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Toxoplasma gondii infection-induced Loss of Innate Aversion to Cat Urine in Mice

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Toxoplasma gondii infection-induced Loss of Innate Aversion to Cat Urine in Mice

By

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Committee in charge:

Professor Ellen Robey, Co-Chair
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Abstract

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Toxoplasma gondii chronic infection in rodent secondary hosts has been reported to lead to a loss of innate, hard-wired fear toward cats, its primary host. However the generality of this response across *T. gondii* strains and the underlying mechanism for this pathogen-mediated behavioral change remains unknown. To begin exploring these questions, we evaluated the effects of infection with two previously uninvestigated isolates from the three major North American clonal lineages of *T. gondii*, Type III and an attenuated strain of Type I. Using an hour-long open field activity assay optimized for this purpose, we measured mouse aversion toward predator and non-predator urines. We show that loss of innate aversion of cat urine is a general trait caused by infection with any of the three major clonal lineages of parasite. Surprisingly, we found that infection with the attenuated Type I parasite results in sustained loss of aversion at times post infection when neither parasite nor ongoing brain inflammation were detectable. This suggests that *T. gondii*-mediated interruption of mouse innate aversion toward cat urine may occur during early acute infection in a permanent manner, not requiring persistence of parasite cysts or continuing brain inflammation. We investigated the role of the mouse immune system in this behavior manipulation and identify that interleukin 4 (IL4) is a key molecule required for the loss of innate aversion to cat urine. Characterization of the source of IL4 in mice following infection has revealed that there are a number of cell types from which the critical IL4 could be produced. Initial T cell transfer experiments suggest that CD4 T cells capable of making IL4 may be partially involved in mediating the behavior manipulation.

Dedication

I dedicate this work to my parents who brought me into this world, Irvin Ingram and Kathryn Orr. I dedicate this work also to my teachers and mentors, including my family, friends, and formal educators, who have guided me and given me knowledge and the tools with which to explore and follow my path. I dedicate this work to greater society within which both the beauty of human nature and the aspects that require further work and improvement inspire my aspirations to contribute my gifts to the world. Ultimately I dedicate this work to the natural world and Nature itself, through the observation and exploration of which lead me in infinite curiosity, reverence, and purpose.

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Chapter 1: Introduction

"Nature's imagination is richer than ours."
- Freeman Dyson

Section 1.1: Parasite manipulations of host behavior

Among the marvels abundant in nature, few capture the imagination like parasites manipulating the behavior of their hosts. There are many harrowing examples of putative mind or body take-overs involving all manner of organisms [1] Rabies, an RNA virus encoding only 5 proteins, frequently causes it's mammalian hosts to develop horror movie like aggression, allowing the abundant viral load found in their frothy saliva to transmit to the next host via bite wounds [2]. *Ophiocordyceps*, a genus of host-specific fungal parasites, secure their spread by quite remarkably causing unfortunate 'zombified' hosts such as ants to brazenly leave their colony, ascend vegetation to specific heights and then engage in a so called 'death grip,' locking them in place in preparation for the fruiting bodies of the parasitic yeast to then sprout forth grotesquely from the body, raining infectious spores down upon those below [3]. Even multicellular organisms such as trematodes have been described parasitizing fish, invading their brains and then causing conspicuous swimming behavior, remaining too close to the surface of the water and flashing their shiny underbelly, making them easy prey for the parasite's primary host, predatory water birds[4]. These are but a mere few examples of the mysterious and often complicated infectious manipulations described, but thus far, poorly understood. The lack of understanding of the detailed mechanisms of manipulations employed by parasitic organisms in the natural world is due to in part the extreme difficulty with which one may study these organisms in a controlled laboratory environment.

Section 1.2: *Toxoplasma gondii*, a master manipulator

Toxoplasma gondii, like the trematode, has a multi-host life cycle involving predation of an intermediate host[5], but is actually perfectly suited to laboratory study. *T. gondii* is a single celled, obligate intracellular apicomplexan protist whose definitive host is the feline[6]. Cats are the only organisms within which the parasite can sexually reproduce. In the cat, they invade the epithelial lining of the gut and produce billions of highly infectious oocysts that are excreted into the environment in the feces and then contaminate soil, water, and vegetation. Any mammal or bird, including mice (a well-studied laboratory model organism), can then be infected by ingesting an oocyst. The parasite will then invade any nucleated cell and asexually reproduce, disseminating throughout the body of the secondary host. This mode of reproduction conveniently allows for *T. gondii* propagation in culture in a laboratory setting as well. In the secondary host, the parasite uses a combination of mechanisms to cross the blood brain barrier (BBB) and targets neurons, within which the parasite transitions to a slower growing form called bradyzoites and encysts in these living neurons, persisting for the

life of the host [7,8]. It was hypothesized and then experimentally supported that rodents, a natural secondary host and natural prey of cats, may lose their innate hard-wired aversion to cat odors following infection in order to enhance their transmission [9,10]. These fascinating descriptions however shed very little light on how the parasite might be accomplishing this. Conveniently, mice and *T. gondii* are both easily raised and studied in the laboratory environment, making them perfectly suited for investigations to reveal the underlying mechanisms of this parasitic behavior manipulation.

Section 1.3: Behavioral changes in the rodent secondary host

A number of studies support the hypothesis that infection with *Toxoplasma gondii* results in a behavior change in rodents that may enhance the transmission of the parasite to its primary host the cat. First it was demonstrated that a loss of aversion to feline urine occurred in *T. gondii* chronically infected rats [9]. Years later, this finding was recapitulated and extended with the observation of loss of aversion to cat odors in infected mice [10–12]. Others have demonstrated that this loss of aversion even appears to be specific for the primary host, felines, and is not generalized to other non-primary host predators such as canines and mink [13,14]. Although evidence for this putative behavioral manipulation has been accumulating, many studies have been published criticizing the validity of these findings.

A number of conflicting studies insist that the loss of innate aversion to cat urine is not a true and specific manipulation caused by *T. gondii* infection. These studies report findings that suggest that the observed behavior changes are merely a by-product of general immune-mediated brain pathology [15–17], or in some cases are not reproducible at all [18]. These conclusions are based on disparate experimental approaches including: host species (rat or mouse) and sex (male or female), infection-resistant (Balb/c) *versus* -sensitive (C57BL/6) hosts, type of parasite strain used, time post infection, and behavioral tests performed, reviewed elsewhere [19,20]. As it stands, there remains an unmet need for a thorough and unbiased assessment of this behavioral phenotype in rodents.

Section 1.4: Mouse innate aversion and behavioral paradigms

The underlying mechanism of hard-wired predator aversion responses in rodents is not yet fully understood, however, there exists a significant amount of research investigating the phenomenon, reviewed in depth elsewhere [21]. In brief, beginning in the early 1900's, laboratory rodents were described as having stereotyped unconditioned defensive behaviors in response to exposure to predators and their odors, including avoidance. Over the decades, the innate aversion displayed by rodents to predator odors, specifically cat odors, has been used to study anxiety and to define the neural pathways underlying this unconditioned response [21–23]. Exposure to cat odor is accepted as a 'partial predator stimulus' and is believed to give rise to a moderate fear response and induce anxiety, resulting in avoidance as well as defensive investigation, whereas exposure to the predator itself gives rise to fear and escape behavior only [24]. Exhaustive neuroanatomical evaluation of rodent circuitry activation following exposure

to cat odor has revealed the involvement of the main olfactory epithelium and vomeronasal organ which, through separate pathways, signal to the medial amygdala [25–30]. This then leads to activation of a number of medial hypothalamic defensive circuits (dorsal premammillary nucleus, anterior hypothalamic nucleus, and dorsomedial part of the ventromedial hypothalamus) and ultimately triggers behavioral output regions (prefrontal cortex, periaqueductal gray, cuneiform nucleus, and locus coeruleus). Recently, specific biological molecules found in cat urine and other excretions have been identified as the putative triggers detected either by the main olfactory epithelium [31] or the vomeronasal organ [32], possibly acting together to elicit innate aversion and other behaviors. Taken together, it is clear that there are many different ways in which *T. gondii* could potentially interfere with the detection, processing, or output of innate avoidance behavior caused by predator odor exposure.

In order to clearly establish if infection with *Toxoplasma gondii* indeed disrupts the innate aversion to cat urine in rodents, the behavior must first be reliably measured. A variety of behavior tests have been utilized by the groups mentioned above arguing either for or against the existence of a parasite-induced loss of aversion in rodents. Summarized in a review [20], the behavior tests used to evaluate rodent innate aversion have varied widely in a number of ways: arena size, arena shape, type and amount of predator odor, single or multiple odors during each test, number of habituation trials, metric of evaluation, and time of experiment (as few as 5 minutes and as long as 'dawn til dusk') [9,10,13,14,18,33,34]. It is understandable that without any consistency between behavioral assays used by various research groups, there remains skepticism in the field that *T. gondii* causes a real and robust behavior change in rodents. Additionally, two key confounding factors must also be taken into account: 1) all of these experiments were performed in the light, even though mice and rats are both considered primarily nocturnal; and, even more importantly, 2) most studies do not demonstrate clear aversion to cat odor in the control uninfected animals. In almost every study, animals are exposed to two or more odors at the same time, resulting in a preference assay rather than an aversion assay [9,13,14,33,34]. Any resulting differences between infected and uninfected animals demonstrate a change in preference. Only one behavior assay exposed animals to bobcat urine alone and in it, the control animals spent approximately 40% ±10% of their time in the cat bisect of the enclosure [10]. Researchers specifically interested in studying the innate aversion of mice toward predator odors evaluate animal behavior for 60 minutes, in the dark, and clearly report that control mice display aversion when exposed to cat odors [32]. In their studies, uninfected control mice spend less than 15% ±1% of their time near the cat odor. In order to evaluate the effect of *T. gondii* infection on this behavior it will be important to establish a robust and consistent behavioral assay that demonstrates natural rodent innate aversion.

Section 1.5: Possible mechanisms of loss of aversion

There are many possible ways in which *T. gondii* may influence host behavior. A few proposed mechanisms have been favored and previously investigated including 1)

neurotransmitter modulation, 2) local effects due to cyst location, and 3) changes in neural circuits.

The first proposed mechanism of behavior change in secondary hosts was infection-induced changes in neurotransmitter levels, specifically dopamine. This was based on measurements of brain dopamine concentrations being 14% higher in chronically infected mice than controls [35]. Later, experiments employing a selective dopamine uptake inhibitor reported effects on "dopamine related" infection-induced behaviors in mice, although these investigators were not specifically assessing loss of aversion to cat odors [36]. Following the sequencing of the *T. gondii* genome, it was discovered that there are two functional and expressed *T. gondii* aromatic amino acid hydroxylase genes (AAH), homologous to mammalian tyrosine hydroxylase, a critical enzyme involved in dopamine metabolism [37]. Another group subsequently reported that *in vivo,* tissue cysts in neurons appear to immunohistochemically stain for dopamine [38]. They also reported that *in vitro, T. gondii* encysted dopaminergic neuron-like cells (PC-12) produce more dopamine than non-infected controls, however, others were not able to recapitulate this finding [39]. More recent studies investigating changes in dopamine and *T. gondii* AAHs, however, have cast doubt on this neurotransmitter playing a key role in behavior manipulation in rodents. In a study comparing neurotransmitter concentrations in selected regions of the brain in both male and female mice, the authors report that while dopamine increased in males, dopamine decreases in females [40]. The loss of innate aversion to cat urine has been reported in both male and female mice and rats, thus suggesting that while dopamine concentration changes may be occurring and influencing behavior in some way, its role in this specific phenotype may not be critical. Most recently, transgenic parasite strains were successfully generated lacking the *T. gondii* AAH genes [39]. The authors concluded that AAH1 is critical for parasite survival in non-dopaminergic cells (likely involved in cyst wall formation) and that lack of AAH2 does not impact global or regional dopamine production *in vivo* in mice. In light of these recent findings and the lack of direct experimental correlation of the loss of innate aversion with dopaminergic changes following infection with *T. gondii*, the involvement of neurotransmitter modulation as a mechanism remains unconvincing.

The second mechanism with which *T. gondii* has been proposed to mediate the loss of innate aversion to cat odors is through highly local effects related to parasite cyst location. Cyst location has long been assumed to play a key role in mediating behavioral changes because, logically, if the chronic infection results in cysts residing inside neurons, the cells most critically known to be responsible for animal behavior, then they likely will be influencing their permanent cellular domicile [41]. Using a luciferase reporter parasite, early data suggested that there was a possible preference for 'amygdalar regions' [10]. However, later studies from the same group reported inconsistent loss of aversion to cat odor in rats, but a positive correlation between cysts found in specific 'forebrain regions' and loss of aversion following infection [42]. Thorough studies analyzing the entire brain following infection mapping cyst density concluded that *T. gondii* exhibits a random distribution pattern throughout the brain with no statistically significant tropism [40,43]. Recent evidence utilizing a cre-recombinase expressing *T. gondii* transgenic system in combination with Cre-GFP reporter mice has

demonstrated that the parasite actually delivers effector proteins into many more host cells than it invades and encysts in and is able to impact their function [44,45]. This could mean that *T. gondii* establishes the necessary molecular impact to alter mouse neurobiology and behavior early during infection and, if permanent, may not require a chronic infection with cysts to mediate ongoing behavioral changes. While no clear cyst localization requirements have been established thus far, the specific cells with which tachyzoites interact may still be an important aspect of *T. gondii* host behavioral manipulation.

A third popular theory of rodent behavioral manipulation by *T. gondii* proposes that significant changes in neural circuits occur following infection. As reviewed elsewhere, there are many direct and indirect effects observable in *T. gondii*-infected rodent brains [46]. A few changes have specifically been suggested to be involved in the observed behavioral manipulation. Using cfos labeling as a proxy for neuronal activation following exposure to cat odor, one group reported that the normal limbic regions of activation are less active in *T. gondii*-infected rats [11]. They further report that proximal limbic regions typically associated with 'sexual arousal,' are more highly activated than uninfected controls. Another group reported that neurons infected with either tachyzoites or chronically infected with cysts display differences in their neuronal activity, with a progressive decrease in normal Calcium (Ca^{2+}) signaling [47]. Changes in neuronal connectivity and dendritic branching have also been reported [46,48]. While intriguing, these studies fail to isolate or specifically ameliorate the observed neurological changes and thus prove their requirement for the loss of aversion to feline odors. Further investigation into the parasite-derived factors leading to these neurological changes is warranted in order to provide convincing evidence of their involvement in the loss of aversion to cat odor.

At this time, there is clear evidence that many measurable changes occur in the brains of rodents following infection with *T. gondii*, however no one theory has garnered fully convincing and mechanistic evidence to explain the loss of innate aversion to cat odor. In fact, some, if not all, of these findings may turn out to be related to the mechanistic pathway triggered by the parasite. With so many different ways to approach this puzzling parasite manipulation, it is odd that such little attention has been given to the aspect of rodent biology most intimately involved with the infection: the immune response.

Section 1.6: Immunological influences on the brain and behavior

Although *Toxoplasma gondii* infection and the host immune response required for control of the parasite has been well studied, the role that the immunes system may be playing in the loss of innate aversion to cat odors has not yet been examined. *T. gondii* is a rare and fascinating example of a neurotropic infection that not only invades the typically immune-privileged central nervous system (CNS), but also results in dramatic, typically permanent, alteration of the CNS immune environment by establishing a chronic infection in the brain [46,49]. Parasite invasion of the CNS results in local activation of brain-resident microglia and astrocytes as well as a permissive

environment for infiltrating peripheral leukocytes [42,50]. It is well established that *T. gondii* infection requires both innate and adaptive immune responses in order to effectively control toxoplasmosis [51–55]. Upon infection, innate cells such as microglia and macrophages typically exhibit M1 activation, and adaptive CD4 and CD8 T cells a robust Th1-polarized immune response in order for the host to not succumb to the infection. This response is characterized in part by the production of inflammatory cytokines such as interferon gamma (IFNγ), interleukin (IL)-12, IL-2, and others. While the M1/Th1 phenotype is definitively required for parasite control, other non-canonical innate and adaptive cell phenotypes and cytokines are reported to influence the infection outcome as well. For example, the M2/Th2 associated cytokine IL4 has been reported to reduce severity of acute infection, promoting IFNγ production during chronic infection, and modifying the replication of tachyzoites in macrophages [56–58]. Host-pathogen interactions are often complicated and nuanced, resulting from extensive co-evolutionary mechanisms [59–61]. The impact of CNS immune activation due to *T. gondii* infection on the brain and behavior of rodents is currently poorly understood and warrants closer study.

It is quite possible that the host immune response to *Toxoplasma gondii* is playing a role in the loss of innate aversion following infection. Immune related cytokines in the periphery and the CNS have long been known to directly result in stereotyped 'sickness' behavior [62–64]. Well-characterized sickness behaviors include lethargy, anxiety, sleep disturbances, and anhedonia. The majority of known behavior-modifying immune molecules are related to M1/Th1 polarized responses, however, there is some circumstantial and correlational evidence implicating Th2 type responses as well in aspects of learning and memory [65]. In fact, recent research has begun to reveal the role of immune molecules in healthy animals. Immune-related complement protein C1q is now known to serve an important developmental role in the CNS, and may have an ongoing role in synaptic plasticity [66,67]. Another group has demonstrated that otherwise healthy mice deficient in IL4 have disturbances in normal learning and memory [68,69]. They go on to demonstrate that transfer of peripheral T cells capable of making IL4 appears to rescue these learning deficits. Additionally, *in vitro* studies suggest that immune cytokines can influence neurogenesis. Microglia treated with IFNγ causes neuroprogenitors to differentiate into neurons while IL4 treatment biases these same precursor cells to become oligodendrocytes [70]. Whether *T. gondii* infection triggers an intended immune-mediated behavior change or instead is causing an inadvertent affect on brain biology due to the unusual invasion of the peripheral immune system into the CNS, it is clearly possible that some aspect of the immune response may be involved in the behavior changes in mice.

Section 1.7: Conclusions and Motivation

Toxoplasma gondii infection is proposed to cause loss of innate aversion to cat urine in rodents. There is a growing body of research that shows there are changes in rodent preference for cat odor following infection, however, there remains ambiguity as to if this is really disrupted innate aversion. It would be useful to the field to develop a robust behavioral assay with which to test uninfected and infected animals. Here in we

describe the design of a robust behavioral assay with which to assess the effect of *T. gondii* infection on the mouse intermediate host.

There are many inconclusive reports demonstrating changes in rodent neurobiology and claiming that these may be related to the parasite-induced behavioral changes. However, no reports have yet employed careful analysis of the host immune response as it directly relates to the loss of innate aversion to cat odor. Here we will describe a previously unknown requirement for the immune system in the parasite-mediated loss of innate aversion to cat odor.

Chapter 2 – Mice Infected with Low-virulence Strains of *Toxoplasma gondii* Lose their Innate Aversion to Cat Urine, Even after Extensive Parasite Clearance

Section 2.1: Abstract

Toxoplasma gondii chronic infection in rodent secondary hosts has been reported to lead to a loss of innate, hard-wired fear toward cats, its primary host. However the generality of this response across *T. gondii* strains and the underlying mechanism for this pathogen-mediated behavioral change remains unknown. To begin exploring these questions, we evaluated the effects of infection with two previously uninvestigated isolates from the three major North American clonal lineages of *T. gondii*, Type III and an attenuated strain of Type I. Using an hour-long open field activity assay optimized for this purpose, we measured mouse aversion toward predator and non-predator urines. We show that loss of innate aversion of cat urine is a general trait caused by infection with any of the three major clonal lineages of parasite. Surprisingly, we found that infection with the attenuated Type I parasite results in sustained loss of aversion at times post infection when neither parasite nor ongoing brain inflammation were detectable. This suggests that *T. gondii*-mediated interruption of mouse innate aversion toward cat urine may occur during early acute infection in a permanent manner, not requiring persistence of parasite cysts or continuing brain inflammation.

Section 2.2: Introduction

Toxoplasma gondii is a highly prevalent and successful neurotropic protozoan parasite that infects mammals and birds and is found nearly everywhere in the world [71,5]. However, the parasite can only sexually reproduce in the feline gut, defining cats as the primary host [72]. When *T. gondii* infects an intermediate host such as rodents or humans, it infiltrates the central nervous system and forms slow-growing cysts inside neurons where it can persist for the life of the host [73]. In order to complete the parasite's life cycle, an infected secondary host must be eaten by a cat. Behavioral studies comparing infected and uninfected mice have suggested that rodents lose their innate, hard-wired fear of cat odors when chronically infected with *T.* [9,10,13], presumably enhancing the transmission of the parasite to its primary host.

While intriguing, these studies had several limitations. Most *T. gondii* strains found in North America and Europe can be categorized into three well-defined clonal lineages called Type I, Type II, and Type III [74–76]. The majority of behavioral studies have used Type II strains, which are known to result in high parasite-cyst loads in the brains of mice and cause correspondingly high levels of immune-mediated brain inflammation [16,17,77]. This can result in general brain pathology causing extraneous motor and behavioral changes in infected mice, complicating the interpretation of predator aversion responses.

Type III parasites, in contrast, result in a lower cyst burden and cause less general brain inflammation [78,79]. Type I parasites are typically highly lethal in mice; however, the recent identification of *ROP5* genes as the mediators of acute virulence and the generation of attenuated parasites lacking this locus [80,81] permits long-term Type I infection studies in mice, perhaps due to a non-persistent infection following Immunity Related GTPase-mediated clearance. Extending mouse innate avoidance studies to include the less virulent Type III and attenuated Type I parasites described above could lead to clearer behavioral results, free of mitigating pathology-related changes.

Section 2.3: Results and Discussion

In order to evaluate the loss of aversion to cat urine in mice, we developed a rigorous high throughput behavioral assay. Mice were placed in a 15-inch by 7-inch enclosure with a small plastic dish affixed to one end. Either bobcat urine or rabbit urine was added to the dish and mice were allowed to explore the cage freely for one hour in the dark. The movements of each mouse were automatically recorded using Motor Monitor Smart Frames by Kinder Scientific, which are comprised of a grid of infrared beams and detectors. Each time the animal crosses a photobeam, the system records a 'beam break,' and provides the position and time spent in each designated area. The time spent close to the dish, defined as 'Near Target', or on the opposite end of the enclosure, the 'Avoidance Area', was assessed for up to 16 individually caged mice simultaneously (Figure 2.1A).

In the process of optimizing our behavioral assay, we confirmed the loss of aversion to cat urine in mice previously reported by others [10,14]. In preliminary experiments with male and female Balb/c mice, chronic infection with two Type II parasite strains (Pru and ME49) caused loss of innate aversion to bobcat urine (unpublished data). Due to slightly higher variability in female mouse behavior, likely due to higher levels of pathology [82], we performed all other experiments exclusively with males.

As anticipated, mice infected with Type II parasites succumbed to infection during both the acute and chronic phase. Surviving mice tended to have more complications associated with the progress of the infection as well as more 'sickness' behavior

Figure 2.1

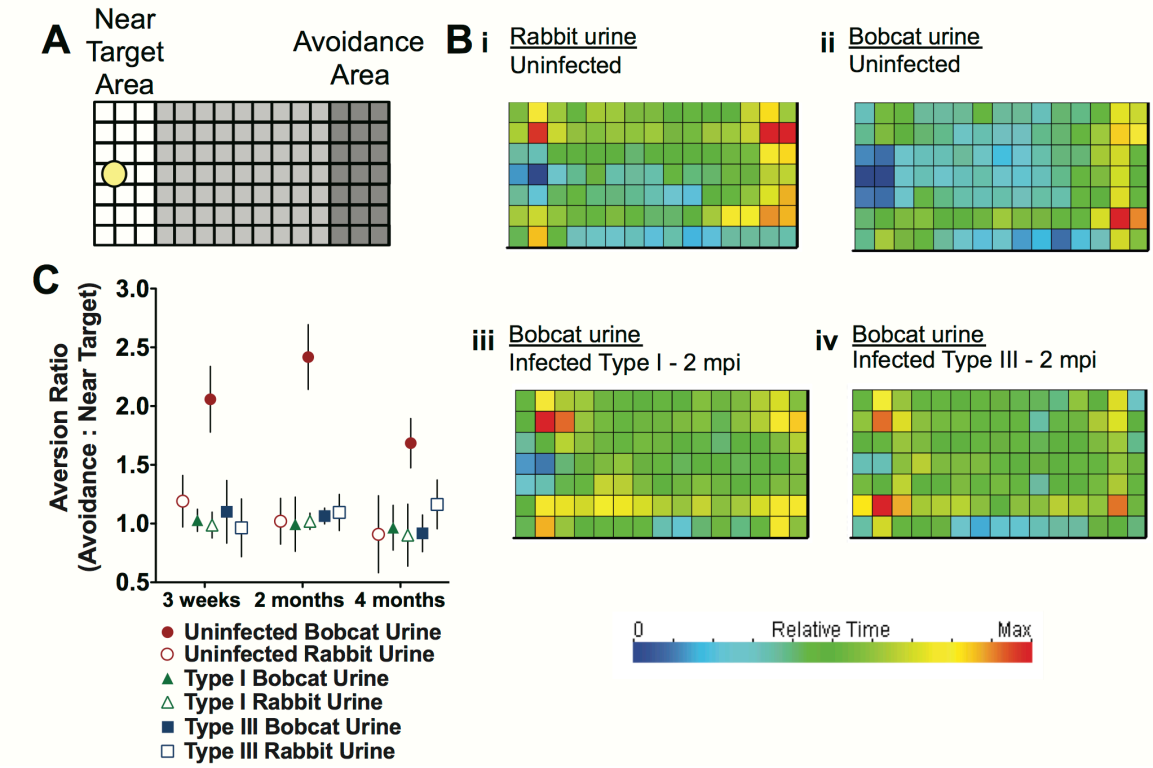

Figure 2.1. Assessment of aversion demonstrates loss of fear toward cat urine in Type I- and Type III-infected mice. [A] Overhead representation of behavioral arena where a small dish containing the 'target' solution (yellow disk) is affixed at one end of the behavioral arena. 'Near Target' is defined as the area of the arena (white) proximal to the target. 'Avoidance' is defined as the most distal region (dark grey) of the enclosure relative to the target. [B] Representative heat maps of mouse place preference during a 60-minute trial of (i) uninfected mice exposed to rabbit urine, and (ii) uninfected, (iii) attenuated Type I-infected, and (iv) low-virulence Type III-infected mice exposed to bobcat urine from trials conducted at 2 months post infection. [C] Aversion ratio, the avoidance time to near target time, of uninfected (red circles), Type I-infected (green triangles), and Type III-infected (blue squares) animals when exposed to bobcat urine (filled shapes) or rabbit urine (open shapes) at 3 weeks, 2 months, and 4 months post infection (n=10 for each group). Error bars are the Standard Error of the Mean (SEM).

characterized in part by lower activity levels. Due to the variety of generalized pathology and since detailed studies of Type II infection as it relates to mouse behavior exist elsewhere, we did not continue investigations with Type II parasites.

To better characterize *T. gondii*'s ability to cause loss of innate aversion to cat urine in mice, we compared two additional parasite strains: an attenuated Type I and a low-virulence Type III. Male mice were assayed at three time points: three weeks, two months, and four months post infection. Uninfected mice showed no place preference when exposed to non-predator control rabbit urine (Figure 2.1Bi), spending close to equal time in the Near Target Area and the Avoidance Area. When exposed to bobcat urine, these same animals exhibited marked aversion, spending more time in the Avoidance Area than in the Near Target Area (Figure 2.1Bii). Type I- and Type III-infected animals behaved similarly to uninfected animals when exposed to rabbit urine, including total movements made, proportion of time spent in the periphery (unpublished data), and lack of aversion (Figure 2.1C). Additionally, we performed a Hidden Cookie Test to evaluate general olfaction and observed no difference between uninfected and infected animals (Uninfected, Type I-, and Type III-infected animals found the cookie on average within 96±14, 109±18, and 123±31 seconds, respectively where variance indicates Standard Error of the Mean). Infection with either attenuated Type I or Type III parasites resulted in complete loss of aversion to bobcat urine (Figure 2.1Biii and 2.1Biv). This effect was observed at all three time points and appears to be an all-or-nothing effect (Figure 2.1C). There were no 'non-responders' in either infection group, nor did the effect diminish with time. These data show that the ability of *T. gondii* to disrupt innate predator aversion extends to all three major parasite clonotypes types.

We next investigated the parasite load and the immune response in the brains of mice infected with attenuated Type I and Type III parasites during chronic infection. Following the final behavior experiment, we sacrificed the cohort over the course of 4 weeks. We carefully harvested the brains and skull cap meninges from each animal and assessed 10% of homogenated brain and meninges for parasite load using semi-quantitative polymerase chain reaction (qPCR) targeting an abundant *T. gondii* gene family, B1 [83]. Type III-infected mice all had readily detectable parasite load, well above uninfected controls (Figure 2.2Ai). In contrast, Type I-infected mice all had undetectable levels of brain-resident parasites (Figure 2.2Aii).

The brain is generally considered an immune-privileged environment and immune cell leukocyte infiltration is known to be tightly regulated [84,85]. Thus, only during an ongoing infection would we expect to find a large number of brain-resident leukocytes such as CD4 and CD8 positive T cells, required to control toxoplasmosis [51,86]. We isolated and compared brain leukocytes isolated from the combined parenchyma and meninges of each animal. Purified cells were surface stained for a variety of leukocyte markers and analyzed using flow cytometry. Type III-infected animals had elevated total brain leukocyte numbers, whereas attenuated Type I-infected animals had equivalent brain leukocyte numbers to uninfected animals (Figure 2.2B). The average proportion of CD4 and CD8 positive T cells in Type III-, but not Type I-, infected animals were much

Figure 2.2

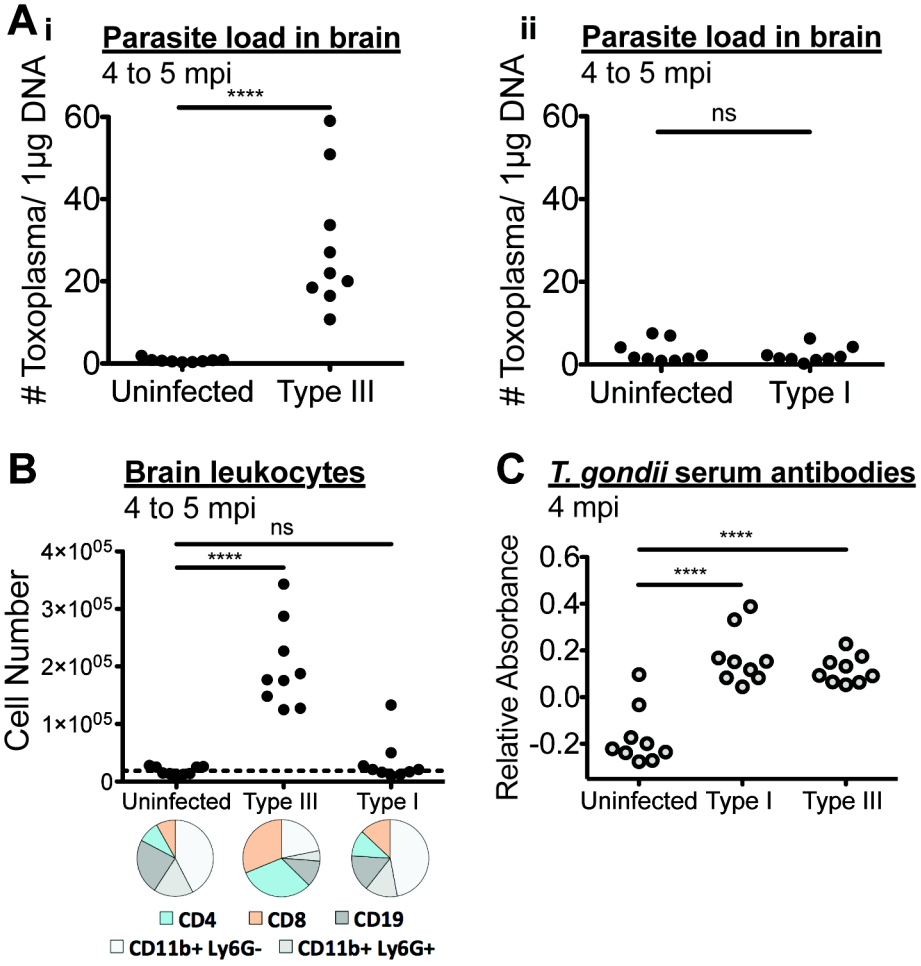

Figure 2.2. Persistent inflammation during chronic infection with Type III but not Type I parasites. [A] Quantitative PCR of genomic DNA prepared from 10% of brain homogenate reveals that parasite DNA was readily detectable in Type III-infected animals (i) but undetectable in attenuated Type I-infected animals (ii). [B] Brain leukocytes were percoll purified from the remaining brain and meningeal homogenate, stained for surface markers CD4, CD8, CD19, CD11b, and Ly6G, and assayed using flow cytometry. Total numbers of cells positive for any of these markers are reported for uninfected, Type I-infected, and Type III-infected animals at 4 to 5 mpi. Average percentages of brain leukocyte populations for uninfected, Type I, and Type III animals are displayed in pie charts below each group. [C] Blood serum was collected following the final behavior experiment 4 months post infection (4 mpi). *T. gondii* specific antibodies were detected using ELISA. Relative absorbance at 405 nm (sample 1:10 dilution absorbance – HRP no serum control absorbance) is reported for uninfected, Type I, and Type III. Each dot signifies one mouse. Significance was determined by Student's T-test for [A] and 1 way ANOVA for [B] and [C] where ns indicates $p>0.05$ and **** indicates $p<0.0001$ (n=9 for each group).

higher than uninfected controls, reflecting ongoing brain inflammation. Brain leukocytes from uninfected controls and Type I-infected animals show a lower proportion of T cells, B cells, and neutrophils and likely correspond to meningeal leukocytes. We assayed blood serum for antibodies specific for *T. gondii* using an enzyme-linked immunosorbent assay (ELISA) and confirmed that both attenuated Type I and Type III parasites had established infection in our mice (Figure 2.2C). Together these data show that permanent loss of aversion to predator urine may not depend on persistent brain infection.

It is possible that *T. gondii* causes a permanent change in the brain during acute infection, thereby not requiring continued parasite presence and an ongoing immune response. To address this possibility, we performed time course experiments, first infecting mice with Type III parasites. As expected, both parasite load and brain leukocyte numbers increased and remained well above uninfected levels (Figure 2.3A and 2.3B). We also infected mice with attenuated Type I parasites and detected parasites in the brain and meningeal homogenate in a number of mice between 5 and 20 days post infection (Figure 2.3D). At later time points, qPCR assays resulted in undetectable levels of these parasites. This early appearance of parasites in the brain may be related to the greater motility of Type I parasites in comparison to other strains [87], or may reflect more rapid dissemination within host cells. Early infection with attenuated Type I parasites was accompanied by a modest increase in total brain leukocytes (Figure 2.3E). While the Type I-infected animals had relatively low brain leukocyte numbers at day 13 post infection, the average percent of CD4 and CD8 positive T cells in these mice was similar to Type III-infected animals. Moreover, the increase in leukocyte infiltration is most striking when considering the total numbers of CD4 and CD8 T cells, providing further evidence for a T cell-mediated immune response in the brain and/or meninges following infection with attenuated Type I parasites (Figure 2.3F). Day 8 through day 20 post infection with attenuated Type I parasites from two separate experiments resulted in brain and meningeal T cell numbers all significantly above uninfected animals. This suggests that infection with attenuated Type I parasites does in fact elicit a transient inflammatory response in the brain and/or meninges following parasite presence.

Combined with previously published studies, our data indicate that infection with all three major North American *T. gondii* clonal lineages results in loss of innate, hard-wired aversion to feline predator urine in mice. Immunological analysis of mice infected with attenuated Type I and low-virulence Type III strains demonstrates that this behavioral change is not directly correlated with parasite load or brain inflammation. Taken together, our studies suggest that permanent interruption of mouse innate aversion to feline urine is a general trait of *T. gondii* infection that occurs within the first three weeks, independent of parasite persistence and ongoing brain inflammation.

Some current models propose that cysts residing in neurons play an active role in mediating loss of predator aversion. For example, it has been posited that *T. gondii* cysts might actively modulate dopamine production [11,37,38,41], or directly interrupt

Figure 2.3

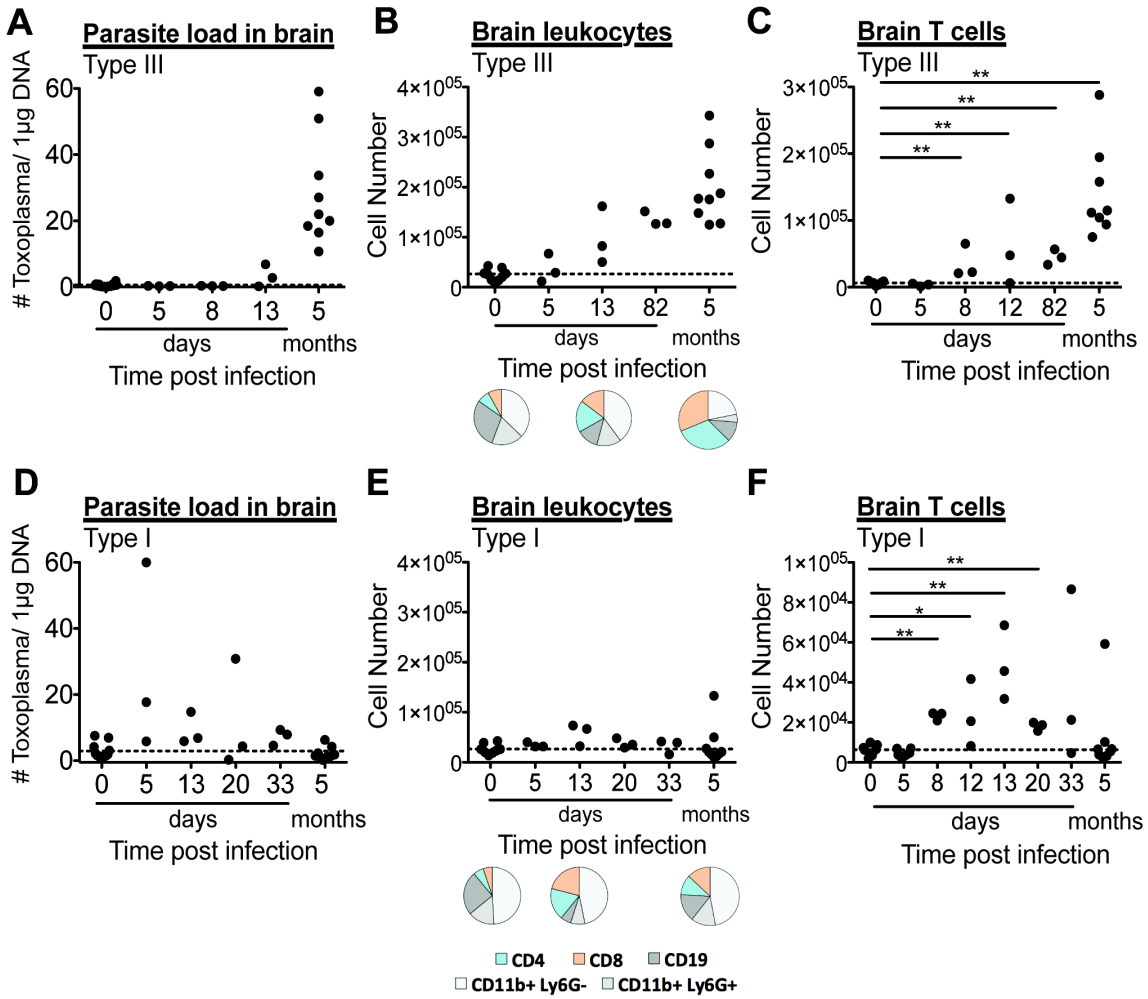

Figure 2.3. Type I and Type III acute infection results in parasite and leukocyte infiltration of the brain region. [A and D] Parasite presence was assessed using semi-quantitative PCR of genomic DNA prepared from 10% of each mouse brain and meninges at various times post infection. For reference, data from Figure 2A is included which was collected from animals that were used in behavior experiments 4 to 5 months post infection. [A] Type III-infected animals have detectable parasite in brain regions early during acute infection, which is sustained over time. [D] Some attenuated Type I-infected animals have detectable parasite in brain regions early during acute infection, decreasing to undetectable levels (average of uninfected indicated by black dashed line). [B and E] Brain leukocytes were percoll purified from the remaining brain and meningeal homogenate, stained for leukocyte surface markers CD4, CD8, CD19, CD11b, and Ly6G, and assayed using flow cytometry. [B] Type III-infected animals have brain leukocyte numbers above uninfected levels (black dashed line) 13 days following infection which continue to increase over time. [E] Average total cell numbers from Type I-infected animals. Average percentages of brain leukocyte populations for uninfected, Type I-, and Type III-infected animals at selected time points are represented in pie charts below the corresponding data in [B and E]. [C and F] Total brain and meningeal T cells (CD4 plus CD8), indicators of brain region inflammation, are reported for Type III- and attenuated Type I-infected animals. Each dot signifies one mouse. Significance was determined for [C and F] by Student's T-test where * indicates $p<0.02$, and ** indicates $p<0.002$.

neuronal activity [48,88]. In line with this notion, some investigators report higher cyst density in amygdalar regions known to be involved in innate fear [10], although this has been challenged by others [43]. In contrast, our results suggest that cysts may not even be required for sustained fear disruption. Moreover, recent studies show that *T. gondii* can deliver effector proteins into cells that it does not invade [44,45], and that these proteins can manipulate host cells without active parasite replication [89]. Thus *T. gondii* may interact with and manipulate its intermediate hosts without the requirement of cyst formation or parasite persistence. In light of these findings and our data reported here, we believe that a new non-cyst-centric model of *T. gondii*-mediated behavior manipulation of the mouse intermediate host is warranted.

Section 2.4: Materials and Methods

2.4.1: Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of UC Berkeley (Protocol #: R165-1212BCR).

2.4.2: Animals

All mice were bred and housed in specific pathogen-free conditions at the Association of Laboratory Care-approved animal facility at the Life Science Addition, University of California, Berkeley, CA. Preliminary experiments used male and female BALB/c mice bred in our facilities ranging in age from 5 to 16 weeks old. Females were housed 5 animals per cage while males were housed between 1 and 5 animals per cage. Males were separated and housed individually if they began to fight on a cage-by-cage basis. Behavior experiments reported here and time course experiments involved male BALB/c mice all 9 weeks old, ordered from The Jackson Laboratory, 10 mice per group. Animals were housed 5 per cage until they began to fight. Upon the first cage needing to be separated, all animals were housed individually. Mice were sacrificed between 5 days and 20 weeks post infection by transcardial perfusion with 20 mL ice-cold sterile phosphate buffered saline following anesthesia with 500µL 2.5% avertin administered intraperitoneally (i.p.).

2.4.3: Behavior Studies

Mouse aversion was assayed using the MotorMonitor SmartFrame System (Kinder Scientific, Poway, CA; Build # 11011-16). We used 7 x 15 High-Density SmartFrames to record up to 16 individually caged animals simultaneously. Animal movement was evaluated in transparent (17 cm X 38 cm) polycarbonate enclosures using the computerized photobeam system MotorMonitor. Animals were habituated for 1 hour the day before each trial in an empty enclosure. For the bobcat and rabbit urine exposure trials, a sterile cell culture dish (35mm x 10mm, treated polystyrene) was affixed to one end of the enclosure using a small amount of Blu-Tack (Bostick). 400 µL of either

bobcat urine (LegUp Enterprises, Lovell, ME) or rabbit urine (Pete Rickard's, Fleming Outdoors, Ramer, AL) was added to the dish. One animal per cage was gently placed in the center of the enclosure and their activity monitored for 1 hour in the dark. The following day, we repeated the experiment with whichever urine sample was not used the previous day, the order of which was semi-randomized. Data was analyzed using MotorMonitor Software. User-defined Zone Maps were generated as described in Figure 2.1A. Recorded beam breaks were used by the software to quantify total movement and time spent in each zone. Heat maps were generated by MotorMonitor using the HotSpots Graphic Comparator for each animal trial (parameters: Time 1 hour, smoothing 0.1, Intensity Cube Rt, Minimum Visibility Normal). For the hidden cookie test, animals were food-deprived overnight (16-17 hours), during which time water was freely available. Testing performed on the following morning consisted of timing the latency for the animal to find an appetizing piece of food (1 Teddy Grahams cookie) buried 1 cm beneath fresh cage bedding.

2.4.4: *T. gondii* infections

Tachyzoites were cultured on monolayers of human foreskin fibroblasts and prepared immediately before mouse infection as previously described [90]. All infections were administered i.p. in 200 µL volumes. Type I parasites used for infection were attenuated RH*Δrop5* (dose: $5x10^5$ parasites). Type II parasites used in preliminary studies were either Prugniaud expressing tandem dimeric tomato red fluorescent protein and OVA peptide (dose: 2500 parasites) or ME49 expressing luciferase (dose: 300 parasites). Type III parasites used for infection were CEP expressing green fluorescent protein (dose: $5x10^5$ parasites). All parasite strains were generously provided by John Boothroyd (Stanford, Palo Alto, CA).

2.4.5: *Ex vivo* analysis of tissue samples

Brains and skull caps were harvested and placed in 10 mL ice cold PBS before being immediately processed. Brains were transferred to 2.5 mL cold RPMI medium 1640 + L-glutamine (Sigma) and meninges were dissected from the skullcaps as described elsewhere [68] and combined with the brain parenchyma. Brains and meninges were crushed using a 3 mL syringe plunger then homogenized by repeated passage through an 18-gauge needle. 10% of the homogenate was removed and stored at -20°C for future PCR analysis. The remaining homogenate was digested in 1 mg/mL Collagenase IA (Sigma) and 0.1 mg/mL DNase I (Roche) for 40 minutes at 37°C. The digested material was filtered through a 70 µm cell strainer and centrifuged at 800*g* for 5 minutes. The brain and meningeal material was resuspended in room temperature 60% (vol/vol) Percoll (GE Healthcare)/RPMI and overlaid with 30% (vol/vol) Percoll/PBS and centrifuged with no acceleration or brakes for 20 minutes at 1000*g*. Mononuclear cells were harvested from the gradient interface and washed twice in PBS before preparation for flow cytometric analysis.

2.4.6: Flow cytometry

Antibodies to mouse CD4 (RM4-5), CD8α (53-6.7), CD19 (eBioID3), CD11b (M1/70), and CD11c (N418) were obtained from eBioscience. Anti-mouse Ly6G (1A8) was obtained from BD Biosciences. Cell viability was assayed using LIVE/DEAD® Fixable Aqua Dead Cell Stain Kits (Invitrogen). Surface staining with anti-mouse CD4, CD8, CD19, CD11b, CD11c, and Ly6G was performed at 4°C for 30 minutes. Cells were fixed and acquisitions were performed using a BD LSR II flow cytometer (BD) and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

2.4.7: Parasite load assay

Whole genome DNA was isolated from brain and meningeal homogenate using DNeasy Blood and Tissue Kit (Qiagen). Parasite burden was assessed using semi-quantitative PCR as described elsewhere [91].

2.4.8: Serum ELISA for *T. gondii*

Blood was collected by mandibular vein bleed prior to animal sacrifice. Samples were incubated for 4 hours at room temperature to allow clot to form, then incubated at 4°C overnight. ELISA 96 well plates were coated with 1 µg/ml Soluble Tachyzoite Antigen (STAg). Plates were washed 3 times with PBS – 0.05% TWEEN and blocked with 5% milk PBS-0.05% TWEEN, then washed 3 times more with PBS-0.05% TWEEN, and 54 µl of 5% milk PBS-0.05% TWEEN was applied to each well. Five serial dilutions, 6µl each, of serum from each sample were incubated overnight at 4°C. Plates were washed three times with PBS-0.05% TWEEN, and once with PBS. Plates were then incubated with rabbit anti-mouse IgG (H+L)-HRP (Jackson Immuno Research) 1:1000 dilution for 2 hours at room temperature. H_2O_2 was added to ABTS substrate (Sigma) at 1:1000, mixed and applied to the plate. Enzymatic reaction times were recorded at 405 nm.

2.4.9: Statistical Analysis

Prism software (GraphPad) was used for statistical analysis. P values were calculated using two-tailed Student's (non-parametric) t-test or 1 way ANOVA as indicated.

Chapter 3: Interleukin 4 is required for *Toxoplasma gondii*-induced loss of innate aversion to cat urine in mice.

Section 3.1: Abstract

Toxoplasma gondii is a brain parasite that has a multi-host life cycle involving the passage from rodent secondary hosts to the feline primary host through predation. It has been demonstrated that *T. gondii* infection can result in the loss of rodent innate aversion toward cat urine. However, the mechanism of this purported behavior manipulation remains unknown. Here we investigate the role of the mouse immune system in this behavior manipulation and identify that interleukin 4 (IL4) is a key molecule required for the loss of innate aversion to cat urine. Characterization of the source of IL4 in mice following infection has revealed that there are a number of cell types from which the critical IL4 could be produced. Initial T cell transfer experiments suggest that CD4 T cells capable of making IL4 may be partially involved in mediating the behavior manipulation.

Section 3.2: Introduction

Toxoplasma gondii is an obligate intracellular neurotropic protozoan parasite whose primary host is the cat [6]. The parasite employs a multi-host life cycle; it travels from cats through their feces to rodent secondary hosts, then back to cats *via* predation. Mice have evolved to detect molecules in predator cues, such as urine and saliva, and exhibit stereotyped innate aversion in response to these. Following *T. gondii* infection in rodents this innate hard-wired aversion disappears [9,10,92]. We have previously demonstrated that three genetically distinct strains of the parasite cause indistinguishable permanent loss of innate aversion. The mechanism of this behavior change, however, remains unknown.

When *T. gondii* infects a rodent secondary host, the parasite disseminates throughout the body, capable of invading any nucleated cell [71]. The host immune system responds with an M1/Th1 polarized response in order to control the systemic acute infection [51]. The parasite quickly crosses the blood brain barrier (BBB) and invades the central nervous system (CNS) [8]. Once the parasite invades the brain, microglia, the brain's innate immune cell population, and astrocytes, glial cells that control many aspects of brain biology, become activated [49]. In order for mice to survive the acute infection, microglia and astrocyte activation must allow for peripheral immune cell infiltration of the brain parenchyma. Millions of immune cells including T cells, B Cells, Neutrophils, and other myeloid cells will cross the BBB and intercalate throughout the brain [92]. These immune cells, especially T cells, are required for control of the chronic infection [51].

The host immune response not only results in cellular recruitment and activation. It also results in the production and secretion of immune molecules called cytokines. Certain immune-related cytokines have been known to not only contribute to host defense against pathogens but to also influence the behavior of the host. Th1 cytokines such as

IL6 and IFNγ induce social withdrawal and immobility [93]. Recently, the Th2 cytokine IL4 has been shown to be required for normal learning and memory [68]. Both IFNγ and IL4 have been shown to differentially activate microglia, which can then influence the developmental fate of neurogenesis [70]. We hypothesize that the host immune response may be playing a role in *T. gondii*-mediated loss of innate aversion to cat urine.

Section 3.3: Results and Discussion

To begin assessment of the role of the host immune response in the loss of innate aversion to cat urine, we compared a variety of immune compromised transgenic mouse strains. Using a robust innate aversion behavioral assay described previously [92], we first tested a variety of uninfected immune compromised animals in order to establish if immune deficits influence the innate aversion towards cat urine. We began with mice deficient in Recombination gene 1 (Rag1), Signal Transducer and Activator of Transcription (STAT) 4, STAT6, and Interleukin (IL) 4 to cover a broad array of immune dysfunctions. Uninfected immune compromised animals all displayed normal innate aversion when exposed to bobcat urine (Figure 3.1A) and no aversion when exposed to control rabbit urine (data not shown). This suggests that hardwired innate aversion to cat odors is not impacted by the lack of T and B cells (Rag1-dependent), defects in the development of Th1 cells (STAT4-dependent), defects in the development of Th2 cells (STAT6-dependent), or milder defects in Th2 cells and lack of mast cells (IL4-dependent).

Though these immune deficiencies did not impact the innate aversion in uninfected mice, we wanted to investigate if these aspects of mouse immunity were playing a role in the parasite-mediated behavior change. To do this, we infected immune compromised animals with the highly attenuated *Toxoplasma gondii* Type I parasite, RHΔRop5, known to cause loss of innate aversion in wild type (WT) mice following a transient infection. Rag1-, STAT4-, and STAT6-deficient mice all succumbed to the acute infection, while WT and IL4-deficient mice survived (Figure 3.1B). Behavioral assessment of IL4-deficient mice revealed that there was no loss of aversion towards bobcat urine following infection with either a transient attenuated Type I parasite or a chronic cyst-forming Type III parasite (Figure 3.1C). It is known that IL4 deficiency can influence the progress and severity of *T. gondii* infection. IL4 has been reported to reduce the severity of acute infection[56], but also to play a role in promoting IFNγ production during chronic infection[57]. It has even been reported to modify the replication of tachyzoites in macrophages [58] Because of these diverse influences on the course of infection, it was critical to confirm that these IL4 deficient animals had been successfully infected. Type I infection was confirmed by detection of *T. gondii* serum antibodies (Figure 3.1Di) and Type III infection was confirmed by assessing brain cyst burden (Figure 3.1Dii). This finding suggests that IL4 is required for the parasite-induced behavior manipulation.

We next sought to characterize the cell type(s) responsible for making IL4 following infection with *T. gondii*. Using an IL4 GFP reporter mouse strain, we isolated and

Figure 3.1

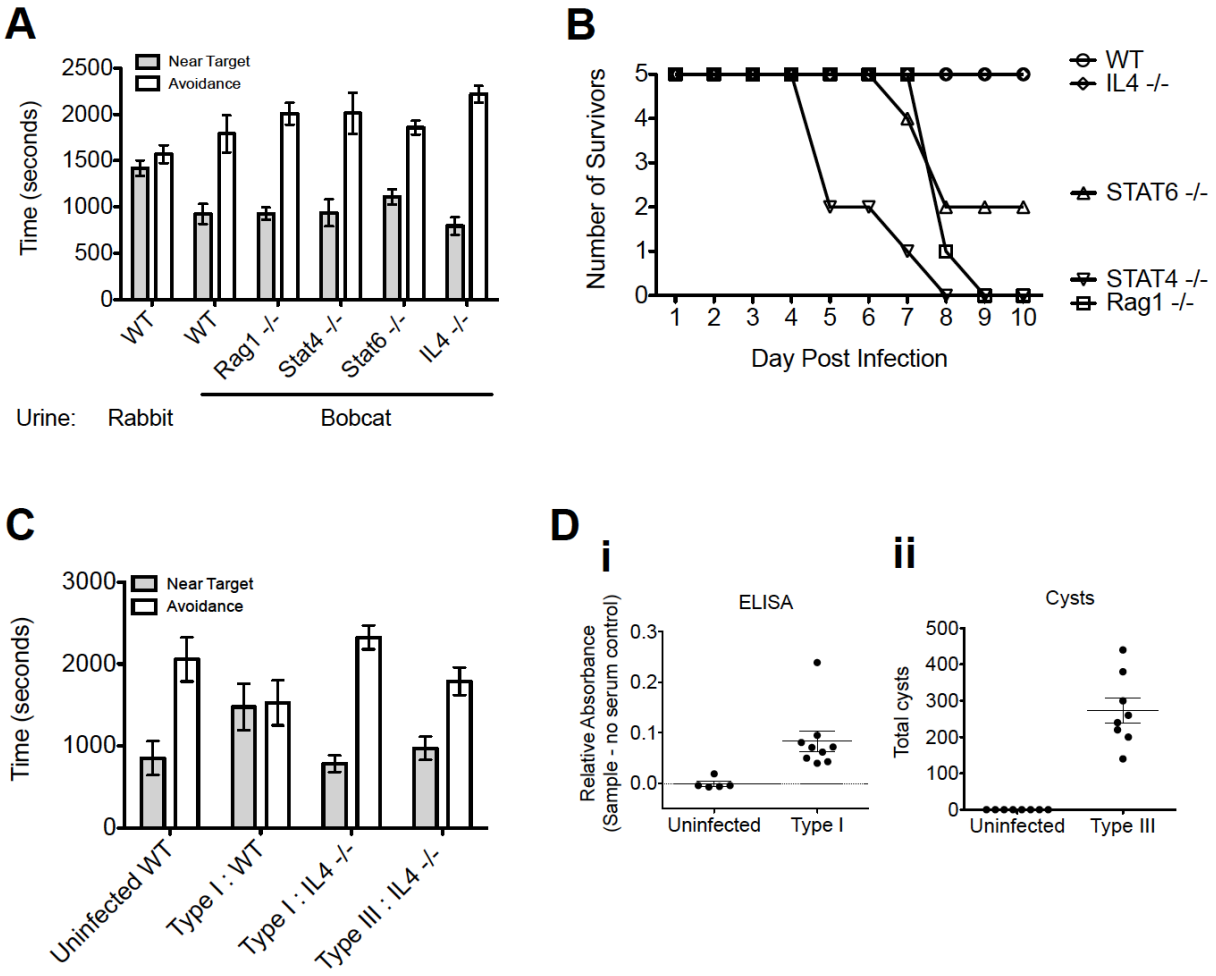

Figure 3.1. IL4 deficient mice do not exhibit behavior changes. [A] Behavior of WT or immune compromised animals when exposed to rabbit or bobcat urine in 1-hour aversion assay (n=10 per group). [B] Survival curve following infection with attenuated Type I parasite (n=5 per group). [C] Behavior of WT or IL4 deficient mice infected with either attenuated Type I or low-virulence Type III parasites (n=5-9 per group). [Di] Blood serum was collected from Type I infected animals. *T. gondii* specific antibodies were detected using ELISA. Relative absorbance at 405 nm (sample 1:10 dilution absorbance – HRP no serum control absorbance) is reported for uninfected and Type I. Each dot signifies one mouse. [Dii] 5% of the brain homogenate of each mouse was collected from uninfected and Type III infected animals and GFP-positive tissue cysts were counted. Total cysts were calculated. Each dot signifies one mouse. Error bars indicate Standard Error of the Mean (SEM).

characterized leukocytes from animals at various time points during infection. Type I and Type III parasites induce the loss of innate aversion within the first two to three weeks following infection [92](data not shown), thus our studies focus on this critical time period.

Flow cytometric analysis revealed three distinct IL4-procuding populations in the brain (including meninges) (Figure 3.2A) and two distinct populations in the spleen (Figure 3.2B) based on CD4 and CD11b surface staining. The total cell number of IL4-producing cells in the brain and the spleen increases transiently with the transient Type I infection (Figure 3.2A, 3.2B), but the proportions of each cell population do not change dramatically. The gating strategy to identify the brain leukocyte IL4-producing cells is shown (Figure 3.2C).

Cell types known to produce IL4 include CD4 T cells, NK T cells, basophils, mast cells, and eosinophils [94]. Recently described immune cells called Type 2 innate lymphoid cells (ILC2) have also been reported to produce IL4 in culture [95–97]. Characterization of brain leukocyte IL4-producing cells in uninfected and Type III infected animals (Figure 3.3A) reveals that the CD4+ subset are also CD3+, indicating that they are CD4 T cells and/or NK T cells. The CD11b+ subset includes a MHCII+ population in uninfected animals and increases surface expression following infection. The CD4- CD11b- subset does not stain for CD117 (c-Kit), a common marker found on ILCs and mast cells. In the spleen (Figure 3.3B), the CD4+ subset co-stain for CD3, indicating they are also CD4 T cells and/or NK T cells. There is a portion of the CD4- CD11b- subset in the uninfected animals that also expresses CD117, but this population disappears or is undetectable at 16 days post infection. Taken together, it is clear from this data that there are a wide variety of cell types that produce IL4 in the brain and the periphery both before and following infection. Any of these IL4-producers may individually, or in combination, be the critical source of the cytokine.

Finally, we sought to identify the critical sources of IL4. We first wanted to assess the role of T cell derived IL4 and therefore needed to choose a T cell deficient mouse model. Rag1 deficient mice lack all T cells due to a defect in thymocyte development. Unperturbed Rag1 deficient mice display normal innate aversion to cat urine, however, they do not survive *T. gondii* infection. We isolated mature adult peripheral lymphocytes and splenocytes from WT mice and transferred these cells intravenously (i.v.) into Rag1 deficient mice. Five days post transfer, we infected the mice with Type III parasites. As expected, the lymphocyte transfer protected the Rag1 deficient mice during the acute infection. Unexpectedly, when we tested innate aversion toward cat urine three weeks post infection, the adoptively transferred animals did not display a loss of innate aversion to cat urine (Figure 3.4A). Rag1-deficient mice are known to not only lack T cells, but because of this deficiency, they suffer from many severe lymphoid organ developmental defects as well. This result indicates that some cell type or immune-related compartment in Rag1-deficient mice does not develop properly which is required for parasite-induced loss of innate aversion to cat odor.

Figure 3.2

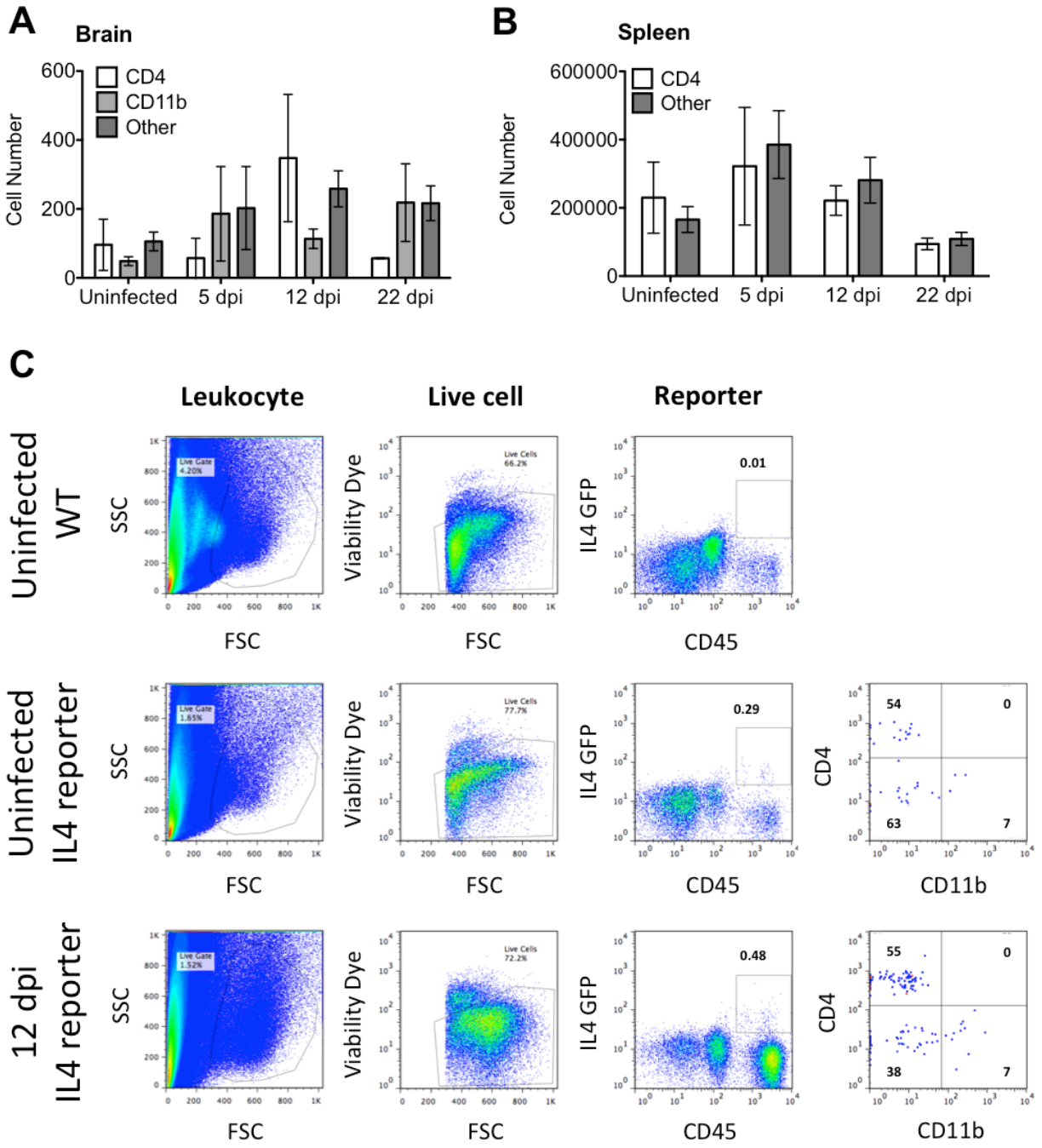

Figure 3.2. IL4 is produced by multiple cell types in the brain and spleen. Total cell numbers of GFP positive percoll-purified brain and meningeal leukocytes [A] and splenocytes [B] in IL4-reporter mice following infection with the transient attenuated Type I parasite. [C] Flow cytometry gating strategy using forward scatter (FSC), side scatter (SSC), AmCyan Viability Dye, Interleukin 4 Green Fluorescent Protein reporter (IL4 GFP) signal, CD45, CD4, and CD11b surface stains. We used a "Leukocyte" gate based on FSC and SSC, a "Live cell" gate based on Viability Dye negative events, and a "Reporter" gate based on GFP positive events. GFP positive cells found in the Reporter gate were then classified based on CD4 and CD11b expression. Percentages for each population from the previous gate are indicated in bold. Uninfected n=4, 5 days post infection (dpi) n=2, 12 dpi n=4, 22 dpi n=2.

Figure 3.3

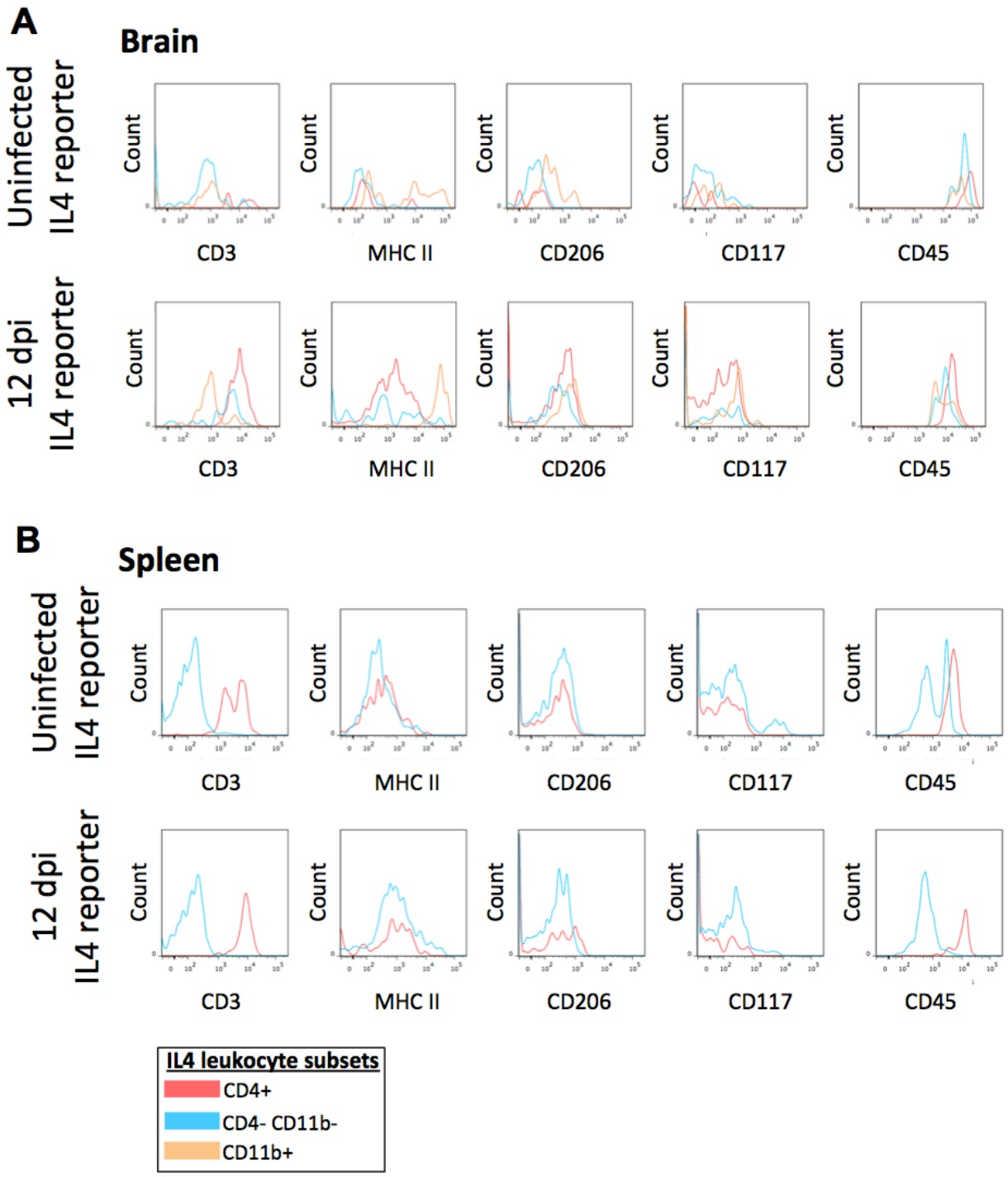

Figure 3.3. IL4 producing cell types. Representative histograms of expression levels of CD3, MHCII, CD206, CD117, and CD45 on three subtypes of IL4-GFP reporter positive cells as identified in Figure 3.2C. Cells were percoll-purified brain and meningeal leukocytes [A] or splenocytes [B] from uninfected and Type III infected animals.

Figure 3.4

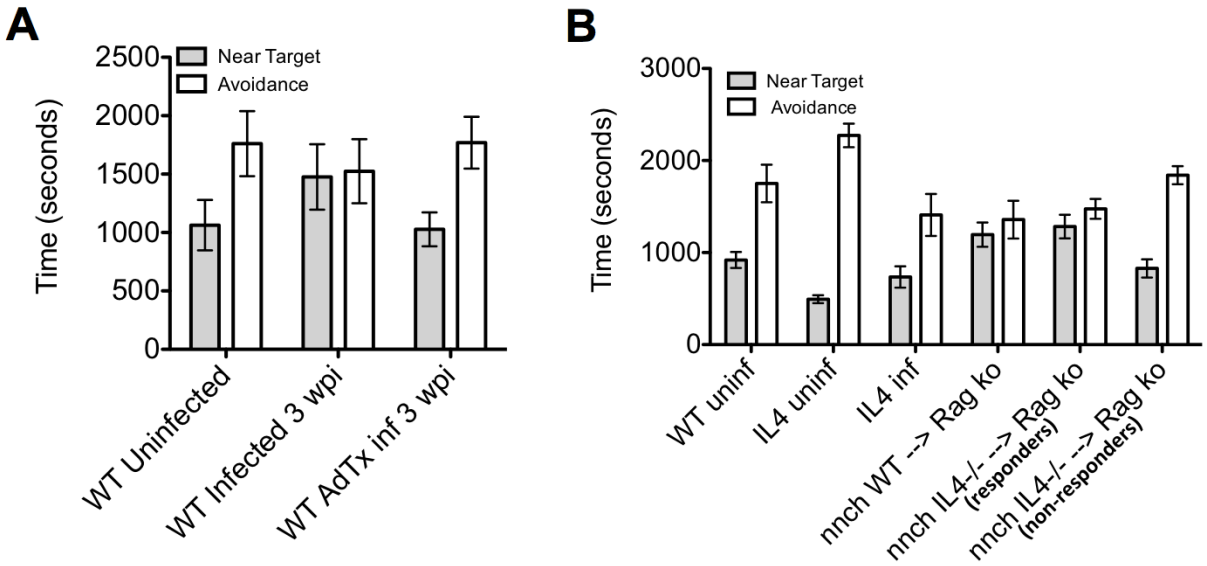

Figure 3.4. Development with IL4 producing T cells permits behavior change. Assessment of innate aversion towards bobcat urine three weeks post infection with Type III parasites. [A] Behavior of WT or adult Rag1-deficient mice adoptively transferred (AdTX) with WT mature lymphocytes and splenocytes. WT uninfected n=5, WT Infected n=5, WT AdTx inf n=4). [B] Behavior of F1 WT, IL4 deficient, or Rag1 deficient neonatal chimeras (nnch) generated with either WT or IL4 deficient (-/-) bone marrow. Behavior was reassessed two additional times and each nnch IL4 -/- → Rag ko animal consistently either displayed a behavior change (responders) or showed no behavior change (non-responders). WT uninf n=5, IL4 uninf n=5, IL4 inf n=4, nnch WT→ Rag ko n=4, nnch IL4 -/- → Rag ko (responders) n=4, nnch IL4 -/- → Rag ko (non-responders) n=5. Error bars are SEM.

In order to address the possibility that Rag1-deficient mice have a developmentally derived defect, we generated neonatal bone marrow chimeric mice. Rag1 deficient neonates that received WT bone marrow survived *T. gondii* infection and displayed loss of innate aversion at three weeks post infection (Figure 3.4B). Because neonatal chimerism of Rag1-deficient hosts with WT bone marrow results in animals that lose their innate aversion towards cat urine following infection, the behavior change seems to require normal development of the animal in the presence of T and B cells.

To test the hypothesis that T cell-derived IL4 is required for the behavior change following infection, we generated IL4 deficient neonatal chimeras and infected them with Type III parasites. Interestingly, at three weeks post infection, approximately half of the IL4-deficient neonatal chimeras displayed loss of innate aversion (responders) and half did not (non-responders). We repeated the behavior experiment two additional times and each animal consistently displayed the same behavior. In these chimeras, the only sources of IL4 are non-T cells. In the animals that did not display loss of innate aversion following infection (non-responders), it appears that T cell-derived IL4 is playing a critical role in the behavior change. However, because a portion of the chimeras (responders) display loss of innate aversion following infection, this suggests that a portion of the critical IL4 involved in parasite-mediated behavior manipulation is coming from non-T cells. In order to determine what cells are responsible for making the required IL4 necessary for the *T. gondii* induced behavior change, it will be important to identify the immune cells that were successfully reconstituted in the animals that exhibited the behavior change but were not in the animals that did not show the behavior change.

Here we began to assess the role of the immune system in *T. gondii*-induced loss of innate aversion to cat urine. We demonstrated that a variety of immune deficiencies do not perturb the innate hardwired aversion towards cat urine. We also show for the first time that IL4 deficient mice do not lose their innate aversion following infection with either transient or persistent strains of *T. gondii,* implicating this immune cytokine as an important mediator of the parasite mediated behavior manipulation. We began characterization of the IL4-producing cells following infection and have identified that CD4 T cells, as well as at least two additional populations of cells, produce the cytokine. Neonatal chimeric mice that developed with IL4-deficient bone marrow displayed a split phenotype following infection. Half of the animals lost their aversion to cat urine and half did not. This curious outcome requires further investigation. It is possible that there is a critical cell type that makes IL4 that did not develop properly in half of the chimeras. However, there may be some other aspect of the immune response that is different between those that changed behavior and those that did not. This work clearly demonstrates that the immune response, specifically the cytokine IL4, is playing a critical role in *Toxoplasma gondii*-mediated loss of innate aversion to cat urine in mice.

Section 3.4: Materials and Methods

3.4.1: Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of UC Berkeley (Protocol #: R165-1212BCR).

3.4.2: Animals

All mice were bred and housed in specific pathogen-free conditions at the Association of Laboratory Care-approved animal facility at the Life Science Addition, University of California, Berkeley, CA. Mice were either bred in our facilities or ordered from Jackson Laboratories. All mice are on a Balb/c genetic background except for animals in Figure 3.4B which are F1 hybrids between Balb/c and C57/B6 genetic background strains. Females were housed up to 6 animals per cage while males were housed between 1 and 6 animals per cage with littermates. Males were separated and housed individually if they began to fight on a cage-by-cage basis. Mice were sacrificed between 5 days and 20 weeks post infection by transcardial perfusion with 20 mL ice-cold sterile phosphate buffered saline following anesthesia with 500µL 2.5% avertin administered intraperitoneally (i.p.).

3.4.3: Behavior Studies

Mouse aversion was assayed using the MotorMonitor SmartFrame System (Kinder Scientific, Poway, CA; Build # 11011-16). We used 7 x 15 High-Density SmartFrames to record up to 16 individually caged animals simultaneously. Animal movement was evaluated in transparent (17 cm X 38 cm) polycarbonate enclosures using the computerized photobeam system MotorMonitor. For the bobcat and rabbit urine exposure trials, a sterile cell culture dish (35mm x 10mm, treated polystyrene) was affixed to one end of the enclosure using a small amount of Blu-Tack (Bostick). 400 µL of either bobcat urine (LegUp Enterprises, Lovell, ME) or rabbit urine (Pete Rickard's, Fleming Outdoors, Ramer, AL) was added to the dish. One animal per cage was gently placed in the center of the enclosure and their activity monitored for 1 hour in the dark. Data was analyzed using MotorMonitor Software.

3.4.4: *T. gondii* infections

Tachyzoites were cultured on monolayers of human foreskin fibroblasts and prepared immediately before mouse infection as previously described [90]. All infections were administered i.p. in 200 µL volumes. Type I parasites used for infection were attenuated RH*Δrop5* (dose: $5x10^5$ parasites). Type III parasites used for infection were CEP*gra6* expressing green fluorescent protein (dose: $5x10^5$ parasites). All parasite strains were generously provided by John Boothroyd (Stanford, Palo Alto, CA).

3.4.5: *Ex vivo* analysis of tissue samples

Brains and skull caps were harvested and placed in 10 mL ice cold PBS before being immediately processed. Brains were transferred to 2.5 mL cold RPMI medium 1640 + L-glutamine (Sigma) and meninges were dissected from the skullcaps as described elsewhere[68] and combined with the brain parenchyma. Brains and meninges were crushed using a 3 mL syringe plunger then homogenized by repeated passage through an 18-gauge needle. 5% of the homogenate was removed and immediately analyzed for cyst load for Type III infections. The remaining homogenate was digested in 1 mg/mL Collagenase IA (Sigma) and 0.1 mg/mL DNase I (Roche) for 40 minutes at 37°C. The digested material was filtered through a 70 µm cell strainer and centrifuged at 800*g* for 5 minutes. The brain and meningeal material was resuspended in room temperature 60% (vol/vol) Percoll (GE Healthcare)/RPMI and overlaid with 30% (vol/vol) Percoll/PBS and centrifuged with no acceleration or brakes for 20 minutes at 1000*g*. Mononuclear cells were harvested from the gradient interface and washed twice in PBS before preparation for flow cytometric analysis.

Spleens were harvested following transcardial perfusion and placed in 2% heat inactivated fetal bovine serum in HBSS (Sigma). Single cell suspensions of splenocytes were made by smashing the spleens through a 70 µm cell strainer with a syringe plunger. Cells were centrifuged at 800*g* for 5 minutes and resuspended in 10 mL PBS.

3.4.6: Serum ELISA for *T. gondii*

Blood was collected by mandibular vein bleed prior to animal sacrifice. Samples were incubated for 4 hours at room temperature to allow clot to form, then incubated at 4°C overnight. ELISA 96 well plates were coated with 1 µg/ml Soluble Tachyzoite Antigen (STAg). Plates were washed 3 times with PBS – 0.05% TWEEN and blocked with 5% milk PBS-0.05% TWEEN, then washed 3 times more with PBS-0.05% TWEEN, and 54 µl of 5% milk PBS-0.05% TWEEN was applied to each well. Five serial dilutions, 6µl each, of serum from each sample were incubated overnight at 4°C. Plates were washed three times with PBS-0.05% TWEEN, and once with PBS. Plates were then incubated with rabbit anti-mouse IgG (H+L)-HRP (Jackson Immuno Research) 1:1000 dilution for 2 hours at room temperature. H_2O_2 was added to ABTS substrate (Sigma) at 1:1000, mixed and applied to the plate. Enzymatic reaction times were recorded at 405 nm.

3.4.7: Flow cytometry

Antibodies to mouse CD3e (145-2C11), CD4 (GK1.5), CD11b (M1/70), CD45 (30-F11), MHCII (M5/114.15.2), and CD117 (ACK2) were obtained from eBioscience. Antibodies to mouse CD4 (RM4-5), CD45 (30-F11), and CD206 (C068C2) were obtained from BioLegend. Cell viability was assayed using Ghost Violet 510 viability dye (TONBO biosciences). Surface staining with anti-mouse CD3e, CD4, CD11b, CD45, MHCII, CD117, and CD206 was performed at 4°C for 30 minutes. Acquisitions were performed

using either a BD LSR II flow cytometer or a BD Bioscience LSR Fortessa and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

3.4.8: Adult T cell transfer

Mature lymphocyte and splenocyte donor cells were harvested from wildtype (WT) mice. Adults 6-12 weeks of age were sacrificed with isofluorane overdose and cervically dislocated. The spleen and all lymphnodes were collected and single cell suspensions were made by smashing the lymphoid organs through a 70 µm cell strainer with a syringe plunger. Cells were centrifuged at 800*g* for 5 minutes. Lymphocytes were resuspended in 5 mL RPMI complete medium and kept on ice. Splenocytes were resuspended in ACK lysis buffer and incubated for 2.5 minutes for red blood cell lysis. The lysis was terminated by diluting the cells in 10 mL RPMI and centrifuged at 800*g* for 5 minutes and resuspended in 5 mL RPMI and combined with the lymphocytes. T cell percentages and numbers were determined by surface staining and flow cytometric analysis. Cells were then centrifuged at 800*g* for 5 minutes and resuspended at a concentration of $5x10^7$ T cells/mL. Each recipient mouse received a tail vein injection of 200 µL using a 27G needle and 1mL syringe.

3.4.9: Neonatal chimera generation

Donor bone marrow was isolated from either WT or IL4 deficient adults aged 6-12 weeks old. Donor animals were sacrificed with isofluorane overdose and cervically dislocated. The hind limbs were harvested and the bone marrow flushed from the bone into a sterile tissue culture dish using cold sterile PBS. Bone marrow cells were passed repeatedly through an 18G needle and then filtered through a 70 µm cell strainer. Cell numbers were assessed using a hemocytometer and cells were diluted to a concentration of $2x10^8$ cells/mL or higher. Neonates (postnatal day 2-8) were injected in their liver with 100 µL of bone marrow cells using a 8mm 31G .3cc insulin syringe (BD).

3.4.10: Statistical Analysis

Prism software (GraphPad) was used for statistical analysis. P values were calculated using two-tailed Student's (non-parametric) t-test or 1 way ANOVA as indicated.

Chapter 4: Closing Remarks

Understanding how *Toxoplasma gondii* infection can result in the behavior manipulation of rodent secondary hosts is both a fascinating study of one of nature's greatest curiosities as well as a useful exploration of animal neuroimmunology. Far too little is known about how the immune system interacts with and impacts the brain and influences animal behavior. Here we have an organism that has co-evolved with rodents and seems to have 'inside knowledge' about how innate aversion to predators can be interrupted. *T. gondii* represents a powerful tool with which to explore the interactions between the brain, the immune system, and behavior.

Here we have carefully explored the previously reported observation that *T. gondii* infection leads to decreased aversion to cat odors. We optimized a mouse behavioral assay to directly assess innate aversion and performed a variety of experiments to characterize the phenotype in mice. We showed that both male and female mice exhibit loss of innate aversion following infection. We then for the first time compared three well-characterized lab strains of the parasite, Type I, Type II, and Type III, and observed that all three cause similar loss of aversion. The Type I parasite strain that we employed was a highly attenuated strain that caused an apparent transient brain infection, yet a permanent behavior change. This finding has implications for both the underlying mechanism of *T. gondii*-mediated behavior manipulation and for infectious disease in general. For the behavior manipulation, it indicates that the parasite is likely causing a permanent change in the neurobiology of the animals early during infection and does not require persistence in the form of cysts in order to continue the manipulation, narrowing the timing of when is best to search for the causative parasite activity that results in this change. For infectious disease, the finding that a transient infection can result in a permanent change in biology in an animal, even after the infectious agent is cleared indicates that the current microbe-centric disease model may be flawed in some circumstances. Modern medicine generally assumes that by clearing an infectious agent from a host with antibiotics or antivirals will result in the amelioration of the symptoms of disease. Here is an example where a profound biological imprint with little evidence of physical damage to the host remains even after the parasite has been cleared, warranting a reconsideration of our approach to disease.

With an interest in explaining the mechanism of *T. gondii* behavior manipulation, we began by investigating the role of the host immune system. Our studies revealed that a certain cytokine, interleukin 4 (IL4), appears to be required for the behavior change to occur. This cytokine has previously been linked to learning and memory in healthy uninfected animals and many cell types within the central nervous system express IL4 receptors. Very little is known about how IL4 may influence neurobiology and behavior, especially in the context of a brain infection. We have begun to characterize what types of cells produce IL4 near the brain prior to and following infection and observe a number of possible sources. Initial investigations aiming to tease apart the essential sources of IL4 in this infection model have resulted in complicated results. It seems that T cells, a major source of IL4, do not reconstitute the behavioral change entirely but are contributing, at least developmentally. Further studies will need to be performed to

identify the cell types responsible for the critical IL4 production and the resulting molecular or immune changes that then lead to changes in the neurobiology and thus behavior of infected mice.

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