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CCR7-Dependent T Cell-Mediated Immunity During *Toxoplasma gondii* Infection

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ABSTRACT OF THE DISSERTATION

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by

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University of California, Riverside, June 2012
Dr. Emma Wilson, Chairperson

Infection with the protozoan parasite, *Toxoplasma gondii* is one of the most prevalent human infections and has been targeted by CDC (Centers for Disease Control and Prevention) as a priority for public health action. Its been recognized as a "silent killer" because of its ability to cause a latent infection in the host brain without any significant symptoms, unless the host is immunocompromised. Thus, it is an important concern to the ever-increasing AIDS (acquired immune deficiency syndrome) community or people on immunosuppressive therapies. In addition, immune responses following Toxoplasma infection in an immunocompetent host represent finely tuned host immunity that grants the parasite survival but robust enough to provide protection against the parasite and thus, avoiding serious illness. A thor-

ough understanding of the key components of this balanced protective immunity may help to design efficient strategies to maximize immune responses for the host benefit.

Chemokines are a family of small molecules secreted by host cells and can induce diverse biological responses in nearby responsive cells. They are important immunomodulatory agents coordinating leukocyte migration during homeostasis and inflammation. This dissertation highlights a lymphoid chemokine receptor CCR7 (C-C chemokine receptor 7) as an essential component of the host protective immunity during both the acute and the chronic phase of Toxoplasma infection although through separate mechanisms. Host protective immunity during Toxoplasma infection is mainly T cell-mediated. This dissertation demonstrates that CCR7 is an absolute requirement for the generation of protective immunity during the acute phase of infection. CCR7-deficient mice succumb in the early phase of infection due to a delay in T cell priming. Further investigation reveals that the requirement of CCR7 to optimize T cell priming is eventually compensated during the chronic phase of infection, however, CCR7 is required for T cell migration *within* the infected CNS that influences their ability to control the parasite burden at this stage. Although there is no defect in the antigen-specificity or the influx of effector T cells in the infected brain, CCR7-deficient mice are unable to mount protective memory responses following secondary infection. This dissertation provides new insights about the hallmarks for protective immunity in such a balanced chronic infection scenario as well as reveals new mechanisms as to how this lymphoid chemokine signaling influences important immunological processes in the peripheral tissues particularly in the CNS.

Contents

List of Tables

List of Figures

Chapter 1

Introduction

1.1 Biology of *Toxoplasma gondii*

1.1.1 Taxonomy and prevalence

Toxoplasma is considered to be the most widespread protozoan parasite in the world, capable of infecting virtually any nucleated mammalian or avian cells [52][53][21]. This obligate intracellular pathogen belongs to the phylum *Apicomplexa*, which includes a number of important human and animal pathogens such as *Plasmodium* (the cause of malaria) and *Eimeria* (causes chicken coccidiosis) [21]. So far, *Toxoplasma gondii* is the only species assigned to the genus Toxoplasma. It was first described in 1908 by Nicolle and Manceaux, in the laboratory of Pasteur Institute in Tunis and by Splendore, in Brazil [21]. The name *T. gondii* is derived from the Greek word "toxon" (meaning "arc") that refers to the crescent shape of the organism, *plasma* from life and "gondii" from the North African rodent (*Ctenodactylus gundii*) from which this parasite was isolated [21]. The findings that Toxoplasma causes pathology in humans or warm-blooded animals have led to extensive research of this parasitic disease in the last three decades.

T. gondii infection in humans is extremely common, over half of the world's population has been exposed to this parasite. The prevalence of toxoplasma infection depends on age, geographical area and food habit with an overall prevalence rate of 10-30% in the US and up to 80% in parts of Europe and South America. The ability to infect a broad range of hosts, invade biological barriers (e.g., eye, central nervous system and placenta) and a capacity to

pass through vertical (mother to fetus) and horizontal (ingestion of oocyst or tissue cyst) transmission is thought to be the "secret" behind this parasitic success [51].

1.1.2 Life cycle and pathogenesis

The infective stage of *T. gondii* is the oocyst or tissue cyst. The oocyst is derived from their sexual reproduction in the stomach of domestic and wild cats, which makes these animals the parasite's only primary host [21][51]. Oocysts excreted from the primary host contaminate soil or water and represent a leading cause of deaths attributed to foodborne illness in the United States. Ingestion of oocysts or tissue cysts (from improperly cooked meat or unwashed vegetables) gives rise to fast replicating tachyzoites that disseminate rapidly throughout the body leading to a systemic infection, however, in immune competent hosts, the parasites are eventually controlled by production of the effector cytokine, IFN-γ [68]. This also initiates the conversion of tachyzoites to slow replicating, cyst forming bradyzoites, enabling *T. gondii* to exist chronically for the lifetime of the host [21][119]. In mice, the tissue cysts are predominantly found in the brain, however, in other hosts, cysts are also found in the eyes, heart, muscles, liver and lungs. In the chronically infected brain, these tissue cysts are mainly located in the frontal cortex, constantly trying to reactivate to tachyzoites [42][191]. A persistent immune response is required in the CNS (Central Nervous System) to keep cysts from reactivating in the brain and the development of pathology [119][202].

Toxoplasma infection is usually subclinical, although it may occasionally cause mild nonspecific symptoms such as fever, rash and lymphadenopathy (swollen/enlarged lymph

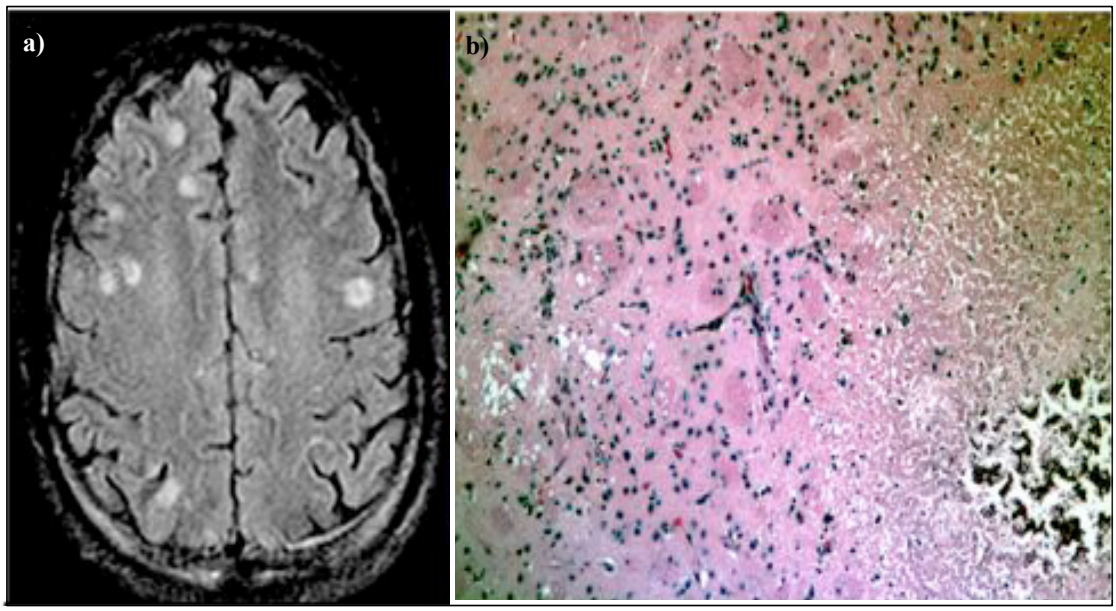


Figure 1.1: Examples of *T. gondii* induced pathology in the brain.

a) Gadolinium-enhanced MRI (Magnetic Resonance Imaging) of HIV patient with re-activating Toxoplasmosis illustrated by ring-enhancing lesions in the brain (source: http://dept.washingtom.edu/hivaids/oit/case3/discussion.html); b) H&E stained brain section from NFκB$^{-/-}$ mouse showing proliferating parasites, necrosis and cell infiltration in the brain.

nodes). As an opportunistic pathogen however, clinical disease is most commonly found in immune compromised individuals or in maternal-fetal transmissions resulting from an acute infection of the expectant mother [21][51]. Depending on the stage of pregnancy when the acute infection occurs, congenital toxoplasmosis may lead to spontaneous abortion or severe neurological disorders in the newborn. Infection with *T. gondii* can cause chorioretinitis

(inflammation in the choroid and retina of the eye) even in immune competent individuals [84]. The most serious clinical manifestation of Toxoplasmosis is intracerebral focal lesions leading to life threatening toxoplasmic encephalitis (TE) (inflammation in the brain) that has been documented in people with immune deficiencies or those on immunosuppressive therapies for organ transplants or cancer [52]. In fact, infection with *T. gondii* has emerged as the most common cause of CNS opportunistic infection in patients infected with human immunodeficiency virus (HIV) leading to TE characterized by ring-enhancing lesions (Figure 1.1), profound neurological symptoms and is often fatal if left untreated [119]. Although the current drugs (pyrimethamine and sulfonamides) can control the tachyzoites along with some toxic side effects, such treatment fails to completely eradicate the chronic bradyzoite cysts. Thus, life-long therapy is needed to prevent reactivation of cysts in immune compromised patients [90].

1.1.3 Immune responses during the acute phase of infection

Toxoplasma actively penetrates the host cells to establish a nonfusigenic compartment called the parasitophorous vacuole within which they multiply, rupture and infect contiguous cells. Toxoplasma can be transported by the migrating antigen presenting cells through the lymphatics or the bloodstream and rapidly disseminate into the deep tissues [111]. Immediately after the first host-parasite contact, *T. gondii* activates macrophages, NK (natural killer) cells or stromal cells (fibroblasts, epithelial or endothelial cells) to secret pro-inflammatory cytokines and chemokines [125][189]. IL (interleukin)-12 and IFN-γ are two crucial cytokines

for the generation of protective immunity during this acute phase of infection [68][215]. The first line of defense is mediated by IL-12 production from macrophages and several DC subsets namely plasmacytoid (pDCs) and conventional dendritic cells (cDCs) [149][217][118][159] that are crucial to trigger IFN-γ production by NK (Natural Killer) cells [193]. Chemokines such as MCP-1 (monocyte chemotactic protein 1) or IL-8 released from the site of infection rapidly recruit macrophages and neutrophils. Neutrophils secret IL-12 and may also participate to attract other immune cells at the site of infection [23]. However, a recent study has demonstrated neutrophils are not a critical source of IL-12 in vivo, rather inflammatory monocytes recruited to the site of infection are crucial to control *T. gondii* infection during the acute phase of infection [54]. TNF-α is another important pro-inflammatory cytokine produced by activated macrophages, monocytes or T cells. TNF-α induces nitric oxide synthase (iNOS) production from IFN-γ-activated macrophages. iNOS generates abundant NO (nitric oxide) which is a potent antimicrobial agent [1]. However *T. gondii* has been found to modulate macrophages and suppress NO production as a possible mechanism to escape killing by this phagocytic cells [74].

The innate immune recognition pathways that trigger IL-12 production have recently been addressed. Toxoplasma expresses ligands (heat shock proteins, profilin) for several TLRs (toll like receptors) such as TLR2, TLR4 and TLR11 [217][3][133]. However, TLR11 is expressed on certain mammalian species including mice but not in humans suggesting TLR11-mediated recognition is not crucial for the protective immunity [153]. *T. gondii* also generates cycophilin-18 that acts as a ligand for the chemokine receptor CCR5 and induces

IL-12 production from DCs [3]. Thus, during *T. gondii* infection, chemokine receptors and TLRs may work together and activate antigen-presenting cells (APCs) to present antigens to T cells via their MHC (major histocompatibility class) molecules. Peptides are presented on Class II MHC molecules to CD4 T cells and CD8 T cells recognize peptides presented on Class I MHC molecules. Antigen-specific activation (a process known as T cell priming) differentiates naive CD4 T cells into effector T cells with different cytokine profiles (Th1, Th2 and Th17) [151]. Toxoplasma infection induces a Th1 dominant adaptive immune response mediated by IFN-γ production from CD4 and CD8 T cells [66][43]. IFN-γ contributes in several anti-toxoplasma effector mechanisms that include NO or reactive oxygen intermediates (ROI), intracellular lysis of the parasites in monocytes via CD40 ligation and IFN-γ inducible GTPases [32][81][134][194]. CD8 T cells and NK cells have been thought to contribute to host defense via the release of cytotoxic granules (perforin, granzymes) that induce apoptosis of the infected cells. However, during the acute phase of infection, the perforin- granzyme pathway seems to have a limited role in controlling parasite replication [45]. Despite severely impaired CTL (cytotoxic T lymphocytes) and NK lytic activity, vaccinated perforin knockout mice are resistant to challenge with the virulent strain RH, which normally causes a lethal acute infection [45]. The production of type 1 cytokines peaks by 7 days post infection and is controlled by anti-inflammatory cytokines, IL-10 ad TGF-β from macrophages and regulatory T cells (Treg) to avoid systemic pathology during acute toxoplasmosis [189][70]. Within two weeks, these effector mechanisms successfully limit the

tachyzoite expansion in the peripheral tissues and convert them to the cyst forms that are predominantly found in the brain (Figure 1.2).

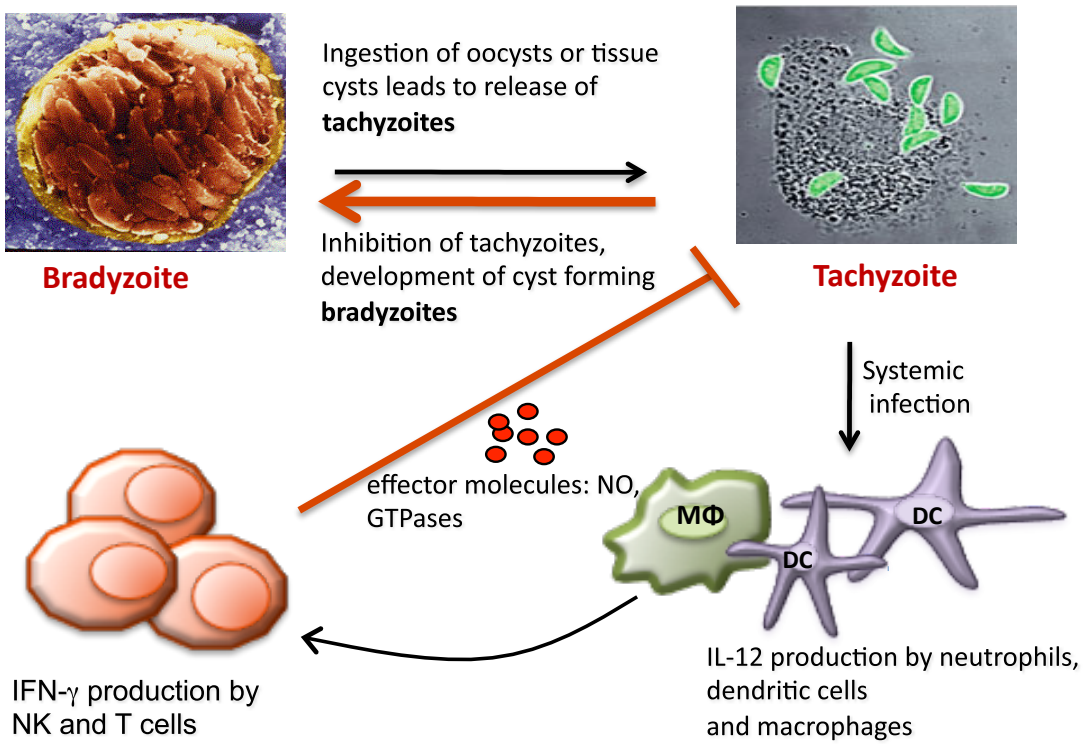


Figure 1.2: Host immune responses and stage conversion of Toxoplasma parasites.

Following infection, fast replicating tachyzoites disseminate rapidly throughout the body leading to systemic infection. The host mounts robust immune responses that involve antigen presentation by macrophages and DCs that induce IFN-γ production, a major cytokine with anti-toxoplasma effector mechanisms. This initiates the conversion of tachyzoites to slow replicating, cyst forming, bradyzoites enabling *T. gondii* to exist chronically for the lifetime of the infected host.

1.1.4 Immunity during chronic infection in the brain

T. gondii elicits a robust immune response, however, it follows several strategies to escape the host immune system and establish a persistent infection in the CNS of the host. During this chronic stage of infection, CNS resident cells and peripheral immune cells collaborate to mount immune responses that are efficient enough to limit tachyzoite emergence but sufficiently controlled to reduce immunopathology. Toxoplasma has been thought to traverse the BBB (Blood Brain Barrier) via highly migratory infected immune cells in a "Trojan horse" manner [109]. Cells expressing CD11b with or without CD11c are strong candidates for the intracellular transport of *T. gondii* across the BBB [109]. Once Toxoplasma is in the brain, they invade endothelial cells, neurons and glial cells. Astrocytes are the predominant cell types found infected in the CNS [207]. At this stage, resistance to *T. gondii* depends on IFN-γ production that stimulates multiple cell types in the brain to inhibit intracellular parasite replication [216]. IFN-γ is predominantly produced by CNS infiltrating T cells, although, IFN-γ production has been reported from blood derived macrophages and microglia in the infected brain [185]. Both CD4 and CD8 T cells produce IFN-γ in mice, CD4 T cells also induce CD8 T cell immunity against the parasite [29]. Thus, both CD4 and CD8 T cells are required to control parasite replication in the brain, which is evident from HIV patients or mouse T cell depletion studies [66][187]. Other than IFN-γ production, perforin and granzymes also play a major role in the protective immunity during the chronic phase of infection. Perforins disrupt the membrane of the target cells and granzymes (death-inducing serine proteases) enter the target cells in a perforin-dependent manner. CD8 T cells have been

demonstrated to control cysts in the brain using the perforin-granzyme pathway [45][186]. CNS infiltrating T cells also take part in several other mechanisms important for the host protective immunity including the production of IL-4, IL-10 and IL-27 the absence of which may lead to the development of immunopathology [139][209][183]. In *T. gondii*-susceptible mouse strains, IFN-γ and TNF-α -dependent iNOS production is also needed to control parasites during chronic infection in the brain [173]. In the absence of iNOS, mice succumb 3-4 weeks following infection due to increased number of cysts and severe TE [172]. Other cytokines such as IL-6 and TNF-α produced by T cells also play important roles to prevent TE [67].

Resident glial cells during Toxoplsma infection

Along with CNS infiltrating T cells, several studies have demonstrated a fundamental requirement of non-hematopoetic CNS resident cells for the protective immunity during the chronic phase of infection [216]. CNS resident astrocytes and microglia are potential candidates. Astrocytes are important structural components of BBB and also "nurse" cells providing nutrients to neurons [138]. Though their immune role is not well understood, among other CNS resident cells, astrocytes are considered as one of the most responsive cell types following *T. gondii* infection [207]. Astrocytes are able to produce both pro- and anti-inflammatory cytokines and thought to be the primary producers of chemokines in the CNS during *T. gondii* infection [182][88]. Also, astrocytes have recently been shown to be necessary for protective immunity against TE [50]. At the peak of the acute infection, systemic cytokines are able to

activate astrocytes even prior to the entry of parasites into the brain that is thought to happen around two weeks post-infection [207]. IFN-γ-stimulated astrocytes control the parasite in a manner uniquely dependent on the activation of IFN-γ-induced GTPase (IGTP) and are thought to minimize dissemination of the parasites in the brain [207][181].

Microglia are the resident macrophage population in the brain. They are considered to be the most immune responsive cell population in the inflamed brain. Microglia are highly activated in the infected brain and can contribute in controlling parasites by phagocytosis and production of oxygen and nitrogen free radicals [100]. However, their requirement in the immune protection during Toxoplasma infection has not been demonstrated yet. Toxoplasma infected microglia do not upregulate MHC class II molecules or co-stimulatory molecules, rather in contrast to astrocytes, they exhibit a hypermotility phenotype [41]. Their migratory phenotype and increased sensitivity to T cell-mediated killing following infection may facilitate rapid dissemination of parasites within the CNS [41].

Cell migration and chemokine production in the infected brain

As in other CNS inflammation models, Toxoplasma infection increases the BBB permeability [109] and recruits several immune cell populations from the peripheral circulation. Among them macrophages and migratory DCs infiltrated in the infected brain are considered to play an important role in antigen presentation and IL-12 production. The infected CNS also allows a continuous recruitment of T cells to maintain this latent stage of infection. CD4 and CD8 T cells are both important in limiting parasite replication; CD4 T cells are needed for long-term

protection [14][69][87]. IL-10 producing infiltrating macrophages and CD4 T cells (Tregs) are also crucial to prevent immunopathology due to uncontrolled production of Th1 cytokines [209] [93] [137].

Following entry, the migration of T cells within the dense CNS parenchyma may be aided by infection-induced proteases [31]. However, the mechanisms that control T cell migration into and within the CNS during Toxoplasma infection or in other models of CNS inflammation is not well understood and currently an area of immense interest. A previous study demonstrated that antigen-specific T cells in *T. gondii* infected brain do not seem to associate with the cysts rather they cluster in the vicinity of isolated parasites that may be derived from cyst rupture [171]. A study by Wilson *et al.,* 2009 investigating the migration behavior of parasite-specific CD8 T cells has revealed their distinct patterns of migration in the infected brain. There are parasite-specific T cells that demonstrate a constrained pattern of migration whereas some CD8 T cells are highly migratory in the brain [206]. This study has also demonstrated the presence of an infection-induced network of fibers in areas of parasite replication and inflammation in the brain [206]. Parasite-specific T cells have been shown to migrate along these fibers visualized by 2 photon live microscopy. However, the composition of these networks is still under investigation.

For decades, chemokines and their receptors have received a great deal of attention as potential candidates to contribute to immune cell recruitment in the inflamed CNS. Chemokines are proteins or cytokines, named due to their ability to induce chemotaxis (cell migration towards the source of chemokines) of nearby cells that express the corresponding recep-

tors. The ability of chemokines to convey remarkably versatile but context-specific signals highlights them as key modulators of immune responses generated following diverse pathogenic or non-infectious insults. Multiple chemokines such as CCL2, CXCR2, CXCL10 and CXCL12 have been implicated in immune cell trafficking to the inflamed CNS [208]. CCR2-CCL2 interactions recruit neutrophil and certain DCs in the CNS [15]. There are evidences indicating the involvement of chemokines in immune cell migration in the brain during Toxoplasma infection [109]. Brain endothelial cells have been shown to induce adhesion molecules and chemokines following *T. gondii* infection *in vitro* [109]. MCP-1 production by infected brain endothelial cells and other cell types may play a role in recruiting peripheral macrophages in the brain [109]. Although, infected DC migration is thought to be chemokine dependent, the exact chemokine is not known yet [111]. Chemokine receptors that are known to be involved in T cell chemotaxis such as CCR7 and CXCR3 are found upregulated in the brain during the chronic phase of infection [8]. In addition, CCL21, a ligand for chemokine receptor CCR7 is upregulated in the *T. gondii* infected brain parenchyma [206]. CNS infiltrating T cells are often found in close association with CCL21 expression in the infected brain [206]. These observations have drawn significant attention to this lymphoid chemokine as a potential candidate to contribute to T cell migration in the infected CNS.

1.2 CCR7 during inflammation and immunity

CCR7 is an extensively studied G-protein coupled receptor, expressed mainly on semi-mature and mature dendritic cells (DCs), naive B and T cells and central memory T cells [158][62] [142][166][188]. CCR7 ligands, CCL19 and CCL21 are constitutively expressed on the high endothelial vessels (HEVs) of the lymph nodes (LN) and by fibroblastic reticular cells (FRC) and follicular dendritic cells (FDC) forming the conduits that guide T cell migration in the LN [11][144]. As such CCR7 is the principal chemokine receptor that controls DC-T cell antigen-specific interactions facilitating optimal priming in the lymph node (LN) (reviewed in [62][132][196][148]) (Figure 1.3). Other than its role in development and maintenance of secondary lymphoid organs (SLOs), CCR7 signaling is involved in a number of immunological processes such as the generation of thymocytes [117][71], central and peripheral tolerance [34][82], Treg function [126][174][104] and T cell homeostasis [154] (Table 1.1). This section focuses on these properties of CCR7/CCL21 signaling and integrates the knowledge of the role of CCR7 in coordinating immune responses between secondary lymphoid organs and peripheral tissue microenvironments particularly in the CNS.

1.2.1 Signaling properties of CCR7 and its ligands

CCR7 is a seven trans-membrane domain receptor protein coupled with pertussis toxin-sensitive Gai components. Signaling of immobilized CCR7 ligands through their receptor causes lymphocytes to go from a “rolling” state to “arrest” on endothelial surfaces and sub-

Table 1.1: Summary of roles of CCR7

Role in the immune system	Mechanism	References
Thymic architecture and function	CCR7 is involved in the recruitment of fetal haematopoietic progenitors and coordinate migratory events of thymocytes at their different maturation and selection ages in the thymus	[117] [127] [35] [197] [152]
Regulatory T cell function	CCR7 is required for Treg cell homing and positioning within the paracortical LN area; Treg function is impaired in CCR7-deficient mice	[126] [174] [104]
T cell priming	Ag-experienced DCs (enter via afferent lymphatics) and T cells (via HEVs) use CCL21 coated stromal networks in the T cell zone to interact with each other and generate the effector T cell pool	[11] [144] [211] [10]
Lymphocyte recirculation in peripheral tissues	CCR7 and CCL21 contribute to T cell recruitment and egress from peripheral tissues, the pleural and the peritoneal cavity	[26] [120] [200] [39] [85]
Peripheral tissue-resident DC trafficking	Tolerogenic (homeostatic) DCs and also inflammation-induced DCs acquire CCR7 expression as they exit from peripheral tissues to present antigens in the draining LN	[142] [166] [91] [92]
DC survival, maturation and antigen uptake	CCR7-mediated signaling positively regulates the survival and the rate of endocytosis of the mature DCs; CCR7 also induces dendritic cytoplasmic extensions that may contribute to their ability to present antigens	[213] [212] [168]
T cell homeostasis	CCR7 ligands support the survival and homeostatic expansion of naive T cells	[154] [116]
B cell help	CCR7 upregulation mobilizes follicular B cells towards the T cell zone in the LN to receive “help” from CD4 helper (Th) cells	[170] [80]

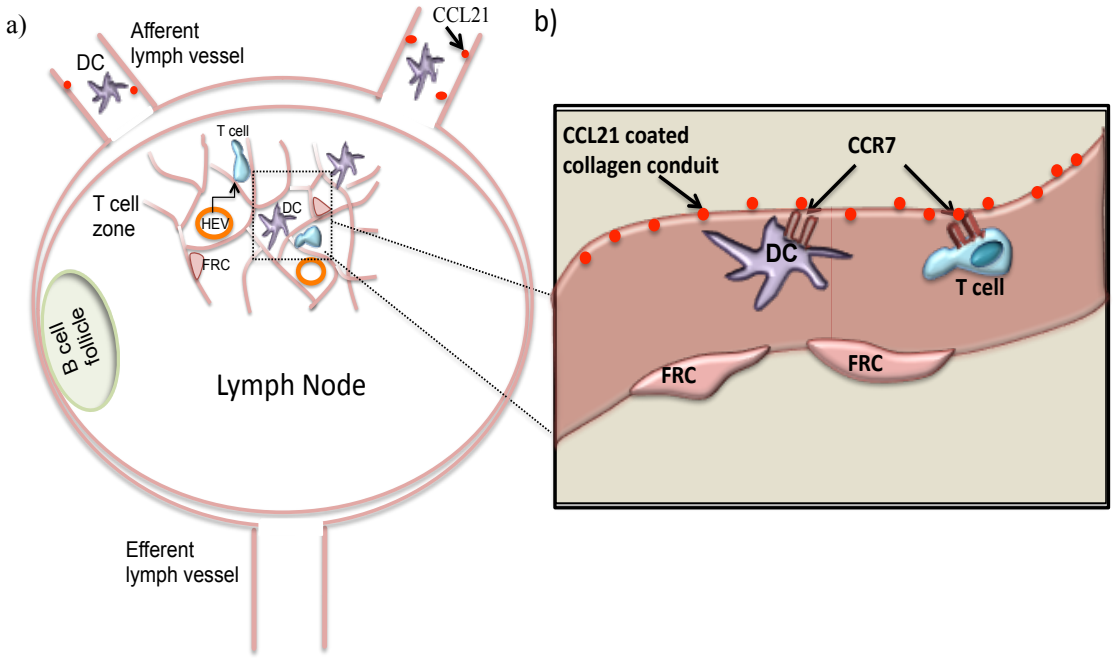


Figure 1.3: CCR7 in guiding lymphocyte migration in the lymph nodes.

a) Mature DCs enter into the lymphatics in a CCR7-dependent manner. Then they are passively carried with the afferent lymph into the draining lymph nodes. T cells enter the lymph node via the high endothelial venules (HEVs). b) T cells move along the fibroblastic reticular cells (FRC) that constitutively produce CCL21 and recruit $CCR7^{+}$ T cells and DCs in the T cell zone of the lymph node paracortex. These CCL21 conduits increase the likelihood of their encounter with DCs presenting cognate antigen and generation of effector T cells.

sequent activation and redistribution of integrin molecules leads to transendothelial migration [62][180]. CCR7 signaling components have multiple features that allow them to govern a wide range of leukocyte functions in different tissue microenvironments. An important aspect of the CCR7 chemokine system is its transcriptional regulation. Though CCR7 has been established as a homeostatic chemokine receptor, CCR7 and its ligands are also inducible

during inflammation to coordinate complex leukocyte trafficking between the peripheral vs the lymphoid tissue [206][140][26]. Importantly, ligation of CCL19 vs CCL21 may allow CCR7 to exert differential effects within tissue since:

- CCL21 has >10 fold higher affinity than CCL19 in binding collagen and other extracellular molecules, thus allowing it to be a better candidate to form an immobilized chemokine gradient [214].

- CCL19 and CCL21 are natural biased ligands of CCR7 with equivalent efficacy for G protein activation but possess differential engagement of GRK/β-arrestin system [223] and hence, presumably differentially phosphorylate CCR7 [40]. Thus, CCL19, not CCL21, binding to CCR7 induces receptor desensitization and clathrin-mediated internalization [145][13] [105] (Figure 1.4). This may result in local changes in the chemokine environment that would optimize directed immune responses.

- Differential expression and localization of CCL19 and CCL21 in different regions of the tissue have a functional significance that can influence the position of lymphocytes and DCs in the lymph node and spleen tissue microenvironment [120][136].

- CCL21 and CCL19 are the only ligands for CCR7. However, CCL21 can also signal through CXCR3, a prominent inflammatory chemokine receptor in Th1 immune responses [157][201].

- CCR7 ligands share only 32% sequence homology. CCL19 is an obligate soluble ligand whereas CCL21 remains membrane bound due to the presence of a GAG (gly-

cosaminoglycan) binding domain [175][218][76][79]. These soluble and immobilized ligands therefore have the potential to induce different functional responses while signaling through the same receptor.

CCR7 downstream signaling uses multiple independent modules to control distinct leukocyte functions [169]. In DCs, chemotaxis toward CCR7 ligands involves Gi (G-inhibitory)-mediated activation of MAPK family members (Figure 1.4) [162][169]. Thus, actin and myosin inhibitors affect only the speed of crawling, whereas pertussis toxin inhibits the directed motion of bone marrow derived DCs [161].

In recent years, considerable effort has been devoted to understanding the mechanisms of how CCR7 signaling components control leukocyte propulsion and directional migration [136][175][161][77]. Recently, there have been significant conceptual advances made based on *ex vivo* and *in vitro* experimental models investigating how soluble versus immobilized CCR7 ligands influence the speed, motion and directionality of leukocyte movement within lymphoid tissues [136][175][77]. Studies conducted using two-dimensional microfluidic devices revealed that CCL19 is apparently 100-fold more potent than CCL21 for DC chemotaxis [161] and is also a more potent chemoattractant than CXCL12 for activated T cells [115]. By contrast, another recent study used microfluidics based approaches to generate chemokine gradients on three-dimensional (3D) matrices mimicking the 3D tissue environment in vivo [77]. This latter study found that when CCL21 and CCL19 are presented on competing overlapping gradients, DC migration will follow CCL21. Furthermore, DCs preferentially chemotax towards CCL21 even if not bound to the tissue matrix [77]. A sim-

ilar preference is seen in human peripheral blood T cells that are chemotactic to CCL21, but not CCL19 under physiological gradient conditions [136]. The differential ability of CCL19 and CCL21 to desensitize the receptor may fine-tune the intensity of CCR7 signaling in coexisting chemokine fields, potentially explaining the preferential migration to CCL21. Data from these studies also indicate leukocyte migration behavior to a specific chemoattractant may vary depending on other coexisting chemokine fields, their physiological gradient strength, state of the ligand (soluble vs. bound) and tissue matrix components [136][175][161][77][115].

1.2.2 CCR7 in the generation of primary host immune responses

The role of CCR7 and its ligands in leukocyte guidance is most evident during T cell entry into the LN via HEVs and DC entry through the afferent lymph vessels [62], [11], [25],[63]. Immature DCs continuously sample the antigen milieu in the tissue. When they sense danger signals, they exit as $CCR7^{+}$ mature DCs via the afferent lymphatics to present antigen in the lymphoid tissues [12][167]. In addition, CCL21 coated stromal networks allow antigen-experienced DCs to establish physical contact with T cells [11][25][63]. CCR7 has also appeared crucial for DC migration from other peripheral tissues such as gut and lung to their corresponding draining lymph nodes [91][92]. These steady state DCs (sDCs) have recently been shown to contribute to T cell homeostasis by producing VEGF (vascular endothelial growth factor) [203]. Thus, they are able to support HEV formation and may facilitate T cell entry into the LNs [203]. Such sDCs further influence T cell populations within the lymph

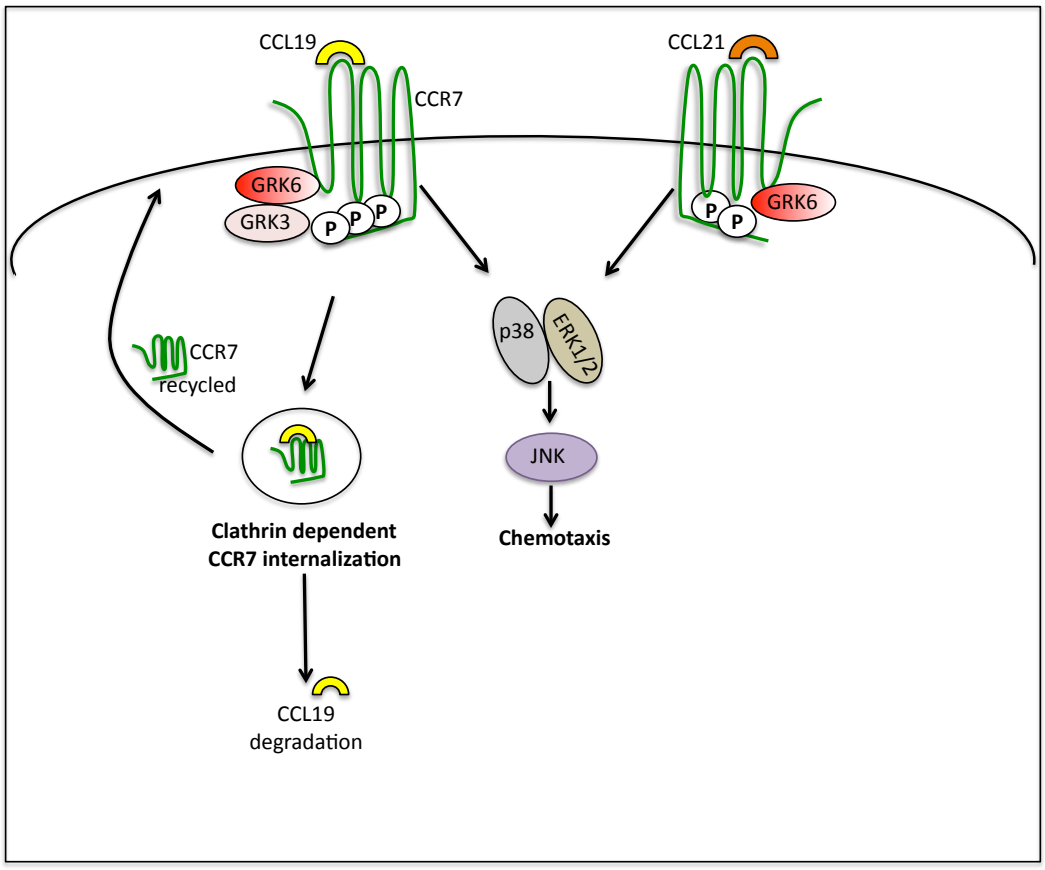


Figure 1.4: CCR7 downstream signaling and differential consequences of CCL19 vs CCL21 ligation to CCR7.

CCR7 signaling activates MAPK signaling module leading to chemotaxis. Ligand binding and activation of CCR7 leads to its phosphorylation by GRKs (G protein coupled receptor kinases). Signaling by both CCL19 and CCL21 causes GRK6 to phosphorylate CCR7. In addition, GRK3 phosphorylates CCR7 following CCL19 ligation only. Differential phosphorylation patterns may lead to the differential ability of CCR7 ligands to induce clathrin-dependent receptor endocytosis and desensitization. Following internalization, CCR7 recycles back to the plasma membrane whereas CCL19 is sorted to lysosomes for degradation.

node by increasing CCL21 expression on fibroblastic reticular cells as well as being able to bind free CCL21 [203]. This binding ability is CCR7-independent, however, will result in the retention of CCL21 in LNs [203]. Whether or not signaling or behavior of antigen dependent

interactions between DCs and T cells is affected when the DC is also presenting chemokine' remains to be investigated.

CCR7-deficient or *plt/plt* mice (lacking CCL19 and CCL21-serine) display gross alterations in the micro-architecture of spleen and thymus; and their DCs and T cells fail to home to spleen and LN T cell zones [62][63][75]. Consequently, CCR7-deficient mice display delayed and impaired adaptive immune responses especially in the absence of large amounts of antigen [62][170]. Since antigen presentation at ectopic sites might also activate T cells and generate host immunity, the requirement of CCR7 for protective immune responses may vary depending on the pathogen. Though, CCR7-deficient mice demonstrate reduced T cell priming following lymphocytic choriomeningitis virus (LCMV) or *Listeria monocytogenes* infection, mice were able to generate sufficient immune responses [98][107]. Unlike CCR7$^{-/-}$, *plt/plt* mice were able to mount unimpaired antiviral CTL responses probably due to the expression of another isoform of CCL21 (CCL21-leu) in lymph vessels and peripheral tissues [97].

T cell circulation between peripheral tissues and lymphoid organs is essential for immune surveillance and host defense of non-lymphoid sites. Upon stimulation by their cognate antigen, naive T cells become activated and change their chemokine receptor expression profile to migrate out of the lymph node via the efferent lymph [55]. Therefore, inflammation results in a great influx of lymphocytes into peripheral tissues from the bloodstream. Like few other chemokine receptors, CCR7 has been suggested as a key regulator of lymphocyte migration from blood to specific peripheral tissues [160][147]. Upregulation of CCR7 and/or

its ligands have been previously demonstrated in inflamed tissues such as liver, lung, kidney and muscle [200][165][192][99] contributing to lymphocyte recruitment and the generation of lymphoid like structures [120][200].

In a number of studies, CCR7 deficiency led to abnormal lymphocyte accumulation in peripheral tissues such as skin and lung where CCR7 is apparently not controlling the entry of activated lymphocytes [26], [39]. These observations led to the idea that lymphocytes may also need CCR7 to egress from the body cavities and extra-lymphoid tissue compartments via the afferent lymph. CCR7 deficiency led to massive accumulation of both CD4 and CD8 lymphocytes in the pleural and peritoneal cavities [85]. CCR7 expression is also required for T cell migration from skin, though CD4 and CD8 lymphocytes show differential requirements [39]. CCR7 determines the tissue exit of antigen-specific T cells and also guides their entry into the mediastinal lymph node via the afferent lymph of asthmatic lung tissue [26]. DCs residing in tissue surfaces exposed to external environment such as in the gut, airway and skin also use CCR7 to migrate out of the tissues towards peripheral lymph nodes [142][91][92]. Recently, two-photon microscopy following intralymphatic microinjection revealed CCR7 is dispensable for the parenchymal entry of lymph-derived T cells [24]. However, CCR7 is absolutely required for the directional migration of both DCs and T cells into the T cell zone in LNs [24]. To summarize, CCR7 orchestrates leukocyte navigation between lymphoid organs and inflamed sites to ensure an efficient adaptive immune response.

1.2.3 CCR7 in T cell memory responses

In response to acute infection or vaccines, naive mature CCR7^{hi}CD62L^{hi} T cells interact with their cognate antigen displayed by APCs and undergo proliferation and differentiation processes to produce short-lived effector T cells and a small pool of long-lived self-renewing memory T cells [166]. Memory T cells are heterogeneous and undergo interconversion between subsets that express markers for distinct homing and migration properties. Central memory T cells (T_{CM}) exhibit expression of lymphoid homing molecule (CD62L, CCR7) and thus, reside in lymphoid organs [166]. T_{CM} produce lower amounts of cytotoxic molecules than the other major subset of memory T cells, effector memory T cells. However, T_{CM} cells tend to have a greater capacity to proliferate upon secondary antigen encounter and down-regulate CD62L and CCR7 as they migrate into non-lymphoid inflamed tissues [166]. By contrast, effector memory T cells (T_{EM}) do not express lymphoid homing molecules, reside in the inflamed peripheral tissues and tend to express more cytotoxic molecules [166]. Upon restimulation with specific antigen, T_{EM} cells re-acquire CCR7 expression that enables them to migrate to the draining lymph node to alert the immune system.

However, during chronic infection, memory T cell development has been thought to follow different dynamics. Due to consistent antigenic stimulation, T cells become exhausted and thus memory T cells during chronic infection predominantly show effector memory phenotypes and depend on the presence of antigen for their survival [178]. However, recent studies demonstrate the presence of a subset of protective memory T cells generated during chronic infection that express CCR7 and thus reside in the lymph nodes. Chronic infections

with *Leishmania major* and *Trypanosoma cruzi* develop a stable, antigen-independent central memory T cells that are able to mediate long-term protective immunity even in the absence of persistent antigenic stimulation [20][220]. Supporting these observations, clinical samples with intermediate clinical form of Chagas disease (caused by *T. cruzi*) show increased CD4 T_{CM} cells and also memory T cell pool generated by drug-induced clearance of this pathogen demonstrates T_{CM} phenotype [61]. These $CCR7^{+}$ memory T cells eventually reach the draining lymph node and may modulate the quality of T cell recall responses through several ways- 1) they proliferate quickly and generate a larger pool of effector and effector memory T cells during persistent infection or following secondary encounter; 2) provide long term protective immunity even if the antigens are completely cleared; 3) CD4 central memory T cells prevent exacerbated inflammatory responses elicited by the infection probably through an immunoregulatory mechanism [20][61][112]. Thus, not only following primary infection, CCR7 holds the key to coordinate the protective immune responses between peripheral tissues and lymphoid compartments but also during secondary antigen encounter.

1.2.4 CCR7 in CNS specific immune responses

CNS immunity requires the production of appropriate immune responses that are robust enough to control pathogens but at the same time contained to prevent damage in an area in which inflammation is physically restrained by the skull and is dense with sensitive neurons not normally replaced in adult life [208][28][131][65]. For decades, the CNS has been considered as an "immune specialized site" with unique strategies to control the influx of pe-

ripheral leukocytes in the brain. Several studies have suggested CCR7 as a potent chemokine signal to control CNS entry and migration of lymphocytes in both healthy and diseased states [206][5][106][156].

During homeostasis, peripheral immune cells continuously survey for ongoing infection or tissue damage in the brain; however, their migration is restricted. The major route for immune cell entry and antigen sampling is via the choroid plexus and the meningeal veins into the subarachnoid space [208]. The secretory epithelium of the choroid plexus produces cerebrospinal fluid (CSF) that circulates through the ventricles of the brain. Constitutive expression of CCL19 has been demonstrated in the CSF [106] and therefore it is proposed that, following integrin-dependent adhesion, CCR7 signaling may mediate further activation and firm arrest of lymphocytes on the endothelium to facilitate transendothelial migration [57]. Recent studies on the cellular composition of CSF indicate that the majority of lymphocytes in the CSF express a central memory phenotype with high levels of CCR7 and L-selectin [37][103]. These data, along with evidence of the presence of CCL19 in the CSF of healthy individuals suggest that perhaps as part of routine surveillance, CCR7-CCL19 interactions regulate the entry of central memory T cells into the subarachnoid space [106]. When these cells recognize their cognate antigen they switch to an effector/activated phenotype and hence gain access to the CNS parenchyma [102].

Following inflammatory insults, activated CNS resident cells induce the expression of adhesion molecules and chemokines that allow circulating leukocytes to bind and extravasate from the cerebral vasculature into the CNS parenchyma [208][49][7]. In experimental au-

toimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), encephalitogenic T cells extravasate from post-capillary CNS venules [5][57]. Functional expression of CCL19 and CCL21 has been reported in these inflamed CNS venules [5], [33]. Blocking of CCR7 signaling reduced binding of T cells to inflamed venules of EAE brain sections [5][57]. In addition, during EAE, $CCR7^{+}$ cells accumulate in perivascular cuffs and meningeal infiltrates of the brain [33] corroborated further in MS patients in whom CSF has been found enriched with $CCR7^{+}$T cells and DCs. By contrast, T cells in MS lesions do not express CCR7, an indication that downregulation of CCR7 following BBB transmigration may occur [102]. Similarly, CCL19 transcripts have been found upregulated in active and inactive MS specimens [106]. In relapsing-remitting and secondary progressive MS patients, CCL19 transcript levels correlated with intrathecal IgG production [106]. These data suggest that CCL19 may be involved in B cell trafficking and expansion in the inflamed CNS. Thus, CCR7 is a potential candidate along with other current B cell-selective therapeutic approaches to reduce humoral responses in MS patients [124][6].

Independent of its role in T cell migration, CCR7 signaling has been implicated in the differentiation of T cells [108]. During allergic rhinitis, the absence of CCR7 ligands results in aberrant Th2 responses due to a reduction of Tregs in the cervical lymph nodes (cLN) [190]. By contrast, CCR7 signaling has been demonstrated to stimulate DCs to produce IL-23 and IL-12 and generate Th17 and Th1 cells [108]. Thus, deficiency of CCR7 ligands was protective against development of EAE due to a defect in IL-23 dependent induction of Th17 cells [108]. Therefore the role of CCR7 in polarizing the immune response is context specific

and may act more as a co-stimulatory signal as previously reported for CCR5 and CXCR4 [128].

In recent studies, CCR7 has emerged as a potent regulator of neuroimmune crosstalk during inflammation in the brain. CNS resident glial cells induce CCR7 ligands in response to inflammatory insults in the brain [72]. Although few reports suggest CCR7 expression by glial cells [72][47], the predominant cell types infiltrating the CNS are $CCR7^{+}$ lymphocytes and dendritic cells [103][102]. Administration of CCL21 or CCL19 restored T cell dysfunction observed in lymphotoxin-$\alpha^{-/-}$ (LT$\alpha^{-/-}$) mice during viral infection and increased their resistance to encephalitis [58]. Also, a profound change in CCL21 expression has been detected in the CNS during the chronic phase of *T. gondii* infection [206]. CCL21 induction appeared as fibrous strands associated with migrating T cells and has been hypothesized to act as a migratory network guiding T cell migration within the brain parenchyma (Figure 1.5a) [206].

CCL21 has also been implicated in glial activation in non-infectious CNS insults [157][38]. However, several findings in these models contrast with what has been established from models of autoimmune responses or situations in which ongoing inflammation is present due to CNS infection. Firstly, CNS infection or autoimmune models report expression of CCR7 ligands on glial cells, brain endothelial cells or choroid plexus epithelium [106][102]. By contrast, in both cerebrovascular ischemia and glutamate-mediated damage, neurons are the source of CCL21 production [38][18]. Supporting this finding, endangered cortical and hippocampal neurons have been shown to release CCL21 *in vitro* [38]. However, no CCL21

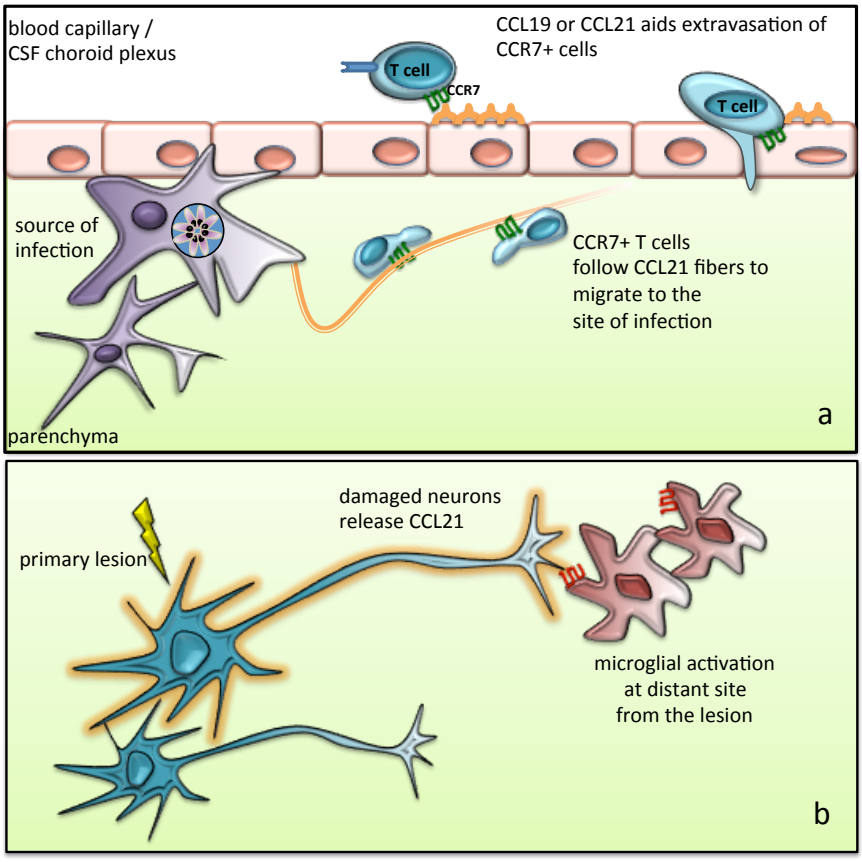


Figure 1.5: Models proposing the role of CCL21 during inflammation in the brain.

a) CCL19 and CCL21 expression at the blood-brain barrier (BBB) may aid extravasation of $CCR7^{+}$ leukocytes. During pathogenic insults CNS resident glial cells induce CCL21. CCL21 may also guide T cell migration in the CNS parenchyma towards the source of infection to keep pathogens under control; b) Ischemia or glutamate-mediated damage causes neurons to release CCL21 from the primary lesion site to activate microglia through CXCR3 receptor. CXCR3-CCL21 signaling-mediated neuroglial communication is a potent mechanism to activate glial cells that are present at a distant site from the lesion.

mRNA was detected from cultured astrocytes or microglia [18] [110]. Secondly, although neuronal release of CCL21 leads to microglial activation, this is not via CCR7 but rathar another low affinity receptor, CXCR3 [157][18][48]. In other models of neurological damage such as during peripheral nerve or spinal cord injury, CCL21 is released by damaged neu-

rons [221][19]. This stress-induced CCL21 can be transported as secretory vesicles along neuronal processes and be released at distant sites from the source of injury [38]. This phenomenon indicates a direct mechanism by which damaged neurons communicate and remotely activate microglia or other CCL21-responsive cells (Figure 1.5b) [221][19]. Thus, CCL21-mediated signaling contributes during neuronal-immune-glial cell interactions and is required to mount appropriate immune responses during both infectious and non-infectious insults.

1.2.5 CCR7 in protective immunity during Toxoplasma infection

The CCR7 chemokine system has pleiotropic effects in leukocyte function and migration that are relevant to protective immunity during pathogenic or non-pathogenic insults. This dissertation aims to understand the contribution of CCR7 in the generation of protective immune responses during Toxoplasma infection. Despite the role of CCR7 in T cell priming and initiating adaptive immunity, due to the systemic nature of antigens during the acute phase of Toxoplasma infection, CCR7-dependent T cell priming in the lymphoid organs could be dispensable. Thus, CCR7 may or may not be essential for the generation of protective immune responses following Toxoplasma infection. In this dissertation, Chapter 2 investigates the importance of CCR7-mediated interactions in the generation of protective immune responses during the acute phase of *T. gondii* infection. Data suggest that despite the systemic nature of this infection and the presence of abundant antigen, CCR7 is an absolute requirement for host protective immune responses during the acute phase of infection [140]. This is associ-

ated not only with delayed T cell responses including decreased IFN-γ production but also a significant defect in the recruitment of inflammatory macrophages [140]. This points to either a direct or indirect role for CCR7-interactions in the recruitment of effector cells from the bone marrow.

As discussed above, the CNS has been considered as an immune-privileged site due to the presence of the blood brain barrier and the absence of a draining lymphatic system limiting the sampling of antigens present in the brain [28]. However, the current scientific literature has demonstrated that the CNS is not immune isolated. Rather it has intrinsic and extrinsic components to modulate immune responses to maximize their efficiency. Understanding mechanisms that control immune cell migration and their function in Toxoplasma infected CNS will help to define important criteria for such a balanced protective CNS immunity. Upregulation of CCR7 and CCL21 in *T. gondii* infected brain indicates them as a potential chemokine axis to control T cell migration at and within the infected CNS. However, the sources of this chemokine and chemokine receptor as well as the mechanisms of how CCR7/CCL21 influences T cell migration in the CNS are largely unknown. Chapter 3 investigates the expression of CCR7 on the inflammatory infiltrates and defines possible CCL21-responsive cells in the infected brain. Data presented here discuss the cellular sources of CCL21 in the brain as well as the infection-associated stimuli that induce CCL21 following infection. Studies described in Chapter 3 also establish a particular role of CCR7-CCL21 interactions in T cell migration in the infected CNS. Data will be presented demonstrating

that CD4 T cell migration in the chronically infected brain parenchyma is CCL21-dependent and infection is not controlled in its absence [156].

In Chapter 4, we focus on the requirement of CCR7 in generating protective memory responses during Toxoplasma infection. Data presented here suggest that a significant proportion of CCR7$^+$ T cells in the *T. gondii* chronically infected brain represent memory T cells. More importantly, in the absence of CCR7, chronically infected mice succumb to secondary infection indicating the requirement of CCR7 for the generation efficient memory responses. A defect in the memory T cell population is evident in the brains of CCR7-deficient mice following secondary infection. All together, these data highlight the importance of CCR7 in T cell activation and generation of memory phenotypes in the brain in addition to their role previously known for T cell priming in the lymph nodes.

1.3 Methods and Materials

Mice

C57Bl/6 mice and congenic CD45.1 mice (C57BL/6 background) were obtained from the Jackson Laboratory (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). CCR7$^{-/-}$ (C57BL/6 background) and *plt/plt* (BALB/c background) were bred and maintained in a pathogen free environment under IACUC established protocols at the University of California, Riverside. GFAP-CCL21 transgenic mice were generated by standard methodologies [59]. In brief, the previously reported CCL21 transgene was cloned into glial fib-

rillary acidic protein (GFAP) expression cassette (into the single BamHI transgene insertion site [59][179]. Transgenic offspring (GFAP-CCL21) were identified by PCR analysis of genomic DNA. GFAP-CCL21 mice were backcrossed onto BALB/c genetic backgrounds for at least ten generations prior to the experiments reported in this study. To control parasite numbers, mice were treated with sulfadiazine at 200 mg/L (Sigma, St. Louis, MO) in their drinking water starting from 4 days post-infection and continued for two weeks.

Parasites

All *T. gondii* strains (Prugniaud, Prugniaud-OVA and RH- OVA) were maintained *in vitro* in Human Foreskin Fibroblasts (HFF) grown in DMEM complete (90% DMEM, 10% fetal bovine serum (FBS), 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). Prugniaud-OVA and RH- OVA strains (transgenic parasites expressing ovalbumin) were maintained with the presence of chloramphenicol in D10 media. Parasites were purified by passing through a 22.5-gauge needle, followed by passage through a 5.0 μm nylon filter, centrifuged at 3000 rpm for 10 minutes at 4°C. After removing supernatant, parasites were resuspended in 1 ml sterile PBS and counted. 10,000 parasites were intraperitoneally injected in 200 μl of 1xPBS. All reagents, tissues and experiments involving *T. gondii* were conducted under Biosafety Level 2 (BSL2) conditions and were performed in compliance with approved Biological Use Authorized protocols.

Blood Cytokine Measurement

Blood was collected from the tail vein at 7 days post infection and again from the heart at time of sacrifice (day 14). Samples were centrifuged at 14,000 rpm for 10 minutes at 4°C and the serum was collected and used to measure the cytokines: IFN-γ, CCL2, Interleukin-12p70 (IL-12p70), Interleukin-10 (IL-10), Interleukin-6 (IL-6) and TNF-α using the BD^{TM} Cytometric Bead Array (according to manufacturer's instructions) on a BD FACSCanto^{TM} II Flow cytometer with FlowJo analysis software v.8.7.3 (Ashland, OR, USA).

Cytospins

At the time of sacrifice, peritoneal exudate cells (PECs) from the site of infection were removed in 5 ml intraperitoneally injected PBS. Cytospins were prepared using 100 μl of the cell mixture from each mouse in a cytocentrifuge and stained with HEMA 3 Staining Kit R (Fisher Scientific Company, Pittsburgh, PA, USA).

Restimulation Assay and ELISA

Single cell suspension of splenic cells were counted and diluted with RPMI complete to a cell density of 5×10^6/ml in a final volume of 100 μl. Cells were added in triplicate to a round bottom 96-well culture plate. Cultures were then left in media alone or stimulated with soluble Toxoplasma Antigen (sTAg) at a final concentration of 25 μg/ml and incubated for 48 hours at 37°C and 5% CO_2. ELISA plates (Costar, Corning, NY, USA) were prepared by coating primary monoclonal anti-mouse-IFN-γ (eBioscienceR Clone AN-18 at 0.5 mg/ml) in sterile

PBS and left to incubate overnight at 4°C. Following incubation, plates were washed 3X in wash buffer (PBS, 0.05% Tween-20, Sigma-Aldrich). Samples and standards (rIFN-γ, eBiosciences) were added in 50 μl and incubated for a further 2hrs at 37°C. Plates were washed as before and secondary biotinylated antibody added at 0.5 mg/ml (eBiosciences) for 1hr at 37°C, following a further wash streptavidin peroxidase at 0.5 μg/ml (Jackson ImmunoResearch) was added in 100 μl/well and incubated for 30 minutes at 37°C. Following washing, 100 μl/well of the substrate ABTS (SouthernBiotech, Birmingham, AL, USA) was added and absorbance measured plate reading spectrometer at 405 nm.

Real-time PCR

Total RNA from the liver and brain tissue samples was extracted with TRIzol reagent (Invitrogen). DNase1 treatment and first-strand cDNA synthesis was performed using cDNA synthesis kit (Fermentas Life Sciences) according to the manufacturer's instructions. CCR7, CCL19 and CCL21 specific primers for real-time PCR were purchased from IDT's primer Quest (http://www.idtdna.com/Scitools/ Applications/Primerquest/). Primer sequences are listed in Table 1.2. real-time PCR was performed using the iQ5 real-time PCR Detection System (Bio-Rad) in total 25 μl reaction mixture with 12.5 μl SYBR Green/Rox qPCR Master Mix (2$\times$) and 300nM primer. The reaction conditions were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The HPRT (Hypoxine Phosphoribosyl-Transferase) primers were used as an endogenous control. Quantified results represent the fold induction of target gene expression at different days post infection in comparison to

Table 1.2: Primers used for real-time PCR

Gene of interest		Primer
CCR7	Forward	5′-TCATTGCCGTGGTGGTAGTCTTCA-3′
	Reverse	5′-ATGTTGAGCTGCTTGCTGGTTTCG-3′
CCL19	Forward	5′-ATGTGAATCACTCTGGCCCAGGAA-3′
	Reverse	5′-AAGCGGCTTTATTGGAAGCTCTGC-3′
CCL21	Forward	5′-TGAGCTATGTGCAAACCCTGAGGA- 3′
	Reverse	5′-TGAGGGCTGTGTCTGTTCAGTTCT-3′
HPRT	Forward	5′-CCCTCTGGTAGATTGTCGCTTA-3′
	Reverse	5′-AGATGCTGTTACTGATAGGAAATTGA -3′
T. gondii B1 gene	Forward	5′-TCCCCTCTGCTGGCGAAAAGT-3′
	Reverse	5′-AGCGTTCGTGGTCAACTATCGATTG-3′

the target gene expression in naive cDNA samples. Analysis on 1.5% agarose gels were performed to exclude nonspecific amplification. NTC, no-template control (reagent alone without template) was included in each assay as to detect any possible contamination of the PCR reagents.

Quantification of *T. gondii* burden by real-time PCR

Parasite burden was measured by amplifying the *T. gondii* B1 gene by real-time PCR, using SYBR® GreenER SuperMix for iCyclerR (InvitrogenTM , Carlsbad, CA, USA) in a 30 μl reaction volume, 2 μg of total template DNA from each organ, and 1.50 μl of 0.5 mM primer (Table 1.2) and water for a final volume of 30 μl. The gene was amplified in an iCyclerR RT-PCR machine (Bio-Rad Laboratories, Hercules, CA, USA) using a 10-min initial denaturation at 95°C followed by 50 cycles that consisted of 15 s denaturation at 95°C, 30 s annealing at 60°C, and 30 s extension at 72°C. The melt curve was generated to check for

primer-dimers and threshold values (Ct) were acquired and analyzed using the Bio-Rad iQ5 2.0 standard Edition Optical System Software v 2.0.148.060623.

Immunohistochemistry

Immediately following excision, brains (bisected sagittally), lung and liver tissue samples were flash-frozen in cold isopentane. Frozen organs were then put into standard Tissue-Tek cryomold and filled with Optimal Cutting Temperature (OCT) solution (also from Tissue-Tek manufactured for Sakura, Torrance, CA, USA) and put on dry ice and subsequently stored at -80°C. Serial sections of 10-20 μm were prepared on a standard Cryostat machine (LEICA/CM1850, Simi Valley, CA, USA). For H&E staining, 10 μm sections were stained with H&E (Harris Modified Hematoxylin from Fisher Scientific Company and Eosin from ProtocolR, Kalamazoo, MI). For immunohistochemistry assays, frozen tissue sections were fixed 75% acetone/25% ethanol then blocked in 10% donkey serum (10-30 min, room temperature) prior to incubation with purified antibodies. For T cell staining, conjugated antibody against CD4 or CD8 (5 μg/ml, from Biolegend, Inc) was used. Biotinylated CD45.2 was purchased from eBioscience, Inc and used at 1:200 dilution followed by streptavidin- Alexa 568 (Invitrogen) diluted at 1:500. Primary or conjugated T cell antibodies were incubated with tissue samples for 3 hr at room temperature or overnight at 4°C. Purified antibodies against murine CCL21 (15 μg/mL, Peprotech), GFAP (8 μg/mL, Invitrogen), VCAM-1 (5 μg/mL, Ebioscience) or laminin (1:50, Cedarlane) were followed with appropriate secondary antibodies at 2 μg/mL, 1hr incubation at room temperature. Samples were mounted in Pro-

long Gold with DAPI (Invitrogen) for nuclear counterstaining. Images were collected on a Leica SP2 scanning confocal microscope (Leica Optics, Germany), and analyzed using Improvision Volocity 5.0 (Perkin-Elmer, Waltham, MA).

Adoptive Transfer

In Chapter 2, Figure 2.5, T cells were purified from naive WT (wild-type, C57BL/6 background) and CCR7$^{-/-}$ splenic cells using T cell enrichment columns (R&D Systems). Purity of T cells was assessed by flow cytometric analysis of splenic cells (pre-isolation) and purified T cells using T cell specific marker (CD3) and at least 88% of the purified population was CD3^{+}. WT or CCR7$^{-/-}$ T cells were resuspended in PBS and 5×10^6 cells (in 200 μl of PBS) were injected intraperitoneally (IP) into mice. In Chapter 3, Figure 3.8, data were obtained from 5×10^6 WT or CCR7$^{-/-}$ T cell transfer (in 200 μl of PBS, IP) into CD45.1 mice 1 day prior to infection. In this experiment, T cells were purified form naive spleens and LNs using T cell enrichment columns (R&D Systems). In studies testing the requirement of CCR7 on KLRG1 expression on CD8 T cells (Chapter 4, Figure 4.5), magnetic particles were used to isolate CD8 T cells from spleen and LNs from CCR7-deficient mice in a negative selection method (immunomagnetic isolation using EasySepTM CD8 T cell enrichment kit, Stemcell technologies). These naive CCR7-deficient CD8 T cells (3×10^6 cells in 100 μl PBS) were transferred IV (intravenous) into CD45.1 mice 1 day prior to infection. In both studies, purity of T cells or CD8 T cells was confirmed by flow cytometric analysis of pre- and post-isolation samples.

Preparation of splenocyte/lymph node cell suspensions

Spleens and cervical lymph nodes were harvested from anesthetized mice and homogenized via passage through a 40 μm cell strainer (BD Falcon^{TM}). The resultant single cell suspensions were washed with RPMI complete (10% FBS, 1%penicillin/streptomycin, 1% glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 0.1% β-mercaptoethanol) and centrifuged for 5 minutes at 1200 rpm at 4°C. Red blood cells were lysed by addition of a hypotonic salt solution (0.86% ammonium chloride) for 5 minutes on ice and washed and centrifuged again. The leukocyte-enriched pellet was resuspended in RPMI complete and kept on ice.

Preparation of brain mononuclear cell suspensions (BMNCs)

Following sacrifice and perfusion with ice cold sterile PBS the brains were harvested and placed in 4 ml of RPMI on ice. Tissues were then minced with a razor blade and passed multiple times through an 18-gauge needle. The suspension was then incubated with 100 μl of collagenase/dispase (10 mg/ml) (Roche Diagnostics, Indianapolis, IN) for 45 min at 37°C and then a further 45 min at 37°C with 300 μl DNAse (10 mg/ml) (Sigma). Following enzymatic digestion, the cell suspension was passed through a 70 μm cell strainer, resuspended in 40 ml of RPMI complete and centrifuged at 1200 rpm for 10 min at 4°C. The pellet was then resuspended in 60% isotonic percoll (GE Healthcare Bioscience, Uppsala, Sweden) solution (in RPMI complete) and overlaid with 30% percoll solution made in 1$\times$ sterile PBS. The percoll gradient was centrifuged at 2000 rpm for 25 min at 25°C without brakes. After

centrifugation, the myelin layer on top of the gradient was removed. BMNCs (lymphocytes, macrophages, dendritic cells and microglia) were harvested from the 30%-60% percoll interphase and washed twice in complete RPMI medium before further analysis.

Restimulation Assay to analyze IFN-γ^+ T cells by flow cytometry

Single cell suspension of splenic cells/ BMNCs (brain mononuclear cells) were counted and diluted with RPMI complete to a cell density of 4×10^6/ml. Cells were added in triplicate to a round bottom 96-well culture plate in 200 μl final volume per well. Cultures were then left in media alone or stimulated with PMA (50 ng/ml) and Ionomycin (1 μg/ml) and incubated for 4-5 hours at 37°C and 5% CO_2. Brefeldin A (10 μg/ml) was added 2 hrs prior to harvest in order to block secretion of cytokines.

Chemotaxis assay

A transwell chemotaxis assay was employed for the studies described in Chapter 3, Figure 3.1(c-f). BMNCs and splenic cells, harvested from the chronically infected mice were incubated at 37°C, 5% CO_2 (1hr) and washed, resuspended in assay medium (RPMI with 0.5% bovine serum albumin and 25mM HEPES). Recombinant mouse CCL21 (R&D Systems) was diluted in assay medium at different concentrations starting from 50-2000 ng/ml. 5×10^5 cells were applied (in 100 μl assay medium) in each transwells (6.5 mm diameter; 5 μm pore size, Costar) coated with fibronectin (10 μg/ml). The lower compartments of the wells were filled with 600 μl assay medium with CCL21, 0-2000 ng/ml (three replicates for

each concentrations of CCL21). For the "input" count, there was 500 μl assay medium in an empty well (without transwell insert) with 100 μl of 5×10^6 cell suspension. Following a 1.5-hr incubation at 37°C and 5% CO_2, cells were collected from lower compartments of the wells. The BD FACSCanto^{TM} II flow cytometer was used for quantification and phenotypic analysis of the cells that had migrated though the transwells. Numbers of migrating cells were quantified using count beads (20 um Fluoresbrite (FITC) Yellow-Green microspheres, Polysciences), 5000 beads were counted per sample.

Flow cytometric analysis

For flow cytometric analysis, cells were counted, resuspended at 1×10^6/ml in RPMI complete. 1×10^6 cells were transferred in a FACS tube (BD Falcon^{TM}, MA, USA) and centrifuged at 1200 rpm for 5 minutes at 4°C and resuspended in FACS buffer (1$\times$ 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. 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 analysis software v.8.7.3. Cytokines staining was performed using intracellular staining (IC). To perform intracellular staining, cells were stained with surface expression markers and then fixed with 4% PFA (paraformaldehyde). Following overnight fixation, cell were permeabilized with 0.3% saponin and stained with anti-mouse IFN-γ (from eBioscience) for 30 minutes on ice.

Cells were then washed with the permeabilization buffer (0.3% saponin in FACS buffer) followed by a wash with FACS buffer. MHC class II tetramer specific to toxoplasma epitope (AVEIHRPVPGTAPPS) was generously provided by Dr. Marion Pepper, University of Washington, Seattle and also by the NIH tetramer core facility. For MHC class II tetramer staining, cells were stained with the tetramer (diluted at 1:300) at room temperature for 40 mins prior to staining with other surface expression markers. MHC class I tetramer specific for SIINFEKL (OVA peptide) was purchased from iTAg^{TM} MHC tetramer, Beckman Coulter, CA or Immudex, CA and was used according to manufacturer's instructions.

Chapter 2

CCR7-dependent immunity during acute *Toxoplasma gondii* infection

2.1 Abstract

The chemokine receptor CCR7 is a well established homing receptor for dendritic cells and T cells. Interactions with its ligands, CCL19 and CCL21, facilitate priming of immune responses in lymphoid tissue, yet CCR7-independent immune responses can be generated in the presence of sufficient antigen. In these studies we investigated the role of CCR7 signaling in the generation of protective immune responses to the intracellular protozoan parasite *Toxoplasma gondii*. The results demonstrated a significant increase in the expression of CCL19, CCL21 and CCR7 in peripheral and CNS tissues over the course of infection. Unexpectedly, despite the presence of abundant antigen, CCR7 was an absolute requirement for protective immunity to *T. gondii*, as CCR7$^{-/-}$ mice succumbed to the parasite early in the acute phase of infection. Although serum levels of IL-12, IL-6, TNF-α, and IL-10 remained unchanged, there was a significant decrease in CCL2/MCP-1 and inflammatory monocyte recruitment to the site of infection. In addition, CCR7$^{-/-}$ mice failed to produce sufficient IFN-γ, a critical Th1 associated effector cytokine required to control parasite replication. As a result there was increased parasite dissemination and a significant increase in parasite burden in the lungs, livers and brains of infected mice. Adoptive transfer experiments revealed that expression of CCR7 on the T cell compartment alone is sufficient to enable T cell priming, increase IFN-γ production, and allow survival of CCR7$^{-/-}$ mice. These data demonstrate an absolute requirement for T cell expression of CCR7 for the generation of protective immune responses to Toxoplasma infection.

2.2 Introduction

The chemokine receptor CCR7 and its ligands, CCL19 and CCL21, are known to be critical for a number of essential immunological processes throughout the development of an immune response. These include the generation of thymocytes [117] [34], central and peripheral tolerance, T cell homeostasis [154], Treg function [126][174][104] and the activation and homing of CCR7 expressing dendritic cells to lymph nodes [62][142][121]. This central role is in part due to the constitutive expression of CCL19 and CCL21 in primary and secondary lymphoid organs and their role in guiding the migration of developing, naive and central memory T cells. CCL21 is expressed on the high endothelial vessels (HEVs) of the lymph node and by fibroblastic reticular cells (FRC) and follicular dendritic cells (FDC) forming the conduits that are the structural core of the lymph node [11][144]. These structures enable the migration and interaction of $CCR7^{+}$ cells, namely naive or memory T cells and dendritic cells (DCs). Following CCR7-mediated entry into the lymphatics, DCs present antigen to naive T cells within the lymph node paracortex. T cells enter the lymph node via HEVs and show a "haptokinetic" mode of migration on the FRC surface in response to immobilized CCL21 [25]. These chemokine interactions therefore influence the velocity of T cell migration and the duration and likelihood of their contact with DCs presenting cognate antigen. As such, in the absence of the receptor, ($CCR7^{-/-}$) or it's ligands, (*plt/plt* mice), lymph nodes have poorly organized T cell and B cell zones and a drastic decrease in the numbers of T cells and DCs in their secondary lymphoid organs [62][75]. Despite the ap-

parent requirement for CCR7 during the priming of adaptive immune responses in the lymph node, studies have demonstrated robust immune responses in the absence of CCR7-CCL21 signaling during lymphocytic choriomeningitis virus [98], [97] and *Listeria* [107] infection, models of contact hypersensitivity, autoimmune disease, and asthma [146][73][155][129]. Indeed, the consensus is that in the presence of a large antigen dose, CCR7 is dispensable for protective immune responses [62].

Toxoplasma gondii is an obligate intracellular parasite that induces a strong systemic immune response. During primary acute infections, quickly replicating tachyzoites can invade any nucleated cell and disseminate rapidly throughout the body. This stage is associated with systemic Th1 immune responses. Production of IL-12 by DCs, macrophages and neutrophils triggers IFN-γ production from NK and T cells [118][150][89][44][114]. IFN-γ production is the major mediator of anti-toxoplasma effector mechanisms that includes Nitric oxide production and expression of IFN-γ inducible genes such as those encoding IGTP and LRG-47, which are necessary for controlling parasite growth during the acute infection phase [32][194]. This control of parasite replication initiates conversion of tachyzoites to cyst-forming bradyzoites that exist chronically for the lifetime of the infected host.

Previous work has demonstrated profound changes in the expression of CCL21 in the lymph node during acute *T. gondii* infection [94] and in the brain during chronic infection [206]. However, as yet, there has been no demonstration of the requirement for CCR7/CCL21 signaling during Toxoplasma infection. To rectify this, we analyzed the kinetics of CCR7, CCL21 and CCL19 expression in the brain and peripheral organs, and infected mice deficient

in CCR7 and monitored their ability to control infection. In contrast to other infectious models [107][97], CCR7$^{-/-}$ mice succumbed during acute infection, associated with a failure to control parasite replication. Transfer of WT cells indicates that CCR7 expression in the T cell compartment is sufficient to increase IFN-γ production and control infection.

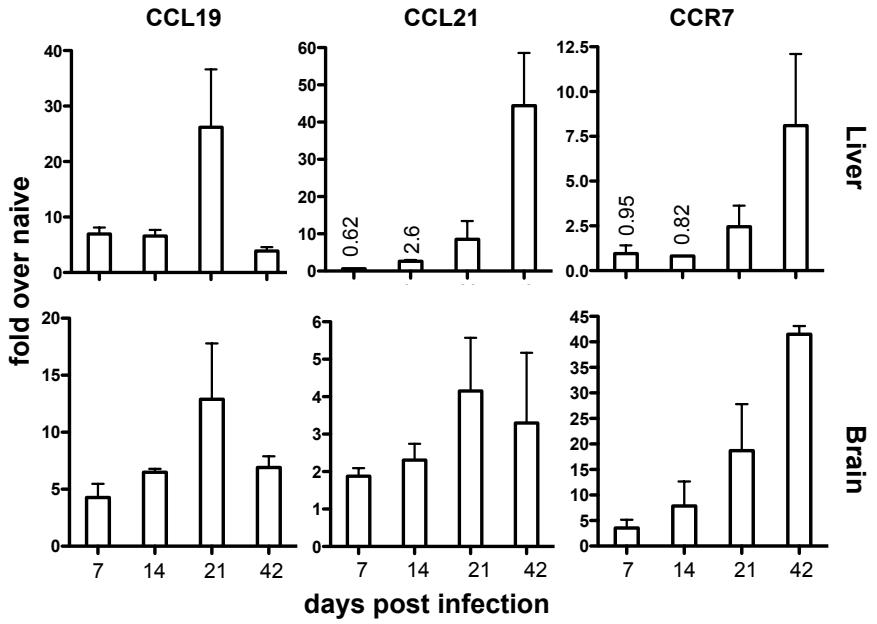
2.3 Results

Upregulation of CCR7 and ligands at sites of infection

It has previously been reported that CCL21 is upregulated in the brain following *T. gondii* infection [206]. To establish the kinetics of CCR7 expression and its ligands in non-lymphoid organs during infection, mice were infected with the Prugniaud (Pru) strain of *T. gondii*, and real-time PCR was conducted on livers and whole brains at various time points following infection (Figure 2.1a and Figure 2.1b). At 7 days post infection, a time point considered to be the peak of the acute systemic immune response, there was a significant increase in the level of CCL19 in the livers of infected mice compared to those of naive uninfected mice. By 21 days postinfection, transcript levels have reached a peak of $>$20 fold above that of naive mice and by day 42, once chronic infection has been established, CCL19 had been downregulated in the liver to levels similar to those in early infection. In contrast, CCL21 transcript was delayed, with no significant increase detected until 14 days after infection. CCL21 continued to increase over the course of infection, reaching a $>$40 fold increase at 42 days postinfection. Transcript levels of CCR7 followed a pattern similar to that of CCL21,

reaching a >8 fold increase in the liver at day 42 postinfection. At this later time point parasites had been controlled in the periphery and were predominantly found in the brain of the host. Kinetics of transcript levels for all three molecules were mimicked in the brain, although the fold increases and copy number ratio of CCR7 were significantly greater in the brain at all time points, reflecting the accumulation of parasites and inflammation in this area. At all time points in the brain, copy number ratio of CCL19 is greater than that of CCL21 (Figure 2.1b). These data demonstrate that following Toxoplasma infection there is a significant upregulation of CCR7, CCL19 and CCL21 in peripheral tissues associated with sites of infection.

a)


CCL19
CCL21
CCR7
fold over naive
Liver
Brain
0.62
2.6
0.95
0.82
7
14
21
42
days post infection

b)

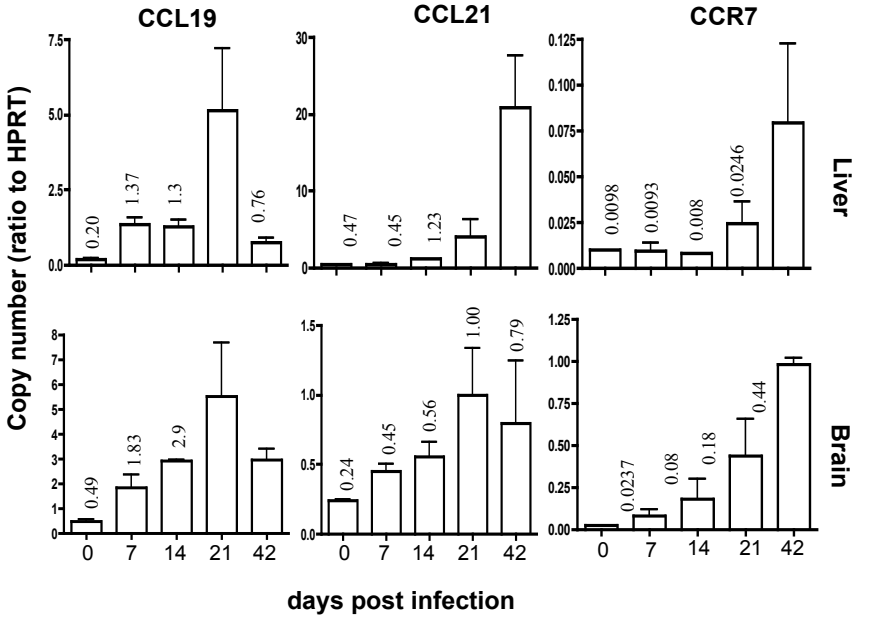

CCL19
CCL21
CCR7
Copy number (ratio to HPRT)
Liver
Brain
0.20
1.37
1.3
0.76
0.47
0.45
1.23
0.0098
0.0093
0.008
0.0246
0.49
1.83
2.9
0.24
0.45
0.56
1.00
0.79
0.0237
0.08
0.18
0.44
0
7
14
21
42
days post infection

Figure 2.1: CCL19, CCL21 and CCR7 are upregulated during the course of *T. gondii* infection.

Infected mice were sacrificed at days 7, 14, 21, 42 postinfection and total RNA extracted from livers and brains. Real-time PCR was conducted to determine the absolute copy numbers of CCL19, CCL21 and CCR7 using a standard curve, and they were compared to the internal reference gene, HPRT. The data are represented as mean fold changes over naive (a) and copy number ratios to HPRT (b); with error bars representing standard errors of the mean (SEM) of at least 3 biological replicates.

CCR7$^{-/-}$ mice produce significantly less IFN-γ in response to infection

To determine if CCR7 interactions are required for the generation of protective immune responses to *T. gondii*, CCR7$^{-/-}$ mice were infected and serum levels of cytokines were compared to wild-type (WT) C57BL/6 mice. At day 7 post infection, the peak of the systemic immune response, equivalent concentrations of IL-6, IL-12p70 and TNF-α were present in the serum of CCR7$^{-/-}$ mice compared to control mice (Figure 2.2a). Measurement of IL-10, required to prevent infection-induced immunopathology [209][204], demonstrated a trend for decreased production although this did not reach statistical significance. However, the concentrations of CCL2 and the pro-inflammatory cytokine IFN-γ were significantly decreased in mice deficient for CCR7. CCL2 is required for the recruitment of monocytes to the site of infection [163], while IFN-γ, produced by NK and Th1 cells during *T. gondii* infection, is required for the activation of monocytes and ultimately effector mechanisms, against the parasite [22][222]. In addition, the defect in IFN-γ production remained following restimulation of splenic cells from naive mice with Toxoplasma antigen (sTAg). Thus, although both WT and CCR7$^{-/-}$ cultures increased the production of IFN-γ compared to cultures from

uninfected naive mice, cultures from infected CCR7$^{-/-}$ mice remained deficient in IFN-γ compared to cells from WT controls (Figure 2.2b).

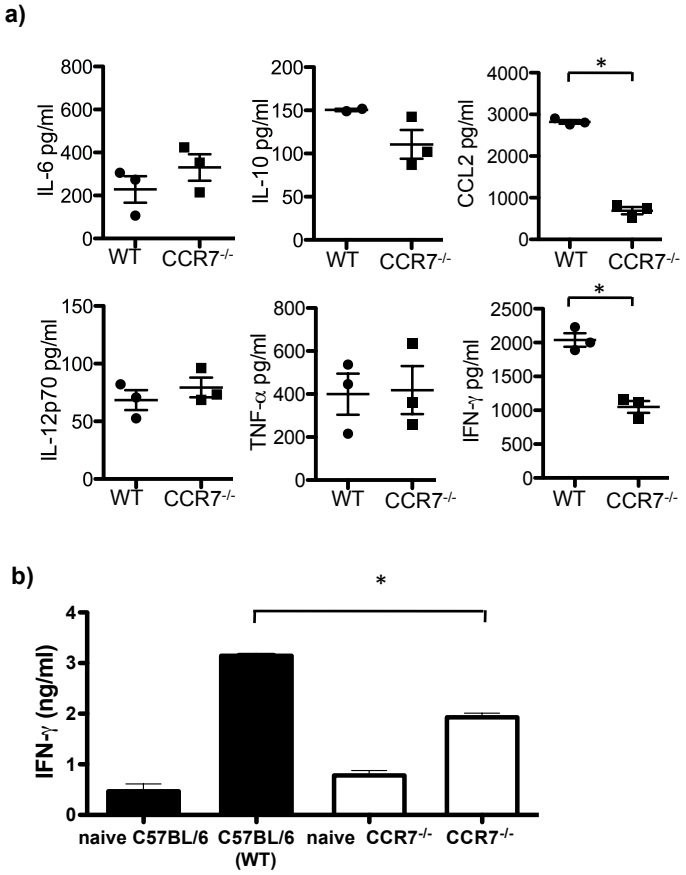


Figure 2.2: Deficiency in pro-inflammatory cytokine production during acute infection in CCR7$^{-/-}$ mice.

a) Serum samples were collected from C57BL/6 (WT) and CCR7$^{-/-}$ mice at day 7 postinfection and protein levels of IFN-γ, CCL2, IL-12p70, IL-10, IL-6 and TNF-α were measured. Levels for individual mice (n=3) are plotted with average $\pm$ SEM. b) Single cell suspensions were generated from naive and infected spleens and restimulated with sTAg. IFN-γ production was measured after 48 hrs using ELISA. Average $\pm$ SEM of at least 3 biological replicates are plotted and are representative of 3 independent experiments containing a minimum of 3 biological replicates *p$<$0.001.

CCR7$^{-/-}$ mice are susceptible to Toxoplasma infection

Survival and parasite burden were monitored in CCR7$^{-/-}$ and WT mice. Consistent with the failure to produce IFN-γ, CCR7$^{-/-}$ mice succumbed to infection at around 10 days post infection (Figure 2.3a). Histological analysis of liver and lung revealed increased inflammation and necrosis (Figure 2.3b). Examination of the inflammatory infiltrate at the site of infection revealed increased numbers of cells in the peritoneal cavity (PECS), many of which were infected, and replicating parasites were readily detectable compared to infected WT controls (Figure 2.3b). To quantify this increase in parasite burden, real-time PCR was conducted for the parasite specific B1 gene in peripheral and CNS tissues. At 7 days post infection, parasites could be detected in WT mice in the lungs but remain undetectable in the liver and brain. By day 14, a time point at which we saw contraction of systemic infection and inflammation, parasites could be detected only in the brain (Figure 2.3c). In contrast, parasites in CCR7$^{-/-}$ mice were detectable in the liver and were significantly increased in the lung compared to infected control mice. In addition, although parasites had not yet migrated to the brain by day 7, by day 14 there was a significant increase in the number of parasites in the brain compared to control mice (Figure 2.3c). These data demonstrate that CCR7 is required to generate protective immune responses to *T. gondii*, as in its absence, there is increased parasite survival and dissemination and a failure to survive infection.

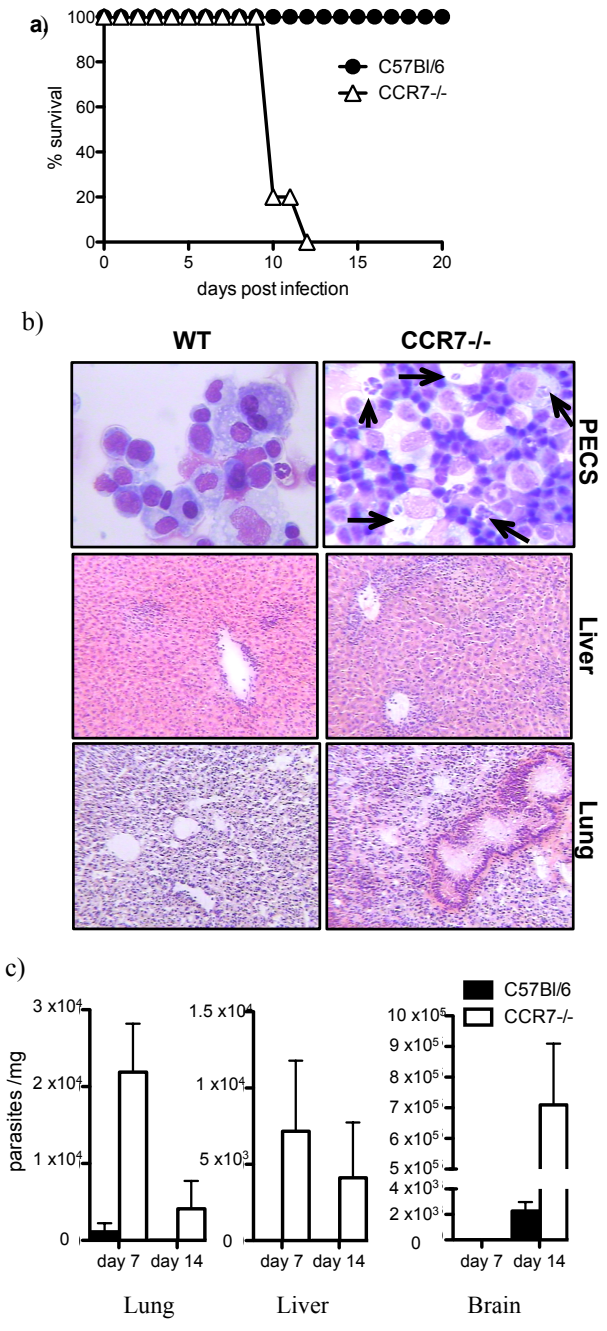

a)
100
80
60
40
20
0
% survival
0
5
10
15
20
days post infection
C57Bl/6
CCR7-/-
b)
WT
CCR7-/-
PECS
Liver
Lung
c)
3 x10⁴
2 x10⁴
1 x10⁴
0
parasites /mg
day 7
day 14
Lung
1.5 x10⁴
1 x10⁴
5 x10³
0
day 7
day 14
Liver
10 x10⁵
9 x10⁵
8 x10⁵
7 x10⁵
6 x10⁵
5 x10⁵
4 x10³
2 x10³
0
day 7
day 14
Brain
C57Bl/6
CCR7-/-

Figure 2.3: CCR7$^{-/-}$ mice fail to survive acute Toxoplasma infection due to uncontrolled parasite replication.

a) C57Bl/6 WT (n=5) and CCR7$^{-/-}$ mice (n=5) were infected with 10^4 *T. gondii* parasites and survival monitored daily. b) PECs (top panel)(arrows indicate replicating parasites), liver and lung histology at day 7 post infection indicating increased parasite burden and inflammation in CCR7$^{-/-}$ compared to C57Bl/6 mice. b) Measurement of parasite DNA in lung, liver and brain at days 7 and 14 post infection using real-time PCR as described in Materials and Methods. Average $\pm$SEM of 3 biological replicates are plotted.

Defective recruitment of effector cells in CCR7$^{-/-}$ mice

CCR7 and its ligands can play roles in multiple cell types. To determine which cells during Toxoplasma infection require CCR7 signaling, analysis of the cellular infiltrate was conducted using flow cytometry. Following *T. gondii* infection, neutrophils and inflammatory monocytes, differentiated by their expression of Ly6C and Ly6G, are amongst the first effector cells at the site of infection and are actively involved in killing the parasite [54][56]. Analysis of inflammatory monocytes (CD45$^+$CD11b$^+$Ly6G$^-$Ly6C^{hi}) and neutrophils (CD45$^+$CD11b$^+$Ly6G^{hi}Ly6C^{med}) [71] at day 7 postinfection, revealed significant populations of both cell types in the peritoneal cavity and increased numbers of these cells in the lymph node following infection (Figure 2.4) (Hi and Med are high and medium levels of expression, respectively). In contrast, CCR7$^{-/-}$ mice had a significant defect in the proportion of inflammatory monocytes at the site of infection, with on average a 50% reduction in the percentage of CD45$^+$CD11b$^+$Ly6G$^-$Ly6C^{hi} cells in the peritoneal cavity and decreased numbers in the lymph node (Figure 2.4a and c). These data are consistent with the significant

defect in serum levels of CCL2 (Figure 2.2a) and this is a likely cause of the delay in the control of parasite replication [54][135].

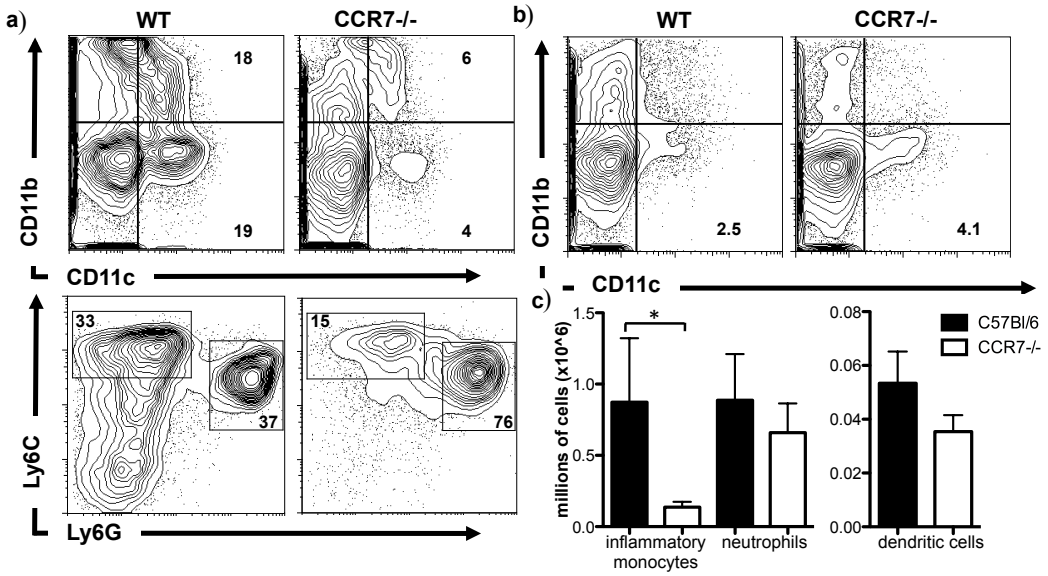


Figure 2.4: Analysis of cellular composition using flow cytometry at a) the site of infection (PECs) and b) draining lymph nodes from 7 day infected C57Bl/6 and CCR7$^{-/-}$ mice.

Numbers represent proportions of dendritic cells (CD11c^{+}) as a percentage of live cells (top panel) and proportions of inflammatory monocytes (CD45^{+}CD11b^{+}Ly6C^{hi}Ly6G^{-}) and neutrophils (CD45^{+}CD11b^{+} Ly6^{med}Ly6G^{hi}) as a percentage of CD11b^{+} cells from PECs (bottom panel, a). c) Absolute numbers of neutrophils and dendritic cells in the draining lymph node from 7 day infected C57Bl/6 and CCR7$^{-/-}$ mice.

As previously published, phenotypic analysis of cells in lymphoid compartments revealed very few T cells in the lymph nodes of naive CCR7-deficient mice and increased

numbers of CD4 and CD8 T cells in peripheral sites, including the peritoneal cavity before and after infection [25], [39]. Analysis of the proportion of recently activated T cells ($CD44^{hi}CD62L^{lo}$) that are present at these sites reveals little to no defect in their activation status (WT: 24.9$\pm$1.0; $CCR7^{-/-}$: 20$\pm$0.9) suggesting that the small numbers of T cells that are in the lymph node are capable of getting primed. CCR7 is also required for DC migration into the lymph node and migration within the T cell zone. As a result DC numbers in the lymph nodes of $CCR7^{-/-}$ mice are vastly reduced during inflammation [82], [92][211]. Analysis of the dendritic cell population at the site of Toxoplasma infection revealed a substantial decrease in the number of $CD45^{+}CD11b^{-}CD11c^{+}$ cells in the peritoneal exudate cells (Figure 2.4a). However, analysis of lymph nodes following infection revealed an increase in the proportion of $CD45^{+}CD11b^{-}CD11c^{+}$ dendritic cells compared to WT control mice (Figure 2.4b), although absolute cell numbers never reached the levels of WT controls (Figure 2.4c). Unlike some viral infections [170], dendritic cells play an intrinsic role in the generation of immunity to Toxoplasma via production of IL-12 and antigen presentation [3][150]. CCR7- independent recruitment of dendritic cells during infection has previously been demonstrated [135]. To determine if the inflammation induced dendritic cells present in the lymph nodes of $CCR7^{-/-}$ mice were sufficient to generate protective immune responses, adoptive transfer of WT CCR7 expressing T cells was conducted. One day following transfer of WT or CCR7-deficient T cells, $CCR7^{-/-}$ mice were infected as before and monitored for survival (Figure 2.5). As previously shown CCR7-deficient mice succumbed to infection within the first two weeks. $CCR7^{-/-}$ mice that received additional $CCR7^{-/-}$ T cells also

succumbed to infection, with similar kinetics. However, mice that received CCR7 expressing T cells had significantly increased survival rates (Figure 2.5a). In addition, measuring serum IFN-γ from wild-type, CCR7$^{-/-}$ mice and CCR7$^{-/-}$ mice that received WT T cells, revealed a significant increase in the concentration of IFN-γ following T cell transfer (Figure 2.5b). These data demonstrate that CCR7^{+} T cells can be primed in CCR7$^{-/-}$ mice, producing IFN-γ during *T. gondii* infection that is sufficient to rescue CCR7-deficient mice.

2.4 Discussion

Previous reports have established CCR7 as a crucial chemokine receptor for promoting T cell migration and coordinating antigen presentation by DCs in the lymph node [144][211]. Despite this central role, multiple studies have demonstrated that, although CCR7-deficient mice can show delayed kinetics, CCR7 signaling is not an absolute requirement for effective immune responses during experimental models of inflammation [174][73][129] or for protective immunity during infection [62][98][107][97]. The prevailing consensus is that during strong inflammatory responses with abundant antigen the requirement for antigen presentation in the lymph node, and thus the need for CCR7, can be bypassed. This present study investigated the role of CCR7 for the generation of protective immune responses to the intracellular pathogen *Toxoplasma gondii*. This infection is often described as the "atomic bomb" of Th1 immune responses, characterized by the invasion of any nucleated cell by fast-replicating parasites, resulting in systemic infection and tissue damage during the acute phase

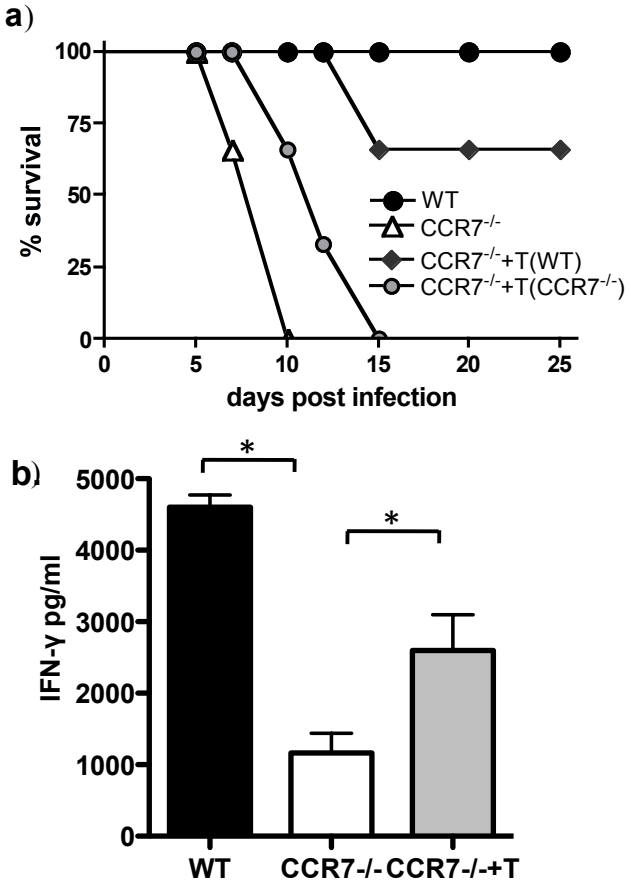


Figure 2.5: CCR7$^+$ T cells are sufficient for protective immune responses in CCR7$^{-/-}$ mice.

a) T cells were isolated from spleen and lymph nodes of naive C57Bl/6 or CCR7$^{-/-}$ mice and 5×10^6 cells were adoptively transferred into CCR7$^{-/-}$ mice (n=5). C57Bl/6 (n=5) and non-transferred CCR7$^{-/-}$ mice (n=5) were sham injected with PBS. Survival curve is representative of four independent experiments with similar results. b) Serum IFN-γ levels at day 7 post infection.

of infection. Despite this high antigen load we found that CCR7$^{-/-}$ mice were incapable of mounting protective immune responses, with severe defects in inflammatory monocyte recruitment and in the production of the key effector cytokine IFN-γ.

The decrease in CCL2 and monocyte recruitment to the site of infection has a significant detrimental role in the ability CCR7$^{-/-}$ mice to innately control parasite replication. Inflammatory monocytes are required for protection during infection of many intracellular pathogens, including Toxoplasma [54][163][56]. These cells are recruited into inflamed sites and can directly kill pathogens by production of nitric oxide [163]. In addition, as they are capable of differentiating to dendritic cells they may play an important role in shaping the adaptive immune response [56][71]. Inflammatory monocytes are not thought to express CCR7, and therefore, the decrease in recruitment of these cells at day 7 following infection may reflect an absence of sustained Th1 immune responses. To our knowledge, this defect in inflammatory monocyte recruitment in CCR7$^{-/-}$ mice has not previously been reported. The lack of inflammatory monocytes most likely leads to impaired parasite killing and a significant increase in parasite numbers and dissemination in CCR7-deficient mice.

In addition to this defect in the innate immune response, CCR7$^{-/-}$ mice while producing equivalent levels of IL-12, failed to mount a significant type-1 adaptive immune response. A recent paper has characterized the dynamic early responses following Toxoplasma infection in the lymph node [94]. These studies demonstrate significant recruitment of naive CCR7^{hi} CD8 T cells and sustained contact with DCs at the sub-capsular region alongside profound structural changes in lymphoid architecture. The studies presented here address whether an

alternate priming site is enough for efficient T cell-DC interaction that is required for the robust Th1 immune response following *T. gondii* infection. Despite the significant numbers of T cells at the site of infection in CCR7$^{-/-}$ mice, the low levels of IFN-γ suggest that they are not sufficiently activated. This implies a dominant role for the lymph node in antigen presentation and T cell priming during Toxoplasma infection. To support this, adoptive transfer of CCR7^{+} T cells, and not CCR7$^{-/-}$ T cells, lead to a significant increase in IFN-γ and increased survival of CCR7$^{-/-}$ mice. Thus, CCR7 signaling on only T cells enabled sufficient T cell priming to produce protective immunity in *T. gondii* infected mice. This is likely aided by the presence of inflammatory-induced DCs at the site of infection and their migration to the lymph node [135]. Analysis of the cellular infiltrate demonstrated that although numbers of dendritic cells were never equivalent to that of WT mice there was an increase in these cells in the lymph node following infection. Toxoplasma is known to induce CCL2 [163][64] and is known to stimulate the migration of blood derived inflammatory dendritic cells to the lymph node in a CCR7-independent manner [135]. This process would enable the priming of CCR7^{+} T cells in CCR7-deficient mice. Intriguingly, the expression of CCR7 and its ligands accumulate in tissue during chronic infection, suggesting that these molecules are not only required for priming of T cells, but may play a role in chronic immune responses at the site of infection [206].

To summarize, these studies have established a requirement for CCR7 signaling for the generation of protective immune responses during Toxoplasma infection. T-cell expression of

CCR7 is sufficient to facilitate priming of a type-1 adaptive immune response and generation of IFN-γ.

Chapter 3

CCL21-dependent CD4 T cell migration plays a role in protective immune responses against Toxoplasma infection in the brain

3.1 Abstract

Kinetics of CCR7 and CCL21 expression during the course of Toxoplasma infection revealed increases of their transcripts in the infected brain. Here we investigate the purpose of increased CCR7 and CCL21 during chronic Toxoplasma infection in the brain. Studies conducted here focus on understanding the cellular sources as well as the infection-associated stimuli of this chemokine and more importantly, the contribution of this chemokine in T cell migration in the brain. CCL21 upregulation was detected on CNS resident astrocytes following direct infection and CCL21 induction appeared to be related to active parasite replication. Flow cytometric data indicated CNS infiltrating T cells are the possible responders of this chemokine in the brain as a significant proportion of CNS infiltrating T cells express CCR7 and are able to respond to CCL21 *in vitro*. Investigation into the exact role of CCR7/CCL21 interactions in the brain revealed, T cell extravasation into submeningeal, perivascular and ventricular sites of infected CNS was not CCL21-dependent, occurring even in CCL19/CCL21-deficient mice. Supporting this data, CCR7-deficient T cells migrate into the brain in the same numbers as CCR7 sufficient cells demonstrating that CCR7/CCL21 is not required for T cell entry across the blood brain barrier. However, migration of extravasated CD4, but not CD8 T cells from extra-parenchymal CNS sites into the CNS parenchyma is CCR7/CCL21-dependent. CD4 T cells preferentially accumulate at perivascular, submeningeal and ventricular spaces in the brain in the absence of CCR7 or its ligands. Our studies demonstrate that the absence or overexpression of CCL21 leads to

increased CD4 T cells but a failure to control parasite replication indicating a requirement of organized CCL21 induction for protective immunity during Toxoplasma infection in the brain.

3.2 Introduction

CNS immune responses are regulated in a tissue-specific manner [28][36]. A high threshold for initiating antigen-specific T cell responses from within the CNS does exist; yet, large numbers of T cells readily enter the CNS in response to a wide variety of insults. Thus, the presence of activated T cells within the CNS is also not rare in the human population. Considering just the example of *T. gondii*, the CDC estimates that $\sim$30% of the world population ($\sim$20% in the US and $\sim$40% in western Europe) is chronically infected by this parasite [95][83]. Thus $\sim$30% of the world's population has a lifelong influx of IFN-γ-producing T cells in their CNS without overt signs of neurodegeneration. Even in the absence of CNS infection, T cells routinely enter and survey the healthy CNS. The apparent function is not only aimed at pathogen detection. T cells also play critical cytoprotective roles in maintaining neuronal function during injury and stress [179][27][46][176]. Thus, defining mechanisms regulating T cell influx is important both to prevent unwanted neurotoxic inflammation as well as to promote neuroprotective inflammatory responses.

Data from Chapter 1 suggest that in the absence of CCR7 expression, *T. gondii* infection cannot be controlled and leads to early lethality following infection [140]. Restoration of

CCR7 expression solely in T cells is sufficient to restore immune mediated control of the parasite and survival of the infected host. CCR7 expressing T cells are found within the CSF and lesions of individuals with active multiple sclerosis (reviewed in [62][110] and Chapter 1, Introduction). Moreover, constitutive and induced CNS expression of CCL21 is hypothesized to facilitate T cell entry from the CSF and blood into the CNS parenchyma [208]. Ischemic injury, infection or experimental autoimmune triggers all rapidly induce increased CCL21 expression within the CNS roughly co-incident or just prior to the appearance of T cells within the CNS [38][17][4]. Finally, CCL21 immunoreactivity has been observed in *T. gondii* infected mice associated with migratory pathways from the points of T cell influx into the CNS (perivascular and meningeal spaces) to brain regions surrounding *T. gondii* cysts [206]. Thus, CCL21 is a candidate molecule for regulating T cell influx into and within the CNS.

Here we investigate possible cellular sources of CCR7 and CCL21 in the infected brain. Flow cytometric analysis of CCR7 expression on the inflammatory infiltrates identifies a CCR7 expressing leukocyte population in the chronically infected brain. The majority of them were CNS infiltrating T cells. CNS resident astrocytes were found to be a major source of CCL21 induction following infection. These data indicate CCL21 induction by resident glial cells from the source of infection may guide T cell migration to maximize T cell-mediated protective immunity in the CNS.

This chapter also investigates the role of CCR7/CCL21 in T cell influx *into* and *within* the CNS. Following infection, lymphocytes infiltrate the CNS in a two-step procedure. First, the cells extravasate from the blood into the perivascular or extraparenchymal (submeningeal

and ventricular) spaces. Second, extravasated immune cells may either accumulate in these extraparenchymal sites or infiltrate the parenchyma. Studies aimed to understand the involvement of CCR7/CCL21 in each of these steps of T cell infiltration were conducted in this section. Data suggest that CCR7 expression is dispensable for T cell influx into the brain but required for efficient T cell migration within the infected CNS. Furthermore, CNS expression of CCL21 augmented the migration of CD4 T cells from perivascular spaces into the CNS parenchyma of *T. gondii* infected mice. These data contribute to the growing literature demonstrating that the immune privileged CNS can regulate T cell-mediated immunity.

3.3 Results

Brain mononuclear cells (BMNCs) from the chronically infected brain express CCR7 and respond to CCL21

To investigate the immune cell populations that can respond to infection-induced CCL21 in the brain, the expression of CCR7 was analyzed on the inflammatory infiltrates harvested from the chronically infected brain. Flow cytometric analysis at 4 weeks post infection revealed a significant proportion of $CCR7^+$ leukocytes. Indeed 22% of CD4 T cells and 13% of CD8 T cells in the infected CNS expressed CCR7 (Figure 3.1a). Analysis of CCR7 expression on $CD11c^+$ cells revealed $\sim$13% of this population expressed CCR7 (Figure 3.1a). Further analysis of CCR7 expression on different subsets of DC populations in the infected brain revealed that there was a population of $CCR7^+$ cells in all subsets namely myeloid

$CD8^-$and $CD8^+$ DCs as well as plasmacytoid DCs (pDCs) (Figure 3.1b). All these different subsets of DCs have distinct functions during *T. gondii* infection including antigen processing, cytokine production and cross-presentation of antigens [159][150][122].

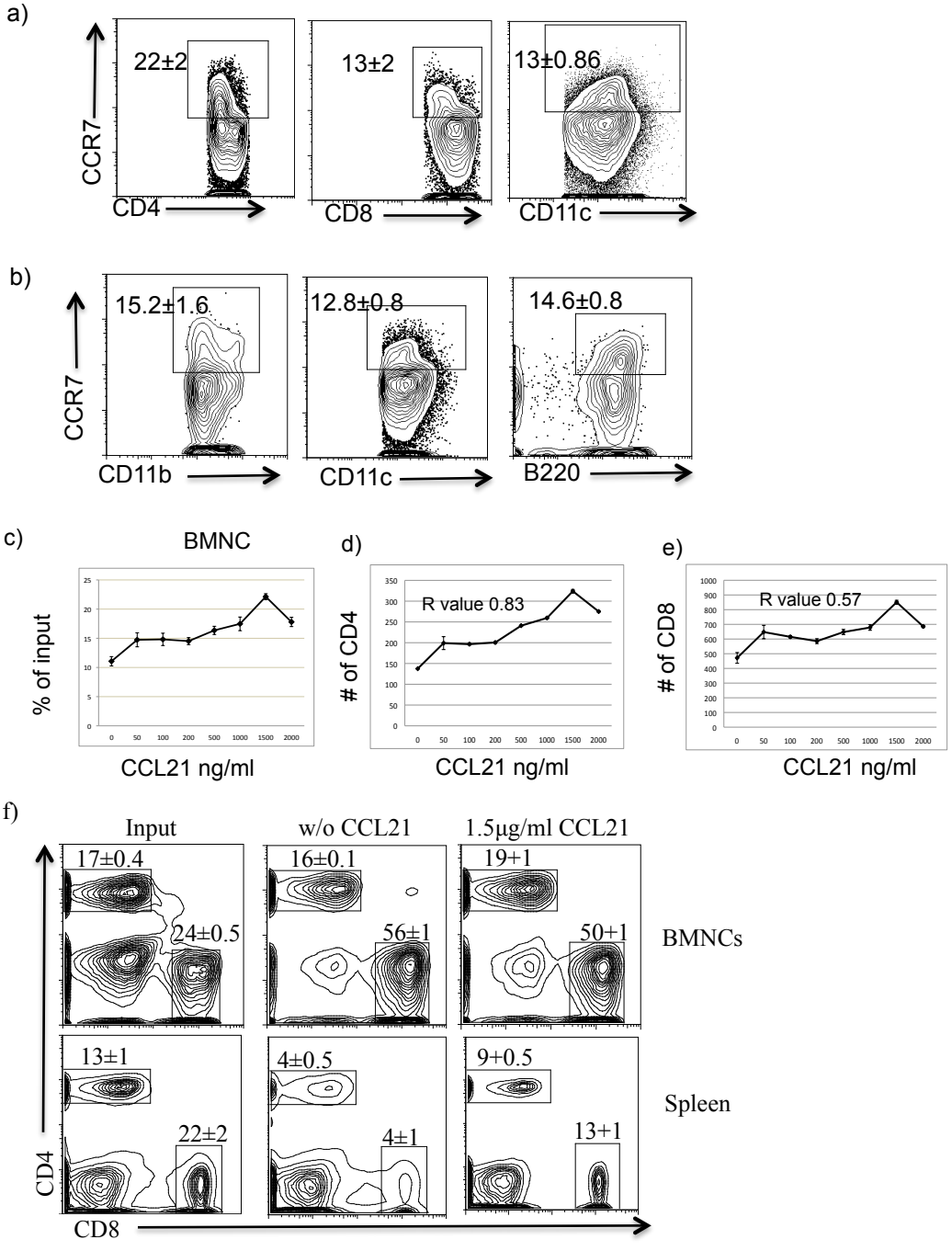

a)
22±2
13±2
13±0.86
CCR7
CD4
CD8
CD11c
b)
15.2±1.6
12.8±0.8
14.6±0.8
CCR7
CD11b
CD11c
B220
c)
BMNC
% of input
CCL21 ng/ml
d)
R value 0.83
# of CD4
CCL21 ng/ml
e)
R value 0.57
# of CD8
CCL21 ng/ml
f)
Input
w/o CCL21
1.5μg/ml CCL21
17±0.4
24±0.5
16±0.1
56±1
19+1
50+1
BMNCs
13±1
22±2
4±0.5
4±1
9+0.5
13+1
Spleen
CD4
CD8

Figure 3.1: CCR7 expression on BMNCs and their chemotactic response to CCL21.

a) proportions of CCR7$^+$ population on CD4$^+$, CD8$^+$ T cells and CD11c$^+$ cells harvested from the brains of 4 week infected mice; b) CCR7 expression on different DC subsets- CD8$^+$ DCs (gated on CD11b$^+$CD8$^+$), conventional DCs (gated on CD11b$^+$CD11c$^+$) and plasmacytoid DCs (gated on CD11b$^-$CD11c$^+$) respectively; c) percentage of BMNCs compared to the number of cells in the "input" sample and total number of CD4 T cells (d); and CD8 T cells (e) migrated through the transwells at each different CCL21 concentrations (0-2000 ng/ml). Absolute CD4 or CD8 T cell count is calculated form the percentage of CD4 or CD8 T cells and the total number of cells migrated in that corresponding well. f) proportions of CD4 and CD8 T cells- in the "input" sample (left), in the cells that had migrated in response to 1500 ng/ml CCL21 (right) or without any chemokine (middle).

Although CCR7$^+$ leukocytes were evident in the infected brain, they may or may not be responsive to the presence of CCL21. To test if CCR7$^+$ cells remain responsive to the presence of CCR7 ligands, chemotaxis assays were performed on the cells harvested from the infected brain. Data suggest a significant proportion of BMNCs (up to 23% of the total "input" or cells present in transwells) responded to increasing concentrations of CCL21 (Figure 3.1c). Phenotypic analysis of the cells that had migrated through the transwell inserts suggest that T cells were the major responders ($\sim$75-80%) to CCL21. There was an increasing number of total CD4 (Figure 3.1d) and CD8 (Figure 3.1e) T cells migrating in response to CCL21 with peak cell migration at 1.5 μg/ml CCL21. A small number of cells also migrated in a chemokine-independent manner (Figure 3.1c-e). The proportions of CD4 or CD8 T cells were compared between the cells harvested from infected brains and spleens; and those that had migrated in the presence (1.5 μg/ml) or absence of CCL21 (Figure 3.1f). Interestingly, in contrast to splenic CD8 T cells, the proportion of CD8 T cells in the brain cell suspension that had migrated without CCL21 was significantly increased than that of the corresponding

"input" sample (Figure 3.1f). Particularly, there was a two-fold increase in the proportion of CD8 T cells where cells migrated through the transwell inserts without the presence of any chemokine compared to the proportion of CD8 T cells in the BMNCs "input" sample (Figure 3.1f, top panel). Thus, CD8 T cells from the chronically infected brain were apparently more active and migratory compared to CD8 T cells from the infected spleen. However, there was also a chemokine-dependent CD8 T cell migration which was evident from an increase of the proportion of CD8 T cells migrated at 1.5 μg/ml CCL21 compared to the BMNC "input" (Figure 3.1f, top panel) and as well as the total number of CD8 T cells responding to increasing CCL21 concentrations (Figure 3.1d). The proportion (Figure 3.1f) and the total number (Figure 3.1e) of CD4 T cells migrated at 1.5 μg/ml CCL21 were also increased than those of cells migrated in chemokine-independent manner. These data indicate CD4 T cells demonstrate a strong chemotactic response towards CCL21. In fact, CD4 T cells appeared to be more responsive to increasing concentrations of CCL21 than CD8 T cells as indicated by R^2 (the coefficient of determination) values 0.84 and 0.57 respectively. All together these data indicate that a significant proportion of inflammatory infiltrates from the Toxoplasma infected brain express CCR7 and actively respond to the presence of CCL21.

CNS resident astrocytes induce CCL21 in the infected brain

CCL21 upregulation was detected on the blood vessels, meningeal area as well as in the brain parenchyma even in the early phase of chronic infection (2 weeks post infection) (data not shown). Preliminary data suggested that CNS resident astrocytes are the primary cells

responsible for CCL21 production following infection. Thus, immunohistochemistry (IHC) of CCL21 with the astrocyte specific protein, GFAP was carried out on the chronically infected brain tissue. In the infected brain, CCL21 expression frequently colocalized with GFAP, however, not all astrocytes expressed CCL21 (Figure 3.2a). This observation led to the investigation of infection associated stimuli for CCL21 induction in the brain.

We hypothesized that CCL21 induction by astrcoytes may represent a directional signal for CNS infiltrating T cells towards the active areas of infection in the brain. Thus, CCL21 induction by astrocytes during infection could be due to a) direct parasite invasion or b) stimulation from the surrounding inflammatory milieu. Direct infection by *T. gondii* tachyzoites can both induce and inhibit cell signaling and such manipulations of the host immune responses may favor parasite or host survival [113]. In addition, direct invasion by tachyzoites can lead to chemokine production by many cells [125][207][64][184][96]. To determine if CCL21 induction in astrocytes is due to direct invasion by parasites or if CCL21 is induced in response to bystander events, primary astrocyte cultures were exposed to the Pru or RH strain of *T. gondii* or stimulated by IFN-γ or soluble Toxoplasma antigen (sTAg) (Figure 3.2b).

Astrocytes are primary components of the blood brain barrier [138]. It is likely, due to systemic concentrations of IFN-γ in the blood following infection, that many astrocytes are exposed to IFN-γ even before becoming infected. Astrocytes that have been primed with IFN-γ can effectively control parasite replication [78]. IFN-γ may not induce CCL21 expression as it suggests the presence of effector T cells and thus, the requirement for their recruitment is diminished. In fact, no significant induction of CCL21 was detected from astrocytes

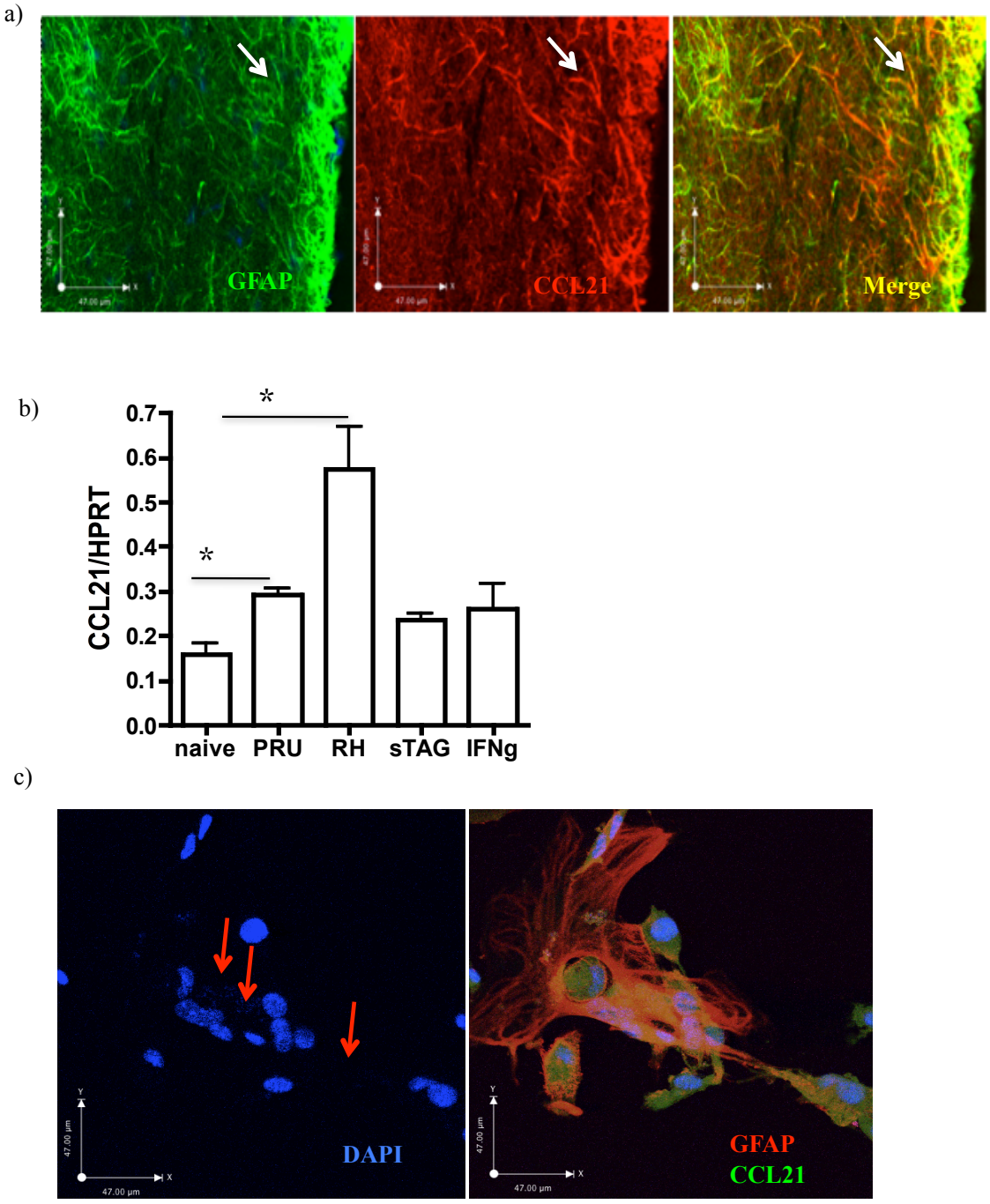


Figure 3.2: Investigating the infection associated stimulus for CCL21 production.

a) Real-time PCR data demonstrates significant induction of CCL21 by astrocytes following direct infection with Pru and RH strain of *T. gondii*; b) CCL21 expression in the chronically infected brain tissue (21 days post-infection) often colocalizes with astrocytes, GFAP (green), CCL21 (red); c) CCL21 expression by infected astrocytes *in vitro*, arrows indicate Toxoplasma tachyzoites.

stimulated with IFN-γ ((Figure 3.2b). This implies that continued parasite invasion would lead to heightened CCL21 production which was evident by a significant increase of CCL21 copy numbers following direct infection with *T. gondii* whereas stimulation with sTAg was not an efficient inducer of CCL21 (Figure 3.2b). CCL21 expressing astrocytes were identified that had been directly infected with *T. gondii* suggesting direct invasion induces CCL21 (Figure 3.2c). In addition, there was $\sim$2-fold more CCL21 transcripts following infection with the virulent and actively replicating RH strain compared to CCL21 transcripts following infection with the Pru strain of *T. gondii* (Figure 3.2b). These data support the idea that continued parasite replication might be responsible for CCL21 induction by astrocytes.

CCR7 is not essential for T cell entry into the infected CNS

CCR7 has been suggested as an essential regulator of T cell infiltration during CNS inflammation. However, cells may change their CCR7 expression following entry into the CNS as observed in MS lesions [102]. Thus, our data suggesting that not all T cells harvested from the brain express CCR7 is not sufficient to prove that T cells do not require CCR7 to enter into the Toxoplasma infected brain. To determine the requirement for CCR7 for T cell infiltration during Toxoplasma infection, T cells isolated from CCR7-deficient mice were adoptively transferred into WT congenic mice prior to infection with the Pru-OVA strain of *T. gondii*. By 4 weeks post infection CD45.2$^+$ (CCR7$^{-/-}$) T cells were detectable in the infected brain. To exclude any possible defect on T cell activation and priming of CCR7$^{-/-}$ T cells that may impact their ability to enter the brain, the generation of OVA-specific CD8 T

cells was investigated in the recipient mouse brain as well as in the spleen (Figure 3.3a). Data from this experiment indicated that the proportion and total number of Ag-specific CD8 T cells in the brain or spleen did not differ significantly during WT or CCR7$^{-/-}$ T cell transfer. Both the CD4 and CD8 T cell populations, proportions (Figure 3.3b) and absolute numbers (Figure 3.3c) of adoptively transferred cells were equivalent between WT T cell transfer and CCR7-deficient T cell transfer indicating CCR7 is not required for T cell entry into the infected brain.

To confirm these findings, the requirement of CCL21 in recruiting inflammatory infiltrates in the infected brain was also tested using *plt/plt* mice. Furthermore, the consequence of CCL21 overexpression on accumulation of inflammatory infiltrates in the brain was analyzed using transgenic GFAP-CCL21 (CCL21 expression driven under GFAP expression cassette) mice. Quantification of inflammatory infiltrates isolated from the brains following 2 weeks (early chronic phase of infection) and 6 weeks post-infection revealed no significant defect in the total number of inflammatory infiltrates in the brains of *plt/plt* mice (Figure 3.4a). In fact, there was an overall increase in total cells isolated from the brains of both *plt/plt* mice or GFAP-CCL21 mice compared to the WT mice. Flow cytometric analysis was conducted to analyze the proportions and numbers of immune cell populations in the absence or in the presence of overexpression of CCL21. There was no statistical difference between CD45^{hi} macrophages nor CD8 T cells isolated from the brains of chronically infected wild-type, *plt/plt* and GFAP-CCL21 mice (Figure 3.4b). By contrast, 2-fold more CD4 T cells were isolated from the brains of both *plt/plt* and GFAP-CCL21 mice than from WT mice

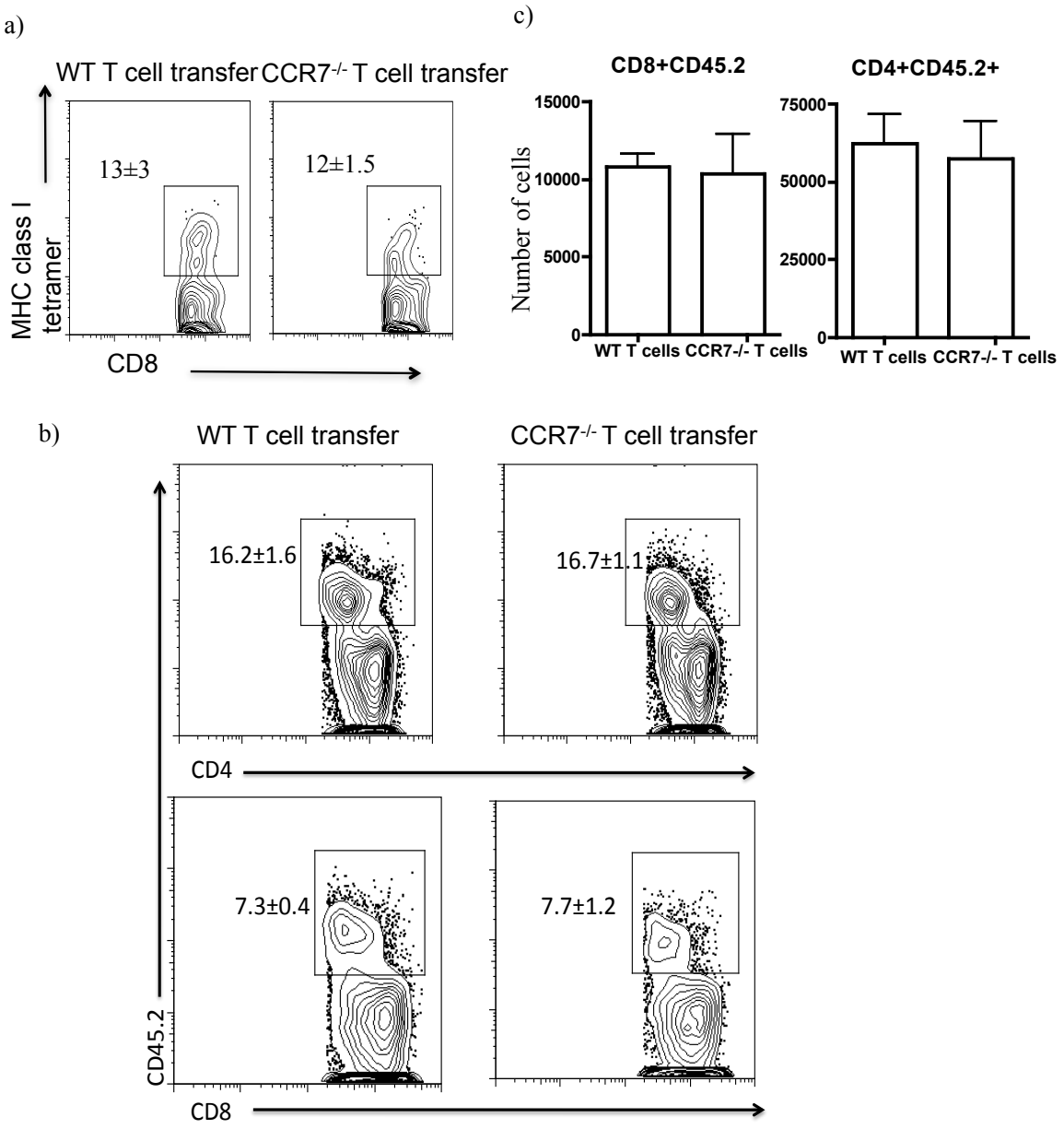


Figure 3.3: CCR7-independent T cell infiltration in the infected brain.

T cells from naive WT and CCR7-deficient mice were isolated and adoptively transferred into CD45.1 congenic mice 1 day prior infection with Pru-OVA strain of *T. gondii.* a) Proportions of OVA-specific CD8 T cells in transferred (CD45.2$^+$) CD8 T cells and b) proportions of transferred cells (CD45.2$^+$) in total CD4 or CD8 T cells and absolute numbers of CD45.2$^+$ CD4 or CD8 T cells isolated from the brains of 4 week chronically infected CD45.1 mice during WT versus CCR7$^{-/-}$ T cell transfer.

(Figure 3.4b). In the absence of CCL19/CCL21, T cells were inefficient at limiting and controlling *T. gondii* infection in peripheral tissues and brain resulting in significantly higher parasite burdens ($\sim$3-fold in *plt/plt* versus WT mice) (Figure 3.4c). Thus, the higher levels of CD4 T cells in *plt/plt* mice may reflect more robust stimulation of the immune response provided by the higher parasite burden. However, these data also indicate that in the presence of strong antigenic stimulus such as *T. gondii*, extravasation of activated myeloid cells and lymphocytes from the blood into extraparenchymal spaces is CCL21-independent.

CCR7-CCL21 interaction is required for efficient CD4 T cell migration from perivascular sites into the CNS parenchyma

The finding that both CCL21 deficiency and CCL21 overexpression recruited significantly more CD4 T cells into the infected brain, led to the investigation of the locations and distributions of inflammatory infiltrates in these mouse brains. Histologic examination of brains during the early phase of infection (14 days post-infection) revealed CCL21-dependent accumulation of inflammatory infiltrates at perivascular and meningeal sites (Figure 3.5). Inflammatory infiltrates could be detected under the meninges of all strains of mice (Figure 3.5a-c). However, accumulation of inflammatory infiltrates was much greater and much more extensive in CCL19/CCL21-deficient mice than in non-transgenic WT mice. Similarly, accumulation of inflammatory infiltrates at perivascular sites within the brain parenchyma was readily detected in infected WT and *plt/plt* mice (Figure 3.5d-e); But, perivascular infiltrates were

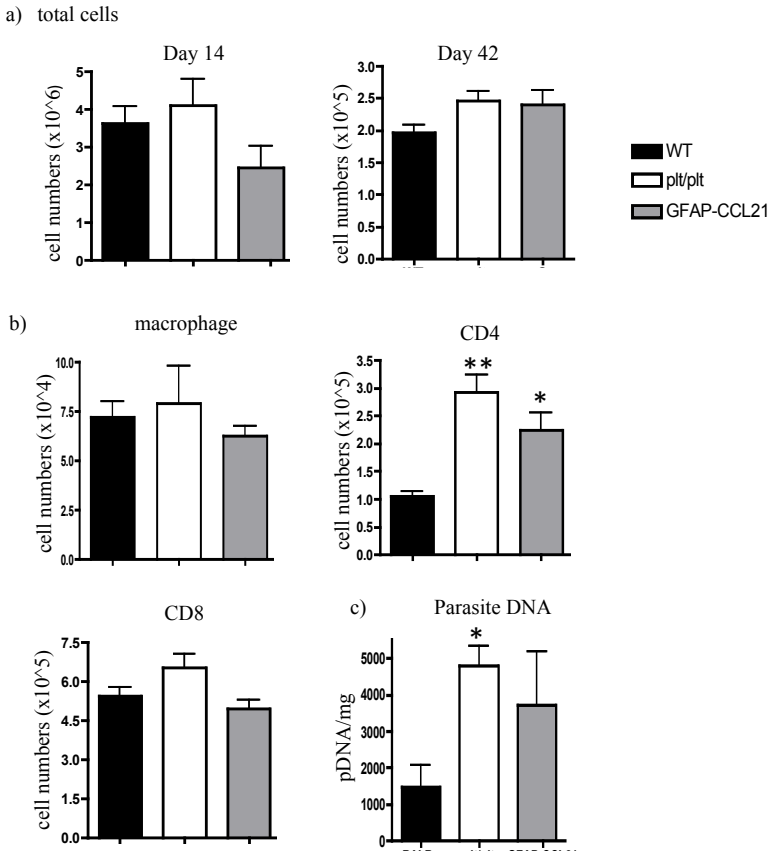


Figure 3.4: CCL21 is not required for CD4 T cell extravasation in the chronically infected brains.

a) The total number of cells isolated from the CNS of WT, CCL19/CCL21-deficient (*plt/plt*) and GFAP-CCL21 mice isolated 2 weeks and 6 weeks following *T. gondii* infection. b) The absolute numbers of $CD45^{hi}$ macrophages, CD4 T cells and CD8 T cells harvested from chronically (6 week) infected mice, determined using flow cytometric analysis of BMNCs c)) the burden of parasite DNA per mg brain weight. In all panels, the error bars represent mean $\pm$SEM. Significant difference from WT was determined using a 2-tailed Student's T test: *p$<$0.05, **p$<$0.01.

much greater in infected *plt/plt* mice. Conversely, perivascular accumulation of inflammatory infiltrates in the parenchyma of GFAP-CCL21 was rare and difficult to detect (Figure 3.5f).

Both CD4 and CD8 T cells can respond to CCL21 via high affinity interactions with CCR7 (reviewed in [154]). In addition, both CD4 and CD8 T cells infiltrate the brain in response to *T. gondii* infection ([66] and Figure 3.5g-i). Strikingly, while CD8 T cells infiltrated the parenchyma of all three strains of infected mice, CD4 T cells preferentially accumulated at perivascular and extraparenchymal sites in *plt/plt* mice. Specifically, the ratio of CD4 to CD8 T cells in extraparenchymal sites was more than 2-fold greater in *plt/plt* mice than in either WT or GFAP-CCL21 (Figure 3.5j). Conversely, the ratio of CD4 to CD8 T cells within the parenchyma was reduced more than 2-fold in *plt/plt* mice as compared to both WT or GFAP-CCL21 (Figure 3.5k). Lastly, confirming our flow cytometric data, no inflammatory infiltrates, CD4 or CD8 T cells could be detected by histologic analysis in any of the three strains of mice in the absence of infection [156]. These data indicate that there is a dependency on CCL21 expression for CD4, but not CD8 T cell migration from perivascular to intraparenchymal sites, even in the presence of a strong antigenic signal presented in an inflammatory context.

We therefore examined the CCL21-dependence of inflammatory infiltrate accumulation at the stable, chronic phase of infection (Figure 3.6-3.7). At 6 weeks post-infection, inflammatory infiltrates were detected in all three strains of mice by hematoxylin and eosin histology (Figure 3.6). Notably, the magnitude of inflammatory infiltrate accumulation at extra-parenchymal sites (submeningeal, ventricular and perivascular) in the brains of *plt/plt*

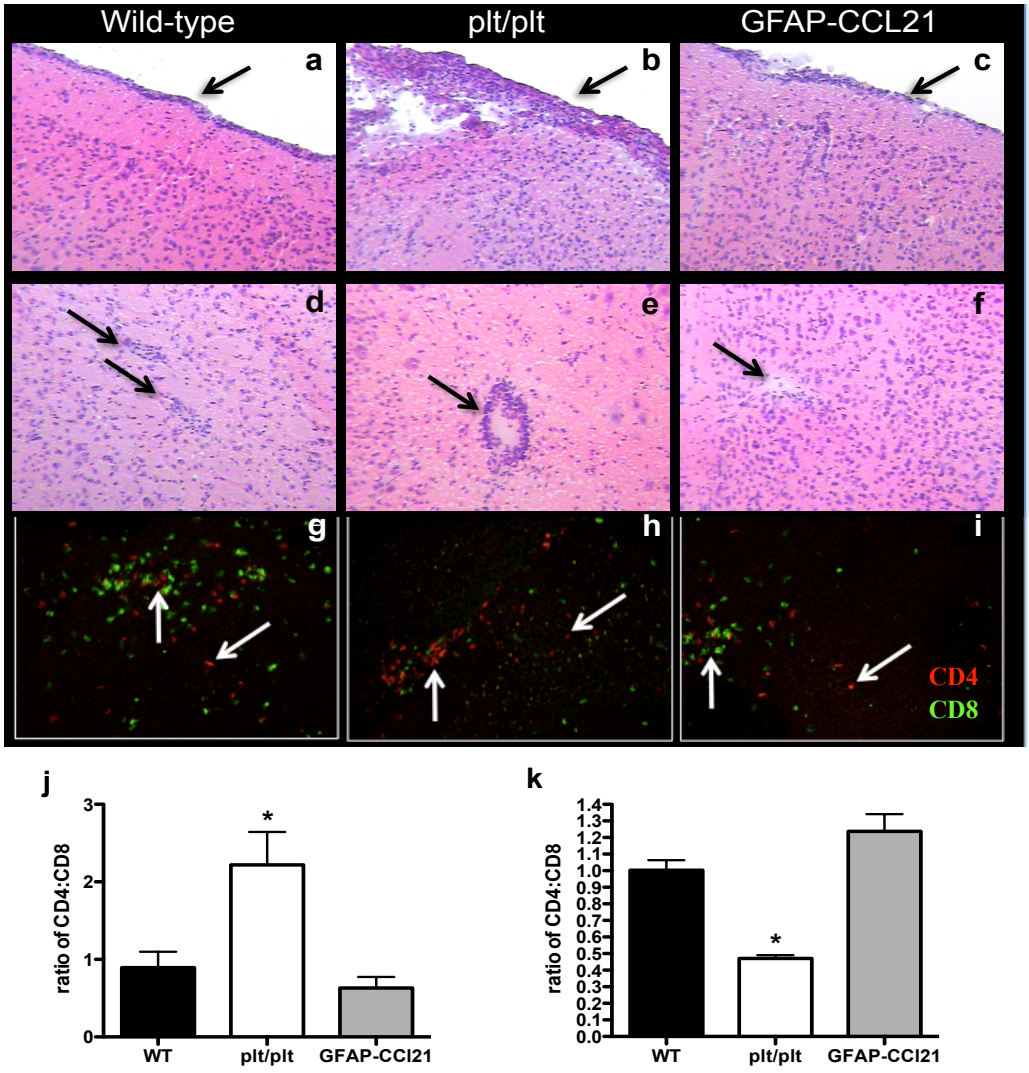


Figure 3.5: CCL21 is required for efficient migration of CD4 T cells into *T. gondii*-infected brain.

Hematoxylin and eosin histology of meningeal (panels a-c) and cortical parenchymal (panels d-f) brain sections from WT (panels a, d, g), CCL19/21-deficient (*plt/plt*, panels b, e, h) and GFAP-CCL21 (panels c, f, i) mice 14 days postinfection. Left downward pointing arrows indicate meningeal inflammatory infiltrates (panels a-c). Right downward pointing arrows indicate perivascular regions (panels d, f, e). In panels g-i, CD4 T cells are visualized in red with PE-conjugated anti-CD4 antibodies CD8 cells are visualized in green with FITC-conjugated anti-CD8 antibodies. Upward pointing arrows indicate CD4 T cells in perivscular spaces, downward pointing arrows indicated CD4 T cells in parenchymal spaces. Quantitation of the ratio of CD4 T cells /CD8 T cells is depicted in panels j (ratio in extraparenchymal spaces) and k (ratio within parenchyma). In both panels, error bars represent mean +/- SEM. Significant difference from WT was determined using a 2-tailed Student's T test: *$p<0.05$.

mice was greater than that observed in WT and GFAP-CCL21 mice (Figure 3.6). Immunohistochemistry of CD4 T cells confirmed the CCL21-dependent accumulation of CD4 T cells in stable, chronically infected (6 week) *plt/plt* mice brain (Figure 3.7), as observed in early phase of chronic infection (Figure 3.5). However, this was not a simple consequence of higher numbers of CNS-infiltrating cells in the brains of *plt/plt* mice. Although similar numbers of CD4 T cells were present in infected *plt/plt* and GFAP-CCL21 mice, they were not similarly distributed within the chronically infected brain (Figure 3.7). While at least some CD4 T cells were found in the parenchyma of all chronically infected mice, the proportion of CD4 T cells in extraparenchymal (submeningeal and ventricular, Figure 3.7a-f) versus parenchymal sites was greatest in *plt/plt* mice (Fig, 3.7g-i). The few CD4 T cells detected within the brain parenchyma of *plt/plt* mice were often detected in close proximity to VCAM-1 expression that is indicative of activated vascular endothelium and sites of lymphocyte influx. Furthermore, the ratio of CD4 T cells in extraparenchymal sites versus intraparenchymal sites was $\sim$6-fold higher in *plt/plt* mice than in either WT or GFAP-CCL21 mice at 6 weeks post-infection (Figure 3.7j).

To determine whether the effect of CCL21 on CD4 T cell migration is mediated by CCL21 signaling through its primary receptor CCR7, analysis of the location and distribution of adoptively transferred CCR7-deficient T cells was conducted in the brain tissues of infected CD45.1 mice. This experiment also allowed us to verify that the migratory defect of CD4 T cells we observed during chronic infection in the brain was not due to any possible defect in the periphery during acute infection in *plt/plt* mice. Laminin staining was per-

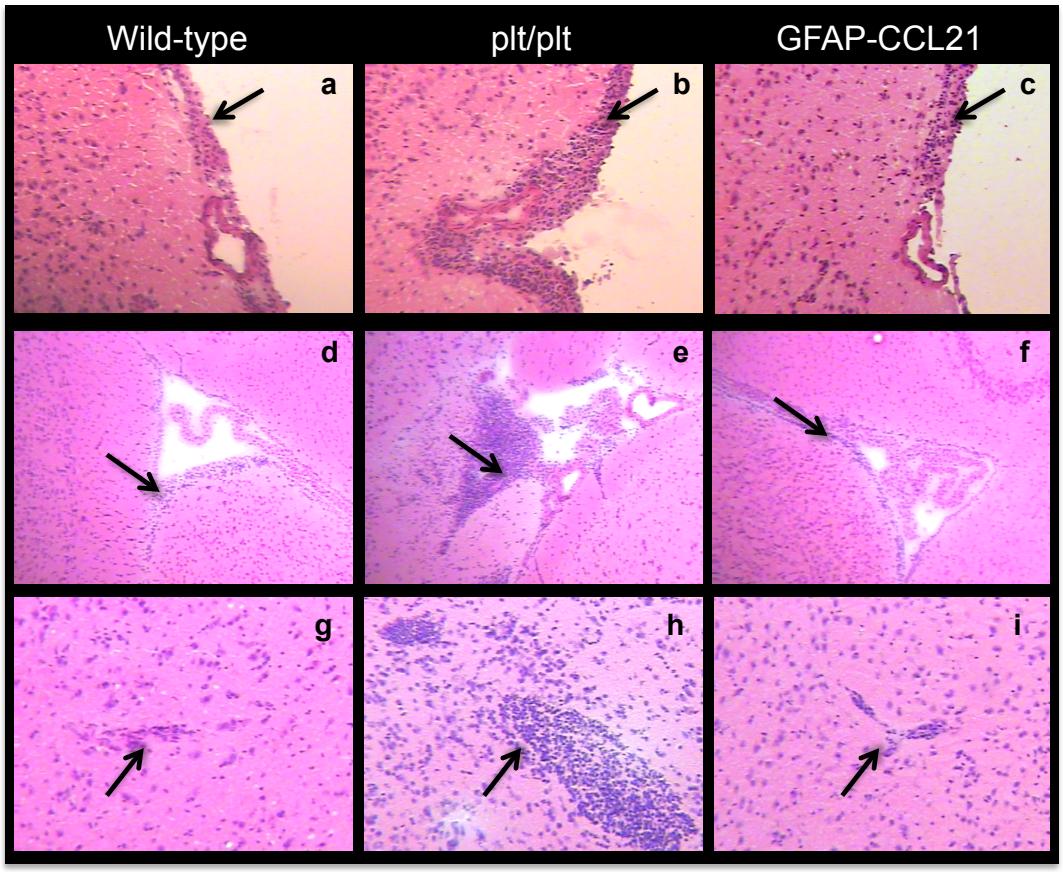


Figure 3.6: Requirement for CCL21 continues in chronic *T. gondii*-infected brain.

Hematoxylin and eosin histology of meningeal (panels a-c), ventricular (panels d-f) and cortical parenchymal (panels g-i) brain sections from WT (panels a, d, g), CCL19/21-deficient (*plt/plt*, panels b, e,h) and GFAP-CCL21 (panels c, f, i) mice 6 weeks postinfection. Left downward pointing arrows indicate meningeal inflammatory infiltrates (panels a-c). Right downward pointing arrows indicate ventricular/subventricular regions (panels d and e). Right upward pointing arrows indicate perivascular regions (panels g-i).

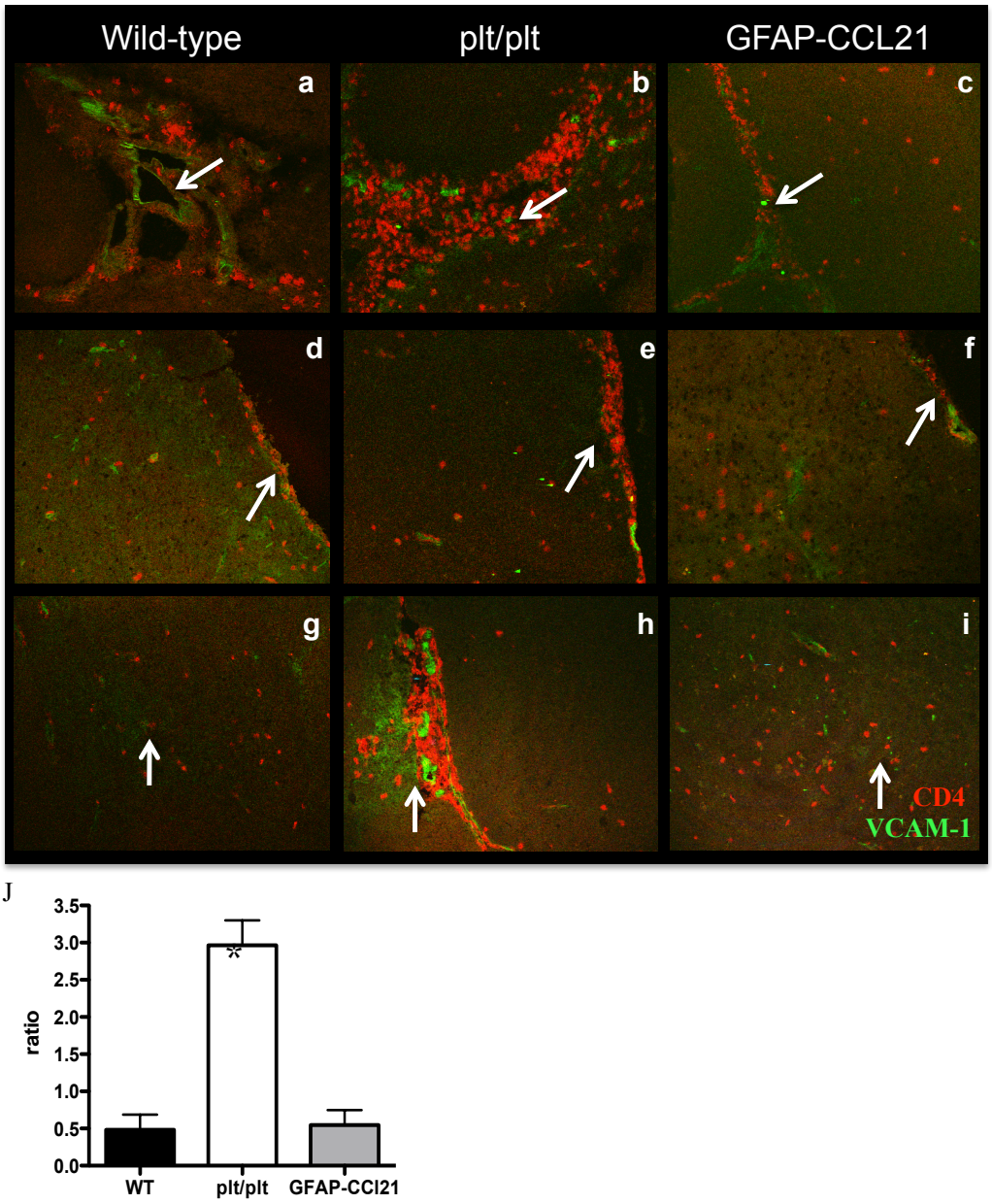


Figure 3.7: CD4 T cells preferentially accumulate in non-parenchymal sites of chronically infected brains in the absence of CCL21.

Immunofluorescence of CD4 T cells (red) and VCAM-1 (green) at meningeal (panels a-c), ventricular (panels d-f) and parenchymal (panels g-i) brain regions from WT (panels a, d, g), CCL19/21-deficient (*plt/plt*, panels b, e, h) and GFAP-CCL21 (panels c, f, i) mice 6 weeks postinfection. Arrows indicate CD4 T cells in the chronically infected brain. Panel J represents the quantitation of the ratio of CD4 T cells in extraparenchymal sites/CD4 T cells within parenchymal sites. Error bars represent mean +/- SEM. Significant difference from WT was determined using a 2-tailed Student's T test: *$p<0.05$.

formed to visualize T cells between the inner and outer limits of perivascular space (Figure 3.8a). Quantification of the ratio of the adoptively transferred CD4 or CD8 T cells present in extraparenchymal spaces to within the CNS parenchyma (Figure 3.8b) revealed significantly more CD45.2^{+}CD4 T cells in the perivascular area during CCR7$^{-/-}$ T cell transfer compared to WT T cell transfer. Thus, similar to the results seen in *plt/plt* mice, the ability of CCR7-deficient CD4 T cells to migrate from the perivascular areas was significantly diminished (Figure 3.8b). Of note there was no significant difference in CD45.2^{+}CD8 T cell migration in the infected brain tissue during WT and CCR7$^{-/-}$ T cell transfers (Figure 3.8b). To summarize, CCR7-CCL21-mediated interactions facilitate CD4 T cell migration from perivascular sites into the CNS parenchyma.

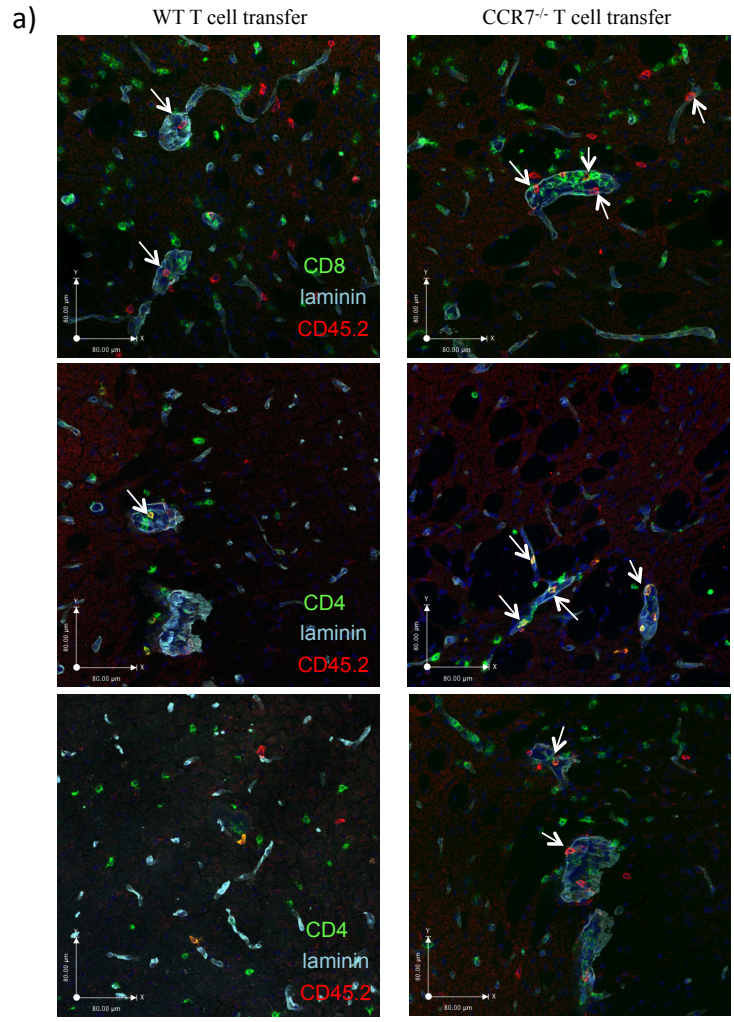

a)
WT T cell transfer
CCR7-/- T cell transfer
CD8
laminin
CD45.2
CD4
laminin
CD45.2
CD4
laminin
CD45.2
b)
CD4
CD8
ratio
1.25
1.00
0.75
0.50
0.25
0.00
1.0
0.8
0.6
0.4
0.2
0.0
*
WT
CCR7-/-
WT
CCR7-/-

Figure 3.8: Involvement of CCR7 in CCL21-dependent CD4 T cell migration within the CNS parenchyma.

T cells form naive WT and CCR7-deficient mice were isolated and adoptively transferred into CD45.1 congenic mice 1 day prior infection. Mice were sacrificed at 4 weeks post-infection and brain tissues were stained with laminin (cyan), CD45.2, to detect transferred cells (red) and CD4 or CD8 T cells (green) (a). Arrows indicate the transferred CD4 T cells in the perivascular areas in the brain during WT versus CCR7$^{-/-}$ T cells transfer. b) Quantitation of the ratio of CD4 T cells in extraparenchymal sites to CD4 T cells within parenchymal sites, quantified in histologic images from three biological replicates per condition. Error bars represent mean +/- SEM. Significant difference from WT was determined using a 2-tailed Student's T test: *$p<0.05$.

A new insight to CCL21 and CNS autoimmunity

Outside the CNS, endogenous and transgenic CCL21 expression is sufficient to lead to accumulation and organization of large numbers of T cells into lymph node-like structures in most tissues [110][59]. In addition, increased CCL21 expression within tissues is sufficient to pre-activate auto-reactive T cells and trigger autoimmunity by promoting homeostatic proliferation [154][59]. Other tissues such as the skin are refractory to lymphoid formation even in the presence of transgenic CCL21 expression [30]. Thus, CCL21 has been thought to contribute to the development of follicle-like structures observed in the meningeal sites of MS patients [4]. However, studies described in this chapter using GFAP-CCL21 mice indicate that the CNS is rather refractory in CCL21 driven lymphoid neogenesis. In the transgenic murine model of astrocytic driven CCL21 expression (GFAP-CCL21 mice), CCL21 expression was detected only in the CNS. Although in GFAP-CCL21 mice, CNS-produced CCL21 was bioavailable and CCL21 expression was even higher than it's level in the in-

fected non-transgenic mouse brain, in the absence of pathogenic stimuli lymphocytes could not be detected in the perfused brain by histologic or flow cytometric measures. It is possible that in the absence of infection, CD4 T cells could not gain access to the CNS. Despite the presence of $\sim$2.5-fold higher numbers of CD4 T cells in the brain parenchyma of infected GFAP-CCL21 mice than in WT mice, CD4 T cells did not form organized lymphoid-like structures. Instead they remained dispersed throughout the CNS. Thus, there is no essential link between CCL21 expression and ectopic lymphoid tissue formation in the CNS.

3.4 Discussion

CCL21 is a potent chemoattractant and functional regulator of CD4 T cells [62][154][59]. For example, in non-immune privileged organs, CCL21 plays at least three distinct roles regulating CD4 T cell function. First, CCL21 promotes homeostatic proliferation of CD4 but not CD8 T cells [154]. Second, CCL21 recruits CD4 T cells and other immune cells involved in adaptive immunity to the sites of expression [59] [30]. Third, CCL21 expression is sufficient to organize chronic inflammatory infiltrates into lymphoid-like structures [154] [30]. In the CNS, CCL21 expression is induced by a broad array of injury, infectious and autoimmune triggers [110]. The consequences of the presence of CCR7 and CCL21 within the CNS are ill defined. Here we report the sources of CCR7/CCL21 expression in the infected CNS as well as the contribution of CCR7-CCL21 interactions in the chronically infected CNS.

Previous studies have revealed an uneven distribution of Toxoplasma reactivation in the brain. Following immunosuppression the foci of parasite recrudescence were predominantly found in frontal and parietal cortex [42]. Analysis of inflammatory infiltrates at these parasite foci demonstrated abundant lymphocytes in the vicinity of replicating parasites and microvasculature. Thus, guided T cell migration towards these localized foci of parasites would be a possible immune strategy of CNS protective immunity. Understanding how peripheral cells are directed to the site of infection and still prevent immunopathology in the CNS has direct relevance to controlling the CNS infectious diseases. Yet the current literature is primarily limited to the adhesion molecules and chemokines that facilitate entry to this site and not the factors that influence inflammatory cells once within the brain. This chapter provides a CCL21-dependent mechanism of directing T cell migration in the infected CNS and highlights the contribution of infection-induced CNS resident glial cells in this process.

This study identifies $CCR7^{+}$T cells in the infected CNS and demonstrates that CCL21 is a potent chemoattractant especially for CD4 T cells that have infiltrated in the brain. This led to the investigation of the possible roles of CCR7 signaling in controlling T cell migration in the chronically infected CNS. Outside the CNS, expression of CCL21 is not required to recruit lymphocytes to the sites of infection or autoimmune triggers [62][155]. Thus, it was not surprising that lymphocytes could be recruited to extra-parenchymal sites within the brains of CCL21/CCL19-deficient mice in response to *T. gondii* infection. However, analysis of the location and distribution of T cell infiltrates in the absence of CCR7 or CCL21 revealed a role for this chemokine signaling in regulating T cell migration within the CNS.

In CCL19/CCL21-deficient mice, most CD4 T cells (not CD8 T cells) failed to enter the infected CNS parenchyma, while in GFAP-CCL21 mice nearly all CD4 T cells entered the infected CNS parenchyma. Similarly, compared to CD4 T cells from WT mice, adoptively transferred CCR7$^{-/-}$ CD4 T cells were less efficient at migrating into the CNS parenchyma. Therefore, we conclude that CCR7/CCL21 facilitates CD4 T cell migration into the CNS parenchyma.

In addition, despite the high numbers of CD4 T cells present in the CNS of GFAP-CCL21 mice, no organization of T cells and/or inflammatory infiltrates into neo-lymphoid structures could be detected. Instead, CD4 T cells were found dispersed throughout the infected CNS during chronic inflammation. This data also contributes to the current knowledge of CNS autoimmunity indicating that, the CNS remains refractory to CCL21-driven organization of chronic inflammatory infiltrates even in the presence of constitutive CCL21 expression, chronic influx of CD4 T cells and ongoing pathogenic stimulus. Lastly, CD4 T cell influx into the parenchyma of infected GFAP-CCL21 was much greater than in WT and was detected in all brain regions. These data may in part explain the highly variable parasite burden detected in GFAP-CCL21 mice at 6 weeks post-infection that did not correlate with CD4 T cell or inflammatory infiltrate numbers. Specifically, we speculate that in GFAP-CCL21 mice expression of the CCL21 transgene throughout the brain and spinal cord recruits lymphocytes into the parenchyma of all brain regions (infected and non-infected). Therefore, GFAP-CCL21 mice may be less efficient at localizing CD4 T cell responses to the sites of

infection than WT mice indicating the importance of CCL21 expression from the areas of infections that is evident by CCL21 induction in astrocyte cultures following direct infection.

Chapter 4

CCR7 is dispensable for T cell priming but required for protective T cell memory responses during chronic infection with *Toxoplasma gondii*

4.1 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. CCR7 has been considered as an important coordinator of T cell circulation between lymphoid organs and peripheral tissues during inflammation. CCR7^{+} T cells have previously been identified in the inflamed CNS in several neurological disorders. However, their exact role in T cell-mediated protection in the CNS is not clear. Our previous study defines a CCR7/CCL21-dependent mechanism of T cell migration in the brain. However, whether or how this chemokine-dependent migration contributes in generating protective immune responses following infection remains to be explored. Data presented in this chapter suggest that generation of antigen-specific T cells during the chronic phase of infection is not defective in CCR7-deficient mice. These data indicate the defect in T cell priming we observed during the acute phase of infection (Chapter 2) is a delay rather than an inability to generate antigen-specific T cells in the absence of CCR7. Despite this equivalent T cell priming we establish that the presence of CCR7 is required to maintain an IL-7R^{lo}KLRG1^{+} T cell effector or effector memory population in the brain although not in the draining LNs or spleen. Data will be presented suggesting CCR7 expression on the T cell compartment is necessary to generate the KLRG1^{+} CD8 T cells in the brain, previously defined as a major subpopulation of effector and memory CTLs during *T. gondii* infection. Though CCR7 is not essential to survive chronic infection with Toxoplasma, CCR7-deficient mice are unable to mediate efficient parasite killing following

a secondary infection. All these data point to a vital role of CCR7-mediated signaling at the site of infection to generate protective immunity during Toxoplasma infection.

4.2 Introduction

Chemokines are a major component of the host immune system to shift immune cells from the lymphoid organs to the non-lymphoid peripheral tissues during infection. The chemokine receptor, CCR7 signaling through its ligands CCL19 and CCL21 impacts DC and lymphocyte migration and positioning to ensure optimal T cell priming [62][11]. CCR7 has been implicated in host immunity in multiple ways such as the generation of antigen-specific T cells [62][144], memory T cell recirculation [166] and continuous antigen sampling of DCs from peripheral tissues [91] [92]. However, little is known about the exact mechanisms by which expression of this lymphoid chemokine receptor in non-lymphoid tissues, particularly in the CNS contributes to the generation of host immune responses.

Infection with the intracellular protozoan parasite, *T. gondii* leads to a lifelong chronic infection in the CNS of its host. At this point, *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) [68], [90][177]. However, 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 min-

imum damage to the CNS [208][28]. During *T. gondii* infection and other models of brain inflammation, CNS resident glial cells produce chemokines that recruit leukocytes to the site of inflammation [182]. In such scenarios, CCR7/CCL21 interactions have been thought of as a potential chemokine system to regulate T cell influx and migration in the inflamed CNS.

Our study on the contribution of CCR7 during acute *T. gondii* infection demonstrated that despite high antigen burden during systemic infection, there is an absolute requirement for CCR7 expression on T cells to generate protective immune responses. A profile of CCR7 and its ligands (CCL19 and CCL21) revealed increased transcripts of all of these components in the infected brain (Chapter 2 and [140]). $CCR7^+$ T cells were identified in the chronically infected brain. Investigation into the role of CCR7 ligands revealed that in the absence of CCL19/CCL21 (*plt/plt* mice), CD4 T cells were unable to migrate into the parenchyma of the brain and essentially remained in the perivascular areas (Chapter 3 and [156]). By contrast. the over expression of CCL21 entirely in the CNS (GFAP-CCL21 transgenic mice) led to an increase in CD4 T cell numbers in the brain however, perivascular cuffing of CD4 T cells was not evident. These data indicated CCL21 interactions facilitate CD4 T cell migration from perivascular spaces into the brain parenchyma. Here we investigate the requirement and the contribution of $CCR7^+$ T cell populations in protective immunity during the chronic phase of infection.

Similar to our observation during *T. gondii* infection, several studies on MS and other neurological disorders have demonstrated the presence of $CCR7^+$ inflammatory infiltrates in the CSF and sites of inflammation in the CNS [106][102]. $CCR7^+$ T cells have been de-

fined as circulating central memory T cells that are able to enter the inflamed CNS following activation upon antigen encounter [102]. However, the requirement of CCR7 expression on T cell-mediated protective immune responses in the infected brain is still under investigation. Our data suggest CCR7$^+$ T cells co-express activation and memory T cell markers and contribute to the maintenance of memory populations in the infected brain. Thus, despite equivalent T cell priming in the absence of CCR7 mice are unable to generate sufficient protective responses to a secondary infection. This is associated with an intrinsic requirement for CCR7 expression on CD8 T cells for KLRG1 (Killer cell lectin-like receptor subfamily G member 1) expression at the site of infection. These data highlight the importance of CCR7-mediated interactions at the site of infection to generate protective immune responses during chronic infection in the brain.

4.3 Results

CNS infiltrating CCR7$^+$T cells are a heterogeneous population with effector and memory phenotypes

Antigen-experienced T cells down regulate LN homing receptors as they migrate to non-lymphoid tissues, a subset of effector T cells re-acquire CCR7 expression and become central memory T cells [55][130]. Thus, CCR7$^+$T cells in the inflamed brain are thought to be central memory T cells circulating between the non-lymphoid tissues and secondary lymphoid organs. To further characterize the CCR7$^+$ T cells that have infiltrated the brain during

chronic infection, flow cytometric analysis was conducted to analyze the surface expression of T cell activation and memory markers on $CCR7^+$ as well as $CCR7^-$ T cells (Figure 4.1a). CXCR3 is a Th1 activation associated chemokine receptor, required for controlling parasite replication and T cell migration during chronic infection with *T. gondii* [141] (Harris TH *et al.,* article in press). Analysis of CXCR3 expression reveals that almost all $CCR7^+$ T cells in the brain also express CXCR3. There is a distinct population up to 5% of CD4 or CD8 T cells in the infected brain that are $CD62L^+$ (lymph node homing adhesion molecule), all of which express CCR7 and therefore likely to be a central memory population. CD44 is a widely used memory T cell marker that is known to augment the generation of memory Th1 cells by promoting effector T cell survival [9]. As previously published all T cells harvested from the infected brain express CD44 in line with their activated status required to get into the brain [60]. The expression of KLRG1 is another T cell activation associated phenotype, used to define terminally differentiated effector or effector memory T cells [164][198]. KLRG1 expressing T cells are efficient producers of cytokines, however, with limited proliferative potential as KLRG1 engagement with cadherin molecules inhibits antigen-induced cell division [195]. About 20-25% of CD4 or CD8 T cells express KLRG1. About 22-26% of $KLRG1^+$ CD4 or CD8 T cells express CCR7 that is less than one-third of total $CCR7^+$ T cells in the infected brain.

IL-7R expression on memory T cells is known to be a survival signal allowing IL-15 and IL-7 signaling and is important for long-term immunity during Toxoplasma infection [16]. In the infected brain there is a distinct population of CD4 and CD8 T cells ($\sim$15% and 7%

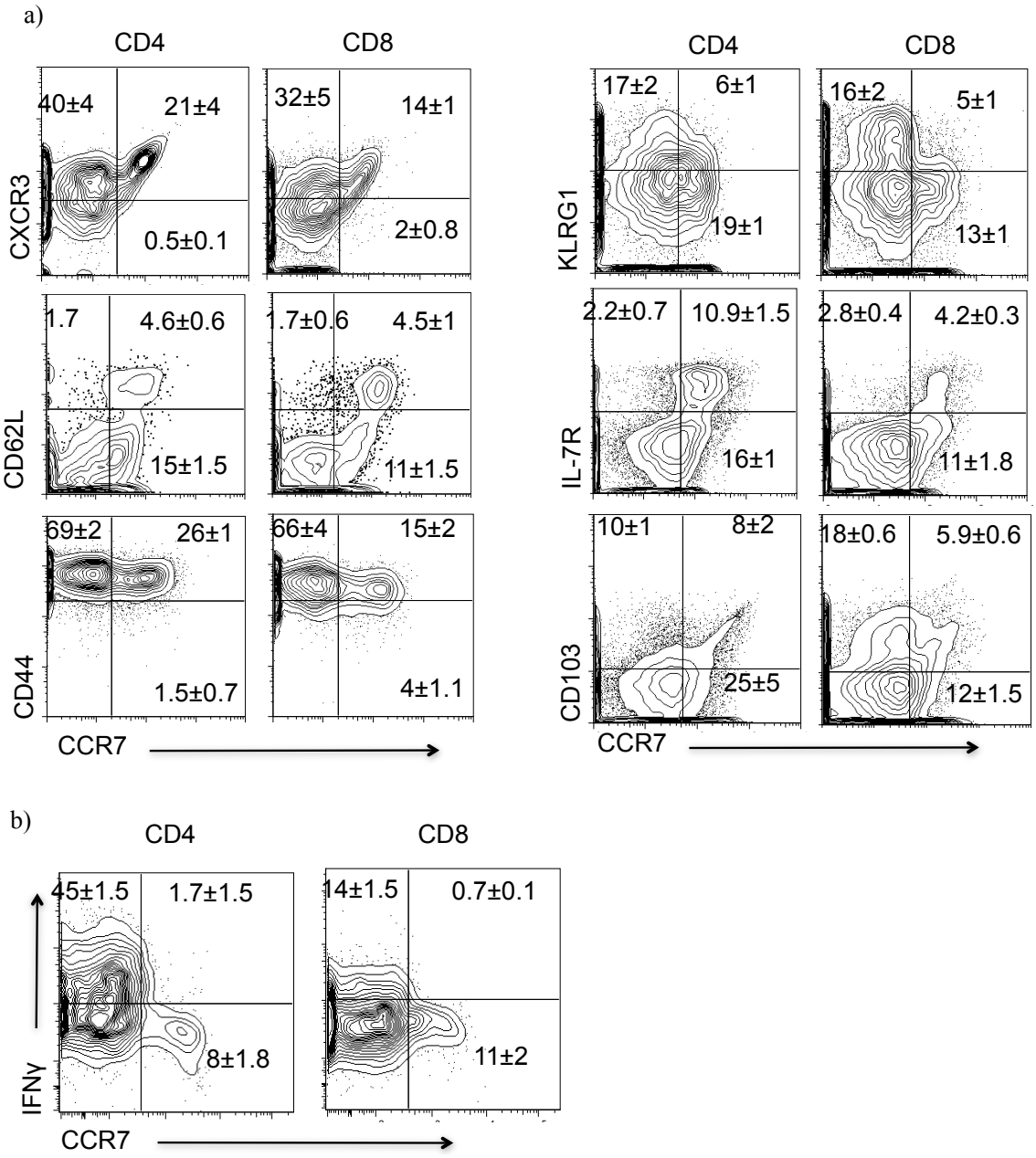


Figure 4.1: CCR7$^+$ T cells in the brain demonstrate effector and memory phenotype.

BMNCs were isolated from the brains of 4 week chronically infected mice. a) *Ex vivo* expression of memory and activation markers versus CCR7 expression were analyzed on T cells gated on CD4 or CD8 T cells; b) BMNCs were stimulated with PMA/ionomycin to induce cytokine production *in vitro*. Following stimulation, intracellular staining was performed to analyze IFN-γ^+ cells versus CCR7 expression on T cells gated on CD4 or CD8 T cells.

respectively) that express IL-7R. The majority of IL-7R$^+$ T cells also express CCR7 in line with the evidence suggesting CCR7 expression on memory T cell populations. A further marker of memory T cells is CD103 (Integrin, alpha E) that has been identified as a marker for tissue-resident memory T cells in the brain [199]. Approximately, 20% of CD4 T cells and $\sim$25% of CD8 T cells in the infected brain express CD103. About one-half of CD4$^+$CD103$^+$ T cells are also positive for CCR7 which is $\sim$25% more compared to CCR7 expression on CD8$^+$CD103$^+$ T cells. Overall, one-third of CCR7$^+$CD4 or CD8 T cells express CD103. All these data indicate CCR7$^+$ T cells in the infected brain are an activated T cell population with a mixed phenotype of effector and memory T cells.

IFN-γ is the major cytokine required to control parasite replication [43]. To investigate the ability of CCR7$^+$T cells to produce this effector cytokine, BMNCs from the chronically infected brain were stimulated *in vitro*. Following stimulation with PMA/ionomycin, 45% of CD4 T cells and 15% of CD8 T cells harvested from the chronically infected brain were able to produce IFN-γ. However, IFN-γ^+ T cells were largely negative for CCR7 expression (Figure 4.1b). A decrease in the proportion of CCR7$^+$ T cells was evident in both CD4 and CD8 T cells following stimulation *in vitro*. Thus, it is also possible that, following stimulation T cells down regulate CCR7 expression that limits our ability to detect all CCR7$^+$T cells that produce effector cytokines.

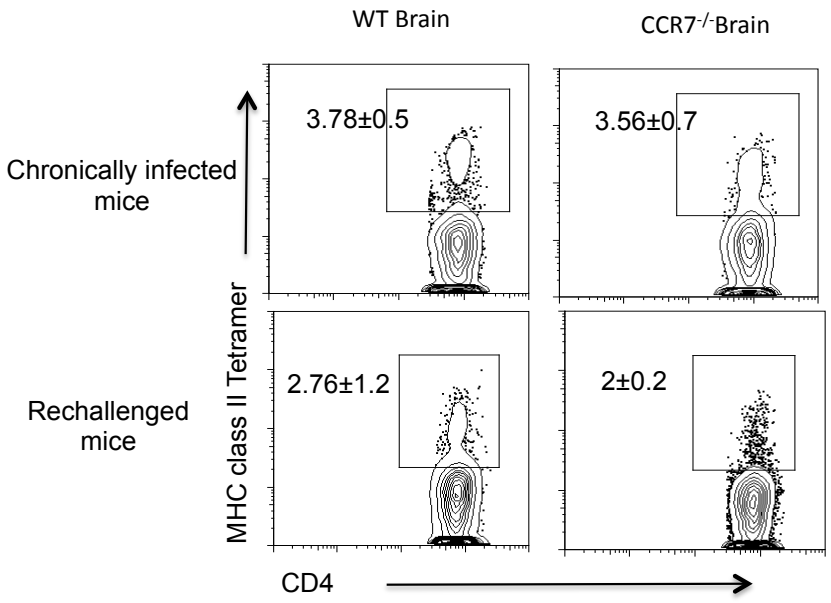
CCR7 is dispensable for T cell priming but required for efficient memory responses during chronic infection with *T. gondii*

Data described above indicated that a significant proportion of CCR7$^+$ T cells in the infected brain demonstrate the phenotype of central or effector memory T cells. Thus, we hypothesized these CCR7$^+$ T cells may play a vital role in generating memory responses to Toxoplasma infection. To test this hypothesis the ability of CCR7-deficient mice to generate sufficient immune responses was tested following a secondary infection with RH-OVA, a highly virulent strain of *T. gondii*. As CCR7 is an absolute requirement during acute *T. gondii* infection, CCR7-deficient mice were treated with sulfadiazine during acute infection phase that allowed them to keep parasites under control and let them establish a chronic infection. Following sulfadiazine treatment for two weeks, CCR7-deficient mice did not succumb to infection even when they were left untreated for at least another 6 weeks. This suggests CCR7 is not absolutely necessary for ongoing control of parasites and to maintain the chronic stage of infection.

Since CCR7 is crucial for optimal antigen presentation to T cells [62], the generation and maintenance of endogenous parasite-specific T cells was investigated in the absence of CCR7 during the chronic phase of infection with Pru-OVA and also following rechallenge infection with RH-OVA. Multivalent MHC molecules loaded with a single parasite-specific peptide (MHC tetramers) were used to mimic the presentation of peptides by endogenous antigen- presenting cells and enable the identification of T cells recognizing a single epitope. Toxoplasma-specific MHC class II and OVA-specific MHC class I molecules were used to

analyze the parasite-specific CD4 (Figure 4.2a) and CD8 T cell population (Figure 4.2b) during the chronic phase of infection (6 weeks following primary infection) with (bottom panel) or without (top panel) secondary infection. The helper T cells that are specific for a particular peptide are extremely rare within the total T cell population. Thus, only about 2-3.5% of total CD4 T cells in the brain were found to recognize the peptide, AVEIHRPVPGTAPPS from *T. gondii* hypothetical protein (TGME49_012300605-619) presented on MHC class II molecules (Figure 4.2a). In contrast, 10-15% of CD8 T cells in the infected brain were specific for the OVA peptide (Figure 4.2b). Data from these studies suggest the proportions of antigen-specific CD4 or CD8 T cells are equivalent between WT and CCR7-deficient mouse brain (Figure 4.2) and spleen (data not shown). Our previous study suggested there is defect in T cell priming in CCR7$^{-/-}$ mice during the early phase of infection [140]. Thus, these new data suggest that this is a delay rather than their inability to generate antigen-specific T cell populations entirely. Thus, T cell priming is equivalent during the chronic phase of infection that is evident even by 4 weeks post infection (Chapter 3, Figure 3.3a).

a)


WT Brain
CCR7-/-Brain
Chronically infected mice
Rechallenged mice
3.78±0.5
3.56±0.7
2.76±1.2
2±0.2
MHC class II Tetramer
CD4

b)

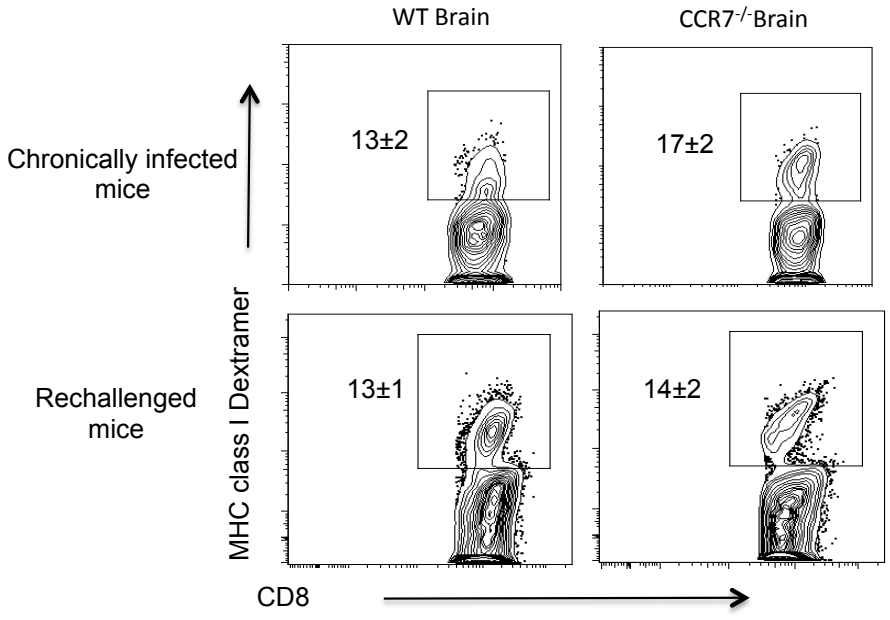

WT Brain
CCR7-/-Brain
Chronically infected mice
Rechallenged mice
13±2
17±2
13±1
14±2
MHC class I Dextramer
CD8

Figure 4.2: Generation of antigen-specific T cells during chronic infection in the presence or absence of CCR7.

CCR7$^{-/-}$ mice (n=8) and WT control mice (n=8) were infected with Pru-OVA strain of *T. gondii*. Following 4 weeks post infection one group of CCR7$^{-/-}$ (n=4) and WT (n=4) chronically infected mice were rechallenged with another virulent strain of *T. gondii* (RH-OVA). All mice (with or without rechallenge infection) were sacrificed 6 weeks following infection with Pru-OVA. Parasite specific peptides embedded in MHC class I or class II molecules (MHC tetramers) were used to study the generation of parasite specific CD4 or CD8 T cells respectively. Toxoplasma epitope specific MHC class II molecules (a) and OVA-specific MHC class I molecules (b) were used to analyze the proportion of antigen-specific CD4 T cells (a) and CD8 T cells (b) in the WT versus CCR7$^{-/-}$ mouse brain during chronic infection with Pru-OVA and following rechallenge with RH-OVA strain of *T. gondii*.

To test the ability to mount protective memory responses, 4 week chronically infected WT and CCR7$^{-/-}$ mice were rechallenged with another virulent strain, RH-OVA. A single parasite of RH strain is able to kill a naive mouse. However, with a previous exposure to Toxoplasma, WT mice are able to generate protective memory responses and survive the infection with RH strain. Thus, the ability of Pru-OVA infected CCR7-deficient mice to survive a secondary infection with RH stain, would be indicative of their ability to mount an intact memory response. Two weeks post rechallenge infection, their parasite burden and tissue inflammation was analyzed. Following rechallenge, chronically infected WT mice were able to generate sufficient memory response to control the secondary infection. Despite equivalent T cell priming, 70% of the CCR7$^{-/-}$ mice succumbed to infection within 10-16 days of rechallenge infection (Figure 4.3a). To quantify the parasite burden, real-time PCR was conducted for the parasite specific B1 gene in the lung and brain tissue samples of rechallenged mice. Significantly more parasite burden was detected in the brain tissue of CCR7$^{-/-}$ mice compared to rechallenged control mice (Figure 4.3b). Parasite burden in

CCR7$^{-/-}$ mice lung tissue was higher on average, however, not significant due to variations in the biological replicates. Increased inflammation and necrosis was evident in CCR7$^{-/-}$ mouse brain and liver tissue following rechallenge infection (Figure 4.3c). All these data suggest CCR7 signaling is not essential to maintain the chronic phase of infection rather it is needed to control reactivating parasites.

In the absence of CCR7, the IL-7R^{lo}KLRG1^{hi} population is defective at the site of infection

CCR7$^{-/-}$ mice were unable to survive a secondary infection without any defect in T cell priming. This led to further investigation of the phenotype of inflammatory infiltrates recruited to the infected brain, cervical LN and spleen during rechallenge. During acute infection, there was a defect in the effector cell recruitment at the site of infection [140]. Thus, we analyzed the recruitment of inflammatory infiltrates, neutrophil and CD11c^{+} cells and peripheral macrophages in the CCR7$^{-/-}$ mouse brain, cLNs and spleen during secondary infection. The proportions and the activation status of resident microglia in the rechallenged brain were also analyzed. None of these effector cell populations were defective in proportion nor activation in the absence of CCR7 (data not shown). However, phenotypic analysis of T cells revealed that the proportion of IL-7R^{lo}KLRG1^{hi} population was significantly decreased in the brain. The proportion of CD4^{+} IL-7R^{lo}KLRG1^{hi} in CCR7$^{-/-}$ mouse brain was only one-third of that present in the WT rechallenged mice (Figure 4.4a). Further, there was a 45% reduction of IL-7R^{lo}KLRG1^{hi} CD8 T cells in the absence of CCR7 (Figure 4.4a).

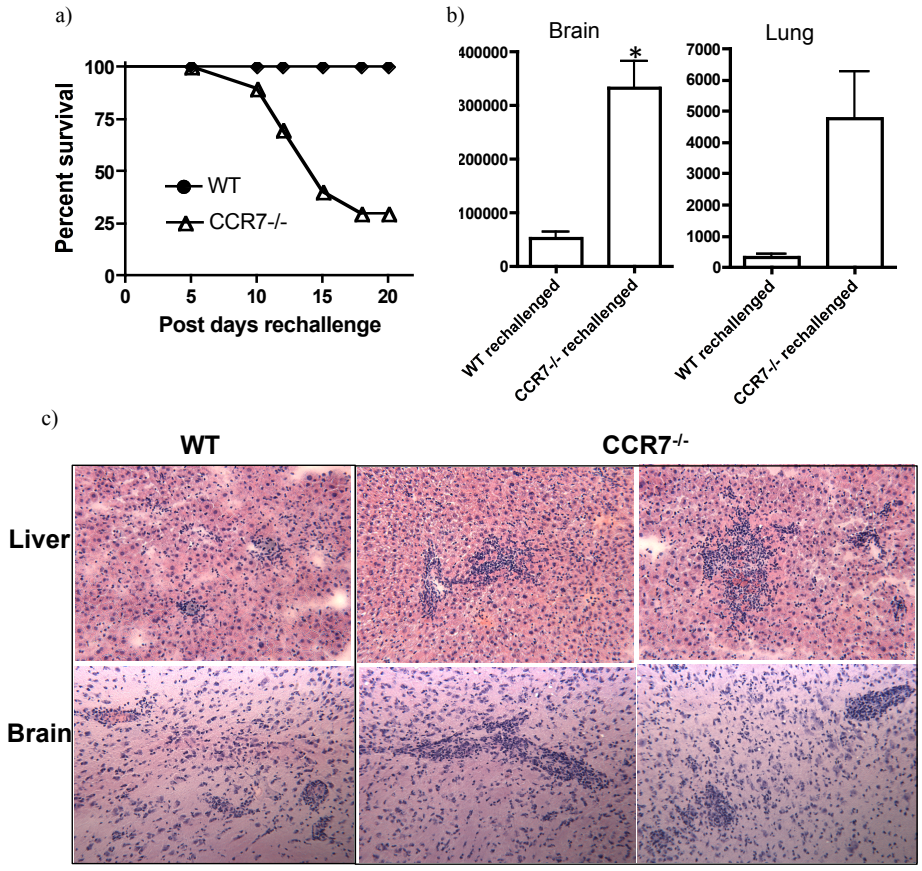


Figure 4.3: CCR7 is required for efficient memory responses during chronic infection with *T. gondii*.

CCR7$^{-/-}$ mice and WT control mice were infected with Pru-OVA strain of *T. gondii*. Following 4 weeks post infection, chronically infected mice were rechallenged with RH-OVA. a) Survival curve of the WT versus CCR7$^{-/-}$ rechallenged mice, representing data from two independent experiments with at least 5 biological replicates in each group; b) parasite burden in the brain and lung; c) histology of liver and brain tissue samples two weeks following rechallenge infection.

However, this defect was only present at the site of infection and was not evident in the cervical LNs (Figure 4.4a). A similar defect was evident in *plt/plt* mice suggesting CCR7-CCL21 signaling is required to maintain this IL-7R^{lo}KLRG1^{hi} T cell population in the brain (Figure 4.4b). Interestingly, this defect in KLRG1^{+} population was more prominent in the *plt/plt* brain than in CCR7$^{-/-}$-deficient mouse brain. There was only 0.7% of CD4 and 4% of CD8 T cells with IL-7R^{lo}KLRG1^{hi} phenotype compared to 20% and 23% of CD4 and CD8 T cells, respectively in WT control mouse brain (Figure 4.4b). As observed in CCR7-deficient rechallenged mice, there was no defect of this population in the cLNs of *plt/plt* rechallenged mice. To summarize, in the absence of CCR7 or CCL21, the IL-7R^{lo}KLRG1^{hi} T cell population is defective in the brain during infection with *T. gondii*.

There is a CD8 intrinsic requirement of CCR7 signaling at the site of infection

Our previous study identified a particular role of CCR7 in CD4 T cell migration in the chronically infected brain ([156] and Chapter 3). Thus, the defect observed in CD8 IL-7R^{lo}KLRG1^{hi} T cell population could be due to the requirement of CCR7 signaling on CD4 T cells. We tested whether KLRG1 expression on CD8 T cells requires CCR7 signaling on the CD8 T cell compartment or if it is a secondary defect due to the absence of CCR7 in other immune cell populations. To test this, CD8 T cells isolated from CCR7$^{-/-}$ naive mice were adoptively transferred into congenic WT mice prior to the day of primary infection. Four weeks after primary infection, these mice were rechallenged with RH-OVA strain of *T. gondii* as described before (Figure 4.5). Two weeks post-rechallenge, the expression of KLRG1 and

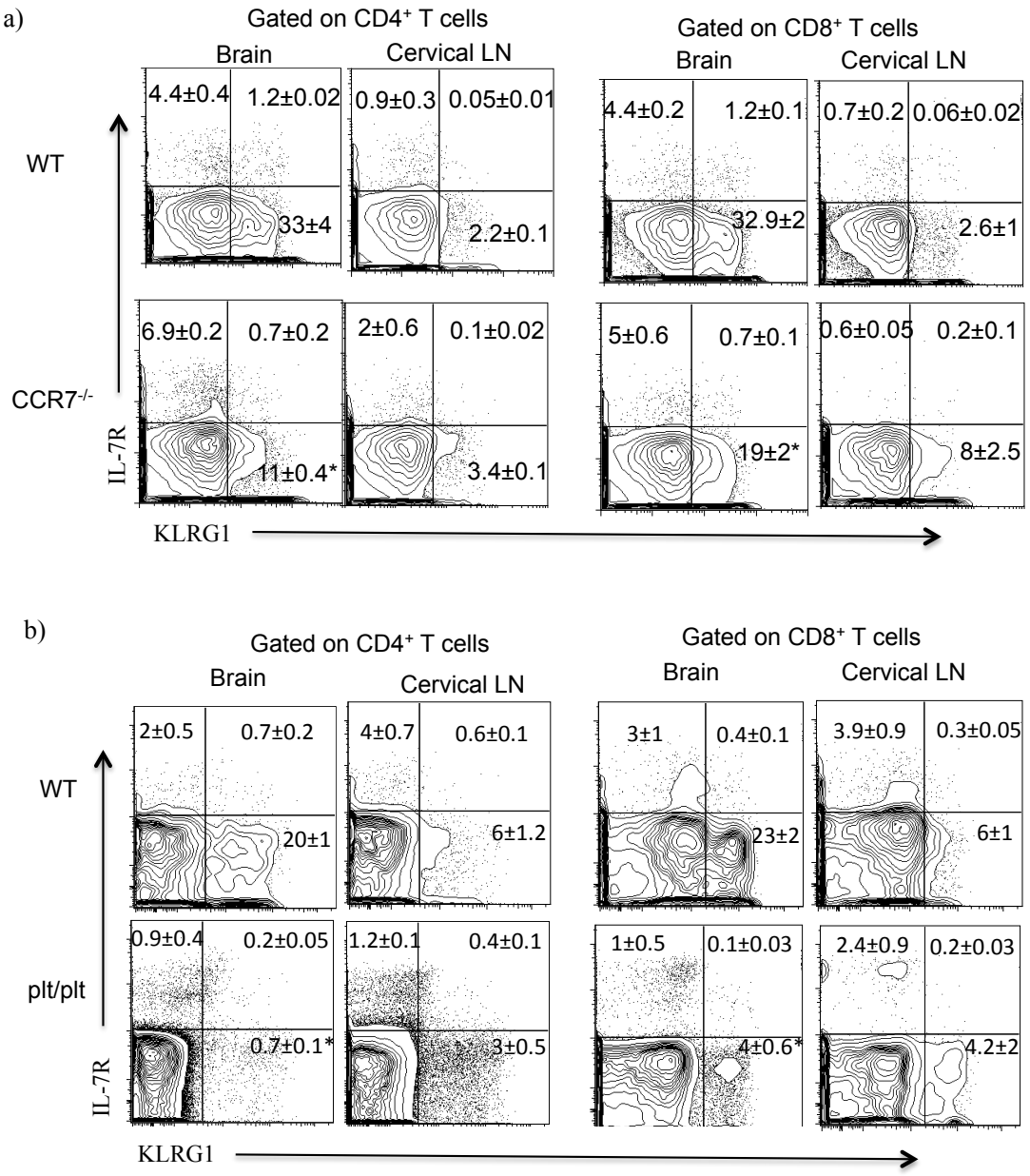
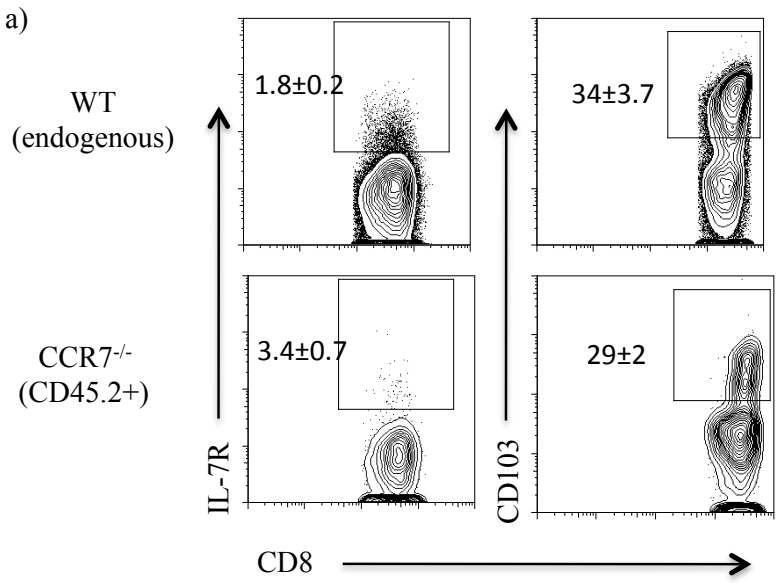


Figure 4.4: Defective IL-7R^{lo}KLRG1^{hi} population in the brain following rechallenge infection in CCR7-deficient mice.

CCR7$^{-/-}$, *plt/plt* and WT control mice were infected with Pru-OVA strain of *T. gondii* and 4 week chronically infected mice were rechallenged with RH-OVA as described in Figure 4.3. Two weeks after rechallenge infection, mice were sacrificed and BMNCs were harvested. Analysis of the expression of IL-7R versus KLRG1 on CD4 or CD8 T cells in the brain or in the cervical LNs in CCR7$^{-/-}$ mice (a) and *plt/plt* mice (b) along with the WT controls demonstrate a significant reduction of IL-7R^{lo}KLRG1^{hi} CD4 or CD8 T cell populations in the CCR7$^{-/-}$ or *plt/plt* rechallenged mouse brain.

other memory markers, IL-7R and CD103 expression were compared between the adoptively transferred cells (CCR7$^{-/-}$) and the endogenous (WT) T cell population (Figure 4.5). IL-7R and CD103 expression on endogenous cells were similar to that on transferred cells in the brain (Figure 4.5a) and in the periphery (data not shown). The expression of KLRG1 on CD8 T cells was also comparable between the transferred cells and the endogenous CD8 T population in the spleen and cLNs. However, in the brain KLRG1 expression on transferred (CCR7$^{-/-}$) CD8 T cells remained defective (Figure 4.5b). These data suggest that there is a CD8 intrinsic requirement for CCR7 signaling at the site of infection to maintain KLRG1^{hi} effector/memory population.


a)
WT
(endogenous)
CCR7-/-
(CD45.2+)
1.8±0.2
34±3.7
3.4±0.7
29±2
IL-7R
CD103
CD8

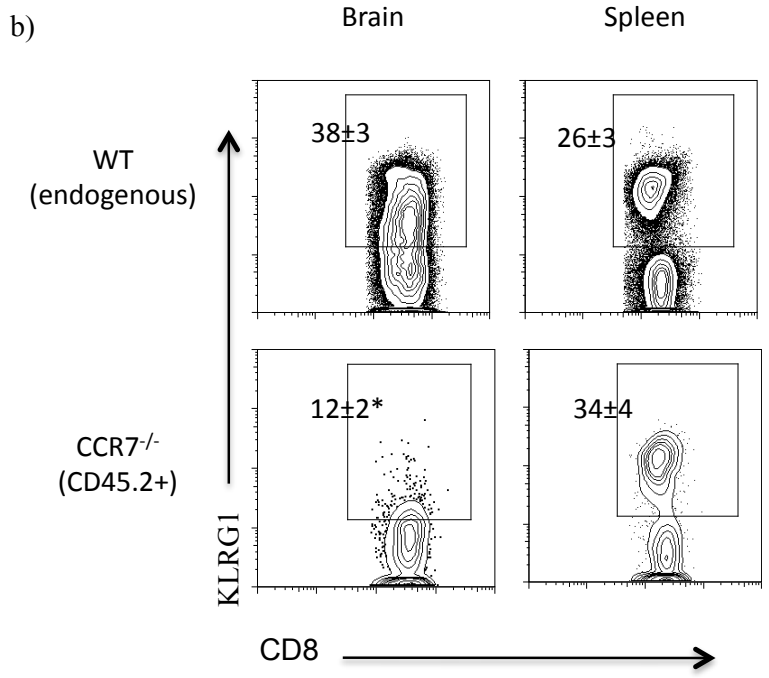

b)
Brain
Spleen
WT
(endogenous)
CCR7-/-
(CD45.2+)
38±3
26±3
12±2*
34±4
KLRG1
CD8

Figure 4.5: Investigating the requirement of CCR7 signaling to restore KLRG1 expression on CD8 T cells at the site of infection.

To investigate whether or not CCR7 signaling on the CD8 T cell compartment is required to generate KLRG1^{+}CD8 T cell population in the brain following rechallenge infection, CD8 T cells from CCR7$^{-/-}$ naive mice were adoptively transferred (IV) into CD45.1 congenice mice (n=4) 1 day prior infection. CD45.1 WT mice were then infected with Pru-OVA and 4 weeks following primary infection, mice were rechallenged with RH-OVA. Two weeks following secondary infection mice were sacrificed and proportions of IL-7R^{+}, CD103^{+} and KLRG1^{+} cells were compared between the adoptively transferred (gated on CD45.2^{+} CD8^{+}) cells versus endogenous (gated on CD45.2^{-}CD8^{+}) cells. a) IL-7R and CD103 expressions in the brains were comparable between WT (endogenous) versus CCR7$^{-/-}$ (CD45.2^{+}) CD8 T cells; b) proportions KLRG1^{+} cells were equivalent on WT or CCR7$^{-/-}$ CD8 T cells isolated from spleens of rechallenged mice but remained defective on CCR7$^{-/-}$ (CD45.2^{+}) CD8 T cells harvested from the brains of rechallenged mice.

4.4 Discussion

Following primary infection, selective survival of a subset of effector T cells generates heterogeneous memory T cells with different migratory capacity and effector functions [166]. The expression of CCR7 on central memory T cells marks their ability to circulate through the lymphoid organs, in contrast to effector memory T cells that reside in the peripheral tissues [166][98]. During LCMV infection, CCR7 deficiency affects the distribution of memory T cells between the lymphoid and non-lymphoid organs. But CCR7-deficient mice stably maintain memory T cells predominantly in the non-lymphoid tissues and are able to mount protective recall responses [98][178]. However, the quality and the requirements for protective immunological memory in a chronic infection setting may be different compared to other infection models where antigens are completely cleared following primary infection

[178]. It is likely that the activated T cell pool in the presence of persistent antigenic stimulation would consist of mainly effector and effector memory T cells [143]. Thus, CTL differentiation during *T. gondii* infection is skewed towards an effector-cell rich subpopulation ($CD62L^{lo}KLRG1^{hi}$) and the $KLRG1^{+}$ subset represents a major subpopulation of toxoplasma-specific memory CTLs [205]. Supporting these findings, our data also suggest that the T cell pool in the infected brain is predominantly activated effector or effector memory T cells expressing CD44, CXCR3 and down regulated CD62L. A small proportion of T cells are central memory like ($IL\text{-}7R^{hi}$ $CD62L^{+}$) T cells during Toxoplasma infection in the brain. Thus, $CCR7^{+}$ T cells in the brain are likely to be a mixed phenotype of central memory T cells transitioning to effector or effector memory T cells which is evident from our detailed phenotypic analysis.

In this study, we demonstrate that CCR7 is required to maintain $KLRG1^{+}$ effector and effector memory population at the site of infection despite any defect of this population in the lymphoid organs in the absence of CCR7. CCR7 expression defines a long-lived memory cell whereas $KLRG1^{+}$ T cells are terminally differentiated cells with very low proliferation potential [164]. Thus, quite expectedly, only few CD4 or CD8 T cells were found to express both CCR7 and KLRG1. However, our *in vitro* stimulation data indicate that following stimulation T cells down regulate CCR7. Thus, CCR7 expression may facilitate T cell migration to areas with infection-induced CCL21 where T cells receive further antigenic stimulation that may eventually result in CCR7 down regulation and KLRG1 expression because of excessive antigen-induced proliferation.

Several studies demonstrated that effector T cells undergo antigen-specific stimulation and proliferation to maximize their effector potential at the site of ongoing infection [123][86]. In fact, during pulmonary infection, memory CD4 T cell differentiation into activated effector T cells preferentially occurs at the peripheral site of infection rather than in the draining lymph nodes [210]. This might be an important immune mechanism to ensure rapid and local immune responses against pathogens at the site of infection while limiting damage to other tissues. An increased proportion of $CCR7^+$ T cells in the infected brain compared to in the blood (data not shown) indicate that there is a selective enrichment of $CCR7^+$ T cells in the brain that are largely negative for KLRG1 expression. These cells may undergo further differentiation to become highly activated $KLRG1^+$ population following stimulation in the infected areas of the CNS. Thus, in the absence of CCR7 expression, T cells fail to differentiate into highly activated, cytokine producing $KLRG1^+$ populations in the infected brain.

Another possibility is defective recruitment or activation status of antigen-presenting populations in the CCR7-deficient brain that causes defective generation of $KLRG1^+$ T cells. Defective recruitment of inflammatory monocytes at the site of ongoing infection has been observed before in CCR7-deficient mice during the acute phase of infection with *T. gondii* [140]. Moreover, $CD11b^+$ inflammatory DCs have been demonstrated to interact with effector T cells in the CNS or other peripheral tissues that enhance effector T cell function and ensure rapid activation effector memory T cells in the non-lymphoid tissues [101][219][2]. However, we have not detected any defect in the numbers and activation of these antigen-

presenting populations in the chronically infected brain in the absence of CCR7. In addition, adoptive transfer of CCR7-deficient T cells into the CCR7-sufficient, WT environment was not able to restore the KLRG1 expression on CD8 T cells in the infected CNS. These data indicate this defect of KLRG1 expression is not due to the defective expression of CCR7 on the antigen-presenting DCs or CD4 T cells that might influence CD8 T cell activation and differentiation. Thus, we conclude that CCR7 expression on T cells that are present at the site of infection augments the generation of KLRG1^{+} T cells that is crucial to ensure protective immunity following secondary infection with Toxoplasma.

Chapter 5

Concluding remarks

Along with the public health importance, host immunity during Toxoplasma infection is also an excellent example where the immune competent host is able to induce robust immune responses that successfully check parasite replication at the earlier stages of infection. Also, during the latent stage, the host ensures sufficient immune responses in the CNS to prevent the reactivation of these opportunistic pathogens. This dissertation emphasizes the chemokine-dependent modulation of immune cell function and migration behavior that is crucial to enable the host to maintain such a balanced immunity during Toxoplasma infection.

Generation of pathogen-specific T cells is crucial to initiate adaptive immunity following infection. This T cell priming process generally occurs in the LNs and CCR7 is a well-known chemokine receptor that directs T cell migration and antigen-specific activation in the LNs. Following infection, antigen abundance as well as the presence of APCs might help this priming to occur in the spleen or at the site of infection. In contrast to this general consensus, this dissertation enforces the requirement of T cell priming in the LNs to ensure sufficient effector function of T cells in the early stage of Toxoplasma infection. Furthermore, restoration of protective immunity with CCR7 expression only on T cells indicated the necessity of CCR7 signaling on T cells in the acute stage of infection (Chapter 2).

Data presented in this dissertation is an important addition to the current understanding of CNS immunity in the context of chronic infection. Previous studies indicated the involvement of CCL21 in neuron-glial interactions following non-infectious insults. However, the requirement of CCR7 or CCL21 expression in the inflamed CNS was largely unknown. One might

speculate that during this chronic infection setting in the "immune restricted" CNS there will be checkpoints that control the access as well T cell functions to maintain continuous T cell surveillance in the CNS. One such mechanism has been thought to be through controlling T cell entry. In contrast to other CNS inflammation models where CCR7 is considered to regulate the entry of encephalitogenic T cells, we have not detected any requirement of CCR7 signaling to infiltrate the infected brain. However, studies described in this dissertation revealed two other important checkpoints -T cell migration within the CNS parenchyma and local activation and generation of memory phenotype and indicated the requirement of CCR7 in these processes.

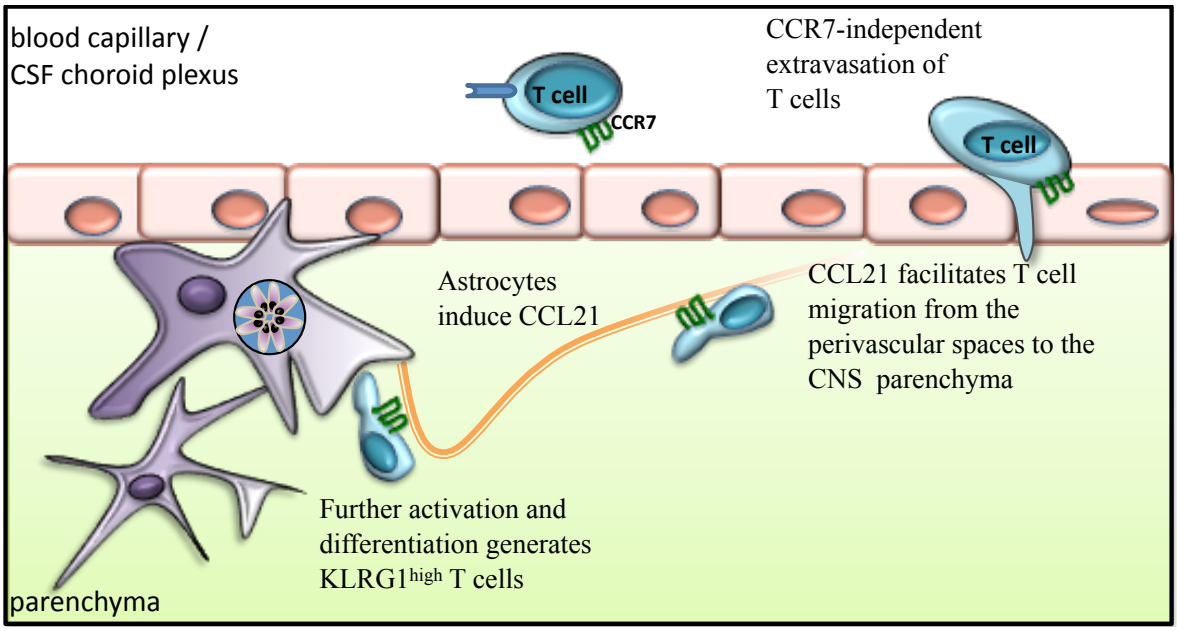


Figure 5.1: The role of CCL21 during Toxoplasma infection in the brain.

Following infection, CNS resident astrocytes induce CCL21. CCR7 is not essential for T cell entry in *T. gondii* infected brain, however, CD4 T cell migration from the perivascular area is CCL21-dependent. Within the CNS parenchyma, T cells may undergo local activation to generate the highly activated KLRG1^{hi} effector/effector memory T cell population and keep pathogens under control.

The mechanisms that drive T cell migration in the dense CNS parenchyma have been a long-standing question. Here we demonstrate that resident glial cells induce CCL21 following infection and there is a CCR7/CCL21-dependent mechanism of T cell migration within the CNS parenchyma. Thus, CCL21 induction by infected cells aids the recruitment of T cells towards the source of infection. While directing T cells towards areas of active parasite replication, CCR7 signaling also facilitates further activation and generation of highly activated KLRG1^{+} T cells to mediate efficient killing of parasites. Such mechanisms provide new insights about host immune strategies to ensure localized distribution and activation of effector T cells to maximize effector killing and limit inflammation in other areas which is crucial for CNS immunity. In addition, this CCR7-dependent mechanism contributing to T cell migration and the generation of memory phenotypes is evident only at the site of ongoing infection, not in the LNs or spleen. However, whether it is a CNS-intrinsic immune mechanism is yet to be determined.

Toxoplasmosis currently affects millions of people all over the world and is a serious concern for individuals who have weakened immune system such as in AIDS patients. Pin pointing the essential components of host protective immunity will be useful for developing new therapies targeting the complete elimination of these parasites from the host. In addition, delineating the mechanisms that control T cell migration and their function in the CNS will lead to improved therapies by manipulating host immune mechanisms during CNS inflammation or autoimmunity towards more protective and efficient immune responses. In light of the data presented in this thesis, CCL21 upregulation following infection appears as a direc-

tional signal to guide as well as activate CNS infiltrating T cells to ensure efficient parasite control. Such an infection-induced mechanism of CCL21 upregulation is crucial to facilitate directed T cell migration and parasite control as indicated by an overall increase in parasite burden in the ubiquitous presence of CCL21 transgene in the brain (Chapter 3). Therefore, a thorough understanding of such mechanisms of CNS protective immunity is required to design efficient strategies to manipulate host immunity and avoid pathological responses during pathogenic or non-pathogenic insults.

Bibliography

[1] L. B. Adams, Y. Fukutomi, and J. L. Krahenbuhl. Regulation of murine macrophage effector functions by lipoarabinomannan from mycobacterial strains with different degrees of virulence. *Infect Immun*, 61(10):4173–81, 1993.

[2] Jr. Aldridge, J. R., C. E. Moseley, D. A. Boltz, N. J. Negovetich, C. Reynolds, J. Franks, S. A. Brown, P. C. Doherty, R. G. Webster, and P. G. Thomas. Tnf/inos-producing dendritic cells are the necessary evil of lethal influenza virus infection. *Proc Natl Acad Sci U S A*, 106(13):5306–11, 2009.

[3] J. Aliberti, C. Reis e Sousa, M. Schito, S. Hieny, T. Wells, G. B. Huffnagle, and A. Sher. Ccr5 provides a signal for microbial induced production of il-12 by cd8 alpha+ dendritic cells. *Nat Immunol*, 1(1):83–7, 2000.

[4] F. Aloisi and R. Pujol-Borrell. Lymphoid neogenesis in chronic inflammatory diseases. *Nat Rev Immunol*, 6(3):205–17, 2006.

[5] C. Alt, M. Laschinger, and B. Engelhardt. Functional expression of the lymphoid chemokines ccl19 (elc) and ccl 21 (slc) at the blood-brain barrier suggests their involvement in g-protein-dependent lymphocyte recruitment into the central nervous system during experimental autoimmune encephalomyelitis. *Eur J Immunol*, 32(8):2133–44, 2002.

[6] J. Antel and A. Bar-Or. Roles of immunoglobulins and b cells in multiple sclerosis: from pathogenesis to treatment. *J Neuroimmunol*, 180(1-2):3–8, 2006.

[7] V. C. Asensio and I. L. Campbell. Chemokines in the cns: plurifunctional mediators in diverse states. *Trends Neurosci*, 22(11):504–12, 1999.

[8] H. Aviles, J. Stiles, P. O'Donnell, J. Orshal, J. Leid, G. Sonnenfeld, and F. Monroy. Kinetics of systemic cytokine and brain chemokine gene expression in murine toxoplasma infection. *J Parasitol*, 94(6):1282–8, 2008.

[9] B. J. Baaten, C. R. Li, M. F. Deiro, M. M. Lin, P. J. Linton, and L. M. Bradley. Cd44 regulates survival and memory development in th1 cells. *Immunity*, 32(1):104–15, 2010.

[10] M. Bajenoff, J. G. Egen, L. Y. Koo, J. P. Laugier, F. Brau, N. Glaichenhaus, and R. N. Germain. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity*, 25(6):989–1001, 2006.

[11] M. Bajenoff, J. G. Egen, H. Qi, A. Y. Huang, F. Castellino, and R. N. Germain. Highways, byways and breadcrumbs: directing lymphocyte traffic in the lymph node. *Trends Immunol*, 28(8):346–52, 2007.

[12] J. Banchereau and R. M. Steinman. Dendritic cells and the control of immunity. *Nature*, 392(6673):245–52, 1998.

[13] G. Bardi, M. Lipp, M. Baggiolini, and P. Loetscher. The t cell chemokine receptor ccr7 is internalized on stimulation with elc, but not with slc. *Eur J Immunol*, 31(11):3291–7, 2001.

[14] M. H. Beaman, F. G. Araujo, and J. S. Remington. Protective reconstitution of the scid mouse against reactivation of toxoplasmic encephalitis. *J Infect Dis*, 169(2):375–83, 1994.

[15] L. Benevides, C. M. Milanezi, L. M. Yamauchi, C. F. Benjamim, J. S. Silva, and N. M. Silva. Ccr2 receptor is essential to activate microbicidal mechanisms to control toxoplasma gondii infection in the central nervous system. *Am J Pathol*, 173(3):741–51, 2008.

[16] R. Bhadra, H. Guan, and I. A. Khan. Absence of both il-7 and il-15 severely impairs the development of cd8 t cell response against toxoplasma gondii. *PLoS One*, 5(5):e10842, 2010.

[17] K. Biber, A. Rappert, H. Kettenmann, N. Brouwer, S. C. Copray, and H. W. Boddeke. Neuronal slc (ccl21) expression: implications for the neuron-microglial signaling system. *Ernst Schering Res Found Workshop*, (39):45–60, 2002.

[18] K. Biber, A. Sauter, N. Brouwer, S. C. Copray, and H. W. Boddeke. Ischemia-induced neuronal expression of the microglia attracting chemokine secondary lymphoid-tissue chemokine (slc). *Glia*, 34(2):121–33, 2001.

[19] K. Biber, M. Tsuda, H. Tozaki-Saitoh, K. Tsukamoto, E. Toyomitsu, T. Masuda, H. Boddeke, and K. Inoue. Neuronal ccl21 up-regulates microglia p2x4 expression and initiates neuropathic pain development. *EMBO J*, 30(9):1864–73, 2011.

[20] L. M. Bixby and R. L. Tarleton. Stable cd8+ t cell memory during persistent trypanosoma cruzi infection. *J Immunol*, 181(4):2644–50, 2008.

[21] M. W. Black and J. C. Boothroyd. Lytic cycle of toxoplasma gondii. *Microbiol Mol Biol Rev*, 64(3):607–23, 2000.

[22] S. K. Bliss, L. C. Gavrilescu, A. Alcaraz, and E. Y. Denkers. Neutrophil depletion during toxoplasma gondii infection leads to impaired immunity and lethal systemic pathology. *Infect Immun*, 69(8):4898–905, 2001.

[23] S. K. Bliss, Y. Zhang, and E. Y. Denkers. Murine neutrophil stimulation by toxoplasma gondii antigen drives high level production of ifn-gamma-independent il-12. *J Immunol*, 163(4):2081–8, 1999.

[24] A. Braun, T. Worbs, G. L. Moschovakis, S. Halle, K. Hoffmann, J. Bolter, A. Munk, and R. Forster. Afferent lymph-derived t cells and dcs use different chemokine receptor ccr7-dependent routes for entry into the lymph node and intranodal migration. *Nat Immunol*, 12(9):879–87, 2011.

[25] S. K. Bromley, T. R. Mempel, and A. D. Luster. Orchestrating the orchestrators: chemokines in control of t cell traffic. *Nat Immunol*, 9(9):970–80, 2008.

[26] S. K. Bromley, S. Y. Thomas, and A. D. Luster. Chemokine receptor ccr7 guides t cell exit from peripheral tissues and entry into afferent lymphatics. *Nat Immunol*, 6(9):895–901, 2005.

[27] S. C. Byram, M. J. Carson, C. A. DeBoy, C. J. Serpe, V. M. Sanders, and K. J. Jones. Cd4-positive t cell-mediated neuroprotection requires dual compartment antigen presentation. *J Neurosci*, 24(18):4333–9, 2004.

[28] M. J. Carson, J. M. Doose, B. Melchior, C. D. Schmid, and C. C. Ploix. Cns immune privilege: hiding in plain sight. *Immunol Rev*, 213:48–65, 2006.

[29] L. Casciotti, K. H. Ely, M. E. Williams, and I. A. Khan. Cd8(+)-t-cell immunity against toxoplasma gondii can be induced but not maintained in mice lacking conventional cd4(+) t cells. *Infect Immun*, 70(2):434–43, 2002.

[30] S. C. Chen, M. W. Leach, Y. Chen, X. Y. Cai, L. Sullivan, M. Wiekowski, B. J. Dovey-Hartman, A. Zlotnik, and S. A. Lira. Central nervous system inflammation and neurological disease in transgenic mice expressing the cc chemokine ccl21 in oligodendrocytes. *J Immunol*, 168(3):1009–17, 2002.

[31] R. T. Clark, J. P. Nance, S. Noor, and E. H. Wilson. T-cell production of matrix metalloproteinases and inhibition of parasite clearance by timp-1 during chronic toxoplasma infection in the brain. *ASN Neuro*, 3(1):e00049, 2011.

[32] C. M. Collazo, G. S. Yap, G. D. Sempowski, K. C. Lusby, L. Tessarollo, G. F. Woude, A. Sher, and G. A. Taylor. Inactivation of lrg-47 and irg-47 reveals a family of interferon gamma-inducible genes with essential, pathogen-specific roles in resistance to infection. *J Exp Med*, 194(2):181–8, 2001.

[33] S. Columba-Cabezas, B. Serafini, E. Ambrosini, and F. Aloisi. Lymphoid chemokines ccl19 and ccl21 are expressed in the central nervous system during experimental autoimmune encephalomyelitis: implications for the maintenance of chronic neuroinflammation. *Brain Pathol*, 13(1):38–51, 2003.

[34] A. C. Davalos-Misslitz, J. Rieckenberg, S. Willenzon, T. Worbs, E. Kremmer, G. Bernhardt, and R. Forster. Generalized multi-organ autoimmunity in ccr7-deficient mice. *Eur J Immunol*, 37(3):613–22, 2007.

[35] A. C. Davalos-Misslitz, T. Worbs, S. Willenzon, G. Bernhardt, and R. Forster. Impaired responsiveness to t-cell receptor stimulation and defective negative selection of thymocytes in ccr7-deficient mice. *Blood*, 110(13):4351–9, 2007.

[36] D. S. Davis and M. J. Carson. When the tail can't wag the dog: the implications of cns-intrinsic initiation of neuroinflammation. *ASN Neuro*, 1(2), 2009.

[37] M. T. de Graaf, P. A. Smitt, R. L. Luitwieler, C. van Velzen, P. D. van den Broek, J. Kraan, and J. W. Gratama. Central memory cd4+ t cells dominate the normal cerebrospinal fluid. *Cytometry B Clin Cytom*, 80(1):43–50, 2011.

[38] E. K. de Jong, I. M. Dijkstra, M. Hensens, N. Brouwer, M. van Amerongen, R. S. Liem, H. W. Boddeke, and K. Biber. Vesicle-mediated transport and release of ccl21 in endangered neurons: a possible explanation for microglia activation remote from a primary lesion. *J Neurosci*, 25(33):7548–57, 2005.

[39] G. F. Debes, C. N. Arnold, A. J. Young, S. Krautwald, M. Lipp, J. B. Hay, and E. C. Butcher. Chemokine receptor ccr7 required for t lymphocyte exit from peripheral tissues. *Nat Immunol*, 6(9):889–94, 2005.

[40] K. A. DeFea. Beta-arrestins as regulators of signal termination and transduction: how do they determine what to scaffold? *Cell Signal*, 23(4):621–9, 2010.

[41] I. Dellacasa-Lindberg, J. M. Fuks, R. B. Arrighi, H. Lambert, R. P. Wallin, B. J. Chambers, and A. Barragan. Migratory activation of primary cortical microglia upon infection with toxoplasma gondii. *Infect Immun*, 79(8):3046–52, 2011.

[42] I. Dellacasa-Lindberg, N. Hitziger, and A. Barragan. Localized recrudescence of toxoplasma infections in the central nervous system of immunocompromised mice assessed by in vivo bioluminescence imaging. *Microbes Infect*, 9(11):1291–8, 2007.

[43] E. Y. Denkers and R. T. Gazzinelli. Regulation and function of t-cell-mediated immunity during toxoplasma gondii infection. *Clin Microbiol Rev*, 11(4):569–88, 1998.

[44] E. Y. Denkers and A. Sher. Role of natural killer and nk1+ t-cells in regulating cell-mediated immunity during toxoplasma gondii infection. *Biochem Soc Trans*, 25(2):699–703, 1997.

[45] E. Y. Denkers, G. Yap, T. Scharton-Kersten, H. Charest, B. A. Butcher, P. Caspar, S. Heiny, and A. Sher. Perforin-mediated cytolysis plays a limited role in host resistance to toxoplasma gondii. *J Immunol*, 159(4):1903–8, 1997.

[46] N. C. Derecki, E. Privman, and J. Kipnis. Rett syndrome and other autism spectrum disorders–brain diseases of immune malfunction? *Mol Psychiatry*, 15(4):355–63, 2010.

[47] I. M. Dijkstra, A. H. de Haas, N. Brouwer, H. W. Boddeke, and K. Biber. Challenge with innate and protein antigens induces ccr7 expression by microglia in vitro and in vivo. *Glia*, 54(8):861–72, 2006.

[48] I. M. Dijkstra, S. Hulshof, P. van der Valk, H. W. Boddeke, and K. Biber. Cutting edge: activity of human adult microglia in response to cc chemokine ligand 21. *J Immunol*, 172(5):2744–7, 2004.

[49] S. Doerck, K. Gobel, G. Weise, T. Schneider-Hohendorf, M. Reinhardt, P. Hauff, N. Schwab, R. Linker, M. Maurer, S. G. Meuth, and H. Wiendl. Temporal pattern of icam-i mediated regulatory t cell recruitment to sites of inflammation in adoptive transfer model of multiple sclerosis. *PLoS One*, 5(11):e15478, 2010.

[50] K. Drogemuller, U. Helmuth, A. Brunn, M. Sakowicz-Burkiewicz, D. H. Gutmann, W. Mueller, M. Deckert, and D. Schluter. Astrocyte gp130 expression is critical for the control of toxoplasma encephalitis. *J Immunol*, 181(4):2683–93, 2008.

[51] J. P. Dubey. Toxoplasmosis–an overview. *Southeast Asian J Trop Med Public Health*, 22 Suppl:88–92, 1991.

[52] J. P. Dubey. Advances in the life cycle of toxoplasma gondii. *Int J Parasitol*, 28(7):1019–24, 1998.

[53] J. P. Dubey, D. S. Lindsay, and C. A. Speer. Structures of toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev*, 11(2):267–99, 1998.

[54] I. R. Dunay, A. Fuchs, and L. D. Sibley. Inflammatory monocytes but not neutrophils are necessary to control infection with toxoplasma gondii in mice. *Infect Immun*, 78(4):1564–70, 2010.

[55] L. M. Ebert, P. Schaerli, and B. Moser. Chemokine-mediated control of t cell traffic in lymphoid and peripheral tissues. *Mol Immunol*, 42(7):799–809, 2005.

[56] C. E. Egan, W. Sukhumavasi, A. L. Bierly, and E. Y. Denkers. Understanding the multiple functions of gr-1(+) cell subpopulations during microbial infection. *Immunol Res*, 40(1):35–48, 2008.

[57] B. Engelhardt. Molecular mechanisms involved in t cell migration across the blood-brain barrier. *J Neural Transm*, 113(4):477–85, 2006.

[58] S. K. Eo, U. Kumaraguru, and B. T. Rouse. Plasmid dna encoding ccr7 ligands compensate for dysfunctional cd8+ t cell responses by effects on dendritic cells. *J Immunol*, 167(7):3592–9, 2001.

[59] L. Fan, C. R. Reilly, Y. Luo, M. E. Dorf, and D. Lo. Cutting edge: ectopic expression of the chemokine tca4/slc is sufficient to trigger lymphoid neogenesis. *J Immunol*, 164(8):3955–9, 2000.

[60] D. Fee, A. Crumbaugh, T. Jacques, B. Herdrich, D. Sewell, D. Auerbach, S. Piaskowski, M. N. Hart, M. Sandor, and Z. Fabry. Activated/effector cd4+ t cells exacerbate acute damage in the central nervous system following traumatic injury. *J Neuroimmunol*, 136(1-2):54–66, 2003.

[61] J. A. Fiuza, R. T. Fujiwara, J. A. Gomes, M. O. Rocha, A. T. Chaves, F. F. de Araujo, R. C. Fares, A. Teixeira-Carvalho, O. A. Martins-Filho, G. G. Cancado, and R. Correa-Oliveira. Profile of central and effector memory t cells in the progression of chronic human chagas disease. *PLoS Negl Trop Dis*, 3(9):e512, 2009.

[62] R. Forster, A. C. Davalos-Misslitz, and A. Rot. Ccr7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol*, 8(5):362–71, 2008.

[63] R. Forster, A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. Ccr7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*, 99(1):23–33, 1999.

[64] A. E. Fouts and J. C. Boothroyd. Infection with toxoplasma gondii bradyzoites has a diminished impact on host transcript levels relative to tachyzoite infection. *Infect Immun*, 75(2):634–42, 2007.

[65] I. Galea, I. Bechmann, and V. H. Perry. What is immune privilege (not)? *Trends Immunol*, 28(1):12–8, 2007.

[66] R. Gazzinelli, Y. Xu, S. Hieny, A. Cheever, and A. Sher. Simultaneous depletion of cd4+ and cd8+ t lymphocytes is required to reactivate chronic infection with toxoplasma gondii. *J Immunol*, 149(1):175–80, 1992.

[67] R. T. Gazzinelli, I. Eltoum, T. A. Wynn, and A. Sher. Acute cerebral toxoplasmosis is induced by in vivo neutralization of tnf-alpha and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. *J Immunol*, 151(7):3672–81, 1993.

[68] R. T. Gazzinelli, F. T. Hakim, S. Hieny, G. M. Shearer, and A. Sher. Synergistic role of cd4+ and cd8+ t lymphocytes in ifn-gamma production and protective immunity induced by an attenuated toxoplasma gondii vaccine. *J Immunol*, 146(1):286–92, 1991.

[69] R. T. Gazzinelli, M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri, and A. Sher. Parasite-induced il-12 stimulates early ifn-gamma synthesis and resistance during acute infection with toxoplasma gondii. *J Immunol*, 153(6):2533–43, 1994.

[70] R. T. Gazzinelli, M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. In the absence of endogenous il-10, mice acutely infected with toxoplasma gondii succumb to a lethal immune response dependent on cd4+ t cells and accompanied by overproduction of il-12, ifn-gamma and tnf-alpha. *J Immunol*, 157(2):798–805, 1996.

[71] F. Geissmann, S. Jung, and D. R. Littman. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*, 19(1):71–82, 2003.

[72] D. Gomez-Nicola, N. Pallas-Bazarra, B. Valle-Argos, and M. Nieto-Sampedro. Ccr7 is expressed in astrocytes and upregulated after an inflammatory injury. *J Neuroimmunol*, 227(1-2):87–92, 2010.

[73] D. Grinnan, S. S. Sung, J. A. Dougherty, A. R. Knowles, M. B. Allen, 3rd Rose, C. E., H. Nakano, M. D. Gunn, S. M. Fu, and Jr. Rose, C. E. Enhanced allergen-induced airway inflammation in paucity of lymph node t cell (plt) mutant mice. *J Allergy Clin Immunol*, 118(6):1234–41, 2006.

[74] L. V. Guillermo and R. A. DaMatta. Nitric oxide inhibition after toxoplasma gondii infection of chicken macrophage cell lines. *Poult Sci*, 83(5):776–82, 2004.

[75] M. D. Gunn, S. Kyuwa, C. Tam, T. Kakiuchi, A. Matsuzawa, L. T. Williams, and H. Nakano. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med*, 189(3):451–60, 1999.

[76] M. D. Gunn, K. Tangemann, C. Tam, J. G. Cyster, S. D. Rosen, and L. T. Williams. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive t lymphocytes. *Proc Natl Acad Sci U S A*, 95(1):258–63, 1998.

[77] U. Haessler, M. Pisano, M. Wu, and M. A. Swartz. Dendritic cell chemotaxis in 3d under defined chemokine gradients reveals differential response to ligands ccl21 and ccl19. *Proc Natl Acad Sci U S A*, 108(14):5614–9, 2011.

[78] S. K. Halonen, T. Woods, K. McInnerney, and L. M. Weiss. Microarray analysis of ifn-gamma response genes in astrocytes. *J Neuroimmunol*, 175(1-2):19–30, 2006.

[79] T. M. Handel, Z. Johnson, S. E. Crown, E. K. Lau, and A. E. Proudfoot. Regulation of protein function by glycosaminoglycans–as exemplified by chemokines. *Annu Rev Biochem*, 74:385–410, 2005.

[80] S. Hardtke, L. Ohl, and R. Forster. Balanced expression of cxcr5 and ccr7 on follicular t helper cells determines their transient positioning to lymph node follicles and is essential for efficient b-cell help. *Blood*, 106(6):1924–31, 2005.

[81] S. Hayashi, C. C. Chan, R. Gazzinelli, and F. G. Roberge. Contribution of nitric oxide to the host parasite equilibrium in toxoplasmosis. *J Immunol*, 156(4):1476–81, 1996.

[82] G. Hintzen, L. Ohl, M. L. del Rio, J. I. Rodriguez-Barbosa, O. Pabst, J. R. Kocks, J. Krege, S. Hardtke, and R. Forster. Induction of tolerance to innocuous inhaled antigen relies on a ccr7-dependent dendritic cell-mediated antigen transport to the bronchial lymph node. *J Immunol*, 177(10):7346–54, 2006.

[83] A. Hofhuis, W. van Pelt, Y. T. van Duynhoven, C. D. Nijhuis, L. Mollema, F. R. van der Klis, A. H. Havelaar, and L. M. Kortbeek. Decreased prevalence and age-specific risk factors for toxoplasma gondii igg antibodies in the netherlands between 1995/1996 and 2006/2007. *Epidemiol Infect*, 139(4):530–8, 2010.

[84] G. N. Holland. Reconsidering the pathogenesis of ocular toxoplasmosis. *Am J Ophthalmol*, 128(4):502–5, 1999.

[85] U. E. Hopken, S. Winter, A. H. Achtman, K. Kruger, and M. Lipp. Ccr7 regulates lymphocyte egress and recirculation through body cavities. *J Leukoc Biol*, 87(4):671–82, 2010.

[86] M. M. Hufford, T. S. Kim, J. Sun, and T. J. Braciale. Antiviral cd8+ t cell effector activities in situ are regulated by target cell type. *J Exp Med*, 208(1):167–80, 2011.

[87] C. A. Hunter, M. J. Litton, J. S. Remington, and J. S. Abrams. Immunocytochemical detection of cytokines in the lymph nodes and brains of mice resistant or susceptible to toxoplasmic encephalitis. *J Infect Dis*, 170(4):939–45, 1994.

[88] C. A. Hunter, C. W. Roberts, M. Murray, and J. Alexander. Detection of cytokine mrna in the brains of mice with toxoplasmic encephalitis. *Parasite Immunol*, 14(4):405–13, 1992.

[89] C. A. Hunter, C. S. Subauste, and J. S. Remington. The role of cytokines in toxoplasmosis. *Biotherapy*, 7(3-4):237–47, 1994.

[90] C. A. Hunter, Y. Suzuki, C. S. Subauste, and J. S. Remington. Cells and cytokines in resistance to toxoplasma gondii. *Curr Top Microbiol Immunol*, 219:113–25, 1996.

[91] C. Jakubzick, F. Tacke, J. Llodra, N. van Rooijen, and G. J. Randolph. Modulation of dendritic cell trafficking to and from the airways. *J Immunol*, 176(6):3578–84, 2006.

[92] M. H. Jang, N. Sougawa, T. Tanaka, T. Hirata, T. Hiroi, K. Tohya, Z. Guo, E. Umemoto, Y. Ebisuno, B. G. Yang, J. Y. Seoh, M. Lipp, H. Kiyono, and M. Miyasaka. Ccr7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol*, 176(2):803–10, 2006.

[93] D. Jankovic, D. G. Kugler, and A. Sher. Il-10 production by cd4+ effector t cells: a mechanism for self-regulation. *Mucosal Immunol*, 3(3):239–46, 2010.

[94] B. John, T. H. Harris, E. D. Tait, E. H. Wilson, B. Gregg, L. G. Ng, P. Mrass, D. S. Roos, F. Dzierszinski, W. Weninger, and C. A. Hunter. Dynamic imaging of cd8(+) t cells and dendritic cells during infection with toxoplasma gondii. *PLoS Pathog*, 5(7):e1000505, 2009.

[95] J. L. Jones and J. P. Dubey. Waterborne toxoplasmosis–recent developments. *Exp Parasitol*, 124(1):10–25, 2010.

[96] C. H. Ju, A. Chockalingam, and C. A. Leifer. Early response of mucosal epithelial cells during toxoplasma gondii infection. *J Immunol*, 183(11):7420–7, 2009.

[97] T. Junt, H. Nakano, T. Dumrese, T. Kakiuchi, B. Odermatt, R. M. Zinkernagel, H. Hengartner, and B. Ludewig. Antiviral immune responses in the absence of organized lymphoid t cell zones in plt/plt mice. *J Immunol*, 168(12):6032–40, 2002.

[98] T. Junt, E. Scandella, R. Forster, P. Krebs, S. Krautwald, M. Lipp, H. Hengartner, and B. Ludewig. Impact of ccr7 on priming and distribution of antiviral effector and memory ctl. *J Immunol*, 173(11):6684–93, 2004.

[99] A. Kahnert, U. E. Hopken, M. Stein, S. Bandermann, M. Lipp, and S. H. Kaufmann. Mycobacterium tuberculosis triggers formation of lymphoid structure in murine lungs. *J Infect Dis*, 195(1):46–54, 2007.

[100] H. Kang and Y. Suzuki. Requirement of non-t cells that produce gamma interferon for prevention of reactivation of toxoplasma gondii infection in the brain. *Infect Immun*, 69(5):2920–7, 2001.

[101] S. S. Kang, J. Herz, J. V. Kim, D. Nayak, P. Stewart-Hutchinson, M. L. Dustin, and D. B. McGavern. Migration of cytotoxic lymphocytes in cell cycle permits local mhc i-dependent control of division at sites of viral infection. *J Exp Med*, 208(4):747–59, 2011.

[102] P. Kivisakk, D. J. Mahad, M. K. Callahan, K. Sikora, C. Trebst, B. Tucky, J. Wujek, R. Ravid, S. M. Staugaitis, H. Lassmann, and R. M. Ransohoff. Expression of ccr7 in multiple sclerosis: implications for cns immunity. *Ann Neurol*, 55(5):627–38, 2004.

[103] P. Kivisakk, B. Tucky, T. Wei, J. J. Campbell, and R. M. Ransohoff. Human cerebrospinal fluid contains cd4+ memory t cells expressing gut- or skin-specific trafficking determinants: relevance for immunotherapy. *BMC Immunol*, 7:14, 2006.

[104] J. R. Kocks, A. C. Davalos-Misslitz, G. Hintzen, L. Ohl, and R. Forster. Regulatory t cells interfere with the development of bronchus-associated lymphoid tissue. *J Exp Med*, 204(4):723–34, 2007.

[105] T. A. Kohout, S. L. Nicholas, S. J. Perry, G. Reinhart, S. Junger, and R. S. Struthers. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and erk1/2 activation by the two endogenous ligands for the cc chemokine receptor 7. *J Biol Chem*, 279(22):23214–22, 2004.

[106] M. Krumbholz, D. Theil, F. Steinmeyer, S. Cepok, B. Hemmer, M. Hofbauer, C. Farina, T. Derfuss, A. Junker, T. Arzberger, I. Sinicina, C. Hartle, J. Newcombe, R. Hohlfeld, and E. Meinl. Ccl19 is constitutively expressed in the cns, up-regulated in neuroinflammation, active and also inactive multiple sclerosis lesions. *J Neuroimmunol*, 190(1-2):72–9, 2007.

[107] M. Kursar, U. E. Hopken, M. Koch, A. Kohler, M. Lipp, S. H. Kaufmann, and H. W. Mittrucker. Differential requirements for the chemokine receptor ccr7 in t cell activation during listeria monocytogenes infection. *J Exp Med*, 201(9):1447–57, 2005.

[108] T. Kuwabara, F. Ishikawa, T. Yasuda, K. Aritomi, H. Nakano, Y. Tanaka, Y. Okada, M. Lipp, and T. Kakiuchi. Ccr7 ligands are required for development of experimental autoimmune encephalomyelitis through generating il-23-dependent th17 cells. *J Immunol*, 183(4):2513–21, 2009.

[109] S. M. Lachenmaier, M. A. Deli, M. Meissner, and O. Liesenfeld. Intracellular transport of toxoplasma gondii through the blood-brain barrier. *J Neuroimmunol*, 232(1-2):119–30, 2011.

[110] S. J. Lalor and B. M. Segal. Lymphoid chemokines in the cns. *J Neuroimmunol*, 224(1-2):56–61, 2010.

[111] H. Lambert, N. Hitziger, I. Dellacasa, M. Svensson, and A. Barragan. Induction of dendritic cell migration upon toxoplasma gondii infection potentiates parasite dissemination. *Cell Microbiol*, 8(10):1611–23, 2006.

[112] J. R. Lees and D. L. Farber. Generation, persistence and plasticity of cd4 t-cell memories. *Immunology*, 130(4):463–70, 2010.

[113] J. Leng, B. A. Butcher, and E. Y. Denkers. Dysregulation of macrophage signal transduction by toxoplasma gondii: past progress and recent advances. *Parasite Immunol*, 31(12):717–28, 2009.

[114] L. A. Lieberman and C. A. Hunter. The role of cytokines and their signaling pathways in the regulation of immunity to toxoplasma gondii. *Int Rev Immunol*, 21(4-5):373–403, 2002.

[115] F. Lin and E. C. Butcher. T cell chemotaxis in a simple microfluidic device. *Lab Chip*, 6(11):1462–9, 2006.

[116] A. Link, T. K. Vogt, S. Favre, M. R. Britschgi, H. Acha-Orbea, B. Hinz, J. G. Cyster, and S. A. Luther. Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive t cells. *Nat Immunol*, 8(11):1255–65, 2007.

[117] C. Liu, T. Ueno, S. Kuse, F. Saito, T. Nitta, L. Piali, H. Nakano, T. Kakiuchi, M. Lipp, G. A. Hollander, and Y. Takahama. The role of ccl21 in recruitment of t-precursor cells to fetal thymi. *Blood*, 105(1):31–9, 2005.

[118] C. H. Liu, Y. T. Fan, A. Dias, L. Esper, R. A. Corn, A. Bafica, F. S. Machado, and J. Aliberti. Cutting edge: dendritic cells are essential for in vivo il-12 production and development of resistance against toxoplasma gondii infection in mice. *J Immunol*, 177(1):31–5, 2006.

[119] B. J. Luft and J. S. Remington. Toxoplasmic encephalitis in aids. *Clin Infect Dis*, 15(2):211–22, 1992.

[120] S. A. Luther, A. Bidgol, D. C. Hargreaves, A. Schmidt, Y. Xu, J. Paniyadi, M. Matloubian, and J. G. Cyster. Differing activities of homeostatic chemokines ccl19, ccl21, and cxcl12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J Immunol*, 169(1):424–33, 2002.

[121] A. MartIn-Fontecha, S. Sebastiani, U. E. Hopken, M. Uguccioni, M. Lipp, A. Lanzavecchia, and F. Sallusto. Regulation of dendritic cell migration to the draining lymph node: impact on t lymphocyte traffic and priming. *J Exp Med*, 198(4):615–21, 2003.

[122] M. Mashayekhi, M. M. Sandau, I. R. Dunay, E. M. Frickel, A. Khan, R. S. Goldszmid, A. Sher, H. L. Ploegh, T. L. Murphy, L. D. Sibley, and K. M. Murphy. Cd8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by toxoplasma gondii tachyzoites. *Immunity*, 35(2):249–59, 2011.

[123] J. McGill and K. L. Legge. Cutting edge: contribution of lung-resident t cell proliferation to the overall magnitude of the antigen-specific cd8 t cell response in the lungs following murine influenza virus infection. *J Immunol*, 183(7):4177–81, 2009.

[124] E. Meinl, M. Krumbholz, and R. Hohlfeld. B lineage cells in the inflammatory central nervous system environment: migration, maintenance, local antibody production, and therapeutic modulation. *Ann Neurol*, 59(6):880–92, 2006.

[125] F. J. Mennechet, L. H. Kasper, N. Rachinel, W. Li, A. Vandewalle, and D. Buzoni-Gatel. Lamina propria cd4+ t lymphocytes synergize with murine intestinal epithelial cells to enhance proinflammatory response against an intracellular pathogen. *J Immunol*, 168(6):2988–96, 2002.

[126] A. Menning, U. E. Hopken, K. Siegmund, M. Lipp, A. Hamann, and J. Huehn. Distinctive role of ccr7 in migration and functional activity of naive- and effector/memory-like treg subsets. *Eur J Immunol*, 37(6):1575–83, 2007.

[127] A. Misslitz, O. Pabst, G. Hintzen, L. Ohl, E. Kremmer, H. T. Petrie, and R. Forster. Thymic t cell development and progenitor localization depend on ccr7. *J Exp Med*, 200(4):481–91, 2004.

[128] B. Molon, G. Gri, M. Bettella, C. Gomez-Mouton, A. Lanzavecchia, A. C. Martinez, S. Manes, and A. Viola. T cell costimulation by chemokine receptors. *Nat Immunol*, 6(5):465–71, 2005.

[129] S. Mori, H. Nakano, K. Aritomi, C. R. Wang, M. D. Gunn, and T. Kakiuchi. Mice lacking expression of the chemokines ccl21-ser and ccl19 (plt mice) demonstrate delayed but enhanced t cell immune responses. *J Exp Med*, 193(2):207–18, 2001.

[130] B. Moser, M. Wolf, A. Walz, and P. Loetscher. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol*, 25(2):75–84, 2004.

[131] P. Mrass and W. Weninger. Immune cell migration as a means to control immune privilege: lessons from the cns and tumors. *Immunol Rev*, 213:195–212, 2006.

[132] S. N. Mueller and R. N. Germain. Stromal cell contributions to the homeostasis and functionality of the immune system. *Nat Rev Immunol*, 9(9):618–29, 2009.

[133] H. S. Mun, F. Aosai, K. Norose, M. Chen, L. X. Piao, O. Takeuchi, S. Akira, H. Ishikura, and A. Yano. Tlr2 as an essential molecule for protective immunity against toxoplasma gondii infection. *Int Immunol*, 15(9):1081–7, 2003.

[134] H. W. Murray, B. Y. Rubin, S. M. Carriero, A. M. Harris, and E. A. Jaffee. Human mononuclear phagocyte antiprotozoal mechanisms: oxygen-dependent vs oxygen-independent activity against intracellular toxoplasma gondii. *J Immunol*, 134(3):1982–8, 1985.

[135] H. Nakano, K. L. Lin, M. Yanagita, C. Charbonneau, D. N. Cook, T. Kakiuchi, and M. D. Gunn. Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute t helper type 1 immune responses. *Nat Immunol*, 10(4):394–402, 2009.

[136] S. Nandagopal, D. Wu, and F. Lin. Combinatorial guidance by ccr7 ligands for t lymphocytes migration in co-existing chemokine fields. *PLoS One*, 6(3):e18183, 2011.

[137] L. E. Neyer, G. Grunig, M. Fort, J. S. Remington, D. Rennick, and C. A. Hunter. Role of interleukin-10 in regulation of t-cell-dependent and t-cell-independent mechanisms of resistance to toxoplasma gondii. *Infect Immun*, 65(5):1675–82, 1997.

[138] Y. Ni, E. B. Malarkey, and V. Parpura. Vesicular release of glutamate mediates bidirectional signaling between astrocytes and neurons. *J Neurochem*, 103(4):1273–84, 2007.

[139] M. B. Nickdel, R. E. Lyons, F. Roberts, F. Brombacher, C. A. Hunter, J. Alexander, and C. W. Roberts. Intestinal pathology during acute toxoplasmosis is il-4 dependent and unrelated to parasite burden. *Parasite Immunol*, 26(2):75–82, 2004.

[140] S. Noor, A. S. Habashy, J. P. Nance, R. E. Clark, K. Nemati, M. J. Carson, and E. H. Wilson. Ccr7 dependent immunity during acute toxoplasma gondii infection. *Infect Immun*, 2010.

[141] K. Norose, A. Kikumura, A. D. Luster, C. A. Hunter, and T. H. Harris. Cxcl10 is required to maintain t-cell populations and to control parasite replication during chronic ocular toxoplasmosis. *Invest Ophthalmol Vis Sci*, 52(1):389–98, 2010.

[142] L. Ohl, M. Mohaupt, N. Czeloth, G. Hintzen, Z. Kiafard, J. Zwirner, T. Blankenstein, G. Henning, and R. Forster. Ccr7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity*, 21(2):279–88, 2004.

[143] D. Ojdana, K. Safiejko, A. Lipska, P. Radziwon, J. Dadan, and E. Tryniszewska. Effector and memory cd4+ and cd8+ t cells in the chronic infection process. *Folia Histochem Cytobiol*, 46(4):413–7, 2008.

[144] T. Okada and J. G. Cyster. Cc chemokine receptor 7 contributes to gi-dependent t cell motility in the lymph node. *J Immunol*, 178(5):2973–8, 2007.

[145] C. Otero, M. Groettrup, and D. F. Legler. Opposite fate of endocytosed ccr7 and its ligands: recycling versus degradation. *J Immunol*, 177(4):2314–23, 2006.

[146] A. Pahuja, R. A. Maki, P. A. Hevezi, A. Chen, G. M. Verge, S. M. Lechner, R. B. Roth, A. Zlotnik, and D. G. Alleva. Experimental autoimmune encephalomyelitis develops in cc chemokine receptor 7-deficient mice with altered t-cell responses. *Scand J Immunol*, 64(4):361–9, 2006.

[147] K. A. Papadakis, J. Prehn, V. Nelson, L. Cheng, S. W. Binder, P. D. Ponath, D. P. Andrew, and S. R. Targan. The role of thymus-expressed chemokine and its receptor ccr9 on lymphocytes in the regional specialization of the mucosal immune system. *J Immunol*, 165(9):5069–76, 2000.

[148] M. Papatriantafyllou. Trafficking: Tracking immune cells on the lymph node map. *Nat Rev Immunol*, 11(10):644, 2011.

[149] M. Pepper, F. Dzierszinski, A. Crawford, C. A. Hunter, and D. Roos. Development of a system to study cd4+-t-cell responses to transgenic ovalbumin-expressing toxoplasma gondii during toxoplasmosis. *Infect Immun*, 72(12):7240–6, 2004.

[150] M. Pepper, F. Dzierszinski, E. Wilson, E. Tait, Q. Fang, F. Yarovinsky, T. M. Laufer, D. Roos, and C. A. Hunter. Plasmacytoid dendritic cells are activated by toxoplasma gondii to present antigen and produce cytokines. *J Immunol*, 180(9):6229–36, 2008.

[151] M. Pepper and M. K. Jenkins. Origins of cd4(+) effector and central memory t cells. *Nat Immunol*, 12(6):467–71, 2011.

[152] H. T. Petrie. Cell migration and the control of post-natal t-cell lymphopoiesis in the thymus. *Nat Rev Immunol*, 3(11):859–66, 2003.

[153] R. Pifer and F. Yarovinsky. Innate responses to toxoplasma gondii in mice and humans. *Trends Parasitol*, 27(9):388–93, 2011.

[154] C. Ploix, D. Lo, and M. J. Carson. A ligand for the chemokine receptor ccr7 can influence the homeostatic proliferation of cd4 t cells and progression of autoimmunity. *J Immunol*, 167(12):6724–30, 2001.

[155] C. Ploix, R. I. Zuberi, F. T. Liu, M. J. Carson, and D. D. Lo. Induction and effector phase of allergic lung inflammation is independent of ccl21/ccl19 and lt-beta. *Int J Med Sci*, 6(2):85–92, 2009.

[156] C. C. Ploix, S. Noor, J. Crane, K. Masek, W. Carter, D. D. Lo, E. H. Wilson, and M. J. Carson. Cns-derived ccl21 is both sufficient to drive homeostatic cd4+ t cell proliferation and necessary for efficient cd4+ t cell migration into the cns parenchyma following toxoplasma gondii infection. *Brain Behav Immun*, 2010.

[157] A. Rappert, K. Biber, C. Nolte, M. Lipp, A. Schubel, B. Lu, N. P. Gerard, C. Gerard, H. W. Boddeke, and H. Kettenmann. Secondary lymphoid tissue chemokine (ccl21) activates cxcr3 to trigger a cl- current and chemotaxis in murine microglia. *J Immunol*, 168(7):3221–6, 2002.

[158] K. Reif, E. H. Ekland, L. Ohl, H. Nakano, M. Lipp, R. Forster, and J. G. Cyster. Balanced responsiveness to chemoattractants from adjacent zones determines b-cell position. *Nature*, 416(6876):94–9, 2002.

[159] C. Reis e Sousa, S. Hieny, T. Scharton-Kersten, D. Jankovic, H. Charest, R. N. Germain, and A. Sher. In vivo microbial stimulation induces rapid cd40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to t cell areas. *J Exp Med*, 186(11):1819–29, 1997.

[160] Y. Reiss, A. E. Proudfoot, C. A. Power, J. J. Campbell, and E. C. Butcher. Cc chemokine receptor (ccr)4 and the ccr10 ligand cutaneous t cell-attracting chemokine (ctack) in lymphocyte trafficking to inflamed skin. *J Exp Med*, 194(10):1541–7, 2001.

[161] B. G. Ricart, B. John, D. Lee, C. A. Hunter, and D. A. Hammer. Dendritic cells distinguish individual chemokine signals through ccr7 and cxcr4. *J Immunol*, 186(1):53–61, 2010.

[162] L. Riol-Blanco, N. Sanchez-Sanchez, A. Torres, A. Tejedor, S. Narumiya, A. L. Corbi, P. Sanchez-Mateos, and J. L. Rodriguez-Fernandez. The chemokine receptor ccr7 activates in dendritic cells two signaling modules that independently regulate chemotaxis and migratory speed. *J Immunol*, 174(7):4070–80, 2005.

[163] P. M. Robben, M. LaRegina, W. A. Kuziel, and L. D. Sibley. Recruitment of gr-1+ monocytes is essential for control of acute toxoplasmosis. *J Exp Med*, 201(11):1761–9, 2005.

[164] S. H. Robbins, S. C. Terrizzi, B. C. Sydora, T. Mikayama, and L. Brossay. Differential regulation of killer cell lectin-like receptor g1 expression on t cells. *J Immunol*, 170(12):5876–85, 2003.

[165] N. Sakai, T. Wada, H. Yokoyama, M. Lipp, S. Ueha, K. Matsushima, and S. Kaneko. Secondary lymphoid tissue chemokine (slc/ccl21)/ccr7 signaling regulates fibrocytes in renal fibrosis. *Proc Natl Acad Sci U S A*, 103(38):14098–103, 2006.

[166] F. Sallusto, D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. Two subsets of memory t lymphocytes with distinct homing potentials and effector functions. *Nature*, 401(6754):708–12, 1999.

[167] F. Sallusto, B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, and A. Lanzavecchia. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur J Immunol*, 29(5):1617–25, 1999.

[168] N. Sanchez-Sanchez, L. Riol-Blanco, G. de la Rosa, A. Puig-Kroger, J. Garcia-Bordas, D. Martin, N. Longo, A. Cuadrado, C. Cabanas, A. L. Corbi, P. Sanchez-Mateos, and J. L. Rodriguez-Fernandez. Chemokine receptor ccr7 induces intracellular signaling that inhibits apoptosis of mature dendritic cells. *Blood*, 104(3):619–25, 2004.

[169] N. Sanchez-Sanchez, L. Riol-Blanco, and J. L. Rodriguez-Fernandez. The multiple personalities of the chemokine receptor ccr7 in dendritic cells. *J Immunol*, 176(9):5153–9, 2006.

[170] E. Scandella, K. Fink, T. Junt, B. M. Senn, E. Lattmann, R. Forster, H. Hengartner, and B. Ludewig. Dendritic cell-independent b cell activation during acute virus infection: a role for early ccr7-driven b-t helper cell collaboration. *J Immunol*, 178(3):1468–76, 2007.

[171] M. Schaeffer, S. J. Han, T. Chtanova, G. G. van Dooren, P. Herzmark, Y. Chen, B. Roysam, B. Striepen, and E. A. Robey. Dynamic imaging of t cell-parasite interactions in the brains of mice chronically infected with toxoplasma gondii. *J Immunol*, 182(10):6379–93, 2009.

[172] T. M. Scharton-Kersten, G. Yap, J. Magram, and A. Sher. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen toxoplasma gondii. *J Exp Med*, 185(7):1261–73, 1997.

[173] D. Schluter, M. Deckert-Schluter, E. Lorenz, T. Meyer, M. Rollinghoff, and C. Bogdan. Inhibition of inducible nitric oxide synthase exacerbates chronic cerebral toxoplasmosis in toxoplasma gondii-susceptible c57bl/6 mice but does not reactivate the latent disease in t. gondii-resistant balb/c mice. *J Immunol*, 162(6):3512–8, 1999.

[174] M. A. Schneider, J. G. Meingassner, M. Lipp, H. D. Moore, and A. Rot. Ccr7 is required for the in vivo function of cd4+ cd25+ regulatory t cells. *J Exp Med*, 204(4):735–45, 2007.

[175] K. Schumann, T. Lammermann, M. Bruckner, D. F. Legler, J. Polleux, J. P. Spatz, G. Schuler, R. Forster, M. B. Lutz, L. Sorokin, and M. Sixt. Immobilized chemokine

fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity*, 32(5):703–13, 2010.

[176] M. Schwartz and I. R. Cohen. Autoimmunity can benefit self-maintenance. *Immunol Today*, 21(6):265–8, 2000.

[177] A. Sher, E. Y. Denkers, and R. T. Gazzinelli. Induction and regulation of host cell-mediated immunity by toxoplasma gondii. *Ciba Found Symp*, 195:95–104; discussion 104–9, 1995.

[178] H. Shin and E. J. Wherry. Cd8 t cell dysfunction during chronic viral infection. *Curr Opin Immunol*, 19(4):408–15, 2007.

[179] A. K. Stalder, M. J. Carson, A. Pagenstecher, V. C. Asensio, C. Kincaid, M. Benedict, H. C. Powell, E. Masliah, and I. L. Campbell. Late-onset chronic inflammatory encephalopathy in immune-competent and severe combined immune-deficient (scid) mice with astrocyte-targeted expression of tumor necrosis factor. *Am J Pathol*, 153(3):767–83, 1998.

[180] J. V. Stein, A. Rot, Y. Luo, M. Narasimhaswamy, H. Nakano, M. D. Gunn, A. Matsuzawa, E. J. Quackenbush, M. E. Dorf, and U. H. von Andrian. The cc chemokine thymus-derived chemotactic agent 4 (tca-4, secondary lymphoid tissue chemokine, 6ckine, exodus-2) triggers lymphocyte function-associated antigen 1-mediated arrest of rolling t lymphocytes in peripheral lymph node high endothelial venules. *J Exp Med*, 191(1):61–76, 2000.

[181] W. Stenzel, S. Soltek, D. Schluter, and M. Deckert. The intermediate filament gfap is important for the control of experimental murine staphylococcus aureus-induced brain abscess and toxoplasma encephalitis. *J Neuropathol Exp Neurol*, 63(6):631–40, 2004.

[182] A. Strack, V. C. Asensio, I. L. Campbell, D. Schluter, and M. Deckert. Chemokines are differentially expressed by astrocytes, microglia and inflammatory leukocytes in toxoplasma encephalitis and critically regulated by interferon-gamma. *Acta Neuropathol*, 103(5):458–68, 2002.

[183] J. S. Stumhofer, A. Laurence, E. H. Wilson, E. Huang, C. M. Tato, L. M. Johnson, A. V. Villarino, Q. Huang, A. Yoshimura, D. Sehy, C. J. Saris, J. J. O'Shea, L. Hennighausen, M. Ernst, and C. A. Hunter. Interleukin 27 negatively regulates the development of interleukin 17-producing t helper cells during chronic inflammation of the central nervous system. *Nat Immunol*, 7(9):937–45, 2006.

[184] W. Sukhumavasi, C. E. Egan, and E. Y. Denkers. Mouse neutrophils require jnk2 mapk for toxoplasma gondii-induced il-12p40 and ccl2/mcp-1 release. *J Immunol*, 179(6):3570–7, 2007.

[185] Y. Suzuki, J. Claflin, X. Wang, A. Lengi, and T. Kikuchi. Microglia and macrophages as innate producers of interferon-gamma in the brain following infection with toxoplasma gondii. *Int J Parasitol*, 35(1):83–90, 2005.

[186] Y. Suzuki, M. A. Orellana, R. D. Schreiber, and J. S. Remington. Interferon-gamma: the major mediator of resistance against toxoplasma gondii. *Science*, 240(4851):516–8, 1988.

[187] Y. Suzuki and J. S. Remington. Dual regulation of resistance against toxoplasma gondii infection by lyt-2+ and lyt-1+, l3t4+ t cells in mice. *J Immunol*, 140(11):3943–6, 1988.

[188] V. Szanya, J. Ermann, C. Taylor, C. Holness, and C. G. Fathman. The subpopulation of cd4+cd25+ splenocytes that delays adoptive transfer of diabetes expresses l-selectin and high levels of ccr7. *J Immunol*, 169(5):2461–5, 2002.

[189] E. D. Tait and C. A. Hunter. Advances in understanding immunity to toxoplasma gondii. *Mem Inst Oswaldo Cruz*, 104(2):201–10, 2009.

[190] K. Takamura, S. Fukuyama, T. Nagatake, D. Y. Kim, A. Kawamura, H. Kawauchi, and H. Kiyono. Regulatory role of lymphoid chemokine ccl19 and ccl21 in the control of allergic rhinitis. *J Immunol*, 179(9):5897–906, 2007.

[191] Y. Takashima, K. Suzuki, X. Xuan, Y. Nishikawa, A. Unno, and K. Kitoh. Detection of the initial site of toxoplasma gondii reactivation in brain tissue. *Int J Parasitol*, 38(5):601–7, 2008.

[192] M. Tateyama, K. Fujihara, T. Misu, and Y. Itoyama. Ccr7+ myeloid dendritic cells together with ccr7+ t cells and ccr7+ macrophages invade ccl19+ nonnecrotic muscle fibers in inclusion body myositis. *J Neurol Sci*, 279(1-2):47–52, 2009.

[193] C. M. Tato, A. Villarino, J. H. Caamano, M. Boothby, and C. A. Hunter. Inhibition of nf-kappa b activity in t and nk cells results in defective effector cell expansion and production of ifn-gamma required for resistance to toxoplasma gondii. *J Immunol*, 170(6):3139–46, 2003.

[194] G. A. Taylor, C. M. Collazo, G. S. Yap, K. Nguyen, T. A. Gregorio, L. S. Taylor, B. Eagleson, L. Secrest, E. A. Southon, S. W. Reid, L. Tessarollo, M. Bray, D. W. McVicar, K. L. Komschlies, H. A. Young, C. A. Biron, A. Sher, and G. F. Vande Woude. Pathogen-specific loss of host resistance in mice lacking the ifn-gamma-inducible gene igtp. *Proc Natl Acad Sci U S A*, 97(2):751–5, 2000.

[195] M. S. Tessmer, C. Fugere, F. Stevenaert, O. V. Naidenko, H. J. Chong, G. Leclercq, and L. Brossay. Klrg1 binds cadherins and preferentially associates with ship-1. *Int Immunol*, 19(4):391–400, 2007.

[196] S. J. Turley, A. L. Fletcher, and K. G. Elpek. The stromal and haematopoietic antigen-presenting cells that reside in secondary lymphoid organs. *Nat Rev Immunol*, 10(12):813–25, 2010.

[197] T. Ueno, F. Saito, D. H. Gray, S. Kuse, K. Hieshima, H. Nakano, T. Kakiuchi, M. Lipp, R. L. Boyd, and Y. Takahama. Ccr7 signals are essential for cortex-medulla migration of developing thymocytes. *J Exp Med*, 200(4):493–505, 2004.

[198] D. Voehringer, C. Blaser, P. Brawand, D. H. Raulet, T. Hanke, and H. Pircher. Viral infections induce abundant numbers of senescent cd8 t cells. *J Immunol*, 167(9):4838–43, 2001.

[199] L. M. Wakim, A. Woodward-Davis, and M. J. Bevan. Memory t cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc Natl Acad Sci U S A*, 107(42):17872–9, 2010.

[200] O. Wald, I. D. Weiss, E. Galun, and A. Peled. Chemokines in hepatitis c virus infection: pathogenesis, prognosis and therapeutics. *Cytokine*, 39(1):50–62, 2007.

[201] H. R. Weering, A. P. Jong, A. H. Haas, K. P. Biber, and H. W. Boddeke. Ccl21-induced calcium transients and proliferation in primary mouse astrocytes: Cxcr3-dependent and independent responses. *Brain Behav Immun*, 2009.

[202] L. M. Weiss, Y. F. Ma, P. M. Takvorian, H. B. Tanowitz, and M. Wittner. Bradyzoite development in toxoplasma gondii and the hsp70 stress response. *Infect Immun*, 66(7):3295–302, 1998.

[203] M. Wendland, S. Willenzon, J. Kocks, A. C. Davalos-Misslitz, S. I. Hammerschmidt, K. Schumann, E. Kremmer, M. Sixt, A. Hoffmeyer, O. Pabst, and R. Forster. Lymph node t cell homeostasis relies on steady state homing of dendritic cells. *Immunity*, 35(6):945–57, 2011.

[204] U. Wille, E. N. Villegas, L. Craig, R. Peach, and C. A. Hunter. Contribution of interleukin-12 (il-12) and the cd28/b7 and cd40/cd40 ligand pathways to the development of a pathological t-cell response in il-10-deficient mice. *Infect Immun*, 70(12):6940–7, 2002.

[205] D. C. Wilson, G. M. Grotenbreg, K. Liu, Y. Zhao, E. M. Frickel, M. J. Gubbels, H. L. Ploegh, and G. S. Yap. Differential regulation of effector- and central-memory responses to toxoplasma gondii infection by il-12 revealed by tracking of tgd057-specific cd8+ t cells. *PLoS Pathog*, 6(3):e1000815, 2010.

[206] E. H. Wilson, T. H. Harris, P. Mrass, B. John, E. D. Tait, G. F. Wu, M. Pepper, E. J. Wherry, F. Dzierzinski, D. Roos, P. G. Haydon, T. M. Laufer, W. Weninger, and C. A. Hunter. Behavior of parasite-specific effector cd8+ t cells in the brain and visualization of a kinesis-associated system of reticular fibers. *Immunity*, 30(2):300–11, 2009.

[207] E. H. Wilson and C. A. Hunter. The role of astrocytes in the immunopathogenesis of toxoplasmic encephalitis. *Int J Parasitol*, 34(5):543–8, 2004.

[208] E. H. Wilson, W. Weninger, and C. A. Hunter. Trafficking of immune cells in the central nervous system. *J Clin Invest*, 120(5):1368–79, 2010.

[209] E. H. Wilson, U. Wille-Reece, F. Dzierszinski, and C. A. Hunter. A critical role for il-10 in limiting inflammation during toxoplasmic encephalitis. *J Neuroimmunol*, 165(1-2):63–74, 2005.

[210] E. L. Wissinger, W. W. Stevens, S. M. Varga, and T. J. Braciale. Proliferative expansion and acquisition of effector activity by memory cd4+ t cells in the lungs following pulmonary virus infection. *J Immunol*, 180(5):2957–66, 2008.

[211] T. Worbs, T. R. Mempel, J. Bolter, U. H. von Andrian, and R. Forster. Ccr7 ligands stimulate the intranodal motility of t lymphocytes in vivo. *J Exp Med*, 204(3):489–95, 2007.

[212] Y. Yanagawa and K. Onoe. Ccl19 induces rapid dendritic extension of murine dendritic cells. *Blood*, 100(6):1948–56, 2002.

[213] Y. Yanagawa and K. Onoe. Ccr7 ligands induce rapid endocytosis in mature dendritic cells with concomitant up-regulation of cdc42 and rac activities. *Blood*, 101(12):4923–9, 2003.

[214] B. G. Yang, T. Tanaka, M. H. Jang, Z. Bai, H. Hayasaka, and M. Miyasaka. Binding of lymphoid chemokines to collagen iv that accumulates in the basal lamina of high endothelial venules: its implications in lymphocyte trafficking. *J Immunol*, 179(7):4376–82, 2007.

[215] G. Yap, M. Pesin, and A. Sher. Cutting edge: Il-12 is required for the maintenance of ifn-gamma production in t cells mediating chronic resistance to the intracellular pathogen, toxoplasma gondii. *J Immunol*, 165(2):628–31, 2000.

[216] G. S. Yap and A. Sher. Effector cells of both nonhemopoietic and hemopoietic origin are required for interferon (ifn)-gamma- and tumor necrosis factor (tnf)-alpha-dependent host resistance to the intracellular pathogen, toxoplasma gondii. *J Exp Med*, 189(7):1083–92, 1999.

[217] F. Yarovinsky, S. Hieny, and A. Sher. Recognition of toxoplasma gondii by tlr11 prevents parasite-induced immunopathology. *J Immunol*, 181(12):8478–84, 2008.

[218] R. Yoshida, M. Nagira, M. Kitaura, N. Imagawa, T. Imai, and O. Yoshie. Secondary lymphoid-tissue chemokine is a functional ligand for the cc chemokine receptor ccr7. *J Biol Chem*, 273(12):7118–22, 1998.

[219] D. J. Zammit, L. S. Cauley, Q. M. Pham, and L. Lefrancois. Dendritic cells maximize the memory cd8 t cell response to infection. *Immunity*, 22(5):561–70, 2005.

[220] C. Zaph, J. Uzonna, S. M. Beverley, and P. Scott. Central memory t cells mediate long-term immunity to leishmania major in the absence of persistent parasites. *Nat Med*, 10(10):1104–10, 2004.

[221] P. Zhao, S. G. Waxman, and B. C. Hains. Modulation of thalamic nociceptive processing after spinal cord injury through remote activation of thalamic microglia by cysteine cysteine chemokine ligand 21. *J Neurosci*, 27(33):8893–902, 2007.

[222] Y. Zhao, D. Wilson, S. Matthews, and G. S. Yap. Rapid elimination of toxoplasma gondii by gamma interferon-primed mouse macrophages is independent of cd40 signaling. *Infect Immun*, 75(10):4799–803, 2007.

[223] D. A. Zidar, J. D. Violin, E. J. Whalen, and R. J. Lefkowitz. Selective engagement of g protein coupled receptor kinases (grks) encodes distinct functions of biased ligands. *Proc Natl Acad Sci U S A*, 106(24):9649–54, 2009.