STAT1 signaling as a regulator of microglial activation and resistance to CNS infection

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by

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Abstract

During central nervous system (CNS) infection, the immune system must balance between combating an invading pathogen and preventing excessive inflammation that destroys neural tissue. This balance of control is seen in the case of CNS infection by the ubiquitous neurotropic parasite, *Toxoplasma gondii*. While relatively benign in immunocompetent individuals, CNS infection with *T. gondii* must be tightly regulated by the immune system to prevent fatal toxoplasmic encephalitis. In comparison to the extensive characterization of microglia, the resident immune cells of the brain, in neurodevelopmental and neurodegenerative contexts over the past decade, the topic of microglial functional roles during infection models has been surprisingly limited. Several studies have demonstrated brain-resident microglia are functionally distinct from blood-derived myeloid cells that infiltrate the brain and resemble microglia during neuroinflammation. These findings have prompted the need to investigate these distinct myeloid populations independently during infection.

In order to study microglial immune function, we used a genetic targeting approach to generate mice with disrupted IFN-γ-STAT1 signaling specifically in brain-resident microglia. Genetic deletion of *Stat1*, a transcription factor that is critical for generating cellular responses against intracellular infections, induced a loss of parasite control specific to the brain and uniform lethality in mice challenged with *T. gondii*. We found that STAT1-deficient mice displayed efficient parasite clearance and immune responses in multiple tissues outside of the brain, and observed that brain-specific pathology occurred despite increased anti-parasitic immune activation in both blood-derived myeloid and T cells within the brain. These results indicated that STAT1-deficiency within the brain-resident microglial compartment was sufficient to induce severe toxoplasmic encephalitis (TE) for which the peripheral immune compartment was unable to

compensate – thus underscoring the importance of microglial STAT1-mediated activation in anti-microbial defense.

Contrary to previous *in vitro* reports, we also found that microglia do not express inducible nitric-oxide synthase (iNOS) during *T. gondii* infection *in vivo*. Instead, transcriptomic analyses of microglia reveal that STAT1 regulates both (i) a transcriptional shift from homeostatic to "disease-associated microglia" (DAM) phenotype conserved across several neuroinflammatory models, including *T. gondii* infection, and (ii) the expression of anti-parasitic cytosolic molecules that are required for eliminating *T. gondii* in a cell-intrinsic manner. Finally, we show that microglial STAT1-deficiency results in the overrepresentation of the highly replicative, lytic tachyzoite form of *T. gondii*, relative to its quiescent, semi-dormant bradyzoite form typical of chronic CNS infection. Our data suggest an overall protective role of CNS-resident microglia against *T. gondii* infection, illuminating (i) general mechanisms of CNS-specific immunity to infection (ii) and a clear role for IFN-STAT1 signaling in regulating a microglial activation phenotype observed across diverse neuroinflammatory disease states.

Acknowledgements

The last five years have been challenging and at times overwhelming, but have culminated in a dissertation that reflects everything that I set out to gain when I began graduate school. I have grown immensely from a crystalized knowledge standpoint, but more importantly I have used this foundation of knowledge to learn and practice the art of critical thinking to produce my own novel scientific discoveries. Thanks to the steadfast support of friends and family, colleagues, and the greater research community at UVA, I have grown tremendously as an individual.

I want to start by thanking Dr. Akin Abayomi for serving as my earliest scientific role model. Uncle Akin has, without a doubt, been my most obvious inspiration for becoming a scientist and for venturing into the field of infectious disease. Whether he is saving lives as a hematologist, leading a multi-national emerging infectious disease consortium, speaking at the United Nations about biosecurity and biosafety, or serving as Lagos State Commissioner for Health during a global pandemic, he is a constant inspiration for what one can accomplish in this field through innovative thinking, dedication, and perseverance. Thank you, Uncle Akin, for taking time over the years to support and mentor me, nurture my fondness for scientific writing, and for actively guide my professional trajectory.

My inspiration for pursuing neuroscience was undoubtedly Dr. Cedric Williams, my undergraduate research mentor. Professor Williams was the first black neuroscientist I had ever met (and at the time the only black neuroscientist at UVA). Taking his course on learning and memory was a transformative experience for me. For reasons that are likely to uniquely resonate with black Americans in STEM fields, I found myself studying a little bit harder, thinking more deeply about course material, asking more insightful questions, and sitting up a little bit straighter in his class. I asked Professor Williams if I could join his lab as I handed him my final exam, which

was the best decision my awkward teenage self had ever made. For the second half of college, he took me under his wing. His laboratory became a home to me, wherein I learned the ins and outs of critical thinking and experimental design, made beautiful life-long friendships, and in many ways, grew into an adult. Importantly, he instilled in me the confidence that I could pursue advanced academic study in neuroscience – something for which I will be forever grateful.

I am also especially lucky to have had a boss and mentor after college who went out of his way to help prepare me for graduate school. Dr. David Hill provided me with the opportunity to experience what full-time research looked like. Dave and Dr. Chengsan Sun equipped me with a range of wet-lab and critical thinking skills that I would need down the line, and Dave took the time to meet with me to look and discuss different graduate programs. Dave's advice to just "follow the science," and a serendipitous project in his lab also sparked my interest in microglia, and he connected me to scientists and their work at UVA's Center for Brain Immunology and Glia (BIG). After starting graduate school and eventually joining the BIG Center, Dave continued to be supportive of me – chairing my thesis committee for most of my PhD, helping me expand my professional network, and meeting with me for coffee during what I imagine was an increasingly hectic schedule as he got tapped for increasingly prestigious administrative positions at our university. Dave's straightforward yet creative and elegant way of performing and interpreting science, devoid of the bells and whistles of politics, continues to serve as a gold standard for how I hope to carry on my career.

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I am also grateful to have had the current director of the BIG Center, Dr. Tajie Harris, as my graduate school advisor. I first met Tajie at the panel interview during the NGP admissions process. When asked by another faculty member on the panel who at UVA I might be interested in working with throughout grad school, I pointed to Tajie, saying: "her." This gesture wasn't an attempt to be bold or stand out during the interview process – it was an honest answer to a simple question in light of my desire to marry my interest in neuroscience with that of infectious disease research. While my initial eagerness to work with Tajie stemmed from me just "following the science," I've come to grow a deep appreciation for her mentorship and for her as a person. Just as it has been transformative for me to have black role models in science, having a female thesis advisor – one in a STEM leadership position who is capable of commanding a room with her soft-spoken yet confident and remarkably intelligent delivery – has been an absolute joy. To Tajie – the elegance with which you have trained me to think critically about the world around me is a gift that I will forever cherish. Without a doubt, your mentorship has opened opportunities for me to pursue a fuller rest of my life. I am proud to be a product of your lab.

The research environment that Tajie has cultivated over the years has been one that encourages close friendships and collaborative work amongst graduate students. In the Harris lab

I had mentorship and support from senior graduate students – Drs. Jeremy Thompson, Carleigh O'Brien, Katherine Still, and Samantha Batista. Jeremy was my graduate student mentor during my rotation in the Harris lab and I have him to thank for initially showing me the ropes. Carleigh taught me flow cytometry and took time out of her weekend to help me with my first big experiment – a day I remember fondly. Kate and Sam are two women who actively shaped my science and also became good friends. Kate has a wisdom and wholesome personality that made me, like many others, drawn toward her. Sam is a gentle, soft-spoken intellectual with whom I found it so easy to connect once she opened up. Michael Kovacs, who may best be described as my lab twin, joined the Harris lab around the same time that I did, and he has navigated both pre- and post-pandemic laboratory life beside me. A "true Gemini," Mike is studious, professional, and possesses serious grit – but also sometimes radiates a hilariously chaotic energy that easily rivals that of my own.

These graduate students helped me so frequently with experiments that at one point, I remember telling Tajie at one point that I felt almost undeserving of their support. Her response was to "pay it forward," which I tried my best to do as I gained the opportunity to mentor rotating graduate students and helped those who joined our lab. Both Kate and Sam graduated with their PhDs during a time in the COVID-19 pandemic that was severely limited in both social gatherings and basic collaborative research tasks. The additions of Isaac Babcock, Lydia Sibley, and eventually Sydney Labuzan on the other side of these restrictions did wonders to restore a dynamic work environment. Being in a mentorship role, whether it's training a rotation student or working with my trusty undergraduate mentee Ish Sethi, who I have depended on over the years, has been a formative element of my graduate training. The Harris lab has taught me that "following the

science," can be both incredibly fun and fulfilling when you have a group of great people by your side.

During my first year of graduate school, Dr. Francis Collins –director of the NIH – gave a talk at UVA. One thing I remember him saying was something along the lines of all students in biomedical research needing to learn computer programming skills to enhance their work. I distinctly remember thinking, "hell no," at his apparent attempt to make getting my PhD more complicated than was necessary. Fast forward five years, and I can say that statistical programming has been integral to analyzing my data, and I am now planning a career transition to the field of data science. I have two key people to thank for this – Dr. Marieke Jones and Will Rosenow. Marieke, another example of female excellence in STEM, has been incredibly patient in introducing me to statistical programming tools I needed for my work, instructing coding workshops I have benefited from, suggesting online courses to help me learn R, and has inspired me to transition to the field of data science post-graduation. While he was at UVA Research Computing, Will Rosenow regularly met with me to teach me computational pipelines for analyzing my RNA sequencing data, which absolutely elevated the quality of my work. I feel lucky to have had the opportunity to work with such kind and supportive data scientists who understood my initial intimidation and guided me into realizing that this field was fully accessible to me – as long as I was willing to take the leap.

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I've had an enriching and fulfilling PhD experience, which has had much to do with the incredible people who make up my community. To my family, mentors, professors, instructors, and friends of Charlottesville – thank you!

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List of Abbreviations

AD	Alzheimer's Disease
ALS	Amyotrophic lateral sclerosis
APC	Antigen presenting cell
ATP	Adenosine 5′-triphosphate
BBB	Blood-brain barrier
C3	Complement component 3
CD	Cluster of differentiation
CNS	Central nervous system
CR3	Complement receptor 3
CSF1R	Colony stimulating factor 1 receptor
DAM	Disease-associated microglia
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DIM	Disease Inflammatory Macrophage
DPI	Days post-infection
EAE	Experimental autoimmune encephalomyelitis
FACS	Fluorescence-activated cell sorting
GBP	Guanylate binding protein
GWAS	Genome-wide association studies
H&E	Hematoxylin and eosin
IHC	Immunohistochemistry

IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRG	Immunity-related GTPase
JEV	Japanese encephalitis virus
LCMV	Lymphocytic Choriomeningitis Virus
MHC	Major histocompatibility complex
MHV	Mouse hepatitis virus
MS	Multiple sclerosis
NO	Nitric oxide
STAT1	Signal transducer and activator of transcription 1
T. gondii	*Toxoplasma gondii*
TAM	Tamoxifen
TBI	Traumatic brain injury
TCR	T cell receptor
TREM2	Triggering receptor expressed on myeloid cells 2
TMEV	Theiler's murine encephalomyelitis virus
TNF	Tumor necrosis factor
WNV	West Nile virus
qPCR	Quantitative polymerase chain reaction
WT	Wild-type
YAM	Youth-associated microglia

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Chapter 1 – Introduction

1.1 The immunoparasitology of *Toxoplasma gondii*

Toxoplasma gondii is a ubiquitous, obligate intracellular protozoan parasite first identified over a century ago and has since been found to be capable of infecting any nucleated cell in virtually all homeothermic animals - terrestrial, arboreal, avian, and aquatic[1,2]. The parasite is the causative agent of the infectious disease known as toxoplasmosis, and an estimated one-third of the global human population is infected by *T. gondii*[3,4]. An apicomplexan, *T. gondii* is phylogenetically related to *Plasmodium*, the protozoan family that causes malaria, and *Cryptosporidium*, the pathogenic agent for cryptosporidiosis[5]. In immune-competent hosts, *T. gondii* establishes an acute infection that is typically mild or asymptomatic and effectively cleared by the immune system following dissemination to peripheral tissues[6]. However, following infection, the parasite establishes a chronic infection in immune-privileged sites such as skeletal muscle and brain for the lifetime of a host, during which constant local immune pressure is required to prevent fatal reactivation-induced destruction of the CNS. Why *T. gondii* is able to persist chronically in the brain despite being cleared from peripheral tissues is unclear, but this gap in understanding highlights the importance of studying mechanisms of immunity in immune-privileged sites.

1.1.1 Parasite stages and life cycle

The first form of *T. gondii* to be identified by Nicolle and Manceaux (1908) was its fast-replicating, lytic tachyzoite form, which replicates asexually through the internal budding process

of endodyogeny, whereby two daughter cells emerge from a parent cell, destroying it in the process[1,7]. Tachyzoites are the predominant form of the parasite during the initial acute phase of infection, and they are capable of undergoing a stage conversion to a bradyzoite, a slowly-replicating and metabolically quiescent form of the parasite contained intracellularly within tissue cysts of infected animals[8–10]. Within the host, these cysts can spontaneously reactivate into tachyzoites, lyse from host cells, and infect neighboring cells. This process thereby spreads parasite throughout infected tissue – a phenomenon clinically observed in congenital toxoplasmosis and in patients living with AIDS[11–13]. Latent infection of bradyzoite-containing tissue cysts in brain and skeletal muscle can lead to transmission of the parasite to intermediate hosts via carnivory of infected prey[14]. In addition to the parasite's proclivity for spreading from one intermediate host animal to another through its bradyzoite form, *T. gondii* is specialized for infecting one known clade of definitive hosts, *Felidae,* the "true cats"[15,16]. Within enterocytes of the feline gut, *T. gondii* completes its life cycle by sexual recombination, and a single feline host is capable of shedding millions of highly infectious, yet highly stable oocysts throughout its environment via its feces during a defined prepatent infectious period[17–21]. Both an incredibly wide host range and ability to spread via oocyst dispersion and through the food chain help explain *T. gondii*'s ubiquity across the globe and underscore its success as a parasitic organism.

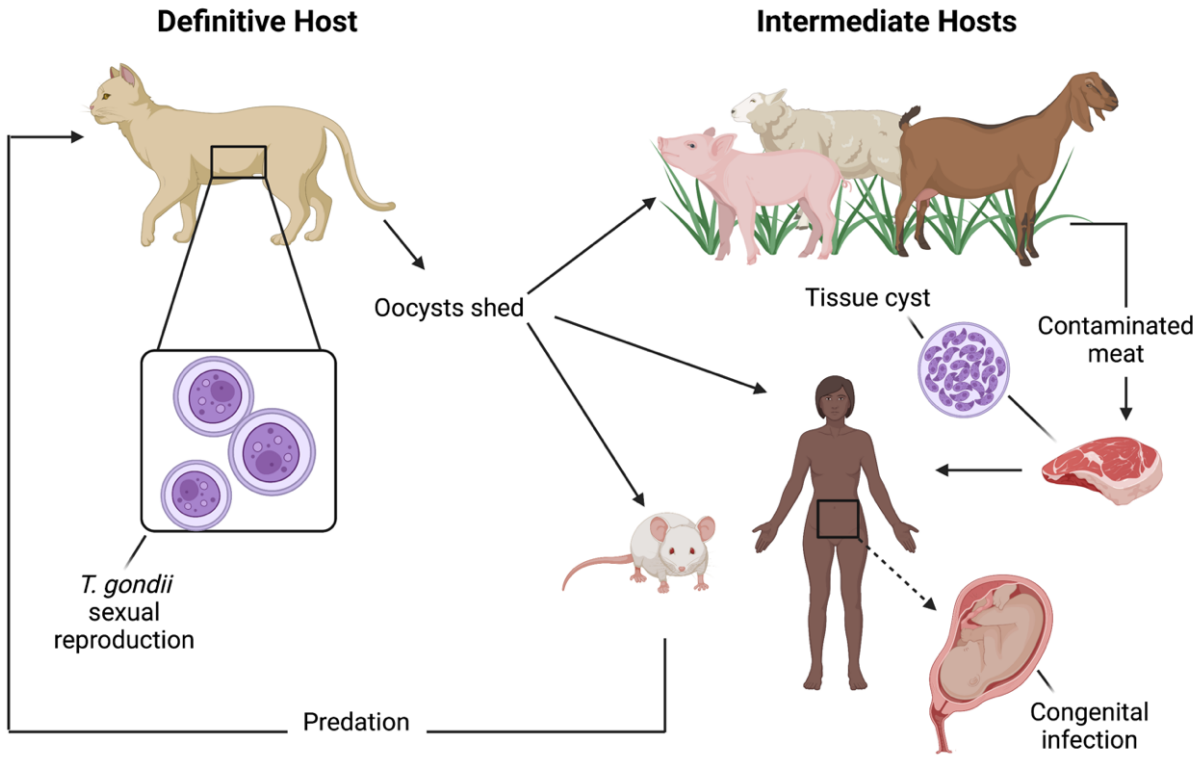

Figure 1.1. *T. gondii* life cycle and transmission.

T. gondii is a protozoan parasite whose definitive hosts are members of the family *Felidae*, and whose intermediate hosts include a wide range of warm-blooded animals, including humans and mice. Sexual recombination takes place within the feline digestive tract, from where the diploid form of the parasite, the oocyst, can be shed into the environment through cat feces. Intermediate hosts such as humans can be infected by ingesting oocysts in contaminated water or more commonly, by ingesting contaminated meat that harbors bradyzoite-stage tissue cysts. Congenital infection also occurs in cases of acute infection that occur during pregnancy. Predation of intermediate hosts such as mice by cats allows for life cycle completion.

1.1.2 Clinical and behavioral manifestations of toxoplasmosis

In the immunocompetent host, *T. gondii* infection is mild to asymptomatic, lays dormant in the brain, and does not lead to serious disease, explaining why an estimated third of the human population harbors the parasite chronically, typically without any major concerns[3,6,22,23]. This ubiquitous pathogen, however, is opportunistic and became a significant public health threat during the 1980s at the height of the AIDS epidemic, during which up to half of HIV-positive individuals who were chronically infected *T. gondii* suffered from severe and oftentimes fatal toxoplasmic encephalitis due to inadequate CD4+ T cell counts necessary to prevent parasite spread throughout brain tissue[24]. In response to this global public health crisis, a wealth of research characterizing both the innate and adaptive immune mechanisms that confer resistance to both acute and chronic *T. gondii* infection emerged. These immune mechanisms are largely centered on the cellular and molecular induction and response to the critical cytokine IFN-γ, best characterized in governing Th1 immune responses to intracellular pathogens[25,26]. During *T. gondii* infection, T cell-derived IFN-γ is required for priming an effector myeloid cell response that is involved in applying constant local immune pressure to control fatal parasitemia and toxoplasmic encephalitis[6,23,27].

In the absence of this local immune pressure, patients diagnosed with toxoplasmic encephalitis typically present in the clinic with headache, confusion, muscle weakness, and fever – although symptoms ranging from personality changes to seizures can also occur[13,24]. Another clinical manifestation of *T. gondii* infection occurs in the case of congenital toxoplasmosis, wherein pregnant women who become infected with the parasite are able to transmit the infection on to their fetuses[28]. Congenital infection may also result in severe neurological and developmental symptoms – fetuses that survive the infection may suffer from microencephaly, blindness, and

seizures[11]. The standard treatment for toxoplasmic encephalitis and congenital infection includes pyrimethamine and sulfadiazine, which have been shown to inhibit parasite replication[29–31].

CNS infection with *T. gondii* is also associated with well-characterized behavioral effects in both humans and mice[32,33]. In humans, *T. gondii* seropositivity is linked with an increased risk of vehicular accidents[34], suicide attempts[35,36], and increased behavioral aggression and impulsivity[37,38]. *T. gondii* seropositivity has also been identified as a psychiatric risk factor for the development of schizophrenia[32,39]. Rodents infected with *T. gondii* have been found to lose their instinctive aversion to cat urine, instead developing a mild attraction to feline pheromones[40–43]. The "behavioral manipulation hypothesis" posits that *T. gondii* has adapted to manipulate rodents in a way that leads to their predation by the parasite's feline definitive host, permitting completion of its sexual cycle[33]. Some work has suggested that *T. gondii*-driven behavioral manipulation, is highly specific to an attraction to cat urine, and batteries of behavioral tests have revealed no differences in amygdala-dependent fear conditioning, hippocampal spatial memory, neophobia of food and novel scents[41]. More recent work, however, has suggested that this behavioral modification displays a pan-predator effect, which extends to a rodent attraction to fox odors[43].

T. gondii-infected rodents exhibit hyperactive and anxiolytic behavior, which may be related to inflammatory responses mounted in the brain against the infection[44]. Guanabenz, an alpha-adrenergic medication, has been shown to reduce pro-inflammatory cytokine expression in the brain in tandem with a behavioral rescue from hyperlocomotion in mice[44]. Some work also suggests that behavioral changes during infection being linked to disrupted neurotransmission throughout the brain[45], as chronically-infected mice display increased excitatory glutamatergic neuronal activity[46] as well as widespread changes to machinery required for GABA synthesis[47]. Much remains unknown about how *T. gondii* influences these changes to host behavior, but

together, these studies highlight complex ways in which the brain, immune system, and parasite itself interact during CNS infection.

1.1.3 Parasite invasion and dissemination

T. gondii is able to infect virtually any nucleated cell, as its invasion process does not depend on specific cell-surface receptors that drive its entry[22]. Instead, this process requires *T. gondii* surface antigen (SAG) proteins, which drive an initial lateral interaction with a host cell[48,49], microneme organelles that form an apical attachment[50], and rhoptry proteins that are discharged during invasion allowing the parasite to create an invagination of the host cell plasma membrane which comprises the parasitophorous vacuole (PV) in which the parasite resides and maintains with additional components such as dense granule proteins[51–53]. The fact that the PV is derived from host cell plasma membrane is thought to serve as one of many approaches for evading detection by the immune system[54].

There are three major hypotheses for how *T. gondii* crosses the BBB to disseminate into the brain to begin a chronic infectious stage: (1) transcellular migration, (2) paracellular migration and (3) the "Trojan Horse" hypothesis[55]. *T. gondii*, as a lytic parasite capable of infecting any nucleated cell, may enter into the brain by a series of replication and egression cycles through cells leading into the brain parenchyma, including via the observed infection and lysis of endothelial cells comprising the brain vasculature[56]. The paracellular migration hypothesis posits that *T. gondii* is able to cross the BBB using its gliding motility mechanism, similarly to its observed ability to cross the gut epithelium *ex vivo*[57]. Alternatively, the Trojan Horse hypothesis proposes that infected leukocytes from the periphery extravasate and introduce parasite to the brain. Evidence to support a role for this third hypothesis comes from *in vitro* studies in which infected DCs acquire

hypermotility and the ability to cross endothelial monolayers in the absence of chemotactic cues[58,59]. Additionally, adoptive transfer of infected DCs leads to more rapid dissemination of parasite relative to free tachyzoites transferred at the same multiplicity of infection *in vivo*, suggesting that the parasite may be able to hijack immune cells for transport throughout the host[58]. It is likely that a combination of these three models synergize to facilitate entry of *T. gondii* into the brain.

1.1.4 Tachyzoite-to-bradyzoite stage conversion and reactivation

While tachyzoites are the predominant form of *T. gondii* during the acute phase of infection, the parasite undergoes a stage conversion to its bradyzoite form after seeding the CNS[9]. This stage conversion is characterized by the development of a cyst wall rich in lectin-binding glycoproteins through structural changes within the parasitophorous vacuole[60,61], and transcriptional changes that suppress genes driving parasite replication and metabolism[62]. IFN-γ-mediated protection is critical in tachyzoite parasite clearance throughout most host tissues, but parasite in "immune-privileged" sites such as the brain, retina, and skeletal muscle can be found to convert to its cystic, bradyzoite form and persist for the lifetime of the host[63].

The determinants of the tachyzoite-to-bradyzoite stage conversion are poorly understood and have been largely examined in *in vitro* studies[9]. The tachyzoite-to-bradyzoite stage conversion is preceded by a slowed but not abrogated cell cycle, as evidenced by pharmacological cell cycle blockade failing to result in bradyzoite differentiation[64,65]. Experimental conditions that favor this stage conversion in culture include alkaline pH[66], heat-shock treatment[66], nutrient withdrawal[67], and treatment of host cells with IFN-γ[68] or sodium nitroprusside (a nitric oxide donor)[8]. These studies suggest that both environmental stress and host immunity are able to induce expression of

genes that tachyzoite conversion to semi-dormant bradyzoites. It is unclear whether *T. gondii* is able to directly sense stressors and adaptively undergo its stage conversion to optimize survival, or whether the stimuli that generate these stressors themselves govern this transition.

Previous studies have indicated that during chronic infection, most parasite cysts in the brain are observed in neurons within the cortex[69], although there are some mixed reports of the regional distribution of cysts within the brain[41,70]. Why neurons are the predominant cellular host for parasite cysts also remains unclear. It was initially thought that neurons were susceptible to latent *T. gondii* infection due to an inability to mount cell-autonomous protective IFN-γ-mediated immune responses to clear intracellular parasite. However, recent work using a fate-mapping approach for infected cells suggest that neurons are able to clear infection *in vivo*, with additional *in vitro* work suggesting that this process is mediated by the IFN-γ-inducible genes *Irga6*, *Irgm1*, and *Irgm3*[71]. Another study also indicated that presentation of *T. gondii* antigen to CD8+ T cells via neuronal MHC I is required for control of brain parasite burden, suggesting that infected neurons are able to trigger their own cytotoxic killing via T cells to restrict parasite growth[72]. Whether these parasite restriction programs are merely less efficient in post-mitotic, non-regenerative neurons relative to most other cell types, or neurons instead possess other qualities that render them the ideal cellular reservoir for the tachyzoite to bradyzoite transition is yet to be determined.

While bradyzoite-containing cysts are typically regarded as quiescent and not sensed by the immune system, they have been noted to undergo a process termed "reactivation." Reactivation refers to the process by which a cyst spontaneously ruptures from an infected cell, and bradyzoites convert back to their highly-replicative tachyzoite form and go on to infect surrounding tissue[73]. Reactivation can be readily observed in immunocompromised patients[13,73,74], and can be induced

by the withdrawal or blockade of local immune signals known to trigger the tachyzoite-to-bradyzoite stage conversion, such as IFN-γ[26,75]. Reactivation can be regarded as a highly inflammatory event, as it liberates intracellular host cell contents such as DAMPs and is typically associated with focal recruitment of blood-derived immune cells and development of reactive microglial nodules within the brain[76,77].

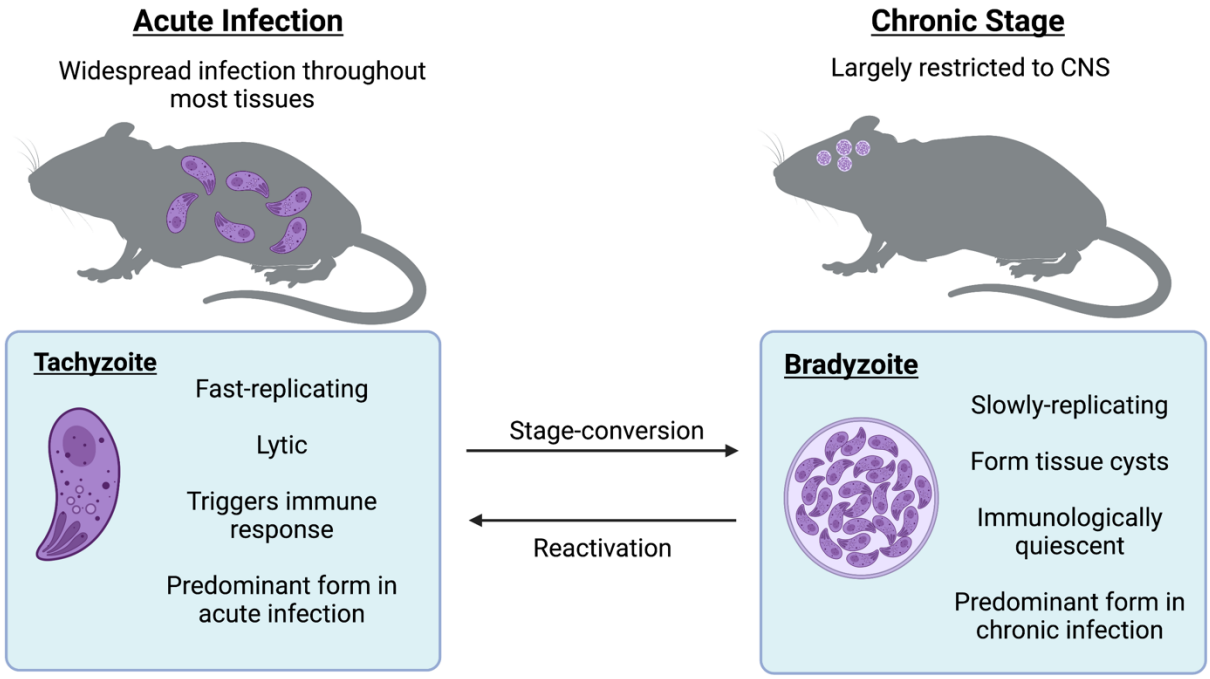

Figure 1.2. Stages of *T. gondii* infection in the intermediate host.

T. gondii infection in the intermediate host is characterized by two stages. The acute stage is the initial phase of infection, wherein the parasite rapidly disseminates throughout most host tissues while in its fast-replicating and lytic tachyzoite form. IFN-γ-mediated immunity drives clearance of the acute infection from peripheral tissues and potentially the tachyzoite-to-bradyzoite stage conversion observed within immune privileged sites such as the brain. Bradyzoite-containing tissue cysts are typically observed within neurons during chronic CNS infection. Parasite reactivation is a process that can be readily observed in instances of immunosuppression, whereby cysts stochastically reactivate and liberate intracellular parasite, which goes on to infect neighboring cells.

1.1.5 Parasite sensing by the innate immune system

Both dendritic cells (DCs) and monocytes / macrophages play critical roles in the initial innate sensing of *T. gondii*. Murine DCs express the endosomal TLRs 11 and 12, which have been shown to recognize the parasite's profilin, a protein that guides actin polymerization and is thus an essential molecule for the parasite's invasion into a host cell[78,79]. While TLR 11/12 signaling serves as a central sensing mechanism for *T. gondii* in mice, TLR 11 is a pseudogene in humans, and TLR12 is not encoded in the human genome, therefore making it unclear as to how *T. gondii* is sensed in humans. Deficiencies in a single TLR lead to increased parasite burden in infected mice, and eliminating signaling of multiple TLRs via deletion of the convergent downstream adaptor signaling protein, MyD88, results in animal mortality during the acute phase, by 9 days post-infection (DPI)[80]. Similarly, triple-deficient (3d) mice that harbor a loss-of-function mutation to UNC93B1, a protein that plays a role in trafficking TLRs 3, 7, 9 within the cytosol in addition to TLR 11 signaling, are highly susceptible to *T. gondii* infection, and they succumb 10 DPI[81]. TLRs 7 and 9 may further play a role in sensing *T. gondii*-derived nucleic acids, or alternatively in sensing disturbances to gut flora arising from oral challenge with *T. gondii*[82,83]. In addition to TLR signaling, *T. gondii* infection is able to activate both the NLRP1 and NLRP3 inflammasome sensors capable of inducing pyroptosis in mice, a form of pro-inflammatory cell death that is required for limiting parasite replication in infected hosts[84–86].

1.1.6 Initiation of the immune response via Interleukin-12

During *T. gondii* infection, TLR signaling serves as the gateway to the innate immune response. Activation of TLRs 11 & 12 leads to the robust production of the pro-inflammatory cytokine, IL-12, which promotes the development of a Th1-polarized adaptive immune response[87].

While multiple myeloid cell types including DCs, monocytes, and neutrophils are able to produce IL-12 during infection, CD8α+ DCs have been identified as the major producers of this cytokine in response to *T. gondii* challenge[88]. Irf8 or Batf3 KO mice, which fail to develop CD8α+ DCs, are capable of producing WT levels of TNF-α, IL-1, and IL-6 in response to *T. gondii* challenge, but fail to produce IL-12 and succumb during the acute phase of infection[88,89]. This defect is rescued by treating these knockout mice with recombinant IL-12, which restores Th1-polarization and IFN-γ production, thus highlighting the importance of IL-12-mediated immunity during infection[88]. DC-derived IL-12 has been shown to act on both NK cells early during the acute phase[90], and CD4+, and CD8+ T cells throughout the infection, to drive IFN-γ production and protective systems-level immunity[91].

1.1.7 TNF-α signaling in myeloid cells

DCs and other immune cell populations are actively involved in the immune response to *T. gondii*, yet two major types of immune cells serve as the key effectors in controlling infection both in peripheral tissues and in the brain: (1) T cells, and (2) myeloid cells (monocytes / macrophages)[6]. IFN-γ is widely appreciated as one of the most critical cytokine mediators of resistance during both acute and chronic *T. gondii* infection by promoting a Th1-polarized immune response that activates and mobilizes monocytes / macrophages, but other cytokines are also required in both inducing and negatively regulating inflammation. The pro-inflammatory cytokine TNF-α, serves as an important signal that acts synergistically with IFN-γ to activate monocytes and macrophages for intracellular killing of pathogens by inducing activity of the transcription factor NF-κB[92]. TNF-α is thought to provide a "second hit" required for anti-parasitic nitric oxide

production, as TNF-α neutralization via blocking antibody administered during chronic infection results in lower levels of the anti-parasitic protein iNOS, decreased activation of infiltrating myeloid cells, and severe toxoplasmic encephalitis[75,93].

1.1.8 Interferon-γ and STAT1

Interferon-gamma (IFN-γ), also known as type II interferon, is the major pro-inflammatory cytokine that governs the immune response to intracellular pathogens, and its effector functions are required to mount an effective immune response to *T. gondii* infection, during both acute and chronic phases of infection[23,26,94,95]. IFN-γ KO mice uniformly succumb acutely to avirulent *T. gondii* by 10 DPI[96], and blocking IFN-γ with a neutralizing antibody in chronically infected mice leads to unchecked reactivation of parasite in the brain and mortality 7-8 days later[26]. CD4+ and CD8+ T cells are the predominant producers of this critical cytokine during chronic *T. gondii* infection, and depletion of these cellular sources via antibody blockade during chronic infection in mice is similarly sufficient for driving severe toxoplasmic encephalitis and rapid animal mortality[26].

IFN-γ has intrinsic anti-toxoplasmic effects on non-hematopoietic cells, by inducing the degradation of the amino acid, tryptophan in host cells, which is required *T. gondii* growth, but the parasite is unable to autonomously acquire[97]. This IFN-γ -induced degradation of host cell tryptophan is dependent on the enzyme IDO, and its inhibition leads to increased parasite burden and mortality in mice during chronic *T. gondii* infection[98,99]. Many IFN-γ-driven anti-parasitic effects, however, are orchestrated through the hematopoietic cell compartment, and are centered

on priming myeloid cells such as monocytes and macrophages, for intracellular parasite destruction[6].

The binding of IFN-γ to its surface receptor, leads to the phosphorylation of Janus kinases 1 and 2 (JAK1/2), which in turn phosphorylation of the downstream transcription factor, signal transducer and activator of transcription 1 (STAT1)[100]. While STAT1 is activated by signaling through both type 1 and type 2 interferons, *T. gondii*-infected STAT1-KO mice phenocopy the IFN-γR-KO mice in terms of susceptibility, whereas IFNAR-KO mice do not succumb to infection[96], or show comparatively mild pathology[101]. These findings suggest that type II, but not type I interferon plays primary role in controlling infection. IFN-γ-STAT1 signaling is required during *T. gondii* infection for: (1) the upregulation of antigen presenting molecules to prime an effective T cell response, (2) the recruitment of immune cells to site of infections using STAT1-dependent chemokines, (3) the production of reactive nitrogen species, and (4) the upregulation of anti-parasitic machinery such as immunity-related GTPases (IRGs) and guanylate binding proteins (GBPs) that lead to direct targeting and clearance of the parasite intracellularly from infected hematopoietic cells[27,102]. The remainder of this section will review IFN-γ-mediated effector mechanisms in controlling *T. gondii* infection, with an emphasis on the immune response localized to the brain.

1.1.9 CXCL9 and CXCL10 and T cell recruitment

CXCL9 and CXCL10 are STAT1-dependent T cell chemoattractants that bind to their shared receptor, CXCR3[103]. During chronic *T. gondii* infection, CXCL9 and CXCL10 become robustly expressed in resident microglia, and CXCL10 in astrocytes[104]. These two CXCR3 ligands recruit CD4+ and CD8+ T cells to focal areas of parasite replication in the CNS[105–107]. Constant

CXCL10 production in the brain is required to maintain the T cell population during chronic infection, and its blockade leads to a dramatic reduction of T cell numbers in the brain after a short treatment period, and a resultant increase in parasite burden[106]. One other notable T cell chemoattractant, CCL5, is also produced by myeloid cells during chronic *T. gondii* infection, also in a STAT1-dependent manner[104].

While T cells are the major producers of IFN-γ, which primes macrophage activation, they also serve additional roles in controlling the spread of parasite. Both CD4+ and CD8+ T cells are able to cytolytically destroy tachyzoite-infected cells as well as extracellular parasite in an antigen-restricted manner *in vitro*[108–111]. Perforin-KO mice survive acute *T. gondii* infection, but display increased susceptibility later during the chronic stage (~75 DPI), suggesting that loss of cytotoxic T cell activity may lead to a gradual accumulation of parasite in the brain, but is compensated for in peripheral tissues[112]. While tertiary in importance during *T. gondii* challenge relative to IFN-γ production, T cell-mediated cytolytic killing, may be more important as classically described in CD8+ T cells *in vivo*, as CD8+ T cell depletion also results in more severe toxoplasmic encephalitis than is observed with CD4+ T cell depletion[26].

1.1.10 Production of reactive nitrogen and oxygen species

Nitric oxide (NO) is a nitrogenous free radical produced by myeloid cells (monocytes, macrophages, DCs, and neutrophils) via the enzymatic activity of inducible nitric oxide synthase (iNOS). The iNOS promoter contains an IFN-γ-activated site (GAS), and while cytokines and immunogenic stimuli such as TNF-α, IL-1β, and LPS can lead to NO production, full expression of iNOS *in vitro* requires that STAT1 translocates to the GAS sequence to drive transcription[113,114]. *Nos2*$^{-/-}$ mice acquire fatal toxoplasmic encephalitis during late acute/early chronic infection,

displaying necrotic lesions throughout the brain and a heightened brain parasite burden, indicating that NO production seems to play a critical role in controlling *T. gondii* replication specifically in the brain[115]. In the chronically infected mouse brain, infiltrating monocytes and monocyte-derived macrophages express iNOS, with Ly6C+ inflammatory monocytes representing the largest producers of reactive nitrogen[115,116]. The reports of a microglial subset capable of producing NO in the brain during chronic *T. gondii* infection have relied on crude flow cytometric gating of cells based on CD45 expression[116] rather than newer fate-mapping tools[117,118], and may represent contamination from border-associated macrophages[119,120] or infiltrating monocytes[118,121].

iNOS uses L-arginine and NADPH as substrates in a series of reactions that leads to the production of nitric oxide (NO), which has been shown to (1) block viral replication by nitrosylating viral proteases, and (2) target intracellular bacteria by blocking bacterial ribonucleotide reductase activity and creating DNA double-strand breaks[122–125]. NO production is thought to starve parasite growth by depleting arginine in the host cell, as *T. gondii* is an arginine auxotroph[126,127]. Additionally, supplementing macrophages *in vitro* with exogenous NO via the addition of sodium nitroprusside, a nitric oxide donor, has been shown to limit parasite replication, indicating direct anti-toxoplasmic effects independent of arginine deprivation[128].

Similarly to reactive nitrogen, reactive oxygen species (ROS), such as superoxide, have been shown to possess anti-toxoplasmic properties in isolated human macrophage cultures[129]. Superoxide is produced by the enzyme NADPH oxidase, which is both upregulated and functions optimally upon macrophage activation with LPS, an inducer of NF-κB signaling[130]. Superoxide also serves as a precursor to antimicrobial molecules such as hydrogen peroxide, hydroxyl, and peroxynitrite[131]. Relative to NO, the role of ROS during *T. gondii* is far less explored, but treatment of macrophages *in vitro* with superoxide dysmutase and catalase to scavenge and

neutralize superoxide leads to a loss of toxoplasmicidal capacity and increased parasite replication, suggesting a potential ROS-driven role in controlling infection[129,132]. While phagocytes express NADPH oxidase constitutively and its activity is associated with the "respiratory burst" seen with phagocytosis, IFN-γ has been shown to increase the affinity of NADPH oxidase for its substrates, leading to increased superoxide production in macrophages *in vitro*[133,134]. Work investigating ROS generation have traditionally relied on *ex vivo* detection assays, but one recent study has shown that genetic deletion of NADPH oxidases 1 and 2 (encoded by the genes *Nox1* and *Nox2*) results in moderately increased *in vivo* susceptibility observed during the acute phase of infection[135]. Additional work on the specific cellular sources, relevant time points of anti-parasitic control, and mechanisms of action of IFN-γ-mediated ROS during *T. gondii* infection requires further exploration.

1.1.11 Immunity-related GTPases and guanylate-binding proteins

The finding that IFN-γR$^{-/-}$ mice succumb early during acute *T. gondii* infection, whereas iNOS$^{-/-}$ mice succumb later to the chronic stage of infection, points to the existence of IFN-γ-dependent, iNOS-independent mechanisms of resistance during the acute phase of infection. Some evidence also suggests that these mechanisms may play major roles in providing immunity during chronic infection in the brain. One such example are the immunity-related GTPases (IRGs), a family of IFN-γ induced proteins that are critical drivers in the clearance of *T. gondii* and other intracellular pathogens such as *Chlamydia*, *Leishmania*, *Salmonella*, *Listeria*, and *Trypanosoma* genera[136,137]. While the human genome only expresses one IRG, there have been seven identified IRGs in mice that are recruited to the parasitophorous vacuole during *T. gondii* infection (Irgm1, Irgm2, Irgm3, Irgb6, Irgb10, IrgD, and Irga6), which cooperatively drive vacuole rupture and

exposure of the parasite to the cytosol, where the parasite can be detected by immune sensors or cleared via autophagy[136,138–142]. Genetic deletions of IRG-family proteins has been so far associated with either increased parasite burden in the brains of mice, or increased animal mortality during *T. gondii* challenge[143,144]. For instance, *Igtp*$^{-/-}$ mice succumb to parasitemia by 10 DPI, very closely phenocopying IFN-γR and STAT1$^{-/-}$ mice, thus highlighting the necessity of IRGs in controlling *T. gondii* infection[143].

A second family of IFN-γ-STAT1-dependent proteins involved in the clearance of *T. gondii* from infected cells is comprised of the guanylate-binding proteins (GBPs). The mouse genome contains 13 GBPs, six of which are expressed in humans and thought to contribute to immunity against *T. gondii*[102,145]. Genetic deletion of the entire cluster of six GBP genes located on chromosome 3 of the mouse genome leads to increased parasite load and susceptibility to avirulent *T. gondii* strains, with animal mortality beginning at 10 DPI during the acute phase of infection, despite displaying intact IFN-γ and IL-12 production [146,147]. Yamamoto's group has discovered that genetic deletion of mouse chromosome 3 GBPs also resulted in defective recruitment of Irgb6 and Irgb10 to the parasitophorous vacuole in isolated murine peritoneal macrophages, indicating that IRGs and GBPs function cooperatively to destroy *T. gondii* intracellularly. Rescue experiments were performed to further show that individual genetic complementation of *Gbp1, Gbp5*, or *Gbp7*, via retroviral expression vectors leads to incremental restoration of IFN-γ-induced toxoplasmicidal activity in macrophages lacking GBPs on mouse chromosome 3[147]. GBP family proteins have been implicated in driving pyroptotic cell death in infected murine macrophages via the inflammasome sensors NLRP1 and NLRP3, *in vitro*[85,86]. Recently, it was also discovered that in human macrophages, the IFN-γ-induced upregulation and multimerization of GBP1, leads to AIM2 and caspase-8-dependent apoptosis[148]. These studies

collectively present the GBPs as regulators of cell death of infected cells, thereby depriving *T. gondii* of replicative niche to disseminate the infection, and in the case of pyroptotic murine cells, driving a form of pro-inflammatory cell death capable of priming the initiation or potentiation of an immune response.

While there are not yet any studies investigating the roles of IRGs or GBPs specifically during chronic *T. gondii* infection in the brain, there is decent evidence suggesting that in addition to nitric oxide production in monocytes, these programs may serve as principal anti-parasitic effectors. Importantly, GBP activity does not appear to be restricted to hematopoietic cell types. GBP protein expression is a hallmark of activated endothelial cells *in vivo*[149], where it could possibly play an important role in regulating parasite entry to the brain, since *T. gondii* has been shown to replicate in and traverse through endothelial cells to establish chronic infection in the CNS[56]. GBP1 has also been shown to restrict *T. gondii* in both lung epithelial cell lines[150] and mesenchymal stromal cells[151] *in vitro*. In the brain, GBP transcripts are amongst the most highly upregulated astrocytic genes in response to IFN-γ stimulation[152]. STAT1-induced IRG and GBP expression may be critical for resistance to cerebral toxoplasmosis, as astrocyte-specific STAT1 KO animals succumb to infection around 30 DPI[152]. In the future, genetic deletions of specific IRGs and GBPs across brain-resident cell types will be able to clarify their relative contributions in the brain.

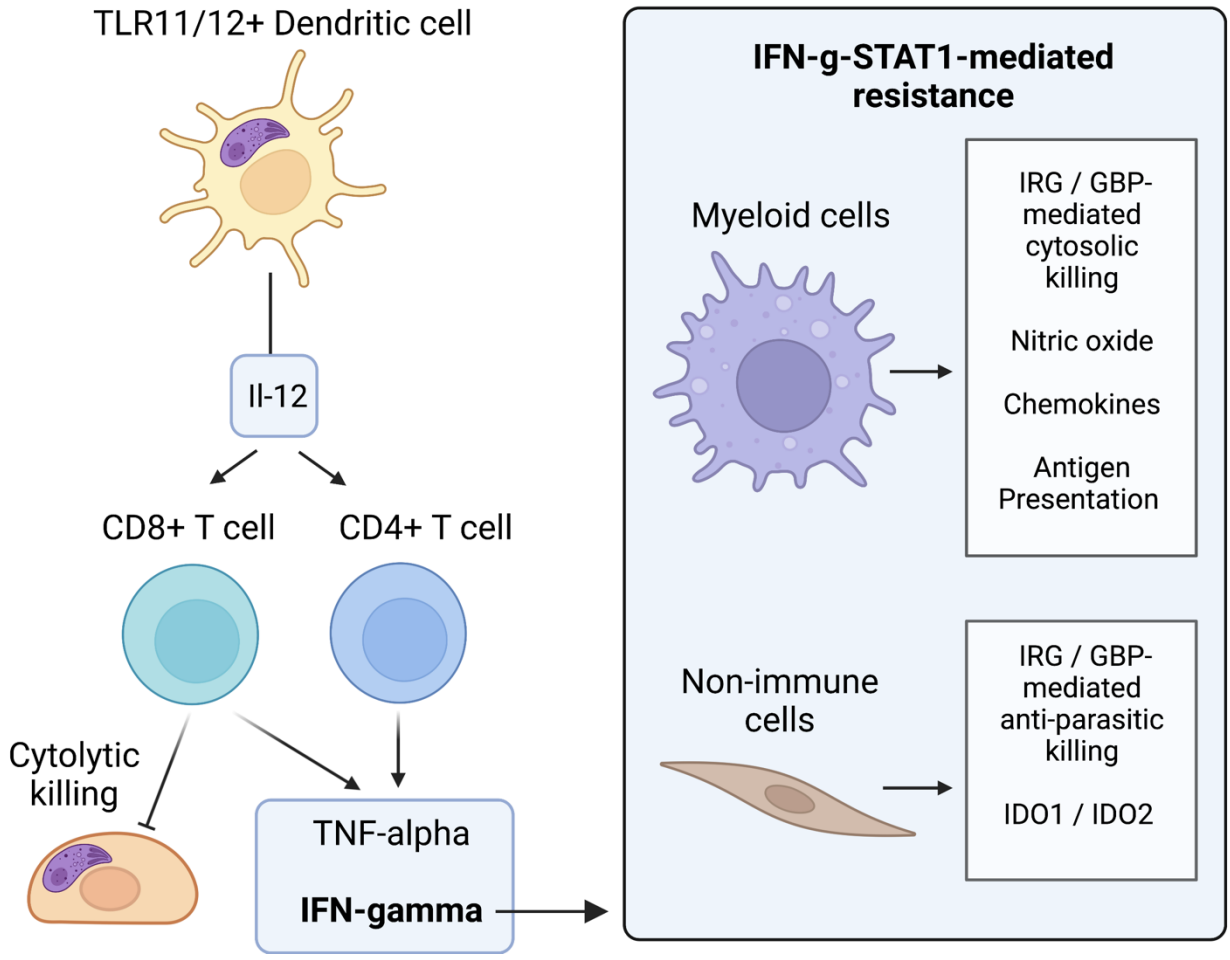

Figure 1.3. Overview of major cellular and cytokine mediators of resistance against *T. gondii* infection.

Parasite sensing occurs through TLR signaling in CD8α+ dendritic cells, which stimulates the production of the cytokine IL-12. IL-12 signaling initiates a Th1-mediated immune response from T cells, which produce pro-inflammatory cytokines such as TNF-α (which signals through NF- κB) and IFN-γ (which signals through STAT1). IFN-γ, in particular, is essential in shaping anti-parasitic immune responses on the cellular and systems-levels, and both hematopoietic (immune) and non-hematopoietic cells are able to employ IFN-γ-STAT1-dependent antimicrobial programs. Myeloid cells are especially potent responders to IFN-γ, and they express pro-inflammatory cytokines, chemokines, nitric oxide, and antigen presentation machinery to restrict parasite both directly and indirectly. Non-hematopoietic cells are also equipped with specialized anti-parasitic programs in response to IFN-γ signaling such as the expression of IDO1/2 and anti-parasitic killing machinery.

1.1.12 Monocytes, astrocytes, and alarmins

Monocytes and monocyte-derived macrophages have well-characterized effector roles during both acute and chronic *T. gondii* infection, many of which are dependent on priming by IFN-γ to actively destroy and contain parasite[6]. Monocytes highly express the chemokine receptor CCR2, which binds the ligand CCL2 that is required for them to leave the bone marrow and enter the blood before entering tissues and differentiating into macrophages[153]. $CCR2^{-/-}$ mice, whose inflammatory monocytes are unable to leave the bone marrow and mobilize to infected tissues, succumb to *T. gondii* infection acutely[154,155]. Similarly, CCR2 antibody blockade during chronic *T. gondii* infection, during which the parasite is largely restricted to the brain, results in both increased parasite burden and fatal toxoplasmic encephalitis due to an inability of anti-parasitic monocytes to seed the site of infection[116].

Monocytes that infiltrate into the brain during *T. gondii* infection are found densely localized to areas suggestive of active parasite replication, typified by focal tachyzoite rather than latent bradyzoite presence[76]. A recently established mechanism by which the peripheral myeloid compartment may recruit cells to sites of replicating parasite has been recently elucidated by recent work using a CCL2 fluorescent reporter mouse, finding that CCL2 is expressed focally by astrocytes in these same areas of active parasite[76]. These studies showed that the induction of CCL2 expression by astrocytes is dependent on IL-33-ST2 signaling, whereby the alarmin IL-33 is expressed by both astrocytes and oligodendrocytes during chronic CNS infection with *T. gondii*. IL-33 is a cytokine normally sequestered within the nucleus of expressing cells, and its release upon aberrant forms of cell death such as necrosis serves as an inflammatory "alarm" to alert the immune system of potential threats[156].

Because *T. gondii* is a lytic parasite and its replication and reactivation results in necrotic cell death, brain-resident alarmins such as IL-33 may serve as the necessary initiating signals for recruiting blood-derived immune cells to participate in a concerted immune response in immune-privileged sites such as the brain[76]. Additional work from our group has shown that the alarmin, IL-1α is expressed by microglia to activate local brain vasculature and drive the infiltration of blood-derived immune cells to restrict brain parasite burden[77]. This topic is explored in further detail in the following section. Other mechanisms that the brain uses to alert and direct the immune system to specific sites of local infection currently remain unexplored, may include or be centered around the versatility of additional damage-associated, rather than strictly pathogen-associated signals.

1.2 Microglia in CNS infections: insights from *Toxoplasma gondii* and other pathogens

Maureen N. Cowan, Ish Sethi, and Tajie Harris

1.2.1 Microglia: The resident immune cells of the CNS

Microglia, the resident immune cells of the brain, have commanded a plethora of research investigations in the rapidly expanding field of neuroimmunology. Microglia are tissue-resident macrophages (TRMs) – long-lived myeloid cells displaying transcriptional phenotypes shaped by their unique tissue microenvironments[157]. Unlike most TRM populations which primarily differentiate from fetal liver monocytes via the process of definitive hematopoiesis, microglia arise solely from embryonic yolk sac macrophages through the distinct developmental pathway of primitive hematopoiesis[117,158,159]. Microglia are dynamic cells with complex morphology and highly motile arborizations. Microglial filopodia-like processes survey the entire brain volume every few hours, a process involving the detection of extracellular purines – notably adenosine-family molecules[160–162]. Research over the past decade has revealed roles for microglia in both developmental and homeostatic processes, and as drivers of neuroinflammation in neurodegenerative models[163]. In the context of infection, however, our understanding of the roles of microglia as immune cells that provide protective immunity has been far less explored.

Previous research on the role of microglia in infection has heavily relied on *in vitro* systems to model CNS infection. The utility of these models, however, has been recently challenged due to the emergence of literature revealing that microglia rapidly de-differentiate when removed from their tissue microenvironment and plated on plastic[164]. This de-differentiation includes not only a

loss of major transcription factors that maintain microglial homeostasis, but also the increased expression of pro-inflammatory markers not observed *in vivo*[165,166]. Hippocampal slice cultures allow for short-term *ex vivo* microglial pharmacological targeting, although this approach induces microglial activation over the course of several hours[167,168]. Over the last decade, however, innovations in microglial genetic targeting systems have advanced considerably for *in vivo* studies[118,120,169–171]. Importantly for CNS infection, these tools are needed to distinguish microglia from brain-infiltrating myeloid cells, which are commonly present during neuroinflammation and have historically confounded microglial-specific population analyses. Here, we will: (1) review current and emerging tools for studying microglia, (2) outline microglial innate and adaptive immune functions characterized across disease states, and (3) highlight emerging research investigating microglial roles in specific infection models, with an emphasis on parasitic CNS infection with *Toxoplasma gondii* and neurotropic viral models. We emphasize two key themes across these different disease states: (1) brain-resident microglia display CNS responses that are often non-redundant with that of brain-infiltrating macrophages, and (2) whether these functions are neuroprotective or detrimental to CNS health, depend on the specific context.

1.2.2 Microglial physiology and experimental approaches

Microglia display a transcriptional signature that distinguish them from other myeloid populations in both humans and mice[172–175]. The brain is unique amongst most organs, in part, due to its limited capacity for regeneration following damage incurred by either invading pathogens or the immune system itself[176]. Microglia express transcriptional repressors, such as Sall1, Sall3, and Mef2a, which are thought to regulate their identity by restricting their immune activation[171,174,177]. The microglial transcriptional signature also includes genes such as *P2ry12, P2ry13, Hexb, and*

TMEM119 (**Fig 1.4**)[164,172–174,178]. Even when blood-derived monocytes are permitted to engraft and differentiate in the brain with the use of artificial pharmacological or irradiation systems that deplete the brain's macrophage niche, the resulting engrafting macrophage population fails to acquire the classical microglial transcriptional signature defined by multiple groups[172–174]. Further developmental transplantation studies have revealed that neither fetal liver nor bone marrow progenitors, which arise from the process of definitive hematopoiesis and differentiate into TRM populations outside of the brain, are able to fully adopt a microglial signature within the brain[172]. These studies emphasize that microglia are shaped by their unique yolk sac ontogeny in addition to their local tissue microenvironment. Microglia are the primary hematopoietic cell population in the steady-state brain, which may poise them for rapid responses against neurotropic pathogens.

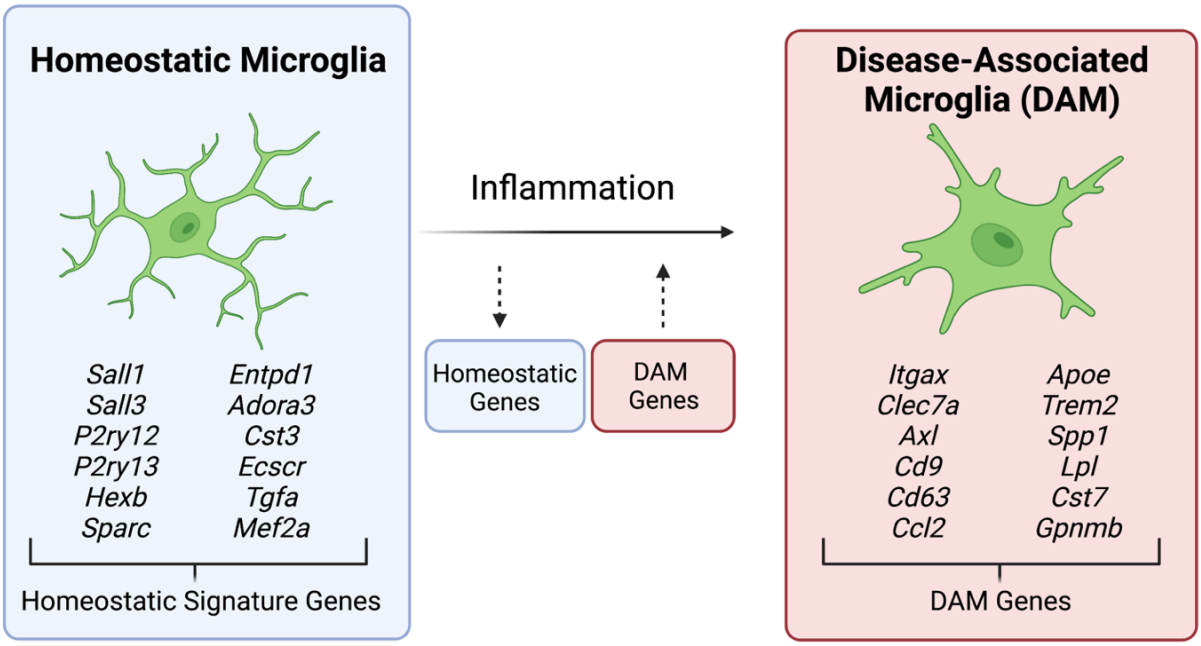

Figure 1.4. Transcriptional changes underlying the transition of homeostatic microglia to disease-associated microglia (DAM).

Homeostatic microglia express signature genes under steady state conditions that distinguish them from macrophage populations outside of the brain parenchyma. These homeostatic signature genes are downregulated upon neuroinflammatory challenge, with a concomitant upregulation of DAM genes that have been identified during neurodegenerative states. Large networks of transcriptional changes have been identified with the transition of homeostatic microglia to DAMs, with most common genes for each state highlighted.

Arguably, the most versatile tool currently used for manipulating microglia *in vivo* utilizes a tamoxifen-inducible cre recombinase system. Under these systems, a targeted gene of interest is flanked by *loxP* sites and a cre-ERT2 recombinase is expressed, typically under the *Cx3cr1* promoter[118,121]. Tamoxifen is delivered to mice harboring these transgenes, to induce cre recombinase activity that excises the target gene within a temporally restricted window of drug administration. The power of using an inducible cre system to study microglia stems from findings that microglia are long-lived and self-renew from a brain-resident CX3CR1-expressing population[179]. In contrast, CX3CR1-expressing myeloid cells that are recruited to the brain during inflammatory insults are short-lived, and renew from CX3CR1-negative progenitors located in bone marrow[179]. Hence, by restricting cre activity to a defined window and subsequently permitting peripheral immune cell turnover, microglia can be genetically targeted separately from bone marrow-derived immune cells[118,121] **(Figure 1.5A-C)**. This strategy is particularly valuable when studying microglia in infection or other inflammatory models in which a non-resident, monocyte-derived macrophage population infiltrates the brain[118,180]. While valuable in targeting microglia differentially to monocyte-lineage cells, the inducible CX3CR1-inducible cre system additionally targets tissue-resident macrophages along CNS interfaces, which include meningeal, perivascular, and choroid plexus macrophages (collectively referred to as CNS border-associated macrophages, or BAMs)[120,181]. As a result, alternative promoters for cre expression, including a novel Hexb cre,

and binary cre systems that provide improved targeting specificity have been recently developed[120,170].

Another widely-used approach for manipulating microglia *in vivo* includes pharmacological antagonism of CSF1R, a receptor whose signaling is vital for macrophage maintenance and survival[182]. The CSF1R antagonists, PLX3397 and PLX5622, lead to near-complete microglial elimination within days to weeks (depending on the formulation) when administered via chow[182] **(Fig 1.5D)**. PLX5622 allows for increased microglial depletion and CSF1R specificity relative to PLX3397, as the latter additionally antagonizes KIT and FLT3, and also displays decreased brain penetrance[183]. Multiple genetic tools have similarly been used to deplete microglia, including cre-lox-mediated *Csf1r* deletion and mice deficient for *Il34*, the gene encoding the primary CSF1R ligand in the brain[173,184–186]. Multiple CNS viral infection studies have used PLX5622 to deplete microglia[187–191]. These studies, in the context of murine picornavirus, alphaherpes, and coronavirus infections, cumulatively highlight that mice lacking microglia display increased viral load and mortality, and they are unable to promote optimal T cell responses during CNS infection. However, there are multiple caveats to consider when interpreting the results from microglia depletion studies during neuroinflammation. Initial depletion studies suggested that microglial depletion does not appear to grossly impact animal behavior or general health, but more recent literature suggests that depletion may impair neurovascular vasodilation[192]. PLX5622 requires a few days for optimal microglial depletion, and rapid repopulation and return

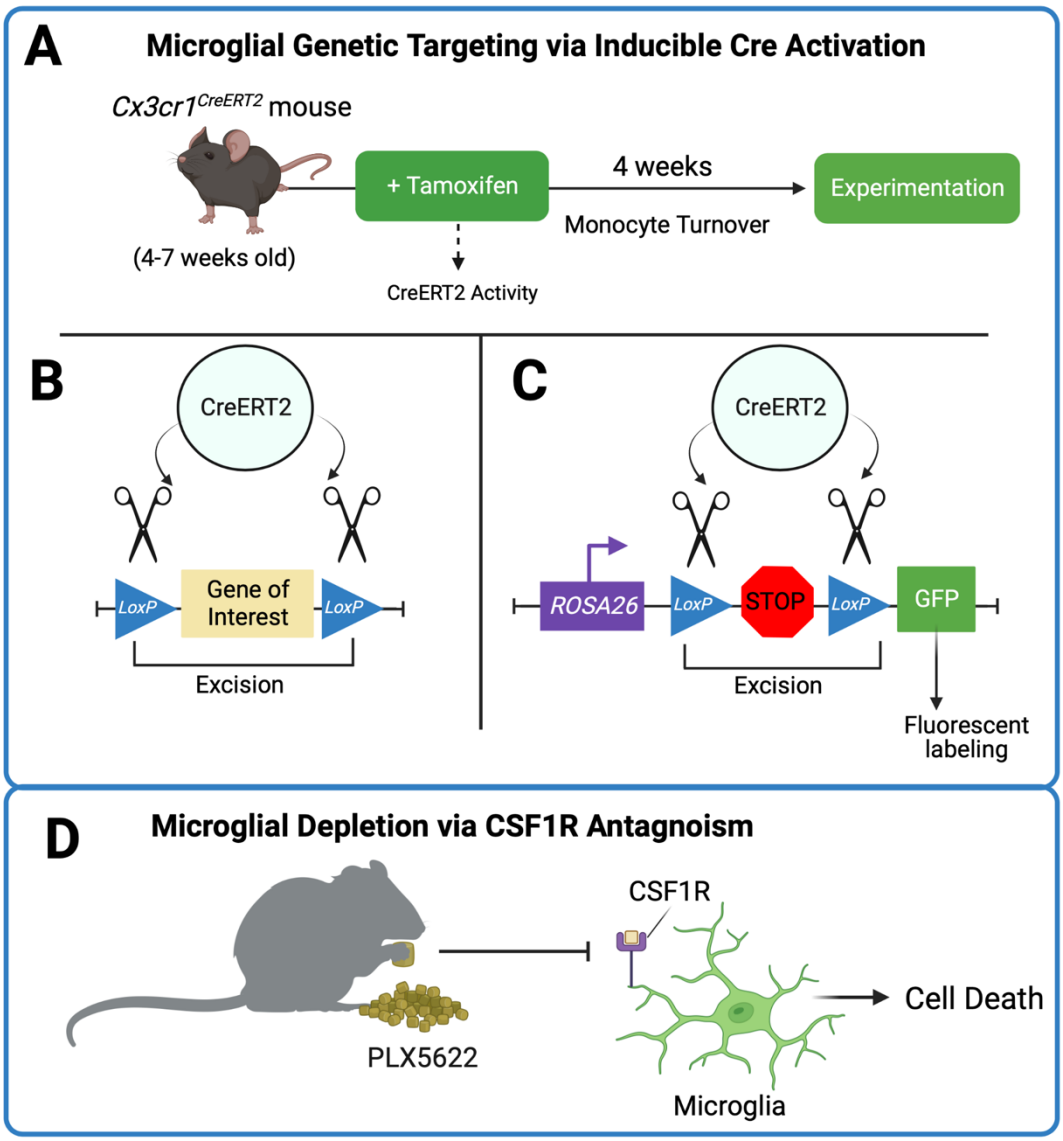

Figure 1.5. Experimental approaches for targeting microglia.

(**A**) Microglia can be genetically targeted using the CX3CR1 inducible cre system. A tamoxifen-inducible cre recombinase is transgenically expressed under the *Cx3cr1* promoter. Waiting four weeks post-tamoxifen administration restricts cre recombinase activity largely to self-renewing CNS macrophages and their progeny, without targeting the renewed circulating myeloid compartment. **(B)** Cre activity drives genetic recombination by excising an experimental gene of interest flanked by loxP sites (a "floxed" gene). **(C)** Cre activity can be used to excise a floxed stop codon inserted in the *ROSA26* locus, permitting permanent fluorophore expression in CNS macrophages and their progeny. **(D)** Microglia can be pharmacologically depleted using a CSF1R antagonist, such as PLX5622. CSF1R antagonism leads to systemic macrophage cell death, due to the blockade of survival signals in all macrophage populations.

to baseline levels is similarly observed after treatment is stopped[189]. Additionally, CSF1R antagonism, even in models without inflammatory insult, results in system-wide depletion of several macrophage populations, disrupts dendritic cell proliferation, and results in increased astrogliosis and pro-inflammatory cytokine production– all potentially confounding observed findings in these published works[190,193–195]. CSF1R antagonism has also been shown to deplete BAMs in some studies, although results across are mixed[189,196,197]. Further, CSF1R antagonism during both West Nile virus (WNV) and Theiler's murine encephalomyelitis virus (TMEV) infections has been shown to decrease CD11c+ and MHC II+ antigen presenting cell (APC) numbers in blood, spleen, and lymph nodes, which may impair systemic T cell priming and activation independently of microglial roles in the brain[190,191]. Thus, caution is necessary when depleting microglia in disease models that rely on functional APC-T cell interactions.

1.2.3 Microglial innate immune programs

Microglial spatial positioning throughout the brain and their complex "sensome" poises them to respond to several chemokines and cytokines released by other cells in response to inflammation, damage, and infection in the CNS[77,198,199]. RNA sequencing of microglia in homeostatic and neuroinflammatory contexts have revealed substantial differential gene expression upon activation[200,201]. One component of the activated microglial signature is the upregulation of complement system molecules, a cluster of innate immune proteins typically used in opsonizing and phagocytosing microbial pathogens or cellular debris[202,203]. In the healthy developing brain, numerous studies have shown that microglia express the complement proteins C1q and C3, and engage in the pruning of neuronal synapses via classical complement-dependent,

receptor-mediated phagocytosis[204–206]. This synaptic pruning process, while required for the sculpting of functional synaptic circuits during development, has been shown to manifest aberrantly during neurodegenerative and infectious disease states, where complement activation leads to excessive synaptic pruning[185,207,208].

During neurodegenerative disease models for Alzheimer's disease (AD), Multiple Sclerosis (MS), and amyotrophic lateral sclerosis (ALS), microglia transition to a disease-associated microglia (DAM) phenotype[202,209]. DAMs are characterized by a downregulation of microglial homeostatic signature genes (including *P2ry12, P2ry13, TMEM119,* and *Hexb),* with a concomitant upregulation of inflammation-associated genes (*Itgax, Apoe, Ccl2, Clec7a,* and *Spp1*) **(Fig 1.4)**. Transcriptomic analyses suggest that the DAM phenotype is regulated by both phagocytic clearance of cellular debris, and possibly interferon signaling[210]. Some research suggests that DAMs may be protective against neurodegeneration, but how they ultimately affect disease progression is unclear[209]. Mice that are deficient for or harbor the R47H human variant of TREM2 have microglia that are unable to acquire a full DAM phenotype, display increased neuronal dystrophy and amyloid beta plaque burden and microglial activation in the 5XFAD mouse model of early-onset AD[211–213]. However, additional studies have paradoxically shown that depleting microglia entirely in this same AD model results in amyloid plaque formation in the brain vasculature rather than the brain parenchyma, with no change in net brain amyloid load or effects on hippocampal learning and memory[183]. DAMs are poorly understood at the systems-level, but their discovery and characterization has expanded our understanding of microglial innate immune capacity on the cellular level. Indeed, a recent study highlights features of the DAM signature that are shared across infectious disease models, including LCMV and potentially a mouse model of SARS-Cov-2 infection – suggesting a conserved microglial phenotype shared

between neurodegeneration and infection[214]. CNS infection models may present intriguing opportunities for uncovering whether the pro-inflammatory activation signature of DAMs yields protective functional outcomes in other disease states.

1.2.4 Microglia as activators of adaptive immunity

Given their myeloid lineage, microglia are presumed to serve as the professional antigen-presenting cells (APC) of the CNS. However, literature is both limited and mixed regarding their APC capacity. Under basal conditions and unlike most myeloid populations, only a small subpopulation (about 3%) of microglia express MHC class II, the critical antigen presentation molecules required for activating antigen-specific CD4+ T cells[215]. With inflammation, microglia upregulate MHC II across several conditions, yet its functionality in initiating and maintaining adaptive immune responses is unclear.

Different models of CNS infection, including CNS challenge with mouse hepatitis virus (MHV), can result in demyelination by self-reactive T cells following infection. During MHV infection, brain-infiltrating macrophages, but not brain-resident microglia, are the APC population capable of activating self-reactive CD4+ T cells, as evaluated by *ex vivo* assays[216]. *In vivo* approaches for investigating microglial APC capacity have also been performed during sterile experimental autoimmune encephalomyelitis (EAE) and cuprizone-mediated demyelination. In these settings, type 2 conventional dendritic cells (cDC2s) are the potent APC population that activates encephalitogenic T cells, as microglial-specific genetic deletion of MHCII bears no impact on CD4+ T cell activation or clinical symptom development[215,217]. These studies collectively highlight intrinsic differences between microglia and other myeloid populations present in the brain during CNS infection, and their capacity to influence adaptive autoimmunity.

Whether microglial antigen presentation provides protective immunity against microbes more poorly understood. Two-photon imaging coupled with flow cytometric analysis of APCs during *T. gondii* infection has revealed that a large majority of microglia upregulate the traditionally dendritic cell-associated integrin, CD11c[218]. These live-imaging studies captured both transient and stable physical interactions between CD11c+ cells and antigen-specific CD8+ T cells, consistent with T cell receptor (TCR)-MHC I interactions[218]. However, given: (1) the presence of bone marrow-derived CD11c+ dendritic cells during CNS infection with *T. gondii* and (2) limitations associated with *in vitro* microglia assays, these studies are unable to determine whether these potential microglial-T cell interactions represent antigen presentation. Transient, non-apoptotic physical interactions between T cells and microglia have also been observed via intravital imaging during LCMV infection, with these studies also sharing similar caveats[219]. However, recent studies using an inducible CX3CR1-cre system was used to show that H-2D^b (MHC I) in CNS-resident macrophages is required for mediating CD8+ T cell recruitment and optimal viral control during Theiler's murine encephalomyelitis virus (TMEV) infection[191]. In this latter study, it remains unclear whether microglia were a necessary APC population for CD8+ T cell activation, or if perivascular macrophages were instead the cell type most relevant to T cell priming and recruitment from the blood[181]. Emerging genetic tools that allow for more specific targeting of microglia independently of border-associated macrophages, such as a split cre or Hexb cre-driven systems, will be necessary to clarify the field's understanding of microglia-specific APC capacity during CNS infection models[120,170].

1.2.5 Microglia promote neuroinflammatory processes

Microglia serve as a key source of cytokines within the CNS at baseline and in disease contexts. Animal models of AD, MS, ALS, and LCMV infection have been used to study activated microglia *in vivo*, where both microgliosis and morphological changes are observed in these cells[202,209,219–221]. During EAE, deletion of *MAP3K7* (the gene encoding the NF-κB modulator, TAK1), results in the abrogation of autoimmune inflammation and clinical pathology in mice[118]. Arguably, most studies of microglial inflammatory responses are neurodegeneration-focused, where a pattern has emerged that overexuberant microglial activation results in tissue damage[118,165,185,207]. In some cases this immunopathology is indirect, as in the instance of microglia driving the formation of neurotoxic astrocytes in neurodegenerative models through the release of C1q, IL-1, and TNF-α[165]. Research in CNS infection is currently emerging that provides insights to how these pro-inflammatory processes may translate to neuroprotection during CNS infection, rather than observed neurotoxic and degenerative phenotypes in sterile contexts.

1.2.6 Microglia in CNS infection models

Current research efforts to understand the intricate interactions of microglia with the rest of the immune system in the infected CNS suggest that microglia have many ways to respond to infection and drive innate immunity. Here, we summarize the currently identified roles of microglia in several experimental infection states, and explore their roles in cytokine release, synaptic pruning, and driving an innate immune response.

Cerebral Toxoplasmosis

Toxoplasma gondii is a well-studied intracellular protozoan parasite capable of infecting nucleated cells in virtually all warm-blooded vertebrates, including 25-30% of the global human population[23,222]. As a neurotropic parasite, there are several key advantages of using *T. gondii* as a model to study microglia during CNS infection. Notably, *T. gondii* traffics to the brain following peripheral inoculation, and the establishment of CNS infection does not require intracranial injection[23,222]. Peripheral inoculation spares the brain from the robust CNS injury response and resulting microglial activation well-described with transcranial insult, and it also allows the peripheral immune system to be primed before parasites reach the brain[199,202]. *T. gondii* infection in both humans and mice occurs in two phases: (1) an acute infection that disseminates widely throughout host tissues, and (2) a chronic, latent infection largely restricted to the immune-privileged CNS[23,222]. While acute *T. gondii* infection has been most heavily studied, both phases of infection converge on a protective immune response governed by the critical T cell-derived cytokine, interferon-gamma (IFN-γ)[23].

Most hematopoietic and non-hematopoietic cell types are capable of responding to IFN-γ via signaling through the downstream transcription factor, STAT1[6,223]. IFN-γ-STAT1 signaling is essential for the upregulation of anti-parasitic molecules, such as guanylate-binding proteins (GBPs) and immunity-related GTPases (IRGs)[6,23]. These cytosolic proteins are encoded by large families of genes and are required for cooperatively targeting and disrupting the *T. gondii* parasitophorous vacuole or promoting parasite elimination via autophagy[138,148,224,225]. Monocyte-derived cells additionally require IFN-γ-STAT1 signaling for the upregulation of specialized cell-intrinsic and cell-extrinsic anti-toxoplasmic programs, including nitric oxide production via the enzyme, inducible nitric oxide synthase (iNOS), and the upregulation of the antigen presentation

molecules required for T cell activation and further IFN-γ production from T cells[223,226]. The specific IFN-γ-STAT1 mechanisms of resistance employed by microglia during CNS infection with *T. gondii* have not yet been defined, but may in part parallel those of monocyte-derived cells, including the recruitment of immune cells via the T-cell chemoattractants CXCL9 and CXCL10[104,106,107]. Microglia produce both of these chemokines during *T. gondii* infection, and CXCL10 is required for CD8+ T cell recruitment to the CNS, where its inhibition leads to increased brain parasite burden[105,106]. Together, previous studies strongly suggest that IFN-γ-STAT1 signaling in microglia may be required for controlling *T. gondii* in the brain (**Fig 1.6**).

Alarmins are damage-associated molecular patterns (DAMPs) sequestered intracellularly and canonically released upon tissue damage or inflammasome activation[227,228]. A recent paper has revealed that microglia release the cytosolic alarmin, IL-1α, to control CNS infection with *T. gondii*[77]. Microglial IL-1α was shown to upregulate adhesion molecules on the brain vasculature to permit the infiltration of anti-parasitic monocytes from the blood into the brain to control CNS parasite burden (**Fig 1.6**). This study used an inducible *Cx3cr1*-driven fluorescent reporter as a tool to discriminate microglia from blood-derived myeloid cells, revealing that microglia are the key source of IL-1α in both the steady-state and *T. gondii*-infected brain. *Batista et al*. showed that microglia release IL-1α *ex-vivo*, in a Gasdermin-D-dependent manner. While microglial-derived IL-1α is required to control *T. gondii* parasite burden during chronic infection, IL-1α-deficient mice do not succumb to infection. Surprisingly, IL-1α but not IL-1β is required for controlling *T. gondii* parasite burden in a manner that phenocopies IL-1R1-deficient mice, despite the two cytokines being both expressed in the brain and sharing the same receptor. These studies also revealed that IL-1α signaling via IL-1R1 does not alter brain IFN- γ levels or lead to animal mortality, but instead regulates infiltrating monocyte number and ability to express iNOS.

Interestingly, this microglial-specific control of parasite burden occurred despite a dampened NF-κB signature in resident microglia relative to blood-derived myeloid in the brain. This study thus highlighted physiological distinctions between the two key macrophage populations responding to *T. gondii* infection, despite a shared tissue micro-environment in the inflamed brain. Because Gasdermin-D has been characterized an essential driver of inflammasome-mediated pyroptosis, this work suggests that microglial IL-1α release and control of infection is mediated by cell death[229]. The potential triggers of microglial cell death and the specific inflammasome sensor(s) required for IL-1α release remain unidentified. Alarmin release by resident glial cells may serve as a key effector program in prompting immune activation and parasite control in discrete regions of the CNS, without resulting in unrestrained, tissue-wide neuroinflammation and resulting immunopathology.

Microglia may be especially poised to respond to lytic cell death stemming from focal sites of *T. gondii* replication, in part through purinergic-mediated chemotaxis. Several studies have demonstrated that the microglial "sensome" includes purinergic receptors that regulate their motility, including P2RY12, P2RY13, P2RY6, and P2RX4[161,198]. These receptors collectively mediate cellular chemotactic responses to ATP and adenosine-family purines. Notably, a loss of purinergic signaling due to genetic deletion of these genes or the delivery of purinergic inhibitors results in a loss of the rapid microglial chemotactic responses to sites of acute injury, in addition to markedly reduced baseline motility[161,199,230]. While microglia are evenly spatially distributed throughout the steady-state brain, they lose their uniform tiling and migrate into nodules surrounding *T. gondii* replication in the infected brain[231,232]. While not yet explored, *T. gondii*-induced cell lysis and the resulting liberation of purinergic alarmins may mobilize and position microglia to focal sites of parasite replication to spatially target and restrict pathogen growth. Their

resident status in the CNS, coupled with a unique sensome not shared by other myeloid populations, may thus position microglia to serve as first responders to CNS infection, possibly by pinpointing discrete regions of the brain that require recruitment and activation of blood-derived immune cells for parasite restriction.

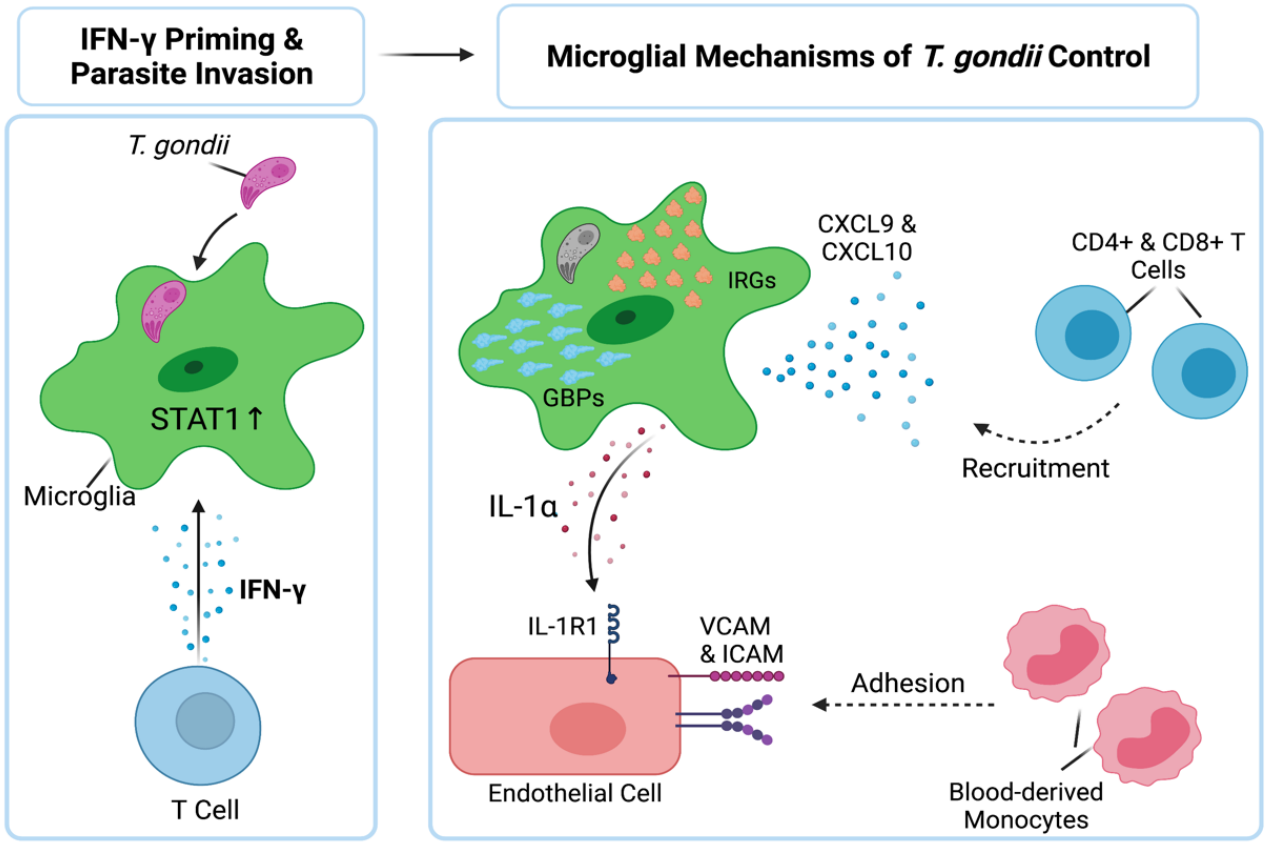

Figure 1.6 Microglial responses to CNS infection with *Toxoplasma gondii*.

Microglia respond to T cell-derived IFN-γ via the downstream transcription factor, STAT1. IFN-γ-STAT1 signaling primes microglia for cellular responses against intracellular parasitic infection. Upon parasite invasion, IFN-γ-STAT1 signaling confers microglia with the cell-intrinsic ability to clear parasite via Immunity-Related GTPases (IRG) and Guanylate-Binding Proteins (GBP) activity. Microglial-derived CXCL9 and CXCL10 further recruit T cells, also in a STAT1-dependent manner. In addition to IFN-γ-mediated mechanisms of parasite control, microglia have been recently shown to release the pro-inflammatory cytokine, IL-1α, to activate CNS endothelial cells, driving the recruitment of anti-parasitic monocyte-derived cells from the blood into the CNS.

West Nile Virus and Japanese Encephalitis Virus

West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV) are mosquito-borne, neurotropic flaviviruses that cause widespread morbidity and mortality throughout the human population [233]. A WNV neuroinvasive disease model determined that microglial activation drives complement-mediated synapse elimination and neurocognitive impairments in mice (**Fig 1.7**). IL-34-deficient mice, which lack most microglia, were shown to be protected from synapse loss and the cognitive sequelae stemming from WNV infection[185]. In both WNV and JEV, microglia appear to be critical for preventing disease progression, as microglial depletion with the CSF1R antagonist PLX5622 results in increased CNS viral load and mortality[190,234]. This neuroprotection may be mediated by phagocytosis and the release of proinflammatory chemokines and cytokines by activated microglia, including CXCL10, CCL5, CCL2, TNF-α, and IL-6 as suggested by *ex vivo* spinal cord culture[235]. Whether microglia primarily respond to pro-inflammatory cytokines in an autocrine manner or protective immune responses are mediated cell-extrinsically by other brain-resident or infiltrating cells remains unclear. However, these studies together suggest that microglia may serve protective roles in controlling neurotropic viral infection, and also highlight that microglia activation leads to synapse loss and neuronal dysfunction as is similarly observed during classical neurodegenerative models.

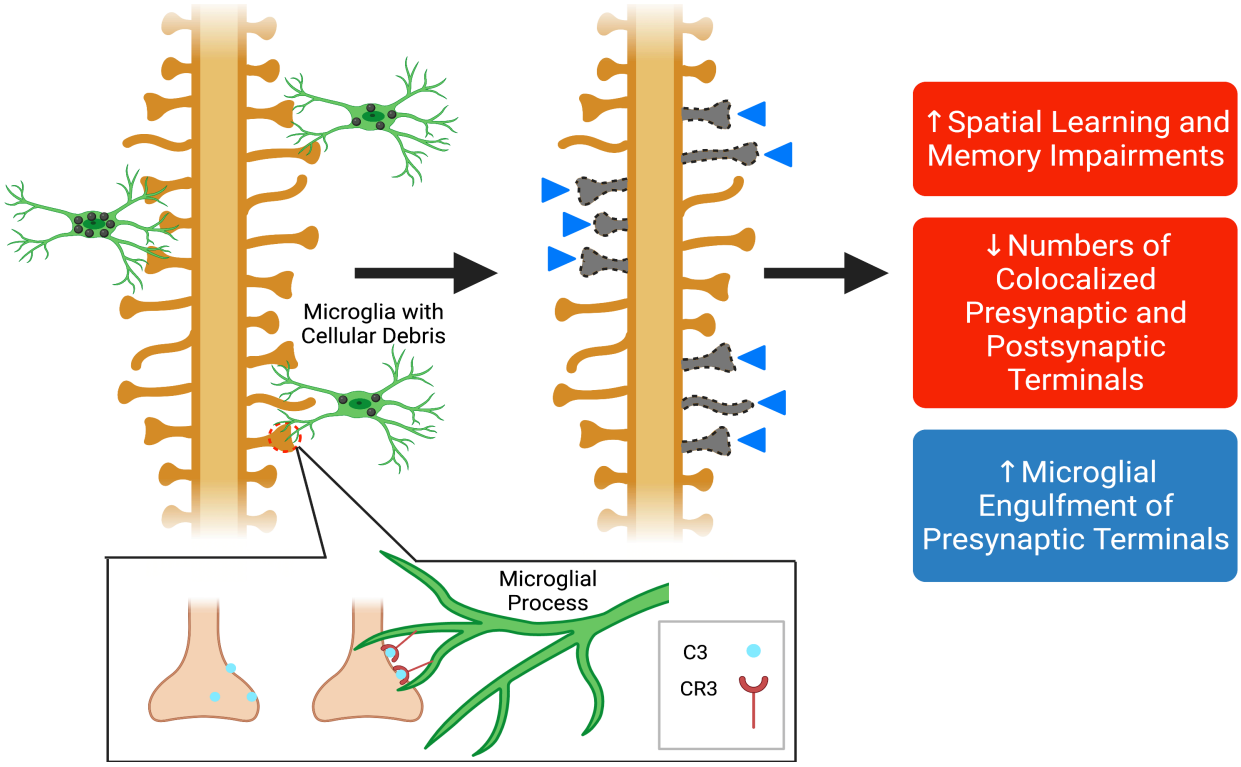

Figure 1.7. Microglial-mediated synaptic pruning in West Nile Virus (WNV) drives neurocognitive sequelae.

The microglial-derived complement protein, C3, mediates microglial engulfment of synaptic terminals (synaptic pruning) via CR3-dependent receptor-mediated phagocytosis during WNV infection. Hippocampal CA3 synaptic terminals are excessively pruned following infection-induced microglial activation, leading to impaired spatial learning acquisition and memory recall.

Coronaviruses

Coronaviruses have gained strong global interest due to the COVID-19 pandemic caused by the SARS-CoV-2 virus. Unsurprisingly, microglial activation is observed in CNS infection with various coronaviruses. Infection with the J2.2v-1 neuro-attenuated strain of mouse hepatitis virus (MHV), a murine coronavirus, drives acute encephalitis and chronic de-myelination in mice[216,236–

[238]. A 2018 study showed that microglial depletion with PLX5622 resulted in increased MHV viral protein expression in neurons and mortality, along with diminished CD4+ and CD8+ brain T cell counts and IFN- γ production[189]. This research lends supports the notion that microglia may serve as APCs or promote the recruitment, retention, or survival of T cells in the brain during viral infection.

Additional studies using pharmacological depletion of microglia with the J2.2v-1 strain of MHV found similar results with respect to viral load, associated with increased white matter damage in both brain and spinal cord[237]. This latter research reported more modest decreases in CD4+ T cell activation and concomitant increases in both CD8+ T cell and B cell populations[237]. The mixed results of these separate MHV studies may relate to differences in the timing of pharmacological depletion of microglia between the two studies. However, these reports remain consistent with an expanding pool of literature across several viral models in recent years supporting a role for microglia in controlling CNS viral replication.

In addition to viral clearance, the role of microglia in coronavirus-induced demyelination has been examined by multiple groups[236–239]. While targeted depletion of peripheral macrophages does not alter demyelinating disease progression following JHV infection, an intact microglial population, is critical for neuroprotection[237,239]. Depleting microglia via PLX5622 during late stages of viral clearance leads to exacerbated white matter damage, impaired oligodendrocyte function, and the persistence of myelin debris, each consistent with this neuroprotective role[237,239]. These findings underscore fundamental functional differences between resident microglia and peripherally-derived macrophages that are recruited to the CNS during infection.

The role of various CNS cells in SARS-CoV-2 infection has only been preliminarily investigated, but there is clear evidence of microglial activation in post-mortem human COVID-

19 brain tissue[240,241]. Similarly to CNS infection with *T. gondii*, microglial nodules, or the aggregation of activated microglia within discrete foci, are observed in post-mortem brain samples of COVID-19 patients[241–243]. SARS-CoV-2 infection in the CNS is largely restricted to ACE2-receptor-expressing cells within the olfactory bulb and brainstem vasculature – presenting the paradox of what parenchymal microglia may be responding to within nodules[242]. A potential explanation may involve microglial migration to sites of blood-brain-barrier damage stemming from vascular viral replication or robust CD8+ T cell infiltration to the brain[242]. Multiple studies have highlighted microglial purinergic-dependent juxtapositioning with the brain vasculature, either upon induced injury, or under steady-state conditions in micro-segments of the neurovascular unit displaying limited astrocytic endfoot coverage[192,230,244]. A leaky blood-brain-barrier caused by the cytokine storm resulting from SARS-CoV-2 infection may result in microglial nodule formation in areas displaying blood-brain-barrier disruption, where CD8+ T cells may contribute to vascular immunopathology[242]. Whether microglial activation mediates the poorly understood neurological sequelae of COVID-19, as is the case in WNV infection, is yet to be determined. Given the role of microglia in driving neuroinflammation via pro-inflammatory cytokine release, promoting neurotoxic astrocyte development, and promoting excessive synaptic pruning, these may take center stage in studies exploring the new clinical phenomenon of "neuro-COVID."

1.2.6 Summary: microglia, infection, and neuroinflammation

Recent studies investigating microglial function during CNS infection highlight two main themes: (1) microglia as a key mediator of neuroprotection against invading pathogens and (2) microglia as inflammatory drivers of neuropathology and degeneration. In several infection

models, microglia are collectively protective in combatting pathogen growth, by releasing and responding to proinflammatory cytokines, as well as via recruiting anti-parasitic and anti-viral blood-derived immune cells[77,187,189]. This immune activation can simultaneously prove detrimental, as inflammation caused by microglial activation may result in neurotoxicity and neurodegeneration[118,165,185,207]. Current work highlights this central need for the immune system to strike a balance between pathogen control and immune tolerance during CNS infection in order to prevent neuropathology. The field of neuroimmunology is continuing to learn the ways in which microglial ontogeny and their unique transcriptional identity as CNS-resident cells influences functional responses that differ from blood-derived immune cells[77,172–174]. With the advent of emerging experimental tools for studying microglia, further research will expand our growing understanding of how microglial physiology confers neuroprotection against CNS infection and other disease states.

1.3 Project rationale

Thus far, the introduction of this dissertation has provided an overview of the existing literature on the immunoparasitology of *T. gondii*, and what is known about microglia within the contexts of neuroinflammation and CNS infection. Given that *T. gondii* is an extensively well-studied pathogen that serves as a reliable and robust model for microbial neuroinflammation, we have used it throughout our experimental studies to investigate microglial immune responses to infection. Because STAT1 signaling downstream of IFN-γ is: (i) well-established as serving as the cornerstone of the immune response to *T. gondii*, (ii) regulates the transcription of large families of genes in both hematopoietic and non-hematopoietic cells in a cell type-specific manner, and (iii) exerts specialized anti-parasitic responses in myeloid cells, we hypothesized that STAT1 signaling in microglia was required for host resistance to CNS infection with *T. gondii*. We thus aimed to investigate the following research questions:

<u>STAT1-mediated cell-intrinsic responses</u>:

1. Does STAT1 signaling regulate microglial capacity for intracellular killing via classical anti-parasitic machinery such as iNOS, IRGs, and GBPs?
2. Do STAT1-activated microglia share similar or non-overlapping anti-parasitic effector functions with those of STAT1-activated blood-derived macrophages?

<u>STAT1-mediated cell-extrinsic responses</u>:

1. Does STAT1 signaling in microglia shape brain-infiltrating immune cell responses?
2. How critical is the upregulated expression of antigen presentation molecules on microglia for antigen-specific T cell functions in the brain?

Chapter 3 of this dissertation reported and discussed the results of our work investigating the cellular and systems-level effects of microglial STAT1 deletion during *T. gondii* challenge. Key findings include that microglia-specific STAT1 deletion results in fatal encephalitis, and unlike blood-derived myeloid cells that infiltrate the brain during neuroinflammation, microglia do not express the anti-parasitic protein iNOS. In Chapter 3 we also reported the finding that the disease-associated (DAM) signature that is transcriptionally conserved across several disease models of neurodegeneration is activated in microglia during CNS infection in a STAT1-dependent manner – this is discussed within the context of follow-up experiments in Chapter 4. Experiments designed to explore a limited capacity for microglial-extrinsic parasite control via the STAT1-dependent antigen presentation molecule MHC II is also discussed in Chapter 5. Chapter 6 contains an in-depth discussion of major themes that emerged from this work, including the topics of (i) mechanisms underpinning parasite killing, (ii) microglial identity in the *T. gondii*-infected brain, (iii) microglial parasite sensing, and (iv) microglial STAT1 signaling across health and disease contexts.

Chapter 2 – Materials and Methods

Resource and reagent sharing:

Informational inquiries or requests for resources or reagents can be directed to the lead contact and laboratory principal investigator, Tajie Harris (tajieharris@virginia.edu).

Mice. CX3CR1^{CreERT2} (#020940) and ROSA26$^{Ai6/Ai6}$ (#007906) mouse lines were originally purchased from Jackson Laboratories and cross-bred to generate CX3CR1^{CreERT2} x ZsGreen$^{fl/stop/fl}$ mice used as controls. These control mice were subsequently cross-bred with STAT1$^{fl/fl}$ mice (provided by Lothar Hennighausen, NIH) to generate STAT1$^{fl/fl}$ x CX3CR1^{CreERT2} x ROSA26$^{Ai6/Ai6}$ (MG$^{STAT1\Delta}$) mice. Age- and sex-matched mice were intraperitoneally administered tamoxifen (4 mg per 20 g body weight, Sigma-Aldrich) between 4-7 weeks of age for 5 consecutive days to induce STAT1 deletion. Four weeks following tamoxifen treatment, mice were challenged with the type II *T. gondii* strain Me49 or proceeded with naïve experiments. Parasite was passaged through CBA/J and Swiss Webster mice (Jackson Laboratories), and mice used in infection experiments were intraperitoneally challenged with 10 Me49 cysts from CBA/J brain homogenate. *Stat1* excision was confirmed by qPCR, flow cytometry, or immunohistochemistry. All mice were housed in University of Virginia specific pathogen-free facilities with a 12h light/dark cycle, with ambient temperature between 68 and 72 F, and 30-60% humidity. Mice used in experiments were euthanized by CO_2 asphyxiation if they showed weight loss greater than 20% of their baseline, pre-recorded weight. All procedures were approved and conducted in accordance with the University of Virginia Institutional Animal Care and Use Committee approval of protocol 3968.

Immunohistochemistry. PBS-perfused brain hemispheres were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 24 hours at 4°C. Brains were then cryopreserved in a 30% sucrose solution for 48 hours at 4°C, flash-frozen in OCT (Sakura), on dry ice, and cryosectioned at a thickness of 50 μm. Free-floating sections were blocked for 1 hour at room temperature in a 1x PBS solution containing 0.1% Triton, 0.05% Tween 20, 5% BSA, and 0.1% BSA. Sections were incubated in primary antibodies overnight at 4°C. Sections were washed with a 1x PBS, 0.05% Tween solution prior to incubation in primary antibodies for one hour at RT. Following secondary washes, sections were incubated in DAPI (Thermo Fisher Scientific) for 5 minutes and mounted on glass coverslips (Thermo Fisher Scientific) in Aquamount (Lerner Laboratories) mounting media. Dilutions were performed at a 1:200 concentration for primary antibodies and a 1:1,000 concentration for secondary antibodies and DAPI staining. All immunohistochemical micrographs were captured using a Leica TCS SP8 Confocal microscope. Images were analyzed using Imaris or ImageJ software. Primary antibodies used in experiments included goat anti-Iba1 (Abcam), rabbit anti-Me49 (gift from Fausto Araujo), and rat anti-MHC II (Cat. #: 14-5321-82; Thermo Fisher Scientific). Secondary antibodies used in experiments included: Alexa Fluor 594 Donkey Anti Rabbit IgG (Cat. #: 711-585-152; Jackson ImmunoResearch), Alexa Fluor 647 Donkey Anti-Rabbit IgG (Cat. #: 711-605-152; Jackson ImmunoResearch), Cy3 AffiniPure Donkey Anti Rat IgG (Cat. #: 712-165-153; Jackson ImmunoResearch).

Sholl Analysis. Sholl analysis was used to analyze microglial morphological complexity as a readout for their activation state, in accordance with the protocol published in *Norris et al., 2014* [245]. In brief, confocal photomicrographs of naive WT and MG$^{STAT1\Delta}$brains with ZsGreen+ microglia

were captured and analyzed in Fiji with the Sholl Analysis plugin software. Images were processed in binary pixels, microglia were manually selected, and shells were inserted in 5 μm concentric circles, starting from 10 μm outside the center of the soma, and ending at the limit of the longest arborization for each analyzed microglia. The number of dendritic intersection points was plotted for each distance away from the soma, and data was analyzed via 2-way ANOVA with Sidak's multiple comparison test.

H&E Tissue staining. Brains were fixed in formalin, prior to being embedded in paraffin, sectioned, and stained with hematoxylin and eosin at the UVA Research Histology Core. Following mounting, sections were imaged on a DM 2000 LED brightfield microscope (Leica Biosystems).

Tissue Processing. Mice were given an overdose of ketamine/xylazine and transcardially perfused with 30 mL of ice-cold 1X PBS. Brains and spleen were collected and placed in cold complete RPMI media (cRPMI) (10% FBS, 1% sodium pyruvate, 1% non-essential amino acids, 1% penicillin/streptomycin, and 0.1% 2-ME). Brains were minced with a razor blade, passed through an 18G needle, and enzymatically digested with 0.227 mg/mL collagenase/dispase and 50U/mL DNase (Roche) for 45 minutes at 37C. If brain samples were used to quantify parasite burden or for gene expression analysis, aliquots were removed and frozen for downstream analysis prior to addition of digestion enzymes. If brain cells were being used for downstream RNA sequencing for WT vs. $MG^{STAT1\Delta}$ analysis, Actinomycin D (Sigma-Aldrich) was added to the digestion buffer at a concentration of 45 μM during incubation and 3 μM during washes to inhibit upregulation of immediate early activation genes associated with subsequent FACs sorting. All brain samples were

resuspended in 20 mL of 40% percoll and spun for 25 minutes at 650 xg to remove myelin. Following myelin removal, samples were washed with and subsequently resuspended in cold cRPMI. Blood collected for flow cytometric analysis was isolated from the heart prior to transcardial perfusion and transferred into 1x PBS + EDTA (Thermo Fisher Scientific). Blood was then processed with RBC lysis and resuspended in cold cRPMI prior to staining and fixation for flow cytometry. For peritoneal lavage experiments, 5 mL of cold 1X PBS was injected through the membrane encasing the peritoneal cavity via a 26G needle and withdrawn with a 22G needle. Lavage fluid was washed and suspended in cRPMI.

Flow cytometry. Following generation of a single-cell suspension, cells were plated in a 96-well plate and incubated for 10 minutes in 50 μL Fc block (1 μg/mL 2.4G2 Ab (BioXCell), 0.1% rat γ-globulin (Jackson ImmunoResearch) at room temperature. Cells were incubated in primary antibodies at a concentration of 1:200, and AF-780 viability dye (eBioscience) at a concentration of 1:800 for 30 minutes at 4°C. Antibody clones used for experiments included: MHC II (M5/114.15.2), CD11b (M1/70), Ly6C (HK1.4), iNOS (CXNFT), CD45 (30-F11), TREM2 (237920), CD11c (N418), CD3e (145-2C11), CD4 (GK1.5), CD8a (53-6.7), and IFN-γ (XMG1.2) (Thermo Fisher Scientific, R&D Systems). In experiments analyzing antigen-specific CD4+ T cell responses, cells were pre-incubated with PE-conjugated MHC II I-A^b AS15 tetramer (NIH Tetramer Core Facility) for 15 minutes at room temperature before surface staining with antibodies. After staining for surface markers, cells were washed and fixed overnight in 2% PFA at 4°C, before being washed and intracellularly stained, if quantifying cytosolic protein. For intracellular cytokine staining (IFN-γ), initial single cell suspensions were incubated with Brefeldin A (Selleckchem) for 5 hours at 37°C prior to blocking and staining. For intracellular

cytokine analysis, cells did not receive any additional stimulation *ex-vivo*. For intracellular staining, cells were permeabilized with Permeabilization Buffer (eBioscience) and stained for 30 minutes at room temperature. Cells were washed with FACS buffer and transferred into 5 mL FACS tubes, then were analyzed on a Gallios flow cytometer (Beckman-Coulter). Flow cytometry data was analyzed using FlowJo.

Cell Sorting / Enrichment. For RNA sequencing and analysis of microglial *Stat1* relative expression to validate excision, brains were processed into a single cell suspension, as described above. Cells were then magnetically labeled with CD11b-conjugated beads diluted in MACS buffer for 15 minutes, per manufacturer's instructions. Following a wash with 2 mL of MACS buffer, samples were spun at 1500 RPM for 5 minutes and resuspended in 600 μL of MACS buffer. Myeloid cells were then positively selected for using anti-CD11b-conjugated magnetic beads enrichment (Miltenyi). Cells were resuspended and lysed in Trizol for RT-qPCR analysis, or incubated for 10 minutes in 50 µL Fc block (1 µg/mL 2.4G2 Ab (BioXCell), 0.1% rat γ globulin (Jackson ImmunoResearch) at room temperature for RNA-sequencing. Cells were stained with the following antibodies (Thermo Fisher Scientific) for 30 minutes at 4°C: CD11b-Percp Cy5.5 (Cat. #45-0112-82), MHCII-eFluor 450 (Cat. # 48-5321-82), CD45-APC (Cat. #17-0451-81), Ly6C-PE Cy7 (Cat. #12-5932-80), CD3e-PE Cy7 (Cat. #25-0031-81), NK1.1-PE Cy7 (Cat. #25-5941-81), CD19-PE Cy7 (Cat. #25-0193-81). Live cells were analyzed and sorted using a BD Aria flow cytometer at the University of Virginia Flow Cytometry Core facility. Cells were sorted based on ZsGreen and dump gating ($CD3e^-$ $NK1.1^-$ $CD19^-$ $Ly6C^-$) directly into Trizol (Invitrogen) for RNA extraction and RNA sequencing. For $MG^{STAT1\Delta}$ mice, MHC II^{neg} microglia were gated in order to positively select for STAT1-deficient cells.

Quantitative RT-PCR. For tissue-level analysis, one-fourth of a mouse brain was placed in 1 mL Trizol (Ambion), mechanically homogenized using 1 mm zirconia/silica beads (Biospec) for 30 seconds using a Mini-BeadBeater 16 (BioSpec). For gene expression analysis of magnetically-enriched cells, cells were homogenized in Trizol by pipetting. RNA was extracted from Trizol according to manufacturer's instructions (Invitrogen). High Capacity Reverse Transcription Kit (Applied Biosystems) was used to generate cDNA. Quantitative PCR was performed using 2X Taq-based Master Mix (Bioline) and TaqMan gene expression assays (Applied Biosystems), or custom primers (Integrated DNA Technologies), run on a CFX384 Real-Time System thermocycler (Bio-Rad Laboratories). Murine *Hprt* and *T. gondii Act1* were used for normalization for analyzing host and parasite gene expression, respectively, and relative expression is reported as $2^{(-\Delta\Delta CT)}$. The following Thermo Fisher mouse gene probes were used: *Stat1* (Mm00439518_m1), *Hprt* (Mm00446968_m1), *Ifng* (Mm01168134_m1), *Nos2* (Mm00440502_m1), *Il6* (Mm00446190_m1), *Icam1* (Mm00516023_m1), *Vcam1* (Mm01320970_m1), *Tnfa* (Mm00443258_m1), *Ccl2* (Mm00441242_m1), *Ccl5* (Mm01302427_m1), *Cxcl9* (Mm00434946_m1), *Cxcl10* (Mm00445235_m1). Custom primers for used for analyzing *T. gondii* genomic DNA and gene expression were used and are provided in **S1 Table**.

RNA sequencing analysis. RNA reads from FASTQ files were trimmed and filtered using Trimmomatic (v0.39) paired-end set to phred 33 quality scoring. Adapters were trimmed, and reads with a minimum quality score of 15, leading and trailing quality scores of 3, and minimum fragment length of 36 were used for analysis. FastQC (v0.11.9) was used to verify quality of

sample reads. Trimmed and filtered reads were aligned to the GENCODE M13 reference genome using Salmon (v0.8.2) and output as .sam files. Transcript abundance files were imported into R (v4.1.1) and converted to gene abundances using Tximport (v1.24.0). The R Bioconductor package, DESeq2 (v1.36.0), was used to perform differential expression analysis. DESeq2-normalized data was visualized using the following R packages: EnhancedVolcano (v1.14.0), pHeatmap (v1.0.12), and ggplot2 (v3.3.6). Gene names were converted from mouse ENSEMBL gene identifiers to gene symbols using the Bioconductor BiomaRT (v2.52.0) database. Labeled genes were manually selected from significantly differentially expressed genes from the DESeq2 results data frame. All genes with a Benjamini-Hochberg (BH) adjusted p-value below 0.05 were considered significantly upregulated if they had a log2FC > 0, and downregulated if they had a log2FC < 0. In order to determine Gene Ontology (GO) enrichment analysis for biological processes was performed by running a list of significantly differentially expressed genes and their p-values through TopGO (v2.48.0). Enrichment score is reported as the $-\log_{10}$ of enrichment p value, based on Kolmogorov-Smirnov (KS) analysis. Significantly enriched GO Terms were selected and plotted based on biological interest. For analysis of the DAM signature, the full list of common genes upregulated (disease-associated microglia genes) and downregulated by microglia (homeostatic genes) across disease conditions in *Krasemann et al., 2017* was analyzed in our dataset, and significantly differentially expressed genes from this list were plotted. For targeted analysis of anti-parasitic genes, the full list of unique annotated genes in the GO:0042832 Term ("Defense Response to Protozoan") was analyzed within our dataset, and all significantly differentially expressed genes were plotted.

Statistics. Data from multiple experiments within a given time-point were aggregated to reflect biological variability from different infections. When data from multiple infection cohorts were compiled, a randomized block ANOVA test (two-way) was performed in R. This statistical test was selected to evaluate the effect of treatment group, while controlling for infection date as a variable that was statistically modeled as a random effect contained in our datasets. Data from Kaplan-Meier curves, flow cytometric analyses, and qPCR results were graphed using GraphPad Prism, and data related to transcriptomic analysis were graphed using R. Error bars indicate standard error of the mean (SEM). Statistical tests used for each reported experiment are detailed within figure legends. Normal distributions for each population are assumed for statistical testing.

Data Availability. All RNA sequencing data files are available from the GEO database (accession numbers GSE146680, GSE204751, and GSE203655). For graphs that visualize compiled experimental group means, data points for individual mice are available in a source data file on Dryad: doi:10.5061/dryad.fttdz08w2. The remaining data are within the manuscript and its Supporting Information files.

Chapter 3 – Microglial STAT1-sufficiency is required for resistance to toxoplasmic encephalitis

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3.1 Introduction

Toxoplasma gondii is a ubiquitous protozoan parasite that traffics to the brain, where it establishes a chronic, life-long infection in both humans and mice. Infection with *T. gondii* occurs in two phases: (i) an acute infection wherein parasite disseminates widely throughout host tissues and (ii) a subsequent chronic infection largely restricted to the immune-privileged CNS[23]. Protective immunity during both the acute and chronic phases of *T. gondii* infection converge on cellular responses to the pro-inflammatory cytokine, interferon (IFN)-γ, and its downstream signaling through the transcription factor signal transducer and activator of transcription 1 (STAT1)[246,247]. *Stat1*$^{-/-}$ mice phenocopy *Ifng*$^{-/-}$ mice, succumbing to *T. gondii* infection during the acute stage of infection[96]. Similarly, antibody blockade of IFN-γ during chronic *T. gondii* infection leads to animal mortality, illustrating that interferon signaling is critical for immune resistance during both stages of infection[26].

During both health and disease, microglia, the tissue-resident macrophages of the central nervous system (CNS), play diverse roles in maintaining tissue homeostasis and potentiating inflammation[248]. A large pool of research has focused on studying microglia during

neurodevelopmental and neurodegenerative models, yet their functional roles during CNS infection *in vivo* have been less explored. Part of this gap in understanding stems from recent findings that yolk sac-derived microglia are ontogenically, transcriptionally, and functionally distinct from the bone-marrow derived macrophage population that infiltrates the immune-privileged brain during neuroinflammatory states, including CNS infection[77,172–174,249]. Microglia have further been shown to rapidly de-differentiate and adopt an inflammatory signature when removed from the brain's unique tissue microenvironment, making *in vitro* approaches inadequate for recapitulating microglial physiology *in vivo*[164,250].

Several studies utilizing pharmacological depletion of microglia by targeting CSF1R during viral CNS infections have collectively revealed that microglia provide protection in controlling viral burden and against host mortality[187–191,237]. However, CSF1R antagonism alone also depletes macrophages systemically, with multiple studies also pointing to impaired maturation and function of myeloid populations in circulation and lymphoid organs[190,193,194]. CSF1R antagonism has also been reported to elevate baseline levels of pro-inflammatory cytokines in the brains of treated mice[195]. These local and systemic effects thus serve as confounding variables for cell type-specific functional interpretations.

Recently, our group utilized a tamoxifen-inducible cre recombinase system commonly used in neuroinflammatory mouse models to label and assay microglia independently of bone marrow-derived cells[77]. These experiments revealed that microglial but not peripheral macrophage-derived IL-1α is required for brain parasite burden control. We have extended this experimental paradigm to investigate how the microglial response to IFN-γ impacts neuro-immunity by genetically deleting *Stat1* from this cell type. Given that (i) microglia are the primary hematopoietic cell population present in the steady-state brain, and (ii) IFN-γ-STAT1 signaling is

essential for anti-parasitic functions across a wide array of cell types, we hypothesized that microglia serve as the brain's first responders in restricting early CNS infection with *T. gondii* via STAT1-mediated signaling.

Here, we report that despite efficient parasite clearance from peripheral tissues during the acute stage of *T. gondii* infection, mice with microglial-specific *Stat1* deletion succumb to severe toxoplasmic encephalitis following uncontrolled parasite replication within the brain. We also find that, despite this severe pathology observed with microglial genetic targeting, the brain-infiltrating immune compartment displays a robust anti-parasitic activation profile. Our studies thus implicate cell-intrinsic roles for microglia in controlling CNS infection with *T. gondii* and highlight an inability for the peripheral immune system to compensate for STAT1 deficiency in the microglial compartment.

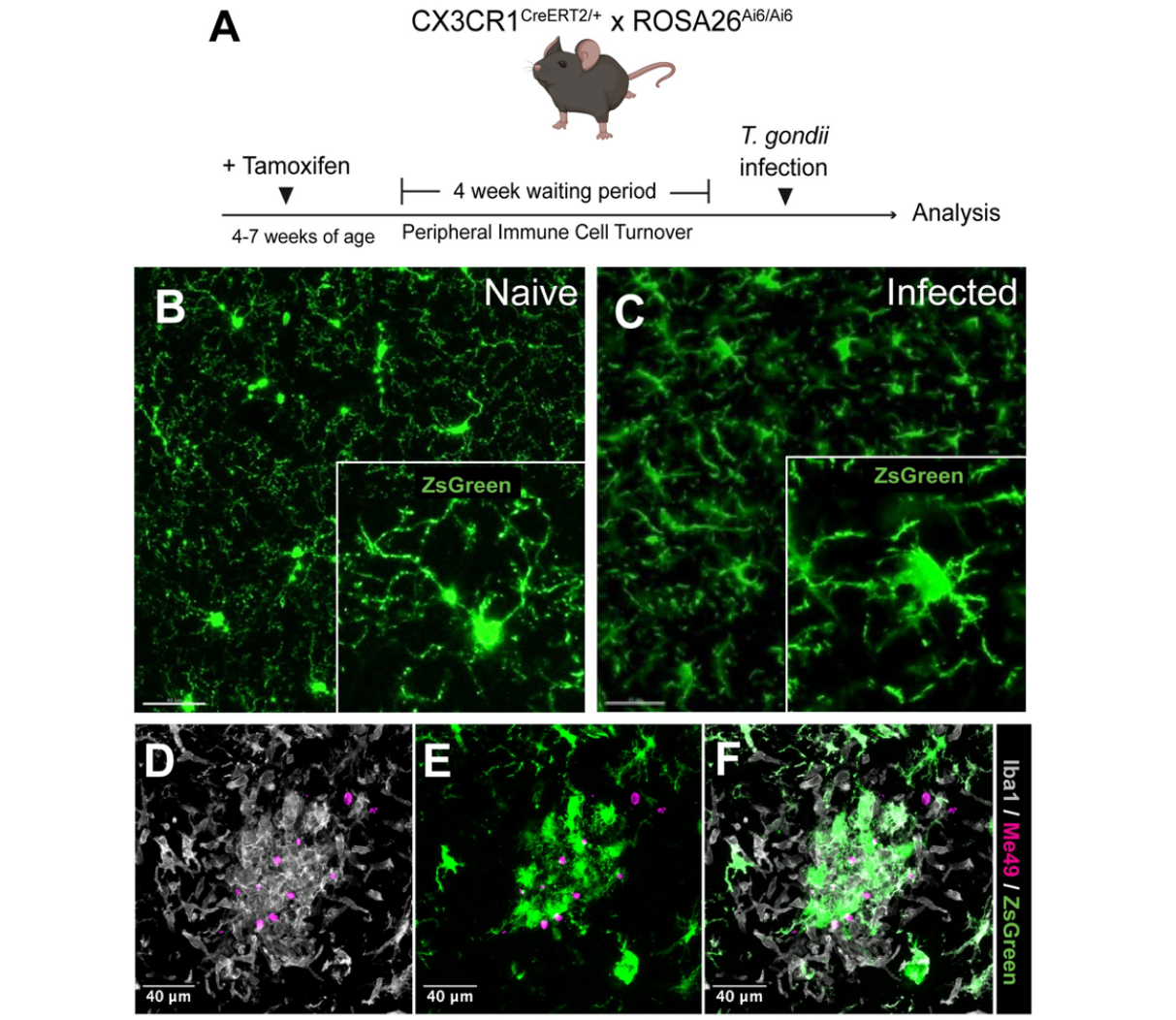
3.2 Results

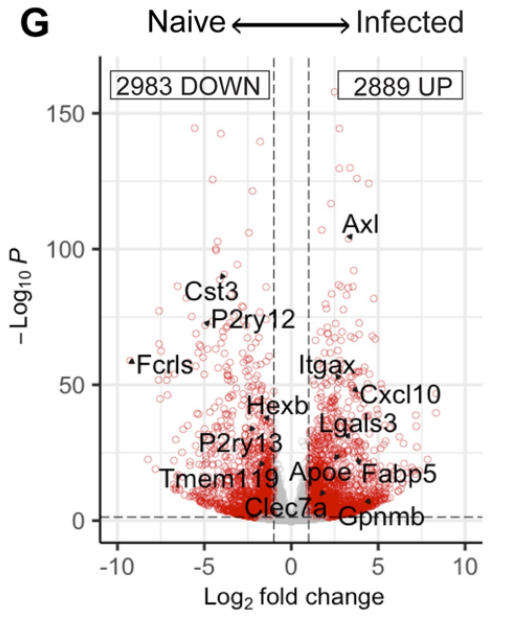
Microglial activation and recruitment to T. gondii foci within the brain.

Previous studies have revealed that myeloid cell recruitment from the blood to the *T. gondii*-infected brain are required for preventing fatal toxoplasmic encephalitis [116]. Because microglia and monocyte-derived macrophages are difficult to discriminate via immunohistochemistry, which offers spatial resolution of the infected brain, we generated CX3CR1$^{CreERT2/+}$ x ROSA26$^{Ai6/Ai6}$ (WT) mice to specifically fluorescently label microglia with ZsGreen and discriminate them from monocyte-derived macrophages in the inflamed brain parenchyma[118] (**Fig 3.1A**). We opted to use a genetic *in vivo* approach throughout our studies due to multiple studies characterizing rapid microglial de-differentiation and increased activation when removed from the CNS and analyzed in *vitro*[164,178]. Immunohistochemical analyses illustrated that

following intraperitoneal challenge and progression to chronic *T. gondii* infection, microglia attain a classic "amoeboid" morphology throughout the brain, typical of activation during neuroinflammatory states (**Fig 3.1B** and **3.1C**). We further observed disrupted microglial spatial tiling and increased microglial recruitment to foci of parasite growth (**Fig 3.1D-F**). Our fluorescent labeling approach allowed for detection and discrimination of resident microglia from infiltrating macrophages during CNS infection by both confocal microscopy and flow cytometry, permitting the interrogation of microglial functional roles during *T. gondii* challenge (**Fig 3.1B-3.1F, Supplementary Fig 1A-D**).

In order to identify transcriptional programs activated specifically by microglia during *T. gondii* challenge, we FACS-sorted and performed bulk RNA-sequencing on microglia isolated from naïve and chronically-infected mice using our ZsGreen fluorescent reporter. Differential gene expression analysis revealed an enriched IFN-γ response signature in microglia purified from infected, relative to naïve mice **(Fig 3.1G** and **3.1H)**. We identified 2,889 upregulated genes and 2,983 downregulated genes in microglia that were significantly differentially expressed during *T. gondii* challenge, relative to uninfected controls. Interestingly, we observed that during infection, microglia displayed a transcriptional activation state suggestive of a disease-associated microglia (DAM) phenotype, which is commonly associated with neurodegeneration and typified by the downregulation of microglial homeostatic genes (including *P2ry12*, *P2ry13*, *Hexb*, *Tmem119*, and *Fcrls*) and concomitant upregulation of microglial disease-associated markers (including *Itgax*, *Apoe*, *Axl*, *Clec7a*)[202,251] (**Fig 3.1G**). This phenotype emerged when we compared our list of genes differentially expressed by wild-type microglia isolated from naïve and infected mice against a core list of common genes that are differentially expressed across multiple neurodegenerative conditions, including mouse models for Alzheimer's Disease, multiple sclerosis, amyotrophic


A
CX3CR1CreERT2/+ x ROSA26Ai6/Ai6
+ Tamoxifen
4 week waiting period
T. gondii infection
Analysis
4-7 weeks of age
Peripheral Immune Cell Turnover
B
Naive
ZsGreen
C
Infected
ZsGreen
D
E
F
40 µm
40 µm
40 µm
Iba1 / Me49 / ZsGreen


G
Naive ⟷ Infected
2983 DOWN
2889 UP
−Log10 P
Log2 fold change
Axl
Cst3
P2ry12
Fcrls
Itgax
Cxcl10
Hexb
Lgals3
P2ry13
Tmem119
Apoe
Fabp5
Clec7a
Gpnmb

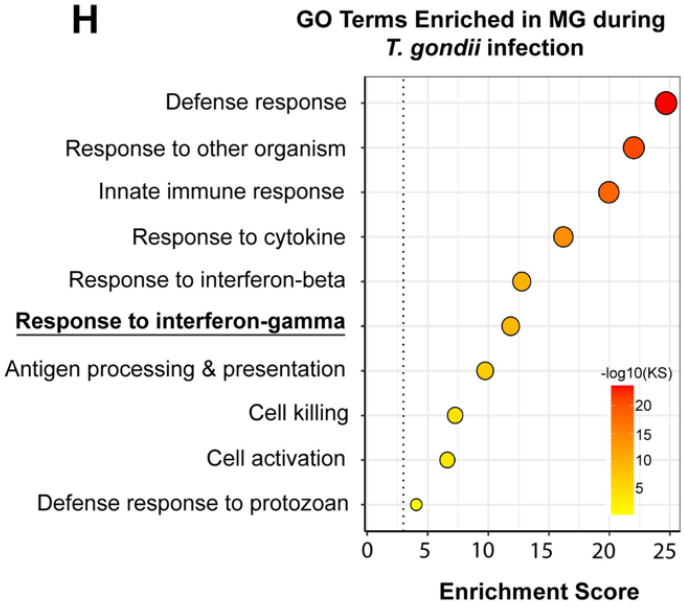

H
GO Terms Enriched in MG during T. gondii infection
Defense response
Response to other organism
Innate immune response
Response to cytokine
Response to interferon-beta
Response to interferon-gamma
Antigen processing & presentation
Cell killing
Cell activation
Defense response to protozoan
-log10(KS)
Enrichment Score

Figure 3.1. Microglial activation and recruitment *to T. gondii* foci within the brain.

WT microglia reporter mice were infected with 10 cysts of the Me49 strain of *T. gondii* and were analyzed against naïve controls using IHC and bulk RNA-sequencing. **(A)** Schematic outline for generating microglia reporter mice. **(B, C)** Representative confocal micrographs with zoomed insets of ZsGreen+ microglia (green) in naïve mice **(B)** and at 15 DPI **(C),** illustrating transition to amoeboid morphology. **(D – F)** Representative confocal micrograph of microglial / macrophage clustering in *T. gondii* foci**.** Brain sections were immuno-stained for Iba1 (gray) and Me49 (magenta). **(G)** Volcano plot indicating differentially expressed gene expression and **(H)** gene ontology terms for biological processes statistically over-represented in RNA-sequenced microglia at 28 DPI, relative to naïve state. GO terms were selected based on interest and plotted with enrichment scores that indicate the -$\log_{10}$ of enrichment *p* value, based on Kolmogorov-Smirnov (KS) analysis. Scale bars indicate 20 μm **(B – C)** or 40 μm (**D-F**). *n* = 4-5 mice per group (**G-H**).

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lateral sclerosis, and normal aging[210]. As observed in neurodegeneration models[210], we observed that during *T. gondii* challenge, microglia downregulate homeostatic genes (81% of reported genes) and upregulate DAM genes (71% of reported genes). These data illustrate strong concordance between microglial transcriptional state in *T. gondii* infection and the classical DAM phenotype observed in neurodegeneration (**Supplementary Fig 2A-B**). Given that IFN-γ-STAT1 signaling serves as a dominant pathway driving immune activation and host defense during intracellular parasitic infection[97,104,252,253], we focused our efforts on evaluating (i) how microglial *Stat1* deletion impacts host susceptibility to *T. gondii*, (ii) the systems-level effects of this deletion during *T. gondii* challenge, and (iii) a potential role for STAT1 signaling in regulating DAM activation during infection.

***Mice with STAT1-deficient microglia succumb to fatal toxoplasmic encephalitis*.**

Myeloid cells are particularly potent responders to IFN-γ through the production of reactive nitrogen species[113,114,254] and are capable of serving as antigen-presenting cells during

infections[255]. To determine if microglial function during *T. gondii* infection is a similarly essential immune program for defense, we used a *Cx3cr1*-driven tamoxifen (TAM)-inducible system to genetically delete STAT1 from brain-resident microglia, generating $MG^{STAT1\Delta}$ mice (**Fig 3.2A**).

Excision of STAT1 from microglia in $MG^{STAT1\Delta}$ mice was assessed using multiple approaches, including: (i) ZsGreen reporter expression, (ii) direct quantification of *Stat1* relative mRNA expression, and (iii) quantification of STAT1 functional readouts. On average, 99.5% of $CD45^{int}$CD11b+ microglia expressed ZsGreen across TAM-treated animals harboring the microglia reporter construct (**Supplementary Fig 3A-C**). We also observed a 70-85% knockdown in *Stat1* mRNA expression in microglia isolated from naïve tamoxifen-treated (+TAM) compared to corn oil vehicle-treated (No TAM) littermate controls by RT-qPCR (**Supplementary Fig 3D**). Because STAT1 is a key transcriptional regulator of major histocompatibility proteins[256–258], MHC II expression served as a functional readout of microglial STAT1 excision. We observed that while 98% of WT microglia expressed MHC II at 12 days post-infection (DPI), 18% of microglia from $MG^{STAT1\Delta}$ mice were MHC II+ at this time point, allowing us to use MHC II expression in microglia as a reliable readout for STAT1 excision across experiments (**Fig 3.2B, Supplementary Fig 1E-F**). In line with previous literature[118,259], these data collectively illustrate that the *Cx3cr1*-driven tamoxifen-inducible system is efficient in targeting brain-resident myeloid cells during CNS infection.

In comparison to WT reporter controls, which display robust resistance to *T. gondii*, we found that $MG^{STAT1\Delta}$ mice succumb to *T. gondii* infection, starting at 17 DPI (**Fig 3.2C**). $MG^{STAT1\Delta}$ mice also display increased brain parasite burden at 12 and 15 DPI, despite equal brain parasite burden when parasite invades the brain around 8 DPI (**Fig 3.2D-F**). Quantitative PCR of *T. gondii* genomic DNA indicated that our observed increase in parasite burden was largely restricted to the

brain, as parasite burden in lung, liver, and heart tissue revealed no difference in parasite burden at 8 DPI, around the peak of the acute peripheral infection (**Supplementary Fig 4A-C**). Furthermore, lung, liver, and heart parasite burden decreased substantially from 8 to 15 DPI, indicating robust peripheral clearance of parasite despite increased parasite burden in the CNS (**Supplementary Fig 4D-F**). Histological examination of hematoxylin and eosin-stained brain slices prepared from mice at 15 DPI additionally revealed widespread liquefactive necrosis in $MG^{STAT1\Delta}$ mice treated with tamoxifen (but not vehicle-treated littermate controls), a hallmark of parasite replication-induced tissue destruction (**Fig 3.2G-H)**.

Given the severe pathology seen in *T. gondii*-infected $MG^{STAT1\Delta}$ mice relative to controls, we analyzed several additional parameters to examine the specificity of our experimental model. We analyzed ZsGreen expression in circulating immune cells in both WT and $MG^{STAT1\Delta}$ mice by flow cytometry to identify potential off-target STAT1 deletion in brain-infiltrating immune cells. In contrast to >99% ZsGreen labeling efficiency of brain-resident microglia (**Supplementary Fig 3C**), we observed that ~1% of total circulating immune cells were ZsGreen+ at 12 DPI, and we observed low frequencies of ZsGreen expression across myeloid and T cell populations (**Supplementary Fig 3E**). No differences were observed in T cell or myeloid cell counts in circulation at this time point (**Supplementary Fig 5A-E**). As an additional parameter for evaluating the CNS tissue-specificity of our model, we analyzed the peritoneal immune response during the acute phase of infection (8 DPI). We observed no changes in the number of myeloid cells, T cells, or their iNOS and interferon-γ production at this time point, indicating that functionally equivalent protective immune responses are able to effectively form at the peripheral site of inoculation (**Supplementary Fig 5F-J**). To assess the potential for differences in microglial activation due to genetic deletion of STAT1 prior to *T. gondii* challenge, we performed

morphometric Sholl analysis of microglia in naïve WT and MG$^{STAT1\Delta}$ mice[245]. We found no statistically significant differences in microglial morphology, suggesting comparable baseline microglial activation (**Supplementary Fig 6A-C**). These findings collectively highlight that STAT1-deletion from a single cell type, brain-resident microglia, results in a CNS-specific loss of parasite restriction following infection with *T. gondii*.

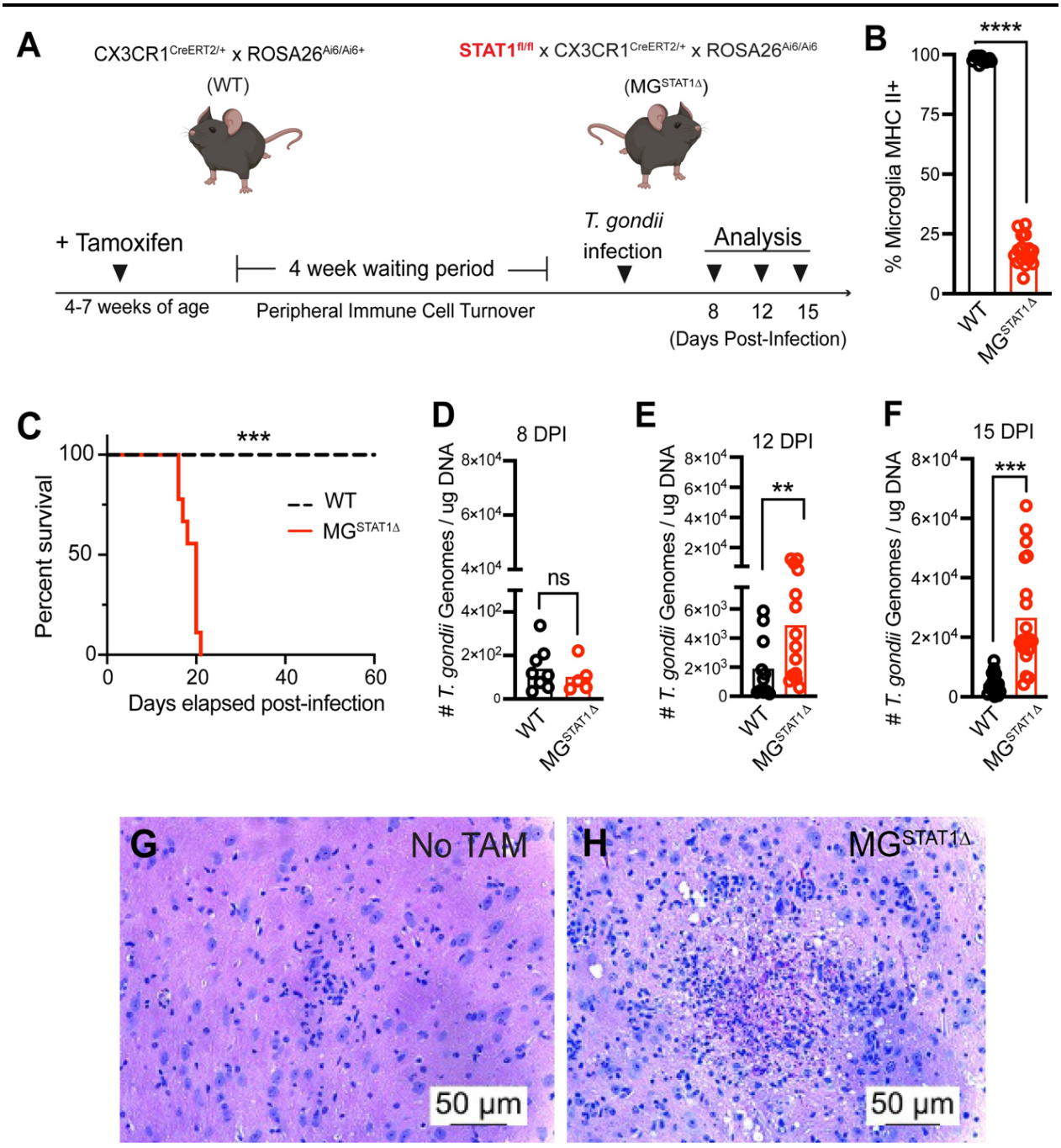

Figure 3.2. Mice with STAT1-deficient microglia succumb to fatal toxoplasmic encephalitis.

Tamoxifen-treated CX3CR1$^{CreERT2/+}$ x ROSA26$^{Ai6/Ai6}$ (WT) and STAT1$^{fl/fl}$ x CX3CR1$^{CreERT2/+}$ x ROSA26$^{Ai6/Ai6}$ (MG$^{STAT1\Delta}$) mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii* and analyzed at 8, 12, and 15 DPI. **(A)** Schematic for generating WT and MG$^{STAT1\Delta}$ mice. **(B)** Flow cytometric quantification of microglial MHC II expression at 12 DPI, as a functional readout for STAT1 excision. **(C)** Animal survival curve of WT and MG$^{STAT1\Delta}$ following *T. gondii* challenge. **(D – F)** qPCR analysis of *T. gondii* brain parasite burden quantified by qPCR analysis of homogenized brain tissue at 8- **(D)**, 12- **(E)**, and 15- **(F)** DPI. (**G, H**) Representative brain histopathology observed via H&E staining in litter-mate vehicle **(G)** or tamoxifen-treated MG$^{STAT1\Delta}$ mice at 15 DPI. ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 10^{-4}$. Statistical significance was determined by two-way randomized block ANOVA **(B, D-F)** or Kaplan-Meier analysis with n = 12-16 mice per group from 4 experiments **(B)**, n = 6-9 mice per group from 2 experiments (**C**), n = 5-8 mice per group from 2 experiments **(D)**, n = 11-15 mice per group from 3 experiments **(E)**, and n = 18-20 mice per group from 4 experiments (**F**). Scale bar = 50 um.

Brain-infiltrating myeloid cells, but not brain-resident microglia, express iNOS during *T. gondii* challenge.

Nitric oxide is a potent reactive nitrogen molecular species thought to restrict parasite replication by depleting host cell arginine[127] and targeting parasite proteases[260]. Expression of inducible nitric oxide synthase (iNOS) is the primary mechanism by which cells are capable of producing nitric oxide, and the expression of this synthase is regulated by IFN-γ-STAT1 signaling[261]. Previous studies have implicated microglia in restricting *T. gondii* replication via nitric oxide production [116,262]. Further, iNOS-deficient mice display pathology during chronic CNS but not acute peripheral infection, typified by a loss of parasite restriction and necrotizing lesions throughout the brain – suggesting that microglial-derived nitric oxide serves as a specialized anti-toxoplasmic resistance mechanism specific to brain tissue[115]. However, flow cytometric quantification of iNOS+ cells with the use of our microglia-specific fluorescent reporter indicated that brain-resident microglia fail to express iNOS *in vivo*, regardless of STAT1-sufficiency (**Fig**

3.3A, 3.3E and **3.3I**). Instead, we found that > 95% of iNOS expression in the brain is accounted for by the brain-infiltrating myeloid compartment, based on $CD45^{hi}$ $CD11b^{+}$ $Ly6C^{+}$ expression (**Fig 3.3C** and **Supplementary Fig 1G-I)**. These findings underscore that microglia are not producers of this STAT1-driven mechanism despite a shared tissue micro-environment that triggers potent iNOS expression in other myeloid cells. In addition to the absence of microglial iNOS expression, we quantified and detected no significant changes in microglial number from the brains of WT and $MG^{STAT1\Delta}$ mice at 12 or 15 DPI across several experiments (**Fig 3.3D** and **3.3H**).

Microglial STAT1 deletion does not impair the global CNS immune response to T. gondii.

To examine the potential for microglial STAT1 deletion to impair the development of anti-parasitic effector mechanisms in the brain-infiltrating immune compartment of $MG^{STAT1\Delta}$ mice, we immunophenotyped both infiltrating-myeloid and T cell populations. Previous studies have indicated that both monocyte-derived cells and T cells must traffic into the CNS to prevent fatal toxoplasmic encephalitis[26,116]. This concept is clearly illustrated by studies revealing that antibody blockade of CD4+ and CD8+ T cells or monocytic CCR2 results in an inability for these protective immune populations to enter the brain and a subsequent loss of parasite control and animal mortality during chronic CNS infection with *T. gondii*[26,116].

Brain-infiltrating myeloid cells.

At 12 DPI, we observed no difference in the number of brain-infiltrating myeloid cells based on $CD45^{hi}$ $CD11b^{+}$ $Ly6C^{+}$ expression, indicating that circulating myeloid cells are able to effectively traffic to the *T. gondii*-infected brain despite microglial STAT1-deletion (**Fig 3.3F**). We further found a statistically significant increase in the iNOS expression of these myeloid cells,

suggestive of increased, rather than decreased, activation and effector functions of the infiltrating myeloid compartment in MG$^{STAT1\Delta}$ mice relative to WT controls (**Fig 3.3G**). At 15 DPI, there was a clear increase in brain-infiltrating myeloid cell recruitment to the brain, with a further increase in their ability to express the anti-parasitic effector protein, iNOS (**Fig 3.3J-K**).

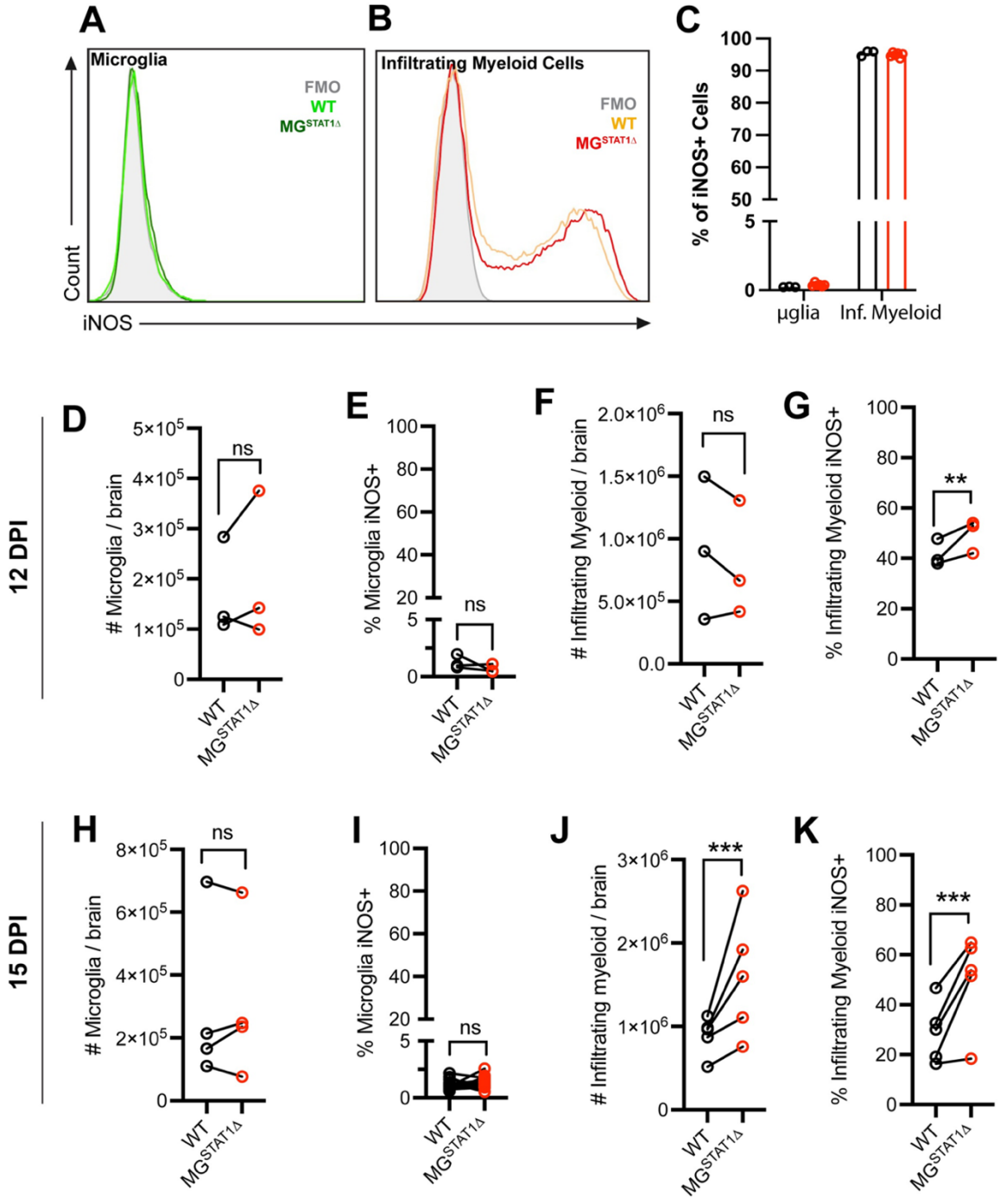

Figure 3.3. Brain-infiltrating myeloid cells, but not brain-resident microglia, express iNOS during *T. gondii* challenge.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii* and brains were analyzed by flow cytometry. Representative histograms indicating iNOS expression in ZsGreen+ CD11b+ CD45int brain-resident microglia **(A)** and CD11b+ CD45hi Ly6C+ infiltrating myeloid cells **(B)** at 12 DPI. **(C)** Flow cytometric quantification of the total brain iNOS+ cells by myeloid population at 12 DPI. 12 DPI analysis of total number of microglia isolated per brain **(D)**, microglial iNOS frequency **(E)**, number of infiltrating myeloid cells isolated per brain **(F),** and infiltrating myeloid iNOS frequency **(G).** 15 DPI quantification of total number of microglia isolated per brain **(H)**, microglial iNOS frequency **(I)**, number of infiltrating myeloid cells isolated per brain **(J),** and infiltrating myeloid iNOS frequency **(K)**. ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 10^{-4}$. Statistical significance was determined by unpaired *t* test (**C**) or two-way randomized block ANOVA (**D-K**). *n* = 3-5 (**C**), *n* = 9-10 (**D & F**), *n* = 9-13 (**E & G**), *n* = 15-16 (**H-I**), and *n* = 18-20 (**J-K**) mice per group**. (B-K)** Biological replicates are individual mice, with group means from individual experiments plotted as open circles with black lines connecting experimental and control groups. Data are pooled from 3 experiments (**D-G**), 4 experiments (**H-I**), or 5 experiments (**J-K**). Source data (**D-K**) are provided in a source data file.

Brain-infiltrating CD4+ and CD8+ T cell activation.

In contrast to most peripheral macrophage populations, only a small subset (< 3%) of brain-resident microglia express the professional antigen presentation molecule, MHC II, at baseline[217]. Because STAT1 is a primary transcriptional regulator of MHC II expression [256–258], we investigated MHC II expression on microglia, along with the expression of additional antigen presentation-related molecules (MHC I, CD80, and CD86) that are required for antigen-specific T cell activation [256,257]. In naïve mice, we find that few microglia express any of these antigen presentation molecules (**Supplementary Fig 7A**). During *T. gondii* infection, however, the expression of MHC I, MHC II, and CD86 was upregulated by microglia in a STAT1-dependent manner (**Supplementary Fig 7A-D**). Despite the STAT1-dependency of these antigen presentation molecules, we quantified no impairment in T cell activation at multiple time-points during infection in MG$^{STAT1\Delta}$ mice. At 12

DPI, MG$^{STAT1\Delta}$ mice displayed no statistically significant changes in $CD3^+CD4^+$ or $CD3^+CD8^+$ T cell numbers within the brain, and they showed a two-fold increased frequency of IFN-γ+ expression of both T cell sub-types by flow cytometry, relative to WT controls (**Fig 3.4A-D** and **Supplementary Fig 1J-M**). By 15 DPI, MG$^{STAT1\Delta}$ mice had a three- and two-fold increase in $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell numbers in the brain, respectively, with an increased frequency of CD4+ T cell IFN-γ production (**Fig 3.4E-H**). To analyze antigen-specific T cell responses, we used a tetramer approach to quantify CD4+ T cells specific for the *T. gondii* peptide AS15 by flow cytometry[263]. We observed a statistically significant increase in the number of AS15 tetramer CD4+ T cells in MG$^{STAT1\Delta}$ mice, relative to WT controls (**Supplementary Fig 8A-C**). Collectively, analyses of the T cell presence and functional responses in MG$^{STAT1\Delta}$ mice indicate increased, rather than decreased, T cell activation. Further, these results do not suggest that a loss of microglial antigen presentation machinery underpinned the inability to restrict parasite burden in MG$^{STAT1\Delta}$ brains.

Whole brain RNA analysis.

We performed RT-qPCR analysis of mRNA isolated from homogenized brain tissue to examine the expression of a larger panel of immune mediators of resistance against *T. gondii*. In addition to increased *Nos2* (iNOS) gene expression at the tissue-level, MG$^{STAT1\Delta}$ mice showed an overall equivalent (12 DPI) or increased (15 DPI) expression of several chemokines (*Ccl2, Ccl5, Cxcl9, Cxcl10*), pro-inflammatory cytokines (*Ifng*, *Il6*, *Tnfa*), and adhesion molecules (*Icam1*, *Vcam1*) identified in previous literature as conferring resistance against *T. gondii* infection[26,77,106,264–268] (**Supplementary Fig 9A-B**). Together, these data indicate that a robust set of anti-parasitic immune mediators are present at the tissue-level, and suggest that neither the brain-

infiltrating immune compartment nor other brain-resident cell types are able to compensate for a STAT1 signaling defect within the brain's microglial compartment. We thus focused further efforts on identifying microglial-intrinsic mechanisms of *T. gondii* restriction.

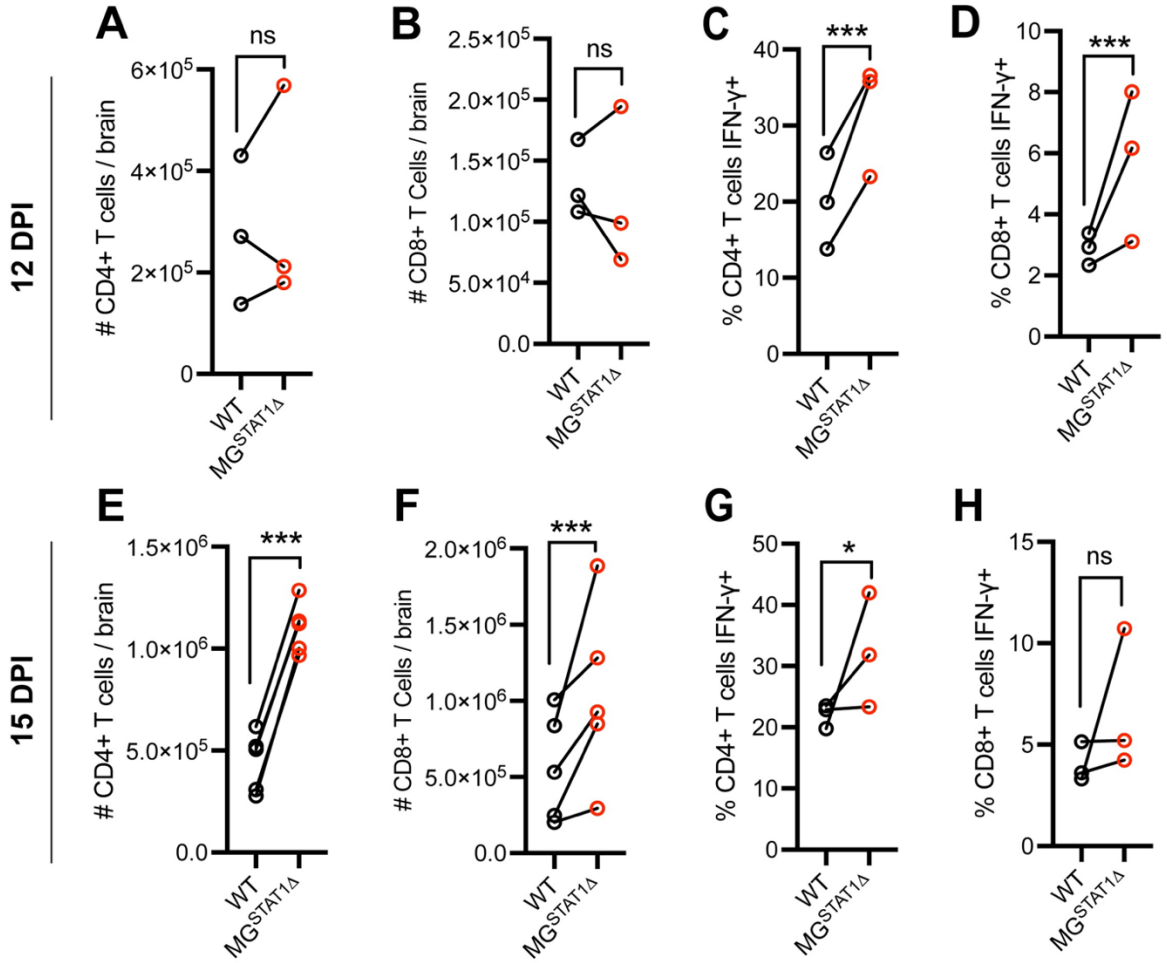

Figure 3.4. Brain T cell responses are increased in MG$^{STAT1\Delta}$ mice relative to WT controls.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii* and brains were analyzed by flow cytometry at 12 and 15 DPI. (**A-B**) CD3+CD4+ and CD3+CD8+ cell count enumerated for whole brain and (**C–D**) IFN-γ protein expression at 12 DPI. (**E-F**) CD3+CD4+ and CD3+CD8+ cell count enumerated for whole brain and (**G-H**) IFN-γ protein expression at 15 DPI. *ns* = not significant, and *** = $p < 0.001$. Statistical significance was determined by two-way randomized block ANOVA. Data are pooled from 3 (**A-D** & **G-H**), or 4 (**E-F**) separate experiments, with $n = 9$-10 (**A-B**), $n = 11$-13 (**C-D**), $n = 12$-16 (**E-F**), and $n = 9$-12 (**G-H**) mice per group. Biological replicates are individual mice, with group means from individual experiments plotted as open circles with black lines connecting experimental and control groups. Source data (**A-H**) are provided in a source data

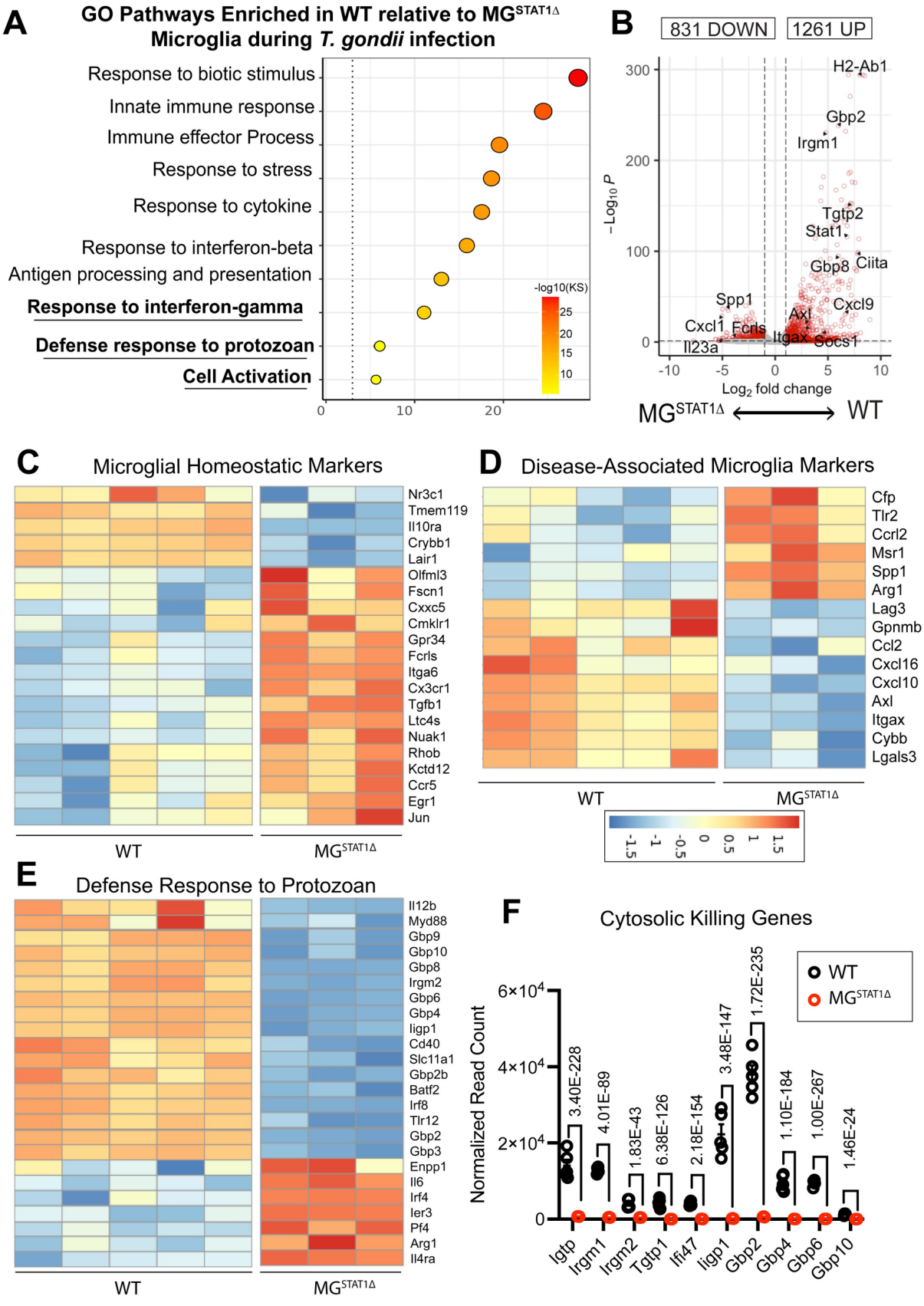
A
GO Pathways Enriched in WT relative to MG^STAT1Δ Microglia during T. gondii infection
Response to biotic stimulus
Innate immune response
Immune effector Process
Response to stress
Response to cytokine
Response to interferon-beta
Antigen processing and presentation
Response to interferon-gamma
Defense response to protozoan
Cell Activation
-log10(KS)
25
20
15
10
0
10
20
B
831 DOWN
1261 UP
H2-Ab1
Gbp2
Irgm1
Tgtp2
Stat1
Gbp8
Ciita
Spp1
Cxcl9
Axl
Cxcl1
Fcrls
Itgax
Socs1
Il23a
-Log10 P
Log2 fold change
MG^STAT1Δ
WT
C
Microglial Homeostatic Markers
Nr3c1
Tmem119
Il10ra
Crybb1
Lair1
Olfml3
Fscn1
Cxxc5
Cmklr1
Gpr34
Fcrls
Itga6
Cx3cr1
Tgfb1
Ltc4s
Nuak1
Rhob
Kctd12
Ccr5
Egr1
Jun
WT
MG^STAT1Δ
D
Disease-Associated Microglia Markers
Cfp
Tlr2
Ccrl2
Msr1
Spp1
Arg1
Lag3
Gpnmb
Ccl2
Cxcl16
Cxcl10
Axl
Itgax
Cybb
Lgals3
WT
MG^STAT1Δ
-1.5
-1
-0.5
0
0.5
1
1.5
E
Defense Response to Protozoan
Il12b
Myd88
Gbp9
Gbp10
Gbp8
Irgm2
Gbp6
Gbp4
Iigp1
Cd40
Slc11a1
Gbp2b
Batf2
Irf8
Tlr12
Gbp2
Gbp3
Enpp1
Il6
Irf4
Ier3
Pf4
Arg1
Il4ra
WT
MG^STAT1Δ
F
Cytosolic Killing Genes
WT
MG^STAT1Δ
Normalized Read Count
6×10^4
4×10^4
2×10^4
0
3.40E-228
4.01E-89
1.83E-43
6.38E-126
2.18E-154
3.48E-147
1.72E-235
1.10E-184
1.00E-267
1.46E-24
Igtp
Irgm1
Irgm2
Tgtp1
Ifi47
Iigp1
Gbp2
Gbp4
Gbp6
Gbp10

Figure 3.5. STAT1-deficient microglia fail to upregulate genes encoding critical anti-parasitic cytosolic proteins.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii*, and brain-resident microglia were FACS-sorted and RNA-sequenced at 12 DPI. (**A**) Gene ontology (GO) terms statistically enriched in WT relative to MG$^{STAT1\Delta}$ microglia. GO terms were selected based on interest and plotted enrichment scores indicate the -$\log_{10}$ of enrichment p value, based on Kolmogorov-Smirnov (KS) analysis. (**B**) Volcano plot indicating differential gene expression between microglia isolated from WT and MG$^{STAT1\Delta}$ mice. (**C-D**) Heat maps displaying VST-normalized, hierarchically-clustered significantly differentially expressed microglial homeostatic genes (**C**) and disease-associated microglia genes (**D**) previously reported as markers of disease-associated microglia (DAM) across neuroinflammatory conditions. (**E**) Heatmap displaying genes from the GO Term "Defense response to protozoan" (GO:0042832) in WT vs. MG$^{STAT1\Delta}$ mice. (**F**) Normalized read count of IRG- and GBP-family proteins expressed by microglia and differentially regulated by STAT1, with *p* values indicating BH-adjusted *p*-values from the full gene expression analysis in DESeq2. *n* = 3-5 mice per group (**A-F**).

STAT1-deficient microglia display transcriptionally impaired cell-intrinsic immune activation and effector capacity.

In order to attain an unbiased transcriptional overview of how STAT1 regulates microglial immune effector capacity, we FACS-sorted and performed RNA-sequencing on brain-resident microglia from *T. gondii*-infected WT and MG$^{STAT1\Delta}$ mice. (**Fig 3.5A-F**). Gene ontology (GO) analysis for biological pathways suggested that relative to WT microglia, STAT1-deficient microglia failed to display the transcriptional signatures of immune defense responses, with GO terms including "response to interferon" and "defense response to protozoan" (**Fig 3.5A**). Paired differential gene expression analysis indicated that 1,261 genes were significantly enriched in WT microglia, and 831 genes were significantly enriched in STAT1-null microglia (**Fig 3.5B**). Two key themes emerged from our differential gene expression analysis of microglia isolated from *T. gondii*-infected brains: (i) STAT1-null microglia display an impaired ability to acquire a disease-

associated microglia (DAM) phenotype observed across several neuroinflammatory disease states[202,251], and (ii) these cells further fail to express key cytosolic genes required for killing intracellular parasite in a cell-intrinsic manner.

STAT1-deficient microglia fail to acquire a cell activation phenotype conserved across diverse neuroinflammatory states.

Given our observations that microglia in the *T. gondii*-infected brain display strong transcriptional overlap with DAMs observed during neurodegeneration, we sought to determine whether STAT1 signaling regulated the DAM phenotype (**Fig 3.1G, Supplementary Fig 2**). Similarly to our transcriptomic analysis of WT naïve vs. infected microglia, we compared our WT vs $MG^{STAT1\Delta}$ differential gene expression dataset to a dataset of DAM genes shared across several neurodegenerative disease states [202]. Because the DAM phenotype is characterized by both the downregulation of microglial homeostatic genes and the upregulation of unique disease-associated markers during neuroinflammation, we explored both features of the transcriptional signature. We observed during *T. gondii* challenge that relative to wildtype microglia, STAT1-deficient microglia: (i) failed to downregulate 23.5% of all microglial "homeostatic genes" (including *Cx3cr1*, *Tgfb1*, and *Fcrls*) and (ii) failed to upregulate 32% of all DAM-specific genes (including *Itgax*, *Axl*, *Gpnmb*, and *Cybb*) reported in *Krasemann et al. 2017* (**Fig 3.5C-D**). In addition to highlighting that STAT1-deficient microglia retain a more homeostatic and less inflammatory transcriptional signature relative to controls during *T. gondii* infection, these data point to STAT1 signaling as an integral component of a shared microglial transcriptional profile that is conserved across varying models of neuroinflammation.

STAT1-deficient microglia fail to upregulate genes encoding critical anti-parasitic cytosolic proteins.

To determine specific STAT1-dependent mechanisms that could explain a functional loss of parasite restriction in $MG^{STAT1\Delta}$ mice, we performed further differential gene expression analysis. We analyzed each of the genes within the Gene Ontology term, "Defense response to protozoan" (GO:0042832) and found that 44% of annotated genes were expressed at significantly lower levels in STAT1-null microglia (**Fig 3.5E**). Amongst the most highly differentially expressed genes between WT and $MG^{STAT1\Delta}$ microglia, we observed robust STAT1-dependent expression of several cytosolic anti-parasitic killing genes (**Fig 3.5E-F**). These genes included immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs), both families of which have been implicated in cooperatively contributing to the mechanical killing and intracellular clearance of *T. gondii* from both hematopoietic and non-hematopoietic cell types[138,143,146,150,269]. We identified several additional IRGs (*Igtp*, *Irgm1*, *Tgtp1*, *Ifi47*) and *Gbp10* that were expressed in microglia in a STAT1-dependent manner (**Fig 3.5F**). These data identifying the lack of large families of cell-intrinsic parasite killing genes, in tandem with a lack of observable effects of microglial STAT1 deletion on the global, cell-extrinsic immune response, support the hypothesis that cell-intrinsic parasite killing may serve as a primary mechanism of STAT1-dependent microglial parasite restriction. In the absence of this cytosolic restriction, we hypothesized that microglial STAT1-deficiency would generate a replicative niche in which *T. gondii* can expand.

Microglial STAT1-deficiency results in a skewing of T. gondii toward its replicative form.

Consistent with the hypothesis that microglial STAT1 deletion provides a replicative niche for the parasite, we observed increased microglial co-localization with large foci of reactive *T.*

gondii lesions in the brains of MG$^{STAT1\Delta}$ mice, which has been previously described as characteristic of the highly-replicative and lytic tachyzoite form of *T. gondii,* rather than its semi-dormant bradyzoite (cystic) form[231] (**Fig 3.6A-B**). We then analyzed the prevalence and ratio of these two infectious forms of the parasite in MG$^{STAT1\Delta}$ and WT mice via RT-qPCR. Three primers were designed for *T. gondii*-specific genes: (i) *Sag1*, a gene expressed selectively in tachyzoites (replicative form); (ii) *Bag1*, expressed by slowly replicating bradyzoites (quiescent form); and (iii) *Act1*, expressed by both forms of *T. gondii*[270–272]. While WT brains displayed a one-fold increase in *Bag1* to *Act1* ratio relative to MG$^{STAT1\Delta}$ brains, we observed a nearly 150-fold increase in the tachyzoite gene *Sag1* normalized to *Act1* at 15 DPI in MG$^{STAT1\Delta}$ relative to WT brains, and thus a 300-fold increase in the ratio of *Sag1* relative to *Bag1* with STAT1-deficiency (**Fig 3.6C-E**). These data confirm that relative to WT controls, MG$^{STAT1\Delta}$ brains are highly skewed toward a replicative and lytic form of *T. gondii*. Further consistent with microglial STAT1-deletion permitting a parasite replicative niche, we were able to identify microglia filled with tachyzoite rosettes via confocal microscopy in the brains of MG$^{STAT1\Delta}$ but not WT mice (**Fig 3.6F-G**).

3.2 Discussion

Here, we find that abrogation of STAT1 signaling in brain-resident microglia results in a severe susceptibility to CNS infection with *T. gondii*, despite robust immune effector functions in the brain-infiltrating immune compartment and efficient parasite clearance in peripheral tissues – highlighting a requirement for microglial-intrinsic parasite control. Importantly, we show that contrary to interpretations from previous literature[262], microglia do not express iNOS during *in vivo T. gondii* challenge. Instead, transcriptomic analysis suggests that a loss of STAT1-regulated cytosolic killing genes, including those from the IRG and GBP superfamilies, may normally play

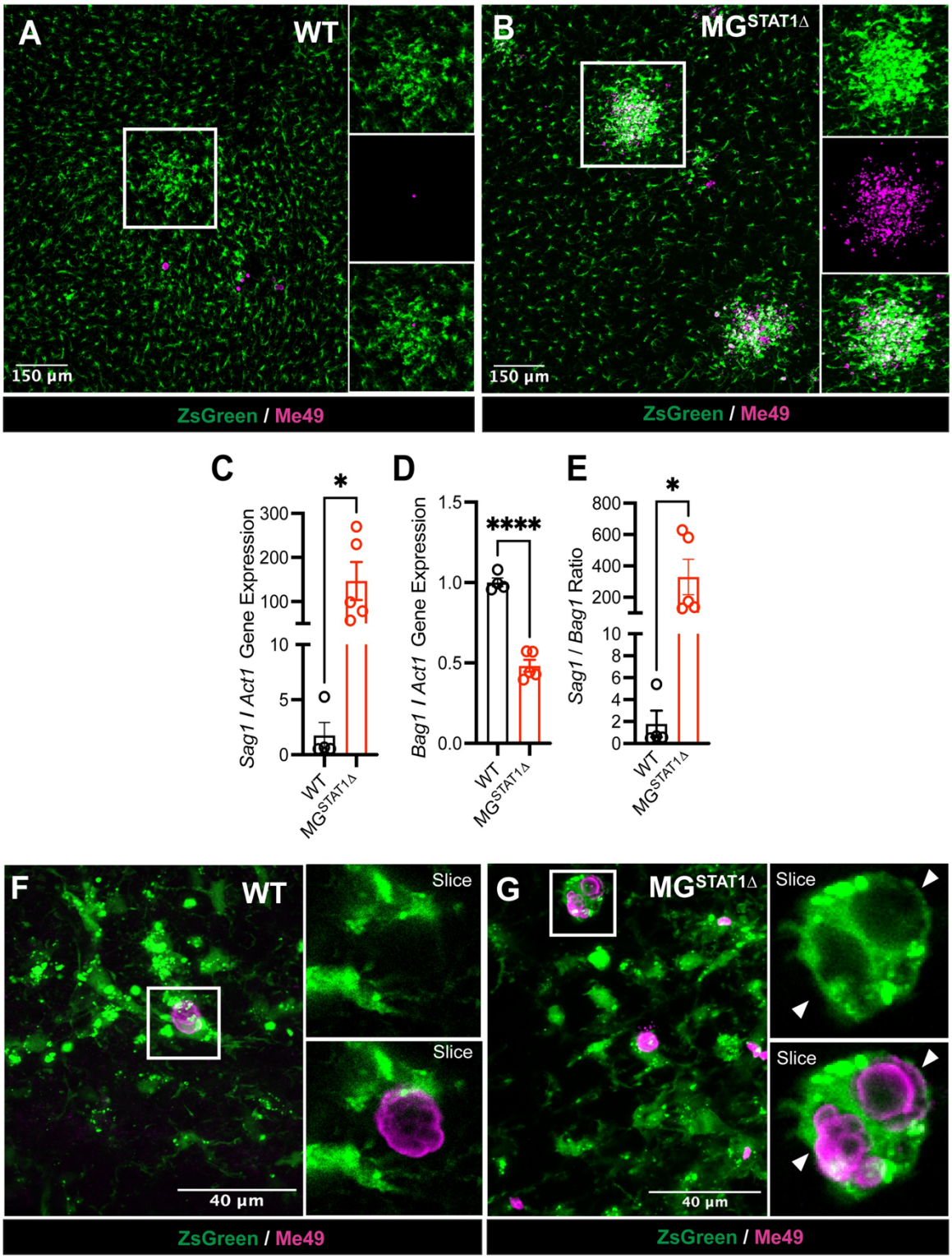

Figure 3.6. Microglial STAT1-deficiency results in a skewing of *T. gondii* toward its replicative form.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii* and brains were analyzed by confocal microscopy and RT-qPCR at 15 DPI. (**A-B**) Representative 10x immunofluorescent confocal Z-stack images of *T. gondii* inflammatory foci in infected brains. White square indicates example foci, with insets providing zoomed detail. (**C-E**) RT-qPCR analysis of *T. gondii* relative gene expression of *Sag1* (tachyzoite-specific gene), *Bag1* (bradyzoite-specific gene), and *Act1* (non-stage-specific gene) analyzed using *t* tests. (**F-G**) Representative 40x Immunofluorescent images of microglial-parasite interactions in infected brains. White square indicates *T. gondii* vacuole(s) in maximum projection view, with insets providing zoomed detail in single-plane view (Slice). (**A-B**) and (**F-G**) ZsGreen is indicated green, and anti-Me49 staining is indicated in magenta. Scale bar = 150 μm (**A-B**) and 40 μm (**F-G**). n = 3-4 mice per group (**C-E**). ns = not significant, * = $p < 0.05$, *** = $p < 0.001$.

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a role in preventing microglia from serving as a cellular niche in which *T. gondii* can freely replicate. Consistent with this model, we observe that STAT1-deficiency in microglia results in an increased brain parasite burden and a skewing of parasite towards its fast-replicating and lytic tachyzoite form within the brain.

As with any study, there are caveats and limitations that must be appropriately considered in interpreting the presented data. Importantly, the CX3CR1^{CreERT2} system allows for substantial turnover of short-lived circulating immune cells [118,121] but is expected to additionally target other tissue-resident macrophage populations[121,181]. Experiments were performed to verify effective parasite clearance and immunity in peripheral tissues, but there exists a relatively small but long-lived CX3CR1+ population of border-associated CNS macrophages (BAMs), located along CNS interfaces[181]. We expect that tamoxifen treatment may yield STAT1-deficient BAMs, such as perivascular and choroid plexus macrophages, which may be spatially positioned to encounter *T. gondii* before it breaches into the brain parenchyma. While we did not observe increased parasite burden at 8 DPI, when parasite is seeding the brain between MG$^{STAT1\Delta}$ and WT mice, future

experiments are necessary to determine whether BAMs play an active role in parasite restriction, and whether these roles are similar to those of microglia.

While STAT1 is activated downstream of multiple interferons and IFN-γR activation was not directly investigated in the current study, multiple lines of evidence support the hypothesis that a defect in the ability for microglia to respond to IFN-γ is the primary driver of the pathology observed in $MG^{STAT1\Delta}$ mice during *T. gondii* challenge. IFN-γ is essential during both acute and chronic *T. gondii* infection, with STAT1-/- mice closely phenocopying IFN-γ-/- mice in uniformly succumbing during the acute phase of infection[96], and pharmacological depletion of IFN-γ specifically during chronic infection similarly resulting in uniform lethality[26]. In contrast to IFN-γ, IFN-α/β appear to carry a more limited protective role during *T. gondii* infection[96,101]. In an oral model of infection, global genetic deletion of their shared receptor, IFNAR, results in increased brain parasite burden but more modest levels of animal mortality (~50% mortality in IFNAR-/- vs. ~20% mortality in wild-type controls by 50 DPI)[101]. Together, this literature supports a predominant role for IFN-γ-STAT1 signaling in the current study. However, future experiments will be necessary to determine to what extent IFN-α/β, or other potential activators of STAT1 such as IL-27 and IL-6[273–276], could play compounding roles or are independently capable of shaping the microglial response to *T. gondii* infection. Lastly, our RNA-sequencing data did not suggest appreciable levels of IFN-λR on microglia, suggesting limited relevance of type III interferons to this cell type (**Fig A2**).

Our finding that STAT1-deficiency in microglia leads to a 300-fold increase, relative to WT controls, in the replicative tachyzoite form of *T. gondii*, is well-framed within the context of a previous study that examined the role of STAT1 signaling in astrocytes[152]. We observe multiple similarities between our data and this study: (i) increased animal mortality, (ii) increased brain

parasite burden, (iii) liquefactive necrosis throughout the brain, and (iv) a loss of IRG and GBP expression with concomitant susceptibility of the targeted STAT1-deficient cell type to *T. gondii* parasitization. Our study dovetails with this previous publication in illustrating the essential role of STAT1 signaling in both hematopoietic and non-hematopoietic cell types, and suggests that many different cell types may be able to serve as replicative niches for this opportunistic pathogen in the absence of STAT1. However, there are important differences observed between microglial and astrocytic STAT1 deletion during *T. gondii* infection that may be informative in better understanding cell type-parasite interactions.

First, the animal mortality observed in $MG^{STAT1\Delta}$ mice in our study occurs more rapidly (~17 DPI) than as reported with astrocytic STAT1-deletion (~25 DPI), and with greater penetrance, indicating more severe disease pathology[152]. Second, $MG^{STAT1\Delta}$ mice display increased, rather than equivalent, T cell and infiltrating myeloid functional responses[152]. Third, astrocytic STAT1-deletion appears to promote the bradyzoite form of *T. gondii*, whereas $MG^{STAT1\Delta}$mice show strong bias toward the tachyzoite form of *T. gondii*[152]. Different physiological properties of these two glial populations may offer insights into these key differences in phenotype. Microglia, relative to astrocytes, are highly motile cells with a complex sensome that drives chemotactic responses to tissue damage or disruption[161,199,230,277], as is evident in our confocal images of microglia abandoning their evenly-tiled territories and clustering around *T. gondii* tachyzoites at multiple time-points, regardless of STAT1-sufficiency. Rapid migration of STAT1-sufficient microglia to sites of lytic tachyzoite egress may thus position these cells to serve as an immunological "cellular buffer" – becoming actively infected and subsequently clearing intracellular infection via STAT1-dependent cytosolic killing molecules. In the absence of STAT1 signaling, this chemotactic response may contrastingly permit the parasite to be more rapidly

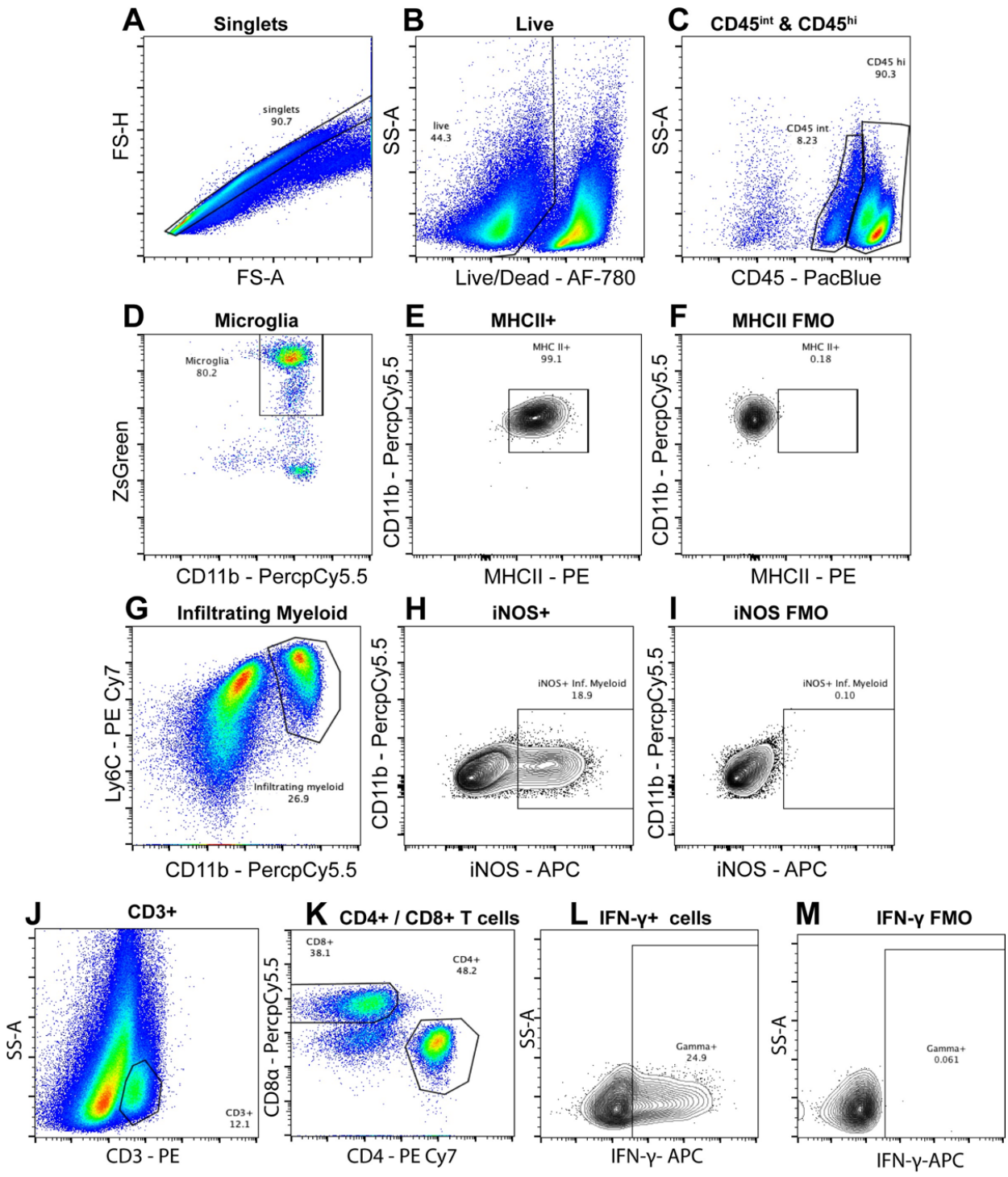
passaged through a hospitable cell type that is unable to mount a cell-intrinsic immune response, thus potentially overwhelming brain tissue. Future studies will be needed to understand if the observed skewing of parasite form toward its tachyzoite state reflects an inability for tachyzoites to convert to bradyzoites due to intrinsic properties of microglia, or alternatively reflect an interaction of STAT1 signaling abrogation with unknown biological factors of early-chronic infection (15 DPI).

While the precise determinants of tachyzoite relative to bradyzoite enrichment in $MG^{STAT1\Delta}$ mice remain unclear, the observation that brain-infiltrating T cell and myeloid responses are increased in $MG^{STAT1\Delta}$ relative to control mice may best be explained as a consequence of increased brain parasite burden, rather than a direct result of microglial STAT1 deletion. We expect that increased parasite burden stemming from impaired microglia-mediated tachyzoite killing would lead to increased T cell stimulation due to an increase in antigen availability in the brain that can be presented by STAT1-competent infiltrating myeloid cells. Consistent with this view, we observed an increase in *T. gondii* antigen-specific CD4+ T cells in the brain, measured by flow cytometry using an MHC II tetramer, as well as increased CD4+ and CD8+ T cell IFN-γ production. Moreover, increased parasite replication and egress, a lytic process[278], may also increase the release of inflammatory signals such as alarmins that drive further recruitment of immune cells from circulation[76,77]. The inability of a robustly activated brain-infiltrating immune compartment to compensate for microglial STAT1 deficiency thus underscores the necessity for STAT1 signaling in brain-resident populations during *T. gondii* challenge.

Our data also provide multiple insights to a broader understanding of microglial identity and their physiology across disease states. While most macrophages typically display remarkable plasticity attuned to their tissue microenvironments[172,279,280], the observation that microglia do not

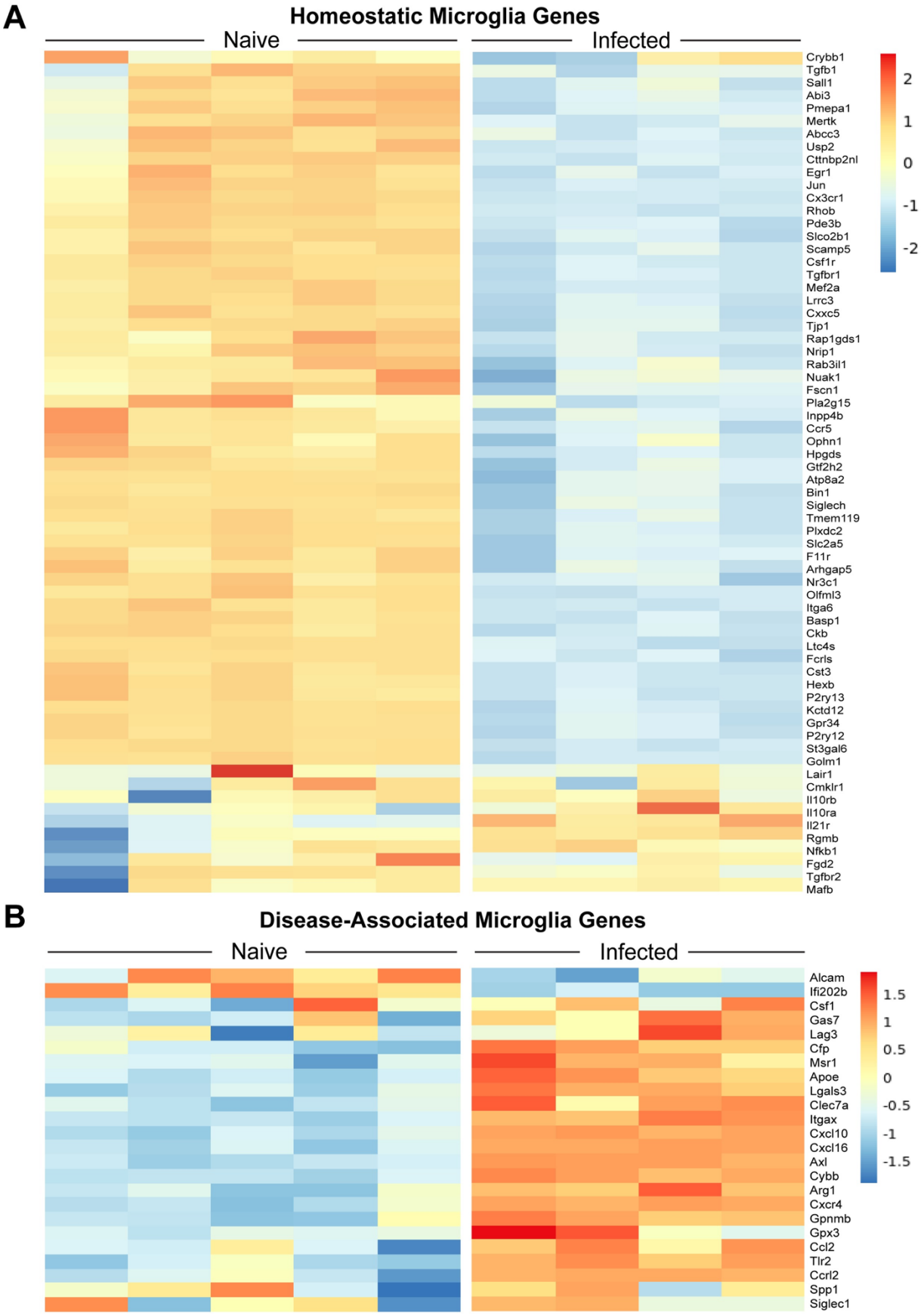
express iNOS, despite sharing an interferon-primed environment with iNOS+ infiltrating myeloid cells, indicates that these two cell populations provide differential immune effector functions during *T. gondii* infection. This differential expression of immune effector mechanisms mirrors previous work from our lab showing that microglia, unlike infiltrating macrophages: (i) express low levels of IL-1β during chronic infection, and (ii) display a comparatively dampened NF-κB signature during *T. gondii* challenge[77]. In the present study, we also find that microglia acquire a neurodegenerative-like DAM signature during *T. gondii* infection, and that this signature is partially regulated by STAT1 signaling during parasitic infection. While caution must be exercised in attributing the DAM phenotype to parasite restriction, this gene expression signature highlights an inability of STAT1-deficient microglia to acquire the cell type-specific transcriptional hallmarks of immune activation conserved across other neuroinflammatory models. Future studies targeting genes such as TREM2 or APOE, which have been shown to regulate the microglial transition to DAMs, may be needed to discern whether DAMs are, in themselves, neuroprotective during CNS infection. Similarly, future experiments using $MG^{STAT1\Delta}$ mice in models of neurodegeneration may additionally yield novel insights into how the microglial response to interferon shapes or mitigates disease progression in a wide range of disease models.

3.3 Supplementary Figures



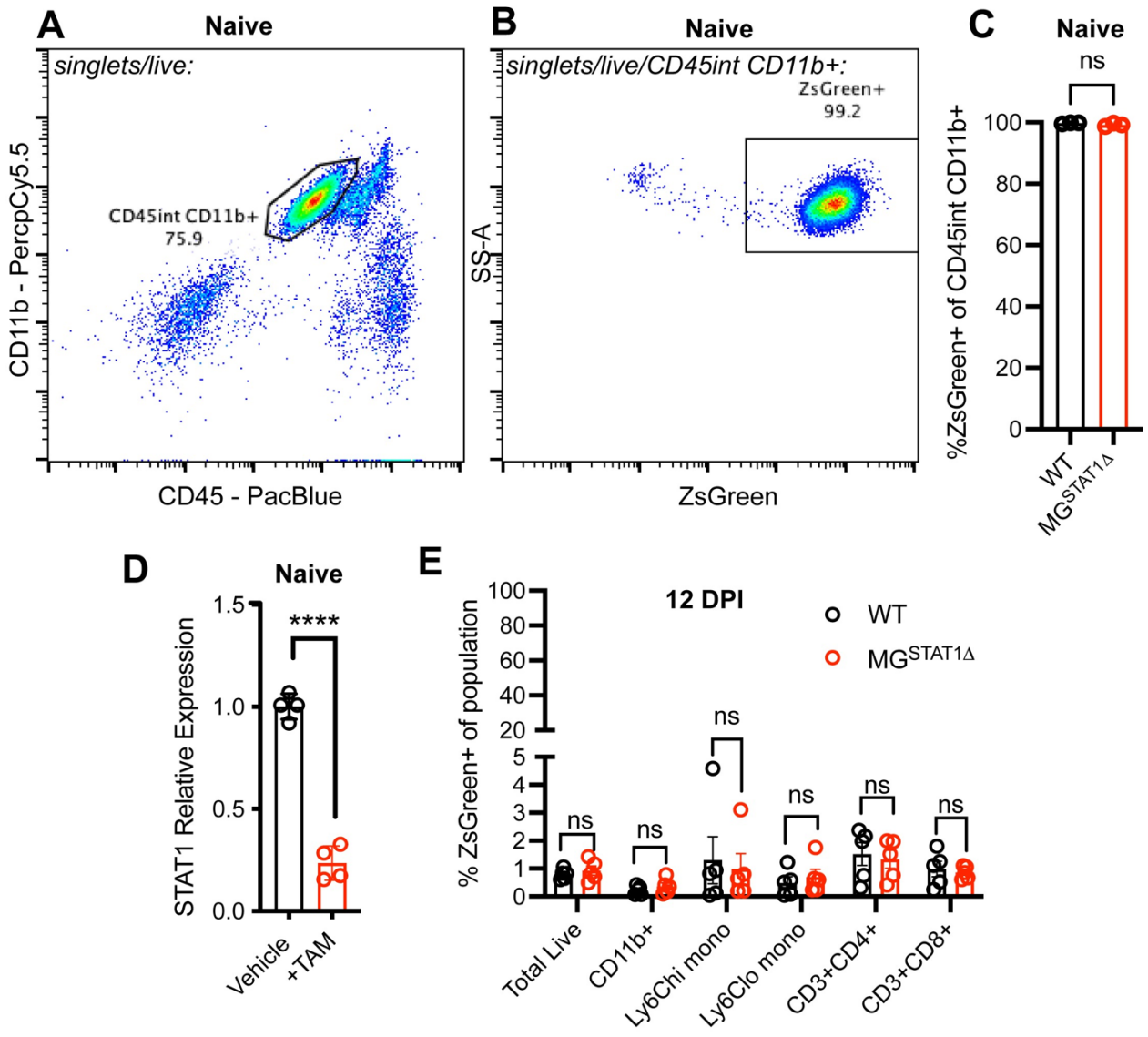
Supplementary Figure 1. Example gating strategy for brain immune cells.

Myeloid and T cells isolated from mice with ZsGreen+ microglia were analyzed via flow cytometry. (**A-C**) For all panels, cells were pre-gated on singlets (**A**), then live cells using a viability dye (**B**). (**C**) Cells were gated to identify CD45 hi (brain-infiltrating) and CD45 int (brain-resident) immune cells. (**D**) Microglia were gated based on CD45 intermediate expression, ZsGreen and CD11b expression, and (**E-F**) MHC II positivity was assessed using FMO. (**G**) Infiltrating myeloid cells were gated based on CD45 hi expression, and the expression of both CD11b and Ly6C. (**H-I**) iNOS expression was determined via FMO gating. (**J-K**) T cells were gated based on the expression of CD3 and CD4 or CD8. (**L-M**) IFN-γ expression on CD3+CD4+ and CD3+CD8+ cells was determined based on FMO positivity.


A
Homeostatic Microglia Genes
Naive
Infected
Crybb1
Tgfb1
Sall1
Abi3
Pmepa1
Mertk
Abcc3
Usp2
Cttnbp2nl
Egr1
Jun
Cx3cr1
Rhob
Pde3b
Slco2b1
Scamp5
Csf1r
Tgfbr1
Mef2a
Lrrc3
Cxxc5
Tjp1
Rap1gds1
Nrip1
Rab3il1
Nuak1
Fscn1
Pla2g15
Inpp4b
Ccr5
Ophn1
Hpgds
Gtf2h2
Atp8a2
Bin1
Siglech
Tmem119
Plxdc2
Slc2a5
F11r
Arhgap5
Nr3c1
Olfml3
Itga6
Basp1
Ckb
Ltc4s
Fcrls
Cst3
Hexb
P2ry13
Kctd12
Gpr34
P2ry12
St3gal6
Golm1
Lair1
Cmklr1
Il10rb
Il10ra
Il21r
Rgmb
Nfkb1
Fgd2
Tgfbr2
Mafb
2
1
0
-1
-2
B
Disease-Associated Microglia Genes
Naive
Infected
Alcam
Ifi202b
Csf1
Gas7
Lag3
Cfp
Msr1
Apoe
Lgals3
Clec7a
Itgax
Cxcl10
Cxcl16
Axl
Cybb
Arg1
Cxcr4
Gpnmb
Gpx3
Ccl2
Tlr2
Ccrl2
Spp1
Siglec1
1.5
1
0.5
0
-0.5
-1
-1.5

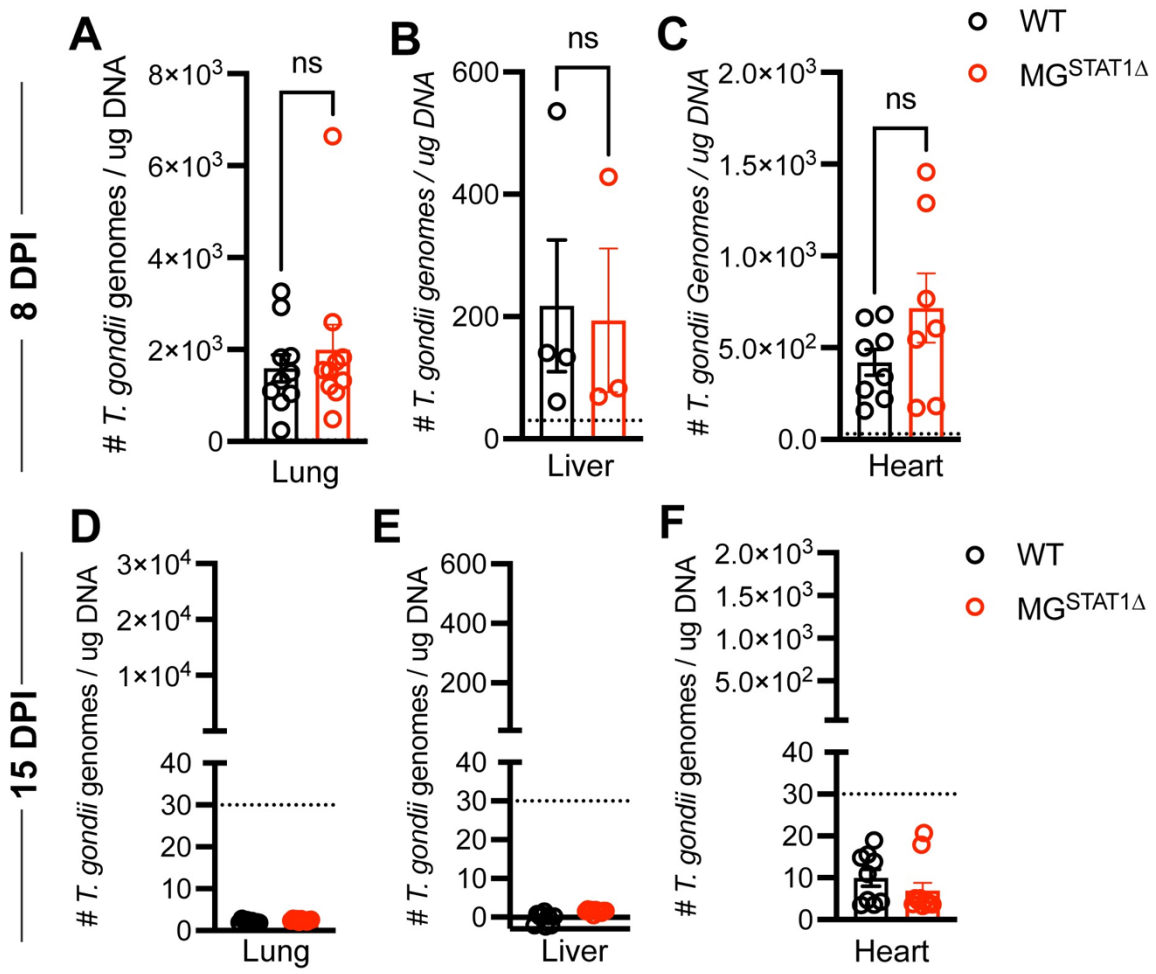
Supplementary Figure 2. RNA sequencing analysis of naïve vs. *T. gondii*-infected WT microglia.

Microglia from wild-type naïve or wild-type mice infected with *T. gondii* for 4 weeks were FACS-sorted and RNA-sequenced at 4 weeks post-infection. (**A-B**) Heat maps displaying hierarchically-clustered gene expression from regularized log transformed gene abundance counts. Heatmap data display the full set of significantly differentially expressed microglial homeostatic genes (**A**), or disease-associated microglia genes (**B**), shared across neurodegenerative models investigated in *Krasemann et al., 2017* and reflected in the naïve vs. T. *gondii*-infected DESeq2 dataset. Statistical significance was defined in the differential gene expression analysis as a BH adjusted p value < 0.05. n = 4-5 mice per group.



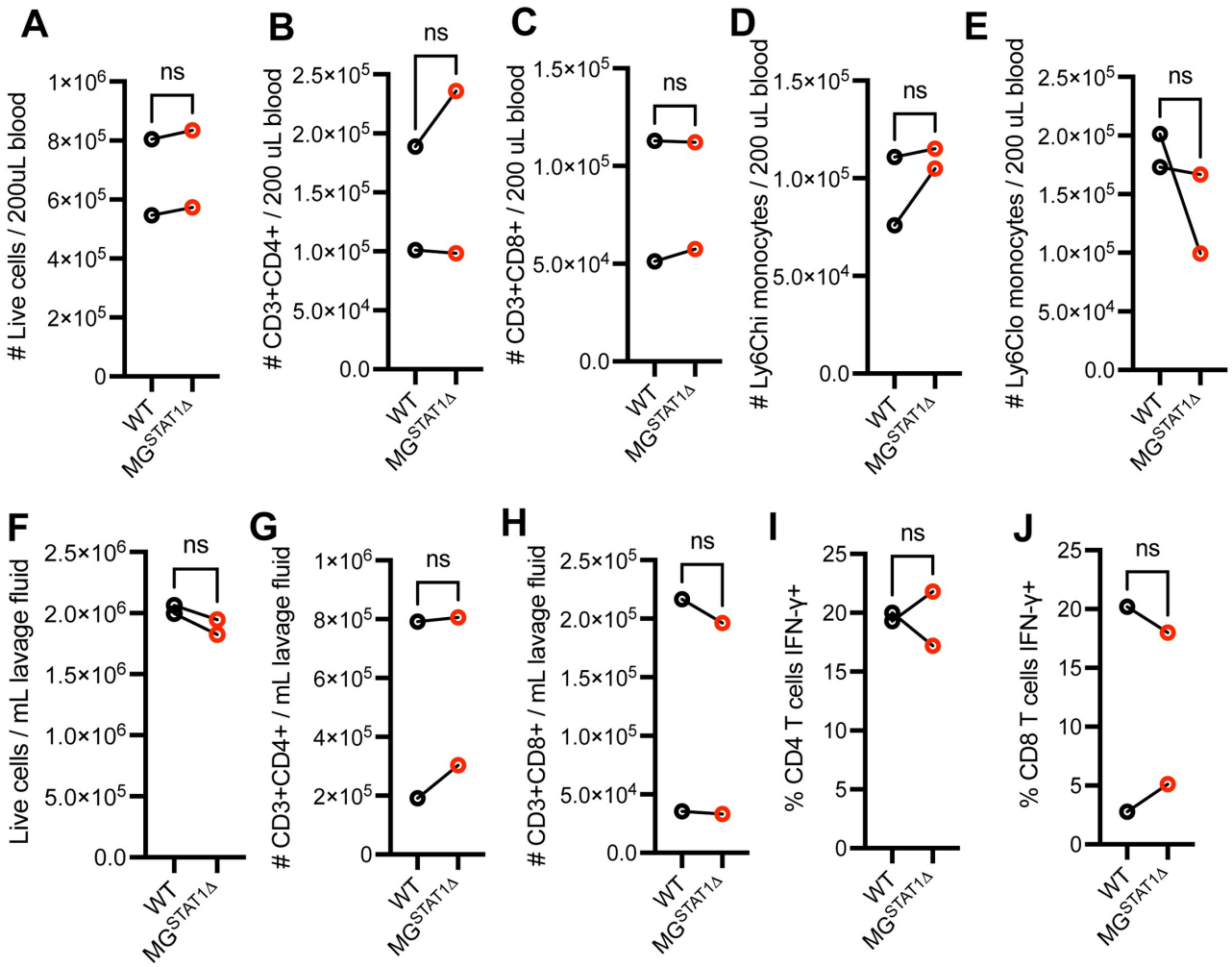
Supplementary Figure 3. Validation of cre activity and STAT1 excision in MG$^{STAT1\Delta}$ mice.

Naïve microglia and microglia isolated from brains at 12 DPI were analyzed by flow cytometry or RT-qPCR for relative gene expression. (**A-B**) Representative FACS plots indicating gating strategy for validating microglial ZsGreen expression in naïve mice. (**C**) Flow cytometric quantification of ZsGreen expression in total CD45^{int} CD11b+ cells in naïve WT or MG$^{STAT1\Delta}$ mice. (**D**) RT-qPCR quantification of *Stat1* relative expression in microglia that were magnetically enriched from naïve vehicle or tamoxifen (TAM)-treated MG$^{STAT1\Delta}$ mice. (**E**) Flow cytometric quantification of ZsGreen expression in various immune populations isolated from blood at 12 DPI, in WT or MG$^{STAT1\Delta}$ mice. Statistical significance was determined via unpaired *t* test, with *n* = 3-5 mice per group (**C-E**). ns = not significant;



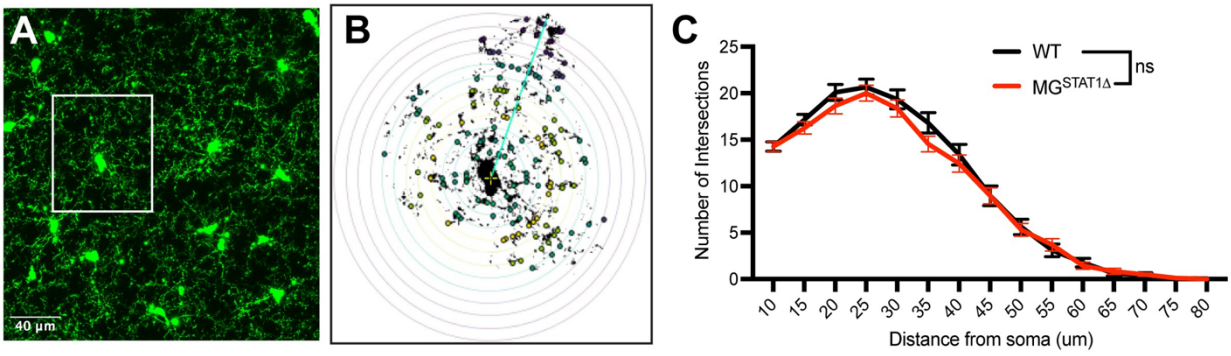
Supplementary Figure 4. *T. gondii* burden in peripheral tissues in WT and MG$^{STAT1\Delta}$ mice.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii*, and peripheral tissues were harvested and analyzed by qPCR for parasite genomic DNA, relative to total tissue DNA. Parasite burden was quantified at 8 DPI in lung (**A**), liver (**B**), and heart (**C**) tissue. Parasite burden was quantified at 15 DPI in lung (**D**), liver (**E**), and heart (**F**) tissue. (**A-C**) Statistical significance was determined via randomized block ANOVA using compiled data from 2-3 experiments with n = 10 mice per group (**A**), n = 7-8 mice per group (**C**), n = 9-11 mice per group (**D-F**), or via unpaired t test with n = 3-4 mice per group (**B**). Dotted line on y axis denotes assay limit of quantification, based on lower limit of standard curve. ns = not significant.



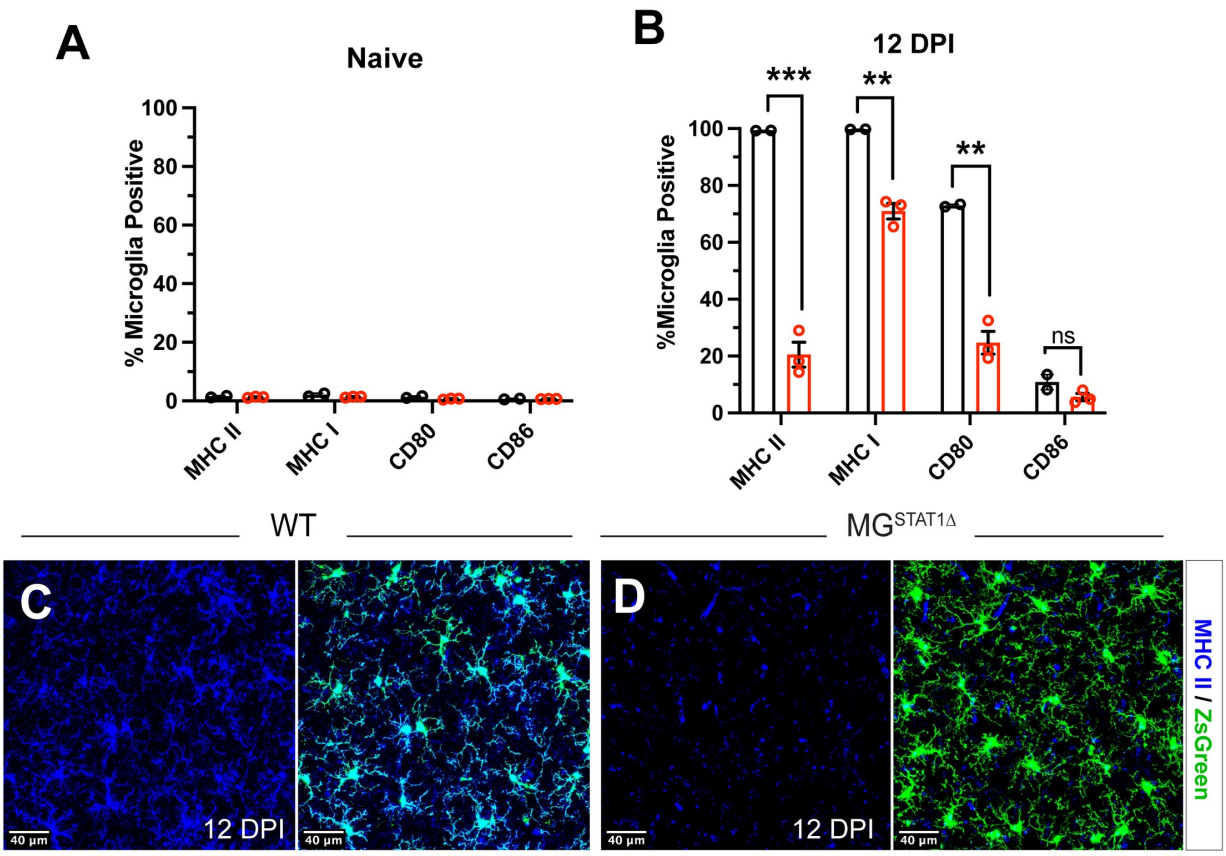
Supplementary Figure 5. WT and MG$^{STAT1\Delta}$ mice display equivalent immune activation in peripheral tissues during *T. gondii* challenge.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii,* and immune cells from blood and peritoneal fluid were analyzed by flow cytometry. (**A-E**) Flow cytometric quantification of total live immune cells (**A**), CD3+CD4+ T cell count (**B**), CD3+CD8+ T cell count (**C**), CD11b+Ly6Chi monocytes (**D**), and CD11b+Ly6Clo monocytes (**E**), calculated from blood. (**F-J**) Quantification of total live cells (**F**), number of CD3+CD4+ T cells, (**G**) number of CD3+CD8+ T cells (**H**), and CD4+ or CD8+ T cell expression of IFN-γ (**I-J**) isolated from the peritoneal cavity at 8 DPI. Statistical significance was determined by two-way randomized block ANOVA (**A-J**). ns = not significant, n = 11 per group from two pooled experiments (**A-E**), or n = 7-9 mice per group from two pooled experiments (**F-J**). Biological replicates are individual mice, with group means from individual experiments plotted as open circles with black lines connecting experimental and control groups. Source data (**A-H**) are provided in a source data file.



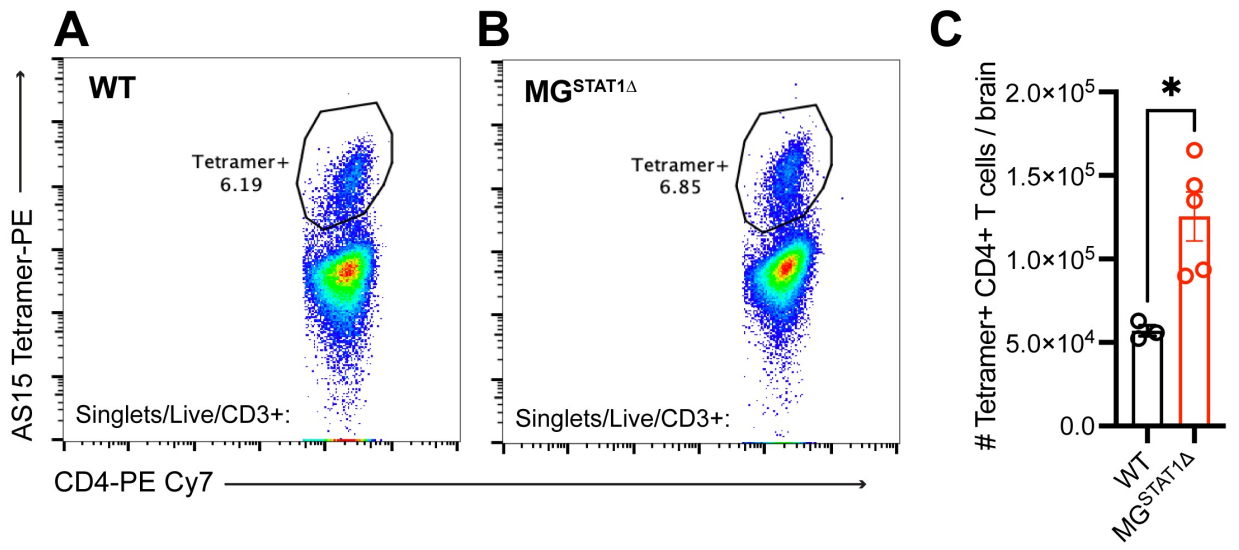
Supplementary Figure 6. Morphometric analysis of WT and STAT1-deficient microglia.

To analyze microglial activation, Sholl analysis was performed on microglia from the somatomotor cortex of naïve WT and $MG^{STAT1\Delta}$ mice. (**A**) ZsGreen+ microglia were imaged using a confocal microscope, and images were processed into a maximum projection using Fiji. (**B**) Images were made binary, microglia were manually isolated to determine cell process continuity, and the Sholl analysis Fiji plugin was executed to record intersections at varying soma distances. (**C**) Quantification of Sholl data via two-way ANOVA with Sidak's multiple comparisons test, n = 64-67 microglia from 3 mice per group. (**A**) Scale bar = 40 μm.



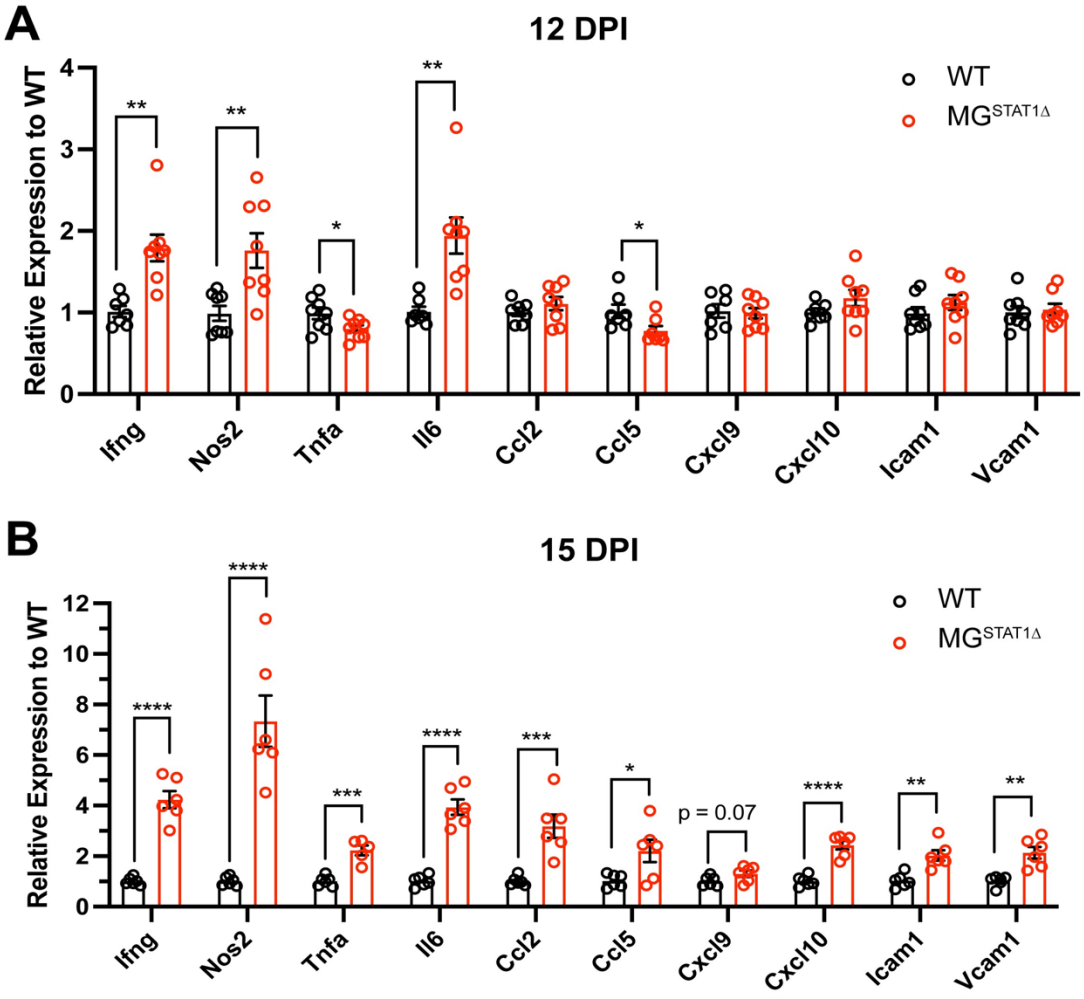
Supplementary Figure 7. Microglial antigen presentation machinery is regulated by STAT1 during *T. gondii* infection.

Microglia were isolated from naïve or 12 DPI infected WT or MG$^{STAT1\Delta}$ mouse brains and analyzed by flow cytometry and confocal microscopy. Flow cytometric analysis of microglial major histocompatibility complex and co-stimulatory molecules in (**A**) naïve brains, and (**B**) 12 DPI brains. (**C-D**) Immunohistochemical analysis of MHC II positivity by confocal microscopy; scale bar = 40 um, blue indicates MHC II, and green indicates ZsGreen fluorescence. n = 2-3 per group, unpaired t test (**A-B**). ns = not significant, ** = p <0.01, *** = p < 0.001.



Supplementary Figure 8. Antigen-specific CD4+ T cell responses are increased in MG$^{STAT1\Delta}$ mice.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii*, and brain-infiltrating CD4+ T cells were analyzed by flow cytometry for MHC II I-A^{b} AS15 tetramer positivity at 15 DPI. (**A-B**) Representative FACS plots indicating tetramer gating in WT (**A**) and MG$^{STAT1\Delta}$ mice (**B**). (**C**) Quantification of CD3+CD4+ tetramer+ cells isolated from brains at 15 DPI via unpaired *t* test, $n = 3$ and 5 mice per group. * = $p < 0.05$. Error bars indicate standard error of the mean.



Supplementary Figure 9. Whole brain RNA analysis of anti-parasitic defense genes.

Whole brain homogenate from WT and MG$^{STAT1\Delta}$ mice was analyzed by RT-qPCR for a panel of various anti-parasitic genes. (**A**) 12 DPI and (**B**) 15 DPI immune effector profile. Statistical significance was determined by unpaired *t* test for one experiment (**A**), and two-way randomized block ANOVA from two pooled experiments (**B**). $n = 8$ per group (**A**), and $n = 9$-10 mice per group (**B**). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 10^{-4}$. Error bars indicate standard error of the mean.

Chapter 4 – STAT1 regulation of disease-associated microglia activation during *T. gondii* infection

This chapter contains unpublished data.

4.1 Introduction

Disease-associated microglia (DAM) are a highly phagocytic subtype of microglia that were first identified in mouse models of Alzheimer's disease (AD), where they aggregate around amyloid-beta plaques within the brain parenchyma in both humans and mice[209]. Since their identification in 2017, multiple papers have shown them to be present in the contexts of mouse models for MS, ALS, facial nerve axotomy, and normal aging[202]. Genome-wide association (GWAS) studies investigating genetic risk factors for AD development in particular have been influential in the identification of microglial genes that contribute to DAM development in a broad range of neurodegenerative contexts[281]. For instance, human single nucleotide polymorphisms located at *Trem2*[282–287], *Apoe*[288–292], and *Cd33*[293,294] genetic loci are associated with increased late-onset AD risk development. Deletion of these genes that are highly expressed by microglia in models of familial AD such as APP-PS1 and 5xFAD mouse models results in decreased markers of neurodegeneration such as AB-induced pathology in the case of CD33, and increased pathology in the case of loss-of-function TREM2 or APOE mutant mice[202,209,295]. These studies thus collectively implicate the involvement of microglia, the resident immune cells of the immune-privileged brains in protection against AD-mediated pathology.

Previous work has identified both TREM2 and APOE as a requirement for the transcriptomic transition of resting, homeostatic microglia to the DAMs observed in different models of neurodegeneration, within a shared pathway[202,209]. CD33 has also been proposed to modulate this pathway, as the protection from AD pathology including deficits to learning and memory observed in *Cd33*-/- mice is lost when those mice are bred to a *Trem2*-/- background. Because disrupted operation of the TREM2-APOE pathway yields worsened AD pathology[202,209], these studies, in conjunction with more recent high-throughput statistical modeling and integration of several sc-RNA-seq microglia datasets, have contributed to the hypothesis that DAMs are a neuroprotective microglial subtype during neurodegeneration[296].

In addition to outlining the importance of STAT1 signaling in microglia during CNS infection with *T. gondii*, the previous chapter utilized a bioinformatics approach to introduce two novel concepts related to microglial physiology during neuroinflammation. The first is that the disease-associated microglia (DAM) signature, classically defined as a conserved microglial transcriptomic state across neurodegenerative conditions and previously coined a "universal sensor of neurodegeneration[297]," is also largely present during CNS infection. The second concept is that STAT1 is required for the full transition of homeostatic microglia to DAMs during *T. gondii challenge*. This chapter further expands on the current understanding of DAMs, and includes a brief report of follow-up studies that offer an explanation of where and how STAT1 signaling fits into an expanding understanding of this microglial activation state.

4.2 Results

Bulk RNA-sequencing is able to provide insights into population-level gene expression, but it is not able to provide an indication of prevalence of unique subtypes within a population. Consequently, we sought to quantify how prevalent DAMs are during *T. gondii* infection, using CD11c as a marker for DAMs. Previous research has indicated that few microglia express CD11c in the naïve brain, but in transgenic AD mice, up to 22.3% of microglia co-express CD11c with the pan-macrophage marker, Iba1[209]. We calculated that during *T. gondii* challenge, an average of 76.7% of WT microglia were CD11c+ by flow cytometry at 15 DPI (**Fig 4.1A-C**). In contrast, only 7.2% of microglia from tamoxifen-treated $MG^{STAT1\Delta}$ mice were CD11c+, with a pattern that mirrored that of STAT1 excision as quantified by expression of the STAT1-dependent molecule MHCII (**Fig. 4.1A-C**). These data: (i) support our previous transcriptomic identification of the classical DAM marker, CD11c, as a STAT1-regulated gene, and (ii) provide evidence that DAMs are highly prevalent within the *T. gondii*-infected brain.

Our approach for generating mice with STAT1-deficient with the use of tamoxifen results in mice with incomplete levels of excision (**Fig 3.2B** and **Fig 4.1B**). We leveraged this technical limitation as a tool to further understand the relationship between STAT1 and DAMs, as $MG^{STAT1\Delta}$ mice have a subpopulation of microglia that are expected to retain STAT1, as evidenced by their ability to upregulate the STAT1-dependent molecule MHC II[226,256,258]. Microglia from $MG^{STAT1\Delta}$ mice were thus binned according to MHC II positivity, and the frequencies of surface CD11c expression was analyzed. We found that on average, only 2% of MHC II-negative microglia expressed CD11c, in contrast to 47.6% of MHC II+ microglia (**Fig 4.1D**). These data indicate that within a single $MG^{STAT1\Delta}$ mouse, CD11c expression is largely restricted to the small subpopulation

of microglia that retain some level of STAT1 function – likely "escapees" from cre recombinase activity.

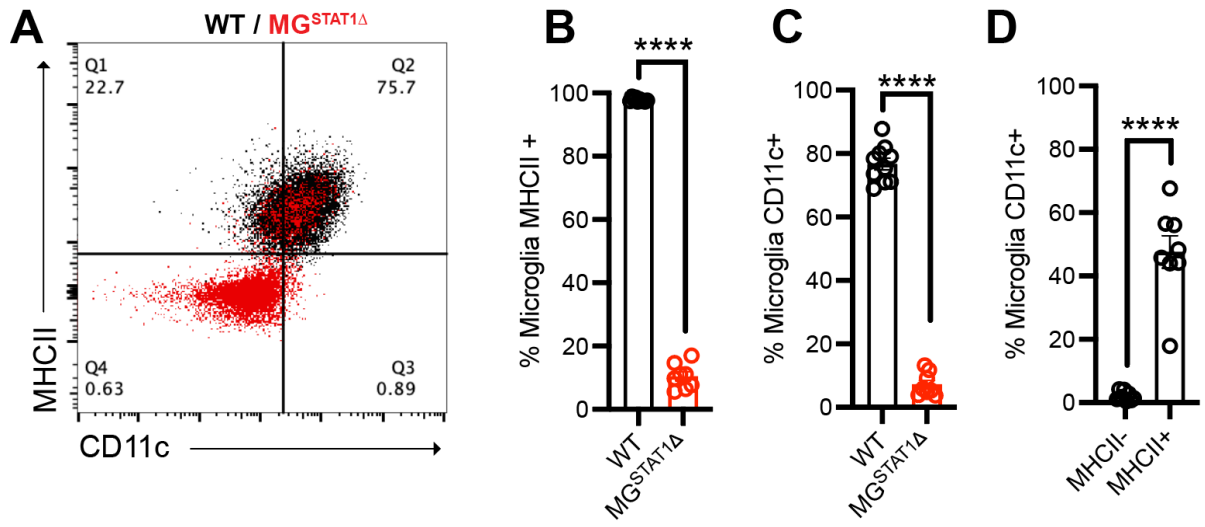


Figure 4.1. STAT1 is required for microglial CD11c expression at the protein level.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii* and brains were analyzed by flow cytometry at 15 DPI. (**A**) Representative FACS plot of MHC II and CD11c expression in microglia isolated from WT and MG$^{STAT1\Delta}$ mice. Quantification of microglia (**B**) MHC II expression, as a readout for STAT1 excision and (**C**) CD11c expression as an indicator of DAM prevalence. (**D**) Flow cytometric analysis of CD11c frequency from data-binned MHC II-negative and -positive microglia in MG$^{STAT1\Delta}$ mice. n = 8-10 mice per group from two pooled experiments (**B and C**), or n = 8 mice total for intra-mouse microglial analysis. (**D**). Data was pooled from two independent experiments and statistical significance was determined via unpaired t test. **** = $p < 10^{-4}$.

We next sought to determine whether STAT1 was related to previously identified regulators of the DAM signature. Previous literature has indicated that TREM2, APOE, and CD33 operate within a shared pathway to drive the expression of what have been referred to as "Stage 2 DAMs." Intriguingly, flow cytometric analysis of microglia isolated from WT and MG$^{STAT1\Delta}$ mice

indicated revealed that STAT1 was not required for TREM2 expression. Instead, we found that nearly all microglia from both groups of mice were TREM2-positive, and MG$^{STAT1\Delta}$ mice displayed higher TREM2 expression, as quantified by geometric mean fluorescence intensity (MFI) (**Fig 4.2A-C**). These data, in tandem with differential expression of TREM2-independent genes by STAT1-deficient microglia, suggest that STAT1 regulation of the DAM signature during *T. gondii* challenge is TREM2-independent.

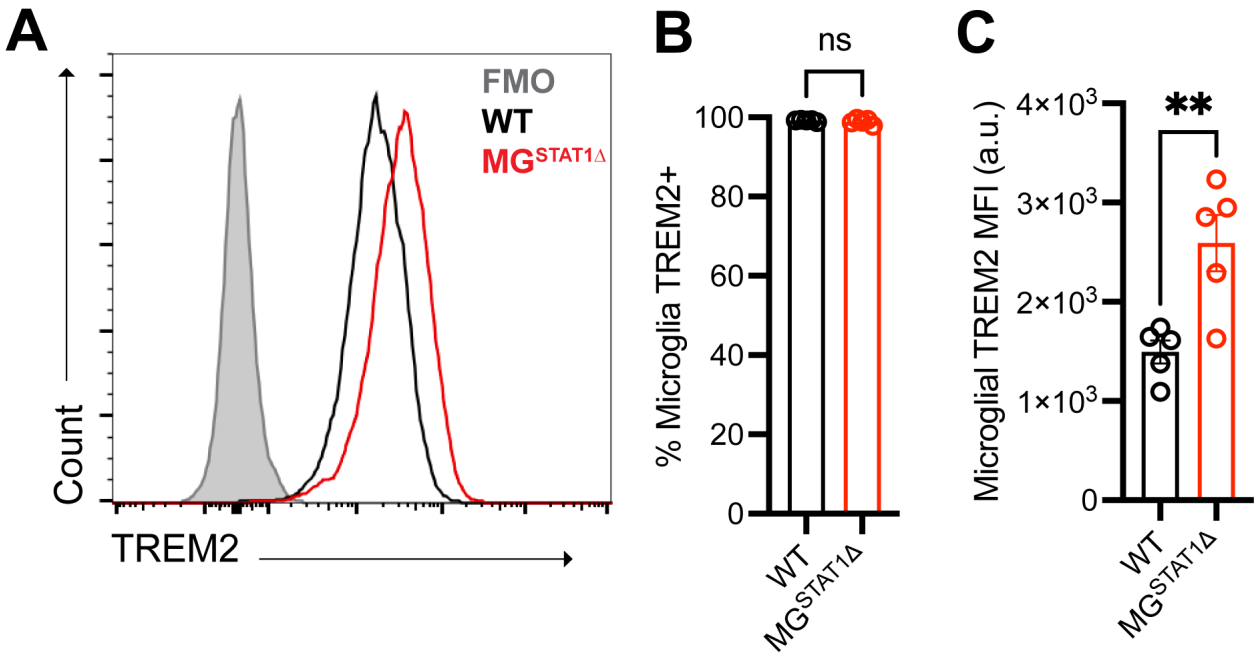


Figure 4.2. STAT1 is not required for TREM2 expression in microglia.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii* and brains were analyzed by flow cytometry at 15 DPI. (**A**) Representative FACS histograms of microglial TREM2 expression, with gating based on a FMO negative control (grey). Quantification of microglial TREM2 frequency (**B**), and geometric mean fluorescence intensity (MFI) (**C**). Statistical significance was determined via unpaired t test. ns = not significant, ** = $p < 0.01$. $n = 5$ mice per group (**B-C**).

4.3 Discussion

Our use of flow cytometry to characterize the relationship between the DAM marker CD11c and STAT1 is valuable for multiple reasons. First, while we observed that STAT1 regulates *Itgax* (CD11c) at the gene level, additional protein-level analysis suggests a binary relationship between the two molecules – rather than simply modulating the level of CD11c expression, STAT1 appears to be a strict requirement for any level of CD11c expression in microglia. Second, previous single-cell seq analysis that marked the initial discovery and characterization of DAMs reported that there are no microglia with a DAM signature that are negative for CD11c expression, underscoring its utility in defining this subpopulation[209]. These previous findings, paired with our current data thus suggest that a pre-requisite of the DAM transition is a cellular response to interferon in the brain, and that interferon-STAT1 signaling is a shared feature of microglial activation in diverse models for neuroinflammation. Consistent with this hypothesis, in the case of CNS infection with *T. gondii*, IFN-γ is the predominant cytokine that activates STAT1, but in neurodegenerative mouse models for AD, multiple studies suggest IFN-α/β, which also signal through STAT1, may serve as the relevant cytokine driving DAM development.

Our flow cytometric and bioinformatic analysis of microglial TREM2 expression in WT and $MG^{STAT1\Delta}$ mice also serves as an important first step in understanding the relationship between STAT1 and other known regulators of the DAM signature. Keren-Shaul et al. initially showed that the transition from homeostatic microglia to DAM is a two-step process involving TREM2 – with the TREM2-dependent portion of the DAM signature primarily modulating the upregulation of disease-associated markers, rather than the downregulation of homeostatic genes[209]. Previous studies have suggested that TREM2, APOE, and CD33 operate within a shared pathway to initiate the DAM phenotype in microglia, again largely implicating TREM2-dependent molecules.

However, while we find that while STAT1 is required for microglial upregulation of CD11c during *T. gondii* challenge, its larger contribution to DAM development is seen in the restriction of homeostatic, TREM2-independent genes.

The data presented in this chapter are useful in constructing a model to understand how STAT1 influences DAM development. Rather than contributing solely to the TREM2-APOE-CD33 pathway, STAT1 is to our knowledge, the first protein identified in microglia that influences the expression of both TREM2-depenent and -independent DAM genes, as identified through our bioinformatic analysis in Chapter 3. Our CX3CR1 inducible cre system for labeling microglia also eliminates the potential confound of classifying other blood-derived CD11c-expressing cells in the inflamed brain as DAMs, as recent literature has identified in older datasets[296]. Still, there are some caveats that must be considered in interpreting our data. While CD11c has been used as a robust marker of DAMs, its utility as a stand-alone marker in their identification is limited, since DAMs represent a broader transcriptional signature. Either single cell RNA sequencing or the inclusion of a more comprehensive flow cytometry or mass cytometry panel incorporating both homeostatic and DAM markers will be needed to definitively classify DAMs in the *T. gondii*-infected brain.

Many other questions remain unanswered, including (i) whether the DAM signature, irrespective of STAT1-dependent genes, are protective during *T. gondii* challenge, (ii) whether the STAT1-dependency of CD11c plays a functional anti-microbial role, and (iii) clarity on the relevant activators of STAT1 signaling in other disease models. These topics are explored in Chapter 6 ("*Open questions pertaining to DAMs during T. gondii infection*," and "*Beyond CNS infection – microglial STAT1 signaling across health and disease*.")

Chapter 5 – An analysis of microglial-mediated antigen presentation capacity via MHC II

This chapter contains unpublished data.

5.1 Introduction

Antigen presentation is an essential mechanism by which the innate immune system is able to selectively prime and activate an antigen-specific adaptive T cell-mediated immune response[255]. T cell-mediated immunity is central to the immune response against chronic CNS infection with *T. gondii*[26], and previous literature has revealed that unlike other tissues, antigen must be specifically expressed in the CNS to license T cells and facilitate their entry[298]. Thus, understanding which cell types serve this function in the brain is critical for understanding how adaptive immune responses – either protective or pathological – are initiated in the CNS. Here, we focus on the function of MHC II, a STAT1-dependent molecule that is required for antigen-specific TCR stimulation and downstream T cell activation[226,256,258].

Throughout our studies, the professional antigen presentation molecule MHC II, expressed by myeloid cells, has served as a robust and reliable readout for STAT1 activity in brain-resident microglia. While < 3% of microglia express MHC II in the naïve brain, this number approaches 100% at just 12 DPI (**Fig S7**). Our results from Chapter 3 suggested that a loss of MHC II expression in microglia was unable to explain the severe toxoplasmic encephalitis observed in $MG^{STAT1\Delta}$ mice, since it was associated with increased, rather than decreased, T cell activation in these animals (**Fig 3.4**). As a result, we hypothesis that microglial antigen presentation was not a primary STAT1-dependent anti-microbial function during *T. gondii* infection. Here, to further test

this hypothesis, we have generated a new mouse strain to specifically examine microglial-mediated antigen presentation by MHC II as a standalone STAT1-dependent function in brain-resident macrophages during *T. gondii* challenge.

5.2 Results

To investigate a potential role for microglial antigen presentation-mediated activation or maintenance of CD4+ T cells during *T. gondii* infection, we generated MHC II $^{fl/fl}$ x CX3CR1$^{CreERT2/+}$ x ROSA26$^{Ai6/Ai6}$ (MG$^{MHCII\Delta}$ mice). Similarly to the MG$^{STAT1\Delta}$ mice used in Chapters 3 and 4, MG$^{MHCII\Delta}$ mice harbor a tamoxifen-inducible cre recombinase under the expression of the *Cx3cr1* promoter, and they received five consecutive i.p. injections of tamoxifen (TAM) to induce target gene excision as previously described. In contrast to MG$^{STAT1\Delta}$ mice which succumb to infection starting at 17 DPI, we found that MG$^{MHCII\Delta}$ mice survived well into the chronic phase of *T. gondii* infection – beginning to succumb to infection at around 40 DPI. However, we observed a statistically significant increase in MG$^{MHCII\Delta}$ animal mortality relative to No-TAM litter-mate controls receiving corn oil vehicle treatment. These data are consistent with microglial MHC II-mediated antigen presentation promoting extended lifespan, although the potential confound of tamoxifen treatment in experimental but not control mice in this experiment will need to be investigated.

For subsequent experiments, we sought to control for potential experimental effects of tamoxifen on the immune system by pairing TAM-treated MG$^{MHCII\Delta}$mice with age-matched, TAM-treated CX3CR1$^{CreERT2/+}$ x ROSA26$^{Ai6/Ai6}$ (WT) control mice as was performed throughout our analyses of MG$^{STAT1\Delta}$ mice. Thus, we harvested the brains of these mice at 4 weeks post-infection (WPI) by flow cytometry to quantify brain parasite burden and analyze microglial, infiltrating

myeloid, and T cell responses. We observed no statistically significant differences in brain parasite burden, as evaluated by cyst counts. There was similarly no significant difference in total live cells purified from the brains of $MG^{MHCII\Delta}$ animals, relative to controls. These data suggest that microglial MHC II deficiency does not result in severe toxoplasmic encephalitis (as seen in $MG^{STAT1\Delta}$ mice), although a closer analysis of tachyzoite and bradyzoite enrichment may be warranted in subsequent analyses.

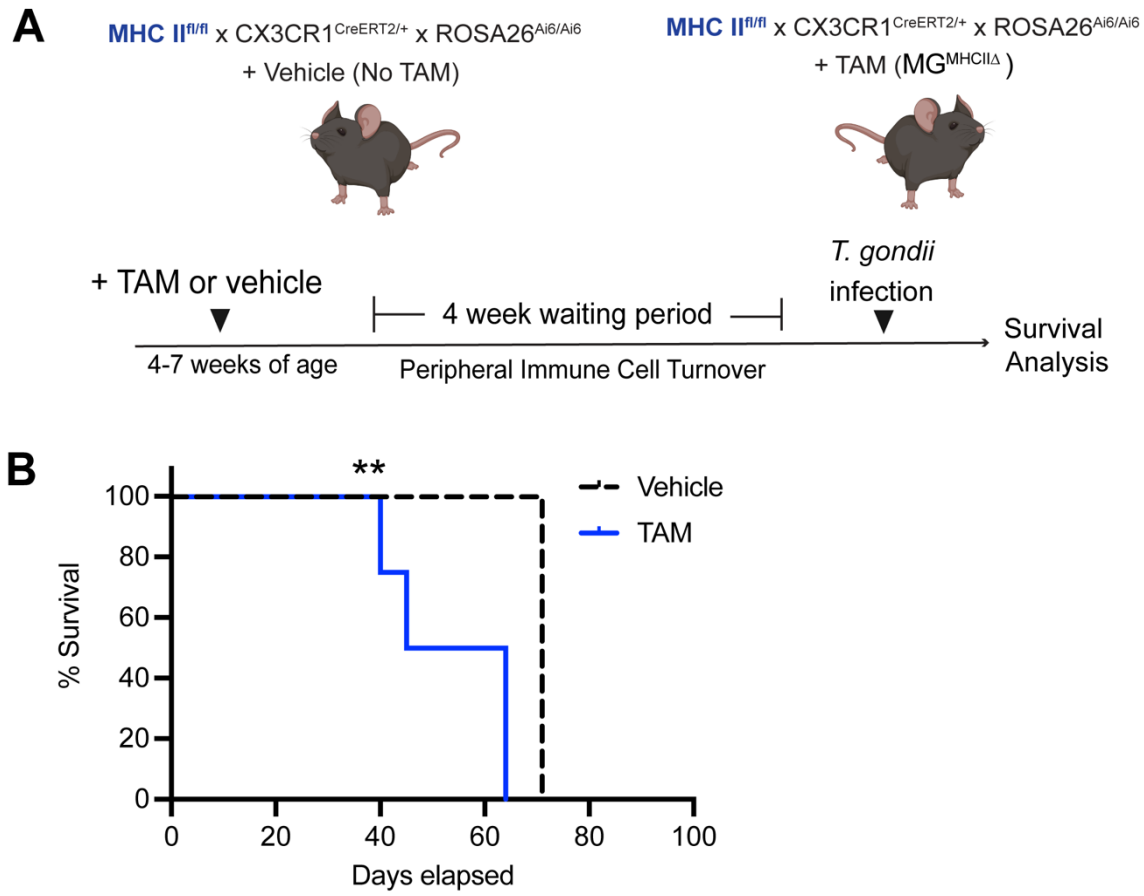


Figure 5.1. TAM-treated $MG^{MHCII\Delta}$ mice display accelerated mortality relative to vehicle-treated littermates during *T. gondii* challenge.

Vehicle and tamoxifen (TAM)-treated $MG^{MHCII\Delta}$ mice were i.p. infected with 10 cysts of the Me49 strain of *T. gondii* and were analyzed for survival over time. **(A)** Schematic outline for generating vehicle and TAM-treated $MG^{MHCII\Delta}$ mice. (**B**) Kaplan-Meier survival analysis of control (black) and experimental (blue) mice, with $n = 4$ mice per group. ** = $p < 0.01$.

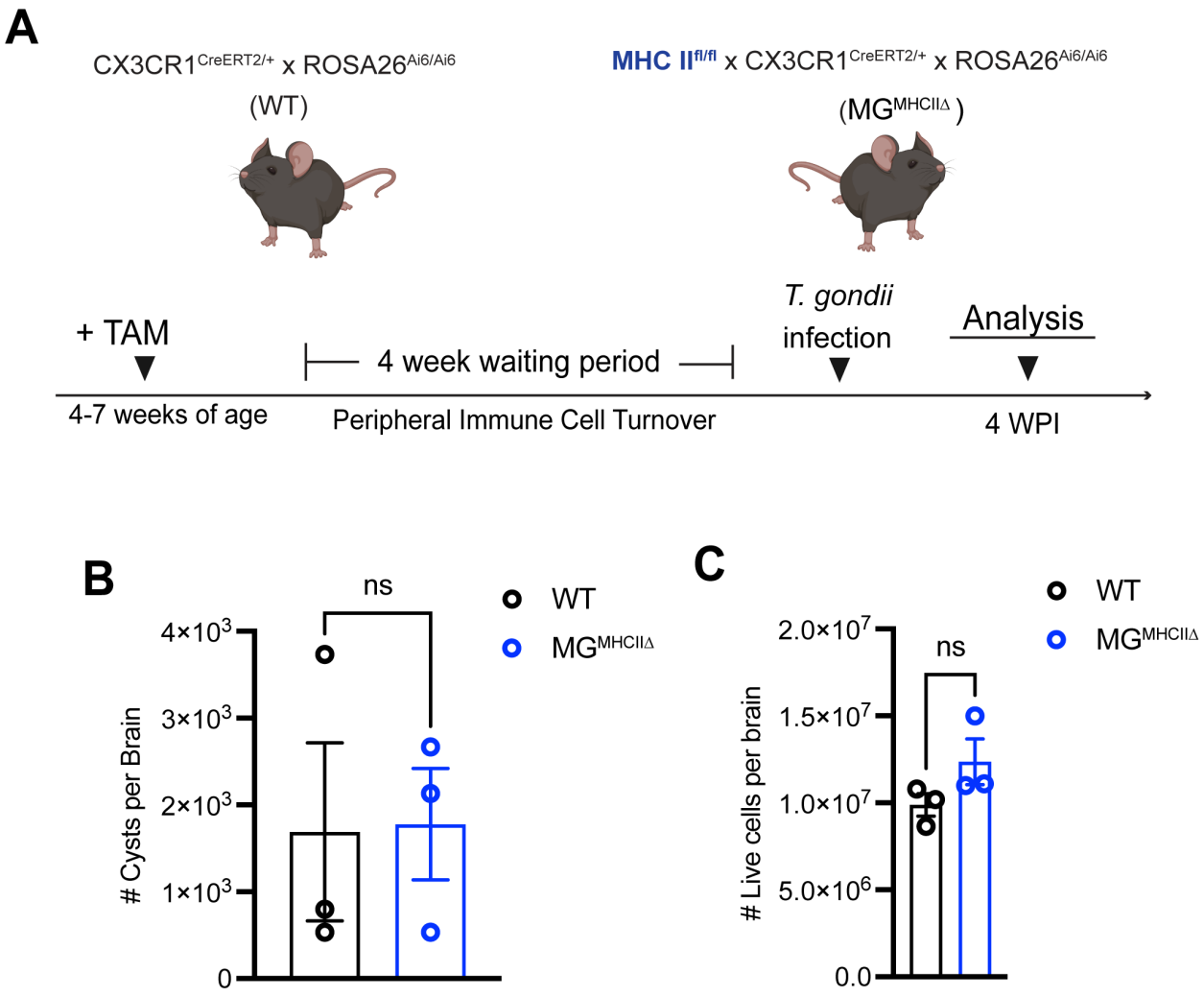


Figure 5.2. MG$^{MHCII\Delta}$ mice display no changes in brain parasite burden relative to WT mice.

WT and TAM-treated MG$^{MHCII\Delta}$ mice were i.p. infected with 10 cysts of the Me49 strain of *T. gondii* and were analyzed at 4 weeks post-infection (WPI). (**A**) Schematic outline of optimized strategy for generating WT and MG$^{MHCII\Delta}$ mice for experiments. (**B**) Enumeration of *T. gondii* cysts from brain homogenate via brightfield microscopy. (**C**) Enumeration of total live cells isolated from WT and MG$^{MHCII\Delta}$ brains, following red blood cell exclusion. Statistical significance was determined via unpaired *t* test, with $n = 3$ mice per group. ns = not significant.

We next sought to validate excision of MHC II from microglia and evaluate microglial expression of additional proteins associated with antigen presentation (MHC I, CD80, CD86) and

activation (CD11c). We observed no differences in total microglial count (**Fig 5.3A**), but there was an ~80% reduction in microglial MHC II expression (**Fig 5.3B-C**), consistent with efficient excision. Microglia isolated from $MG^{MHCII\Delta}$ brains displayed increased expression of MHC I by geometric mean fluorescent intensity (MFI), but no changes in the expression of the antigen presentation co-stimulatory molecules CD80 and CD86 (**Fig 5.3D-F**). CD11c was also expressed by microglia at significantly higher levels in $MG^{MHCII\Delta}$ mice relative to controls. Together, these data suggest that of the major proteins required for antigen presentation, only MHC II expression is compromised in microglia in $MG^{MHCII\Delta}$ mice.

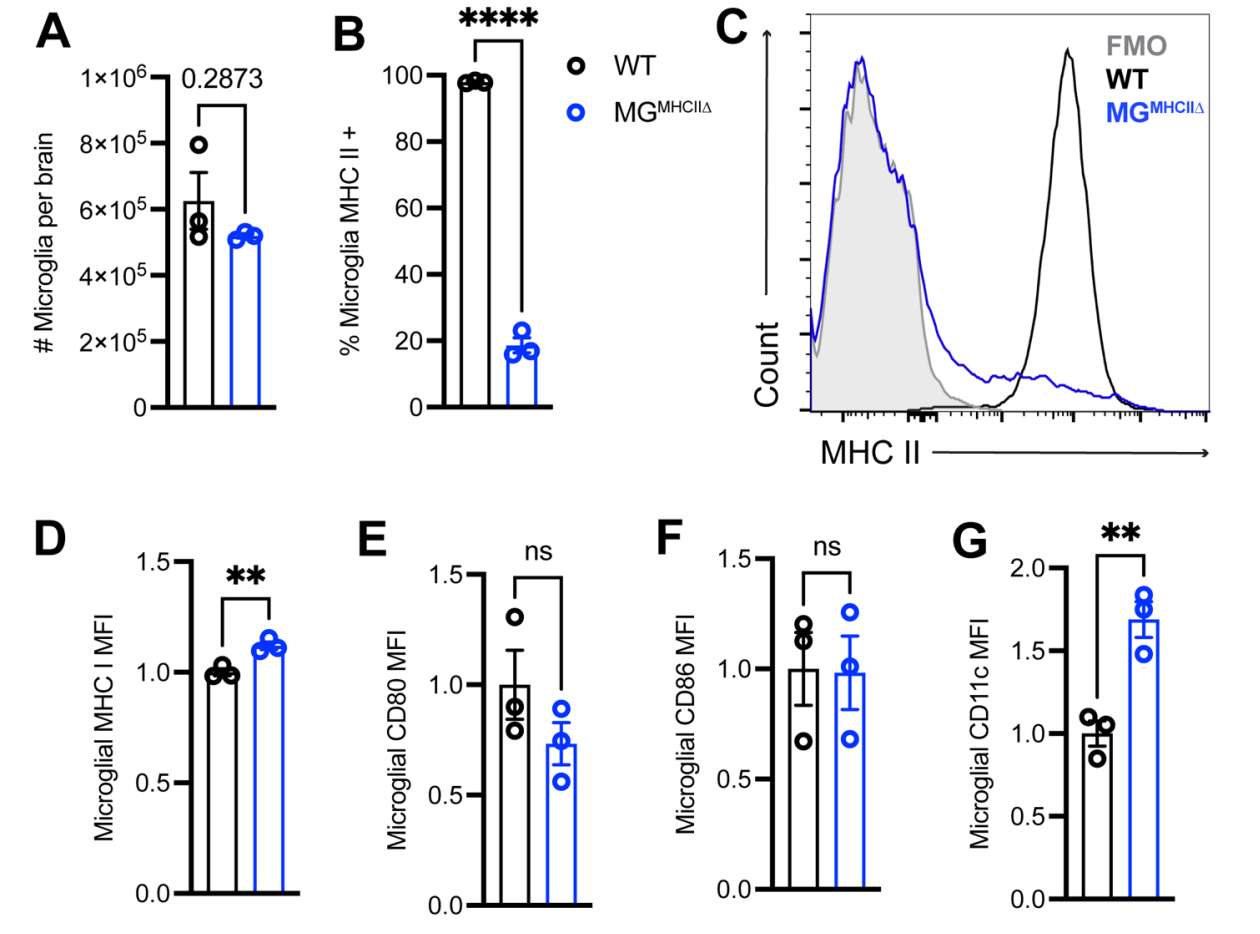


Figure 5.3. Validation of MHCII excision and analysis of antigen presentation / activation-related molecules in microglia.

WT and TAM-treated MG$^{MHCII\Delta}$ mice were i.p. infected with 10 cysts of the Me49 strain of *T. gondii* and were analyzed by flow cytometry at 4 weeks post-infection (WPI). (**A**) Total number of microglia isolated from brains of WT and MG$^{MHCII\Delta}$ mice. (**B**) Quantification of microglial MHC II expression frequency as a readout for target gene excision. (**C**)Representative FACS histogram of microglial MHC II expression, with FMO designating negative control sample in gray. (**D-G**) Quantification of geometric mean intensity of microglial MHC I (**D**), CD80 (**E**), CD86, and CD11c (**G**). Statistical significance was determined via unpaired *t* test, with $n = 3$ mice per group. ns = not significant, ** = $p < 0.01$.

To determine the specificity of MHC II deletion in MG$^{MHCII\Delta}$ mice, we also examined the brain-infiltrating myeloid population, marked by the expression of both CD11b and Ly6C. This population was selected for analysis because blood-derived monocytes express low levels of *Cx3cr1*, the gene whose promoter is used to drive cre recombinase expression in our experiments. We observed no statistically significant differences in infiltrating myeloid cell counts or their ability to express the anti-parasitic protein iNOS (**Fig 5.4A-B**). We did, however, observe a slight but statistically significant change (4.7% reduction) in the frequency of infiltrating myeloid cells that expressed MHC II in MG$^{MHCII\Delta}$ mice, relative to WT controls (**Fig 5.4C**). Further examination of the MHC II-positive fraction of infiltrating myeloid cells did not reveal any statistically significant difference in the amount of MHC II expression on a per cell basis, suggesting that haplo-insufficiency is not observed in this cell population in MG$^{MHCII\Delta}$ mice (**Fig 5.4D**). In light of the large population of infiltrating myeloid cells observed in both groups of mice, these data are largely consistent with considerable specificity of MHC II deletion to microglia (80% excision), and not infiltrating myeloid cells.

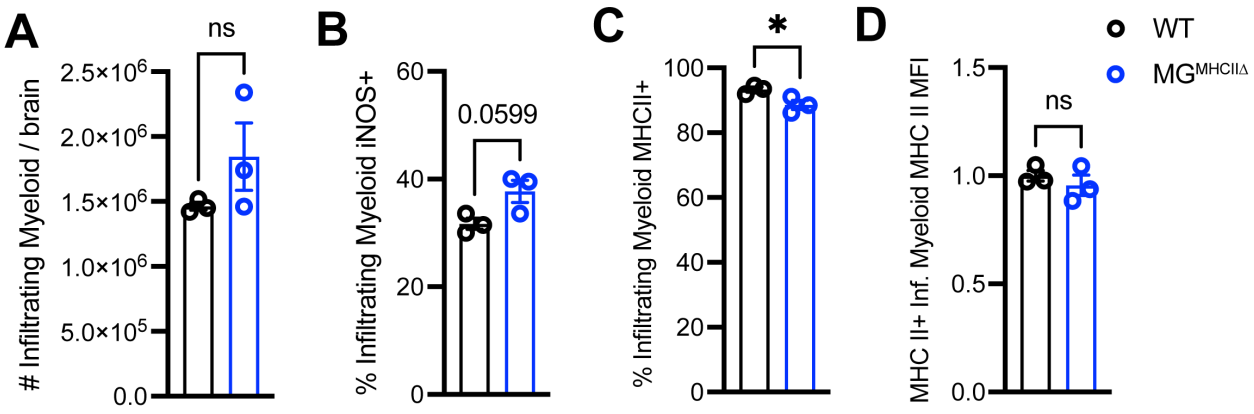


Figure 5.4. Analysis of brain-infiltrating myeloid responses in MG$^{MHCII\Delta}$ mice.

WT and TAM-treated MG$^{MHCII\Delta}$ mice were infected with 10 cysts of the Me49 strain of *T. gondii* and were analyzed at 4 weeks post-infection (WPI) by flow cytometry. (**A**) Quantification of infiltrating myeloid cells, defined by positive expression of CD11b and Ly6C, isolated from *T. gondii*-infected brains. (**B-C**) Quantification of infiltrating myeloid cells that display positive expression of iNOS (**B**), and MHCII (**C**). Geometric mean fluorescent intensity (MFI) of MHC II protein expression in infiltrating myeloid cells, of the MHCII-expressing fraction. Statistical significance was determined via unpaired *t* test, with $n = 3$ mice per group. ns = not significant, * = $p < 0.05$).

We next analyzed brain-infiltrating T cell responses to determine if microglia MHC II deficiency impacted T cell number or activation in the brain. We observed no statistically significant changes to either CD4+ or CD8+ T cell count in MG$^{MHCII\Delta}$ mice, relative to controls, nor their expression of the proliferation marker Ki67, which is induced by TCR-MHC engagement (**Fig 5.5A-D**). These data are consistent with a model in which microglial antigen presentation by MHC II is not a strict requirement for CD4+ T cell entry, priming, or retention during chronic CNS infection with *T. gondii*.

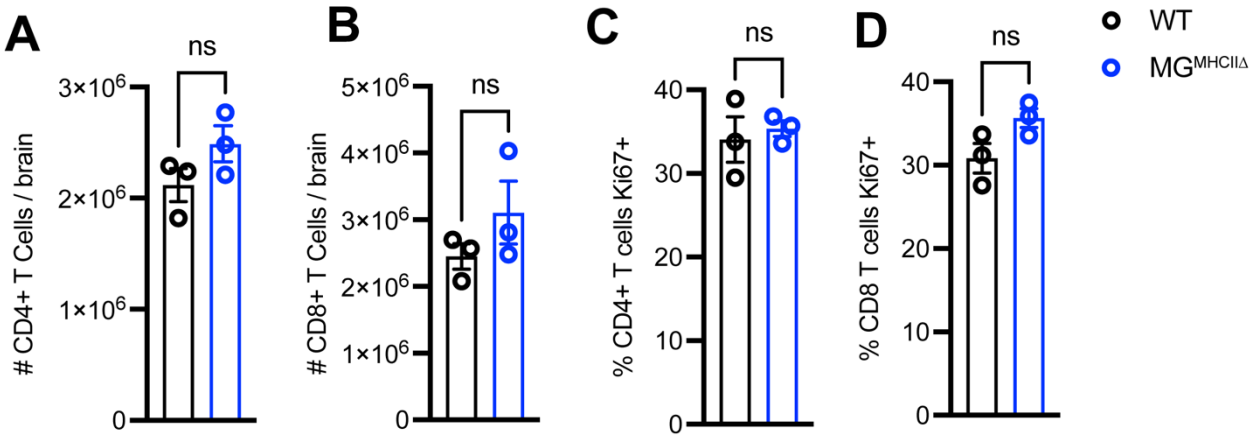


Figure 5.5 Analysis of brain-infiltrating T cell responses in MG$^{MHCII\Delta}$ mice.

WT and TAM-treated MG$^{MHCII\Delta}$ mice were i.p. infected with 10 cysts of the Me49 strain of *T. gondii* and were analyzed at 4 weeks post-infection (WPI) by flow cytometry. (A-B) Flow cytometric enumeration of CD4+ (**A**) and CD8+ (**B**) T cells isolated from WT and MG$^{MHCII\Delta}$ brains. (**C-D**) Quantification of CD4+ (**C**) and CD8+ (**D**) T cell expression of intracellular Ki67 in WT and MG$^{MHCII\Delta}$ brains. Statistical significance was assessed via unpaired *t* test, with $n = 3$ mice per group. ns = not significant.

5.3 Discussion

The results of these data provide valuable insights that help frame our understanding of microglial STAT1-mediated effector functions during CNS infection with *T. gondii*. A key takeaway from these results is that, in contrast to the rapid mortality displayed by MG$^{STAT1\Delta}$ animals during *T. gondii* challenge, MG$^{MHCII\Delta}$ mice display extended lifespan and similar brain parasite burden to WT controls. These findings are consistent with our interpretation in Chapter 3 that a loss of potential microglial antigen presentation capacity due to STAT1 deletion, is unable to account for the fatal toxoplasmic encephalitis observed in MG$^{STAT1\Delta}$ mice. Thus, we continue to hypothesize that cell-intrinsic parasite restriction, and not cell-extrinsic mechanisms for shaping

systems-level immunity, such as antigen presentation, comprises the predominant and most relevant STAT1-dependent microglial functional role for controlling CNS infection with *T. gondii*.

While the brains of $MG^{MHCII\Delta}$ mice are suggestive of much less severe infection than $MG^{STAT1\Delta}$ mice, there are some intriguing differences between $MG^{MHCII\Delta}$ and WT mice that have emerged from the experiments presented in this chapter. First, the observation that $MG^{MHCII\Delta}$ mice show accelerated mortality is suggestive of increased susceptibility to *T. gondii*, although follow-up experiments that control for tamoxifen treatment will be required to more clearly support this interpretation. When tamoxifen is controlled for, however, we observe in $MG^{MHCII\Delta}$ mice the significantly increased expression of microglial MHC I and CD11c – two proteins whose encoding genes are both regulated by IFN-γ and serve as DAM markers during neuroinflammation[202,209,297]. These data thus provide a clear impetus for future studies determining whether microglial MHC II deletion leads to increased IFN-γ by T cells in the brains of $MG^{MHCII\Delta}$ mice. Whether increased microglial expression of DAM markers serves as sensitive method for detecting subtle changes in IFN-γ in the brain is also of interest for future applications.

In turn, a closer examination of parasite burden including an analysis of tachyzoite gene expression may be warranted, as brain cyst counts do not account for the tachyzoite form of *T. gondii*. In such a scenario, increased parasite reactivation could sustain increased T cell activation and production of IFN-γ, should MHC II-sufficient infiltrating myeloid cells serve a compensatory role for a loss of antigen presentation in microglia during infection. An analysis of brain-infiltrating T cell number, tetramer positivity, and IFN-γ production at earlier time points might also be valuable in clarifying if resident CNS macrophage antigen presentation by MHC II plays a role in *T. gondii* challenge. To this end, future experiments could examine earlier time points in chronic infection, such as 12 DPI, when there is a large T cell population in the brain but a comparatively

smaller population of infiltrating myeloid cells that could potentially exert a compensatory antigen presentation function in the brain (**Figs 3.3 -3.4**).

An important consideration in more strictly evaluating microglial antigen presentation by MHC II is the use of a more specific cre driver. In the current study, the CX3CR1 inducible cre would be expected to additionally target CNS border-associated macrophages (BAMs), including perivascular macrophages. While perivascular macrophages would be expected to constitute a small percentage of CNS macrophages targeted by this cre system, their positioning at the blood-brain-barrier may make them a particularly relevant cell type candidate for T cell priming and licensing for entry into the brain parenchyma. While we have focused on microglial antigen presentation under the broader lens of possible STAT1-mediated functions in this section, a more specific cre driver, such as the Hexb cre[170], will be necessary to determine whether more subtle phenotypes associated with $MG^{MHCII\Delta}$ mice are indeed specific to microglia. In this case, use of an existing binary cre system shown to differentially target microglia and BAMs may be an attractive alternative, but it is limited in application by sacrificing target gene excision for improved cell type specificity[120].

Chapter 6 – BIG picture discussion and future directions

6.1 Summary of dissertation findings

This dissertation work contributes to the fields of neuroimmunology, glial biology, and infectious disease by investigating how microglia, the resident immune cells of the brain, mediate protection against CNS infection. Yolk sac-derived microglia, in contrast to their bone marrow-derived macrophage cousins in other tissues, have been extensively characterized as serving specialized developmental and neuroprotective roles in the immune-privileged brain. A growing body of work has also characterized these cells as drivers of pathology during instances in which they inappropriately mount inflammatory responses that cause destruction to the CNS. Here, we have used the ubiquitous neurotropic pathogen, *Toxoplasma gondii,* as a model for microbial neuroinflammation to show that microglial immune activation is required for host resistance to CNS infection. In doing so, we have highlighted a predominantly cell-intrinsic role in microglial parasite clearance driven by the transcription factor STAT1.

We decided to study STAT1 signaling in microglia for several reasons. STAT1 is required for cellular responses to interferon, notably IFN-γ, which serves as the master regulator of immune resistance against *T. gondii* and other intracellular pathogens. Previous research has identified a requirement for IFN-γ-STAT1 signaling at both the organismal level and within specific cellular compartments during acute and chronic infection with *T. gondii*, highlighting the importance of this pathway against this parasite both within and outside of the CNS. Our pilot experiments suggested an intimate spatial association between microglia and inflammatory clusters containing the replicative tachyzoite form of the parasite, as well as clear evidence of a strong IFN-γ-driven transcriptional signature in microglia during *T. gondii* infection. We thus hypothesized that

microglia were critical anti-parasitic responders against *T. gondii* and that STAT1 signaling would be integral to their prospective functional responses during infection.

The topic of microglial identity has been marked by a series of influential papers that have shaped our current-day understanding that microglia are ontogenically and functionally distinct from brain-infiltrating monocyte-derived macrophages that arise from bone marrow. With this literature in mind, we used a tamoxifen-inducible genetic approach for studying microglia to specifically fluorescently label and target microglia for STAT1 excision. We performed several validation studies to confirm the specificity of our genetic deletion, including analysis of other immune cell populations and parasite clearance in multiple tissues outside of the brain at various time points post-infection. These experiments cumulatively indicated that during *T. gondii* challenge, STAT1 deletion from microglia results in CNS-specific pathology that is marked by an inability to restrict brain parasite burden, necrotizing lesions throughout the brain, and uniform animal mortality.

We then went on to immunophenotype the brains of mice with STAT1-deficient microglia to show that this CNS-specific pathology occurs despite robust anti-parasitic immune responses from brain-infiltrating myeloid and T cell compartments, which are critical for parasite control in the brain. While we initially thought that STAT1 deletion would impair the brain's global immune response due to a loss of potential microglial supportive roles such as chemokine production or antigen presentation, these experiments instead supported the hypothesis that the primary mechanism(s) by which microglia restrict *T. gondii* are cell-intrinsic. Previous literature supported the notion that this cell-intrinsic parasite restriction by microglia would be mediated by inducible nitric oxide production, a potent anti-parasitic effector response. However, accurate and efficient fluorescent labeling of microglia revealed that microglia do not express iNOS during *T. gondii*

challenge, regardless of STAT1-sufficiency. This finding sharply contrasted with the observation that high levels of iNOS expression were induced in brain-infiltrating macrophages within a shared tissue microenvironment, and builds on previous work from our group supporting a delineation in functional roles between brain-resident and brain-infiltrating macrophage populations within the *T. gondii*-infected brain.

Keeping in mind that IFN-STAT1 signaling is an ancient and evolutionarily conserved pathway capable of differentially regulating the expression large transcriptional networks, we turned to a bioinformatics approach for an unbiased interrogation of potential cell-intrinsic mechanisms of microglial-mediated parasite control. Our transcriptomic data resulted in the identification of several well-described parasite-killing molecules belonging to the immunity-related GTPase (IRG) and guanylate-binding protein (GBP) superfamilies that are differentially regulated by STAT1 in microglia. Since these proteins have been shown to play a role in restricting parasite replication, we analyzed the brains of mice with STAT1-deficient microglia for patterns of *T. gondii* gene expression associated with parasite replication vs. latency. In mice with STAT1-deficient microglia, we observed a 300-fold increase in the ratio of replication-associated tachyzoite gene expression relative to the quiescent-associated bradyzoite gene expression that is classically associated with CNS infection. Consistent with the hypothesis that microglial STAT1-deficiency results in the formation of a niche in which *T. gondii* can replicate due to the absence of IRG and GBP-mediated cytosolic killing, we observed increased microglial cytosolic infection in our experimental mice.

In this work, we also used RNA sequencing to construct a transcriptomic dataset of genes that are differentially expressed by microglia in naïve and *T. gondii* brains. This analysis showed strong overlap in the microglial transcriptional signature that is induced by CNS infection and that

which is also induced during diverse neurodegenerative disease states. These data indicate that the disease-associated microglia (DAM) transition, which is induced in AD, MS, ALS, and the natural CNS aging process, is activated in the *T. gondii*-infected brain. Further analysis revealed that the transcription factor we have been studying in microglia, STAT1, is required for full expression of the DAM signature seen during infection, pointing to interferon signaling as an activator of this shared microglial activation state. In doing so, we have also highlighted the potential value of using *T. gondii* as a novel model for studying DAMs. Collectively, completion of these studies has provided a better-developed understanding of how STAT1 signaling in microglia regulates cellular and systems-level immune responses during parasitic CNS infection. Our work has also added to a better understanding of the molecular cues that drive a conserved microglial activation state that is induced across neuroinflammatory contexts.

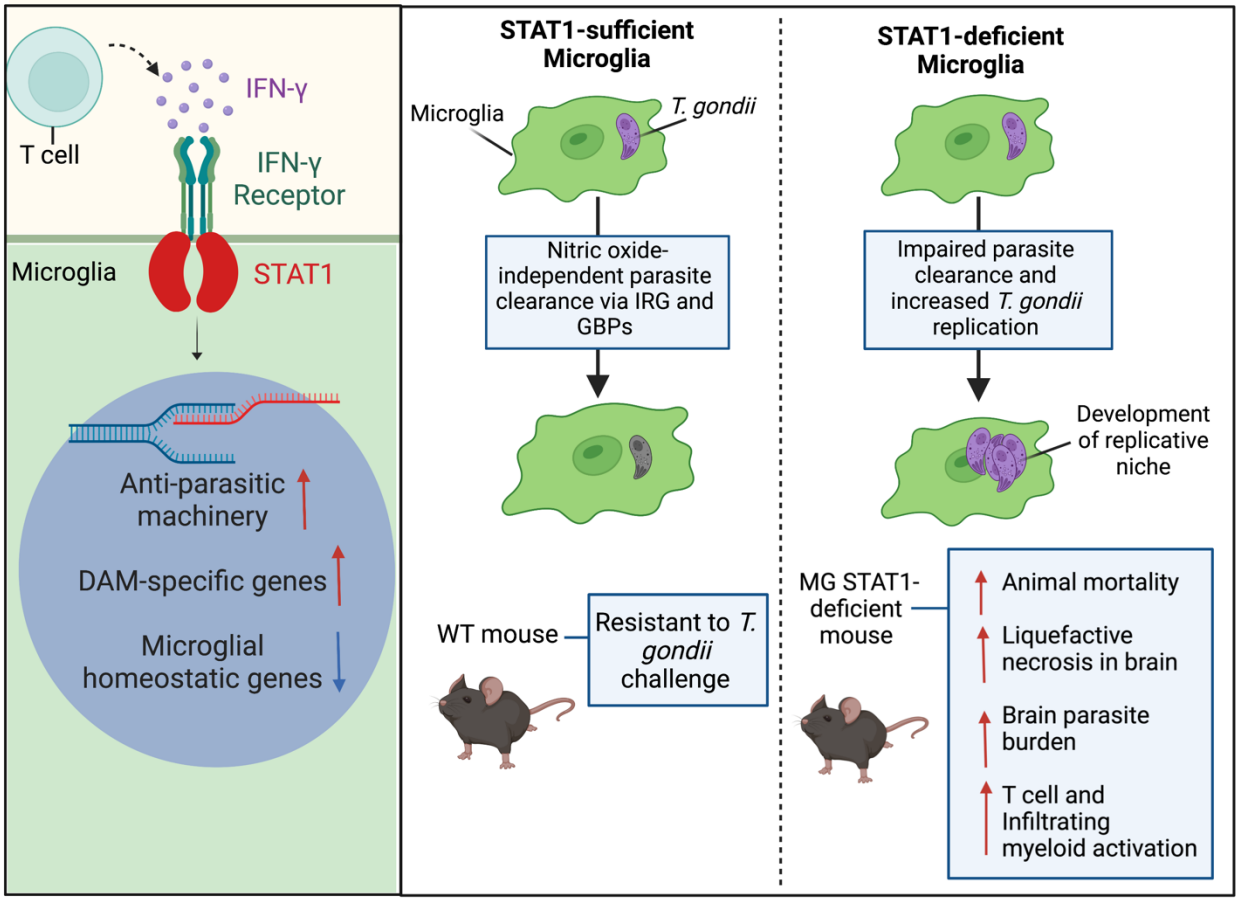


Figure 6.1. Working model and schematic summary of major dissertation findings.

6.2 Questions surrounding microglial-intrinsic parasite killing

Our studies implicate a STAT1-mediated, cell-intrinsic, and nitric oxide-independent mechanism of microglial parasite clearance during CNS infection with *T. gondii*. However, the precise STAT1-dependent mechanisms by which microglia kill parasite remain unclear. This section discusses potential effector candidates that drive microglia-mediated resistance to CNS infection with *T. gondii*, with a focus on immunity-related GTPase (IRG) and guanylate-binding protein (GBP) family proteins, microglial phagocytosis, and follow-up efforts to quantify infected microglia.

6.2.1 IRG and GBPs revisited

Based on both previous literature and our transcriptomic analysis, we hypothesized that IRG and GBP-family proteins in microglia contribute to parasite killing. Of the several IRG family genes that have been found to localize at the protein level to the *T. gondii* parasitophorous vacuole in mice[136], *Igtp*, *Irgm1*, *Irgm2*, *Tgtp1*, *Ifi47*, and *Iigp1* were all expressed by microglia in a STAT1-dependent manner (**Fig 3.5E-F**). The GBP-family proteins *Gbp2*, *Gbp5*, *Gbp6*, and *Gbp10* were similarly expressed by microglia during *T. gondii* challenge with a clear STAT1 dependency (**Fig 3.5E-F**). Previous work has implicated both IRG and GBP proteins in decorating the parasitophorous vacuole (PV) in which *T. gondii* is housed, effecting its mechanical rupture, and inducing autophagic processes to clear intracellular infection in both hematopoietic and non-hematopoietic cell types[136]. STAT1-regulated IRG and GBP effector function thus represents a promising candidate mechanism for microglial-intrinsic parasite control.

Endeavors to pinpoint which IRG or GBP proteins are most relevant to microglia-mediated parasite clearance are currently limited by the cooperative nature in which these proteins confer

resistance, and are also compounded by limited genetic tools to efficiently excise larger segments of chromosomes within a particular cell type[299]. These concepts are highlighted in a study from Yamamoto et al., in which mice were engineered to carry a global knockout for a cluster of six GBPs located on Chromosome 3[147]. Using this chromosomal engineering approach, the authors observed increased animal mortality in these knockout mice when challenged with *T. gondii*[147]. This increase in animal mortality was not observed with deletion of a single isoform of GBP2 (*Gbp2ps*), or with deletion of *Gbp2ps* in combination with *Gbp5*[147]. Subsequent *in vitro* studies in which isolated peritoneal macrophages had individual GBPs genetically restored via retroviral transfer resulted in an incremental rescue of parasite control[147]. While an elegant approach for producing global knockout animals, there would be a need to use an inducible cre-lox approach when applying the deletions to microglia[118,121]. Such an approach could yield insufficient or unpredictable excision results, as cre recombinase efficiency declines as a factor of targeted genomic distance[299].

As previously discussed, genetic targeting of microglia *in vivo* is also typically preferred over *in vitro* approaches, since the latter results in rapid de-differentiation and loss of microglial identity. However, because it is not currently feasible to use an inducible cre recombinase system to genetically delete several genes selectively from the microglial population at once, there would be some value in incorporating select *in vitro* approaches[164] to guide candidate gene selection to subsequently study in microglia *in vivo*. For instance, *in vitro* studies utilizing small interfering RNAs that decrease expression of these genes during *T. gondii* challenge may thus be informative in determining which permutation of two to three IRG and GBP combination genes should be subsequently targeted in an *in vivo* setting using an inducible cre system to produce a specific and reproducible phenotype.

Bioinformatics may play an integral role in designing future experiments to understand which IRGs and GBPs may exert strongest anti-parasitic effector functions in microglia. Our transcriptomic analysis suggests that *Gbp2*, *Igtp*, and *Irgm1* are three IRG/GBP-family genes that are amongst the most highly expressed by wildtype microglia during *T. gondii* challenge (**Fig 3.5F**), and they may thus serve as the most relevant genes to target. Consistent with this view, mice with global *Igtp* deletion succumb to toxoplasmosis with a survival curve similar to that of IFN-γ and STAT1-deficient mice[96,144]. Similar studies have also implicated GBP2 in restricting parasite growth in multiple cell types[146,300]. *Irgm1*, the gene encoding LRG-47, negatively regulates effector IRG activation and its deficiency is associated with deficits in both phagosome maturation and autophagy[301]. With our current knowledge, *Gbp2*, *Igtp*, and *Irgm1* may thus serve as promising STAT1-regulated genes to excise from microglia to more closely probe the mechanics of microglial-intrinsic parasite restriction.

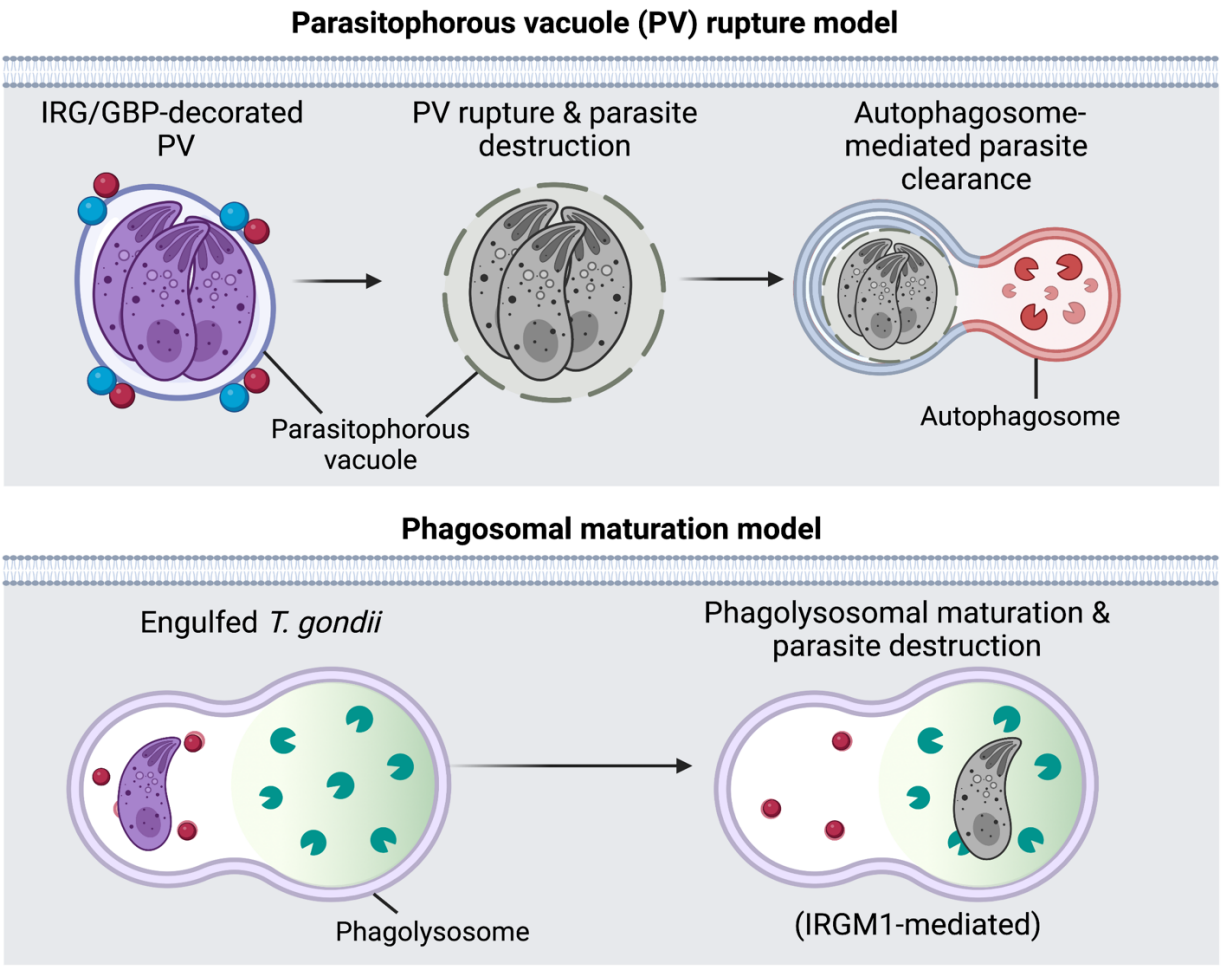


Figure 6.2. Two models of IRG and GBP family protein activity in promoting cell-intrinsic parasite killing.

IRG and GBP family proteins are STAT1-regulated molecules that cooperatively contribute to parasitophorous vacuole (PV) membrane disruption and subsequent parasite killing. Several of the genes encoding these molecules, including *Igtp*, *Irgm1*, *Irgm2*, *Tgtp1*, *Ifi47*, and *Iigp1* are differentially regulated in microglia by STAT1 during *T. gondii* infection. In the PV rupture model, IRG and GBP proteins decorate the parasitophorous vacuole in response to IFN-γ, and exposure of *T. gondii* to the cytosol allows clearance via autophagy machinery. In the phagosomal maturation model, the regulatory IRG, LRG-47 (*Irgm1*), is required for normal parasite destruction following phagocytosis.

6.2.2 Phagocytosis

As the resident macrophage and professional phagocyte population of the brain, microglia display strong phagocytic capacity that distinguishes them from non-professional phagocytes. Activated microglia are equipped with phagocytic machinery such as the TAM receptors Axl and Mer, as well as a suite of complement, scavenger, and Fc receptors[198,302]. Work has been done to identify microglial phagocytosis of cellular debris[159,203,302,303], neuronal synapses[185,204,207,304,305], and amyloid aggregates[306–309], but less is known about whether microglial phagocytosis can serve as an anti-microbial effector function. This gap in understanding likely stems from the limited availability of tools to study phagocytosis of microbes *in vivo*, although recent technologies to more comprehensively explore these functions are beginning to emerge[310]. However, both research linking STAT1 to improved phagocytic capacity[311–313] and literature investigating *T. gondii*-phagocyte interactions[314–316] may present some clues as to whether microglial-mediated phagocytosis serves as a cell-intrinsic protective program during CNS infection.

In order for a phagocytosed microbe to undergo degradation within a macrophage, a phagosome containing that microbe must mature in a series of steps that results in membrane fusion with endocytic compartments and increased phagolysosomal acidification[317]. Studies have shown that treatment of cultured macrophages with recombinant IFN-γ increases phagosome acidification and enhances proteolytic and hydrolytic degradation of phagocytosed cargo[313]. Moreover, STAT1-deficient macrophages display impaired acidification of *Leishmania*-containing phagosomes relative to wildtype cells, even in the absence of IFN-γ – suggesting a potential dual role for STAT1 signaling in phagosome maturation[311]. Work has been performed showing that *T. gondii* is highly susceptible to low pH, yet intriguingly, the parasite may possess some innate ability to block phagosome acidification[314]. For instance, antibody-coated or dead *T.*

gondii treated with a pH-sensitive reporter permits phagosome acidification, whereas phagosomes containing live parasite show little capacity for acidification-induced toxoplasmicidal activity unless macrophages were pre-activated[314], likely due to the secretion of a network of proteins later shown to modify the phagosome itself [315]. Some work also suggests that avirulent strains of *T. gondii* have evolved some capacity to actively evade the macrophage phagosome following initial phagocytosis in what has been termed the phagosome to vacuole invasion (PTVI) pathway[318]. PTVI appears to rely on some degree of phagosome acidification, although at what pH range or whether this phenomenon selectively occurs under specific conditions is unclear[318]. Together, these studies (i) highlight a relationship between STAT1 and phagosome maturation, (ii) suggest that the phagosome may serve as an inhospitable cellular compartment for supporting parasite growth, and (iii) support the hypothesis that STAT1-enhanced phagocytic processes may serve as a candidate mechanism of microglial-intrinsic *T. gondii* destruction.

In light of this literature, the topic of whether impaired phagosome maturation is sufficient to drive unrestricted *T. gondii* growth is intriguing. We have observed via confocal microscopy that microglia from $MG^{STAT1\Delta}$ mice harbor intracellular tachyzoite structures, typically prompting the question as to whether this phenomenon represents active invasion or phagocytosis (**Fig 3.6F-G**). Initial attempts to answer this question via immunohistochemistry have proved challenging due to a staining artifact that likely stem from parasite expression of a surface Fc receptor on tachyzoites that binds antibodies in a non-specific manner – proposed as an evolutionary adaptation to evade antibody-mediated host immunity within an infected host[319]. However, given the literature that phagocytosis does not necessarily result in a "dead end" for *T. gondii*[318], perhaps a more suitable initial question is: "are parasites within STAT1-deficient microglia viable?" Experiments that address this question may focus on cytoarchitectural analyses of parasitophorous

vacuole integrity via electron microscopy, or *in situ* staining approaches that assess the nuclear localization of *T. gondii*-specific proliferation or viability markers within infected STAT1-deficient microglia. Alternatively, the use of LysoTracker[320], a pH-sensitive dye, may be helpful in determining if the intracellular parasite is located within an acidified lysosome. Because microglia specialize as the professional phagocytes of the CNS[198], *T. gondii* infection may thus serve as a useful system for better understanding STAT1 signaling as a candidate driver of enhanced phagocytic capacity in microglia as it pertains to anti-microbial control or even sterile contexts.

6.2.3 Insights from attempts to quantify *T. gondii*-infected microglia

A key qualitative observation from Chapter 3 was that of potentially increased susceptibility of STAT1-deficient microglia to parasitization at 15 DPI (**Fig 3.6F-G**). We thus performed follow-up experiments to provide a quantitative measurement for this phenomenon in *T. gondii*-infected WT and $MG^{STAT1\Delta}$ mice. Using flow cytometry, we analyzed the frequency of cells isolated from brain that stained positively for intracellular *T. gondii*. Because prior confocal microscopy analysis indicated that microglia and macrophages in particular contained vacuolar parasite antigen that conformed to neither tachyzoite or tissue cyst morphology, possibly consistent with phagocytosis, we used optical side-scatter as a preliminary approach for distinguishing infected cells from phagocytic debris at various stages of degradation. This approach allowed for gating of a clearly defined population of intracellular *T. gondii* staining with low optical side-scatter. While more advanced flow cytometry with imaging capability (i.e. ImageStream[321]) will be necessary to definitively determine that this population correspond to infected cells, multiple lines of evidence support this interpretation. These include: (i) relative absence of the high side-

scatter population posited to correspond to phagocytic debris in CD45-negative cells in contrast to cells with robust phagocytic capacity such as microglia and macrophages, and (ii) an increase in fluorescence intensity specific to the tentative "infected" but not "debris" FACS gate in cells isolated from heavily-infected $MG^{STAT1\Delta}$ brains (**Fig A3**).

Based on this analysis, we found that each of the isolated cell populations that were analyzed – CD45-negative cells, microglia, infiltrating myeloid cells, and lymphocytes – displayed a significant increase in *T. gondii* "infection rate," as operationally defined (**Fig A4)**. The total percentage of infected cells, however, differed widely across cell populations (**Fig A4)**. Infiltrating myeloid cells and lymphocytes had a relatively low infection rate in either mouse genotype, whereas CD45-negative (non-immune) cells displayed the highest rate of infection amongst analyzed cell types within both WT and $MG^{STAT1\Delta}$ mice (**Fig A4)**. Microglia displayed the largest increase in infection rate between groups of mice, with microglia from $MG^{STAT1\Delta}$ mice displaying a ~10-fold increase in infection rate relative to WT controls (**Fig A4)**. These data are consistent with the hypothesis that STAT1-deficiency in microglia supports parasite replication.

The observation that cell types other than microglia display increased rates of infection, however, presents a paradox. It is unclear why multiple cell types would display increased susceptibility to *T. gondii*, since our genetic manipulation is largely restricted to the microglial compartment (**Supplementary Fig 3.2A-E**). One potential explanation for this finding may be that high rates of parasite replication within and egress from microglia due to impaired STAT1-mediated cell-intrinsic killing may generate a sufficiently high parasite burden to permit parasitization of several different cell types, some which may be fully competent in resolving their own cytosolic infection over time via STAT1-mediated effector functions. Since *T. gondii* is an obligate intracellular parasite, it would not be expected to live extracellularly following a lytic

replication cycle, and a high parasite burden must be somehow distributed across cells within the brain. Now that we have used a high-throughput approach for quantifying infected cells with some degree of success, an analysis of earlier time points may be helpful in resolving these details. 15 DPI is a time point very close to the onset of mortality in $MG^{STAT1\Delta}$ mice, and is also when their brains display histological evidence of widespread liquefactive necrosis (**Fig 3.2G-H**). This time point was selected for preliminarily quantifying infected microglia because we were previously able to confirm that this phenomenon occurs at this time point via confocal microscopy (**Fig 3.6F-G**). However, an analysis of an earlier time point may be sufficient for experimentally controlling for unknown confounds that may be associated with terminal toxoplasmic encephalitis. While these data certainly raise new questions related to cell-intrinsic anti-parasitic in multiple cell types in $MG^{STAT1\Delta}$ mice, they are also consistent with the hypothesis that microglial STAT1-deficiency supports parasite replication. These data thus frame a qualitative observation from Chapter 3 (**Figure 3.6F-G)** with a preliminary quantitative analysis that is central to our understanding of microglial-intrinsic parasite control.

6.3 Microglial STAT1-deficiency as a replicative niche

The hypothesis that microglial STAT1-deficiency generates a replicative niche in which *T. gondii* can expand is supported by multiple lines of evidence. This evidence includes the loss of several STAT1-dependent IRG/GBP genes that play roles in cell-intrinsically restricting parasite replication (**Fig 3.5E-F**), a 300-fold increase in the highly replicative tachyzoite form of *T. gondii* relative to its quiescent bradyzoite form (**Fig 3.6D-E**), and an increase in the rate of microglia harboring intracellular parasite in $MG^{STAT1\Delta}$ mice (**3.6F-G**). The Hunter laboratory has also previously shown that astrocytic STAT1 deletion similarly results in increased parasite burden and

animal mortality during *T. gondii* challenge, although with apparently reduced disease severity as compared to microglial STAT1 deletion[152]. This previous literature, however, sets a precedent for our hypothesis that STAT1 deletion may be linked to formation of a replicative niche for intracellular parasites.

6.3.1 Experimental approaches for testing the "replicative niche hypothesis"

Our proposed "replicative niche hypothesis" may posit that STAT1-deficient microglia are more maladaptive to anti-microbial immunity than would the total absence of microglia within the same brain. I thus hypothesize that microglial ablation during *T. gondii* challenge would lead to impaired parasite control relative to non-ablated WT animals, but not to the level of infection severity observed in $MG^{STAT1\Delta}$ mice. A combination of multiple microglial depletion strategies may be used to test this hypothesis, such as: (i) CSF1R antagonism via PLX5622[187–191] and (ii) genetic ablation of IL-34, a tissue restricted ligand for CSF1R[184]. PLX5562 is easily administered to mice via chow and can be formulated to deplete >99% of microglia in a matter of days[183]. This pharmacological treatment may thus serve as a feasible and effective first-look in determining if microglial STAT1-deficiency as a replicative niche is a primary driver of the fatal toxoplasmic encephalitis seen in $MG^{STAT1\Delta}$ mice. For instance, if parasite burden is reduced or host survival is extended in *T. gondii*-infected $MG^{STAT1\Delta}$ mice that are treated with PLX5622, this may suggest that clearance of a STAT1-deficient replicative niche allows for some degree of resolution of toxoplasmic encephalitis.

Many caveats exist with global CSF1R antagonism, including a lack of understanding of how microglia undergo cell death, mixed reports of induced inflammation, systemic macrophage depletion, and compromised circulating antigen presentation cell maturation at baseline[190,193–195].

As a result, a complementary approach to further understand the relationship between microglial STAT1-deficiency and parasite growth may employ the use of *Il34*$^{-/-}$ mice. IL-34 is a tissue-restricted ligand for CSF1R expressed in both the brain and skin in the absence of CSF1R's alternative ligand, mCSF[184]. As a result, *Il34*$^{-/-}$ mice lack forebrain microglia as well as Langerhaans cells, but possess typical macrophage number and function in other tissues[184–186]. IL-34-deficient mice were previously used in a mouse model for WNV infection, wherein microglial depletion by this method prevented overexuberant synaptic stripping and rescued consequent neurocognitive impairment – highlighting their utility in infection models[185]. An experiment in which *Il34*$^{-/-}$ mice are challenged with *T. gondii* may reveal that they fail to acquire a level of parasite burden comparable to that of MG$^{STAT1\Delta}$ mice, especially at early time points prior to blood-derived macrophage infiltration into the brain. Such an observation would be consistent with the view of microglial STAT1-deficiency as a replicative niche.

IL-34-deficient mice are not commonly used throughout the microglia field, likely due to the genetic deletion impairing developmental functions of microglia such as synaptic pruning[185,204,207,304,305]. While this may serve as a limitation for research efforts that seek to better understand the developing brain or focus on behavioral readouts, it may be less of a concern when studying the fundamentals of pathogen-host interactions with a parasite capable of infecting virtually any nucleated cell, such as *T. gondii*. A unique experimental advantage of using IL-34-deficient mice would be that while these mice lack forebrain microglia, they display relatively normal numbers of microglia in the cerebellum, a region that produces the alternate CSF1R ligand, mCSF[184,186]. Should PLX5622 and IL-34-deficiency result in a relative reduction in brain parasite burden compared to MG$^{STAT1\Delta}$ mice, more complex genetic approaches could be used. For instance, crossing IL-34-deficient and MG$^{STAT1\Delta}$ mice would yield mice with STAT1-deficient cerebellar

microglia and an absence of microglia in forebrain structures. In this case, one could compare parasite burden and density in the different regions both within the same mouse and in comparison to mice of *Il34* $^{-/-}$ and MG$^{STAT1\Delta}$ genotypes. These proposed experiments may thus prove valuable in framing a hypothesized artificially-created replicative niche within the context of total microglial immune effector capacity, thus contributing to a better understanding of microglial anti-microbial defense and elucidating how an opportunistic neurotropic pathogen interacts with brain-resident cell types. Understanding how microglial depletion impacts common opportunistic CNS infections such as *T. gondii* may be important in preventing reactivation, since microglial depletion is currently being used in an ongoing Phase 1 clinical trial by Janssen Pharmaceutica as an intervention for Alzheimer's disease and mild cognitive impairment (Trial ID #: NCT04121208).

6.3.2 STAT1 signaling and cell death

In addition to a lack of intracellular immune pressure in the form of IRG and GBPs to prevent parasite replication in STAT1-deficient microglia, an impairment in the ability to undergo programmed forms of cell death may also support sustained parasite replication[322]. IFN-STAT1 signaling has been strongly implicated as an inducer of both apoptosis[323–326] and pyroptosis[327–329] in different cell types. Several studies have shown that STAT1 activation promotes pro-apoptotic signaling by upregulating the expression of caspases and cell death receptors and ligands such as Fas/FasL and TRAIL/TRAIL-R[330–334]. Independent of transcription, STAT1 has also been found to bind with TNFR1 and TRADD to inhibit pro-survival NF-κB-mediated pathways[335]. We have found that STAT1 regulates the differential expression of *Casp4* and *Casp9* in microglia, and is similarly required for the expression of *Bcl2l14*, a pro-apoptotic gene. Both Caspases 4 and 9 have defined roles in driving pyroptosis and intrinsic apoptosis, respectively, and the increased

expression of *Bcl2l14* has been shown to induce apoptosis[336–338]. STAT1-deficiency has also been previously shown to impair the proteolytic processing of Caspases 1 and 11, two molecules with clear roles in executing pyroptotic cell death[339,340]. These latter studies are intriguing, because previous work from our laboratory has suggested that microglia undergo pyroptosis to release the alarmin IL-1α to control *T. gondii* parasite burden. Should STAT1 excision inhibit pyroptotic cell death, STAT1-deficient microglia could facilitate parasite growth while simultaneously displaying a decreased capacity for alerting and priming neighboring cells.

Pyroptosis, first described in 1992, is an inflammatory form of cell death that is mediated by a multi-protein complex referred to as the inflammasome[341,342]. The inflammasome can be activated by sensors that respond to a variety of PAMPs and DAMPs, and canonically leads to the activation of Caspase-1, the cleavage of cytokines such as IL-1β and IL-18 into their active forms, and the activation of the pore-forming molecule Gasdermin-D, which disrupts plasma membrane integrity[342]. The pores formed by Gasdermin-D allow for the release of active forms of pro-inflammatory interleukins and cytosolic molecules and can lead to cell swelling and lysis due to a dysregulation of cellular osmolarity[342]. Previous work has shown that the NLRP1 and NLRP3 inflammasomes provide resistance to *in vitro* and acute *T. gondii* infection, highlighting the importance of this cell death pathway during parasitic infection[84,343].

A transcriptomic analysis of pyroptosis-associated molecules indicates that STAT1 in microglia is not required for the expression of NLRP3, but is required for full expression of NLRP1α, Caspase-1, and Gasdermin-D. Regardless of the specific inflammasome sensor that may function in microglia, pyroptotic machinery may not be biologically useful if it cannot lead to the proteolytic cleavage of Gasdermin-D by Caspase-1 to initiate cell death and promote replicative niche clearance. Previous work from our group has also indicated that the pore-forming molecule

Gasdermin-D is required for microglial release of IL-1α and downstream vascular priming and leukocyte infiltration to the brain[77]. However, our work in Chapter 3 also shows that $MG^{STAT1\Delta}$ mice display robust levels of vascular and infiltrating immune activation, suggesting that impaired recruitment and priming may be compensated for by other mechanisms in these mice (**Fig 3.3**, **Fig 3.4**, and **Supplemental Figure 9**). As a result, the potential functional protection provided by microglial pyroptosis during *T. gondii* infection may be most strongly rooted in the ability to eliminate the parasite's replicative niche.

Future experiments can provide insights into whether STAT1 signaling in microglia facilitates programmed cell death pathways such as apoptosis or pyroptosis, and whether replicative niche elimination is important for restricting brain parasite burden. Confocal or flow cytometric analysis of cleaved Caspase-3 in microglia isolated from WT and $MG^{STAT1\Delta}$ brains during *T. gondii* challenge could provide an indication of whether STAT1-deficiency leads to reduced capacity for apoptosis. Similarly, reagents such as FLICA and YVAD-AFC may be used to detect cleaved Caspase 1 activity, to understand if microglial STAT1-deficiency prevents pyroptosis. Because STAT1-excision in $MG^{STAT1\Delta}$ mice does not reach full penetrance with our tamoxifen-inducible model, the occurrence of cleaved forms of Caspase-1 or Caspase-3 in STAT1-sufficient and -deficient microglia can also be compared within the same brain. Should such experiments generate traction, intravital two-photon imaging of dual-fluorescent microglial-parasite interactions could also present interesting opportunities for qualitatively or quantitatively evaluating the fate of infected microglia as a variable of STAT1-sufficiency.

6.4 Sensing *T. gondii*: A potential role for purinergic signaling

Another question generated from our work pertains to how microglia sense parasite during *T. gondii* challenge. Perhaps one of the most-studied element of the complex microglial sensome is purinergic signaling. Microglia are especially motile cells, and their repertoire of P2X and P2Y receptors, as well as ectonucleases collectively drives rapid chemotactic responses and cytoskeletal modifications in response to extracellular purines[198]. Purines such as ADP and ATP are fundamental molecules to cellular energetics and are present in the cytosol of all cells[344]. They are also released during necrotic forms of cell death, therein prompting microglial chemotaxis[161]. This microglial response is often studied using models of microscopy-induced focal laser injury to brain tissue, wherein microglia project their filopodia-like processes toward the induced lesion, forming a spherical enclosure at its boundary[161]. Because *T. gondii* is a lytic intracellular pathogen, its egress from cells would be expected to liberate intracellular purines from a necrotic host cell, which could then be sensed by microglia. Such a system of microglial chemotactic responses to parasite-induced damage rather than necessarily the parasite itself may be neuroprotective by equipping microglia with a mechanism that allows them to indirectly sense a wide range of pathogenic microbes.

While clear disruptions to microglial spatial tiling during *T. gondii* challenge are reported in Chapter 3, this phenomenon warrants a closer look (**Fig 3.1B-F** and **Fig 3.6A-B**). In the steady-state brain, microglia are typically evenly spatially distributed in non-overlapping territories[345]. At 15 DPI, microglia display a more rounded and simplified morphology and localize to nodules containing clusters of *T. gondii* (**Fig 3.1B-F** and **Fig 3.6A-B**). However, our unpublished data shows that at 8 DPI, a time around when parasite is first enters the brain parenchyma[56], microglia display process extension response that radially converges toward these parasite foci (**Fig A5A-**

A5B). Importantly, this response qualitatively mirrors the well-studied microglial process extension response observed following experimentally-induced laser injury both in other studies[161,230] and our own hands in both WT and MG$^{STAT1\Delta}$ mice (**Fig A5C-A5D**). These observations suggest that purinergic signaling may modulate microglial spatial interactions with *T. gondii* – an important requisite for parasite sensing.

If purinergic signaling drives microglial spatial contact with *T. gondii*, it may intersect with STAT1 signaling in multiple ways. First, interferon signaling, either directly or through interferon-regulated factor 8 (IRF8), has been found to regulate the expression of purinergic molecules involved in the chemotaxis of microglia or other cell types, including P2X4R, P2Y6R, P2Y13R, ENTPD1/CD39, and ECSR[346]. Of these molecules, our transcriptional analysis of WT and STAT1-deficient microglia suggest that STAT1 might be required for normal levels of P2X6R and the ectonuclease CD39 during *T. gondii* infection (**Fig A6**). Initial attempts to study baseline purinergic-mediated microglial motility, however, indicate that STAT1-deficient microglia do not show changes to baseline motility, as evaluated by intravital two-photon imaging of naïve mice (**Fig A7**). These preliminary results may either indicate that STAT1-deficient microglia do not have impaired purinergic responses, or could instead suggest that changes to motility may be preferentially observed during *T. gondii* infection – a disease state that leads to strong levels of sustained interferon-STAT1 signaling due to a large T cell presence and IFN-γ production in the brain[26]. This pilot experiment also involved a small cohort of mice, and may additionally lack the statistical power to parse out these effects.

The observation that microglia in MG$^{STAT1\Delta}$ mice appear to display the distinct locomotive and process extension response associated with purinergic signaling suggests that there may be at least some level of purine-driven chemotaxis (**Fig A6**). Thus, a second avenue on which purinergic

signaling may intersect with STAT1-mediated responses in microglia relates back to our replicative niche hypothesis. In a wildtype brain, parasite egress from nearby cells may initiate purinergic-mediated process extension and chemotaxis toward focal sites of parasite replication. Should this rapid motile response lead to rapid microglial parasitization by *T. gondii*, or position microglia to surround the focal lesion and become infected after a subsequent round of lytic replication, IRG and GBP-family proteins may allow for effective parasite clearance by STAT1-sufficient microglia. Conversely, purinergic-mediated chemotaxis may increase the incidence of STAT1-deficient microglia becoming infected by parasite at these focal sites, essentially providing *T. gondii* with a consistent and optimal reservoir of IRG/GBP-deficient cells in which it can replicate. Such a scenario is consistent with the large parasite burden observed in focal clusters in $MG^{STAT1\Delta}$ mice (**Fig 3.6A-B**). This hypothesis may also help explain why STAT1-deficiency in astrocytes, which outnumber microglia in the brain but display comparably decreased motility, does not lead to as severe toxoplasmic encephalitis as is seen in $MG^{STAT1\Delta}$ mice[152].

Analyzing what functional roles microglial purinergic signaling may play during *T. gondii* infection could utilize a combination of genetic and imaging strategies. For instance, *P2ry12* is a highly expressed microglial signature gene[173,198], and genetic knockouts for this gene or the use of pharmacological inhibitors of its encoding protein result in impaired neurovascular sealing in response to laser injury[230]. Pharmacological inhibition of P2Y12R with the drug MeSAMP also prevents resting microglia from acquiring highly motile and phagocytic "honeycomb" and "jellyfish" phenotypes characterized in a closed-skull mouse model of traumatic brain injury (TBI)[347] – highlighting its role in activation states that display rapid and complex cytoskeletal rearrangements. Thus, feasible initial attempts to examine microglial purinergic signaling during infection may include analyzing *P2ry12* -/- brains for potential changes to parasite burden, as well

as potential changes to microglial process extension and engagement. Further analyzing infected brains from these mice by confocal microscopy could also yield insights into whether P2Y12R is required for the proper formation of microglial nodules – aggregates of microglia at focal sites of replicating parasite. Parameters such as: (i) nodule sphericity, (i) the number of microglia that display process extension as a function of total microglial density, (iii) the number of processes extended per microglia, (iv) the radius from which microglia are able to extend their processes, (v) and whether extended microglial processes are preferentially infected, may all provide interesting clues about how purinergic signaling might modulate microglial interactions with *T. gondii*, especially at early time points of CNS infection.

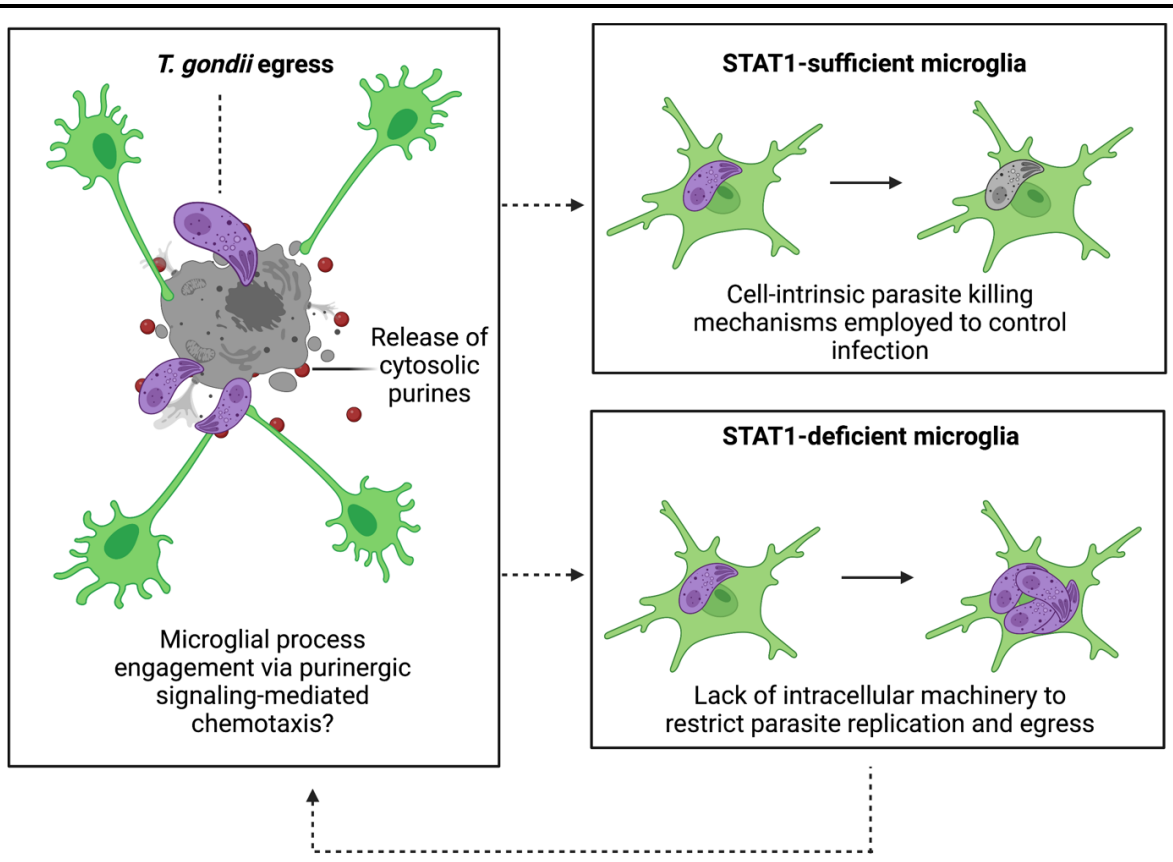


Figure 6.3. Hypothesized model for purinergic signaling in driving microglial activity as a cellular buffer during *T. gondii* infection.

Microglial chemotactic capacity is driven by a complex sensome which includes a repertoire of purinergic receptors and ectonucleases. Microglia have been shown to rapidly extend their filopodia-like processes to sites of laser injury in a purinergic-dependent manner, and a similar phenomenon is observed at early time points of CNS infection with *T. gondii*. Should purinergic signaling play a role in microglial physical association with parasite during lytic egression events from nearby infected cells, this process may increase the likelihood of microglia becoming infected by *T. gondii*. A lack of cytosolic anti-parasitic machinery including IRG and GBP-family proteins in STAT1-decient microglia is expected to lead to an inability to constrain parasite replication within the host microglia, thus allowing purinergic signaling-mediated chemotaxis to facilitate an optimal and consistent replicative niche in which *T. gondii* can proliferate. Whether STAT1-deficiency and purinergic-driven microglial chemotaxis synergize to result in the severe toxoplasmic encephalitis observed in $MG^{STAT1\Delta}$ mice is an area of great interest for future studies.

6.5 Microglia, macrophages, and iNOS

Another interesting finding from Chapter 3 is that even in the presence of T cell-derived IFN-γ, brain-resident microglia do not express the anti-parasitic effector protein iNOS – required for the inducible production of anti-parasitic reactive nitrogen species[125,261] (**Fig 3.3**). This lack of iNOS in microglia stood in stark contrast to that of a large population of iNOS+ infiltrating macrophages, which we were able to discriminate from microglia using a ZsGreen fluorescent reporter. Since both the microglial and infiltrating macrophage populations in this system share a tissue microenvironment, it is curious as to why these cell types differ in their anti-parasitic effector molecule expression, especially in light of literature illustrating that macrophages typically display marked plasticity in response to their environments. This finding, however, does build on existing themes in the literature on microglia possessing a unique identity and functional properties that distinguish them from other macrophage populations[77,172–174]. This literature includes previous work from our group showing that during chronic CNS infection with *T. gondii*, microglia display a distinct transcriptional signature from that of macrophages, and were enriched in molecules such

as IL-1α, while simultaneously displaying decreased markers of NF-κB activation when compared to macrophages[77].

These differences in microglial and infiltrating myeloid activation in a shared tissue microenvironment may be related to the unique ontology of microglia, relative to other immune cells. Microglia are derived from embryonic yolk sac, in contrast to the predominant fetal liver origin of other tissue resident macrophages, and the bone marrow origin of monocyte-derived macrophages that infiltrate the brain in states of neuroinflammation[117,121,348]. In mammals, a cell type referred to as the primitive erythromyeloid progenitor cell eventually gives rise to the microglial population prior to formation of the BBB and definitive hematopoiesis in peripheral tissues[117]. This progenitor cell also generates the large and nucleated red blood cells seen during fetal development, which contrast with the anucleated red blood cells generated through the process of definitive hematopoiesis post-gestation[349] – highlighting unique physiological qualities of this branch of immune cells that are largely replaced in tissues outside of the CNS. Whether the establishment of the mature microglial pool from this primitive hematopoietic cell type is what fundamentally impacts the nature and range of possible immune responses amongst macrophage populations is not currently known. Some literature suggests that transcriptional repressors such as *Sall1*, a microglial signature gene, may be responsible for suppressing microglial activation in the immune-privileged brain[171,177]. Indeed, microglial-specific *Sall1* deletion results in an increased microglial activation and consequent disruptions to neurogenesis – suggesting that immunological quiescence is necessary for steady-state brain function[171]. It is intriguing, however, to speculate on whether inhibiting transcriptional repressors such as *Sall1* during states in which inducing microglial-mediated inflammation may be beneficial, such as CNS infection or even malignant brain tumors, could yield neuroprotective effects.

A recent paper that integrated several single-cell RNA sequencing datasets[201,209,296,350,351] that focus on microglia across the lifespan and during neurodegeneration also provides some new insights into the identities of microglia vs. macrophages during neeuroinflammation[296]. This study found that during both normal aging and in mouse models of Alzheimer's disease, bone marrow-derived monocytes accumulate in the brain, where they display a pro-inflammatory transcriptional profile marked by the elevated expression of markers such as TNF-α, IL-1, and IL-6[296]. These cells, termed disease inflammatory macrophages (DIMs), were reported to express genes previously deemed specific to yolk sac-derived microglia, presenting a potential experimental confound to previously analyzed microglia and macrophage datasets in the literature[296]. Throughout our own studies, our use of a tamoxifen-inducible genetic labeling system in young mice confers well-established fidelity in studying bona fide microglia[118,121]. However, this recent study echoes the consistent observation that microglia and blood-derived macrophages that infiltrate the brain during neuroinflammation are functionally distinct cell populations. Importantly, this study revealed that relative to monocyte-derived DIMs, DAMs of microglia origin display a less inflammatory signature[296].

Because they have so far been described within the contexts of neurodegeneration and aging, whether these recently discovered DIMs are similar to the infiltrating macrophages seen during CNS infection is a new question that may be answered as the field achieves a broader understanding of their identity. The discovery of disease-associated microglia (DAM), was marked by the interpretation that they were unique to neurodegeneration[209,297], yet our results in Chapters 3 and 4 indicate that DAMs are present during CNS infection. Thus, efforts to understand what molecular patterns may result in DIM generation or accumulation in a variety of contexts are warranted. For instance, if bona fide DIMs are sorted from aged 5xFAD mouse brains and injected

into the brain of a *T. gondii*-infected mouse, one might hypothesize that they would take on an anti-parasitic transcriptional state marked by the expression of iNOS. In this light, DIMs could thus be seen as the monocyte's "answer" to disease-associated microglia (DAM) in the brain – in this case, a monocytic activation state shaped by its tissue microenvironment, yet constrained by fundamental properties stemming from its cellular lineage.

6.6 Open questions pertaining to DAMs during *T. gondii* infection

The presence of DAMs in the brain during *T. gondii* infection strongly suggest that there are shared molecular cues that link infection and neurodegeneration. Our work from Chapters 3 and 4 suggests that interferon-STAT1 signaling is embedded in the core list of genes that underpins transition of homeostatic microglia to DAM, and thus IFN is one of the molecular cues that shapes this transition (**Fig 3.5C-D** and **Supplementary Fig 1**). In the case of *T. gondii* infection, the functionally predominant source of IFN-STAT1 signaling appears to be T cell-derived IFN-γ. However, during some neurodegenerative diseases, type I interferon may also serve as a more relevant activating signal, such as during AD. *In vitro* and *ex vivo* studies indicate that AD-associated amyloid beta is able to induce IFN-α/β production in both neurons and mixed glial populations, prior to the upregulation of other pro-inflammatory cytokines[352–355]. Similarly, there is strong evidence of type I interferon signaling in contexts ranging from Parkinson's disease to ALS[356].

What insights could be generated from studying DAMs during T. gondii infection?

Whether DAMs are neuroprotective or instead drivers of pathology remains an open area of debate and may depend on the specific disease, stage of disease progression, specific marker of

pathology, and whether increased immune activation presents a therapeutic target. For instance, pro-inflammatory cytokine production and peripheral immune cell recruitment through the production of the DAM-associated chemokine CCL2 and its downstream signaling through CCR2 is associated with worsened disease outcomes during mouse models for MS[118,357]. At the same time, literature suggests that DAM activation through the molecular switch TREM2 is required for healthy tissue repair through myelin debris clearance in cuprizone models of demyelination[358]. In the case of AD, preventing microglia from transitioning to DAMs by specifically deleting TREM2 results in failed compaction of neurotoxic amyloid beta plaques and consequent increases in neuritic dystrophy[212,309,359]. However, the transition from homeostatic microglia to DAM also marks an increase in phagocytic capacity and activity, something that is thought to drive excessive microglia-mediated synaptic stripping during sustained inflammation in multiple models of AD[354,355,360]. Our ability to interpret these observations are further complicated by the recent finding that a portion of the presumed DAMs in these models may actually be monocyte-derived DIMs that accumulate in the brain and display a comparatively pro-inflammatory profile[296]. In light of this transcriptomic profiling suggesting that DAM, relative to DIM, display a neuroprotective and anti-inflammatory profile, it is also possible that some of the immunopathology attributed to DAMs have been conflated with that of DIMs.

Because our functional understanding of DAMs is so far limited, studying them within the context of *T. gondii* infection could be beneficial. Based on initial estimations that used CD11c as a marker for DAMs, this microglial activation state may be especially prevalent in the *T. gondii*-infected brain, representing over two-thirds of total microglia isolated (**Fig 4.1A-D**). Whether this proportion increases due to sustained interferon signaling past 15 DPI infection is also a topic of interest. Because it can be experimentally difficult to quantify small but significant changes

effected by rare cell subpopulations, having a large population of DAMs in the brain may be beneficial for probing their functional roles – both STAT1-dependent and STAT1-independent.

What other molecular cues might link CNS infection with neurodegenerative disease states?

A well-supported theme that may bridge the presence of DAMs during both *T. gondii* challenge and neurodegeneration is the presence of tissue microenvironmental cell death[76,77,202]. In examining potential etiologies of DAMs, Krasemann et al. performed an elegant series of experiments highlighting the link between microglial-mediated cellular debris clearance and DAM activation[202]. In this paper, it was reported that microglial phagocytosis of apoptotic neurons, but not zymosan particles or *E. coli*, was sufficient to upregulate the expression of *Apoe* and drive a DAM transcriptional phenotype[202]. The notion that microglial-mediated apoptotic cell clearance induces DAM activation is consistent with literature illustrating that TREM2, a molecule required for the DAM transition[202,209] and is a strong risk factor for AD development[282–287], binds anionic and zwitterionic membrane-associated lipids including phosphatidyl serine (PS), which is expressed on the surface of apoptotic or damaged cells[309]. Multiple reports indicate that CNS immune activation and *T. gondii* itself results in increased levels of cell death in both the brain and retina during infection[76,77,361], presenting a potential mechanism for DAM induction during infection. While apoptotic cells were specifically examined in Krasemann et al. study, it may also be reasonable to hypothesize that other forms of cell death that would lead to the externalization of membrane "eat-me" signals such as phosphatidylserine and "find-me" signals such as ATP – in the case of pyroptosis – may similarly be able to initiate the DAM transition during *T. gondii* infection. Moreover, lytic parasite replication and egress from infected cells would be expected to generate cellular membrane debris that may be cleared by microglia, thus potentially prompting

the DAM transition via physical interactions with necrotizing cells. Lastly, the presence of large numbers of blood-derived myeloid and T cells in the brain during *T. gondii* infection[76,77,106,116], and studies showing that their ongoing recruitment is required for parasite control throughout life long chronic infection[106,116], could suggest that efferocytosis may be a requirement for the maintenance of these potentially shorter-lived immune cell populations in the brain. Whether microglia, the resident professional phagocytes of the brain, are equipped with this task is currently unknown.

What hypothesized roles might DAMs play in the T. gondii-infected brain?

While we have shown that microglial STAT1-sufficiency is both required for resistance to fatal toxoplasmic encephalitis and for the full transition from homeostatic microglia to DAM during *T. gondii* infection, we have been careful not to overgeneralize these potentially independent findings to conclude that DAMs are in themselves, anti-microbial. One interpretation of these results posits that independent of anti-microbial killing, the microglial transition to DAM could be necessary for clearing apoptotic, pyroptotic, or otherwise necrotic cellular debris, thereby maintaining tissue homeostasis or even playing a role in tissue repair during *T. gondii* infection. This hypothesis resonates with literature highlighting phagocytosis as both an initiating and key effector function of DAMs during neurodegeneration[202].

Most of our studies have focused on the effects of increased parasite burden as a readout for pathology in our model of CNS infection, yet there is extensive literature showing that deficits in efferocytosis (phagocytosis of apoptotic cells) results in secondary necrosis[362,363] and worsened clinical pathology in a variety of models, including infection[364], autoimmunity[365–367], atherosclerosis[368,369], cystic fibrosis[370], myocardial infarction[371,372], and chronic obstructive pulmonary disease[373,374]. Notably, the process of efferocytosis is generally anti-inflammatory and

leads to the release of cytokines such as TGF-β and IL-10 by macrophages, both of which have defined roles in promoting wound healing processes[375]. Thus, it is plausible to hypothesize that some of the pathology observed in $MG^{STAT1\Delta}$mice could stem from a combination of unconstrained parasite replication in tandem with an overexuberant immune response due to insufficient anti-inflammatory or tolerogenic cues. *Axl*, a DAM gene involved in microglial-mediated phagocytosis of amyloid proteins[376], encodes one of the three TAM receptor tyrosine kinases that is also involved in the phagocytosis-mediated clearance of apoptotic debris, and is required for efferocytosis along neurogenic niches of the brain[302]. *Axl* is expressed at relatively low levels in the naïve brain[302], but its RNA expression in microglia increases during *T. gondii* infection, in a STAT1-dependent manner (**Fig 3.5D** and **Supplementary Fig 3.2B**). Genetically targeting *Axl* in microglia may provide an opportunity to understand whether efferocytosis by microglia is broadly required for maintaining tissue homeostasis during CNS infection. Alternatively, the enhanced phagocytic capacity during the transition from homeostatic microglia to DAM may equip microglia with the ability to more effectively ingest and degrade *T. gondii*, should phagocytosis serve as a cell-intrinsic mechanism of parasite restriction in this cell type.

Because we have extensively illustrated the importance of microglial STAT1 signaling during *T. gondii* infection, attempts to study the larger STAT1-independent portion of the DAM signature may elucidate DAM functional roles during infection while simultaneously preventing the loss of STAT1-dependent anti-parasitic mediators such as IRGs and GBPs. To this end, perhaps the most suitable gene to target in microglia would be *Apoe* – a gene that (i) displays a polymorphisms in humans conferring increased risk for developing AD[288–292], (ii) is highly upregulated by microglia during *T. gondii* challenge, (iii) is required downstream of TREM2 for the transition from homeostatic microglia to DAM[202], and (iv) has been shown to augment

microglia and macrophage-driven efferocytosis to maintain tissue homeostasis[202,377]. To probe whether DAMs are intrinsically anti-microbial, initial experiments could include *in vitro* attempts to interrogate whether *Apoe* deficiency in cultured microglia leads to greater susceptibility to parasite replication in tandem with decreased expression of the STAT1-dependent anti-parasitic IRG/GBP-family proteins identified in microglia in Chapter 3. The subsequent transition to an *in vivo* system with the use of mice with microglial-specific *Apoe* deficiency could then provide a systems-level indication of whether the DAM transition helps control brain parasite burden or immune priming of other cell types.

Targeting *Apoe* in microglia may be more strategic than targeting other genes required for the homeostatic microglia to DAM transition, because in contrast to transmembrane receptors such as TREM2, APOE protein can be exogenously supplemented to genetically deficient mice[378]. This approach has been utilized as a therapy in a mouse model for spinal cord injury, wherein exogenous APOE supplementation decreased cell death and subsequent inflammation, restored blood-brain-barrier integrity, and promoted spinal cord tissue repair in mice with a global *Apoe* deletion[378]. This approach may prove especially powerful should blocking DAM activation through microglial *Apoe* deletion result in impaired efferocytosis and subsequent excessive inflammation, and should APOE supplementation rescue the resulting pathology.

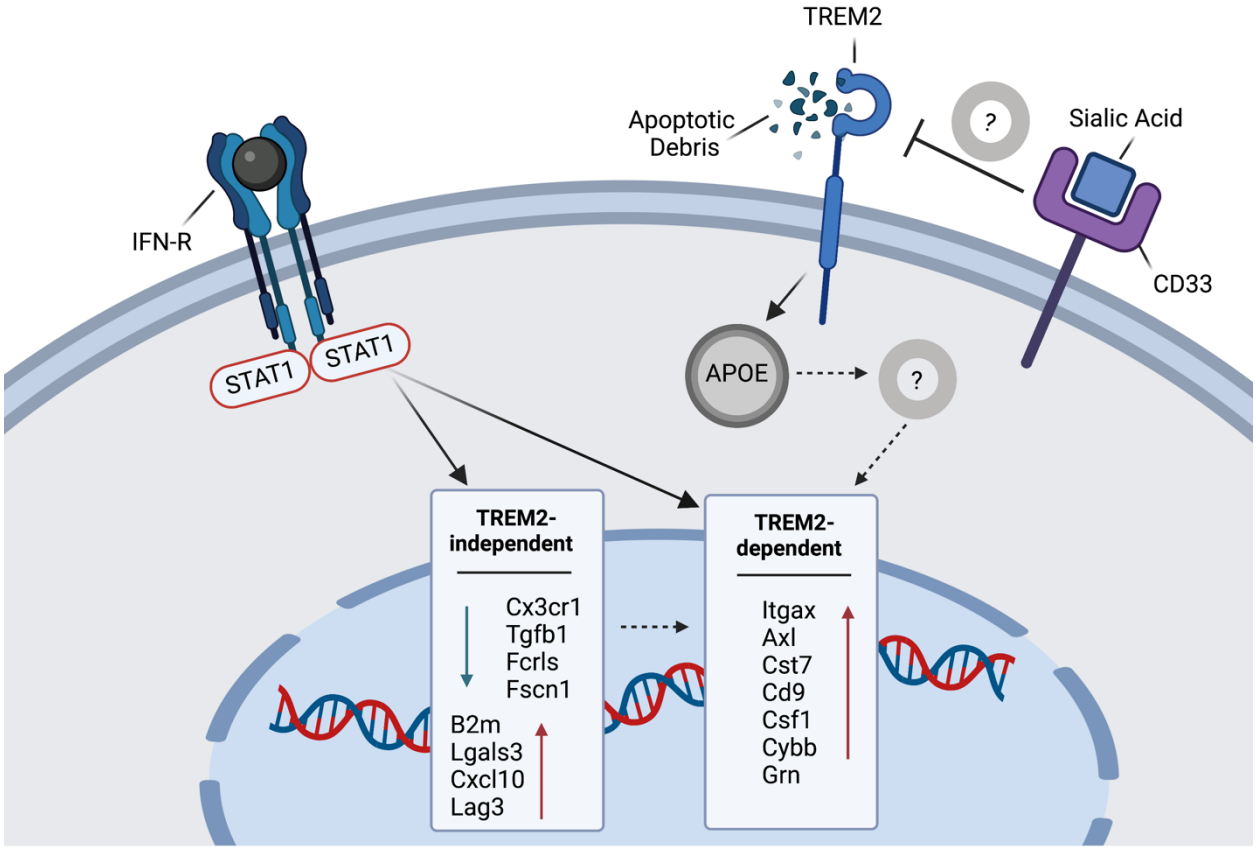


Fig 6.4 Hypothesized model for the relationship between STAT1 and the disease-associated microglia (DAM) transcriptional signature.

The disease associated microglia (DAM) transcriptional signature, commonly associated with neurodegeneration in both humans and mice, has been proposed to activate in response to microglial sensing of apoptotic cells. Previously identified regulators of the DAM signature include TREM2, APOE, and CD33, which have been hypothesized to operate within a shared pathway. Interferon signaling via STAT1 represents a novel contribution to DAM signature acquisition, as it is associated with the differential regulation of both TREM2-independent and TREM2-dependent genes.

The finding that homeostatic microglia transition to highly phagocytic, neurodegeneration-linked DAM during *T. gondii* challenge illustrates similarities in their activation state in both infection and neurodegeneration. The etiology of DAM is currently hotly debated, but appear

tightly linked to phagocytic clearance of dead cells present in diverse disease states. Because STAT1 signaling is both embedded in the DAM transcriptional signature and fundamental to protection against *T. gondii*, a current open question is whether DAMs are in themselves, anti-microbial, or they are instead an artifact of shared molecular cues in the brain during inflammation. I hypothesize that DAM may develop from *T. gondii*- or inflammation-induced cell death during infection, and a primary functional role in conferring neuroprotection may be through the process of efferocytosis to maintain tissue homeostasis and preventing overexuberant immune responses in the brain.

6.7 Beyond CNS infection – microglial STAT1 signaling across health and disease

IFN-STAT1 signaling is a remarkably evolutionarily conserved cytokine signaling arm of the vertebrate immune system that is shared across diverse phylogenetic classes – from jawed fish to mammals[379]. This dissertation has focused on how IFN-STAT1 signaling in microglia is critical for protection against the ubiquitous protozoan parasite, *Toxoplasma gondii*, and is also integral to the DAM transcriptional state observed during diverse neurodegenerative conditions involving inflammation. Our work suggests that IFN-STAT1 signaling contributes to a conserved microglial activation and is thus fundamental to our understanding of microglial physiology in pathological contexts. The use of $MG^{STAT1\Delta}$ mice may thus present intriguing opportunities for manipulating microglial function in different neuroinflammatory disease states. Direct evidence of increased type I and/or type II IFN signaling, which both converge on the transcription factor STAT1, is seen in many neurological conditions – including AD[380], MS[381], Parkinson's disease[382], stroke[383], ALS[356], TBI[384], and acute spinal cord injury[385]. This section frames our understanding of microglial

STAT1 signaling during *T. gondii* infection with a discussion of potential areas in which this signaling manifests in health and disease.

As previously described, the literature regarding microglial functional roles in AD is both complex and mixed. Multiple studies have shown that microglial containment of neurotoxic amyloid plaques in the brain parenchyma requires a TREM2-dependent DAM transition, yet a recent study revealed that blocking IFNAR-STAT1 signaling either pharmacologically or specifically in microglia results in a rescue from phagocytosis-mediated post-synaptic synapse loss[360]. Together, these two findings suggest that during AD, microglia may display contrasting roles – STAT1-independent microglial activation may be beneficial in preventing plaque-induced neuritic dystrophy, while STAT1-dependent microglial activation may be detrimental by driving phagocytosis-mediated synapse loss. Consistent with this interpretation, IFNAR-deficient microglia, which displayed the loss of nuclear STAT1 staining, engulf equivalent amounts of amyloid beta in AD mice when compared to WT microglia on the same genetic background, but display protection against excessive microglial-mediated synapse loss[360].

Interestingly, pharmacological IFNAR blockade in aged 5xFAD mice has been shown to result in the decreased expression of the DAM gene *Clec7a*, but not with more specific microglial genetic IFNAR deletion, which would not be expected to impact monocyte-derived cells[360]. The DAM gene and phagocytic receptor, *Axl*, which our work in *T. gondii* infection indicates is indeed STAT1-dependent, however, did decrease under both conditions[360]. In light of recent work illustrating that DIMs are present in the brain, it is plausible to hypothesize that in the absence of inducible cre-driven genetic targeting systems or single cell RNA sequencing to validate myeloid cell identity, conflating DIMs with DAMs could explain the differential targeting of *Clec7a* gene expression in “microglia” with IFNAR blockade. The use of a standard inducible cre microglial

genetic targeting approach, coupled with maintenance of mice on such tamoxifen to sustain differential STAT1 deletion or inhibition in microglia and all CNS macrophages could potentially experimentally resolve these differences. Developing a better understanding of microglial functional roles during neuroinflammation moving forward may thus require careful attention to some of the themes discussed throughout this chapter – the use of microglia-specific genetic approaches, cognizance of functional differences between microglia and blood-derived macrophages, and a clear definition of microglia as a yolk sac-derived macrophage population with long-term residence within the brain parenchyma.

Because STAT1 is a key transcription factor that drives immune responses to diverse intracellular infections in a wide spread of cell types, inhibiting STAT1 itself may not be a reasonable therapeutic target during neuroinflammation. Indeed, STAT1 loss-of-function mutations are associated with high susceptibility to ubiquitous viruses, such as herpetic infections[386]. However, specific STAT1-dependent genes in microglia that are associated with either protection or pathology could instead serve as druggable targets. For instance, the STAT1-dependent DAM gene, *Axl*, has also been hypothesized to contribute to synapse loss in the 5xFAD mouse model of AD[360]. In AD mice, Axl shows colocalization with its ligand Gas6 and the post-synaptic terminal marker PSD95[360]. *Axl* mRNA levels also show statistically significant positive and inverse correlations with PSD95 engulfment by microglia and ThioS plaque area, respectively[360]. While correlational, these findings are further consistent with a model wherein the STAT1-dependent portion of the DAM signature is a driver of synapse loss pathology during AD.

An EAE study also found that pharmacological inhibition of Axl resulted in attenuation of animal paralysis, suggesting this STAT1-dependent molecule may be also inappropriately activated during MS. Interestingly, however, STAT1 signaling is broadly therapeutically targeted

in some EAE patients who receive interferon beta-1a therapy under brand name Avonex[387]. The precise mechanisms of action of Avonex is unclear, but it is a helpful therapy in reducing the severity of relapsing-remitting forms of MS by regulating blood-brain-barrier permeability and inhibiting pro-inflammatory (IL-17) and elevating anti-inflammatory (IL-10) cytokines[387,388]. Whether some of the therapeutic effects of Avonex during MS are at all related to STAT1 signaling in microglia – specifically as they may pertain to a resolution of efferocytosis and tissue homeostasis or repair – may be of interest for future studies.

A pre-print from the Molofsky lab has also used a combination of bioinformatics and traditional wet-lab approaches to highlight a shared relationship between microglia in the developing brain and a "conserved microglial state" driven by interferon signaling and activated in mouse models of development, LCMV, stroke, AD, demyelination, ALS, normal aging, and even COVID-19 infection[389]. This work helps frame a recent study illustrating that the DAM transcriptomic signature observed during neurodegeneration map closely onto transcriptomic signatures of P7 CD11c+ microglia, also referred to as youth-associated microglia (YAM)[296]. At first glance, the finding that microglia undergo what was described as "fetal-like reprogramming"[296] during neurodegeneration and aging is puzzling, but studies like our own and from the Molofsky group showing that IFN-STAT1 signaling drives a conserved microglial activation state shed light on IFN being a fundamental molecular driver of neuroinflammation. Indeed, IFNs as pro-inflammatory signals that boosts microglial phagocytic capacity during development may be needed to resolve the large quantities of cell death and tissue remodeling that occurs over the course of healthy brain development[185,204,207,304,305,390]. Thus, in some cases, this traditionally anti-microbial signal appears to be appropriately activated during CNS development

or infection, yet in others, it is inappropriately activated during neurodegeneration due to microenvironmental cell death.

In summary, our research has focused on microglial STAT1 signaling during *T. gondii* infection under a largely anti-parasitic lens, yet it has also emphasized that IFN-STAT1 signaling is a common molecular pathway shared across contexts related to both health and disease. While DAMs are generally described as neuroprotective[202,251,296,297], there is some evidence suggesting that the STAT1-dependent portion of the DAM signature is a contributor to disease in some contexts[360]. When one contrasts these observations with our work showing that microglial STAT1-deficiency results in uniform animal mortality during protozoan infection, it highlights a fascinating theme of the immune system – a need for balancing immune activation and pathogen restriction with immune tolerance and preventing immunopathology. In light of high rates of *T. gondii* seropositivity on the global scale[3,4], this theme may be especially important to consider should inhibiting microglial STAT1-mediated pathways become a therapeutic avenue for any disease.

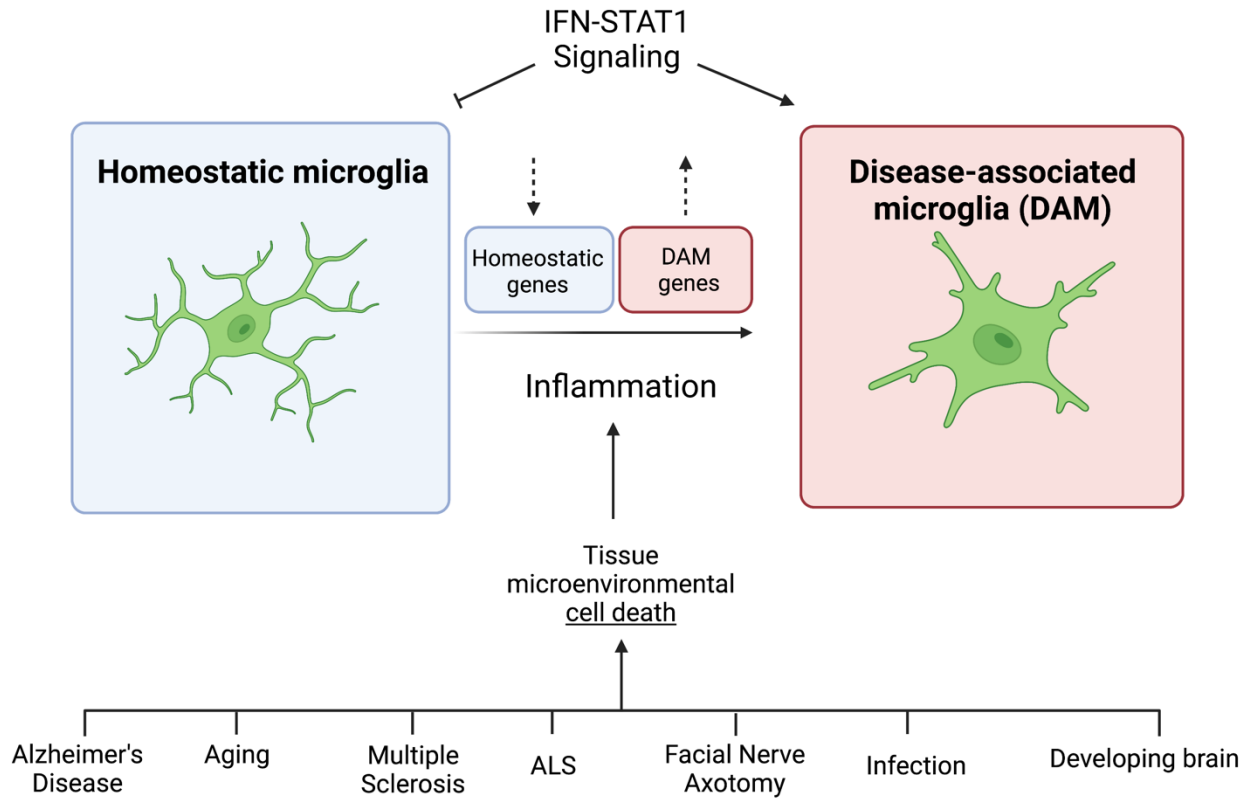


Figure 6.5 Schematic summary of STAT1 signaling and DAM signature involvement in health and disease.

An IFN-STAT1-driven microglial signature is observed across several different contexts, including multiple models of neurodegeneration, aging, infection, and normal CNS development. Across these contexts, neural cells undergo various forms of cell death, and tissue-resident microglia display subsequent enhanced phagocytic activity. In cases in which phagocytic activity is beneficial for tissue repair, remodeling, or homeostasis, STAT1-activated microglia may play a neuroprotective role. However, there is evidence that the STAT1-portion of the disease-associated microglia (DAM) signature can result in inappropriate or overexuberant synaptic pruning, in which case microglia have been implicated as a driver of neurodegeneration-associated pathology. Linking IFN-STAT1 signaling to the DAM phenotype broadens our understanding of what molecular cues drive microglial activation during diverse neuroinflammatory contexts.

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Appendix

Gene Name	Base Mean	Log2 Fold Change	LFC SE	padj	Category
H2-Aa	35912.5162	8.29817317	0.219514	< 2.23E-308	AP
H2-Q6	8250.83676	6.93213086	0.18428688	< 2.23E-308	AP
H2-Ab1	82148.4533	8.01867923	0.20684215	< 2.23E-308	AP
H2-Eb1	20988.8187	8.48277395	0.23152278	2.35E-290	AP
Gbp6	6009.67517	7.09983288	0.20177845	8.90E-268	GBP
Gbp2	23967.5429	5.98520021	0.1813321	1.48E-235	GBP
Ifit2	3425.81152	6.65647473	0.20428943	1.38E-229	AV
Irgm1	8269.4505	4.77907555	0.14709182	2.50E-228	IRG
Gbp4	5941.13141	7.12056046	0.2435718	1.10E-184	GBP
Ifit3	2124.3032	6.96852014	0.23934728	3.21E-183	AV
Fgl2	16422.0269	4.56718829	0.15844276	1.28E-179	P
Irf7	4746.1071	7.29710094	0.25743367	1.09E-173	TF
Rtp4	1847.58204	7.5542156	0.26689524	3.25E-173	AV
Oasl2	2775.13359	6.52592664	0.23310021	1.77E-169	AV
H2-T23	19771.4278	4.36197483	0.15631617	2.13E-168	AP
Cd74	157694.578	5.75829603	0.20835543	3.44E-165	AP
Ifi47	2563.74531	6.2605993	0.23428136	2.08E-154	AV
Tap1	9716.9378	4.30969335	0.16186827	2.68E-153	AP
Tgtp2	4982.20556	7.12087043	0.26981245	1.22E-150	IRG
Ifi211	1918.82672	7.28605696	0.27646299	3.12E-150	AV
H2-Q7	9184.34392	6.80958092	0.26051827	8.68E-148	AP
H2-Q7	9184.34392	6.80958092	0.26051827	8.68E-148	AP
Iigp1	14004.2326	7.02527008	0.26934941	3.60E-147	IRG
Usp18	1978.7127	6.81576655	0.26451653	1.24E-143	IR
Ly6a	13443.8594	7.23174654	0.28274927	1.59E-141	AM
Il18bp	3978.18806	6.30566093	0.25253671	7.24E-135	NRC
Zbp1	3435.83642	6.54111559	0.26677938	4.91E-130	AV
Gm12250	1710.38569	5.73485473	0.2364033	2.72E-127	IRG
Tgtp1	2649.19669	6.82295706	0.28279288	6.37E-126	IRG
Xaf1	1408.66058	5.38248818	0.22317856	7.77E-126	CD
Rnf213	2370.20194	3.56735642	0.14793214	7.99E-126	U
Samhd1	5710.86676	3.96767064	0.16908892	4.11E-119	AV
Stat1	15276.2298	6.8065692	0.29665565	7.20E-114	TF
Irf1	8630.1314	3.81304614	0.16734801	2.68E-112	TF
Il12rb1	1799.59423	7.91778264	0.34847751	1.12E-111	CR
Isg15	978.100062	5.86589117	0.2659625	3.33E-105	AV
Sp100	1902.57103	4.75397853	0.22256231	1.19E-98	P
Ciita	1803.83854	7.84506493	0.37039059	5.36E-97	AP
Ifi206	998.928918	7.79450149	0.37143148	3.25E-95	AV
Gbp8	1340.20194	5.96385987	0.28750642	4.95E-93	GBP

Ifitm3	11310.2326	5.18485222	0.25423362	6.48E-90	AV
Igtp	9056.86505	4.28812303	0.21118844	3.91E-89	IRG
Ifi205	1118.39666	6.37241236	0.31490031	1.53E-88	AV
Phf11d	1753.6914	3.74159706	0.18929703	1.87E-84	U
Nampt	3373.91737	2.8943121	0.1466313	3.13E-84	M
Ifit1	608.076261	5.98653786	0.30532054	4.08E-83	AV
Oas3	1182.08596	7.49875857	0.39099475	1.66E-79	AV, CD
Ifi27l2a	6030.17252	6.34505088	0.33180962	4.78E-79	AV
Oas1a	2115.91074	6.16544963	0.32302984	9.38E-79	AV, CD
Klrk1	1126.94714	7.6864036	0.40429855	3.82E-78	AV

Table A1. Top 50 genes differentially-expressed by STAT1 during *T. gondii* challenge.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii*, and brain-resident microglia were FACS-sorted and RNA-sequenced at 12 DPI. Top 50 differentially expressed genes were exported from DESeq2. Base Mean indicates averaged normalized read count across samples. Log2Fold Change indicates the fold change effect size, with positive values indicating enrichment in WT mice. LFC SE indicates standard error of the log fold change for each gene padj indicates the Benjamini-Hochberg (BH) adjusted *p*-value associated with each gene. Adjusted p values below 0.05 are statistically significant, with the lowest p value that can be calculated in R set at 2.23E-308. The functional biological category for each gene is manually assigned.

AP = antigen presentation-associated, GBP = guanylate binding protein, IRG = immunity related GTPase, TF = transcription factor, CR = cytokine receptor, AV = anti-viral, NRC = negative regulator of cytokine signaling, AM = adhesion molecule, CD = regulator of cell death, M = metabolic function, P = pleiotropic function, U = unknown.

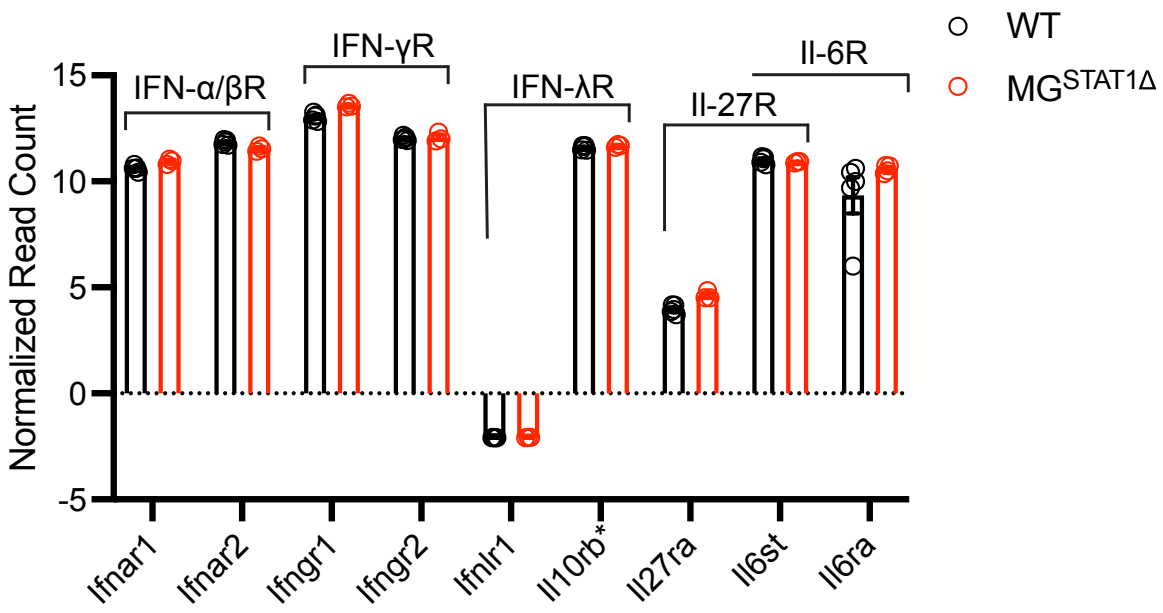

Figure A1. Microglial expression of receptor subunits associated with downstream STAT1 activation during *T. gondii* challenge.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii*, and brain-resident microglia were FACS-sorted and RNA-sequenced at 12 DPI. Regularized log-transformed read counts are displayed for receptor subunits that are associated with STAT1 activation and phosphorylation upon ligand binding. Annotation above bars specify subunit requirement for specific cytokine receptor at the protein level.

Note: *Il10rb* is also a shared unit of Il-10R.

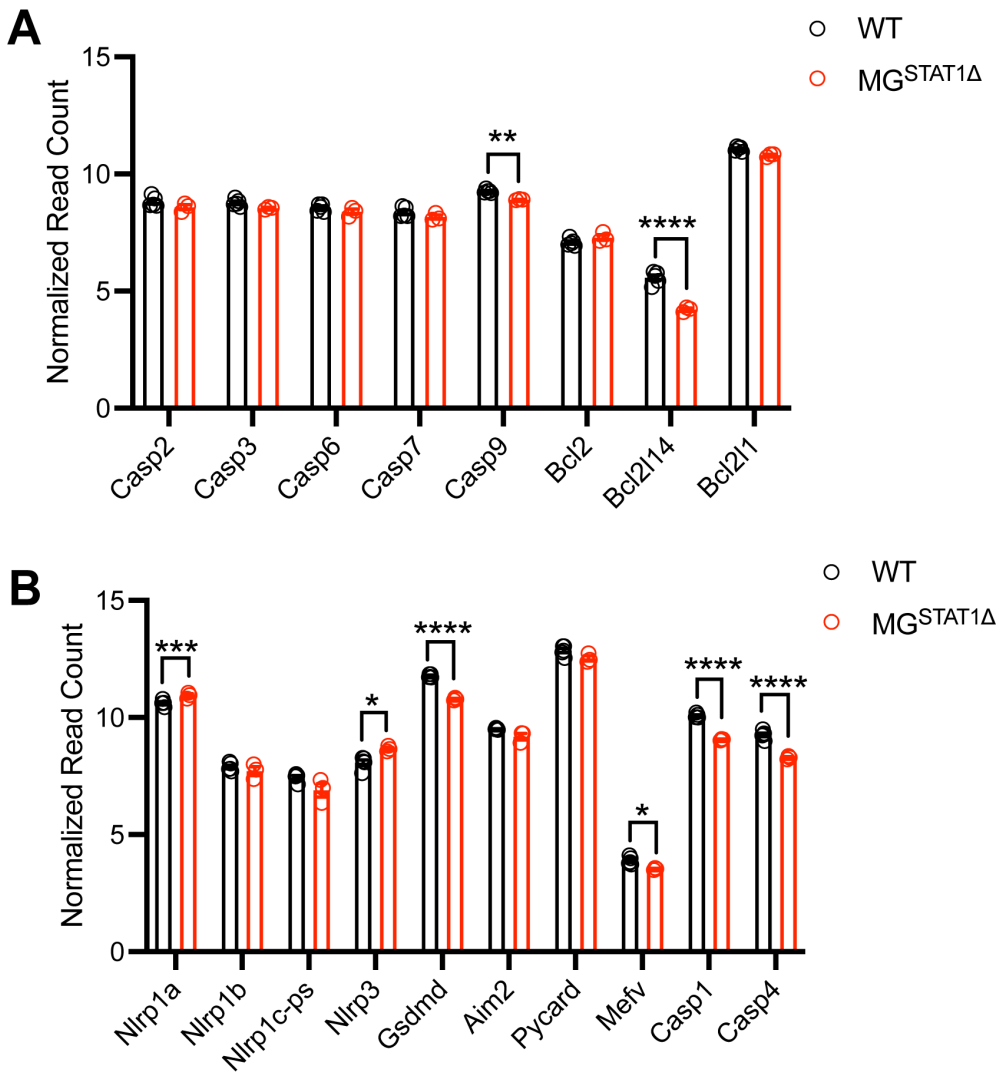

Figure A2. Microglial expression of cell-death associated genes in WT and MG$^{STAT1\Delta}$ mice.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii*, and brain-resident microglia were FACS-sorted and RNA-sequenced at 12 DPI. Regularized log-transformed read counts are displayed for (**A**) molecules associated with apoptosis and (**B**) pyroptosis pathways. n = 3-5 mice per group. p values indicate BH-adjusted p values from the full gene expression analysis in DESeq2. * = $p < 0.05$, ** $p < 0.01$, *** = $p < 0.001$, and **** = $p < 10^{-4}$.

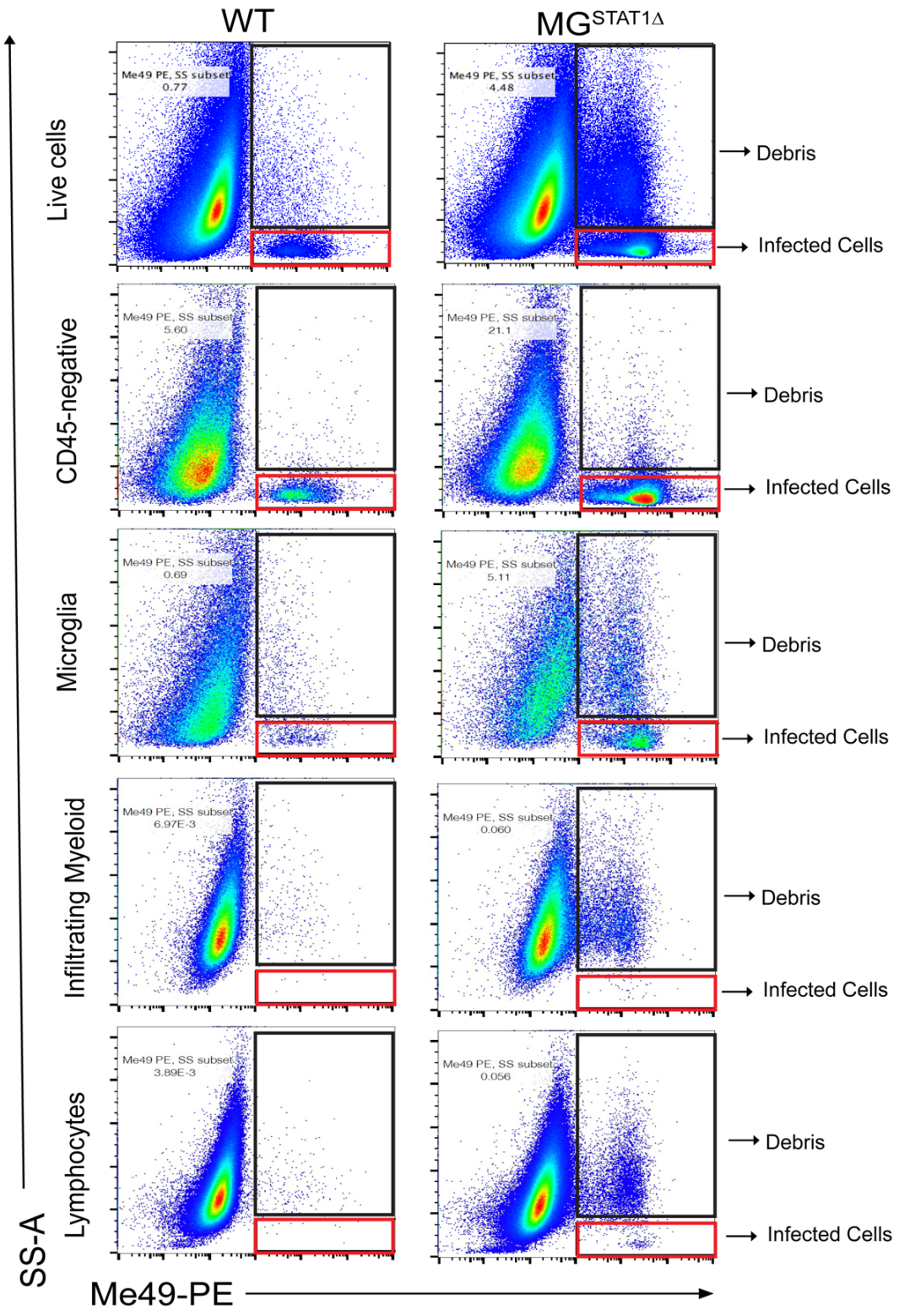
WT
MG STAT1Δ
Live cells
CD45-negative
Microglia
Infiltrating Myeloid
Lymphocytes
Me49 PE, SS subset 0.77
Me49 PE, SS subset 4.48
Me49 PE, SS subset 5.60
Me49 PE, SS subset 21.1
Me49 PE, SS subset 0.69
Me49 PE, SS subset 5.11
Me49 PE, SS subset 6.97E-3
Me49 PE, SS subset 0.060
Me49 PE, SS subset 3.89E-3
Me49 PE, SS subset 0.056
Debris
Infected Cells
SS-A
Me49-PE

Figure A3. Example gating strategy for identifying *T. gondii*-infected cells isolated from brain.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii* and brains were analyzed by flow cytometry at 15 DPI. Cells were intracellularly stained for Me49, and pre-gated for singlets and live cells. Microglia were then pre-gated as CD45-intermediate and ZsGreen positivity. Infiltrating myeloid cells were gated based on CD11b and Ly6C expression, and lymphocytes were gated based on high levels of CD45 and CD11b negativity. Red rectangle identifies Me49-positive, side scatter-low events consistent with infected cells, and black rectangle identifies Me49+, side scatter-high events consistent with phagocytic debris or cargo.

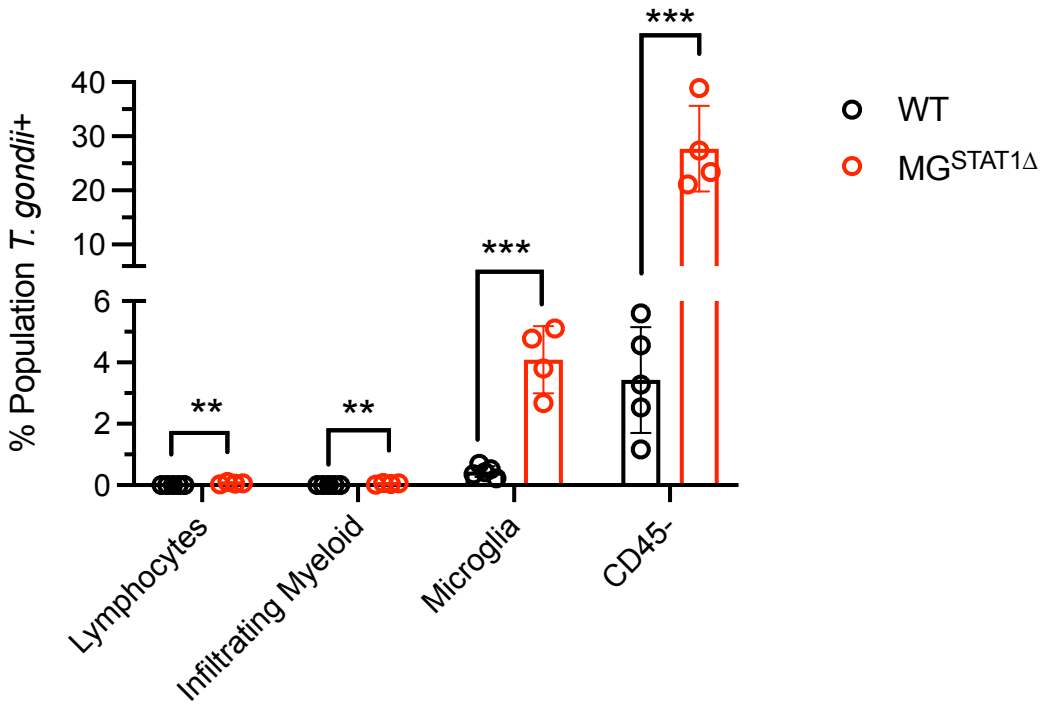

Figure A4. Microglia and other cell types show increased rates of intracellular *T. gondii* in MG$^{STAT1\Delta}$ mice.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii* and brains were analyzed by flow cytometry at 15 DPI, as described in Figure A3. Cell populations generated from single cell suspensions were quantified for intracellular T. gondii positivity. Statistical significance was determined via unpaired *t* test. ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 10^{-4}$.

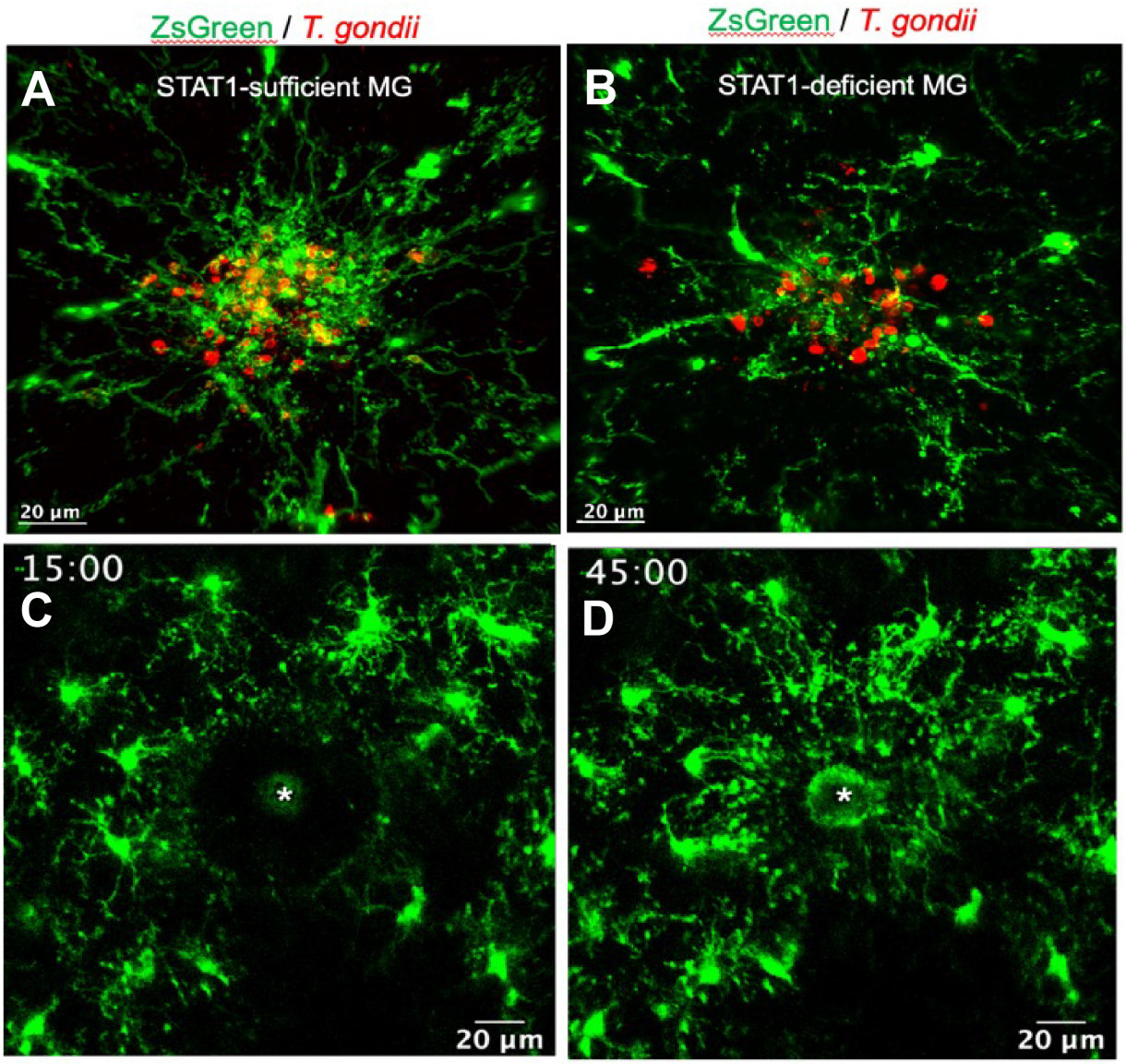

Figure A5. Microglial process extension during early CNS infection shares qualitative similarities with microglial responses to laser injury.

STAT1-sufficient microglia from WT fluorescent reporter mice and STAT1-deficient microglia from $MG^{STAT1\Delta}$ mice were analyzed by confocal or two-photon intravital microscopy. (**A-B**) Confocal micrographs of WT and $MG^{STAT1\Delta}$ microglia at 8 DPI following *T. gondii* challenge. *T. gondii* staining is indicated in red. (**C-D**) Two-photon intravital imaging analysis of the microglial response to laser injury, induced in (**C**) and captured 15 minutes later in (**D**), in a single WT fluorescent reporter animal. Asterisk denotes site of microscope-induced laser injury, with microglial process extensions observed approaching this focal site over time. All scale bars = 20 μm.

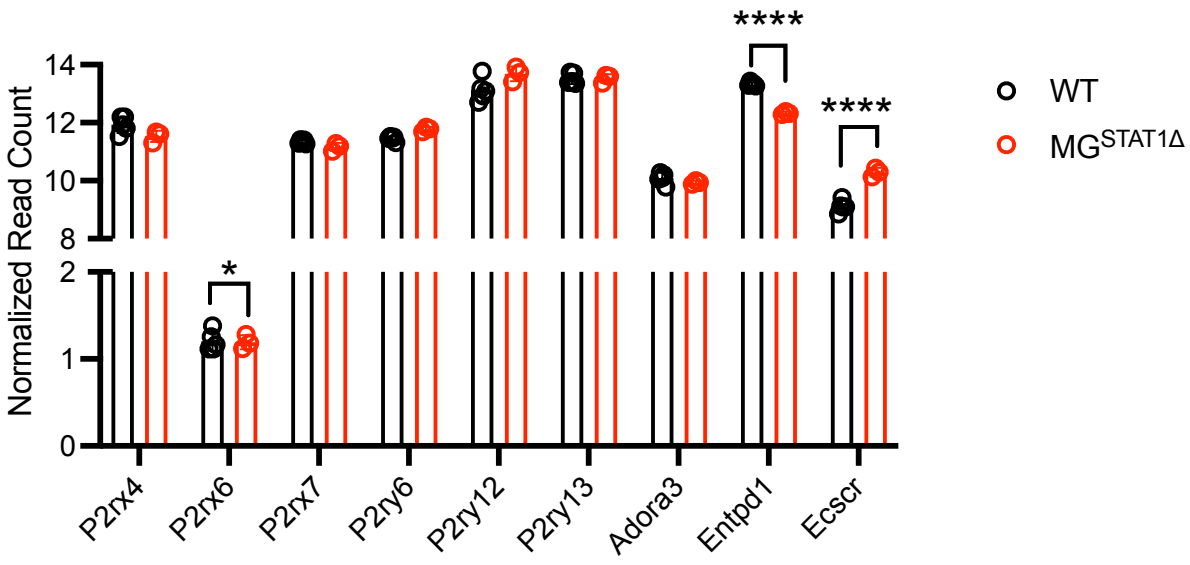

Figure A6. Microglial expression of purinergic receptors and ectonucleases with potential STAT1-dependency during *T. gondii* challenge.

WT and MG$^{STAT1\Delta}$ mice were intraperitoneally infected with 10 cysts of the Me49 strain of *T. gondii*, and brain-resident microglia were FACS-sorted and RNA-sequenced at 12 DPI. Regularized log-transformed read counts are displayed for purinergic receptors and ectonucleases that have been previously described as being regulated by IFN signaling in the literature. *p* values indicate BH-adjusted *p* values from the full gene expression analysis in DESeq2. * = $p < 0.05$ and **** = $p < 10^{-4}$.

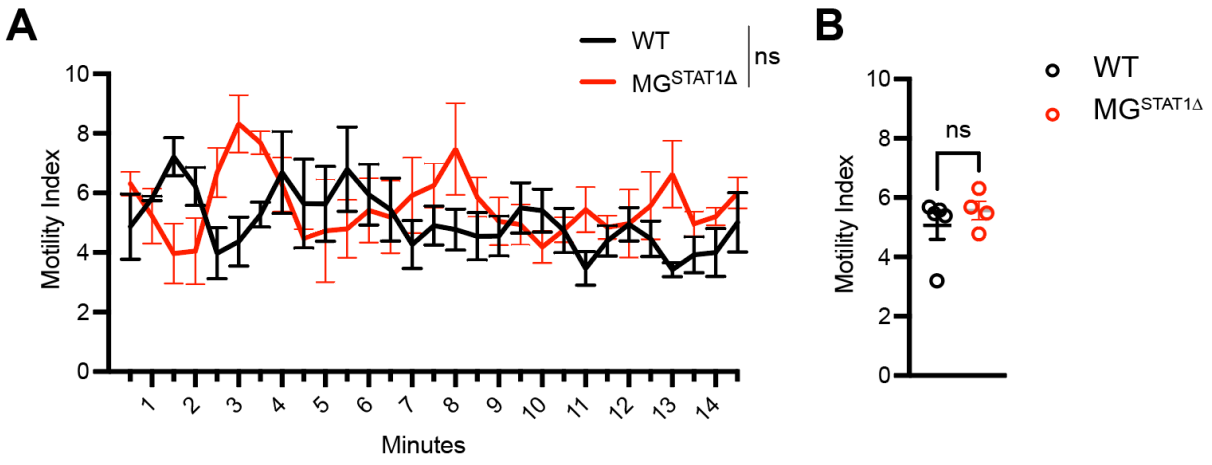

Figure A7. Baseline motility of naïve microglia in WT and MG$^{STAT1\Delta}$ brains.

Microglia from naïve WT and MG$^{STAT1\Delta}$ mice were analyzed via intravital two-photon imaging. Time-lapse images were recorded every 30 seconds for 15 minutes. Motility index was calculated as the change in ZsGreen area in the imaging frame every 30 seconds (**A**) and averaged over time for a total index value (**B**). Results were analyzed via two-way ANOVA (**A**) and unpaired *t* test (**B**). *n* = 4-5 mice per group. *ns* = not significant.