IL-1α and cell death in the brain: promoting CNS immunity to infection

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Abstract

Toxoplasma gondii is a protozoan parasite that infects large portions of the human and animal populations, and which persists in the brain of its host. Like many chronic CNS infections, *T. gondii* requires consistent immune pressure to prevent symptomatic disease. Large numbers of T cells and myeloid cells infiltrate the brain during this infection to control parasite replication. But the brain also harbors microglia, resident macrophages of the CNS, which are thought to be first-line defenders against CNS infections. Whether microglia and peripheral macrophages in the same environment respond to the infection in similar or distinct ways is still not fully understood. This is due, in part, to the fact that microglia and infiltrating macrophages have historically been difficult to distinguish. Now, however, there are newer tools available to discriminate the functions of these two populations. In order to identify the specific function of microglia in the brain during infection, we sorted microglia and infiltrating myeloid cells from infected microglia reporter mice. Using RNA-sequencing, we found that microglia and blood-derived macrophages differ in their inflammatory profiles during infection, with strong NF-κB signatures overrepresented in macrophages versus microglia. Interestingly, we also found that IL-1α is overrepresented in microglia and IL-1β is enriched in macrophages. We confirmed at the protein level that microglia express IL-1α but not IL-1β. These results were especially interesting since the role of IL-1 signaling in the brain during chronic *T. gondii* infection had not yet been addressed.

We went on to show that mice lacking IL-1R1 have impaired parasite control and immune infiltration specifically within the brain. Using bone marrow chimeras, we have shown that IL-1R1 expression on a radio-resistant cell population is necessary to recruit iNOS-expressing monocyte-derived macrophages into the brain to mediate protective inflammation. Our evidence suggests that this occurs through IL-1-dependent activation of the blood vasculature in the brain.

Surprisingly, we found that the alarmin IL-1α, not IL-1β, mediates the pro-inflammatory effects of IL-1 signaling in our model. Further, by sorting purified populations from infected brains, we were able to show that microglia, not peripheral cells, release IL-1α *ex vivo*. This implicates microglia as the source of IL-1α in the brain and illuminates a microglia-specific function during infection.

Using knockout mice as well as chemical inhibition, we have shown that *ex vivo* IL-1α release is gasdermin D-dependent, and that mice lacking gasdermin D have an impaired response to chronic *T. gondii* infection. These results implicate pyroptosis, or at least inflammasome activation, as a mechanism of IL-1α release. Indeed, we have demonstrated the presence of ASC specks in infected brain tissue, indicating inflammasome formation. We have also shown that caspase-1/11 double-deficient mice have an impaired response to chronic *T. gondii* infection. We have further used single knockout mice to show that our phenotype is likely driven by caspase-1 rather than caspase-11. In addition, we have ruled out NLRP3 and AIM2 as inflammasome sensors involved in IL-1α release, or in the control of chronic *T. gondii* infection.

Together, our results have demonstrated that microglia and macrophages are differently equipped to propagate inflammation, and that in chronic *T. gondii* infection, microglia specifically can release the alarmin IL-1α, driving neuroinflammation and parasite control. We have shown that this occurs in a manner dependent on gasdermin D, possibly downstream of an inflammasome and subsequent caspase-1 activation. This work has demonstrated a role for a single alarmin in promoting the CNS response to *T. gondii* infection.

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These past five years have been challenging and they have been rewarding. I made the decision to come to UVA for grad school on a feeling that was hard to explain, and I have never doubted that it was the right choice for me. I have experienced an incredible amount of personal growth over these years; in terms of skills in research (which were quite sparse before), but also in terms of my ability to think and to question. I owe any success to the support of many people, a few of which I'll mention here.

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List of Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's Disease
AIDS	Acquired Immunodeficiency Syndrome
AIM2	Absent in melanoma 2
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing CARD
BMDMs	Bone marrow-derived macrophages
CLR	C-type lectin receptor
CNS	Central nervous system
CR3	Complement receptor 3
CSF	Cerebrospinal fluid
CX3CL1	Fractalkine
CX3CR1	Fractalkine receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-associated cell sorting
GBP	Guanylate binding protein
GLP-1	Glucagon-like peptide 1
GSDMD	Gasdermin D
H&E	Haemotoxylin & Eosin

HA	Hemagglutinin
HIV	Human Immunodeficiency Virus
HMGB1	High-mobility group box protein-1
i.p.	Intra-peritoneal
ICAM-1	Intercellular adhesion molecule 1
ICE	Interleukin-1 converting enzyme
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRG	Immunity-related GTPase
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MECP2	Methyl-CpG-binding protein-2
MLKL	Mixed lineage kinase domain-like
MMP	Matrix metalloproteinase
MS	Multiple Sclerosis
NET	Neutrophil extracellular trap
NK	Natural killer
NLR	NOD-like receptor
NLS	Nuclear localization sequence

NO	Nitric oxide
NSA	Necrosulfonamide
p.i.	Post-infection
PAMP	Pathogen-associated molecular pattern
PI	Propidium iodide
PIT	Pore-induced intracellular trap
PML	Progressive multifocal leukoencephalopathy
PRR	Pattern recognition receptor
PS	Phosphatidylserine
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
RLR	RIG-I-like receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSV	Respiratory Syncytial Virus
TCR	T cell receptor
TE	Toxoplasmic Encephalitis
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TREM2	Triggering receptor expressed on myeloid cells-2

VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen-4
WT	Wild-type

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CHAPTER 1

INTRODUCTION

1.1 Innate Immunity

Humans have been living alongside microbes from the beginning of our existence. Countless species of bacteria, viruses, fungi, and protozoa reside around us, as well as on us and within us, contributing to our health and disease.[1-6] Our immune system functions to protect us from pathogenic invaders. The innate immune system mounts early defense against infection, providing protection before the adaptive immune response is in full swing.

1.1.2 Pattern Recognition

The innate immune response is able to be mobilized quickly because it relies on the recognition of broad patterns rather than mounting an antigen-specific response to a particular pathogen. This involves a category of molecules known as pattern recognition receptors (PRRs). PRRs are considered innate because they are germline encoded and do not require genetic rearrangement.[7] PRRs may be secreted and bind to pathogens extracellularly to initiate anti-microbial cascades.[7] They may also be expressed on the cell surface or intracellularly to promote such processes as internalization of pathogens, pro-inflammatory programs, anti-microbial programs, as well as cell death cascades.[7] PRRs generally recognize PAMPs, or pathogen-associated molecular patterns. These can include molecules on the surface of pathogens, like LPS, as well as microbial DNA and RNA.[7] It is now appreciated that PRRs can also recognize endogenous signals, known as damage-associated molecular patterns, or DAMPs.[8]

A large family of pattern recognition receptors are toll-like receptors (TLRs). TLRs are expressed on the cell surface as well as on membranes within the cell, and recognize a wide range of PAMPs, with most TLRs able to recognize multiple factors and even multiple classes of pathogens.[7,8] Downstream of PAMP engagement with a TLR is a conserved signaling cascade dependent on the adaptor molecule MyD88. MyD88 interacts with receptors through interactions

with the shared TIR domain. MyD88 then recruits IRAK, through interactions with their shared death domain, to form a complex that also includes TRAF6. IRAK then autophosphorylates and activates IKKβ, which in turn phosphorylates and inactivates IκB, allowing for the nuclear activity of NF-κB (Fig. 1.1).[7] This signaling cascade is conserved downstream of all TLRs as well as IL-1 family receptors. However, there are other downstream pathways that may add more nuance and specificity to the downstream signaling through different receptors.[7] Stimulation of certain TLRs may also lead to different downstream cytokine signatures in different cell types.[8]

Other families of PRRs include NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs).[8] These families of receptors all differ from each other in the molecules they recognize, their expression patterns, and the downstream signaling pathway, but will generally all recognize PAMPs and/or DAMPs and potentiate a transcriptional program promoting innate inflammation.[8]

1.1.3 Microbial Killing

Another important aspect of innate immunity is the ability to directly kill pathogens. One mechanism innate immune cells use to kill pathogens, including bacteria, protozoa, and viruses, is the creation of reactive oxygen and nitrogen species (ROS and RNS).[9,10] In fact, the use of ROS and RNS in host defense is widely conserved among both animals and plants.[11] In innate immune cells, enzymes localized in phagosomes create molecules including nitric oxide (NO) and superoxide (O_2^-), which creates a discrete compartment with potent cell killing activity.[10] However, NO and RNS can be less spatially restricted than ROS and therefore can impact microbial killing in more places.[10] Importantly, ROS and RNS often work in concert to kill pathogens.[10,12] In addition to their antimicrobial activities, ROS and RNS can also cause harm to

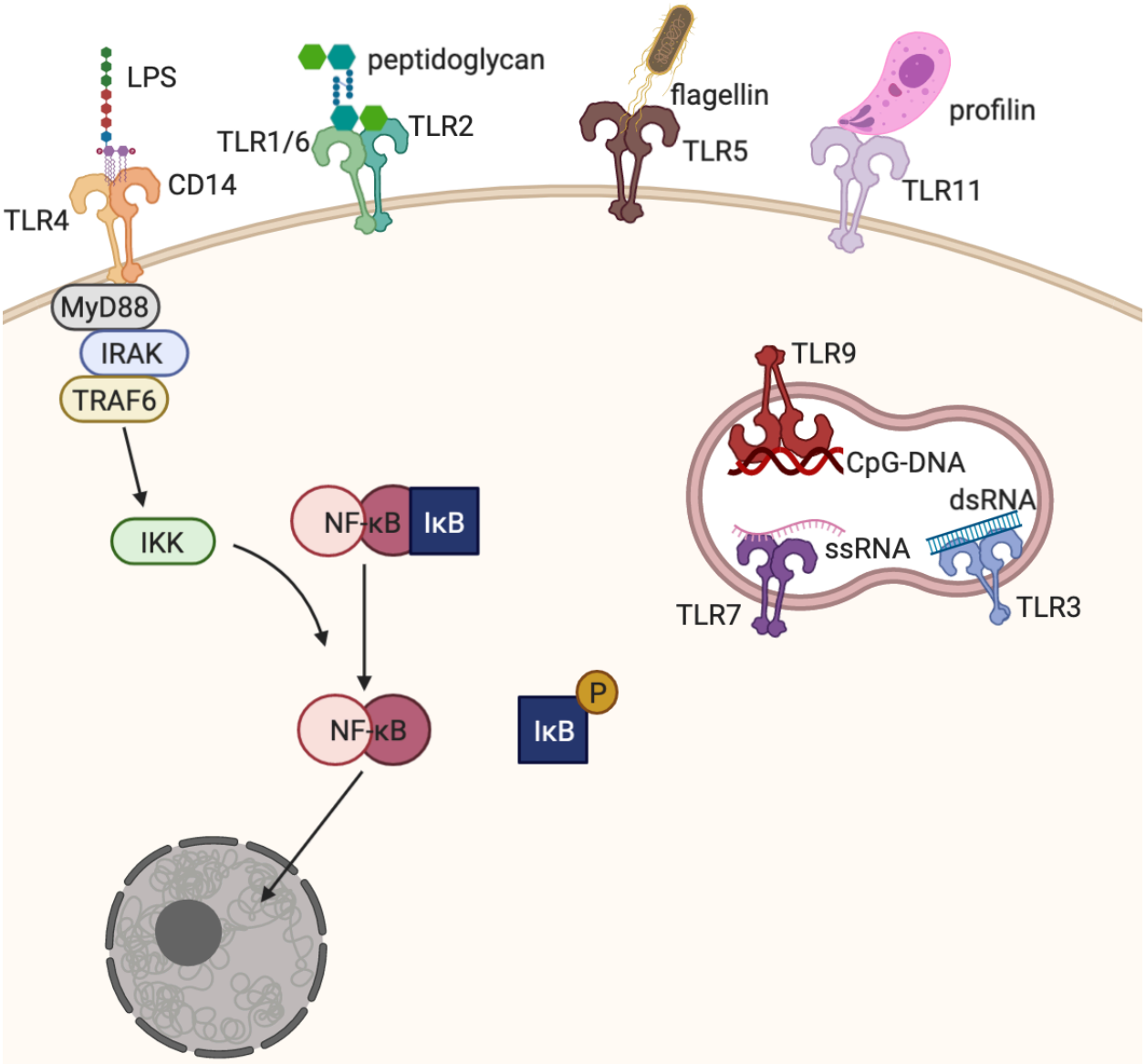


Figure 1.1 Overview of basic TLR signaling This figure shows a variety of mouse TLRs, their subcellular localization, as well as examples of their ligand recognition. Also shown is one basic signaling pathway conserved downstream of TLR activation. TIR domains in the receptor recruits MyD88, which leads to the subsequent recruitment and activation of IRAK and TRAF6. This leads to the activation of IKK-β, which phosphorylates IκB, allowing the release and nuclear translocation of NF-κB dimers.

host tissues, therefore their creation must be tightly controlled to balance control of infection and host damage.

In addition to oxidative mechanisms of killing bacteria and other pathogens, innate immune cells also possess antimicrobial proteins. Neutrophils are one innate immune cell type equipped with granules containing potent anti-microbial molecules. One of these is a protease called elastase.[13] Neutrophil granules can fuse either with the phagolysosome or cell membranes, releasing contents including elastase into cellular compartments containing pathogens or into the extracellular space (Fig. 1.2). Neutrophil elastase has been shown to directly kill gram-negative bacteria by degrading the outer membrane.[13] Macrophages also possess an elastase (also known as matrix metalloproteinase 12 or MMP12).[14] Most MMPs are known to function extracellularly and are not thought to possess direct antimicrobial activity. However, MMP12 (macrophage elastase) has been shown to exert direct antibacterial activity within macrophages, specifically by disrupting bacterial outer membranes.[14] Elastase is potent at degrading microbes, but has also been implicated in host tissue damage.[13,14]

In addition to these mostly intracellular mechanisms of killing pathogens, innate immune cells also possess methods to kill pathogens in the extracellular space. Neutrophils in particular are not only able to release the contents of their antimicrobial granules into the extracellular space, but are also able to employ a fascinating weapon called a neutrophil extracellular trap, or NET.[15] NETs are produced by activated neutrophils and contain a mix of DNA, histones, and antimicrobial proteins, including elastase and myeloperoxidase (Fig. 1.2).[15] NETs have been shown to be able to directly kill pathogens, specifically bacterial pathogens. They seem to accomplish this by trapping bacteria in the fibrous network of DNA while antimicrobial molecules work locally to degrade virulence factors.[15] However, not only bacteria induce NETs, as they have also been

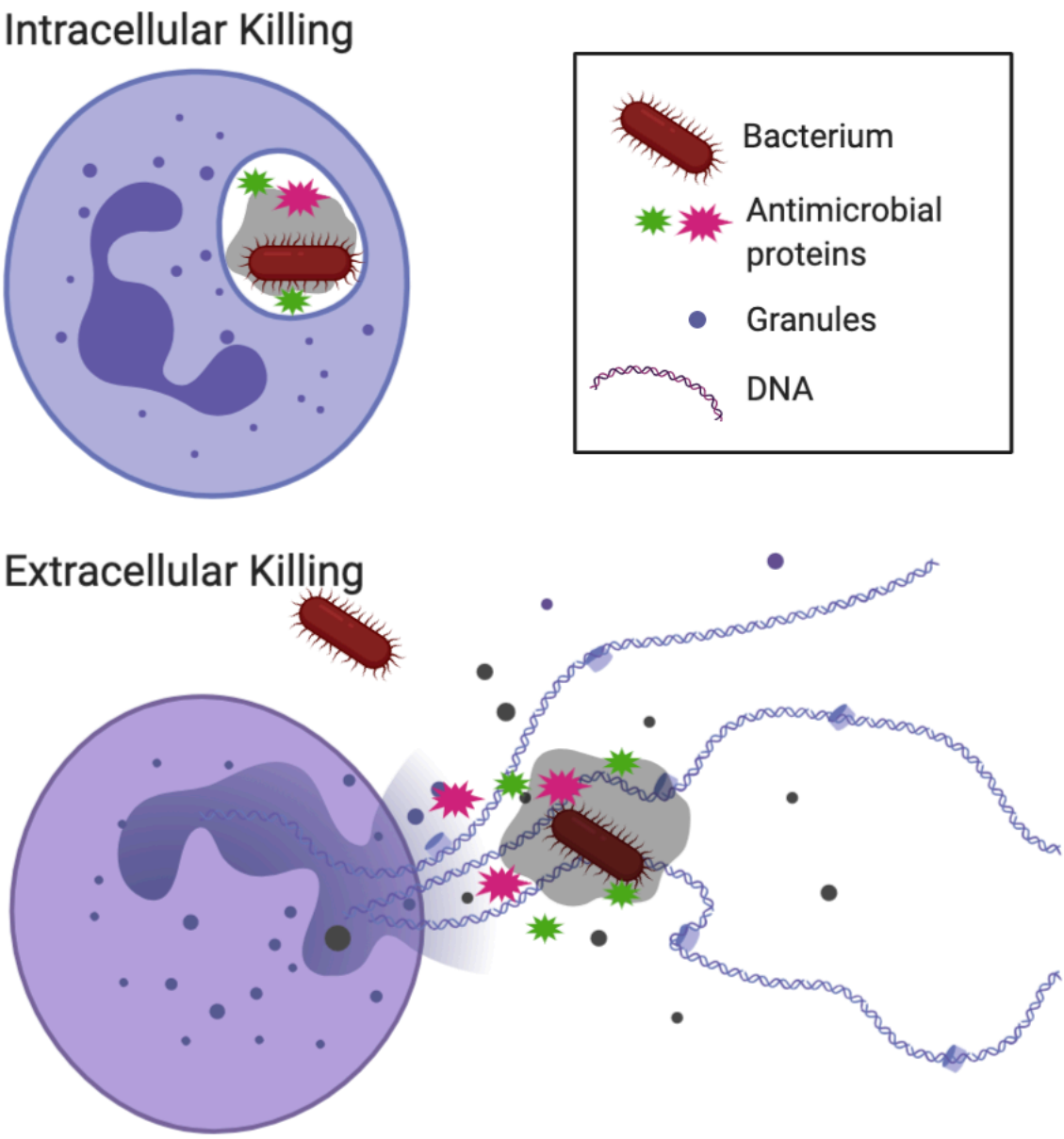


Figure 1.2 Intracellular and Extracellular Neutrophil Killing This graphic displays a basic example of intracellular and extracellular microbial killing mechanisms, using neutrophils as the model immune cell. Intracellularly, granules containing antimicrobial proteins such as elastase and myeloperoxidase can be released into phago-lysosomes to kill intracellular bacteria. The example of extracellular bacterial killing depicts NETosis, an extrusion of DNA, histones, and local accumulation of antimicrobial molecules which trap and kill extracellular bacteria.

shown to combat infections with fungi, protozoa, and viruses.[16] NETs may also contribute to host defense by sequestering pathogens so that they cannot spread, as well as keeping the potentially damaging high concentrations of antimicrobial molecules in a defined space.[15]

Finally, another mechanism of eliminating microbes is through the induction of cell death. By killing itself, a host cell can eliminate a niche of microbial replication and survival, thus contributing to the overall protection of the host. Some forms of cell death go beyond just getting rid of a niche, and actually trap pathogens, facilitating their clearance. In this section, NETs were summarized as a pathogen killing mechanism, but NETs are actually often associated with a form of cell death called NET-osis.[17] Net-osis is a ROS dependent cell death program in which neutrophils release NETs to sequester and kill extracellular microbes prior to, and during, their death.[17] It has also been shown that the programmed, inflammatory cell death pathway known as pyroptosis fits into this category as well. Pyroptosis occurs when active caspase-1 (or caspase-11) leads to the formation of pores in the plasma membrane. These pores allow for the release of soluble cytosolic contents, and eventually cell death, but the skeleton of the cell that's left behind can actually trap intracellular bacteria so they may be found and killed by other immune cells.[18] This was termed a pore-inducted intracellular trap, or PIT.[18]

1.1.4 Interplay with Adaptive Immunity

The innate immune system does not exist in a vacuum, but rather in conjunction with the antigen-specific arm of immunity: the adaptive immune system. These two arms of the immune response are intimately associated. The innate immune response is involved, both directly and indirectly, in the generation, refinement, targeting, and amplification of the adaptive response.

One major way innate immunity promotes adaptive immunity is through the process of antigen presentation. While many cell types can present antigen to T cells, dendritic cells (DCs)

are the most efficient 'professional' antigen presenting cell (APC).[19] These cells are resident in many tissues and sample their environment, taking up antigen. When they are also presented with an inflammatory stimulus, either sensing of infection through PRR ligation or sensing of damage to cells in the surrounding tissue, they begin the process of mobilization and maturation. In short, they exit tissues and travel to lymph nodes, where they engage directly with T lymphocytes, presenting antigen in the form of peptides within either an MHC I or MHC II molecule, which can be recognized by the T cell receptor (TCR).[19] This allows for the clonal expansion of antigen specific T cell populations which can then mobilize to sites of infection.

In addition to TCR binding of peptide-MHC complexes, successful activation of T cells requires costimulation, which involves binding of other receptor/ligand pairs expressed by the T cell and the APC. Some of these interactions that have described and ascribed importance in T cell activation include LFA/ICAM-1; CD28, CTLA-4/CD80, CD86 (B7); and CD40L/CD40.[20-23] One mechanism by which costimulation promotes T cell activation, is that these interactions have been shown to stabilize the contact surface between the T cell and the APC, mobilizing more receptors to this interface and allowing for longer, amplified interactions.[20,21] Another effect of costimulation is the activation of downstream signaling pathways that ultimately promote transcription and stabilization of activation-related programs.[21,23]

Innate immune cells also promote the generation of an adaptive response through the production of cytokines. With antigen recognition by TCR being signal 1, costimulation being signal 2, cytokine production from innate immune cells can be seen as a signal 3 for complete activation of T cells. [24] This signal can also dictate the type of effector the T cell will differentiate into, including the master transcription factor it will express and the cytokines it will produce. For example, there are different subsets of $CD4^+$ T cells that produce different cytokines after

activation. These include Th1, Th2, and Th17 cells.[25,26] Th1 cells that are characterized by IFN-γ production are potently induced by IL-12 produced by innate immune cells.[25] On the other hand, Th2 cells which are characterized by production of IL-4, IL-5, and IL-13, are induced by IL-4 produced by innate immune cells.[25] In addition, IL-17-producing Th17 cells can be differentiated in the presence of IL-23 and the absence of IL-4 and IFN-γ.[26]

Innate immunity also coordinates the adaptive response through the production of chemokines. Chemokines produced by dendritic cells as well as stromal cells organize lymphocytes in lymph nodes, bringing them to the appropriate areas where they can interact with other cells and undergo activation.[27] Chemokines produced in infected or damaged tissues by either innate immune cells or tissue resident cells function to attract lymphocytes into these sites.[27]

Thus, there are many ways in which the innate immune response influences the adaptive immune response. However, adaptive immunity is also able to influence the innate system as well. Cytokines produced by activated lymphocytes in a tissue can lead to the production of chemokines which attract innate immune cells to the site of an infection.[27] Cytokines produced by activated lymphocytes can also act directly on innate immune cells to facilitate activation and microbial killing. An excellent example of this is the ability of IFN-γ, produced by T cells, to activate macrophages, changing their metabolism and leading to the production of antimicrobial ROS.[28,29] IFN-γ and type I IFN also induce the expression of a class of intracellular effector proteins called Immunity-Related GTPases (IRGs).[30] IRGs have been shown to promote pathogen clearance, specifically cell-autonomous clearance of intracellular pathogens, likely through a role in degradation of pathogen containing vacuoles, with which they tend to colocalize.[30] These are just a few examples of a multitude of ways in which the innate and adaptive immune responses interact with, and influence, each other.

1.2 Microglia

1.2.1 Origins and Identity

Microglia are essentially the resident macrophages of the central nervous system (CNS). The ontogeny of microglia as well as their transcriptional identity seems to be unique compared to other macrophages, and their role in CNS homeostasis and disease is still being uncovered. Microglia are derived from yolk sac progenitor cells, and seed the neural tissue early in embryonic development.[31] Unlike macrophages in other compartments, the adult microglia population is maintained without blood input, with minimal to no contribution from fetal or bone marrow hematopoiesis.[31] This population is maintained predominantly through self-renewal.[32,33] While the brain environment is unquestionably important to microglial identity,[34-36] microglia ontogeny also imparts much of this identity. Indeed, it was shown through transplantation studies, that when any other macrophages that are not of yolk sac origin engraft into the brain environment, they never fully attain microglia identity.[34] It has also been shown that in the context of microglia depletion without irradiation, bone marrow-derived cells can engraft the brain long-term, but they remain transcriptionally and functionally distinct from microglia.[37] There are many factors that contribute to the induction and maintenance of microglial identity, including transcription factors such as Runx1, PU.1, and IRF8; cytokine/receptor pairs such as IL-34/CSF1R; and microRNAs such as miR-124.[38-48] Some of these factors, such as Runx1, PU.1, and CSF1R, promote myeloid/macrophage identity and sometimes activation.[38,40,41,43] On the other hand, IRF8 and miR-124 likely regulate microglia population dynamics and activation respectively.[46-48] Though these factors could be reflective of a general macrophage identity, at a more minute level, the enhancers in microglia show enrichment for different transcription factors (specifically *Mef2c*) than those of

other tissue macrophages.[35,49] *Sall1* is another transcription factor that seems to be important in specifically defining microglia identity, as it is enriched in microglia compared to other macrophages.[50-52] Absence of *Sall1* also leads to loss of the homeostatic microglia signature and morphology.[50,53]

1.2.2 Functions of Microglia in Development and Homeostasis

During development as well as adulthood, microglia produce trophic factors that contribute to the health and survival of neurons.[54-57] One important example is microglial release of IGF-1 (insulin-like growth factor-1), which promotes survival of neurons, but also, interestingly, promotes the survival of oligodendrocytes.[58-60] Thus, at baseline, microglia support not only neuronal health, but a healthy CNS environment in general. On the other side of the coin, cell death is also a necessary process for proper development and homeostasis. Microglia have roles in cell death as well as cell survival. There is evidence that microglia actively induce neuronal death in certain brain regions.[61-63] Microglia also have an important role in cleaning up after neuronal death through phagocytosis.[63-66] This is largely a non-inflammatory process which involves receptors on microglia including TREM2 (triggering receptor expressed on myeloid cells-2) and soluble factors from neurons including CX3CL1 (fractalkine).[64,67]

On a more minute, but equally important, scale, microglia also contribute to the health and pruning of synapses in the brain.[68,69] Microglial factors such as DAP12 can promote the proper functioning of synapses.[70] Microglia also closely associate with synapses and can modulate their fine architecture.[71,72] Just as with cell death, to maintain a healthy state, defective synapses must be eliminated. This occurs through a microglia-dependent process called pruning, which seems to be reliant on the complement system and microglia expression of CR3.[69,73,74]

1.2.3 Murky Waters - Microglia in Disease

Microglia are likely involved in countless diseases. Rett syndrome is one, which results from a mutation in the transcriptional repressor MECP2 (methyl-CpG-binding protein-2).[75] Cell type specific mutations or rescue experiments have shown intrinsic roles for neurons and astrocytes in this disease,[76-78] but microglia have also been implicated. Introduction of wild-type bone marrow into MECP2$^{-/-}$ mice after irradiation led to myeloid cell engraftment and amelioration of disease.[79] Though we know that engraftment of peripheral cells does not occur under steady state, and that these cells are different in nature, further study of this process could be therapeutically beneficial.

It is likely that microglia play a role in neurodegenerative diseases including Alzheimer's disease (AD), and a lot of work has been done to investigate this. However, whether microglia are protective, damaging, or just responding to their environment remains unclear and highly debated. Microglia express several risk genes associated with AD, including CD33, mutations in which have detrimental impairments on microglia phagocytosis of amyloid beta (Aβ).[80,81] In models of AD, stimulation of pattern recognition receptors such as TLR2, TLR4, and CD36 is thought to promote beneficial phagocytosis by microglia.[82-86] On the other hand, it was reported in a mouse study that microglia depletion did not impact Aβ plaques.[87] In addition to phagocytosis, microglia may release pro-inflammatory cytokines. As a result of blockade studies, inflammatory cytokine production is believed to be pathogenic, and is assumed to come from microglia.[88,89] However, it is important to note that though effects of PRR signaling and cytokine production have been attributed to microglia, other myeloid cells are capable of engaging in these pathways. Therefore, more complete understanding of potential immune infiltration into the CNS during AD will be necessary to assign specific functions to microglia.

Microglia may also be involved in the response to CNS infections. Microglia express pattern recognition receptors, and can respond to bacterial components and TLR stimulation.[90-93]

In the context of bacterial infection, signaling through these receptors has been shown to lead to both neuropathology as well as pathogen control.[94-101] Microglia have also been shown to exert neuroprotective effects in response to bacterial infection.[102-105] In addition to bacteria, many viruses also infect the CNS and can impact, and be impacted by, microglia function. In the context of viral infection, costimulatory molecules and antigen presentation machinery are expressed by microglia.[106-109] Microglia have even been demonstrated to present antigen to T cells, though not as efficiently as DCs.[106-108,110] Another important component of antiviral immunity is the type I interferon response, and microglia have demonstrated the ability to produce and respond to these factors.[111-113] Importantly, microglia can also be infected by viruses. For example, in HIV infection, microglia are a reservoir of the virus during latent stages.[114] During active encephalopathy, microglia may also promote the spread of HIV throughout the CNS through migratory behavior.[115,116] Evidence for both pro-inflammatory and anti-inflammatory roles for microglia during infections could indicate context-specific functions, a temporal switch in the microglia response, or heterogeneity in the microglia population.

1.3 Cell Death

There are a variety of different mechanisms leading to the death of a cell. These can be programmed or non-programmed, and inflammatory or immunologically silent.

1.3.1 Apoptosis

The most well-known of these is apoptosis, which is a programmed, immunologically quiet form of cell death, that is conserved and ubiquitous. Apoptosis was defined first in terms of morphology[117], and then signaling pathways were discovered that characterize this type of death.

During apoptosis, there is condensation of chromatin, blebbing of the plasma membrane, and formation of apoptotic bodies, but there is no exposure of intracellular contents.[117] Regardless of the initiating signals, the common terminal effectors of apoptosis are caspases, a family of cysteine proteases.[118] Upstream death-inducing signals typically result in the activation of so-called initiator caspases (either caspase-8 or caspase-9), which leads to the activation of the effector caspase (caspase-3).[118] There are two main death-inducing pathways. One, which is commonly referred to as the intrinsic pathway, involves sensing of intracellular stress. This is mediated through the finely coordinated activity of both pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins, which regulate the activity of the adaptor molecule Apaf-1 to activate caspase-9 (Fig. 1.3).[118] The other pathway, commonly referred to as the extrinsic pathway, involves extracellular-derived signals. This is generally mediated through ligation of TNF family receptors, including Fas and TNFR1, which recruit the adaptor protein FADD to activate caspase-8 (Fig. 1.3).[118] Both the caspase-8 and caspase-9-dependent pathways converge on the activation of caspase-3, which then interacts with and cleaves cellular proteins leading to apoptosis.[118]

1.3.2 Necrosis

Necrosis is another type of cell death, typically defined as not displaying the hallmarks of any other defined type of cell death. It seems to be the opposite of apoptotic death, in that it appears to be non-programmed, it is caspase-independent, and it can be inflammatory.[119] However, there do appear to be some defining characteristics of necrosis. Rather than the shrinking and blebbing seen in apoptosis, necrotic cells display swelling and plasma membrane rupture.[119] There may additionally be more consistencies that accompany necrosis that we do not understand yet. Cell death used to be defined as either non-inflammatory apoptosis, or inflammatory necrosis, but we now know that there are a variety of other mechanisms in between, as discussed below. The term

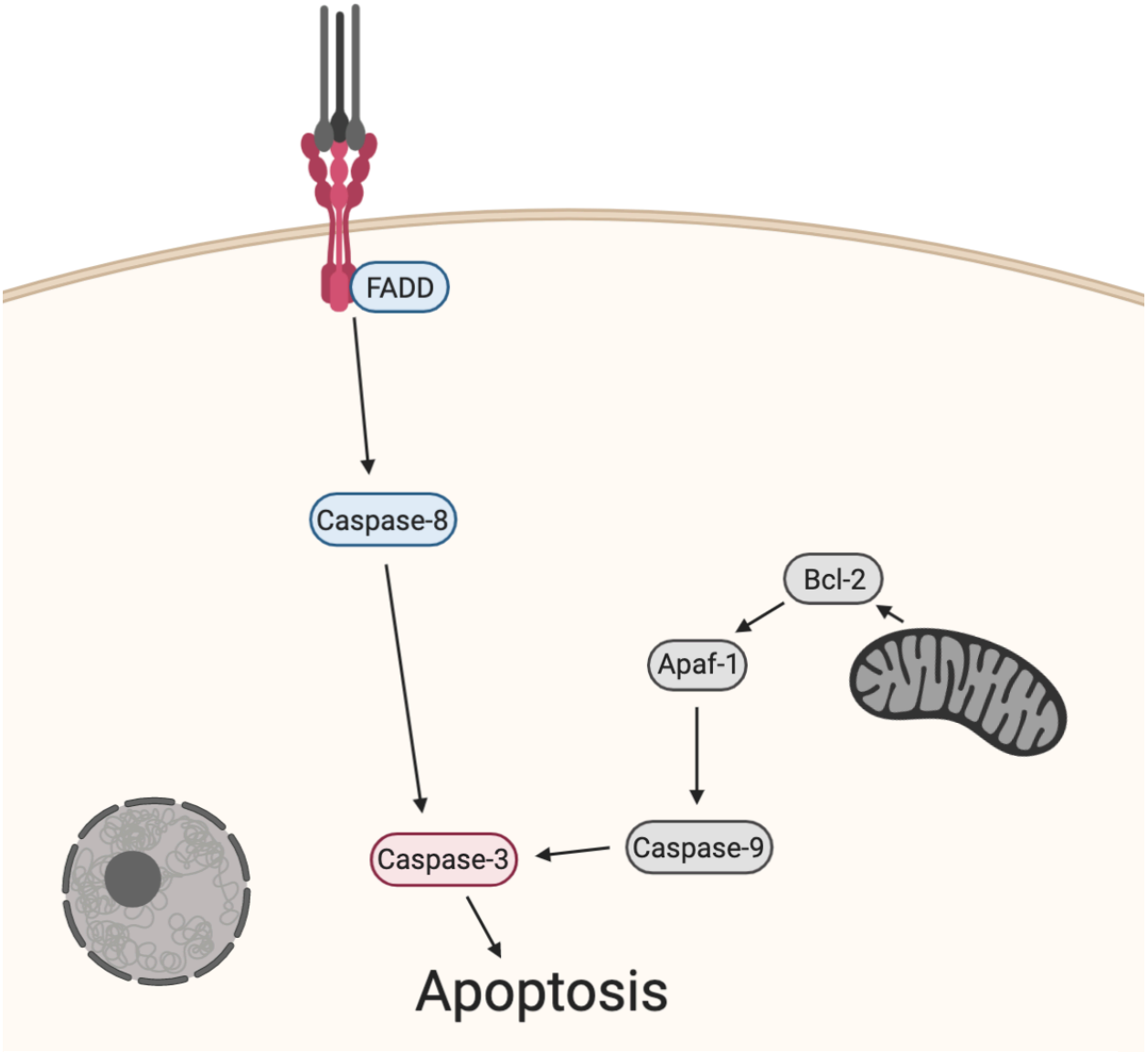


Figure 1.3 Intrinsic vs. Extrinsic Apoptosis

necrosis may come to mean, not a single mechanism of explosive cell death, but rather a category of programmed death pathways.

1.3.3 Necroptosis

Necroptosis can be defined as a kind of programmed necrotic cell death, which is dependent on RIPK3 (receptor-interacting protein kinase 3).[120,121] Necroptosis is a caspase-independent form of cell death, and has actually been studied mostly in the context of caspase inhibition.[120] Downstream of the same death-inducing signals that initiate the extrinsic apoptotic pathway, for example TNF signaling, if caspase-8 is inhibited, RIPK1 forms a complex with RIPK3 called the necrosome.[120] Active RIPK3 is then able to phosphorylate the pseudokinase MLKL (mixed lineage kinase domain-like) which effects necroptosis, likely through the formation of membrane pores.[120,122] In addition to death receptor signaling, pattern recognition of PAMPs has also been suggested to initiate necroptosis, especially during caspase inhibition.[121] Necroptosis, unlike apoptosis, promotes inflammation.[120] It is tempting to think that an inflammatory form of cell death which can seemingly only be achieved when interventions are taken to inhibit apoptosis would not be physiologically relevant. However, a number of intracellular pathogens produce effectors that inhibit caspases and/or apoptosis, likely making necroptosis biologically important as a means to inhibit pathogen replication as well as promote inflammation in response to infection.[120] In addition to its relevance during infection, necroptotic cell death has been suggested to occur during insults such as ischemia-reperfusion injury, and thus there is therapeutic interest in targeting this pathway using tools such as necrostatin 1, a small molecule inhibitor of RIPK1.[121]

It should be noted that many of the same signals can lead to different outcomes when it comes to cell death. Specifically, as described in this section, TNF signaling through TNFR1 can

lead to either cell survival, apoptosis, or necroptosis, depending on the context and on the balance of downstream signaling pathways within a cell (Fig. 1.4).

1.3.4 Pyroptosis

Pyroptosis is a caspase-dependent form of programmed cell death, but it is not dependent on the apoptotic caspases. Rather, it was originally defined by its dependence on caspase-1.[123] This mechanism of cell death also involves rupture of the plasma membrane and the release of inflammatory mediators from within the dying cell.[123,124] The terminal effector of pyroptosis is a protein called gasdermin D. Downstream of caspase-1 (or caspase-11) activation, gasdermin D is cleaved, allowing its N terminal fragment to oligomerize and form pores in the plasma membrane.[125,126] The signals leading to pyroptosis usually include recognition of a pathogen or of DAMPs which then initiates inflammasome activation. These pathways as well as the consequences of pyroptosis will be discussed in more depth in later sections.

1.3.5 Cleaning up after Death

After a cell dies, its remains need to be dealt with by other cells in the system. During the process of death, different molecules may be exposed on the surface of, or released from, the dying cell. These will include signatures constituting “find me”, “eat me”, and potentially “danger” signals. The distinct combination of these signals will determine which cells respond to this death, and what action they take in response.[121] Apoptotic cells release “find me” signals like ATP, and display “eat me” signals like exposed phosphatidylserine (PS), usually in the absence of any danger signals. This facilitates their consumption by macrophages or other phagocytes through phagocytosis, without overt inflammation.[121] Necrotic cells, on the other hand, are often consumed through macropinocytosis, a process of membrane ruffling and intake of cell material as well as fluid content.[121] Necrotic cells have the ability to release a variety of inflammatory mediators

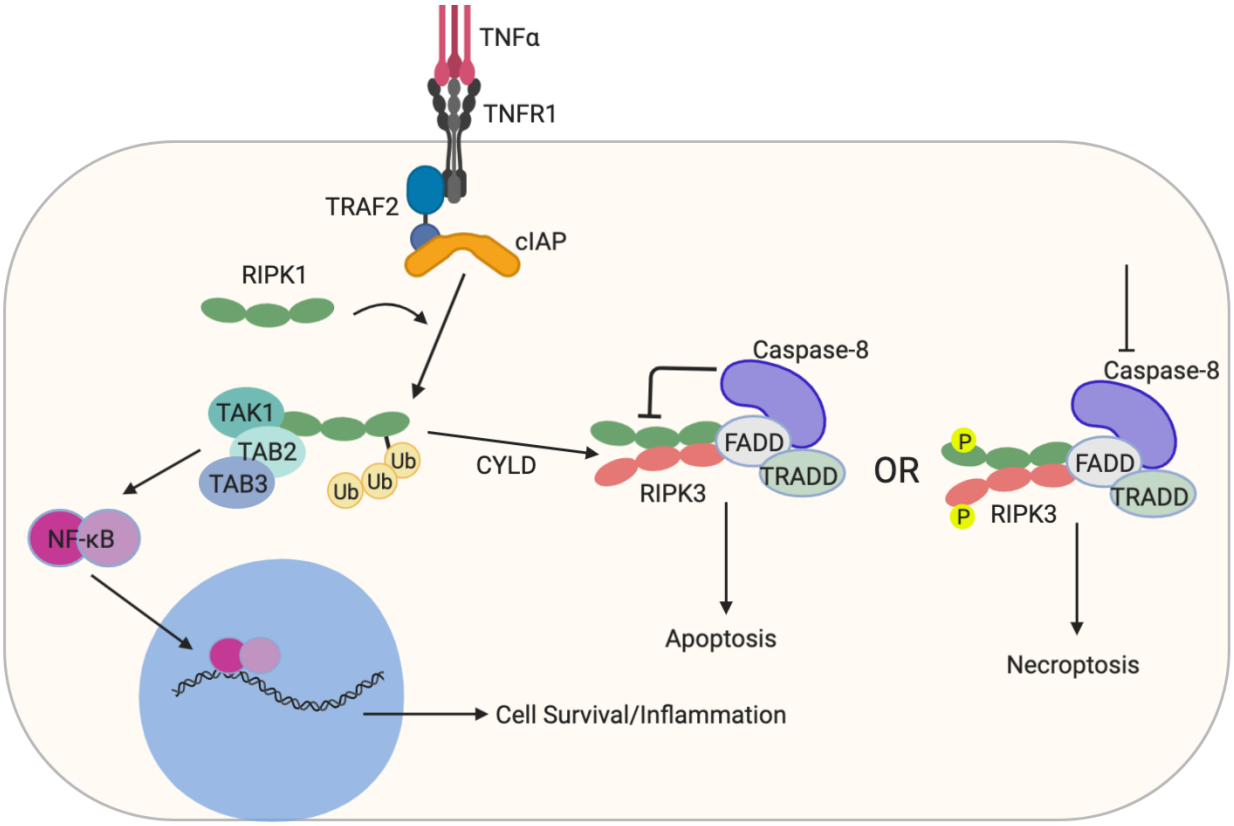


Figure 1.4 TNF and Cell Death Overview of the different outcomes of TNF signaling. Downstream of TNFR1, formation of complex I with the ubiquitination of RIPK1 leads to the activation of NF-κB. De-ubiquitination of RIPK1 leads to the formation of complex IIa with caspase-8, which drives caspase-3-dependent apoptosis. During caspase-8 inhibition, this becomes complex IIb in which active RIPK3 drives MLKL-dependent necroptosis.

during death due to the membrane permeability as well as the processes that proceed these types of cell death, which can initiate inflammatory reactions.[121]

1.4 Inflammasomes

Inflammasomes are multi-protein complexes serving as a platform for caspase activation, and were initially described as being integral to IL-1β processing.[127] These complexes generally consist of a sensor (or pattern recognition receptor), an adaptor protein, and caspase-1.[127,128] Inflammasomes are now known to lead to the processing and maturation of IL-18 as well as IL-1β, and also to an inflammatory form of cell death known as pyroptosis.[128]

1.4.1 Sensors

The first step in inflammasome activation is the activation of a sensor through binding of a PAMP or DAMP. There are five well characterized inflammasome sensors: NLRP1, NLRP3, NLRC4, AIM2, and pyrin.[128] The NLRP1 inflammasome has been shown to sense *Bacillus anthracis* lethal toxin as well as *Toxoplasma gondii*.[128] The NLRP3 inflammasome is activated by a wide array of circumstances, including infection with different types of organisms as well as sterile insults, but the unifying event leading to its activation appears to be potassium efflux.[128] The NLRC4 inflammasome has been shown to be activated by bacterial flagellin as well as components of bacterial type 3 secretion systems (T3SS).[128] AIM2 senses cytosolic DNA and thus this inflammasome is activated by viral infection, intracellular bacteria, as well as host DNA.[128] The pyrin inflammasome is activated by a number of bacterial proteins, and may become activated as a result of disturbed actin cytoskeletal dynamics during infection.[128] Through the functions of these, and potentially other, PRRs, inflammasomes can be formed in response to a wide variety of stimuli.

1.4.2 Assembly and Functions

In order to provide a platform for caspase-1 activation, following activation of a sensor, inflammasomes form through a complex series of protein-protein interactions. NLRs, including NLRP1 and NLRP3, oligomerize following activation through interactions between their NOD domains.[128] The NLRC4 inflammasome nucleates slightly differently, in that the initial sensor is a NAIP molecule. However, the NAIPs themselves do not oligomerize, but rather one binds to NLRC4, which then oligomerizes through NOD interactions.[128] In the case of AIM2, which does not contain a NOD domain, it is thought that DNA itself provides a platform for AIM2 nucleation through DNA binding of AIM2 HIN domains.[128] Oligomerization of the receptor is an important step in inflammasome formation because it then allows for ASC polymerization.[128] ASC, or apoptosis-associated speck-like protein containing CARD, is an adaptor molecule present in most inflammasomes. ASC contains both a PYD and a CARD domain. Depending on which of these domains each sensor expresses, it will associate with ASC through homotypic interactions.[128] ASC oligomerizes into filaments through its PYD domains, and recruits caspase-1 via CARD-CARD interactions.[128] This all brings caspase-1 molecules in close enough proximity to each other to lead to auto-cleavage and activation.[128]

Functions of inflammasome activation, downstream of caspase activation, include processing and maturation of cytokines IL-1β and IL-18, as well as processing of gasdermin D leading to pyroptosis.[128] IL-1β and IL-18 need to be cleaved in order to have biological activity, and caspase-1, when active, cleaves these cytokines into their active forms. Active caspase-1 also cleaves gasdermin family member gasdermin D (GSDMD).[128,129] The structure of GSDMD facilitates autoinhibition, but once it is cleaved by inflammatory caspases, the N-terminal domain promotes inflammatory cell death known as pyroptosis.[129] GSDMD-N does this by inserting into

the inner leaflet of the plasma membrane and oligomerizing to form pores.[125] These pores allow for the release of cytoplasmic contents, including IL-1 family cytokines that do not contain a conventional secretion signal, and eventually lead to cell lysis (Fig. 1.5).[125,128,129]

1.4.3 Non-canonical Inflammasome

Summarized above is the activation of the canonical inflammasome involving activation and oligomerization of a receptor, binding of the adaptor protein ASC to the receptor, nucleating polymerization, and activation of caspase-1, ultimately leading to the processing of IL-1β, IL-18, and GSDMD. However, there is another, related, pathway known as the non-canonical inflammasome. The non-canonical inflammasome relies on the activation of caspase-11 (or caspases-4 and -5 in humans) rather than on caspase-1.[128,130] Unlike the canonical inflammasome pathway, the activation of caspase-11 does not require a separate sensor, nor does it require ASC.[130,131] Rather, caspase-11 has been shown to respond to infection with gram-negative bacteria by recognition of intracellular LPS. Caspase-11 has been demonstrated to directly bind LPS, which facilitates its oligomerization and activation.[131] Like caspase-1, active caspase-11 is also able to cleave GSDMD leading to membrane pores and pyroptosis.[132] Unlike caspase-1, caspase-11 is not thought to cleave IL-1β and IL-18, but through induction of pyroptosis can lead to the release of any active cytokines in the cytosol including IL-1α.[130] However, caspase-11 is proposed to be able to activate the NLRP3 inflammasome, indirectly leading to the activation of caspase-1 and IL-1β.[130,132] This likely occurs as a result of potassium efflux through GSDMD pores activating NLRP3 (Fig. 1.5).[130,132]

1.4.4 Inflammasomes in Disease

Canonical and non-canonical inflammasome activation as well as pyroptosis have been implicated in many disease models. Non-canonical inflammasome activation by caspase-11 has

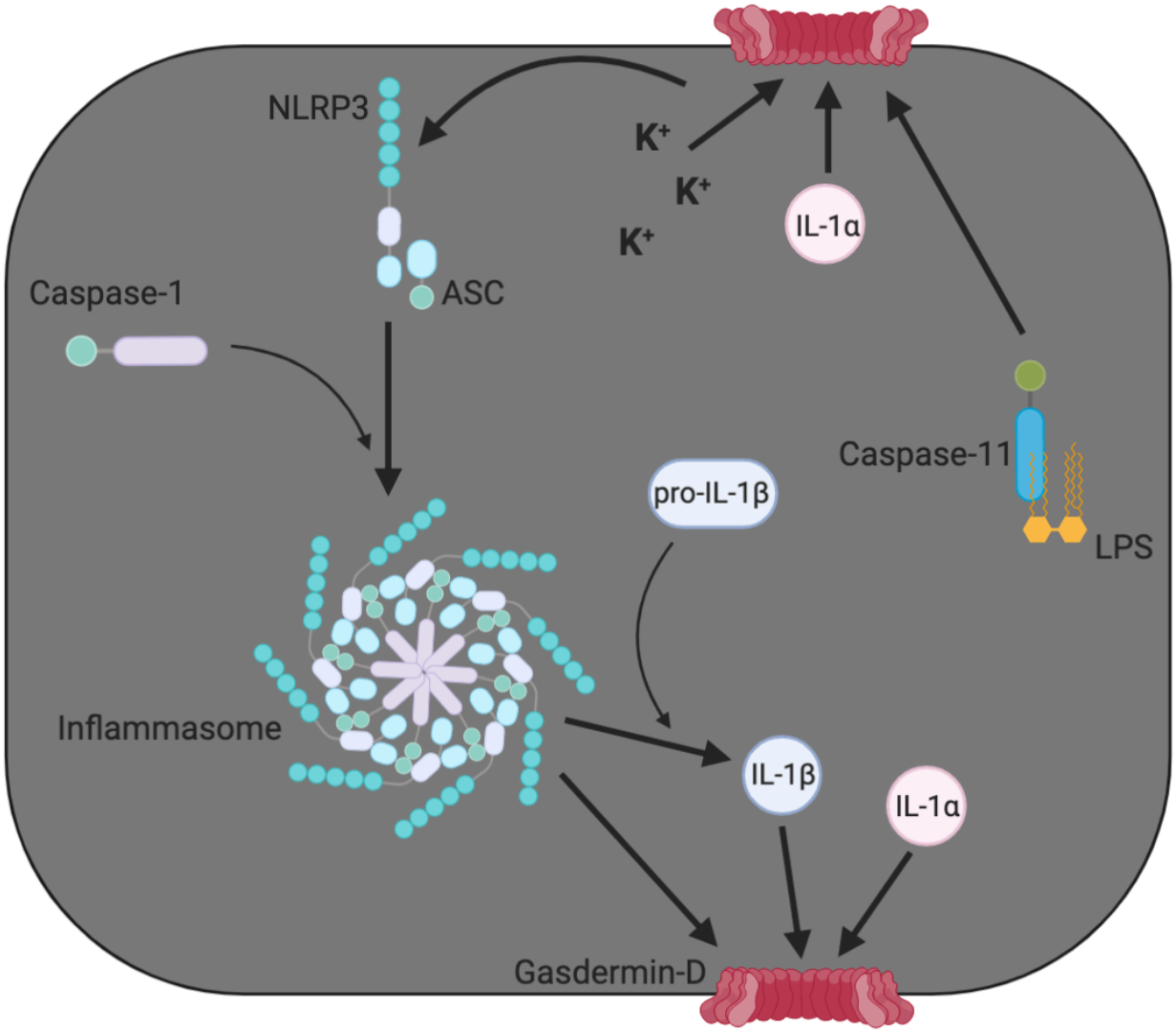


Figure 1.5 Inflammasome Pathway This graphic shows the canonical and non-canonical inflammasome pathways. In the canonical pathway, activation of an inflammasome sensor leads to oligomerization and formation of the inflammasome complex which activates caspase-1. Caspase-1 cleaves IL-18 and IL-1β, as well as gasdermin-D which forms pores in the plasma membrane. In the non-canonical pathway, direct activation of caspase-11 leads to cleavage of gasdermin-D and membrane pores. Potassium efflux through these pores can subsequently activate NLRP3 and the canonical inflammasome.

been shown to be crucial for pathology in models of endotoxic shock and severe sepsis.[131,133] Multiple lung infection models have established roles for inflammasomes in these diseases. NLRP3 and AIM2 inflammasomes have been shown to drive pathological inflammation in patients with protracted bacterial bronchitis.[134] On the other hand, the NLRP3 inflammasome has also been demonstrated to be important in the generation of adaptive immunity to influenza A virus infection in the lung.[135] In addition, ASC-dependent inflammasomes have been shown to facilitate viral/bacterial lung superinfection and lead to worsened lung pathology.[136] As referenced earlier, GSDMD-dependent pyroptosis has also been shown to trap intracellular bacteria such as *Salmonella* and facilitate its clearance.[18]

There are also a number of non-infectious disease contexts in which inflammasome activation has been implicated. GSDMD has been shown to be necessary for pathology in the chronic autoinflammatory disease Familial Mediterranean Fever.[137] Inflammasome priming of retinal pigment epithelial cells sensitizes these cells to photooxidative damage and pyroptotic death.[138] Additionally, inflammasomes and caspase-1-dependent pyroptosis have been implicated in traumatic brain and spinal cord injury leading to worse pathology.[139,140]

1.5 IL-1

The IL-1 family of molecules is large and diverse, including cytokines as well as receptors, and both pro-inflammatory and anti-inflammatory molecules.[141] Here we will focus on IL-1α, IL-1β, and their shared receptor (IL-1R1). IL-1α and IL-1β are pro-inflammatory cytokines that signal in the same way, however, their expression and the conditions mediating their release differ in important ways.

1.5.1 IL-1α vs β: Expression, Processing, and Release

Both IL-1α and IL-1β are synthesized as proteins of about 31 kDa that can be cleaved into products of about 17 kDa.[142] Neither of these proteins contain a signal sequence.[142] IL-1α is constitutively present in certain cell types and can be expressed upon stimulation as well.[143] Cells of barrier tissues often express IL-1α at baseline, and it can be further induced in both immune and non-immune cells, largely in response to NF-κB activation.[143] It can be found in the cytosol as well as the nucleus, as the N-terminal pro-piece contains a nuclear localization signal (NLS) and can bind to DNA.[143-145] It is thought to influence transcription of inflammation-related genes through interactions with chromatin, independent of signaling through its receptor.[143,144] Nuclear versus cytosolic localization of IL-1α varies with cell type as well as stimulation, with some inflammatory stimuli leading to increased nuclear localization.[143] Full length IL-1α can also be found on the surface of cells, anchored in the plasma membrane.[142,143] IL-1β is not constitutively expressed, but it can be induced in innate immune cells by inflammatory stimuli.[142] Following translation, pro-IL-1β is mainly cytoplasmic in localization.[142]

Both IL-1α and IL-1β can be cleaved intracellularly. IL-1α is cleaved by calpains within the cell, while IL-1β is cleaved by caspase-1 (formerly known as IL-1β converting enzyme or ICE).[142,143] Both of these cytokines can also be cleaved extracellularly by inflammation-associated proteases.[142,143] Importantly, IL-1α does not need to be cleaved for its activity as both the pro-form and the processed 17 kDa form are able to signal through its receptor, but IL-1β must be cleaved in order to be active, as its pro-form does not signal through its receptor.[146] Because it requires cleavage by caspase-1, IL-1β maturation is closely linked to the inflammasome pathways discussed above.

Because neither of the IL-1 molecules contains a signal peptide, they cannot be conventionally secreted. Release of both of these cytokines is coupled with loss of plasma

membrane integrity.[143] Expression at baseline, no need for processing, and release upon cell death make IL-1α a classic alarmin. However, because IL-1β must be induced, and then further processed, it is not generally considered to be an alarmin. As mentioned in the previous section, IL-1β release is generally associated with inflammasome activation, when it can be cleaved by active caspase-1 and can be released through GSDMD pores. However, the association of IL-1α release with inflammasome activation is less straight forward. It is now generally accepted that IL-1α is not directly dependent on inflammasomes for cleavage, but inflammasome-dependent pyroptosis is one way in which it can be released.[143] It has also recently been shown that release of IL-1 through GSDMD pores can occur in a manner distinct from pyroptotic cell death.[147]

1.5.2 Regulation of IL-1 Production

IL-1 molecules are potent inducers of inflammation; therefore, it makes sense that their genesis and release is tightly regulated. As discussed above, cells need to be exposed to certain stimuli even to kickstart expression of IL-1β transcript. Transcription of IL-1β does not always lead to its translation unless the cells experience a further stimulus.[141,142] Once translated, pro-IL-1β is still not biologically active. It needs to be cleaved by active caspase-1, which becomes activated as a result of inflammasome activation with its own regulation to overcome. Even if processed, IL-1β is ultimately unable to be released from a cell without pore formation or a loss of membrane integrity. Therefore, a variety of specific signals need to coincide and converge on the same cell for active IL-1β release to be achieved.

IL-1α also must overcome certain regulatory mechanisms in order to signal. Though IL-1α is constitutively present in some cell types, other cells may need to be exposed to inflammatory stimuli in order to upregulate expression. In addition, as mentioned above, the pro-piece of IL-1α contains an NLS. Nuclear localization likely has effects on transcription, but it may also function

as a way of sequestering IL-1α. Indeed, as a cell undergoes apoptosis or experiences genotoxic or other stress, IL-1α is increasingly localized to the nucleus as opposed to the cytosol.[143,148] Calpain-mediated cleavage of IL-1α, though not required for activity, does separate the NLS-containing pro-piece from the C-terminal mature species. Thus, cleaved IL-1α will not be sequestered in the nucleus upon the event of cell death.[143] Some cells also express a kind of decoy receptor, IL-1R2, which can bind IL-1α, but cannot signal. When present in the cytosol, IL-1R2 bound to IL-1α can prevent its cleavage.[149] It can also remain bound to Il-1α after release during necrosis, thus inhibiting signaling.[149] Interestingly, active caspase-1 can cleave IL-1R2, freeing IL-1α for cleavage and signaling.[150] This is another way in which inflammasome activation can promote IL-1α activity.

1.5.3 IL-1 Receptor and Signaling

Once they have been released, IL-1α and -β can bind to their receptor. IL-1R1 is expressed on the cell surface. Once bound by either IL-1α or IL-1β, IL-1R1 undergoes a conformation change that allows it to recruit the IL-1 receptor accessory protein (IL-1RAcP).[151] Recruitment of IL-1RAcP is necessary for signal transduction, and it is also a part of the receptor complex for a number of IL-1 family cytokines.[151] Upon assembly of this complex, MyD88 and IRAK4 are recruited through interactions with TIR domains.[151] This allows for the subsequent activation of IRAK1 and 2 as well as TRAF6.[151] This culminates in the activation of p38 MAPK as well as NF-κB and AP-1 transcription factors, leading to the initiation of a broad inflammatory program (Fig. 1.6).[151] Another ligand for IL-1R1 is the IL-1 receptor antagonist, IL-1RA, which binds to IL-1R1 but does not propagate a signal.[141,151] It should be noted that signaling downstream of IL-1R1 may change in different cell types depending on the downstream machinery they express. For example,

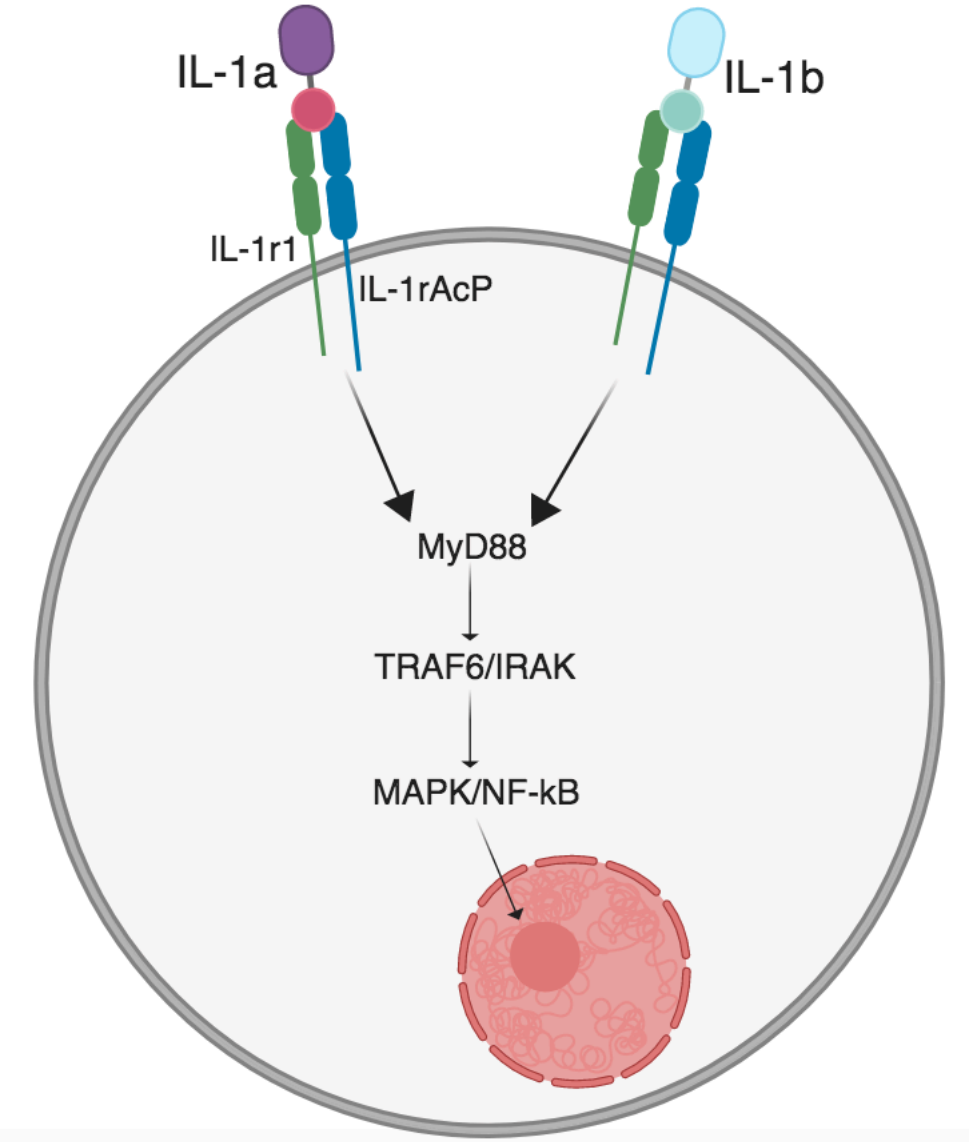


Figure 1.6 IL-1 Signaling Summary

IL-1 signaling in neurons may be atypical because neurons express a different isoform of IL-1RAcP, termed AcPb.[152,153]

1.5.4 IL-1 Signaling in Models of Disease

As broadly pro-inflammatory innate cytokines, IL-1 molecules have been shown to be involved in the immune response to various injury and infection models. One way in which IL-1 promotes inflammation seems to be through modulation of vascular activation. In models of respiratory syncytial virus (RSV) infection, IL-1α released from infected epithelial cells was shown to induce expression of adhesion molecules, particularly ICAM-1, on vascular endothelial cells.[154] In a mouse model of ischemia, platelet aggregation in brain vessels led to activation of the endothelium. It was further shown that IL-1α from the platelets led to increased expression of adhesion molecules and neutrophil chemoattractant in brain endothelial cells, promoting immune cell entry.[155] IL-1R1 expression on spinal cord blood vessels has been shown, and further, it was demonstrated that these vessels respond to immune cell-derived IL-1β by producing a number of pro-inflammatory cytokines and propagating inflammation in a model of autoimmune encephalomyelitis (EAE) in mice.[156] In addition, IL-1 signaling specifically on endothelial cells in the brain was recently shown to be sufficient to induce sickness behavior in mice.[157]

Outside of specific effects on vasculature, IL-1 has also been shown to play a role in many infectious contexts. It has been demonstrated that IL-1 signaling, specifically IL-1α, drives pathology in a neonatal mouse model of sepsis.[158] IL-1 signaling through IL-1R1 was shown to be necessary for control of pulmonary *Mycobacterium tuberculosis* infection on both hematopoietic and non-hematopoietic cell populations.[159] IL-1 has also been implicated in models of central nervous system (CNS) infection with West Nile virus. IL-1 signaling through IL-1R1 was shown to be important specifically in CNS control of West Nile virus.[160] The authors demonstrated that

IL-1 signaling was necessary for optimal dendritic cell activation of $CD8^+$ T cells within the CNS.[160] Also in a model of West Nile virus infection, another group has shown that IL-1β in particular, as a result of NLRP3 inflammasome activation, restricts viral replication.[161] Further, in modeling recovery from West Nile virus infection, it has been suggested that IL-1 signaling in the brain promotes preferential astrogenesis over neurogenesis, leading to cognitive impairment following infection.[162]

IL-1 also plays a role in non-infectious inflammatory conditions. Peripheral inflammation in rheumatoid arthritis (RA) has been shown to coincide with central IL-1β production, as measured in the CSF of patients.[163] IL-1 signaling was also implicated as one factor downstream of glucagon-like peptide 1 (GLP-1) activity that promotes decreased food intake and weight loss.[164] In mouse models of sterile lung injury using inhaled silica nanoparticles, IL-1α release was shown to initiate pathological lung inflammation including later IL-1α-dependent IL-1β release.[165] IL-1α release from microglia has also been shown to promote death of oligodendrocytes and inflammation in a mouse model of spinal cord injury.[166]

1.6 *Toxoplasma gondii*

Toxoplasma gondii is a protozoan parasite with a broad host range, able to infect many animals as well as humans. Infection with this parasite is very prevalent worldwide.[167] *T. gondii* is an apicomplexan parasite, belonging to the phylum Apicomplexa. There are at least 6000 known apicomplexan species and likely many more that have not yet been identified.[168] Apicomplexan parasites cause disease in human and animal populations. Apicomplexans are morphologically similar, and share many specialized organelles.[169] All of these parasites have an essential organelle called the apicoplast, which is similar to a chloroplast (though not photosynthetic) and is thought

to have been acquired at some point from green algae or to be a vestige from a photosynthetic ancestor.[169-172] The apical polar ring is another structure found in all apicomplexa, which functions as a microtubule organizing center.[169,173] These parasites also contain rhoptries and micronemes which are secretory structures that facilitate movement, host cell invasion, and the creation of the parasitophorous vacuole (PV).[169,174] *T. gondii*, like many apicomplexans, is an obligate intracellular parasite which establishes a unique niche within host cells; the PV. This vacuole is created from host cell membrane upon invasion, and thus contains host lipids, but the incorporation of host proteins into this membrane is tightly regulated by the parasite.[169,175-177] The vacuole is non-fusigenic, and thus is not part of the endosomal pathway and does not acidify or fuse with lysosomes.[178-180] The PV protects the parasite while also allowing it to acquire resources it needs from the host cell.[181] The parasite relies on the host cell for things such as amino acids including arginine and tryptophan, purines and pyrimidines, and certain lipids.[182-186]

There are three different stages in the *T. gondii* life cycle, all of which are infectious: tachyzoites, bradyzoites, and sporozoites.[187] Tachyzoites are a stage of the parasite that is rapidly replicating, and can replicate in any nucleated cell within its host.[187] Bradyzoites are much more slowly replicating parasites that form intracellular tissue cysts within the host.[187] Sporozoites are parasites contained within oocysts.[187] The life cycle of *T. gondii* is fairly complex. Felines are the only definitive host for this parasite, meaning that only within cats (specifically within the feline intestinal tract), the parasite can undergo its full sexual reproduction. After its replicative cycle, an oocyst wall forms around the parasite, the infected intestinal epithelial cells rupture, and oocysts are shed in cat feces.[187] This form of the parasite is very stable, and can infect other intermediate hosts when their food and/or water sources have been contaminated by cat feces containing oocysts.[188] In intermediate hosts, the parasite exists in either the tachyzoite or bradyzoite forms.

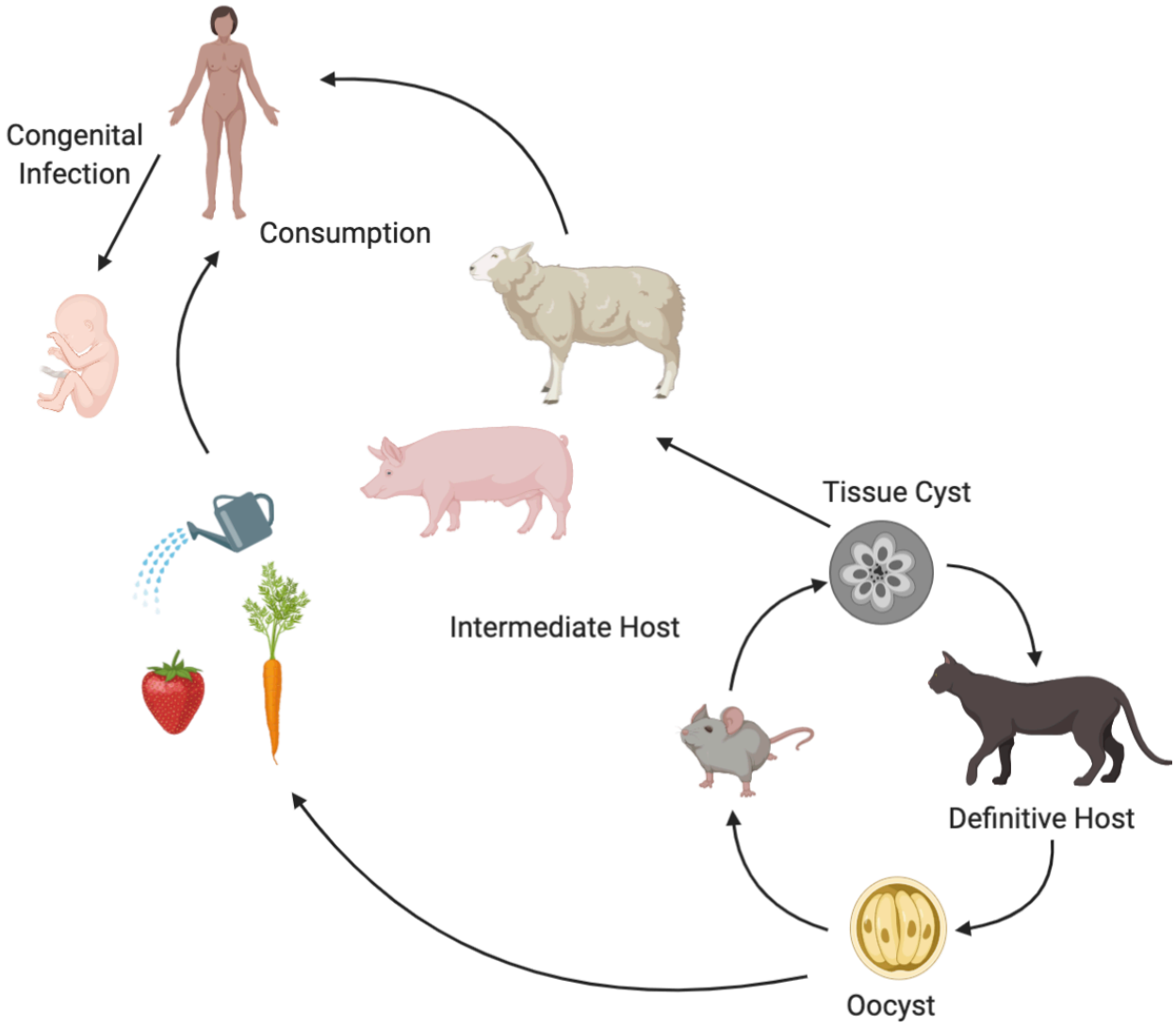


Figure 1.7 ***Toxoplasma gondii*** **Transmission**

There are different strains of *T. gondii* that express different effectors and therefore have different levels of virulence, though these have been defined mainly through mouse studies. The most well studied clonal lineages include type I, II, and III parasites. While all lineages infect humans, most human infections are due to type II strains.[189] Prevalence of type II strains is higher in human AIDS patients than in animals, prevalence of type I strains is higher in human congenital infections than animal infections, and type III strains are found more in animal infections than human infections.[189] In the nomenclature defined by mouse studies, type I strains are highly virulent, type II strains are intermediate in virulence, and type III strains are avirulent.[190] Several parasite antigens are polymorphic between strains, possibly contributing to differences in virulence; for example, type II parasites promote a strong T cell response through DC activation, whereas this is not as strong in infections with type I parasites.[190] This is associated with higher levels of NF-κB activation in infections with type II parasites.[190] There are many differences between the parasite strains that contribute to differences in virulence, but one effector that seems to contribute to this difference is a *T. gondii* rhoptry protein ROP18. ROP18 is expressed highly in type I and II strains, but at very low levels in type III strains, and complementation is able to enhance virulence of type III parasites.[190] ROP18 functions to inhibit NF-κB activation.[191] Another parasite effector that leads to strain differences is the dense granule protein GRA15 which is expressed by type II strains of *T. gondii*, leading to increased NF-κB activation and IL-12 production in these infections.[190] Overall, type I strains are defined as highly virulent because of their ability to escape mechanisms of parasite killing intracellularly while also inhibiting the induction of type I immunity until it is too late to be effective. This ultimately means that mice succumb to type I infections before a chronic infection can be established.[190] Type II strains appear to have a mix of virulent and avirulent alleles for some effector proteins, meaning that they can

escape killing to a certain extent, but also induce protective immunity. Thus, type II strains establish a chronic infection as peripheral infection is cleared and bradyzoite parasites form tissue cysts.[190] Type III strains are avirulent because ultimately, though they do not potently stimulate inflammation, they are readily cleared by the host.[190]

1.6.1 Disease Relevance

Worldwide, infection prevalence for this parasite ranges from 10 to 90 percent.[167,192] This parasite is primarily acquired through ingestion, either of food or water that has been contaminated with oocysts shed in the feces from a feline, or of tissue cysts present in undercooked meat.[188] For most immunocompetent individuals, infection with *T. gondii* is asymptomatic. However, these infections can cause severe disease and even death when they are congenitally acquired or when they reactivate in immunocompromised people, such as in patients with late-stage AIDS.[193-195]

Congenital infection occurs when a mother experiences an initial *T. gondii* infection while she is pregnant. Congenital infection with *T. gondii* can lead to prenatal death of the fetus, post-natal death of the offspring, and in surviving offspring can lead to illness with ranging severity including cerebral and ocular disease, with manifestations such as hydrocephalus, intracranial calcifications and chorioretinitis.[193,196,197]

Because maintained immunity is required for control of chronic *T. gondii* infection, a major example of severe manifestation of disease is seen in patients suffering from late-stage AIDS who have a depleted $CD4^+$ T cell population. Toxoplasmic encephalitis (TE) is a common and severe symptom experienced by such immunosuppressed individuals, which often includes the formation of necrotic lesions within the brain.[188,194,195]

1.6.2 Immune control of acute infection

Acute infection in intermediate hosts is characterized by spreading of replicating tachyzoites throughout most host tissues. The immune response to *T. gondii* during this stage displays a strong type I polarization, characterized by reliance on interferon-γ (IFN-γ) for parasite control.[198] Infection of innate immune cells, including dendritic cells and macrophages, by *T. gondii* or the recognition of pathogen associated molecular patterns (PAMPs) by these cells leads to the production of IL-12.[199,200] IL-12 then acts on both natural killer (NK) cells and T cells to elicit the production of IFN-γ.[198,199] Both IL-12 and IFN-γ have been shown to be essential for controlling infection with *T. gondii*.[198-200] During the acute stage of infection, killing of infected cells by parasite-specific $CD8^+$ T cells plays a role in limiting infection, however, the production of IFN-γ also primes many cell types to be able to kill parasites intracellularly upon infection (Fig. 1.8).[28,29,201,202] In myeloid cells as well as other non-hematopoietic cells, IFN-γ signaling through STAT1 activates genes including interferon regulated GTPases (IRGs) as well as guanylate binding proteins (GBPs) that compromise the integrity of the parasitophorous vacuole, in addition to reactive oxygen and nitrogen species (ROS and RNS).[30,203-208] IRGs appear to be important in mice as an intermediate host to *T. gondii* as interestingly, wild mice with polymorphic IRG alleles are able to survive infection with virulent strains which kill laboratory mice.[209] IFN-γ can also lead to the sequestration of tryptophan within cells, starving parasites of this essential host-derived nutrient.[210] This IFN-γ-driven response is effective at clearing the parasite from most tissues, however the parasite is then able to seed selected tissues including the brain and the muscle and persist for the lifetime of the host.[211,212]

1.6.3 Immune control of chronic infection

By at least four weeks post infection with *T. gondii*, the parasite mainly resides in the brain and the muscle, and this is known as the chronic stage of infection. There have been two theories

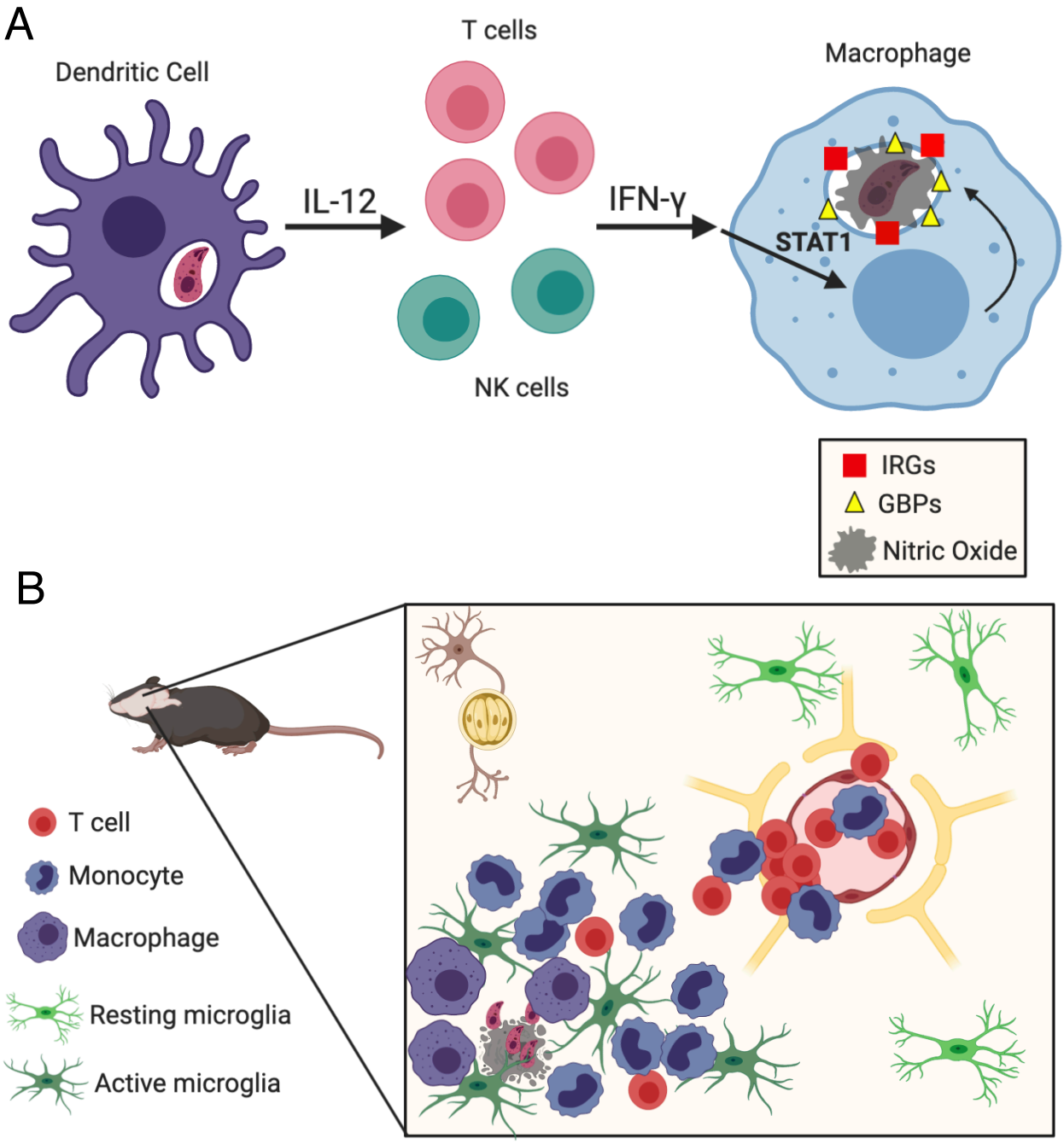


Figure 1.8 Response to acute and chronic *T. gondii* infection A) Basic summary of the immune response to acute infection: parasite recognition leads to IL-12 production, which initiates IFN-γ production from lymphocytes, which primes cells to kill *T. gondii*. B) Overview of chronic infection: immune cells are present in perivascular spaces and brain parenchyma, and cluster around active parasite.

put forth as to how the parasite ends up in the brain, both with supporting evidence. One is the trojan horse hypothesis, in which infected myeloid cells traffic to the brain.[211,213] The other is a free parasite hypothesis, in which parasites in the circulation infect and lyse a brain endothelial cell, crossing onto the other side of the blood brain barrier.[212] During this stage, the parasite is found in intracellular bradyzoite cysts which are relatively quiescent and are thought to be unrecognized by the immune system. However, these cysts in the brain are also known to stochastically reactivate, releasing replicating parasites.[198,199,214] This is understandably dangerous in a tissue like the brain, and in immunocompetent hosts immune cells mobilize to these areas of active parasite and restrict the parasite. Whole brain RNA-sequencing has shown that infected mice differ in gene expression from uninfected mice, and exhibit enrichment for immune and inflammation-related pathways.[215] Brain resident cells such as astrocytes have been demonstrated to produce chemokines and cytokines in the brain that promote inflammation and parasite control, as well as to be able to directly kill parasites *in vitro*.[216-223] In culture systems, primed microglia have also been shown to limit parasite replication.[224-226] *In vitro* and *in vivo* studies have also shown that microglia can produce chemokines and cytokines in the brain during infection and can also display migratory behavior and cluster around parasite reactivation.[216,221,227-231] It should be noted however, that much of this work has been unable to distinguish between microglia and blood-derived macrophages. It has been shown through depletion studies that T cells and the IFN-γ that they produce are still required during chronic infection to maintain control of the parasite.[232] This is also why reactivation is seen in AIDS patients, particularly when their $CD4^+$ T cell count drops below a certain threshold.[233-235] Myeloid cells, particularly cells of the monocyte/macrophage lineage, have also been shown to be recruited into the brain parenchyma during chronic infection and to be important for the long term control of *T. gondii* as mice treated

with an anti-CCR2 antibody succumb to infection.[214] Therefore, sustained innate and adaptive responses to *T. gondii* throughout life are necessary for host defense (Fig. 1.8).

1.6.4 Pattern recognition in *T. gondii* infection

There is evidence supporting the role of pattern recognition in the immune response to *T. gondii* infection. One of the earliest pieces of evidence to support this was the finding that MyD88$^{-/-}$ mice displayed increased mortality and a decreased ability to control parasite replication.[200] This was originally attributed solely to a role for TLR signaling, however, this pathway is now known to be more broad. TLR11 and TLR12 have both been shown to recognize *T. gondii* profilin (Fig. 1.9).[236-239] Dendritic cell recognition of profilin through TLR11 has been shown to potentiate IL-12 production *in vivo*.[236] TLR12 and TLR11 have also been shown to induce IL-12 production in DCs in a manner dependent on IRF8.[237] TLR7 and TLR9 have also been demonstrated to recognize parasite-derived nucleic acids and to contribute to host immunity to infection (Fig. 1.9).[239] Since humans to do not possess functional TLR11 or TLR12, it may be the case that nucleic acid sensing is more important in the response of human cells to *T. gondii* infection.[239,240] Beyond TLR recognition of the parasite, its activation of other PRRs and the consequences of this activation has been fairly controversial. The role of inflammasome pathways has been studied in *in vitro* models using rodent macrophages as well as rodent models of acute *T. gondii* infection. One study showed that variants of NLRP1 correlate with differences in rat macrophage cell death following *in vitro* infection with *T. gondii* across different strains.[241] This study also showed that macrophages susceptible to death after infection also released IL-18 and, when primed with LPS, IL-1β.[241] Intriguingly, allelic differences in the corresponding human inflammasome sensor NALP1 have been shown to be associated with susceptibility to congenital toxoplasmosis.[242] Another study showed IL-1β release from LPS-primed rat and mouse macrophages infected *in*

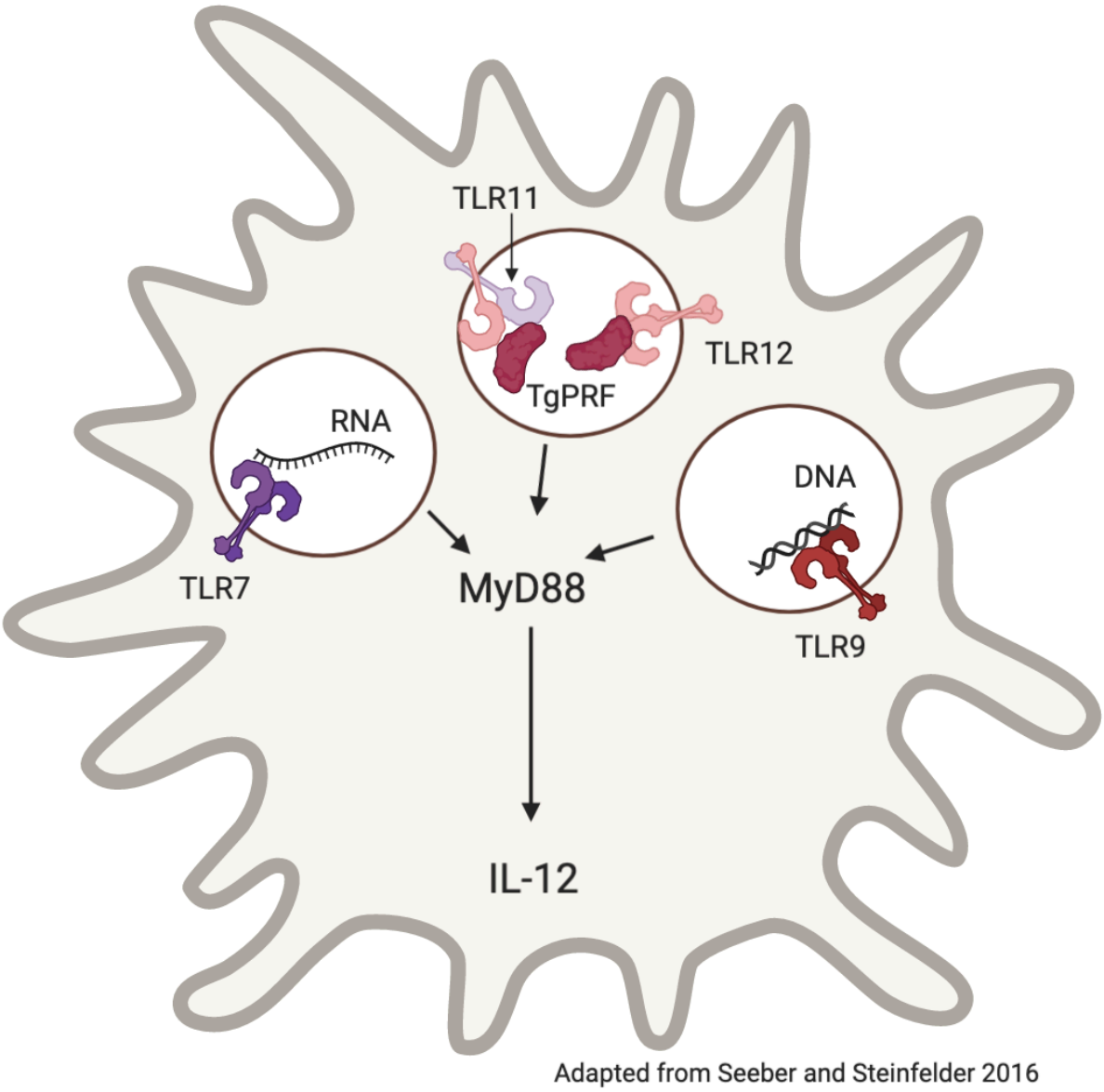


Figure 1.9 TLR activation in acute *T. gondii* infection

	Majority of experiments	NLRP1	NLRP3	ASC	casp-1/11	IL-1R	IL-18	IL-1β
Ewald et al. 2013	*in vitro*	Yes	No	Yes	Yes	n/a	n/a	Yes
Cirelli et al. 2014	*in vitro*	Yes	n/a	n/a	n/a	n/a	Yes	Yes
Gorfu et al. 2014	*in vivo*	Yes	Yes	Yes	Yes	Yes	Yes	No

Table 1.1 Summary of previous findings This table summarizes the published findings related to inflammasome and IL-1 pathways in *T. gondii* infection, and indicates relevance found for these proteins in each model.

vitro with *T. gondii*, and that this was dependent on NLRP1 and not NLRP3.[243] This study also showed that caspase-1/11$^{-/-}$ mice lost more weight than WT mice and had increased parasite burden in the brain, intestine, and testes compared to controls.[243] Another study showed that unprimed mouse BMDMs exposed to a type II strain of *T. gondii in vitro* release IL-1β in a manner dependent on caspase-1/11, ASC, and NLRP3, but independent of cell death.[244] This study reported inability to detect IL-1β release *in vivo*, but did show detection of IL-18 in serum of *T. gondii*-infected mice at early time points.[244] In this study, it was further reported that serum IL-18, parasite control, and survival during the acute stage of infection were dependent on caspase-1/11, ASC, IL-1R, NLRP1, and NLRP3.[244] One study did broadly examine the requirement for caspase-1/11 during early chronic stages of infection, only noting increased inflammation and parasite burden in the brain as determined by histology.[245] Still, the role of these pathways, especially IL-1 signaling, in the brain during chronic infection remains to be addressed.

1.7 Project Rationale

As discussed above, work has been done examining the role of innate pattern recognition and IL-1 pathways in acute *T. gondii* infection and in *in vitro* models, however, very little is known about how these pathways may function in the chronic stages of infection in the brain. Constant immune infiltration into the brain needs to be maintained during chronic infection for the survival of the host. We know that both T cells and myeloid cells are important for maintaining this control of the parasite, but the events within the brain that initiate inflammation in response to local parasite are not understood. Since IL-1 is a cytokine associated with host cell damage and has been established as a pro-inflammatory mediator which can promote immune cell recruitment, we hypothesized that it would play a role in the chronic neuroinflammation in *T. gondii* infection.

Thus, we investigated this *in vivo* using genetic knockout mice (Chapter 3). These studies also led us to examine the apparent differences between the functionality of microglia and blood-derived myeloid cells in the same tissue microenvironment, and what those differences may mean for the inflammatory response (Chapter 3). Much of the work done on microglia in the context of *T. gondii* infection has either been done *in vitro* or does not distinguish between microglia and other macrophages. Using newer tools, we are able to investigate whether microglia perform specific, non-overlapping functions distinct from infiltrating macrophages.

Chapter 3 key findings (published in Batista et al. 2020) (Fig. 1.10):

- Microglia and macrophages have different inflammatory signatures
- IL-1 signaling promotes brain inflammation and parasite control
- IL-1-dependent inflammation is driven by IL-1α
- Microglia release IL-1α
- IL-1α release is dependent on gasdermin-D

Our investigation into the role of IL-1 and the inflammasome pathway in promoting inflammation in the brain during chronic *T. gondii* infection, also led us to the interesting question of what the host senses to activate the inflammasome, whether it is host cell damage or the parasite itself. This includes the related question of how the host senses this signal, in other words, which inflammasome sensor is involved. We have begun to perform experiments to address these questions, but more work is needed to fully elucidate this mechanism (Chapter 4).

Chapter 4 key findings (unpublished):

- Caspase-11 is not necessary for inflammation, parasite control, or IL-1α release
- NLRP3 is not necessary for inflammation, parasite control, or IL-1α release
- AIM2 is not necessary for inflammation, parasite control, or IL-1α release

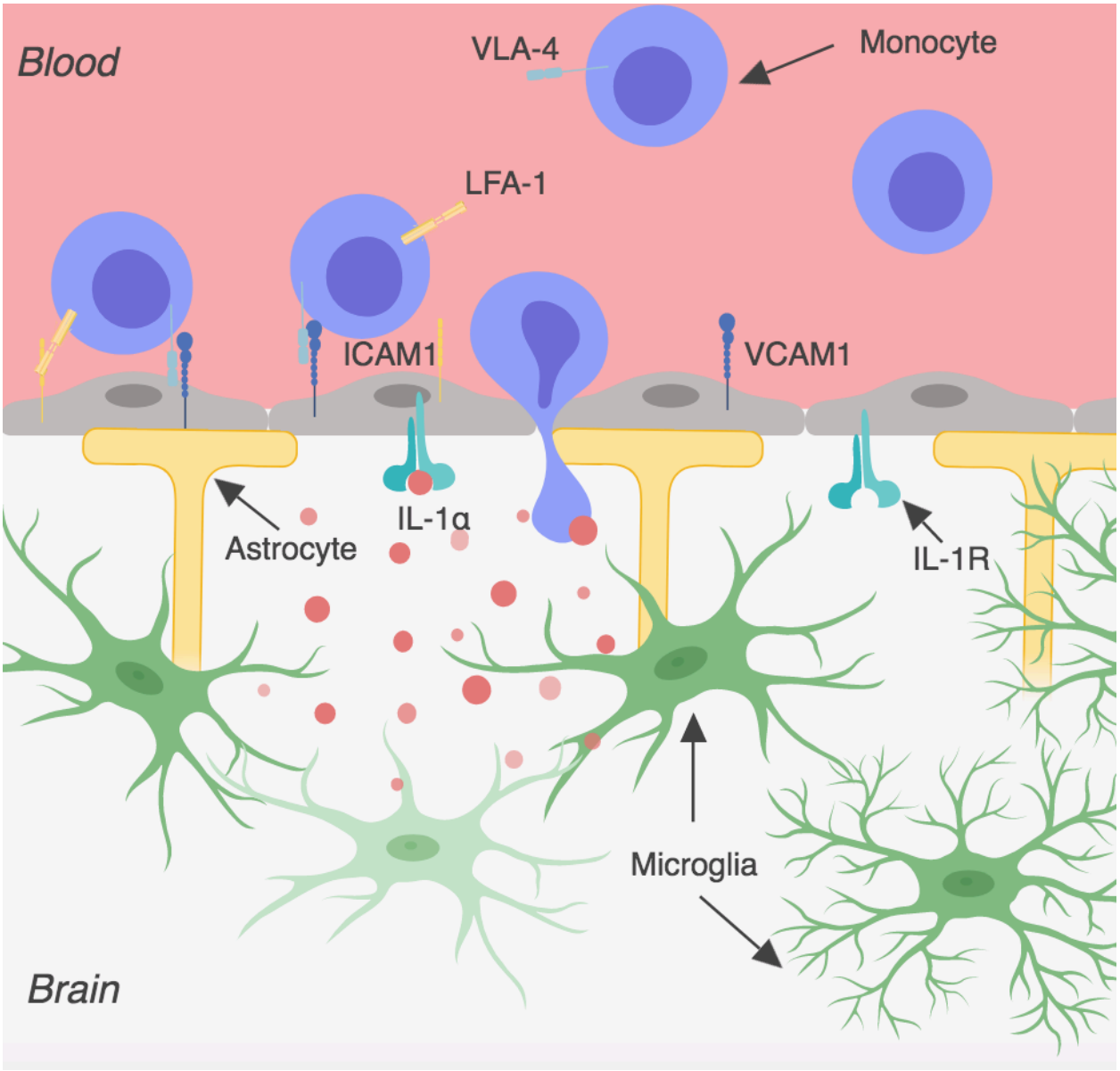


Figure 1.10 IL-1-driven brain inflammation In response to a currently unknown stimulus, microglia in the infected brain release IL-1α in a gasdermin-D-dependent manner. This then signals through IL-1R1, likely on brain vasculature, to activate the endothelium and promote the infiltration of immune cells from the blood.

All of these studies have also piqued our interest in other mechanisms of cell death and the role they may play in immunity to infection. We have begun very preliminary studies looking into the role of necroptosis as well as apoptosis in the response to *T. gondii* infection (Chapter 5). Though they are preliminary at this stage, I think these studies hold a lot of interest going forward.

Chapter 5 key findings (unpublished):

- RIPK3$^{-/-}$ and caspase-8$^{-/-}$RIPK3$^{-/-}$ mice have increased brain inflammation
- RIPK3$^{-/-}$ mice have a modest increase in brain parasite burden
- Caspase-8$^{-/-}$RIPK3$^{-/-}$ mice have an extreme increase in brain parasite burden

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice and Infections

C57BL/6 mice were purchased from The Jackson Laboratory or bred within our animal facility in specific pathogen-free facilities. All mice were age- and sex-matched for all experiments. Infections used the type II *T. gondii* strain Me49, which was maintained in chronically infected Swiss Webster mice (Charles River Laboratories) and passaged through CBA/J mice (The Jackson Laboratory) for experimental infections. For the experimental infections, the brains of chronically infected (4-8 week) CBA/J mice were homogenized to isolate tissue cysts. Experimental mice were then injected i.p. with 10 Me49 cysts. All procedures followed the regulations of the Institutional Animal Care and Use Committee at the University of Virginia.

2.2 Sampling

Within a single experiment, when multiple parameters were assessed in the same tissue, the same samples were used (i.e. for analysis of multiple immune cell populations by flow cytometry, cells from the same brain sample were used). Representative IHC images accompanying flow cytometry data were taken from distinct brain samples.

2.3 *T. gondii* Cyst Counts

Brain tissue was placed in complete RPMI, minced with a razor blade, and then passed through an 18-gauge needle. 30 μL of homogenate was placed on a microscope slide and covered with a coverslip. Cysts were counted manually on a brightfield DM 2000 LED microscope (Leica Biosystems).

2.4 Tissue Processing

Immediately after sacrifice mice were perfused with 30 mL of cold 1X PBS. Brains and spleens were harvested and put into cold complete RPMI media (cRPMI) (10% FBS, 1%

penicillin/streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, and 0.1% 2-ME). If peritoneal lavage fluid was collected, prior to perfusion, 5 mL of cold 1X PBS was injected through the intact peritoneal membrane with a 26-gauge needle, and removed with a 22-gauge needle. If serum was collected, blood from the heart was collected and allowed to clot at 4°C overnight to separate serum.

After harvest, brains were minced with a razor blade, passed through an 18-gauge needle, and then enzymatically digested with 0.227 mg/mL collagenase/dispase and 50 U/mL DNase (Roche) at 37°C for 45 minutes. After digestion, brains homogenate was passed through a 70 μm filter (Corning) and washed with cRPMI. To remove myelin from samples, filtered brain homogenate was then resuspended with 20 mL of 40% Percoll and spun at 650 x g for 25 minutes. Myelin was aspirated, samples were washed with cRPMI, and then resuspended in cRPMI. Spleens were mechanically homogenized and passed through a 40 μm filter (Corning). Samples were washed with cRPMI and then resuspended in 2 mL of RBC lysis buffer (0.16 M NH_4Cl) for 2 minutes. Cells were then washed with cRPMI and then resuspended. Peritoneal lavage fluid was washed with cRPMI, pelleted and resuspended.

2.5 Cytospin

Peritoneal lavage fluid samples were diluted to 1 x 10^5 cells/200 μL which was added to the upper chamber of the slide attachment (Simport). Samples were spun onto slides using a Cytospin 4 (Thermo Scientific), and then H&E stained.

2.6 Flow Cytometry and Cell Sorting

Single cell suspensions from tissue samples were plated in a 96-well U-bottom plate. Cells were initially incubated with 50 μL Fc block (1 μg/mL 2.4G2 Ab (BioXCell), 0.1% rat γ globulin (Jackson ImmunoResearch)) for 10 minutes at room temperature. Cells were then surface stained

with antibodies and a Live/Dead stain for 30 minutes at 4°C. After surface staining, cells were washed with FACS buffer (0.2% BSA and 2 mM EDTA in 1X PBS) and fixed at 4°C for 30 minutes with either 2% paraformaldehyde (PFA) or a fixation/permeabilization kit (eBioscience). Cells were then permeabilized and stained with any intracellular markers for 30 minutes at 4°C. Samples were then washed, resuspended in FACS buffer, and run on a Gallios flow cytometry (Beckman Coulter). Analysis was done using FlowJo software v.10. Antibody clones used include: CD31 (390), CD45 (30-F11), MHC-II (M5/114.15.2), NK1.1 (PK136), CD19 (1D3), CD3 (17A2), CD4 (GK1.5), CD11c (N418), CD11b (M1/70), Foxp3 (FJK-16s), Ly6G (1A8), Ly6C (HK1.4), CD8a (53-6.7), IFN-γ (XMG1.2), NOS2 (CXNFT), IL-1α (ALF-161), and pro-IL-1β (NJTEN3).

For cell sorting, CX3CR1cre^{ERT2} x ZsGreen$^{fl/stop/fl}$ mice were used. After surface staining, live cells were analyzed on a BD Aria in the UVA flow cytometry core facility. Cells were sorted based on ZsGreen expression, into serum-containing media for *ex vivo* culture, or into Trizol for RNA-sequencing.

2.7 *ex vivo* Culture Experiments

To assess IL-1 release, brain single-cell suspensions were plated in a 96-well plate in complete RPMI media with no additional stimulation. They were then incubated at 37°C for 4 hr to overnight (indicated in figure legends). For gasdermin-D inhibition assays, 20μM necrosulfonamide (NSA) was added to wells during incubation. Cells were pelleted and supernatants were collected for analysis. For sorted cell populations, equal numbers of microglia and macrophages were plated for analysis.

2.8 Quantitative RT-PCR

Approximately ¼ of a mouse brain was put into 1mL TRIzol (Ambion) in bead-beating tubes (Sarstedt) containing 1 mm zirconia/silica beads (BioSpec). Tissue was homogenized for 30

seconds using a Mini-bead beater (BioSpec). RNA was extracted according to the manufacturer's instructions (Ambion). High Capacity Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis. qRT-PCR was done using 2X Taq-based Master Mix (Bioline) and TaqMan gene expression assays (Applied Biosystems). Reactions were run on a CFX384 Real-Time System (Bio-Rad Laboratories). HPRT was used as the housekeeping gene for all reactions and relative expression was calculated as $2^{(-\Delta\Delta CT)}$.

2.9 Immunohistochemistry

Brains from mice were harvested and placed in 4% PFA for 24 hours. Following PFA fixation, brains were moved to a solution of 30% sucrose for 24 hours, and were then embedded in OCT and flash frozen on dry ice. Samples were stored at -20°C until cutting. After cutting, sections were blocked in 1X PBS containing 0.1% triton, 0.05% Tween 20, and 2% goat or donkey serum (Jackson ImmunoResearch) for 1 hour at room temperature. Sections were then incubated with primary Abs overnight at 4°C. Sections were then washed with PBS, and incubated with secondary Abs for 1 hour at room temperature. Sections were then washed, and nuclear stained with DAPI (Thermo Fisher Scientific) for 5 minutes at room temperature. Finally, sections were mounted, covered in Aquamount (Lerner Laboratories), and covered with coverslips (Thermo Fisher Scientific). All images were captured using a Leica TCS SP8 Confocal microscopy system. Images were analyzed using either ImageJ or Imaris software.

2.10 H&E Tissue Sections

Brains from mice were submerged in formalin and sent to the UVA Research Histology Core, where they were embedded in paraffin and sectioned. They were then imaged on on a brightfield DM 2000 LED microscope (Leica Biosystems).

2.11 ELISAs

Samples for ELISAs were obtained by harvesting mouse brains and processing them to form a single cell suspension. Cells were then plated in 96-well plates and incubated at 37°C either overnight or for 5 hours (indicated in figure legends). Supernatants were then collected and stored at -20°C until use. ELISAs for IL-1α and IL-1β (BioLegend), as well as for IFN-γ, were performed according to the manufacturer's instructions. Briefly, Immunolon 4HBX ELISA plates (Thermo Fisher Scientific) were coated with capture antibody at 4° overnight. Plates were then washed and blocked with buffer containing BSA at room temperature for 1 hour. After washing, standards and samples were added and incubated at room temperature for 2 hours. After washing, biotinylated detection antibody was added and incubated for 1 hour at room temperature. Plates were washed and incubated with avidin-HRP for 30 minutes at room temperature. Finally, plates were washed and incubated with ABTS peroxide substrate solution (SouthernBiotech) for 15 minutes or until color change occurred. Immediately after color change, plates were read on an Epoch Biotek plate reader using Gen5 2.00 software.

2.12 Ab Blockade Experiments

Chronically infected mice (4 weeks p.i.) were treated on days 1 and 3 of the treatment regimen with 200 μg i.p. each of anti-LFA-1 and anti-VLA-4 blocking antibodies (Bio X Cell) or control IgG. They were then sacrificed and analyzed on day 5.

2.13 Propidium Iodide Injection

Chronically infected mice (4 weeks p.i.) were injected i.p. with 0.4 mg of propidium iodide. 24 hours after injection, mice were sacrificed and their brains were PFA fixed and analyzed by confocal microscopy.

2.14 Microglia Depletion

For studies involving microglia depletion, mice were fed either control chow or chow containing PLX5622 *ad libitum* for 12 days prior to harvest.

2.15 Bone Marrow Chimeras

At 8 weeks of age, C57B6/J and *Il1r1*$^{-/-}$ mice were irradiated with 1000 rad. Bone marrow cells isolated from WT and *Il1r1*$^{-/-}$ mice were then i.v. transferred (by retro-orbital injection) into the irradiated recipient mice. Mice were allowed to recover for 6 weeks after irradiation and reconstitution and were then infected. 4 weeks post infection, mice were sacrificed and tissues were harvested for analysis.

2.16 RNA Sequencing Data Analysis

Pre-Processing/Primary Analysis:

Read quality profiles for raw FASTQ files was performed with FastQC (v0.11.5) before and after trimming and filtering. Read filtering and trimming was accomplished with Trimmomatic (v0.39) paired-end set to phred33 quality scoring. Reads were trimmed according to a four-base sliding window with a minimum quality score of 15 and minimum leading and trialing quality scores of 3. The minimum fragment length was set to 36. Trimmed and filtered reads were mapped to the GENCODE M13 genome and transcript abundances were quantified using Salmon (v0.8.2). Quantified transcript abundances were imported into the R programming environment and converted into ENSEMBL gene abundances with Tximport (v1.4.0). All pre-processing steps were performed within the Pypiper framework (v0.6.0) with Python version 2.7.14.

Secondary and Tertiary Analysis:

Differential expression testing was performed using the R Bioconductor package DESeq2 (v1.16.1) at a preset alpha value of 0.05. Log2 fold change values were shrunken using a normal prior distribution. Any results that lacked the replicates or had low counts were thrown out of the

dataset prior to differential expression testing. Results of differential expression testing were visualized using the R package EnhancedVolcano (v1.2.0) to display transformed p-values (-log10) against the corresponding log2 fold change values. All labeled genes were manually selected from significantly differentially expressed genes in the DESeq2 results list.

Differential expression testing results were labeled as "upregulated" or downregulated" for a given pairwise comparison. All genes with a log2 fold change value above 0 and a BH adjusted p-value below 0.05 were designated upregulated and all genes with a log2 fold change value below 0 and a BH adjusted p-value below 0.05 were designated downregulated. Gene names for the differential expression results tables were converted from mouse ENSEMBL codeas to gene symbols with AnnotationDbi (v1.46.0). In order to determine the functional profile of the gene lists, the R package clusterProfiler (v3.12.0) was used to apply Fisher's exact test with respect to over-representation of GO terms for biological processes at all levels of the Gene Ontology Consortium hierarchy. The lists were tested against a background distribution that consisted of all genes that returned a p-value in differential expression testing. Significant GO terms had a BH adjusted p-value below 0.05.

GO terms were manually selected from the results output in the clusterProfiler package for plotting with the pheatmap package (v1.0.12). Each GO term-specific heatmap displays rlog-transformed abundance values that have been Z-score normalized with respect to each gene. The genes displayed were selected from clusterProfiler results for enrichment of GO terms for biological processes. Significantly enriched GO terms were also selected and plotted using the clusterProfiler dotplot function.

2.17 Statistics

Statistical analysis comparing two groups at a single time point was performed in Prism software using an unpaired Student's T test. When data from multiple experiments were combined, to show natural biological variability between infections, a randomized block ANOVA was performed using R v.3.4.4 software. This test was designed to assess the effect of experimental group while controlling for any effect of experimental date, by modeling the group as a fixed effect and date as a random effect. Tests used for each figure is shown in the figure legend. All data were graphed using Prism software. Distributions were assumed to be normal. All graphs show the mean of the data, or the mean along with individual values. Error bars indicate standard deviation.

CHAPTER 3

Gasdermin-D-dependent IL-1α release from microglia promotes protective immunity during chronic *Toxoplasma gondii* infection

Samantha J. Batista, Katherine M. Still, David Johanson, Jeremy A. Thompson, Carleigh A. O'Brien, John R. Lukens, and Tajie H. Harris

3.1 INTRODUCTION

Numerous brain infections cause significant morbidity and mortality worldwide. Many of these pathogens persist in a chronic latent form in the brain and require constant immune pressure to prevent symptomatic disease. As the only resident immune cell, microglia are widely assumed to play an integral role in controlling CNS infections, but in many contexts their specific role remains poorly understood. One CNS-tropic pathogen is *Toxoplasma gondii*, a eukaryotic parasite with a broad host range that infects a large portion of the human population.[167,192,194,195,233,246] *T. gondii* establishes chronic infections by encysting in immune privileged organs, including the brain.[247,248] Without sufficient immune pressure, an often fatal neurological manifestation of this disease toxoplasmic encephalitis can occur.[194,195,233]

Studies done in mice, a natural host of this parasite, have elucidated many aspects of the immune response that are essential for maintaining control of the parasite during chronic stages of infection. T cell-derived IFN-γ is one essential element.[198,201,232] IFN-γ acts on target cells to induce an anti-parasitic state, allowing for the destruction of the parasite through a number of mechanisms including the recruitment of immunity-related GTPases (IRGs) and guanylate binding proteins (GBPs) to the parasitophorous vacuole, as well as the production of nitric oxide (NO).[28,29,203,205,206,210] Large numbers of monocytes and monocyte-derived macrophages, a target population for IFN-γ signaling,[28] are recruited into the brain parenchyma during chronic *T. gondii* infection in mice, and these cells are also necessary for maintaining control of the parasite and host survival.[214] Though microglia occupy the same environment as these cells in the infected brain, have an activated morphology, their role in chronic *T. gondii* infection has not been fully elucidated. Indeed, whether microglia and recruited macrophages respond in similar ways to brain infection is an open question.

In this work, we have focused on IL-1, its expression by microglia and macrophages, as well as its role in the brain during chronic *T. gondii* infection. IL-1 molecules include two main cytokines: IL-1α and IL-1β. IL-1α can function as a canonical alarmin, which is a pre-stored molecule that does not require processing and can be released upon cell death or damage, making it an ideal candidate for an early initiator of inflammation.[143,249] In contrast, IL-1β is produced first as a pro-form that requires cleavage by caspase-1 in order for it to be biologically active, rendering IL-1β dependent on the inflammasome as a platform for caspase-1 activation.[126,128,250] Both of these cytokines signal through the same receptor (IL-1R), a heterodimer of IL-1R1 and IL-1RAcP, with similar affinity.[251] They also lack signal sequences and thus require a loss of membrane integrity to be released. Caspase-mediated cleavage of gasdermin molecules has been identified as a major pathway leading to pore formation and IL-1 release.

The role of IL-1β and inflammasome pathways in *T. gondii* infection has been studied *in vitro* as well as in rodent models of acute infection. In sum, these studies suggest roles for IL-1β, IL-18, IL-1R, NLRP1 and /or NLRP3 inflammasome sensors, the inflammasome adaptor protein ASC, and inflammatory caspases-1 and -11.[241,243-245] However, the role of IL-1 signaling in the brain during chronic infection has not been addressed.

Here, we show that though they are present in the same tissue microenvironment in the brain during *T. gondii* infection, monocyte-derived macrophages in the brain have a stronger NF-κB signature than brain-resident microglia. Interestingly, we also find that while IL-1α is enriched in microglia, IL-1β is overrepresented in macrophages, suggesting that these two cell types are able to contribute to IL-1-driven inflammation in different ways. We go on to show that IL-1 signaling is, indeed, important in this model as *Il1r1*$^{-/-}$ mice chronically infected with *T. gondii* are less able to control parasite in the brain, and additionally, these mice have deficits in the

recruitment of inflammatory monocytes and macrophages into the brain in comparison to wild-type mice. We find IL-1R1 expression predominantly on blood vasculature in the brain, and observe IL-1-dependent activation of the vasculature during infection. Further, IL-1-dependent control of *T. gondii* is mediated through IL-1R1 expression on a radio-resistant cell population. Interestingly, the pro-inflammatory effect of IL-1 signaling is mediated via the alarmin IL-1α, not IL-1β. We show that microglia, but not infiltrating macrophages, release IL-1α *ex vivo* in an infection- and gasdermin-D-dependent manner. We propose that one specific function of microglia during *T. gondii* infection is to release the alarmin IL-1α to promote protective neuroinflammation and parasite control.

3.2 RESULTS

Microglia lack a broad inflammatory signature compared to macrophages in the infected brain

As the resident macrophages in the brain microglia are assumed to play a significant role in infections and insults to the brain. *T. gondii* infection results in robust, sustained brain inflammation that is necessary for parasite control. This inflammation in marked by the infiltration of blood-derived T cells and monocytes into the brain as well as morphological activation of microglia. Blood-derived monocytes have been demonstrated to be important for host survival during infection,[214] but whether microglia perform similar functions is still unknown. Previous work from our lab has observed that while blood-derived monocytes and macrophages express high levels of the nitric oxide-generating enzyme iNOS in the brain during *T. gondii* infection, microglia markedly lack this anti-parasitic molecule.[252] This observation led to the hypothesis that even though they are in the same tissue microenvironment, microglia are unable to respond to the infection in the same way as infiltrating macrophages. Thus, we used a $CX3CR1^{Cre\text{-}ERT2}$ x $ZsGreen^{fl/stop/fl}$ mouse line that has been previously described as a microglia reporter line.[253] Reporter mice were treated with tamoxifen to induce ZsGreen expression and rested for 4 weeks after tamoxifen injection to ensure turnover of peripheral CX3CR1-expressing cells. We have consistently used this mouse line in our lab to label over 98% of microglia in the brain. Perivascular macrophages will also be labeled by this method, but are not purified by our isolation protocol as evidenced by a lack of $CD206^{+}$ cells. Following infection, FACS was used to sort out $CD45^{+}CD11b^{+}$ $ZsGreen^{+}$ microglia and $ZsGreen^{-}$ blood-derived myeloid cells from brains of infected mice for RNA sequencing analysis (Fig. 3.1a).

Analysis of differentially expressed genes shows that these two cell populations segregate clearly from each other, confirming that they are fundamentally different cell types (Fig. 3.1b). Analysis of pathway enrichment displayed a striking lack of an inflammatory signature in microglia compared to macrophages (Fig. 3.1c), and we further show a selection of genes that were differentially expressed, showing a clear enrichment for inflammation associated genes in the macrophage population (Fig. 3.1d). Interestingly, an NF-κB signature seemed to be one factor differentiating the macrophages from the microglia (Fig. 3.1c-d). A difference in expression of NF-κB genes could provide the basis for functional differences between microglia and macrophages and their ability to respond to the infection. Thus, we aimed to validate this at the protein level in infected mice. Indeed, in brain sections from infected microglia reporter mice, both RelA and Rel were distinctly absent from $ZsGreen^{+}$ microglia (Fig. 3.1e-f) but these molecules were present in $ZsGreen^{-}Iba1^{+}$ macrophages (Fig. 3.1g-h). This suggests that some aspects of microglia identity may inhibit upregulation of a certain inflammatory signature during infection, including a strong NF-κB response.

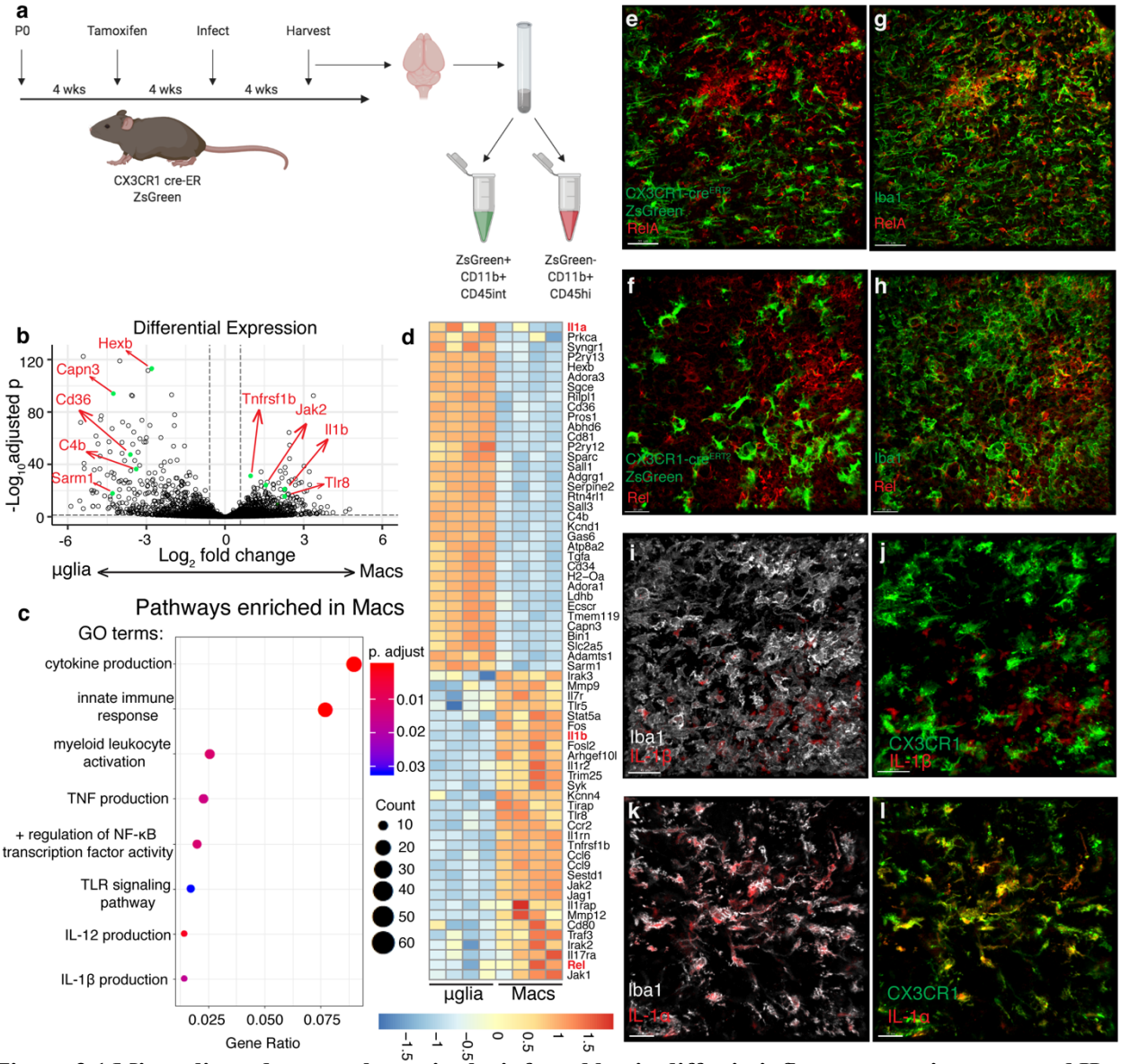
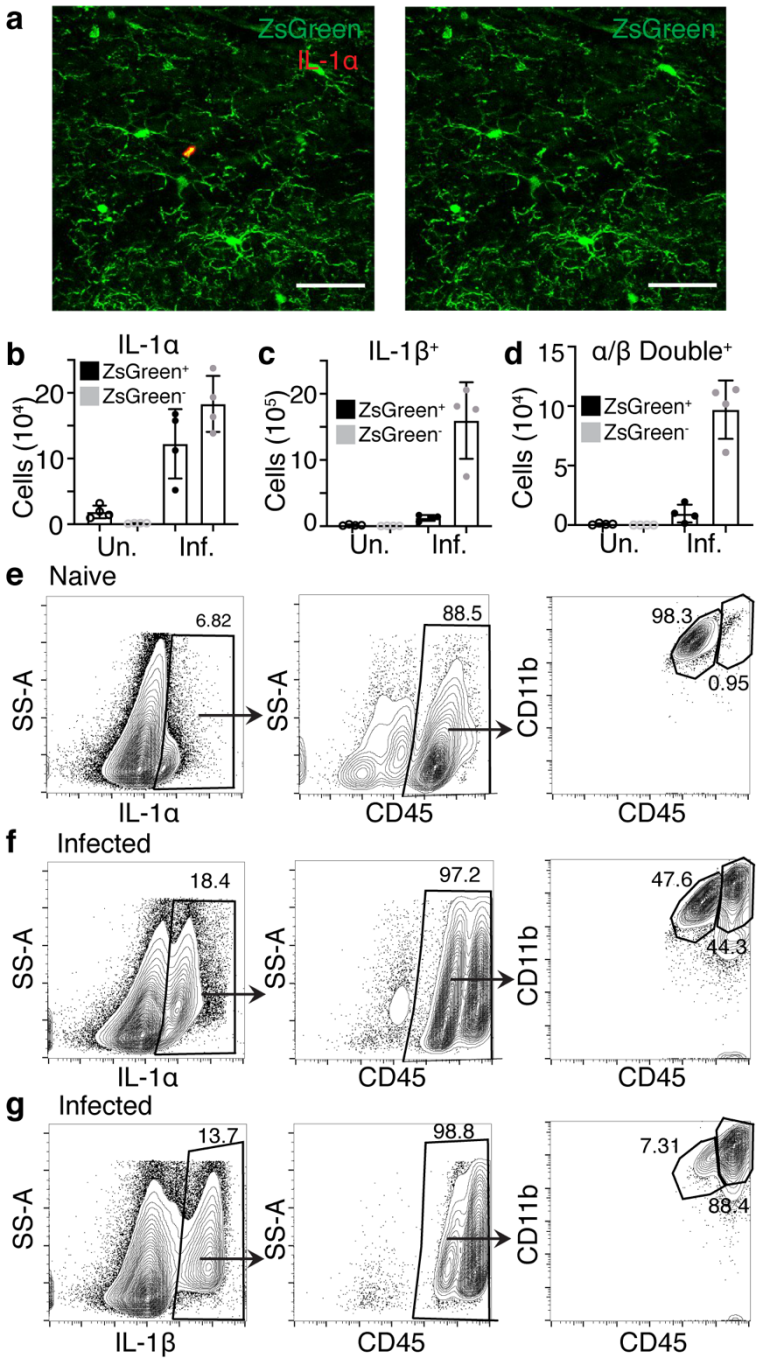


Figure 3.1 Microglia and macrophages in the infected brain differ in inflammatory signature and IL-1 expression a-d, Chronically infected $CX_3CR_1^{Cre\text{-}ERT2}$ x $ZsGreen^{fl/stop/fl}$ mice were sacrificed and brains were harvested and processed for flow cytometry (n = 4 mice). Samples were run on a BD Aria, gated on live/singlets/$CD45^+$/$CD11b^+$ from which $ZsGreen^+$ and $ZsGreen^-$ populations were gated and sorted. Sorted cell populations were subjected to RNA sequencing. **a,** Experimental setup. **b,** Differential abundance testing was performed and results were plotted in R to produce a volcano plot showing differentially expressed genes between microglia and macrophage populations. Example genes are labeled in red corresponding to green dots. **c,** GO terms statistically over-represented in macrophages compared to microglia were generated and a selection of significantly enriched pathways of interest were plotted using

R. **d,** Significantly differentially expressed genes between the two cell populations were selected based on interest and plotted in a heatmap using complete-linkage clustering of a euclidean distance matrix of all samples. **e-l,** Representative images of brain sections from chronically infected $CX_3CR_1^{Cre\text{-}ERT2}$ x $ZsGreen^{fl/stop/fl}$ mice. ZsGreen is shown in green (**e-f,j,l**) and sections were stained for Iba1 (green, **g-h**; gray, **i,k**), RelA (**e,g** red), Rel (**f,h** red), IL-1β (**i-j** red), and IL-1α (**k-l** red). Scale bars indicate 30 μm.

IL-1 genes are differentially expressed by microglia and macrophages

The sequencing data showed that many inflammatory cytokine and chemokine signatures were also enriched in the macrophages compared to the microglia. Of note, it was observed that the IL-1 cytokines segregated differently between these populations. IL-1α was enriched in the microglia population, while IL-1β was enriched in the macrophage population (Fig. 1d). This suggests that these two cell types may be differently equipped to propagate innate inflammatory signals. The lack of microglia expression of pro-IL-1β was validated at the protein level in sections from infected microglia reporter mice, which also showed its expression by ZsGreen^{-}Iba1^{+} cells (Fig. 1i-j). On the other hand, staining of tissue sections from chronically infected microglia reporter mice show IL-1α expression generally in Iba1^{+} cells (Fig. 1k), and further confirm microglial expression of IL-1α (Fig. 1l). These results were further confirmed using flow cytometry analysis on the brains of both WT and microglia reporter mice. IL-1α protein is present in the brain prior to infection where it is found in ZsGreen^{+} microglia and microglia defined by CD11b^{+}CD45^{int} (Fig. S3.1a-b,d). During chronic infection, it is expressed by both ZsGreen^{+} microglia and ZsGreen^{-} myeloid cells (Fig. S3.1b) also defined by CD11b^{+}CD45^{int} and CD45^{hi} (Fig. S3.1f). IL-1β was not detected in uninfected brains, but was detected in the brain during chronic *T. gondii* infection (Fig. S3.1b,f). During chronic infection, pro-IL-1β and was not significantly expressed by ZsGreen^{+} cells, but was rather seen in ZsGreen^{-} myeloid cells (Fig. S3.1c) also defined as CD11b^{+}CD45^{hi} cells (Fig. S3.1g). It was also apparent that while ZsGreen^{-} blood-derived myeloid cells can express both IL-1α and pro-IL-1β, very few ZsGreen^{+} microglia were double positive (Fig. S3.1d). These data suggested that microglia and macrophages may play different roles in an IL-1 response. Thus, we aimed to investigate the potential importance of an IL-1 response in *T. gondii* infection.



Supplementary Figure 3.1 Microglia and macrophages in the infected brain differ in IL-1 expression. **a-d,** $CX_3CR_1^{Cre\text{-}ERT2}$ x $ZsGreen^{fl/stop/fl}$ mice were left naïve or infected with 10 cysts of Me49 strain *T. gondii* parasites for 4 weeks. (n = 4 mice per group) **a,** Representative image of IL-1α in naïve brain colocalizing with microglia, scale bar is 50 μm. **b-d,** Brains were harvested and analyzed by flow cytometry with intracellular cytokine staining. Numbers of IL-1α$^+$ (**b**), IL-1β$^+$ (**c**), and double positive (**d**) cells were quantified in both ZsGreen$^+$ and ZsGreen$^-$ populations in naïve and infected mice. Cells were pre-gated on

singlets/live/ZsGreen. **e-g,** Brains from naïve or chronically infected mice were analyzed by flow cytometry. **e,** Representative plots of IL-1α expression for naïve samples, previously gated on live/singlets. **f-g,** Representative plots of IL-1α (**f**) and IL-1β (**g**) expression for infected samples.

Il1r1$^{-/-}$ mice have an impaired immune response to *T. gondii* infection

To determine if IL-1 signaling plays a role in chronic *T. gondii* infection, we infected mice lacking the IL-1 receptor (IL-1R1), which is bound by both IL-1α and IL-1β. Six weeks post-infection (p.i.) *Il1r1*$^{-/-}$ mice displayed an increase in parasite cyst burden in the brain (Fig. 3.2a). An increase in parasite burden is often due to impaired immune responses. Indeed, *Il1r1*$^{-/-}$ mice also have a decrease in the number of CD11b^{+}CD45^{hi} cells of the monocyte/macrophage lineage in the brain during chronic infection (Fig. 3.2b, f-g). Microglia typically express intermediate levels of CD45 compared to the high levels expressed by blood-derived myeloid cells, thus we use this marker as a proxy to define these populations by flow cytometry.[254] The cells we defined as infiltrating monocyte/macrophages are also Ly6G^{-}, CD11c^{-}, and Ly6C^{+}. Infiltrating myeloid cells are important producers of nitric oxide, a key anti-parasitic molecule, and thus we assessed their expression of inducible nitric oxide synthase (iNOS). *Il1r1*$^{-/-}$ mice had significantly decreased expression of iNOS in the brain compared to WT mice (Fig. 3.2c, h-i), which was observed specifically in focal areas of inflammation (Fig. 3.2j-k). Of note, though there were decreases in CD4^{+} and CD8^{+} T cells (Fig. 3.2d-e), the reduced iNOS expression did not appear to be due to reductions in IFN-γ production from the T cell compartment within the brain, which was unchanged between groups (Fig. S3.2a-b). Together, these data suggest that the CNS immune response is affected in *Il1r1*$^{-/-}$ mice, with striking deficits particularly in the myeloid response.

Importantly, these differences were restricted to the site of infection, as there were no deficits in any immune cell compartments in the spleens of *Il1r1*$^{-/-}$ mice (Fig. S3.2c-h). In fact, T cell and macrophage responses were slightly elevated in the spleen. The immune deficits in *Il1r1*$^{-/-}$ mice are also specific to chronic infection as *Il1r1*$^{-/-}$ mice analyzed earlier during infection (12 dpi) displayed no deficit in their monocyte/macrophage or T cell populations compared to WT in

the peritoneal cavity or the spleen (Fig. S3.3a-b). IFN-γ levels in the serum were, if anything, increased in *Il1r1*$^{-/-}$ mice at this time point, indicating that this response is not impaired (Fig. S3.3c). The only immune defect detected during this early phase of infection in *Il1r1*$^{-/-}$ mice was a decrease in neutrophils recruited to the peritoneal cavity (Fig. S3.3a). In sum, these results show that mice lacking IL-1R1 have an impaired response of blood-derived immune cells in the brain, leading to increased parasite burden. This suggests that IL-1 signaling promotes immune responses in the brain during chronic *T. gondii* infection.

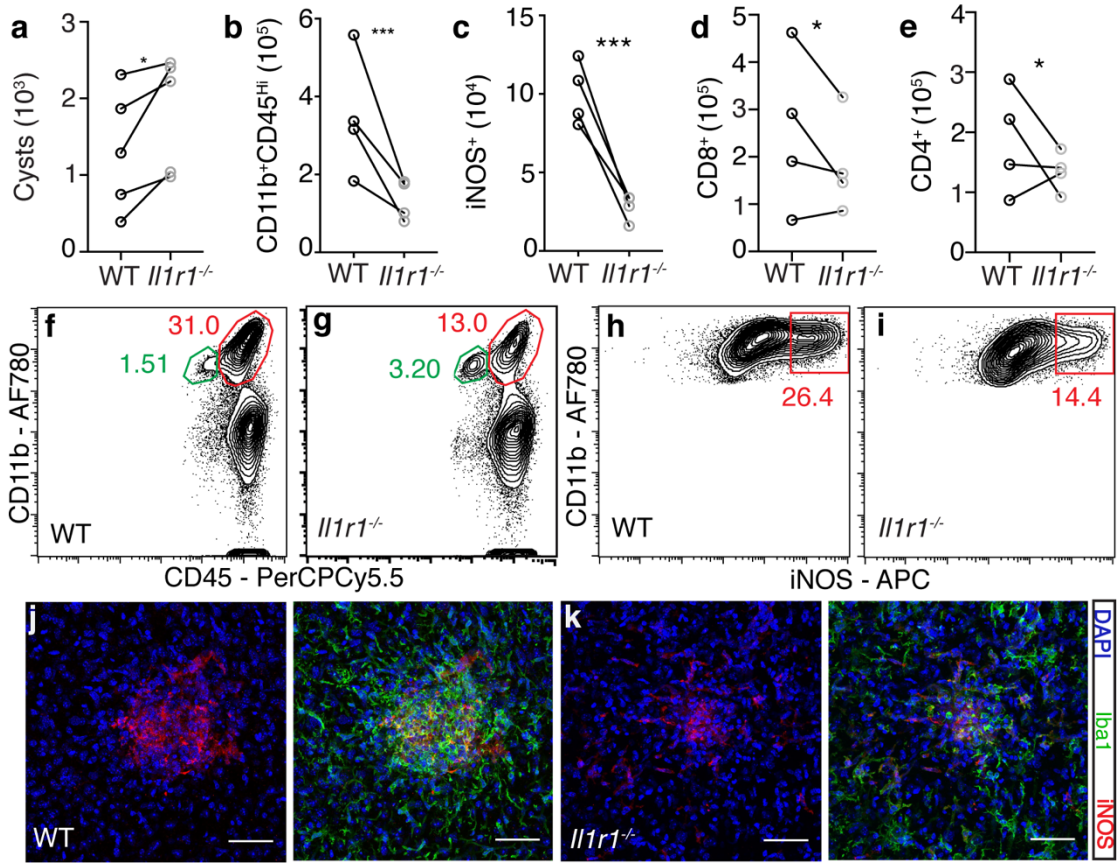
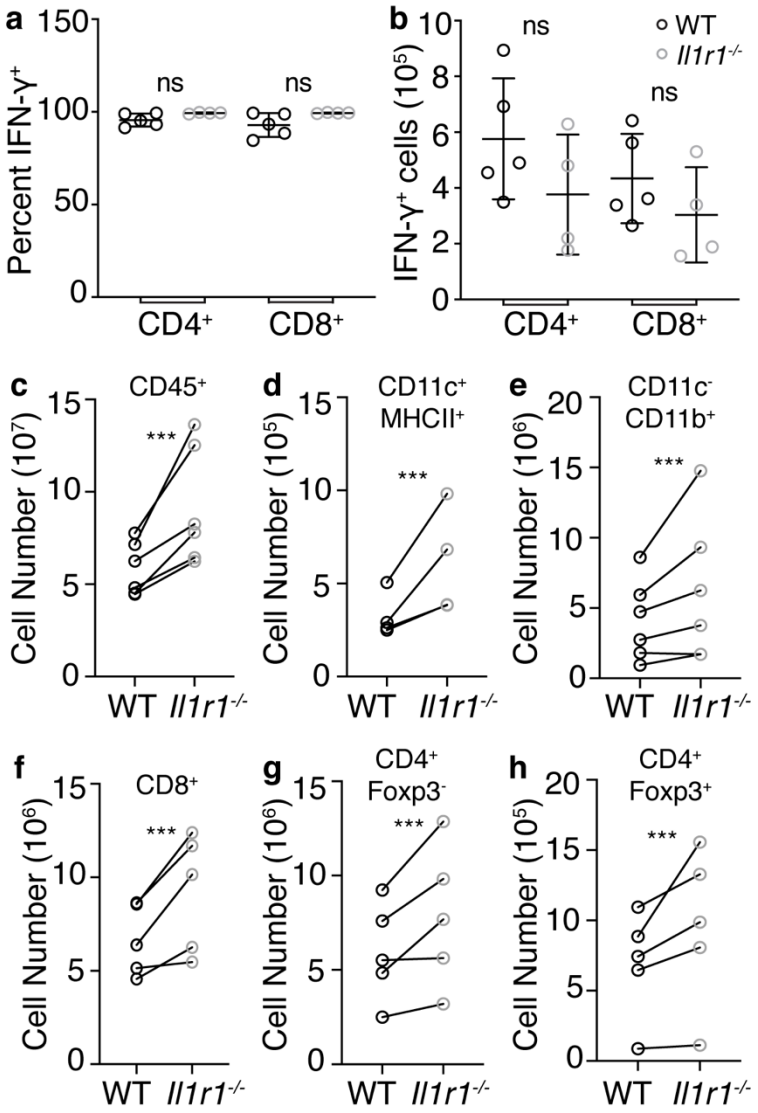
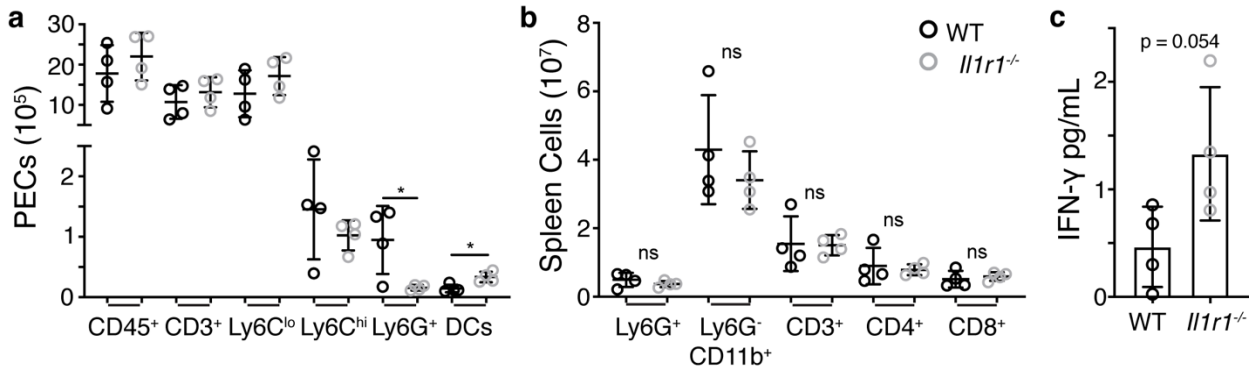


Figure 3.2 IL-1R$^{-/-}$ mice have an impaired immune response to *T. gondii* infection. WT C57B6/J or *Il1r1*$^{-/-}$ mice were infected i.p. with 10 cysts of the Me49 strain of *T. gondii*. 6 weeks p.i. brains were harvested and homogenized. (n = 3-5 mice per group per experiment) **a,** Cyst burden per brain was determined by counting cysts in brain homogenate on a light microscope. Paired averages from 5 experiments are shown, and statistics were performed using a randomized block ANOVA. **b-g,** Brains from the same mice were processed to achieve a single cell suspension and analyzed by flow cytometry. Data compiled from 4 experiments; statistics were performed using a randomized block ANOVA. **b,** Blood derived myeloid cells were defined as CD11b^{+}CD45^{hi}, cells were pre-gated on singlets/live/CD45^{+}/CD11c^{-}, representative flow plots are shown in (**f-g**). **c,** The number of iNOS^{+} cells per brain were calculated, pre-gated on singlets/live/CD45^{+}/CD11c^{-}/CD11b^{+}CD45^{hi}, representative flow plots are shown in (**h-i**). **d-e,** CD8^{+} and CD4^{+} T cell numbers were calculated, pre-gated on singlets/live/CD3^{+}. **j-k,** Representative confocal images of focal areas of inflammation in chronically infected brains of WT (**j**) and IL-1r1$^{-/-}$ (**k**) mice. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Scale bars indicate 50 µm.



Supplementary Figure 3.2 Brain IFN-γ responses and peripheral immune responses are not impaired in *Il1r1*$^{-/-}$ mice during chronic *T. gondii* infection. WT and *Il1r1*$^{-/-}$ mice were infected i.p. with 10 cysts of the Me49 strain of *T. gondii*. 6 weeks p.i. brains (**a-b)** and spleens (**c-h**) were harvested and processed for flow cytometry. Immune cell populations were enumerated. **a-b,** Brains were harvested and digested. Isolated cells were incubated at 37°C for 5 hours with a mix of PMA/ionomycin and brefeldin A. Intracellular cytokine staining was performed and analyzed by flow cytometry. Cells were pre-gated on singlets/Live/CD3$^+$ and percent (**a**) and number (**b**) of IFN-γ$^+$ CD4 and CD8$^+$ T cells were determined. **c,** Total immune cells, pre-gated on singlets/live. **d,** DCs, pre-gated on singlets/live/CD45$^+$/Dump$^-$ (CD3/NK1.1/B220). **e,** Monocytes/ macrophages, pre-gated on singlets/live/ CD45$^+$/CD11c$^-$ /CD11b$^+$/CD45^{hi}. **f,** CD8$^+$ T cells, pre-gated on singlets/live/CD3$^+$. **g,** Effector CD4$^+$ T cells, pre-gated on singlets/live/CD3$^+$. **h,** Tregs, pre-gated on singlets/live/CD3$^+$. **a-b,** A representative experiment is shown

and statistics were performed using a Student's T test. (n = 4-5 mice per group) **c-h,** Paired averages compiled from 3-6 experiments. Statistics were performed using a randomized block ANOVA. (n = 3-5 mice per group per experiment) * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.



Supplementary Figure 3.3 IFN-γ response and monocyte/macrophage response during acute infection are not impaired in *Il1r1*$^{-/-}$ mice. WT and *Il1r1*$^{-/-}$ mice were infected i.p. with 10 cysts of the Me49 strain of *T. gondii*. Mice were sacrificed 12 days p.i. **a,** Peritoneal lavage was performed and peritoneal exudate cells (PECs) were isolated and analyzed by flow cytometry. **b,** Spleen cells were isolated and analyzed by flow cytometry. **c,** Serum was harvested at the time of sacrifice and IFN-γ in the serum was analyzed by ELISA. A representative experiment is shown (n = 4 mice per group). Statistics were performed using a Student's t-test between groups for each measure.

IL-1R1 is expressed by brain vasculature during chronic *T. gondii* infection

Having established a role for IL-1 signaling in promoting the immune response to chronic *T. gondii* infection in the brain, we next wanted to determine which cells in the brain could respond to IL-1 in the brain environment. We performed immunohistochemical staining for IL-1R1 on brain sections from chronically infected mice. We found that IL-1R1 was expressed principally on blood vessels in the brain, as marked by laminin staining which highlights basement membranes of blood vessels (Fig. 3.3a-b). Interestingly, expression is not seen continuously along vessels (Fig. 3.3a-b), nor on all vessels (Fig. 3.3b). This suggests a degree of heterogeneity among endothelial cells and perhaps in their ability to respond to IL-1. We detected IL-1R1 expression specifically on $CD31^+$ cells by IHC (Fig. S3.4a) and by flow cytometry (Fig. S3.4b-c). To test whether endothelial expression of IL-1R1 is required in this infection, we first assessed potential contributions from radiosensitive (hematopoietic) and radio-resistant (non-hematopoietic) cells. To do this, we created bone marrow chimeras with *Il1r1*$^{-/-}$ mice. We lethally irradiated both WT and *Il1r1*$^{-/-}$ mice, and then i.v. transferred bone marrow cells from either WT or *Il1r1*$^{-/-}$ mice. We allowed 6 weeks for reconstitution before infecting the mice with *T. gondii*, and we performed our analyses at 4 weeks post infection (Fig. 3.3c). We found that *Il1r1*$^{-/-}$ recipients that had received WT bone marrow, had a higher cyst burden in their brain than WT recipients that had received either WT or *Il1r1*$^{-/-}$ bone marrow (Fig. 3.3d). Consistent with this, *Il1r1*$^{-/-}$ recipient mice, regardless of their source of bone marrow displayed a decrease in total leukocyte numbers in the brain compared to WT recipients (Fig. 3.3e). Taken together, these data suggest that IL-1R1 expression on a radio-resistant cell population is required for host control of the parasite, which is consistent with our hypothesis that the relevant expression is on brain endothelial cells.

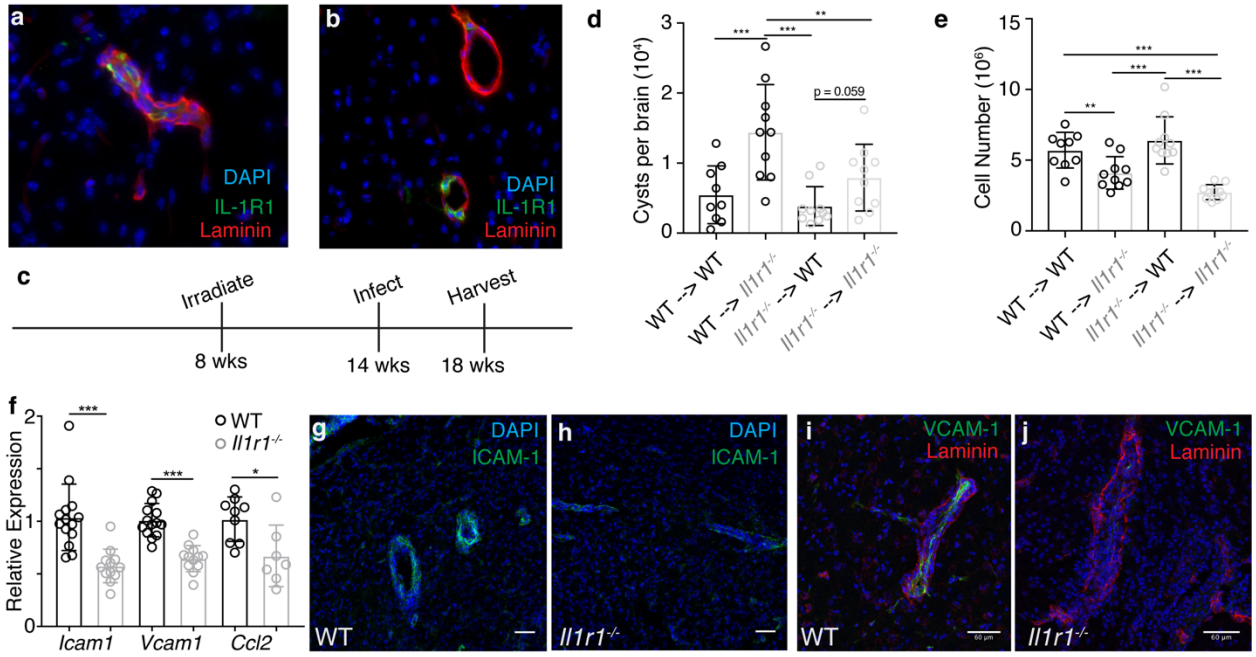
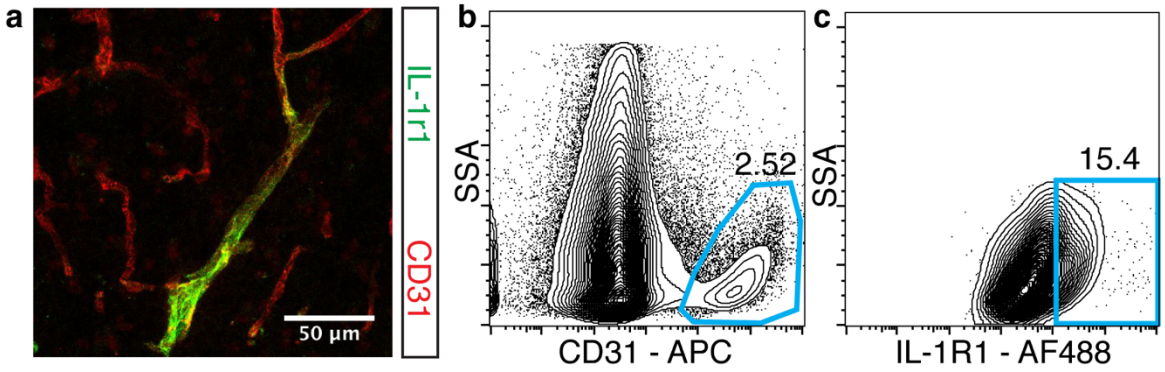


Figure 3.3 IL-1R1 is expressed on brain vasculature during chronic *T. gondii* infection. a-b, Brains from chronically infected C57B6/J WT mice were sectioned and stained with DAPI (blue) and antibodies against laminin (red) and IL-1r1 (green), showing parenchymal blood vessels. **c-e,** WT (CD45.1) and *Il1r1*$^{-/-}$ (CD45.2) mice were lethally irradiated and then reconstituted with bone marrow from either WT or *Il1r1*$^{-/-}$ mice. Mice were allowed to reconstitute for 6 weeks and then were infected i.p. with 10 cysts of the Me49 strain of *T. gondii*. 4 weeks p.i. mice were sacrificed and their brains were harvested for analysis. (n = 3-6 mice per group per experiment) **d,** Brains were homogenized and cysts were counted by light microscopy. **e,** Brains were processed for flow cytometry and the numbers of total leukocytes were calculated. Cells were pre-gated on singlets/live. **d-e,** Data compiled from 2 experiments, statistics performed using a randomized block ANOVA. **f,** WT and *Ilr1*$^{-/-}$ mice were infected i.p. with 10 cysts of the Me49 strain of *T. gondii*. 6 weeks p.i. the mice were sacrificed and brains were homogenized, RNA was extracted, and qPCR analysis was performed. Data compiled from 2 (CCL2) or 3 (ICAM1, VCAM1) experiments; statistics performed using a randomized block ANOVA. (n = 3-5 mice per group per experiment) **g-j,** Brains from chronically infected WT and *Il1r1*$^{-/-}$ mice were sectioned and stained for either ICAM-1 (**g-h**) or VCAM-1 (**i-j**). Representative images of blood vessels are shown. **g-h,** scale bars are 50 μm and (**i-j**) scale bars are 60 μm. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.



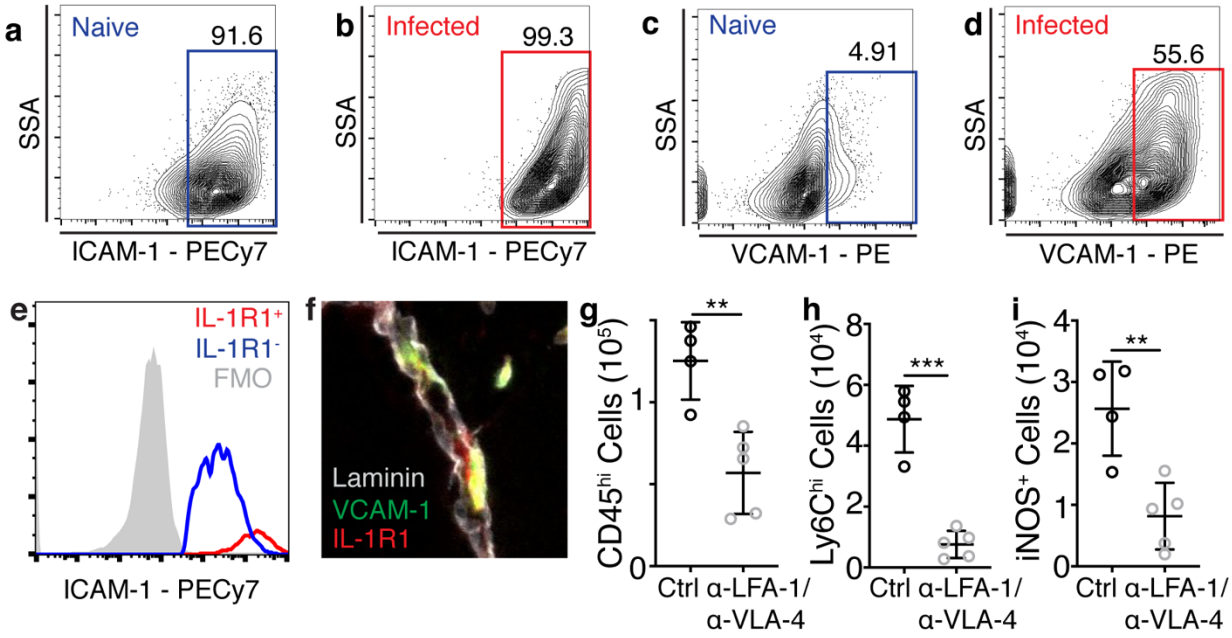
Supplementary Figure 3.4 IL-1R1 is expressed by endothelial cells in the brain. a, Brains from chronically infected C57B6/J mice were harvested, fixed, and stained with antibodies against CD31 (red) and IL-1R1 (green). **b-c,** Brains from uninfected C57B6/J mice were harvested and processed for flow cytometry analysis. Cells were previously gated on Singlets/Live/CD45^{-} and then were gated on CD31^{+} (**b**) and IL-1R1 (**c**) expression on the CD31^{+} population.

Vascular adhesion molecule expression in the brain is partially dependent on IL-1R1 signaling and is necessary for monocyte infiltration

During chronic *T. gondii* infection continual infiltration of immune cells into the brain is necessary for maintaining control of the parasite. One step in getting cells to successfully infiltrate the brain, as in other tissues, is the interaction with activated endothelium expressing vascular adhesion molecules as well as chemokines. Indeed, the brain endothelium is activated during chronic *T. gondii* infection compared to the naïve state, as seen by increased expression of ICAM-1 and VCAM-1 molecules on brain endothelial cells (Fig. S3.5a-d). Our data show that ICAM-1 is expressed to a higher extent by endothelial cells that express IL-1R1 compared to cells that do not in the naïve state (Fig. S3.5e), and that IL-1R1^{+} endothelial cells also express VCAM-1 in infected tissues (Fig. S3.5f).

We investigated the dependence of these molecules on IL-1 signaling in our model, and found that their expression is dependent in part on IL-1 signaling. *Il1r1*$^{-/-}$ mice displayed decreased mRNA expression of *Icam1*, *Vcam1*, and *Ccl2* in the brain (Fig. 3.3f) as assessed using whole brain homogenate from chronically infected mice. To more specifically address effects on the CNS vasculature, we examined expression of ICAM-1 and VCAM-1 protein in brain sections of WT and *Il1r1*$^{-/-}$ mice during chronic infection using IHC (Fig. 3.3g-j). Representative images show a marked decrease in ICAM-1 and VCAM-1 reactivity on blood vessels in the brains of *Il1r1*$^{-/-}$ mice compared to WT (Fig. 3.3g-j). Together, these data show that the increased expression of vascular adhesion molecules, and potentially chemokine, in the brain that is characteristic of chronic *T. gondii* infection is partially dependent on IL-1 signaling. The modulation of adhesion molecule expression may be one mechanism by which IL-1 promotes the infiltration of immune into the brain during chronic *T. gondii* infection.

To determine the importance of ICAM-1 and VCAM-1 in the recruitment of infiltrating monocytes during chronic *T. gondii* infection, we used antibody treatments to block their ligands (LFA-1 and VLA-4 respectively) *in vivo*. We treated chronically infected WT mice with a combination of α-LFA-1 and α-VLA-4 blocking antibodies, giving a total of two treatments. After 5 days of treatment, mice receiving blocking antibody displayed decreases in the number of infiltrating myeloid cells isolated from the brain compared to control treated mice (Fig. S3.5g). Specifically, we observed deficits in the $Ly6C^{hi}$ population (Fig. S3.5h), indicating a lack of blood-derived monocytes. The decrease in monocyte entry translated into fewer $iNOS^{+}$ cells in the brain as well (Fig. S3.5i). These data show that interactions with ICAM-1 and VCAM-1 are necessary for monocyte infiltration into the brain during chronic infection, and that IL-1 signaling promotes the expression of these adhesion molecules.



Supplementary Figure 3.5 The brain endothelium is activated during chronic *T. gondii* infection. a-d, WT C57B6/J mice were either left naïve or infected i.p. with the Me49 strain of *T. gondii*. 4 weeks p.i. mice were sacrificed and brains were harvested for flow cytometry analysis. (n = 2 mice per group) **a-b,** Samples were pre-gated on singlets/live/Hoescht^{+}/CD45^{-}/CD31^{+} and then ICAM-1 expression was assessed. Representative plots from naïve (**a**) and infected (**b**) mice are shown. **c-d,** Samples were pre-gated as in **a** and then VCAM-1 expression was assessed. Representative plots from naïve (**c**) and infected (**d**) mice are shown. **e,** Histogram showing ICAM-1 expression on IL-1R1 positive and negative endothelial cells, the FMO is shown in filled gray **f,** Brains from chronically infected C57B6/J mice were harvested, fixed, and stained with antibodies against laminin (gray), IL-1r1 (red), and VCAM-1 (green). **g-i,** C57B6/J mice were infected i.p. with 10 cysts of the Me49 strain of *T. gondii*. 4 weeks p.i. mice were treated with either control IgG or 200 μg each of α-LFA-1 and α-VLA-4 blocking antibodies on days 1 and 3 of treatment, and were sacrificed on day 5. Brains were harvested and processed for flow cytometry. (n = 4-5 mice per group) **g,** Cells were previously gated on singlets/live/CD11c^{-}/CD45^{+} and the numbers of CD11b^{+}CD45^{hi} cells are shown. Of the CD45^{hi} cells numbers of Ly6C^{hi} cells (**h**) and iNOS^{+} cells (**i**) were enumerated. Statistics were performed using a Student's T-test. * = $p < 0.05$ ** = $p < 0.01$, *** = $p < 0.001$.

IL-1α$^{-/-}$ but not IL-1β$^{-/-}$ mice have an impaired immune response to *T. gondii* infection

IL-1α and IL-1β both bind to and signal through IL-1R1. Having established a role for IL-1 signaling in promoting the myeloid response in the brain during chronic *T. gondii* infection, we next sought to determine whether this effect was mediated by one or both of these cytokines, given that IL-1α and IL-1β are expressed by different populations of myeloid cells in the infected brain. To address this, we infected mice lacking either IL-1α or IL-1β and analyzed the cellular immune response and parasite burden during chronic phase of infection. At six weeks post-infection, IL-1α$^{-/-}$ mice displayed an increase in parasite burden compared to WT as measured by qPCR analysis of parasite DNA from brain homogenate (Fig. 3.4a). IL-1β$^{-/-}$ mice, however, showed no change in parasite burden compared to WT (Fig. 3.4a). This suggests that, rather unexpectedly, IL-1α is involved in maintaining control of the parasite during chronic infection, while IL-1β is not.

IL-1α$^{-/-}$ mice displayed fewer focal areas of inflammation compared to WT (Fig. 3.4b-c), as seen by clusters of immune cells in H&E stained brain sections. We further found that IL-1α$^{-/-}$ mice, like *Il1r1*$^{-/-}$ mice, have decreases in peripheral monocyte/macrophage populations infiltrating the brain as well as a decrease in the number of iNOS-expressing cells compared to WT (Fig.3. 4d-e). They also had a decrease in CD8^{+} T cells in the brain (Fig. 3.4f-g). On the other hand, IL-1β$^{-/-}$ mice displayed no difference from WT in the number of peripheral myeloid cells infiltrating the brain during chronic infection, or in the number of these cells that are expressing iNOS across multiple experiments (Fig. 3.4h-i), which is consistent with no change in parasite burden in these mice. IL-1β$^{-/-}$ mice also showed no defect in T cell infiltration (Fig. 3.4j-k). Together, these results suggest that the role of IL-1 signaling in promoting immune responses in the brain during chronic *T. gondii* infection is mediated by IL-1α, rather than by IL-1β.

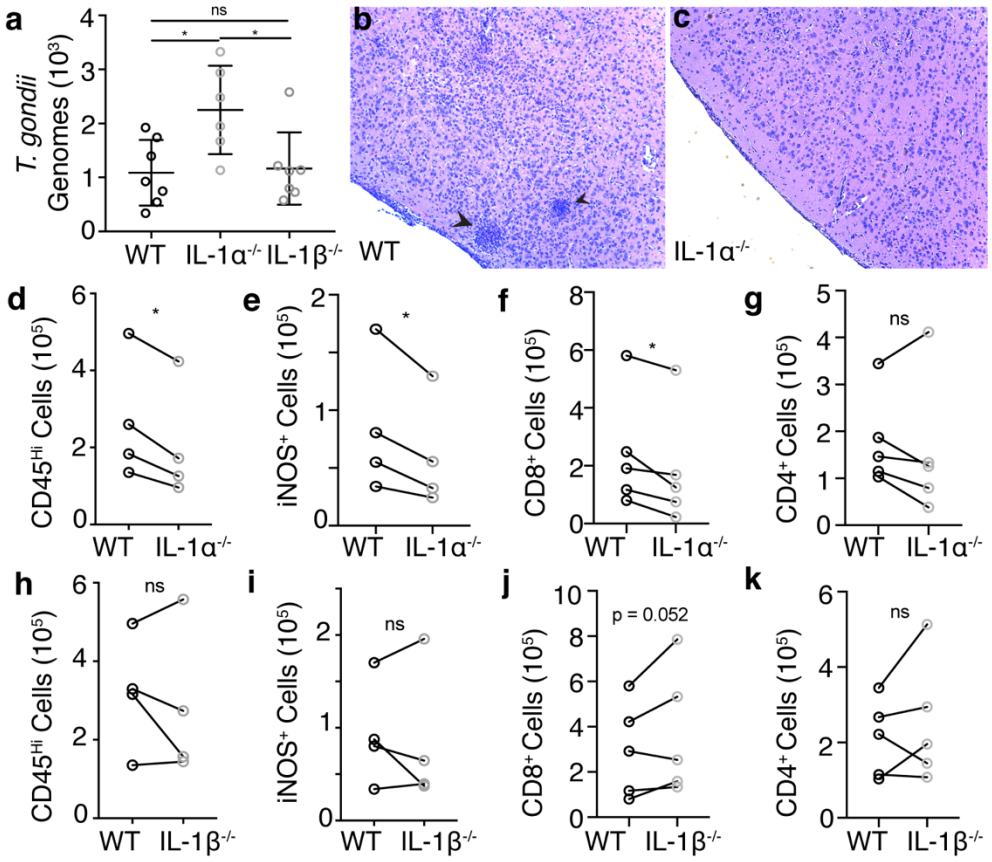


Figure 3.4 IL-1α$^{-/-}$ but not IL-1β$^{-/-}$ mice have an impaired response to *T. gondii* infection. WT C57B6/J, IL-1α$^{-/-}$, and IL-1β$^{-/-}$were infected i.p. with 10 cysts of the Me49 strain of *T. gondii*. 6 weeks p.i. brains were harvested and analyzed. **a,** Genomic DNA was isolated from brain homogenate, and parasite DNA was quantified using real-time qPCR. Data compiled from 2 experiments; statistics performed using a randomized block ANOVA. (n = 3-4 mice per group per experiment) **b-c,** Brain slices from WT (**b**) and IL-1α$^{-/-}$ (**c**) were H&E stained and representative images are shown. Arrow heads indicate clusters of immune cells. **d-k,** Brains were processed to obtain a single cell suspension, and analyzed by flow cytometry. Paired averages from 4 or 5 compiled experiments, statistics performed using a randomized block ANOVA. (n = 3-5 mice per group per experiment) **d,h,** Blood-derived myeloid cells per brain as defined by CD11b^{+}CD45^{hi}. Cells were pre-gated on singlets/live/CD45^{+}/CD11c^{-}. **e,i,** iNOS^{+} cells per brain were quantified, pre-gated on singlets/live/CD45^{+}/CD11c^{-}/CD11b^{+}CD45^{hi}. **f-g, j-k,** CD8^{+} and CD4^{+} T cells were quantified, pre-gated on singlets/live/CD3^{+}. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

IL-1α is released *ex vivo* from microglia isolated from *T. gondii* infected brains

Our results demonstrate a role for IL-1α in chronic *T. gondii* infection. We have also shown that microglia in the infected brain are enriched in IL-1α compared to macrophages, though it is expressed by both populations. Thus, we aimed to determine which cell type releases IL-1α in this model. Uninfected mice treated with PLX5622 for 12 days to deplete microglia lost almost all IL-1α mRNA expression in the brain (Fig. 3.5a), consistent with flow cytometry and immunohistochemistry data detecting IL-1α in microglia in naïve mice (Fig. S3.1a-b,d). To further examine IL-1α release during infection, we first established an assay to measure IL-1α release from isolated brain cells *ex vivo*. A single cell suspension was generated from brain homogenate, brain mononuclear cells were washed and plated in complete media for 18 hours, and supernatant was collected for analysis by ELISA. Using this method, we found that cells isolated from mouse brain can indeed release IL-1α in an infection-dependent manner (Fig. 3.5b). It should be noted that isolated spleen cells from infected animals did not release detectable IL-1α. We then used our microglia reporter model to FACS sort ZsGreen^{+} microglia and ZsGreen^{-} myeloid cells from infected mice. Equal numbers of microglia and peripheral myeloid cells were plated and supernatant was collected to measure IL-1α release. We observed a very clear difference in these populations; purified microglia released IL-1α *ex vivo*, while purified monocytes/macrophages released negligible amounts of this cytokine (Fig. 3.5c). We show that this difference in IL-1α release does not appear to do due to overall increased death in microglia *ex vivo* as blood-derived cells actually released more LDH (Fig. 3.5d). We also show that IL-1α release is inhibited when membrane integrity is preserved with glycine treatment (Fig. 3.5e) as well the total possible IL-1α release from isolated brain mononuclear cells *ex vivo* (Fig. 3.5f). Taken together, these findings

show that microglia from infected mice have the capability to release IL-1α, which could suggest that microglia and macrophages may undergo different types cell death.

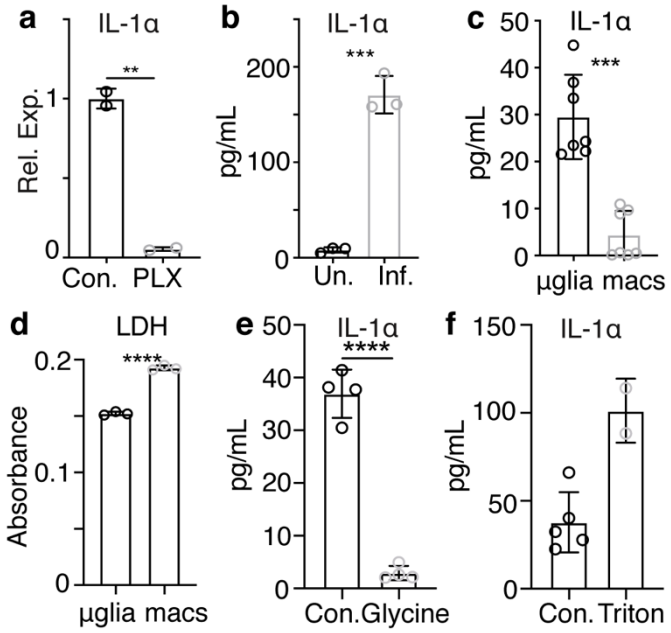


Figure 3.5 IL-1α is released *ex vivo* from microglia from *T. gondii*-infected brains. a, Uninfected mice were fed either control chow or chow containing PLX5622 for 12 days prior to sacrifice. mRNA levels of IL-1α were determined by RT-qPCR on whole brain homogenate. (n = 2 mice per group) **b,** 6 weeks p.i. brains from WT mice were harvested and processed to a single cell suspension. Cells were plated in a 96 well plate and incubated at 37°C overnight. IL-1α release was then measured by ELISA. (n = 3 mice per group) **c-d,** Chronically infected $CX_3CR_1^{Cre\text{-}ERT2}$ x $ZsGreen^{fl/stop/fl}$ mice were sacrificed and brains were harvested and processed for flow cytometry. Samples were run on a BD Aria, gated on live/singlets/$CD45^+$/$CD11b^+$ from which $ZsGreen^+$ and $ZsGreen^-$ populations were gated and sorted. Cells from 6 mice were pooled. Equal numbers of each cell population were plated and incubated overnight at 37°C. (n = 3-4 wells per group) Supernatants were collected and analyzed by ELISA for IL-1α (**c**) and LDH (**d**) (plotted as absorbance at 490nm-680nm). For (**c**) results from two experiments are shown. **e-f,** Assay was performed as in **b**, with some wells treated with glycine to stop membrane permeability (**e**) or triton-containing lysis buffer to show total possible release (**f**). Statistics were performed using Student's T test (**a-b, d-e**) or a Randomized Block ANOVA (**c**). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Caspase-1/11$^{-/-}$ mice have an impaired response to *T. gondii* infection

To begin to address whether inflammatory cell death could release IL-1α in the brain during chronic *T. gondii* infection, we first took a broad look at cell death in the brain. 4 weeks p.i., mice were injected intraperitoneally (i.p.) with propidium iodide (PI). 24 hours after PI injection, mice were sacrificed for analysis. PI uptake in cells, which is indicative of cell death or severe membrane damage, was observed in the brains of *T. gondii* infected mice, and appeared in focal areas (Fig. 3.6a-b), suggesting that there is cell death occurring in the brain during chronic infection.

Inflammasome activation has been implicated *in vitro* and during acute *T. gondii* infection, and could potentially be involved in IL-1α release. IL-1α, like IL-1β, is not canonically secreted and requires cell death or significant membrane perturbation to be released extracellularly.[143,255-257] Unlike IL-1β, IL-1α does not need to be processed by the inflammasome platform for its activity, however, because permeabilization of the plasma membrane is required for IL-1α to be released, inflammasome-mediated cell death may still contribute to its release. To look for evidence of inflammasome activation the brains of mice chronically infected with *T. gondii*, we infected ASC-citrine reporter mice, in which the inflammasome adaptor protein apoptosis-associated speck-like protein containing CARD (ASC) is fused with the fluorescent protein citrine. Upon inflammasome activation, the reporter shows speck-like aggregates of tagged ASC. In the brain during chronic *T. gondii* infection, ASC specks were observed around areas of inflammation in Iba1^{+} microglia or macrophages (Fig. 3.6c). We further crossed the ASC-citrine mouse line to the microglia reporter mouse line. Following infection, ASC specks were observed contained within microglia in the infected brain (Fig. 3.6d).

To further investigate a role for inflammasome-dependent processes in chronic *T. gondii* infection, we infected caspase-1/11$^{-/-}$ mice. Six weeks p.i., mice lacking these inflammatory

caspases had an increased number of parasite cysts in their brains (Fig. 3.6e), indicating impaired parasite control. Caspase-1/11$^{-/-}$ mice also have a decrease in the number of cells of the monocyte/macrophage lineage in the brain during chronic infection (Fig. 3.6f), as well as significantly fewer infiltrating myeloid cells expressing iNOS in the brain compared to WT mice (Fig. 3.6g). These mice also displayed decreases in CD4^{+} T cells (Fig. 3.6h-i). In addition to an increased overall cyst burden, caspase-1/11$^{-/-}$mice had more instances of clusters of parasite cysts compared to WT (Fig. 3.6j-k), likely indicating a lack of parasite control in areas of parasite reactivation. Together, these results are similar to those observed in infected *Il1r1*$^{-/-}$ mice and show that caspase-1/11 activity is important for host control of *T. gondii* infection.

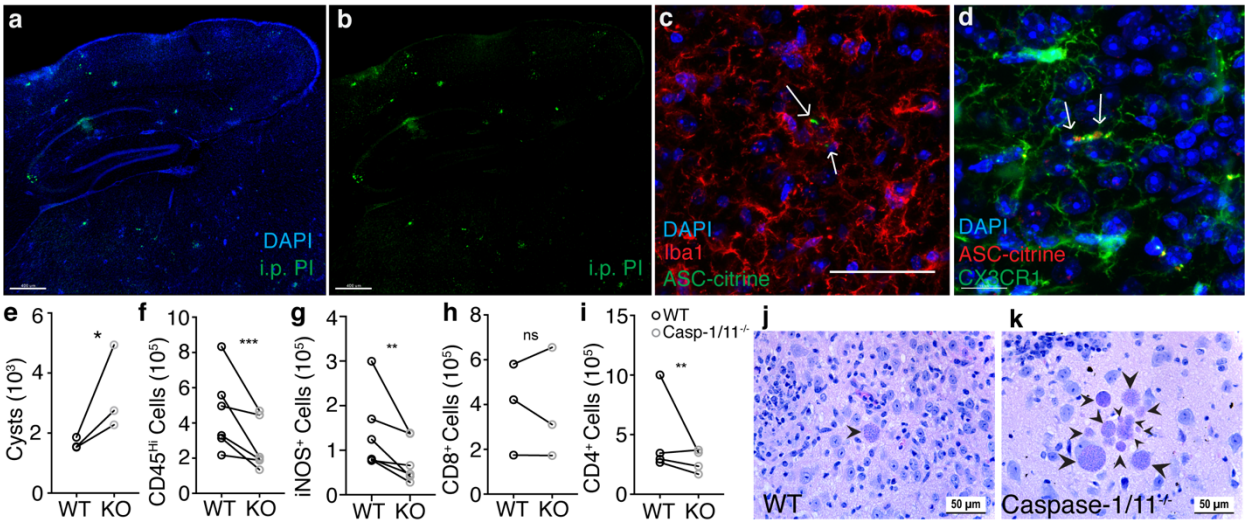


Figure 3.6 Caspase-1/11$^{-/-}$ mice have an impaired response to *T. gondii* infection. a-b, Chronically infected C57B6/J mice were injected i.p. with 20 mg/kg propidium iodide. 24 hrs later, mice were sacrificed and brains were imaged with confocal microscopy. A representative image is shown. **c-d,** Mice expressing ASC-citrine (**c**) or ASC-citrine and CX3CR1-cre^{ERT2}ZsGreen (**d**) were infected with 10 cysts of the Me49 strain of *T. gondii*. 4 weeks post infection brains were harvested, cryopreserved, stained, and imaged. Arrows indicate ASC aggregates in Iba1^{+} cells (**c**) or in ZsGreen^{+} microglial cells (**d**). **e-i,** WT and casp-1/11$^{-/-}$ mice were infected with 10 cysts of the Me49 strain of *T. gondii*. 6 weeks p.i. brains were harvested and analyzed. Paired averages for 3-6 experiments are shown. (n = 3-5 mice per group per experiment) (**e**) Cyst burden per brain was determined by counting cysts in brain homogenate on a light microscope. **f,** Infiltrating myeloid cell populations were quantified by flow cytometry. Cells were pre-gated on singlets/live/CD45^{+}/CD11c^{-}. **g,** iNOS^{+} cell populations were quantified, cells were pre-gated on singlets/live/CD45^{+}/CD11c^{-}/CD11b^{+}/CD45^{hi}. **h-i,** CD8^{+} and CD4^{+} T cell populations were quantified, cells were pre-gated on live/singlets/CD3^{+}. **j-k,** Brain slices from WT (**j**) and caspase-1/11$^{-/-}$ (**k**) mice were H&E stained and representative images are shown. Arrow heads indicate parasite cysts. Statistics were performed using a randomized block ANOVA (**e-i**). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Scale bars in (**a-b**) are 400 µm, scale bar in (**d**) is 15 µm, all other scale bars are 50 µm.

Gasdermin-D$^{-/-}$ mice have an impaired response to *T. gondii* infection and impaired IL-1α release

Our data implicate an inflammasome-dependent processes in the control of *T. gondii* in the brain, thus we investigated the importance of gasdermin-D, the pore-forming executor of pyroptosis.[125,126,137,258] We utilized gasdermin-D (gsdmd)$^{-/-}$ mice to specifically assess the importance of pyroptosis. Six weeks p.i., gsdmd$^{-/-}$ mice displayed a significant increase in parasite cyst burden compared to WT (Fig. 7a). Like *Il1r1*$^{-/-}$, IL-1α$^{-/-}$, and caspase-1/11$^{-/-}$ mice, gsdmd$^{-/-}$ mice also displayed a decrease in the number of immune cells infiltrating the brain (Fig. 7b).

To directly assess the contribution of pyroptosis to IL-1α release, brain mononuclear cells were isolated from gsdmd$^{-/-}$ mice and *ex vivo* IL-1α release was determined by ELISA. Cells isolated from the brains of gsdmd$^{-/-}$ mice released significantly less IL-1α into the supernatant than cells from WT mice, about a 70 percent reduction in IL-1α release (Fig. 7c). We also utilized necrosulfonamide (NSA), which has been shown to be a specific inhibitor of gsdmd in mice.[259] Brain cells isolated from WT mice were analyzed for *ex vivo* IL-1α release under control conditions, or incubated with 20μM NSA (Fig. 7d). Strikingly, NSA inhibited *ex vivo* IL-1α release, indicating that release is dependent on gsdmd. Taken together, these results suggest that IL-1α is released from cells from infected brains in a gsdmd$^{-/-}$-dependent manner, and promotes the infiltration of anti-parasitic immune cells into the *T. gondii* infected brain.

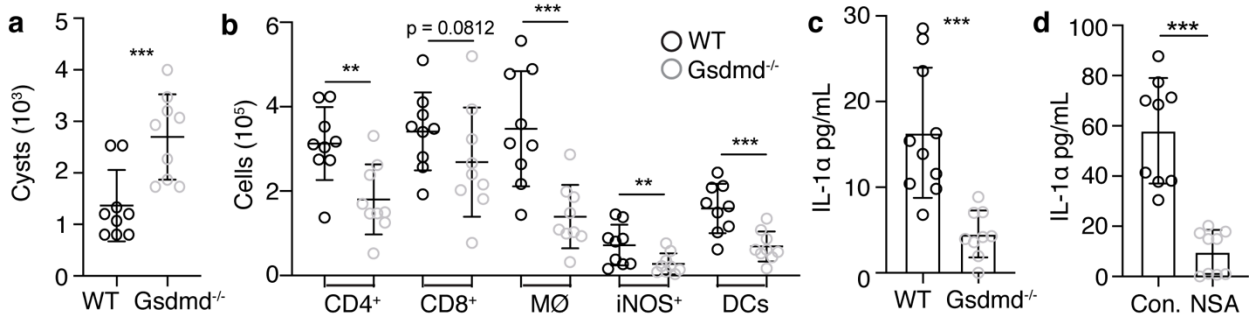
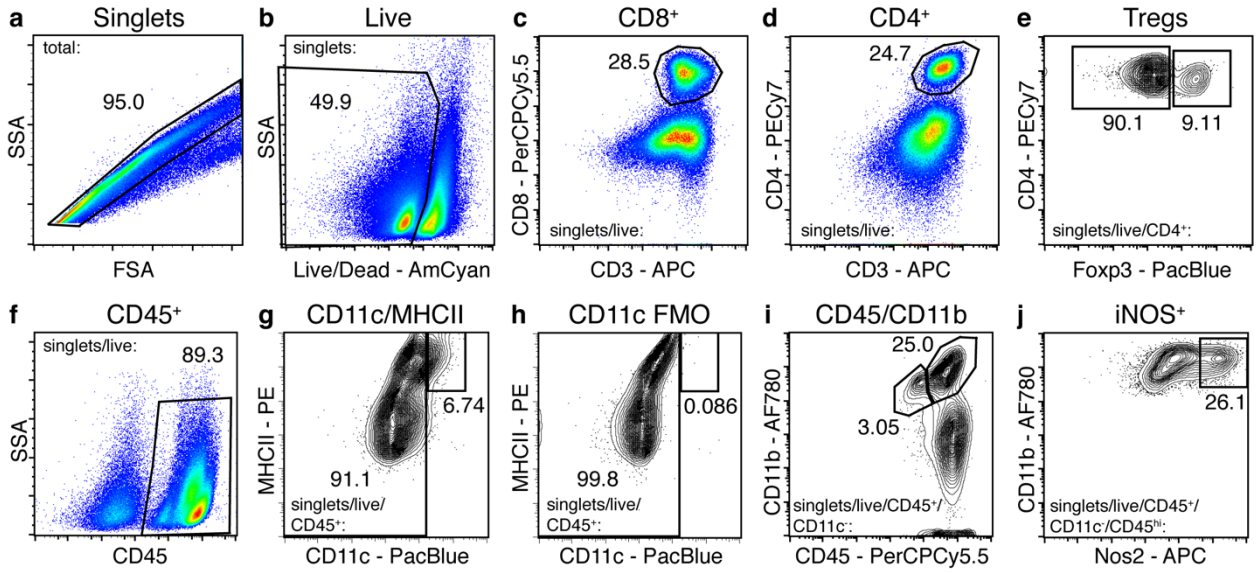


Figure 3.7 Gasdermin D$^{-/-}$ mice have an impaired response to *T. gondii* infection and impaired IL-1α release. a-c, C57B6/J and Gasdermin D$^{-/-}$ mice were infected i.p. with 10 cysts of the Me49 strain of *T. gondii*. 6 weeks p.i., mice were sacrificed and tissues were harvested for analysis. Data from two experiments are shown (n = 4-5 mice per group) **a,** Cyst burden per brain was determined by counting cysts in brain homogenate on a light microscope. **b,** Brain tissue was processed for flow cytometry analysis and immune cell populations were quantified. All populations were previously gated on live/singlets. CD4$^+$ and CD8$^+$ were pre-gated on CD3$^+$ T cells; DCs were pre-gated on CD45$^+$ cells; infiltrating macrophage/monocytes (Mϕ) are defined as CD11c$^-$CD11b$^+$CD45^{hi}; iNOS$^+$ cells were gated within the Mϕ gate. **c,** Single cell suspension from brain homogenate from WT and Gsdmd$^{-/-}$ mice was plated in a 96 well plate and incubated at 37°C overnight. Supernatant was isolated and analyzed by ELISA for IL-1α. **d,** Brain homogenate from WT mice was plated as in **c**, with either control media or 20 μm necrosulfonamide (NSA). IL-1α release from two experiments is shown (**d**). (n = 4 wells per group per experiment) Statistics were performed using a randomized block ANOVA. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.



Supplementary Figure 3.6 Example gating strategy for brain immune populations. Myeloid and T cell populations were identified using two separate panels. **a-b,** for all panels, samples were first gated on singlets and then cells which excluded the live/dead dye. **c-e,** to identify T cell populations, live cells were plotted to gate on either CD8+CD3+ (**c**) or CD4+CD3+ (**d**) cells. **e,** to identify Tregs, CD4+ T cells were gated on Foxp3+. **f-j,** to identify myeloid cell populations live cells were first gated on CD45+ (**f**). CD45+ cells were then gated on CD11c and MHCII (**g**), CD11c+MHCII hi cells were called DCs. CD11c- cells were then gated by CD45 and CD11b (**i**). CD11b+CD45int cells were called microglia, and CD11b+CD45hi cells were called infiltrating myeloid cells. CD45hi cells were then gated on iNOS+ (**j**).

3.3 DISCUSSION

Toxoplasma gondii establishes a chronic brain infection in its host, necessitating long-term neuroinflammation.[194,195,260] Much is known about the immune response to this parasite, but the role of the brain-resident microglia is still largely unknown. Early studies using culture systems of murine and human microglia showed that IFN-γ and LPS treatment prior to infection inhibited parasite replication.[224-226] However, understanding of microglia-specific functions in brain infections has been hindered by the fact that microglia rapidly lose their identity in culture.[36] Moreover, culture techniques do not recapitulate the complex interactions microglia have during infection with other cells or the tissue architecture of the brain. Thus, we aimed to examine microglia and macrophages within the brain to begin to uncover their function.

Through RNA-seq analysis as well as staining of the infected brain tissue, we find that there is an NF-κB signature present in brain-infiltrating monocytes/macrophages, that is largely absent in microglia in the same environment. These two cell types are likely exposed to the same signals within the brain, which suggests that the ontogeny of these cells has long lasting implications for their functional capacity. Transcription factors, including *Sall1*, that have been accepted as defining microglia identity may be shaping the transcriptional landscape, repressing certain loci that could potentially lead to damaging inflammation in the brain.[50] We suggest that these differences are evidence of a division of labor, with microglia and blood-derived macrophages contributing in different ways to inflammation in the brain during infection with *T. gondii*. While blood-derived cells display a classic inflammation-associated NF-κB response, microglia may be better suited to contributing to inflammation through the release of alarmins, rather than through upregulation of a broader NF-κB-dependent program that may be injurious to the local tissue.

We find the alarmin IL-1α expressed in microglia, though they notably lack expression of IL-1β which is found in infiltrating myeloid cells. This suggests that both of these cell types may be able to participate in an IL-1 response, but in fundamentally different ways. Importantly, we show that host immunity is dependent on the activity of IL-1α rather than IL-1β, and that IL-1α is released *ex vivo* from microglia but not from infiltrating macrophages. In general, IL-1β has been the subject of more study than IL-1α, and has a history of being implicated when IL-1 signaling is discussed. More recently, IL-1α has been shown to contribute to certain inflammatory environments. IL-1α release from lymph node macrophages in an inflammasome-independent death event has been shown to enhance antigen presentation and humoral responses to influenza vaccination.[261] IL-1α has been shown to initiate lung inflammation in a model of sterile inflammation using silica.[165] Recently, some reports have suggested IL-1α rather than IL-1β drives sepsis pathology.[158] IL-1α activity in the CNS has begun to be studied, with a deleterious role for the cytokine shown in spinal cord injury.[166] IL-1β has been implicated in some infection models, but IL-1α activity in brain infection has not previously been reported. As an alarmin expressed in the brain at baseline, IL-1α is ideally placed to initiate inflammation in response to early damage caused by the parasite before there is robust immune infiltration.

In this work, we also show that IL-1α likely signals on brain vasculature, promoting the infiltration of immune cells. We found that IL-1R1 expression on brain vasculature displays a mosaic pattern. This could suggest that there are functionally distinct sub-populations of endothelial cells capable of becoming activated in response to different signals.[262] There is ample evidence in the literature to support IL-1R1 expression on endothelial cells as well as the responsiveness of CNS vasculature to IL-1.[154-157,263,264] However, IL-1 has also been shown to signal on immune cells.[265-268] We found that IL-1R1 expression on radio-resistant cells is what is

important in our model, which is supportive of endothelial cells being the relevant responders, but there has also been evidence put forth that other brain resident cells can respond to IL-1. It has been suggested that microglial IL-1R1 expression plays a role in self-renewal after ablation.[269] Microglia are partially radio-resistant and do experience some turnover after irradiation and repopulation. It has also been suggested that IL-1 can act on neurons, though it should be noted that it has also been reported that neurons express a unique form of IL-1RAcP which affects downstream signaling.[152] It is unclear whether astrocytes express IL-1R1, but astrocytes represent another radio-resistant cell population in the brain that has the ability to affect immune cell infiltration through chemokine production.[221,252]

Infiltrating immune cells express pro-IL-1β, but we have not detected a role for IL-1β in promoting inflammation in this model. We have also shown that they do not release IL-1α *ex vivo* even though they express it, suggesting that they may die in an immunologically quiet way such as apoptosis, while microglia may undergo a more inflammatory form of cell death, including pyroptosis. If these two cell types do in fact undergo different forms of cell death, it is of great interest how microglia activate gasdermin-D to release inflammatory factors. It is possible that microglia in an area of parasite reactivation in the brain become infected, sense parasite products in the cytoplasm, and undergo death to eliminate this niche for parasite replication. NLRP1 and NLRP3 have both been described as sensors for *T. gondii* and could be the potential sensors in microglia.[241,243,244] AIM2 is another inflammasome sensor that can recognize DNA and could therefore be activated if parasite DNA becomes exposed to the cytosol.[270] However, we and others have not been able to observe direct infection of microglia in chronically infected mice.[247] On the other hand, microglia migrate to sites of parasite reactivation and may recognize products resulting from host cell death or damage, such as ATP, and undergo death that will promote inflammation.

The presence of ASC specks in infected brains suggests the formation of the caspase-1-dependent canonical inflammasome, but it remains unclear if the ASC specks are directly linked to IL-1α release from microglia. Moreover, the canonical inflammasome can even be activated downstream of non-canonical inflammasome-driven gasdermin-D pores.[271] Thus, the sensors upstream of caspase-dependent cleavage of gasdermin-D in microglia are of great interest.

CHAPTER 4

Inflammasome sensors and IL-1α release in chronic *Toxoplasma gondii* infection: unanswered questions

This chapter contains unpublished data

4.1 INTRODUCTION

The work detailed in the previous chapter illustrates that IL-1α contributes to the inflammation in the brain during chronic infection with the parasite *Toxoplasma gondii*. The data in chapter 3 also show that IL-1α is released *ex vivo* from brain mononuclear cells and that mice lacking gasdermin D display decreased *ex vivo* IL-1α release as well as impaired inflammation and parasite control *in vivo*. This suggests that IL-1α release occurs downstream of inflammasome activation, but which inflammasome components are involved in this process remains unclear. Thus, we aimed to investigate which caspases and sensors contribute to IL-1α release as well as overall immunity to *T. gondii* infection. We show that IL-1α release is not dependent on the caspase-11-driven non-canonical inflammasome. We also show that neither NLRP3 nor AIM2 appear to be responsible for IL-1α release, and importantly, that mice lacking either of these sensors display no defect in their inflammatory response or parasite control.

4.2 RESULTS

Caspase-11$^{-/-}$ mice do not have an impaired response to *T. gondii* infection or impaired IL-1α release

Figure 3.7 shows that *ex vivo* IL-1α release from infected brain tissue is at least partially gasdermin-D-dependent, and that mice lacking gasdermin D have an impaired response to *T. gondii*. However, gasdermin D can be activated by both caspase-1 and caspase-11. Figure 3.6 shows that mice lacking both caspase-1 and -11 have an impaired response to *T. gondii* similar to the gasdermin-D-deficient mice. Figure 3.6 also shows the formation of ASC aggregates in infected brains, which is indicative of canonical inflammasome activation. However, we were unable to directly link the formation of ASC specks with IL-1α release. Therefore, we investigated the potential role of non-canonical inflammasome activity by utilizing caspase-11-deficient mice.

Six weeks p.i. caspase-11$^{-/-}$ mice displayed no difference in parasite burden compared to WT mice (Fig. 4.1a). Consistent with this, caspase-11$^{-/-}$ mice displayed no defect in immune cell recruitment to the brain (Fig. 4.1b). There were, in fact, even more immune cells present in the brain in caspase-11$^{-/-}$ mice, which is a phenomenon we do not yet understand. We further examined *ex vivo* IL-1α release from brain mononuclear cells, and found no decrease in the release of this cytokine from cells isolated from caspase-11$^{-/-}$ mice (Fig. 4.1c). These results suggest that caspase-11 activity is not necessary for IL-1α release, nor is it necessary for the maintenance of chronic inflammation or parasite control.

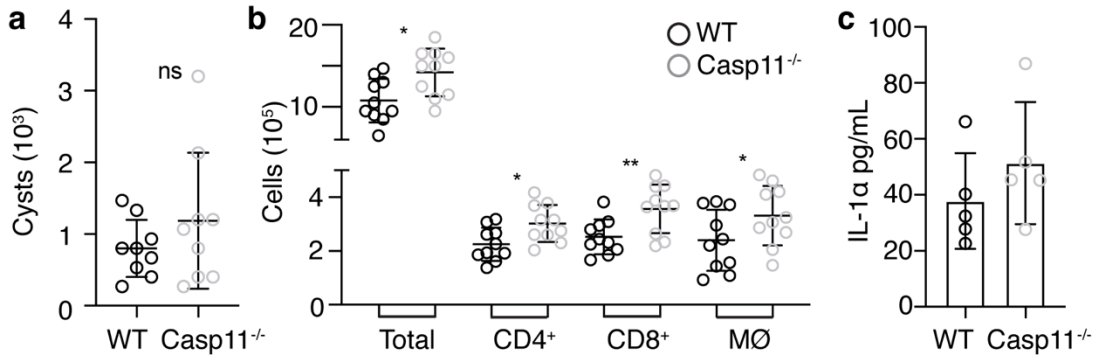


Figure 4.1 Caspase-11$^{-/-}$ mice do not have an impaired response to *T. gondii* infection or impaired IL-1α release. WT and caspase-11$^{-/-}$ mice were infected i.p. with 10 cysts of Me49 parasites for 6 weeks. **a,** Cyst burden per brain was determined by counting cysts in brain homogenate on a light microscope. **b,** Brain tissue was processed for flow cytometry analysis and immune cell populations were quantified. All populations were previously gated on live/singlets. CD4^{+} and CD8^{+} were pre-gated on CD3^{+} T cells; infiltrating macrophage/monocytes (Mϕ) are defined as CD11c^{-}CD11b^{+}CD45^{hi}; iNOS^{+} cells were gated within the Mϕ gate. **a-b,** Data from two experiments are shown, statistics were performed using a randomized block ANOVA. **c,** Single cell suspension from brain homogenate from WT and caspase-11$^{-/-}$ mice was plated in a 96 well plate and incubated at 37°C overnight. Supernatant was isolated and analyzed by ELISA for IL-1α. Statistics were performed using a Student's t test.

NLRP3$^{-/-}$ mice do not show an impaired response to *T. gondii* infection or impaired IL-1α release

Our data support that IL-1α is released downstream of pyroptosis, and the non-canonical inflammasome does not appear to be involved in this process. Thus, we aimed to investigate canonical inflammasome sensors for contribution to this pathway. NLRP3 is a logical candidate to interrogate. NLRP3 can be activated by a broad range of stimuli.[128] In addition, NLRP3 has been implicated as a sensor of *T. gondii* infection.[244] Our RNA-seq data shows that NLRP3 is expressed in microglia in the brain during infection, though not as highly as infiltrating macrophages (Fig. 4.2a). We can also see NLRP3 protein expression in infected brain tissue, but importantly, it does not colocalize with ASC specks in the brain (Fig. 4.2b). Consistent with this result, six weeks p.i., NLRP3$^{-/-}$ mice displayed no difference in parasite load in the brain compared to WT mice, and no defect in immune cell recruitment to the brain (Fig. 4.2c-d). Cells isolated from brains of WT and NLRP3$^{-/-}$ mice were also assayed for *ex vivo* IL-1α release. IL-1α release was found not to be dependent on NLRP3 (Fig. 4.2e).

An inhibitor of NLRP3 is available which was also used to answer this question. MCC950 or vehicle was administered to chronically infected mice i.p. every other day for 12 days before brains were harvested. Mice that received MCC950 treatment displayed no significant difference in parasite burden or immune cell infiltration (Fig. 4.2f-g). In a separate experiment, cells were purified from chronically infected WT brains and incubated with either MCC950 or vehicle. In this paradigm, MCC950 treatment did not impair IL-1α release from cells (Fig. 4.2h). Taken together, these results suggest that NLRP3 is not necessary for IL-1α release, nor is it necessary for the maintenance of chronic inflammation or parasite control.

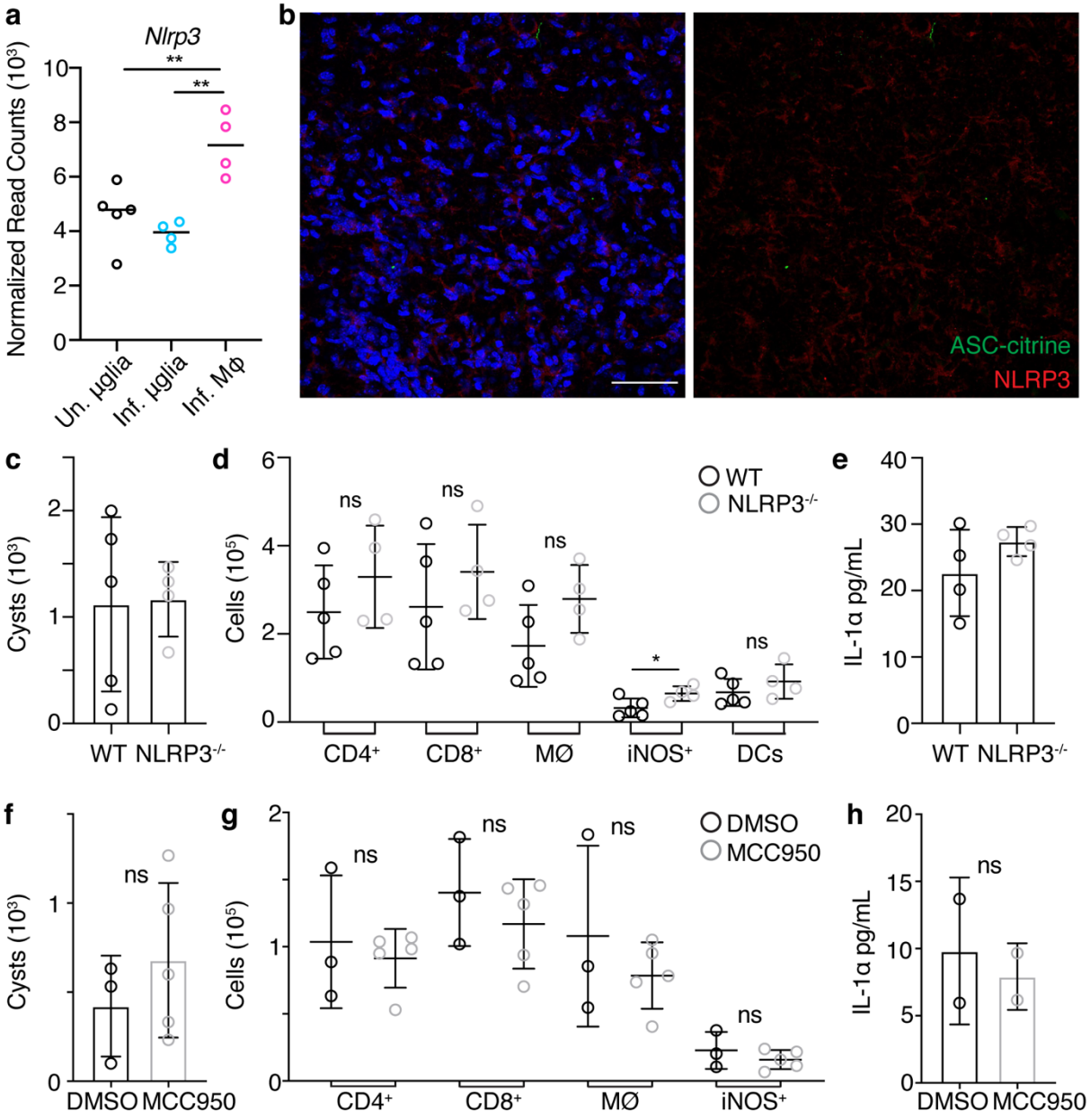


Figure 4.2 NLRP3 is not necessary for IL-1α release or control of chronic infection. a, Normalized counts of *Nlrp3* expression from the RNA-seq data set were plotted **b,** Brain sections from chronically infected ASC-citrine (green) mice were stained with DAPI (blue) and an antibody against NLRP3 (red). **c-e,** WT and NLRP3$^{-/-}$ mice were infected for 6 weeks. **f-h,** Chronically infected WT mice were treated with vehicle or 200 μg of MCC950 i.p. every other day for 12 days prior to harvest. **c & f,** Cyst burden per brain was determined by counting cysts in brain homogenate on a light microscope. **d & g,** Brain tissue was processed for flow cytometry analysis and immune cell populations were quantified. All populations were previously gated on live/singlets. CD4^{+} and CD8^{+} were pre-gated on CD3^{+} T cells; DCs were pre-gated on

$CD45^+$ cells; infiltrating macrophage/monocytes (Mϕ) are defined as $CD11c^-CD11b^+CD45^{hi}$; $iNOS^+$ cells were gated within the Mϕ gate. **e,** Single cell suspension from brain homogenate from WT and $NLRP3^{-/-}$ mice was plated in a 96 well plate and incubated at 37°C overnight. Supernatant was isolated and analyzed by ELISA for IL-1α. **h,** Brain homogenate from WT mice was plated as in **e**, with either DMSO or MCC950. Representative experiments are shown. Statistics were performed using a Student's t test (**c-h**).

AIM2$^{-/-}$ mice do not show an impaired response to *T. gondii* infection or impaired IL-1α release

The data shown have suggested that gasdermin D promotes immunity to *T. gondii* infection and that it is also involved in IL-1α release. Our search for an inflammasome sensor has revealed that NLRP3 does not contribute to this phenotype. We decided to examine whether the AIM2 inflammasome could be involved in this process. AIM2 senses DNA[128] and thus we reasoned that if there are parasites present within a cell that are being degraded and have their DNA exposed to the host cell cytosol, AIM2 could potentially be activated. We do find that AIM2 is expressed at the RNA level in both microglia and infiltrating macrophages in the brain (Fig. 4.3a). However, using AIM2$^{-/-}$ mice, we observe no significant difference in parasite burden or immune cell infiltration in the brain compared to WT mice (Fig. 4.3b-c). Additionally, cells isolated from AIM2$^{-/-}$ brains displayed no defect in IL-1α release (Fig. 4.3d). These data show that AIM2 is not potentiating IL-1α release *ex vivo*, and does not appear to contribute to the response to chronic *T. gondii* infection.

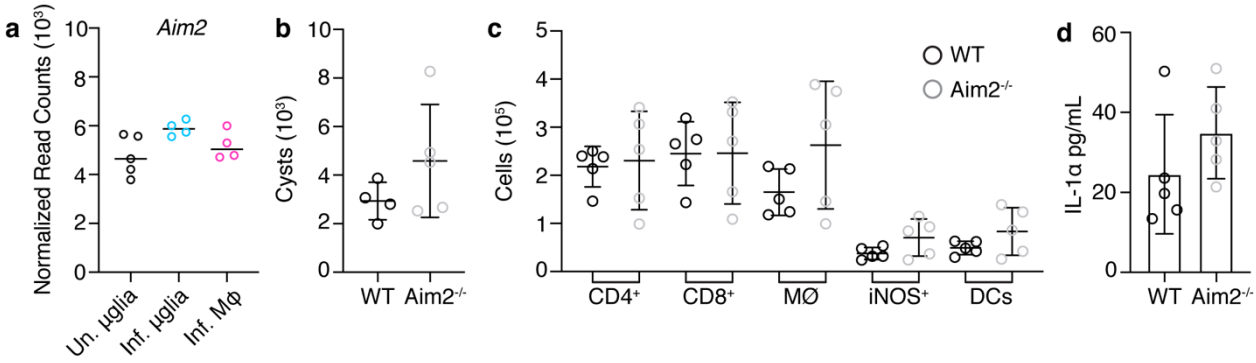


Figure 4.3 AIM2 is not necessary for IL-1α release or for control of chronic infection. a, Normalized counts of *Aim2* expression from the RNA-seq data set were plotted. **b-d,** WT and AIM2$^{-/-}$ mice were infected i.p. with 10 cysts of Me49. **b,** Cyst burden per brain was determined by counting cysts in brain homogenate on a light microscope. **c,** Brain tissue was processed for flow cytometry analysis and immune cell populations were quantified. All populations were previously gated on live/singlets. CD4^{+} and CD8^{+} were pre-gated on CD3^{+} T cells; infiltrating macrophage/monocytes (Mϕ) are defined as CD11c^{-} CD11b^{+}CD45^{hi}; iNOS^{+} cells were gated within the Mϕ gate. **d,** Single cell suspension from brain homogenate from WT and AIM2$^{-/-}$ mice was plated in a 96 well plate and incubated at 37°C overnight. Supernatant was isolated and analyzed by ELISA for IL-1α. A representative experiment is shown. Statistics were performed using a Student's t test (**b-d**).

4.3 DISCUSSION

The data displayed in chapter 3 demonstrate that gasdermin D is involved in promoting inflammation in response to chronic *T. gondii* infection and in the release of the alarmin IL-1α. This suggested to us that the inflammasome was likely activated leading to IL-1α release and subsequent inflammation. Thus, we aimed to investigate a few candidate molecules to see which inflammasome could be mediating this effect.

NLRP3 has been shown to recognize *T. gondii* infection as well as a broad range of conditions leading to cellular stress and damage.[128,244] However, our results show that mice lacking NLRP3 do not have defects in inflammation in the brain or in parasite control during chronic infection. Importantly, through the use of NLRP3$^{-/-}$ mice as well as chemical inhibition we have shown that NLRP3 also does not appear to mediate IL-1α release in this model. AIM2 is another inflammasome sensor that could potentially be activated if parasite DNA was to become exposed to the cytosol.[270] However, the results in this chapter show that AIM2 does not appear to be necessary for chronic inflammation or for IL-1α release in this model.

Gasdermin D can be cleaved by caspase-11 as well as by caspase-1, and in addition, the canonical inflammasome can be activated downstream of caspase-11 activation.[271] Thus, we investigated whether the caspase-11-dependent non-canonical inflammasome was involved in IL-1α release and the downstream inflammation. Mice lacking caspase-11 had no defect in parasite control or in IL-1α release. Caspase-11$^{-/-}$ mice interestingly had increased numbers of immune cells present in their brains, which may suggest that caspase-11 is somehow involved in immune cell die-back or in regulation of their entry. One study that examined caspase-11$^{-/-}$ mice during chronic *T. gondii* infection also showed increased inflammation in the brain, though they did this simply though H&E staining.[245] This study also reported an increased cyst load in capsase-11$^{-/-}$

mice which we do not observe, but this was reported only as the percentage of animals with tissue cysts present.[245]

Together the results in this and the previous chapter suggest that IL-1α release in this model of chronic *T. gondii* infection occurs in a mechanism dependent on canonical inflammasome activation of caspase-1, and subsequently, gasdermin D. The inflammasome sensor does not appear to be NLRP3 or AIM2, but it is possible that multiple sensors contribute to parasite control through the same pathway and that there is compensation when one is missing. But there are also a number of other potential candidates that could be involved. NLRP1 could be a reasonable option to test since it has been shown to recognize *T. gondii* and the human NALP1 has been implicated in congenital infection with *T. gondii*.[241-243] It also remains unknown whether the inflammasome would be activated in response to direct parasite invasion, or as a result of the recognition of inflammatory products released from host cell death or damage due to infection.

CHAPTER 5

Investigating necroptosis and apoptosis in chronic *Toxoplasma gondii* infection

This chapter contains unpublished data

5.1 INTRODUCTION

The work outlined in the previous chapters has begun to address how pyroptosis contributes to the host response to *T. gondii* infection. This has prompted our interest in other mechanisms of cell death, and how they may promote to host immunity to infection. Necroptosis is another form of programmed cell death which has the potential to stimulate inflammation. Necroptosis results when death receptor signals are received under caspase-8 inhibition, leading to RIPK1 activation of RIPK3, which goes on to phosphorylate MLKL ultimately leading to membrane perturbation and cell death.[120-122] As many intracellular pathogens utilize caspase inhibition, necroptosis may be a relevant mechanism to prevent pathogen replication or spread, as well as to stimulate inflammation in an effort to control infection.

Apoptosis is the most well-known and studied form of programmed cell death, which is not thought to be inflammatory. However, apoptosis could still be vastly important to the host response to infection, not only through its effect on the longevity of the immune cells fighting the infection, but also through its potential to eliminate a niche for pathogen survival in infected cells.

RIPK3$^{-/-}$ mice have been developed which are unable to undergo necroptosis, making them a useful tool to study the importance of this pathway in the context of disease. It should be noted that at baseline RIPK3-driven necroptosis may be involved in the homeostatic die-back and turnover of lymphocytes, leading to an accumulation of lymphocytes in its absence.[272,273] To study the importance of apoptosis, specifically extrinsic apoptosis, mice lacking caspase-8 can be used, however, they must be on a background of RIPK3 deficiency or the lack of caspase-8 is embryonic lethal.[272] Thus, we can use caspase-8$^{-/-}$RIPK3$^{-/-}$ mice for our studies. Our preliminary data show that both necroptosis and caspase-8-dependent apoptosis promote parasite control in chronic *T. gondii* infection, but that the lack of extrinsic apoptosis is much more detrimental in this model.

5.2 RESULTS

RIPK3$^{-/-}$ mice have impaired parasite control and increased immune cell numbers in the brain during chronic *T. gondii* infection

To begin to assess the importance of necroptosis in our model of chronic *T. gondii* infection, WT and RIPK3$^{-/-}$ mice were infected. The mice survived to chronic infection and were then assessed. The number of cysts in the brains of RIPK3$^{-/-}$ mice was increased compared to WT mice, indicating a role for RIPK3 activity in parasite control (Fig. 5.1a, f-g). In addition, both infiltrating myeloid cells as well as T cells were increased in the brains of RIPK3$^{-/-}$ mice compared to WT (Fig. 5.1 b-e). This could be the result of an increased response to a higher parasite load, or it could be indicative of a RIPK3-dependent death mechanism that normally regulates the infiltrating immune cell pool in the brain or even of a RIPK3-dependent death mechanism that regulates the number of these cells at baseline.[272,273] Overall, these data, though preliminary, suggest that RIPK3 promotes parasite control in *T. gondii* infection.

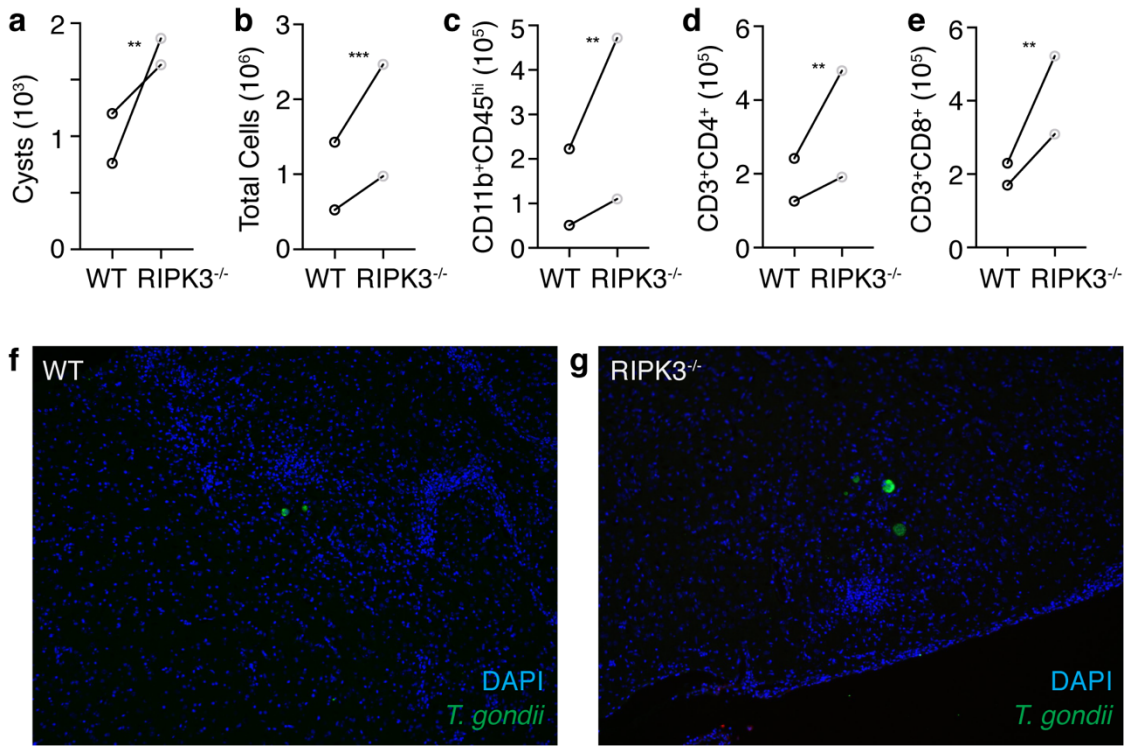


Figure 5.1 RIPK3$^{-/-}$ mice have impaired parasite control and increased immune cell numbers in the brain during chronic *T. gondii* infection. a-e, Data compiled from two experiments are shown. **a,** Cyst burden per brain was determined by counting cysts in brain homogenate on a light microscope. **b-e,** Brain tissue was processed for flow cytometry analysis and immune cell populations were quantified. All populations were previously gated on live/singlets. **b,** Total number of live, singlet cells. **c,** infiltrating macrophage/monocytes, defined as CD11c^{-}CD11b^{+}CD45^{hi}. **d-e,** CD4^{+} and CD8^{+} were pre-gated on CD3^{+} T cells. Statistics were performed using a randomized block ANOVA. **f-g,** Representative images of WT (**f**) and RIPK3$^{-/-}$ (**g**) brain sections stained with DAPI (blue) and antibody recognizing Me49 parasites (green).

Caspase-8$^{-/-}$RIPK3$^{-/-}$ mice have severely impaired parasite control and increased immune cell numbers in the brain during chronic *T. gondii* infection

To try to assess the importance of apoptosis in our model of chronic *T. gondii* infection, WT and Casp8$^{-/-}$RIPK3$^{-/-}$ mice were infected. Mice survived to chronic infection, and were then assessed 4 weeks p.i.. Casp8$^{-/-}$RIPK3$^{-/-}$ mice displayed a very significant increase in parasite burden compared to WT mice (Fig. 5.2). This high density of cysts can also be appreciated through images of brain tissue stained for the parasite (Fig. 5.2f-h). As seen with the RIPK3$^{-/-}$ mice, Casp8$^{-/-}$RIPK3$^{-/-}$ mice also had increased numbers of immune cells present in the brain (Fig. 5.2b-e), the reason for which we do not know. Interestingly, though we and others have not been able to observe direct infection of macrophages in the brain during chronic infection,[247] in brains from Casp8$^{-/-}$RIPK3$^{-/-}$ mice it was an easily observable phenomenon (Fig. 5.2i). Together, these data show that a caspase-8-dependent process is likely important for parasite control in the brain.

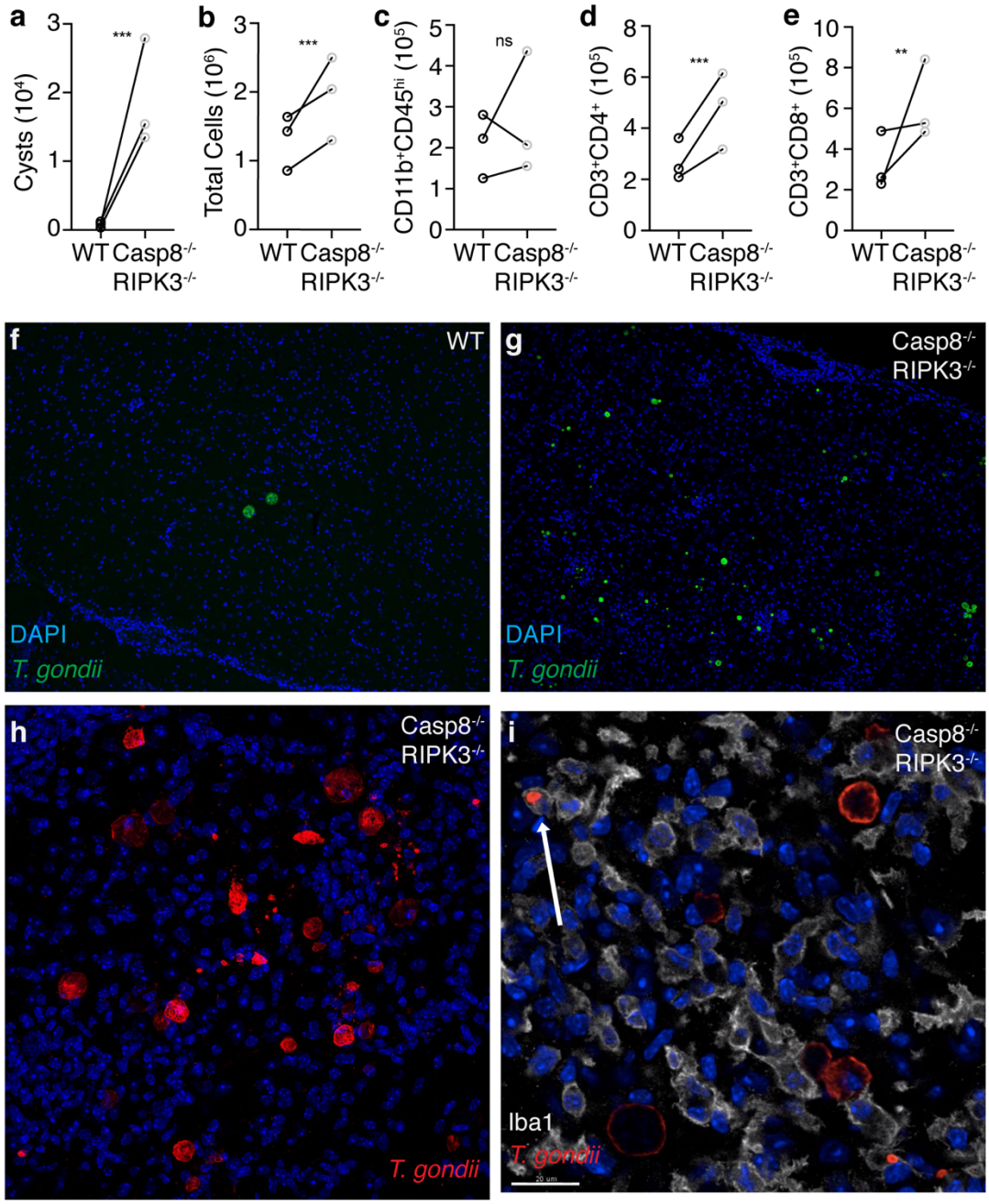


Figure 5.2 Caspase-8$^{-/-}$RIPK3$^{-/-}$ mice have severely impaired parasite control and increased immune cell numbers in the brain during chronic *T. gondii* infection. a-e, Data compiled from 3 experiments are shown. **a,** Cyst burden per brain was determined by counting cysts in brain homogenate on a light microscope. **b-e,** Brain tissue was processed for flow cytometry analysis and immune cell populations were quantified. All populations were previously gated on live/singlets. **b,** Total number of live, singlet cells. **c,** infiltrating macrophage/monocytes, defined as CD11c^{-}CD11b^{+}CD45^{hi}. **d-e,** CD4^{+} and CD8^{+} were pre-gated on CD3^{+} T cells. Statistics were performed using a randomized block ANOVA. **f-h,** Representative images

of WT (**f**) and Casp8$^{-/-}$RIPK3$^{-/-}$ (**g-h**) brain sections stained with DAPI in blue and antibody recognizing Me49 parasites in green (**f-g**) or red (**h**). **i,** A representative image of one z plane in a Casp8$^{-/-}$RIPK3$^{-/-}$ brain section stained with DAPI (blue), *T. gondii* (red), and Iba1 (gray), showing infection of an Iba1^{+} cell (indicated by arrow).

5.2 DISCUSSION

Results in the previous chapters outlined a role for pyroptosis in promoting control of *T. gondii* infection. In this chapter, we aimed to investigate the potential role of other cell death mechanisms, particularly apoptosis and necroptosis. To study necroptosis, we used RIPK3$^{-/-}$ mice, and performed analyses after 4 weeks of infection. We found increased parasite load in mice lacking RIPK3 as well as more immune cells in the brain. As mentioned in the previous section, this increase in immune cells could be due to increased antigen load/parasite-induced tissue damage, absence of a death pathway that takes place in the brain during infection to allow turnover of immune cells, or absence of death pathway that takes place at baseline in the periphery or during development that limits immune cell numbers. Increased T cells in mice lacking RIPK3 has been previously reported,[272,273] and to determine the relevance of this phenotype in our model the RIPK3$^{-/-}$ mice should be evaluated at baseline in our hands. The increased parasite burden in the brain in these mice is interesting and we do not know how this is mediated. It is possible that RIPK3-dependent necroptosis stimulates protective inflammation normally during infection. In fact, a death-independent role for RIPK3 in promoting neuroinflammation has been shown in a model of West Nile virus infection.[274] However, because there is no loss of inflammation in these mice this is unlikely in our model. Another, perhaps more likely, explanation is that RIPK3-dependent necroptosis limits the parasite itself, possibly through eliminating infected cells. A pathogenic role for necroptotic death in the brain has been demonstrated in human and mouse models of Alzheimer's disease.[275] One study has looked at RIPK3$^{-/-}$ mice during acute *T. gondii* infection. They found that RIPK3$^{-/-}$ had no difference in survival compared to WT mice, nor did they have any differences in immune infiltration or parasite load in the peritoneum early during

infection.[276] Thus, our data are the first showing a role for this pathway during chronic *T. gondii* infection.

To study apoptosis, specifically extrinsic apoptosis, we used caspase-8$^{-/-}$RIPK3$^{-/-}$ mice, and performed analysis 4 weeks after infection. As with the RIPK3$^{-/-}$ mice, the double knockout mice displayed an increase in parasite load in the brain as well as an increased number of immune cells. Interestingly, the increase in immune cell numbers was about the same for the RIPK3$^{-/-}$ and caspase-8$^{-/-}$RIPK3$^{-/-}$ mice compared to WT (which was also observed directly comparing all groups in the same infection, not shown). This may suggest that the increase in immune cell presence is not a direct result of the increased parasite burden, because the caspase-8$^{-/-}$RIPK3$^{-/-}$ mice have a much higher increase in parasite. More likely, what we are observing is lack of a RIPK3-dependent death mechanism (necroptosis) in both cases. The increase in parasite is very interesting in the caspase-8$^{-/-}$RIPK3$^{-/-}$ mice, because the magnitude of the increase is quite high, much higher than that seen in the RIPK3$^{-/-}$ mice. This suggests that extrinsic apoptosis is important for controlling parasite. This could be occurring in infected neurons or glia or in immune cells that become transiently infected, or a combination of these circumstances (Fig. 5.3). Interestingly, it was fairly easy to find infected macrophages in the brains of caspase-8$^{-/-}$RIPK3$^{-/-}$ mice, which is not typical in WT mice during chronic infection. This could indicate that these cells get infected and normally die very quickly in a caspase-8-dependent manner, or that there is just so much parasite in the system that more cells are being exposed to it than normally would. It should be noted that caspase-8$^{-/-}$RIPK3$^{-/-}$ mice have been looked at during acute *T. gondii* infection. They were reported to have impaired survival compared to WT mice, as well as impaired inflammation and increased parasite in the peritoneum early in infection.[276] This was determined to be due to a defect in IL-12 production in the absence of caspase-8, rather than anything to do with apoptosis.[276] In this study,

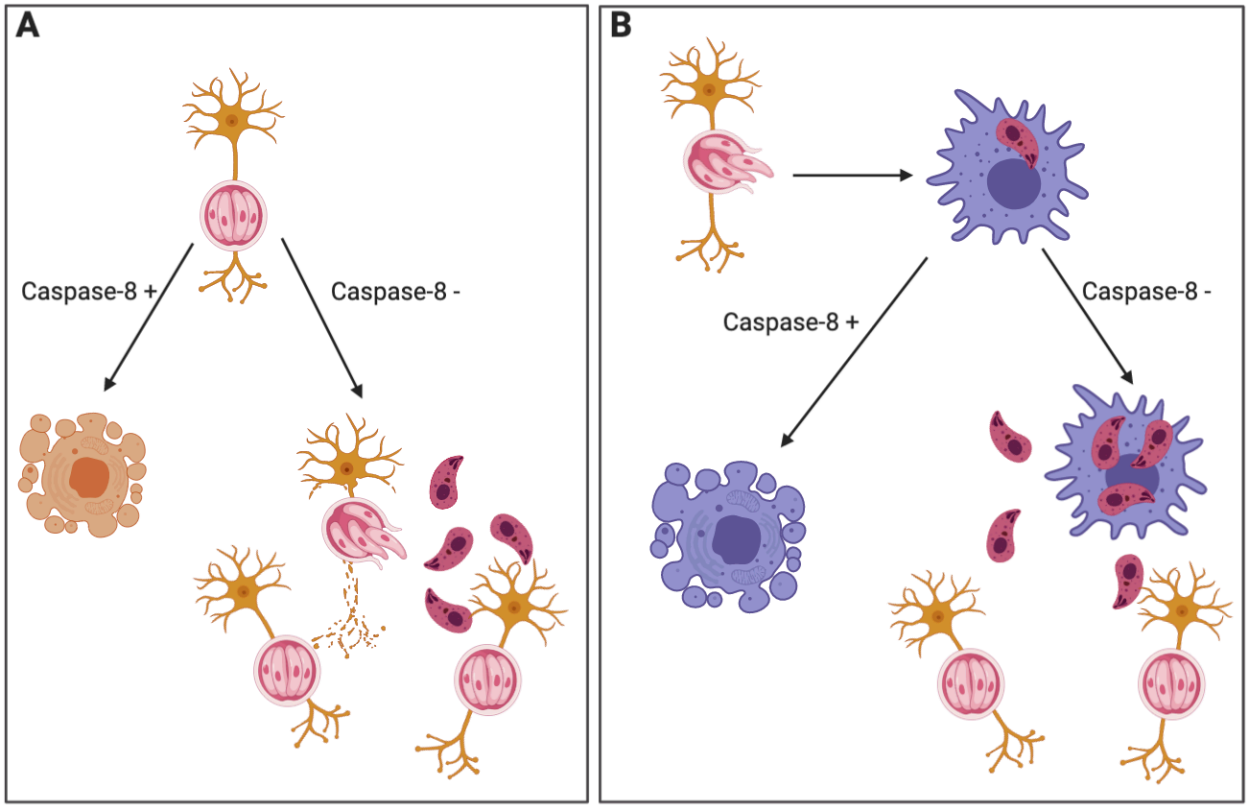


Figure 5.3 Visual Hypothesis for the Function of Apoptosis in *T. gondii* infection

the caspase-8$^{-/-}$RIPK3$^{-/-}$ mice were reported to die by about day 10 p.i., but it should be noted that in our hands these mice survive to chronic infection, so there may be fundamental differences in the models. However, we can address whether our phenotype is a result of this acute parasite proliferation by analyzing the caspase-8$^{-/-}$RIPK3$^{-/-}$ mice during acute infection in our hands, as well as by administering IL-12 to the mice until chronic infection. Overall, these data are very preliminary, but may provide the foundation for interesting studies going forward.

CHAPTER 6

Conclusions, Future Directions, and Final Remarks

6.1 Summary

In this work, we began by investigating the contribution of one alarmin to neuroinflammation, which then led us to interesting questions and findings regarding the differential functions of microglia and brain-infiltrating macrophages as well as the contribution of cell death to an immune response. These studies were bred from an interest in how innate factors can initiate and maintain a chronic, local immune response, specifically within the brain.

In order to study factors promoting neuroinflammation, we used a mouse model of infection with *Toxoplasma gondii*. *T. gondii* naturally establishes a chronic infection in the CNS which is controlled by sustained inflammation and immune cell recruitment to the brain.[194,195,233,247,248] Though both infiltrating myeloid cells and T cells are known to be required to maintain control of chronic infection[198,201,214,232], the local events within the brain that initiate inflammation are less understood. We began by investigating IL-1, a pro-inflammatory cytokine that has been associated with host cell damage. Using mice lack that IL-1R1 and cannot respond to IL-1, we found that these mice had increased parasite burden and decreased immune infiltration, specifically within the brain during chronic infection. We also found that IL-1 signaling promotes activation of the brain endothelium, an important step for the successful infiltration of immune cells, and that it likely does this by signaling on the vasculature itself. (Fig. 6.1)

IL-1α and IL-1β signal through the same receptor complex[251] and neither molecule is conventionally secreted. But while IL-1α functions as an alarmin,[249] IL-1β activity is dependent on caspase-1 cleavage.[126,250] We found that IL-1α$^{-/-}$ mice exhibited impaired parasite control compared to WT mice, as well as impaired immune cell infiltration. Interestingly, this phenotype was not observed in IL-1β$^{-/-}$ mice which were unchanged from WT. This finding was interesting to us because we have shown that both of these molecules are present in the brain during infection,

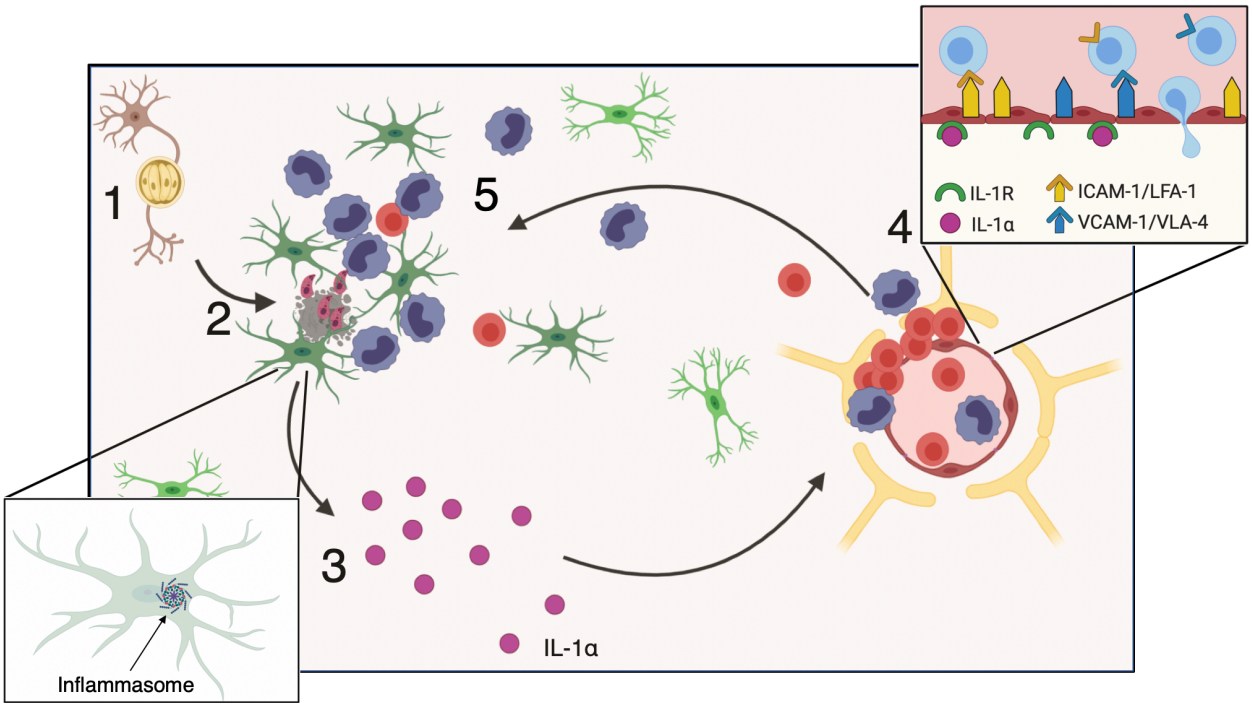


Figure 6.1 Working Model

and it is known that they signal in the same manner, all of which suggests that this difference is likely driven by cell type differences in expression. Indeed, we found that while brain-infiltrating monocytes/macrophages express both IL-1α and IL-1β, brain-resident microglia seem to express predominantly IL-1α. When we sorted these two macrophage populations from infected brains and analyzed them *ex vivo*, the microglia, but not the infiltrating cells, released detectable IL-1α. This further supports microglia as the relevant source of IL-1α in the brain during chronic *T. gondii* infection.

The finding that both microglia and infiltrating myeloid cells express IL-1α, but that microglia specifically can release it, led us to question how the release is occurring. IL-1α cannot be canonically secreted and is usually thought to require membrane permeability to be released. We decided to investigate whether the pyroptosis machinery is involved in this process. Caspase-$1/11^{-/-}$ as well as gasdermin-$D^{-/-}$ mice were found to display a similar impairment in the control of infection to that seen in mice lacking IL-1R1 or IL-1α. Additionally, *ex vivo* IL-1α release from brain cells was found to be gasdermin-D-dependent. This led us to the hypothesis that microglia may be more susceptible to pyroptosis than other cells present in the brain during infection. (Fig. 6.1)

These differences in the capabilities of microglia versus macrophages are especially interesting given that they are present in the same tissue microenvironment in the brain and likely exposed to the same signals. RNA-seq analysis on these two populations during infection confirmed differences in IL-1 expression as well as many other differences. Notable to us, was the absence of an NF-κB signature in microglia, which was present in brain-infiltrating monocytes/macrophages. This was confirmed through staining of infected brain tissue, which showed lack of expression of both Rel and RelA specifically in microglia. The difference in this

NF-κB signature alone likely has important implications for the functions of these cells during infection, and we think that they would contribute to inflammation in the brain in different ways. As a brain-resident cell, microglia may be better suited to alarmin release, rather than activation of a broad inflammatory program that could me more damaging to the local environment. (Fig. 6.2)

We have also performed preliminary studies to further investigate the mechanism of IL-1α release in the brain during chronic *T. gondii* infection. We have shown that this process is dependent on gasdermin-D, and thus, likely involves inflammasome activation. We examined the possible contribution of the non-canonical inflammasome by utilizing caspase-11$^{-/-}$ mice. We found that IL-1α release was not dependent on caspase-11, and neither was immune recruitment or parasite control. Since caspase-1/11$^{-/-}$ mice displayed a defect in inflammation and parasite control, which was not seen in caspase-11$^{-/-}$ mice, we hypothesize that canonical inflammasome activation of caspase-1 is mediating this effect. We have plans to study caspase-1$^{-/-}$ mice in the future to address this. We have begun to investigate which inflammasome sensor could be potentiating IL-1α release in this model. We have tested for the importance of NLRP3 as well as AIM2, and have found that neither of these is necessary for IL-1α release or for immune cell recruitment.

Finally, our findings showing that pyroptosis contributes to the host response to chronic *T. gondii* infection led us to question the possible contributions from other forms of cell death. We have begun by characterizing the response of RIPK3$^{-/-}$ and caspase-8$^{-/-}$RIPK3$^{-/-}$ mice during chronic infection. RIPK3 deficiency prevents necroptosis, and caspase-8/RIPK3 double deficiency prevents necroptosis and extrinsic apoptosis, thus, by comparing the two, we can determine the importance of each pathway.

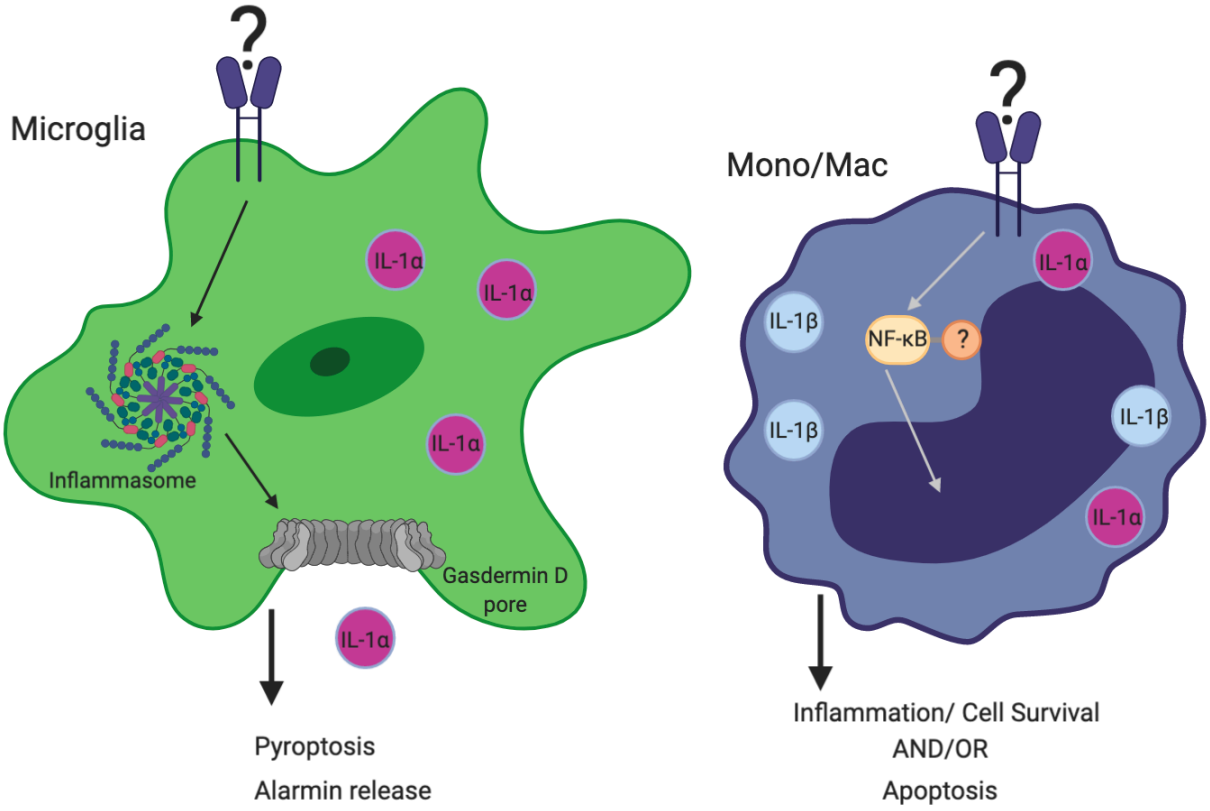


Figure 6.2 Response of Microglia versus Monocyte/Macrophages to Inflammatory Signals in the Brain

We found that there is an accumulation of immune cells in the brain in the absence of necroptosis, the exact mechanism of which we do not understand yet. We also found that there is an increase in parasite load in the brain in the absence of necroptosis, indicating that this process limits parasite to some extent. Interestingly, we found a significant overgrowth of parasite in the brain in the absence of extrinsic apoptosis, indicating that at some stage apoptosis is very important for parasite control. These initial studies will provide the groundwork for continued investigation into these processes.

6.2 Inflammasomes Revisited

There are a variety of inflammasome sensors that have been described, each recognizing a repertoire of different stimuli. In addition to the well-studied inflammasomes, new sensors are still being discovered that can potentiate similar downstream events in response to certain triggers. The list of ligands for the known inflammasome sensors is also continually being updated. Add to this that any one infection or insult could release a number of different molecules that could stimulate different inflammasome pathways. This may mean that it could be exceedingly difficult to pinpoint the exact mechanism of inflammasome activation in our model. However, there are still some avenues that could be explored and remaining questions that we can try to answer.

One thing we are going to do is to perform experiments using caspase-$1^{-/-}$ mice. The evidence we have gathered thus far all seems to point to the relevance of caspase-1 in our model, but using the single knockout mice will conclusively answer this question. Our data have shown that caspase-1/11 double deficient mice have defects in inflammation as well as parasite control in the brain during chronic *T. gondii* infection (Fig. 3.6). We further showed that caspase-11 single knockout mice did not display these same defects (Fig. 4.1), suggesting that it was the loss of caspase-1 that mediated the phenotype we observed. We also observed ASC specks in infected

brain tissue, indicating the formation of inflammasome complexes (Fig. 3.6), which is further evidence of canonical inflammasome activation. I plan to infect caspase-$1^{-/-}$ mice and analyze the immune response and parasite burden in the brain during the chronic stage of infection. Additionally, we have shown that gasdermin-$D^{-/-}$ mice exhibit impaired IL-1α release (Fig. 3.7), which was not observed in caspase-$11^{-/-}$ mice (Fig. 4.1). Thus, I will assess IL-1α release in caspase-$1^{-/-}$ mice. My hypothesis is that caspase-$1^{-/-}$ mice will display an increase in parasite burden correlating with decreased immune infiltration, as well as impaired IL-1α release. If this is not the case, then it may be that something else is leading to the activation of gasdermin-D. Though the well-defined activation mechanism of gasdermin is cleavage by either caspase-1 or capsase-11,[129,132] it is now known that gasdermin-D can also be cleaved by caspase-8,[277] so there may be even other mechanisms that we are unaware of as of yet.

Additional studies could be performed investigating other inflammasome sensors to see if they are important for the immune response to chronic *T. gondii* infection and for IL-1α release. I think that examining NLRP1 knockout mice would be a good place to start. NLRP1 has been described as a sensor *T. gondii* infection and has been shown to affect cell death and susceptibility to infection in models of acute infection as well as *in vitro*.[241,243,244] Additionally, human NALP1 has been shown to influence susceptibility to congenital *T. gondii* infection.[242] NLRP1 deficient mice have not been studied specifically in the context of chronic *T. gondii* infection in the brain. Because IL-1α is likely being released by microglia during infection, when looking for an inflammasome sensor potentiating IL-1α release we should consider expression by microglia. According to our RNA-seq data set, NLRP1b is expressed by microglia in the brain during infection, though it should be noted that infiltrating macrophages express similar levels (Fig. 6.3a). The pyrin inflammasome could be investigated in this model and is expressed by macrophages in

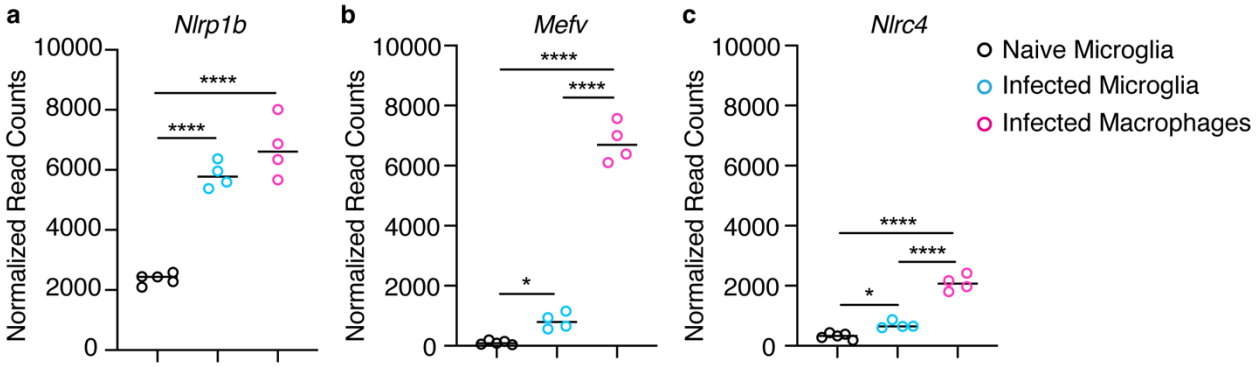


Figure 6.3 Read Counts of Inflammasome Sensors from RNA-seq data

the brain, but mostly by infiltrating macrophages rather than microglia (Fig. 6.3b). NLRC4 is another sensor that could be studied, and it is expressed by macrophages in the brain during infection, but to a lesser extent than previously mentioned inflammasome sensors and not to a high extent by microglia specifically (Fig. 6.3c).

It would also be possible to use a more unbiased biochemical approach to find the relevant inflammasome sensor(s). The ASC-citrine reporter mice that were used in chapter 3 also have an HA tag fused to the ASC protein. Thus, it may be possible to perform an HA pull down on homogenized brain tissue, and then submit the bound fraction for mass spectrometry analysis. One potential problem with this approach could be columns being overwhelmed by soluble, un-aggregated ASC and thus not being enriched for actual inflammasomes. Most immune cells present in the brain during infection will contain ASC even though it is not involved in inflammasome formation. Another potential problem is the aggregates of interest being too big for column purification. ASC may also be able to nucleate aggregation of proteins outside of the inflammasome platform, and thus in homogenized tissue it may be exposed to binding partners that are not actually relevant *in vivo*.

I think that understanding which inflammasome sensor(s) is being activated in microglia in the brain during infection is valuable, not only because it will provide further insight into the mechanism of IL-1α release, but also because it will lead us closer to uncovering what is being sensed in the brain to activate the inflammasome. I think that what this signal is, and whether it is parasite- or host-derived is an important and interesting question.

6.3 Cell Death

We have shown that pyroptosis, or at least elements of the pyroptotic machinery, is involved in the release of inflammatory mediators that promote inflammation during *T. gondii*

infection. We have also begun to study how necroptosis and apoptosis may be involved in the response to *T. gondii* infection, potentially through elimination of a niche for parasite survival and/or replication.

This work has demonstrated that mice lacking RIPK3 have a modest increase in parasite burden in the brain and a notable increase in immune cells in the brain, but the mechanism behind both of these phenotypes is unclear. As discussed in chapter 5, it seems likely that the increase in parasite burden is due to a loss of necroptosis rather than death-independent effects of RIPK3 on neuroinflammation. However, to directly answer this question, we could utilize mice lacking MLKL and compare them to mice lacking RIPK3. If the effect on parasite burden is indeed due to a loss of necroptosis, then $MLKL^{-/-}$ mice would display the same phenotype as $RIPK3^{-/-}$ mice. The possibility of necroptosis being physiologically relevant to *T. gondii* infection actually does make sense, since *T. gondii* has been shown to be able to inhibit apoptotic caspases, which is precisely when necroptosis generally becomes important.[278,279] If, however, $MLKL^{-/-}$ mice do not have an increase in parasite burden comparable to $RIPK3^{-/-}$ mice, then we could assess the quality of the inflammatory response. Though we have shown that rather than less inflammation, there are actually more immune cells in the brains of $RIPK3^{-/-}$ mice, we have not evaluated their function. If the wrong types of cells are being recruited, or the immune cells in the brain are not being properly activated or maintained, then there could feasibly be an impairment in parasite control.

The increase in immune cells in $RIPK3^{-/-}$ mice is also interesting. This difference may be something that is systemic and present at baseline, thus we should evaluate the immune compartment of these mice prior to infection. The accumulation of immune cells in the brain could also be due to intrinsic differences in the immune cells that only becomes important in the context of the infection. It is also possible that both of these hypotheses could contribute the phenotype.

One way to determine the importance of necroptosis to the turnover of immune cells in the brain specifically during infection, would be to create bone marrow chimeras. If WT mice that received RIPK3$^{-/-}$ bone marrow had a similar accumulation of immune cells, that would indicate that regardless of systemic accumulation of immune cells through development, RIPK3 in immune cells contributes their population dynamics in the brain during infection. If, on the other hand, the opposite is true and RIPK3$^{-/-}$ mice that received WT bone marrow displayed an increase in immune cells in the brain during infection, that would suggest that necroptosis of non-hematopoietic cells somehow regulates immune cell accumulation in the brain. Of course, it could also be the case that neither chimeric mouse recapitulates the phenotype seen in total RIPK3$^{-/-}$ mice, which would suggest that it is simply the accumulation of immune cells throughout development in these mice that contributes to increased neuroinflammation during infection. Bone marrow chimera studies would also be illuminating for the effect of RIPK3 deficiency on parasite burden.

This work has also uncovered that caspase-8$^{-/-}$RIPK3$^{-/-}$ mice have severely impaired parasite control. This could mean that extrinsic apoptosis is important for controlling infection. It must be considered that caspase-8$^{-/-}$RIPK3$^{-/-}$ mice have been shown in one study to have an impaired response early in infection in the peritoneal cavity,[276] so it is possible that the increase in parasite burden is due to increased seeding of the brain following poor control during acute infection. However, in that study, the caspase-8$^{-/-}$RIPK3$^{-/-}$ mice were reported to succumb to infection by two weeks[276], a dramatic phenotype which we do not see, as in our hands they survive to chronic infection. Thus, I think that it is worth fully investigating the phenotype seen in these mice. We should analyze the peripheral immune response during acute infection to see if we also observe defects. What will be probably even more important is measurement of parasite burden peripherally during acute infection, to see if early increases could account for the phenotype seen

in the brain. If severe defects are not seen acutely, then study of a brain-specific phenomenon can proceed. But if severe defects are seen early on, there are still ways that we could study the role of caspase-8 during chronic infection. In the published study, the problems in early infection were reported to be due to a lack of IL-12 in the caspase-$8^{-/-}$RIPK$3^{-/-}$ mice.[276] So one thing that could be done is to treat mice with exogenous IL-12 until chronic infection (or even continually through chronic infection) to eliminate that variable.

If we find caspase-8 deficiency does cause bona fide loss of parasite control in the brain during chronic infection, then we will want to determine how this occurs. As discussed in chapter 5, there are many cell types which could be dependent on apoptosis to limit parasite. One way to address this would be create bone marrow chimeras. As described with RIPK$3^{-/-}$ mice, this would show the contribution of immune cells and non-immune cells to apoptosis-dependent parasite control. Another approach would be to identify the cells harboring the excess cysts and free parasites in caspase-$8^{-/-}$RIPK$3^{-/-}$ mice. I have tried to identify the cell types harboring cysts in brain sections of these mice, but it is technically difficult. To achieve this, we could make use of cre-expressing parasites that can be used to drive fluorescent labelling of infected host cells.[247]

Another interesting question these studies could lead to is what signals are normally leading to apoptosis in the infected brain. Because we are examining a caspase-8-dependent pathway, this would be extrinsic apoptosis, which is usually initiated by signaling through death receptors. I think that TNF is a good candidate for involvement in this pathway. TNF-α is expressed in the brain during *T. gondii* infection, so it is plausible that it could be important (Fig. 6.4). Additionally, once again looking at our RNA-seq data, TNF receptor signaling pathways are highly expressed in macrophages in the brain during infection, in contrast to Fas, which could also trigger apoptosis, but which is less highly expressed, at least in macrophage populations (Fig. 6.5). An interesting

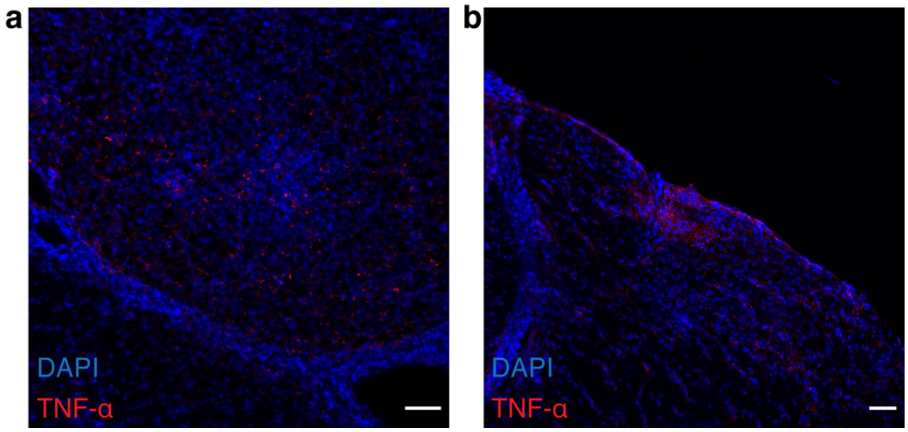


Figure 6.4 TNF staining in *T. gondii*-infected brains

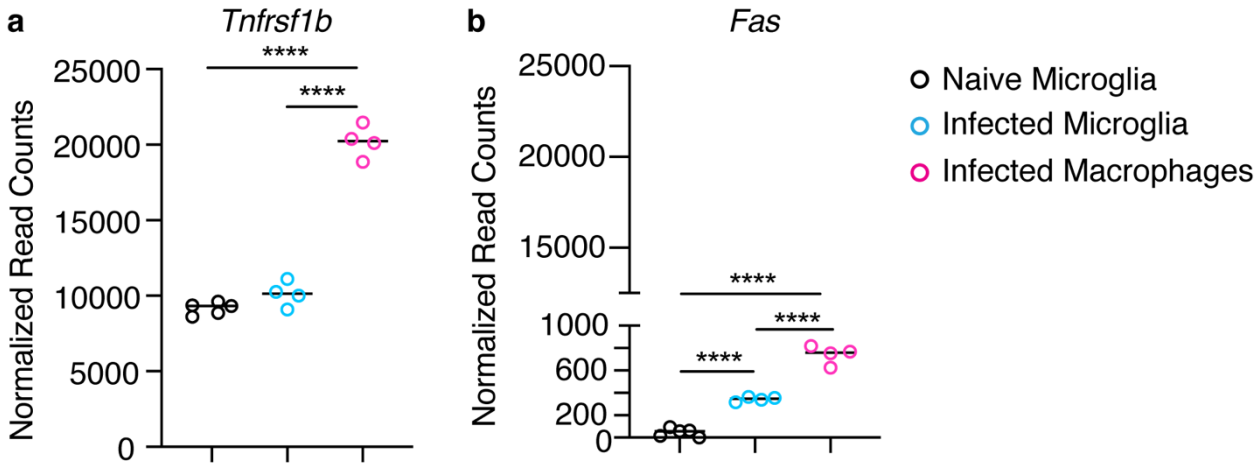


Figure 6.5 Death receptor pathway expression

experiment to perform would be to block TNF during chronic infection and to see if the effects of such a blockade are similar to caspase-8 deficiency.

I am also interested in determining whether in caspase-$8^{-/-}$RIPK$3^{-/-}$ mice there would be more necrosis occurring. I plan to address this by purifying cells from the brains of infected WT and caspase-$8^{-/-}$RIPK$3^{-/-}$ mice, and culturing them briefly *ex vivo*. I will then measure LDH release into the supernatant to assess levels of cell death. If there is increased necrosis in the absence of apoptosis and necroptosis, there would be more LDH released. A shift to necrosis would also likely have consequences for the response to infection *in vivo*.

I have come to appreciate that cell death is a vastly complicated subject and that there is a surprising amount of interplay between what are considered to be different mechanisms of cell death. In the world of cell death and recognition of these events, context is everything. I think that understanding what leads to different types of cell death and how different cell types may be fundamentally different in their death pathways is important and will contribute to our ability to manipulate inflammation in clinical settings, including but extending beyond infections to autoimmune and autoinflammatory disorders as well as cancers.

6.4 Alarmin-gly Complicated

This work has shown the role of one single alarmin in the response to *T. gondii* infection in the brain, and the fact that loss of one such molecule has measurable effects on inflammation and on parasite burden is somewhat remarkable. However, the loss of IL-1α leads only to partial impairment of parasite control, indicating it is one of multiple factors performing similar functions. Our lab has also demonstrated similar results with another alarmin IL-33.[252] But there are likely many more of these early innate cytokines that work together to potentiate the proper immune response to *T. gondii* in the brain.

Though it is not technically an alarmin, IL-18 is another IL-1 family cytokine that could be a good candidate for an innate initiator of inflammation. IL-18 has been studied in *in vitro* as well as acute *in vivo* models of *T. gondii* infection. IL-18 has been shown to be released from rat BMDMs *in vitro* in response to *T. gondii* infection, and this was suggested to be dependent on the NLRP1 variant of the rat strain.[241] Using mouse BMDMs, another study was unable to detect IL-18 release following *in vitro* infection.[244] However, they were able to detect IL-18 in the serum of *T. gondii*-infected mice, and found that mice lacking IL-18 were more susceptible to infection and died by about 10 dpi.[244] This suggests a role for this cytokine in controlling the infection during acute stages, but it is unknown if it would play a role in the brain during chronic infection. It is possible that we would not be able to study IL-18 in chronic infection using knockout mice because the previous paper reported that they do not survive past acute infection. However, many other strains reported in that paper to display early mortality, survive to chronic infection in our hands, including IL-1R$^{-/-}$, capsase-1/11$^{-/-}$, and NLRP3$^{-/-}$ mice. Therefore, I think it would be worth trying the preliminary experiments using IL-18$^{-/-}$ mice to see if there is any effect on parasite control or inflammation in the brain. According to our RNA-seq data, IL-18 is not highly expressed by microglia or infiltrating macrophages in the infected brain (Fig. 6.6a). However, according to the brain RNA-seq database from Stanford, the highest expression of *Il18* in the brain is seen in astrocytes, though it is important to note that this data set is taken from developmental samples.[51] Therefore, if IL-18 seems to contribute to the response to chronic infection, its source and which cells respond to it would be interesting questions to investigate. However, I have preliminary data showing that *Il18* expression from whole brain homogenate does not increase very much in infected samples compared to naïve (Fig. 6.7A). *Il1a* levels in the same samples are shown for comparison (Fig. 6.7B). This could suggest that there are low levels of IL-18 that do not increase

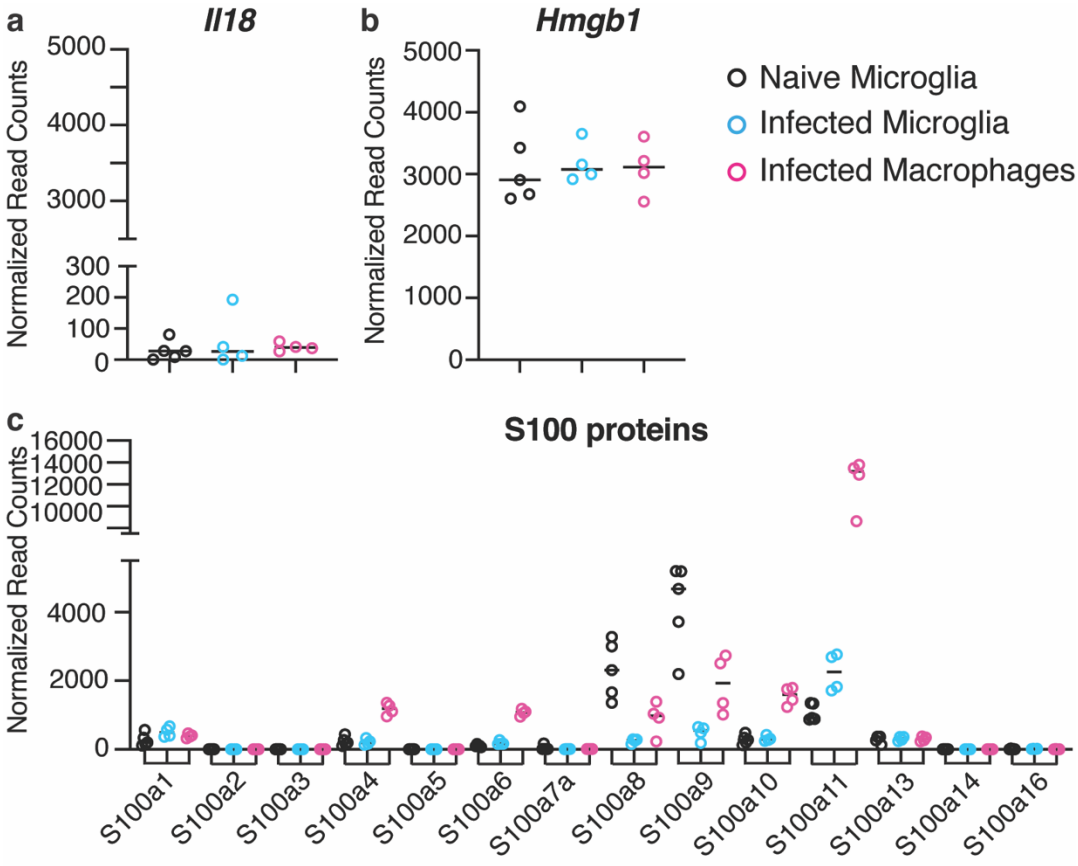


Figure 6.6 Normalized read counts of alarmins from RNA-seq data

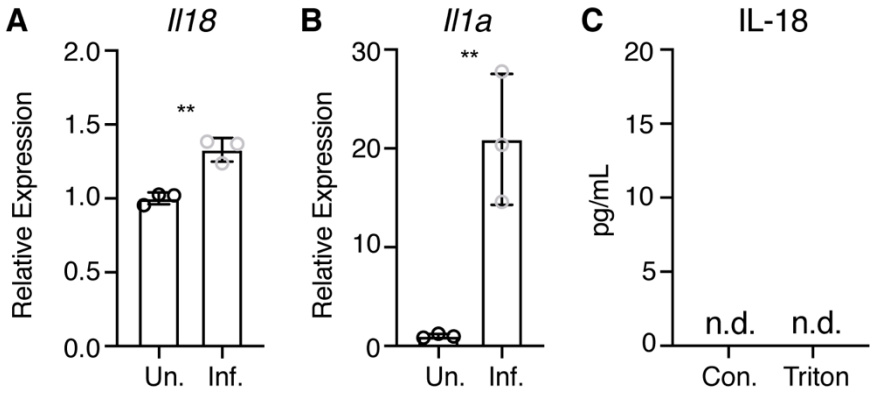


Figure 6.7 Little expression changes and no release of IL-18 in chronically infected brains

much after infection, or that there are significant levels of expression to begin with that is not changed by infection. A more illuminating result is that isolated cells from infected brain samples did not release detectable IL-18 *ex vivo* (Fig. 6.7C). This means that whether or not IL-18 is present in cells, it is not being released from these cells, as we know IL-1α is. Further, treating these samples with a triton-containing lysis buffer also did not lead to detectable IL-18 release (Fig. 6.7C), perhaps suggesting that it is not present in cells in the infected brain to begin with.

HMGB1 (high mobility group box-1) is a canonical alarmin that could be interesting to investigate. According to our sequencing data, it is expressed in the brain both prior to, and during, infection by microglia and macrophages, and to a much higher extent than IL-18 (Fig. 6.6b). According to the Stanford database, *Hmgb1* is expressed highly by many cell types in the brain,[51] therefore there are many possibilities for it to be released. HMGB1 is a nuclear, chromatin-associated protein.[280-282] HMGB1 is fairly ubiquitously expressed and is released from cells upon necrotic death promoting inflammation, though it can also be actively released from immune (or even non-immune) cells through non-canonical secretory pathways.[280,283-285] Cells can be induced to release HMGB1 by stimulation with cytokines, cellular stressors, or recognition of PAMPs.[280,285-289] HMGB1 has specifically been shown to be released in response to treatment with cytokines such as IFN-γ, TNF-α, and IL-1, all of which we know are present in the brain during chronic *T. gondii* infection, not to mention possible parasite-induced stress or damage to cells that could potentiate its release. HMGB1 can bind to RAGE (receptor for advanced glycation end products) and stimulate activation of NF-κB.[280,290-293] HMGB1 has also been shown to induce signaling downstream of multiple TLRs.[294,295] An HMGB1-TLR4 pathway has been shown to promote MHCI expression in a model of experimental autoimmune myositis.[296] HMGB1 has also been shown to facilitate intracellular localization of LPS via internalization through RAGE.[133]

Whether or not it could function as a delivery system for other pathogen-derived molecules is an interesting question. In addition to its effects on immune cell populations, HMGB1 has also been shown to promote inflammation by activating endothelial cells.[297,298]

Another family of canonical alarmins are the S100 proteins. Our RNA-sequencing data does show expression in microglia or macrophages of some of these proteins, most notably S100A11 and S100A8/A9 (Fig. 6.6c). Because of the ubiquitous nature of these proteins, they are likely expressed by many other cell types in the brain during infection as well. S100A11 and S100A8/A9 would probably be the members of this family to begin investigating, as well as S100B due to its demonstrated role in brain damage.[299-301] At baseline, S100 proteins are important intracellularly for maintaining Ca^{2+} homeostasis.[299,302] The expression of these proteins is dependent on cell type as well as stimuli.[299,302] S100 proteins form either homo- or heterodimers, which is necessary for their functions within a cell as well as their signaling extracellularly.[299] Like HMGB1, S100 proteins can either be released passively during necrotic cell death, or actively through a non-canonical mechanism.[299,303] Extracellularly, S100 proteins can bind to a variety of cell surface receptors, including RAGE as well as TLR4.[299,301,304-312] It is important to note that post-translational modifications can change the function of these proteins, so expression will not be the whole story.[299,313,314] S100A8/A9 specifically has been shown to contribute to inflammation, and often harmful inflammation, in multiple models. These molecules have been studied in models of colorectal, gastric, and prostate cancers, particularly acting through activation of NF-κB.[299,306,308,309,315] S100A8/A9 has also been implicated in worsening endotoxic shock.[311] This heterodimer has also been implicated in rheumatoid arthritis, as well as a biomarker of severity in juvenile rheumatoid arthritis.[303,316] S100A8/A9 has also been studied in the skin, specifically in psoriasis.[317] Notably, S100A11 has been demonstrated to promote CCL2-dependent monocyte

recruitment into the peritoneal cavity during acute *T. gondii* infection, thus it would be interesting to asses a potential role for this alarmin in the brain.[318]

There are many alarmins and early innate cytokines, particularly those associated with cell death and damage, that could contribute to the immune response to *T. gondii* in the brain. We have begun to illuminate single pathways that contribute to immunity, but there is likely a coordinated, multi-pronged "alarmin" response to infection in the brain that is yet to be fully understood.

6.5 Microglia vs Macs

Through RNA-sequencing analysis, our work has begun to illuminate differences between microglia and infiltrating macrophages in the brain in the context of infection. Our microglia reporter mouse model has allowed us to distinguish between these two populations which is otherwise difficult during inflammation due to morphological and phenotypic similarities. In addition to differences in IL-1 expression, the RNA-seq revealed differences in NF-κB expression. These differences were very striking and have interesting implications for the functions of these cells in an inflammatory response. Thus far, we have evaluated expression of Rel and RelA in microglia and macrophages, and have found that they are largely absent in microglia. However, there are other NF-κB transcription factors that we have not yet investigated. It would be a worthwhile effort to uncover exactly which factors may or may not be active in these cells. I think it would also be important to isolate and culture these two cell populations, with various stimuli, to asses not only expression, but nuclear translocation of these transcription factors which is ultimately necessary for their function.

We also do not know what is regulating expression of such transcription factors in microglia. Because the infiltrating macrophages are localized in the same areas as the microglia in the tissue, I think it is less likely to be a difference in local signals, and is more likely to be a

function of the origin of these cells. There are some transcription factors that seem to define microglial identity, such as *Sall1*. Expression of certain inflammatory genes, including NF-κB transcription factors, may be inhibited by transcriptional repression mediated by *Sall1* or another such factor. This may be difficult to test, but a good place to start might be to assess the chromatin landscape surrounding these areas, and to look for *Sall1* binding sites. It is also important to note that the expression patterns we observe in our infection model may not apply to other models of neuroinflammation, as these things may be very context-specific.

Our investigation into IL-1 expression and release has led us to interesting questions about microglial propensity to cell death. We observed that both microglia and infiltrating macrophages express IL-1α, but we only observed release of this cytokine from microglia. Because we have evidence that IL-1α release in our model is dependent on gasdermin D, this implies that microglia undergo pyroptosis and infiltrating macrophages in this model do not. We do not yet understand why this is the case, especially because bone marrow-derived cells are well-defined as being able to undergo pyroptosis following certain stimuli. One possibility is that microglia, but not infiltrating macrophages, become infected during a reactivation event and undergo pyroptosis after intracellular sensing of the parasite. If this is the case, this must be a rare or very transient event because it is difficult to observe in chronically infected tissue sections, a situation not aided by the high density of cells in areas of parasite reactivation. Another possibility is that both microglia and infiltrating cells receive the same signals, either direct infection or environmental cues, but that something about the microglia identity skews them toward pyroptosis while the macrophages skew toward either survival or non-pyroptotic death. Both of these possibilities could potentially be tested *in vitro*, by assessing inflammasome activation, death, and cytokine release in the two populations following infection or other stimulation; assuming that microglia could retain their

unique identity long enough for study. It is also possible that this response by microglia is not specific to our infection model, but could represent a general property of activated microglia: that they are just more likely to die by pyroptosis when they do die. This is something that could be tested using other models of inflammation and *ex vivo* analysis of microglia.

6.6 Modulating Neuroinflammation: *Toxoplasma* and Beyond

Our work has shed light on mechanisms of initiating and maintaining an immune response in the brain. In the case of *T. gondii* infection, maintained immune pressure is necessary for controlling the infection, so a tolerable level of neuroinflammation is a good thing. It is interesting to note, however, that even though our studies have shown that lacking the alarmin IL-1α results in decreased neuroinflammation and increased parasite burden, the increase in cysts in the brain is not enormous, and is tolerated by the mice. This may indicate that though this pathway does promote control of the parasite in the brain, its absence may alleviate some of the inflammation without leading to a fatal increase in parasite, which could ultimately be beneficial. But this would require further investigation to confirm.

Importantly, in other infectious and non-infectious circumstances, neuroinflammation is pathological and contributes to disease severity. Thus, understanding the underlying processes involved in promoting neuroinflammation may be useful in designing therapeutics to ameliorate this inflammation in other contexts, such as in MS (multiple sclerosis). MS is a debilitating disease characterized by neurodegeneration following demyelination events, which also features pathological inflammation of the CNS. Genetic studies of MS patients versus controls have shown that many risk alleles are associated with immune-related genes, including the genes encoding the IL-2 receptor, the IL-7 receptor, and the HLA locus.[319] Therapies that have been considered as treatments for MS also highlight the importance of inflammation to disease progression. For

example, clinical studies of the benefits of recombinant IFN-β treatment have shown that the clinical improvements correlate with increased CSF levels of IL-10 as well as fewer immune cells present in the CSF, suggesting an anti-inflammatory effect of treatment.[320,321] A monoclonal antibody against CD52, which targets lymphocytes, was also shown to have some efficacy in limiting relapses and disability in MS patients.[322] Additionally, there is the fascinating story of natalizumab, a monoclonal antibody targeting α4β1 integrin, which allows for immune cell entry into the CNS. Natalizumab was shown to be effective in treating MS, and was approved but then quickly removed from the market due to cases of progressive multifocal leukoencephalopathy (PML).[323] This could potentially indicate that complete loss of immune cell trafficking to the CNS, though effective at limiting inflammation-driven MS symptoms, also allows for the reactivation of latent CNS infections. Thus, therapies that take advantage of pathways, like IL-1 signaling, that contribute to immune infiltration but are more finely tuned than blocking adhesion molecules, could be valuable.

Our work has also illuminated differences between brain-resident microglia and brain-infiltrating monocytes/macrophages during infection. Analyzing these cells from the same microenvironment shows cell type-specific functions; uncovering what microglia are poised to do and, importantly, what microglia seem unable to do. Understanding the functions and limitations of this CNS-resident cell type could be important for modulating neuroinflammation therapeutically. There could be instances in which it would be beneficial to inhibit the activity of microglia or, alternatively, instances in which it would be beneficial to harness the function of blood-derived cells within the CNS.

Understanding mechanisms of cell death and their relationship to neuroinflammation is also very important. Cells die in the brain during development, during homeostasis, and definitely

during disease. Defining how and why certain contexts lead to specific types of death could provide therapeutic insights. The response to cell death also affects the balance of inflammation and tissue homeostasis and repair. Thus, these pathways may be good targets to modulate the course of infections, developmental disorders, neurodegenerative diseases, and cancers.

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