.3 Localization of cysts in the brain

The location of cysts at 2 and 6 months post infection were mapped in the mouse brain (Table 2). Cysts were found throughout the brain at both timepoints. The majority of cysts, at both 2 and 6 months post infection were found in the cerebral cortex. Cysts were also prevalent in the hippocampus at both 2 and 6 months. At 2 months, cysts were numerous and more widely distributed in the brain, being found in the thalamus, hypothalamus, amygdala, caudate putamen, and the cerebellum (Table 2). This suggests that cyst numbers were declining with time, either due to reactivation and destruction by the immune response or due to direct effects of astrocytes or other effector cells on these cysts.

Brain region	2 months ¹	6 months ²
Cerebral cortex	34	57
Hippocampus	10	25
Thalamus	5	—
Hypothalamus	6	3
Amygdala	25	9
Caudate putamen	12	6
Cerebellum	8	—

¹ number of cysts observed = 67;

² number of cysts observed = 32.

Table 2: Localization of cysts in the brain. Percentage of cysts located in different brain regions from serial sections of mouse brain at 2 and 6 months post infection.

4 Discussion

Previous *in vitro* studies have indicated that astrocytes, in addition to neurons, can be host cells for the cyst stage of *T. gondii* in the brain. In this study, however, we found that cysts in a chronic infection of *T. gondii* in murine brain were found exclusively in neurons. No cysts were identified in astrocytes, using antibody staining for the astrocyte marker, GFAP. While GFAP is being the cell specific marker for astrocytes, levels of GFAP vary in astrocytes depending upon astrocyte subtype and location in the cell and thus it is possible that some cysts are located in astrocytes but were not identifiable due to amounts of GFAP that were below the detection level of antibody labeling. However, from this study, it appears that the majority of cysts in a chronic infection in murine brain are found in neurons. Results from this study confirm previous ultrastructural studies that also identified cysts as located primarily in neurons [11, 12, 31].

Astrocytes are known to play an important role in the immunopathogenesis of *T. gondii* in the brain [17]. Astrocyte activation, for example, is a hallmark of Toxoplasmic encephalitis with activated astrocytes encircling the inflammatory infiltrates around *T. gondii*

cysts [17,23,32]. Additionally, recent studies found that activated astrocytes are critical for control of Toxoplasmic encephalitis, playing a role in restricting lesion size, parasite load, and prevention of necrosis [6,17,32]. Collectively, these studies indicate that astrocytes are an important immune effector cell controlling parasite replication in the brain. Additionally, numerous *in vitro* studies have found that *T. gondii* is able to infect astrocytes [5,9,15,18] and *in vivo* studies have found that tachyzoites are prevalent in astrocytes during the early stages of invasion of the brain [4,16]. *In vitro* studies have shown that tachyzoite infection of astrocytes causes a disappearance of gap junctions resulting in a loss of intercellular communication amongst astrocytes and induces production of chemokines and cytokines, suggesting that infected astrocytes may be capable of attracting and communicating with T cells and cytokine-activated astrocytes are capable of intracellular killing of tachyzoites [5,15,19]. Collectively, these studies indicate that astrocytes are an important host cell for tachyzoites and suggest that astrocytes play a number of significant roles during *Toxoplasma* infection [5,15]. Data from this study, however, indicate that astrocytes are not a prevalent host cell for the cyst stage in the brain. However, another *in vivo* study found that astrocytes were host cells for the cyst stage in mice that were immune suppressed, suggesting that astrocytes may be a host cell for the cyst stage depending upon the host immune status [4,22].

While our study did not identify cysts in astrocytes, astrocytic processes in close association with large intact cysts were commonly observed. Astrocytes are now recognized as specialized glial cells that exert many essential functions in the healthy central nervous system, such as maintaining homeostasis in the brain and associating with neurons, including neuronal synapses, with bidirectional communication known to occur between neurons and astrocytes [1,3,28,29]. It is also now recognized that disruption of mechanisms involved in astrocyte-neuron signaling may result or be involved in brain diseases [1,2]. Association of activated astrocyte processes with cysts has been described in other studies [16] but cyst-associated astrocytes may also have other roles. The possible role(s) of astrocyte interaction with the intracellular neuronal cysts is largely unknown and deserves further study.

The patterns of cyst growth found in this study are consistent with other studies that have found that cysts in the brain grow uniformly in size up to 2 to 3 months post infection and persist in the brain for many months post infection [7]. Additionally, in this study we found that young cysts, about 5 μm in diameter, are present up through 6 months post infection, and clusters of cysts of varying sizes were frequently found, indicating that continual formation of new or second-generation tissue cysts in the brain occurs during the chronic infection. The mechanism of generation of these

new tissue cysts in chronically infected mice is not well understood. Studies of bradyzoite differentiation and cyst formation *in vitro*, however, found that intracellular bradyzoites are motile within host cells, being able to invade surrounding cells and initiate new cysts, and that the cysts can proliferate by fission indicating that bradyzoites and cysts are highly dynamic and suggesting a mechanism of parasite dissemination during the chronic infection [8]. The patterns of cyst clusters of varying sizes and small/young cysts in close proximity to larger cysts, observed in this study, are consistent with this proposed mechanism of parasite dissemination in the brain in a chronic infection.

Finally, in this study we found that cysts were located throughout the brain with most cysts found in the cerebral cortex but also prevalent in the hippocampus, basal ganglia, and amygdala. This cyst distribution in murine brain is similar to that found in *T. gondii*-infected rats in which cysts were found to be prevalent in the limbic area and medial areas of the brain [16]. *T. gondii*-infected rats exhibited lower anxiety and the predominant invasion in limbic areas, brain areas that modulate emotional processes, was suggested to account for the changes in the rat lower anxiety authors [16]. Thus, cyst distribution in mice and rats is supportive of recent theories suggesting that chronic *T. gondii* infection can have effects on the behavior of the host. The predominant location of cysts in neurons and the effects of astrocyte interaction(s) with the infected neurons may also have effects on the brain physiology and host behavior.

In conclusion, in this study we found that cysts were found to occur almost exclusively in neurons throughout chronic *Toxoplasma* infection. While astrocytes were not found to be a prevalent host cell for cysts in the brain, astrocyte processes were frequently observed around neuronal cysts. Cysts with predominant astrocytic processes surrounding them usually have well-defined cyst walls with no indication of cyst rupture, indicating that the cysts were intact. These results suggest a mechanism for neuronal cyst interactions to affect glial cell physiology in chronic *Toxoplasma* infection that may possibly have neuropathological effects.

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