Initiation of immunity to parasite infection of the brain: a role for astrocytes in sensing damage

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by

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Abstract

The initiation of immune responses is thought to occur more slowly in the brain than in other tissues, owed to the blood brain barrier and the challenge of a lack of circulating immune cells to sense pathogens and other forms of danger. Thus, it is of great interest to understand how brain cells sense invading pathogens in order to initiate signals which bring immune cells into the brain. In this thesis work, we have used the parasite *Toxoplasma gondii* as a model pathogen to better understand these early sensing mechanisms. *T. gondii* reliably traffics to the brain of its hosts and persists there for the host's lifetime, kept in constant check by the immune system. Therefore, *T. gondii* infection is an excellent model for better understand the process of initiating immunity in the brain.

We hypothesized that recognition of damage to brain tissue caused by *T. gondii* plays a significant role in sounding the alarm to recruit immune cells. This was based on histological evidence of brain cell loss in areas where parasite was quickly replicating. We focused on the role of one candidate damage signal, IL-33, which is highly expressed in the brain and spinal cord at baseline. We found that during *T. gondii* brain infection, IL-33 can signal locally – it is released into the CSF, it induces chemokine within brain tissue, and it can bring immune cells into brain tissue and support their anti-parasitic function. Importantly, we found that IL-33 signals on astrocytes, and not on immune cells, to achieve these effects. Therefore, IL-33 is one local signal by which brain resident cells can sense *T. gondii* and promote blood-derived cells to enter the brain and control parasite. In addition, our work highlights an important role for astrocytes in promoting immunity, as

we found they are chief producers of the chemokine *Ccl2* during chronic infection. When we removed this chemokine from astrocytes alone, it resulted in significant impacts on immune cell recruitment and parasite burden. This work opens new doors to better understanding the importance of astrocytes in controlling *T. gondii*, and other infections that impact the CNS.

Acknowledgements

Getting my Ph.D. has been the hardest and most rewarding thing I've ever done. Over the past five and a half years, I have learned how to think critically and be a real presence in my own science and the science of others. Sometimes it's easy for me to look back on formidable experiences in my life and think it wasn't that difficult, perhaps because significant time has passed. But this degree was hard-earned and I had a lot of help getting here. I am sincerely grateful for a number of people that, without whom, this experience would not have been as worthwhile, or would not have been seen to completion. This section is long-winded, but I think that is appropriate. In many ways, gratitude is one of the most important parts of this journey.

First, I want to thank my undergraduate mentor, Bob McKown. He single-handedly inspired me to become a scientist. When I went door to door asking PIs at JMU to join their labs, in truth to boost my resume for medical school, he took me in. Until then, my experience with hands-on science consisted of chemistry and biology lab, where I followed cookie-cutter worksheets to complete lab experiments. Bob shared with me a simple but powerful perspective – that research can allow you to learn brand new things about the world. I can picture him walking around with his coffee cup on a weekend, excited to be making the walk from his office to the lab. He gave me significant responsibility, which energized me, and he went out of his way to lift myself and other young scientists up. He brought us to consortium meetings with his collaborators (at UVA!), to international conferences, put us up for awards, and gave us a shot at contributing to manuscripts. Maybe more important than I realized at the time, he always took the time to make sure we

celebrated big and small achievements. He had the lab over to his home regularly and he treated us like adults. Bob, I am so thankful for you.

I also want to acknowledge Harrisonburg Hospice, where I volunteered caring for terminally ill patients after college. The experience taught me a lot about grief and compassion, and also helped shape my research interests. Many of my patients had some form of dementia which led me to learn more about neurodegenerative diseases. The more I read the scientific literature, the more I realized they all had an immunological component – and so did many other neurological diseases. That clinical interest was the reason I fell in love with the rapidly growing field of neuroimmunology.

When I decided to do a post-baccalaureate program to gain more research experience, I worked at NIH, under mentors Robert Kreitman and Evgeny Arons. At the time, those two years felt like I was lagging behind my peers who had already set off for professional schooling. But looking back, my time at NIH were instrumental to my success in graduate school. During that time, I increased my arsenal of lab techniques and I started to think of them as means to answer interesting questions rather than intimidating protocols I had yet to learn. I think that gave me confidence during my rotations at UVA. And I had the time to continue thinking about medical school versus graduate school. During my two years at NIH, I developed a clear picture that I wanted to go to graduate school, and that I wanted to work in neuroimmunology.

That brings me to the reason I applied to UVA – the Brain Immunology and Glia (BIG) center – created and led by Jony Kipnis. When I was researching schools to apply to, my first consideration was how many labs I could see myself joining at that institution. Naturally, after years of careful thought, I was focused on neuroimmunology. I came across

the BIG center at UVA and realized there was nothing like it at any other school in the country. Once I was accepted at UVA, I was given advice not to spend all three of my rotations within the same subject matter, but I couldn't help it, and never regretted rotating within the center. I want to thank Jony for creating a true community – of shared equipment, reagents, ideas and dreams. We have had rigorous group meetings every week that unquestionably improved my science. And we have shared hikes, laughs, conferences, beers, retreats together. Some of my best friends in graduate school I met in the center. Sometimes communities like the BIG center can be taken for granted, but I have paid attention when chatting with alumni at conferences, and noticed how much they missed the environment. I have brought up this community of scientists to family, friends, recruits, and many others and I know I will miss it.

Next, I want to thank my mentor, Tajie Harris. She has had such a big impact on my life that it might be easy to generalize and say "thank you for everything." But there are some specific things I want to highlight about Tajie. When I interviewed at UVA, I already felt supported by her, even though she was a stranger. She was on the panel that interviewed me and she gave me this look that said – I can see something in you. My first year of graduate school I suffered quite a bit from imposter syndrome and Tajie was a calming presence that reminded me I deserved to be there. I remember panicking about a class I thought I may not do well in and she laughed and said – "don't worry – I have a feeling I will be proud of whatever you do." Such a small statement meant so much to me at that juncture in my training.

Perhaps the thing I most appreciate about Tajie is that she values individuality. She does not subscribe to a one-size-fits-all mentality as to how a scientist should be. Graduate

school rewards being quick on your feet, and being interactive, which has always been difficult for me. I prefer to ruminate on topics before I ask a question or start drafting a scientific document. She recognized that and validated my learning process. When I was nervous about committee meetings, she did in-depth practice talks with me – fielding questions to me and helping me sort through my thoughts. She never made me feel as if I was not where I needed to be, she just accepted me for me. And she gave me and my lab-mates freedom to work when we needed to work and decompress when we needed to decompress. That freedom to be myself without having to fit into a box was instrumental to my success in graduate school. At the same time, Tajie was always direct. I always appreciated that I didn't have to guess what she was thinking and that we had an open dialogue.

Tajie is also one of the most intelligent, efficient, elegant scientists I have ever met. She taught me to be sure that an experiment is watertight before sinking time and resources into it. She created an environment that fostered critical thinking in journal club and lab meetings. And she taught us all, by example, how to ask tough questions during group meetings without being combative. Above all, she taught us to value the science more than the splash the science makes, for which I will always be grateful. Although I like to think I would have made it through graduate school under any leadership, I'm not sure that's true. Thank you to Tajie for all of the little and big things she did to keep me on track.

I also want to thank my committee which has provided the infrastructure for my science to grow the past five and a half years. Kevin Lee, the chair of my committee, has been fair and diplomatic and kept me progressing towards my degree. When I had lost my voice from a nasty cold and was presenting my thesis proposal, he informed everyone that

we would have to adjust and stay within a stricter time limit while my voice was still audible. He didn't shy away from the tough questions and he provided real mentorship in how to navigate interpersonal relationships in graduate school. He also gave me the opportunity to participate in one of the most worthwhile activities I have done in graduate school – picking fruit to donate to local food banks and churches at the height of food insecurity during the pandemic. There are some times when you think, "this is what life is about," and touring around the orchard with Kevin and other department members on a beautiful summer day was one of them. Next, thank you to Jony Kipnis, who not only founded the BIG center but also took an interest in my personal journey. Jony always challenged me to move towards the surprising, to look for big holes in the literature. He taught me never to accept the status quo and there is always merit in improvement. I appreciate his outlook that a project should be an investment and not a means to an end in completing graduate school. I remember meeting with him individually after a committee meeting and he told me he had been tough because his expectations had been high. I would so much rather my advisors push me because they care than let me coast. I would also like to thank Sarah Ewald, who brought with her knowledge of infectious disease and an understanding of the importance of social connection. I remember having a picnic with her lab and having drinks with an invited speaker at Sarah's house. Sometimes, you learn more at those events than many hours of formal training. She has also asked insightful questions at my committee meetings, and I often find myself thinking of her feedback long after our meetings. Scott Zeitlin has now stepped in twice in my committee meetings, to fill in for Kevin at one meeting and for Jony at this final meeting. I am so grateful for his willingness to join my committee and provide valuable insight. He also was there when I needed

someone to submit a brain awareness video to a contest at the Society for Neuroscience at the last minute. I think it says a lot about someone if they are willing to help those that will not be able to help them back in the immediate future. And last but certainly not least, John Lukens. In writing these acknowledgements I get a little bit emotional as I come to John. I rotated with John, I had committee meetings and classes with him, and I worked next door to him these past five and a half years. I think what stands out most about John is he has done so much more for me than was ever required of him. My first year, he told me he heard I had done well in core course and that he was excited to see the progress I would make in graduate school. Such a seemingly small gesture meant so much to me. I have not always known if I was on the right track and hearing it from John always kept me going. When I wrote a Working Life essay for *Science* about a challenge I faced in graduate school, I worried that my advisors would see me as weak and take the essay as a personal affront to their mentorship. But John publicly supported me as he always has. That meant something not only to me but to my family. John also got to know the person who means the most to me in graduate school, my partner Pedro. Sure, they have similar senses of humor, and sure, Pedro visited my lab giving John the opportunity to meet him. But John goes out of his way to build meaningful relationships and I always felt that he cared about me as a person. This is all in addition to his valued feedback on my science. This past year I felt like I knew a couple of celebrities when he and Cat Lammert published in *Nature, Nature,* ever heard of it? Going forward, I know that John will continue to be a valued mentor and I am so thankful to have someone like him in my corner.

And now to thank my lab mates, past and present. There is no relationship quite like a lab mate – since they intimately understand your day to day life - personally,

technically, and scientifically. When I first joined Tajie's lab, I met Nik Hayes, Jeremy Thompson, and Carleigh O'Brien. I must say I was struck by their competency and ease. All of them set the tone for what graduate school could be and set the bar high for what I could achieve. Nik worked with me throughout my rotation to take the most beautiful images on the microscope, and I never got the sense that we were rushed or we were under pressure to produce a result. He just genuinely cared about his observations and I think that rubbed off on me when I would later take charge of the project. Nik was incredibly inventive and would come up with a new project almost every week. I wish we had written down all of his ideas. Jeremy brought me much needed levity throughout my training. Since I did not work with him directly during my rotation, we talked about anything and everything else. Jeremy understood my sense of humor and I think I took for granted our laughing fits and coffee runs. Jeremy was also a respected colleague. He has an incredible command of the literature and a deep understanding of everything he does. I remember thinking that perhaps one day I would be able to rattle off author name and year of big papers like he did, but that day never came. And Carleigh – who began as my teacher and also became a close friend. Carleigh is likely the most mentally-strong student I have met in graduate school. When experiments did not work, or when she had to come in for off-hours time points, she never complained. She accepted that science was hard and she took joy in the process. I always looked up to her for that. She taught me everything she knows about flow cytometry, which was a ton. And I can say that she genuinely cheered me and my science on. Graduate school can feel competitive at times, but she was always the first to light up when I presented a cool result. Carleigh also became a close friend outside the lab and we spent a significant amount of time together on weekends exploring

Charlottesville and being indecisive about with movies we wanted to watch. When I officially joined the lab, Sam Batista joined along with me. Sam has been like my lab twin, or sister, throughout this process. Our projects were similar and our senses of humor were similar. My first week in the lab with Sam I was blown away by how open she was with me. We talked about our struggles and triumphs every step of the way these past five and a half years. With Sam, I never felt I had to act as if everything was fine and I always knew that I could trust her. We also had a big hand in each other's projects and if we ever needed help we knew who to ask. The first thing most people would say about Sam is that she is also very intelligent, and I always knew that if I had an idea that she agreed was interesting, then I might have something. Although it was always our goal to graduate around the same time, and I am late to my end of the bargain, I am glad that I have only had one semester at UVA without Sam. More recently, Mike Kovacs and Maureen Cowan joined the lab. Mike has been an inspiration in that he is always willing to put in the effort to thoroughly answer a question – even if it means committing to experiments that extend well beyond normal work hours. He also is one of the best listeners I have met in graduate school. He has been there for some of the more challenging moments of my journey and he has exercised good judgement in when to lighten the mood with jokes and when to just be there. And Maureen has been responsible for much excitement in the lab the past couple of years. She could probably convince a rock to become interested in microglia, as she literally floats around lab after getting an interesting result. She has a strength that has shown me it's okay to have boundaries and to stand up for myself when needed. I wish all of my lab mates, near and far, the best in their journeys and I will always be missing them!

To my friends in Charlottesville – thank you for making my life whole. I started out graduate school spending 100% of my time in the lab and focused on my studies, and that was not sustainable. You all have taught me that people matter most in life, which has taken me approximately 25 years to learn. I especially want to thank Catherine Lammert and Tori Osinski – who I look up to as scientists and people. At times, graduate school, and female friendships in general, can feel competitive. But I have never had anyone genuinely listen to me and share in my successes and my failures as Cat and Tori have. They always ask follow-up questions and they have taught me how to be a better friend and listener. All of the workouts and beers and face masks didn't hurt either! To Cat – we trained for the half ironman at a time when my project was most challenging and I was unsure if I would ever graduate. Spending nearly every day with you after work reset me for the next day and I learned so much about pushing my physical and mental boundaries. And what an achievement to look back on! I know I will have a lifelong friendship with them both. I also want to thank Mike Raymond, who was an endless supply of much needed humor throughout graduate school. Especially during core course, when I was stressed and often wondering if I belonged to be there, you brought levity that helped me realize everything would be alright. Still now, our group with Pedro have turned my day around on many occasions. You are also a respected colleague with possibly the coolest project I have watched develop throughout graduate school. And thank you to many more friends along the way – especially Phil for getting me out and doing things I love – like backpacking, trips to your family's cabin, and lunches on the corner. Thank you to Dana and Elizabeth for being examples of strong women that care about their impact on the world. Our impromptu hangouts – spanning chats in the lab, making Christmas crafts,

attending arm wrestling matches, and going for bike rides have significantly enriched my life. And to the many others who I have spent time with, thank you.

And I want to thank my family, who have made me who I am. My parents gave me the stability and infrastructure to become an educated person with career options. But they are also responsible for all the intangible aspects of my successes. My dad has taught me many valuable lessons but there are two I want to highlight here. First – he taught me that a person's character is defined in quiet moments, when you will not get credit for an action. He has taught me to always treat everyone with respect, even if the person is not in a position to help me or return the favor. He has also impressed upon me that happiness is not necessarily some end goal or state of being. This is particularly relevant to a career in science where the challenges keep coming. Instead, he has encouraged me to think of happiness as little moments – a successful experiment, a coffee break, the wind on my face. Both of these lessons have reminded me of who I am when school has been challenging. My mom has shown me that women can have it all. She was an incredibly respected woman in her career at the CIA and I am sure that from an early age I had ambitious dreams because I knew they were possible. My mom has taught me self-sufficiency on all levels. In some of the earliest videos of me as a kid she is saying "Come on, you can do it (yourself)!" She has empowered me to know what I want, to make no apologies for myself, and to stand up for myself (although I am still working on that part). More so than anyone else, she has lived every moment with me – cried when I cried, celebrated when I celebrated. Finally, my brother, and this is no exaggeration, is the smartest person I know. Throughout graduate school, he has taken an intellectual interest in my studies and the entire machine that is science. More than anyone outside of my schooling, he has made an effort to understand

my project and my thoughts about science as a whole. I have been a proud sister to watch him develop in his career alongside me, and I am continually grateful for our close relationship.

Finally, I want to thank the only person without whom I sincerely may not have completed graduate school – my partner, Pedro. I met Pedro in core course, and early on, he offered perspective about what was important in grad school. While I was worried about my grades, he reminded me that the point of core course was to absorb important lessons about biology, but also to form meaningful relationships. I consider myself to have good intuition and emotional intelligence, but Pedro exceeds my capacity in that area. He has inspired me to strengthen all of my relationships, with family, old friends, and colleagues. We sometimes joke that he is the unofficial mayor of Charlottesville, because of the sheer number of people that wave to him in the hallways. The amazing thing is that all his relationships are built off of a genuine interest he has in people's lives – their pets, their hobbies, their families, in addition to their science. I have learned a great deal from him about how to be more of a presence in the lives of other people, which is in some ways, the only thing that matters in this life. In addition to this, Pedro is a prolific (how on earth does he have ~25 papers?) and talented scientist, and I have sought his advice at all levels of my science - from completing experiments to presentations. He always has valuable insight that are typically in my blind spots. We also have similar outlooks on the fundamentals of science and I have loved our many in-depth discussions about how the scientific process, from funding to publishing paradigms, can be improved. But perhaps the biggest contribution Pedro has made to my life these past five and a half years is he has kept me on an even keel. I have a tendency to overthink and overwork, and some days I come home

and can spiral into negative thoughts. Pedro has been my rock each and every day and has allowed me to reset and look at the next day with fresh eyes. He always gives me his honest opinion and never gets sick of listening to me. He supported me by taking care of many household duties when I was giving all of myself to work, and he has been a caring cat dad to our Virginia (Ginny) Mae. I can't put into words how much his support has meant besides to say that I am not confident I would have finished graduate school without him, and certainly not in as strong a mental state. Ruth Bader Ginsburg said that she owed much of her success to a supportive partner who believed in her, and I can't be more grateful to have found that in Pedro.

List of Abbreviations

AD – Alzheimer's Disease
AIDS – acquired immunodeficiency syndrome
APC – antigen presenting cell
ASC – apoptosis-associated speck-like protein
BBB – blood brain barrier
CD – cluster of differentiation
CSF – cerebrospinal fluid
DC – dendritic cells
EAE – Experimental Autoimmune Encephalitis
ER – endoplasmic reticulum
GBP – guanylate binding proteins
GPI – glycosylphosphatidylinositols
HMGB1 – high mobility group box 1
HSV – Herpes Simplex Virus
i.c.v. – intracerebroventricular
i.p. – intraperitoneal
IL – Interleukin
IL-1R – IL-1 receptor
IL-1RacP – IL-1 receptor accessory protein
IL1RL1/ST2 – IL-1 receptor like-1
ILC – Innate lymphoid cell
iNOS – inducible nitric oxide synthase
IRG – immunity-related GTPases
ISF – interstitial fluid
ISF – interstitial fluid
LPS – Lipopolysaccharide
MHC – Major histocompatibility complex
MOG – myelin oligodendrocyte glycoprotein

MS – Multiple Sclerosis

NLR – Nod-like receptor

PAMP – Pathogen associated molecular pattern

PARP – Poly(ADP-ribose) polymerase

PCR – polymerase chain reaction

PI – propidium iodide

PV – Parasitophorous vacuole

RAGE – the receptor for advanced glycation end-products

ROS – reactive oxygen species

SNP – single nucleotide polymorphism

TBI – traumatic brain injury

TCR – T cell receptor

TIR – toll-interlukin 1 receptor

TLR – Toll-like receptor

T. gondii – *Toxoplasma gondii*

WNV – West Nile Virus

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Chapter 1 - Introduction

Toxoplasma gondii, a CNS-tropic pathogen

Toxoplasma gondii has captured the attention of many scientists over the past century, perhaps because it is an incredibly successful pathogen. *T. gondii* can infect many cell types, and a wide range of hosts throughout the world, including mice and humans. *T. gondii* also persists for the lifetime of the host, striking a balance of benefiting from the host without becoming too detrimental. Central to my work, *T. gondii* reliably traffics to the brain, inciting a powerful immune response which keeps the parasite in check within this tissue. Thus, *T. gondii* can be used as a model to better understand neuro-immunological mechanisms. These might include understanding how immune cells are recruited to the brain, how brain infection is controlled, and how parasite can impact brain function.

1.1.1 *Discovery of T. gondii*

Toxoplasma gondii was incidentally discovered in 1908 by French researchers Nicolle and Manceaux studying *Leishmania* in Tunisia, Africa[1-3]. They observed *T. gondii* in tissues of the rodent *Ctenodactylus gundi,* and named the parasite *Toxoplasma* - Greek for "arc form", based on the bow-like morphology of the parasite[1-3]. *Gondii* is thought to have arisen from a misspelling of the host species in which it was discovered - *Ctenodactylus gundi*[1-3]. In the immediate years following its discovery, *T. gondii* presence was noted in infected rabbits, sparrows, canaries, dogs, and guinea pigs[4]. *T. gondii* was first isolated

from a human in 1938, from autopsy of an infant girl who died one month after birth of severe brain, eye, and spinal cord pathology[5]. By 1941, it was shown that isolated *T. gondii* could be pathogenic across host species, and that serum could confer protective immunity between hosts[6]. These studies confirmed that the same strain of *T. gondii* could infect animals and humans. From 1940 to 1970, after clinical and veterinary significance was established, research focus on *T. gondii* boomed. Foundational studies were published in these years describing clinical manifestations of *T. gondii* in humans, the *T. gondii* life cycle, and components of protective immunity to *T. gondii*[1-3,7] that will be highlighted in the coming sub-sections.

1.1.2 *T. gondii strains*

T. gondii undergoes sexual reproduction, thus giving rise to genetically distinct strains. Considerable effort has been made to categorize strains by geographical region and virulence. In 1995, *T. gondii* genetic variability was characterized throughout North America and Europe[8]. A pattern of clonality emerged, in which strains fell into three principal lineages, with little genetic diversity within lineages[8]. Approximately 50 strains have been identified that fall within one of these three lineages - I, II, and III[9]. With the advent of more advanced DNA sequencing technology, and with the integration of data from over one thousand *T. gondii* isolates around the world, close to two hundred unique strains have now been identified, from more than fifteen lineages[9-14]. The majority of genetically unique strains have been isolated from Central/South America[12,15-17]. These data support a model in which *T. gondii* either originated in Central/South America, or in which the tropical environment in Central/South America supports a greater diversity of *T.*

gondii hosts[9]. Nevertheless, the nomenclature of types I, II, and III, has predominated as *T. gondii* strains isolated from North America and Europe have been widely used in research[18]. Studies in mice have revealed important distinctions in virulence between strain types I-III, due to genetic variation relatively few loci which impact the parasite's capability to invade host cells, to subvert the host immune response, and capacity for growth[18]. Type I strains have been described as highly virulent and are lethal to mice. Type I strains generally have increased resistance to breakdown of the vacuole in which *T. gondii* is contained, can prevent adequate antigen presentation, and can antagonize beneficial immune signatures by activating STAT transcription factors that benefit the parasite[18]. Type II strains are described as having intermediate virulence. While type II strains typically invade cells successfully, they induce a relatively strong immune response, and are thus able to be cleared intracellularly, although immunopathology may occur. These strains typically exhibit a controlled, but chronic infection in the mouse[18]. Finally, type III strains are readily cleared in primed cells, and do not typically induce a strong immune response, thus avoiding immunopathology. The strain type used in all studies for this thesis is the Type II strain, Me49. We hypothesize that type II strains most closely model human infection, as human infection is chronic, typically not lethal, and most human isolates obtained from North America and Europe are categorized as Type II[19-21].

1.1.3 *Life cycle: reproduction, transmission, host cell invasion*

Reproduction

T. gondii is capable of undergoing both sexual reproduction and asexual reproduction. Significant clonal expansion of *T. gondii* strains suggests that *T. gondii* sexual reproduction is somewhat limited[8]. Several pathogens, including fungal pathogens and protozoan parasites, have adopted limited sexual reproduction as a survival strategy[22]. Maintaining low levels of sexual reproduction provides opportunity to adapt to environmental challenges, while propagating well-adapted strains via asexual reproduction[22]. Indeed, *T. gondii* is thought to have evolved relatively recently, within the past 10,000 years, increased capability for oral transmission, thus greatly expanding host range[23]. In the 1970s, several researchers independently discovered that *T. gondii* preferentially undergoes sexual reproduction in felines – thus describing felines as definitive hosts[24]. Specifically, *T. gondii* can form zygotes, called "oocysts" within the feline intestine (Fig 1.1). Last year, in 2019, it was discovered that linoleic acid is required for oocyst development, and felines are the only mammals that lack enzymes which break down linoleic acid, leading to an excess within the feline intestine[25]. Exhaustive testing of other species in the 1970s revealed that all other warm blooded animals capable of being infected can only support asexual reproduction of the parasite, and are therefore called intermediate hosts[24] (Fig 1.1).

Transmission

Oocysts from cats can be shed in their feces[26]. Once shed, oocysts can undergo meiosis, creating sporulated oocysts, a form of the parasite which is highly resistant to

degradation[27]. Shed oocysts can contaminate food and water supply, thus leading to ingestion by intermediate hosts, including all warm blooded vertebrates[18]. Animals can also become infected via carnivory of other animals, consuming *T. gondii* within infected tissue[28,29] (Fig 1.1). Carnivory is thought to be the primary mode of transmission to humans, and is especially prevalent in cultures which consume undercooked meat[29]. Finally, *T. gondii* can be spread via congenital infection, if a mother becomes infected while pregnant[30,31] (Fig 1.1). For this reason, expecting mothers are warned against scooping cat litter and consuming undercooked meat.

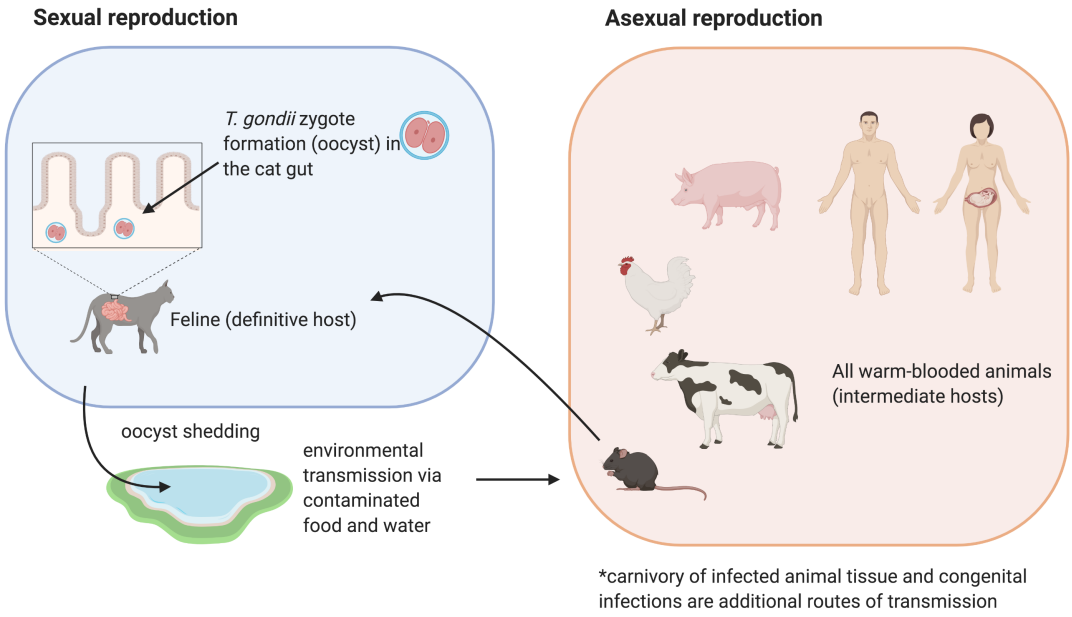

Fig 1.1. *T. gondii* reproduction and transmission

T. gondii can only sexually reproduce and introduce genetic variation specifically within the feline intestine. Feline intestinal tracts lack an enzyme that breaks down a nutrient *T. gondii* needs to sexually reproduce. The diploid form of *T. gondii,* called an oocyst, is highly infectious and can be shed by cats into the environment from their feces. *T. gondii* can then be transmitted through contaminated food or water to any warm-blooded animal, including humans and mice. The most common route of infection for humans is carnivory of infected animal tissue, but the parasite can also be transmitted congenitally if a woman becomes infected while pregnant. *T. gondii* can then re-infect cats, completing its life cycle, if a cat consumes an infected animal, such as a mouse.

Invasion of host cells

T. gondii is an obligate intracellular parasite, and has the incredible ability to infect all nucleated cells. *T. gondii* invasion begins with actin-myosin based motility of the *T. gondii* cytoskeleton, which allows the parasite to move in a gliding fashion and encounter host cells[32-34]. Adhesion to the host cell does not appear to be dependent on receptor recognition, but rather, by small, secreted proteins, called mironemes which have adhesive capability[35,36]. Micronemes provide continual to the host cell via many weak interactions at once and allows the parasite to be propelled forward by actin remodeling[37]. The parasite then begins to invaginate the membrane of the host cell, which forms around the parasite as it invades, thus creating a parasitophorous vacuole (PV) which is excluded from the host cell cytosol[38]. Next, the parasite secretes proteins from specialized organelles, called rhoptries, which serve to stabilize the PV and promote an environment conducive to parasite replication, including by effecting host cell signaling, including dampening anti-parasitic immune signaling pathways[18,39]. It is not clear whether or not secreted proteins require host cell invasion, as rhoptries have been reported to be injected into cells the parasite does not actively invade[40]. *T. gondii* also secretes dense granule proteins, which have begun to be more recently characterized, and contribute to nutrient acquisition by creating pores in the PV, the maintenance of the PV, interruption of host cell immune signaling, and host cell egress[18,41-43]. Once within the PV, *T. gondii* is protected from the endocytic pathway and has the capacity to replicate by co-opting host nutrients. As complicated a process as host cell invasion by *T. gondii* is, so are the immune processes the host employs to break down the PV and clear intracellular parasite, discussed in future sections. After rounds of replication, the parasite can egress from host cells in what is a

lytic event *in vitro*[44]. The signals which initiate egress are poorly understood, but lead to immediate infection of nearby cells[44].

1.1.4 *T. gondii infection prevalence*

The prevalence of *T. gondii* infection is high among animals and humans, and is a leading cause of illness contracted from food[45]. Worldwide, it is estimated that approximately one third of the human population is infected with *T. gondii*, as measured by sero-prevalence, ranging widely by geographical location and culture, from 10% to 90% by country[46,47]. For example, high prevalence is observed in Europe, hovering around 50% depending on the country[48-52], whereas the U.S. and Canada exhibit <20% seroprevalence[46,53,54]. Some of the highest rates of infection, above 60%, are in African countries[55-57]. It is important to note that many of these studies have focused solely on pregnant women, which may not be reflective of the overall population[46]. High rates of *T. gondii* sero-positivity are thought to be a result of eating undercooked meat, socioeconomic status of the country, including water and soil purification, contact with felines, and varied prevalence of infection within farm animals themselves by country, potentially owed to housing conditions and antibiotic treatment[46,47,58]. With recognition of these factors, *T. gondii* prevalence has declined over time in western countries[46,59-62]. In the U.S., a prevalence of approximately 15% in 1988-1994 dropped to approximately 10% by 2004[61]. Nevertheless, *T. gondii* still remains a highly successful and globally-relevant pathogen, infecting a large portion of the human population.

1.1.5 *T. gondii mouse models: strains and mode of infection*

Susceptibility of mice by strain

T. gondii lends itself to laboratory study, since *T. gondii* strains can infect both humans and animals naturally. Experimentally infected mice can live long-term, and control *T. gondii* infection, thus shedding light on immunity to *T. gondii*. But there are variations between mouse models in severity and course of infection, depending on mouse strain, *T. gondii* strain, infection dose, and mode of delivery. First, certain mouse strains are inherently more resistant or susceptible to *T. gondii* due to genetics. In 1990, there was an observation that Balb/c mice are significantly more resistant to infection when subjected to the same *T. gondii* strain and infection dose than C57BL6 mice[63]. On average, C57BL/6 mice harbored fifty times the parasite burden of Balb/c counterparts[63]. Susceptibility was mapped to major histocompatibility complex (MHC) genes, important for antigen presentation. Specifically, the two strains differed in haplotype of H2-L genes, linked with MHC class 1[63]. Variation in susceptibility of mouse strains to *T. gondii* has been exploited for the purpose of passaging parasites, to maintain a pool for laboratory infection *in vivo*. For instance, Swiss-Webster mice, which are on average less susceptible to infection than C57BL/6 mice, can be infected to maintain the parasite long-term, and the susceptible CBA/J strain, which typically succumbs to infection, can be used as an intermediate to yield large amounts of parasite before infecting C57BL/6 mice[64,65].

Mode of infection

Mode of infection can also impact disease course. Some of the earliest studies on immunity to *T. gondii* in mice employed intraperitoneal injection of the parasite[66,67]. In these studies,

wildtype infected mice lived for extended periods of time, thus highlighting increased mortality in mice with manipulated immune systems[66]. Since then, it has been argued that mice should be infected orally with *T. gondii*, as a more natural route of infection. This has been accomplished both by consumption of material containing *T. gondii*, and by oral gavage. Oral infection is likely inconsistent in infection course[65], with oral gavage proving to be more variable in infection outcome[68]. In many cases, oral infection leads to more severe disease in mice. Several studies have highlighted the intestines as a major site of parasite replication, and lethal pathology, following oral infection[69-71]. Destruction of gut tissue following oral infection has been connected with immunopathology, requiring IFN-γ, TNFα and nitric oxide[69,72,73]. Pathology has also been shown to be dependent on commensal microbiota[72]. It appears that dysbiosis occurs in *T. gondii* infection, leading to outgrowth and translocation of bacterial species that can be detrimental to gut tissue[72,74,75]. There is evidence for both the immune system causing changes in the microbiota[75], and for microbiota influencing the immune response, as an adjuvant[72,76]. In contrast to oral infection, intraperitoneal injection of parasite also results in infection of intestinal villi, but to a lesser degree, suggesting that different routes of infection will contact different tissues at different timepoints[71,77].

In this thesis, *T. gondii* infection was done intraperitoneally in C57BL/6 mice, which results in a chronic infection with significant parasite burden in the brain, that is properly controlled long term with an intact immune system.

1.1.6 *Stages of infection*

Acute phase of infection

When an intermediate host, such as a mouse, becomes infected with *T. gondii*, the first stage of infection is characterized by widespread dissemination of *T. gondii* throughout peripheral tissues. During this stage, the parasite becomes highly replicative and invasive. Individual parasites have the capability to infect any nucleated cell and are called "tachyzoites", named for their fast replication[78]. Tachzyoites are crescent shaped, and very small, roughly 1 x 5 υm[79](Fig 1.2). Tachzyoites reproduce asexually, typically via asynchronous cell division. But if synchronous, tachyzoites can form rosettes[79]. Uncontrolled parasite replication can cause lysis of the host cell, propagating infection of nearby cells[44,79]. Within the first two weeks of infection, the parasite disseminates throughout most tissues, with high burdens in the intestine, liver, spleen, peritoneal exudate, and kidney, depending on route of infection[77,80]. Following recognition of the parasite and induction of a strongly polarized immune response, peripheral infection is ultimately controlled[81]. The protective immune response to *T. gondii* in mice will be described in detail in future sections. The acute phase in mice is considered to last the first two weeks, which is the amount of time necessary to prime an adaptive immune response and control parasite in most peripheral tissues[77,80] (Fig 1.2).

Chronic phase of infection

After the immediate threat of acute infection has been controlled, *T. gondii* that has disseminated via the blood persists chronically in its hosts in a slowly-replicating cyst form, known as the "bradyzoite"[78]. Cysts consist of a wall, decorated by carbohydrates[82], and can

house many individual parasites[79], averaging a size of 70-100υm depending on tissue residence[83] (Fig 1.2). Cyst formation preferentially occurs in specific tissues, including the brain, eye, spinal cord, and muscle tissue[84,85]. The factors which promote cyst formation *in vivo* are poorly understood, but studies *in vitro* suggest that arrest of host cell proliferation supports bradyzoite formation, thus selecting for terminally differentiated cells[86]. Other exogenous factors which could influence bradyzoite formation include alkaline pH[87], heat[88], nutrient deprivation[89], and host immunity, including nitric oxide production[90,91]. Other possibilities include parasite-intrinsic slowing of replication in the absence of exogenous signals, or host cell type-specific factors, including inability to clear the parasite, and cell type microenvironment[37]. Cyst residence in muscle cells is especially important from a transmission perspective, since carnivory is a major route of infection, as discussed previously. However, ongoing responses to parasite during the chronic phase in muscle tissue is relatively understudied. In the brain, cre-reporter mice infected with cre-secreting parasites has shown that neurons are the primary cell type to harbor cysts[92], and a high number of cysts are associated with the cerebral cortex. It is unknown if other factors could influence this finding, including quickly-occurring cell death of other infected cell types. Nevertheless, cysts can be visualized in the processes of neurons, typically far away from the soma, again suggesting that surface area or nutrients could play a role in cyst formation[92]. Another mystery concerning bradyzoites are their impact on host cell processes, although cysts are generally thought to be quiescent, due to a lack of tissue damage and inflammation surrounding them, and their degree of motility is also poorly understood[93]. Most of what is known about bradyzoite prevalence and formation has been studied *in vitro*, or in animals, because despite high prevalence of Toxoplasma

seropositivity in humans, cysts have rarely been observed in human tissue incidentally by biopsy, which likely requires sampling of large amounts of tissue[94]. Experimentally, mice harbor many bradyzoites in their brain during chronic infection, depending on the strain[63]. A typical time point for assessing chronic infection in mice is four weeks post infection, after peripheral infection has been controlled (Fig 1.2).

Bradyzoite conversion: T. gondii reactivation

During chronic infection, bradyzoites can revert to individual fast-replicating parasites – a poorly understood process termed "reactivation." *In vitro*, bradyzoites have converted to tachyzoites with removal of the same factors that stimulate cyst formation, such as cytokine, increased heat, and alkaline pH[93]. *In vivo*, reactivation has been documented in human congenital infection[95] and in AIDS patients[96,97], thus underscoring the importance of immune pressure in controlling infection. A similar phenomenon is seen in mice, where mice lacking the critical cytokine known to control *T. gondii* succumb to infection with large amounts of reactivated parasite[67,98]. Reactivation can also occur occasionally in immune-competent hosts, and has been documented in the human retina[99] and in mice[100], including by our lab[101]. Reactivation, although poorly understood, is an important concept that has implications for host cell damage, due to either cyst rupture, lytic replication of recently converted parasite, or secondary inflammation[102]. Reactivation as an instigator of damage is a central theme in my project, which revolves around the importance of damage signaling in control of *T. gondii* infection.

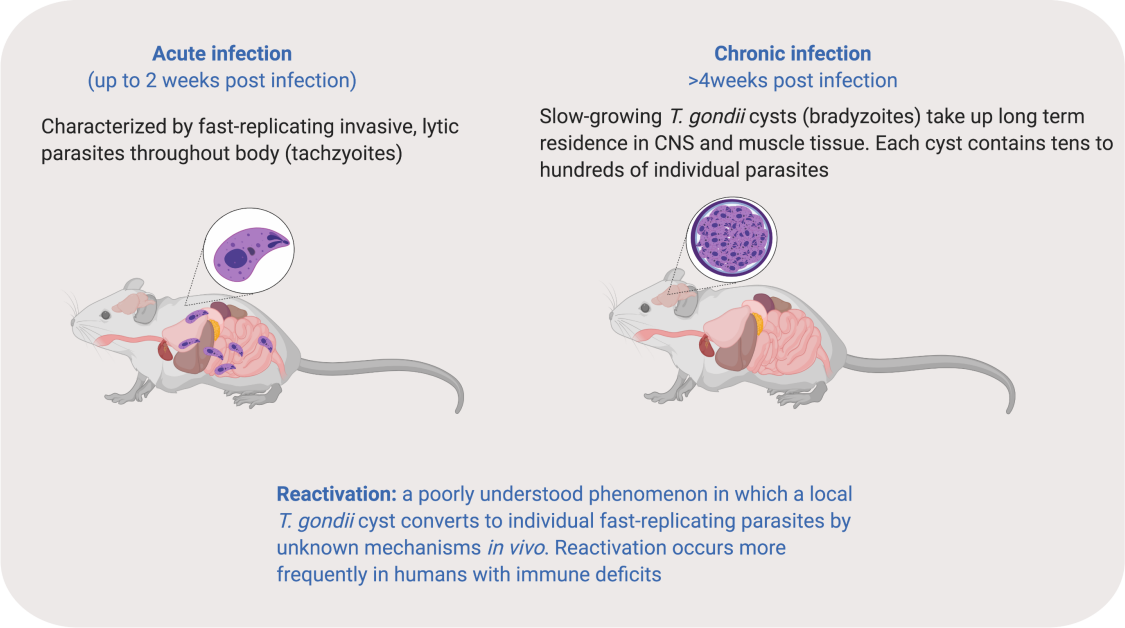

Fig 1.2. *T. gondii* stages: acute infection, chronic infection, and reactivation.

Once a host becomes infected with *T. gondii*, the parasite circulates indiscriminately throughout host tissues. The first two weeks of infection is called the acute stage, characterized by a crescent-shaped form of *T. gondii* called the "tachyzoite," which is fast replicating and invades many host tissues. Approximately two-weeks post-infection, the majority of the parasite throughout the body has been cleared, and *T. gondii* persists as a slow-growing cyst form in skeletal muscle, heart tissue, and in the brain, for reasons that are not fully understood. *T. gondii* persists in these tissues for the lifetime of the host. For researchers studying brain infection, 4 weeks post-infection is typically denoted as chronic infection, where the parasite is largely restricted to the brain in its cyst form. For reasons we don't yet understand, *T. gondii* cysts can periodically convert, or "reactivate" to individual fast replicating parasites within brain tissue, even in immunocompetent hosts.

1.1.7 *Host susceptibility to T. gondii infection*

Pathology

Since humans are tested for *Toxoplasma* infection based on seropositivity, it is often difficult to know when infection first occurred. But based on the high percentage of the human population that is infected, infection is presumed to be asymptomatic, or coincide with mild flu-like symptoms[18]. However, there are several instances in which *T. gondii* infection can have severe consequences. Immunocompromised patients, such as those with

acquired immunodeficiency syndrome (AIDS), can suffer from toxoplasmic encephalitis, resulting in widespread necrosis of brain tissue and death[97,103]. *T. gondii* pathology typically occurs in late-stage AIDS, when T cell counts drop significantly, thus underscoring the importance of immunity in controlling *T. gondii*[104]. *T. gondii* can either be acquired as an opportunistic infection, or can reactivate with reduced capability for immunity[104]. *T. gondii* cause of death in these patients can be surmised with evidence of serological positivity for *T. gondii*, post-mortem brain pathology, and radiology, including observation of ring-enhancing lesions in brain tissue[104,105]. In some instances, *T. gondii* has been detected by polymerase chain reaction (PCR) in the cerebrospinal fluid (CSF) of infected AIDS patients, highlighting the potency of parasite in these cases[106]. Immunosuppressed patients undergoing transplant surgeries present with similar phenomena, and can succumb to opportunistic or reactivated *T. gondii* infection due to widespread necrosis of brain tissue[107]. Congenital infection can also result in severe neurological disease[30,95,108], including abortion of the fetus, and surviving fetuses may exhibit blindness, seizures, microcephaly, and developmental issues[109]. Although transmission rate to a fetus is directly correlated with the point of gestation, severity of congenital infection is inversely correlated with gestational age, with transmission to a younger fetus resulting in worsened clinical outcomes[109]. Finally, both immunocompetent and immunosuppressed individuals can exhibit ocular toxoplasmosis, which in its most severe form, can lead to necrotizing retinitis, retinal detachment, edema, glaucoma, and cataracts[110-113]. In all cases, severity of disease is correlated with immunity. Mice with impacted immune systems also succumb to infection, and will be discussed in detail in future sections. In general, there is evidence that experimentally infected mice harbor more

cysts in their brains than immunocompetent humans proportionally, and the mouse model can be thought of as intermediate between immune competent human infection and AIDS-induced toxoplasmic encephalitis[100]. Although there is no evidence of shortened lifespan of immune-competent hosts infected with *T. gondii*, infection has been correlated with altered behavior in mice and humans, which will be discussed in the next section.

Treatment

A combination treatment of antibiotics, including sulfadiazine, pyrimethamine, clindamycin, clarithromycin, azithromycin, and others, have been most commonly used to treat *T. gondii* infection clinically[114,115]. Unfortunately, these drugs only impact tachyzoites, not bradyzoites, and often have adverse effects, such as liver toxicity and inflammation of the gut[115]. During experimental infection of mice, antibiotics have been used to help mice survive acute infection in cases of immune perturbation, such as IL-10 deficiency[116].

1.1.8 *T. gondii impact on CNS tissue*

Parasite trafficking to brain tissue may occur by direct infection and replication in BBB endothelial cells[117]. A popular alternative hypothesis is that the parasite traffics to the brain within an infected cell[118-121], and although i.v. transfer of infected dendritic cells results in earlier infection of the brain[119], crossing of infected immune cells into the brain has not been visualized *in vivo* using live imaging. A common mechanism other pathogens use to reach the brain is via retrograde transport from peripheral nerves, but this is not typically achieved with high fidelity[122]. *T. gondii* is one of the few infections that reliably traffics to the brain from the periphery and models chronic brain involvement without requiring

intracerebral injection[123]. Although *T. gondii* has been described as CNS-tropic, it is possible that rather than preferentially infecting the brain, the parasite is just maintained in this tissue due to an inability to clear cysts harbored in neurons[124]. There appears to be an increased prevalence of *T. gondii* in the cerebral cortex, amygdala, and striatum[92,125], but cysts and reactivated parasite can be found in any brain region[126]. The impact of *T. gondii* infection on brain functioning is relatively understudied, but there is some evidence that brain physiology is disrupted.

Blood brain barrier permissiveness

For one, there is evidence of increased blood brain barrier (BBB) permeability during infection, as measured by Evans Blue leakage into the parenchyma from the blood[127]. Although the BBB typically permits small lipophilic molecules and gases, larger, >400-500 Da hydrophilic substances are restricted[128], including Evans Blue, a dye which is approximately 68kDa[129]. Evans blue leakage in *T. gondii* infection peaks during early chronic infection and subsides later on, approximately 6 months post infection[127]. The same study found that blood vessels during chronic infection were less able to dilate, and that blood flow was restricted, potentially explaining why introduction of *T. gondii* prevents induction of cerebral malaria, where the parasite replicates within blood vessels[130]. Antibodies injected peripherally can also have observed effects in the brain during *T. gondii* infection[67,131,132], suggesting BBB permissiveness of up to 150kDa.

Neurotransmission

There is also some evidence that Toxoplasma brain infection can alter neurotransmission. Two studies have found increased levels of dopamine in the brain during chronic

infection[133,134], although this may be a result of dopamine production by infiltrating immune cells[135]. *In vitro*, tachyzoites have the ability to manipulate calcium stores in cultured neurons[136]. Other studies have described increased excitability in *T. gondii* infected brains, possibly owed to increased extracellular glutamate, an excitatory neutrotransmitter[137], or mis-localized machinery for the synthesis of GABA, an inhibitory neutrotransmitter[138]. These studies may shed light on an increased propensity of *T. gondii*-infected animals to seize[138], but it is important to note that altered neurotransmission has been studied on a global scale, throughout the brain, rather than on a per cell basis[135]. Therefore, it is poorly understood how infected neurons may differ in function by neuron type, or from uninfected neurons in basic functioning. Finally, it is possible that *T. gondii* brain infection results in demyelination, via neuronal loss or altered synapse connectivity, which has been reported via imaging of white matter tracts in the somatosensory cortex by MRI, and more specifically, by observation of reduced dendritic spines and pre- and post-synaptic proteins in this region compared to uninfected controls[139].

Behavior

Several studies argue that infected mice and rats have reduced aversion to predator urine[125,140-142], one claiming a specificity to cat urine[125], another arguing for a pan-predator effect[141]. These studies have formed the basis for the "manipulation hypothesis", that the parasite induces risk-seeking behavior in infected hosts, which is advantageous for infection rate[143], although there is little direct evidence of the parasite directly manipulating this behavior[144]. It is not clear whether an increased interaction with predator urine in infected animals is due to reduced anxiety, decreased olfaction, increased locomotion or

increased social behavior[144,145], but the behavior does seem to be positively correlated with parasite load[141]. Another possibility is that loss of aversion to predator urine is associated with a loss of memory of fear-conditioning[134]. Consensus on *T. gondii* impact on mouse behavior is murky, with some studies arguing for decreased anxiety upon infection, assessed by elevated plus maze and open field[146], while other studies find increased anxiety and cognitive loss[147]. Variability in mouse strain, *T. gondii* strain, and behavioral tests performed may underlie contrasting conclusions[145]. In adult humans, several studies have correlated *T. gondii* infection with changes in behavior, including increased anxiety[148] and increased psychomotor activity[149]. There is also some correlation with neurological disease, most notably a 2-3-fold increased seropositivity in schizophrenic populations[150-153], and some enrichment for *T. gondii*-seropositivity in epilepsy[154,155]. The link between *T. gondii* and schizophrenia is perhaps the strongest of any psychiatric disease, evidence for which dates back to 1896[156]. More recent studies have noted correlations between *T. gondii* infection and suicidal behavior[157,158]. Some hypotheses for mechanism of altered human behavior include increased testosterone observed in *T. gondii*-infected individuals, increased dopamine, and injury to astrocytes[148,156,159]. It is important to note that human studies are correlative, and that a link to neurological disease may be tenuous.

Necrosis

As described earlier in "Bradyzoite conversion: *T. gondii* reactivation", *T. gondii*, either directly, or indirectly, via toxic inflammation, can damage brain tissue. This is most readily observed in hosts with an impaired immune response[67,97,107,160], where reactivation of parasite is common. Dating back to the 1980s, Jacob Frenkel noted that "when cysts that

maintain chronic infection disintegrate, they often give rise to lesions with intense inflammation…Although only a single host cell is destroyed by the cyst, many surrounding cells may under necrosis"[102]. Although necrosis is a general term used in pathology, any damage, however classified, is likely detrimental to delicate brain tissue containing terminally differentiated cells. These reactivation events can be observed in mice, both stochastically and in immunocompetent mice[100]. Specific loss of brain resident cell markers has also been reported in the *T. gondii* infected brain, including the astrocyte marker GFAP[161]. The *T. gondii* infected brain is characterized by widespread gliosis, and morphologically activated astrocytes. With immunofluorescent staining, focal loss of GFAP staining can be observed in infected brain tissue[161]. We observe a similar phenomenon, with oligodendrocytes in addition to astrocytes[162] making *Toxoplasma gondii* CNS infection an excellent model of focal tissue damage.

Initiation of immunity: pattern recognition

1.2.1 *Sensing of pathogens: toll like receptors*

TLRs and what they recognize

In 1996, a fruit fly gene named *toll* was found to be important for fly fungal immunity[163]. *Toll* was named in German, meaning "great" or "amazing" because of its initially discovered role in establishing the dorsal-ventral axis of flies during development[164]. All receptors since the discovery of *toll,* with significant homology, have been named "toll-like receptors (TLRs)," the majority of which have been discovered with mutational analysis upon challenge with pathogens[165]. TLRs, in general, recognize pathogen

associated molecular patterns (PAMPs), and each TLR has the capacity to recognize multiple ligands[165]. Thirteen TLRs have been discovered to date. In the late 1990s and early 2000s, there was an explosive characterization of the ligands for the various TLRs. TLRs1, 2, 4, 5, 6, and 11, can be grouped together as extracellular receptors and primarily recognize ligands exposed on pathogen surfaces[166]. TLR4 was the first toll-like receptor characterized, and recognizes lipopolysaccharide (LPS), found on the outer membrane of bacteria[167], as well as *Mycobacterium tuberculosis* antigen, Respiratory Syncytial Virus, bacterial lipotechoic acids, and the endogenous ligand, heat shock protein 60[165]. TLRs 1 and 6 each form heterodimers with TLR2 to sense a wide range of ligands[168,169], including peptidoglycan on gram-positive bacteria, bacterial lipopeptides, atypical LPS structures, parasitic Glycosylphosphatidylinositols (GPI) proteins, yeast glucose, and other[165]. Toll-like receptor 5 recognizes bacterial flagellin[170]. TLRs11 and 12 specifically recognizes an actin-associated protein of *T. gondii*, called profiling, and will be discussed in more detail in future sections[171,172]. While TLRs 1-10 share significant homology with humans, TLRs 11 and 12 are a pseudogene and nonexistent in the human genome, respectively[173], and are thus left out of most TLR-focused reviews. TLRs 3, 7, 8, and 9, recognize intracellular components of pathogens[166]. TLR3 recognizes viral double-stranded RNA[174], murine TLR7 and human TLR8 recognize single stranded viral RNA[175], and TLR9 recognizes CpG motifs in bacterial DNA[176]. TLR13 is also endosomal and recognizes bacterial RNA, but is not expressed in humans[177]. TLRs are expressed by a variety of cell types, principally immune cells, and barrier cells[165], with a propensity for expression by antigen presenting cells (APCs)[178]. In considering tissue specificity, peripheral blood is the only tissue in which the majority of TLRs are expressed[178]. In instances where TLRs form heterodimers

with each other, such as TLRs 1 and 6 with TLR2, TLRs 1 and 6 are widely and basally expressed by many cell types, while TLR2 requires more activation and is more restricted[165]. Although the overwhelming majority of TLR ligands are exogenous, TLRs can also sense endogenous ligands, such as fibrinogen, heat shock proteins, and HMGB1 under conditions of cellular stress in which endogenous ligands are liberated[179]. Most endogenous ligands can signal on the two-most diversified TLRs, TLR2 and TLR4[179]. Implications for activation of TLRs via endogenous ligands, such as heat shock proteins, hyaluronan, HMGB1, and nucleic acids, like mitochondrial DNA, walk the line of pathological and beneficial like all other pattern recognition pathways – with the potential to further injury in ischemia/reperfusion contexts, to promote regeneration in other injury contexts, and can contribute to autoimmune conditions such as arthritis[179].

TLR Signaling

After TLRs form either homodimers, or heterodimers with other TLRs, and non-TLRs, to recognize a wide variety of ligands. For instance, TLR2 and TLR4 have increased transmembrane partners compared with other TLRs, which give them an increased capacity to recognize diverse ligands[165]. Following ligand recognition, TLRs undergo signaling pathways that lead in one of two directions: upregulation of inflammatory cytokine genes, or type I interferon genes[180] (Fig 1.3). Upregulation of inflammatory cytokine genes occurs following recruitment of a series of adaptors, including toll-interleukin 1 receptor adaptor protein (TIRAP) and MyD88, which associate with each other using toll-interleukin 1 receptor (TIR) domains, which then signal via additional adaptor proteins, including TRAF6, ultimately leading to NF-kB and AP-1-driven inflammatory cytokine gene

expression[180]. Alternatively, signaling through adaptors TRAM/TRIF, instead of TIRAP/MyD88, ultimately leads to IRF3 and IRF7-mediated type I interferon expression. Type I interferon genes are induced downstream of TLRs3, 4 7, and 9, following recognition of genetic material of pathogens, and MyD88 is activated downstream of every TLR with the exception of TLR3[180].

TLRs in the CNS

Reports on brain resident cell expression of TLRs vary by technique used to detect TLR expression, and markers and purification used to isolate brain resident cells. TLRs 2 and 4 are the most highly studied TLRs by far, perhaps for their wide range of ligands and available tools. There is consensus that microglia, in mice and humans, have been shown to express nearly all TLRs, by *in situ* hybridization, and RNA sequencing[181-185]. To study microglia TLR responses, many studies have isolated microglia from wildtype and TLR knockout mice, and then challenged them with ligands of that TLR[185]. This experimental paradigm is problematic, since purified cells, especially microglia, may not maintain their intrinsic properties *ex vivo*[186]. Nevertheless, these studies have found that purified microglia respond to many TLR2 and TLR4 ligands *ex vivo*, including gram negative and positive bacteria, and components of their membranes, such as LPS, and make pro-inflammatory cytokines which are dependent on TLR expression[183-185]. Other studies on TLRs in the CNS have injected TLR agonists and observed increased expression of TLRs within the CNS, including increased TLR4 expression following LPS administration[187]. In these instances, it is very difficult to separate expression by brain resident cells, versus infiltrating, TLR-expressing immune cells. Finally, a third category of studies on TLRs in

the CNS have used whole body knockouts of TLRs, in the context of CNS-specific infections or injury. These types of studies have found that TLR2 is necessary for control of Pneumococcal meningitis, and for recruiting immune cells and lowering bacterial burden during *Staph aureus* CNS infection[185]. Roles for TLRs in parasitic and viral infections are more rare, but knockout studies have shown TLR2 to be important for controlling herpes simplex virus (HSV), and that TLR3 is detrimental for control of West Nile Virus (WNV)[185]. Additional knockout studies have found that TLR4 is necessary for recruitment of cells to the brain during EAE, TLR4 is also necessary for behavioral sensitivity to injury, TLR2 for the recruitment of peripheral to cells to the brain following axotomy, and TLRs2 and 4 worsen hypoxic injury in stroke models[185,187]. In global knockout contexts, it is unclear if intracerebral administration of TLR ligands, or TLR signaling in the periphery, affects interpretation of results. More recent advancements have begun to restrict the importance of TLRs to CNS tissue, including a bone marrow chimera of TLR4 deficiency, which showed that non-hematopoeitic cells are responsible for peripheral immune cell recruitment to the brain following endotoxin challenge[188]. TLR4fl/fl mice have been developed and used to find that endothelial TLR4 is important for permissivity of immune cells getting into the retina[189] and for neurotransmitter output in neurons in the paraventricular nucleus in the context of added beta adrenergic agonists[190]. Consensus on the relevance of TLRs on brain resident cells besides microglia has, generally, not been reached. There have been several reports that mouse and human astrocytes can express TLR3, which is expressed basally and highly in the CNS, and appears to be important for production of anti-inflammatory cytokines by astrocytes[191,192]. These studies have similar limitations to those listed above. Endothelial cells follow as the

third highest expressers of TLRs, including TLRs 3, 4, and 12 by RNA sequencing studies[181,182], but were generally not sampled in earlier studies. Endothelial cells have most often been studied in the context of TLR4, as mentioned above. Some studies have reported expression of TLRs in neurons, especially human neurons, although the level of expression is controversial[193,194]. Neurons isolated *ex vivo* have been shown to express TLRs 2 and 4, and upregulate expression of these receptors with IFN-γ stimulation[195]. It is also possible that neuronal TLR expression plays a role during development, where TLRs 7 and 9 are most highly expressed by neurons, and transiently[196]. All in all, it appears that more work is needed to better understand what specific brain-resident cell types are capable of doing downstream of TLR signaling. This will require conditional deletion of TLRs, brain-specific pathologies, and pure isolation of brain resident cells, or precise marking of brain-resident cell types within tissue, to assess their functional capability following TLR stimulation.

1.2.2 *Sensing of pathogens and cellular stress: nod-like receptors*

Nod-like receptors and inflammasome assembly

Another major arm of response to pathogens or cellular stress is the intracellular assembly of "inflammasomes". First proposed in 2002, an inflammasome is a multi-protein assembly which leads to the activation and release of IL-1 and IL-18, two IL-1 family members that promote pro-inflammatory immune signatures not unlike TLR signaling[197]. Most inflammasome sensors described to date are cytosolic Nod-like receptors (NLRs), which contain a C-terminal ligand-binding domain, a central self-oligomerization domain, and a

varied N-terminal domain – which typically allows for the recruitment of caspases, or an adaptor which recruits caspases (ASC)[198]. NLRs which recruit ASC via pyrin protein-protein interactions are named "NLRP" for their associated pyrin domain[198]. NLRP3 is the most widely studied NLR. To increase expression of NLRP3 beyond a basal level, an extracellular signal, called "signal 1" activates NF-kB. Signal 1 also upregulates expression of IL-1B, IL-18, and caspases. Signal 1 could be a TLR ligand, such as bacterial-derived LPS, TNF signaling, IL-1 receptor signaling, etc[199-201]. Then, NLRP3 senses a cytosolic readout of stress, such as potassium efflux, chlorine influx, calcium influx, mitochondrial distress, endosome breakdown, and production of reactive oxygen species (ROS)[201]. Sensing of cellular stress is broad and can occur downstream of pathogen invasion, pore formation in the membrane in response to extracellular ATP or pore-forming toxins, aggregation of proteins such as beta-amyloid[201]. Once NLRP3 is activated and oligomerized, it sets off a chain of events, including recruitment and oligomerization of the adaptor protein ASC. While ASC is present in the cytosol at basal levels in the cell, it can be detected as an oligomer – typically called an "ASC speck" [199,200,202]. ASC specks can theoretically function intracellulary as well as extracellularly[203], to recruit caspase 1, which is able to cleave IL-1B and IL-18 into active versions for extracellular signaling, as well as activate gasdermin-D, which forms pores in the cellular membrane and is associated with inflammatory cell death known as pyroptosis, a pro-inflammatory programmed death named for fire "pyro"[199,201,204]. To give another example of NLR sensing, another NLRP family member, NLRP1, can recognize cytosolic *Bacillus anthracis* toxin or *T. gondii* infection [205-207]. NLRC4, another Nod-like receptor which lacks the pyrin domain, and instead has the ability to recruit caspase 1 independent of ASC, can be activated by bacteria

that has entered the cytosol[208,209]. There are also non-NLR inflammasome sensors - AIM2 and pyrin, like which can interact with ASC, but have differing central and C-terminal regions[198,199]. AIM2 can recognize cytosolic double-stranded DNA, including pathogen and host DNA[210,211], while pyrin recognizes disturbances in the cytoskeleton caused by bacterial toxins[212,213]. Although there are additional inflammasome sensors, these five are the most heavily studied. Finally, there is canonical and non-canonical inflammasome signaling. Canonical inflammasome signaling is mediated via caspase 1, whereas non-canonical inflammasome signaling involves caspase 11 (in mice)[214], in which intracellular pathogen components such as LPS can cause oligomerization of caspase 11, which can then directly cleave Gasdermin, or activate NLRP3 to do so[214]. Additionally, assembly of inflammasome components can lead to other forms of cell death, such as caspase-8-mediated apoptosis[215].

NLRs & inflammasomes – sensing infection in the CNS

The literature surrounding inflammasome assembly and function in the CNS is somewhat more scarce than TLR signaling, perhaps because the inflammasome is a more recent concept. But, similarly to TLR expression, microglia express the majority of Nod-like receptors, NLRP1, NLRP3, NLRC4, and AIM2[181,182,198]. AIM2, in mice and humans, seems to be most diverse in cell type expression, and between the two species has been reported to be expressed in OPCs, fetal astrocytes, microglia/macrophages, and endothelial cells[181,182]. Isolated microglia/macrophages treated with the bacteria *Legionella pneumophila*, activate caspase 1, IL-1B, and IL-18. Inflammasome responses in macrophages to *L. pneumophila* are thought to be via NLRC4-mediated recognition of

flagellin, although this was not directly tested[216]. Another study showed that Japanese encephalitis virus can induce IL-1B, IL-18, and caspase 1 activation *in vivo,* and that these readouts were dependent on NLRP3 in a macrophage cell line *in vitro*[217]. It has also been shown that NLRP3, IL-1B, and ASC, are necessary for controlling CNS WNV infection, a phenomenon that was relatively specific to the CNS using time courses[218,219]. Inflammasome components including IL-18, caspase-1, and IL-1B, have been detected in HIV-infected human brains, and HIV-1 infection induced IL-1B release from human microglia *ex vivo*[220]. More recent studies have implicated NLRP3, and even gasdermin-D in sensing HIV or its specific envelope proteins, but have done so in cultured macrophage cell lines[221,222]. Differential expression and physiological relevance of inflammasome sensors between microglia, other tissue resident macrophages, and infiltrating monocyte-derived cells during pathology, are not well understood[198]. This will require careful labeling, perhaps through microglia-specific genetic reporters, or temporally-restricted labeling of microglia vs other macrophages. Very recent work from our lab has differentiated microglia from peripheral macrophages based on time of turnover, using an inducible CX3CR1cre reporter, and has shown that recently isolated microglia release IL-1α, while peripheral macrophages do not, in a Gasdermin-D-dependent manner during chronic *Toxoplasma gondii* infection[223].

Aside from microglia and macrophages, astrocytes have been shown to express NLRP2 (in humans)[224], and human fetal astrocytes have been shown to express a range of inflammasome sensors, including NLRP1, AIM2, and NLRC4[225] pointing to a role for astrocytic inflammasomes in development. There is also evidence for inflammasome activation in neurons, most commonly NLRP1 and NLRP3[198], although the majority of

these studies focus on neuronal responses to cellular stress, with the exceptions of neurons isolated *ex vivo* and stimulated with West Nile Virus, that appear to produce IL-1B[218]. Oligodendrocytes and endothelial cells have been reported to express inflammasome components, but extensive work in infection has yet to be done[225]. Further evidence, beyond gobal knockouts, of inflammasome assembly in the CNS during infection includes visualization of ASC-speck formation, seen in cerebral malaria[226] and chronic *T. gondii* infection[223]. Further evidence of pyroptotic cell death in the CNS will be discussed in the cell death detection *in vivo* section.

1.2.3 *Sensing of host cell damage: alarmin signaling*

Damage associated molecular patterns – history and expression

In the early 2000s, at the same time that TLR and NLR ligand discovery was peaking, Polly Matizinger, an immunologist, argued that the immune system is more concerned with detecting danger than foreign molecules[227]. She wondered how proteins produced in breast-feeding mothers, which are present for a restricted time, weren't rejected as non-self[227]. Since then, self-antigens released upon cell death or damage have been widely studied for their ability to engender an immune response. These are host-derived damage-associated molecular patterns (DAMPs). DAMPs are constitutively expressed and reside in the cytosol, nucleus, or granules of a cell, and when released extracellularly, without processing, can initiate or potentiate immune responses[228]. These proteins or small molecules are thought to be passively released in response to damage, and can also be called alarmins, owed to their ability to alert the immune system. Examples of DAMPs include IL-1α[229], IL-33[230], high mobility group protein B1(HMGB1)[231], S100 proteins[232],

mitochondrial DNA[233], ATP[234], uric acid[235,236], primarily neutrophil and eosinophil-derived granule proteins such as defensins[228], and heat shock proteins – although their categorization as DAMPs is controversial[237]. HMGB1, IL-1a, and IL-33 can be differentiated from other DAMPs in that they are well studied, are restricted by cell type, and are expressed in the nucleus under homeostatic conditions[238]. Nuclear roles for these proteins are still poorly understood. Whole-body HMGB1 deletion is lethal, underscoring the importance of HMGB1 under homeostatic conditions, and nuclear HMGB1 appears to have roles in DNA repair and nucleosome movement along DNA[239]. IL-1a is important for transcription related to cell growth and differentiation[240]. IL-33's role as a transcriptional repressor or activator is unclear, since it has been shown to associate with both heterochromatin and euchromatin[241,242]. Some studies argue that IL-33 has no global impact on gene expression[243], and several studies have proposed that IL-33 nuclear localization prevents it from being released under homeostatic conditions, since disrupting the nuclear localization peptide results in extracellular IL-33-mediated amplification of immune responses[243,244]. All three alarmins are generally expressed by barrier cells, such as epithelial, endothelial cells, and fibroblasts, as well as myeloid-lineage immune cells, in most tissues of the body[238]. S100 proteins, while ubiquitously expressed, are a large family, and some S100 proteins are also cell-type restricted. S100A8-S100A12, for example, are primarily expressed in myeloid cells, but can be upregulated upon activation in barrier cells[245-247]. Although DAMPs are highly expressed constitutively, their expression can be increased both within a cell and can be made by additional cell types in inflammatory environments[228,238]. Additionally, DAMP localization is regulated. Nuclear alarmins can be modified post-translationally at their nucleus-localization sequence, by de-acetylation,

acetylation, etc. in response to cellular stress, resulting in shuttling of the DAMP to the cytosol[248], poised for extracellular release.

DAMP signaling – release, extracellular modification, and receptors

DAMPs typically do not have secretion signal peptides, and are traditionally thought to be released passively, via necrotic cell death, or actively via vesicular release[228,238,248]. Recent studies have argued for secretion of DAMPs in the absence of necrotic death, in conditions where cells are stressed metabolically, pharmacologically, are in the presence of inflammatory cytokine, or are undergoing another type of cell death such as pyroptosis[238,248]. DAMPs have also been proposed to be secreted and have roles in homeostasis, including remodeling of fat in adipose tissues, and remodeling of synapses during brain development[249,250]. It has begun to be proposed that apoptosis is not as silent a form of cell death as once thought, and alarmins such as HMGB1 may be released during later stages of apoptosis or upon phagocytosis of cells undergoing apoptosis[238]. Some of these studies have been conducted *in vitro*, and may not represent the ability of DAMPs to be secreted *in vivo*. Either way, once released, DAMPs are heavily regulated, and can be activated by oxidation, proteolytic cleavage, intracellular calcium concentrations, DAMPs more frequently are de-activated, to prevent prolonged amplification of immune responses. Several alarmins can be deactivated by oxidation, binding partners which prevent cognate receptor binding, binding of decoy receptors, autophagy, and proteolytic cleavage[228,238,248]. HMGB1 is perhaps the most promiscuous ligand in all of pattern recognition and can bind to many receptor combinations. HMGB1 can bind directly to TLR4-MD-2 complexes, can

bind LPS to signal through TLR4-CD14 heterodimers, it can bind the receptor for advanced glycation end-products (RAGE)[238]. HMGB1 can also complex with IL-1B, thus signaling through the IL-1 receptor (IL-1R), it can bind CXCL12 and signal through CXCR4, and can bind nucleosomes to signal through TLR2[238]. Perhaps the ability of HMGB1 to bind other ligands explains its promiscuity, thus requiring a potent inflammatory milieu to have a large impact. HMGB1 receptors are expressed primarily by immune cells, and depending on the receptor involved, HMGB1 signaling promotes chemokine expression, expression of other pro-inflammatory cytokines, survival, proliferation, and differentiation[238]. Once extracellular, IL-1α and IL-33 signal relatively similarly. IL-1α signals through one cognate receptor, IL-1R which forms a heterodimer with IL-1 receptor accessory protein (IL-1RacP)[238]. IL-33 also signals through one receptor, a heterodimer of an IL-1like receptor, ST2, and IL-1RacP[238]. These receptors incite a signaling paradigm very similar to TLR signaling, in which TIR domains signal through MyD88, TRAF6, and NF-κB to initiate pro-inflammatory gene expression[238]. Particular S100 proteins, most notably S100B, S100A8,A9,A11,and A12 have been shown to amplify the immune response upon extracellular release[238]. These S100 proteins can signal through RAGE and TLR4/MD2 to achieve similar ends as listed above, such as inflammatory cytokine expression, migratory ability, and proliferation. Other DAMPs induce similar expression signatures – mitochondrial DNA is thought to signal through TLR9, extracellular ATP activates the NLRP3 inflammasome by signaling via purinergic receptors to open membrane pores leading to ion flux, as well as promoting chemotaxis directly via purinergic receptors, and high levels of extracellular uric acid can form crystals which promote potassium efflux, thus activating the NLRP3 inflammasome[201,251-253].

DAMPs in the CNS & CNS infection

Expression of alarmins and their receptors in the CNS differ somewhat from the periphery, and there are fewer CNS-specific studies of alarmin signaling than TLR or NLR signaling. As in the rest of the body, HMGB1 signaling in the brain is theoretically the most promiscuous of alarmin signaling. By RNA, HMGB1 is expressed relatively highly by nearly every cell in the brain, including neurons, astrocytes, oligodendrocytes, endothelial cells, and microglia[181,254]. But studies on HMGB1 signaling during brain infection have been highly correlative – mostly focused on detecting HMGB1 in the serum and CSF. Increased HMGB1 has been detected systemically in cerebral malaria[255], bacterial meningitis[256], and WNV patients[257]. Aside from correlative studies, injection of HMGB1 into brain tissue increased BBB permissivity[258], and HMGB1 was found to be released from cultured epithelial cells in a bacterial meningitis model[259]. Studying the importance of HMGB1 is difficult, since whole body knockouts are embryonic lethal[238]. HMGB1fl/fl mice are cryopreserved, but no studies have utilized these mice in CNS infection. To make matters more complicated, HMGB1 has the capability of signaling on a number of cell types in the brain and no receptor is specific to HMGB1 signaling. RAGE has been shown to be expressed on a range of cell types, differing by isoform and method of detection, but has been reported on neurons, endothelial cells, and astrocytes[260]. TLR4, as discussed earlier, is primarily expressed by microglia but is not restricted to HMGB1 signaling. IL-1a is expressed by microglia and macrophages, often requiring an inflammatory stimulus to be readily detected[181,182,261]. By several accounts, the highest expresser of the IL-R is

endothelial cells[181,182,262-264], and has been directly implicated in sickness behavior and WNV encephalitis[218,265]. But in studying the IL-1R, many of these studies have either not differentiated IL-1α from IL-1β signaling, or have specifically injected IL-1β. Even in studying IL-1 release into the serum during neurological disease, IL-1β is most commonly measured[266]. It is also often difficult to say if IL-1 signaling is specifically relevant locally in the brain, similarly to whole body knockouts of TLRs. One study used an IL-1R reporter/knockout construct to detect low levels of IL-1R on brain resident cells and dissect its importance[264], finding that endothelial expression of IL-1R was critical in responding to IL-1β to recruit immune cells to the brain, but that low levels were detected on astrocytes and necessary for IL-6 production, and low levels were also found on neurons[264]. Very recently, a study from our lab has implicated IL-1α, and not IL-1β in control of chronic *T. gondii* infection, mediated through IL-1R signaling on the vasculature[223]. Comparatively, very little is known about S100 proteins in the CNS, in infections or otherwise. Increased extracellular S100B in particular has been linked to traumatic brain injury (TBI)[267,268], and S100B is expressed by oligodendrocytes and astrocytes by RNA sequencing[181]. But specific functions of S100 proteins as alarmins during CNS infection has yet to be fully explored. Similar limitations exist in studying these proteins as HMGB1, since they play integral roles in the cell at baseline and do not have a dedicated receptor that is specific[238]. ATP, although expressed in nearly all cells with integral roles intracellularly, can activate the NLRP3 inflammasome extracellularly, which was discussed in the previous section, but can also activate purinergic receptors in microglia to initiate chemotaxis[254]. This has been observed in injury, but has also been shown *in vitro* in an infection setting by co-culturing p2y12 sufficient and deficient microglia with herpes-infected neurons[269], and

with direct injection of *Porphyromonas gingivalis* into the brain [270]. IL-33, although expressed in barrier tissues throughout the body, is expressed in oligodendrocytes and astrocytes throughout the brain parenchyma[181,250,271]. There is significantly more literature on IL-33 in CNS infection than other alarmins, perhaps because its signaling paradigm is simple and easy to manipulate, having one cognate receptor and few critical roles of the cytokine at baseline. Because IL-33 is the protein of interest for this thesis, its roles in the CNS will be discussed in detail in future sections. For IL-33 and other alarmins in general, the biggest mystery of CNS signaling are relevant responder(s). Although alarmins principally signal on immune cells in the periphery, immune cells are restricted from the brain under homeostatic conditions, likely necessitating capability of brain-resident cells to respond. It is also interesting to consider differential expression of these alarmins by cell type as making up a patchwork within the tissue to ensure that any brain resident cell can serve as a sentinel of damage.

1.2.4 *Overlap of pattern recognition pathways*

The diversity of pattern recognition mechanisms allows for tailored immune responses to a particular insult. And yet, there is considerable overlap in pattern recognition pathways and there is opportunity for multiple pattern recognition pathways to be active in a single context, such as *T. gondii* infection. Although a majority of ligands recognized by TLRs are pathogen derived, host molecules can also signal through TLRs[179,238] (Fig 1.3). One example is TLR4, which recognizes a number of pathogen-derived signals such as LPS, but can also recognize HMGB1 and heat shock proteins through the help of various co-receptors[238,272]. This allows for an integrated sensing of pathogen associated patterns,

and host-derived signs of danger, but can also lead to aberrant autoimmunity to self-molecules. In addition to TLRs, cytosolic inflammasome sensors represent true hybrids of pattern recognition, since they are activated by extracellular TLR signals, and are capable of recognizing both pathogens and intracellular stress[199,201].

The ultimate consequences, in terms of downstream signaling, can be very similar across pattern recognition receptors (Fig 1.3). As discussed previously, within varied TLRs, generally, two primary pathways are induced – pro-inflammatory NF-kB driven signatures like IL-12, IL-6, TNF, etc., and IRF3/7 –driven Type 1 interferon[180]. It makes some sense that different intracellular pathogen can invoke similar signaling paradigms, since some effector functions are shared in clearing intracellular pathogens. Additionally, alarmin signaling can be very similar to toll-like receptor signaling. In fact, TIR domains which allow for adaptor interactions, are literally named "Toll-interleukin receptor" domains, because they allow for similar outcomes, like connecting with the MyD88 adaptor[180,238]. So, a variety of unique pathogen signals, either exogenous, or through host cell damage, can enact similar pathways. Recognition of pathogens within an endosome, which leads to differential adaptor recruitment and IRF3 and 7-depednent type 1 interferon signaling[180], can also be beneficial in controlling multiple pathogens. Although type 1 interferon signaling was first characterized in viral infection, it generally restricts pathogen replication and promotes antigen presentation, which could be helpful in many contexts[273].

Extracellular pathogens which require an entirely different mode of control, either through mucus secretion or tissue turnover, still signal through similar pathways. Helminth infection of the lungs for instance, can result in alarmin release such as IL-33, which, at face value, signals very similarly to TLRs [274,275]. But there is also regulation at the level of

receptor-expressing cell types. In this scenario, IL-33 signals on restricted receptor-expressing cells such as ILC2s, alternatively activated macrophages, eosinophils, etc., which are uniquely equipped due to the environmental milieu and cell-type-specific transcriptional regulation to expel an extracellular pathogen[274,275].

Thus, specific pathogen signals, location of pathogen detection, level of tissue destruction, and cell-type-specific transcriptional regulation can all tailor an immune response to a specific pathogen, while pattern recognition as a whole, and its consequences across methods of detection, has many commonalities.

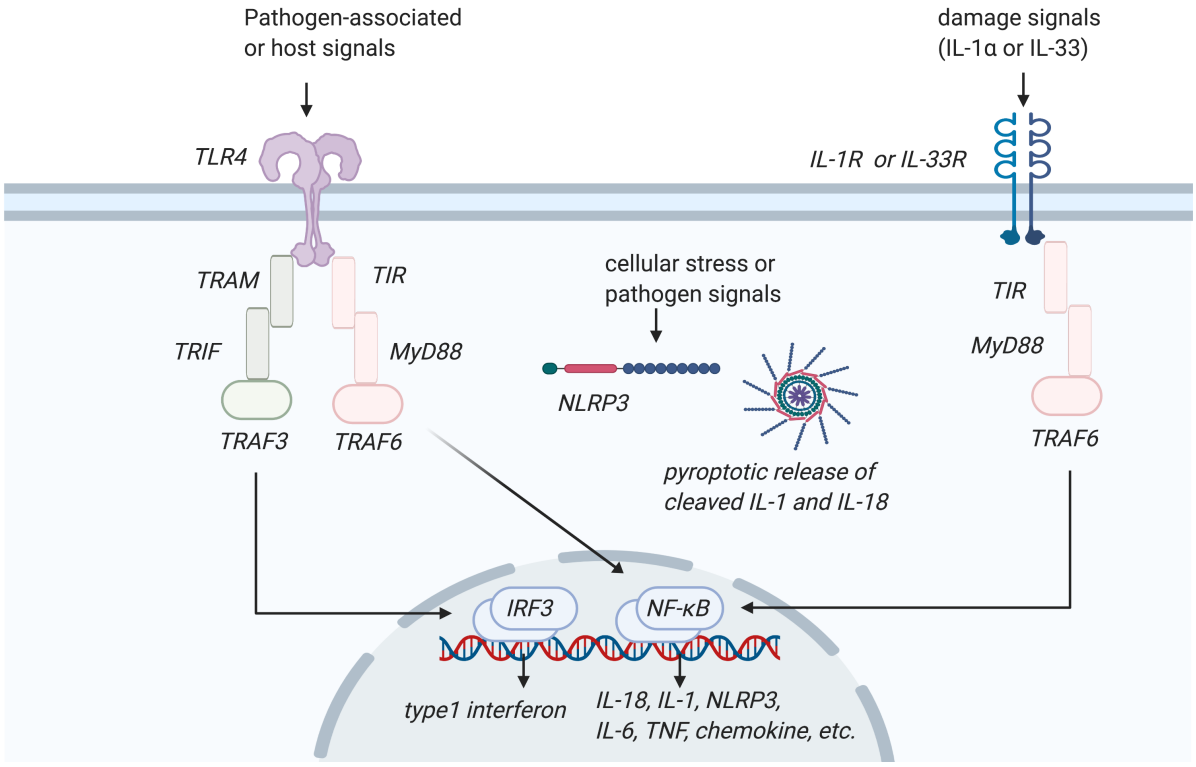

Fig 1.3. Overlap of pattern recognition pathways

There are a number of ways by which pathogen infection can be recognized by the immune system. Toll-like receptors (TLRs) often recognize pathogen-specific patterns, like components of a bacterial membrane, or in the case of *T. gondii*, a *T. gondii*-specific actin cytoskeletal protein. Infection can also be sensed by the stress it causes to the cell, including changes in ion flux, mitochondrial stress, etc., which are typically recognized by cytosolic sensors called Nod-like receptors (NLRs). Finally, infection can be recognized by the damage it causes to the cell, which can lead to release of molecules typically housed inside the cell but have the signaling capacity to induce immunity, called alarmins, or damage signals. Interestingly, there is a lot of overlap between these pattern recognition pathways. TLRs, for instance, can recognize host signals, like host protein HMGB1, in addition to pathogen components. Likewise, while NLRs are good at sensing cell stress, they can also recognize specific pathogen components in the cytosol, like bacillus anthracis toxin.
In addition, these signaling pathways have similar impacts on the cell. All three pathways can lead to NF-kB-mediated pro-inflammatory gene expression – inducing expression of innate cytokines, like IL-18, IL-6, and IL-6, chemokines, etc. Thus, immune cells like dendritic cells and macrophages are equipped with machinery to sense a wide range of pathogens, but can ultimately converge on similar cellular responses to initiate immunity.

1.2.5 *Detecting cell death in vivo*

Cell death is intimately entwined with pattern recognition, either downstream of pattern recognition, as is the case with pyroptosis, or can precede the extracellular release

of damage-associated patterns. Definitive detection of cell death is difficult, especially *in vivo*, for several reasons. First, visualization of a cell dying in real time is often difficult in a living animal. Second, identifying a specific effector of cell death is difficult, since proteins often have dual functions, or are not specific to a type of cell death. Third, it is difficult *in vivo*to manipulate cell death pathways in a physiological away, since global knockouts will affect tissues aside the tissue of interest, and administration of inhibitors can also have unintended effects. Nevertheless, cell death is an integral part of a host's response to infection. There are several categories of cell death, all of which could theoretically occur in the context of *T. gondii* infection.

Apoptosis, a programmed form of cell death which occurs during development, and in the absence and presence of inflammation, is thought to be immunologically tolerogenic[276]. Morphologically, apoptotic cells round and shrink, condense their chromatin, and form membrane blebs[277], and can release extracellular vesicles and, more recently, a number of soluble metabolites, which promote phagocytosis, regeneration, and anti-inflammatory signatures in neighboring cells[278,279]. Apoptosis can be initiated intrinsically, in response to mitochondrial stress within the cell, or extrinsically, in response to extracellular binding of ligands such as TNF[280]. Practically, apoptosis is distinguished from other modes of cell death by activity of initiator caspase 8 and 9-mediated cleavage of the executioner caspase 3[281]. Cleaved caspase 3 activity can cause morphological changes in the cell membrane[281] and can be stained for in tissue sections. TUNEL is another available tool which recognizes double stranded DNA nicks, by the machinery required to repair them, but doesn't necessarily differentiate apoptosis from other causes of DNA

damage[282]. Our lab has shown increased cleaved caspase 3 and TUNEL positivity in lesions of reactivated *T. gondii* in the brain[223].

In direct contrast to apoptosis, unregulated necrosis is an accidental form of cell death characterized by cell swelling. Permeabilization of the cellular membrane is unprogrammed and lytic[281]. This form of cell death is thought to result in passive release of intracellular inflammatory signals, such as alarmins[238,248]. Necrosis can occur in response to ischemic conditions, direct toxicity by reactive oxygen or nitrogen species, or from physical distress, in this case, from something like uncontrolled *T. gondii* replication within a cell that leads to lysis[44,283]. It has been proposed that apoptotis and necrosis can be differentiated from each other using Annexin V, which binds phosphatidylserine exposed on cells undergoing with perturbed membranes, and propidium iodide (PI), which should only enter a cell if the outer membrane is fully compromised. While both types of cell death can result in Annexin V positivity, PI is thought to be excluded from apoptotic cells[284]. This technique can be used to label cells *ex vivo* by flow cytometry, and some studies, including from our lab during *T. gondii* infection, have administered PI i.v. *in vivo*, to detect membrane permissivity and therefore death[223,285]. Theoretically, PI should detect any form of cell death featuring a loss of membrane integrity and is not specific to necrosis.

Necroptosis is a form of regulated necrosis in that it is lytic and employs kinases. There is no way to distinguish it morphologically from necrosis[281]. Here, external signals such as TNF signaling, a shared input to extrinsic apoptosis, cause activation of the kinase RIPK1, which phosphorylates RIPK3, activating MLKL, an executioner of membrane lysis. Caspases active in apoptosis de-activate these kinases and thus, necroptosis is thought to occur in the absence of apoptosis[283,286]. Necroptosis is difficult to definitively

demonstrate since RIP kinases can phosphorylate other substrates and might induce cell death independently of MLKL[287]. Deletion of RIPK3 from mice is embryonic lethal, making it difficult to study on its own, but deletion of RIPK3 in conjunction with apoptotic caspase 8 is viable. Our lab has found that co-deletion of RIPK3 and caspase 8 results in higher parasite burdens in the CNS during *T. gondii* infection, as well as increased numbers of immune cells. It is difficult in this case to separate the contribution or prevalence of apoptosis from necrosis.

A fourth major form of cell death is pyroptosis. Pyroptosis is a lytic, programmed cell death which releases pro-inflammatory cytokines processed by caspases, as discussed previously. It involves coordinated activity of a cytosolic pattern sensor, an adaptor molecule ASC, and a canonical (Caspase 1) or non-canonical caspase (11)[200]. Caspases cleave precursor versions of IL-1β and IL-18 to active forms, and Caspase 1 can activate gasdermin-D, which forms pores in the plasma membrane and is an effector of cell lysis[281]. It is difficult to use caspases as markers of death since they might have other functions, and typically Gasdermin-D is most convincingly used to detect pyroptosis. Recent literature suggests, however, that even following Gasdermin-D activity, cells maybe viable for a period of time, therefore uncoupling gasdermin-D pore formation from death in some situations[288]. Our lab has observed a dependence on gasdermin-D for alarmin release (IL-1α) from microglia during chronic *T. gondii* infection[289]. Interestingly, not all IL-1α release from microglia was gasdermin-D-dependent, leaving room for conventional necrosis or necroptosis in releasing this alarmin[223].

Autophagy can occur in conjunction or just prior to cell death, although it is rarely thought to be the cause of cell death[290]. Autophagy occurs in infected macrophages during

T. gondii infection[291], and interestingly, the parasite has evolved mechanisms of interfering with autophagy in certain cell types, like endothelial cells, by stimulating the EGFR pathway which counteracts autophagy[292].

Additional understudied types of programmed necrosis have been described, including NETosis, a granulocyte-specific form of inflammatory cell death where extracellular traps are deployed to kill pathogens, pyroptosis that doesn't appear dependent on caspases 1 or 11, ferroptosis, cell death following overactivation of Poly(ADP-ribose) polymerase (PARP) proteins, cell death following mitochondrial permeability, and others[286]. Work is still needed in understand overlap and differing characteristics of these inflammatory forms of cell death, which are difficult to distinguish morphologically. *T. gondii* may be an excellent model for better understanding death of brain-resident cells.

1.2.6 *Pattern recognition during T. gondii infection*

Most of what is known about *T. gondii* pattern recognition has been studied in the context of controlling peripheral acute infection. Early recognition of *T. gondii* presence provides the basis for skewing a strongly Th1-polarized immune response necessary for intracellular *T. gondii* killing[81]. It also facilitates recruitment of other immune cells through the production of chemokine. During acute *T. gondii* infection, the major factor that kicks off the immune response by stimulating IFN-γ production is IL-12[81]. IL-12 is critical for control of *T. gondii* infection, specifically made by dendritic cells[293,294]. IL-12, IFN-γ and all other components to *T. gondii* resistance will be addressed at length in a future section. Much like the history of pattern recognition itself, the first wave of studies on *T. gondii* recognition focused on TLRs. IL-12 production is induced by dendritic cell expression of

the TLRs 11and 12, which sense profilin, an actin-binding protein involved in *T. gondii* cytoskeletal turnover[171,172]. TLR12 deficiency, as well as TLR11/12 deficiency result in drastically reduced IL-12 production and mortality of infected mice, leading to the concept that TLRs 11 and 12 work in conjunction as a heterodimer[172]. Although TLR11-deficiency did not result in mortality during acute infection in the original study, TLR11 on its own has since been shown to be critical for acute survival, perhaps with a varied infectious dose, mouse model used, and other factors[295]. Other TLRs expressed by innate cells include TLRS 2, 3, 4, 7, and, which recognize broader patterns that *T. gondii* shares with other pathogens. For instance, TLRs 7 and 9 sense prevalent uridine in RNA sequences and DNA with unmethylated cytosines[81]. TLRs 2 and 4 are thought to recognize *T. gondii* GPI anchors heat shock proteins, respectively[81]. Although no TLRs with the exception of TLR12 are required for resistance individually, they are important in combination[296]. For instance, deletion of a chaperone protein which allows for endosomal sensing of *T. gondii* nucleic acids by TLRs 3, 7, and 9 results in mortality[296]. TLRs 2 and 4 have never been shown to be major inducers of IL-12[297,294], but there is some evidence that TLR2 signaling initiates monocyte chemoattractant signaling[298].

Although TLRs appear, at least in concert, to be necessary for control of *T. gondii* infection in mice, their importance for control of human *T. gondii* infection is not clear. TLRs 11 and 12 are a pseudogene and nonexistent in the human genome, respectively[173]. In addition, MyD88-deficient mice are acutely more susceptible to *T. gondii* infection than individual and combination TLR knockouts, leaving room for other pattern recognition pathways[294]. Aside from TLR sensing of *T. gondii* infection, several studies have underscored the importance of the inflammasome during *T. gondii* infection. First,

inflammasome sensing appears to be important to human infection, since NLRP1 deficiency in human macrophages results in parasite outgrowth, and single nucleotide polymorphisms (SNPs) in *nalp1* and the *p2xr7*, involved in ATP-mediated ion flux activation of NLRP3, are associated with susceptibility to congenital *T. gondii* infection[299,300]. In mice, the nod-like receptors NLRP1 and NLRP3 have been implicated in sensing *T. gondii*[206,207]. Mice deficient in elements of these inflammasomes succumb during acute infection due to an inability to control parasite replication[207]. In these and other studies, macrophages were shown to respond to *T. gondii* in culture by producing IL-1β[206,207]. It is unclear at this time which stimuli NLRP1 and NLRP3 sense in this system, and signals could be exogenous or endogenous. Dependence on caspases 1/11 and ASC during *T. gondii* infection is more amplified in the absence of TLR11[295]. As mentioned in previous sections, work from our lab demonstrates ASC assembly in the brain during chronic infection, IL-1 release from microglia, and increased parasite burden in the brains of infected caspase 1/11-deificent mice, implicating the inflammasome during chronic infection, although the relevant inflammasome sensor is unclear[223]

Recently, it was shown that alarmin recognition is important in human macrophages, which can sense S100A11 when released from other infected monocytes and macrophages in a caspase 1-dependent manner[246]. IL-1R signaling and IL-33 receptor signaling, encompassing two other alarmins, appears to be detrimental to acute infection in mice. Knockouts of these receptors are associated with reduced immunopathology[301,302]. There are several hypotheses underlying this, including neutrophil recruitment, IL-22 suppression, and cachexia mediated by these cytokines[74,301,302]. These data may frame the finding that TLR11-defiency can lead to severe immunopathology during acute *T. gondii*

infection, suggesting that alternative pattern recognition pathways, potentially alarmin signaling, leads to an overly powerful immune response[303].

Studies focused on recognition of *T. gondii* during chronic infection are scarce. TLR11-deficient mice that survive the acute phase exhibit seven-fold higher cyst burden in the brain compared to wildtype controls, but it is unclear if this is a result of more parasite seeding the brain, or if TLR11-sensing occurs in brain tissue[171]. The same can be said for IL-12 deficiency. When given recombinant IL-12 to survive the acute phase, IL-12-deficient mice succumb to *T. gondii* at approximately seven weeks post infection[304], whereas IL-12 deficient mice which are not supplemented succumb to infection by 10 days[304]. Given this protracted mortality curve during chronic infection, it is unclear how important local IL-12 is within the brain following initial priming of immune cells initially. Furthermore, there is a paucity of information on expression of major pattern recognition receptors in the brain – some RNA sequencing studies finding TLR11 not to be expressed by any major brain resident cells[181], others finding TLR11 and TLR12 expression by neurons and microglia[305]. As covered in previous sections, microglia are the highest expressers of other pattern recognition machinery that may be relevant to *T. gondii* infection, including other TLRs, and inflammasome sensors. Because knocking out some pattern recognition receptors is lethal, and because *T. gondii* is administered peripherally before reaching the brain, discerning the specific impact of pattern recognition during chronic infection is difficult, and will require either temporal deletion, or brain cell-specific promoter deletion of pattern recognition components. This gap in the literature forms the basis for my project, focused on a brain-centric role for IL-33 signaling during chronic infection.

1.2.7 *Protective immunity during acute T. gondii infection*

Pattern recognition, antigen presentation, and costimulation are all first mechanisms of defense during the acute phase of *T. gondii* infection. These mechansims skew the immune response towards a Th1 paradigm where IFN-γ facilitates intracellular killing (Fig 1.4). IL-12, which is produced downstream of TLR11 and 12 sensing of *T. gondii*, primarily by CD8a+ dendritic cells, is required for early control of infection[293]. IL-12 deficient animals succumb to infection within ten days and have significantly reduced IFN-γ production[294,304,306]. Pattern recognition by inflammasome sensors can also result in IL-18 processing and release, which is also required for survival acutely, shown with IL-18-deficient animals[207]. Mechanistic studies have shown, with IL-18 depletion and supplementation, that IL-18 is important for driving IFN-γ production, likely from NK cells[307], although IL-12 seems to be able to compensate for IL-18 deficiency, and be somewhat dominant in inducing IFN-γ production[308].

Another mechanism for stimulating T cells to produce IFN-γ is antigen presentation. Dendritic cells (DCs), again, are known to be the chief antigen presenting cells[81], although the most predominant mechanism by which dendritic cells sample antigen is unclear[81]. Some lines of evidence suggest that DCs which are infected are most able to present antigen via MHC class 1 to CD8+ T cells[309,310], and that this requires exchange of antigen from the parasitophorous vacuole to the host endoplasmic reticulum (ER). Electron microscopy imaging has shown host ER recruitment to the parisitophorous vacuole, and exchange between the two compartments, which is likely beneficial for the parasite in receiving host nutrients[311]. One *T. gondii* associated dense granule protein in particular, GRA6, can result

in CD8+ T cell stimulation by DCs following processing by an ER-specific enzyme[312]. Pulsing of this immune-dominant peptide protected mice from lethal *T. gondii* challenge[312]. Interestingly, host ER interaction was not seen with phagosomes, and possible connections between phagocytosis and antigen presentation are unclear[311]. Imaging of DC interactions with CD8+ T cells during acute infection demonstrated that cognate antigen and DC interactions were necessary to slow and prime CD8+ T cells in the lymph node, but that activation of T cells was largely mediated by uninfected DCs, pointing either to disparity of DC infection rates/antigen presentation depending on strain, or to secretion of parasite effectors which may affect uninfected, bystander cells[40,313].

A third major mechanism by which T cells could be activated is co-stimulation. Although co-stimulation may be important, no reports as of yet have implicated individual costimulatory molecules in survival of the acute phase. CD28 appears indispensable for *T. gondii* infection survival[314,315], and yet blockade of CD80 and CD86 results in reduced IFN-γ production[316]. Some co-stimulatory molecules appear to be required for either survival or parasite burden during the acute phase, including CD40L-deficiency, and ICOS-deficiency[317,318]. PD-1-PDL-1 interactions also seem to come into play during chronic infection, where blockade can re-invigorate CD8+ T cells and prevent *T. gondii* reactivation[319].

All of these priming mechanisms are important for inducing IFN-γ production, which is the critical cytokine required for *T. gondii* control[81,306,320,321] (Fig 1.4). IFN-γ–deficient mice are extremely susceptible to acute infection, dying by ten days post infection[322-324]. T cells are major producers of IFN-γ and depletion of CD4+ and CD8+ T cells together

results in mortality during the acute phase[321]. IFN-γ production by NK cells early in acute infection is also required for control, discovered when MHC1-deficient mice still produced high levels of IFN-γ that was abrogated with NK1.1 depleting antibodies[325-327]. More recent reports suggest that ILC1s, and neutrophils in the absence of TLR signaling, may contribute to IFN-γ production as well, underscoring several layers of redundancy for production of this critical cytokine[323,328].

IFN-γ is critical because it can induce chemokine and adhesion factor expression, as described at length in subsequent chronic infection sections – but it can also upregulate anti-parasitic effector mechanisms within cells. Early studies demonstrated that cultured infected human macrophages were significantly more capable of destroying parasite once stimulated with recombinant IFN-γ[81]. It has been shown *in vitro* that IFN-γ can aid in macrophage-degradation of the amino acid tryptophan[329], a nutrient used by the parasite. *T. gondii* is an auxotroph for several essential amino acids, requiring them for its replication. IFN-γ-dependent depletion of tryptophan was found to require IDO several years later[330]. IFN-γ can also stimulate iNOS production by macrophages, which depletes arginine required by the parasite and can be directly toxic[81]. iNOS knockouts die at the start of the chronic phase, but it is unclear if nitric oxide production is selectively important in the brain, or, is a manifestation of earlier consequences, including increased parasite seeding the brain[322,331]. Another prominent mechanism of cell-mediated resistance to intracellular pathogens in mice is the immunity-related GTPases (IRGs). Mice deficient in IRGs IGTP (Irgm3) or LRG-47 (Irgm1) do not survive acute infection whereas mice deficient in IRG-47 (Irgd) show reduced survival during chronic stages of infection[332,333].

IRGs accumulate on the parasitopherous vacuole (PV) membrane encapsulating *T. gondii*, vesiculate and rupture the PV membrane, and the parasite is ultimately eliminated by autophagy[334,335]. Interestingly, there are 23 variants of IRGs in mice, but only two in humans, neither of which is induced by IFN-γ[336-338]. Guanylate binding proteins (GBPs) are a subset of IFN-γ-inducible GTPases which are also recruited to the PV and are required to control infection[339]. Macrophages in mice lacking GBP genes fail to promote PV membrane vesiculation, suggesting that these proteins may be involved in disrupting the parasitopherous vacuole. Co-immunoprecipitation experiments have revealed that GBPs can physically interact with IRGs, likely working in concert with one another[340]. Unlike IRGs, GBPs are more conserved across the vertebrate lineage[341].

Aligned with the many roles macrophages can play in clearing *T. gondii*, a series of studies in the past decade have attempted to characterize the importance of Gr1+ cells, including inflammatory monocytes and neutrophils. These studies were done in the midst of the earliest distinction between inflammatory monocytes and those that were "tissue resident"[342]. In 2005, it was found that the chemokine CCL2 peaks four days post infection in peritoneal lavage fluid[343]. CCL2 is important for recruiting CCR2+ inflammatory monocytes from the blood. In models of oral and i.p. infection, CCR2 and CCL2 deficient mice succumbed to acute infection with increased parasite burdens[344]. There was severe intestinal pathology in these mice, including ileum lesions tied to increased neutrophil infiltration. Several years later, the same group has also specifically depleted neutrophils and have found them to be more involved in tissue damage than host protection in the acute stage[345].

In light of these findings, it is important to mention that although the immune response to *T. gondii* is largely protective, immunopathology can occur, outside of neutrophil recruitment, and the immune response must be kept in check. IL-10 is absolutely required to prevent immunopathology driven by CD4+ T cells, TNFα, and IFN-γ, in both the acute and chronic phases of infection[69,131,324]. Immunopathology in peripheral tissues occurs more often during oral infection than intraperitoneal infection, thought to be caused by interplay between the microbiota and overproduction of the same inflammatory cytokines[69,70,72].

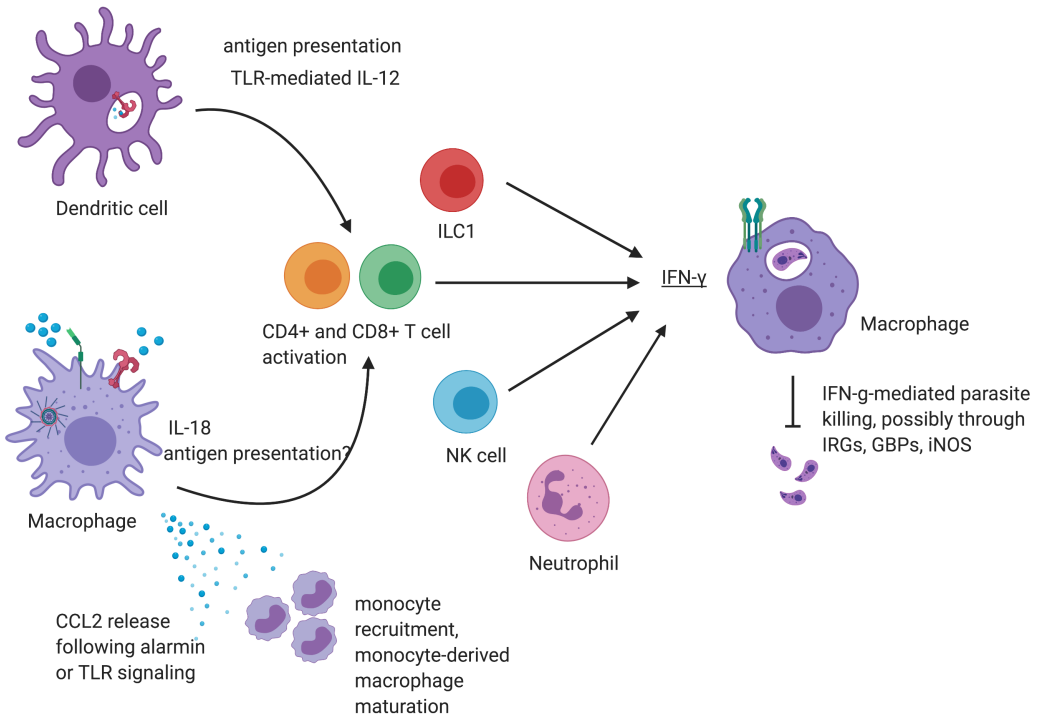

Fig 1.4. Acute infection - priming and control

Once *T. gondii* infects host tissue, dendritic cells and macrophages can sense the presence of *T. gondii* via TLRs, NLRs, and alarmin receptors. Recognition of *T. gondii* then leads to IL-12 and IL-18 production, and possibly to upregulation of antigen presentation machinery, which can all educate other cells to start producing IFN-γ, the critical mediator in controlling *T. gondii.* IFN-γ can then act on many cells to clear intracellular parasite, aids in chemokine production, and adhesion factor expression.

Mounting an immune response to T. gondii in the brain parenchyma

1.3.1 *The brain as an "immune privileged" site*

The brain is an immunologically-unique organ. Unlike other tissues, the brain parenchyma is devoid of circulating immune cells in the steady state, and has long been thought to be incapable of mounting immune responses, largely because animal transplant studies in the early 20th century showed that heterologous tissue grafted into the brain was not rejected[346,347]. This gave rise to the label that the brain is "immune privileged." Later, the brain was found to be capable of mounting an immune response following peripheral immunization, but occurs more slowly than in other tissues[348].

There are several unique challenges to mounting an immune response in brain tissue. A major structural challenge is the blood brain barrier (BBB)[349] - cells circulating in the blood must traverse the blood vessel endothelial layer, the basement membrane, and the glia limitans before entering the brain parenchyma[350]. Immune cells could also enter from the CSF, which would require them to cross the innermost layer of meninges, the pia mater[350]. Another challenge to mounting an immune response in the brain is low MHC expression which is important for antigen presentation. Although MHCI plays a role in synaptic plasticity during development[351], MHC expression in the adult unperturbed brain is difficult to detect. The brain also lacks resident migratory dendritic cells[352-354]. These cells are known to collect antigen in other inflamed tissues and traffic to secondary

lymphoid structures[354]. Finally, until recently, the brain was thought to lack traditional lymphatic vessels. But in 2015, lymphatic structures were found to line the dural sinuses that are capable of draining CSF to the deep cervical lymph nodes[355]. Immune cells were found in these vessels, hinting they may have homeostatic roles in the adult CNS[355]. Indeed, there are described roles for T cells and the cytokines they produce in CNS homeostatic processes, including cognition[356], learning and memory[357], and social behavior[358] - even while T cells are restricted to the meninges in the physiological state. Since 2015, meningeal lymphatic vessels have been shown to be important in disease, including but not limited to experimental autoimmune encephalitis (EAE)[359], Alzheimer's models[360], and brain cancer[361]. In additional to dural lymphatic vessels, a glymphatic system has been described, which is a route of waste clearance, although it could also carry antigen, by which CSF/ interstitital fluid (ISF) washes the brain parenchyma[362]. At this time, it is unclear, anatomically, if the glymphatic and lymphatic systems interact, since it is not clear how CSF accesses lymphatic vessels in the dura[363]. Thus, how specific antigens are communicated to the periphery to maintain immune responses in the brain are still not fully understood.

Although immune cells are not present in the brain normally, they will enter the parenchyma in disease contexts, and their roles are often contentious in these settings. The environment into which a cell infiltrates, the stage of the immune response at which it does so, and how broad its mechanisms are, are all factors that complicate this picture, especially in a delicate, highly structured environment such as the brain with complicated circuitry. For instance, nitric oxide produced by macrophage responders to injury is considered neurotoxic, yet phagocytic clearance of debris mediated by these cells is beneficial[254]. In

contrast to sterile injury, immune responses to CNS infection can limit the spread of pathogen, but can also cause severe pathology and must be carefully regulated[364,365]. The existence of peripheral immune cells are also hallmarks of CNS autoimmune and neurodegenerative diseases, and regardless of their functions in these contexts, mechanisms of their recruitment is of interest.

1.3.2 *Factors required to surmount the BBB for immune cell entry*

The BBB is highly regulated to prevent molecules greater than approximately 500Da from entering the brain[127]. For an immune cell to cross the barrier, it requires coordinated expression of a number of factors – namely, chemokine, and adhesion molecules expressed on the vasculature as well as the immune cells themselves, all of which can be upregulated in a pathological situation, mainly through circulating cytokine. The two most common pathological environments where immune cell trafficking has been studied are EAE and infection[123,366]. In these contexts, the importance of individual chemokines and adhesion molecules has been tested.

In a rat model of EAE, injection of neutralizing antibodies to the integrins VLA-4 and LFA-1, expressed on immune cells, resulted in a buildup of cells within the CSF, suggesting decreased entry to brain tissue[367]. In the context of chronic *T. gondii* infection, blocking the same integrins led to a halving of infiltrating leukocytes into the brain[223]. Blockade of one integrin, VLA-4, also results in decreased immune cell recruitment in AIDS encephalitis and Borna virus encephalitis[123]. Interestingly, a drug was developed in humans, named natalizumab, that blocks VLA-4 interactions for treatment of multiple sclerosis (MS), which resulted in reactivation of latent infections in the brains of patients,

presumable due to a lack of leukocyte trafficking[123]. Blockade of vasculature-expressing adhesion factors individually also leads to reduced adherence or recruitment of immune cells to the brain in pathological settings, including individual blockade of P selectin[368-370], and VCAM1, although blockade of VCAM1 on its own seems to mostly contribute to reduced leukocyte adhesion rather than extravasation[371-373]. All of these steps may be important, as leukocyte transmigration is thought to happen following rolling, and adhesion to the vasculature before transmigration[123].

Chemokine is another major arm of immune cell recruitment to the brain. Because chemokine receptors are more restricted by cell type than integrins, chemokine may be the major determinant in cell-type specific recruitment to brain tissue. Although many studies have focused on the role of chemokine receptors in tissue infiltration, many of these receptors, such as CCR2, are necessary for immune cell entry to the blood, and therefore can have confounding effects[374]. Thus, studying the chemokines themselves may shed more light on their importance. For all their importance, it is still not fully understood how chemokines in the brain work to recruit cells – whether they work primarily as stop signals, whether they increase speed of leukocytes to have a higher chance of encountering antigen, if they directly impact the permissivity of the BBB, or all of the above[374-376]. Furthermore, we lack spatial resolution on the importance of chemokine at the BBB, it is unclear of chemokine crosses the BBB, resulting in a gradient within the blood, or if it is strictly important within the tissue or for bringing cells into the parenchyma from perivascular spaces[377,378]. Blocking chemokine signaling generally with injection of pertussis toxin results in decreased immune cell recruitment during EAE and CNS infection[367,375,379]. Individual chemokines are also hugely important for the recruitment of specific cell types,

as shown with individual blockade of CXCL10, CXCL1, CCL2, and others[375,380-383]. Specific deletion of chemokines within the brain using brain-specific cre drivers like GFAP have shown that production of chemokines, such as CXCL10, and CCL2, from one cell type, is required to recruit adequate numbers of immune cells to the brain during EAE[384-386].

As with any studies claiming specificity to the brain, it is important to note than in many instances, blockade of BBB factors has been administered peripherally, and therefore may have impacts elsewhere in the body. Additionally, length of blockade and the time at which the blockade is administered should be noted. Nevertheless, it appears that individual adhesion factors/integrins and individual chemokine signaling are all strikingly necessary to bring immune cells into the brain on their own. These data support a model in which there are layers of regulation on bringing immune cells into the parenchyma, and every step is required.

Aside from chemokine and adhesion factor expression, it is unclear to what degree antigen presentation is involved in T cell activation for entry, or maintenance within CNS compartments[123]. In LCMV, i.v. blockade of MHCI resulted in reduced T cell infiltration and increased velocity of T cells in the subarachnoid space, presumably due to fewer antigen presentation contacts[387]. Generally, T cells purified from the brain are highly activated, suggesting antigen presentation as a requirement for their entry or maintenance. Additionally, aside from active immune cell recruitment, it is often unclear to what degree the blood brain barrier has increased permissivity in pathological settings, due to a loosening of tight junctions from matrix metalloproteinases, chemokine or cytokine expression, etc[388,389]. For instance, as mentioned earlier, during *T. gondii* infection,

peripherally-administered antibodies can enter the brain, which are large molecules with unregulated entry[67,131,132].

Regardless of how immune cells enter the brain*, where* immune cells enter the parenchyma, and whether there is a primary route of entry is poorly understood and hotly debated[350]. Three main routes have been hypothesized, entry through the meninges, the choroid plexus, or across blood vessels within the brain parenchyma[350,390]. To answer this question requires live imaging of an immune cell crossing into the parenchyma. It has thus far been impossible to get the spatial and temporal resolution necessary, including labeling of parenchyma, basement membranes/pia, and leptominges/blood, simultaneously, to visualize the trafficking event[367].

1.3.3 *T. gondii infection as a model of immune cell recruitment to the brain*

Many factors required to surmount the BBB are massively upregulated during chronic *T. gondii* infection, including chemokine and adhesion factors. Some chemokines, such as *ccl2* and *cxcl10,* are upregulated several hundred-fold, others, such as *cxcl9*, over-one thousand-fold[101,375,391]. Most chemokines during chronic *T. gondii* infection are expressed by astrocytes, myeloid cells, or the vasculature[391]. Vascular adhesion factors are also increased during chronic infection, although they are generally expressed at baseline[162,392,393]. All of these components are upregulated by circulating IFN-γ[391,392,394]. It follows that many immune cells are recruited to the brain during chronic *T. gondii* infection and which are required to control infection. This concept is further underscored by reactivation of *T. gondii* in immunosuppressed patients, as discussed earlier. Peripheral

infiltration in mouse brains is obvious by hematoxylin and eosin stain – infected brains feature meningitis (Fig. 1.5D), perivascular cuffing (Fig. 1.5E), and the formation of inflammatory foci (Fig. 1.5F). Indeed, a substantial increase in immune cells can be isolated from the infected mouse brain compared with naïve controls - boosting from approximately 300,000 purified cells, primarily microglia, to over one million[162,395]. Inflammatory foci containing monocytes, macrophages, and T cells[101]. Functionally, these are the major cell types required for control of chronic *T. gondii* infection, which will be discussed in the next section, and are also the most highly recruited[124]. Approximately 50-60% of recruited immune cells to the brain are effector T cells, which begin entering the brain approximately two-weeks post infection[101,396]. Regulatory T cells constitute approximately 6% of immune cells purified from the brain, are restricted to the meninges and perivascular spaces, and enter later, approximately 3-weeks post infection[396]. Approximately 10% of cells purified from the brain are other lymphocytes, likely mostly B cells, approximately 8%, and a very low number of NK cells[101,397]. Myeloid cells present in the brain are largely made up of monocytes, and monocyte-derived macrophages and dendritic cells[124,132,162], with a low percentage of neutrophils and a low level of neutrophil chemokine induced[101,132]. A lack of recruited neutrophils may be adaptive by the host, since they predominantly cause immunopathology during acute infection[345]. The predominance of other immune populations, such as ILCs, during chronic *T. gondii* infection is unclear. Ttype 2 innate lymphoid cells (ILC2s), which have been detected in the meninges in the homeostatic state[398], diminish in number with onset of chronic *T. gondii* infection (Carleigh O'Brien, unpublished).

Interestingly, immune cell presence in the brain during chronic *T. gondii* infection does not significantly subside with time[399]. Although it is possible that recruited cells are long-lived, there are several lines of evidence to suggest that recruitment is continual, including trafficking of i.v.-injected cells, and continual expression of markers of newly recruited cells, including the monocyte marker CCR2[101,132,313,375,395].

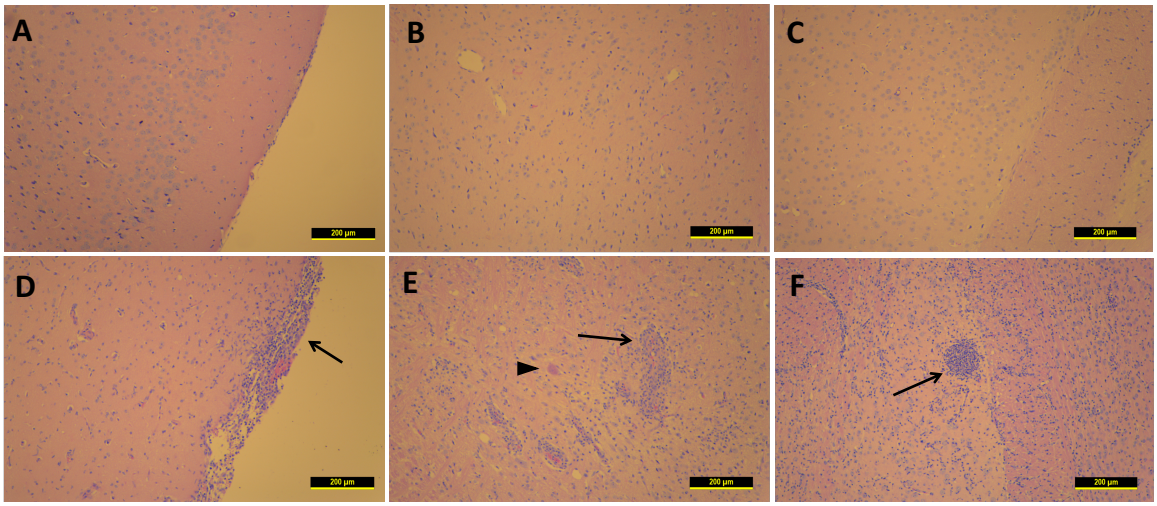

Fig 1.5. *T. gondii* infection features peripheral immune cell recruitment to the brain

Tissue sections from wildtype C57BL/6 uninfected (A-C) and 4wk-infected mice (D-F) were stained with hematoxylin and eosin. Infected mice exhibit meningitis (D), perivascular cuffing (E), and inflammatory foci within the brain parenchyma (F), all of which are not present in naïve controls (A-C). Cells do not cluster around cysts (E, arrowhead), and are instead likely responding to free parasite.

1.3.4 *Protective immunity during chronic brain infection*

Significantly less is known about control of *T. gondii* brain infection compared with acute infection. This could be due, in part, to the fact that whole-body deficiency in some major immune effectors are lethal acutely, and would require conditional deletion from brain resident cell types, or temporal blockade of these factors. Initiation of an immune response to *T. gondii* when it reaches the brain is not well understood, or if "initiation" is

the right term, considering immune cells will be primed during peripheral infection. IL-12 is required for survival of chronic infection, suggesting an importance of pattern recognition, although these mice do not succumb immediately during chronic infection[304]. The role individual TLRs, NLRs, and other pattern recognition receptors play, specifically in the CNS, has not been tackled. Much like acute infection, IFN-γ and simultaneous depletion of CD4+ and CD8+ T cells are critical for control of chronic infection[67] (Fig 1.6). In contrast to acute infection, there do not seem to be other major sources of IFN-γ in the brain beyond CD4 and CD8 T cells, such as NK cells[101]. Depletion of CD8+ T cells alone had a more profound effect on survival than CD4+ T cells alone, suggesting that CD8+ T cells have additional functions to CD4+ T cells[67]. Indeed, CD8+ T cells have the unique ability to make perforin, which is required for survival during chronic infection, since knockouts succumb close to eight weeks post infection with significantly increased parasite burden[400]. In addition, transferred perforin-deficient CD8+ T cells were not able to restrict cysts in infected mice otherwise deficient for T cells, while CD8+ T cells sufficient for perforin, but lacking IFN-γ, were able to restrict parasite growth[401]. Although CD8+ T cells seem to be more capable of impacting parasite burden, recent studies suggest that they rely on CD4 help for proper functioning during chronic infection[402]. Both CD4+ and CD8+ T cell CD40-CD40L interactions with macrophages are important for control of chronic infection, where CD40L-deficient mice succumb during early chronic infection[317]. B cells are also important for survival in the chronic phase (Fig 1.6); υMT mice succumb at four weeks post infection, displaying higher cyst and tachyzoite burdens, suggesting that these cells may be important earlier, in the transition to chronic infection[403]. Exact B cell mechanisms of *T. gondii* control are poorly described *in vivo*, but antibodies they secrete

could be important for opsinization of parasites for phagocytosis or for activating the complement cascade. Interestingly, CD4+ T cells likely play a supportive role to B cells, since increased susceptibility in CD4+ T cell deficient mice can be correlated with antibody[404]. NK cells are not present in the brain during chronic infections in large numbers and do not appear to make much IFN-γ like they do in the earliest stage of infection[101]. In fact, NK cells may be detrimental, by reducing CD8+ T cell functionality, and depletion of NK cells during chronic infection is protective[405].

Several cell types that can respond to IFN-γ have been implicated in control of *T. gondii* brain infection, including cells which are brain resident. For one, our lab has found that resident macrophages, likely including both microglia and perivascular macrophages, are required for control of infection (Maureen Cowan, unpublished). Specifically, when these cells lack IFN-γ-driven STAT1 responses, mice succumb to infection by day 17 (Maureen Cowan, unpublished). STAT1-deficient astrocytes are also susceptible during early chronic infection, exhibiting a lack of parasite control by approximately double[406]. In both of these instances, microglia and astrocytes lacking STAT1 were found to harbor parasites, a rare if not impossible event to catch in wildtype infected mice[406]. These results suggest, as would be expected, that IFN-γ is likely required for infected cells in the brain to clear parasite. The machinery brain resident cells possess to do so, is unclear. Additionally, it is entirely unknown how brain resident cells respond to signals beyond IFN-γ during chronic infection, including PAMPs and DAMPs. One study from our lab has shown that microglia are a major source of IL-1α, and selectively release it, which is required for control of parasite burden[223]. Aside from microglia and astrocytes, the

importance and capability of other brain resident cells including endothelial cells, to clear parasite, has not been studied. Neurons are of particular interest, since they are most commonly observed to harbor cysts[92].

Infiltrating myeloid subsets, which respond to IFN-γ peripherally, can theoretically do the same during chronic infection[81]. As mentioned earlier, infiltrating myeloid subsets in the brain during chronic *T. gondii* infection are mostly monocyte-derived. Monocytes have been shown to be critical for control of chronic infection, as administration of a depleting CCR2 antibody at the start of chronic infection results in immediate mortality[132]. Work from our lab has shown that monocytes and monocyte derived cells make the overwhelming majority of inducible nitric oxide synthase (iNOS) compared with microglia and other cell types[101]. Separating contributions of infiltrating myeloid cells from microglia is quite difficult, since these cells express many similar markers, and unique gene expression of microglia has just begun to be explored. Elegant studies require labeling of microglia prior to infection, allowing time for peripherally-labeled cells to turn over[101]. Aside from iNOS, many pro-inflammatory capabilities that microglia should have, as macrophages, are downregulated during infection, including chemokine, for reasons we do not yet understand[223]. iNOS is one effector mechanism, likely of many, that infiltrating myeloid cells employ to control infection, which interestingly, is selectively required for survival during chronic infection[322,331] (Fig 1.6). During chronic infection, iNOS is expressed in foci within brain tissue, and is not present in the blood or elsewhere, suggesting that macrophages obtain the ability to express iNOS locally[101,331]. It is most likely a good thing that nitric oxide production is spatially restricted during chronic infection, as it could have toxic effects if widespread. Aside from iNOS, infiltrating

myeloid cells can express innate cytokines like IL-6 and TNFα, which are both required for survival during chronic infection. IL-6 deficient mice succumb by day 21 post infection, while TNFα−deficient mice succumb at day 28 post infection[407,408]. Other mechanisms that myeloid cells employ to control *T. gondii* infection acutely have not been studied during chronic infection, including IRG and GBP activity.

While we understand that some cell types and their effector functions are required for control of chronic *T. gondii* infection, the importance of many other cell types, their localization, and the delineation of their effector functions remain to be explored. For instance, it is unclear why some immune cell types exist predominantly in meningeal and perivascular spaces, like regulatory T cells, while others readily infiltrate the parenchyma. Furthermore, we understand little about how immune cells are primed, or re-primed, within brain tissue, if at all. For instance – practically all T cells purified from brain tissue during chronic infection are highly activated, leaving little room for activation of naïve T cells within the brain. Whether or not these cells require a second hit after initial activation in the periphery is not well understood. Availability of antigen over time, and the importance of antigen presentation, are unclear. It is not clear exactly how, acutely or chronically, macrophages and dendritic cells sample antigen[18]. Although T cells are always present during chronic *T. gondii* infection, to what degree this is a result of longevity, local proliferation, or continual recruitment, is not understood. The concept of parasite reactivation, providing theoretically constant antigen access, discourages the idea of prominent memory T cell populations in the brain, and there has not been definitive identification of a memory T cell during chronic *T. gondii* infection. Transferring and fate-mapping T cells may be necessary to better understand their turnover and the signals they

encounter in the brain. Understanding how myeloid cells acquire anti-parasitic characteristics in brain tissue is also somewhat of a mystery. *In vitro* studies suggest that IFN-γ is one signal required for macrophage-expression of iNOS[81], but there is no evidence that IFN-γ expression is restricted spatially, although iNOS expression is[101,331]. Detecting IFN-γ *in vivo*is difficult, due to a lack of tools, such as reporters with temporal resolution. These and other open questions concerning control of chronic *T. gondii* infection will be explored at length in future sections.

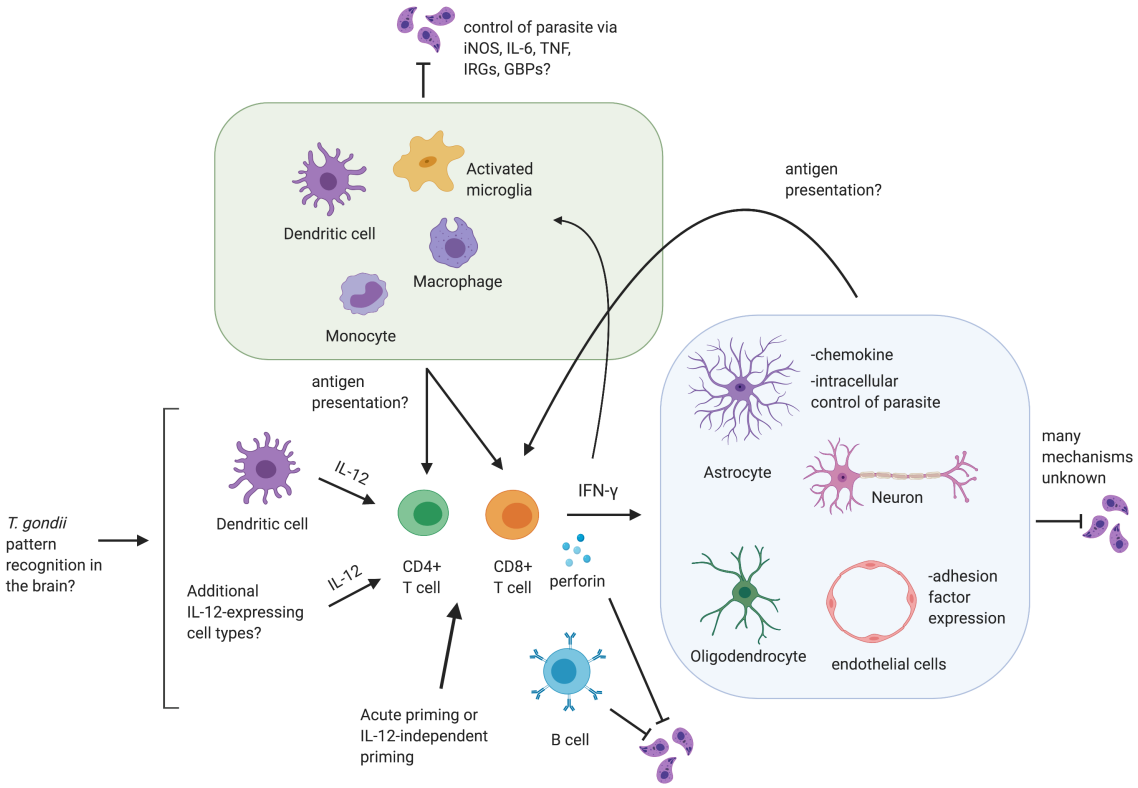

Fig 1.6. Control of chronic *T. gondii* infection

There are many unknowns about how *T. gondii* is controlled in brain tissue. Unlike during acute infection, it is largely unclear how and if *T. gondii* is sensed in brain tissue – whether predominantly by TLRs, NLRs, or alarmin receptors, and it is unclear if brain resident cells possess much of this machinery to sense *T. gondii*. IL-12, however, is produced and is important for controlling infection long-term, suggesting that pattern recognition does occur. Monocyte-derived myeloid cells, B cells, and T cells infiltrate the brain from the blood in large numbers, and all three have been shown to be required for mice to survive chronic infection. In particular, iNOS production, IFN-γ, and antibody responses are required. To what degree brain resident cells can clear parasite and contribute to immunity is understudied - and our lab is very interested in these questions.

IL-33: a damage associated molecular pattern highly expressed in the CNS

1.4.1 *Discovery of IL-33, IL-33 expression, and nuclear function*

Lagging behind the discovery of TLR and IL-1R family ligands, IL-33 was not discovered until 2005[230]. IL-33's receptor, ST2, or IL-1-receptor-like-1 (IL1RL1), was discovered prior, in 1989, and was called an "orphan receptor" for sixteen years until IL-33 was discovered[230,275,409]. IL-33 was discovered by searching human and mouse genome databases for sequences similar to other IL-1 family members. Next, IL-33 was co-precipitated with ST2 and was shown to induce NF-κB reporter expression in ST2-positive cells[230].

IL-33 is expressed throughout the body by a variety of cell types. Highest levels were first noted in mice in the spinal cord, brain, and barrier tissues, such as gut, lung and skin[230], and at lower levels in the kidney, heart, and lymphoid tissues[230]. IL-33 has since been shown to also be expressed in mouse female reproductive tissue, including the vagina, uterus, and ovaries[249,410-412], and in adipose tissue[249]. Human IL-33 expression follows a similar trend, expressed highly in the lung, gut, skin, and female reproductive tissues, as well as detectable levels of IL-33 in the brain, kidney, pancreas, lymphoid tissues, and muscle tissue according to the Human Protein Atlas[413,414]. By cell type, IL-33 is expressed in most tissues by non-hematopoeitic cells, mostly endothelial cells, such as in the endometrium, the kidney, and female reproductive organs, in epithelial cells in barrier tissues, and stromal cells, such as fibroblasts, in the endometrium, bone marrow, and lymph nodes[249,274,275,415]. Myeloid cells have also been shown to express low levels of IL-33,

mostly in the context of allergic inflammation[230,249]. IL-33 expression in brain tissue tends to diverge from elsewhere in the body, in that it is expressed by glia – namely, oligodendrocytes and astrocytes in the developed brain[250,254]. Interestingly, the source of IL-33 expression tends to change with development in mice, one study showing that astrocytes are the major producers of IL-33 early in life, but that this starts to shift after P15 towards oligodendrocytes[250]. For some reason, it appears that mature oligodendrocytes are the major expressers of IL-33 past this timepoint, with IL-33 positivity being most dense in myelinated regions[101,254], although astrocytes, particularly gray matter astrocytes, express IL-33 and this expression can vary by brain region[101,254]. A very recent report has also suggested IL-33 expression in the hippocampus by neurons[250]. The role of neuronal IL-33 expression is still unclear, and inconsistencies between reports may be due to technical differences. IL-33 expression by neurons has been found using an IL-33 reporter, while other reports have used IL-33 antibody in brain tissue[101,250,271]. It is important to note that these reports are also limited to the brain parenchyma. Anecdotally, I have observed IL-33 expression in the meninges that come attached to the brain by immunohistochemistry. Although I have not pursued the identity of these cells, they may be a more traditional IL-33-positive cell type, including vasculature or stromal cells, and could reside preferentially within the dura or the subarachnoid layers. In addition to brain tissue, IL-33 is also expressed by a glial cell type in the retina – by Müller cells, and signals in an autocrine fashion[416].

In most cell types, IL-33 is expressed constitutively. This, along with the fact that IL-33 does not require processing to be active, and does not seem to have a signal peptide for release, gave rise to the concept that IL-33 acts as an alarmin[417,418]. At baseline, IL-33

is expressed in the nucleus but its impact on gene expression, if any, is not well understood. IL-33 has been shown to associate with both heterochromatin and euchromatin[241,242] via protein-protein interactions, but some studies have reported no impact of IL-33 on global gene expression[243]. There is some evidence that IL-33 expression in the nucleus serves to antagonize its release extracellularly, since disrupting the nuclear localization peptide results increased plasma IL-33 levels and IL-33-mediated amplification of immune responses[243,244].

Despite being constitutively expressed at high levels in the nucleus, IL-33 expression is often increased during inflammation, although it is sometimes unclear if this is on a per-cell basis, or if new cells are recruited, or have proliferated, that express IL-33. Increases in IL-33 mRNA expression have been observed during asthma, allergy, and helminth infection in mice, and during CNS disease, although fold-increases in IL-33 expression range from approximately 1.5 to 5-fold, and are thus considerably more modest than upregulation of other inflammatory mediators, such as IL-1β[274]. How IL-33 expression in the nucleus is regulated, it still unclear[249].

1.4.2 *IL-33 release and extracellular signaling*

All of the conditions under which IL-33 is released, whether or not release requires cell death, and the type of cell death, are not fully understood. Traditionally, IL-33 is hypothesized to be released upon passive necrotic cell death, partially because IL-33 lacks a signal peptide that would allow it to be secreted traditionally[249]. Additionally. IL-33 can be cleaved into less active forms by caspases involved in apoptotic and pyroptotic cell death, and does not require processing to be fully active[419] (Fig 1.7). Induction of necrosis

in cell culture has resulted in IL-33 release[419,420], and IL-33 was detected in the CSF following necrotic spinal cord injury[271]. These findings do not rule out necroptosis, and increased extracellular IL-33 has been noted in an inflammatory paradigm driven by necroptosis in Ripk1-deficient mice, but was absent in RIPK1$^{-/-}$RIPK3$^{-/-}$ mice unable to undergo necroptosis[421]. Although the overwhelming majority of studies implicate IL-33 release from dying or damaged cells, this is difficult to prove *in vivo*, and evidence is mounting that suggests that IL-33 can be released in the absence of cell death. *In vitro*, IL-33 levels spiked in culture supernatant in response to the airborne allergen *Alterneria*. Cells in this model did not exhibit changes in membrane permeability measured by LDH activity and EthD-1 staining[234]. Similarly, mechanical cyclic strain in cultured cells resulted in IL-33 release from murine fibroblasts without cell loss[242]. The same has been demonstrated *in vivo*by inducing pressure overload in myocardial tissue and monitoring a loss of IL-33 detection in intact cardiomyocytes[242,422]. Additionally, Müller cells undergoing phototoxic stress in culture have also exhibited IL-33 release without cell loss[416]. More recent work has implicated an importance for IL-33 in situations where necrotic or necroptotic cell death are not thought to occur, including during brain development and remodeling of adipose tissue[249,250].

Once IL-33 is released into the extracellular space, it can be regulated in a number of ways. IL-33 is made up of three domains – the nuclear binding domain, the central domain, and the IL-1-like cytokine domain[274]. The central domain can be cleaved by mast cell proteases, including chymase and tryptase, and neutrophil-derived proteases, including elastase and cathepsin G, to an approximately 30-fold more biologically active form[423,424]. IL-33 must be carefully regulated to avoid systemic inflammatory effects. It can be

inactivated by caspases 3 and 7 in apoptotic environments[420], and by caspase 1[419] (Fig 1.7). It has been recently shown that the biological activity of IL-33 is rapidly terminated in the extracellular environment by the formation of two disulphide bridges, resulting in a conformational change that disrupts receptor binding. This finding has aided in differentiating reduced (active) and oxidized (inactive forms) of IL-33, and has been studied in lung gavage of asthma patients[425] (Fig 1.7).

Once IL-33 binds its cognate receptor ST2, IL-1R accessory protein (IL1RAcP) is recruited and is required for signaling[274]. Crystal structure comparison of the IL-33-ST2 complex has revealed that IL-33 serves to stabilize ST2, which directly interacts with IL1RAcP[426,427]. This is in direct contrast to IL-1 signaling, where the cytokine itself is the main driver of interaction with IL1RAcP[426,427]. These differences may explain how IL1RAcP, which is shared between the two signaling complexes, recognizes protein domains that are unique to the different signaling complexes. After binding to ST2, a signaling cascade is initiated, working through the adaptor molecules MyD88, IRAK1/4 and TRAF6 to initiate NF-kB and AP-1-mediated gene expression[274,275,415] (Fig 1.7). ST2 is expressed on many immune cells, including but not limited to mast cells, basophils, ILC2s, Th2 T cells, eosinophils, other myeloid cells, regulatory T cells, NK cells, and CD8+ T cells. ST2 is also expressed by specialized non-hematopoeitic cells in the eye, heart, and brain, including Müller cells, cardiomyocytes, astrocytes, endothelial cells, and microglia[274]. ST2 expression, and the impact of IL-33-ST2 signaling depend heavily on the context of disease and factors present in the environmental milieu, but IL-33 signaling generally leads to amplification of immune responses, via enhanced survival of immune

cells, proliferation, and pro-inflammatory cytokine production, including chemokine expression[274,275,415] (Fig 1.7).

There are several layers of regulation on IL-33-ST2 signaling. A soluble splice variant of transmembrane ST2, sST2, acts as a decoy receptor that circulates in the blood[428], blocks IL-33 signaling[429], and is serum a biomarker for several diseases, including heart disease, Alzheimer's disease, asthma, and others[430-433] (Fig 1.7). Little is known about soluble ST2 regulation, besides that it seems positively correlated with inflammation, and that NF-kB drivers like LPS and lysophasphatidic acid can upregulate its expression in ST2 expressing cells, such as mast cells and cardiomyocytes[434,435]. It makes good sense that with inflammation, regulatory factors like soluble ST2 also increase. The ST2 dimerization process itself can also be inhibited by a single immunoglobulin domain IL-1R-related molecule (SIGIRR, also known as TIR8), which also antagonizes IL-1R signaling and TLR signaling, but little is known about how it does so, and it is still considered an orphan receptor[436,437] (Fig 1.7). IL-33 and ST2 can also be internalized following ubiquitination and phosphorylation of ST2[438] (Fig 1.7). Finally, helminths can secrete products, such as microRNAs, which divert IL-33 signaling[439].

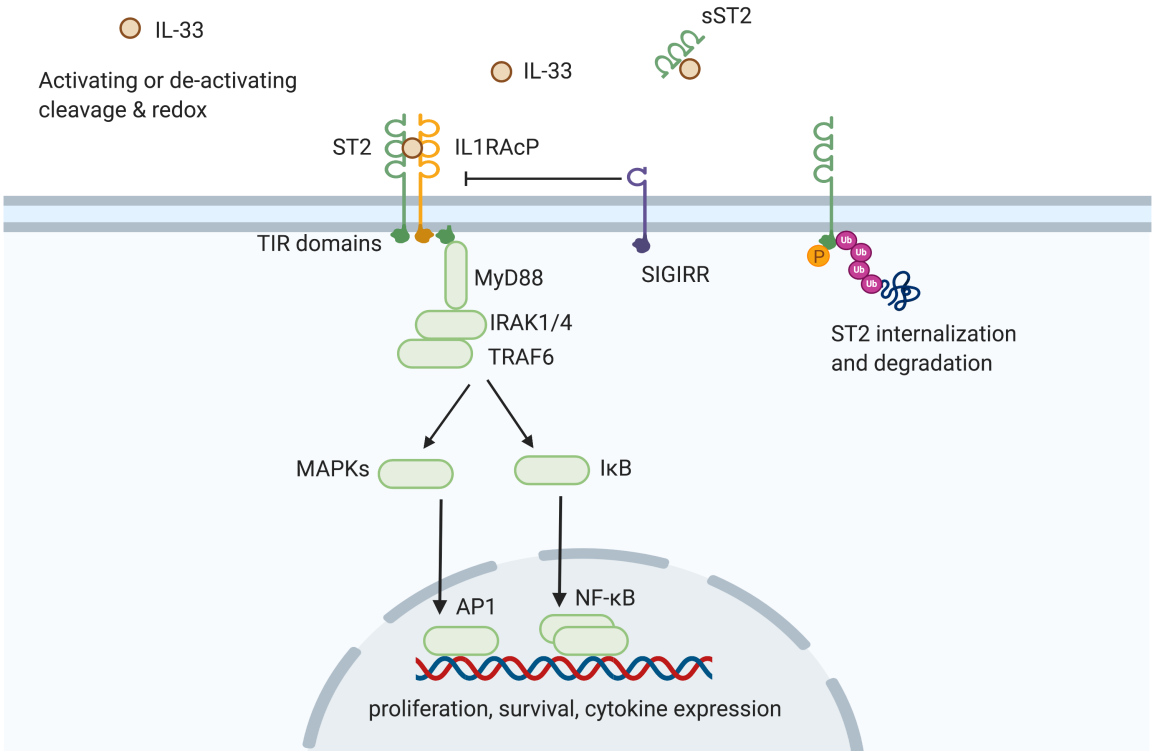

Fig 1.7. IL-33-ST2 signaling

Once IL-33 is released extracellularly, it can undergo a number of fates. First, IL-33 can be activated, via cleavage by mast cell and neutrophil proteases, or it can be deactivated, after cleavage from caspases, becoming reduced in the extracellular environment, or being sopped up by its soluble decoy receptor, ST2. Full length IL-33 that has not been cleaved is fully active, and can bind to its cognate receptor ST2 which dimerizes with IL1RAcP. Il-33 then initiates MyD88/NF-kB signaling to promote immune cell recruitment, activation and survival to fit the environmental milieu.

1.4.3 *Roles for IL-33 in homeostasis*

Although the majority of described roles for IL-33 are inflammatory, IL-33 can also promote homeostatic remodeling. IL-33-ST2 signaling is thought to play an important role in maintenance of resident immune cell populations in white adipose tissue, including ILC2s, alternatively activated macrophages, and regulatory T cells, which limit infiltration of more inflammatory immune subtypes[440-442]. Thus, loss of IL-33-ST2 signaling worsens obesity and insulin sensitivity[443]. IL-33 has also been shown to influence conversion of

white adipose tissue to brown and beige adipose tissue, which serve as heat and energy sources, also through ILC2-derived cytokine remodeling[444,445]. IL-33 plays similar roles in muscle tissue, promoting muscle regeneration following acute injury by expanding local regulatory T cells[446].

IL-33 signaling also contributes to homeostasis in female reproductive tissues. IL-33 is expressed in uterine endometrial cells[410], increases with pregnancy, can stimulate ILC2 populations and has been linked with formation of the placenta[410,447,448]. On the flip side, IL-33 has also been implicated in mediating endometriosis[449]. IL-33 is also thought to be involved in routine breakdown of ovarian follicles, which is required for proper ovulation, and IL-33-deficient mice tend to have reproductive issues, including decreased reproductive time windows[412,450].

There is also a report of IL-33 relevance during brain development, in which IL-33-deficient mice exhibit an excess of synapses and dysregulated thalamic function[250]. IL-33 is thought in this instance to signal, at least in part on mature macrophages and microglia to induce synapse engulfment[250]. In a follow-up study this year, the same group showed that IL-33 can be expressed not only by astrocytes and oligodendrocytes, but by neurons in the hippocampus. With further work, the story evolved, showing that ST2 on microglia may be important for gobbling up extracellular matrix that allows for synaptic plasticity[451]. In this follow-up study, prevention of IL-33 signaling led to fewer dendritic spines in hippocampal neurons. These developments expand our understanding of IL-33 signaling, and the authors posit that either nuclear or extracellular IL-33 signaling impacts development and cognitive function. At this time, it is unclear how IL-33 is released in the absence of disease, but like other roles for IL-33 in homeostasis, it is possible it could be

secreted or that there is ongoing, unappreciated inflammatory cell death in the brain at baseline, as has been shown this year by the Lukens lab at UVA[452].

Very recently, there are described roles for IL-33 in development of some immune cell populations. Erythrocyte-derived IL-33, in conjunction with heme, can signal to encourage the development of monocytes into red pulp macrophages the spleen. Red pulp macrophages are a small, specific population of cells that then maintain erythrocytes and iron levels. Without IL-33 signaling, red pulp macrophage frequencies dropped in the spleen, and iron increased, which got worse with age[453]. IL-33 may also be expressed by B cells during their development, and seems to hinder their ability to proliferate, thought to be mediated by IL-33 nuclear expression and not extracellular signaling, since B cells did not express ST2[454]. Finally, IL-33 causes ILC2, eosinophil, basophil, and mast cell accumulation in the lungs shortly after birth, thought to contribute to allergic responses early in life[455].

In many of these cases, IL-33 is proposed to act extracellularly on immune cells to promote homeostasis. How IL-33 is released in these instances, is not understood, and may be due to currently undescribed metabolic, hormonal, or mechanical stimuli present during tissue turnover, and may be dependent on cell death, or secretion through a non-canonical pathway[249].

1.4.4 *Roles for IL-33 in peripheral disease*

Infection, asthma, and allergy

In the case of infection, IL-33 has been shown to be beneficial in clearing both intracellular and extracellular pathogens. Some of the earliest studies have focused on IL-

33-mediated clearance of extracellular helminths, or worms. During helminth infection, the pathogen is thought to disrupt barrier tissues, in the lung and gut, leading to IL-33 release from epithelial cells, endothelial cells, or fibroblasts. Then, IL-33 works by signaling on a number of ST2-positive cells, such as ILC2s, mast cells, and basophils[456-459]. ILC2s can produce IL-5, an eosinophil attractant, and IL-13, which stimulates mucus-producing goblet cells in the lung or gut to regenerate and turnover, thus expelling extracellular worms[460]. Eosinophils can then produce innate cytokines like IL-6, IL-12, and TNF-α to activate dendritic cells, can release cytotoxic granules, or can present antigen to CD4+ T cells, resulting in an amplification of type 2 cytokines like IL-4, as well as directly producing IL-4 capable of skewing nearby macrophages[461]. IL-33 signaling on ST2-expressing mast cells can result in mast cell release of prostaglandin D2, which acts as a bioactive lipid to recruit more components of a type 2 response – Th2 cells, eosinophils, and basophils[462], as well as producing IL-4, IL-5, and IL-13, similarly to IL-33 signaling on basophils[463]. Interestingly, early studies on IL-33 during helminth infection led to the first description of ILC2s, first called "nuocytes[456]." Nuocytes were characterized as expanding in response to IL-33 and producing type 2 cytokines, yet did not belong to any major category of cell types[456].

IL-33 has also been studied in the context of intracellular infection, although literature in this area in peripheral tissues is significantly more scarce compared with the study of helminths. In some cases, such as the intracellular pathogens *Leishmania donovani,* IL-33 has been reported to be detrimental, by promoting a detrimental type 2 skew, while ST2-deficient mice produced higher levels of cytokines IFN-γ IL-12, and CCL2 necessary to control the pathogen[464]. These results are of interest because they are

in direct opposition to what I have observed and undermine a role for IL-33 in broadly potentiating immune responses to suit any environment. We and other have found that IL-33 is necessary during peripheral *T. gondii* infection for inducing IFN-γ production from ILC1s and Th1 cells[101] (unpublished collaborator data). IL-33 importance has also been studied in CNS protozoan infections, where it is largely protective, including in chronic *Toxoplasma gondii* infection, and cerebral malaria, among others, and will be covered in the next section.

IL-33's impact on fungal, bacterial, and viral infection, varies in beneficial and detrimental effects and is often pathogen and tissue-specific[274]. Inhibition of extracellular IL-33 signaling during respiratory syncytial virus infection limited type 2-driven immunopathology, maintaining IL-33's link with type 2 responses[465]. IL-33's connections with type 2 immunity is further strengthened by a study showing that IL-33 signaling is detrimental in clearing the bacteria *S. aureus*, by promoting a Th2 skew, showing that ST2-deficient animals produced more IFN-γ [466]. Recently, IL-33 has further been connected with accumulated ILC2s and type 2 immunity which is beneficial during *C. difficile* expansion[467].

However, *in vitro* infection of bone-marrow derived macrophages with herpes simplex virus demonstrated IL-33's ability to induce TNFα and IL-6 production[468]. There are a handful of other cases in which IL-33 signaling results in type 1-skewed pro-inflammatory cytokine production. Recently, in superinfections consisting of *S. aureus* along with influenza, IL-33 is protective in the lung by promoting type 1 immunity and IL-33 recruits neutrophils which help to clear *S. aureus*[469]. The effects of IL-33 on influenza itself is contested – since a common adjuvant used in influenza vaccines, HP-β-CD, has

been shown to be beneficial by liberating IL-33, while IL-33 has also been connected with airway immunopathology during influenza infection by dampening anti-viral responses and inducing NETosis, leading to granular release by neutrophils[470,471]. Finally, IL-33 has been shown *in vitro* and *in vivo* to signal on CD8+ T cells, which express ST2 transiently during lymphocytic choriomeningitis viral infection. ST2 expression was dependent on STAT4 and the Th1 transcription factor T-bet in culture[472]. *In vivo* readouts for this study were conducted in the spleen and other peripheral organs. These studies are the first to report IL-33 significance in a type 1 infectious context[472]. Still, it is unclear if cytotoxic CD8+ T cells can express ST2 long-term during infection.

In both asthma and allergy, IL-33 is chronically released and worsens disease. Much like during helminth infection, IL-33 can act on ILC2s and mast cells to skew type 2 cytokine production that exacerbates histamine release and mucous secretion. In this way, IL-33 creates a negative feedback loop of epithelial hypersensitivity[473,474].

In cardiovascular disease, serum soluble ST2 can be used as a clinical prognostic marker[432,433,475]. Endothelial cell secretion of IL-33 in the heart prevents cellular hypertrophy following induced myocardial pressure[242,422]. One study has linked IL-33 with skewing immune responses from type 1 to type 2 in high fat diet, which dampens atherosclerotic-associated inflammation[476]. These findings combined with *in vitro* adipocyte stimulation, and knowledge of IL-33's role in white adipose tissue, may ultimately connect IL-33 with prevention of obesity.

Rheumatoid arthritis is one of the few disease contexts that features IL-33 potentiation of type 1 responses. In early arthritis, IL-33 follows a typical pattern of type 2

skewing, acting on Th2 cells and ILC2s. But later arthritic responses involve mast cell production of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα[477].

IL-33 is also capable of promoting tissue repair following cardiac stress, infection, tissue transplants, and acute injury, by signaling on Tregs which make anti-inflammatory cytokines like IL-10 and TGF-β, and pro-repair factors like Amphiregulin, which acts as a growth factor[249,478]. These pathways can become detrimental when chronic, resulting in IL-33 mediated allergy and tissue scarring[249]. Timing may also be important, as exogenous IL-33 can exacerbate graft versus host disease when delivered post-tissue transplant, but facilitate tissue repair when delivered prior, due to an expansion of Tregs[479].

IL-33 also has roles in cancer, which are varied depending on which flavor of immune response IL-33 induces - some studies argue that IL-33 supports tumor growth, while others maintain that IL-33 boosts anti-tumor immunity[274]. Since IL-33 has more-so been labeled as tumorigenic, IL-33 is sometimes considered a biomarker for certain cancers[480]. Some studies have found that IL-33 can act on cancer cells themselves, like tissue epithelial or endothelial cells, to directly induce proliferative programs or growth factor signaling[481-483]. Some of these findings are difficult to interpret, however, as they have been performed *in vitro* with exogenous addition of IL-33[480]. There is also some evidence that IL-33 promotes angiogenesis that can support local tumor growth[483,484]. Like in other disease states, IL-33 can also induce chemokine expression to recruit a whole host of immune cells, like macrophages, which may become alternatively activated either in direct response to IL-33 or from the tumor microenvironment, and thus are permissive to tumor growth by producing their own growth factors and suppressive cytokines like IL-10[485-487]. IL-33 can also directly signal on regulatory T cells which have an

immunosuppressive, and therefore tumorigenic, effect[488]. Alternatively, in both IL-33 injection and receptor knockout studies, IL-33 has been shown to potentiate IFN-γ production from NK cells and T cells, which has been connected with halting tumor growth[489,490]. It is unclear in many instances if IL-33 could be acting directly on IFN-γ-expressing cells, or if IL-33's role in pro-inflammatory cytokine production is indirect. Some studies support an indirect model, where IL-33 promotes antigen presentation, including increased MHC expression and promotion of cross-presentation by dendritic cells[491,492].

Other major areas of IL-33 study include inflammatory bowel disease and sepsis. IL-33-knockout mice are very susceptible to colitis, thought to be important for anti-inflammatory macrophage polarization and Treg expansion[493-496]. However, as always, some studies have reported the opposite, that IL-33 can be detrimental during acute stages by initiating neutrophil influx[497,498]. In sepsis, IL-33's role is also muddy, having been studied in several sepsis models, including experimental endotoxemia and infection-induced sepsis[499]. For the most part, IL-33 has been deemed detrimental, by amplifying immune responses that contribute to sepsis, including pro-inflammatory macrophage activation following LPS[500-502] and vascular activation for immune cell recruitment in general[503]. However, in some instances, in particular for abdominal sepsis, IL-33 can promote neutrophil recruitment which helps clear pathogen, and by unknown mechanisms can prevent organ damage[504-506].

It is clear from IL-33's study in peripheral tissues that its roles have been expanding for some time. Although the overwhelming majority of cases still argue for type 2 signaling, type 1 roles for IL-33 are emerging. A recent review on the roles of IL-33 in T

cell function and homeostasis argues that IL-33 will eventually be found to potentiate any T cell program[507]. It argues that the environment into which IL-33 is released governs response skew. This commentary is reminiscent of other IL-1 family members that were first studied in type 2 immunity contexts.

1.4.5 *Roles for IL-33 in CNS disease*

In just the past 5 years, publications covering IL-33 in the CNS have skyrocketed. Two important early IL-33 CNS studies identified a role for IL-33 in spinal cord contusion[271,508]. Both reports agreed that IL-33 facilitated recovery via "alternatively activated" macrophage recruitment[271,508]. Gadani *et al* found that IL-33 peaked in the CSF one day post injury[271,509], and acted on a CD11b-negative glia population to induce expression of the monocyte chemoattractant, *ccl2*. Recently, the same group has shown that the newly described ST2-expressing cell type, ILC2s, are present in the brain meninges and are responsive to intraperitoneal IL-33 injection[398]. Future studies will reveal if ILC2s are important responders following IL-33 release within the brain parenchyma.

A 2015 study described a role for IL-33 in recruiting mononuclear phagocytes to the retina. Increased IL-33 expression was detected in human age-related macular degenerationtissue[416], and degeneration was modeled with chronic light exposure in mice. Phototoxic stress led to IL-33 release from Müller cells, and Müller cells were also capable of responding to IL-33 to induce pro-inflammatory expression of *ccl2*, *il1β*, and *il6*. Recruitment of myeloid cells worsened degeneration and led to photoreceptor cell loss.

These results stand out in the field as the first description of IL-33 release and signaling on the same cell type within the CNS.

There are several conflicting reports of IL-33 function in experimental autoimmune encephalitis (EAE). Jiang *et al* in 2012 demonstrated that IL-33 promoted a beneficial, non Th1/Th17 skew in diseased mice. ST2 knockouts experienced exacerbated EAE, and IL-33 treatment increased IL-5 and IL-13 cytokines in cultured supernatants of lymph node and spleen cells[510]. It is important to note that IL-33-conferred protection in this model was rather mild and IL-33-treated animals still developed disease. A contrasting account from Li *et al* painted IL-33 as detrimental. Using an unconventional approach of anti-IL-33 blocking, this group saw inhibited onset and severity of disease with abrogation of IL-33 signaling, accompanied by decreases in IFN-γ and IL-17[511]. Additionally, increased *il33* transcript has been reported in the CSF and serum of multiple sclerosis patients compared with healthy controls, although IL-33 protein was not measurably increased[512]. Targeted, mechanistic studies are required to better understand which cells might be responding to IL-33 in models of multiple sclerosis. Conflicting results in EAE may be a consequence of different methods used to perturb IL-33 signaling and at what stage in EAE progression they were utilized. Another report in 2012 agreed with the findings of Jiang et al, that IL-33 is typically beneficial, since $ST2^{-/-}$ BALB/c animals, which are usually resistant to EAE, suddenly developed the disease after being stimulated with MOG (myelin oligodendrocyte glycoprotein), owed, the authors hypothesized, to increased antigen presentation capabilities in dendritic cells and increased circulating pro-inflammatory T cells[513]. Another study, by Chen et al in 2015, argued that IL-33 was released during EAE in spinal cord interstitial fluid, and that IL-33 was beneficial, although the study used general

homogenate, not unperturbed supernatants, to detect extracellular IL-33[514]. Lending more credence to the idea that IL-33 is beneficial during EAE, Xiao *et al* in 2018 showed that IL-33$^{-/-}$ mice are more susceptible to EAE, and have decreased Treg populations, along with increased IFN-γ+ and IL-17+ effector T cells, in agreement with the earliest Jiang study[515]. It is worth noting that the drug anacacardic acid, when it comes into contact with OPCs, is thought to promote myelination, and also results in an increase of IL-33 protein[516]. Therefore, IL-33 may play a role in directly repairing the brain, although the mechanisms by which this occurs, and whether nuclear or extracellular IL-33 are involved, are unclear[516]. Finally, one group showed that there is an increased number of ILC2s, and increased IL-33 expression, in male mice, which is protective and may explain sexual dimorphisms in EAE pathogenesis[517]. Therefore, despite one conflicting study to the contrary, it appears that IL-33 is likely beneficial overall in dampening autoimmunity in mouse EAE models. In multiple sclerosis patients, since mechanistic studies are not possible, IL-33 has simply been shown to be elevated in the CSF and serum of patients, although it is not clear if this is beneficial or detrimental to disease[518,519].

Polymorphisms in IL-33 have been linked with another disease in humans - Alzheimer's Disease[520]. A 2016 paper by Fu *et al* followed up in mice and found that i.p. injection of IL-33 rescued synaptic impairments in APP/PS1 mice. Reduced amyloid beta plaque burden was reported with IL-33 injection, and increased plaque burden was observed with administration of soluble ST2[430]. The same group built on this observation in 2020, finding that injected IL-33 upregulated MHCII expression in microglia/macrophages[521]. While these results are promising, the role of endogenous IL-33 signaling remains unclear. Mechanistic studies are needed to better understand cellular

changes which are linked to improved memory. Additional behavioral tests could be employed, such as the Morris water maze and the Barnes maze, to confirm cognitive rescue with IL-33 treatment. Another study in 2017 showed that IL-33$^{-/-}$ mice spontaneously develop tau aggregates, increased neuronal degeneration, and cognitive impairment compared with wildtype counterparts at 60-80 weeks of age[522], further asserting that IL-33 guards against cognitive decline.

Several studies have set out to understand IL-33's role in stroke. Like other disease states, IL-33 is elevated in the serum of stroke patients and may be a biomarker for disease[523]. However, release of IL-33 does not necessarily indicate a negative impact of IL-33 signaling, and nearly all stroke studies in mice have shown that IL-33 is beneficial. An experimental stroke model employing middle cerebral artery occlusion reported decreased IL-33 mRNA and protein with stroke. There are few if any studies which cite reductions in IL-33 with disease[524]. One explanation for this exception could be the sheer number of cells lost to ischemia during stroke. IL-33's beneficial effects in this system peaked one day post-stroke, consistent with the timing of macrophage recruitment. IL-33's existence was associated with increases in IL-4 and reduced infarct volume[524]. Another study in 2017 showed that ST2$^{-/-}$ mice had increased infarct volume in a stroke model[525]. This study performed bone marrow chimera experiments to find that the relevant responder to IL-33 was radio-resistant, and therefore likely non-hematopoetic. The authors focused on microglia and astrocytes, detecting expression of ST2 on microglia and astrocytes[525]. Then, adding IL-33 to microglia-like cells *in vitro*, found that IL-33 pushed microglia towards an anti-inflammatory phenotype, producing IL-10[525]. Since 2017, other studies have come out to show that microglia in culture do not express their core signature genes,

and it is therefore unclear if these cells resemble microglia, or in the *in vivo* environment. In 2018, another report showed that IL-33 injection is beneficial for infarct size and induces Th2 cytokines in the brain[526]. Finally, yet another study in stroke showed an expansion of ST2+ Tregs in the brain following IL-33 injection and prior to stroke induction, which could be another mechanism of protection, although IL-33 was injected intracerebroventricularly (i.c.v), and it is unclear if endogenous IL-33 is beneficial in this manner[527].

A handful of studies have implicated IL-33 in neuropathic pain. Measuring hyperalgesia following spinal cord constriction injury by hind limb flexion in response to stimuli, one study found that IL-33 signaling resulted in increased hyperalgesia, by assessing $ST2^{-/-}$ mice, mice injected with the decoy receptor soluble ST2, and mice injected with IL-33[528]. The same group showed, using similar methods, that IL-33 promoted pain following carrageenen injection, pro-inflammatory molecules that induce IL-6, TNF, and CXCL1 expression among other cytokines[529]. Other studies by another research group have shown similar results, in pain models induced by inflammatory agents and cancer[529-531]. These results are consistent with IL-33's ability to induce inflammation of various flavors, including pro-inflammatory cytokine production.

IL-33 in CNS infection follows a familiar trend, it is beneficial, and potentiates type 2 immune responses. Rocio virus-induced encephalitis responded poorly to abrogation of IL-33 signaling[532]. ST2 knockouts displayed increased inflammatory cytokine production, and increased infiltration of macrophages, aligned with worsened pathology and higher viral loads. Interestingly, experimental cerebral malaria has also been reported to benefit from an IL-33-induced type 2 skew. Cerebral malaria is caused by an intracellular,

protozoan parasite that belongs to the same phylum as *T. gondii*. It is controlled peripherally with a strong induction of type 1 cytokines, but this response must be carefully managed and is thought to be responsible for vascular leakage and pathology in the CNS. Cerebral malaria is also interesting in that it is restricted to the brain vasculature. IL-33 injection has been shown to subvert development of cerebral malaria upon infection modeled with blood stage *plasmodium berghei* ANKA[533]. IL-33 was thought to act on ILC2s and Tregs in this model, since ILC2 transfer mirrored Type 2 protection, and depletion of Tregs in IL-33-injected mice canceled out its protective effect. Although injected IL-33 was protective, it may not be protective at baseline, since a conflicting study showed that $ST2^{-/-}$ mice respond to cerebral malaria much better than wildtype mice, exhibiting reduced mortality, reduced pathology in the brain, and reduced BBB breakdown[534]. A follow-up study by this group showed that inflammatory cytokines were much reduced in the brains of infected $ST2^{-/-}$ mice, an observation the authors suggested could account for improved neurological symptoms, although it does not appear that any behavioral tests were done[535].

Most CNS infections, like cerebral malaria, LCMV, and others, with the exception of West Nile Virus and *T. gondii,* are relegated to the meninges and vasculature. Therefore, pathology and immune cell recruitment to the parenchyma would not be well modeled with most infections. IL-33 signaling on immune cells sequestered in these areas may not be relevant to infection-induced damage within the parenchyma. While IL-33 has not been studied in the context of West Nile Virus, it has been studied in *T. gondii* CNS infection. ST2 knockout mice infected via oral consumption of *T. gondii* exhibited severe necrosis of brain tissue and pronounced weight loss in early chronic stages of infection[536]. Cytokine

expression by whole brain homogenate qRT-PCR yielded mixed results. All pro-inflammatory cytokines associated with *T. gondii* infection were increased in ST2 knockout mice with the exception of IL-12. A correlative study in ocular toxoplasmosis was also undertaken in 2015, reporting increased IL-33 and ST2 mRNA with infection, as well as increased type 1 associated cytokines[537]. While these studies have hinted at IL-33's importance during *T. gondii* infection, they lack clear mechanism. Definitive IL-33 responders during *T. gondii* infection have not been identified, and changes in immune cell populations have not been addressed.

Some neurological diseases have not seen huge impacts of IL-33 yet, such as autism spectrum disorder – where IL-33 levels are unchanged in the patient plasma[538,539], and detailed mechanistic studies in mouse models have not yet been done. And there are a couple of new lines of study yet to be fully developed, including IL-33's role in epilepsy and traumatic brain injury. sST2 levels are increased in the serum of epilepsy patients[540], and IL-33 was very recently implicated in epilepsy, in a study where injected IL-33 significantly decreased TUNEL+ cells in an epileptic model, by about half[541]. This result is not one I have come across often and it will be interesting to understand in the future how IL-33 might prevent cell death in this model. The same group found a similar result in a traumatic brain injury (TBI) model, where IL-33 injection improved motor and spatial function post-TBI and increased Bcl-2 expression and reduced cleaved caspase-3 expression, although IL-33 seemed to have the greatest impact over PBS injections in the uninjured brain. It is also unclear if endogenous IL-33 plays a significant role in traumatic brain injury[542]. Another study of traumatic brain injury in 2017 showed that in ST2-/- mice, only one half the number of macrophages were recruited to the brain, and there was a strong

reduction in pro-inflammatory cytokines within brain tissue in these mice, although the impact on the outcome of brain tissue was not discussed[543]. It is interesting to think that IL-33 could be beneficial in TBI, considering it may potentiate pro-inflammatory cytokine production. Especially in comparison to stroke, which could produce similar necrotic lesions in brain tissue. This field will require more study in the future.

An overall theme that encapsulates many of these studies is that IL-33 is usually beneficial – potentiating immune responses that fit the context. This is even true in EAE, a disease characterized by autoimmunity. The biggest mystery in the CNS is IL-33's signaling mechanism. More specifically, the responding cell type(s) are often unclear. Many studies focus on expression of IL-33 and the receptor upon disease, and on overall clinical impact, and less on how readouts such as cytokine expression, immune cell activation are impacted by IL-33. Several brain resident cells, if not all, express the co-receptor for IL-33, IL-1RacP, and infiltrating immune cells, as well as microglia, astrocytes, and other not yet characterized cells may express the cognate receptor ST2. Additionally, since IL-33 and its receptor are expressed in many bodily tissues, it is often unclear how peripheral IL-33 signaling impact brain disease. This is especially true in cases of IL-33 injection, and whole body knockout studies. For this reason, we have focused a significant amount of attention on identifying responding cell type(s) in our manuscript.

Chapter 2 - Methods

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tajie Harris (tajieharris@virginia.edu).

Experimental mice

C57BL/6, CCL2-RFP^{flox} (Jackson Stock No: 16849), CCR2^{RFP} (Jackson Stock No: 017586), GFAPcre (Jackson Stock No. 024098), and CX3CR1cre mice (Jackson Stock No. 025524) were purchased from Jackson Laboratories. B6.SJL-Ptprc^{a} Pepc^{b}/BoyCrCl (C57BL/6 Ly5.1) mice were purchased from Charles River (Code 564). il1rl1$^{-/-}$ mice were generously provided by Andrew McKenzie (Cambridge University). il33$^{-/-}$ animals were obtained from Amgen by Elia Tait Wojno, currently at the University of Washington and previously at Cornell University. All IL-33$^{-/-}$ animals were housed at Cornell University. il1rl1-floxed embryos were received from KOMP repository (RRID MMRRC:048182-UCD). Importantly, we received notice after fully breeding these mice to cre-expressing mice, that the deposited floxed embryos contain a copy of an inducible, cardiomyocyte-driven cre [422], unknown to the KOMP repository. Upon receiving notice, we did detect the presence of MYH6cre^{ERT2} alleles throughout our *il1rl1*$^{fl/fl}$ colony. Importantly, we deleted *il1rl1* under the control of constitutive cre drivers and never administered tamoxifen to these mice. We performed statistical tests to determine the effect of MYH6cre expression, and did not find evidence of an effect in experiments where the MYH6cre was present in

some animals and not others (S11 Fig). Moreover, in the experiments reported here (Fig 6), all animals were positive for the MYH6cre allele. All animals were housed in a UVA specific pathogen-free facility with a 12h light/dark cycle. Mice were age and sex matched for each experiment, and were sacrificed in parallel. Animals were infected with *T. gondii* at 7 to 9 weeks of age and were housed separately from breeding animals. All procedures adhered to regulations of the Institutional Animal Care and Use Committee (ACUC) at the University of Virginia and Cornell University.

Human brain tissue

Healthy human brain samples from adult patients were obtained from the UVA Human Biorepository and Tissue Research Facility. Samples were preserved on paraffin embedded slides. Patient identification and medical background was withheld and therefore IRB approval was not required.

Parasite strains

The avirulent, type II Toxoplasma gondii strain Me49 was used for all infections (gift from Christopher Hunter, University of Pennsylvania). *T. gondii* cysts were maintained in chronically infected (1-6 months) Swiss Webster (Charles River) mice. To generate cysts for experimental infections, CBA/J (Jackson Laboratories) mice were infected with 10 cysts from brain homogenate of Swiss Webster mice by i.p. injection in 200µl PBS. 5-30 cysts from 4 week-infected CBA/J brain homogenate were then used to infect animals in all experiments.

Immunohistochemistry

Mouse Tissue Immunofluorescence:

Reporter mice were perfused with 30 mL PBS followed by 30 mL 4% PFA (Electron Microscopy Sciences). All non-reporter strains were only perfused with PBS. Brains were cut along the midline and post-fixed in 4% PFA for 24h at 4°C. Brains were then cryoprotected in 30% sucrose (Sigma) for 24h at 4°C, embedded in OCT (Tissue Tek), and frozen on dry ice. Samples were then stored at -20°C. 40 µm sections were cut using a CM 1950 cryostat (Leica) and placed into a 24-well plate containing PBS. Sections were blocked in PBS containing 2% goat or donkey serum (Jackson ImmunoResearch), 0.1% triton, 0.05% Tween 20, and 1% BSA for 1h at RT. Sections were then incubated with primary antibody (anti-mouse IL-33 R&D systems Cat#AF3626 1:100, anti-human IL-33 R&D systems Cat#MAB36253 1:100, anti-mouse Olig2 EMD Millipore Cat#AB9610 1:1000, Anti-mouse GFAP Invitrogen Cat#13-0300 1:500, Anti-*T.gondii* was a gift from Fausto G. Araujo, Palo Alto Medical Foundation, Anti-mouse RFP Abcam Cat#ab62341 1:400, Anti-mouse iNOS Invitrogen Cat# PA5-16855 1:400, Anti-mouse mCherry Abcam Cat# ab167453 1:400, Anti-mouse Iba1 Abcam Cat#ab5076 1:500, Anti-mouse MHCII Invitrogen Cat#14-5321-85 1:500, Anti-mouse CC1 Millipore Sigma Cat#OP80 1:200, Anti-mouse CD3 ThermoFisher Cat#140032-82 1:500) diluted in blocking buffer at 4°C overnight. Sections were washed the following day and incubated with secondary antibody (Donkey anti-goat AF488 Jackson Immunoresearch Cat#705-545-147 1:1000, Donkey anti-rat Rhodamine Jackson Immunoresearch Cat# 712-296-153 1:1000, Donkey anti-rabbit AF657 Jackson Immunoresearch Cat# 711-605-152 1:1000) in blocking buffer at room temperature for 1h. Sections were then washed and incubated with DAPI (Thermo

Scientific) for 5 min at RT. Sections were then mounted onto Superfrost microscope slides (Fisherbrand) with aquamount (Lerner Laboratories) and coverslipped (Fisherbrand). Slides were stored at 4°C before use. Images were captured using an TCS SP8 confocal microscope (Leica) and analyzed using Imaris (Bitplane) software. Volumetric analysis was achieved using the surfaces feature of Imaris.

Human tissue immunofluorescence

Slides containing 4 μm sections of human brain tissue were received from the UVA Biorepository and Tissue Research Facility and de-paraffinized in a gradient from 100% xylene (Fisher) to 50% ethanol (Decon Laboratories). Slides were then washed in running water and distilled water. Antigen retrieval was performed by incubating slides in antigen retrieval buffer (10 mM sodium citrate, 0.05%Tween-20, pH 6.0) in an Aroma digital rice cooker for 45 min at 95°C. Slides were then washed in running water followed by PBS-TW. Slides were then incubated with primary and secondary antibodies as described above for mouse brain tissue. Prior to imaging, Autoflourescence Eliminator Reagent was applied per the manufacturer's instructions (EMD Millipore Cat#2160).

Propidium Iodide injection

Adult naïve or 4-week infected mice were injected intraperitoneally with 20mg/kg propidium iodide (Invitrogen, cat#P1304MP). 24 hours post injection, the mice were sacrificed and their brains were fixed in 4% PFA and imaged for endogenous fluorescence by confocal microscopy.

Tissue processing and flow cytometry

Whole PBS-perfused brains were collected into 4 mL of complete RPMI (cRPMI)(10% fetal bovine serum, 1%NEAA, 1%Pen/Strep, 1%Sodium Pyruvate, 0.1%-β-mercaptoethanol). Papain digestion was performed for the chimera experiment. To perform papain digestion, brains were cut into 6 pieces and incubated in 5 mL HBSS containing 50U/mL DNase (Roche), and 4U/mL papain (Worthington-Biochem, Cat#LS003126) for 45 min at 37°C. Tissue was triturated first with a large bore glass pipette tip, and twice with a small-bore pipette tip every 15 min. In all other experiments collagenase/dispase was used to digest brain tissue. To perform collagenase/dispase digestion, perfused brains were minced using a razor blade and passed through an 18-gauge needle. Brains were then digested with 0.227mg/mL collagenase/dispase (Sigma-Aldrich Cat#11097113001) and 50U/mL DNase1 (Millipore Sigma Cat#10104159001) for 1h at 37°C. Following digestion, homogenate was strained through a 70 μm nylon filter (Corning, Cat#352350). Samples were then pelleted and spun in 20 mL 40% Percoll (GE Healthcare Cat#17-0891-09) at 650g for 25 min. Myelin was aspirated and cell pellets were washed with cRPMI. Finally, samples were resuspended in cRPMI and cells were enumerated. Meninges were collected from peeling from the brain and scoring from the skull cap and pooled. Meninges were passed through an 18-gauge needle five times and mashed through a 70μm filter. Samples were spun at 1500rpm for 10 min, and resuspended. Spleens were collected into 4 mL cRPMI and macerated through a 40 μM nylon filter (Corning, Cat#352340). Samples were pelleted and resuspended in 2 mL RBC lysis buffer (0.16 M NH_4Cl) Samples were then washed with cRPMI, and resuspended for counting and staining. In cases of acute infection, 4mL of peritoneal lavage fluid was pelleted and

resuspended in 2mL of cRPMI for counting and staining. Single cell suspensions were pipetted into a 96 well plate and pelleted. Samples were resuspended in 50 μL Fc Block, made in FACS buffer (PBS, 0.2% BSA, and 2 mM EDTA) with 0.1 μg/ml 2.4G2 Ab (BioXCell, Cat#CUS-HB-197) and 0.1% rat gamma globulin (Jackson Immunoresearch, Cat#012-000-002), for 10 min. Cells were then surface stained in 50 μL FACS buffer for 30 min at 4°C with directly conjugated antibodies (from eBioscience: Fixable viability dye eFluor 506 Cat#65-0866-18, CD3 FITC Cat#11-0031-85, CD62L FITC Cat#11-0621-85, CD8 ✔ PerCp-Cy5.5 Cat#45-0081-82, CD4 PE-Cyanine-7 Cat#25-0041-82, CD44 AF780 Cat#47-0441-82, MHCII FITC Cat#11-5321-82, CD45 PerCp-Cy5.5 Cat#45-0451-80, Ly6C PE-Cyanine-7 Cat#25-5932-82, CD11b AF780 Cat#47-0012-82, CD45.1 eFluor 450 Cat#48-0453-82, NK 1.1 FITC Cat#11-5941-85, CD19 FITC Cat#11-0193-82, ST2 PE Cat#12-9335-82, Thy1.2 (CD90) PB Cat#48-0902-80, FcεR1 APC Cat#17-5898-80; BD biosciences: CD45.2 FITC Cat#561874). Fixable viability dye was used at 1:800, all other antibodies were used at 1:200 dilution. Following surface staining, cells were fixed for at least 30 min at 4°C with a fixation/permeabilization kit (eBioscience Cat#00-5123-43 and Cat#00-5223-56) and permeabilized (eBioscience Cat#00-8333-56). Samples were then incubated with intracellular antibodies in permeabilization buffer for 30 min at 4°C (eBioscience: IFN-γ PerCp-Cy5.5 Cat#45-7311-82, IFN-γ APC Cat#17-7311-82, Ki67 APC Cat#17569880, iNOS APC Cat#17-5920-80, Foxp3 PB Cat#48-5773-82). For intracellular cytokine, samples were first incubated with PMA/ionomycin (Sigma-Aldrich Cat#P1585, Cat#I0634) and Brefeldin A (Selleckchem Cat#S7046) for 5 hours at 37°C

before surface staining. Samples were washed, resuspended in FACS buffer, and run on a Gallios flow cytometer (Beckman Coulter), and analyzed using Flowjo software, v. 10.

qRT-PCR

Perfused brain tissue (100 mg) was placed into bead beating tubes (Sarstedt, Cat# 72.693.005) containing 1mL Trizol reagent (Ambion, Cat#15596018) and zirconia/silica beads (Biospec, Cat#11079110z). Tissue was homogenized for 30 seconds with a Mini-bead beater (Biospec) machine. RNA was extracted following homogenization per the Trizol Reagent manufacturer's instructions. Complementary DNA was then synthesized using a High Capacity Reverse Transcription Kit (Applied Biosystems, Cat#4368813). Taqman gene expression assays were acquired from Applied Biosystems (*Ccl2* Cat#Mm00441242_m1, *Ccl5* Cat#Mm01302427_m1, *Cxcl9* Cat#Mm00434946_m1, *Cxcl10* Cat#Mm00445235_m1, *Cxcl1* Cat#Mm04207460_m1, *Vcam* Cat#Mm1320970_m1, *Icam* Cat#Mm00526023_m1, *Il33* Cat#Mm00505403_m1) or IDT (*Il1rl1*, S Table 1). A 2X Taq-based mastermix (Bioline, Cat#BIO-86005) was used for all reactions and run on a CFX384 Real-Time System (Bio-Rad). Hprt was used as the brain housekeeping gene (Applied Biosystems, Cat#Mm00446968_m1) and relative expression to wildtype controls was calculated as $2^{(-\Delta\Delta CT)}$.

For qRT-PCR assessment of parasite burden, brain tissue was placed in complete RPMI post-harvest, minced with a razor blade, and passed through an 18-gauge needle five times. Brain homogenate (300μL) was then put in a tube with zirconia/silica beads (Biospec, Cat#11079110z) and homogenized in a Mini-bead beater (Biospec) machine. Homogenate

was then incubated at 65C for 3 hours with Lysis buffer from the Genomic DNA Isolate II Genomic DNA Kit (Bioline, Cat#BIO-52067). DNA was isolated from brain samples following the manufacturer's instructions. A 2X Taq-based mastermix (Bioline Cat#BIO-86005) was used for all reactions and run on a CFX384 Real-Time System (Bio-Rad), using *T. gondii* genomes as a standard curve, ranging in 10-fold dilutions from 300,000 genomes to 30 genomes. *T. gondii* genomes were isolated from cultured, infected human foreskin fibroblast cells. DNA from brain samples was diluted to 500ng/well. The Taqman assay used to detect *T. gondii* has been described previously[544].

***T. gondii* cyst counts**

Brain tissue (100 mg) was minced with a razor in 2mL cRPMI. Brain tissue was then passed through an 18-gauge and 22-gauge needle. 30 uL of resulting homogenate was pipetted onto a microscope slide (VWR) and counted on a Brightfield DM 2000 LED microscope (Leica). Cyst counts were extrapolated for whole brains.

Bone marrow chimera

Wildtype B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (C57BL/6 CD45.1) and *il1rl1*$^{-/-}$ C57BL/6 CD45.2 mice were irradiated with 1000 rad. Irradiated mice received 3x10^{6} bone marrow cells from CD45.1 and CD45.2 donors the same day. Bone marrow was transferred by retro-orbital i.v. injection under isoflurane anesthetization. All mice received sulfa-antibiotic water for 2 weeks post-irradiation and were given 6 weeks for bone marrow to reconstitute. At 6 weeks, tail blood was collected from representative mice and assessed for reconstitution by flow cytometry. Mice were then infected for 4 weeks prior to analysis.

Brain homogenate *ex vivo* supernatant collection

Brains were harvested from naïve and infected mice. Brain tissue was processed down to a single cell suspension as outlined in "tissue processing and flow cytometry". Cells from half a brain were incubated in a 96-well plate in 200µL for 4 hours at 37°C. Cells were then pelleted at 1500rpm for 5 min, and supernatant was stored at -80°C for measurement of IL-33 by ELISA.

CSF collection

Naïve and infected mice were anesthetized with ketamine/xylazine, their necks were shaved and obstructing skin and tissue surrounding the dura of the cisterna magna was removed. A small glass capillary was inserted into the dura of the cisterna magna, allowing approximately 10µL of CSF to fill the capillary. CSF from 4-5 mice was pooled and frozen at -80°C.

IL-33 ELISA

The IL-33 Quantikine ELISA kit was purchased from R&D Systems (Cat#M3300) and manufacturer instructions were followed. 50µL of either brain homogenate supernatant (*ex-vivo* assay) or CSF was used as sample volume. 4-5 CSF samples from individual animals were pooled to reach 50µL sample volume. IL-33 standards were serially diluted to a minimum of 30pg/mL. Final values of standards and samples were read on a spectrophotometer at 450nm.

IFN-γ and IL-12 ELISAs

For ELISAs from day 7 post-infection, tail blood was taken from infected animals and incubated with 30U/mL heparin (Sigma, H3149-10KU) before being spun at 2000g for 10 min at 4°C to obtain plasma. For the IFN-γ ELISA done at 12 days-post infection, blood was taken from cardiac puncture and allowed to sit for 30 min at room temperature before spinning at 2000g for 10 min at 4°C to obtain serum. Quantikine ELISA kits for IFN-γ and IL-12p40 were purchased from R&D Systems (Cat# MIF00, Cat#M1240) and manufacturer instructions were followed, using 50µL of plasma or serum samples diluted twenty-fold**.** Final values of standards and samples were read on a spectrophotometer at 450nm.

ACSA-2/CD11b magnetic enrichment

Brain tissue was harvested from mice and processed down to a single cell suspension as described in "tissue processing and flow cytometry" methods, except myelin removal beads (Miltenyi Cat#130096733) were used in place of Percoll. The Anti-ACSA2 and CD11b Microbead kits, purchased from Miltenyi (Cat#130097678, Cat#130093634), were used to enrich for astrocytes and macrophages, respectively, over magnetic columns (Miltenyi Cat#130-042-401). In the case of enriching for astrocytes, both kits were used to first remove CD11b+ cells and then enrich for ACSA-2+ cells. Double-purifications were performed in all cases, which was optional, but recommended, by Miltenyi's protocol. Cells were pelleted at 1500rpm and stored in 300µL Trizol (Ambion) at -80°C until further use.

Statistical analyses

Statistical analyses comparing two groups at one time point were done using a student's t-test in Prism software, v. 7.0a. Statistical analyses comparing more than two groups within the same timepoint or infection were done using a one-way Anova. In instances where data from multiple infections were combined, all from the same time-point post infection, a randomized block ANOVA was performed using R v. 3.4.4 statistical software to account for variability between infections. Genotype was modeled as a fixed effect and experimental day as a random effect. P values are indicated as follows: ns=not significant $p>.05$, * $p < .05$, ** $p < .01$, *** $p < .001$. The number of mice per group, test used, and p values are denoted in each figure legend. Data was graphed using Prism software, v.7.0a.

Chapter 3 – Results

3.1 *Astrocytes promote a protective immune response to brain Toxoplasma gondii infection via IL-33-ST2 signaling*

3.1.1 *Project rationale*

Recruitment of immune cells to the brain during infection is a highly orchestrated process, requiring concerted expression of a number of chemokines and adhesion factors at the blood-brain barrier[123]. But the cues which precede these factors are less well understood. In particular, in many cases, it is unclear if brain resident cells possess the machinery to detect the presence of pathogens to promote the recruitment of peripheral cells. Murine infection with the eukaryotic parasite *Toxoplasma gondii* (*T. gondii*) features continual recruitment of blood-derived immune cells to the brain and serves as an excellent model for better understanding immune responses at this site.

T. gondii is a globally relevant pathogen which infects most warm-blooded vertebrates, including one-third of the human population[18,46,47]. Upon initial exposure of hosts to *T. gondii* through contaminated food or water[545], an early stage of infection occurs, called the acute phase, during which *T. gondii* disseminates throughout peripheral tissues[80]. By two-weeks post-infection, parasite has been largely cleared or controlled in most tissues, but ultimately persists in the brain of its hosts for their lifetime[18,79-81,545,546]. Mortality from *T. gondii* infection is associated with an increased prevalence of replicating

parasite in brain tissue, documented in immunosuppressed patients undergoing transplant surgeries[107], and in HIV-AIDS patients[97,547,548], highlighting the importance of the immune response in controlling *T. gondii*. Indeed, control of brain *T. gondii* infection requires a Th1-dominated immune response[18,81], whereby CD4+ and CD8+ T cells and the IFN-γ they produce are required for survival[67]. Macrophages also exhibit anti-parasitic effector mechanisms which are necessary to control the parasite[81,89,322,340,341,549-551].

It is not known, however, how the parasite is sensed in the brain to create an environment that promotes immune cell entry, stimulation, and maintenance. During the acute phase of infection in the periphery, dendritic cells and macrophages can sense either the parasite itself or host signals to initiate chemokine and cytokine expression which recruits and skews a strong Th1 immune response[81,171,172,206,207,246]. However, resident dendritic cells and peripheral immune cells do not exist in brain tissue under steady-state conditions[352,353] and it is unclear if *T.gondii*-specific molecular patterns are sensed in this tissue. During chronic *T. gondii* infection of the brain, necrotic lesions form, characterized by the presence of replicating parasite, loss of brain-resident cell markers, and infiltration of immune cells, suggestive of tissue damage and alarmin release[67,95,100,102,161,395]. We hypothesized that indirect sensing of *T. gondii* infection, via recognition of host cell damage caused by the parasite, is an important step in instructing the immune response to *T. gondii* in the brain. Here we focused on the nuclear alarmin, IL-33, as a candidate orchestrator of the immune response to *T. gondii*. IL-33 is highly expressed in brain tissue[230], and IL-33 signaling has been shown to be protective against tissue pathology during chronic *T. gondii* infection, but the mechanism by which IL-33 signals, and the immune mechanisms underlying this protection, were not studied in detail[536].

IL-33 is categorized as an alarmin because it is known to amplify immune responses upon signaling through its receptor ST2, also known as *il1rl1*, without a requirement for secretion or cleavage. A role for IL-33 in stroke[524,525], neurodegeneration[430,552], EAE[510,511], and CNS infection[533,535,553] has been described, but mechanistic understanding of IL-33 signaling during brain disease is limited. It is unclear in many instances which cell type(s) is the relevant responder to IL-33 during disease in the brain. While brain-resident macrophages have been shown to express the IL-33 receptor at baseline[250], astrocytes have been shown to upregulate it during pathology [271,525]. In addition, IL-33 receptor is also expressed on immune cells which can infiltrate the brain from the blood during disease[274,415]. Here, we separate the relative contribution of astrocytes, microglia/macrophages, and infiltrating immune cells in response to IL-33 release during brain pathology, finding that IL-33 can signal strictly within the brain to promote protective immunity to *T. gondii*.

3.1.2 *Results*

IL-33 is released during *T. gondii* brain infection and is required to control parasite

To study *T. gondii* brain infection, we infected mice by intra-peritoneal injection with the avirulent *T. gondii* strain Me49 and waited four weeks post-infection for a natural, chronic brain infection to be established. At this timepoint, infection of most other tissues throughout the body, also known as acute infection, has been controlled[80,546]. For all experiments, brain tissue was harvested at four weeks post-infection unless otherwise specified.

Consistent with its role as a pre-stored alarmin, IL-33 is highly expressed in the brain at baseline[230], and only mildly increases with infection (Fig 3.1A). Although astrocytes are the major source of IL-33 during development, oligodendrocytes become a significant contributor by postnatal day 30[181,230,250,271]. In the *T. gondii*-infected brain, we found nuclear IL-33 to be expressed predominantly by mature, CC1+ oligodendrocytes and also by astrocytes, the frequencies of which varied by brain region (Figs 3.2A, 3.2B and Fig 3.1A). We found IL-33 to be expressed almost exclusively by oligodendrocytes in white matter tracts, such as the corpus callosum, while IL-33 expression in gray matter, such as the cortex, was split more evenly between astrocytes and oligodendrocytes (Figs 3.2A and 3.2B). These data align with IL-33 expression in the uninfected mouse brain[271], indicating that the major sources of IL-33 in the brain do not change with *T. gondii* infection. Collectively, these results indicate that IL-33 is expressed by glia in the *T. gondii* infected mouse brain parenchyma. Importantly, we also detected IL-33 protein expression in astrocytes in healthy human brain tissue, as has been shown previously on the transcript level[182] (Figs 3.1C and 3.1D).

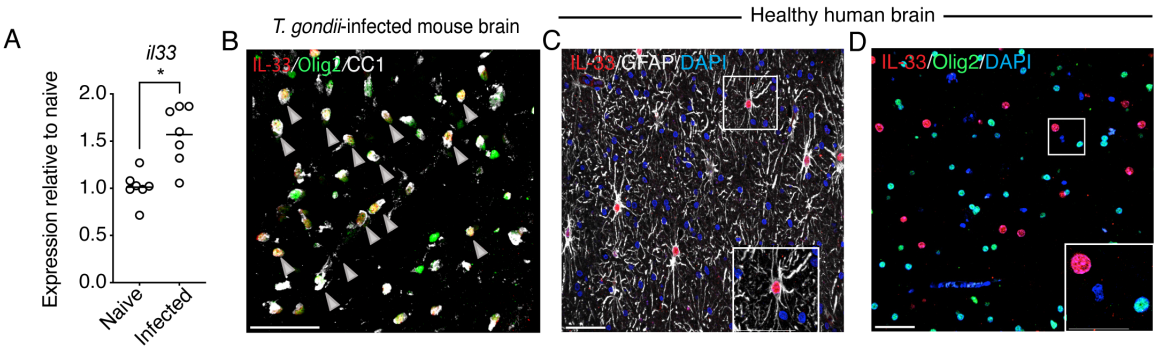

Fig 3.1 IL-33 expression in mouse and human brain tissue

(A) Real time PCR for *il33* transcript from whole brain homogenate at 4 weeks post infection compared to naïve brain tissue. **(B)** Colocalization, denoted by gray arrows, of nuclear IL-33 protein (red) with mature oligodendrocytes, marked by nuclear Olig2 expression (green), and CC1(white) by confocal fluorescence microscopy of infected mouse brain tissue. **(C and D)** Confocal fluorescence microscopy of nuclear IL-33 stain present in astrocytes (C) but not oligodendrocytes (D) in the temporal lobe of human brain tissue from patients that did not succumb to toxoplasmic encephalitis (healthy). Statistical significance was determined by randomized block ANOVA (A), which shows data pooled from two independent experiments *= p<.05, **= p<.01, ***= p<.001. Scale bars indicate 50μm.

Since IL-33 does not need to be cleaved to be active, it is classically thought of as an alarmin that is released by necrotic cell death[419,460]. During *T. gondii* infection, the parasite itself as well as the inflammatory environment provide opportunity for host cell damage and alarmin release. During chronic infection, *T. gondii* predominantly exists in the brain as an intracellular cyst form which is slow growing[79,80] (Fig 3.2C) and does not appear to pose an immediate risk to cells, due to a lack of observed tissue destruction and inflammation surrounding cysts (Fig 3.2C). But, for reasons not fully understood, cysts can reactivate anywhere in the brain, releasing individual parasites previously contained within the cyst wall[79,100] (Fig 3.2C). Individual parasites can invade surrounding cells, replicate, and can lyse the cell or form a new cyst[44]. The presence of individual replicating parasites is correlated with morbidity in humans, and is thought to cause necrotic lesions in immunocompromised patients[97,107,547,548].

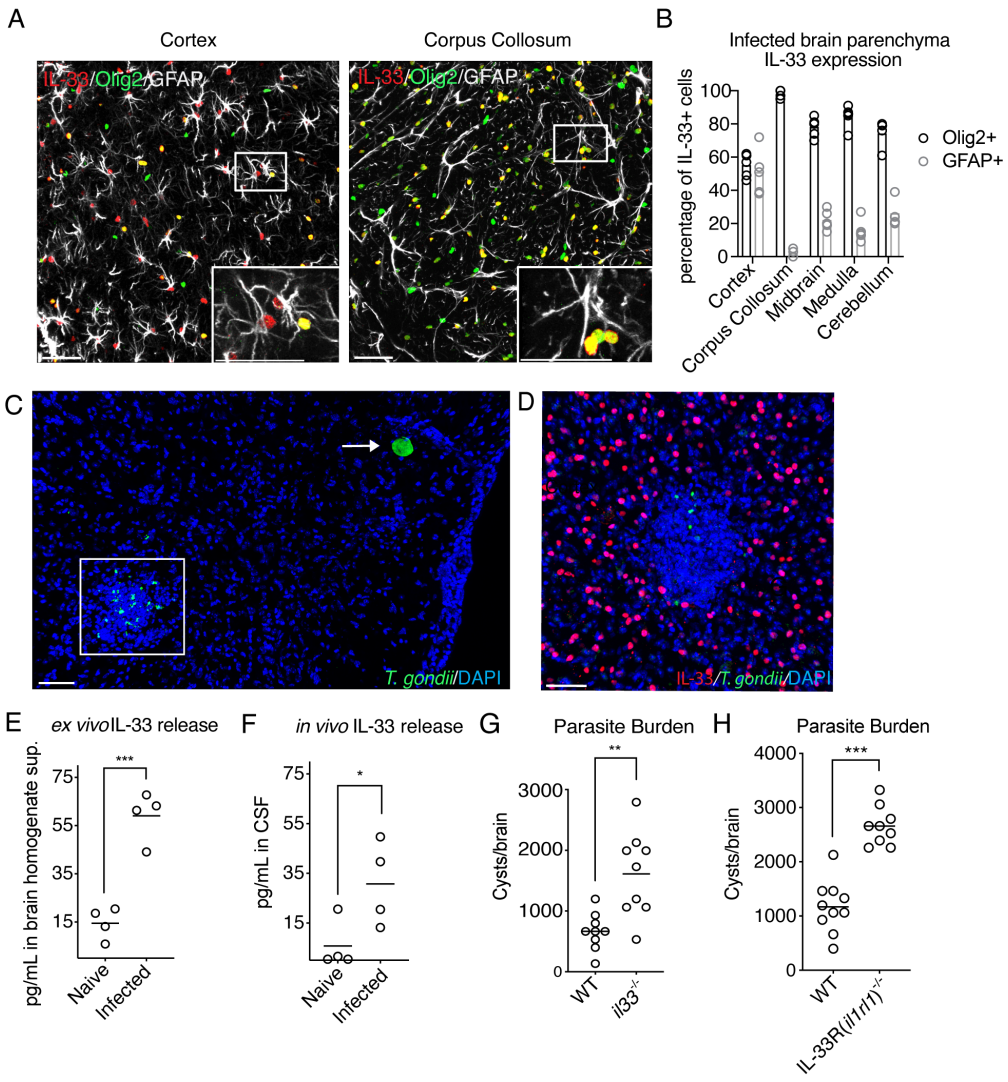

Fig 3.2 IL-33 is expressed by oligodendrocytes and astrocytes during brain *T. gondii* infection, is released during infection, and is required for control of parasite

(A and B) Representatitive images (A) and percentage quantification (B) of nuclear IL-33 protein expression (red) by cell type four weeks post-infection in various brain regions by confocal fluorescent microscopy. IL-33 is costained with the nuclear oligodendrocyte marker, Olig2 (green) or activated astrocyte marker, GFAP, (white). **(C and D)** Representative images of *T. gondii* in brain tissue, either in cyst form (arrow), or reactivated individual parasites (box)(C). IL-33-positive cells are absent from inflammatory foci containing replicating parasites (D). **(E and F)** Extracellular IL-33 release as measured by ELISA after *ex vivo* incubation of all cells isolated from infected brain tissue (E), or from CSF samples, each dot represents pooled CSF from 4-5 individual mice from a separate infection, displaying four infections in total (F). **(G and H)** Parasite burden as measured by cyst count from brain homogenate of IL-33-deficient (G) and IL-33 receptor (*il1rl1*)-deficient mice (H). Statistical significance was determined by two-tailed t-test (E) or randomized block ANOVA, when results from multiple independent experiments are shown (F-H). *= p<.05, **= p<.01, ***= p<.001. Scale bars indicate 50μm.

Interestingly, we found clusters of immune cells, including T cells and macrophages, surrounding individual replicating parasites but not *T. gondii* cysts (Figs 3.3A, 3.3B and 3.2C). Therefore, we hypothesized that either lytic *T. gondii* replication[44] or local inflammation, can cause release of local damage signals, such as IL-33. Corroborating this hypothesis, while IL-33 tiles evenly throughout uninfected brain tissue[271], we noted focal loss of IL-33 staining, as well as oligodendrocyte and astrocyte markers, at the center of inflammatory lesions containing parasite (Figs 3.2D, 3.3C and 3.3D).

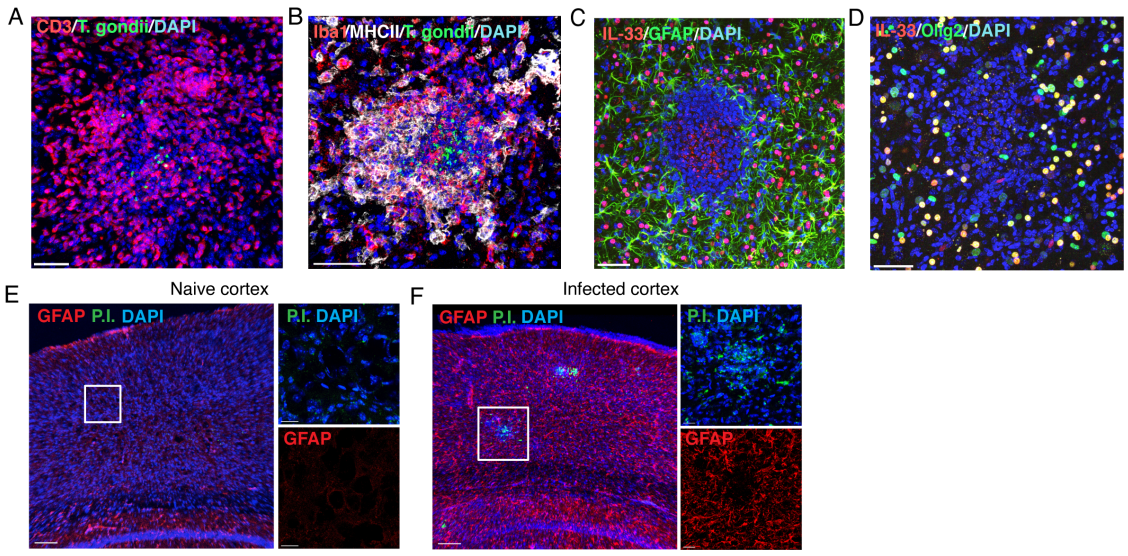

Fig 3.3 Characterization of inflammatory lesions in *T.gondii*-infected brain tissue

(A and B) Representative images of immune cells surrounding foci of individual replicating parasites (green) in cortical brain tissue, including CD3+ T cells (red) (A), and MHCII+ (white) Iba1+ (red) myeloid cells (B). **(C and D)** Representative images of necrotic foci, featuring a loss of brain resident cells which express IL-33 (red), including GFAP+ astrocytes (green) (C), and Olig2+ oligodendrocytes (green) (D). **(E and F)** Representative images of propidium iodide fluorescence in brain tissue, 24 hours post i.p. injection into naïve and infected mice. Staining depicts propidium iodide (green), and GFAP+ astrocytes (red). Scale bars indicate 50μm in A-D, and 100μm or 30μm (insets) in E and F.

To assess if host cell death occurs in the *T. gondii* infected brain which could facilitate IL-33 release, we injected mice intraperitoneally with propidium iodide (PI), which is taken up by cells with loss of membrane integrity. 24 hours post-injection, while we did not observe significant clusters of PI positivity in uninfected (naïve) brain tissue (Fig 3.3E), we found focal areas of PI positivity in chronically infected brain tissue near inflammatory foci, denoted by a lack of GFAP staining and DAPI clustering (Fig 3.3E). These results indicate that infection of the brain by *T. gondii* can cause damage to host cells.

To determine if IL-33 was released during brain *T. gondii* infection, we first utilized an *ex-vivo* assay to measure extracellular IL-33 during infection. We processed naïve and infected mouse brains down to a single cell suspension containing brain resident cells as well as immune cells and parasite. We then incubated the single cell suspensions for four hours at 37°C before taking the supernatant and measuring extracellular IL-33 by ELISA. Strikingly, IL-33 was present in detectable quantities from supernatants of infected brain samples, but not naïve controls, suggesting that *T. gondii* infection has the capacity to induce IL-33 release (Fig 3.2E). We then validated IL-33 release *in vivo*, by sampling cerebrospinal fluid (CSF) from the cisterna magna of infected mice and found detectable IL-33 levels in pooled CSF samples (Fig 3.2F).

We next asked if IL-33 was required to control infection. We infected wildtype and *il33*$^{-/-}$ mice and assessed their brains for parasite burden, enumerating a significantly increased number of cysts in *il33*-deficient mice (Fig 3.2G). We also detected an increased parasite burden in the brains of infected mice that lacked the IL-33 receptor (known as ST2 or *il1rl1*), by cyst count and quantitative PCR (Fig 3.2H and 3.4B), demonstrating a role

for extracellular IL-33 signaling during this infection. At 10 days post-infection, no parasite was detected in the peritoneal cavity of infected *il1rl1*$^{-/-}$ or wildtype animals. Therefore, *il1rl1*$^{-/-}$ mice are not delayed or defective in clearing parasite in the periphery. Additionally, parasite levels were equivalent in the brain at day 12 post infection (12DPI) in *il1rl1*$^{-/-}$ mice in comparison to wildtype mice (Fig 3.4A), further supporting that the control of parasites is intact during the early stages of infection. We did not find *il33*$^{-/-}$ or *il1rl1*$^{-/-}$ mice to succumb to infection. Nonetheless, these results demonstrate that extracellular IL-33 signaling plays a critical role in limiting *T. gondii* during chronic brain infection.

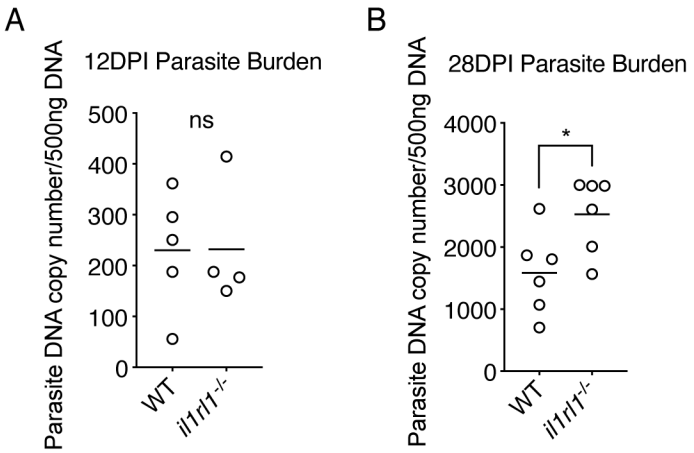

Fig 3.4 Brain parasite burdens at 12DPI (acute infection) versus 28DPI (chronic infection) in *il1rl1*$^{-/-}$ mice

(A and B) Real time PCR for parasite genomic DNA from whole-brain homogenate of infected WT and il1rl1$^{-/-}$ mice at 12 days post infection (DPI) (A) and 28DPI (B). Statistical significance was determined by two tailed t-test (A) or a randomized block ANOVA (B), which shows data pooled from two independent experiments *= p<.05, **= p<.01, ***= p<.001.

IL-33 signaling is required for adequate numbers of functional T cells during chronic *T. gondii* infection

In order to better understand how IL-33 signaling was protective during *T. gondii* infection, we focused on characterizing *il1rl1*$^{-/-}$ mice from this point onward. We first profiled recruited immune cell populations in the brain, beginning with the adaptive immune response, because a strong, Th1-biased immune response is absolutely critical for control of chronic *T. gondii* infection[67]. In the absence of IL-33 signaling during infection, approximately one-third fewer total T cells were present in the brain by flow cytometry (Figs 3.5A and 3.5B), suggesting an inability to recruit or maintain these cells. Approximately 96% of T cells in *T. gondii* infected brains were positive for either CD4 or CD8 markers (Fig 3.5A), and there was no selective decrease in either of these subsets, but rather a general reduction in T cell counts in *il1rl1*$^{-/-}$ mice (Figs 3.5A and 3.5B). A reduction in T cell numbers could be due to reduced recruitment, reduced survival, or reduced proliferation in the absence of IL-33 signaling. Indeed, fewer CD4+ and CD8+ T cells were proliferating in the brain (Figs 3.5C and 3.5D), and fewer of these cells were functional, displaying reduced production of the critical cytokine IFN-γ by *ex vivo* restimulation with PMA/ionomycin (Figs 3.5E and 3.5F).

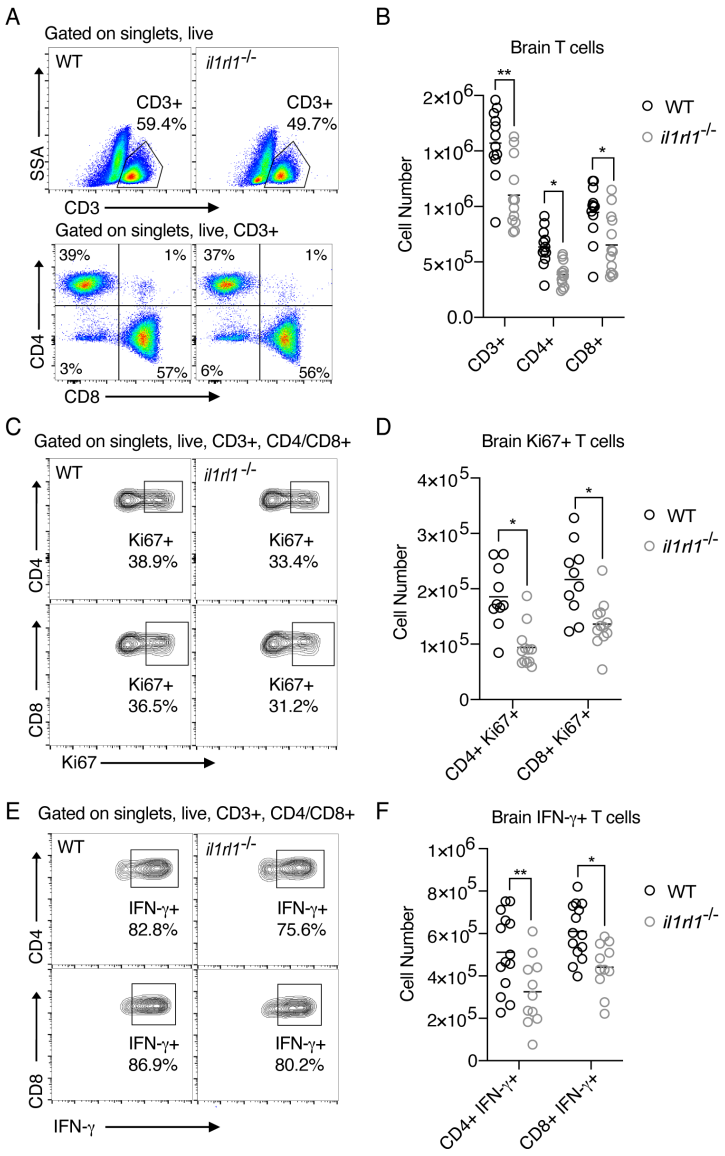

Fig 3.5. IL-33 signaling is required for adequate numbers and functionality of T cells in the brain during chronic *T. gondii* infection

(A and B) Representative flow cytometry plots (A) and quantification (B) of infiltrated total T cells in infected brain tissue four weeks-post infection. **(C and D)** Representative flow cytometry plots (C) and quantification (D) showing frequency of proliferating (Ki67+) T subsets in infected brain tissue. **(E and F)** Representative flow cytometry plots (E) and number (F) of IFN-γ-positive T cell subsets in infected brain tissue. IFN-γ was measured following *ex-vivo* re-stimulation of brain cells, incubated with Brefeldin A and PMA/ionomycin for 5 hours at 37°C (F). Statistical significance was determined by randomized block ANOVA (B,D,F), and each quantified panel displays three pooled independent experiments. *= p<.05, **= p<.01, ***= p<.001

IFN-γ is critical because it induces widespread anti-parasitic changes, such as increasing chemokine production [391,394], adhesion factor expression [392], and intracellular killing mechanisms of infected cell types, such as macrophages[81]. We found that the chief sources of IFN-γ in the brain during infection were CD4+ and CD8+ T cells, with minimal contribution from NK cells, an important source of the cytokine during early stages of acute infection[306] (Fig 3.6A). These results show that extracellular IL-33 signaling is required to maintain an adequate anti-parasitic T cell response in brain tissue.

T cell numbers and function were unaffected at baseline in spleens of *il1rl1*$^{-/-}$ mice, in the spleen and the blood during chronic infection, as well as at sites of inflammation such as the peritoneal cavity during acute infection (Fig S4B-E). We did, however, note a decrease in T cell numbers in the blood at day 10 post-infection in *il1rl1*$^{-/-}$ mice (Fig 3.6F), and decreased serum IFN-γ at day 12 post-infection despite intact IL-12p40 levels (Figs 3.6H and 3.6I). These results indicate that IL-33 signaling does affect the T cell response; however, it does not appear that these defects underlie defects in brain immunity to *T. gondii*, since activated T cell numbers and parasite burden were unaffected in the brain at day 12 in *il1rl1*$^{-/-}$ mice (Figs 3.4A and S6C Figs). Nevertheless, plasma IFN-γ levels at day 7 are intact in *il1rl1*$^{-/-}$ mice (Fig 3.6G), and are likely enough to control parasite early in infection. Furthermore, a time course showed progressive decreases in IFN-γ+ T cell numbers in *il1rl1*$^{-/-}$ mice in comparison to wildtype mice (S6 Fig and 3.5F), demonstrating brain-specific immunity in the absence of IL-33 signaling.

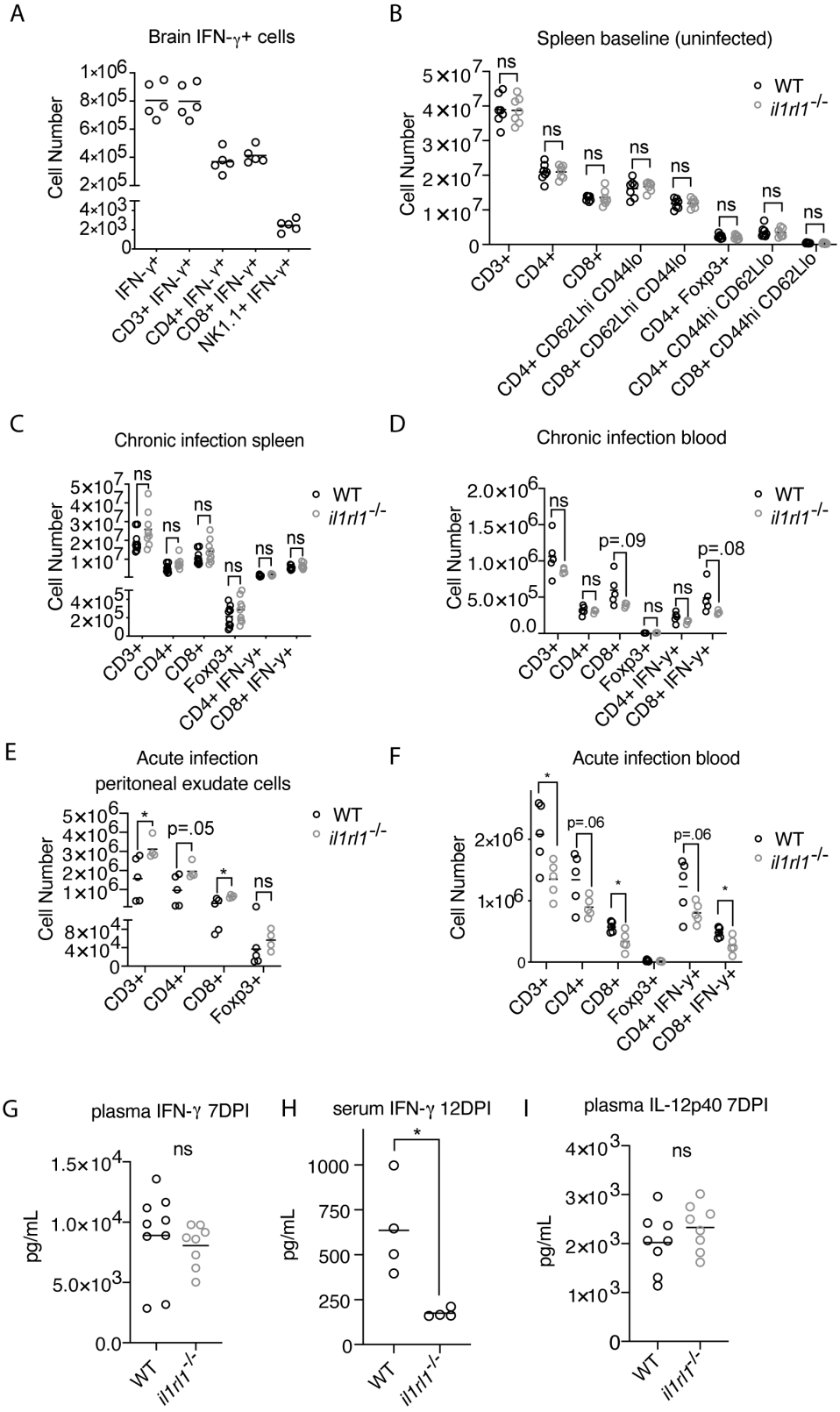
A
Brain IFN-γ+ cells
Cell Number
IFN-γ+
CD3+ IFN-γ+
CD4+ IFN-γ+
CD8+ IFN-γ+
NK1.1+ IFN-γ+
B
Spleen baseline (uninfected)
WT
il1rl1-/-
CD3+
CD4+
CD8+
CD4+ CD62Lhi CD44lo
CD8+ CD62Lhi CD44lo
CD4+ Foxp3+
CD4+ CD44hi CD62Llo
CD8+ CD44hi CD62Llo
C
Chronic infection spleen
D
Chronic infection blood
E
Acute infection
peritoneal exudate cells
p=.05
F
Acute infection blood
p=.09
p=.08
p=.06
G
plasma IFN-γ 7DPI
pg/mL
H
serum IFN-γ 12DPI
I
plasma IL-12p40 7DPI
ns

Fig 3.6. T cells in peripheral tissues and during acute infection in the absence of IL-33 signaling

(A) Breakdown of total IFN-γ+ cells by cell type by flow cytometry in infected brain tissue four weeks post infection. IFN-γ was measured following stimulation *ex vivo* for five hours with PMA/ionomycin. **(B)** Assessment of spleen T cell numbers in *il1rl1*-deficient mice prior to infection by flow cytometry. **(C and D)** assessment of peripheral tissue T cell numbers and activation, including spleen (C) and blood (D) by flow cytometry 4 weeks post infection. **(E and F)** T cell numbers at day 10 acute infection by flow cytometry in the peritoneum (E) and blood (F). **(G-I)** plasma (G,I) or serum (H) ELISAs for IFN-γ (G,H) or IL-12 (I) during acute infection. Statistical significance was determined by randomized block ANOVA when two experiments were pooled (B,C, G, I), or by two-tailed t-test (D, E, F, H) *= p<.05, **= p<.01, ***= p<.001.

It is important to mention that decreased immune activation in *il1rl1*$^{-/-}$ mice, such as decreased IFN-γ production, during the acute or chronic stage of infection, could be beneficial in protecting against immunopathology. Indeed, *il1rl1*$^{-/-}$ mice are protected from weight loss, early mortality, and intestinal pathology during the acute stage of infection when mice are infected orally with *T. gondii*[301]. Infecting with 10 cysts of Me49 parasite intraperitoneally, we do not observe significant mortality or pathology in wildtype mice, but nonetheless, IL-33 signaling could potentiate immunopathology during more severe infections.

IL-33 signaling is required for the recruitment and anti-parasitic function of myeloid cells during brain *T. gondii* infection.

We were also interested in the impact of IL-33 signaling on the myeloid cell lineage since macrophages cluster tightly around replicating parasite in brain tissue and express iNOS, a key molecule invovled in the control of *T. gondii* (Fig 3.3B)[81]. We assessed numbers of CD11b+ myeloid cells in infected *il1rl1*$^{-/-}$ mice by flow cytometry, using CD45hi expression to differentiate infiltrating myeloid cells from CD45int microglia (Figs

3.7A and 3.7B). Many of the CD45hi CD11b+ cells recruited to the *T. gondii*-infected brain are Ly6C+ CD11c- and Ly6G-, indicating that a large portion of recruited myeloid cells are Ly6Chi monocytes and Ly6Clo monocyte-derived macrophages[132]. Importantly, infected *il1rl1*$^{-/-}$ mice displayed a reduced frequency and number of CD45hi myeloid cells, by approximately half, in the brain during chronic infection, while CD45int cell numbers held constant (Figs 3.7A and 3.7B).

To more specifically assess monocyte recruitment, we used CCR2-RFP reporter mice to visualize monocytes in the brain during chronic infection. Monocyte-derived cells are critical for control of brain *T. gondii* infection, as anti-CCR2 antibody administered during chronic infection results in rapid mortality [132]. By immunohistochemistry, we observed large numbers of CCR2+ cells throughout the brain 4 weeks post-infection in wildtype mice (Fig 3.7C). Importantly, we crossed CCR2-reporters to a *il1rl1*$^{-/-}$ background, which revealed a marked reduction in CCR2+ cells (Figs 3.7D and 3.7E). These results confirmed that there is a defect in monocyte recruitment in the absence of IL-33 signaling during infection.

Next, we assessed the anti-parasitic function of the myeloid compartment in the absence of IL-33 signaling, by focusing on inducible nitric oxide synthase (iNOS) expression. Synthesis of nitric oxide by host cells can deprive the parasite of essential amino acids and prevent parasite growth *in vitro*[554]. *In vivo*, iNOS knockout mice succumb to infection during the early chronic phase [322]. Of the myeloid cells that were able to infiltrate the brain, fewer of these cells were making iNOS in the absence of IL-33 signaling (Figs 3.7F-I). Interestingly, we did not detect significant iNOS positivity in any tissues, aside from the brain, in acute or chronic infection (Fig 3.8). This finding is supported by

literature demonstrating that iNOS is particularly important during the chronic phase, but not the acute phase of *T. gondii* infection[322], for reasons that are unclear. No defects in myeloid cell number were found at baseline in uninfected *il1rl1*$^{-/-}$ mice, in peripheral tissues during acute infection, including in the peritoneum, blood, and spleen, or in peripheral tissues during chronic infection (Fig 3.8). In sum, these results, in conjunction with T cell deficits, demonstrate that IL-33 signaling impacts the presence and function of immune cell populations that are necessary for controlling *T. gondii* infection in the brain.

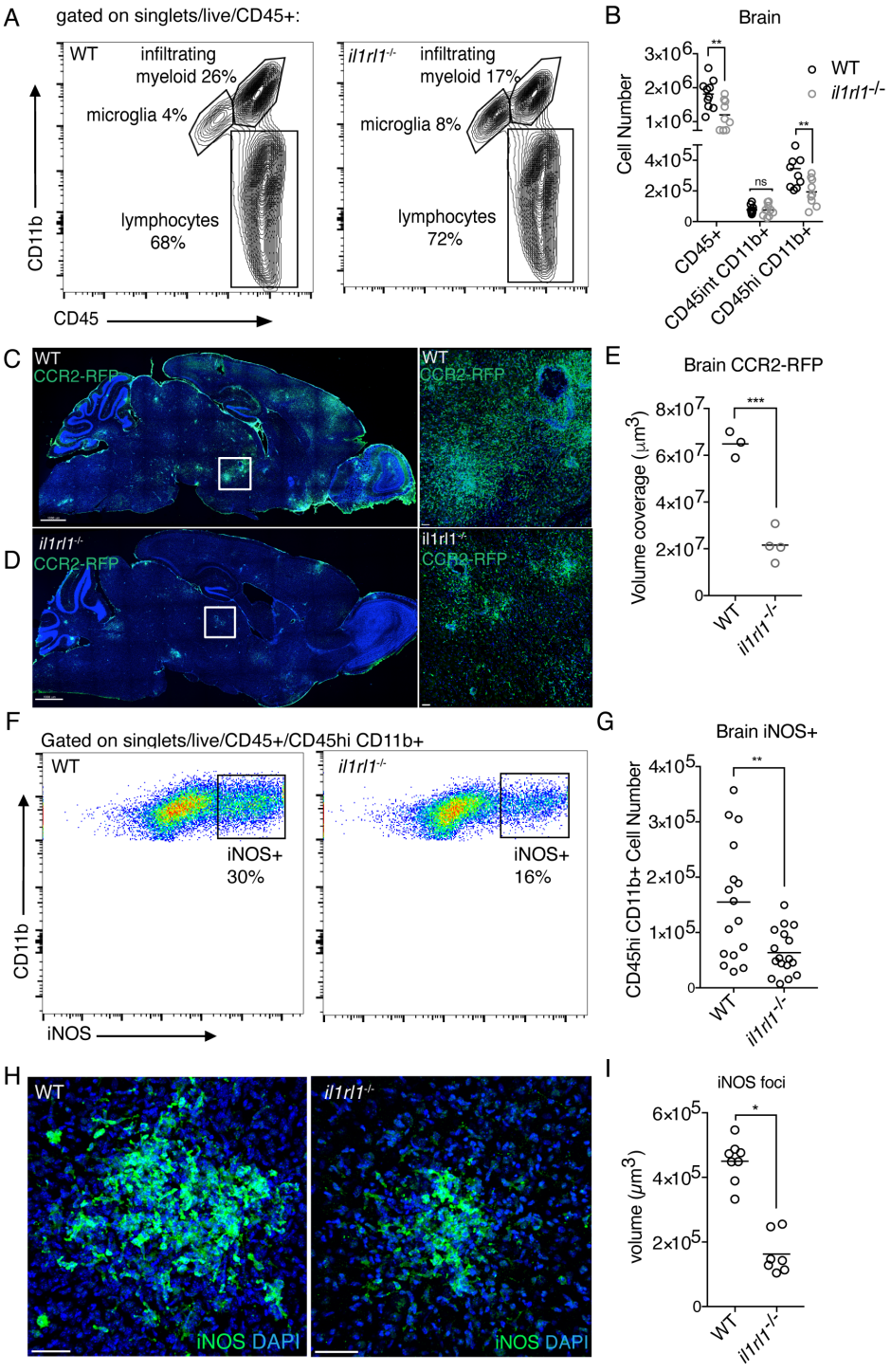
A
gated on singlets/live/CD45+:
WT
infiltrating myeloid 26%
microglia 4%
lymphocytes 68%
il1rl1-/-
infiltrating myeloid 17%
microglia 8%
lymphocytes 72%
CD11b
CD45
B
Brain
Cell Number
WT
il1rl1-/-
CD45+
CD45int CD11b+
CD45hi CD11b+
ns
C
WT
CCR2-RFP
D
il1rl1-/-
CCR2-RFP
E
Brain CCR2-RFP
Volume coverage (μm3)
F
Gated on singlets/live/CD45+/CD45hi CD11b+
iNOS+ 30%
iNOS+ 16%
iNOS
G
Brain iNOS+
CD45hi CD11b+ Cell Number
H
iNOS DAPI
I
iNOS foci
volume (μm3)

Fig 3.7. IL-33 signaling is required for the recruitment and anti-parasitic function of peripheral myeloid cells in the brain during chronic *T. gondi* infection

(A and B) Representative flow cytometry plots (A) and quantification (B) of CD11b+ myeloid cells in the brain 4 weeks post infection. CD45hi expression was used to differentiate infiltrating myeloid cells from CD45int microglia. **(C-E)** Visualization (C and D), and quantification (E) of infiltrated CCR2+ monocytes by fluorescence confocal microscopy of **i**nfected CCR2-RFP reporter mice. *il1rl1*-deficient mice were crossed to CCR2-RFP mice to assess the contribution of IL-33-*il1rl1* signaling to monocyte recruitment. **(F and G)** Representative flow cytometry plots (F) and quantification (G) of iNOS+ CD45hi, CD11b+ infiltrating cells in the brain. **(H and I)** Visualization (H) and quantification (I) of the size of iNOS foci in brain tissue by fluorescence confocal microscopy. Statistical significance was determined by randomized block ANOVA when two or more experiments were pooled (B, G, I), and by a two-tailed t-test (E). *= $p<.05$, **= $p<.01$, ***= $p<.001$. Scale bars indicate 2000μm (C,D) and 50μm (H).

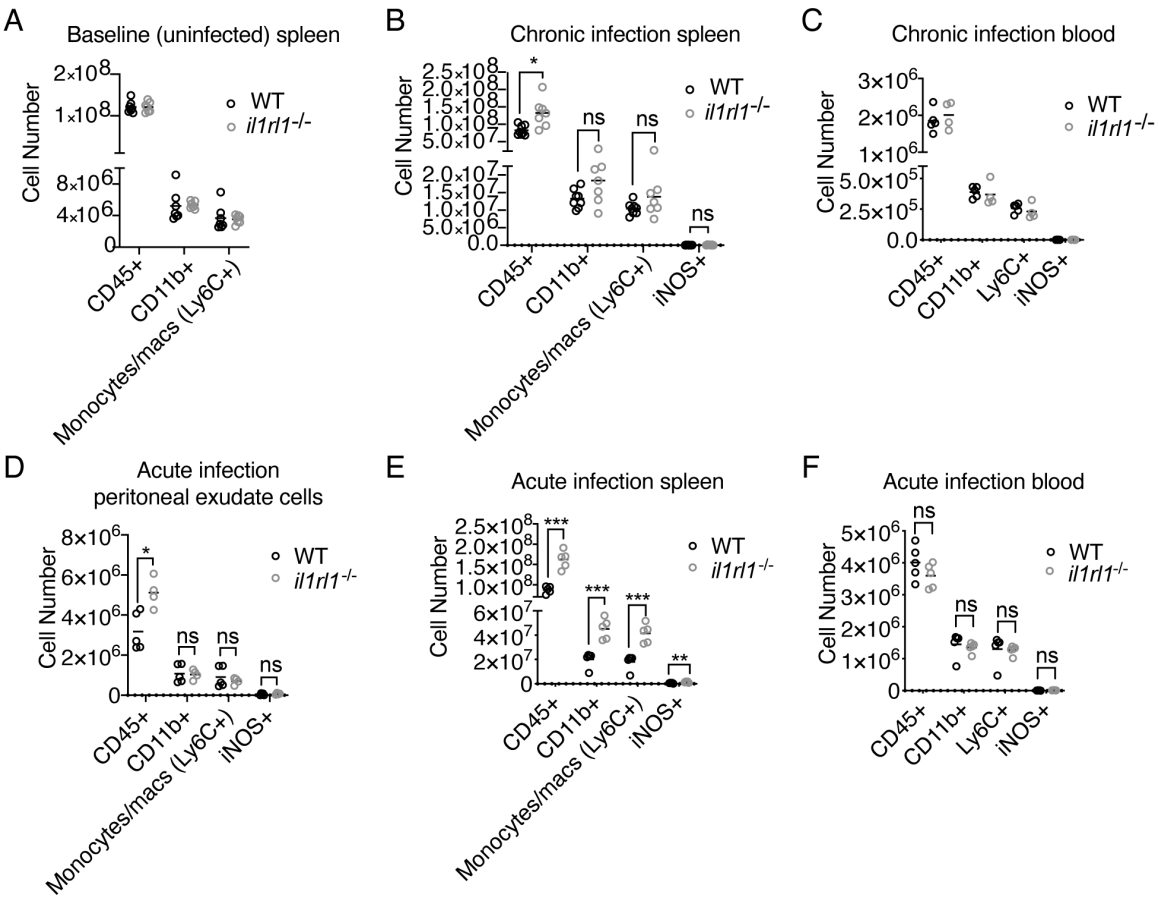

Fig 3.8. The myeloid cell response is intact in peripheral tissues and during acute infection in the absence of IL-33 signaling

(A) Assessment of spleen myeloid cell numbers in *il1rl1*-deficient mice prior to infection by flow cytometry. **(B** and **C)** assessment of peripheral tissue myeloid cell numbers and activation, including spleen (B) and blood (C) by flow cytometry 4 weeks post infection. **(D-F)** Myeloid numbers at day 10 during acute infection by flow cytometry in the peritoneum (D), spleen (E) and blood (F). Statistical significance was determined by randomized block ANOVA when two experiments were pooled (A and B), or by two-tailed t-test (C-F) *= p<.05, **= p<.01, ***= p<.001.

Finally, because we noted defects in both IFN-γ+ T cells and iNOS+ macrophages in *il1rl1*$^{-/-}$ mice, we wanted to better understand the kinetics of the reliance of immune cell populations on IL-33 signaling. Thus, we performed a time course and assessed T cell and myeloid cell numbers and activation in the brain at various stages of infection. We found that at day 12 post-infection, when significant immune populations

are first present in the brain, immunity was largely intact in *il1rl1*$^{-/-}$ mice, measured by IFN-γ+ T cells and INOS+ myeloid cells (Figs 3.9A-E). By day 21, IFN-γ+ T cell numbers were significantly reduced in *il1rl1*$^{-/-}$ mice in comparison to wildtype mice, and infiltrating myeloid cell numbers are reduced as well (Figs 3.9A-E). However, at day 21 post-infection, iNOS frequencies were intact in *il1rl1*$^{-/-}$ mice (Fig 3.9J), in contrast to a significant decrease of iNOS frequency in *il1rl1*$^{-/-}$ mice at day 28 post infection (Figs 3.7F and 3.7G). These results indicate that iNOS expression is one of the last phenotypes to be affected by IL-33 signaling.

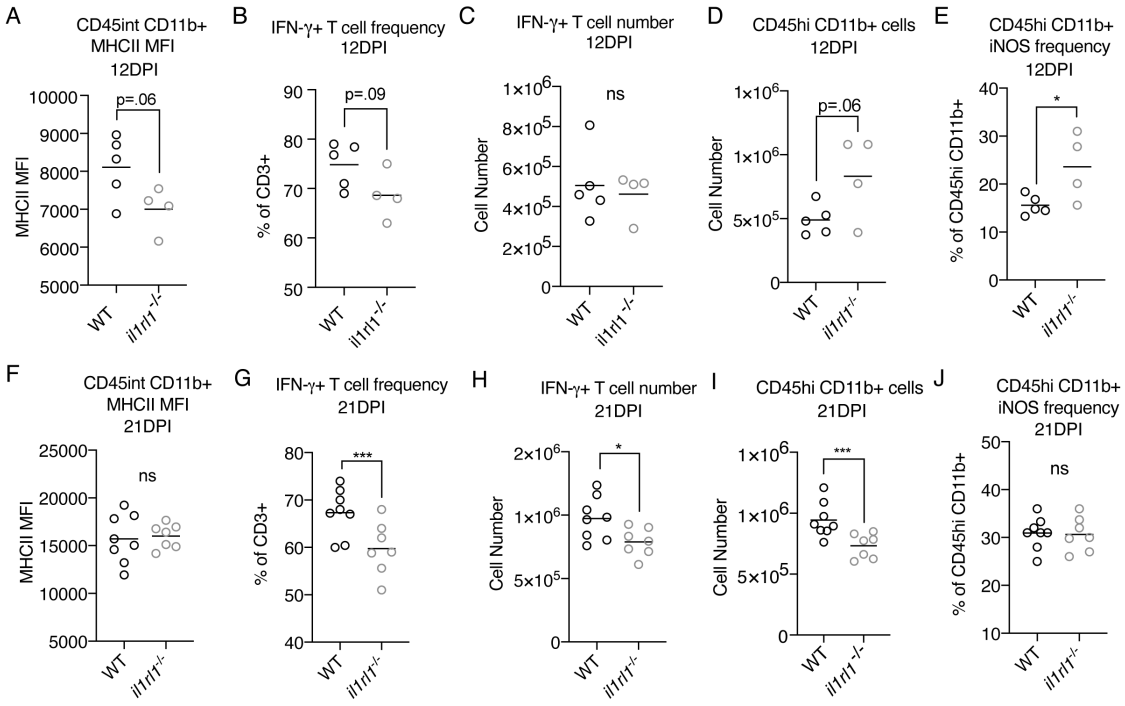

Fig 3.9. Time course of *il1rl1*$^{-/-}$ brain immune cell populations during infection

(A-J) Assessment of T cell and myeloid cell number and activation in the brain by flow cytometry at 12 days post infection (12DPI) (A-E) or 21 days post infection (21DPI) (F-J). Statistical significance was determined by two-tailed t-test (A-E), or by randomized block ANOVA when two experiments were pooled (F-J) *= p<.05, **= p<.01, ***= p<.001.

IL-33 signaling induces expression of factors involved in recruitment of immune cells to the brain

Next, we asked if IL-33 signaling was changing the environment within the brain to make the tissue conducive to immune cell recruitment. Initiation and maintenance of an immune response in the brain requires expression of cytokines, chemokines, adhesion factors, and factors which promote the entry of and maintain proliferation of immune cells[123]. It is well known that *T. gondii* infection induces many of these factors[375,391,392,394],which we validated by whole brain qRT-PCR. We find a profound increase in brain *ccl2*, *cxcl9*, *cxcl10*, *ccl5*, *cxcl1*, *vcam*, and *icam* expression over uninfected controls (Fig 3.10A). When we assessed the same genes in infected *il1rl1*-deficient mice, we saw significantly decreased expression of the chemokines *ccl2*, *cxcl10*, and *cxcl1* (Fig 3.10B), along with smaller reductions in the expression of the adhesion factors *vcam* and *icam* (Fig 3.10B). Interestingly, *ccl2* and *cxcl10* expression has been attributed to astrocytes by *in situ* hybridization during *T. gondii* infection[391]. Although *cxcl1* has not been extensively studied in chronic *T. gondii* infection, it has also been reported in astrocytes during neuroinflammation[555]. However, we did not observe an effect of IL-33 signaling on *cxcl9* expression, a chemokine which is made by PU.1-expressing cells rather than astrocytes[556] (Fig 3.10B). These results suggest that IL-33 signaling, either directly or indirectly, could be inducing chemokine expression in astrocytes during *T. gondii* infection.

We next wanted to identify the cellular source of chemokine in infected brain tissue to better understand where IL-33 was exerting its effects. We focused on studying the expression pattern of a chemokine whose transcript levels were altered to the greatest

degree in the absence of IL-33 signaling, the monocyte chemoattractant *ccl2*. We used immunofluorescence microscopy to image the brain tissue of chronically infected *ccl2*-mCherry reporter mice[557]. We observed *ccl2*-mCherry expression in "hotspots" throughout the brain, implicating a local response to signals in brain tissue (Fig 3.10C). We validated by immunohistochemistry that *ccl2* expression was greatly reduced in *il1rl1*$^{-/-}$ mice by crossing our *ccl2*-mCherry reporters to an *il1rl1*-deficient background (Figs 3.10C-E). Specifically, *ccl2* foci in *il1rl1*$^{-/-}$ mice were much reduced in size compared with wildtype infected mice (Figs 3.10D and 3.10E), while *ccl2* expression was unchanged between groups at baseline in uninfected mice (Fig 3.11A). Of note, we also observed astrocytes to co-express IL-33 and *ccl2*, but only approximately 25% of *ccl2*+ astrocytes in the infected cortex expressed IL-33 (Figs 3.11B and 3.11C). Although multiple signals undoubtedly converge to induce chemokine expression during infection, including other innate cytokines[374], these data suggest that IL-33 is a major contributor to the induction of local *ccl2*, supporting a role for IL-33 in inducing chemokine production to promote immune cell entry to the brain.

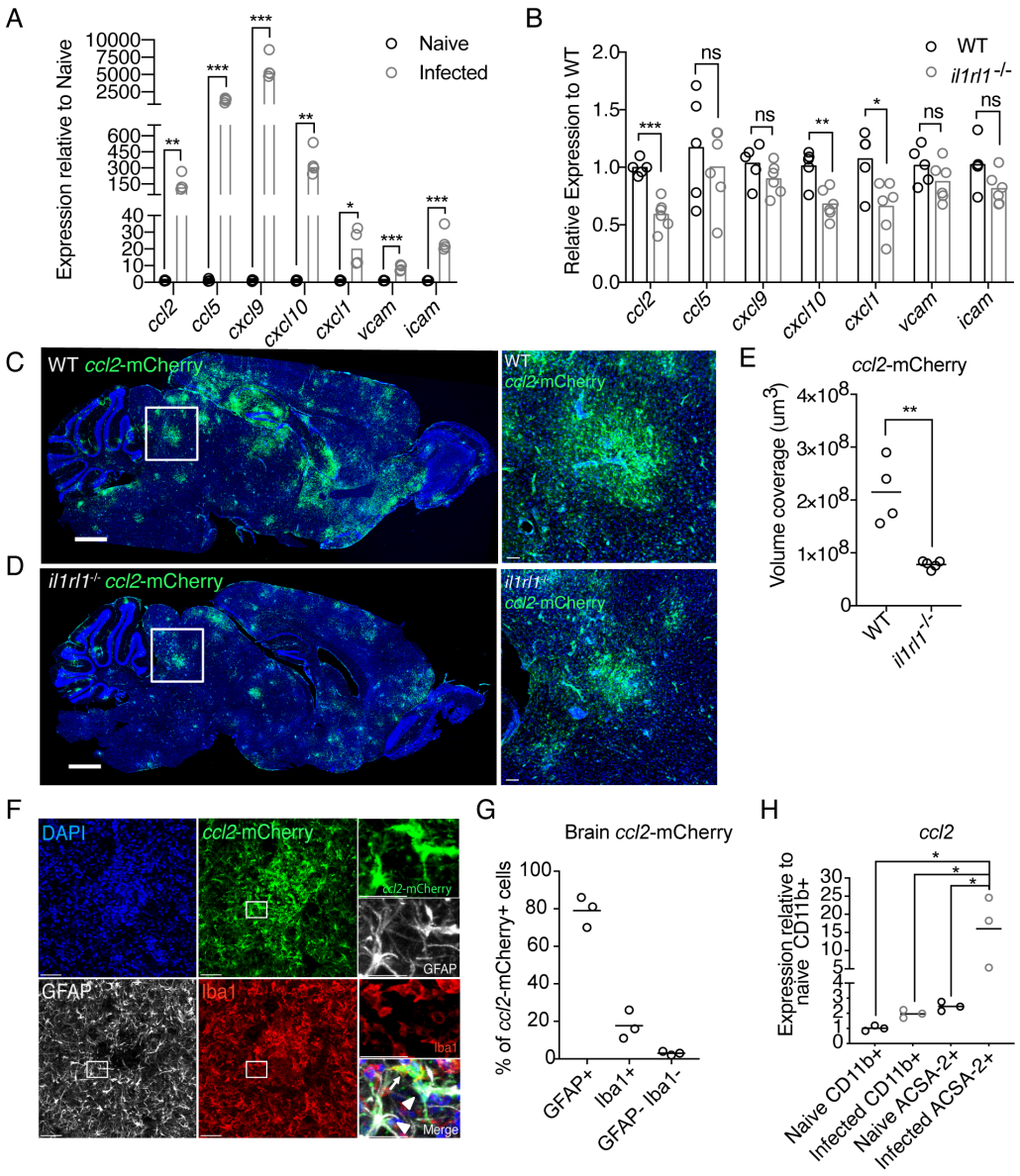

Fig 3.10. IL-33-*il1rl1* signaling induces factors which recruit immune cells to the brain

(A and B) Real time PCR from whole brain homogenate was used to assess changes in chemokine and adhesion factor gene expression from naïve to infected animals (A) and between wildtype and *il1rl1*-deficient animals (B) at four weeks post infection. **(C-E)** Visualization (C,D) and quantification (E) of *ccl2*-RFP reporter expression by mCherry staining (green) in infected brain tissue by confocal fluorescence microscopy. **(F and G)** Breakdown of *ccl2*-mCherry reporter positivity by cell type using confocal microscopy. mCherry (green) is co-localized with Iba1+ macrophages (red) or activated, GFAP+ astrocytes (white). **(H)** Cell-type specific magnetic enrichment for myeloid cells (CD11b+) or astrocytes (CD11b- and ACSA-2+) in naïve and chronically infected brain tissue. Single cell suspensions of enriched cells were resuspended in Trizol, RNA extracted, and run by real time PCR for *ccl2* expression. Statistical significance was determined by two-tailed t-test (A, B, E) or One-way ANOVA with Tukey's test (H). *= p<.05, **= p<.01, ***= p<.001. Scale bars indicate 2000µm (C,D) and 50µm (F).

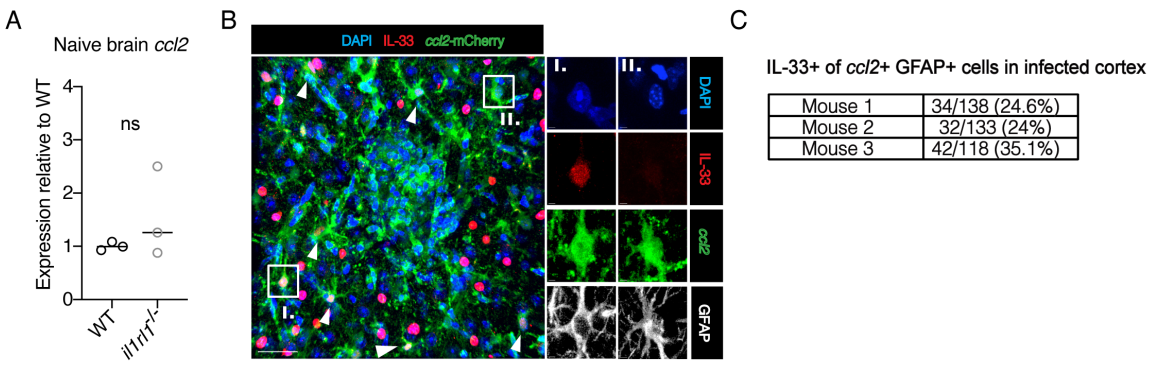

Fig 3.11. Characterization of *ccl2* expression in naïve mice, and co-expression of *ccl2* and IL-33 by astrocytes

(A) Real-time PCR analysis of whole-brain *ccl2* expression in naïve WT and *il1rl1*$^{-/-}$ mice. **(B)** Colocalization, denoted by white arrowheads, of nuclear IL-33 protein (red) with *ccl2* (green), both expressed by GFAP+ astrocytes (insets, white) by confocal fluorescence microscopy. **(C)** Quantification of frequency of colocalization of IL-33 and *ccl2* in cortical astrocytes. Statistical significance was determined by two-tailed t-test (A) *= p<.05, **= p<.01, ***= p<.001. Scale bars indicate 30μm and 3μm (A, insets).

To get a better understanding of which cells might be responding directly or indirectly to IL-33 to induce *ccl2* expression, we next assessed which cells were producing the *ccl2*, and found that *ccl2*-mCherry in infected brain tissue colocalized with both GFAP+ astrocytes and Iba1+ macrophages (Fig 3.10F). Approximately 75% of cells expressing *ccl2* were astrocytes, 22% were Iba1+ macrophages, and 3% of cells did not co-stain with either of these markers (Fig 3.10G). These results were validated by assessing *ccl2* expression in brain cells magnetically enriched for either astrocytes or macrophages. To enrich for astrocytes, we used a previously validated protocol, which first removes CD11b+ cells by negative magnetic isolation, followed by positive isolation of ACSA-2 positive cells[558] (Fig 3.12A-C). At the same time, we enriched for CD11b+ myeloid cells by keeping the CD11b+ magnetically-enriched fraction (Fig3.12 A-C). RNA from these cell populations showed a 10-fold higher *ccl2* expression in astrocytes compared with

macrophages during chronic infection (Fig 3.10H). Thus, because *ccl2* expression is dependent on IL-33 signaling, and because astrocytes are the major producer of *ccl2* in the infected brain, we hypothesized that IL-33 can signal directly on astrocytes to induce chemokine expression, or signals through an intermediate cell type to impact astrocytic chemokine expression.

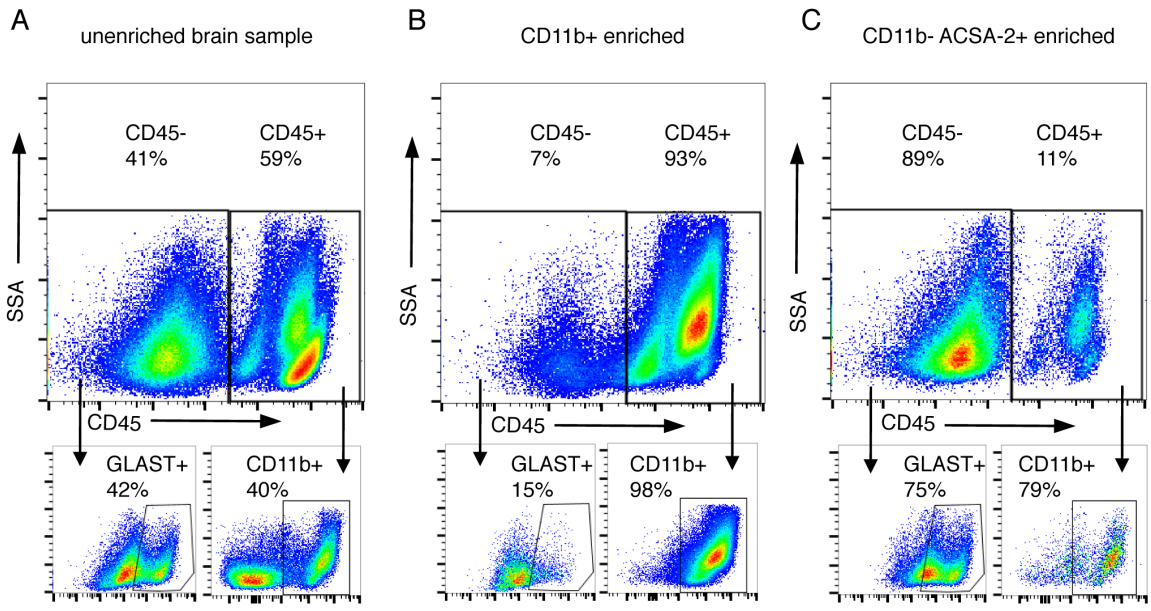

Fig 3.12 Magnetic enrichment for myeloid cells or astrocytes from infected brains

(A) Unenriched single cell suspension of all purified cells from infected brain tissue 4 weeks post infection. **(B and C)** Assessment of purity achieved by enriching for myeloid cells using CD11b+ magnetic beads (B), or astrocytes (C), by negatively selecting for myeloid cells using CD11b+ magnetic beads, followed by positive selection for astrocytes with ACSA-2+ magnetic beads.

IL-33 signals on a radio-resistant cell type to control chronic *T. gondii* infection

In order to further understand IL-33 signaling in the brain, we began to narrow down the cell type(s) that directly responds to IL-33 to support protective immune responses. In studies concerning IL-33 during CNS disease, genetic evidence for a responding cell type to IL-33 is lacking. This is likely due in part to the fact that several

brain-resident cells can express ST2 (*il1rl1)* during disease, such as microglia, astrocytes, and endothelial cells[509,525,559,560], but a wide range of immune cells can also express the receptor, including but not limited to type 2 innate lymphoid cells (ILC2s), regulatory T cells, Th2 cells, mast cells, and macrophages[249,275,415]. During *T. gondii* infection, we did not detect ST2 expression on the immune cells present in highest numbers in the infected brain – effector T cells and monocyte-derived myeloid cells (Fig 3.13). We did, however, detect ST2 expression on ILC2s, which decreased signficiantly in frequency from uninfected to infected brain tissue, as well as mast cells, and regulatory T cells (Fig 3.13).

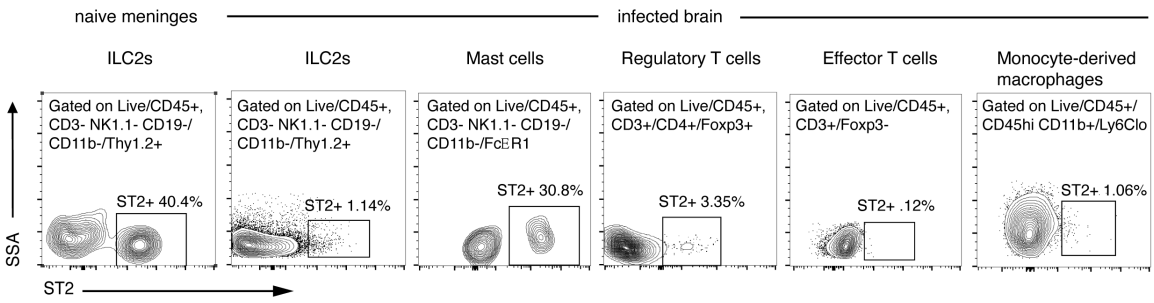

Fig 3.13 Characterization of ST2 expression on immune cells in the *T. gondii*-infected brain

Detection of ST2 expression by flow cytometry of type 2 innate lymphoid cells, mast cells, regulatory T cells, effector T cells, and monocyte-derived macrophages in 4wk *T. gondii*-infected brain tissue.

To determine if IL-33 signals on a radio-sensitive or a radio-resistant cell type to exert its effects during chronic *T. gondii* infection, we lethally irradiated wildtype and *il1rl1*$^{-/-}$ mice and reconstituted these mice with bone marrow from either wildtype donors or *il1rl1*$^{-/-}$ donors (Fig 3.14A). Blood was assessed for reconstitution prior to infection (Figs 3.14B-D), and in all cases, immune cells were >90% positive for the congenic CD45

marker of donor mice, confirming successful reconstitution of the chimera (Figs 3.14B-D). We note that irradiation did alter the severity of infection, resulting in high parasite burdens in all groups, and a high number of infiltrating cells (Figs 3.14E-G) Nonetheless, these experiments revealed that *il1rl1*-deficiency on radio-resistant cells, or in il1rl1$^{-/-}$ recipient, but not donor mice, recapitulated the phenotype of global knockouts, including reduced infiltrating myeloid and T cell numbers, and parasite burden, whereas *il1rl1* expression on donor bone marrow, was dispensable (Figs 3.14E-G). These data demonstrate that IL-33 signals predominantly on non-hematopoeitic cells to control brain *T. gondii* infection, thus largely ruling out immune cells as responders.

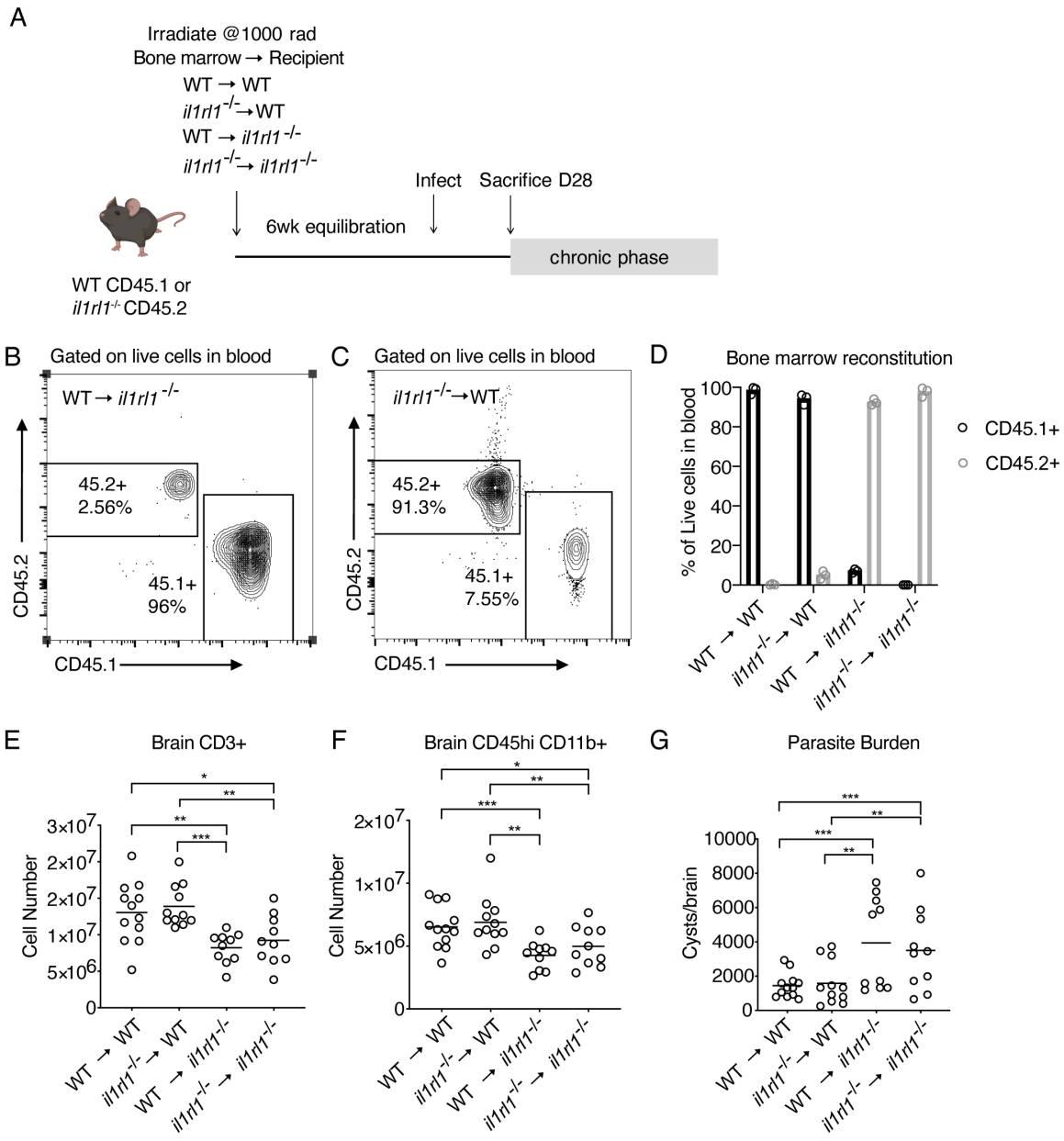

Fig 3.14 IL-33 signals on a radio-resistant responder

(A) Bone marrow chimera experimental setup. **(B-D)** Representative flow cytometry plots (B,C) and quantification (D) of bone marrow reconstitution in blood 6 weeks post irradiation and prior to infection. **(E and F)** Quantification of immune cells, including T cells (E) and infiltrating myeloid cells (F) in the brain at 4 weeks post infection following irradiation. **(G)** Parasite burden as assessed by cyst count of brain homogenate. Statistical significance was determined by randomized block ANOVA (E-G), each panel showing data pooled from two independent experiments *= p<.05, **= p<.01, ***= p<.001.

Importantly, microglia are not fully replenished by bone marrow-derived cells post-irradiation. Therefore, we cannot rule out microglia as responding to IL-33 in the brain. In addition, microglia highly express *il1rl1* at baseline[250,271,509,525], prior to chronic infection. To better understand which radio-resistant cells were capable of responding to IL-33, we assessed *il1rl1* expression on microglia/macrophages, and ACSA-2+ astrocytes, which have also been shown to express the IL-33 receptor, especially during disease[271,509,525]. To do this, we magnetically enriched for these cell types from naïve and chronically infected brain tissue, and found that microglia/macrophages cells express *il1rl1* at high levels in uninfected mice, but downregulate the IL-33 receptor 20-fold upon infection, while astrocytes express low levels at baseline and increase receptor expression with infection (Fig 3.15A). This result indicates a change in capability of cells which are able to respond to IL-33 prior to and following infection.

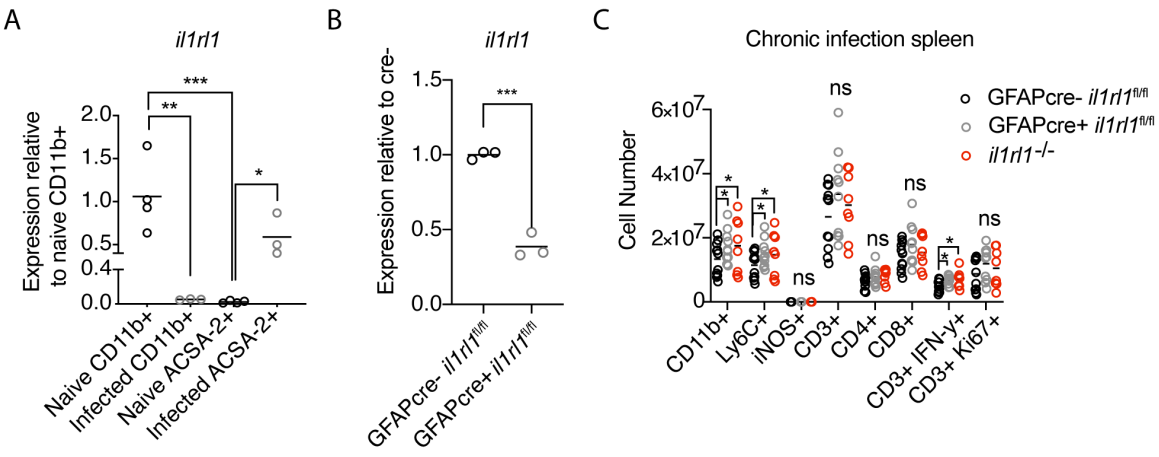

Fig 3.15 Astrocyte *il1rl1* expression with infection, and astrocytic *il1rl1*-dependence for peripheral immune cell numbers

(A) Cell-type specific magnetic enrichment for myeloid cells (CD11b+) or astrocytes (CD11b- and ACSA-2+) in naïve and chronically infected brain tissue. Single cell suspensions of enriched cells were resuspended in Trizol, RNA extracted, and measured by real time PCR for *ilrl1*(st2) expression. (B) Validation of excision of *il1rl1* from magnetically-enriched astrocytes in GFAPcre *il1rl1*$^{fl/fl}$ mice by quantitative PCR **(C)** Assessment of spleen immune cell numbers by flow cytometry four weeks post infection. Statistical significance was determined by one-way ANOVA with Tukey's test (A), a two-tailed t-test (B), or a randomized block ANOVA (C) *= p<.05, **= p<.01, ***= p<.001.

Other brain resident cells, such as endothelial cells, neurons, and oligodendrocytes may express the IL-33 receptor during *T. gondii* infection and contribute to immunity. We chose to focus on microglia/macrophages and astrocytes because they have been previously reported to be the predominant cells types that express the IL-33 receptor[181,250,271,451,509,525,559], are major producers of IL-33-dependent chemokine during *T. gondii* infection [391] (Figs 3.10G and 3.10H), and are required for control of *T. gondii* infection[289,406].

IL-33 signaling on astrocytes, but not microglia/macrophages, potentiates immune responses and limits parasite burden

To determine if IL-33 directly signals on microglia/macrophages or astrocytes, we crossed *il1rl1*$^{fl/fl}$ mice[422], to either constitutive CX3CR1cre mice or GFAPcre mice, respectively. We observed that deletion of *il1rl1* from macrophages did not affect any major phenotype observed in *il1rl1*$^{-/-}$ knockout mice, including brain myeloid cell number and function, T cell number, or parasite burden (Fig 3.16A-C). We observed minor defects in CD4+ T cell number in these mice, including CD4+ T cell proliferation (Fig 3.16A). When we deleted *il1rl1* from astrocytes (Fig 3.15B), while myeloid cell recruitment and activation was relatively unaffected, the adaptive immune response was impacted, including reduced T cell proliferation and IFN-γ production, in mice with IL-33R-deficient astrocytes (Figs 3.16D and 3.16E). In contrast with CX3CR1cre mice, GFAPcre *il1rl1*$^{fl/fl}$ mice exhibited stronger CD8+ T cell proliferation deficits than CD4+ T cells (Fig 3.16D), perhaps suggesting an impact of IL-33 signaling on antigen presentation. Importantly, aligned with a decrease in T cell-derived IFN-γ, which is critical for control of infection[67], GFAPcre *il1rl1*$^{fl/fl}$ mice displayed increased parasite burden (Fig 3.16F). GFAPcre *il1rl1*$^{fl/fl}$ mice did not have defects in immune cell compartments in other tissues, such as the spleen, displaying increased immune cell numbers in the spleen during chronic infection (Fig 3.15C), to a level comparable with whole-body *il1rl1*-deficiency. Collectively, these results show that astrocytes respond to a damage signal, IL-33, to promote a protective immune response to *T. gondii* in the brain.

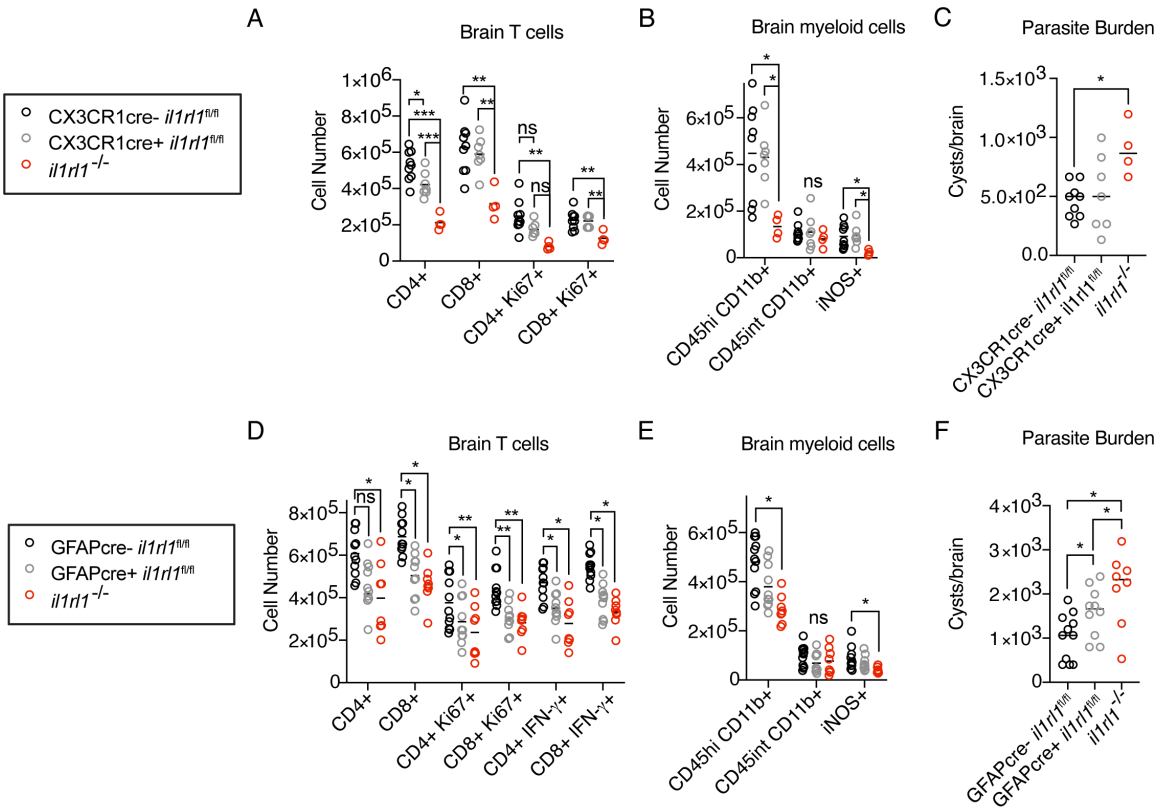

Fig 3.16 IL-33 signaling on astrocytes, but not macrophages, is required to control brain *T. gondii* infection

(A and B) Quantification of brain immune cells in CX3CR1cre *il1rl1*$^{fl/fl}$ mice by flow cytometry – including T cell subsets (A) and myeloid cells (B) at four weeks post infection. **(C)** Parasite burden in CX3CR1cre *il1rl1*$^{fl/fl}$ mice as assessed by cyst enumeration in brain homogenate. **(D and E)** Quantification of brain immune cells in GFAPcre *il1rl1*$^{fl/fl}$ mice by flow cytometry – including T cell subsets (D) and myeloid cells (E). IFN-γ was measured following *ex-vivo* re-stimulation of brain cells, incubated with Brefeldin A, and PMA/ionomycin for 5 hours at 37°C. **(F)** Parasite burden in GFAPcre *il1rl1*$^{fl/fl}$ mice as assessed by cyst enumeration in brain homogenate. Statistical significance was determined by one-way ANOVA with Tukey's test (A-C), or by randomized block ANOVA(D-F), where each panel shows data pooled from two independent experiments *= p<.05, **= p<.01, ***= p<.001.

3.2 *Astrocyte-derived ccl2 is critical for controlling chronic T. gondii infection*

3.2.1 *Project rationale*

Our published work outlined in the previous section demonstrates that astrocytes can respond to IL-33 to promote a protective immune response to *T. gondii*. However, it is unclear what astrocytes can do to help control *T. gondii* infection. One study published in 2016 deleted STAT1 from astrocytes, preventing them from responding to the critical anti-parasitic cytokine IFN-γ[406]. The mice succumbed to infection during the chronic phase, harboring increased parasite load, especially within astrocytes[406]. Interestingly, it is difficult to find infected astrocytes in wildtype animals[92], which suggests that normally, astrocytes clear parasite efficiently in response to IFN-γ. Thus, astrocytes can respond to IFN-γ and are important for controlling infection. But what astrocytes can do to support the immunity is still an open question, since immune cells were recruited in adequate, if not increased, numbers in these mice[406]. In fact, there is limited literature surrounding the capability of astrocytes to respond to any infection in the brain. This may be due to a lack of understanding of what machinery astrocytes possess to activate immune cells, such as antigen presentation capability, cytokine production, etc.

Chemokine is by far the best characterized function astrocytes employ to support immunity. During *T. gondii* infection, *in situ* hybridization has shown that astrocytes are major producers of *cxcl10* and *ccl2*, two critical chemokines for bringing monocytes and T cells into the brain[132,375,391]. Other disease models have demonstrated that astrocytes are major producers of these cytokines *in vivo*, including in EAE, injury, infection, and

others[385,561-567]. Astrocytes have also been consistently shown to express *cxcl1, cxcl2, ccl8, ccl20, cxcl12, and cxcl8 in vivo* in a variety of disease states[555,561,568-574].

To begin to answer the overarching question of how astrocytes promote immunity to *T. gondii*, we first focused on chemokine. Our data with chemokine *ccl2*-mcherry reporter mice suggests that astrocytes produce ~75% of the *ccl2* in the brain (Fig 3.10G), but it is unclear how important chemokine is from astrocytes alone in recruiting and activating immune cells[385,386,575]. Studies from EAE have shown that conditional deletion of *ccl2* from astrocytes results in reduced infiltration of myeloid cells and T cells. To answer this question in *T. gondii* infection, we crossed *ccl2*$^{fl/fl}$ mice to GFAPcre mice, to delete *ccl2* from astrocytes and assessed reduction in *ccl2*, immune cell numbers and activation, localization of immune cells, and parasite burden. These results serve as the beginning of our understanding of how astrocytes promote immunity to *T. gondii* infection, and we plan to follow up with unbiased profiling of astrocyte gene expression during infection to better understand other machinery astrocytes use to promote immunity.

3.2.2 *Results*

To assess the importance of astrocyte-derived chemokine in controlling *T. gondii* infection, we generated mice with conditionally deleted *ccl2* under the GFAP promoter. While wildtype infected *ccl2* reporter mice displayed significant colocalization between *ccl2* and GFAP, accounting for approximately 75% of *ccl2*+ cells (Fig 3.10F and 3.10G), there was no colocalization in GFAPcre *ccl2*$^{fl/fl}$ mice, demonstrating efficacy of deletion of *ccl2* from astrocytes (Fig 3.17B). Remaining *ccl2*+ cells in these mice appeared to be Iba1+ macrophages (Fig. B), a population that makes up only 25% of *ccl2* positivity in wildtype

mice. Next, we assessed *ccl2* expression levels in infected brain tissue by real time PCR, and found that there was a 70% reduction in total brain *ccl2* in GFAPcre+ mice. This result demonstrates that astrocytes are indeed a major producer of this chemokine, and that there is not a significant amount of compensation that occurs when astrocytes are unable to make it. It follows that *ccr2* expression was reduced by half in the brain, suggesting a striking inability to recruit *ccr2*+ cells (Fig 3.17C).

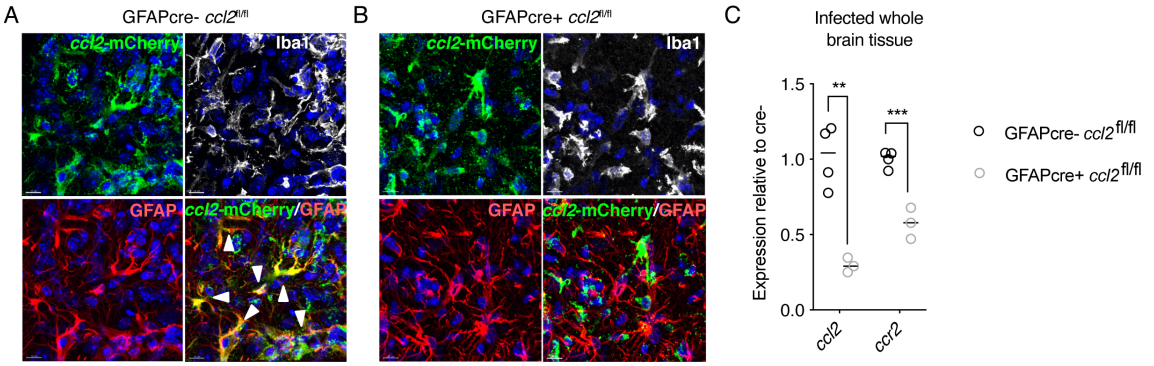

Fig 3.17 Deletion of *ccl2* from astrocytes

(A and B) Representative images of *ccl2*-mcherry reporter expression (green) colocalized with GFAP+ astrocytes (red), white arrows, in wildtype mice (A), while *ccl2* is solely colocalized with Iba1+ macrophages (white) in GFAPcre+ $ccl2^{fl/fl}$ animals (B). **(C)** whole brain homogenate qPCR assessing *ccl2* and *ccr2* expression when *ccl2* has been deleted from astrocytes (C). Statistical significance was determined by a two-tailed t-test (C), *= p<.05, **= p<.01, ***= p<.001.

Next, we measured immune cell numbers in brain tissue of chronically infected GFAPcre+ $ccl2^{fl/fl}$ animals. We observed significant reductions in myeloid cells recruited from the blood, which are traditionally CD45hi CD11b+, while numbers of CD45int CD11b+ cells, typically characterized as mature macrophages/microglia, were unaffected in comparison to wildtype animals. Subsets of infiltrating myeloid cells, including Ly6Chi monoyctes and Ly6G+ neutrophils, which are classically defined to express CCR2[576-578], were reduced by approximately half in GFAPcre+ $ccl2^{fl/fl}$ mice (Fig 3.18A). Unexpectedly, we also

observed a dramatic decrease of T cell numbers in the brains of these mice, including CD4+ and CD8+ subsets and regulatory T cells (Fig 3.18B). It is currently unclear if T cells express CCR2 during *T. gondii* brain infection and how large this population is. Deletion of *ccl2* from astrocytes also resulted in a doubling of parasite burden in brain tissue (Fig 3.18C), suggesting that parasite is unable to be fully controlled in the absence of astrocytic *ccl2*.

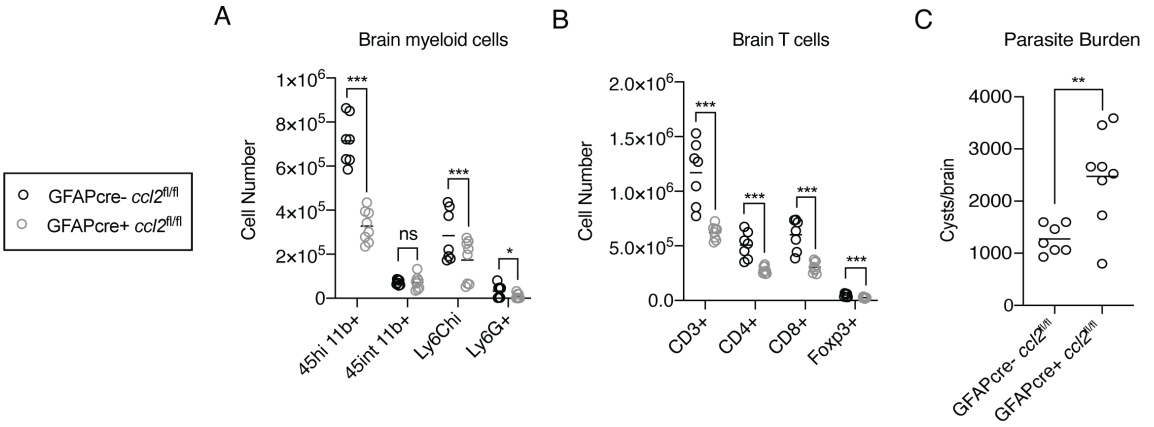

Fig 3.18 Astrocyte-derived *ccl2* is critical for recruiting immune cells to the brain and controlling chronic *T. gondii* infection

(A and B) Quantification of brain immune cells by flow cytometry 4wks-post *T. gondii* infection, of myeloid cell subsets (A) and T cell subsets (B). **(C)** Assessment of parasite burden by cyst enumeration from brain homogenate. Two experiments were pooled and statistical significance was determined by randomized block ANOVA (C) *= p<.05, **= p<.01, ***= p<.001.

To better understand where in the body a lack of astrocyte-derived chemokine was exerting its effects, we assessed immune cell numbers in peripheral tissues. We did not see any difference between genotypes in either the spleen or the blood (Fig 3.19), suggesting

that a lack of immune cells in the brain was not a result of insufficient immune cell numbers or trafficking out of peripheral tissues.

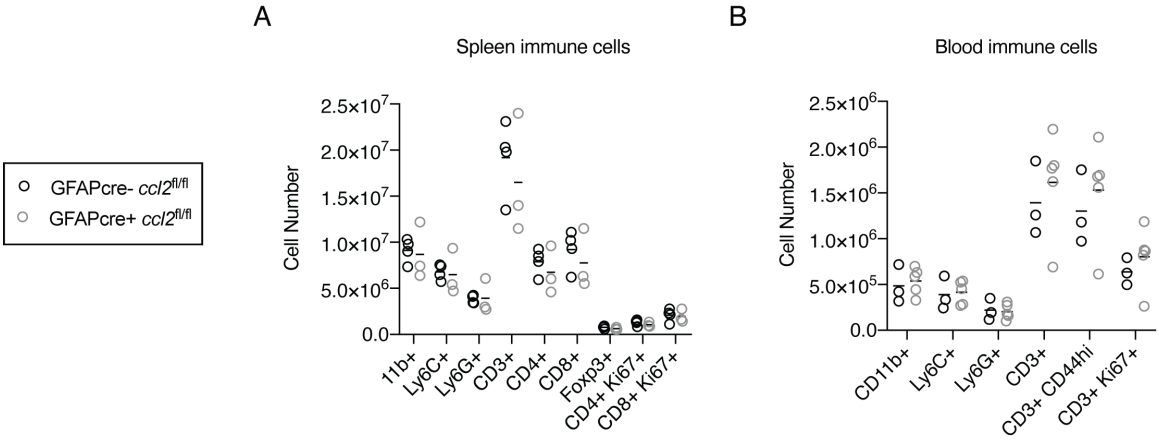

Fig 3.19 Peripheral immune cell numbers are intact during chronic infection of GFAPcre+ *ccl2*$^{fl/fl}$ animals

. (A and B) Quantification of immune cells by flow cytometry 4wks-post *T. gondii* infection, in the speen (A) and blood (B). Statistical significance was determined by a two-tailed t-test (A and B) *= p<.05, **= p<.01, ***= p<.001.

In the brain, however, we observed immune cells stacking up in the meninges and perivascular spaces of GFAPcre+ *ccl2*$^{fl/fl}$ mice, suggesting an inability of these cells to be recruited across the BBB (Fig 3.20).

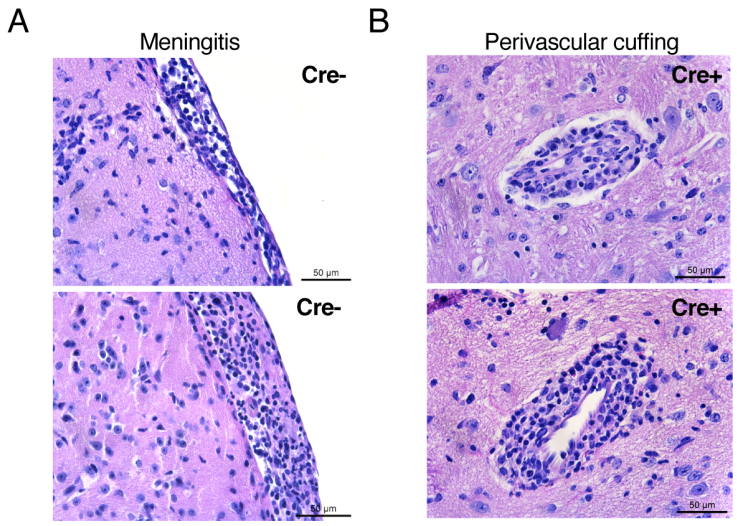

Fig 3.20 In the absence of astrocyte-derived *ccl2*, immune cells accumulate in the meninges and perivascular spaces during brain *T. gondii* infection

(A and B) Representative H&E images of immune cell accumulation in the cortical meninges (A) and in brainstem vessels (B). Scale bars indicate 50µm.

Finally, we also checked if astrocyte-derived *ccl2* impacted the function of immune cells within brain tissue. We hypothesized that *ccl2* is important for recruiting cells to areas of parasite replication, where immune cells could encounter signals – either parasite patterns, damage patterns, cytokines, etc., to become more activated and anti-parasitic. Contrary to this hypothesis we did not see a significant impact of *ccl2* on the frequency of iNOS positivity in infiltrating myeloid cells, or proliferation in T cells (Fig 3.21). This result suggests that while *ccl2* is necessary to bring immune cells into the brain, it may not be necessary for their activation state.

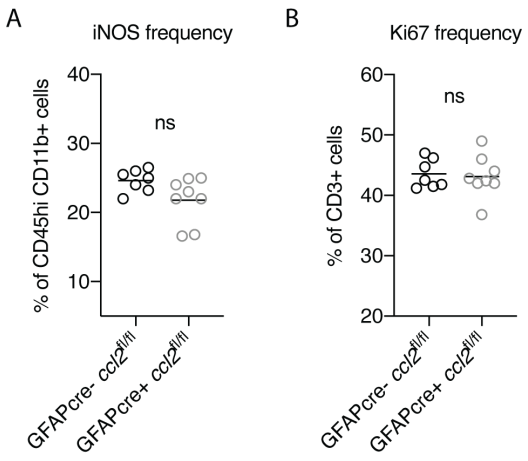

Fig 3.21 Immune cell markers of activation are unchanged in GFAPcre+ *ccl2*$^{fl/fl}$ mice

(A and B) Quantification by flow cytometry from brain tissue at 4wks post infection of iNOS+ frequency of infiltrating, monocyte derived macrophages (A) and frequency of Ki67+ proliferating T cells (B). Two experiments were pooled and statistical significance was determined by randomized block ANOVA *= p<.05, **= p<.01, ***= p<.001.

Chapter 4 – Discussion and future directions

4.1 *Summary of thesis work*

This thesis work contributes to our understanding of how invading pathogens can be sensed within the brain, a tissue with unique challenges to mounting an immune response. The blood brain barrier typically restricts immune cells from entering brain tissue, and a concerted effort of chemokine and adhesion factor expression is required to bring immune cells in[123,579,580]. The signals which precede chemokine and adhesion factor expression, however, which alert brain tissue to an invading pathogen in the first place, are undefined in many brain infections, including *T. gondii* infection. During *T. gondii* infection, we observe lesions in brain tissue where *T. gondii* is replicating, featuring an absence of brain resident cell markers. Thus, we hypothesized that *T. gondii* can cause host cell damage in the brain which can be sensed locally to recruit and/or maintain blood derived immune cells. We were further interested in studying damage signaling for its applicability to other disease states beyond *T. gondi* infection, including responses to other invading pathogens and sterile insults.

We chose to study the protein IL-33, as a candidate orchestrator of immunity to *T. gondii*, since it is highly expressed in brain tissue[230,271,509]. IL-33 signaling has also been previously shown to protect the brain from pathology during chronic *T. gondii* infection[536], but, in this study like many other CNS IL-33 studies, mechanistic insight into IL-33

signaling was lacking. For instance, it is often unclear which cell type IL-33 signals on, and whether or not this cell is brain resident or a recruited immune cell. Determining the local impact of IL-33 signaling is important, since IL-33 can also signal peripherally[249,274,275,415] during the acute stage of *T. gondii* infection. Thus, IL-33 mirrors many other pattern recognition pathways, for which it is unclear what capability the brain has to specifically detect these patterns.

In this work, we show that IL-33 is expressed by oligodendrocytes and astrocytes during *T. gondii* infection, is released locally into the cerebrospinal fluid of *T. gondii*-infected animals, and is required for control of infection. IL-33 signaling promotes chemokine expression within brain tissue and is required for the recruitment and/or maintenance of blood-derived anti-parasitic immune cells, including proliferating, IFN-γ-expressing T cells and iNOS-expressing monocytes. Importantly, we find that the beneficial effects of IL-33 during chronic infection are not a result of signaling on infiltrating immune cells, but rather on radio-resistant responders, and specifically, astrocytes. Mice with IL-33 receptor-deficient astrocytes fail to mount an adequate adaptive immune response in the CNS to control parasite burden – demonstrating, genetically, that astrocytes can directly respond to IL-33 *in vivo*. Furthermore, we find that chemokine made by astrocytes alone is enough to recruit immune cells to the brain and significantly alter control of parasite. Together, these results indicate a brain-specific mechanism by which IL-33 is released locally, and sensed locally, to engage the peripheral immune system in controlling a pathogen.

within the brain parenchyma:

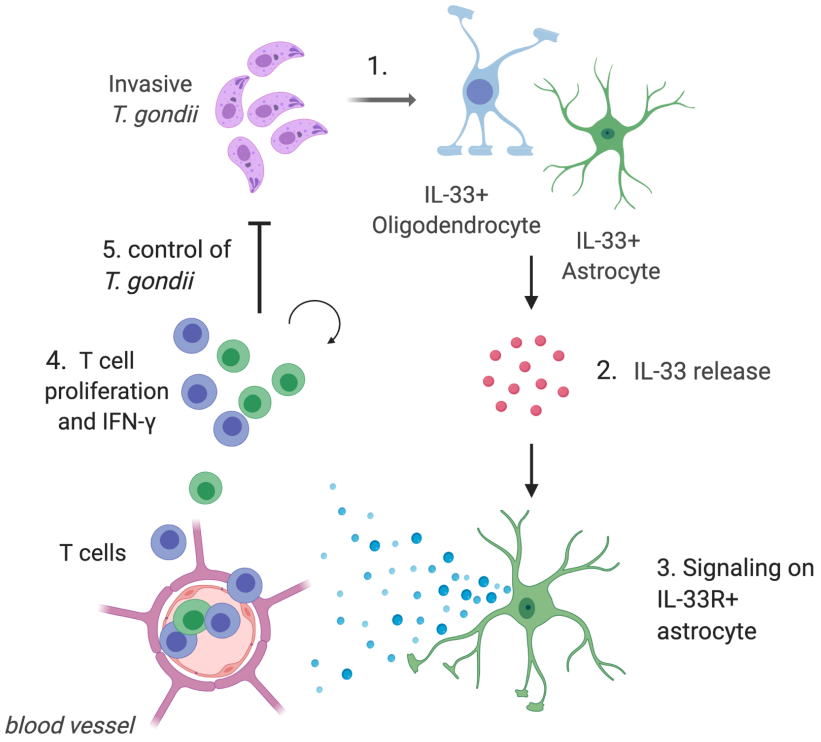

Fig 4.1 Summary schematic of thesis findings

Based on our studies, we propose that during *T. gondii* infection, (1) *T. gondii* can either through direct infection or through bystander inflammation lead to IL-33 release or secretion from oligodendrocytes and astrocytes in the brain (2). Once IL-33 is released, it can signal on IL-33R+ astrocytes (3), which promotes T cell proliferation and IFN-γ production within brain tissue (4). When astrocytes lack the ability to respond to IL-33, parasite burden is increased (5), possibly due to a defect in IFN-γ signaling, the critical regulator of immunity to *T. gondii* infection.

4.2 *How T. gondii is sensed in brain tissue – open questions*

The broadly relevant question this thesis work begins to address is how the presence of parasite is sensed within brain tissue to initiate immunity. This is a difficult question to answer for any pathogen, since most infections impact multiple tissues in the body, making it difficult to isolate the capability of the brain to respond. Our findings demonstrate that

IL-33 signaling is one pattern recognition pathway by which *T. gondii* infection can be sensed that has a local impact in the CNS – since released IL-33 can be measured in the cerebrospinal fluid during infection, IL-33 can induce local changes in chemokine within infected brain tissue, and IL-33 can signal on astrocytes to promote immunity. But there are many other pattern recognition pathways which are likely involved in sensing *T. gondii* – during acute and chronic infection. Here we review current knowledge of *T. gondii* pattern recognition, highlighting current open questions as to how the presence of *T. gondii* is recognized in brain tissue.

During acute *T. gondii* infection, circulating immune cells, namely dendritic cells and macrophages, are essential to sensing *T. gondii* patterns. It has been shown that *T. gondii* profilin protein, a pathogen-associated molecular pattern (PAMP), can be sensed directly by TLR11 and TLR12-expressing dendritic cells, which are capable of initiating protective Th1 immunity by producing IL-12[81,171,172]. Additionally, the NLRP1 and NLRP3 inflammasomes have been shown to be important for control of acute infection. NLRP1 and NLRP3-deficient animals do not survive acute infection, and additional inflammasome components caspase 1/11, and ASC are also required for mice to survive acute infection[207]. Macrophages are likely a key cell type that express NLRP1 and NLRP3 during *T. gondii* infection, since macrophages can produce cleaved IL-1β when exposed to *T. gondii*, and these inflammasome pathways can be specifically manipulated within cultured macrophages[206]. It is not clear at this time what aspect of *T. gondii* infection activates the inflammasome – whether the inflammasome is first primed by host or pathogen signals, and whether cellular stress, and in what capacity, activates these cytosolic sensors. Nevertheless, inflammasomes as a whole are likely critical to controlling acute

infection. Finally, human macrophages have been shown to sense the damage signal S100A11 – identified as a key inducer of *Ccl2* during *T. gondii* infection[246]. It is not explicitly clear if S100A11 is required for control of acute infection, since half of infected S100A11 succumb acutely, and the other half survive[295]. Nevertheless, S100A11 is one mechanism by which *T. gondii* infection can be sensed, and early chemokine production can be initiated. In summary, there are several outlined mechanisms by which acute *T. gondii* infection can be sensed and immunity can be initiated.

The brain, however, is somewhat of a mystery in terms of *T. gondii* pattern recognition for a number of reasons. First, circulating immune cells such as dendritic cells and monocyte-derived macrophages should not be present in the brain under brain under steady-state conditions[581,582]. Thus, when parasite first enters brain tissue, there are likely mechanisms that brain resident cells use to sense *T. gondii*. However, brain resident cells have not been thoroughly profiled for their ability to sense *T. gondii*. According to *in situ* hybridization and sequencing studies, microglia are the major expressers of many TLRS and NLRs at baseline[181-185,198], but whether this changes with infection, and whether microglia are indeed dominant responders during *T. gondii* infection has not been directly tested. In light of the concept that microglia might be the primary pattern recognizers in the brain, our observation that astrocytes can respond to IL-33 to initiate protective immunity during chronic *T. gondii* infection is an interesting development. This work begins to uncover the mechanisms by which the presence of *T. gondii* can be sensed specifically in brain tissue – with one caveat that we need to ensure that our GFAPcre, which we used to target astrocytes, does not impact enteric immunity to *T. gondii* acutely.

Another open question regarding initiation of immunity to *T. gondii* in the brain revolves around the fact that *T. gondii* disseminates throughout host tissue before reaching the brain. It is generally unclear to what degree peripheral immune cells are already primed before reaching the brain, and to what degree priming occurs locally within brain tissue. For myeloid cell activation, it appears that myeloid cells are instructed to make iNOS specifically within brain tissue, since iNOS$^{-/-}$ mice succumb during early chronic infection[322,331], and our studies show that iNOS cannot be detected in the blood during acute or chronic infection. Interestingly, IL-33 appears to be required for iNOS expression, since iNOS expression is significantly reduced in the absence of IL-33 signaling. In the future, it will be interesting to learn which signals in addition to IL-33 are required for myeloid cells to produce iNOS *in vivo*, and how local these signals are to areas of parasite reactivation in the brain. In terms of T cell activation, it will be interesting to separate where antigen presentation is important, which is the subject of an ongoing project in our lab (Michael Kovacs, unpublished data). In the future, our lab plans to separate the relative importance of antigen presentation in lymph nodes versus brain-specific antigen presentation. In addition, it will be interesting to study this at different time-points post infection, to understand if T cells need to be re-primed after a certain period of time post-infection.

Finally, it is remarkable that we were able to see an impact on *T. gondii* infection from losing IL-33 signaling alone, given all of the possible pattern recognition pathways that may be used in the brain to detect *T. gondii* infection. A similar result was seen with the alarmin IL-1α, which was indispensable for controlling *T. gondii* replication and recruiting innate immune cells to the brain[289]. However, lacking either of these pathways

individually did not cause mice to succumb to infection, suggesting that there are multiple pattern recognition pathways required to initiate and maintain immunity to *T. gondii* in the brain. In the future, our lab is interested in teasing apart the impact of other alarmin receptors, TLRs, and NLR pathways individually, using knockout mice for each specific pattern recognition receptor. We are also interested in creating mice that lack multiple receptors at once, such as using IL1RAP-deficient mice, which would prevent signaling of all IL-1 family members. With these mice and other combinations, we would be able to begin to separate the relative importance of damage signaling versus parasite-specific recognition in brain tissue.

4.3 *Astrocytes and oligodendrocyte as sentinels of damage*

Our findings demonstrate that IL-33 is expressed predominantly by astrocytes and mature oligodendrocytes within infected brain tissue. This is also true under healthy conditions in the adult mouse brain[271]. Thus, IL-33 is expressed evenly throughout brain tissue, poised for release during disease. This raises the question of why glia, particularly mature glia like myelinating oligodendrocytes, would be the primary cells that express IL-33 in the brain. It may be that these cell types are particularly sensitive to cell death. There is evidence for this in mature oligodendrocytes, which are particularly sensitive to oxidative stress, since they expend a significant amount of energy producing the myelin sheath which can produce toxic by-products[583-585]. It may make sense that neurons, by contrast, would not be a suitable home for IL-33, since they are long-lived, are integral to cognitive function, and cannot be easily replaced.

Understanding how IL-33 is liberated from astrocytes and oligodendrocytes – whether by cell death or secretion, is a complicated question to answer. Although our results suggest that host cell death occurs during *T. gondii* infection, indicated by propidium iodide staining, the possibility of secretion of IL-33 from living cells cannot be ruled out[249]. Due to a lack of markers that can be used to differentiate types of cell death *in vivo*, mechanisms of glial death during disease have not been extensively studied, and it is unclear if the parasite itself, or if secondary, toxic inflammation can mediate the release of host damage signals. In the future, an *in vitro* system or *in vivo* live imaging will help to visualize the impacts of parasite on a particular cell in real time. Additionally, culturing brain cells lacking caspases involved in apoptosis, pyroptosis, and other executioners of cell death, along with *T. gondii*, will help us to better understand exactly which mechanisms of cell death are prominent in response to *T. gondii* infection. The same can be done *in vivo*, by chronically infecting gasdermin-D or RIPK3 deficient mice, for example, although these mice could have defects at baseline or during acute infection of whole-body deficiency.

Once IL-33 is released, it is also unclear how long-ranging the effects of alarmin signaling are, since extracellular IL-33 is negatively regulated in a number of ways to limit inflammation[419,425,434]. Work on IL-33 in cardiac muscle[422], as well as the retina[416], has indicated that IL-33 often signals very locally, acting in either an autocrine or paracrine manner. For example, Müller cells, specialized astrocytes in the retina, have been implicated in the release and sensing of IL-33 in the retina[416]. Our work further corroborates local IL-33 signaling, as IL-33-dependent *ccl2* expression is found adjacent to necrotic lesions containing replicating *T. gondii*. Thus, IL-33 may be released by

astrocytes or oligodendrocytes, and can signal on astrocytes, which could be the same cell or an adjacent cell. Determining whether or not IL-33 signals on the same cell, adjacent cells, or far-away cells, will require liberating IL-33 from a particular brain region, or even at the resolution of a particular cell, and visualizing its effects on other cells with an ST2 signaling reporter, *in vitro* or *in vivo*.

4.4 *IL-33 signaling – importance during acute vs. chronic infection*

We have observed that IL-33 signaling is necessary for controlling parasite burden within brain tissue during chronic *T. gondii* infection. However, IL-33 signaling does not appear to be necessary during the acute phase of infection. This is a counter-intuitive result since one might imagine that if a pattern recognition pathway is important in one tissue, it should be important in any other tissue when recognizing the same pathogen. Yet, we did not see a significant impact of IL-33 signaling on immune cell numbers, activation, or ability to control parasite during the first two weeks of infection. There are several possible explanations for this finding that are supported by the literature.

It is possible that during acute infection, IL-33 amplifies the immune response in a detrimental manner, contributing to unwanted immunopathology. A study in 2019 demonstrated that IL-33 signaling contributes to neutrophil recruitment in the intestines during acute *T. gondii* infection, which resulted in ileitis and morbidity[301]. Neutrophils, compared with monocytes, have specifically been shown to have a detrimental impact during acute infection, contributing to pathology in peripheral tissues[345]. We have observed supportive evidence that IL-33 signaling is detrimental acutely, during our bone marrow chimera experiments. In these experiments, infection is more severe, likely due to

irradiation, and can result in mortality of mouse strains that would normally survive infection. We observed that while many wildtype mice succumbed to acute infection in these experiments, which does not typically occur, mice which lacked IL-33 signaling survived the acute phase and did not lose as much weight (unpublished data). This is further supported by data on IL-1 signaling acutely, which also appears to coincide with weight loss, neutrophil recruitment, and ileitis[302,544]. And like IL-33, IL-1 signaling is protective chronically[289], suggesting that these pathways are differentially important depending on which tissue is infected.

Why IL-33 signaling might have different impacts acutely and chronically, resulting in a detrimental or beneficial response, is an open question. This may have to do with a difference in cell types present in peripheral tissues versus the CNS. Neutrophils are not recruited to the brain in high numbers during chronic *T. gondii* infection[132] (unpublished data), possibly mitigating the impact of IL-33 signaling on neutrophil recruitment. In the brain, there are also new cell types for IL-33 to signal on, such as astrocytes. In addition, there may be a disparity in the expression and importance of other pattern recognition pathways between the CNS and peripheral tissues. S100A11, for instance, could be a major inducer of *Ccl2* expression acutely and not chronically[246].

There is one commonality between the impact of IL-33 signaling acutely and chronically. Although IL-33 does not impact immune responses in general acutely, or the seeding of parasite to the brain, we did find that IL-33 is important for the number of IFN-γ+ T cells at one time point - day 12, in the serum. This is independent of IL-12 and mirrors our results chronically. In the future, we are interested in uncovering the mechanism by

which IL-33 promotes T cell immunity in multiple tissues in an IL-12-dependent manner – our first hypothesis being antigen presentation.

4.5 *Possible IL-33 responders in the brain*

The question of which cells respond to IL-33 in the brain has been the most difficult aspect of this thesis work to answer. But it was also one of the most important questions to tackle, since mechanistic insight into IL-33 signaling in the brain is sorely lacking for many diseases. Many cells express the IL-33 receptor, including a gamut of immune cells – notably ILC2s, mast cells, and Tregs[274,275,415], as well as brain-resident cells such as astrocytes and microglia. Thus, there are many potential relevant responders to IL-33 within brain tissue which may or may not be present based on disease state and environmental milieu (Fig 4.2) To make matters more complicated, IL-33 receptor expression has not been detected on the protein level in the brain, possibly due to a lack of technical challenges in processing brain tissue and due to a lack of antibodies used for immunohistochemistry staining. Thus, it is difficult to get a quick look at brain tissue to see which cells are expressing the IL-33 receptor.

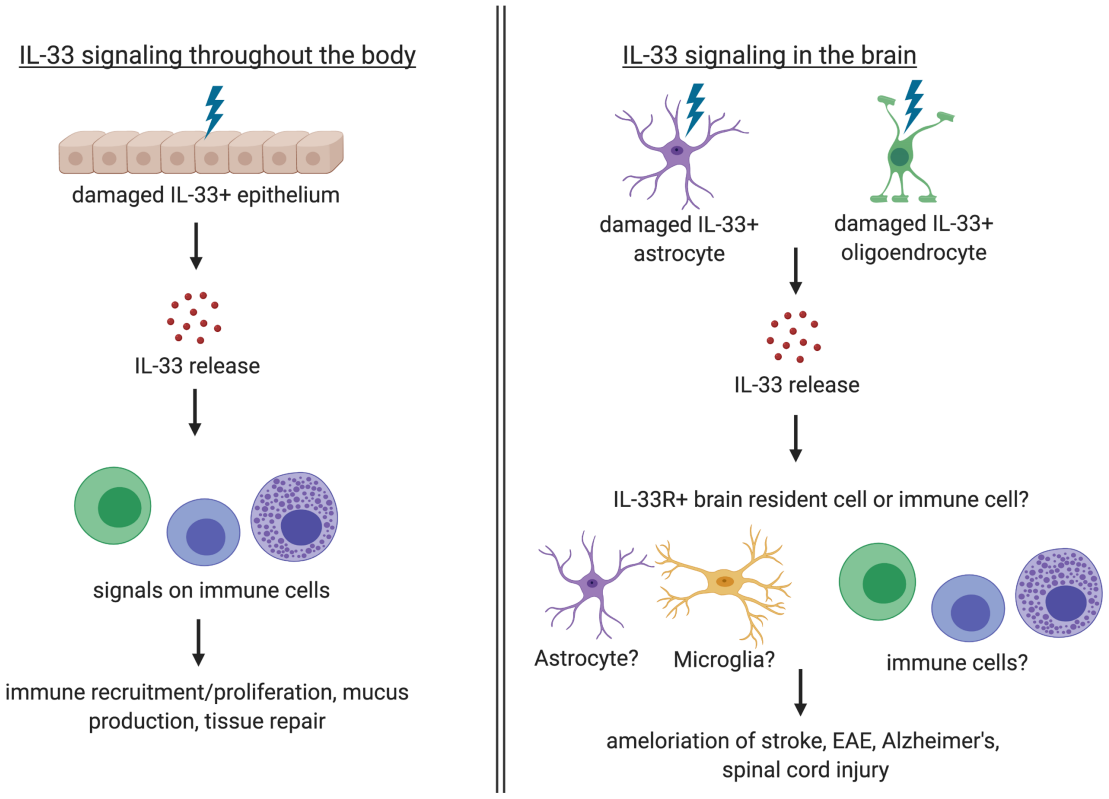

Fig 4.2 The complicated picture of IL-33-responsive cell types

In peripheral tissues throughout the body, IL-33 signaling follows a similar paradigm: release from damaged epithelium and signaling on a number of immune cell types – most commonly ILC2s, mast cells, and Tregs. Signaling on these cells can result in recruitment and skew of more immune cells, generally helping to clear helminth infection while worsening asthma and allergy. With a number of immune cells expressing the IL-33 receptor, it is not always clear which are the principal cell type(s) that respond in a given context and contribute to a particular phenotype. In the brain, this picture is further complicated by the fact that brain resident cells, including microglia at baseline and astrocytes during disease, have been shown to express the receptor. These cells, in addition to immune cells which infiltrate during disease, are all candidate responders in the brain.

We started by asking if IL-33 signals on an immune cell or not. In the lung, gut, and skin, IL-33 has historically been shown to signal almost exclusively on immune cells to orchestrate immunity[415] (Fig 4.2). However, recent work demonstrates that IL-33 can signal on non-hematopoeitic cells – including cardiomyocytes in the heart[422] and endothelial cells in culture[560,586]. To answer this question during *T. gondii* infection, we

took advantage of the fact that immune cells are radiosensitive and irradiated mice to perform bone marrow chimera experiments. We found, consistent with a previous report in stroke[525], that IL-33 signals on a radio-resistant responder during brain *T. gondii* infection. Tissue microenvironment and context likely influence whether IL-33 signaling on immune cells is relevant. Peripheral immune cells are not present in healthy brain tissue due to the blood brain barrier, thus necessitating the capability of brain resident cells to respond to damage. Additionally, the environmental milieu specific to CNS *T. gondii* infection promotes a robust Th-1skewed immune response necessary for intracellular parasite killing, characterized by T-cell derived IFN-γ and macrophage-derived iNOS[81,546]. Thus, the IL-33-dependent mechanisms, specifically those activating type 2 immune responses that drive asthma, allergy, and the expulsion of helminth infections[274,275], may not be relevant to brain infection with an intracellular pathogen.

When we examined brain-resident cell expression of *il1rl1*, we found that microglia express high levels of *il1rl1* in healthy brain tissue, but they downregulate the receptor 20-fold upon infection. A downshift in microglia inflammatory genes has been recently reported by our laboratory during *T.gondii* infection[289], and it is unclear what factors drive this process. However, these results do not rule out the importance of IL-33 signaling on microglia in other settings. Indeed, during development, microglia expression of *il1rl1* is required for microglial phagocytosis of synapses[250]. While we did not find any major impact of microglia/macrophage *il1rl1* expression on control of chronic *T. gondii* infection, it is possible that microglia respond to IL-33 at earlier time-points, such as when the parasite first reaches the brain. If microglia normally respond to IL-33 earlier in infection,

our results indicate that astrocyte responses to IL-33, or additional MyD88-dependent signals, may compensate for the lack of *il1rl1*-signaling in microglia.

In contrast to microglia/macrophages, we found that astrocytes increase expression of the IL-33 receptor upon infection. Astrocytes are critical for controlling CNS infection and potentiate inflammation. For example, astrocyte responses to IFN-γ are necessary to control *T. gondii* infection[406]. We found that IL-33 receptor expression on astrocytes was required for adequate promotion of an anti-parasitic immune response in the brain, including proliferation and IFN-γ production in T cells, which is critical for control of *T. gondii*. This is the first time, to our knowledge, that astrocytes have been directly implicated in an IL-33 response via genetic manipulation. Our results also raise questions regarding the role of astrocytes in supporting T cell responses to infection and whether these responses are direct or indirect. IL-33 signals through the adaptor MyD88[275], and could influence the expression of numerous molecules that support T cell responses, including trophic factors, TCR engagement, chemokines, or blood brain barrier opening for T cell entry. Future directions in this area will be discussed in the next section.

Notably, immune defects in GFAPcre *il1rl1*$^{fl/fl}$ mice did not match the magnitude observed in *il1rl1*$^{-/-}$ mice, and did not significantly impact myeloid cell recruitment. These results either implicate compensation by other cell types when *il1rl1* is deleted from a single population, or the presence of additional *il1rl1*-expressing radio-resistant cell types not considered here, such as neurons, oligodendrocytes, and endothelial cells, which have recently been shown to express *il1rl1* during disease and can be directly activated in response to IL-33 *in vitro*[560,586]. In the future, we plan to image brain tissue using fluorescent RNA-hybridization probes against *il1rl1*, to get an unbiased perspective of

where in the brain *il1rl1* is expressed, and by which cell types. New candidates can then be tested for functional relevance during *T. gondii* infection by deleting *il1rl1* from these cell types using cell-specific promoters.

4.6 *The ability of astrocytes to promote immune responses*

We observed that astrocytes play a key role in responding to IL-33 to support T cell responses during *T. gondii* infection. But, when we dug into the literature to better understand mechanisms by which astrocytes can support T cells, we realized this was a complicated question with a lack of clear supporting literature. These are the makings of a future project – a new question founded on previous work which has not been extensively studied in the literature. Additionally, another project in our lab has led down this road. In Michael Kovacs' unpublished thesis work, he has found that draining of antigen from meningeal lymphatics into the deep and superficial lymph nodes does not play a huge role in supporting T cells and altering the course of infection. Thus, in the future, he is interested in studying local priming of T cells in the brain by brain resident cells, including astrocytes. Here, I will summarize our current knowledge of how astrocytes can support immunity, discuss some preliminary findings of our work in this area, and focus on future directions to develop this project.

Within *T. gondii* infection, it is clear that astrocytes can become infected with *T. gondii*, as seen with a parasite reporter strain[92], although this event is more rarely observed than infection of neurons. There is also evidence that astrocytes can clear intracellular parasite, since deleting STAT1 involved in IFN-γ signaling harbor significantly increased parasite load within astrocytes and mice succumb to infection during the chronic phase[406].

Although there were increased numbers of immune cells in the brains of these infected mice, including neutrophils, monocytes and T cells, their functional capacity was reduced, measured by IL-12 and IFN-γ expression, suggesting a local impact of astrocytes on immune cell activation. These results mirror our results, that astrocyte responses to IL-33 damage signaling support IFN-γ in T cells, but it is entirely unclear how.

One well-documented immune function of astrocytes is production of chemokine. During *T. gondii* infection, we and others have demonstrated that astrocytes are major producers of *ccl2* and *cxcl10*[132,375,391]. This is also true in other disease models, where astrocytes are major producers of these chemokines in EAE, injury, infection, and other disease states[385,561-567]. Astrocytes have also been consistently shown to express *cxcl1, cxcl2, ccl8, ccl20, cxcl12, and cxcl8 in vivo* in a variety of disease states[555,561,568-574]. Although it is well documented that astrocytes can produce chemokine, it is not entirely clear what happens if astrocytes alone lack a single chemokine. Theoretically, other cells can compensate for this loss. Additionally, it is not clear if chemokine is enough to impact immune cell activation within the brain.

We set out to address these questions by deleting a single chemokine, *ccl2*, from astrocytes in the context of *T. gondii* infection. We found that this had a profound effect, including significantly reducing recruitment of monocyte-derived cells and T cells. Although this has been shown during EAE[385,386], it is not entirely expected during *T. gondii* infection. First, chemokines are often made by multiple cell types during infections and removing *ccl2* from astrocytes during *T. gondii* infection could have resulted in a full compensation from other cell types such as macrophages, and possibly endothelial cells[391,561]. Second, it is possible that CCR2+ cells could be recruited adequately by other

chemokines, like CCL7, CCL8, and CCL12, and deletion of *ccl2* may not have a noticeable impact[374].

This data serves as a framework to answer more open questions about astrocyte chemokine in the future. Although chemokines are known to recruit immune cells into the brain, the question of *where* and *how* chemokine impacts trafficking immune cells is less well understood. Is astrocyte chemokine important for bringing cells across the basement membrane of endothelial cells, for immune cells to stop and stick to vessels while circulating in the blood, for immune cell movement or survival within tissue? These questions are difficult to answer because whole body knockouts remove the expression chemokine and chemokine receptors elsewhere from the brain. For example, In the case of the CCL2/CCR2 axis, CCR2-deficient monocytes will not egress from the bone marrow to get into the blood to start with, and CCL2-deficient animals may also have trafficking defects before immune cells would ever be recruited to the brain, especially if the disease model has any involvement of tissues outside the CNS. One study used i.v. administration of anti-CXCL1 in a cerebral HSV-1 model led fewer neutrophils to arrest and stick to the vasculature within the blood[587]. Anti-CXCL1 also made the blood brain barrier less leaky, as measured by extravasation of vascular dyes, suggesting that the chemokine can have direct impacts at the vasculature for bringing immune cells into the brain[587]. But it is not entirely clear how physiological i.v. injection of chemokine is, since chemokine may not reach the blood when released in the brain. In another study, anti-CXCL10 was administered during chronic *T. gondii* infection[375]. In our model, antibodies do often cross the blood brain barrier[131,132,320]. This study found that CXCL10 was critical both for total T cell numbers in the brain, and for behavior within the tissue[375]. CXCL10 was necessary

for T cell movement, making it more likely for T cells to control parasite, likely by increasing T cell exposure to antigen[375]. Still other studies have shown that chemokines like CCL2 can impact the tight junctions between endothelial cells in the blood brain barrier[588,589].Thus, our model of deleting chemokine selectively from astrocytes is valuable to study further, to as a starting point for better understanding the impact of CCL2 on immune cell trafficking to the brain. Now that we have removed a source of the chemokine specifically within brain tissue and seen an impact, follow-up studies including live imaging of the BBB to observe impacts on immune cell trafficking, tracking behavior of immune cells within the tissue, and investigation of the integrity of the blood brain barrier. As tools continue to develop, we can expand this study to other astrocyte-derived chemokines, specifically imaging them during *T. gondii* and deleting them from astrocytes.

Interestingly, we found that deleting *ccl2* from astrocytes did not impact readouts that reflect immune cell activation, such as iNOS and proliferation frequency. In addition, we observed that while deleting the IL-33 receptor from astrocytes impacted T cell number and activation, it did not impact chemokine. These results suggest that astrocytes employ mechanisms beyond chemokine to support immunity in the *T. gondii*-infected brain. What these might be, is somewhat of a mystery (Fig 4.3).

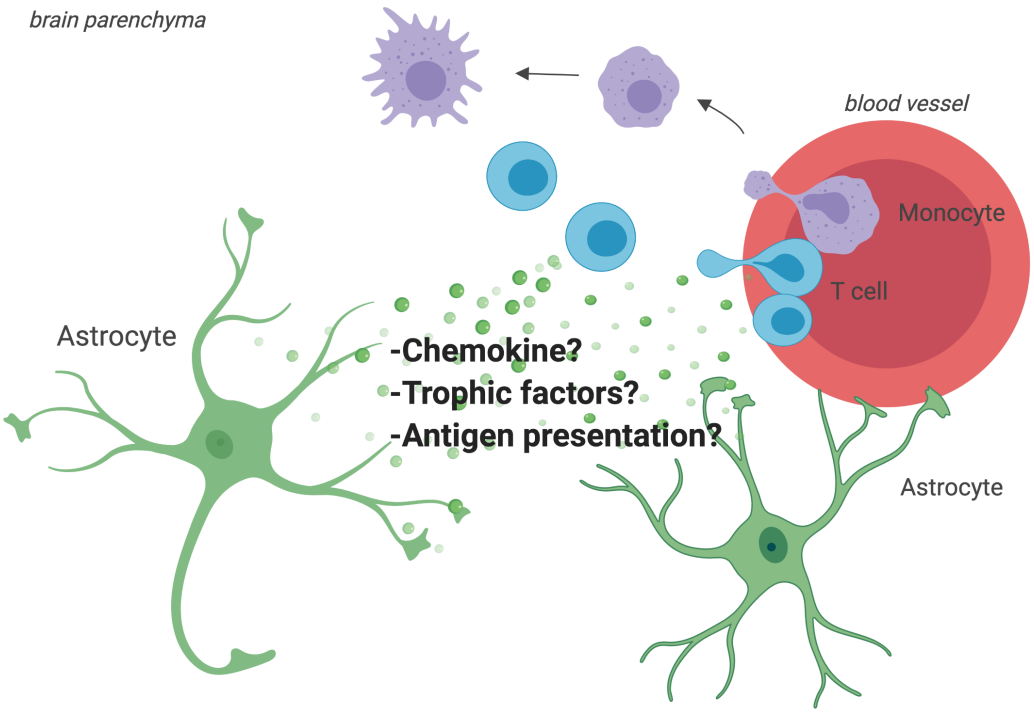

Fig 4.3 What can astrocytes do to support immunity during *T. gondii* infection?

Our findings support a role for astrocytes in promoting T cell responses in the brain – but it is unclear how they may do so. Chemokine is the most well-characterized function of astrocytes, but astrocytes can also produce trophic factors and activating cytokines, and may express antigen presentation machinery and adhesion factors. Our future directions aim to better understand how astrocytes can locally support T cells during chronic *T. gondii* infection.

It is possible that astrocytes produce trophic factors and activating cytokines in the brain. There is evidence in the literature that astrocytes can produce IL-7, IL-2, IL-15, IL-18, IL-6 and TNF-α[161,590-598] in response to IFN-γ, TNF, and IL-1 signaling, all of which are present in the *T. gondii*-infected brain. However, many of these studies were conducted with human or neonatal cultured astrocytes, and it is unclear if astrocytes produce these

cytokines to an appreciable degree in *in vivo* disease settings. Astrocyte cultures can also contain other glia, like microglia, which further complicates this picture.

More contentious is the suggestion that astrocytes can present antigen to T cells. There are some studies that suggest that astrocytes express MHC class 1 and 2 molecules, as well as costimulatory molecules CD80, CD86, and CD40 – while other studies refute these claims, stating that microglia, if any brain resident cells, chiefly express this machinery[595,599-616]. Like the field of trophic factors, many of these studies have been *in vitro* and many have been conducted close to twenty years ago. Progress in *in vivo* tools are needed to specifically delete this machinery from astrocytes – such as beta-2-microglobulin floxed mice, which are now available[617].

Finally, there is some suggestion that astrocytes can directly modulate immune cell entry by expressing ICAM and VCAM, including in a recent *in vivo* study of West Nile Virus[618-622].

To begin to address how astrocytes support immunity during *T. gondii* infection, we will take multiple approaches. First, we will continue to develop data on the importance of astrocyte-derived chemokine, as discussed earlier. Second, we will cross $MHCII^{fl/fl}$ and $B2m^{fl/fl}$ to an astrocyte-targeted cre, either ALDH1L1 or GFAPcre, to understand the impact, if any, of astrocyte-mediated antigen presentation in the brain during *T. gondii* infection.

Finally, we will take an unbiased approach to profiling astrocytes during *T. gondii* infection. We will generate astrocyte-reporter mice, such as ALDH1L1-Ai6 mice, to be used for sorting astrocytes by flow cytometry. Then, we will perform bulk RNA sequencing on these cells and mine the data for expression of known factors, like trophic factors and

innate cytokines, but we will also look for non-classical antigen presentation machinery, and mine the data for new targets which we are not currently envisioning. Because this field is relatively new, it is important not to close any doors on potential mechansisms astrocytes could use to support immunity. After RNA sequencing, we could target specific genes and understand what they do during *T. gondii* infection, using cell-type specific genetic constructs in mice. It would also be interesting to sequence astrocytes not only from naïve and infected wildtype animals, but from animals lacking IFN-g signaling ($STAT1^{fl/fl}$), pattern recognition signaling ($MyD88^{fl/fl}$), and from animals lacking particular TLR, damage signals. These experiments will help separate which signals are necessary to induce expression of chemokines, trophic factors, antigen presentation machinery, and adhesion factors in astrocytes.

A final technical note to consider is that we are interpreting involvement by astrocytes in our phenotype based on a GFAPcre mouse, and GFAP has been shown to be expressed by enteric glia[623-625]. In the future, it will be necessary to test if IL-33 receptor expression on enteric glia is responsible for any portion of our phenotype. We can establish this by looking early in infection in GFAPcre $il1rl1^{fl/fl}$ mice for defects in the immune response. Notably, we do not see many deficits acutely in whole body knockouts, suggesting that it is likely that our GFAPcre data indicates a brain-specific defect, although this experiment will have to be done to say that definitively.

4.7 *Beyond T. gondii infection – local immune activation in the brain*

The mechanistic findings of this thesis work are likely relevant to diseases beyond *T. gondii* infection. Because we chose to study damage signaling in the brain, rather than recognition of parasite-specific proteins, our finding that IL-33 can signal on astrocytes and can induce chemokine locally in the brain may be relevant to any other models where brain resident cells might die a focal, necrotic death and release damage signals. This phenomenon is thought to occur in other infections, such as West Nile Virus, which parallels *T. gondii* in its propensity to infect the brain parenchyma and induce cell death in the brain[287,626-628]. Additionally, focal cell death, particularly to glia, can occur in disease states outside infection. The same is true of reactivation of herpes simplex virus in the brain, although, similarly to *T. gondii* infection, it is unclear to what degree brain resident cells aside from neurons are dying over the course of infection, and from what causes[629]. In addition to cell death mediated-release of alarmins like IL-33, our findings can also help provide context to brain infections where other signaling pathways may lead to increased astrocyte activation, including *ccl2* and *cxcl10* expression, which may point to alarmin involvement.

Outside of infection, our work can be applied to the understanding of neurodegenerative disease. In multiple sclerosis models, which are arguably neurodegenerative, autoimmune, or both, there is an "inside-out" hypothesis, that one of the earliest events to occur is focal degeneration of oligodendrocytes, which leads to free myelin that can activate the immune response depending on individual and severity[630-632]. In these contexts, alarmins may play a large role in bringing immune cells into the brain and activating them once there. Indeed, several studies have demonstrated a protective role

for IL-33 in EAE, by increasing recruitment of Tregs and ILC2s and skewing immune cells away from IL-17 production[510,512,514-517]. But how IL-33 can do this, including which cells IL-33 signals on, has been unclear.

There is also evidence that our work may be applicable to Alzheimer's Disease. (AD) Although neuronal death is the major hallmark of human AD, there are some lines of evidence that glia may also be damaged, including DNA fragmentation within microglia and astrocytes[633], myelin abnormalities in human AD[634], foci of demyelination that occurs near beta amyloid in mouse models[635], and a general loss of Olig2+ cells have been observed in postmortem human AD patients[636]. Beta amyloid has also been shown to directly induce damage to oligodendrocyte cells *in vitro*[634,637,638]. Further, other similar hallmarks are observed in AD compared with *T. gondii* infection, including clustering of Iba1+ macrophages around beta amyloid plaques, although we do not yet understand if these cells are blood derived or microglia[639,640]. Two studies have shown that IL-33 signaling can be beneficial in mouse models of AD, which leads to more clustering of immune cells at plaques, and upregulates antigen-presentation machinery in microglia, which mirrors our findings[430,521]. In the future, our lab plans to study IL-1RacP deficiency in the context of an AD mouse model, to understand how IL-33 and IL-1 converge to promote beneficial outcomes to disease, possibly by recruiting blood-derived immune cells to the brain, as we found in our work.

Thus, in summary, better understanding the signaling mechanism of alarmins based on foundational work from *T. gondii* infection and in other models could help inform more specific therapeutic interventions, to either amplify or abrogate immune cell recruitment to the brain depending on disease context.

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