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Local and Peripheral Cues Driving Heterogeneous Populations of Brain-Infiltrating T Cells During Chronic *Toxoplasma gondii* Infection

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Tyler Landrith

September 2017

Dissertation Committee:
Dr. Emma H. Wilson, Chairperson
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The Dissertation of Tyler Landrith is approved:

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Chapter 1

Landrith TA et al (2015) “Characteristics and critical function of CD8+ T cells in the Toxoplasma-infected brain.” *Seminars in Immunopathology*. 37(3):261-70

Chapter 2

Landrith TA et al (2017) “CD103+ CD8 T cells in the Toxoplasma-infected brain exhibit a tissue-resident memory transcriptional profile.” *Frontiers in Immunology*. doi: 10.3389/fimmu.2017.00335

DEDICATION

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ABSTRACT OF THE DISSERTATION

Local and Peripheral Cues Driving Heterogeneous Populations of Brain-Infiltrating T Cells During Chronic *Toxoplasma gondii* Infection
by
Tyler Landrith

Doctor of Philosophy, Graduate Program in Biomedical Sciences
University of California, Riverside, September 2017
Dr. Emma H. Wilson, Chairperson

T cells form a fundamental component of the protective response to chronic *Toxoplasma gondii* infection. The dire consequences of a failed T cell response in the protective response to this parasite are particularly apparent in AIDS patients, yet there must be a highly coordinated balance to the T cell response in the CNS. First, there must be a balance between pro-inflammatory and anti-inflammatory responses. Next, in a context of persistent antigen there must be a balance between the function of memory and effector subsets that simultaneously occupy the same niche. Finally, the T cell response must adapt to local conditions within the tissue. This dissertation explores these phenomena in the following ways:

In Chapter 1, we discuss what is known about the T cell response to *Toxoplasma gondii* infection. We explore the signals that determine infiltration into the brain, behavior within the brain, and phenotypic diversity. In Chapter 2, we discuss the role of tissue resident memory T cells in the protective response to chronic *Toxoplasma gondii*

infection. Here we use RNA-Seq to determine that the distinct transcriptional profile of brain infiltrating CD103+ CD8 T cells is consistent with tissue resident memory T cells, or T_{RM}. We then use re-stimulation assays to establish that this population has a significantly increased capacity to produce pro-inflammatory cytokines as compared to other populations within the brain. This data supports the presence of a functional population of memory T cells in the response to chronic *Toxoplasma gondii* infection.

In Chapter 3, we examine the requirement of lymph node homing dendritic cells expressing CCR7 for maintenance of effector T cells within the brain. This supports the requirement for ongoing recruitment of brain-infiltrating effector T cells. In Chapter 4 we examine how glutamate dysregulation within the CNS during chronic *T. gondii* infection impacts cytokine production and proliferation in activated T cells. We conclude in Chapter 5 by examining the common themes that emerge from the three primary chapters of the dissertation.

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CHAPTER ONE

Introduction

1.1 Characteristics and critical function of CD8+ T cells in the *Toxoplasma*-infected brain

1.1.2 Abstract

The rise of the AIDS epidemic made the requirement for T cells in our continuous protection from pathogens critically apparent. The striking frequency with which AIDS patients exhibited profound neurological pathologies brought attention to many chronic infections that are latent within the immune-privileged CNS. One of the most common lethal opportunistic infections of these patients was with the protozoan parasite, *Toxoplasma gondii*. Reactivation of *Toxoplasma* cysts within the brain causes massive tissue destruction evidenced as multiple ring-enhancing lesions on MRI and is called toxoplasmic encephalitis (TE). TE is not limited to AIDS patients, but rather is a risk for all severely immunocompromised patients, including recipients of chemotherapy or transplant recipients. The lessons learned from these patient populations are supported by T cell depletion studies in mice. Such experiments have demonstrated that CD4+ and CD8+ T cells are required for protection against TE. Although it is clear that these T cell subsets work synergistically to fight infection, much evidence has been generated that suggests CD8+ T cells play a dominant role in protection during chronic toxoplasmosis. In other models of CNS inflammation, such as intracerebral infection with LCMV and

experimental autoimmune encephalomyelitis (EAE), infiltration of T cells into the brain is harmful and even fatal. In the brain of the immunocompetent host, the well-regulated T cell response to *T. gondii* is therefore an ideal model to understand a controlled inflammatory response to CNS infection. This chapter will examine our current understanding of CD8+ T cells in the CNS during *T. gondii* infection in regards to the (1) mechanisms governing entry into the brain, (2) cues that dictate behavior within the brain, and (3) the functional and phenotypic properties exhibited by these cells.

1.1.3 Introduction

Toxoplasma gondii is an obligate protozoan parasite that can replicate within a wide variety of cell types [1]. The tremendous success of this pathogen rests, in part, in its ability to transition to a cyst form and persist for the lifetime of the host. The effectiveness of our immune response in controlling *T. gondii* is evidenced by the fact that despite a global seroprevalence of about 30 %, symptomatic disease is a rare event [2]. During the AIDS epidemic, individuals with TE revealed not only the consequences of a suppressed immune response, but also revealed the brain as an important site of infection. *Toxoplasma*-induced pathology in these patients nearly always occurs in the CNS, though retinitis is another significant pathology [3, 4]. This points at two possibilities. One possibility, supported by several animal studies, suggests that the brain is a primary site of infection and there is continuous requirement for T cells at this site to prevent reactivation of latent cysts [5–8]. On the other hand, the brain is not the only site of infection and the parasite can be found in other tissues such as muscle. Thus, the

second possibility is that reactivation at these sites or newly acquired infections lead to parasite dissemination to the brain [9]. However, regardless of the scenario, it is clear that to prevent pathology in the CNS, T cell trafficking and migration into the infected brain is critical.

The mouse, a natural host for *Toxoplasma*, has provided an excellent system for studying the immune response to this parasite. Using luciferase-expressing parasites the dissemination and location of the parasite can be tracked in a live mouse over time [8]. Such experiments alongside traditional histological techniques demonstrate that during chronic infection *Toxoplasma* is localized to the brain, where it is observed primarily in the neurons of the frontal cortex [10, 7, 11, 12]. Infiltration into the brain by the immune system is often harmful and the brain is uniquely adapted to regulate this process (for review, see [13]). During chronic *Toxoplasma* infection, dendritic cells, macrophages, NK cells, as well as both T and B cells have all been reported in the brain [14, 15]. The specific role for each cell population is an ongoing area of inquiry, but mouse studies conducted from the late 1980s to early 1990s demonstrated the absolute requirement for T cells and the cytokine IFN-γ to prevent parasite reactivation [5, 6]. These studies showed that mice treated with IFN-γ depleting antibodies displayed pathology indicative of parasite reactivation, including areas of neural necrosis and the presence of free tachyzoites [5, 6]. These cytokine depleting studies were followed not long after by T cell depletion studies demonstrating 100 % mortality rate when mice were treated simultaneously with anti-CD4 and anti-CD8 antibodies [6]. The crucial requirement for T

cells in resistance to *T. gondii* in the CNS has shaped research on TE for over 20 years. Importantly, depletion of CD4+ T cells alone revealed no effect on mortality, in contrast to an observed 50 % mortality upon depletion of CD8+ T cells alone. This suggests that although CD4+ and CD8+ T cells work synergistically to control infection, CD8+ T cells are critical for protection. Supporting this conclusion, resistance in the mouse, maps to the gene encoding the CD8-restricted MHC class I molecule, H-2Ld. Thus, BALB/c mice that express H-2Ld are relatively resistant to chronic toxoplasmosis in comparison to the C57BL/6 mouse which lacks this gene and exhibits higher levels of cyst and tachyzoite numbers, along with inflammation and cytokine production [16, 17].

There have been significant advances in our understanding of CD8+ T cells in the context of chronic toxoplasmosis, but many questions still remain. This chapter will examine areas of ongoing research in three broad categories: entry of CD8+ T cells across the blood-brain barrier and into the brain parenchyma, their behavior and migration once within the tissue, and finally their phenotype and effector capacities for controlling chronic infection. In the majority of CNS inflammatory models, chronic T cell infiltration to the brain is highly pathological [13]. During murine infections with *T. gondii*, millions of T cells can be harvested from the brain, while the mice appear indistinguishable in terms of sickness behavior from their naïve counterparts. This suggests that the immune response to *Toxoplasma* in the CNS is governed by distinct mechanisms that distinguish it from a lethal CNS infection model such as cerebral malaria or LCMV [18, 19]. Thus,

understanding the T cell response to *Toxoplasma* infection in the CNS will provide a greater understanding and new insights into the complex immune responses at this site.

1.1.4 Entry into the brain

Although the brain is highly vascularized, the blood-brain barrier (BBB) limits immune cell infiltration. Tight junctions connect the endothelial cells of the capillaries in the brain. This, along with astrocytic endfeet surrounding the vasculature, allows for highly controlled entry of peripheral molecules and immune cells under normal conditions. Arrays of chemokine receptors, selectins, and integrins have been implicated in T cell entry in other models of CNS inflammation [13]. This includes the requirement of adhesion molecules to slow and facilitate rolling of T cells on the endothelium at the blood/brain interface. PECAM-1, ICAM-1, and VCAM-1 are cellular adhesion molecules that are constitutively expressed on the endothelial cells of the brain vasculature. The upregulation of these molecules in the brain during *T. gondii* infection has been observed as early as the acute stage of infection (days 9–14) [20–22]; implying that their expression precedes parasite or immune cell infiltration of the CNS. Increased expression of VCAM-1 is, at least partially, dependent on IFN-γ signaling [22]; however, the driving force behind the regulation of endothelial cell expression of multiple adhesion molecules and whether these signals are locally derived or due to systemic circulating cytokines is unknown.

One of the ligands of VCAM-1 is the integrin α4β1 (VLA- 4), which is expressed on activated T cells [23]. Adhesion molecules play a central role in the extravasation of T cells into inflamed tissues [13]. The importance of VLA-4 in T cell recruitment to the brain was first observed in the mouse model of multiple sclerosis (MS), experimental autoimmune encephalitis (EAE) [24]. Indeed, antibody-mediated blockade of VLA-4 is the molecular basis of the MS therapeutic natulizumab [25]. During Toxoplasma infection, treatment of chronically infected mice with anti-VLA-4 antibodies, thereby blocking VLA-4/VCAM-1 interactions, inhibits the recruitment of antigen-specific activated CD8+ T cells into the brain and leads to a significant increase in parasite burden [26]. A similar requirement for VLA-4 on CD8+ T cells is seen in a model of *T. gondii* infection that mimics ongoing reactivation [27]. Together, these studies have demonstrated the importance of VLA-4 in the recruitment of T cells to the brain during *T. gondii* infection.

The specific roles of PECAM-1 and ICAM-1 remain to be studied, although LFA-1/ICAM-1 interactions have been implicated in both T cell and dendritic cell recruitment to the brain [15, 27]. In addition to these molecules, PSLG-1 and ALCAM have recently been linked to CD8+ T cell entry in other models of CNS inflammation and perhaps are relevant to TE [20, 28–30].

Once cells have slowed and crossed the endothelium, the cells reach the perivascular space. Thus, specific signals that draw cells from this space into the brain parenchyma are

of interest. CCR7 signaling could provide necessary signals for T cells to migrate into the brain parenchyma from the perivascular space. In spontaneous autoimmunity, CCR7 is involved in T cell infiltration of peripheral tissues, which has in part guided interest in the role of this chemokine receptor in brain entry during chronic toxoplasmosis [31, 32]. In the Toxoplasma-infected brain, the CCR7 ligands, CCL21 and CCL19, are upregulated during the chronic stage. Despite this, CCL21 appears to be uniquely required for CD4+, and not CD8+, T cells to efficiently migrate into the brain parenchyma. Chronically infected plt mice (mice lacking expression of CCL19 and CCL21) have a significantly higher proportion of CD4+ T cells in the perivascular space compared to wild type [33, 26]. CD8+ T cells do not seem to share this requirement, although CCR7 may play a role in CD8+ T cell migration within the brain.

In addition to CCR7, CXCR3 is a chemokine receptor that has been associated with T cell responses during a variety of neuroinflammatory conditions [34–36]. During West Nile Virus infection, CXCL10 expressed by neurons in the brain parenchyma specifically regulates T cell entry to the CNS [37, 38]. Studies have demonstrated a similar upregulation of CXCR3 ligands in microglia and astrocytes [39, 40] within the brain and CXCR3 expression on invading T cells during chronic toxoplasmosis [41]. Treatment with anti-CXCL10 antibodies, leads to a significant reduction (~40 %) in CD8+ T cells in the infected brain [41]. This strongly indicates that in contrast to CCR7, CXCR3 is necessary to maintain the CD8+ T cell population in the CNS. Importantly, one limitation of this data is that it does not definitively establish whether CXCR3 is required for entry

from the perivascular space and/or meninges into the parenchyma or is needed for the retention of these cells. Intravital imaging allows for T cells to be visualized as they cross the BBB and thus will be critical for addressing the key steps involved in the infiltration of T cells into the CNS during TE.

In addition to the requirement for VLA-4 and CXCR3, studies in injury and infectious models of brain inflammation suggest that CD8+ T cell accumulation is antigen-dependent [18, 42]. The requirement for antigen-specific interactions during Toxoplasma infection was tested using parasites expressing the model antigen ovalbumin. Infection with parasites that secrete OVA protein led to the accumulation of OVA-specific CD8+ T cells in the infected brain [26].

However, activation of OVA-specific T cells in vitro or in vivo using OVA protein did not lead to CD8+ T cell accumulation in the brains of mice that were not infected with OVA-secreting parasites. Thus, similar to the LCMV model, where blockade of MHC class I inhibited T cell contacts with meningeal vasculature, this data provides compelling evidence for antigen-specific interactions in generating a CD8+ T cell population within the brain [18]. However, LCMV- specific CD8+ T cells have been shown to enter the CNS during chronic toxoplasmosis, but these cells do not persist, offering support for antigen specificity as a condition for retention and/or survival rather than for entry into the parenchyma [41]. Further studies in the LCMV model shows that antigen presentation to CD8+ T cells leads to their local proliferation in the brain [43]. During Toxoplasma infection, T cell proliferation has also been observed within the brain parenchyma [26].

As this was a rare event, it is clearly not the primary cause of the expansion of the CD8+ T cell population in the CNS. These differences between a controlled protective CD8+ response to Toxoplasma and a pathological one during viral encephalitis may point to the amount of antigen and the degree to which it is presented as an important limiting factor for CD8 expansion in the brain.

In addition to the LCMV model of viral encephalitis, during EAE, there is evidence that T cells interact with meningeal and/or vessel-associated phagocytes and this interaction presumably regulates entry, retention, and/or survival [43–45]. The identity of cells responsible for presenting Toxoplasma antigen at this site remains unknown. Imaging studies have revealed that OVA-specific CD8+ T cells interact with CD11c-expressing cells in the CNS [15]. In addition, numerous APCs, including macrophages and dendritic cells are recruited to the CNS during infection. In the absence of inflammation, the expression of MHC class I in the CNS is minimal, but is upregulated following infection [46]. Furthermore, it is possible that the astrocytes forming the glia limitans could present parasite antigens at this site [47, 48].

A compelling model for CD8+ T cell entry into the brain during *T. gondii* infection posits a three-step process where VLA-4 allows slowing of T cells within the blood vessel, adherence to endothelium and extravasation into the perivascular space. Perivascular APCs ensure retention within the CNS, and expression of CXCL9/10 by microglia and astrocytes and CXCR3 by CD8+ T cells regulates entry into the parenchyma (Fig. 1.1A).

However this model is incomplete and requires further investigation. For example, knockout and depletion methods for these targets do not completely abolish entry into the brain; therefore it is likely that additional molecules are involved. In addition, detailed two-photon microscopy experiments in conjunction with traditional flow cytometric and histological techniques will be required to determine if these signals are required for migration across the BBB or retention of cells once within the infected brain.

1.1.5 Behavior within the brain

Once cells reach the brain parenchyma, less is known about the signals they receive that may direct their behavior and their ability to control infection. T cells could be directed by infection-induced cues or behave using a random search strategy [41]. The ability to monitor the behavior of T cells in live tissue using multi-photon microscopy has dramatically changed the field of immunology, allowing the CD8+ T cell response to *Toxoplasma* to be observed in real time. Imaging of *Toxoplasma* infection in the lymph nodes revealed rapid and significant changes in neutrophil and T cell behavior within the lymph nodes [49, 50]. The first study to look at CD8+ T cells in the CNS during chronic *Toxoplasma* infection in the brain revealed a highly heterogeneous population of cells [26], some of which seemed to interact with cysts and each other. In terms of general migration patterns, the average velocity, displacement, and meandering index of a transferred population of CD8 T cells varied over the course of chronic infection. These parameters were examined in a transferred population of antigen-specific CD8+GFP+ T cells and found that the average velocity of migrating cells ranged between 4 and 8

μm/min with some reaching up to 25 μm/min, with peak velocities reached between 1 and 2 weeks post transfer [26]. Such speeds are similar to those measured in CD8+ T cells in naïve lymph nodes. As discussed in this study, cells exhibited behaviors ranging and transitioning between constrained to motile migration patterns. Cells also clustered, proliferated, and exhibited repetitive or circling behaviors [26]. A further study imaging interactions of T cells, parasites and antigen presenting cells suggest that CD8+ T cells made very few close contacts with intact cysts or CNS resident cells, however, formed significant clusters with CD11b+ cells especially in areas of free parasites. The reduced cell velocity at these sites supports the concept that these are areas containing parasite antigen that is being presented to CD8+ T cells. In chronic toxoplasmosis, there is an extensive network of dendritic cells recruited to the brain, along with activated resident microglia [15]. The role for infiltrating dendritic cells has not been defined, but their association with both the parasite and CD8+ T cells does suggest they may play a role in the cytotoxic response. One possibility is that these dendritic cells serve as a source of soluble chemokine that guides migration; however, their potential role as APC's will be discussed in the following section. In addition to dendritic cells, there are multiple potential sources of chemokines in the brain including neurons, microglia, astrocytes, and infiltrating dendritic cells (Fig. 1.1B). All of these cell populations have been shown to express both chemokines and/or their receptors during CNS inflammation [51, 52].

Of the chemokines that are upregulated during infection, CCL21 appears to be present in fibers that were detected by immunofluorescent staining [26]. Importantly, this in situ staining also reveals that CD8+ cells associate with these fibers. Second harmonic signals

as seen by two-photon microscopy imaging have revealed a fibrous network upon which CD8+ T cells appear to migrate. Although it has not been definitively established, these networks could represent the same linear structures as seen by CCL21 staining. These structures, their regulation and whether or not chemokines actively bind to such extracellular networks are ongoing areas of study; however, such presentation of chemotactic molecules may provide efficient migratory signals to T cells in the CNS. Such roles for chemokine signaling within the infected brain parenchyma have now been established for the CXCR3/CXCL10 system [41]. In addition to its role in recruitment or retention of effector CD8+ T cells, CXCL10 is required to increase the speed of CD8 T cell migration within the brain [41]. CD8+ T cells in mice treated with anti- CXCL10 antibodies migrated at a significantly slower velocity than their untreated counterparts [41]. Blocking all Gαi-coupled receptor signaling with pertussis toxin further reduced the average T cell velocity. These data suggest two things; firstly, that although CXCL10 contributes to T cell velocity, other chemokines are also involved to maintain optimal speed. Secondly, non-chemokine (Gαi- independent) signaling also contributes to T cell migration in the CNS. Rigorous statistical analysis in these studies also revealed that CD8+ T cell migration does not appear to be directed over the observed timescales, suggesting that chemokine gradients may only be present over very short distances [41]. In addition, in contrast to preliminary studies examining the migration patterns of T cells in the lymph node, CD8+ T cell migration in the brain was not well described by a simple random walk or Brownian motion. Instead, a generalized Lévy walk (GLW) described the T cell migration pattern. In this model, CD8+ T cells alternate between runs and

pauses and the length of the runs and pauses is random and drawn from a Lévy distribution. Thus, the movements and pauses by T cells are typically short, with rare long runs and pauses. Surprisingly, CD8+ T cells maintained the GLW behavior in the absence of CXCL10 and chemokine signals, suggesting that chemokines increase the speed, but not the directionality or pattern of migration. Moreover, mathematical modeling of CD8+ T cell migration suggested that chemokines are import for the control of *T. gondii* in the brain, as they shorten the distance that T cells must travel before encountering parasites. This study also raises the question of whether this pattern of migration is characteristic of all T cells. Recent analysis of CD8+ T cells in the uninflamed lymph node revealed that cells do not exhibit GLW behavior [53] suggesting that the activation status of the CD8 T cells during infection or the brain environment may influence the GLW behavior.

Despite the fact that there is no evidence that CD8+ T cells within the brain in the chronic toxoplasmosis model are guided by chemokine gradients or towards an antigenic target in vivo, more research is required to investigate the role of additional chemokines and chemokine receptors. It is likely that these molecules contribute to a protective immune response in diverse ways, as may be expected for the control of a complex infection.

1.1.6 Phenotypic and functional characteristics

Previous work has made clear that CD8+ T cells play a crucial role in control of chronic toxoplasmosis through the actions of perforin and IFN-γ [5, 6, 54–56]. In support of the

H-2Ld data, adoptive transfer of the CD8+ subset of a major IFN-γ- producing population, TCR variant Vβ8, conferred resistance to chronic infection in nude mice [16, 54, 56]. In addition to the requirement for IFN-γ, perforin is required for the control of cyst burden [57, 55]. Importantly, perforin is sufficient for control of cyst burden in IFN-γ−/− mice, suggesting that al- though IFN-γ is required for protection against reactivation, control of cyst burden is uniquely the function of perforin secreted by CD8+ T cells [16]. Nonetheless, there are several details that remain unanswered. For example, the targets of perforin and IFN-γ remain unknown and furthermore, CD8+ T cells rarely directly associate with a cyst [58].

Perhaps the most crucial question regarding CD8 T cells during chronic toxoplasmosis is how they exert effector functions to control infection. Early studies suggested that CD8+ T cells confer protection through perforin secretion [57]. Perforin knockout (PKO) mice succumbed to chronic infection earlier than their wild-type counterparts and exhibited significantly increased parasite burden. However, PKO mice that were vaccinated with ts4 strain parasites were still resistant to challenge with the virulent RH strain. This suggests that perforin is uniquely required for control of cyst burden within the brain (Fig. 1.1C).

More recently, it has been shown that when CD8 T cells from chronically infected BALB/c mice are transferred to their SCID counterparts, there was a significant reduction in cyst burden compared to pre-transfer levels [55]. Importantly, this was still seen in

mice receiving CD8 T cells from IFN-γ−/− donors, suggesting that IFN-γ is not the primary mediator of cyst burden control. Furthermore, recipients given CD8 T cells from PKO mice showed equivalent cyst burdens to pre-transfer numbers [55]. This is compelling evidence suggesting that during chronic infection perforin mediated cell lysis is one of the crucial mediators for the control of cyst burden and parasite reactivation [59]. A recurrent question underlying the discussion of each aspect of the CD8 response during chronic infection is antigen source. It is not currently clear if parasite antigen escapes from infected cells into the brain parenchyma and the periphery or if CD8+ T cells recognize reactivating cysts. The use of parasite-specific MHC I tetramers has provided an important piece to this puzzle by demonstrating that ROP7, but not GRA-4 antigen-specific CD8+ T cells are present in the brain during the chronic stage [60]. ROP7 is expressed by both the bradyzoite and tachyzoites, whereas GRA4 is uniquely expressed by tachyzoites. This supports the possibility that CD8+ T cells are responding to both bradyzoites and tachyzoites within the brain, which is supportive of continuous antigen exposure and presentation.

Live imaging of infiltrating DCs within the infected brain parenchyma demonstrated that these cells both associate with the parasite and maintain prolonged contacts with CD8+ T cells, certainly supporting the idea that DCs are acting as local APCs [15]. Perhaps DCs process and present secreted antigen from infected neurons to CD8+ T cells via cross presentation, although this is by no means the only possibility (Fig. 1.1D). An elegant study in acute toxoplasmosis using a combination of mCherry labeled parasite reporter

and a pH sensitive dye showed that the proportion of dendritic cells directly infected by the parasite were roughly comparable to the proportion that had taken up and processed parasite antigen by phagocytosis [61]. This suggests that during chronic infection, a population of infiltrating dendritic cells could be infected with tachyzoites from reactivating cysts. In this case CD8+ T cells could recognize antigen via the endogenous MHC I pathway, versus cross presentation.

Related to antigen presentation and targeting is the question of where CD8+ T cell priming occurs and whether continuous recruitment/turnover of CD8+ T cells occurs. CD8+ T cells isolated from the brain during chronic toxoplasmosis express high levels of CD44 and low levels of CD62L, characteristic of an activated phenotype [62]. Furthermore, T cells from the brain fail to take up BrdU upon restimulation, suggesting that continuous recruitment may be required to replenish this population [62]. If this is true, then it remains to be defined whether priming occurs in the periphery or perhaps through the actions of perivascular APCs in the CNS. Combining imaging methodologies used for documenting cell migration with readouts of cell function, i.e., NFAT translocation reporter [44], will prove highly useful to visualize these interactions during chronic toxoplasmosis.

Regardless of the mechanism by which this critical CD8+ T cell population recognizes antigen within the parenchyma and exert their effector functions, the response appears dampened or impaired. Notably, T cells in the *Toxoplasma*-infected brain exhibit an

exhausted phenotype that has been observed in other chronic infections [63]. The polyfunctional nature of a single CD8+ T cell means it can exert a variety of effector responses against infected cells, for example, secretion of granzyme B, IFN-γ, IL-2, and TNF-α [64]. In an exhaustion phenotype, this polyfunctionality is lost. Receptors such as PD-1 are upregulated in exhausted CD8+ T cells as a result of persistent engagement of the TCR with antigen [65]. During chronic toxoplasmosis, a proportion of CD8+ T cells in the brain express PD-1 along with reduced proliferative capacity and reduced expression of effector molecules such as IFN-γ and granzyme B [26, 66]. Although this can be temporarily recovered either through blockade of PD-1 or transfer of CD8+ T cells from acute infection, ultimately this exhaustion phenotype persists. [67]. Taken together, this suggests that the CD8+ T cells during chronic infection can acquire progressive loss of effector function that perhaps contributes to the persistence of the parasite within the brain parenchyma.

T cell exhaustion can be considered a direct result of the persistent nature of chronic toxoplasmosis. This persistence also means that the role of memory populations in controlling the parasite, along with the signals that maintain such a population, is currently unknown. As discussed, the T cells present during chronic toxoplasmosis display unique phenotypes comparable to other models of chronic infection, suggesting that they are functionally distinct from the T cells responding to cleared infections [68, 26, 65]. Memory T cells have been described in the CNS, including immune surveillance that occurs in the human CSF predominantly by a circulating central memory phenotype

(CD4+CCR7+) [36]. The discovery of brain resident memory CD8+ T cells in the context of viral infection suggests a potential role for memory in the form of parenchymal surveillance [69].

Regarding the signals that maintain memory CD8+ T cells, the classical view is that IL-7R is expressed by both memory and naïve T cells and helps maintain long-term survival in a quiescent state [70, 71]. The chronic nature of *T. gondii* infection suggests that the signals that maintain memory T cells, as well as the nature of immunological memory itself, may differ from infections that are cleared. Mice immunized with an attenuated strain of *Toxoplasma*, ts4 and treated with IL-15 (a ligand for IL-7R), then challenged with the PLK strain of the parasite showed enhanced protection relative to their untreated counterparts [72]. However, a subsequent study revealed that following vaccination with the ts-4 strain IL-15 is not necessary for protection during a virulent challenge [73]. As ts4 parasites are cleared, this is not reflective of the environment of the chronically inflamed brain, where there is persistent antigen. Additionally, IL-7 may compensate for the function of IL-15. In response to this, IL-15−/− mice were treated with IL-7 depleting antibodies. This study revealed a defect in the ability of CD8+ T cells with a memory phenotype to develop [74]. This suggests that IL-7R is required for the generation of memory CD8+ T cells during chronic toxoplasmosis; however, the role for memory populations in conferring protection at this stage is unknown. Furthermore, the requirement for IL-7R during chronic infection to maintain an effective CD8+ T cell population within the brain has not been tested.

Indeed, it is possible that IL-7R is not required for maintenance of memory populations in the *Toxoplasma*-infected brain at all. Studies of chronic viral infection suggest that the memory CD8+ T cells generated in this context become dependent on their cognate antigen for long-term survival in- stead of IL-15 or IL-7 [68]. The network of dendritic cells that has been discussed in previous sections could support this hypothesis. However in addition to dendritic cells, CD8+ T cells could encounter antigen presented by microglia, astrocytes, and neurons, as all these cell populations express MHC I (Table 1.1) [75, 47, 15]. In other models of brain inflammation, astrocytes have been shown to form synapses with CD8+ T cells as well as to serve as a source of cytokines, and thus they form a compelling candidate to play multiple roles at the site of infection [47, 48]. Experiments similar to those done in acute LCMV infection, visualizing CD8+ T cell interaction with APCs and subsequent activation as measured by proliferation could be useful to establish the targets of CD8+ T cells within the brain. Additionally, studies confirming the survival signals and roles of memory CD8+ T cells during chronic toxoplasmosis have yet to be conducted. The specific role that CD8+ T cell memory populations play in control of any chronic infection has been largely unexplored and *T. gondii* may prove a useful model to answer this question.

1.1.7 Conclusion

As a model of chronic inflammation in the CNS, *T. gondii* infection can both inform and be informed by other models of CNS inflammation. The evidence examined in this

chapter suggests that the CD8+ T cell response at the chronic stage of toxoplasmosis is essential to control of infection, yet there are many questions that remain to be explored. CD8+ T cells are required for control of toxoplasmosis, yet they can exhibit an exhausted phenotype indicating suppression of function. Although blockade of PD-1 can temporarily restore CD8+ T cell function, permanent recovery has not yet been achieved. It is useful to consider whether recovery will enhance the immune response to the parasite, potentially to the point of clearance, or whether this suppression is actually required along with regulatory cytokines to prevent inflammation-related pathology. A robust CD8+ T cell response is a strong correlate of protection in other diseases, and it seems that this is the case in chronic *T. gondii* infection as well, yet the targets and the signals that enhance target location are not fully understood. The infrequency with which CD8+ T cells are observed to contact cyst-infected neurons suggests their response is predominantly against parasite reactivation. The current evidence suggests that whatever the target, chemokines increase the speed at which CD8+ T cells search for said target. However, without a broader understanding of what CD8+ T cells are responding to and how they find what they are responding to, it is important to be cautious in enhancing CD8+ T cell function and behavior towards a therapeutic end. The context within which these CD8+ T cells operate is important, and may define what is protective in one context and pathogenic in another. The study of the CD8+ T cell response during chronic *Toxoplasma* infection within the brain is clearly a useful and informative tool for understanding the generation of protective immunity in the brain during ongoing

inflammation—relevant to a number of infectious and non-infectious immune responses in the CNS.

1.2 A note on CD4+ T cells

Section 1.1 introduces the foundational concepts for this dissertation but with a distinct bias towards the CD8+ subset. Indeed, the evidence discussed in this section supports a critical role for CD8+ T cells in protection at the chronic stage. Nonetheless it is clear that both the CD8+ and CD4+ subsets work synergistically to protect against cyst reactivation and both can serve as sources of IFN-γ [1]. Therefore the introduction to this dissertation would not be complete without a brief discussion of CD4+ T cells, specifically their heterogeneous roles and phenotypes within the chronically infected brain. In many respects the known mechanisms governing entry of CD4+ T cells into the brain and the cues guiding CD4+ migration and behavior within the brain are not substantially different from CD8+ T cells. One important distinction, which has been mentioned previously, is a requirement for the CCR7 ligands CCL19 and CCL21 in regulating efficient entry of CD4+ T cells into the parenchyma [2]. CD8+ T cells do not seem to share a similar requirement however it is not currently understand why.

Like CD8+ T cells, brain-infiltrating CD4+ T cells exhibit a great deal of phenotypic diversity and coordination between these phenotypes to form a protective response is not fully understood [3]. In a broad sense, this diversity is perhaps more pronounced in the CD4+ subset in the sense that CD4+ T cells can exist in Th1, Th2, Th17, and T_{REG}

polarization states whereas CD8+ T cells play a uniformly cytotoxic role. In the context of *Toxoplasma gondii* infection specifically there is evidence that both Th1 and T_{REG} CD4+ T cells are critical for an ongoing protective response by the immune system [1,4,5]. In addition to the previously mentioned studies in which depletion of the Th1 cytokine IFN-γ results in profound and lethal parasite reactivation, mice lacking the regulatory cytokine IL-10 develop a lethal uncontrolled inflammatory response [4].

Multiphoton microscopy has served as a powerful tool for the documentation of T cell localization and migration in the chronically infected brain [6,7]. The discovery of the meningeal/perivascular restriction of the T_{REG} subset is among these findings [8]. In this context, the restriction of the T_{REG} population to these regions is dependent on the presence of co-localized dendritic cells (CD11c+). It is possible that this contributes to control of inflammation via regulation of activated T cell entry. Regardless, it is clear that along with CD8+ T cells, CD4+ T cells are critical for the coordination of a protective response to the parasite during chronic infection.

1.3 Figures and Legends

Figure 1.1: Signals guiding behavior and function of brain-infiltrating T cells

A) VLA-4 and VCAM-1 interactions are required for recruitment of CD8+ T cells to brain. Whether interaction between CXCR3 and its ligands governs entry into the parenchyma during chronic toxoplasmosis has not been established, but has been observed in West Nile Virus. Astrocytes and microglia appear to be sources of CXCL9 and CXCL10 during chronic toxoplasmosis. B) Upon entry into the brain CXCR3 ligands help increase the velocity of Levy walks. Interaction between CCR7 and ligands may also govern migration within the parenchyma. For example, CCL21 is expressed on fibrous networks that appear in the brain parenchyma at the chronic stage of infection. C) Antigen recognition with the tissue may also govern long-term survival/entry. Microglia, astrocytes, and neurons all express MHC I, but CD8+ T cells have been observed to contact infiltrating DCs during Toxoplasma gondii infection. A percentage of DCs may present antigen through cross presentation, or may be directly infected. D) CD8+ T cells contribute to control of reactivation through the actions of IFN-gamma, and control of cyst burden through perforin mediated cell lysis.

CD8+
A
Vasculature
Perivascular Space
Parenchyma
B
C
perforin
IFN-γ
D

MHC I
VLA-4/VCAM-1
Non-antigen specific TCR
CCL21/CCR7
CXCL9, CXCL10/CXCR3
Antigen specific TCR

Table 1.1: Candidate antigen presenting cells in the *Toxoplasma*-infected brain

Cell Type	Description	References	Disease model
Neurons	CD8+ T cells damage neuronal axons in an MHC I dependent manner in vivo	[75]	EAE
	Global elevation in MHC I expression during infection	[46]	Cerebral LCMV
	Toxoplasma cysts are exclusively observed in neurons	[12]	Chronic *T. gondii*
Astrocytes	CD8+ T cells are capable of forming immunological synapses with astrocytes in vivo	[47, 48]	Adenovirus infection
	mice with astrocytes lacking cytokine receptor gp130 succumb to infection	[76]	Chronic *T. gondii*
Microglia	CD8+ T cells observed in contact with CD11c+ cells in brain parenchyma	[15]	Chronic *T. gondii*
	Microglia first to take up antigen during disease progression	[77]	EAE
Infiltrating/perivascular phagocytes	CD8+ T cells observed in contact with CD11c+ cells in the brain parenchyma	[15]	Chronic *T. gondii*
	MHC I elevated in vasculature of brain	[46]	Cerebral LCMV
	CD8+ T cells interact with perivascular APCs	[45, 44]	EAE
	Perivascular MHC I-dependent proliferation of CD8+ T cells	[43]	Cerebral LCMV

1.4 References

1.4.1 Characteristics and critical function of CD8+ T cells in the Toxoplasma-infected brain

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CHAPTER TWO

The maintenance of T_{RM} in the chronically infected brain and their role in protection: insight from RNA-Seq analysis

2.1 Abstract

During chronic infection memory T cells acquire a unique phenotype and become dependent on different survival signals than those needed for memory T cells generated during an acute infection. The distinction between the role of effector and memory T cells in an environment of persistent antigen remains unclear. Here, in the context of chronic *Toxoplasma gondii* infection we demonstrate that a population of CD8 T cells exhibiting a tissue resident memory (T_{RM}) phenotype accumulates within the brain. We show that this population is distributed throughout the brain in both parenchymal and extraparenchymal spaces. Furthermore, this population is transcriptionally distinct and exhibits a transcriptional signature consistent with the T_{RM} observed in acute viral infections. Finally, we establish that the CD103+ T_{RM} population has an intrinsic capacity to produce both IFN-γ and TNF-α, cytokines critical for parasite control within the central nervous system (CNS). The contribution of this population to pro-inflammatory cytokine production suggests an important role for T_{RM} in protective and ongoing immune responses in the infected CNS.

2.2 Introduction

Persistent infections in the brain present an especially daunting challenge for the immune response due to the unique set of rules governing entry of peripheral cells and molecules into this tissue [2]. Immune surveillance of the brain occurs entirely in the cerebrospinal fluid, and T cell infiltration into the parenchyma only occurs during injury and inflammation [3-5]. During chronic infection with the intracellular protozoan parasite

Toxoplasma gondii, there is a well-regulated protective response within the brain mediated by infiltrating T cells, which are required to prevent fatal reactivation [6]. This continuous recruitment and presence of activated CD4+ and CD8+ T cells into the central nervous system represents the generation of protective immunity for the host, but fails to clear the parasite, which continues to reside as cysts within neurons [6,7]. The basis and composition of the long-term protective T cell response in the brain remains an active area of research.

Although protection could be mediated in part by a continuous pool of effector cells there is also evidence of the involvement of memory T cell subsets [8,2,9,10]. Mice chronically infected with *T. gondii* exhibit protection against challenge with the virulent RH strain of the parasite, which is lethal in naïve mice [11]. Persisting antigen is not required for such protection as infection of mice with attenuated strains of *Toxoplasma gondii* confers protection upon rechallenge [11,12]. This suggests that the memory populations generated during chronic *T. gondii* infection are indeed functional, but it is unclear whether there is a distinction in the protection afforded by effector and memory T cell subsets in an environment of persistent antigen. Several subsets of memory T cells have been established, including central memory, effector memory and tissue resident memory (T_{RM}) cells [13,14]. During chronic infection, memory T cells require unique survival signals [15] and can acquire distinct phenotypes, including an exhausted/attenuated phenotype [16]. In chronic *T. gondii* infection, the recent discovery of a T cell population in an intermediate state (T_{INT}) between memory and effector status provides an important

clue to understanding the coordination of the T cell response in this context [10]. Nevertheless, during chronic infection, the unique role for a memory response as opposed to the effector response remains undefined.

The location of the parasite in the parenchyma of the brain offers a potential role for tissue resident memory cells in protection against parasite reactivation. T_{RM} cells have been implicated in the recruitment of peripheral lymphocytes and dendritic cell activation/maturation via secretion of pro-inflammatory cytokines and chemokines [17,18]. The T_{RM} population is characterized by expression of the activation marker CD69, which in tandem with the suppression of the tissue egress axis KLF2/S1PR1, ensures T_{RM} do not re-circulate and remain localized in the tissue. Although not expressed by all T_{RM}, the expression of the integrin CD103 is a defining marker of tissue residency. Typically, CD103 tethers T_{RM} to epithelial tissues through binding to its ligand E-cadherin [19,20]. This positions these cells optimally for a sensing and alarm function at the site of infection [17,18,21,22], suggesting that this memory T cell subset is critical for a first line protective response to localized infection. Much of the work on T_{RM} has been accomplished by studying acute infection models [23-27] where infection is resolved and antigen cleared. This includes viral infection in the CNS [26] and parasitic challenge in the skin and liver [28,29]. Indeed, memory is frequently defined as persistent cells in the absence of infection. Yet during Toxoplasma infection we observed a significant population of CD103+ cells in the brain. This provoked the question of

whether the expression of CD103 defined a T_{RM} population during chronic infection of the CNS or if it represented transient expression by a more common effector population.

Here we show that a population with a T_{RM} phenotype (CD8+ CD69+ CD103+) exists in the brain during the chronic stage of infection, and such a population is not confined to endothelial tissues but is observed throughout the brain. In our model, expression of CD103 defines a transcriptionally distinct population that is consistent with the established literature on T_{RM} [26,27]. Furthermore, this population has a significantly greater capacity to produce the pro-inflammatory cytokines TNF-α and IFN-γ. Thus, even in the context of continuous antigen exposure, recruitment, and exhaustion of effector cells there exists a population of CD8+ CD103+ T cells that exhibit a transcriptional profile characteristic of T_{RM}. Their generation alone is clearly not sufficient to eliminate a chronic parasitic infection from the brain but may be critical nevertheless for host protective immunity.

Therefore, the presence of a population of T_{RM} during *T. gondii* infection is not only relevant to the immune response against the parasite at this stage, but also to more fundamental questions regarding the role of T_{RM} and other memory subsets during chronic infection where significant antigen persists.

2.3 Materials and Methods

2.3.1 Mice and Parasites

Two type II strains of Toxoplasma were used to allow the quantification of parasite specific T cells and to maximize the ability to see cysts in the brain. First, a strain engineered to secrete ovalbumin (Pru-OVA) [30] was maintained *in vitro* in Human Foreskin Fibroblasts (HFF) grown in complete DMEM (90% DMEM, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin). After infecting HFF's, parasites were grown in D10 media (70% DMEM, 20% M199, 10% Fetal Bovine Serum, 5% Penicillin/Streptomycin, 5% Gentamycin) with chloramphenicol. Parasites were purified for infection by passage through a 22.5-gauge needle followed by passage through a 5.0mm nylon filter. After centrifugation at ~2000xg for 10 minutes at 4°C, parasite were counted and resuspended in an appropriate volume of 1xPBS. 10,000 tachyzoites were intraperitoneally (*i.p.*) injected in 200µl of PBS. Second, the Me49 strain of parasites was maintained in CBA mice. For infections, brains were harvested from chronically infected mice and homogenized in 3mL of PBS by needle passage. After counting, cysts were resuspended in an appropriate volume of PBS to infect *i.p.* at 20 cysts per mouse in a 200µl volume. C57Bl/6 and CBA mice were obtained from the Jackson Laboratory (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). Mice were maintained in a pathogen free environment under IACUC established protocols at the University of California, Riverside.

2.3.2 Flow cytometry

Prior to harvest, mice were intracardially perfused with 20mL of ice-cold PBS, with perfusion confirmed by the white appearance of the brain and lack of red blood cells during tissue processing. Mononuclear cells were isolated from the brain by mincing and subsequent homogenization via passage through an 18-gauge needle in complete RPMI (86% RPMI, 10% FBS, 1% Pen/Strep, 1% L-glutamine, 1% NEAA, 1% Sodium pyruvate, <0.01% β-mercaptoethanol). The resulting suspension was incubated at 37°C with 3mg DNAse and 100μg collagenase for 1 hour and 45 minutes. After incubation, the suspension was passed through a 70μm strainer and mononuclear cells were isolated using a density gradient spun at 2000rpm for 25 minutes with no brakes. The density gradient consisted of a 60% Percoll solution in cRPMI overlayed with a 30% Percoll solution in PBS. Brain mononuclear cells (BMNCs) were isolated from the interphase, counted and washed in FACS buffer, blocked for 10 min in F_c block (BD biosciences) then incubated with a panel of antibodies against CD3, CD8, CD4, CD69, and CD103 (eBioscience) for 30 min protected from light. Samples were then washed and fixed in 4% PFA. Dextramer staining was performed as follows: Prior to surface staining, samples were incubated with ovalbumin-specific (SIINKFEKL) MHC I dextramer (Immudex) at room temperature for 45 min, protected from light. The finished samples were resuspended in FACS buffer and analyzed using a FACS Canto from BD Biosciences. Flow cytometry analyses were performed using FlowJo 10.1 and statistical analyses were performed using Prism 6.

For sorting of cell populations for RNAseq BMNCs were isolated as described above. Splenocytes were isolated as follows: spleens were homogenized in a 40μm strainer using the blunt end of a 3mL syringe. The homogenate was washed with cRPMI and red blood cells were lysed using ACK buffer from Lonza. Cells were then washed and counted. CD8+ T cells were isolated from spleen and BMNCs using negative selection columns from R&D systems. The purified CD8+ T cells were then incubated with anti-CD103 antibodies according to the protocol described above. Cells were suspended at a concentration of $3x10^6$ cells/mL, and then sorted using a FACS Aria from BD Biosciences. Cell sorting was performed at the Institute for Integrative Genome Biology at UC Riverside.

2.3.3 In Situ immunofluorescent staining

Immediately following excision, sagitally bisected brain tissue was flash-frozen in a bath of isopentane cooled with dry ice. Frozen organs were then put into a standard Tissue-Tek cryomold, filled with Optimal Cutting Temperature (OCT) solution (also from Tissue-Tek), put on dry ice, and subsequently stored at -80°C. Serial sections of 12μm were prepared on a standard Cryostat machine (LEICA/CM1850). For *in situ* immunofluorescent staining, frozen tissue sections were fixed in 75% acetone/25% ethanol then blocked for 10 min in 10% donkey serum prior to staining. For the primary antibody incubation conjugated anti mouse CD8 and CD103 were added to three panels containing either anti-mouse biotinylated E-cadherin, anti-mouse laminin, or anti-*Toxoplasma gondii*. Antibodies against CD8, CD103, and E-cadherin were from

ebioscience, antibodies against laminin from Cedarlane Laboratories and anti-*Toxoplasma* from Abcam. The sections were incubated for 3 hours at room temperature. After washing, the sections were incubated for 1 hour at room temperature with the appropriate secondary antibodies (streptavidin for anti E-cadherin). Samples were mounted in Prolong Gold with DAPI (Invitrogen) for nuclear counterstaining. Images were collected on either a Leica DMI 6000B epifluorescent or a Leica SP5 scanning confocal microscope (Leica Optics), and data was analyzed and quantified using Volocity 6.1 (Perkin-Elmer).

2.3.4 RNA-Seq Analysis

In order to obtain sufficient RNA for analysis, CD8 T cells were isolated from pooled leukocytes of brain and spleen of at least n=5 mice. This was repeated to obtain 4 trials to serve as biological replicates in analysis. The isolated CD8 T cells were sorted according to expression of CD103 and RNA was extracted from sorted groups using an RNeasy Mini Kit from Qiagen. Multiplexed cDNA libraries were generated, which included brain CD103+, brain CD103-, spleen CD103+ and spleen CD103- CD8 T cells. Of the 4 trials, libraries of sufficient quality for sequencing could not be generated for 2 brain CD103+ samples and 1 brain CD103- sample. RNA concentration and quality was analyzed by running an RNA Nano chip on an Agilent 2100 Bioanalyzer. mRNA was enriched using a RiboGone Mammalian kit from Clontech. cDNA libraries were generated using a SMARTer stranded RNA-Seq kit, also from Clontech. Concentration and quality of libraries was measured with a High Sensitivity DNA chip (Agilent). The resulting

libraries were multiplexed and single end 50bp sequencing was performed at the UC Riverside Genomics Core facility using the Illumina HiSeq 2500. After demultiplexing and QC of the resulting FASTQ files, alignment and differential gene expression analysis was performed with the *SystemPipeR* workflow (https://github.com/tgirke/systemPipeR) [31]. Quality control and trimming was conducted using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and TrimGalore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) respectively. The reads were then aligned to the mouse genome using Tophat2 [32] and transcripts per gene were counted using *GenomicRanges* package in R [33]. Differential gene expression (DEG) analysis was performed following TMM normalization using *edgeR* [34,31]. MetaCore software (Thompson Reuters) was used for functional enrichment analysis. To conduct functional enrichment analysis, only DEGs with a fold change > 2, and FDR < 5% were used. We further excluded genes with very low counts by only selecting gene with a mean RPKM > 1. RPKM (Reads per Kilobase of transcript per Million mapped reads) served as a normalized value for read count and was obtained via *GenomicRanges*.

2.3.5 Microarray analysis

Microarray data from Wakim et al. and MacKay et al. was obtained from the NCBI Gene Expression Omnibus database under accession number GSE39152 [27] and GSE47045 [23]. Differential gene expression analysis was conducted using GEO2R. Genes with a corrected p value < 0.05 were used for subsequent analyses, including functional enrichment analysis. For the Wakim et al. data, differential gene expression analysis was

conducted between brain CD8+ CD103+ (n=5) and brain CD8+ CD103- (n=3) samples. For MacKay et al. data, differential gene expression analysis was conducted between skin T_{RM} (n=3) and spleen T_{CM} plus spleen T_{EM} (n=6) samples.

2.3.6 Restimulation assay

BMNCs were collected from mice by 5 weeks post infection and incubated for 6 hours in complete T cell media (86% DMEM, 10% FBS, 1% Pen/Strep, 1% L-glutamine, 1% NEAA, 1% Sodium pyruvate, <0.01% β-mercaptoethanol) with 10μg/mL BFA. Cells were treated with 10μg/mL αCD3/αCD28 (BD biosciences) antibody or media alone. After restimulation, cells were collected and surface staining was performed as described above. Intracellular cytokine staining was performed with the FoxP3/transcription factor staining buffer kit from ebioscience. Antibodies against IFN-γ and TNF-α were obtained from ebioscience. Flow cytometry analyses were performed using FlowJo 10.1 and statistical analyses were performed using Prism 6.

2.4 Results

2.4.1 Kinetics and Distribution of CD103+ CD8 T cells in the Toxoplasma-infected Brain

T_{RM} are defined as a persisting population of T cells that remain localized to the tissue, that is, are non-recirculating. To determine if such a population exists in the brain during chronic *T. gondii* infection, we investigated whether a stable T_{RM} phenotype could be observed across the course of infection and whether such a phenotype would localize to

the parenchyma. Therefore, we enumerated the distribution and kinetics of CD8 T cells in the brain expressing the residency markers CD103 and CD69. There are no T cells in a naïve uninfected brain [2,35], however by two weeks post infection, representing the late acute stage, there is a small proportion (2±0.4%) of CD8 T cells exhibiting a CD103+ CD69+ phenotype. This population significantly increases ($p<0.0001$) over the course of infection and at the late chronic stage (12 weeks post infection) 38±1% of CD8+ T cells were observed to be positive for CD103 and CD69 (Fig 2.1A, B). Thus, by the late stages of chronic infection, this phenotype forms a substantial percentage of the CD8 subset in the brain relative to all other phenotypes contained in the CD103- group. In the periphery, CD103+ cells are observed to accumulate in the spleen and lymph node over time, however such cells did not express CD69 in significant proportions (Fig S1) and are more transcriptionally consistent with a quiescent/naïve population (Fig S2) [36]. In contrast, the brain has no population of CD103+ CD8 T cells that do not express CD69 (Fig. 2.1A) and therefore the use of this marker for subsequent analysis was redundant.

Previous work has suggested that the majority of T cells in the brain are specific for the parasite and there is little evidence of a bystander population [37]. However, to rule out the possibility that CD103 expressing cells are not directly responding to parasite infection, we used parasites engineered to express ovalbumin along with ovalbumin-specific (SIINKFEKL) MHC I dextramer [30,37] to test the antigen specificity of this population. At 12 weeks post infection, approximately, 3% of CD8+ cells in the brain are bound by the SIINFEKL dextramer, a significant proportion of which express CD103

(Fig 2.1C). This data does not definitively establish that all CD103+ CD8 T cells in the brain are parasite-specific, nor does it indicate the full repertoire of parasite antigens to which CD103+ CD8 T cells could respond. Nevertheless, it does support the view that brain CD103+ CD8 T cells are not simply a bystander population, and is consistent with previous work demonstrating that retention of T cell populations in the brain requires cognate antigen at this site (Fig S7) [37,38].

To determine whether CD103+ CD8 T cells localize to distinct environments within the brain, we used *in situ* immunofluorescent staining. Expression of CD103 by CD8 T cells is observed at entry points to the brain, including the choroid plexus, where it associates with the CD103 ligand E-cadherin (Fig 2.2A). In addition, this population can be found within the perivascular space (Fig 2.2B) and within the parenchyma, despite little expression of E-cadherin at these sites [39]. Finally, this population can be observed in proximity to cysts and individual parasites (Fig 2.2C). Quantification of the distance of CD103+ cells from cysts was highly variable (80-160μm) and there was no correlation between cyst and CD103+ cell number (Fig S3).

Together these data indicate that a population of CD103+ CD8 T cells can be observed throughout the chronic stage and form a substantial proportion of the CD8 subset in the infected CNS. *In situ* immunofluorescent staining revealed that CD103 expression is not confined to a certain area of the brain or specifically localized to regions of infection. Our observation that CD103+ CD8 T cells are present within the perivascular space suggests

this phenotype may be acquired before entry into the parenchyma. Together, these data raised the possibility that CD103 did not define a homogenous population of T cells within the brain but rather a functionally diverse set of cells having in common expression of CD103.

2.4.2 CD103+ CD8 T cells exhibit a Distinct Transcriptional Profile relative to Other CD8 T cells in the Brain and Periphery

The heterogeneity of infiltrating T cells during chronic infection [40,41,9,37] required we exclude the possibility of CD103 expression masking a group of functionally and phenotypically diverse cells that are not T_{RM}. Furthermore expression of CD103 is not the limiting phenotype for T_{RM}, that is, a memory T cell can be resident (non-recirculating) without expressing CD103 [13]. To address whether CD103 defines a transcriptionally distinct population within the brain that is uniquely characteristic of T_{RM}, RNA-Seq analysis was performed on sorted cells from infected mice at four weeks post infection.

Three comparisons between CD8+ subsets were carried out (Fig 2.3A). The first comparison, brain CD103+ to brain CD103-, specified whether CD103 defined a transcriptionally distinct population within the brain. The second, brain CD103+ to spleen CD103+, specified whether CD103 defined a transcriptionally distinct CD8 T cell population when compared to a putatively naïve peripheral population of the same phenotype. Finally the brain CD103+ to spleen CD103- comparison specified whether CD103 defined a transcriptionally distinct population of CD8 T cells when compared to a

putatively activated peripheral population. The comparison of brain CD103+ to spleen CD103- yielded the greatest number of DEGs (2350) (Fig 2.3A). This provided preliminary evidence that the brain CD103+ CD8 T cell group is transcriptionally dissimilar to both local and peripheral CD8 T cells. To confirm this data, principal component analysis was performed to determine the variance in RPKM across all groups (Fig 2.3B). The resulting plots demonstrate that the brain CD103+, brain CD103-, spleen CD103+, and spleen CD103- populations are transcriptionally distinct from each other. PC1 appears to define the organ, spleen versus brain, whereas PC2 appears to define CD103 expression. PC1 had a loading of 12.90 and PC2 had a loading of 14.14. The most variable gene in PC1 was transthyretin (*Ttr*), whereas the most variable gene in PC2 was *S1pr5* (Fig S4). These data lend support to CD103 expression defining a distinct and homogenous population of CD8 T cells within the chronically infected brain.

2.4.3 CD103+ CD8 T cells exhibit a Distinct Transcriptional Profile relative to Brain CD103− CD8 T cells that is consistent with T_{RM}

Next, DEGs were analyzed between CD103+ CD8 T cells and CD103- CD8 T cells within the brain. Functional enrichment analysis revealed that the DEGs enriched to GO terms such as cell migration, proliferation and differentiation (Fig 2.4A, B). These processes are critical aspects of T cell response to infection, T cell signaling, and activation. Additionally, these DEGs had significant over-representation of notable disease terms – "RNA Virus Infection" (Figs 2.4A, C) and "Arteriosclerosis (Figs 2.4A, D). In support of the view that this is a memory population, the terminal differentiation

marker *Klrg1,* expression of which defines terminally differentiated effector cells, is downregulated in the brain CD103+ population (Fold Change (FC)= -3.5) whereas *Il-7r*, a receptor that defines memory cells is upregulated (FC= 2.1) (Fig 2.4C, E). Furthermore, consistent with tissue residency, is the downregulation of *Klf2* (FC= -2.5) and *S1pr1* (FC= -4.4) in the brain CD103+ population (Fig 2.4B-E), inhibiting recirculation of cells back to lymphoid organs [21]. To validate differential expression, flow cytometry of brain mononuclear cells was conducted. This confirms downregulation of both S1PR1 and KLRG1 at the protein level in CD103+ CD8+ T cells (Fig. 2.4F). The percentage of CD103- S1PR1+ CD8 T cells is ~3-fold higher that of the CD103+ S1PR1+ subset (0.88 versus 3.32%) (Fig 2.4F). 37.50% of CD103- CD8 T cells were KLRG1+ whereas 1.05% of CD103+ CD8 T cells were KLRG1+ (Fig 2.4F)

Additional changes of interest in the brain CD103+ population included upregulation of the co-inhibitory receptor *Ctla4* (FC= 2.14) and the transcription factor *Irf4* (FC= 2.1) and downregulation of the transcription factor *Atf3* (FC= -2.6) (Fig 2.4C-E). *Ctla4* is among the first co-inhibitory receptors to be expressed and could provide evidence of increased antigen engagement in this subset [42]. Although several co-inhibitory receptors were upregulated in the brain CD103+ group compared to the spleen CD103+ group, including *Pdcd1* (FC=77.7), *Lag3* (FC= 9.2), and *Tigit* (FC= 27.7) (not shown), only *Ctla4* was uniquely upregulated relative to other CD8 T cells in the brain.

A set of genes associated with extracellular adhesion and migration were upregulated in the brain CD103+ population, including *Cdh1* (E-cadherin, FC= 2.9), *Adam8* (FC= 2.3), *Swap70* (FC= 2.8), *Sema6d* (FC= 3.7), and *Lpar3* (FC= 13.9) (Fig 2.4B, E). Excluding *Sema6d,* these are novel upregulated genes in T cells [43-46]. Both *Sema6d* and the E-cadherin gene were also upregulated relative to peripheral spleen CD8 T cells (both CD103- and CD103+), supporting the possibility that these genes are unique determinants of residency for the brain CD103+ population in our model (Fig S5). This was also the case for the downregulated genes *S1pr1* and *Klf2* (Fig S5).

2.4.4 T_{RM} from the Toxoplasma-infected Brain share a core set of Differentially expressed genes with T_{RM} from Vesicular stomatitis Virus (VSV)

The original studies of T_{RM} in the brain by Wakim and colleagues included microarray analysis to generate a transcriptional profile of T_{RM} in a Vesicular stomatitis virus (VSV) model of acute and resolved brain infection [27]. We compared DEGs from this model to the RNA-Seq data generated in our chronic model, focusing specifically on brain CD103+ CD8 T cells relative to brain CD103- CD8 T cells. The results of our Venn analysis reveal a set of 27 genes (~5%) common to both transcriptional profiles (Fig 2.5A). *S1pr1*, *Klf2*, and *Adam8* are contained within this set, suggesting similar mechanisms of residency between the two models (Fig 2.5B). *Swap70* and *Eomes* are also represented in this group. A remaining 70 genes are unique to VSV, and a remaining 393 genes are unique to *Toxoplasma gondii*. The top enrichment terms for these sets of genes were “immune system process” and “cell adhesion,” respectively (Fig 2.5C-D).

When our data was compared to data from skin T_{RM} generated from HSV infection [23], we observed a similar set of 33 differentially expressed genes, comprising 4.1% of the total DEGs (Fig S6). Although the majority of differentially genes in each model were unique to the model, a core profile of genes associated with tissue residency was common to all models (Fig 2.5, Fig S6).

2.4.5 CD103+ CD8 T cells produce a significantly greater percentage of pro-inflammatory cytokines

Several cytokines and receptors including *Tgfb3*, *Il1a*, *Il1b*, *Il22* and *Csf1r* are downregulated in brain CD103+ CD8 T cells relative to brain CD103- CD8+ T cells (Fig 2.4B-D). The TGF-β receptor was upregulated (FC=2.75) in brain CD103+ CD8 T cells, consistent with previous reports of the requirement for TGF-β in generation of this population [47,48]. Notably, the pro-inflammatory cytokine *Tnf* is significantly upregulated in CD103+ CD8 T cells (FC=2.5 relative to brain CD103-) (Fig 2.4B-D, S5). T_{RM} are reported to produce this cytokine although their ability to do so relative to other CD8 T cell subsets has not been previously validated via flow cytometry [18,27,23].

To validate and confirm the increase in *Tnf* gene expression in the CD103 expressing cells, BMNCs from chronically infected mice were restimulated and production of IFN-γ and TNF-α compared between the CD103+ and CD103- subsets. The proportion of CD103+ T cells producing both IFN-γ and TNF-α was significantly greater than cells not expressing CD103 (19.6±5.3% versus 9.3±3.7%; $p<0.01$) (Fig 2.6A, B). Furthermore, of

CD103+ CD8 T cells expressed more total TNF-α and more total IFN-γ on a per cell basis than their CD103- counterparts (For IFN-γ: 3824±787% vs 3208±746 MFI; $p<0.05$) (For TNF-α : 8778±2165% vs 5966±1171 MFI; $p<0.05$) (Fig 2.6C, D). Since both TNF-α and IFN-γ are critical for protection against Toxoplasmic encephalitis [49,6], these data suggest that the CD103+ T_{RM} population contributes to protection within the brain at the chronic stage.

2.5 Discussion

These data support the conclusions that 1) CD103+ CD8 T cells in the brain during chronic *T. gondii* infection are a transcriptionally distinct population, 2) this population exhibits transcriptional signatures characteristic of T_{RM}, and 3) this population has a distinct contribution to the pro-inflammatory protective response to the parasite. Expression of CD103 can define a resident memory T cell, yet the T_{RM} phenotype is not restricted to CD103 expression, nor is CD103 expression exclusive to this subset [48,50,51,13,52]. This is seen in our data with flow cytometry and immunohistochemistry data revealing expression of CD103 by CD4+ T cells and dendritic cells in the Toxoplasma infected brain as well as CD8+ T cells (Fig. 2; Fig. S7 and data not shown)[53,54]. However, our data suggest that within brain CD8+ T cells expression of CD103 does indeed represent a population of T_{RM} cells that are not restricted to the E-cadherin expressing endothelial cells of the choroid plexus but are also found in parenchymal and extraparenchymal spaces, independently of cyst location [39,24]. Previously, effector T cells have been postulated to be essential for control of chronic

infection, although the presence of a T_{RM} population is in keeping with the requirement to continuously keep cyst reactivation in check.

The reason for CD103 defining a resident memory population has not been addressed and perhaps this is even less clear when brain tissue expresses very little of this integrin's primary ligand, E-cadherin [39]. The expression of N-cadherin is highly prevalent in the brain and CD103 has the potential to bind to this cadherin instead (unpublished data). However, our observation that the E-cadherin gene is upregulated in the CD103+ population suggests residency could be mediated by homotypic pairings within the CD103+ population. Alternatively, our data would support the concept that dendritic cells serve as a binding partner for CD103+ CD8 T cells and mediate residency via formation of the immunological synapse. This could occur via the expression of E-cadherin on brain-infiltrating dendritic cells [55] or, given that our brain T_{RM} upregulate *Sema6d*, via binding to plexins on dendritic cells [45,43]. Independent of cell-cell interactions, CD103+ cells also upregulate *Adam8*. This gene encodes for a metalloprotease implicated in cell-matrix adhesion in human PBMCs and may serve as an additional residency determinant in the brain T_{RM} population [43]. In addition, this gene could play a role in facilitating cytokine shedding [56] and thus provide a mechanistic basis for the increased cytokine production in this population.

TGF-β dependent upregulation of CD103 plays a crucial co-stimulatory role in facilitating TCR-mediated cytokine secretion for tumor specific CD8+ T cells [48]. The

role of TGF-β in the generation and maintenance of CD103+ T_{RM} has been previously reported [24]. Our transcriptional data suggest that CD103- CD8+ T cells may serve as a source of TGF-β and this is strengthened by the concurrent upregulation of *Atf3* in this population [57].

Consistent reports show a dependence on dendritic cell interactions for the priming of or the retention of T_{RM}, and our data is supportive of this. [51,58]. This may extend to other models and tissues, particularly in the context of chronic infections. T_{RM} are observed to accumulate in cases of non-specific inflammation, but during latent viral infections, there is speculation that survival of CD103+ CD8 T cell populations within neural tissues is uniquely dependent on antigen presentation [24,23,47]. A direct comparison of acute and chronic mucosal LCMV infection indicates that chronic viral infection can promote the accumulation of CD4+ T_{RM} in non-lymphoid tissues and upregulation of non-lymphoid tissue homing markers such as CXCR4 [59]. This is in agreement with our data demonstrating the accumulation of T_{RM} in the brain over the course of infection. Finally, in a vaccinia virus model of acute skin infection, cognate antigen is not required for recruitment of CD8+ T_{RM} per se, but the local antigen profile influences the degree of accumulation and TCR repertoire of such T_{RM} [60]. Taken together, these, and our data, support local antigen presentation as a cue for long-term maintenance and accumulation of T_{RM}.

In addition to genes involved in residency determination, we also observed differentially expressed genes that suggest mechanisms of motility within the brain for CD103+ CD8 T cells. These include the GPCR *Lpar3* and the guanidine exchange factor *Swap70*, which are upregulated [44,46]. *Swap70* expression, although typically associated with B cells, has also been shown to initiate membrane ruffling in fibroblasts [44]. Although *Lpar3* expression has not been reported in T cells, it has been reported to mediate motility and proliferation in cancer cells and therefore could play an analogous role in tissue resident T cells [46].

Although the transcriptional profile generated during *Toxoplasma gondii* infection is distinct to the transcriptional profile generated during VSV, the differentially expressed genes shared between the two profiles are consistent with a T_{RM} population [27] and suggest that a T_{RM} subset also exists during chronic infection in the CNS. Our study also demonstrates an important role for these cells in producing effector cytokines. Stimulation of CD103+ T cells compared to CD103- led to an increase in IFN-γ and TNF-α production. Although T_{RM} have been reported to produce TNF-α, our results suggest they have a greater capacity to produce this cytokine, compared to non-CD103+ T cells in the brain [18,27]. TNF-α is crucial to prevent progression of toxoplasmic encephalitis (TE) [61,49,62]. Mice treated with anti-TNF-α antibodies succumb to primary infection [49], and mice lacking TNF-α receptors exhibit necrosis of brain tissue due to parasite reactivation [62,61]. More recently, a study enriching T_{RM} in the gut through depletion of circulating T cells demonstrate a critical function of TNF-α in the

recruitment and activation of dendritic cells [18]. This could serve as an alternate/additional mechanism by which T_{RM} are able to participate in the protective response to infection via production of TNF-α and recruitment of innate and/or antigen presenting cells. Although the contribution of this subset to overall TNF-α production in the BMNC compartment is small the significantly increased capacity to produce this cytokine relative to other CD8 T cells suggests it may be optimized to respond in a robust fashion to inflammatory microenvironments within the brain.

The increased production of IFN-γ in brain CD103+ CD8 T cells relative to other CD8 T cells in the brain also points to a role for T_{RM} in protection against TE. This stimulation occurred by 5 weeks of infection. At this point peak recruitment of effector cells has passed and there is now a considerable population of PD-1^{hi} exhausted effector cells that have a reduced capacity for IFN-γ production [63,9,37]. Thus, the role of T_{RM} as a source of cytokine production has important functional implications. IFN-γ is required for the protective response to chronic *T. gondii* infection, serving to activate microglia, astrocytes and macrophages, to control parasite replication while also playing a role in T cell recruitment [6,64-66]. Overall, the CD103+ population has enhanced production of critical effector cytokines and indicates that this subset of CD8+ T cell remains activated in the presence of ongoing inflammation unlike other subsets in the infected brain.

As expected with a T_{RM} phenotype there is downregulation of the transcription factor *Klf2* that promotes retention within the tissue through down-regulation of *S1pr1*. Our data

shows down-regulation of the transcription factor *Klf3* in addition to *Klf2*. KLF3 has the potential to play numerous roles in a resident population. KLF3$^{-/-}$ mice show significantly increased production of galectin-3, a mediator of diverse actions including chemotaxis and inflammation [67]. Thus, the downregulation of KLF3 further supports brain CD103+ T cells being non-circulating or a highly pro-inflammatory population. It is clear that CD103- T cells in the brain up-regulate a potent array of pro- and anti-inflammatory cytokines, therefore it would be overly simplistic to state that CD103+ T cells in the brain are the sole pro-inflammatory population. Nonetheless, the transcriptional data suggests this is a primary role for this subset. Increased production of pro-inflammatory cytokines is consistent with the view that the CD103+ population is a T cell memory population and increased expression of *Adam8* along with the upregulation of *Ctla4* may reflect increased TCR engagement in this subset [56,42]. Indeed, this potentially fits a model where residency is maintained by continued antigen recognition in the brain via dendritic cells or other APCs, and this in turn upregulates cytokine production.

An important distinction between our model and others where T_{RM} have been studied is the persistence of antigen within the brain where such antigen is continuously visible to the immune system. Previously published data using adoptive transfer of OVA specific CD8+ T cells show that these cells only accumulated in the brain when the infection was conducted with OVA-expressing parasites [37]. This along with the data published here indicates that the CD103+ population is parasite specific. Our data is supportive of

previous studies in chronic infection demonstrating exhaustion of the entire brain CD8 T cell population in the form of upregulation of several co-inhibitory receptors (*Ctla4*, *Pdcd1*, *Lag3*, *Tigit*) relative to spleen CD103+ CD8 T cells [37,9,63]. In addition, *Ctla4* is further upregulated in CD103+ CD8 T cells relative to other CD8 T cells in the brain, suggesting an increased engagement with antigen in this subset as alluded to previously.

The transcription factors *Hobit* and *Blimp-1* have recently been reported to be specifically required for the development of tissue resident memory lymphocytes in the skin, gut, liver and kidney [68,69]. Although we did not observe evidence of differential expression for these transcription factors in our dataset, it is possible the formation of tissue resident memory T cells within the brain as well as other neural tissues may follow a divergent developmental pathway as compared to other tissues. This could be especially true in an environment of a continuous inflammation with persistent antigen. In our data we observe upregulation of the transcription factor *Irf4*. Expression of this gene is dependent on the strength of the initial TCR stimulus and is required for sustained proliferation and expansion of CD8 T cells in the context of influenza virus infection [70]. Expression of this transcription factor by the brain CD103+ population could be particularly beneficial in the context of a chronic infection.

Support for the unique heterogeneity and complexity of the T cell population during chronic *T. gondii* infection is provided by the recent characterization of the T_{INT} population [10]. Such cells serve as a pool from which to quickly generate effector T

cells during persistent infection, and serve as additional evidence that chronic infection produces unique T cell phenotypes as compared to acute infections. We envision a model in which a precursor population gives rise to both T_{RM} and T_{INT} independently. Our data suggests T_{RM} may play a significant part in the protective response to the parasite, which would be a distinct role to that of the T_{INT} population.

It is worthwhile to note that we have reported a substantial population of CD103+ CD69- CD8 T cells progressively accumulating in the secondary lymphoid organs that our data suggest are quiescent. Naïve CD8 T cells are capable of expressing CD103 at intermediate levels, but must down-regulate CD103 upon activation [23]. Although there is precedence for T_{RM} in secondary lymphoid organs during LCMV infection, such cells are uniformly CD69+ and do not express CD103 [21]. Therefore, the accumulation of CD103+ CD69- cells in secondary lymphoid tissue most likely represents a naïve population. Nonetheless our data does not exclude the possibility of T_{RM} in other non-lymphoid peripheral organs. In addition to the brain, the parasite can localize to the skeletal muscle and eye during chronic phase of infection [71], and muscle-infiltrating T_{REG} populations are reported to play a critical role in tissue repair at this site [72]. It is possible that T_{RM} can play a similar role during chronic infection at additional sites to the brain.

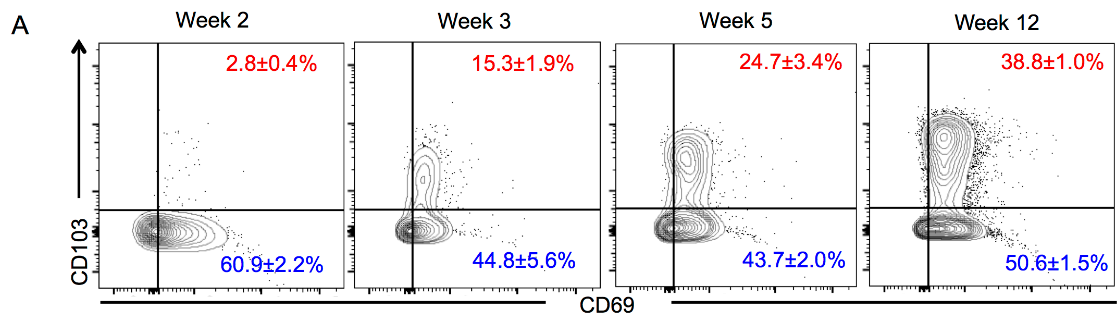
Taken together, these data provide insight into understanding the coordination of effector and memory T cell responses in the context of a chronic localized protozoan infection in

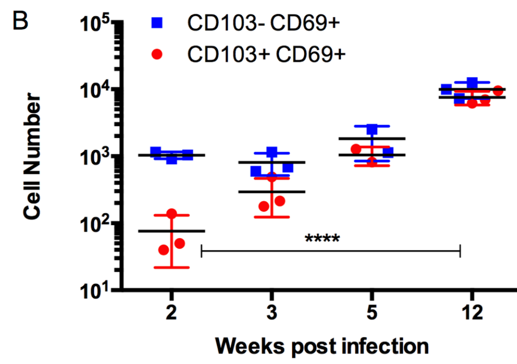
the brain. We show expression of CD103 by brain infiltrating CD8 T cells defines a transcriptionally distinct population during chronic *T. gondii* infection. Furthermore, we show that this population is transcriptionally consistent with tissue resident memory T cells and has an increased capacity for pro-inflammatory cytokine production relative to other CD8 T cells in the brain. Therefore, our data indicates a need for further investigation into a unique protective role for T_{RM} during chronic infection.

2.6 Figures and Legends

Figure 2.1: Kinetics and specificity of brain-infiltrating CD103+ CD8 T cells

A) Brain mononuclear cells harvested from *Toxoplasma gondii*-infected mice at indicated time points and gated on live CD3+ CD8+ cells. Numbers indicate the proportion of CD69+ CD103+ and CD69+ CD103− CD8 T cells in the brain at the indicated time points. B) Absolute cell counts for the data are shown in panel (A). C) Percentage of dextramer+ CD8 T cells in the brain. Numbers indicate average ± SEM of three biological replicates for each time point. D) Percentage of SIINFEKL dextramer+ CD8 T cells expressing CD103 at week 12 postinfection. Significance was determined using two-way ANOVA with multiple comparisons post test. ****p < 0.0001. Data are representative of two independent experiments with similar results.


A
Week 2
Week 3
Week 5
Week 12
2.8±0.4%
15.3±1.9%
24.7±3.4%
38.8±1.0%
60.9±2.2%
44.8±5.6%
43.7±2.0%
50.6±1.5%
CD103
CD69


B
CD103- CD69+
CD103+ CD69+
Cell Number
****
Weeks post infection

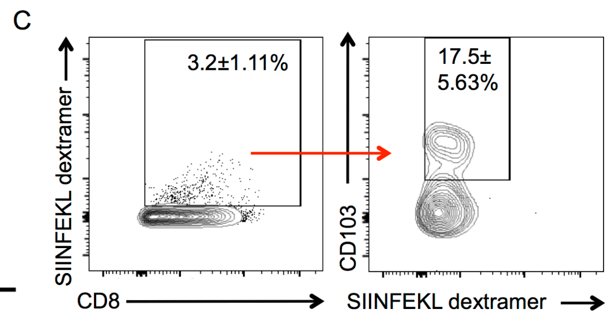

C
3.2±1.11%
17.5±
5.63%
SIINFEKL dextramer
CD8
CD103
SIINFEKL dextramer

Fig 2.2: Distribution of CD103+ CD8 T cells in the *Toxoplasma gondii*-infected brain

Immunofluorescent staining *in situ* for CD8 and CD103 in representative sagittal sections of infected brain at 5 weeks post infection. A) E-cadherin staining in choroid plexus of lateral ventricle, 40× magnification, zoomed in. B) Laminin staining for vasculature of frontal cortex, 40× magnification, zoomed in. C) *T. gondii* staining in the frontal cortex, 25× magnification, zoomed in. All images are representative of n = 3 biological replicates at 5 weeks post infection. White arrows indicate examples of staining positive for both CD8 and CD103. Scale bar indicates 20 μm.

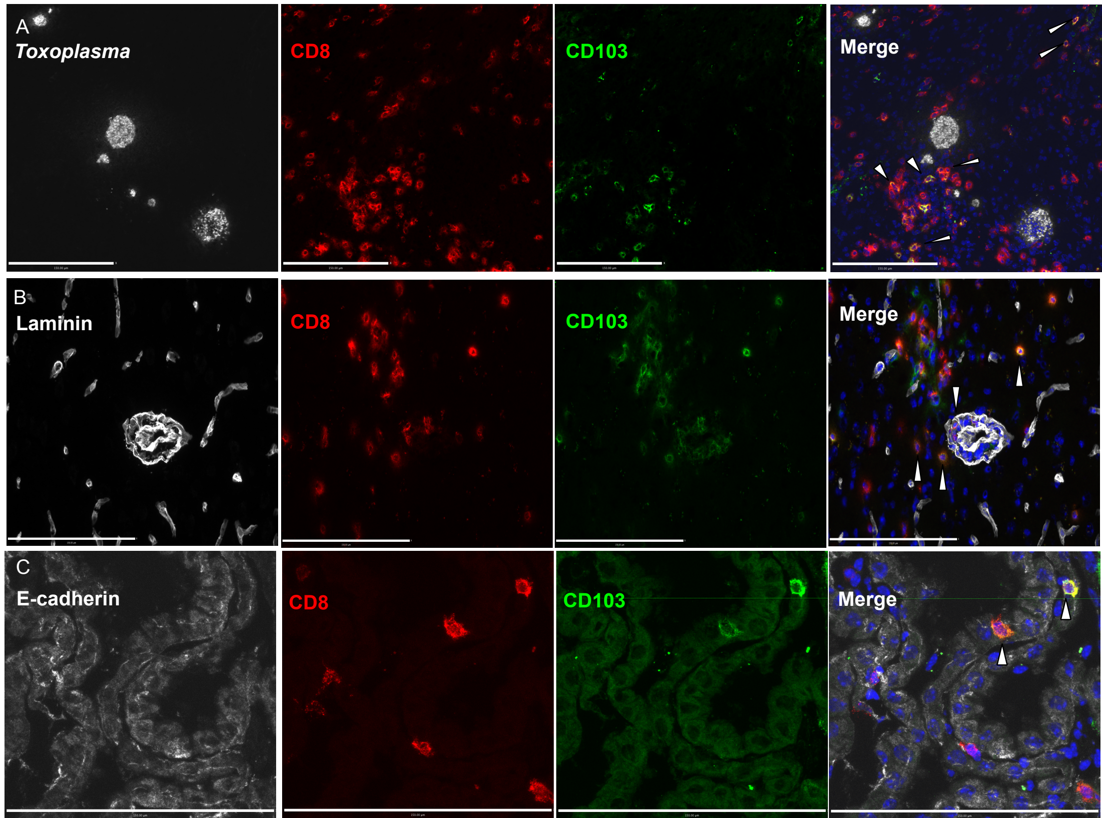

A
Toxoplasma
CD8
CD103
Merge
B
Laminin
CD8
CD103
Merge
C
E-cadherin
CD8
CD103
Merge

Figure 2.3: Transcriptional characteristics of brain CD103+ CD8 T cells

CD8 T cells were isolated from the brain and spleen of chronically infected mice and sorted according to CD103 expression. A) Differentially expressed genes (DEGs) for the three indicated analyses. Numbers indicate combined upregulated and downregulated DEGs with fold change >2 and FDR <5%. B) PCA plot for normalized read counts (reads per kilobase of transcript per million mapped reads) for all samples.

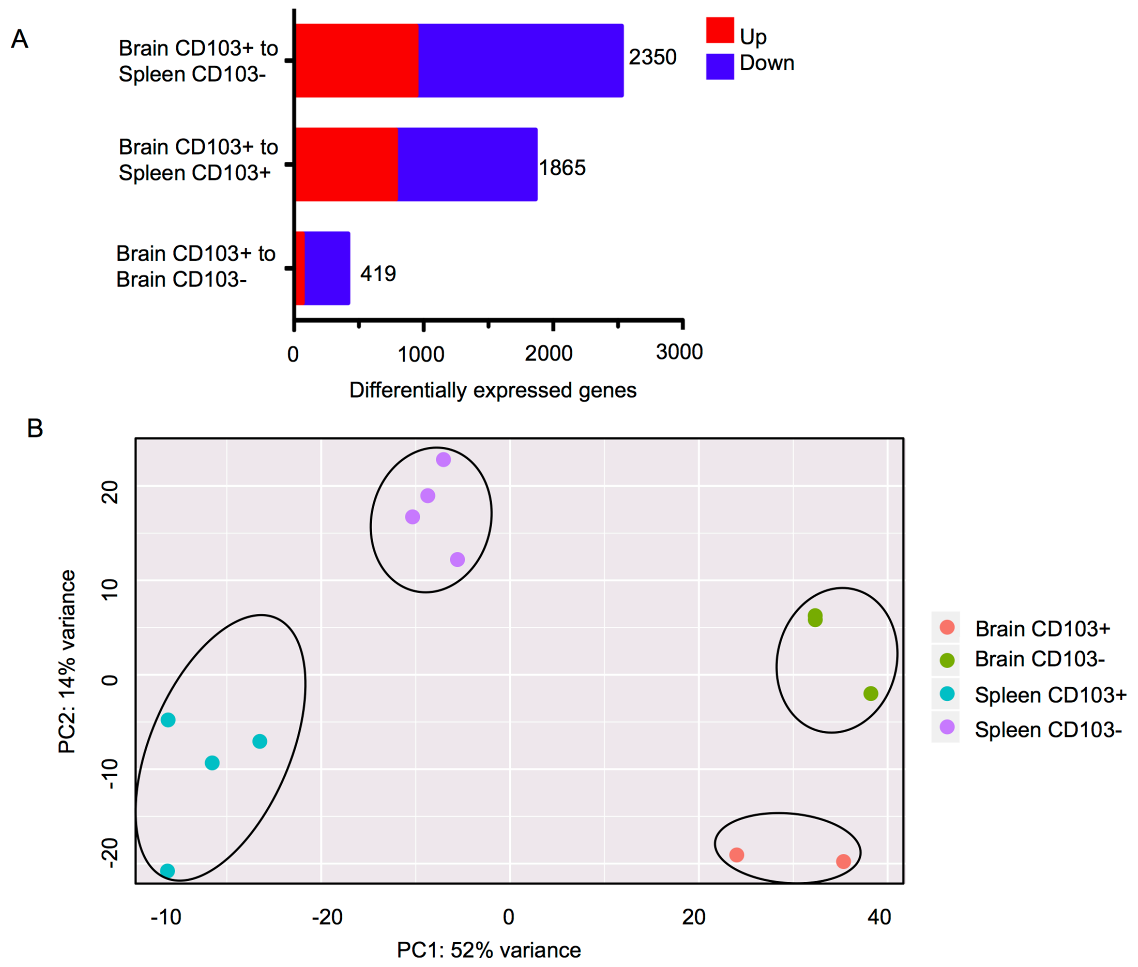
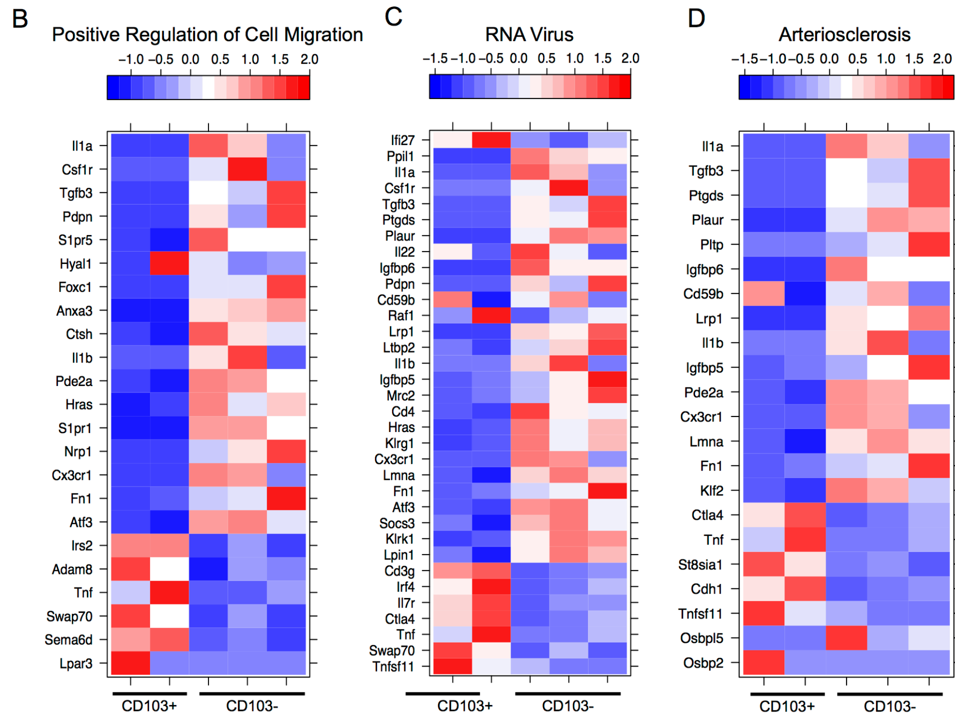

A
Brain CD103+ to
Spleen CD103-
Brain CD103+ to
Spleen CD103+
Brain CD103+ to
Brain CD103-
2350
1865
419
Up
Down
0
1000
2000
3000
Differentially expressed genes
B
PC2: 14% variance
20
10
0
-10
-20
-10
-20
0
20
40
PC1: 52% variance
Brain CD103+
Brain CD103-
Spleen CD103+
Spleen CD103-

Figure 2.4: Distinct transcriptional profile of brain CD103+ CD8 T cells

A) After conducting differential gene expression analysis between brain CD103+ and brain CD103− CD8 T cells, the genes with a fold change >2, FDR <5%, and mean RPKM >1 were input into MetaCore for functional enrichment. Of the 239 genes input, the following terms with FDR <5% were extracted from the categories "Disease by Biomarker" and "GO Process." B) Heatmap of genes under GO process term "Positive regulation of cell migration." C) Heatmap of genes under Disease term "RNA virus." D) Heatmap of genes under enrichment term "Arteriosclerosis." Individual replicates in heatmap are pooled from n = 5 mice. Values in legend are scaled values representative of RPKM. Red indicates a highly expressed gene, and blue indicates a gene with a low expression value. E) Table of fold changes and false discovery rate for genes relevant to tissue residence and memory phenotype. F) Flow cytometry for differential expression of S1PR1 and KLRG1.

A

	Enrichment Term	# of Genes	FDR
GO Process	Regulation of cell differentiation	55	1.35E-09
	regulation of proliferation	52	1.13E-08
	regulation of MAPK cascade	31	6.21E-08
	negative regulation of signal transduction	39	1.14E-07
	positive regulation of cell migration	23	1.16E-07
Disease by Biomarker	RNA virus infection	35	1.15E-09
	Arteriosclerosis	24	8.24E-07



E

Gene	Fold Change	FDR
Lpar3	14.4	9.95E-03
Sema6d	3.9	7.93E-06
Cdh1	2.9	1.55E-04
Swap70	2.9	4.63E-02
Adam8	2.4	2.05E-02
Ctla4	2.14	2.38E-02
Il7r	2.1	2.86E-02
Irf4	2.1	3.36E-02
Klf2	-2.5	1.56E-02
Atf3	-2.7	1.18E-02
Klrg1	-3.6	5.84E-03
S1pr1	-4.4	5.84E-03

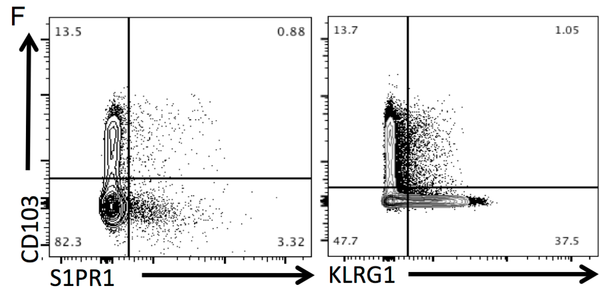
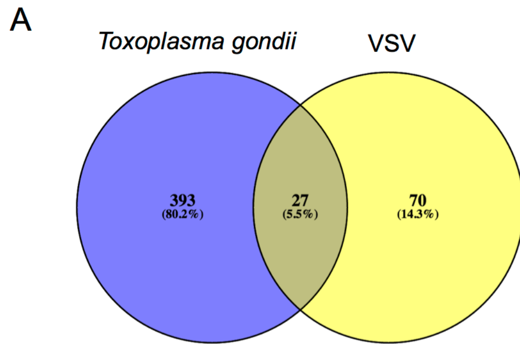


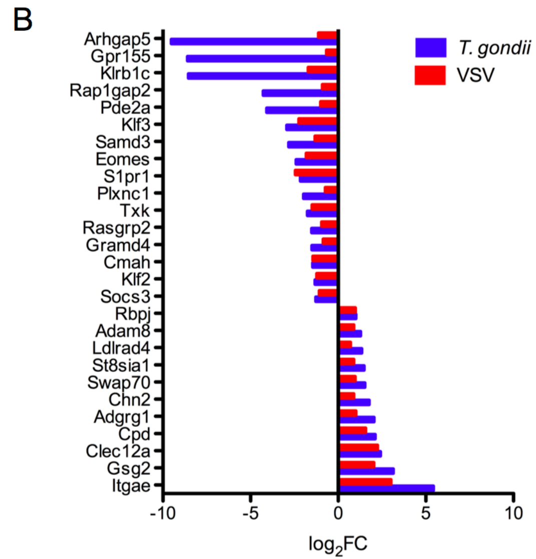
Figure 2.5: Comparison of tissue-resident memory (T_{RM}) from VSV

Microarray data from the study by Wakim et al. were obtained and analyzed via GEO. A) Venn diagram of DEGs in *T. gondii* and VSV (23, 25) for the brain CD103+ CD8 T cells relative to brain CD103− CD8 T cells. B) Comparison of fold changes for the 27 differentially expressed genes common to both models. RNA-Seq DEGs were determined according to the following criteria: fold change >2, FDR <5%, $p < 0.05$, and mean RPKM >1. Microarray DEGs were significant if the p value and adjusted p value were less than 5%. C) Results of enrichment analysis for DEGs unique to microarray analysis of T_{RM} in VSV. D) Results of enrichment analysis for DEGs unique to RNA-Seq analysis of T_{RM} in *T. gondii* infection.

A



B



C

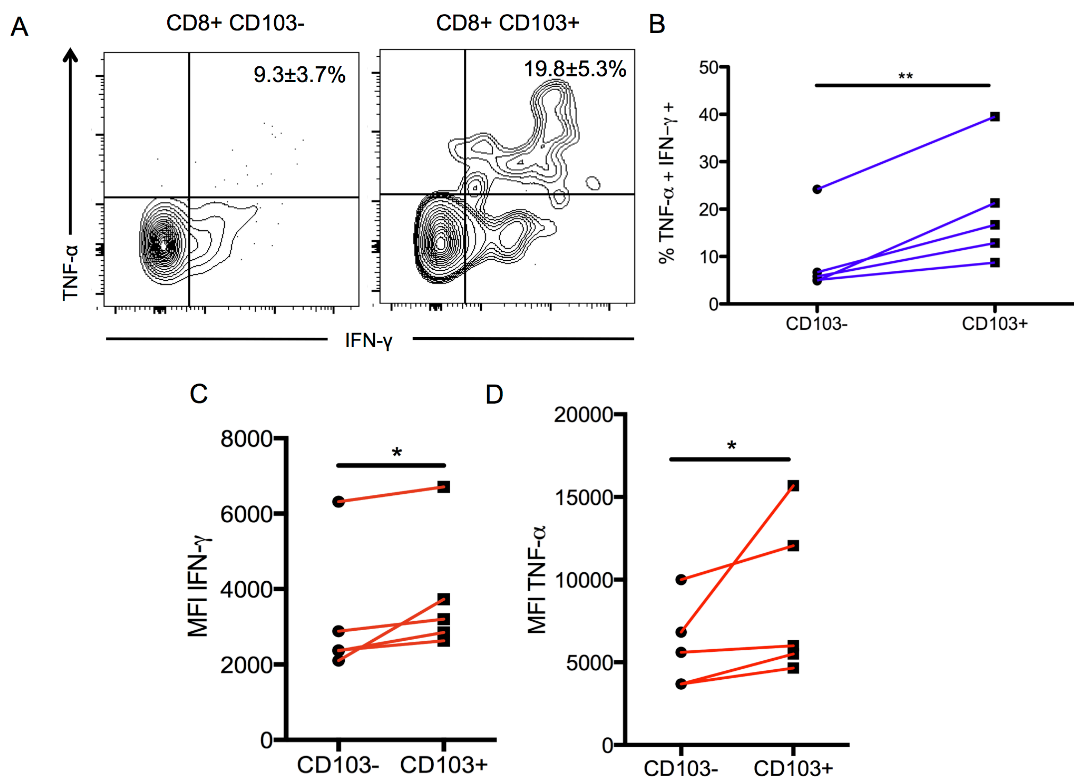
Enrichment Term- VSV	**# of Genes**	**FDR**
immune system process	9	1.69E-04
innate immune response	7	1.39E-02
negative regulation of viral entry into host cell	3	2.09E-02
response to interferon-alpha	3	7.41E-03
response to interferon-gamma	3	4.34E-02

D

Enrichment Term- *T. gondii*	**# of Genes**	**FDR**
cell adhesion	26	3.40E-06
negative regulation of cell proliferation	17	7.33E-03
cell surface receptor signaling pathway	11	4.08E-02
extracellular matrix organization	8	2.20E-02
cell-matrix adhesion	8	4.03E-03

Figure 2.6: Production of pro-inflammatory cytokines in CD103+ CD8 T cells.

Brain mononuclear cells from chronically infected mice were restimulated with α-CD3/α-CD28 antibodies for 6 hours and intracellular cytokine staining was performed. Data were gated on live CD3+ CD8 T cells and then split into CD103+ and CD103- subsets for subsequent analysis. A) Representative flow plot indicating percentage of restimulated CD103+ and CD103- CD8 T cells producing IFN-γ and TNF-α. Numbers indicate mean percentage ± SEM of n=5 biological replicates. B) Paired data plot of the data shown in (A). C) Geometric MFI of IFN-γ production comparing CD103+ to CD103- CD8 T cells subsets. D) Geometric MFI of TNF-α production for the same comparison. Significance was determined using a paired two-sample t-test. *$p<0.05$, **$p<0.01$; p values are one tailed. Data are representative of two independent experiments with similar results.


A
CD8+ CD103-
CD8+ CD103+
9.3±3.7%
19.8±5.3%
TNF-α
IFN-γ
B
% TNF-α + IFN-γ +
**
CD103-
CD103+
C
MFI IFN-γ
*
CD103-
CD103+
D
MFI TNF-α
*
CD103-
CD103+

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CHAPTER THREE

CCR7 Drives Maintenance of T cell Subsets

3.1 Abstract

The chemokine receptor CCR7 and interactions with its ligands CCL19 and CCL21 are well known to facilitate priming of T cells in the lymphoid tissues and such interactions are considered a powerful coordinator of T cell circulation between lymphoid organs and peripheral tissues during inflammation. We have investigated the contribution of CCR7 signaling in the maintenance of T cell subsets in the brain and periphery. Here, we demonstrate that CCR7 expression is uniquely required to generate an optimal IL-7R- KLRG1+ T cell effector population in the brain. Furthermore, our data reveals that CCR7+ dendritic cells are sufficient to restore the IL-7R- KLRG1+ T cell population in CCR7-/- mice. Therefore, although CCR7 appears to mediate efficient coordination of recall responses via dendritic cells, this chemokine receptor is not required for ongoing control of infection. Importantly, this suggests a redundancy in ongoing effector T cell mediated control of *T. gondii* infection in the brain.

3.2 Introduction

Our knowledge of the mechanisms that control chronic infection and infiltration of immune cells into the brain continues to grow. It is well known that *T. gondii* is predominantly controlled by cell-mediated immunity. There is a constant requirement for both CD4+ and CD8+ T cell recruitment in the immune-restricted CNS to control parasite reactivation and replication and to prevent fatal toxoplasmic encephalitis (TE) [1-3]. The cytokines produced by these infiltrating T cells, such as IFN-γ, IL-10, IL-4 and IL-17, are crucial to maintain a balance between inflammation and parasite control [4-7]. Similar to our observations during *T. gondii* infection, several studies on multiple sclerosis and other neurological disorders have demonstrated the presence of CCR7+ inflammatory infiltrates in the cerebrospinal fluid and sites of inflammation in the CNS [8,9]. This provides compelling evidence that CCR7 could play a role in both CNS entry and trafficking within the parenchyma.

Despite this, the specific requirement for CCR7 in T cell mediated protective immune responses during the chronic stage of infection is still under investigation. The immune privileged CNS has been known to follow unique rules to control leukocyte infiltration, antigen presentation and other immunological processes such that protective immunity coincides with minimum damage to the brain [10,11]. We still do not fully understand the mechanisms that govern migration and trafficking within the brain, nor whether activated T cells constantly recirculate or remain constrained to the tissue, nor the role for

memory responses in the presence of persistent antigen. The distinct role that CCR7 plays in any of these processes is an ongoing investigation.

Here we begin to address some of these questions using a CCR7-/- mouse model. We have shown that although cells from the infected brain, in particular T cells, are responsive to CCL21, CCR7 is not required for ongoing control of infection. Expression of CCR7 is required to generate optimal populations of KLRG1+ effector T cells. Adoptive transfer of CCR7+ BMDCs to CCR7-/- mice restores the defective KLRG1+ CD4+ T cell population, thus suggesting that CCR7 plays an important role in coordinating ongoing efficient antigen presentation. Therefore it is likely that CCR7+ dendritic cells improve the efficiency of the effector response, despite not being required for ongoing protection.

3.3 Materials and Methods

3.3.1 Mice and Parasites

_*T. gondii* Prugniaud-OVA and RH strains (transgenic parasites expressing ovalbumin) were maintained *in vitro* in Human Foreskin Fibroblasts (HFF) grown in DMEM complete (90% DMEM, 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin). After infecting HFF's, parasites were grown in D10 media (70% DMEM, 20% M199, 10% Fetal Bovine Serum, 5% Penicillin/Streptomycin, 5% Gentamycin) with chloramphenicol. Parasites were purified by passing through a 22.5-gauge needle,

followed by passage through a 5.0mm nylon filter, centrifuged at ~2000xg for 10 minutes at 4°C. After removing supernatant, parasites were resuspended in 1ml sterile PBS and counted. 10,000 parasites were intraperitoneally injected in 200µl of 1xPBS. To control parasite numbers, mice were treated with sulfadiazine at 200mg/L (Sigma, St. Louis, MO) in their drinking water starting from 4 days post-infection and continued for two weeks. C57Bl/6 mice and congenic CD45.1 mice were obtained from the Jackson Laboratory (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). CCR7-$^{/}$- (C57BL/6 background) and plt/plt (BALB/c background) mice were bred and maintained in a pathogen free environment under IACUC established protocols at the University of California, Riverside.

3.3.2 Flow Cytometry analysis

For flow cytometry analysis, cells were counted, resuspended at $1x10^6$/ml in RPMI complete. 1x10^6 cells were transferred in a FACS tube (BD Falcon^{TM}, MA, USA) and centrifuged at ~300xg for 5 minutes at 4°C and resuspended in FACS buffer (1x PBS containing 1.0% BSA, 0.01% EDTA). After another round of wash with FACS buffer, the cells were incubated with a saturating solution of Fc block (eBioscience, San Diego, USA) for 10 minutes on ice followed by staining with conjugated antibodies for 25 minutes on ice. Antibody against mouse CD4, CD8, CD103, CD45.1, CD45.2, CD11b, CD11c, and KLRG1 were purchased from eBioscience. Following antibody staining, cells were washed and resuspended in 300 µl FACS buffer and analyzed using the BD

FACSCanto^{TM} II flow cytometer and FlowJo version 10.1. Statistical analysis was performed using Prism 6.

3.3.3 Generation of bone marrow derived dendritic cells (BMDCs)

Bone marrow was extracted from the femur and tibia of CD45.1+ mice (Jackson Laboratories). To generate dendritic cells, marrow cells were cultured for 7 days in complete DMEM with 20ng/mL GM-CSF (eBioscience). Cells were dosed with one-half volume, then one-third volume complete DMEM containing GM-CSF every 3 days, respectively. On day 7 of culture, dendritic cells were loaded with 100ug/mL Soluble Toxoplasma Antigen (STAg) and 0.5mg/mL ovalbumin protein (OVA) for 24 hours. In addition to antigen, LPS was added at 100ng/mL to facilitate Th1 activation. Non-adherent cells were isolated for transfer into congenic recipients. Phenotype of transferred cells was confirmed using flow cytometry.

3.3.4 Adoptive Transfer

$5x10^6$ CD45.1+ BMDC in 100ul of PBS were retro-orbitally transferred into CD45.2+ CCR7-/- or WT mice during the chronic stage of infection. Vehicle controls were given PBS only. Mice were sacrificed 2 days post-transfer and brain, cervical lymph nodes and spleen were collected for flow cytometry.

3.4 Results

3.4.1 In the absence of CCR7, IL-7R - KLRG1+ population is deficient at the site of infection

Although CCR7-/- mice display normal populations of antigen specific T cells in the brain upon rechallenge, this did not exclude a potential defect in another infiltrating leukocyte population. Therefore we investigated the phenotype of inflammatory infiltrates recruited to the infected brain, cervical LN and spleen during rechallenge infection. These included neutrophils, CD11c+ cells and peripheral macrophage. The proportions and the activation status of resident microglia in the rechallenged brain were also analyzed using flow cytometry. None of these populations were defective in the absence of CCR7 by any parameters measured including proportion, activation or absolute cell numbers (data not shown). Figure 3.1 provides a summary of the T cell phenotype in CCR7-/- mice[1]. Significant differences in parasite burden were observed in the brain and lung of CCR7-/- mice (Fig 3.1A). No differences were observed in overall numbers of BMNCs, but significant differences were observed in numbers of CD8 and CD4 T cells (Fig 3.1B). Phenotypic analysis of T cells revealed that the proportions of IL-7R- KLRG1+ populations were significantly decreased in the brain in the absence of CCR7. The proportion of CD4+T cells that were IL-7R- KLRG1+ in CCR7-/- mice was only one-third of that present in the WT rechallenged mice (Fig 3.1C). In addition, there was a 45% reduction of CD8+ IL-7R- KLRG1+ cells in CCR7 deficient compared to

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[1] Shahani Noor, unpublished data

control brains (Fig 3.1C). However, this defect was only present at the site of infection and not evident in the cervical LNs (Fig 3.1c). A similar defect was evident in the *plt/plt* mouse brain suggesting CCR7-CCL21/CCL19 mediated interactions are required to maintain this IL- 7R- KLRG1+ T cell population in the brain (Fig 3.1D). Interestingly, the defect in the KLRG1+ population was more prominent in the *plt/plt* brain than in CCR7-/- mouse brain and as in the absence of CCR7 there was no defect in the LN. This data demonstrates that generation of effector T cell populations at the site of infection is critically dependent on CCR7-CCL21 interactions.

3.4.2 Transfer of CCR7+ BMDCs is sufficient to recover the defective KLRG1+ effector population in the brain

Our data suggest that although CCR7 expression is not required to mount effector responses *per se*, it is required to generate an optimal population of effector T cell populations in the brain, and this correlates with an inability to protect against rechallenge. Given that CCR7 is largely not expressed on KLRG1+ T cells, but is expressed on brain-infiltrating DCs, we hypothesized that CCR7 expression on DCs coordinates the efficient interactions required to generate an optimal population of KLRG1+ T cells in the brain. To test this hypothesis, we transferred CCR7+ bone marrow derived dendritic cells (BMDCs) into CCR7-/- recipients at the chronic stage of infection and conducted flow cytometry on the brain, spleen, and cervical lymph nodes to assess localization of transferred dendritic cells and phenotype of T cells in the brain (Fig 3.2A). Transferred dendritic cells were identified in the CD45.2+ recipient as CD11c+

CD11b+ CD45.1+. The transferred cells localized primarily to the cervical lymph nodes and spleen, but significant numbers were not observed to aggregate in the brain (Fig 3.2B). In the brain, no significant differences were observed the proportion of T cells (CD3+), as including CD4+ and CD8+ subsets, between mice that received CCR7+ BMDCs and those that received vehicle (Fig 3.3A-B). However, CCR7-/- mice that received CCR7+ BMDCs exhibited nearly a two-fold increase in proportion of CD4+ KLRG1+ T cells relative to untreated (Fig 3.3C-D). Of note, this significant rescue was not observed in the CD8+ population (Fig 3.3C,E). In CCR7-/- mice that received BMDCs, no significant difference was observed compared to wild type in proportions of KLRG1+ T cells of both CD4+ and CD8+ subsets (Fig 3.3D-E). Taken together these data suggest that CCR7+ dendritic cells are sufficient to recover an effector T cell population at the site of infection, and our observed results are not due to changes in overall proportions of T cells. This is supportive of our hypothesis that via inefficient coordination of DC-T cell interactions, CCR7-/- mice are unable to mount protective responses to rechallenge due to sub-optimal generation of brain infiltrating effector T cells.

3.5 Discussion

Although CCR7 does not appear to be essential for ongoing protection during chronic infection, our data supports a role for CCR7 during chronic infection as a coordinator of efficient priming and maintenance of T cells by APCs. Such an observation finds precedent in the literature. Defective recruitment of inflammatory monocytes at the site

of ongoing infection has been observed in CCR7-/- mice during the acute phase of infection with *T. gondii* [12]. Furthermore CD28/B7 interactions and IL-12 production are critical for generation of terminally differentiated (KLRG1+) T cell subsets and protective T cell responses [13-15]. At the chronic stage of infection, diverse DC subsets within the CNS serve as a primary source of IL-12 [16-18]. Moreover, CD11b+ inflammatory DCs have been demonstrated to interact with effector T cells in the CNS or other peripheral tissues to enhance effector T cell function and ensure rapid activation of effector memory T cells in the non-lymphoid tissues [19-21]. Our data taken together with these observations strongly indicate a similar role for dendritic cells during chronic *T. gondii* infection and includes CCR7 as a critical component of this role.

Our observation that significant numbers of transferred BMDCs were not found in the brain is consistent with previously published literature [22]. Despite primarily localizing to cervical lymph nodes and spleen, transferred CCR7+ BMDCs were still sufficient to recover the effector phenotype at the site of infection. One explanation for these results involves efficient recruitment of CCR7+ BMDCs to the lymph nodes where they activate and replenish the effector population from a local quiescent pool. These cells then migrate to the site of infection and replenish the existing population. This scenario is made problematic by the lack of defect in KLRG1 expression in the lymph node in CCR7-/- mice. One possibility is that delayed priming in the lymph node results in defective recruitment of this specific population to the brain. Another explanation is that splenic BMDCs represent a recirculating population that is able to present antigen in the

perivascular spaces of the brain and therefore activate T cells and replenish the defective effector population at the site of infection.

Regardless, our results clearly demonstrate a role for CCR7 in coordinating efficient generation of effector T cell populations by DCs during chronic infection. Importantly, this suggests that in addition to a requirement for T cells to mediate long-term protection against chronic *T. gondii* infection, there is also an important role for continuous maintenance of brain infiltrating T cell populations by APCs either locally or in the periphery.

3.6 Figures and Legends

Fig 3.1: Deficient effector populations in CCR7-/- mice

Shahani Noor, unpublished data: A) Parasite burden in brain and lung is not significantly different in chronically infected and rechallenged CCR7-/- mouse. B) No difference in total BMNC, but significantly increased infiltration of CD4 and CD8 T cell in brain of CCR7-/- mouse. C-D) No significant difference in proportion of Toxoplasma-specific CD4 and CD8 T cells in brain. E-F) Significant defect in CD4+ KLRG1+ population. At least n=3 mice per group. Data is representative of two independent experiments with similar results. *$p<0.05$

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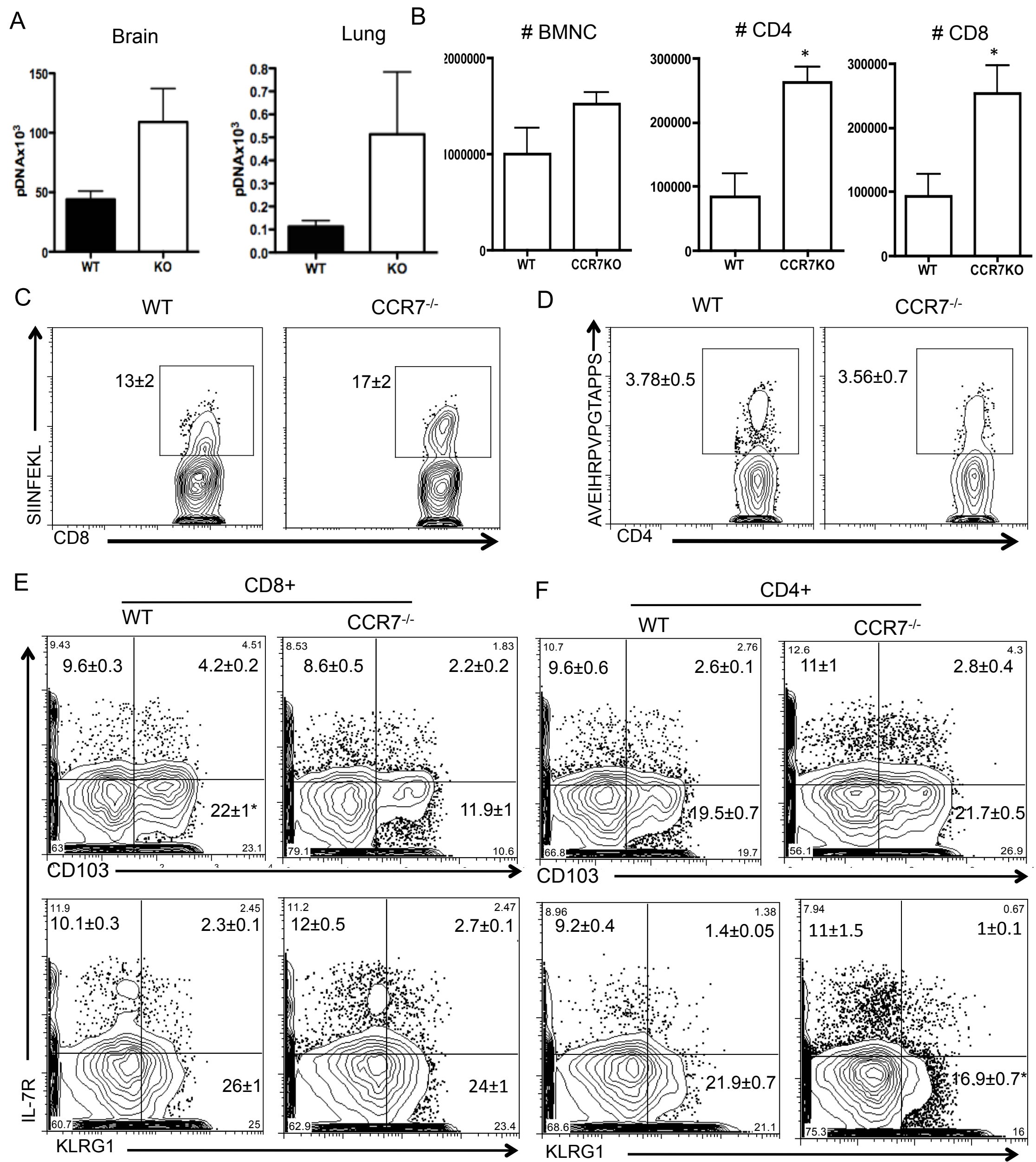
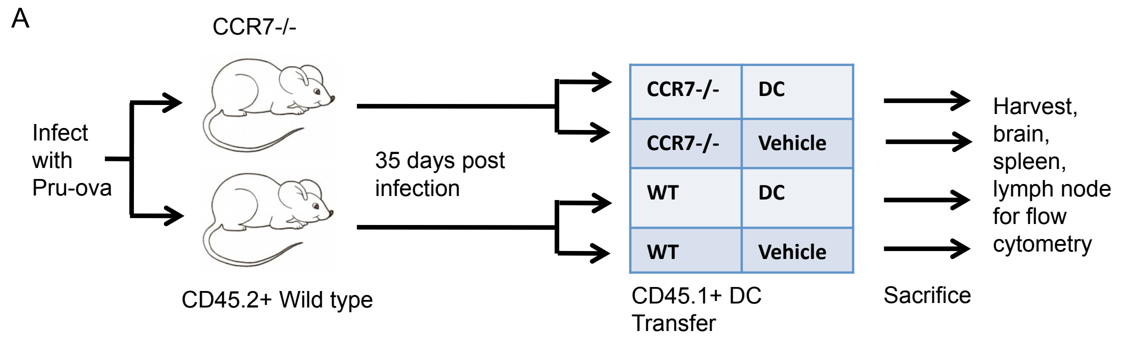


A
Brain
Lung
pDNAx10^3
WT
KO
B
BMNC
CD4
CD8
WT
CCR7KO
C
WT
CCR7-/-
13±2
17±2
SIINFEKL
CD8
D
3.78±0.5
3.56±0.7
AVEIHRPVPGTAPPS
CD4
E
CD8+
F
CD4+
9.6±0.3
4.2±0.2
22±1*
8.6±0.5
2.2±0.2
11.9±1
9.6±0.6
2.6±0.1
19.5±0.7
11±1
2.8±0.4
21.7±0.5
CD103
10.1±0.3
2.3±0.1
26±1
12±0.5
2.7±0.1
24±1
9.2±0.4
1.4±0.05
21.9±0.7
11±1.5
1±0.1
16.9±0.7*
IL-7R
KLRG1

Fig 3.2: Transferred dendritic cells localize to the peripheral lymphoid organs

A) Experimental setup. Two sets of CD45.2+ were mice infected with Pru-Ova. Within each set, one group intravenously received CD45.1+ BMDCs and another was given vehicle (PBS). Two days post transfer; the brain, cervical lymph nodes and spleen were harvested for flow cytometry. B) Localization of BMDCs post transfer in CCR7-/- recipients, compared to vehicle. Transferred dendritic cells observed in spleen and cervical lymph nodes. Numbers in plot represent percentage ±SEM of population that was CD45.2+ CD45.1- and CD45.2- CD45.1+. All results gated on CD11c+ CD11b+ population. n=3 mice per group. At least n=3 mice per group. Data is representative of two independent experiments with similar results. *$p<0.05$


A
CCR7-/-
Infect
with
Pru-ova
35 days post
infection
CD45.2+ Wild type
CCR7-/-
DC
CCR7-/-
Vehicle
WT
DC
WT
Vehicle
CD45.1+ DC
Transfer
Sacrifice
Harvest,
brain,
spleen,
lymph node
for flow
cytometry

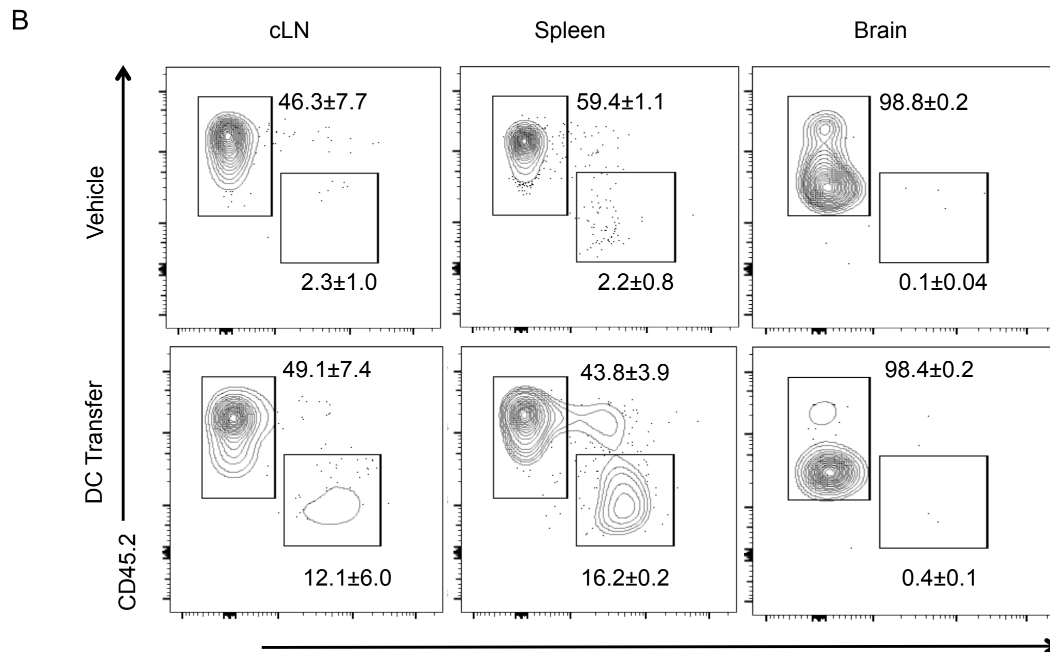
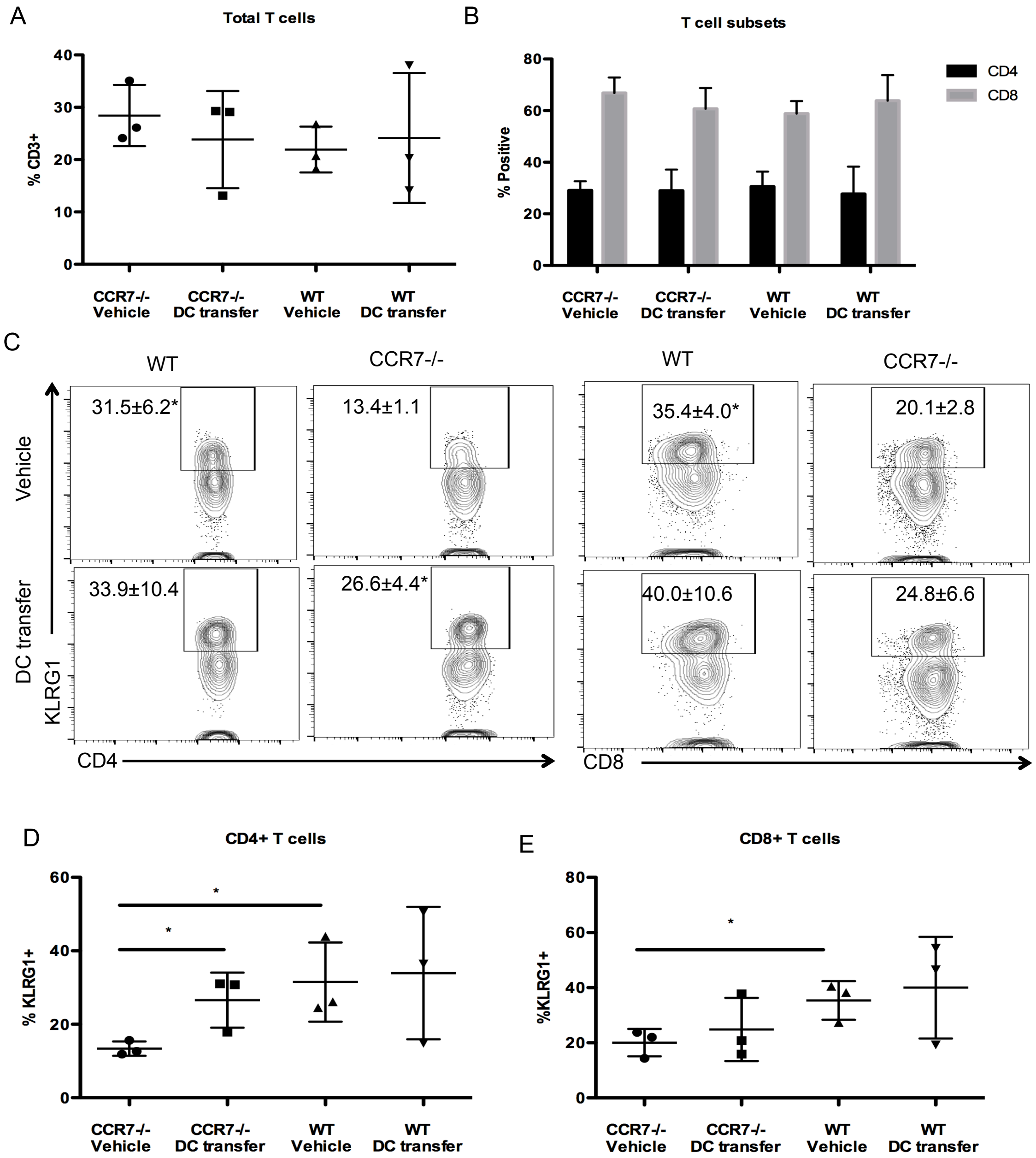

B
cLN
Spleen
Brain
Vehicle
DC Transfer
CD45.2
46.3±7.7
2.3±1.0
59.4±1.1
2.2±0.8
98.8±0.2
0.1±0.04
49.1±7.4
12.1±6.0
43.8±3.9
16.2±0.2
98.4±0.2
0.4±0.1

Figure 3.3: BMDC transfer is sufficient to recover CD4+ KLRG1 expression

A) Proportions of CD3+ T cells are not significantly different between all groups. B) Proportions of CD4+ and CD8+ T cells are not significantly different between all groups. C-E) Results of BMDC transfer. C) KLRG1+ population in CCR7-/- mice receiving DC transfer not significantly different from wild type. D-E) In CD4 subset there is a significant increase in KLRG1+ T cells relative to CCR7-/- mice that received vehicle. For each treatment, top line refers to genotype of recipient mice, and bottom refers to whether vehicle or DCs were transferred. All results were pre-gated on CD3 to exclude populations that were not T cells. Numbers on flow cytometry plots represent percentage of population positive for KLRG1 ±SEM. All data shown is in brain. n=3 mice per group. Data is representative of two independent experiments with similar results. *$p<0.05$ relative to CCR7-/- receiving vehicle.



A
Total T cells
% CD3+
CCR7-/- Vehicle
CCR7-/- DC transfer
WT Vehicle
WT DC transfer
B
T cell subsets
% Positive
CD4
CD8
C
WT
CCR7-/-
Vehicle
DC transfer
KLRG1
CD4
CD8
31.5±6.2*
13.4±1.1
35.4±4.0*
20.1±2.8
33.9±10.4
26.6±4.4*
40.0±10.6
24.8±6.6
D
CD4+ T cells
% KLRG1+
E
CD8+ T cells
%KLRG1+

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CHAPTER FOUR

Role of elevated extracellular glutamate on T cell function in the context of chronic *Toxoplasma gondii* infection

4.1 Abstract

Glutamate dysregulation is reported in many cases of disease and damage to the CNS. We have recently reported the accumulation of extracellular glutamate during chronic *Toxoplasma gondii* infection along with evidence of pathology due to glutamate excitotoxicity. Despite this, there is a well-maintained protective response by the immune system of which T cells are a critical component. It is increasingly appreciated that signals within the tissue play an important role in driving the local immune response. Here we examine the role of extracellular glutamate on the function of T cells responding to chronic *Toxoplasma gondii* infection. We show that T cells in the brain express type I metabotropic gluamate receptors at significantly increased levels compared to peripheral tissues. We show that T cells can modulate expression of these receptors in response to changes in extracellular glutamate in vitro and report associated changes in proliferation and cytokine production. Finally, we examine the effects of signaling through type I metabotropic glutamate receptors on T cells both *in vitro* and *in vivo*. In particular, we provide evidence that mGluR5 may play a role in efficient T cell infiltration into the brain. Our data contributes towards understanding how modulation of glutamate disease states of the CNS may have impacts on the immune compartment that are currently poorly understood. Further studies are needed to examine these impacts.

4.2 Introduction

Dysregulation of the excitatory neurotransmitter glutamate can result in profound neuronal pathologies and is evident in multiple diseases of the CNS including traumatic brain injury, epilepsies, neurodegeneration, and infection [1-3]. Glutamate concentration in the brain is tightly regulated with an extracellular homeostatic concentration of about 1μM. In comparison the plasma concentration of glutamate is quite high, at about 100μM [4]. We have recently described elevated extracellular glutamate in the brain of mice chronically infected with *Toxoplasma gondii* [5]. Such mice displayed evidence of neuronal pathology as measured by dendritic spine count and abnormal neural processing and behaviors as measured by EEG and elevated plus maze. These data support the development of glutamate excitotoxicity during *Toxoplasma gondii* infection. Brain-infiltrating T cells are documented in many CNS disease states including chronic *T. gondii* infection and yet the role of glutamate excitotoxicity on T cell function in this context remains poorly understood.

There is little evidence from the literature that T cells express glutamate transporters, however there is ample evidence that they express both ionotropic and metabotropic glutamate receptors [6]. As their name suggests, ionotropic glutamate receptors (iGluRs) are ion channels gated by glutamate binding. T cells express the Kainate, AMPA, and NMDA ionotropic glutamate receptors and inhibition of these receptors results in decreased $Ca^{2}+$ uptake [7,6]. In addition, inhibition of the Kainate receptors results in a decrease in Kv1.3 activity as measured by patch clamp analysis [8]. Given the critical

role of K+ and $Ca^{2}+$ uptake in the initial stages of T cell activation, this data supports a role for iGluRs in this process. iGluR3, an AMPA receptor, is sufficient to mediate adhesion and chemotaxis of resting T cells *in vitro* [9].

Glutamate is thought to modulate T cell activation via the action of the type I metabotropic glutamate receptors, mGluR1 and mGluR5, however this has only been tested in human T cells [10]. Metabotropic glutamate receptors (mGluRs) are atypical G protein coupled receptors (GPCRs), in reference to their divergent amino acid sequence and structure relative to other GPCRs [11]. Functionally, mGluRs can mediate similar downstream effects to conventional GPCRs. In activated human T cells, mGluR1 agonism results in phosphorylation of ERK1/ERK2, whereas mGluR5 agonism results in an increase of intracellular cAMP and inhibition of proliferation [10]. This gives rise to a model where mGluR1 initiates activating signals, whereas mGluR5 initiates inhibitory signals. Along with the type I receptors, type II and type III receptors form the major subgroups of the mGluR family. The type II receptors include mGluR2 and mGluR3, whereas the type III receptors include mGluR4, mGluR6, mGluR7, and mGluR8. Along with iGluR3 the type II mGLURs appear to play a role in modulating the function of the potassium channel Kv1.3 [6,4]. This implies a critical role for these receptors in regulation of T cell activation. The expression of type III mGluRs in rodent blood lymphocytes has been measured by qPCR, but their role has not been described [6].

As GPCRs, the role of metabotropic glutamate receptors in T cell function could be wide-ranging, including effects on migration, proliferation, and survival [11]. Furthermore there is a great deal of phenotypic diversity among brain infiltrating T cells and the potential role of glutamate in driving this diversity has been unexplored. Here, we provide evidence for the elevated expression of type I metabotropic glutamate receptors on brain-infiltrating T cells in mice chronically infected with *Toxoplasma gondii*. Furthermore we present data indicating that the concentrations of glutamate within the chronically infected brain have a suppressive effect on T cell function. Finally, we discuss the possible role of glutamate as signal for accumulation of tissue resident memory T cells within the brain and the possible role of mGluR5 in CD4+ T cell infiltration into the brain.

4.3 Materials and Methods

4.3.1 Mice and Parasites

C57BL/6 mice (CD45.2 and CD45.1) were purchased from Jackson Laboratories. Mark Geyer of the University of California, San Diego generously donated the mGluR5-/- mice used for the study. Infections were conducted using the Me49 strain of the parasite, which were maintained in CBA mice from Jackson Laboratories. Cysts were isolated from the brains of chronically infected CBA mice by serial passage through 18, 20, and 22 gauge blunt needles. Infections were conducted by intra-peritoneal injection of 20 cysts in 200μL sterile PBS.

4.3.2 In vitro modulation of glutamate concentration

Glutamate free media was generated as described in Pacheco et al 2006 [12]. Briefly, T cell media was heated for 1 hour at 70°C and incubated overnight with 1μg/mL alanine aminotransferase. Elimination of glutamate from the media was confirmed with a colorimetric glutamate assay kit from Abcam. Splenocytes were extracted from naïve C57BL/6 mice, labeled with CFSE (from Invitrogen), and seeded in 96 well round bottom plates at $5x10^5$ cells per well. One set of naïve T cells were cultured in media alone, whereas another set were activated and Th1 polarized by stimulation for 3 days with plate bound αCD3/αCD28 at 10μg/mL and 2.5ng/mL IL-12. At day 3, supernatant was removed and replaced with a 1:2 dilution series of glutamate from 500μM-2μM. One set of Th1 polarized cells and one set of unstimulated cells were given no glutamate as a control. After 2 days, cells were harvested for flow cytometric analysis and the supernatant was taken for IFN-γ ELISA. Proliferation index was calculated as the sum of cells in each generation (peak) divided by the sum of calculated parent cells for each generation [13].

4.3.3 In vitro modulation of type I mGluR signaling

Splenocytes were harvested from a chronically infected mouse and re-stimulated for 6 hours with 10μg/mL αCD3/αCD28 antibodies in 10 μg/mL BFA. Cells were treated either with antibody alone, antibody plus AIDA (mGluR1 inhibitor) [14], antibody plus

MTEP (mGluR5 inhibitor) [15], and media alone. After incubation, cells were harvested and intracellular cytokine staining was performed for flow cytometric analysis. Intracellular cytokine staining was performed using the FoxP3 / transcription factor staining buffer kit.

4.3.4 Adoptive transfer

Leukocytes were extracted from spleen and lymph node of CD45.2+ mGluR5-/- and wild type transfer controls. T cells were isolated using a CD3 negative selection column from R&D Systems and retro-orbitally transferred into a naïve CD45.1+ host. Three days post-transfer both sets of mice were infected. Brain, spleen, lung, liver, peripheral blood, and cervical lymph nodes were harvested and leukocytes were extracted for flow cytometric analysis.

4.3.5 Flow cytometry

Leukocytes were extracted as previously described [16]. Biotinylated antibodies against mGluR5 and mGluR1 were obtained from Bioss Inc, along with streptavidin from ebioscience. Unconjugated antibody against mGluR1 was also obtained from Bioss Inc, along with appropriate secondary antibody from Molecular Probes. Fluorophore-conjugated antibodies against CD3, CD62L, CD4, CD8, KLRG1, CD103, TNF-α, CD45.1, CD45.2, and Annexin V were obtained from ebioscience. Data was acquired

using a FACS Canto from BD Biosciences. Data was analyzed with FlowJo 10.1 and statistical analyses were performed using Prism 6.

4.4 Results

4.4.1 Brain-infiltrating T cells significantly express type I metabotropic glutamate receptors

In order to test whether type I mGluRs were expressed by T cells during chronic *Toxoplasma gondii* infection, and in what proportion, flow cytometric analysis of the brain, spleen, and lungs of wild type mice was conducted at 5 weeks post infection. A significantly ($p<0.05$) higher percentage of type I mGluR expression was observed in CD4+ and CD8+ T cells of the brain, with a smaller but substantial population in the lungs (Fig 4.1). Within each subset, the percentage of T cells expressing mGluR1 was similar to the percentage of T cells expressing mGluR5. CD8+ T cells were 65.3±9.2% mGluR1+ and 63.9±8.1% mGluR5+ respectively (Fig 4.1A-B), whereas CD4+ T cells were 44.4±8.6% mGluR1+ and 46.1±87.1% mGluR5+ (Fig 4.1C-D). Furthermore, nearly all mGluR1+ T cells were also mGluR5+ (Fig 4.2). This double positive population comprised 63.9±9.3% of the CD8+ population (Fig 4.2A), and 44.9±8.1% of the CD4+ population (Fig 4.2B).

Next, the differential expression of type I mGluRs between the CD103+ and KLRG1+ subsets was tested by flow cytometric analysis. We have recently described CD103+

CD8 T cells in the brain of chronically infected mice as exhibiting a transcriptional profile consistent with tissue resident memory T cells, or T_{RM}. Therefore, expression of CD103 defines a distinct memory population, whereas expression of KLRG1 defines a terminally differentiated effector population [16]. In this way, a comparison of type I metabotropic glutamate receptors between these subsets is a useful proxy for a memory versus effector T cell comparison. The proportion of cells that were either CD103+ or KLRG1+ between the mGluR1+ mGluR5+ and mGluR1- mGluR5- subsets was compared. In the mGlur1+ mGluR5+ subset, no substantial population of KLRG1+ T cells was observed whereas there were a significantly greater ($p<0.01$) percentage of CD103+ T cells compared to the mGluR1- mGlur5- subset (Fig 4.2). CD8+ mGluR1+ mGluR5+ T cells were 64.7±4.9% CD103+ as compared to their mGluR1- mGluR5- counterparts, which were 32.7±1.9% CD103+ (Fig 4.2A). Similarly, CD4+ mGluR1+ mGluR5+ T cells were 34.5±2.8% CD103 whereas CD4+ mGluR1- mGluR5- T cells were 10.8±1.0% (Fig 4.2B). Taken together these data support significant expression of type I metabotropic glutamate receptors on T cells at a site of known glutamate accumulation. Furthermore, the increased expression of CD103 in T cells positive for type I mGluRs supports glutamate accumulation as a cue for accumulation of T_{RM} with the brain.

4.4.2 Glutamate suppresses activated T cell function in vitro

The profound expression of type I mGluRs on brain-infiltrating T cells suggested that T cells can modulate expression of these receptors in response to changes in glutamate

concentration. Therefore, we next tested the effect of culturing activated T cells in the presence of specific extracellular glutamate concentrations *in vitro*. T cell activation was confirmed by downregulation of CD62L in proliferated CD3+ cells (Fig 4.3A). To determine percentage of apoptotic cells in the presence of extracellular glutamate, annexin V staining was performed. An increase (3.2%-25.2%) in the annexin V+ T cell population was observed beginning at 30μM extracellular glutamate and the increased percentage was consistent up to 500μM (Fig 4.3B). This also corresponded with an increase in the percentage of CD3+ mGluR5+ cells to about 40% of the total CD3+ population (Fig 4.3C). The upregulation of mGluR5 corresponded to a decrease in IFN-γ production from 1607±85ng/mL at 15μM glutamate to 979.6±32.4ng/mL at 30μM (ns), however production of this cytokine was increased to 2038±200ng/mL starting at 125μM glutamate (Fig 4.3D). Surprisingly this did not correspond to a change in proliferation index as measured by CFSE dilution, rather glutamate treatment resulted in a tonic decrease in proliferation index regardless of concentration (Fig 4.3E). These data support a role for modulation of T cell function in response to changes in glutamate concentration.

4.4.3 Type I metabotropic glutamate receptor signaling modulates cytokine production in activated T cells

In order to determine whether the changes observed in proliferation and cytokine production in response to glutamate were due to signaling through type I metabotropic glutamate receptors, we re-stimulated splenocytes from chronically infected mice in the

presence of either an mGluR1 (AIDA) or mGluR5 (MTEP) receptor antagonist. For both treatments, a non-significant decrease was observed in the percentage of T cells producing TNF-α compared to those treated with αCD3/αCD28 antibodies alone (Fig 4.4A). TNF-α production was above background as measured in the untreated wells. This data suggests that modulation in T cell function in response to changes in glutamate concentration could be mediated through type I metabotropic glutamate receptor signaling pathways.

4.3.4 mGluR5 signaling mediates efficient infiltration of CD4+ T cells into brain

In order to determine the function of metabotropic glutamate receptor signaling on T cells *in vivo*, we adoptively transferred CD45.2+ mGluR5-/- T cells into naïve CD45.1+ hosts. At 5 weeks post infection a significantly smaller percentage of mGluR5-/- T cells were observed in the brain as compared to their wild type counterparts (2.6±0.1% versus 5.0±0.3%, $p<0.05$) (Fig 4.4B). Although similar trends were observed in absolute cell counts, these differences were not statistically significant (Fig 4.4C). No significant difference was observed in the percentage of CD62L+ mGluR5-/- T cells as compared to the wild type controls (Fig 4.4D). Taken together these data suggest that *in vivo*, mGluR5 is dispensable for T cell activation but is required for efficient infiltration of parasite-specific T cells into the brain.

4.5 Discussion

Our data support the view that 1) brain-infiltrating T cells express the type I metabotropic glutamate receptors mGluR1 and mGluR5, 2) activated T cells exhibit changes in cytokine production and proliferation in response to changes in extracellular glutamate concentration *in vitro,* and 3) signaling through mGluR5 could mediate efficient brain infiltration *in vivo* in conjunction with other GPCRs such as CCR7 and CXCR3 [17]. Most notable in our results is the disparity in effect of glutamate on T cell function comparing *in vitro* to *in vivo* experiments. Our *in vitro* results would seem to support the Pacheco et al. model where these receptors serve either a co-inhibitory or co-stimulatory role, whereas our *in vivo* data suggests these could in fact be acting as a kind of chemokine receptor. These disparate results could perhaps be due to the local nature of glutamate excitotoxicity during chronic *T. gondii* infection. Although glutamate can have a suppressive effect on proliferation and cytokine production, initial T cell activation occurs within the lymph nodes, a site low in extracellular glutamate [4]. In contrast, the accumulation of glutamate as a local cue for T cell infiltration via mGluR5 is biologically plausible and supported by studies demonstrating chemotaxis via the ionotropic glutamate receptor iGluR3. Furthermore, although we link inhibition of type I mGluRs to a decrease in cytokine production [18], our data does not reveal a profound effect and would need to be confirmed in future studies, perhaps with adoptive transfer of CFSE labeled mGluR5-/- and mGluR1-/- T cells. In conjunction with this, RNA-Seq analysis of type I mGluR+ T cells could provide functional data by revealing the unique transcriptional signature of T cells expressing these receptors.

The apparent increased percentage of CD103+ T cells expressing type I mGluRs raises the intriguing possibility that glutamate acts as a signal to promote tissue residency. Although expression of CD103 and CD69 define a T cell population with a T_{RM} transcriptional profile the role of CD103 in tissue retention is unclear due to the low abundance of E-cadherin within the brain. Binding of metabotropic glutamate receptors to glutamate could serve as a tissue specific signal for the retention of the CD103+ memory subset within the brain [12]. Future studies might examine the heterogeneity of glutamate accumulation within the brain parenchyma, e.g. is glutamate more highly concentrated in regions lacking expression of GLT-1 [5]? Of course, our current data does not exclude the possibility that as non-circulating cells, CD103+ T cells are simply upregulating type I mGluRs in response to increased extracellular glutamate concentrations and this is not inherently a mechanism of residency. Future studies would need to examine the kinetics of mGluR expression in this subset and how mGluRs as determinants of tissue residency might overlap with their putative role as chemokine receptors *in vivo*.

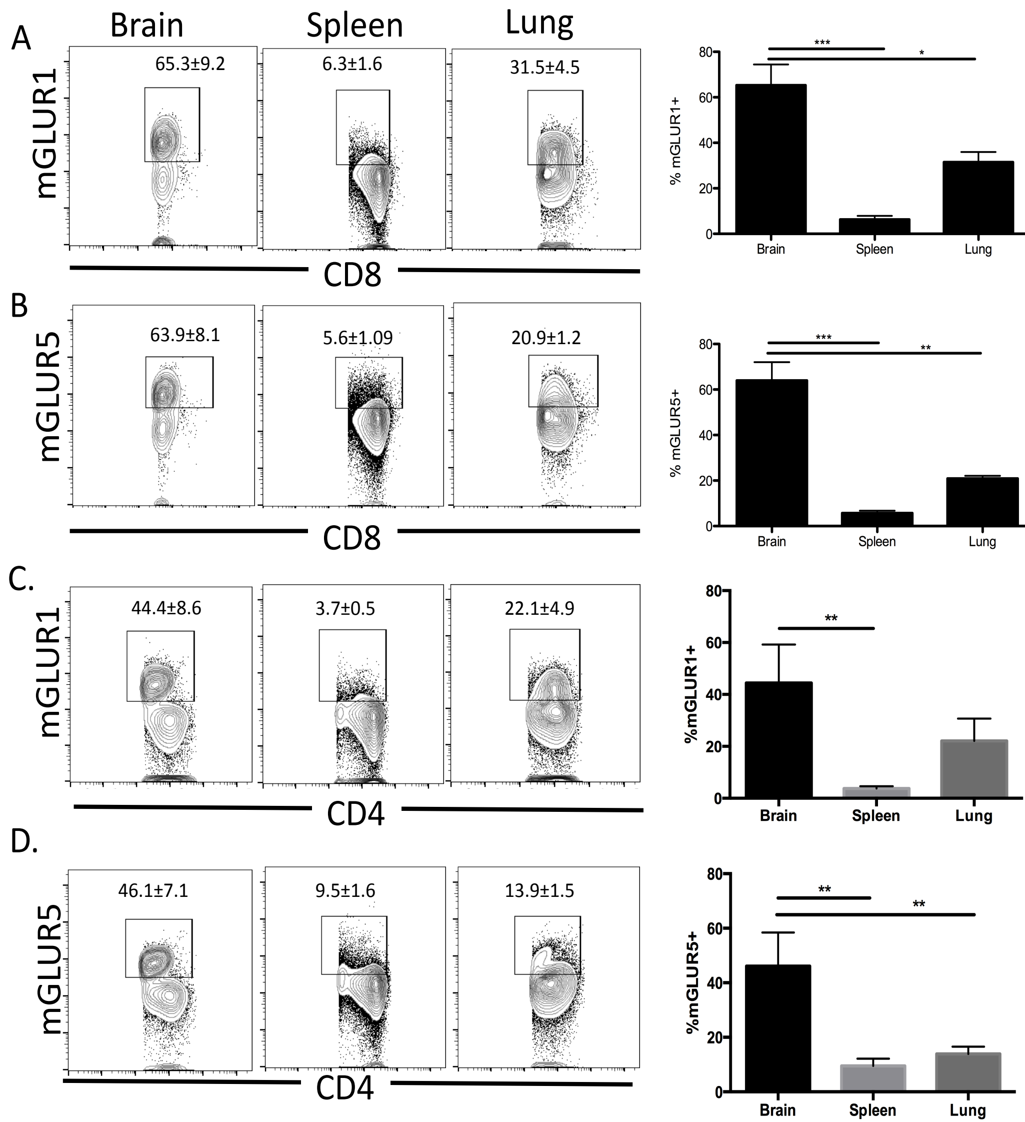
Glutamate exitotoxicity is a ubiquitous response of the diseased or damaged CNS. In addition to *Toxoplasma gondii* infection, glutamate excitotoxicity has been reported in Alzheimer's disease, epilepsy, multiple sclerosis, and cerebral malaria [5,1-3]. In the case of *Toxoplasma gondii* infection it is clear that although this is a disease state, there is a kind of homeostasis established between the host and the parasite. Within a context of

profound pathology due to glutamate excitotoxicity, T cells are still able to mount a protective response to the parasite. Our data suggest it may even be a critical local signal for these cells. Yet the suppressive effects of glutamate observed in this study may serve as a barrier to clearance of the parasite. We hope this will serve as a springboard for further studies that examine the interactions between local metabolites, such as glutamate, and the immune system. Our study indicates that T cells are sensitive to such perturbations in the extracellular metabolic environment. Future studies could certainly have a translational impact for disease states of the CNS.

4.6 Figures and Legends

Figure 4.1: Expression of group I metabotropic glutamate receptors

Expression of metabotropic glutamate receptors by T cells in the brain and periphery. A) Expression of mGluR1 by CD8 T cells in the brain, spleen, and lung at week 8 post infection. B) Expression of mGluR5 by CD8 T cells in the same C) mGluR1 in CD4 T cells D) mGluR5 in CD4 T cells



A
B
C.
D.
Brain
Spleen
Lung
mGLUR1
mGLUR5
CD8
CD4
65.3±9.2
6.3±1.6
31.5±4.5
63.9±8.1
5.6±1.09
20.9±1.2
44.4±8.6
3.7±0.5
22.1±4.9
46.1±7.1
9.5±1.6
13.9±1.5
% mGLUR1+
% mGLUR5+
%mGLUR1+
%mGLUR5+

Figure 4.2: Expression of CD103 in mGLUR1+ mGLUR5+ T cells of the brain

A) Representative plots and percent expression of CD103 in mGLUR1+ mGLUR5+ CD8 T cells relative to mGLUR1- mGLUR5- CD8 T cells B) Same comparison as in (A), but in the CD4+ subset.

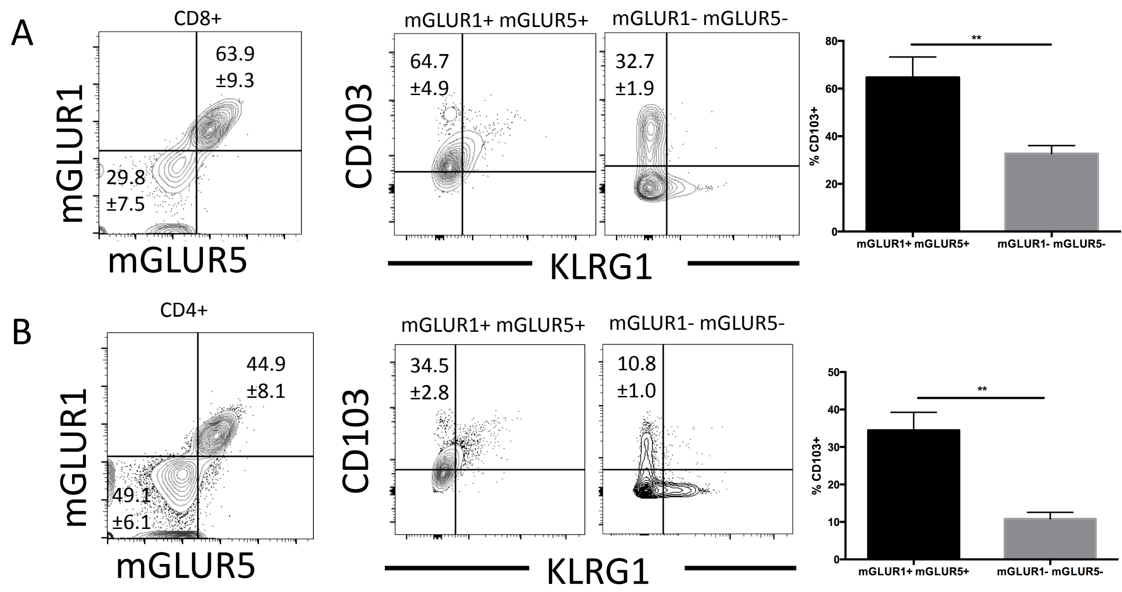
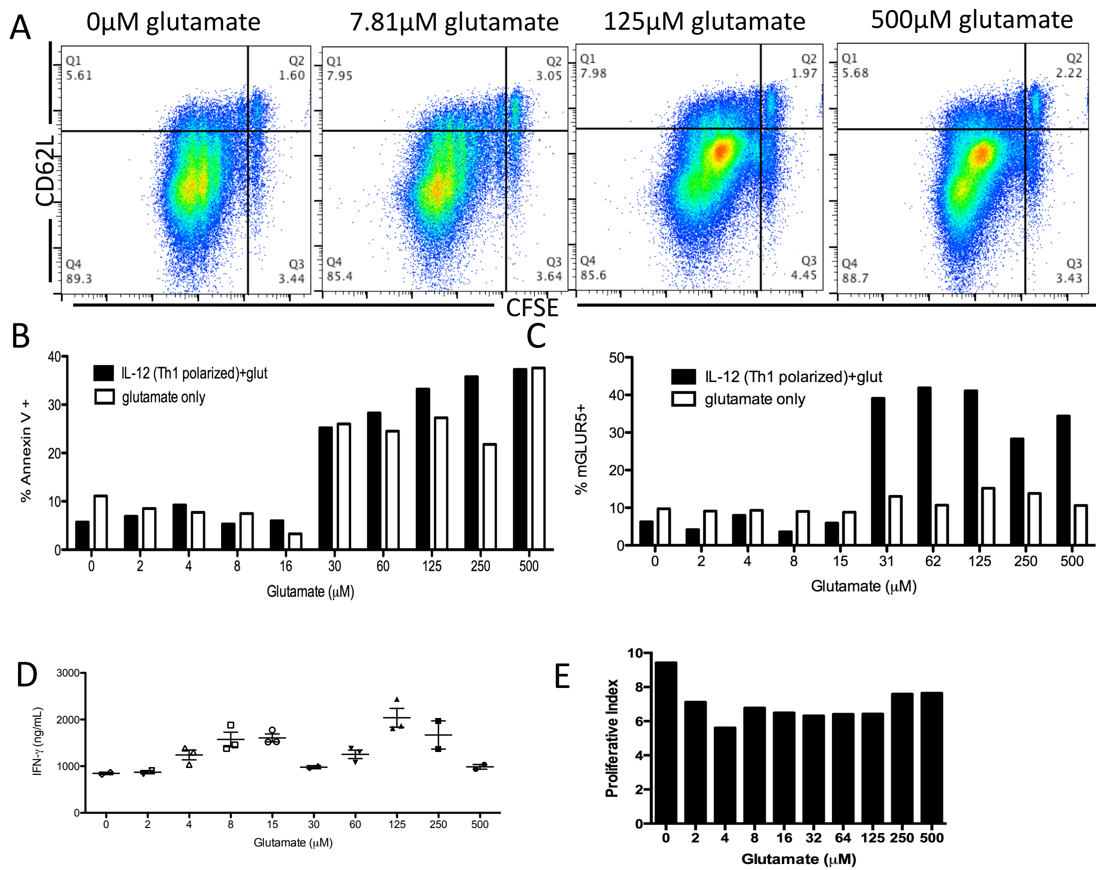

A
CD8+
63.9
±9.3
29.8
±7.5
mGLUR1
mGLUR5
mGLUR1+ mGLUR5+
mGLUR1- mGLUR5-
64.7
±4.9
32.7
±1.9
CD103
KLRG1
% CD103+
mGLUR1+ mGLUR5+
mGLUR1- mGLUR5-
B
CD4+
44.9
±8.1
49.1
±6.1
34.5
±2.8
10.8
±1.0

Figure 4.3: Impact of glutamate modulation on Th1 phenotype and function *in vitro*

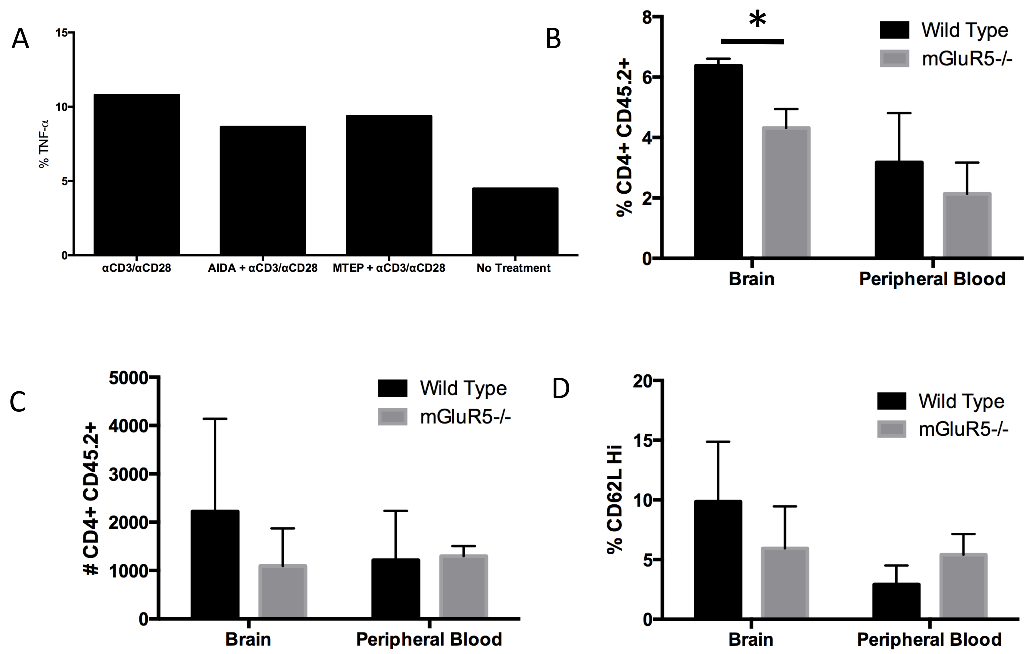
One set of spleen mononuclear cells were cultured in glutamate-free T cell media for 3 days in the presence of anti-CD3, anti-CD28, and IL-12. Another set was cultured in media alone. On day 2, media was removed and replaced with media containing glutamate at the indicated concentrations. A) Expression of CD62L versus CFSE in Th1 polarized cells. B) Expression of Annexin V C) Expression of mGluR5 D) IFN-γ concentration in supernatant E) Proliferation index



A
0μM glutamate
7.81μM glutamate
125μM glutamate
500μM glutamate
CD62L
CFSE
B
% Annexin V +
IL-12 (Th1 polarized)+glut
glutamate only
Glutamate (μM)
C
% mGLUR5+
IL-12 (Th1 polarized)+glut
glutamate only
Glutamate (μM)
D
IFN-γ (ng/mL)
Glutamate (μM)
E
Proliferative Index
Glutamate (μM)

Figure 4.4: Role of type I mGluR signaling in Th1 cell function and phenotype

Cells were cultured in the presence of anti-CD3, anti-CD28, and IL-12 for 2 days, then treated with MTEP (mGluR5 inhibitor), AIDA (mGluR1 inhibitor), or untreated. A) Production of TNF-α for each condition. CD45.2+ mGluR5-/- or wild type T cells were transferred into wiCD45.1+ recipients. B) Percentage of CD45.2+ cells in brain. C) Number of CD45.2+ cells in brain D) Percentage of CD62L+ cells in brain. Gated on live CD3+ T cells. Data is mean ± SEM. *p<0.05


A
% TNF-α
15
10
5
0
αCD3/αCD28
AIDA + αCD3/αCD28
MTEP + αCD3/αCD28
No Treatment
B
% CD4+ CD45.2+
8
6
4
2
0
*
Wild Type
mGluR5-/-
Brain
Peripheral Blood
C
CD4+ CD45.2+
5000
4000
3000
2000
1000
0
Wild Type
mGluR5-/-
Brain
Peripheral Blood
D
% CD62L Hi
20
15
10
5
0
Wild Type
mGluR5-/-
Brain
Peripheral Blood

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CHAPTER FIVE

Conclusions

5.1 Introduction

This dissertation has explored diverse aspects of the T cell response during chronic *Toxoplasma gondii* infection. Yet common themes emerge. Primarily, it is clear that the "local" immune response does not exist in a vacuum. Although the parasite is localized to distinct regions of the brain, there are signals in the global brain and periphery that coordinate the immune response. To illustrate, our data reveals the following:

1) Brain T_{RM} responding to *T. gondii* are not simply associated with the cyst, but also the choroid plexus and perivascular space. We have shown that these cells have an intrinsic capacity to produce IFN-γ and TNF-α and in addition to the direct protective role of these cytokines, it is an intriguing possibility that their production also assists T_{RM} located at strategic entry points to the brain in their role as sentinels.

2) Effector T cells within the brain require expression of CCR7 by peripheral dendritic cells for their optimum maintenance within the brain. Although there is published evidence of dendritic cell infiltration into the brain during chronic infection, the actions of CCR7 in dendritic cells localized to the lymph node are sufficient to restore wild type percentages of KLRG1+ T cells.

3) Accumulation of glutamate within the brain is detected by infiltrating T cells and can modulate their function. Brain-infiltrating T cells uniquely upregulate metabotropic glutamate receptors in response to these increases in glutamate and this is associated with

changes in cytokine production and proliferation. Furthermore, glutamate may act as a chemotactic signal to mediate efficient entry into the brain.

5.2 Future Directions

5.2.1 The role of T_{RM} in the protective response

Chapter two provides evidence that CD103 expression defines a population of T cells that is transcriptionally consist with the tissue resident memory phenotype. We show that such cells have a greater capacity to produce IFN-γ and TNF-α as compared to their CD103- counterparts *in vitro*. Future studies would be needed to examine whether this holds true *in vivo* and the mechanisms of protection mediated by T_{RM}-derived IFN-γ and TNF-α.

There are a few possibilities for the experimental exploration of this question that would involve either enriching for T_{RM} or depleting this population from the brain. In the first case, fingolimod treatment could be a useful strategy. Fingolimod is a competitive inhibitor for S1PR1 and as such prevents T cell egress from the lymph node [1]. It has been used as a treatment for multiple sclerosis for its immunosuppressive effects [2]. Enrichment of T_{RM} via fingolimod treatment is based on the principle that T_{RM} are a population that has downregulated S1PR1 and as such they are non-circulating. Therefore T_{RM} should be insensitive to fingolimod treatment and remain within the brain

after circulating cells have been depleted. Treated mice can then be tested for various readouts of reactivation including parasite burden and sickness behavior.

Another strategy would involve a conditional Cre-Lox strain of mice where Flox sites flank the CD103 gene and Cre-recombinase expression is linked to both the CD3 promoter and expression of, for example, a diptheria toxin receptor. This strategy would be technically difficult but would allow T_{RM} to develop and then be depleted at the chronic stage of infection. Again, similar readouts such as parasite burden and survival could be employed.

Our data also leave open the possibility for studies which examine the potential role of TNF-α in the activation of brain-infiltrating dendritic cells. Indeed, the literature supports a role for local antigen presentation to maintain T_{RM} in neural tissue [3]. We have shown that bead isolated dendritic cells are capable of localizing to the brain and this could be employed to test the role of brain-infiltrating DCs in the maintenance of T_{RM}.

5.2.2 The role of KLRG1+ T cells and CCR7+ dendritic cells in the protective response

In chapter three we provide evidence that CCR7+ dendritic cells in the lymph node are sufficient to maintain a significant population of KLRG1+ effector T cells within the brain. Despite extensive experimentation, we have not been able to establish whether this

has a functional impact on the deficient response to rechallenge observed in CCR7-/- mice. This has been tested using adoptive transfer of wild type dendritic cells into CCR7-/- recipients and subsequently rechallenging the mice to assess survival and parasite burden. It is clear that despite sub-optimal populations of effector T cells in the brain of infected CCR7-/- mice, protection is still ongoing. Furthermore, our data does not address the pleiotropic effects of CCR7 on the KLRG1+ subset including survival and cell migration. Imaging studies may help to clarify the unique sensitivity of this subset to formation of the immunological synapse within the lymph node and the subsequent impact on recruitment to the brain. These data would be further enriched by tracking the developmental pathway of KLRG1+ T cells as compared to other subsets, which could be accomplished through either bone marrow chimeras or adoptive transfer of naïve T cells.

5.2.3 The diverse effects of signaling through type I mGluRs

In chapter four we show that glutamate can modulate cytokine production and profleration in T cells, as well as perhaps serve as local signal for T cell entry. We provide evidence that this is mediated by mGluR5 signaling. Future work could examine the relationship between mGluR1 and mGluR5 signaling and whether these receptors act in a competitive or inhibitory fashion. In addition to small molecule inhibitors, T cells that are mGluR1-/- could also be used. RNA-Seq studies could contribute towards understanding transcriptional differences between T cells which express type I

metabotropic glutamate receptors and those that do not, thus giving some clue to the functional differences between the two groups.

5.3 Discussion

For many immunologists, there is an uneasy awareness that the transcription factors and surface proteins used to identify distinct populations of cells are imperfect delineators. Despite this, they are a useful starting point and in many cases help to define a population of immune cells. There are many studies devoted to understanding the degree to which immune cells commit to a certain fate and the capacity to interconvert between phenotypes [4-6]. One excellent example from studies of the innate response is the evidence of interconversion between macrophages in an M1 (pro-inflammatory) to an M2 (tissue-repair) state and vice versa [7]. Tracking the developmental pathway of the diverse T cell subsets in the brain during *T. gondii* infection would enrich all of the studies in this dissertation. Previous studies have established that naïve T cells have the capacity to differentiate into any subset so cell fate is not pre-determined [8,9]. Furthermore although the process by which T cells are committed to an effector or memory state is not well understood, it is clear that this commitment is stable [6]. However within these categories, whether there is a capacity for interconversion and whether this actually happens is an area of ongoing investigation.

The data in this dissertation brings into focus an analogy of the host-pathogen interactions in chronic *T. gondii* to an ecosystem. No single component of the system, whether it is the tissue, the parasite, or the T cell, is truly independent of the other.

Therefore perturbations in one "compartment" will have significant consequences in another. On the surface this is an obvious conclusion, yet it is easy to adopt a research strategy that treats each component as if it exists within a vacuum. Overall, these studies highlight the interconnectedness of tissue, immune response, and pathogen in the context of *T. gondii* infection and by extension, other models of chronic infection.

5.4 References

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APPENDIX

Supplementary Figures for Chapter 2

Figure S1: CD103+ CD8 specificity and kinetics in the peripheral lymphoid organs

A) CD103+ CD69+ CD8 T cells in the spleen and lymph node. B) Percentage of dextramer+ CD8 T cells in the spleen. Data is representative of two independent experiments with similar results.

Figure S1

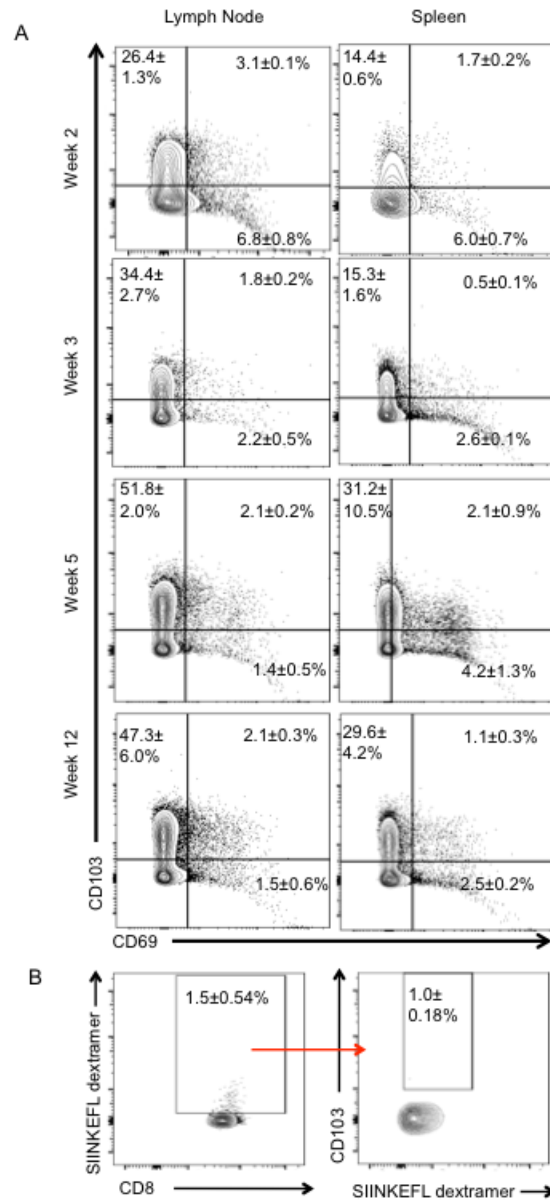


Figure S2: Transcriptional profile of spleen CD103+ population

A) DEGs for the specified comparisons. B) Venn diagram for DEGs for each specified comparison. 290 genes common to the spleen CD103+/spleen CD103- and brain CD103+/spleen CD103+ were input into Metacore. C) Genes under categories "JAK/STAT pathway and inflammation" and "modulation of effector T cell function" were merged into one heatmap. D) Heatmap of the same set of genes as in (C) for the brain CD103+ and spleen CD103+ groups. Individual replicates in heatmap were pooled from n=5 mice. Values in legend are scaled values representative of RPKM. Red indicates a highly expressed gene, blue indicates a gene with a low expression value. DEGs for each comparison were determined according to the following criteria: fold change > 2, FDR < 5%, $p < 0.05$, and mean RPKM > 1.

Figure S2

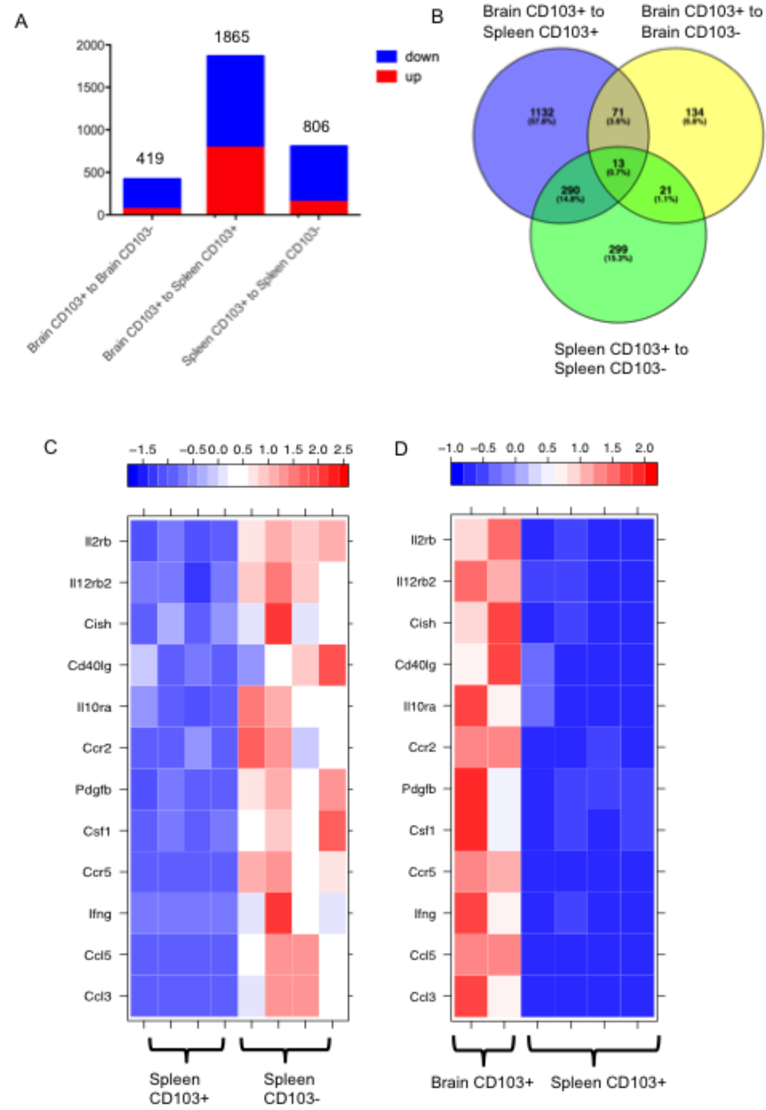
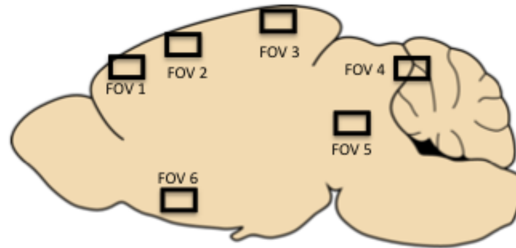


Figure S3: Quantification of distance from cysts to CD8+ CD103+ T cells

A) Schematic of fields of view (FOV) counted in a representative slice. B) Total number of cysts versus total number of $CD8^+$ $CD103^+$ T cells for each field of view analyzed. C) Distance to closest cyst in the field of view for each positive cell. Data is representative of two independent experiments with similar results.

Figure S3

A



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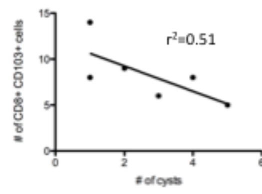

D

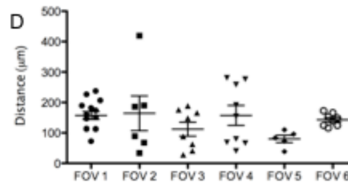

Figure S4: Genes contributing to variability in PCA plots.

A) 50 most variable genes in PC1. B) 50 most variable genes in PC2.

Figure S4

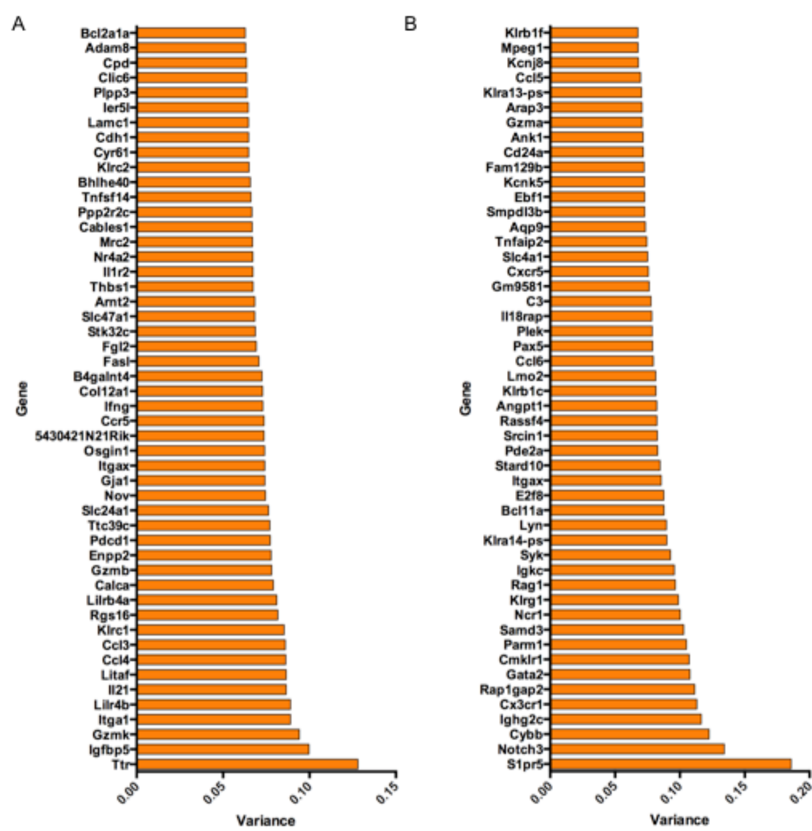

Figure S5: Transcriptional profile of brain CD103+ CD8 T cells

Relative to spleen CD103+ CD8 T cells. A) Venn diagram illustrating unique and shared DEGs for each indicated comparison. B) Heatmap for a subset of the 42 DEGs common to all indicated comparisons, shown in (A). Individual replicates in heatmap were pooled from n=5 mice. Values in legend are scaled values representative of RPKM. Red indicates a highly expressed gene, blue indicates a gene with a low expression value. DEGs for each comparison were determined according to the following criteria: fold change > 2, FDR < 5%, and mean RPKM > 1.

Figure S5

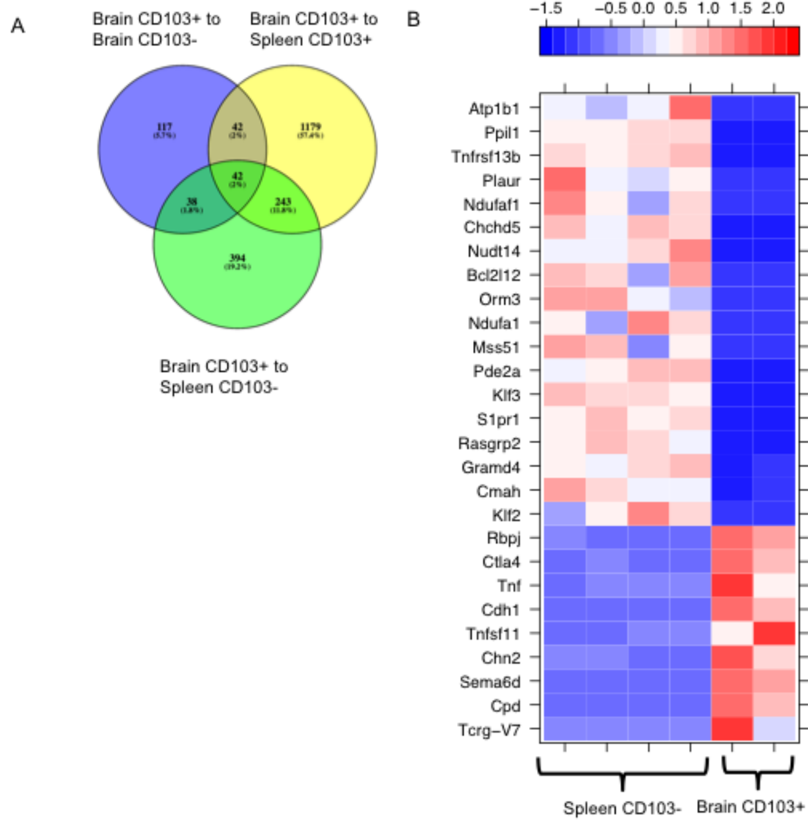

Figure S6: Comparison of T_{RM} from HSV in skin to *T. gondii*.

Microarray data from MacKay et al. was obtained and analyzed via GEO. A) Venn diagram of DEGs in *T. gondii* and HSV for the brain T_{RM} (n=3) relative to spleen T_{CM} and T_{EM} (n=6). B) Comparison of fold changes for the 33 differentially expressed genes common to both models. RNA-Seq DEGs were determined according to the following criteria: fold change > 2, FDR < 5%, $p < 0.05$, and mean RPKM > 1. Microarray DEGs were significant if the p-value and adjusted p-value were less than 5%.

Figure S6

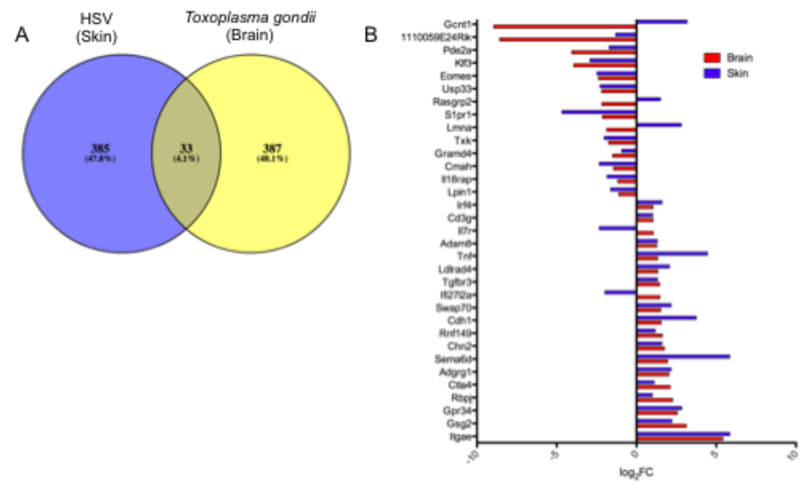

Figure S7: Interaction of brain T_{RM} with CD11c+ cells in the brain

IHC for interactions between CD103+ CD8 T cells and dendritic cells. A) Frontal cortex, 40x. Red arrow indicates CD103+ CD8 T cell in contact with CD11c+ dendritic cell B) Zoomed image of area indicated in A. Representative image of n=3 biological replicates. Scale bar indicates 20μm

Figure S7