Insights into the subset, antigen specificity, and localization of T cells in spatial learning and memory

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ABSTRACT

The notion of "immune privilege" in the central nervous system (CNS) has worked to perpetuate the impression that interactions between the brain and immune system are inherently detrimental. However the view of the CNS as an ivory tower, insulated from the reach and influence of the immune system, has been considerably challenged in recent years. Indeed, many of the immune processes thought to be absent in the CNS have been observed in the meningeal structures that follow the contours, ventricles and vasculature of the brain parenchyma.

A large portion of this thesis is dedicated to T cell behavior in the meninges, which in spite of its profound influence on brain processes, immune surveillance, and neuropathology, remains poorly understood. Part of the original work herein attempts to elucidate the dynamics and the antigenic specificity of T cells within the meningeal space. By employing a parabiotic system of shared circulation we show that the CD4 turnover in the meninges to be slow relative to other tissues, dependent on a memory phenotype, and tightly coupled to the meninges draining deep cervical lymph node.

As our perception of absolute immune privilege has changed, so have our view on the outcomes of T cell-CNS crosstalk. Whereas neuroimmunology was once nearly synonymous with immune-mediated neuropathology, it now encompasses the emerging homeostatic roles of T cell-CNS interactions, including: hippocampal-dependent learning, stress response paradigms, and models of neurogenesis, neurodegeneration, and CNS injury.

This body of work aims to shed light on the observation that lymphopenic mice exhibit significant behavioral impairment in spatial learning and memory tasks. Employing several transgenic mouse models in combination with adoptive transfers, we evidence the necessity of an antigen-specific CD4 T cell compartment in normal special learning and memory in mice. Specifically we show that lymphopenic mice manifest with an impaired spatial learning and memory phenotype that can be rescued by adoptive transfer of wild-type CD4 T cells. We go on to show that a monoclonal T cell population

reactive to CNS self-antigen is sufficient to improve cognitive task performance in otherwise learning impaired animals, and raise the possibility that rescue is mediated by a robust population of meninges infiltrating myeloid derived suppressor cells.

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LIST OF ABREVIATIONS

APC: antigen presenting cell

BBB: blood brain barrier

BDNF: brain derived neurotrophic factor

BMDM: bone marrow derived macrophage

CHS: contact hypersensitivity

CNS: central nervous system

CSF: cerebrospinal fluid

CVO: circumventricular organ

DTH: delayed type hypersensitivity

EAE: experimental autoimmune encephalomyelitis

HEV: high endothelial venule

HPA: hypothalamic pituitary adrenal axis

ISF: interstitial fluid

KLH: keyhole limpet hemocyanin

LGN: lateral geniculate nucleus

LPS: lipopolysaccharide

LTP: long term potentiation

MBP: myelin basic protein

MDSC: myeloid derived suppressor cell

MHC: major histocompatibility complex

MOG: myelin oligodendrocyte glycoprotein

MWM: Morris water maze

OVA: ovalbumin

Rag: recombination activating gene

RSS: recombination signal sequence

SGZ: subgranular zone

SVZ: subventricular zone

T_{CM}: T central memory

TCR: T cell receptor

T_{EM}: T effector memory

Treg: regulatory T cell

T_{RM}: T resident memory

ZO: zonula occluden

I. INTRODUCTION

A. THE MYTH OF CNS IMMUNE PRIVILEGE

Expanding bodies of research, including the studies of this thesis are elucidating the lines of communication between the central nervous system (CNS) and the immune system. While this thesis specifically investigates the phenomenon of T cell involvement in higher brain functions, other studies have demonstrated a role for T cells in models of stress resilience[1], neurogensis[2-5], and CNS injury[6, 7]. Each of these studies has had to contend with the idea of CNS immunoprivelege– the prevailing view of the last century that the CNS is wholly insulated from the immune system[8, 9].

The idea of CNS immune privilege is a product of several fundamental experiments and observations including: the lack of lymphatic vessels or dendritic cells in the brain[8], the uniquely extended survival of foreign tissue grafts in the brain[10], and the blood-brain barrier. While these experiment still hold true, the following three chapters demonstrate that the term immune privilege has been overextended.

In Chapter 1, "The meninges", we describe the structure of the much overlooked CNS tissue that surrounds the brain parenchyma, and review what is known about its immune functions and its contribution to the immunological competency of the CNS. Much of the original experimentation in Chapters 10 and 11 of this thesis, explores the immunology of this poorly characterized compartment where we hypothesize that T cell-CNS crosstalk is taking place. In Chapter 2 we explore the most salient feature of immune privilege, the blood brain barrier (BBB), defining how its structure precludes entry of circulating blood constituents (including immune cytokines/chemokines) from entry into the brain parenchyma, as well as the circumventricular organs at which the BBB is uniquely bypassed. Finally in Chapter 3, "Immune molecules in the CNS", we review select examples of both the

leukocyte independent re-use of “immune” proteins, as well as cases where loss of immune function leads to altered cellular and behavioral biology.

I. INTRODUCTION

A. THE MYTH OF CNS IMMUNE PRIVILEGE

CHAPTER 1: THE MENINGES

The meninges is the collective term for the membrane structures that surround the central nervous system (brain and spinal cord). The meninges is composed of three stacked layers of membrane that follow the complex three-dimensional contours of the underlying neuronal tissue. It not only covers the outer contours of the central nervous system (CNS), but penetrates the parenchyma at the ventricles and choroid plexus, and is functionally contiguous with the perivascular spaces of the CNS[11].

The meninges constitutes the border of the CNS, whether it is considered an actual part of the CNS or not, is largely a matter of semantics. In so much as the meninges directly supports and intimately associates with the CNS parenchyma, it is indeed part of the CNS. Nevertheless, in practice the meninges is viewed independently of the CNS, as most studies (especially as they relate to the immunology of the CNS) overlook or omit the meninges altogether. Here we regard the meninges as part of the CNS, but take care to distinguish between the CNS, which includes the meninges, and the CNS parenchyma, which does not.

As the structure that separates the CNS from overlying bone, one of the immediate functions of the meninges is to cushion the underlying tissue from jostle and impact. The meninges is often referred to as a "cushion" for the brain, but can be more accurately imagined as a three- dimensional semi-gyroscopic layer of cushions that exquisitely protects the brain and spinal cord from the most subtle vibration.

Yet this is not the only function of the meninges. Given its location, it is not surprising that the meninges is also necessary for both the normal development and the homeostatic maintenance of the tissue that it envelops.

The three meningeal Layers

The dura mater is the outermost layer of the meninges. The term originates from the Greek for "tough mother", which aptly portrays it as the dense fibrous tissue that sits directly beneath the bones of the skull and spinal vertebrae (Figure 1.1). It is highly vascularized and carries the dural sinuses that deliver blood from the brain to the heart.

The arachnoid mater (which together with the pia mater constitute the leptomeninges) is the middle tissue layer of the meninges (Figure 1.1). Its Greek etymology means "spider-like", reflecting its webbed structure formed by trabechulae that transverse the arachnoid mater to the pia, creating the cerebrospinal fluid (CSF) filled caverns known as the subarachnoid space. The arachnoid matter is most saliently appreciated as the elastic cushion for the CNS.

The pia mater is the final, innermost layer, of the meninges. The term translates from the Greek meaning "soft mother" describing its thin and delicate structure. The pia directly contacts the underlying CNS parenchyma, and covers the large meningeal blood vessels before they dive laterally into the brain parenchyma[12] (Figure 1.1).

The meninges provides immune surveillance of the CNS.

The CNS parenchyma lacks conventional dendritic cells, it has no direct lymphatic drainage, and rejection of non-autologous transplants made in the brain is dramatically delayed in comparison with transplantation into other host tissues[10, 13]. The evolutionary logic in insulating the CNS from the immune system is clear. Leslie Brent captures the reason perfectly: 'it may be supposed that it is

beneficial to the organism not to turn the anterior chamber or the cornea of the eye, or the brain, into an inflammatory battlefield, for the immunological response is sometimes more damaging than the antigen insult that provoked it'[14]. Nevertheless it would not be beneficial for the organism to be completely impotent against the many bacteria, viruses, and protozoans that are tropic to the CNS, particularly in a tissue that for all intents and purposes lacks regenerative capacity.

Absent from the brain and spinal cord parenchyma are lymphocytes and lymphatic vessels (the hydrodynamic function of lymph is taken over by CSF flow through the meninges[15]), which along with several other attributes discussed later, have led to the view of the CNS as an immune privileged organ[8]. While lymphocytes do well to stay out of delicate neuronal networks, the CNS must still be protected against pathogenic challenge. The meninges acts as an immunological citadel surrounding the 'immune privileged' and consequently pathogenically vulnerable neuronal tissue below. Given that the meninges envelopes the CNS, and that it is an immune-permissive tissue harboring T cells and antigen presenting cells (APCs), it makes a logical candidate for the site of immune-CNS crosstalk that is the subject of this thesis.

As mentioned earlier, the CNS parenchyma lacks both classic tissue resident dendritic cells (DCs) as well as the afferent lymphatic vessels that would otherwise carry them to the draining lymph node[8]. Nevertheless, protective adaptive immune responses are readily generated against CNS-tropic pathogens, as are pathogenic responses to self antigens of the CNS parenchyma[8]. Andersson et al showed that soluble ovalbumin injected into the striatum of rats yields detectable antibody response in the serum 2 weeks after ova microinfusion, demonstrating that CNS derived antigens can indeed elicit adaptive immune responses[16]. Similarly, by injecting radiolabeled sheep red blood cells into the mouse striatum, Widner et al were able to recover antigen specific antibody secreting plasma cells in the spleen and cervical lymph nodes of immunized mice[17].

How does soluble antigen from the CNS reach T and B cells? At least two routes are well appreciated: The first route by which CNS derived soluble antigens gain access to the periphery is through arachnoid granulations (route 1 of Figure 1.2). Brain and spinal cord CSF flowing through the subarachnoid space drains into the venous sinus at specialized projections of the meninges known as arachnoid villi or arachnoid granulations (Figure 1.3 and 1.4). These granulations operate as one way CSF valves from which soluble antigens can exit the meninges, enter the venous circulation and ultimately drain to the spleen[8]. Importantly, the CSF is contiguous with the interstitial fluid (ISF) that occupies the intercellular space within the CNS parenchyma, meaning that subarachnoid granulations can ultimately transport parenchymal antigens to the venous circulation[18]

In the second simultaneous route of soluble antigen escape to the periphery, brain derived-antigens drain to the deep cervical lymph nodes while spinal cord derived antigens drain to the lumbar and intercostal lymph nodes[19] (route 2 in Figure 1.2). In the brain, soluble antigens derived from the CNS parenchyma are picked up by the CSF where they travel through the conduits of the subarachnoid space to the base of the frontal lobe. While several models have been proposed for the anatomical connection between the subarachnoid space and the cervical lymph nodes, the most recent and thorough study to date, shows that drainage first occurs along the meningeal subarachnoid space that covers the olfactory nerve[20]. Upon reaching the cribriform plate the arachnoid and dural meninges of the olfactory nerve fuse into one another to form the new perineurial layer that traverses the cribriform plate. After crossing the cribriform plate, antigen can now drain into the interstitial space of the nasal mucosa where it is picked up by the afferent lymphatics of the deep cervical lymph node[20, 21]. The route of soluble antigen drainage through the cribriform plate is diagramed in Figure 1.5.

Recent studies have elucidated a paravascular waste-clearance pathway termed the “glymphatic system”, in which brain interstitial fluid is cleared from the parenchyma via the paravascular route along veins of the brain parenchyma (route 3 in Figure 1.2)[22]. Movement is generated by arterial

pulsation and CSF-ISF exchange is dependent on Aquaporin-4 expression on brain astrocytes[22, 23]. Importantly the glymphatic system may represent a third route of soluble antigen delivery to the periphery. However the capacity of this new route to initiate immune responses in the periphery has yet to be directly addressed.

So far, two routes of *soluble* antigen delivery from the CNS to the periphery have been discussed. In several early studies, serum antibody titers were consistently higher when immunogen was microinfused into the CNS, as compared to either subcutaneous, or intravenous injections of the same concentration[8, 24-26]. This heightened efficiency suggested that soluble antigens might be arriving at the lymphoid organs not just as soluble antigen, but also as antigen in the context of dedicated antigen presenting cells (APCs) of the meninges.

In a study by Hatterer et al, the fate of bone marrow-derived myeloid dendritic cells was followed after stereotactic injection into the CSF[27]. Cells transferred into the lateral ventricle moved with the passive flow of the CSF and were detected in the subarachnoid space of the meninges, and ultimately the cervical lymph nodes, confirming the anticipated cellular route of antigen drainage suggested by previous studies. Importantly, this route (which is the same as the soluble antigen drainage route described in Figure 1.2) allows for professional APCs of the meninges including dendritic cells and macrophages to sample the contents of the CSF, and generate immune responses in the draining cervical lymph node[28, 29]. Lymphocytes appear to egress from the meninges by the same route. Preparations of entire head and neck cross-sections revealed that GFP^+ lymphocytes injected into the lateral ventricle negotiate the same route of egress, moving along the afferent olfactory nerve, to the nasal mucosa, and finally to the afferent vessels of the deep cervical lymph node[30].

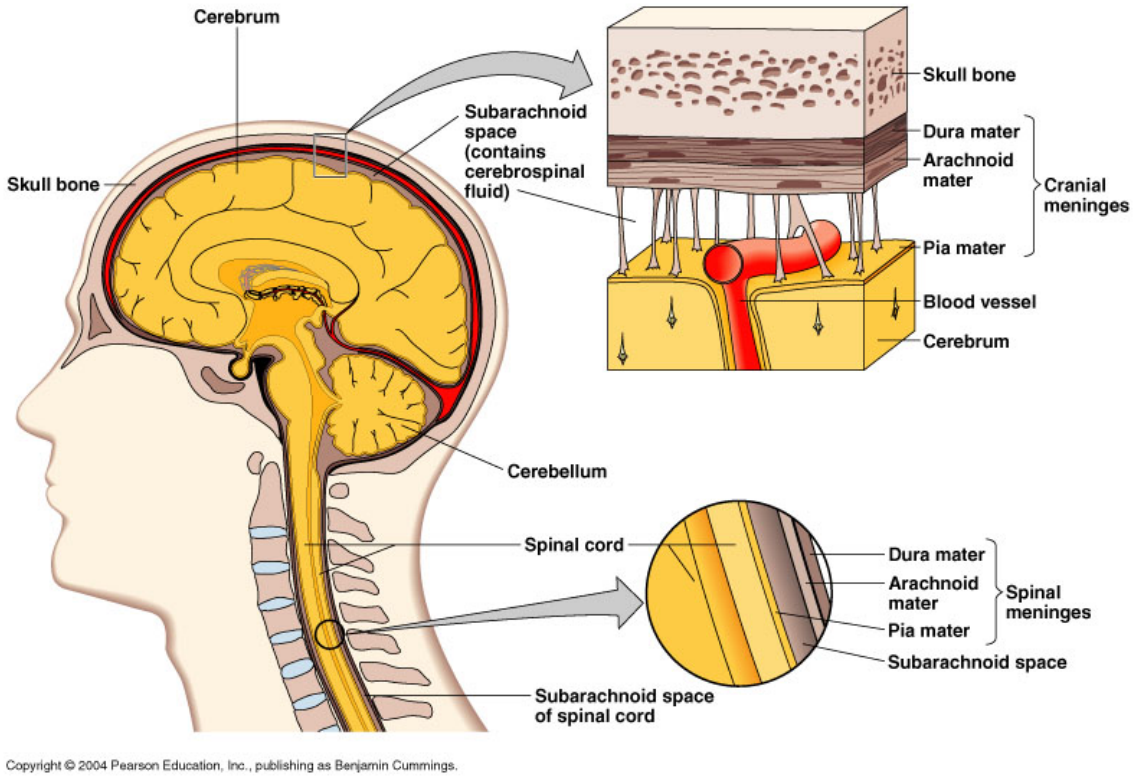


Figure 1.1: Anatomical structure of the meninges.

From: TORTORA, GERARD J.; FUNKE, BERDELL R.; CASE, CHRISTINE L., MICROBIOLOGY: AN INTRODUCTION, 11TH Edition, © 2013. Reprinted by permission of Pearson Education, Inc., Upper Saddle River, NJ

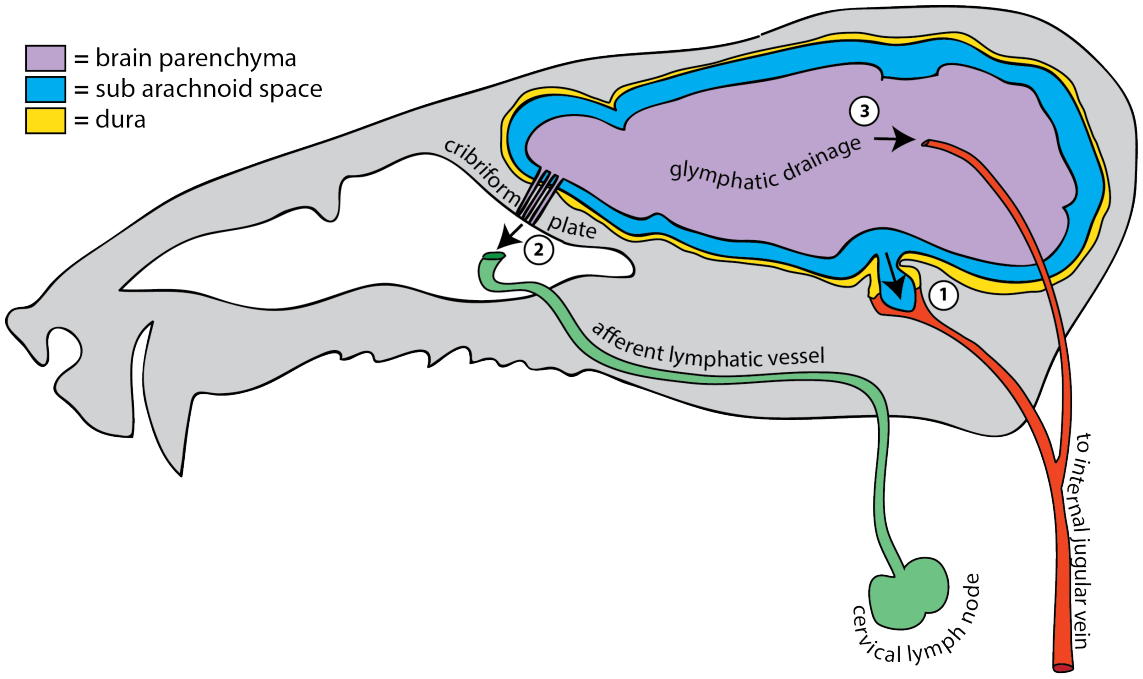


Figure 1.2: Routes of soluble antigen drainage from the brain parenchyma. 1) Soluble antigens in the ISF and CSF flow through the subarachnoid space and drain into the venous sinus at specialized projections into the dura known as arachnoid granulations, from where they drain towards the internal jugular vein. 2) Soluble antigens derived from the CNS parenchyma are picked up by the CSF where they travel through the conduits of the subarachnoid space to the base of the frontal lobe. From here antigens traverse the cribriform plate, moving along the olfactory nerves to the nasal mucosa, where they ultimately gain access to the afferent lymphatics of the deep cervical lymph nodes. 3) The recently appreciated glymphatic route by which ISF is cleared from the parenchyma via the paravascular route along veins of the brain parenchyma.

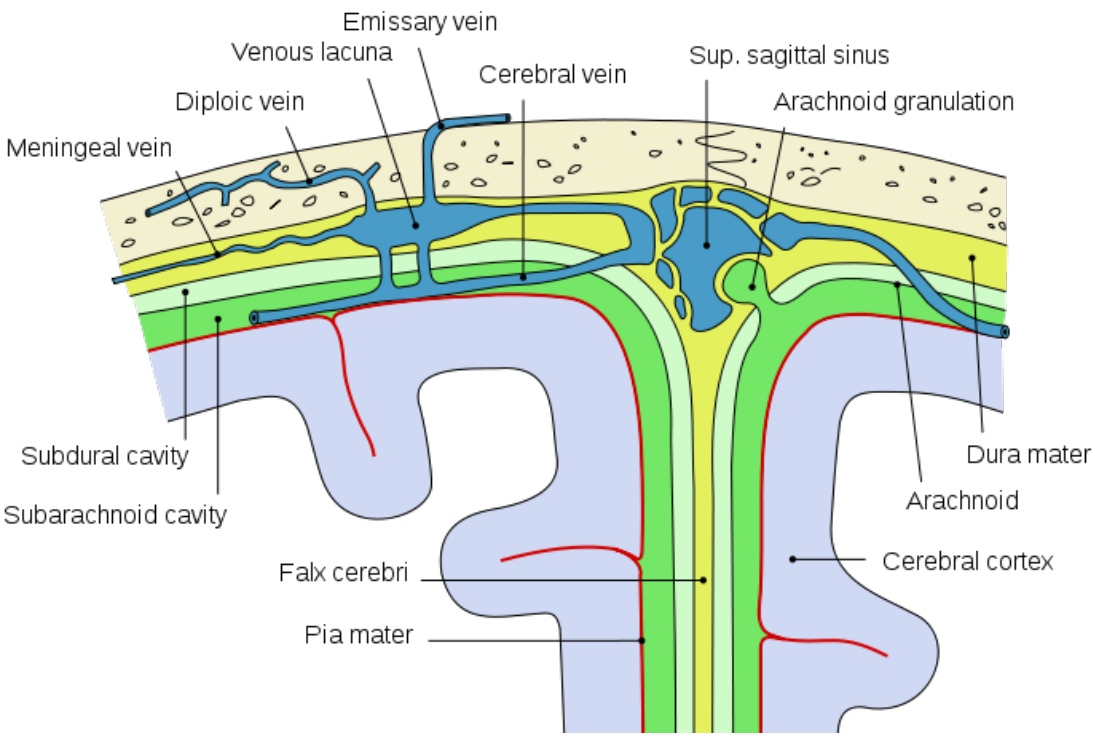


Figure 1.3: Arachnoid granulations of the meninges in a skull cross section.

From: Mysid 2010, Gray769-en: Diagrammatic of a section across the top of the skull, showing the membranes of the brain, etc., image, Retrieved March 21, 2014,from <http://en.wikipedia.org/wiki/File:Gray769-en.svg> Public domain

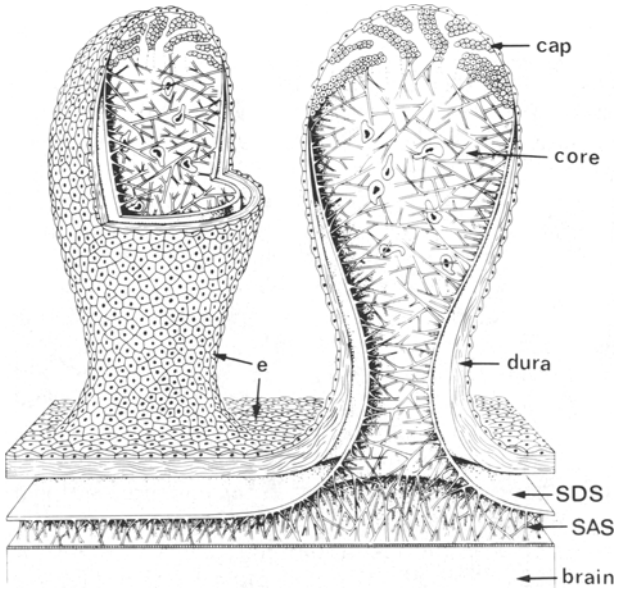


Figure 1.4: **Fine structure of arachnoid granulations of the meninges**. Showing the collagenous core, subarachnoid space (SAS), subdural space (SDS), and sinus endothelium, with CSF channels visible at the cap.

From: Upton M.L. and Weller R.O. The morphology of cerebrospinal fluid drainage pathways in human arachnoid granulations. J Neurosurg 63:867-875 (1985). Reprinted by permission of the publisher

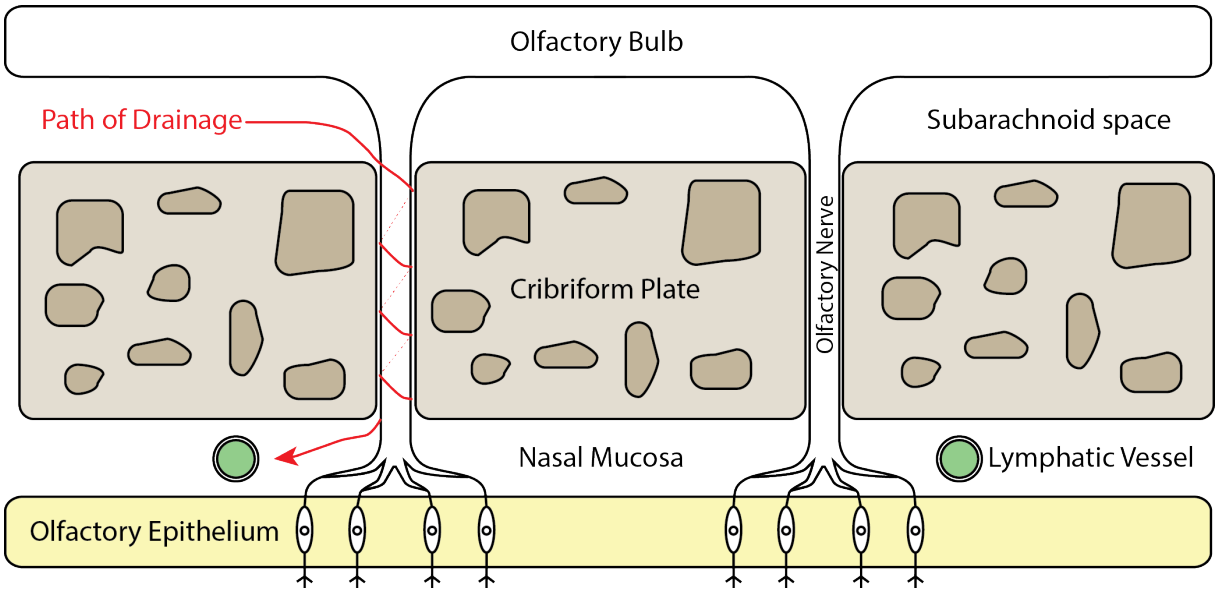


Figure 1.5: The olfactory route of CSF drainage into the peripheral lymphatic system. At the bones of the cribriform plate the route of drainage transitions from the meningeal subarachnoid space surrounding the olfactory nerve to the perineurial space of the nerve. The transition (indicated by dashed lines) occurs uniquely around the olfactory nerve as it traverses the cribriform plate. CSF disperses into the connective tissue at the nasal mucosa and drains into adjacent lymphatic vessels.

Adapted From: Walter B.A. The olfactory route for cerebrospinal fluid drainage into the peripheral lymphatic system. Neuropathology and Applied Neurobiology 32 :338–396 (2006)

I. INTRODUCTION

A. THE MYTH OF CNS IMMUNE PRIVILEGE

CHAPTER 2: THE BLOOD BRAIN BARRIER

The first hints as to the existence of the blood brain barrier came in 1885 when the bacteriologist Paul Ehrlich was experimenting with dye staining of animal tissues. When Ehrlich injected animals intravenously with dye, he observed robust staining in all tissues except the brain and spinal cord. At the time Ehrlich attributed the lack of dye uptake to an intrinsically low affinity of the dye to brain and spinal cord tissues, a theory that would be debunked ten years later by his student Edwin Goldman. Goldman essentially did the reverse experiment, injecting trypan blue dye directly into the animal brain. He observed that the only stained tissues were the brain and spinal cord, proving that there was a unique separation between the CNS and the rest of the body, and not as Ehrlich hypothesized an inability of CNS tissues to take up dye. Fine anatomical understanding of the blood brain barrier (BBB) came in 1967 when Reese and Karnovsky used electron microscopy to implicate tight junctions of the CNS vasculature as the functional unit that precludes entry of intravenously administered horse radish peroxidase[31].

The neurovascular unit

Biologically, the BBB is the collective system that achieves all of the barrier functions unique to the cerebral endothelium throughout the brain (the BBB is present not only in capillaries, but also in arteries, arterioles and veins of the CNS[32]. A less functional, and more discrete definition can be: the BBB *is* the specialized tight junctions of the CNS blood endothelium. Nevertheless, the BBB is a

creation of the CNS, and it would be impossible to think of the BBB outside the context of the underlying neural tissue (Figure 2.1).

At the heart of the BBB are specialized endothelial tight junctions whose central function is to create a barrier to the paracellular diffusion of polar compounds present in the circulation[33]. These junctions effectively block even the diffusion of small ions such as Na^+ and Cl^-, resulting in an electrical resistance across the endothelium that can be 500 times higher in the brain endothelium than in peripheral capillaries[33].

Tight junctions

Like the tight junctions of polarized epithelial cells throughout the body, those of the BBB are composed of a set of transmembrane claudins, occludins, junctional adhesions, as well as cytoplasmic scaffolding proteins such as zonula occludins (ZO) (Figure 2.2).

In 1993 occludin became the first tight junction protein to be described[34]. Structurally, occludin is a hydrophobic protein with four transmembrane domains, two extracellular loops and one intracellular loop (Figure 2.3). The extracellular loops interact with loops on adjacent cells to form the tight junction between cells while the N and C termini of occludin project into the cytoplasm, are highly post-transcriptionally modified[35], and directly interact with many downstream signaling molecules including c-Src, ERK1/2, PP2A, and PP1[36].

The second group of tight junctional proteins to be described was the claudins. Similar to occludins, claudins are hydrophobic proteins with four membrane spanning domains. There are 24 claudins described in humans and mice, with the endothelium in all tissues containing members of the claudin family. The tight junctions of the BBB are set apart from other tissues by the predominant expression of claudin-3, 5, and-12[37-39]. Deletion of claudin 5 results in loss of BBB integrity in mice[38],

whereas its overexpression enhances endothelial cell impermeability[40]. Claudin-3 is selectively lost during conditions of BBB compromise such as experimental autoimmune encephalomyelitis (EAE) and human glioblastoma multiforme[37].

Several cytoplasmic proteins are found in intimate association with tight junction complexes (Figure 2.3). Most salient are the intracellular zonula occludens, ZO-1, ZO-2, and ZO-3. ZO proteins interact with intracellular domains of occludin (via GUK domains[41]), claudins (via PDZ1 domains[42]) and JAMS (via PDZ3 domains[43]), and are essential for their assembly and tethering to the actin cytoskeleton[44]. Collectively the ZO proteins along with other accessory intracellular proteins including Par-3/6[45, 46], and heterotrimeric G-proteins (Gαi)[47, 48], create a complex intracellular platform essential to tight junction integrity, dynamism and signaling.

Astrocytes and the BBB

The tight junctions of the BBB are not static entities. Both their formation and homeostasis is dependent on the neural tissue that they vascularize. The roles of astrocytes (and to a lesser degree pericytes) in formation and maintenance of the BBB have been particularly well established. Culture of endothelial cells in astrocyte-conditioned media induced the formation of numerous, highly organized tight junctions resembling the in vivo BBB[49-51]. The role of astrocytes in the neurovascular unit and the complex cell signaling networks at the BBB are reviewed in NJ Abbot[52].

The circumventricular organs

Segregation of the CNS parenchyma from the periphery by the BBB protects the brain from constituents of the circulation. On the other hand a contiguous BBB effectively blindfolds the CNS parenchyma to the milieu of the blood, which contains valuable information about the state of the animal (e.g. cytokines, hormones) that can in turn be used by the brain for homeostatic regulation.

The central function of the circumventricular organs (CVO) is to reconcile these two competing interests.

The circumventricular organs are grouped into either the sensory CVOs: the subfornical organ, organum vasculosum of the lamina terminalis and the area postrema, or the secretory CVOs: the neurohypophysis, median eminence, intermediate lobe of the pituitary gland, and the pineal gland. Each organ is characterized by capillary endothelium that uniquely lack BBB structures, allowing them to constantly sample constituents of the circulation and relay that information to the relevant areas of the brain. Specifically, the CVOs are characterized by uniquely dense capillary networks with fenestrated endothelium, allowing for direct access of the neural tissue to large molecules flowing through the circulation[53].

The CVOs facilitate the brain's awareness of peripheral immune responses. The receptor for the pro-inflammatory IL-1β cytokine and the endotoxin binding CD14 receptor are present in all sensory CVOs[54-56], and peripheral administration of IL-1β and lipopolysaccharide (LPS) has been repeatedly shown to increase expression of the neuronal activity marker c-fos in the underlying brain tissue[57-59]. Sensing of IL-1β (as well as IL-6, TNF, and LPS) at the CVOs initiates signaling cascades within the brain parenchyma including activation of the hypothalamic-pituitary-adrenal (HPA)-axis and induction of the febrile response (reviewed by Siso[53] and Buller[60]). In-vitro, IL-1β has been shown to depolarize neurons in the SFO which send efferent axonal projections to numerous parenchymal regions including the hypothalamic autonomic control and neuroendocrine centers[61]. Nevertheless, the precise mechanism by which cytokine signals are transduced into action potentials that propagate along neuronal projections into the brain parenchyma are unclear[62]. In addition to their role in sensing of the immune milieu, the CVOs play key roles in many aspects of normal physiology including cardiovascular regulation, fluid balance, energy homeostasis and feeding[63, 64].

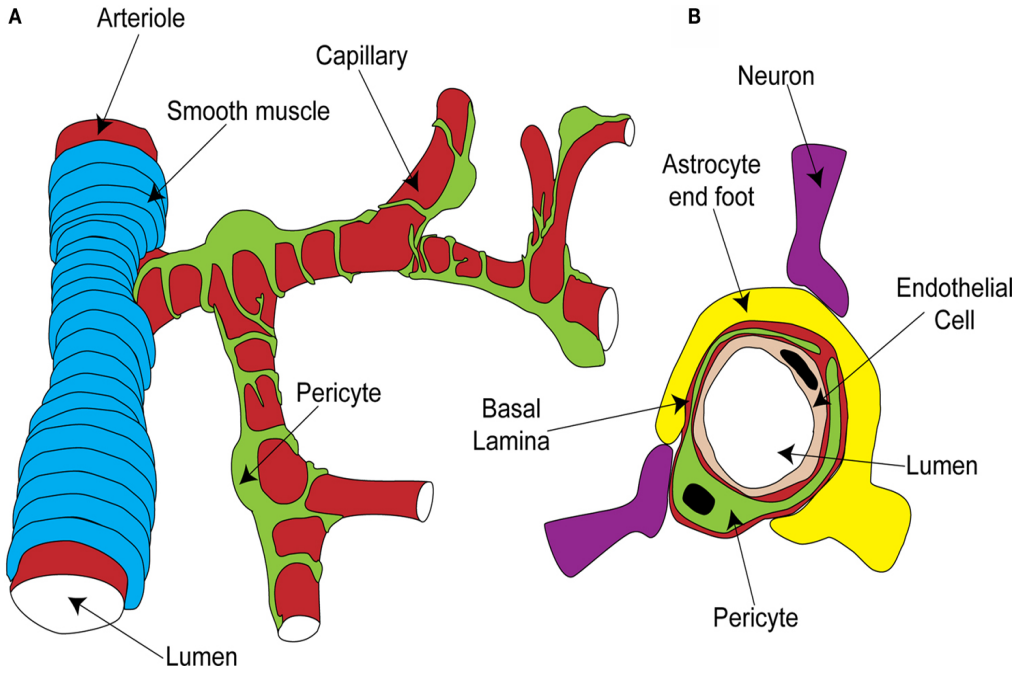


Figure 2.1: **Structure of the neurovascular unit**. It is helpful to envision the BBB in the functional context of the "neurovascular unit" comprised of brain endothelial vessels and the astrocytes, pericytes and neurons that surround it.

From Hamilton, N.B., Attwell, D. & Hall, C.N. Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease 2:5. Front Neuroenergetics (2010). Open Access.

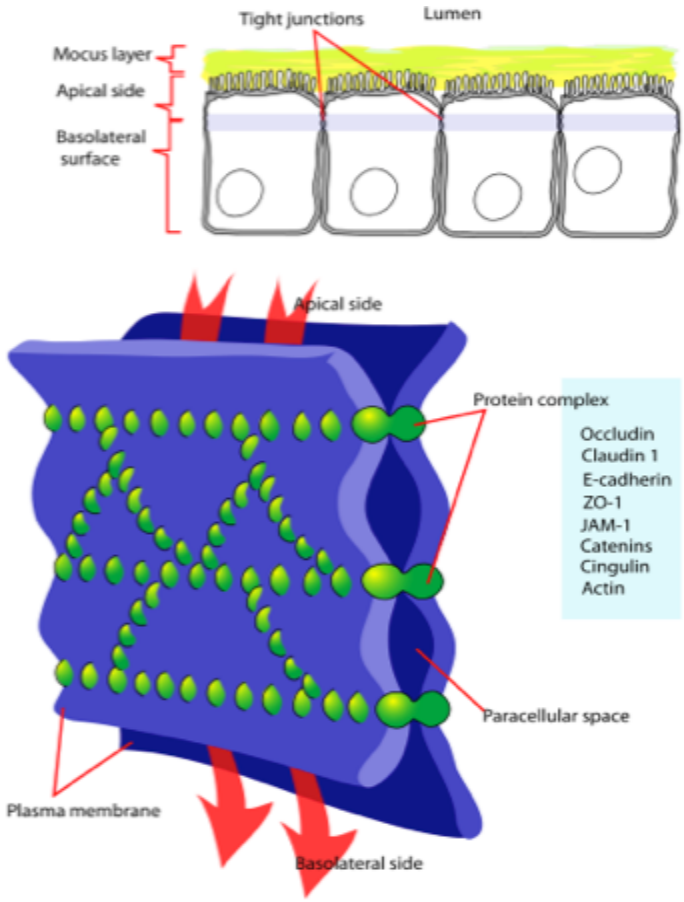


Figure 2.2: **Structure of epithelial tight junctions.**

From: Ruiz, M 2006 Cellular tight junction: diagram showing a tight junction, image, Retrieved March 21, 2014,from <http://en.wikipedia.org/wiki/File:Cellular_tight_junction-en.svg> Public domain.

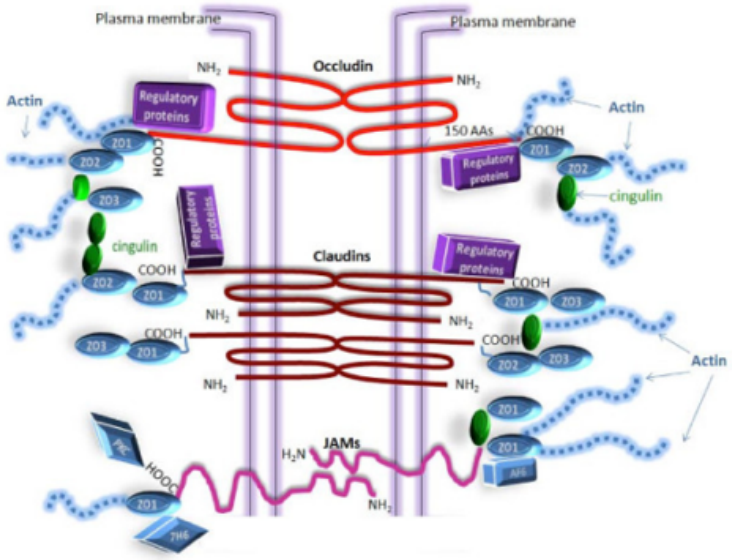


Figure 2.2: **Tight junctional proteins between adjacent endothelial cells.**

From: Redzic, Z. Molecular biology of the blood-brain and the blood-cerebrospinal fluid barriers: similarities and differences. Fluids Barriers CNS 8, 3 (2011). Biomed Central Open access

I. INTRODUCTION

A. THE MYTH OF CNS IMMUNE PRIVILEGE

CHAPTER 3: IMMUNE MOLECULES IN THE CNS

Biology is economical. At every level of organization, complex systems are built from surprisingly few starting pieces. So the involvement of immune molecules such as TNF in the action potentials of neurons[65, 66], or the complement protein C3 in synaptic pruning[67, 68], might not be so surprising after all. However, other immune genes that are important to CNS function/homeostasis (e.g. Rag2 and IL4, among others), are not expressed in the neuronal tissue, and their role in CNS function/homeostasis is attributed to loss of immune function. Here we review select examples of both the leukocyte independent re-use of "immune" proteins, as well as cases where loss of immune function leads to altered cellular and behavioral biology. The homeostatic interaction between T cells and the CNS will be more thoroughly reviewed in chapter 9.

MHC Class I: During critical periods of neural development, the neuronal circuitry is heavily edited, with synapses strengthened, weakened, or destroyed, on the basis of the animal's experiences[69]. In one such process, axonal projections originating from the visual fields of the right and left eye and terminating at the lateral geniculate nucleus (LGN) are initially intermixed. However as the eyes develop shortly after birth these projections begin to segregate in an experience dependent manner[70, 71]. To get at the mechanism of these changes in neural circuitry, Corriveau et al performed an unbiased screen of gene expression changes during this critical developmental period and unexpectedly identified changes in classical and non-classical major histocompatibility complex (MHC) I[72]. Later studies showed MHCI to be expressed on neurons and implicated them as part of the machinery

regulating synaptic remodeling[73-75]. Though much of the mechanism remains unresolved (including which of the 70+ MHCI family members are involved), the immune-independent role of MHCI in neurons has been nicely established[71]. Speculatively, the absence of MHCI-recognizing cell types (like NK cells and CD8 T cells) in the brain may very well be the factor that allows for the free licensing of MHCI in the CNS for completely novel function.

Rag1: The recombination activating gene (Rag)1 (involved in V(D)J recombination during T and B cell development) shares a narrative similar to MHCI. Once thought not to be expressed in the CNS, it was later identified in widespread regions of the embryonic and postnatal brain[76-78]. Rag1$^{-/-}$ mice exhibit several behavioral abnormalities including: increased exploratory behavior[79], reduced aversion towards the center of a brightly illuminated open field[79], increased marble burying behavior[80], and impaired social recognition memory. Our own experiments show significant differences in fear extinction between Rag1 deficient and WT mice (**Figure 3.1**). Importantly, these differences were not rescued by lymphocyte transfer in fear conditioning tests (**Figure 3.2**), and the phenotypes were not observed in Rag2 animals (**Figure 3.3**), again suggesting a lymphocyte independent, CNS-intrinsic requirement of Rag1 in fear extinction. On the other hand, Rag1, Rag2, and DNA-dependent protein kinase, catalytic subunit (Prkdc) knockout mice show defects in spatial learning and memory that *are* rescued by the adoptive transfer of wild-type lymphocytes. So at least in the case of spatial learning and memory, the requirement for the recombination machinery does appear to be lymphocyte dependent[2, 4, 81, 82].

Complement: C1q and C3 are members of the classical complement cascade. Like MHCI, both C1q and C3 are expressed in a punctuate pattern in the developing CNS. Expression of C1q in the developing visual system peaks during the period of activity-dependent remodeling before disappearing in the adult[67]. Together, C1q and C3 tag unwanted synapses for engulfment and

elimination by microglia[68], a process reminiscent of complement mediated clearance in the periphery[83, 84].

A growing list of cytokines is finding non-immune functionality in the developing CNS. While mRNA of many cytokines are expressed in the developing CNS[85, 86], and many more affect proliferation, survival and electrophysiology of neuronal cultures[87], only a small number of cytokines have been functionally characterized in vivo.

TNF: Tumor necrosis factor alpha (TNFα) is another immune cytokine with multiple functions in the CNS. Signaling through the TNFR1 receptor modulates synaptic strength by changing the expression of postsynaptic AMPA receptors (AMPAR)[65]. TNFR1-knockout neurons have markedly reduced AMPAR expression in vitro and in vivo, and exhibit reduced postsynaptic transmission[66].

IL-1β: Aside from its role in the febrile response at the circumventricular organs (reviewed by Siso[53] and Buller[60]), the classic immune cytokine IL-1β is expressed in the CNS by both neurons and glia[88]. IL-1β is up-regulated during long-term potentiation (LTP), both in hippocampal slice cultures and in vivo[89, 90], while application of the IL-1β receptor antagonist, IL-1Ra, has been shown to lead to un-sustained LTP[90]. Moreover, IL-1 receptor type I knockout[91], and overexpressing[92] mice, manifest cognitive deficits, though the mechanisms behind their impairment remain unclear.

TGF-β: transcripts and protein for the immune cytokine TGF-β, as well as its receptors are expressed throughout neural development[93, 94]. Both TGF-β1 and TGF-β2 ligands promote the elongation of neurites in vitro[95], and TGF-β signaling has been shown to regulate axonal development in Drosophila[96]. Furthermore, several human neurodevelopmental disorders have been linked with TGF-β receptors and their downstream signaling complexes, including holoprosencephaly in which the forebrain fails to segregate into two hemispheres[97], and in a familial syndrome of altered

neurocognitive and craniofacial development[98]. More recently, gain-of-function and loss-of-function experiments by Yi et al have shown that the level of TGF-β receptor activity in young neurons dictates axon number, and that TGF-β is sufficient to initiate the differentiation and outgrowth of axons in vivo[99].

IL-4: IL-4, another classic immune cytokine, has been shown to be beneficial in models of learning behavior. IL-4 deficient mice had dramatically impaired learning and memory in both the Barnes and Morris water mazes (MWM)[100]. Importantly, the phenotype was significantly rescued by the transfer of wild-type T cells, and learning deficits in SCID mice were rescued upon reconstitution with wild-type, but not IL-4 deficient, T cells. Lastly, irradiated wild-type mice transplanted with IL-$4^{-/-}$ but not wild-type bone marrow showed impaired MWM performance[100]. Mechanistically, astrocytes of IL-4 deficient mice are attenuated in their production of brain-derived neurotropic factor (BDNF) following MWM training. BDNF has been shown to be vital in learning and long-term memory, supporting the survival, growth, and differentiation, of neurons and synapses[101]. The same study shows that the meningeal myeloid cells of SCID mice have a characteristic pro-inflammatory skew characterized by high intracellular TNF and IL-12, with transfer of wild-type but not IL-4 deficient T cells ameliorating their proinflammatory signature[100]. While these experiments have demonstrated that T cell derived IL-4 appears to be critical in mediating normal MWM behavior, the mechanisms behind the phenomenon remain unknown. A working model of IL-4 function in learning and memory posits that MWM training induces IL-4 secretion by meningeal T cells, which in turn may skew meningeal macrophages towards an M2 phenotype that has been shown to reverse cognitive impairment in SCID mice (see Chapter X). While in-vitro treatment of astrocyte cultures with IL-4 resulted in significantly increase in BDNF mRNA expression[100], the mechanism by which IL-4 might cross the BBB to upregulate BDNF expression in vivo is unknown[102].

Concluding Remarks

The growing list of immune molecules that are expressed in the developing and adult CNS suggests that immune molecule usage in the CNS may be the norm and not the exception. While this chapter highlights a handful of immune molecules associated with CNS function, the vast majority of immune molecules expressed in the developing and adult CNS, as well as those with in vitro effects on neural cultures, remain to be associated with their in vivo CNS functions. Nevertheless, it is clear that while some immune molecules are directly expressed in the brain and serve non-immune functions, others are involved in immune cell-CNS interactions, and at least in the case of IL1- β and Rag1, both immune dependent and independent functions appear to be at play.

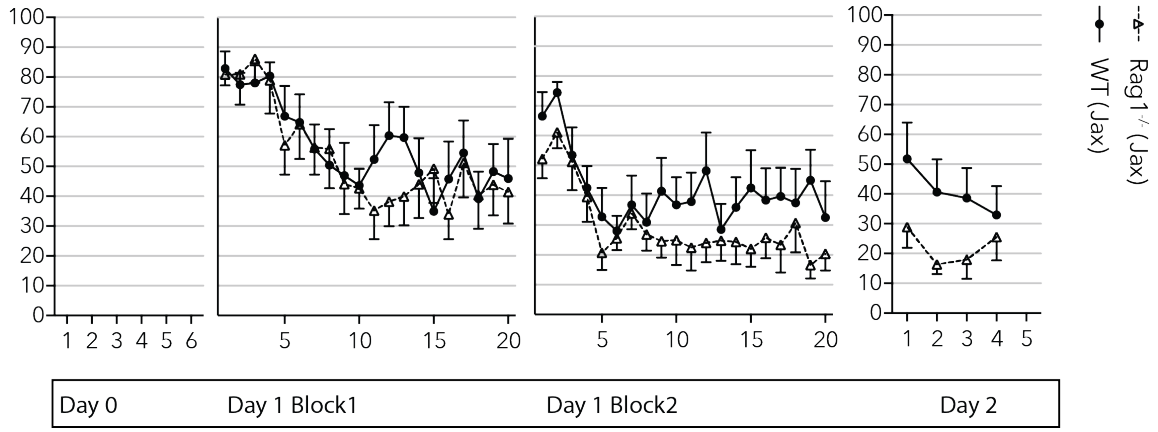


Figure 3.1: Heightened fear extinction in Rag1 deficient mice. Wild-type vs. Rag1$^{-/-}$. Percent of 20s tone mice spent freezing. Day 0; Six tone-shock pairings separated by 100s rest intervals, Day 1; two blocks of 20s tones only, without shock, separated by 100s intervals, Day 2; five 20s tones only (without shock), separated by 100s intervals. (n = 8mice/group $P < 0.05$, Repeated measures ANOVA with Bonferonni post hoc analysis).

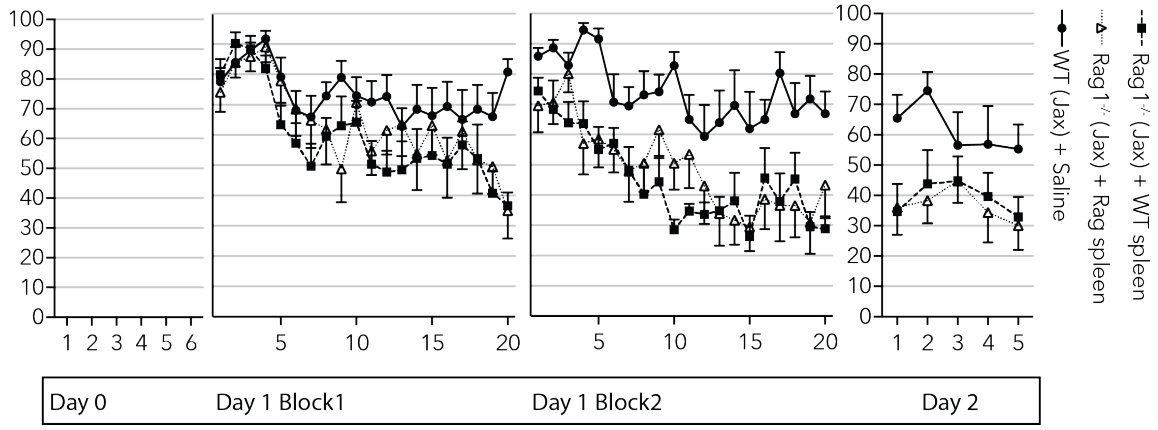


Figure 3.2: No rescue of heightened fear extinction in Rag1 deficient mice after splenocyte adoptive transfer. Wild-type vs. Rag1$^{-/-}$ three weeks reconstituted with Rag1$^{-/-}$ or wild-type splenocytes. Percent of 20s tone mice spent freezing. Day 0; Six tone-shock pairings separated by 100s rest intervals, Day 1; two blocks of 20s tones only, without shock, separated by 100s intervals, Day 2; five 20s tones only (without shock), separated by 100s intervals. (n = 8mice/group WT(Jax)+ Saline v.s. both Rag1$^{-/-}$ groups $P < 0.05$, Rag1$^{-/-}$(Jax)+Rag spleen v.s. Rag1$^{-/-}$(Jax) WT spleen n.s. Repeated measures ANOVA with Bonferonni post hoc analysis).

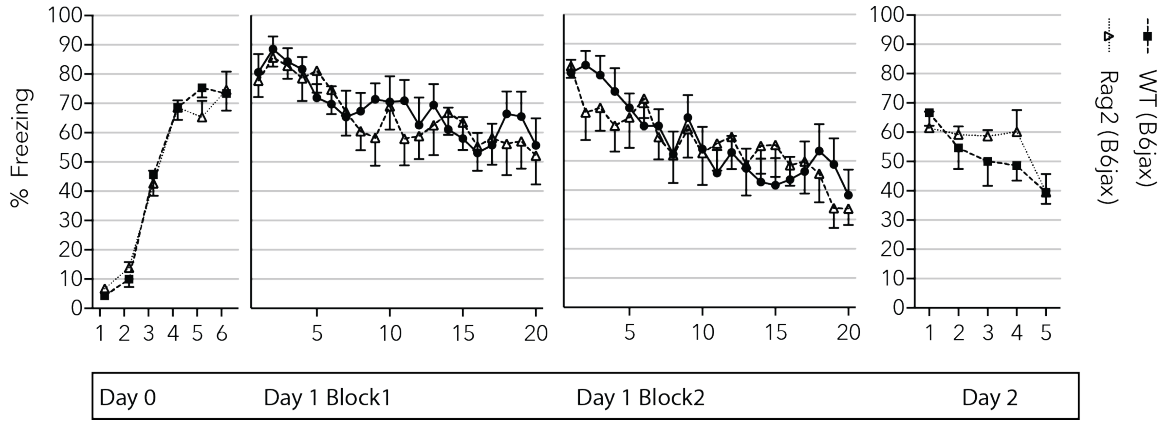


Figure 3.3: Normal fear extinction in Rag2 deficient mice. Wild-type vs. Rag2$^{-/-}$. Percent of 20s tone mice spent freezing. Day 0; Six tone-shock pairings separated by 100s rest intervals, Day 1; two blocks of 20s tones only, without shock, separated by 100s intervals, Day 2; five 20s tones only (without shock), separated by 100s intervals. (n = 8mice/group n.s. Repeated measures ANOVA with Bonferonni post hoc analysis).

I. INTRODUCTION

B. T CELL ANTIGEN SPECIFICITY, HOMEOSTATIC EXPANSION AND PERIPHERAL TRAFFICKING

As the original work of this thesis investigates the influence of T cells on higher brain function, Chapters 4-6 aim to provide the relevant background in T cell biology. One of the central questions of this thesis is the antigen specificity of the T cells that influence higher brain functions. To this end, Chapter 4 introduces the T cell receptor (TCR) and the developmental process by which it is generated and refined. The chapter concludes with discussion of one of the central tools of our experimentation, the TCR transgenic mouse.

Another methodology we utilize throughout the experiments of Chapter 10 and 11 is the adoptive transfer of T cells into lymphopenic, or clonally restricted recipients. Chapter 5 reviews the phenomenon of homeostatic expansion, the proliferative process by which T cells expand to fill the empty T cell niche in lymphopenic animals. This chapter attempts to show the many ways in which the T cell reconstituted lymphopenic mouse is different from its wild-type counterpart, a distinction which we believe has evaded the field and confounded interpretations of schemes in which learning and memory function is rescued by T cell adoptive transfer.

Finally, Chapter 6 provides the relevant background in T cell trafficking and turnover in the peripheral organs. We survey the basic tenants of T cell trafficking in the lymphoid and non-lymphoid tissues to set up the framework for the experiments presented in Chapter 10, which investigate T cell dynamics in the meninges and their connectivity to the deep cervical lymph nodes.

I. INTRODUCTION

B. T CELL ANTIGEN SPECIFICITY, HOMEOSTATIC EXPANSION AND PERIPHERAL TRAFFICKING

CHAPTER 4: THE T CELL RECEPTOR

It has been estimated that there are greater than 10^{15} different possible T cell receptors (TCRs) in the mouse[103], of which around $2x10^6$ are realized in the uninfected mouse spleen alone[104]. This staggering diversity confounds one of the immediate questions to any T cell dependent phenomena – what is the antigenic specificity of the T cells involved? This chapter briefly reviews the generation and shaping of the T cell repertoire in the thymus and periphery and introduces the TCR transgenic mouse as a tool to address the question of antigen specificity of T cell dependent functions.

The TCR binds peptide-MHC complex

It had long been shown that cytotoxic T lymphocytes (CTLs) could bind and lyse infected cells. But it was unclear why these CTLs could not bind free viral particles. Seminal experiments by R.M Zinkernagel and P.C. Doherty in 1974 showed that CTLs recognize viral antigens only in the context of self MHC)[105, 106]. This milestone was followed by isolation of the TCR (now understood to be a heterodimer composed of an alpha and beta chain), and by back-to-back papers in 1984, reporting the cloning and discovery of the TCR genes[107, 108].

Diversity through VDJ recombination

The makeup of the TCR genes explains the staggering diversity of T cell antigen specificity. Deciphering the mechanism of germ-line TCR DNA rearrangement was aided by the discovery of

analogous immunoglobulin gene rearrangement in 1976 by Hozumi and Tonegawa[109]. Briefly, functional V,D, and J gene segments within the unrearranged TCR gene are flanked by 12 or 23 bp recombination signal sequences (RSS) (Figure 4.1). In pre-T cells recombination-activating genes Rag1 and Rag2, upon recognition of RSS, catalyze the joining of V-to-J (alpha chain) and V-to-D-to-J (Beta chain). Diversity of the TCR is generated not only by combinatorial joining of V-D-J segments, but is also further diversified by P and N-region nucleotide addition as well as junctional flexibility. The TCR (now complete with alpha and beta constant regions encoding the non-variable structural domain of the TCR) is transcribed, coupled with CD3, and displayed on the cell surface. Importantly, loss of function mutations in Rag1, Rag2, or any part of the recombination machinery, including Prkdc (the mutation in SCID mice), yield animals that lack any functional T and B cells. The detailed mechanism of TCR gene rearrangement has been extensively reviewed[110-112].

Thymic selection

Selection events in the thymus represent the first safeguard in shaping the peripheral T cell repertoire. T cells are first positively selected when their TCRs bind peptide-MHC complex on cortical epithelial cells of the thymus, in an interaction that is stabilized by accessory molecules and co-receptors on both cell types. It is at this point that the T cell differentiates into either $CD4^+$ (stabilizing the TCR-MHCII-peptide complex) or $CD8^+$ (stabilizing the TCR-MHCI-peptide complex) T cells. T cells bearing rearranged TCRs that fail to bind MHC, or that bind MHC with too high an affinity, are deleted at this stage via apoptosis.

The surviving population of thymocytes now contains T cells reactive against a wide array of self-antigens. Those T cells with high affinity to the self-MHC complexes of thymic stromal cells and APCs are deleted in a process of negative selection. The result is an emigrant population of naïve T cells that has weak or intermediate TCR peptide-MHC interactions[113] (Figure 4.2).

Recently, the scope of thymic selection has been expanded to include deletion of cells that respond to tissue restricted antigens that are presented on MHCII by an AIRE dependent mechanism[114]. Autoimmune regulator (AIRE) knockout mice on the NOD mouse background manifest with autoimmune disease similar to human autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), highlighting the function of AIRE in tolerizing the peripheral T cell repertoire[115]. However, AIRE mediates the ectopic expression of only a small fraction of tissue restricted antigens, and autoreactive T cells exit the thymus in large numbers[116, 117].

Maintaining tolerance in the periphery

Importantly, T cell selection events do not end in the thymus, and the T cell repertoire continues to be shaped by mechanisms operating in the periphery. The post thymic selection events have come to be collectively known as peripheral tolerance, and are ongoing throughout life. Indeed, changes in tissue self-antigen expression that accompany events such as puberty or menopause may suggest that peripheral tolerization is by necessity an ongoing process. Patients who undergo vasectomy see a rise in anti-spermatazoan antibodies, and vasectomy reversal leads to autoimmunity against the testis and fibrosis of the tissue, again suggesting that peripheral tolerance operates to continuously educate the peripheral T cell compartment[118].

The role of regulatory T cells (Tregs) has been well established in maintaining peripheral tolerance. Importantly, dendritic cells that present self-antigen have been found to play a crucial role in tolerance by perpetually maintaining the suppressive phenotype and Foxp3 expression of regulatory T cells. Notably, dendritic cells also control the fate of non Tregs, and are believed to be the central player in mediating peripheral tolerance by presenting self-antigens and inducing deletion or anergy of autoreactive T cells in the periphery[117].

Autoreactive T cell deletion in the periphery arises when T cells chronically engaged by self-peptide MHC complexes apoptose as a consequence of Fas engagement and Bim activation[119, 120]. Peripheral deletion is thought to be particularly relevant for TCR MHC interaction of high avidity, whereas lower avidity interactions between peptide-MHC and TCR (presented in an anti-inflammatory context) are thought to favor anergy[117]. Deletion has been shown to occur via extrathymic expression of peripheral tissue antigens, which appears to operate through both AIRE dependent[121] and independent[122, 123] mechanisms.

Anergy arises when T cells receive antigenic stimulation in the absence of proinflammatory co-signals, rendering them inert to a subsequent challenge. Mechanistically, anergy is thought to arise when the NFAT transcription factor is activated in the absence of AP-1 activity, setting off a cascade of events (mediated in part by Cbl-b, Itch, GRAIL, Caspase 3, and DGKα) that inactivates downstream signaling components of CD28 and the TCR[124]. Additionally, TCR activation in the absence of co-stimulatory signals is thought to silence gene loci that are needed for robust T-cell activation in a mechanism that implicates Egr2, Egr3 and CREM activity[124]. Lastly, anergic T cells are characterized by high surface expression of CTLA4 and PD1, which have structural homology to CD28 but provide an inhibitory signal upon their engagement[125].

The TCR Transgenic mouse model: a tool for studying antigen specificity

The staggering clonal diversity of T cells in the animal significantly confounds efforts at elucidating many immunological phenomena. This dilemma led to the development of TCR transgenic mice. Reducing the total clonal diversity of the T cell population from 10^{13} to just a single clone enables the focused study of antigen specificity. TCR transgenic animals have generally been made by microinjection of transgenic constructs containing a T cell hybridoma-derived alpha and beta

TCR chain along with an endogenous promoter – though more recent methodologies make generation of TCR transgenic mice a more efficient process[126, 127].

The first TCR transgenic mouse was directed against the male H-Y antigen in the context of class I H-2D^{b} [128]. Other TCR transgenic models soon followed, including the OTII TCR transgenic mouse with T cells directed against residues 323-339 of irrelevant chicken egg ovalbumin antigen in the context of Class II I-A^{2} [129]. While the OTII mouse has become a standard immunological tool, there is an important caveat to the model that contributes to a loss of true monoclonality. In models bearing specific TCR-β chains (including Vβ5 of OTII mice), a process of extrathymic Rag re-expression and fresh VDJ recombination as well as intra-thymic receptor editing occurs at low frequency, so that by 10 weeks of age 10–15% of the formerly monoclonal TCR repertoire now bear an additional set of endogenous TCRs[130, 131]. In contrast, OTII.Rag1$^{-/-}$ mice (lacking endogenous TCR rearrangement owing to Rag1 ablation) remain purely monoclonal, with near-perfect 1:1 surface expression of transgenic Vα2/Vβ5 TCR chains. Importantly, the existence of T cell populations bearing endogenous TCRs in TCR transgenic mice is not unique to the OTII model. Indeed, endogenous populations have been characterized in several TCR models[132-135], though they appear to arise by different mechanisms.

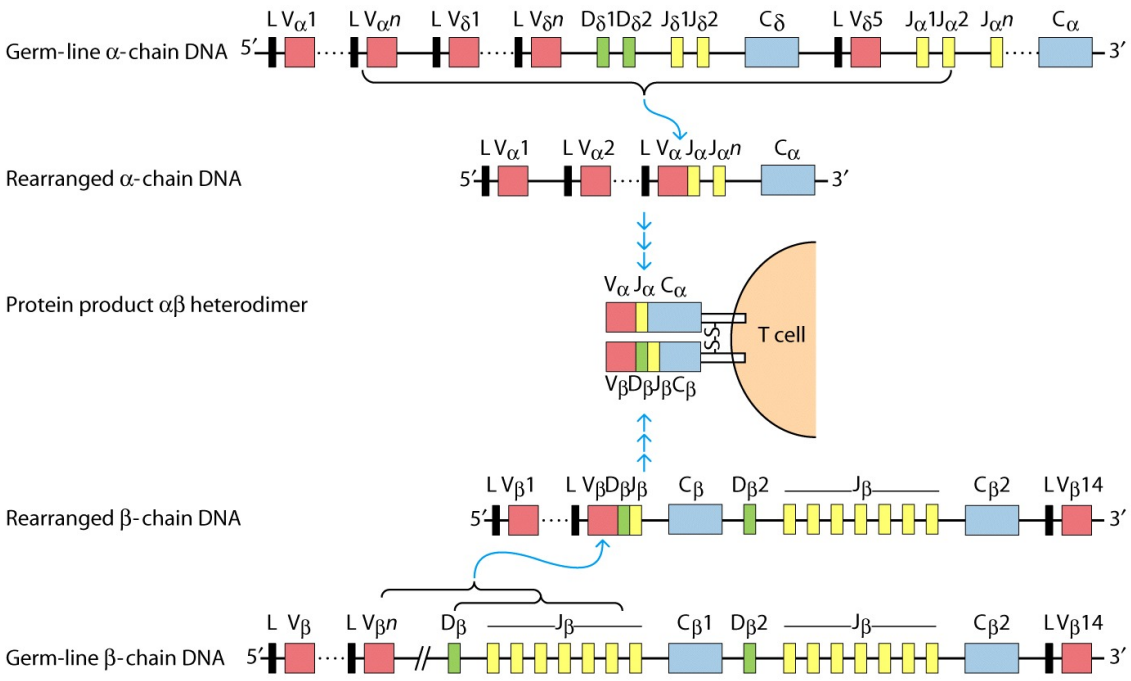


Figure 4.1: Example of gene rearrangements that yield a functional gene encoding the αβ T cell **receptor**.

From Kint T. Goldsby R. and Osborn B. Kuby Immunology 6th edition. New York: W.H. Freeman and Company, 2007. Reprinted by permission of the publisher

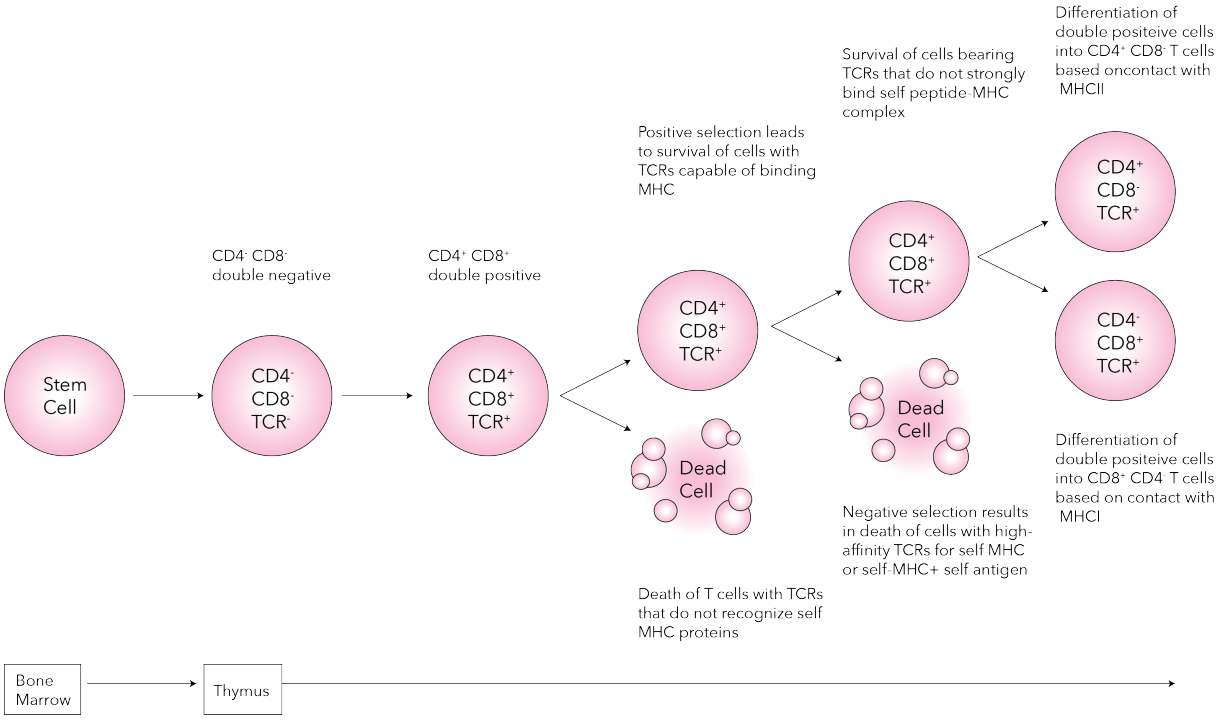


Figure 4.2: ***Stages of T cell development.*** T cell precursors arrive from the bone marrow and enter the thymus, where they undergo the diagramed sequential stages of development to become either CD4 or CD8 T cells.

I. INTRODUCTION

B. T CELL ANTIGEN SPECIFICITY, HOMEOSTATIC EXPANSION AND PERIPHERAL TRAFFICKING

CHAPTER 5: HOMEOSTATIC EXPANSION OF T CELLS IN THE LYMPHOPENIC HOST

Maintaining the proper size of the T cell pool is a crucial point of immune homeostasis. To detect and defend against pathogens, the system must achieve maximal T cell number and diversity, and must do so within the restrictions of physical space. Moreover, the system must be able to accommodate dramatic swings of T cell expansion in response to infection, and to later contract the same pools, which would otherwise usurp the limited space of the T cell niche.

The swells of dynamic T cell expansion and contraction as a result of immune challenge have been extensively reviewed[136-139]. Here we focus on the extreme instances of T cell contraction and expansion. Specifically, in this chapter we explore natural and experimental instances of lymphopenia (the condition of abnormally low lymphocyte levels), and explore the homeostatic driving force that seeks to restore T cell number and clonal diversity.

Setting up the normal T cell pool.

The thymus begins to deliver T cells to the body in utero. In mice, thymic output (and thymic size) peaks at 6-weeks of age, after which output gradually decreases[140, 141]. The initial rise in thymic output represents the need to fill the empty T cell niches of the body, while the gradual decline represents a shift from supplying the T cell pool, to maintaining it. Later in life, replenishment of the

systemic T cell pool comes not only from recent emigrants of the aged thymus, but also from the division of naïve T cells in the periphery[140, 142]. Interestingly, T cell maintenance in humans relies more heavily on peripheral T cell expansion, while recent thymic emigrants make the greater contribution in mice[140, 142, 143].

Tonic TCR-MHC signaling is required for long-term survival of Naïve T cells

It is largely accepted that both naïve CD4[144] and naïve CD8[145] T cells require tonic MHC-peptide stimulation for long-term survival in the periphery. Wild-type fetal thymi grafted into $Rag2^{-/-}$ recipients rapidly fill the lymphopenic niche, whereas transfer into $Rag2^{-/-}$ /$MHCII^{-/-}$ double knockouts, results in naïve T cells that gradually decline in number over a six-month period[144]. Similarly, experiments in which the TCR is inducibly ablated on mature naïve T cells, resulted in T cell death comparable in kinetics to loss of MHC contact[146, 147]. Subsequent studies have again affirmed the need for self-recognition by TCR for the long-term survival of naïve T cells, and have identified lck and fyn, CD4/CD8 as key signals in naïve T cell survival[148-151].

TCR specificity and homeostatic expansion in the lymphopenic host.

Many of the experiments described above relied on the adoptive transfer of monoclonal T cell populations into lymphopenic hosts. Crucially, the proliferative outcome of these systems is dependent on the specific TCR carried by the T cell used in the transfer. Whereas H-Y[145] and OTII[152] TCR transgenic T cells failed to undergo homeostatic expansion, most other TCR transgenic T cells including P-14[153], 1H3.1[154], DO11.10[155], 318[156], OT-1[157-160], A18[161], and 2C[162] readily proliferated in their respective lymphopenic hosts. This seeming discrepancy suggests that the requirement for MHC-TCR signaling for a monoclonal T cell to undergo homeostatic proliferation may in some instances be distinct from that required for its positive selection in the thymus[163], and that the homeostatic proliferation of T cells in the lymphopenic host is specific to the TCR in question.

Bender et al found that homeostatic proliferation of CD4 T cells in irradiated hosts was MHCII dependent, but that when the peptide presented by host MHC II was restricted to the peptide responsible for positive and negative selection in the thymus, homeostatic proliferation of transferred T cells did not occur, concluding that "peptides distinct from those involved in thymic selection control CD4+ T cell expansion in T cell-deficient mice"[163]. However, unlike reconstitution with monoclonal T cells, transfer of polyclonal T cells into lymphopenic hosts always results in robust homeostatic proliferation[152, 154, 156, 157, 160, 161, 163-166].

Phenotypic and functional changes in the T cell repertoire: The T cells seeded into a lymphopenic host are not the same as those recovered from it

While T cell reconstitution of a lymphopenic host can be sufficient to restore the size of the wild-type T cell pool[167], the T cells that repopulate the animal are strikingly different than the transferred population from which they were derived. Many studies report phenotypic conversion of naïve T cells into "memory-like" state, secreting cytokine upon stimulation and up-regulating CD44 and Ly6C memory markers[153, 158, 159, 162]. Moreover, the expanding $CD44^{+}$ T cell pool appears to be phenotypically skewed towards the Th2 phenotype[168]. Milner and colleagues found that $Rag2^{-/-}$ mice developed eosinophilic disease and a strong Th2 bias when they were seeded with low numbers of T cells ($3x10^{4}$). While disease severity was mitigated by transfer of a larger ($2x10^{6}$) inoculum, both groups showed an intrinsic Th2 skew characterized as IL-4, IL-5 and IL-13 production[168].

The TCR repertoire of expanding T cells is also dramatically skewed. Indeed, given the aforementioned homeostatic expansion of some TCR specificities, and not others, as well as the importance of tonic signaling in T cell survival, it may not be surprising that the TCR repertoire is significantly changed and restricted by the homeostatic expansion process[164-166, 169, 170]. The resulting T cell repertoire following T cell reconstitution of lymphopenic mice appears to be altered in function as

well. CD8 T cells undergoing homeostatic proliferation in irradiated mice show enhanced anti-tumor specificity and effector function, against both challenged and established melanoma or colon carcinomas[171, 172]. In fact, studies suggest the possibility of a lymphopenic "window of opportunity" for intervention with anti-tumor immunity following the lymphopenia induced by radio and chemo therapy. As might be expected in any system that promotes anti-tumor immunity, the homeostatic expansion of T cells in the lymphopenic host has been repeatedly associated with an increased susceptibility to autoimmune disease[173-176]. It is likely that the increased T cell self-reactivity and increased susceptibility to autoimmunity in reconstituted lymphopenic mice is a consequence of both the acquisition of memory/effector phenotype [153, 158, 159, 162], as well as the favored expansion of T cells with higher affinity for self-antigens[150, 155, 177].

Space and clonal abundance regulate homeostatic proliferation

A central driving force behind homeostatic expansion is the availability of free space. Indeed, in all the aforementioned studies homeostatic expansion is dependent on the prior absence, ablation, or reduction of T cells. Numerous studies show that cellular divisions in homeostatic expansion can be increased by increasing the degree of lymphopenia in the host, or by decreasing the number of transferred polyclonal T cells[152, 167, 178, 179].

The incredible awareness of the immune system of its own condition does not stop at space and T cell numbers. The system can sense and tune its own clonal diversity. TCR transgenic animals with monoclonal or near-monoclonal T cell repertoires are not lymphopenic per-se. Indeed, the same sensing of space ensures that these monoclonal T cells fill and occupy the entire space of the T cell niche. However, when polyclonal T cells are transferred into these clonally restricted TCR transgenic hosts, the transferred T cells undergo homeostatic expansion at the expense of the transgenic T cell pool. Specifically, naïve polyclonal T cells undergo a similar homeostatic expansion whether

transferred into Rag2$^{-/-}$ or TCR Tg Rag2$^{-/-}$ hosts[155]. A similar homeostatic proliferation, following the transfer of polyclonal T cells into four separate TCR transgenic hosts, led Min et al to call TCR transgenic mice "functionally lymphopenic" [180].

In a subsequent study Jenkins and colleagues arrived at a half-life of 50 days for 1x10^6 polyclonal T cells transferred into wild-type hosts. When the same numbers of monoclonal T cells from D011.10 mice were transferred, the half-life dropped to just 12 days. However when the number of transferred D011.10 T cell was reduced to just 1000 cells, the half-life rose to that of wild-type polyclonal T cells, again suggesting that low clonal abundance favors T cell survival[181].

Regulatory T cells do not block homeostatic proliferation.

Regulatory T cells (whose central function is to inhibit the activation and expansion of self-reactive T cells) create a dilemma in homeostatic proliferation. As the homeostatically proliferating T cell pool is expanding on self-antigens (often distinct from those mediating thymic selection[163]), the functional presence of regulatory T cells would in theory inhibit, or altogether preclude, homeostatic proliferation. On the other hand, if Tregs are functionally absent, the animal would succumb to systemic autoimmunity. Several studies have not only affirmed that Tregs are part of the homeostatically expanding T cell pool, but that the Treg/non-Treg ratio remains normal and may even be slightly elevated[182, 183].

To address the functionality of Tregs during homeostatic expansion, Annacker et al examined the expansion of naïve T cells in Rag1$^{-/-}$ recipients with, or without, the co-transfer of purified CD4$^+$ CD25$^+$ Tregs. Even at a 1:1 ratio of Tregs:Tnaïve cells, Tregs did surprisingly little to stem homeostatic proliferation of the naïve T cells, with CFSE dilution in the naïve pool being only marginally effected by a 1:1 presence of Tregs[184]. While Tregs did not impede homeostatic proliferation, they were necessary and sufficient to prevent autoimmunity; leading to the view that

control of homeostatic expansion and prevention of autoimmunity are independent Treg processes[184, 185].

Homeostatic proliferation induces MDSCs

While expansion of T cells in the lymphopenic host is a homeostatic mechanism aimed at reconstituting the T cell repertoire, it is a process that by its very nature is autoimmunogenic. As referenced previously, the resulting T cell pool acquires an effector/memory phenotype directed against self-antigens, and reconstituted mice are understandably more prone to autoimmune sequelae[173-176, 186]. Furthermore, regulatory T cells may be temporarily suspended during these periods to allow for homeostatic reconstitution, further increasing the risk of autoimmunity. Nevertheless, in spite of rapid self-reactive T cell proliferation and Treg abeyance, most reconstituted lymphopenic hosts do not manifest with overt autoimmunity[174], suggesting that other regulatory mechanisms are afoot.

Myeloid derived suppressor cells (MDSCs) are best characterized in cancer where they have repeatedly been shown to suppress anti-tumor immunity. Tumor infiltrating MDSCs are highly correlated with poor prognosis, while their depletion results in enhanced anti-tumor immunity[187]. MDSC are a heterogeneous population phenotypically defined as $CD11b^+$ $Gr1^+$, and are functionally defined in their ability to suppress T cell activation and proliferation.

As might be expected given the broadly reciprocal relationship between autoimmunity and cancer, MDSCs are thought to be beneficial in the context of preventing autoimmunity. $CD11b^+$ $GR1^+$ MDSCs have been described in numerous models of autoimmunity (reviewed by Cripps[188] and Nicholson[189]). Accumulation of granulocytic MDSC was observed to precede periods of EAE disease remission, and transfer of MDSCs ameliorated EAE etiology, decreasing demyelination, and inhibition of encephalitogenic T cell responses[190]. A protective role of MDSCs was also demonstrated

in a model of collagen induced arthritis in which MDSCs accumulated during peak disease severity[191]. Similar to EAE, adoptive transfer of MDSCs reduced the severity of collagen induced arthritis in vivo, whereas MDSC depletion was found to abrogate spontaneous disease resolution[191]. Finally a similar protective function of MDSCs in autoimmunity has been demonstrated in a model of type 1 diabetes[192]. Furthermore, MDSC populations have been shown to expand specifically as a consequence of total body irradiation-induced lymphopenia[193].

MDSCs can be functionally characterized by their ability to block the proliferation of T cells in co-culture, with increased MDSC:T cell ratio yielding increased suppression. Remarkably, when MDSC are derived from naïve mice that underwent total body irradiation 21 days prior, they failed to suppress T cell proliferation at any ratio, perhaps suggesting that like Tregs, MDSCs can be fine-tuned to allow for homeostatic proliferation while curtailing cytokine production[193] (though this later point has not been tested).

Though much work remains to be done, the aforementioned studies collectively establish a potent suppressive function of MDSCs in T cell mediated autoimmunity. While MDSCs remain best appreciated in their suppression of anti-tumor immunity, tumors do not invent cell-types de novo, but instead recruit, induce, and exploit existing cell types. Indeed, tumors may be exploiting a normal protective function of MDSCs – tempering the autoimmunity that occurs with homeostatic T cell proliferation under physiological conditions of lymphopenia such as viral infection[194-198].

Other key regulators of homeostatic proliferation

Along with TCR-MHC interactions, several other mediators of homeostatic T cell expansion have been identified. Not surprisingly, cytokines play a key role in both survival and homeostatic expansion of T cells. While many cytokines are involved in T-cell homeostasis (reviewed by Takada[199] and Boyman[200]), IL-7 appears to play a crucial, non-redundant role in the homeostatic expansion of T

cells in the lymphopenic host, as the phenomena is severely abrogated in the absence of IL-7, and rescued upon exogenous IL-7 administration[201, 202].

Natural instance of lymphopenia and homeostatic T cell expansion

While the phenomena of homeostatic expansion in the lymphopenic host plays out in many experimental models of adoptive T cell transfer, it also occurs in more natural settings. The induction of lymphopenia is a feature of many viral infections, and while it is best appreciated in AIDS, more transient lymphopenia also occurs after infection with measles[194], influenza[195], VZV[196], viral gastroenteritis[198], and CMV[197]. Lymphopenia has also been associated with non-infectious causalities including autoimmunity (reviewed by Schulze-Koops[175]), silicosis[203], malnutrition[204-206] and chronic stress (Chapter 8). Finally, lymphopenia can result as a consequence of cancer radio/chemotherapy, and graft vs. host disease[207-209].

While the cause, magnitude, and duration of each of the aforementioned conditions varies considerably, homeostatic expansion can begin once the cause of lymphopenia subsides. Even in the extreme lymphopenia induced by AIDS, once anti-retroviral therapy is initiated, the remaining CD4 pool begins its homeostatic expansion and the CD4/CD8 T cell ratio moves towards normalcy[210]. Collectively, the experimental models that engender homeostatic proliferation appear to be exaggerations of the response to transient lymphopenia that is seen in many natural settings.

Concluding remarks

Even from this limited review, it is clear that the subject of T cell homeostatic expansion in the lymphopenic host is complex. While much of the mechanism behind the phenomena remain elusive, it is clear that the immune system is remarkably self-aware. It is able to sense its own

lymphopenia and the clonal abundance of its T cell repertoire, and to dynamically correct itself through a specialized program of proliferation.

Experimentally, it is naïve to think that the adoptive transfer of lymphocytes into a $Rag^{-/-}$ or SCID host will yield a wild-type animal. To the contrary, the studies presented so far would suggest that the result of such a scheme would give rise to a very different mouse. The reconstituted mouse would differ from its wild-type counterpart in the narrowing of its clonal diversity, it would disproportionately bear T cells directed against self-antigens, be heavily Th2 biased and of a memory phenotype, be replete with MDSCs and have a very different cytokine milieu. Moreover, each of these parameters would be nuanced by the degree of starting lymphopenia, and the clonal diversity and number of T cells seeded into the host, and even the makeup of the recipient's intestinal flora. Nevertheless, the resulting mouse will be closer to homeostasis, and competent to perform the central function of the immune systems.

I. INTRODUCTION

B. T CELL ANTIGEN SPECIFICITY, HOMEOSTATIC EXPANSION AND PERIPHERAL TRAFFICKING

CHAPTER 6: T CELL TURNOVER IN THE PERIPHERAL ORGANS

T cell infiltration into the pathologically inflamed tissue has been characterized in many disease models; less is understood about T cell tissue turnover in the non-inflamed homeostatic context. While the testis and brain parenchyma are devoid of any lymphocytes, all other organs of the body appear to contain CD4 and CD8 T cells. Significant numbers of memory T cells migrate through non-lymphoid tissues such as the skin, lung and liver, before returning to the circulation via tissue-draining regional lymph nodes.

Memory T cells

The population of memory T cells generated after activation of naïve T cells rapidly contracts following resolution of inflammation[136-139]. What remains is a population of memory T cells belonging to either $CCR7^{+}$ $CD62L^{+}$ T central memory (T_{CM}) or a $CCR7^{-}CD62L^{-}$ T effector memory (T_{EM}) subsets that are poised to rapidly respond to a subsequent challenge from the sensitizing antigen (Table 6.1)[211, 212]. T_{CM}s have high proliferative capacity, produce IL-2, and circulate in the secondary lymphoid compartments. T_{EM}s on the other hand, are less proliferative, produce effector cytokines upon stimulation and circulate in the spleen and peripheral organs[213]. Finally, a third subset of resident memory T cells (T_{RM}) has been identified, which like T_{EM}s are CCR7 and CD62L negative (Table 6.1). T_{RM}s have been identified in peripheral tissues long after infection is cleared and are

characterized by their terminal differentiation, and minimal turnover and replenishment within the tissue[214-220].

T cell traffic between the blood and lymph nodes

T cells continually circulate throughout the body. In 1964 Gowans and Knight transferred radioactively labeled lymphocytes i.v. into rats and showed that the transferred cells quickly migrated into lymph nodes (Figure 6.1) through specialized post-capillary venules, distinguished by the height of their endothelial structure, and later termed high endothelial venules (HEVs)[221]. Both naïve T cells and T_{CM}s move between the blood and lymph nodes in the manner first described by Gowans and Knight. A naïve T cell or T_{CM} from the blood enters the lymph node through the HEVs that vascularize the lymph node in a process mediated by interactions between CD62L bearing T cells and 6-sulfo sialyl Lewis X ligand on HEVs and chemokine/chemokine-receptor interactions that culminate in transmigration into the lymph node[222]. A naïve T cell will randomly crawl along fibroblastic reticular cell (FRC) networks within the lymph node for several hours before its sphingosine1-dependent egress through the efferent lymphatics[213].

Imprinting of tissue specific trafficking

In contrast to naïve T cells and T_{CM}s, both T_{EM}s and T_{RM}s (which lack both CD62L and CCR7 expression, but upregulate other adhesion markers and chemokine receptors), preferentially migrate into non-lymphoid tissues. While some surface markers are associated with a general capacity to traffic from the blood into the peripheral tissues (e.g CD44, VLA4 and PSGL1[223-227]), entry into specific tissues appears to require distinct combinations of chemokine receptors and adhesion molecules. The specific adhesion marker and chemokine receptors of T cells required for specific trafficking to the skin and gut have been characterized. In addition to the general tissue trafficking markers, traffic to the skin is imprinted by cutaneous lymphoid associated antigen (CLA)[228], CCR4

and/or CCR10[229, 230]. In contrast, trafficking to the gut is dependent on the expression of $\alpha_4\beta_7$ integrin[231] and CCR9[232]. Whether this type of imprinting is unique to the skin and gut, or whether T cells can be directed to other tissues based on yet undefined combinations of chemokine receptors and adhesion molecules is an active area of investigation[233].

Insights from parabiosis

When T cells are isolated from perfused organs it is not immediately clear whether the T cell population is a fixed resident population, or a population that is transiting through the tissue. Parabiosis, the procedure by which the skin of partner mice is surgically conjoined to yield a connected circulatory system[234], allows for quantification of the ratio of host/donor T cells within a tissue compartment, and determination of the tissue residence times and turnover dynamics of cells within the tissues. Though these experiments have focused largely on CD8s and not CD4s, they show significantly varied T cell dynamics between tissues.

Parabiosis models that assess T cell dynamics can be broadly placed into one of two categories: those that assess turnover of T cells in antigen specific infection models, and those that assess turnover in the tissues of naïve mice.

The first broad class of parabiotic models assesses antigen-specific T cell residence and turnover in the context of infection. When lung-tropic hemagglutinin-specific T cells were transferred into naïve mice, they homed to the host lung and did not recirculate in parabiotic experiments[235]. Similarly, long lived, non-circulating populations of CD8s have been observed in skin infection with vaccinia[215] and CNS rabies virus infection[216] long after clearance of virus. These models and others[214, 216-220] collectively point to a system in which antigen-specific memory T cells persist at the tissues where those antigens were encountered, the selective advantage of which is self-evident.

T cell turnover in the tissues of a naïve mouse has been addressed in several studies. In naïve parabiotic pairings (e.g. Ly5.1-Ly5.2, Thy1.1-Thy1.2, WT-GFP) CD4 T cells equilibrate between the bloods of partner mice within two weeks, whereas the T cells derived from at least the gut[236] and NK cells derived from the liver[237], show delayed chimerism relative to the blood. Flushed gut biopsies show roughly 20% turnover 60 days following parabiotic surgery[236]. Interestingly, equilibration was three times slower in CD4 T cells of the small intestinal epithelium than in T cells of the lamina propria of the same tissue, suggesting anatomic sub-compartmentalization of CD4 T cells within the tissue[236].

While these parabiotic studies were done in "naïve" mice, the slow circulating T cell populations of the gut and liver may very well be specific to antigens of the gut flora, and therefore may be similar to antigen-specific T_{RM}s described in the lung and CNS post-infection– thus analysis of these slowly circulating T cells for surface markers of T_{RM}s may prove elucidative. Whether such tissue-resident memory T cells exist in the non-inflamed organ systems that do not readily encounter microbial antigens (i.e. the meninges) is addressed in Chapter 10.

Concluding Remarks

Circulation of memory T cells in the peripheral organs is understood to be a feature of immune surveillance, allowing for rapid T cell responses to pathogenic challenge, which is particularly relevant at sites of pathogen entry such as the skin and mucosal surfaces. However, immune surveillance may not be the only function of T cells within a tissue. Indeed, if the cells are perpetually present in the tissue, they may have a homeostatic role in normal tissue function and integrity. We explore this idea experimentally with the hypothesis that meningeal T cells support higher brain functions.

Memory Subset	Surface Phenotype	Location	Function during infection
T_{CM}	$CD44^{high}$, $CD62L^{+}$, $CCR7^{+}$, $CD127^{+}$, $CD69^{-}$, $CD103^{-}$	Lymph nodes, spleen, blood and bone marrow	Circulating surveillance of secondary lymphoid organs and recall proliferative response
T_{EM}	$CD44^{high}$, $CD62L^{-}$, $CCR7^{+/-}$, $CD127^{+}$, $CD69^{-}$, $CD103^{-}$	Spleen, blood, lung, liver, GI tract, reproductive tract, kidney, adipose tissue, heart	Circulating surveillance of non-lymphoid tissues and recruitment by infection and inflammation
T_{RM}	$CD44^{high}$, $CD62L^{-}$, $CCR7^{-}$, $CD11a^{high}$ $CD69^{+}$, $CD103^{+}$	Skin, gut, and vaginal epithelium, salivary glands, lung airways, brain, ganglia and lung parenchyma	Ongoing control of residual or persisting infection in peripheral tissues of permanent residence

Table 6.1: **Subsets of Memory T cell**. T_{CM}=T central memory, T_{EM}= T effector memory, T_{RM}= T resident memory.

Adapted From Mueller, S.N., Gebhardt, T., Carbone, F.R. & Heath, W.R. Memory T cell subsets migration patterns, and tissue residence. Annu Rev Immunol ***31****, 137-161 (2013). And Gebhardt T, Mueller S.N., & Heather W.R., Carbone F.R., Peripheral tissue surveillance and residency by memory T cells. Trends Immunol. 34(1), 27-32(2013)*

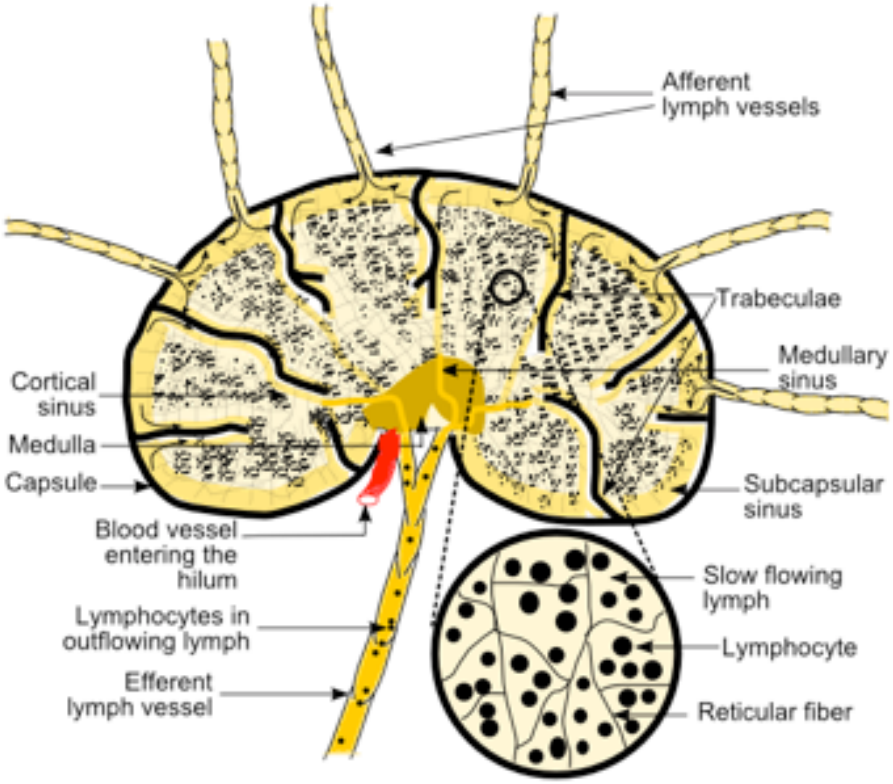


Figure 6.1: **Structure of lymph node and associated lymphatic vessels.**

From: Panchal, KC 2008 Schematic diagram of a lymph node showing flow of lymph through lymph sinuses, image, Retrieved March 21, 2014,from <http://en.wikipedia.org/wiki/File:Schematic_of_lymph_node_showing_lymph_sinuses.svg> Public domain.

I. INTRODUCTION

C. T CELLS IN CNS FUNCTION AND HOMEOSTASIS

This final section of the introduction addresses the role of T cells in CNS function and homeostasis, and in doing so aims to bring the discussions of the preceding two sections together. Chapter 7 reviews what is known about the capacity of meningeal myeloid cells to affect learning behavior, and importantly, the ability of T cells to regulate myeloid cell phenotypes–the leading mechanistic hypothesis for the function of T cells in learning and memory.

While the focus of our own investigation of T cell-CNS axis is on how T cells influence brain function, it is important to emphasize that the axis operates in both directions, i.e. brain processes influence the immune system. In Chapter 8 we briefly explore the effects of both acute and chronic psychological stress on the immune system. Finally, Chapter 9 explores the central subject of this thesis, T cell involvement in higher brain functions. As a starting point for our own investigations, we present what is currently known (and unknown) about T cells in learning and memory, stress responses, and adult neurogenesis.

I. INTRODUCTION

C. T CELLS IN CNS FUNCTION AND HOMEOSTASIS

CHAPTER 7: T CELL INFLUENCE ON MYELOID PHENOTYPE IN THE MENINGES (AND ELSEWHERE)

Myeloid cells refer to hematopoetically derived non-lymphoid cells, and encompass granulocytes and monocytes. The studies referenced in this chapter illustrate the great authority that T cells have over the phenotype of these cells, laying the foundation for a model in which the requirement of T cells in cognition is in maintaining the homeostasis of the myeloid compartment.

Derecki et al examined the cytokine profile of $CD45^{+}$, $CD11b^{+}$ meningeal myeloid cells from wild-type and SCID mice. Following MWM training, meningeal myeloid cells derived from SCID mice showed significantly higher levels of both TNF and IL-12, compared with wild-type controls (whether this skew is present prior to MWM training, or is induced as a result of it remains undetermined). Nevertheless, this pro-inflammatory signature could be reversed by the adoptive transfer of splenocytes from wild-type, but not IL-4 deficient, mice[100].

Myeloid cells can directly affect learning behavior

The striking effect of T cells on inflammatory cytokine production in meningeal myeloid cells raised the possibility that the MWM deficiencies of SCID mice (addressed in detail in Chapter 9) may be directly attributable to the proinflammatory skew of the myeloid compartment. Derecki et al were able to reverse the MWM impairment of SCID mice with M2 macrophages skewed ex-vivo with T cell derived IL-4 cytokine. Conversely, when they transferred M1 macrophages into the same SCID

mice, recipient mice had a trend to do worse on the MWM[238] (though the trend did not reach statistical significance). This study and others led to a generalized "yin-and-yang" model in which an M1 pro-inflammatory skew inhibits cognitive performance while an anti-inflammatory M2 like phenotype appears to promote cognitive performance.

While the work of Derecki et al is the first to report the productive effect of an anti-inflammatory intervention on learning and memory outcomes, the deleterious effects of pro-inflammatory cytokines on the same phenomenon have been well established and extensively reviewed[239-241]. Most prominently IL1α and β, TNFα, and IL-6 have been shown to induce "sickness behavior" in mice. Specifically, both systemic and intracranial administration of IL1-β or TNF resulted in a litany of behavioral changes including depressed locomotor activity, decreased sociability and exploratory behavior, reduced food intake, and impaired learning and memory[241]. While most studies have not directly assessed the effect of cytokine and endotoxin on the meningeal myeloid compartment, pyrogenic doses of LPS were shown to induce IL1β expression specifically in macrophages of the meninges and choroid plexus[242], though the T cell dependence of the phenomena was not addressed.

T cells regulate myeloid phenotypes

While neither M1 nor M2 i.v. transferred macrophages could be recovered from the brain or meninges of recipient mice 2 weeks after transfer, they left lasting changes in the phenotype of endogenous meningeal myeloid cells[241]. M2 recipient SCID mice showed reduced TNFα in both meningeal and splenic $CD45^+$ $CD11b^+$ myeloid populations relative to control SCID mice. Recipients of M1 skewed macrophages, on the other hand showed increased meningeal and splenic TNF production relative to control SCID mice and a trend towards decreased performance in the MWM

spatial task[241]. However, the mechanism by which these macrophages injected i.v. skewed the endogenous meningeal myeloid compartment remains unknown.

The consequence of T cell absence/ablation on the inflammatory state of the myeloid compartment has been reported in several other models. Kim et al demonstrate that Poly IC treated nude and $Rag1^{-/-}$ mice have significantly higher serum TNF and IFN-γ than their wild-type counterparts[243]. Importantly, the heightened serum TNF/ IFN-γ levels of $Rag1^{-/-}$ mice could be significantly tempered by reconstitution of mice with wild-type T cells two days prior to Poly IC treatment. Interestingly when the authors used an in vitro co-culture model, they found that CD4, CD8, OTI and OTII T cells are equally capable of inhibiting TNF production from T cell depleted splenocytes[243].

More recently the inflammasome has been mechanistically implicated in the suppressive effects of T cells on adaptive immunity[244]. Guarda and colleagues co-cultured bone marrow derived macrophages (BMDMs) with Tregs, naïve CD4, naïve CD8, memory CD4 or memory CD8 T cells. LPS was added to induce proIL-1β synthesis by BMDMs, followed by ATP stimulation to activate the NLRP3 inflammasome-mediated processing and release of IL-1β. Surprisingly, both secretion of mature IL-1β and IL-6, as well as abrogation of caspase-1 processing was inhibited only by the presence of memory $CD4^+$, and no other T cell subsets[244]. Finally, regulatory T cells have been repeatedly shown not only to regulate effector T cells, but also to directly influence innate cell function as well[245-247].

Concluding remarks

Modulation of the innate immune system by T cells represents complex bi-directional interactions between both arms of the immune system, with many cell types implicated along the axis of regulation (reviewed by Zhao[248] and Shanker[249]). In light of the modulation of MWM outcomes by

transfer of M1 vs. M2 macrophages, and the pro-inflammatory skew of lymphopenic mice that is associated with cognitive impairment, it may be that the effects of T cells on learning and memory represent the ability of T cells to control the meningeal myeloid compartment. Indeed a model that positions myeloid cells as intermediaries between T cells and the CNS is particularly attractive given the relative abundance of myeloid cells within the meninges compared with CD4 T cells in the same compartment.

I. INTRODUCTION

C. T CELLS IN CNS FUNCTION AND HOMEOSTASIS

CHAPTER 8: THE EFFECT OF PSYCHOLOGICAL STRESS ON THE IMMUNE SYSTEM

A mouse struggling to find food, an elephant seal vying for a mate, a gazelle escaping predation, and a caregiver tending to the chronically ill, are all under stress. These examples illustrate the immense heterogeneity of the term "stress" and explain why most reviews of the subject define stress loosely as "situations that are stressful". Those situations themselves are immensely heterogeneous and have been dissected and compartmentalized under many different schemes of categorization.

Two broad categories of stress

For the purpose of this chapter we adopt the simplest grouping scheme and place a stressor into one of two broad categories, those that are "acute" vs those that are "chronic". While the duration and intensity of many real life stressors may fall somewhere between these extremes, the compartmentalization satisfies most laboratory conditions in which acute stress is defined as stressors lasting on the order of minutes to hours while chronic stressors are defined as lasting on the order of weeks to months.

The effects of both acute and chronic stress have been widely studied in both laboratory animals and humans. While the data generated by these studies is diverse and model-specific, they

collectively evidence an intuitive effect of psychological stress on immune function in which acute stressors enhance immune surveillance, while chronic stress is largely immunosuppressive.

The redistribution of blood leukocytes following acute stress.

The selective advantage of heightened immune surveillance following acute psychological stress is self-evident. An animal escaping predation is a classic example of acute stress. In this stressful predation context, the likelihood of injury and subsequent infection is exponentially higher than when the animal is secure and free of danger. Therefore, it is intuitively understandable how any process of immune up-regulation in anticipation of wounding and infection may be a beneficial adaptation. We can go further to speculate that this type of anticipatory immune up-regulation following acute stress might be most beneficial and prevalent at those anatomical sites which are most likely to encounter wounding during the stress episode (i.e. the skin).

Studies in rodents, fish, primates and humans all evidence changes in the number of circulating blood leukocytes following acute stress, suggesting longstanding evolutionary conservation of the phenomenon (reviewed by Dhabhar[250]). In depth time-course studies show that within minutes acute stress results in an increase in circulating blood lymphocytes followed by a gradual decline, (correlating with an increase in tissue lymphocyte numbers), and finally a return to baseline within four hours[251, 252]. Interestingly this same phenomenon is observed after exercise, and was found to be dependent on (and recapitulated with the administration of) low-dose corticosterone and epinephrine[250]. In contrast, high-dose corticosterone, chronic corticosterone, or low-dose dexamethasone administration significantly suppressed skin delayed type hypersensitivity (DTH)[253].

Importantly, acute stress was found to functionally enhance tissue immunity in the context of immune challenge. When mice underwent 2.5h of restraint stress prior to intradermal challenge with keyhole limpet hemocyanin (KLH), they showed increase in numbers of $CD44^+$ memory T cells in

sentinel lymph nodes 72 hours after primary immunization, and an enhanced immune response upon secondary re-exposure to KLH nine months later[254]. Additionally, experiments by Saint Mezard et al showed an enhancement of the contact hypersensitivity (CHS) response to dinitrofluorobenzene as a result of acute stress, as well as heightened dendritic cell migration from the immunized skin to the draining lymph node relative to unstressed controls[255].

Stress induced changes in the cytokine milieu

Redistribution of leukocytes is not the only immunological outcome of acute stress. Indeed, in both mice and humans, the entire systemic cytokine milieu appears to change as a result of acute stress exposure (Table 8.1) (reviewed by Kronfol[256]). Importantly, the immense diversity of changes in the studies summarized in Table 8.1 reflect the diversity of stress paradigms that are employed, the duration of the stressors, and the time point after stress at which immune parameters are measured. Changes in cytokine milieu are not solely restricted to the blood, as cultured alveolar macrophages isolated from acutely stressed mice showed increased IL1β and TNF, and a strong decrease in nitric oxide production[257]. Finally, stress related hormones have been shown to modulate cytokine production and the Th1/Th2 balance in vitro[258-264]. Administration of glucocorticoids at a dose meant to recapitulate acute stress inhibits the production of Th1 cytokines while upregulating the production of IL-4, IL-10 and IL-13 associated with the Th2 response[260].

Chronic stress is immunosuppressive.

In contrast to the immune enhancing effect of acute stress, Blecha et al showed significant immunosuppression when dinitrofluorobenzene challenged mice were chronically stressed three weeks prior to antigen challenge[265], and the magnitude of leukocyte redistribution observed in acute stress is progressively diminished with chronic stress[266, 267]. Similarly, Saul et al. show that chronically stressed

tumor susceptible mice reached 50% incidence sooner than non-stressed animals and had higher numbers of tumor infiltrating regulatory T cells compared with non-stressed[268].

The effect of chronic stress on immune function is perhaps best appreciated in the context of viral infection. Many studies have shown that Herpes Simplex Virus infection severity, and lesion recurrence is significantly increased in patients with chronic stress[269-273]. Women who reported at least one-week of stress or more had a significantly greater recurrence of genital herpes and re-activation of latent HSV-1 and HSV-2[274]. A strong correlation between chronic stress and the recurrence of herpes zoster (shingles) has been established[275]. In one study the random assignment of elderly adults to a stress mitigating regimen of Tai chi resulted in a 50% increase in varicella-zoster virus-specific T cell immunity compared with control, waitlisted subjects[276].

Together, these human and animal studies show that chronic stress can modulate the steady-state expression of latent HSV, EBV and CMV, downregulating the specific T-cell response to the virus to an extent sufficient to result in viral reactivation (reviewed by Glaser[277]).

Concluding remarks

The studies cited in this chapter utilize various natural and experimental stress paradigms and measure a diverse set of immunological readouts. The variability in these studies reflects the diversity of stress paradigms that are employed, the duration of the stressors, and the timepoint after stress at which immune parameters are measured. Nevertheless, when all the studies are viewed collectively, a general model emerges in which acute stressors enhance immune surveillance, while repeated chronic stress is broadly immunosuppressive. As mentioned earlier, there is likely a strong selective pressure for enhanced immune surveillance during times of stress, as stressful episodes go hand in hand with an increased risk of wounding and infection. Conversely, the immunosuppressive outcome of chronic

stress, which has been evidenced in this chapter, might also be intuited from immunological studies of repeated antigen exposure, tolerization and exhaustion.

While this chapter has focused on the effects that psychological stress brings about in the immune system, part of the following chapter is dedicated to the same axis in the reverse direction – that is, the effect of the immune system on the behavioral stress response. Together these chapters shed light on the complex multi-directional, feedback loaded, communication between the CNS and the immune system in coordinating the physiological response to stress.

Author	Year	Subjects	Stressor	Cytokine	Results
Glaser[278]	1986	Medical students	Examination	IFN- γ	Decreased IFN- γ
Glaser[279]	1990	Medical students	Examination	IL-2, IL-2R	Increased IL-2, decreased IL-2R
Dobbin[280]	1991	Medical students	Examination	IFN- γ -, IL1- β	Decreased IFN- γ, increased IL1- β
Mittwoch-Jaffe[281]	1995	Healthy volunteers	Experimental positive and negative mood induction	IL-1 β, IL-2, IL-3, IL-6, TNF- α	Negative emotions: increased TNF- α, increased IL-2 and IL-3
Kiecolt-Glaser[282]	1995	Chronically stressed individuals matched with control subjects undergoing experimental surgical wound	Alzheimer's disease caregiving	IL-1	Decreased IL-1 slowing of wound healing
Nakano[283]	1998	Taxi drivers in Japan	Economic depression	IL-2, IL-4	Decreased IL-2, increased IL-4
Maes[284]	1998	Medical students	Examination	IFN- γ, TNF- α, IL-4, IL-6, IL-10	Increased IFN- γ, TNF- α, IL-6 and IL-10, for anxious students increased IFN- γ, decreased IL-4 and IL-10
Marshall[285]	1998	Medical students	Examination	IFN- γ, IL-10	Decreased IFN- γ, increased IL-10
Glaser[286]	1999	Healthy volunteers undergoing experimental surgical wound	Perceived level of daily stressors	IL1- α, IL-8	Decreased IL-1 α, decreased IL-8 in αwomen reporting higher perceived stress

Table 8.1: **Effects of acute stress on cytokine production in humans.**

Adapted from: Kronfol, Z. & Remick, D.G. Cytokines and the brain: implications for clinical psychiatry. Am J Psychiatry 157, 683-694 (2000).

I. INTRODUCTION

C. T CELLS IN CNS FUNCTION AND HOMEOSTASIS

CHAPTER 9: T CELLS IN HIGHER BRAIN FUNCTIONS

A role for T cells in higher brain function is not immediately intuitive. To the contrary, elements of "immune privilege", including an absence of T cells in the brain parenchyma, would argue against such an axis. While intuition does not support a role for T cells in higher brain function, experimentation does. A litany of lymphopenic mouse models has repeatedly established significant impairment of these animals in numerous readouts of higher brain function. In this chapter we review the mouse models that link T cells with spatial learning and memory, stress responses, and adult neurogenesis, and explore the proposed mechanistic basis of these phenomena. Finally this chapter reviews a broad spectrum of human CNS etiologies, which may find their causality in immune dysregulation.

T cells in spatial learning and memory:

Spatial learning and memory is the type of memory responsible for recording information about an animal's relation to their spatial environment. A comprehensive review of spatial learning and memory would be outside the scope of this chapter. Instead, we take a brief look at several key features of spatial learning and memory and the experimental systems used to measure them in mice.

The Morris water maze was first employed by R.G.M Morris in 1981 for the study of spatial navigation in rats[287]. The maze represented a simple way to assess the learning and memory of a spatial task (without the confounding effect of odor), and has since become the gold-standard for

assessing defects in spatial learning and memory in both rats and mice. The experiment is initiated by placing the animal in a circular tank of opaque water (Figure 9.1), the only escape being a small circular platform submerged below the waterline, and invisible to the animal being tested. The mouse is allowed repeated attempts over the course of several days to locate the hidden platform, and the time it takes to reach the escape platform is recorded for each trial. While the animal can never actually see the escape platform, a competent learner will employ spatial cues located around the tank to effectively navigate its way to the escape.

Importantly, the MWM task is dependent on the hippocampus, as lesioning this brain region results in the dramatic loss of task performance without resulting in hypo-activity[288, 289]. Moreover the maze is heavily linked to the hippocampal circuitry, long-term potentiation (LTP), and NMDA receptor function[290-293].

Kipnis et al employed the MWM to test the spatial memory of both SCID and nude mice which lack functional lymphocytes due to ablation of the Prkdc and Foxn1 genes respectively necessary for VDJ recombination[81]. Both immunodeficient mice had dramatic impairments in maze performance that were significantly rescued by the adoptive transfer of splenocytes from wild-type hosts. The spatial learning and memory deficits of SCID mice were again demonstrated by Brynskikh[82], Ron-Harel[294], and Derecki et al[100] using the MWM and alternate spatial memory paradigms. In the study by Brynskikh et al, whereas SCID mice reconstituted with wild-type splenocytes recovered MWM performance, no rescue was obtained when splenocytes were treated with pan-T cell depleting antibody prior to transfer. Additionally, when lymphopenia was achieved via split-dose radiation, mice reconstituted with wild-type bone marrow did significantly better in the MWM than those receiving bone marrow from SCID donors. Although these findings were largely phenomenological, they were the first to establish spatial learning and memory deficits in lymphopenic mice. Moreover, the ability to rescue mice with splenocyte reconstitution, as well as the MWM

impairment in irradiated wild-type mice, suggested that the MWM deficits of SCID mice were not developmental.

These initial reports gave rise to many fundamental questions about the nature of the CD3 T cells that appear to be required for normal spatial learning and memory. Crucially, it was still unclear whether these were CD4 or CD8 T cells, and what the phenotype and antigen specificity of the relevant subpopulation might be. Some partial insight into this question came from studies by Wolf et al who showed that treatment of mice with anti-CD4 antibody during the course of the MWM task resulted in poor latency times relative to IgG treated controls[2]. However these results were difficult to interpret given incomplete validation of depletion efficiency, the lack of a CD8 depleted treated group, and the possibility that large scale systemic depletion of any lymphocyte subset would adversely affect cognitive outcomes in the short term.

The same reports also raised questions about the antigenic specificity of the T cells that mediate MWM performance. Based largely on findings that T cells reactive to the CNS specific myelin basic protein (MBP) antigen could promote recovery after CNS injury[295-297], Ziv et al employed the same T cells in the study of neurogenesis and spatial learning and memory. The authors demonstrate that DO11.10 mice on the Balb/c background have significant MWM deficits, suggesting that T cells reactive to irrelevant ovalbumin antigen are not sufficient for normal learning and memory function[4]. Moreover, the authors show that mice transgenic for the anti-MBP TCR (T_{MBP}) performed comparably or even better to their wild-type counterparts in the MWM. The authors use these two findings to suggest that the normal MWM performance of wild-type mice may be mediated by endogenous T cells reactive to CNS self-antigens[4].

While this observation is intriguing, it must be viewed in light of several crucial caveats. First, the DO11.10 and the T_{MBP} mouse models are on different genetic backgrounds, making it difficult to

consolidate conclusions from the two experiments into a single model. Next, the T_{MBP} mice used in the study are likely heterozygous for the TCR transgene (homozygous mice develop spontaneous EAE[134]), and are by no means monoclonal. While approximately 90% of T cells in the T_{MBP} mouse are transgenic, the remaining 10% of T cells bear endogenous TCRs[134, 135]. Therefore it is unclear whether MWM competency is being mediated by T_{MBP} transgenic T cells or by the endogenous T cell pool. The DO11.10 Tg mouse also contains a T cell pool bearing endogenous TCRs, though the endogenous population appears to be much smaller[132, 133]. Comparison of the T_{MBP} and DO11.10 endogenous pools is rendered further confounding by knowledge that the bulk of the endogenous T cell pool of T_{MBP} mice arises from the thymus, whereas that of DO11.10 mice arises from the periphery and co-expresses both endogenous and transgenic TCRs[132, 133]. Therefore it is possible that MWM competency is being mediated by the population of normal thymically derived T cells in heterozygous T_{MBP} mice, and not as the authors argue by the T_{MBP} transgenic T cell population. Importantly, the peripherally derived endogenous T cells of D011.10 mice may not be able to achieve the same effect, possibly owing to reduced relative abundance or a functional skew in the endogenous T cells of D011.10 mice. Indeed, endogenous dual TCR bearing T cells derived from the periphery of Vβ5 TCR transgenic (OTII mice bear Vβ5) as well as DO11.10 mice appear to be skewed towards the Th17 lineage[132, 298], though the consequences of this skew in terms of higher brain function is unknown.

T cells modulate the response to acute psychological stress

In Chapter 8 we reviewed the effect of psychological stress on the immune system. Here we look at the stress axis in the other direction – that is, how T cells (or the lack thereof) affect the normal stress response in mice. Two prominent behavioral models that assess stress in rodents are the elevated plus maze and the prepulse inhibition. Briefly, in the elevated plus maze, a single mouse is placed at the center of a plus shaped maze (Figure 9.2), and the amount of time spent in the "closed" *vs.* "open"

arms is assessed. As might be expected, stress and anxiety are associated with greater time spent in the closed arms of the maze, whereas unstressed animals are curious by nature, and explore the open-arms more readily. In the prepulse inhibition paradigm, mice are habituated in an acoustically insulated chamber and given a series of short acoustic stimuli, to which electronic sensors measure the ensuing startle response. The ability of a previous sound or "prepulse" to inhibit the startle response to a subsequent sound (pulse) is defined as "prepulse inhibition". In a well-adapted mouse, delivery of a prepulse significantly diminishes the startle response to the subsequent test pulse[299].

The elevated plus maze and the prepulse inhibition paradigm were used to assess adaptation to stress in mice that were pre-exposed to a predatory odor[1]. Despite being reared for generations in laboratory housing, exposure of naïve rodents to predatory odors, including cat odor and the fox feces-derived compound trimethylthiazoline, induces quantifiable fear responses including freezing, burrowing, avoidance, and stress hormone release (reviewed by Takahashi[300]). Cohen et al exposed naïve wild-type, SCID and nude mice to cat feces for 10 minutes, followed by behavioral testing in the elevated plus maze and acoustic startle paradigm[1]. Compared to their wild-type counterparts, both SCID and nude mice spent significantly less time in the open arms of the elevated plus maze, and displayed diminished prepulse inhibition–suggesting that both lymphopenic strains exhibit aberrant responses to psychological stress. The neurobiology that underlies the maladaptation to mental stress is complex. While principle brain region through which fear-associated responses occur is the amygdela, many other brain regions also mediate fear responses, including pedunculopontine tegmental nucleus, laterodorsal tegmental nucleus, substantia nigra pars reticulata, and caudal pontine reticular nucleus, each of which receives input from other brain regions[301, 302]. Therefore it is not surprising that the neurobiological underpinnings behind the aberrant stress response of SCID and nude mice are entirely undefined.

Previous findings from models of CNS injury had suggested that T cells reactive to the CNS specific myelin basic protein (MBP) antigen could promote recovery after CNS injury[295-297] Cohen et al hypothesized that the same phenomenon may underlie the necessity of T cells in behavioral outcomes[1]. In pursuit of a model in which T cells reactive to CNS self-antigens are productive for higher brain functions, Cohen et al moved to the B10.PL background and tested wild-type, T_{MBP} and $T_{MBP}/Rag1^{-/-}$ mice in both the prepulse inhibition and elevated plus maze stress response paradigms[1]. Wild-type (B10.PL) and T_{MBP} mice appeared to have high basal stress responses and spent virtually all their time in the closed arms of the plus maze. The truly monoclonal $T_{MBP}/Rag1^{-/-}$ animals (which contain no endogenous TCR bearing T cells) however, spent dramatically more time in the open arms of the maze. The authors argue that the differential between T_{MBP} and $T_{MBP}/Rag1^{-/-}$ mice is a consequence of Tregs that are present in T_{MBP} animals but absent on the $Rag1^{-/-}$ background[134, 135], and go on to show that nude mice reconstituted with wild-type splenocytes depleted of regulatory T cells increase the time that recipients spend in the open arms of the maze, more so than nude recipients of non-depleted wild-type splenocytes. Collectively the authors use these findings to evidence a model in which lymphopenic SCID and nude mice are maladapted to mental stress, and suggest that endogenous non-Treg T cells reactive to CNS self-antigens mediate stress resilience[1]. While the phenomena of a maladapted stress response in the lymphopenic models is robust, the central model derived from behavioral testing of T_{MBP} transgenic animals has several important caveats. While the T_{MBP} to $T_{MBP}/Rag1^{-/-}$ differential may stem from the respective presence and absence of Tregs, it may equally be a result of the $Rag1^{-/-}$ background itself. Indeed, Rag1 (but not Rag2) is expressed in the CNS during development[76-78], and $Rag1^{-/-}$ animals appear to have a higher capacity to extinguish fear memories (Figure 3.1-3.3), have increased exploratory behavior[79], show less aversion towards the center of a brightly illuminated open field[79], increased marble burying behavior[80], and impaired social recognition[303]. Collectively, these behaviors the paint a picture of Rag1 deletion resulting in a "less inhibited" animal, which at least in the case of impaired social recognition[303] and enhanced extinction

of fear memories (Figure 3.1-3.3), appears to be lymphocyte independent (as shown by the use of a $Rag2^{-/-}$ control in these two studies).

T cells in adult neurogenesis:

It was a long held dogma in neuroscience that the adult CNS is terminally differentiated and incapable of regeneration. This view was challenged in the early 1960s by Joseph Altman, who discovered neurogenesis in the cerebral cortex and the dentate gyrus of the hippocampus[304, 305]. Widespread acceptance of adult neurogenesis came over two decades later following a series of experiments by Fernando Nottebohm showing new neurons are involved in song learning by canaries, and are functionally integrated into existing neuronal circuits[306-308].

Neurogenesis occurs in two brain regions throughout life, the subventricular zone (SVZ) of the lateral ventricle, and the subgranular zone (SGZ) of the hippocampus. While neurogenesis in the SVZ is appreciated for its role in the rostral migratory stream and its ability to modify sensitivity to odors[309, 310], the function of neurogenesis in the hippocampus is less established. Importantly, the association between adult hippocampal neurogenesis and spatial learning and memory is a matter of longstanding debate, which has been expertly reviewed[311-313]. Despite the lack of clarity on its ultimate behavioral ramifications, adult neurogenesis in the SGZ of the hippocampus has been repeatedly shown to be modulated by the adaptive immune system[2-5].

Ziv et al were the first to show that SCID mice displayed significant reduction in proliferative BrdU/DCX cells in both the SVZ and the dentate gyrus, and reduced expression of brain derived neurotrophic factor (BDNF) in the hippocampus[4]. Furthermore, environmental enrichment, generated by increasing cage size, and the addition of toys, tunnels, and running wheels, stimulated neurogenesis of wild-type, but not SCID mice. A similar reduction in neurogenesis was observed in nude mice and, importantly, was rescued by the adoptive transfer of wild-type splenocytes[4]. Wolf et al

went on to attribute impaired neurogenesis in Rag1 deficient mice to the absence of a functional CD4 compartment[2]. Rag1$^{-/-}$ mice had impaired neurogenesis that was rescued by adoptive transfer of CD4 but not CD8 T cells, and neurogenesis could be reduced in wild-type mice by CD4 but not CD8 depleting antibody. Interestingly, their data shows that Rag1 knockout animals may have a lymphocyte independent function in neurogenesis as VDJ recombination competent Rag1$^{+/-}$ mice showed an intermediate reduction in neurogenesis[2].

A separate study by Wolf et al addressed the link between peripheral immune activation and neurogenesis[3]. The authors reported increased neurogenesis after induction of peripheral immune response by both staphylococcus enterotoxin B, as well as by adjuvant induced-rheumatoid arthritis initiated at the knee[3]. As neither mode of immune activation is specific to CNS self-antigen, the authors argue for a model in which non-encephalitogenic, non-CNS-specific T cells can promote neurogenesis entirely from the periphery. This model stands in contrast to that championed by Ziv et al who show increased neurogenesis in T_{MBP} and decreased neurogenesis in T_{OVA} transgenic mice and advocate for a CNS specific T cell modulation of neurogenesis[4].

More recently an association between the adaptive immune system and adult neurogenesis was picked up in a high-throughput phenotyping screen of 719 outbred heterogeneous stock mice[5]. The screen identified the blood CD4/CD8 T cell ratio as the strongest correlate of adult neurogenesis, with a lower CD4/CD8 ratio associated with higher neurogenesis. The same body of work went on to show impaired neurogenesis in Rag1$^{-/-}$, TCR$\alpha^{-/}$, and MHCII$^{-/-}$ (but not MHCI$^{-/-}$) deficient animals. While the study failed to show any effect of antibody mediated CD4 depletion on neurogenesis, neurogenesis was significantly rescued in TCR$\alpha^{-/-}$ mice adoptively transferred with CD4 but not CD8 T cells. It is worth noting that the association between a lower CD4/CD8 ratio and increased neurogenesis is seemingly at odds with previous studies that show neurogenesis to be increased by

CD4s, as well as findings in the same study which showed rescue of neurogenesis in TCR$\alpha^{-/-}$ mice adoptively transferred with CD4 but not CD8 T cells.

Cognition and T cell deficiency in humans

While cause and effect relationships are lacking, a litany of neurocognitive disorders in humans are correlated with the collapse of the immune system, particularly within the T cell compartment. The onset of age-associated dementia is consistent with the immunosenescence that characterizes the aged immune system. Similarly, HIV associated dementia is correlated with the concomitant collapse of the T cell compartment, raising the possibility that dementia may not stem from direct viral infection of the CNS[314, 315]. The general phenomena of impaired cognition following radio/chemotherapies known as "chemobrain"[316, 317], may also find its causality in the depletion of T cells necessary for normal homeostasis of the CNS.

Concluding remarks.

While the studies presented in this chapter employ a diversity of behavioral models and immunological manipulations, they collectively affirm robust behavior abnormalities in lymphopenic animals, particularly in hippocampal dependent spatial learning and memory. Nevertheless the same studies leave many fundamental questions unanswered, including the relative contribution of CD4 *vs.* CD8 T cells, the antigenic specificity requirements of the T cells involved, the contribution of hippocampal neurogenesis, and the distinction between peripheral *vs.* meningeal/CNS immunity in mediating behavioral outcomes. Several of these questions are directly addressed in the following chapters.

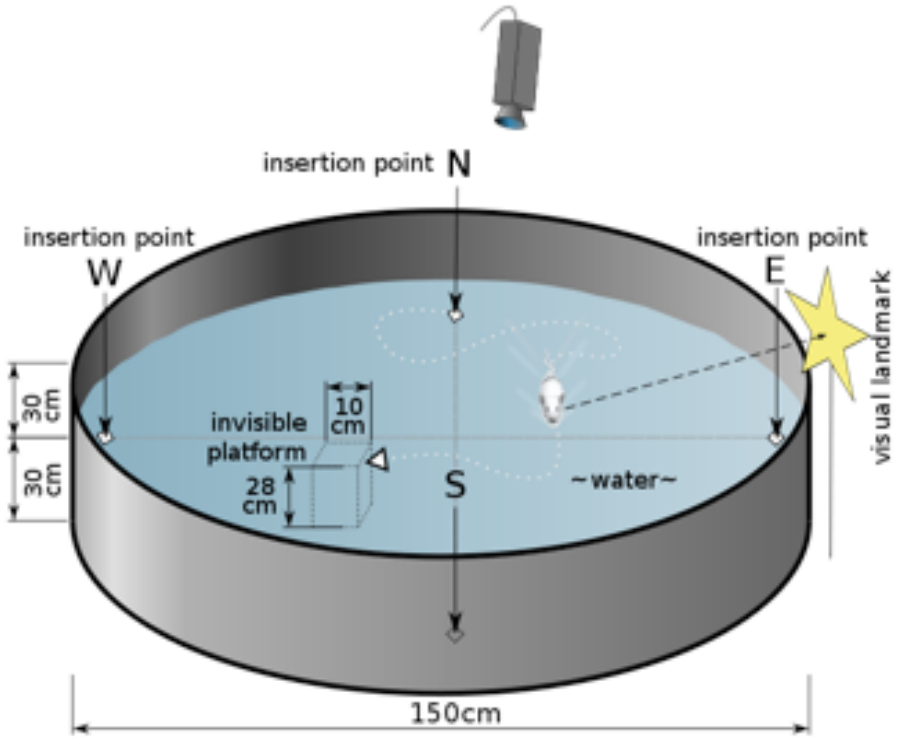


Figure 9.1: **Schematic drawing of the Morris water maze navigation task.**

From: Samueljohn,de 2010 Schematic drawing of the Morris water navigation task, image, Retrieved March 21, 2014, from < http://en.wikipedia.org/wiki/File:MorrisWaterMaze.svg > Public domain.

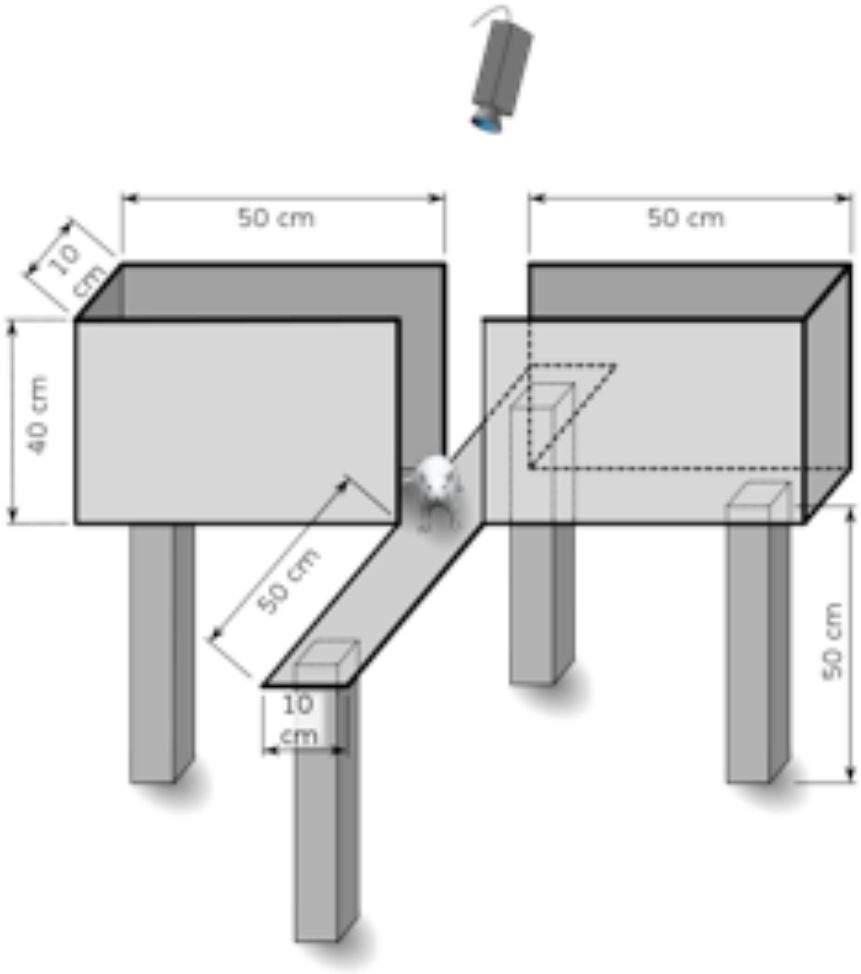


Figure 9.2: **Schematic drawing of the elevated plus maze.**

From: Samueljohn,de 2010 Schematic drawing of the Elevated Plus Maze, image, Retrieved March 21, 2014,from < http://en.wikipedia.org/wiki/File:ElevatedPlusMaze.svg> Public domain.

II. RESULTS

CHAPTER 10: DYNAMICS AND ANTIGEN-SPECIFICITY OF THE MENINGEAL CD4 T CELL REPERTOIRE:

The traditional view of the central nervous system (CNS) as an immune-privileged organ yielded a longstanding perception of such interactions—as seen, for example, in multiple sclerosis — as intrinsically destructive. This notion is changing with the identification of several homeostatic functions attributable to beneficial T-cell/CNS interaction in hippocampal-dependent learning, stress response paradigms, and in models of neurodegeneration and CNS injury. Here we provide insights into the maintenance and dynamics of T-cells in the meningeal space — the candidate site of homeostatic T cell-CNS interaction as well as a critical structure for immune surveillance of the brain. Employing parabiosis, we determine that the meninges contains a small subset of memory CD4 T cells that turnover slowly relative to other tissues in the normal state. While we show that the meninges is normally restrictive to T cell entry, under conditions of homeostatic expansion in lymphopenic hosts, wild-type CD4 T cells can preferentially seed the meningeal space, suggesting that the meninges is not intrinsically restrictive to T cell entry. We go on to show that T cell migration to the meninges is not directly dependent on TCR-specificity, but rather T cell memory status. Finally, we show a tight coupling of meningeal T cells to the draining deep cervical lymph nodes, interruption of which affects T cell composition in the meningeal space, and has consequences for learning and memory task performance. Some of the findings of this chapter have been reported in *Molecular Psychiatry* by Radjavi et al[318].

Introduction and project rational:

T cells have been repeatedly shown to be necessary for normal spatial learning and memory function in mice. Specifically, SCID and nude mice have been shown to exhibit diminished performance in the Morris water maze paradigm, and are each rescued by the adoptive transfer of wild-type T cells (reviewed in Chapter 9). One of the central questions prompted by these observations is the location where productive T cell-CNS interactions take place, and what antigenic specificity if any exists in the T cells of that site.

The most direct location for T cell-CNS interaction would be the CNS itself. However, this is extremely doubtful given the nearly total absence of T cells from the non-inflamed brain parenchyma. While the parenchyma is devoid of any lymphocytes, the brain meninges is permissive to T cells, which have been isolated and characterized by Derecki et al[100]. Importantly, the meninges is not a static bag that covers the brain. Its layers follow the contours of the CNS and the ventricles, and it is contiguous with the interstitial fluid, CSF and perivascular spaces of the brain (Chapter 1). The presence of T cells within the meninges, in addition to its physical juxtaposition against the brain parenchyma, have made the meninges a central candidate for the location of productive T cell CNS interactions.

In part based on experiments by Derecki et al who show a correlation between meningeal T cell numbers and MWM performance, we hypothesized that T cells in the meninges might be permanently resident and expressly dedicated to supporting higher brain function. Studies from other peripheral organ systems have led to the characterization of resident memory T cells (T_{RM}s) that persist within peripheral tissues with little to no egress, or self-renewal. In the naïve mouse, T_{RM}s have been identified in the gut, where they occupy space between adjacent endothelial cells, and have been identified in the lung and brain long after clearance of viral infection. However, detailed phenotypic

characterization of meningeal T cells has not been performed (including assessment of T_{RM} status) and the dynamics of T cell trafficking within the space remains totally undefined.

Recently Baruch et al have characterized T cells in the choroid plexus, which is contiguous with the meningeal space[319]. They use the observation that TCRs of T cells within the choroid plexus resemble CNS-antigen specific T cells to support a model in which CNS self-reactive T cells preferentially reside in the choroid plexus. This interpretation would be correlatively consistent with previous work showing that transgenic mice with TCR directed against the CNS self-antigen myelin basic protein (MBP) are competent in the MWM, whereas D011.10 mice with TCR directed against irrelevant ovalbumin-antigen are not[4]. While there are several major caveats to both sets of interpretations (discussed in Chapter 9), they do raise the intriguing possibility that resident T cells of the meninges and choroid plexus are specific to CNS self-antigens.

This study aimed to address elements of a model in which resident T cells in the meninges are preferentially specific to CNS self-antigens. Specifically we asked two questions, what are the dynamics of T cells in the meninges, and is there any preference in the meninges for CNS self-antigen specific T cells.

Importantly, while these questions were generated in the context of the role of T cells in spatial learning and memory, they are also broadly applicable to fundamental neuroimmunology. The meninges has been repeatedly implicated in the etiology of experimental autoimmune encephalitis (EAE), the mouse model for human multiple sclerosis, in which lymphocyte accumulation in the meninges is thought to precede infiltration into the parenchyma and in which the deep cervical lymph nodes that drain CNS antigens have been shown to play a role in disease onset[25, 320, 321]. In short, findings from this study on the dynamics and antigen specificity of T cells in the meningeal space not

only address a learning and memory paradigm, but also provide valuable insight into important processes of basic neuroimmunology.

Materials and methods:

Animals: Adult (C57BL6, Ly5.1, Thy1.1, OTII, 2D2, MHCII$^{-/-}$) mice were purchased from the Jackson Laboratory and housed in temperature and humidity controlled rooms with 12 hour light/dark cycle and standard diet ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia.

Behavior: Morris Water Maze experiments were conducted in a 100cm diameter pool, maintained between 21 and 22°C. Non-toxic washable tempera paint was added to conceal a 10cm wide platform submerged 1cm below the water line. Bold visible signs were placed on the walls of the testing room (but within 100cm of the tank center) to serve as visual navigation cues, and a curtain was placed between the tank and the experimenter to minimize use of the experimenter as a visual cue. On Day1 mice were placed at a random point along the periphery of the pool and given up to 60 seconds to find the escape platform. If the mouse failed to find the platform within 60 seconds the mouse was lifted and placed onto the platform by the experimenter for a total of 30 seconds for Day1, and 10 seconds for subsequent days. This procedure was repeated for a total of three "acquisition" trials, each trial starting from a unique position along the pool's perimeter, and each mouse given 20-30 minutes of rest between trials. This procedure was carried out for four consecutive days, in a phase collectively termed "acquisition". On the day following the last acquisition, the platform was removed and mice were given a single trial lasting 60s without any available escape. The following day the platform is reintroduced to a novel quadrant of the pool, and mice were again given 3 trials per day for two consecutive days in order to find the escape platform in the "reversal" phase of the MWM. All data was recorded using EthoVision tracking system.

FACS: Mice were thoroughly perfused with heparinized PBS for 5 minutes. Tissues were gently disassociated against and passed through 70μM nylon screens in FACS buffer (PBS+2%BSA+1mM

EDTA). Single cell suspensions were blocked with mouse IgG then stained for extracellular markers (eBiosciences) at 1:200 for 30 minutes, washed thoroughly and resuspended in 300uL FACS buffer. Blood and spleen samples were incubated in RBC lysis buffer, and washed prior to blocking and staining. Dissection of the meninges was carried out as described by Derecki et al [100]. All samples were run on ADP Cyan (Dako) and analyzed with Flowjo (Tree Star).

Parabiosis and adoptive transfer: Mice were anesthetized to full muscle relaxation with xylazine and ketamine HCl (1.67mg per 10g of body weight) by intraperitoneal injection. The corresponding lateral aspects of each mouse were shaved and an incision was made from the olecranon to the knee joint of each mouse, bluntly dissecting the subcutaneous fascia to create 0.5cm of free skin on each side of the incision. The olecranon and knee joints are attached by a single 2-0 silk suture to reduce direct pulling on the sutures of the skin. The dermis of the parabiotic partners are pushed together (excluding epidermal layers in the junction of the dermis) and closed with sutures. Post surgery, systemic analgesics (Ketoprofen 2-5 mg/kg q 24h) were used along with antibiotics (sulfamethaxazole) in the drinking water. All parabiotic pairs (success/survival rate was close to 100%) were sacrificed after 15 days, disjoined, and separately perfused. In adoptive transfer experiments, single cell suspensions of $2.5x10^5$ RBC lysed donor splenocytes derived from Thy1.1 mice were transferred *i.v.* into recipient animals in 200uL PBS.

Cervical lymph node resection and sham surgery: Eight-week-old mice were anesthetized with ketamine/xylazine, shaved at the neck and cleaned with iodine and 70% ethanol and an ophthalmic solution put on the eyes to prevent drying. An incision was made midline 5 mm superior to the clavicle. The SCM muscle was retracted, and the deep cervical lymph node removed with forceps. Sham mice received the incision and had their SCM retracted, but did not have their lymph node removed. Mice were then sutured and allowed to recover on a heating pad until responsive. Post surgery, mice were given analgesic to the drinking water: 50 mg/l for 3 days post surgery and 0.16mg for the next 2 weeks.

Ova immunization: 10 week old OTII.Rag1$^{-/-}$ mice were immunized subcutaneously on the right and left sides of the neck and back with either 100uL of 1mg/mL ovalbumin in saline emulsified 1:1 in incomplete Freund's adjuvant, or 100uL of 1:1 saline: incomplete Freund's adjuvant as control. All mice were sacrificed 15 days post-immunization and single cell suspensions of tissues were subject to FACS analysis.

EAE induction: 100ug of MOG emulsified in complete Freund's adjuvant was injected subcutaneously into B6 recipient mice, with i.p. pertussis toxin administration at day 0 and 1 post-MOG injection. Disease severity was scored as follows. 0. No clinical signs, 1. Tail paralysis 2. Ataxia 3. Partial hindlimb paralysis, 4. Total hindlimb paralysis. 5. Moribund/death

Results:

To study the dynamics of CD4 T cells in the meninges we employed a parabiotic mouse model, enabling us to characterize the relative intra-tissue turnover rate of the CD4 T cell compartment. By day 15, post-parabiotic surgery, CD4 T cells equilibrate across the blood and most lymphoid tissues; however, their turnover in the meninges (and to a lesser extent in the CNS-draining dCLN) was reduced (Figure 10.1 and 10.2), suggesting that there is a constraint on the entry of CD4 T cells into the meninges.

In an attempt to validate this constraint on entry in an adoptive transfer model, we transferred wild-type Thy.1 splenocytes into OTII mice (Figure 10.3). We predicted that given the low CD4 T-cell turnover in the meninges, seeding by transferred wild-type T cells would occur more slowly here than in other tissues. Surprisingly however, the meninges were the most receptive of all the analyzed tissues, with the largest percentage of their total CD4 T cells originating from transferred Thy1.1 splenocytes (Figure 10.3). We interpreted this result on the most basic level to mean the meninges is not globally restrictive to CD4 T-cell entry and residence.

Polyclonal wild-type T cells are known to expand homeostatically under conditions of lymphopenia or low clonal diversity (reviewed in Chapter 5). To determine whether homeostatic expansion could drive T cells from the blood into the meninges, we connected OTII transgenic mice parabiotically with wild-type partners (Figure 10.4). Here, as in the adoptive transfer model, the OTII mouse meninges accepted impressive numbers of donor CD4 T cells from the blood. While this shows that homeostatically proliferating T cells gain access to the peripheral tissues it stops short of identifying the accompanying changes in expression of homing receptors and function that endow homeostatically proliferating T cells with the capacity to migrate to non-lymphoid tissues.

,Whereas 'naive' CD44^{-} T cells equilibrated relatively evenly between parabiotic partners, CD44^{hi} 'activated/memory' T cells did not (Figure 10.5). While naïve T cells likely become CD44hi as a consequence of homeostatic proliferation after moving into the OTII host, these same cells do not appear to efficiently recirculate *back* into the Ly5.1 partner. Not only can this explain the lack of T-cell accumulation in the meninges of the wild-type partner, but also serves as a cautionary tale concerning a major assumption of the parabiotic model, namely that the unified circulatory system guarantees homogenous chimerism of cells in the blood. In fact, mixing of CD11b monocyte populations was also severely diminished (Figure 10.6), suggesting either that some cell types cannot pass through the unified capillary junctions or that they extravasate directly into the site of surgical injury. While the mechanism behind this dramatically attenuated blood chimerism have not been directly tested, a recent study has observed the same phenomenon of restricted monocyte chimerism between the blood of parabiotic partners [322].

Both the uniquely slow T-cell turnover rate in the wild-type meninges and the increased seeding of the meninges of OTII mice by wild-type T cells was also apparent in the dCLNs (Figure 10.1- 10.4). To explore possible coupling of T cells between the meninges and dCLNs, and to find out how interruption of this coupling might affect T-cell egress from the meninges, we surgically resected dCLNs of wild-type mice. Two weeks post-surgery, the numbers of CD4 T cells in the meninges of operated mice were substantially increased (10.7a). To find out what effect, if any, dCLN resection might have on behavioral outcomes, we subjected resected and sham-operated mice to the Morris water maze behavioral task, and observed significant spatial learning and memory impairment in the reversal phase in the dCLN-resected group (Figure 10.7b).

Ly5.1-OTII parabiotic pairings showed that ova-specific OTII T cells were capable of seeding the Ly5.1 (WT) meninges (Figure 10.4), suggesting that entry and retention in the meningeal space may not be an antigen specific, MHCII mediated phenomenon. To continue scrutinizing the model

in which CD4 T cells specific for CNS-self antigen preferentially traffic to the meningeal space, we performed an additional parabiotic pairing of Ly5.1 to TCR transgenic 2D2 homozygous mice. 2D2 homozygous animals have near monoclonality for T cells directed against residues 35-55 of the myelin oligodendrocyte glycoprotein (MOG) antigen[323]. We hypothesized that if T cells specific to CNS-self antigen preferentially traffic to, or are preferentially retained within the meninges, then 2D2 T cells would be overrepresented in the Ly5.1 meninges of parabiotic partner mice. However, this was not the case, as the representative experiment shows 28.7% of the meninges of Ly5.1 mice were reconstituted with 2D2 T cells, which was comparable to both the 26.6% and 25.6% reconstitution observed when Ly5.2 and OTII T cells entered the meninges of Ly5.1-Ly5.2 and Ly5.1-OTII parabiotic pairings respectively (Figure 10.8a). Importantly, the percent blood chimerism between Ly5.1-Ly5.2, Ly5.1-OTII, and Ly5.1-2D2 parabiotic pairs was not significantly different (Figure 10.8b).

To continue testing of the antigen-independence of T cell entry into the meningeal space, we parabiotically connected Ly5.1 to MHCII$^{-/-}$ partner mice. We hypothesized that if TCR and MHCII-peptide complex interactions were necessary for access to the meningeal space, then Ly5.1 T cells would not seed the MHCII deficient meninges of MHCII$^{-/-}$ mice in the parabiotic pairing. Conversely, if TCR and MHCII-peptide complex interactions were not necessary for trafficking into the meningeal space, then we would observe the entry of T cells into the MHCII$^{-/-}$ meninges in numbers comparable to the Ly5.1-Ly5.2 parabiotic pairing. Indeed, we provide evidence for this latter scenario, as approximately 800 Ly5.1 T cells entered the meninges of MHCII$^{-/-}$ animals, roughly the same number that penetrated the Ly5.1 meninges in Ly5.1-Ly5.2 parabiotic pairings, suggesting again that the phenomenon of T cell entry into the meninges was not dependent on antigen specificity, or the presence of MHCII in the meningeal space (Figure 10.9).

Whereas it appeared that T cell entry into the meninges was not dependent on antigen specificity and MHCII, it was unclear if trafficking was dependent on the "activation status" of T cells.

Indeed, the preferential entry of wild-type T cells into the meninges of OTII recipients (Figure 10.3 and 10.4), might be suggestive of activation status dependent trafficking, as homeostatically expanding T cells have a memory phenotype and express CD44 (Chapter 5). To address the possibility that memory T cells can preferentially traffic to the meningeal space, we analyzed the meningeal T cell repertoire of OTII transgenic mice. Specifically, we exploited a unique feature of the OTII mouse model, namely post-thymic T cell receptor (TCR) rearrangement. In models bearing specific TCR-β chains (including Vβ5 of OTII mice), a process of extrathymic Rag re-expression and fresh VDJ recombination as well as intrathymic receptor editing occurs at low frequency[131, 324]. In 10-week-old OTII mice this process of extrathymic Rag re-expression results in ~10–15% of the formerly monoclonal TCR repertoire to become $CD44^+$ memory T cells bearing an additional set of endogenous TCRs (Figure 10.10). In contrast, OTII.$Rag^{-/-}$ mice (lacking endogenous TCR rearrangement owing to Rag1 ablation) remain purely monoclonal, with near-perfect 1:1 surface expression of Vα2/Vβ5 TCR chains (Figure 10.11). The meninges of 10 week old OTII mice showed more than a fourfold enrichment of the $CD44^+$ endogenous TCR subset relative to CD4 T cells in the blood (Figure 10.10), consistent with the notion that T cell traffic to the meninges is correlated with the memory status of T cells.

While the preferential accumulation of $CD44^+$ postrevisional T cells in the meninges of OTII mice shows a correlation between memory status and meningeal trafficking, it does not rule out the possibility that the postrevisional cells in the meninges are reactive to CNS self-antigens. To test whether memory T cells of irrelevant antigenic specificity can traffic to the meninges, we immunized 10-week-old OTII.$Rag1^{-/-}$ mice subcutaneously on the right and left sides of the neck and back with either 100uL of 1mg/mL ovalbumin in saline emulsified 1:1 in incomplete Freund's adjuvant, or 100uL of 1:1 saline: incomplete Freund's adjuvant as control. Fifteen days following immunization we observed a preferential accumulation of $CD44^+$ memory OTII.$Rag1^{-/-}$ T cells to the lung, kidney and

meninges of immunized mice (Figure 10.12 and 10.13), re-affirming the well-known fact that $CD44^{+}$ memory T cells traffic to peripheral tissues, and suggesting that entry to the meninges, like the other peripheral tissues, is predicated on memory phenotype. Interestingly, the proportion of T cells expressing CD44 in ova immunized mice was highest in the meninges, and was uniquely observable in the meninges of IFA treated mice, which together with a preferential accumulation of $CD44^{hi}$ postrevisional cells in the meninges of OTII mice, suggests that the memory phenotype requirement for either entry or retention in the peripheral tissues may be more stringent in the meninges than in other tissues.

These studies are the first to characterize dynamics of T cells within the meninges, a poorly understood tissue with profound influences on brain processes, immune surveillance, and neuropathology. Specifically, we show that CD4 T cells of the meninges exhibit a slow egress relative to other peripheral tissues, and are tightly coupled to the meninges draining deep cervical lymph node. While the normal meninges contains a relatively small number of CD4 T cells, we show that the meninges is not intrinsically restrictive to T cell entry, as homeostatically proliferating T cells infiltrated the meninges in large numbers. Importantly, we show that T cell homing to the meninges is not directly dependent antigen-specificity but rather intimately correlated with CD44 memory phenotype, an observations with important implications for immune surveillance of the CNS. A more thorough and comprehensive discussion of these results are provided in Chapter 12.

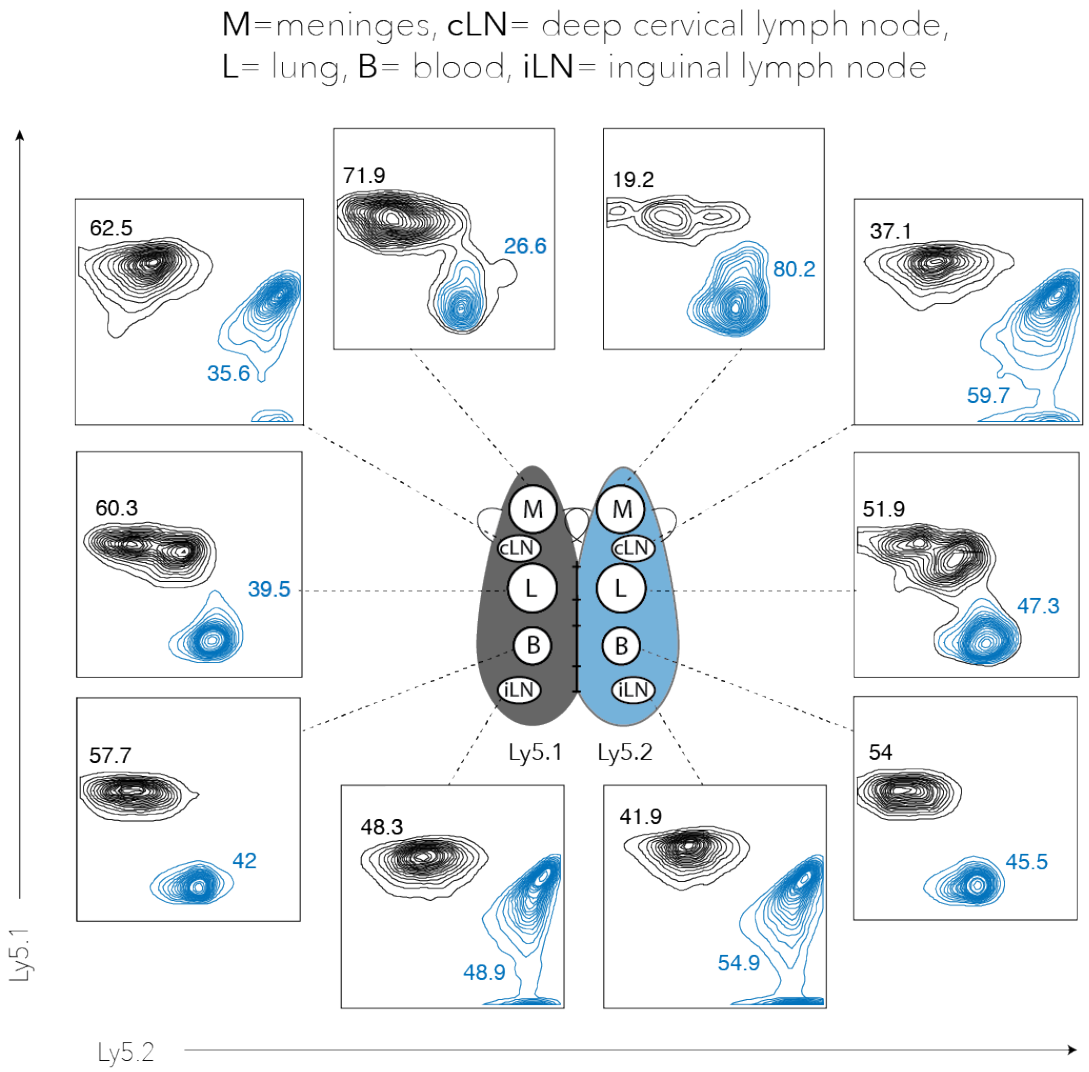


Figure 10.1: Representative experiment of percent CD4 T cell chimerism in the Ly5.1-Ly5.2 parabiotic pairing. Ly5.1 (black) and Ly5.2 (blue) mice were parabiotically joined. After 15 days, each mouse was separately perfused with PBS and the indicated tissues were analyzed for self and donor CD4 T cells by FACS. Data shows reduced T cell chimerism in the meninges relative to the lung, and in the deep cervical lymph node relative to the inguinal lymph node. Representative of *n*=4 pairs.

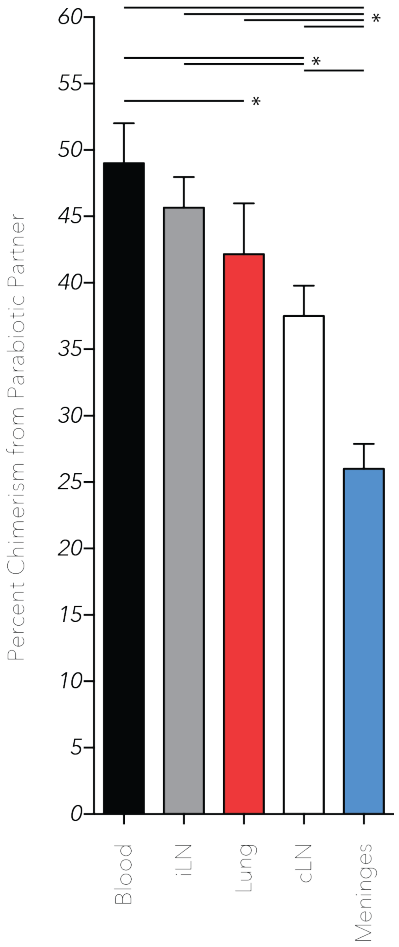


Figure 10.2: Percent CD4 T cell chimerism in the Ly5.1-Ly5.2 parabiotic pairing. Ly5.1 and Ly5.2 mice were parabiotically joined. After 15 days, each mouse was separately perfused with PBS and the indicated tissues were analyzed for percent CD4 T cell chimerism from partner parabiotic mice by FACS. n=4 pairs. *P<0.05; one-way ANOVA with Tukey's multiple comparison test.

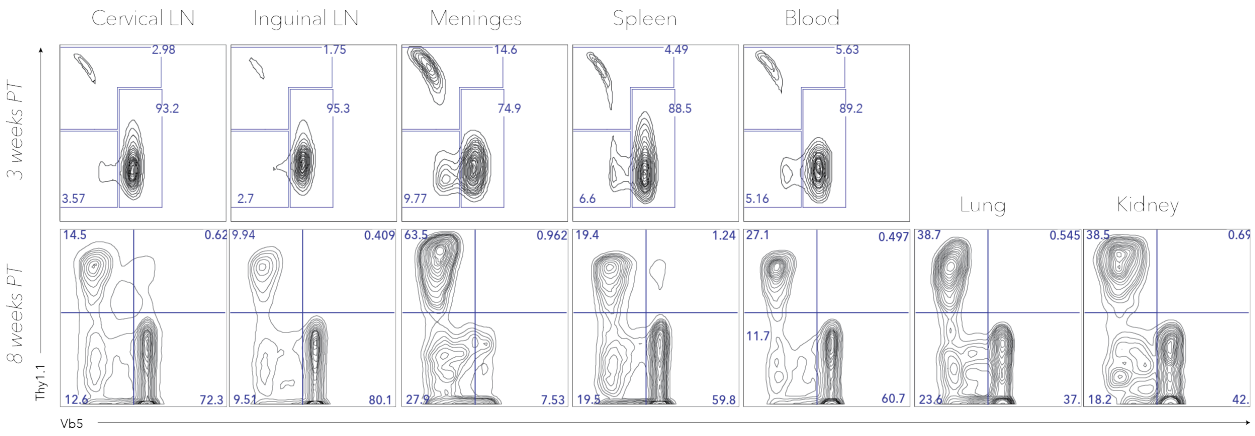


Figure 10.3: Percent Thy1.1 CD4 T cell chimerism following adoptive transfer into OTII recipients. $2.5x10^5$ Wild-type (Thy1.1) splenocytes were adoptively transferred intravenously into OTII recipients and the indicated tissues were analyzed for donor $CD4^+$ T cells 3 and 8 weeks post-transfer. Data illustrates preferential CD4 T cell chimerism in the meninges relative to lung and kidney, and in the cervical lymph node relative to the inguinal lymph node.

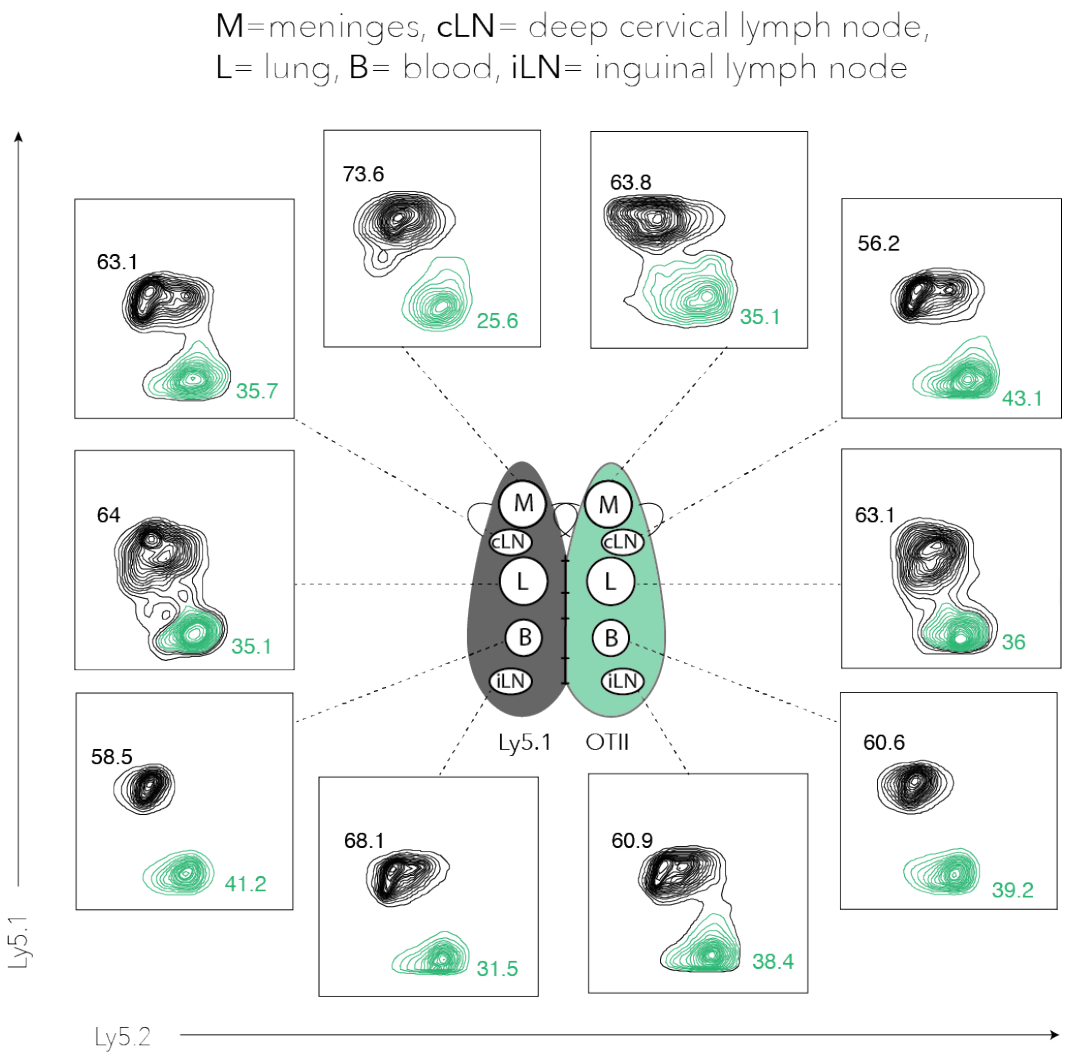


Figure 10.4: Percent CD4 T cell chimerism in the Ly5.1-OTII parabiotic pairing. Ly5.1 (black) and OTII (green) mice were parabiotically joined. After 15 days, each mouse was perfused with PBS and the indicated tissues were analyzed for self and donor $CD4^+$ T cells by FACS. Data illustrates significant chimerism of wild-type T cells in the meninges of OTII mice. Representative of n=3 pairs

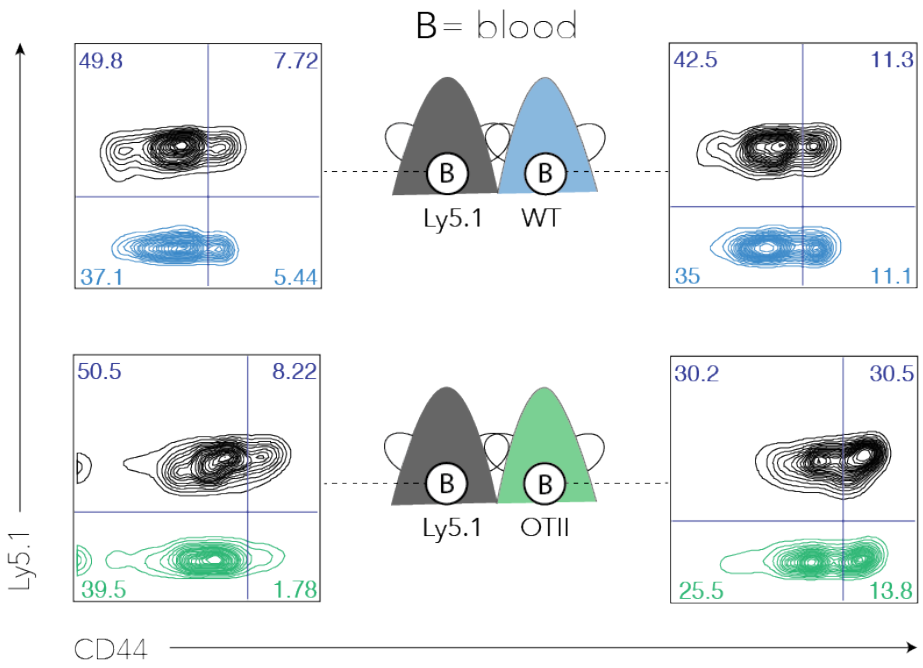


Figure 10.5: Unequal chimerism of CD44^{hi} CD4 T cells between the blood of parabiotic pairs. CD44 memory marker analysis of CD4^{+} T cells from the blood of the indicated parabiotic pairings that were joined for 15 days, showing incomplete homogenization of CD44^{+} T cells between the blood of partner mice. Ly5.1-Ly5.2; Representative of n=4 pairs, Ly5.1-OTII; representative of n=3 pairs

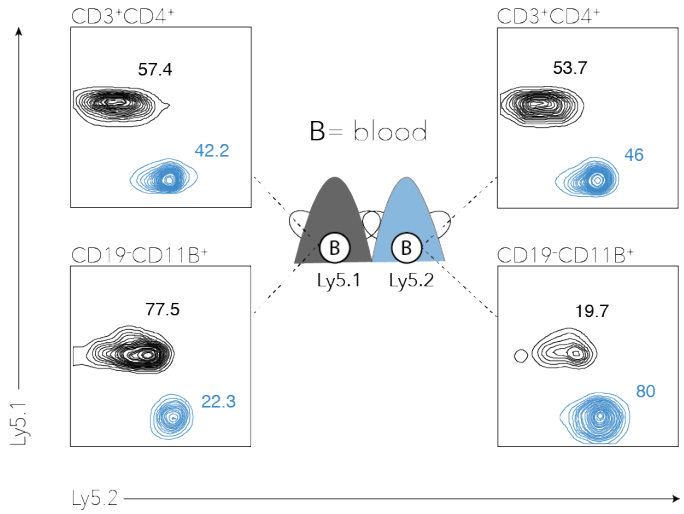


Figure 10.6: Unequal chimerism of CD11B monocytes between the blood of parabiotic pairs. FACS analysis of $CD11B^+/CD19^-$ populations (lower plots) or $CD3^+/CD4^+$ (upper plots) from blood of Ly5.1(left) and ly5.2 (right) mice that were parabiotically joined for 15 days, showing limited monocyte exchange between partners (lower plots). Representative of *n*=4 pairs

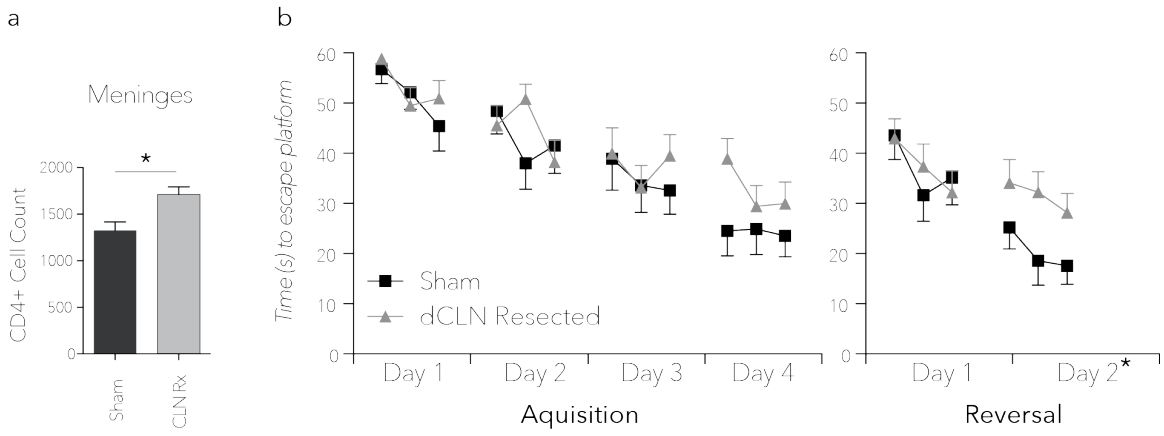
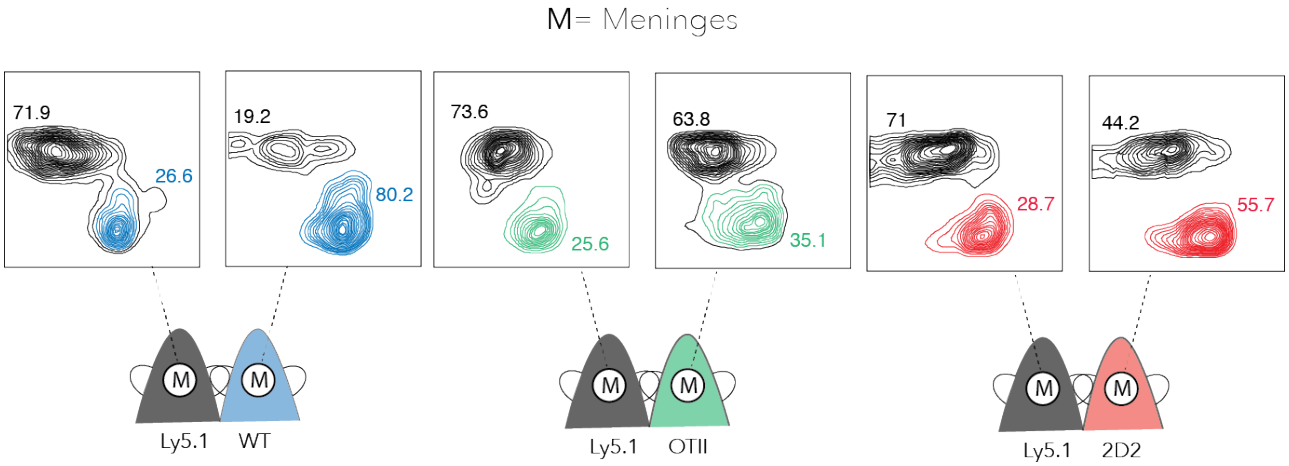


Figure 10.7: Meningeal CD4 T cell numbers and MWM performance following surgical resection of the deep cervical lymph nodes. Total meningeal $CD4^+$ T-cell numbers (a) of dCLN-resected or sham-operated mice 14 days post-surgery showing increased T cell numbers in the meninges of dCLN resected animals, (n=3 mice per group; $^*P$<0.05; Student's t-test). (b) Morris water maze acquisition (left) and reversal (right) of dCLN-resected or sham-operated mice 14 days post-surgery, showing impaired MWM performance following dCLN resection (combined data from three independent experiments; sham, n=16 mice/group; dCLN resection, n=22 mice per group; $^*P$<0.05; two-way repeated measures analysis of variance).

a



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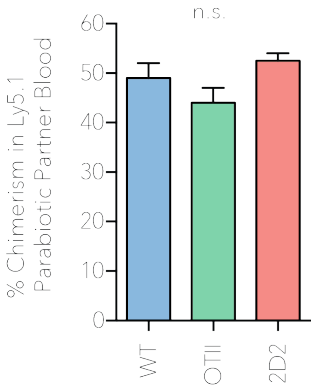


Figure 10.8: Percent CD4 T cell chimerism in the meninges of various parabiotic pairings. Ly5.1 (black), Ly5.2 (blue), OTII (green) and 2D2 (red) mice were parabiotically joined in the indicated pairings. After 15 days, each mouse was separately perfused with PBS and the meninges (a) and blood (b) were analyzed for self and donor $CD4^+$ T cells by FACS , showing comparable entry of WT, OTII and 2D2 T cells into the meninges of Ly5.1 mice (a), without significantly different blood chimerism in respective Ly5.1 partners (b). Representative of *n*=3-4 pairs.

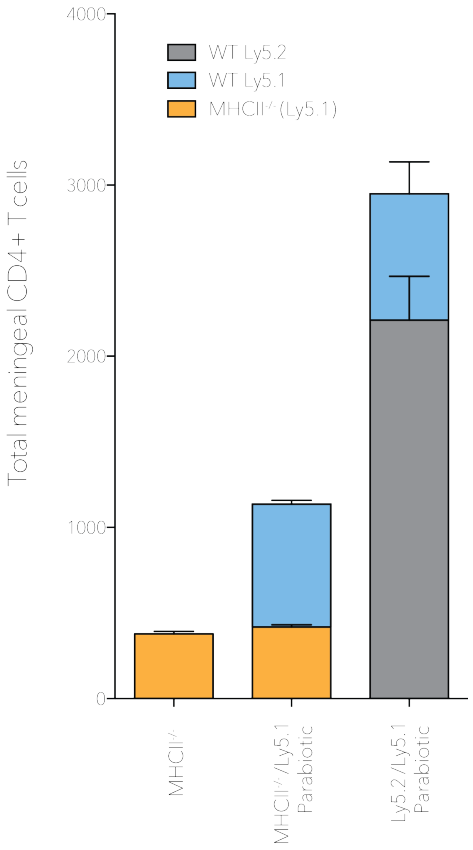


Figure 10.9: Wild-type CD4 T cell chimerism in the meninges of recipient mice is independent of recipient MHCII expression. Total number of isolated $CD4^+$ T cells per meninges from: $MHCII^{-/-}$ mice (n=3), $MHCII^{-/-}$ mice parabiotically joined with Ly5.1 partners for 15 days (n=3), and Ly5.2 mice parabiotically joined with Ly5.1 mice for 15 days (n=4). The data show comparable entry of numbers of Ly5.1 T cells into $MHCII^{-/-}$ and Ly5.2 host meninges in parabiotic partners.

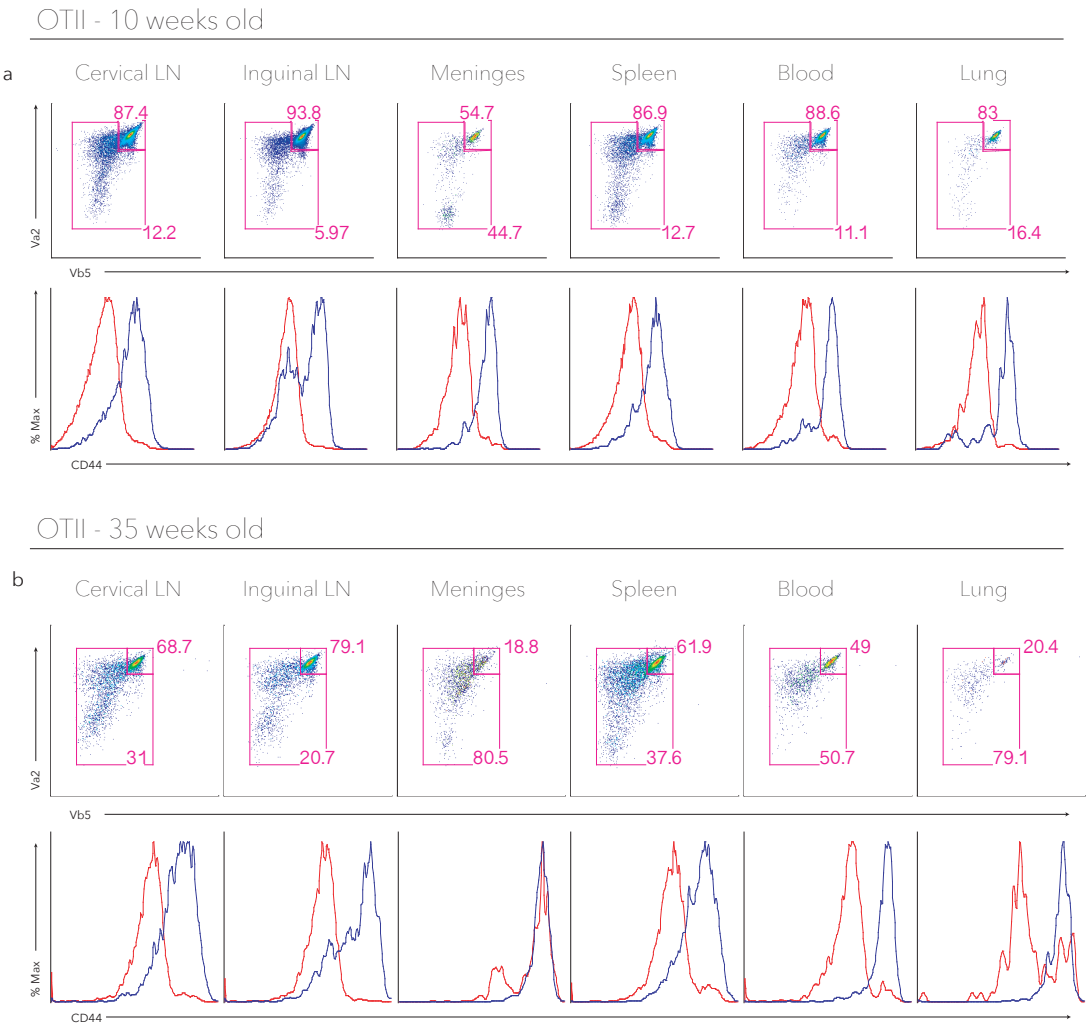


Figure 10.10: Distribution and CD44 expression profile of transgenic and dual TCR bearing endogenous CD4 T cells in the tissues of OTII mice at two ages. a-b: Upper panels; FACS analysis of CD4 gated T cells analyzed for the expression of OTII transgenic TCR chains in the indicated tissues of 10-week-old (a) and 35-week-old (b) mice. Upper right gate encompasses the bulk of monoclonal transgenic T cells ($V\beta5^{hi}/V\alpha2^{hi}$), while the larger leftmost gate represents the population of T cells bearing endogenous TCRs. a-b: Lower panel; CD44 expression on transgenic OTII (red) and endogenous "postrevisional" dual TCR bearing $CD4^+$ T cells (blue) in the indicated tissues of OTII mice. Data illustrates preferential accumulation of $CD44^{hi}$ dual TCR bearing CD4 T cells in the meninges of OTII mice relative to lung, and in the dCLN relative to the iLN, a trend that increases with age.

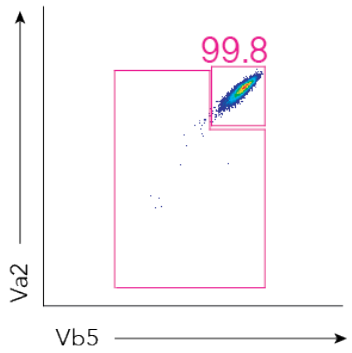


Figure 10.11: Near total monoclonality of CD4 T cells in OTII mice on the Rag1$^{-/-}$ background. Transgenic TCR usage in splenic CD4^{+} T cells of OTII.Rag1$^{-/-}$ mice showing 1:1 Vβ5:Vα2 expression, indicative of pure monoclonality of the CD4 compartment.

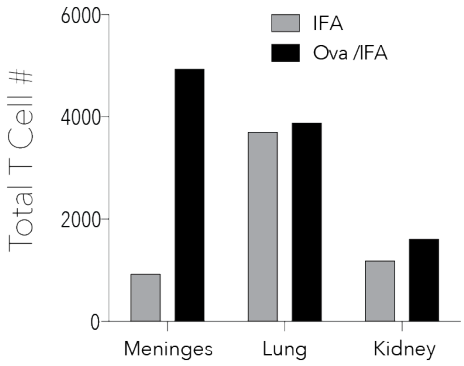


Figure 10.12: T cell accumulation in the tissues of OTII.Rag1$^{-/-}$ mice immunized s.c. with ovalbumin.

Total T cell numbers recovered from the indicated tissues of OTII.Rag1$^{-/-}$ mice immunized s.c. with ova/IFA (black) or IFA alone (gray) 15 days prior, showing preferential accumulation of T cells in the meninges. Representative of *n*=4 mice

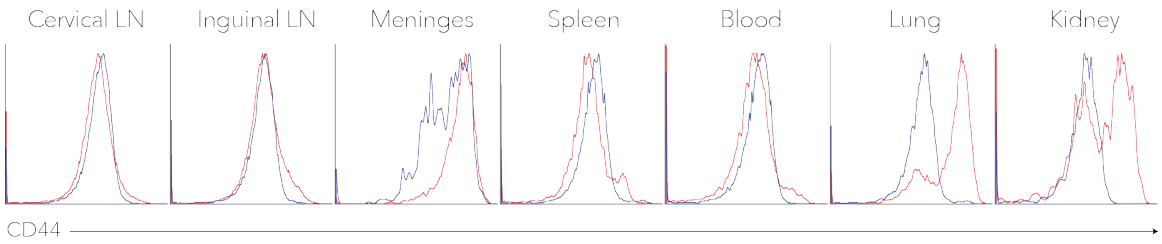


Figure 10.13: CD44 expression profile of T cells in the tissues of OTII.Rag1$^{-/-}$ mice immunized s.c. with ovalbumin. FACS analysis of CD4^{+} T cells from OTII.Rag1$^{-/-}$ mice immunized s.c. with ova/IFA (red) or IFA alone (blue) 15 days prior to analysis, showing preferential accumulation of CD44^{+} OTII.Rag1$^{-/-}$ T cells in the peripheral tissues and meninges in particular. Representative of *n*=4 mice

II. RESULTS

CHAPTER 11: BRAIN ANTIGEN-REACTIVE CD4 T CELLS ARE SUFFICIENT TO SUPPORT LEARNING BEHAVIOR IN MICE WITH LIMITED T CELL REPERTOIRES

Genetic ablation of T cells leads to impairment of spatial learning and memory in mice, while adoptive transfer of whole splenocytes rescues the impairment. However, the precise lymphocyte sub-population mediating the phenomenon had still been uncertain. Using several transgenic mouse models in combination with adoptive transfers, we evidence the *necessity* of an antigen-specific CD4 T cell compartment in normal spatial learning and memory in mice. Specifically, we show that B cell deficient mice are competent learners in the MWM paradigm, and that MHCII deficient animals that are severely deficient in CD4 but not CD8 T cells, are impaired learners[325]. Importantly a purified population of WT CD4 T cells was sufficient to mediate rescue of learning impaired mice. We go on to show that a monoclonal T cell population reactive to the central nervous system (CNS) antigen, myelin oligodendrocyte glycoprotein (MOG), was sufficient to improve cognitive task performance in otherwise impaired OTII mice, and was correlated with the long-term infiltration of a GR1 bearing MDSC in the meninges. A large portion of these studies have been published in *Brain Behavior and Immunity* by Radjavi et al[326].

Introduction and project rationale:

T cell deficient mice have consistently been observed to demonstrate impaired spatial learning and memory (reviewed in Chapter 9). While reconstitution of SCID and nude mice with wild-type splenocytes significantly rescues MWM measured learning and memory deficits, several fundamental questions about both the nature of the baseline behavioral impairment of lymphopenic mice, as well as their rescue by adoptive transfer, remain unanswered. Specifically, it is still unclear what splenocyte population (i.e. CD4 vs. CD8) is necessary for both normal cognitive task performance and the rescue of aberrant task performance in lymphopenic animals. Additionally, while memory status, and not antigen specificity, appears to be necessary for T cell trafficking to the meninges (Chapter 10), it is still unclear what, if any, antigen specificity requirement exists for the mediation and rescue of normal and impaired spatial learning and memory respectively.

Studies by Brynskikh[82], Ron-Harel[294], and Derecki et al[100] have shown that adoptive transfer of wild-type splenocytes can rescue the MWM impairment of lymphocyte deficient mice. While Derecki et al have shown that IL-4 deficient T cells cannot mediate rescue[100], and Cohen et al have shown that treatment of splenocytes with anti-CD3 antibody prior to adoptive transfer abrogates rescue in a mouse model of PTSD[1], it is still unclear whether CD4 T cells, CD8 T cells or both are required for either the baseline MWM performance or the rescue of impaired lymphopenic performers. This chapter hones in on the relevant lymphocyte population by showing that B cell deficient mice are competent learners in the MWM paradigm, and that MHCII deficient animals that are severely deficient in CD4 T cells owing to ablation of CD4/MHCII interactions while their CD8 T cell compartment remains functionally intact, are impaired learners[325]. Finally we show that a purified population of WT CD4 T cells are sufficient to mediate rescue of learning impaired mice–collectively suggesting that CD4 T cells are necessary for normal spatial learning and memory behavior, and that CD4 T cells are sufficient to rescue the impairment of functionally lymphopenic animals.

At least two groups have advocated a model in which the T cells that mediate higher brain function are directed against CNS self-antigens (Chapter 9). In support of this model Ziv et al show that transgenic mice with TCR directed against the CNS self-antigen myelin basic protein (MBP) are competent in the MWM, whereas D011.10 mice with TCR directed against irrelevant ovalbumin antigen are not[4]. Importantly, their interpretation is based on the assumption that the two transgenic strains are monoclonal, and that the learning differential between these two mice is attributable to T_{MBP} and T_{OVA} T cells. However, the T_{MBP} transgenic mice employed in their studies are by no means monoclonal. Indeed, approximately 10% of T cells in the T_{MBP} TCR transgenic mouse bear endogenous TCRs[134, 135]. Therefore it is unclear whether MWM competency is being mediated by T_{MBP} transgenic T cells or by the endogenous T cell pool.

In this study we obviate the limited interpretability of studies by Ziv et al, by adoptively transferring T cells directed against CNS self-antigen into MWM impaired OTII mice. While this scheme does not address the model in which T cells directed to CNS self-antigens endogenously mediate higher brain functions, it does show that a monoclonal population of T cells directed against CNS self-antigen can *rescue* MWM behavior in otherwise impaired mice.

There have been two general experimental observations in the study of T cell influence on higher brain function. One, that lymphopenic mice have impaired spatial learning and memory, and two, that impairment can be rescued by the transfer of a wild-type splenocyte population (reviewed in Chapter 9). Understandably, both observations have been viewed as manifestations of a single phenomenon, that is, the specific function of T cells in learning and memory is lost in lymphopenic mice, and the same function is restored following adoptive transfer. However, this chapter presents data that challenges this basic assumption, raising the possibility that T cells may rescue MWM impairment by a different mechanism than they use to achieve baseline MWM competency. Specifically, we show here that the common denominator in each successful MWM rescue paradigm

is not the presence of CD4 T cells in the meninges, but rather the emergence of a new $GR1^+$ $CD11b^+$ myeloid population in the meningeal space, whose identity and functions are discussed in Chapter 12.

Materials and methods:

Animals: Male adult mice on the C57BL background were purchased either from the Jackson Laboratory (C57BL/6J, OTII, μMT, $Rag2^{-/-}$, $MHCII^{-/-}$), or Taconic (OTII.Rag1) and housed in temperature and humidity controlled rooms with 12 h light/dark cycle, with standard diet ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia.

Behavior: Morris water maze experiments were conducted in a 100 cm diameter pool, maintained between 21 and 22 ˚C. Non-toxic washable tempera paint was added to conceal a 10 cm wide platform submerged 1 cm below the water line. Bold visible signs were placed on the walls of the testing room (but within 100 cm of the tank center) to serve as visual navigation cues, and a curtain was placed between the tank and the experimenter to minimize use of the experimenter as a visual cue. On day1 mice were placed at a random point along the periphery of the pool and given up to 60 s to find the escape platform. If the mouse failed to find the platform within 60 s the mouse was lifted and placed on the platform by the experimenter for a total of 30 s for Day1, and 10 s for subsequent days. This procedure was repeated two more times, for a total of three "acquisition" trials, each trial starting from a unique position along the pool's perimeter, and each mouse given 20–30 min of rest between trials. This procedure was carried out for four consecutive days or until wild type mice reached the platform within 20 s, in a phase collectively termed "acquisition". On the day following the last acquisition, the platform was removed and mice were given a single trial lasting 60 s without any available escape. The following day the platform is reintroduced to a novel quadrant of the pool, and mice were again given 3 trials per day for 2–3 days in order to find the escape platform in the "reversal" phase of the MWM. All data was recorded using EthoVision tracking system, statistical analysis was performed with repeated measures ANOVA and the Bonferroni/Tukey post hoc test. All groups were blinded to the

experimenter and run in shuffled orders. uMT MWM experiment was conducted once, all other experiments were repeated 2–3 times.

FACS: Mice were thoroughly perfused with heperinized PBS for 5 min. Tissues were passed through 70 μM nylon screens and single cell suspensions were stained for extracellular markers (eBiosciences). Dissection of the meninges was carried out as described by Derecki et al. (2010). All samples were run on ADP Cyan (Dako) and analyzed with Flowjo (Tree Star).

Adoptive transfer: In adoptive transfer experiments, cultured T cells, negatively selected CD4 T cells or single cell suspensions of RBC lysed Donor splenocytes, were transferred i.v. into recipient animals in 200 μL PBS. MOG specific CD4 T cells were a gift from Avraham Ben-Nun (Weizmann Institute of Science, Israel) and were cultured as described previously (Krishnamoorthy et al., 2009).

Results:

Previous studies have shown impaired learning and memory in lymphocyte deficient SCID, nude, and Rag1 knockout mice[2, 4, 81, 82]. To reinforce the conviction that this behavioral phenotype is caused by lymphocyte deficiency, and to eliminate confounding effects of Rag1 expression in the brain[76-78] on the cognitive impairment displayed in the MWM, we tested $Rag2^{-/-}$ knockout mice in the MWM paradigm. Consistent with SCID, nude and $Rag1^{-/-}$ mice, $Rag2^{-/-}$ showed a significant impairment in both the acquisition and reversal phase of the MWM (Figure 11.1a and 11.1b). In line with previous observations[4, 82], the major difference between the groups was detected on the second day of the reversal phase (Figure 11.1b). While Rag1 deficient mice have lymphocyte independent cognitive phenotypes (Chapter 3) they too are impaired in the MWM paradigm (Figure 11.1c).

Splenocytes have been shown to improve learning behavior of immune deficient mice, however the role of B cell deficiency has not yet been addressed or ruled out. Importantly, the MWM impairment in $Rag2^{-/-}$ mice is not attributable to B-cell deficiency as B cell-deficient (μMT) mice demonstrated latencies comparable to their wild type counterparts (Figure 11.2), suggesting that the relevant lymphocytes are contained within the T cell compartment.

To differentiate between the contributions of CD4 and CD8 T cells, we repeated the above experiments using MHCII knockout mice. A flow cytometric gating strategy (Figure 11.3) was utilized to demonstrate the severe deficiency of $MHCII^{-/-}$ mice in the CD4 T cell compartment owing to ablation of CD4/MHCII interactions[325], while their CD8 T cell compartment remains intact (Figure 11.4a and b). As can be seen in Figure 11.4, the ablation of CD4 T cells in the MHCII-/- mouse is dramatic but not absolute. Indeed The presence of a small remaining CD4 T cell population was described in the original report on $MHCII^{-/-}$ mice[327], though their origin and function are not entirely certain[328-330]. Nevertheless $MHCII^{-/-}$ have a dramatic and systemic reduction of CD4 T cell

numbers., and the significant impairment of MHCII$^{-/-}$ mice in the MWM task compared to matched wild type controls (Figure 11.5), suggests that CD4 T cells are required for normal task performance.

Adoptive transfer of CD4 T cells isolated from OTII/Rag1$^{-/-}$ donors into Rag1$^{-/-}$ recipients led to partial reconstitution of splenic but not meningeal T cells, whereas adoptive transfer of CD4 T cells derived from wild type donors resulted in substantial reconstitution of both splenic and meningeal T-cell compartments (Figure 11.6). Importantly the number of T cells in the meninges of Rag1$^{-/-}$ mice reconstituted with WT T cells exceeds the number found in the normal wild-type animal by several orders of magnitude. This differential is a consequence of homeostatic proliferation of WT T cells in the Rag1$^{-/-}$ host (OTII T cells do not homeostatically expand in lymphopenic hosts[152]) and its implications are discussed in Chapter 12. Finally, we showed that the MWM performance of Rag1$^{-/-}$ mice could be partially rescued after adoptive transfer of negatively selected CD4 T cells derived from wild-type but not with those from OTII/Rag1$^{-/-}$ donor mice (Figure 11.7), collectively suggesting that polyclonal donor CD4 T cells are sufficient for both meningeal homing and mediation of MWM performance in otherwise impaired mice.

To address the antigen-specificity requirement of CD4 T cells in mediating normal MWM task performance, we tested OTII and OTII.Rag1$^{-/-}$ mice along with their wild-type controls, in the MWM (Figure 11.8). Emphasized in the reversal phase, OTII mice showed impaired task performance, suggesting that their limited T cell repertoire cannot fully support normal learning behavior. Interestingly, when OTII mice were crossed to Rag1$^{-/-}$ mice yielding a completely naïve T cell population with a monoclonal T cell repertoire (Figure 10.11), learning behavior was further impaired, most prominently in the reversal phase (Figure 11.8b). However it is difficult to directly attribute the MWM impairment seen in in OTII.Rag1$^{-/-}$ mice relative to their OTII counterparts to further restriction of the TCR repertoire, as Rag1 is expressed in the CNS and appears to have lymphocyte independent effects on behavioral outcomes [76-79] (Figure 3.1-3.3). Nevertheless, the

cognitive impairment of OTII mice could be reversed by the transfer of wild-type splenocytes four weeks prior to MWM training (Figure 11.9). While the effect of wild-type splenocyte transfer into OTII mice is not noticeable in the acquisition phase, a significant difference between OTII control mice and OTII mice injected with wild-type splenocytes is obtained in the reversal phase (Figure 11.9b). These results demonstrate that a limited T cell repertoire is associated with impaired learning – a phenotype partially, but significantly, remedied by transfer of wild-type splenocytes.

In the absence of candidate antigens, no unbiased and robust assay exists for determining antigenic specificities of CD4 T cell populations. Therefore, to examine whether T cells recognizing CNS-specific self-antigens can improve learning behavior, we injected OTII mice with a clonal population of resting cultured MOG-specific CD4 T cells (Tmog) derived from 2D2 mice bearing TCR transgenes directed against MOG_{35-55} antigen[323]. The MWM performance of OTII mice receiving Tmog cells was significantly improved eight weeks post-transfer relative to Tova recipients (Figure 11.10), suggesting that at least in animals with clonally restricted T cell repertoires, CD4 T cells directed against CNS-derived self-antigens are sufficient to rescue MWM task performance.

Following MWM testing, the peripheral tissues of Tmog and Tova recipient OTII mice were subject to flow cytometry to determine the homing of the transferred T cells. Interestingly, while Tmog cells were able to rescue MWM impairment in OTII mice (Figure 11.10), they were not recoverable from the meninges two-weeks post-transfer. However, a small population of surviving Tmog cells bearing Vα2.1 and Vβ11 were found in the spleens of recipient mice (Fig. 11.11).

As the phenotype of meningeal myeloid cells had been previously shown to influence behavioral outcomes (Chapter 7 and 9), we examined the meninges of Tmog and Tova recipients for differences in the myeloid compartment between the two recipients. Surprisingly, while there was no difference between mice in the T cell compartment 11 weeks after transfer, the meninges of Tmog

recipients contained a dramatic proportion of GR-1^{hi} cells (Figure 11.12). As autoimmune cells in general, and Tmog cells in particular, have been associated with the tissue infiltration of GR1^{hi} myeloid derived suppressor cells (MDSCs)[331], we assessed these GR1^{hi} cells for expression of CD11b and Ly6C, which are indicative of a granulocytic MDSC population (Figure 11.13). Interestingly, the emergence of the purported MDSC population was not a systemic phenomenon, as GR1^{hi} cells were not observed in the lungs of Tmog immunized mice (Figure 11.12). The identity of these GR1^{hi} cells (i.e granulocytic MDSCs, *vs*. neutrophils), as well as a possible function of these cells for conditioning the meninges and influencing higher CNS functions are discussed in chapter 12.

In light of the aforementioned discovery, we retroactively analyzed FACS data of tissues from Rag1$^{-/-}$ mice reconstituted with 3 × 10^5 purified CD4 T cells derived from wild-type or OTII.Rag1$^{-/-}$ donors (Figure 11.7). Indeed, a similar GR1^{hi} cell population was observed in the meninges of Rag1$^{-/-}$ animals receiving wild-type T cells (Figure 11.14). Interestingly, while the transfer of Tmog cells resulted in a meninges specific accumulation of GR1^{hi} cells (Figure 11.12), transfer of wild-type T cells into Rag1$^{-/-}$ recipients resulted in GR1^{hi} cell accumulation in both the meninges *and* lung (Figure 11.14). Indeed, this would be consistent with the view that MDSCs traffic to tissues in response to autoimmune T cell infiltration[188, 189, 193, 331, 332], as transfer of polyclonal T cells into lymphopenic hosts results in significant skewing of the T cell repertoire to systemic self-antigens (Chapter 5), which would include those derived from the meninges and lung.

Collectively, the studies of this chapter evidence the necessity of an antigen-specific CD4 T cell compartment in normal special learning and memory in mice. We go on to show that a monoclonal T cell population reactive to CNS self-antigen is sufficient to improve cognitive task performance in otherwise learning impaired animals, and raise the possibility that rescue is mediated by a robust population of meninges infiltrating myeloid derived suppressor cells. A comprehensive discussion of results is the subject of the following chapter.

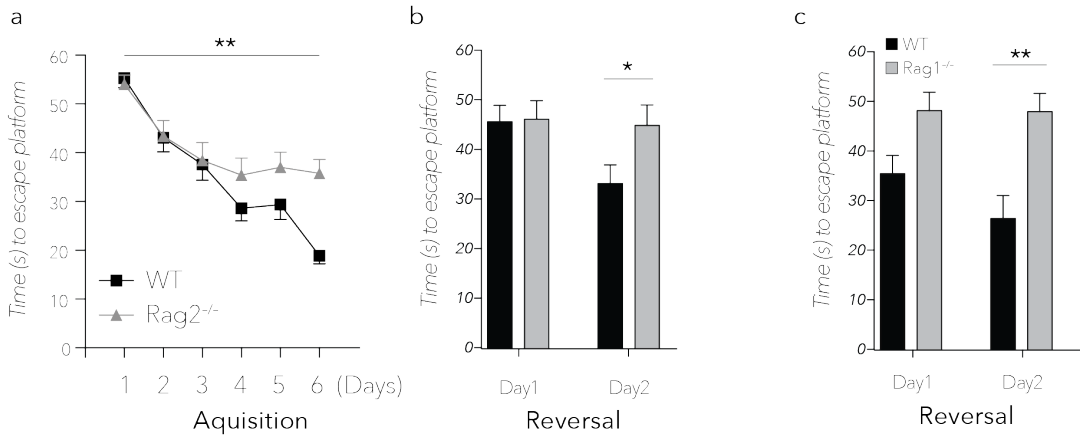


Figure 11.1: Impaired spatial learning and memory in Rag1 and Rag2 deficient mice. (a) Acquisition and (b) reversal of Rag2$^{-/-}$ and wild-type mice in the MWM spatial learning and memory task. (n = 9mice/group ** = P < 0.01 * = P < 0.05; repeated measures ANOVA with Bonferonni post hoc analysis). (c) Reversal latency times of Rag1$^{-/-}$ and wild-type mice (n = 8mice/group, P > 0.5; Repeated measures ANOVA with Bonferonni post hoc analysis).

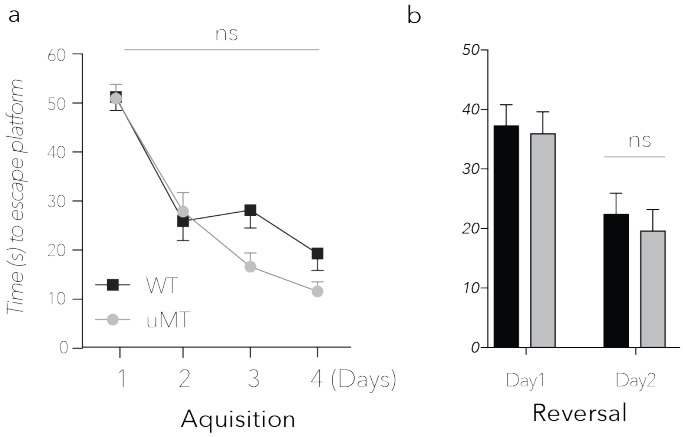


Figure 11.2: Normal spatial learning and memory B cell deficient mice. (a) Acquisition and (b) reversal of B cell deficient uMT and wild-type mice in the MWM spatial learning and memory task. (n = 8mice/group; repeated measures ANOVA with Bonferonni post hoc analysis).

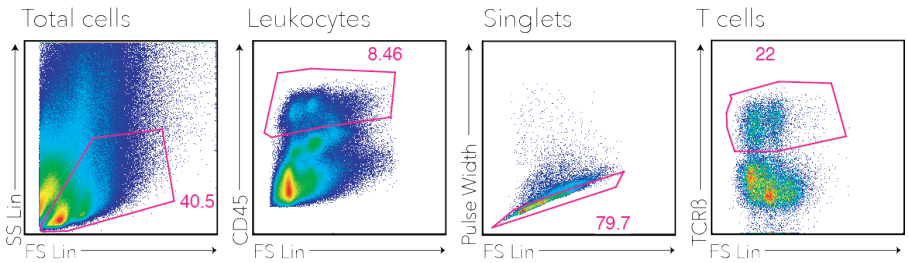


Figure 11.3: FACS gating strategy for meningeal T cells. Representative flow cytometry gating strategy for meningeal T cells beginning with meningeal single cell suspensions. A similar gating strategy is applied for all meningeal samples and other tissues throughout the chapter often using CD3 in place of TCRβ, and the inclusion of viability dye.

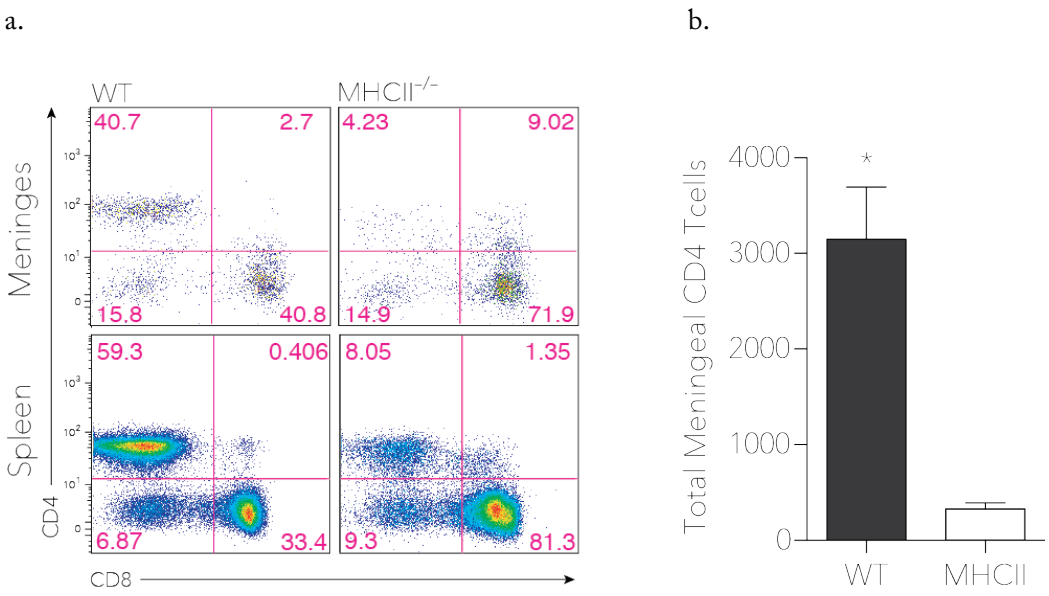


Figure 11.4: Reduction of CD4 T cells in the meninges of MHCII deficient animals. a) FACS analysis of CD4/CD8 expression in meningeal (upper panel) and splenic (lower panel) single-cell suspensions from wild-type and MHCII$^{-/-}$ mice. Representative of n=2 mice/group. b) Total numbers of CD4 T cells in the meninges of WT and MHCII$^{-/-}$ mice n=2 mice/group * = $P < 0.05$; student's T-test.

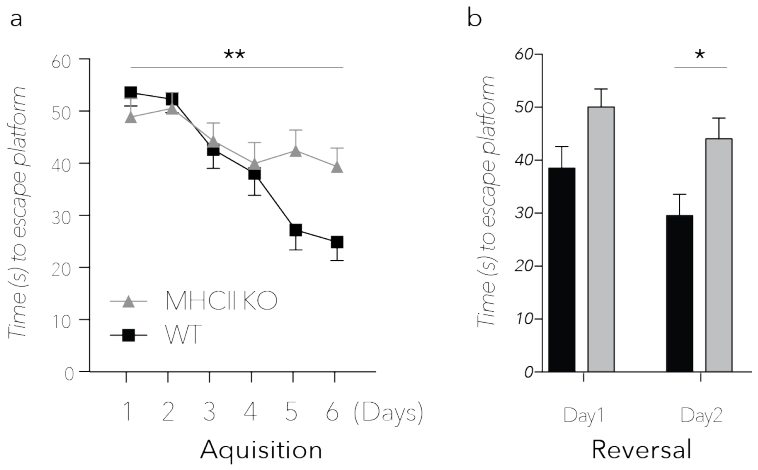


Figure 11.5: Impaired spatial learning and memory in MHCII deficient mice. (a) Acquisition and (b) reversal of B cell deficient MHCII$^{-/-}$ and wild-type mice in the MWM spatial learning and memory task. (n = 8mice/group ** = P < 0.01 * = P < 0.05; repeated measures ANOVA with Bonferonni post hoc analysis).

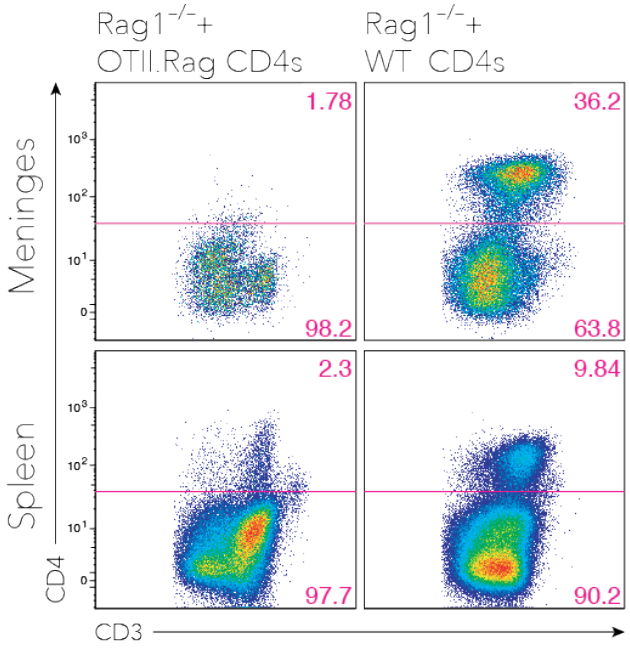


Figure 11.6: Reconstitution of Rag1 deficient mice with either purified polyclonal wild-type CD4 T cells, or monoclonal T cells derived from OTII.Rag1$^{-/-}$ mice. Representative FACS analysis of CD4/CD3 expression in meningeal (upper panels) and splenic (lower panels) single-cell suspensions from Rag1$^{-/-}$ mice receiving 3×10^5 purified CD4$^+$ T cells from wild-type (right) or OTII.Rag1$^{-/-}$ (left) donors.

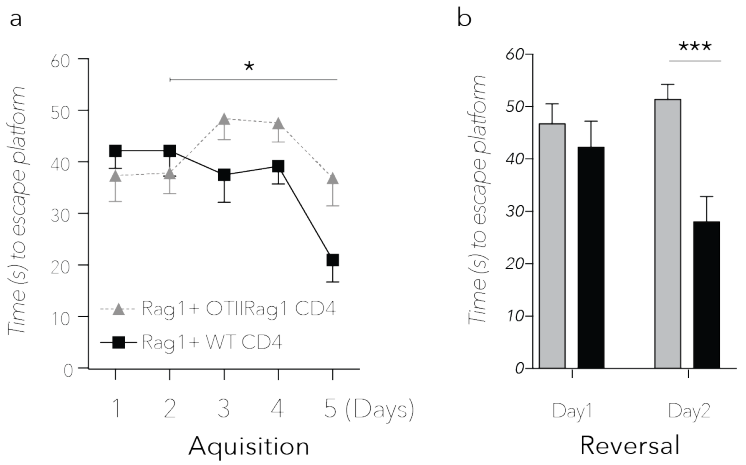


Figure 11.7: MWM performance of Rag1 deficient mice reconstituted with either purified polyclonal wild-type CD4 T cells, or monoclonal T cells derived from OTII.Rag1$^{-/-}$ mice. MWM Acquisition (a) and reversal (b) latency time of Rag1$^{-/-}$ mice receiving 3 × 10^5 purified CD4 T cells from wild type (right) or OTII.Rag1$^{-/-}$ (left) donors (n = 4mice/group * = P < 0.05, *** = P < 0.001; Repeated measures ANOVA with Bonferonni post hoc analysis, significance in acquisition between groups only achieved between days 2–5).

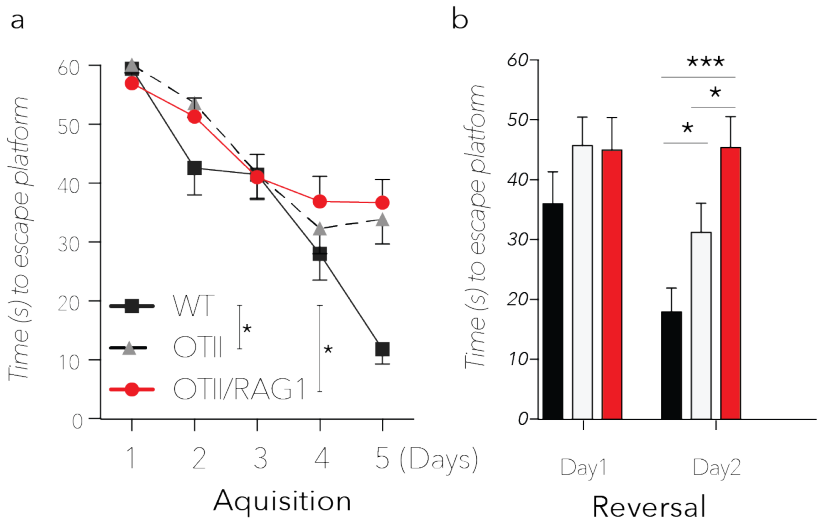


Figure 11.8: MWM performance of WT, OTII and OTII.Rag1$^{-/-}$ mice. (a) MWM acquisition and (b) reversal latency time of OTII, OTII.Rag1$^{-/-}$ and wild type mice (n = 8mice/group * P < 0.05, *** P < 0.001; Repeated measures ANOVA with Tukey post hoc analysis).

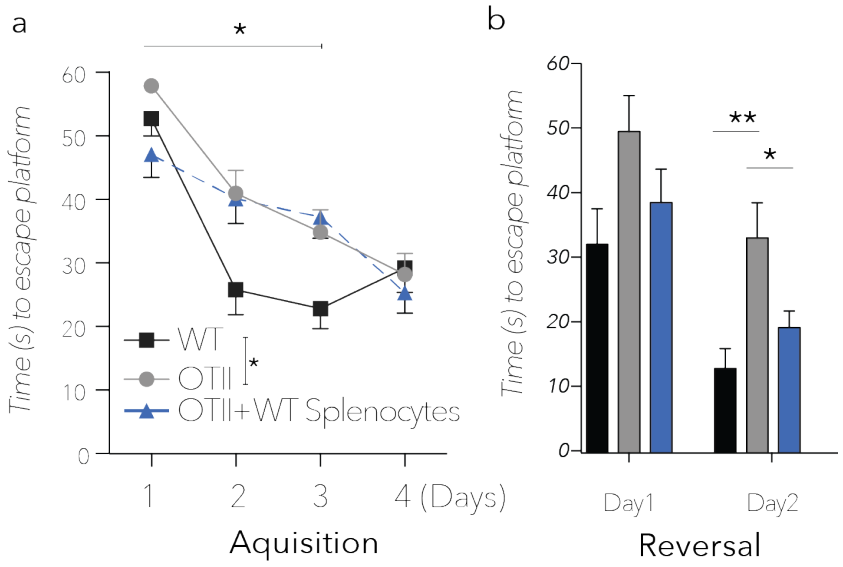


Figure 11.9: MWM performance of WT, OTII and OTII mice reconstituted with wild-type splenocytes. (a) MWM acquisition and (b) reversal latency time of wild type and OTII mice, either untreated or reconstituted with wild type splenocytes four weeks prior to training. (n = 7mice/group * = $P < 0.05$, ** = $P < 0.01$; Repeated measures ANOVA with Tukey post hoc analysis, significance in acquisition only achieved between days 1–3)

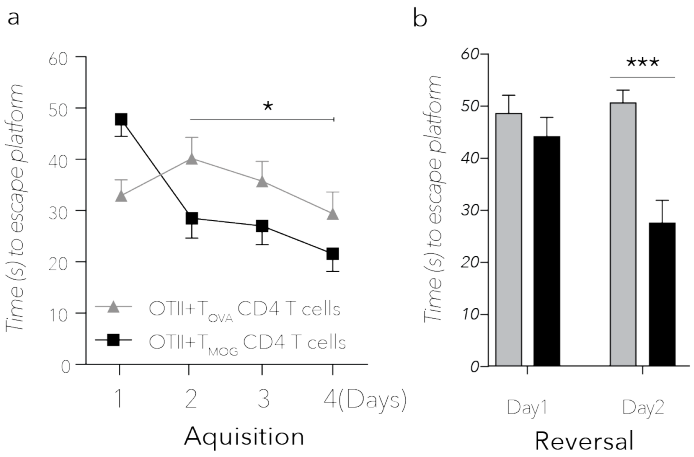


Figure 11.10: MWM performance of OTII mice receiving transfers of Tova and Tmog monoclonal CD4 T cells. MWM Acquisition (a) reversal (b) latency time of OTII mice receiving 2×10^6 purified MOG or OVA specific $CD4^+$ T cells (n = 5mice/group * $P < 0.05$, *** $P < 0.001$; Repeated measures ANOVA with Bonferonni post hoc analysis significance in acquisition only achieved between days 2–4).

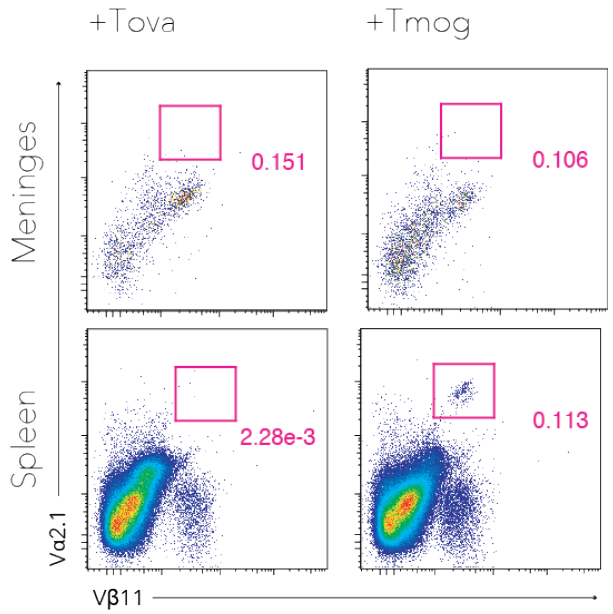


Figure 11.11: FACS determination of Tmog homing to the spleen and meninges of OTII recipient mice on the basis of Vα2.1 and Vβ11 Tmog transgenic TCR usage. FACS analysis of $CD4^+$ T cells in the meninges and spleens of OTII mice receiving either 2×10^6 purified MOG or OVA specific $CD4^+$ T cells, gated on the Tmog Vα2.1 and Vβ11 TCR. Tmog T cells were only recoverable from the spleen of recipient mice 2 weeks after transfer. Representative of three independent experiments.

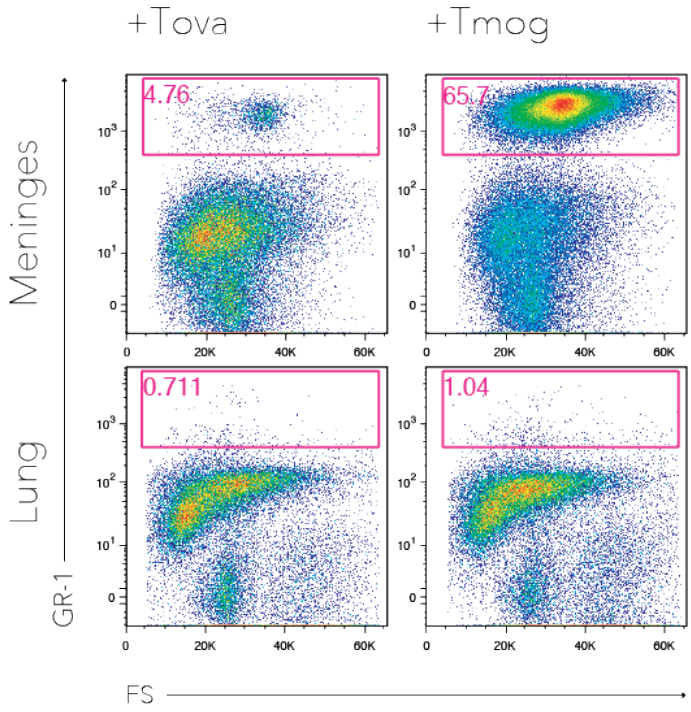


Figure 11.12: FACS analysis showing meninges specific infiltration of GR1^{hi} cells in the meninges of Tmog but not Tova recipient OTII mice. Representative FACS analysis of CD45+ cells from the meninges and lung of OTII mice, 11-weeks after receiving either 2×10^6 purified MOG specific (Tmog) or OVA specific (Tova) CD4$^+$ T cells.

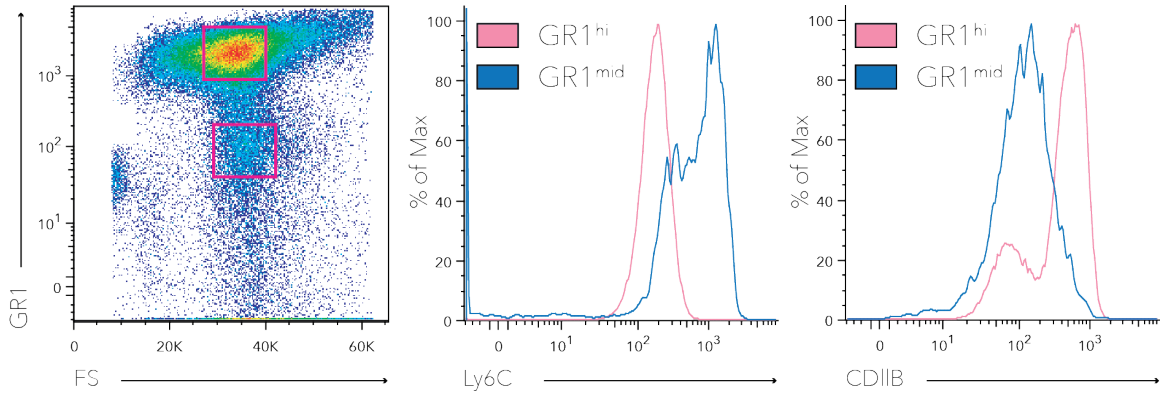


Figure 11.13: FACS analysis of GR1^{hi} cells in the meninges of Tmog recipient OTII mice. Representative FACS analysis of meningeal GR1^{hi} and GR1^{mid} T cells from Rag1$^{-/-}$ mice reconstituted with 3 × 10^5 purified CD4$^+$ T cells from spleens of wild-type donors.

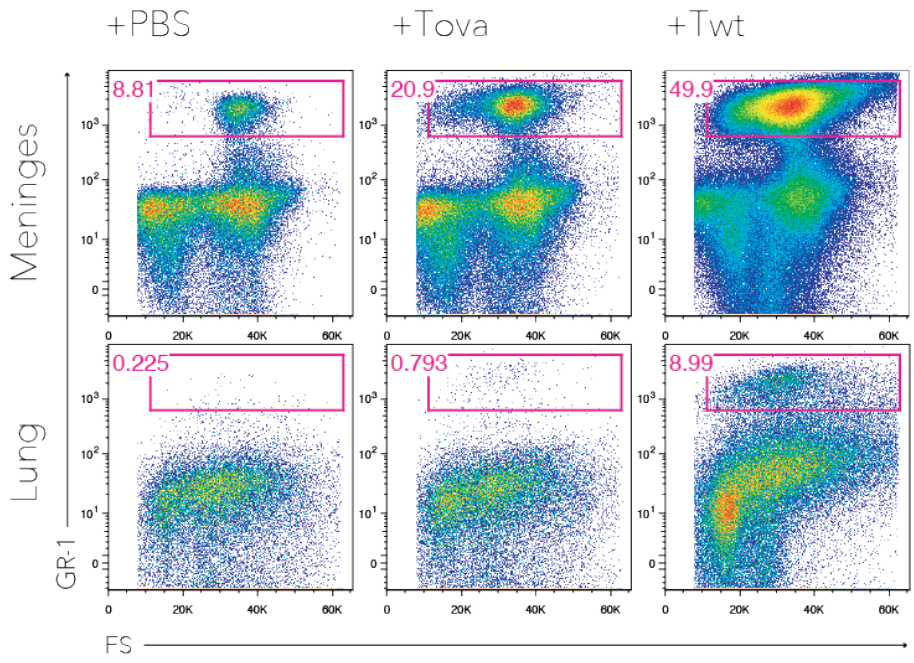


Figure 11.14: FACS analysis showing preferential accumulation of GR1^{hi} cells in the meninges and lung of Rag1$^{-/-}$ mice reconstituted with wild-type CD4 T cells. Representative FACS analysis of total CD45^{+} cells from the meninges and lung of Rag1$^{-/-}$ mice receiving either PBS, 3 × 10^{5} purified CD4 T cells from OTII.Rag1$^{-/-}$ (Tova), or wild-type (Twt) donors.

III. RESULTS

CHAPTER 12: CONCLUSIONS AND FUTURE DIRECTIONS

In Chapter 10 we determined the turnover dynamics of CD4 T cells in the meningeal compartment and tested the effects of T cell antigen specificity on recruitment of T cells to that space. In Chapter 12 we showed that antigen specific CD4 T cells were *necessary* for normal spatial learning and memory in mice, and that rescue of MWM impaired OTII mice can be achieved by both WT T cells and a monoclonal T cell population reactive to the central nervous system (CNS) antigen, myelin oligodendrocyte glycoprotein (MOG). The implications of these findings, both in terms of learning and memory, as well as fundamental immune-surveillance of the brain, are a major focus of this chapter, as is the contribution of these studies in elucidating the role of T cells in higher brain function. With respect to this latter point, I present a model in which the role of T cells in mediating (or maintaining) normal learning and memory in the WT animal, and the role of T cells in rescuing the impairment of lymphopenic mice are mechanistically independent phenomena. With this distinction in mind, I present our working model of T cell involvement in higher brain function, and discuss the directions of future experimentation.

T cell dynamics in the meningeal space:

Our initial interest in the meninges as the site of pro-cognitive T cell–CNS interaction was largely a product of abductive reasoning. As spatial learning and memory are functions of the CNS, we thought that any productive or homeostatic influence of T cells on this function would occur at, or near the brain itself. As the non-pathological brain parenchyma is virtually devoid of any lymphocyte populations, the meninges presented itself as the next best candidate site for the T cell-CNS

interactions that support higher brain function. Indeed, the meninges is permissive to T cell entry, and treatment of mice with FTY720 or anti-VLA4 had been shown to result in a reduction of meningeal T cell numbers that was correlated with MWM impairment[100]. Additionally, a pro-inflammatory skew of the CD11b^{+} meningeal myeloid compartment of SCID mice, characterized by intracellular TNF and IL-12 had been shown to similarly correlate with impaired MWM task performance[4, 82]. Finally, the anatomy of the meninges–a structure that is contiguous with the contours of the CNS, the ventricles, CSF and interstitial fluid (Chapter 1), made regulation of higher brain function by the meningeal immunity a plausible hypothesis.

While the meninges is a logical candidate for the site of pro-cognitive T cell-CNS interactions, we were well aware of studies pointing to a more long-range, systemic T cell influence on brain functions. Studies by Wolf et al had shown that intraperitoneal administration of staphylococcus enterotoxin B increased hippocampal neurogenesis in mice, as did the induction of adjuvant-induced-arthritis at the knee. A similar study addressed spatial learning and memory directly; showing that systemic administration of staphylococcal enterotoxin A superantigen could improve MWM performance in recipient mice. Importantly, these studies never investigated meningeal immunity following treatment, leaving open the possibility that systemic treatment also induced changes in the meningeal myeloid compartment that ultimately produced changes in neurogenesis and MWM performance. Nevertheless, a productive systemic effect of immune activation on higher brain functions has its own evolutionary appeal, as it is easy to envision how an animal would benefit from remembering the location of a water supply that made it sick, or the identity of a pathogenic food source.

Whether the effect of T cells on higher brain function is executed in the meninges or in the periphery, understanding the dynamics of T cells in the meningeal space had its own intrinsic value. The meninges is the site of lymphocyte immune surveillance for the brain, and is implicated in a litany

of pathologies from bacterial and viral meningitis to multiple sclerosis[56, 105, 320, 333-336]. Ultimately, the experiments in Chapter 10 allowed us to test elements of a model of meningeal T cell immunity in learning behavior and at the same time to generate data that would be broadly relevant to the field of neuroimmunology. Indeed, the studies achieved this dual aim — refining a model of meningeal T cell involvement in learning and memory, and also providing invaluable understanding of the CD4 T cell behavior in a poorly understood, yet pathologically relevant, anatomical compartment.

We wished to understand the nature of T cell traffic in the meningeal compartment. At one end of the spectrum was the possibility that T cells in the meninges were more or less fixed in the tissue, with little to no turnover. This is exactly the case for CD8 T cells in the gut[236] and NKT cells of the liver[237], which show dramatically attenuated chimerism between parabiotic partners relative to other tissues. This type of long-term peripheral tissue residence of T cells is also observed in models of immune challenge where adoptively transferred lung-tropic hemagglutinin-antigen specific T cells homed to the host lung, and did not recirculate into partner mice after parabiosis[235]. Similarly, long-lived, non-circulating populations of CD8 T cells have been observed in skin infection with vaccinia[215], and in persistent patches in the brain parenchyma after CNS-tropic rabies virus infection[216] long after clearance of virus. At the other end of the spectrum was the possibility that T cells in the meninges readily recirculate with high intra-tissue turnover. This appears to be the case for T cells of the naive lung and liver in which CD4 T cells show a relatively robust equilibration between partner parabiotic animals[237].

The results in Figure 10.1 and 10.2 supported the later scenario. After 15 days of parabiotic surgery approximately 25% of the host meninges was seeded with T cells derived from partner mice. As the total number of meningeal T cells did not appear to be significantly affected by parabiosis (data not shown), we could assume that the rate of influx and egress (addressed later in this chapter) were comparable. If for the sake of simplicity, we also assume that T cells equilibrated in the blood at a

perfect 1:1 ratio instantaneously after parabiotic surgery, we would arrive at a intra-meningeal T cell turnover rate of approximately 50% within 15 days (assuming 25% of Ly5.2 T cells in the meninges have also turned over).

However the idealized model can be significantly refined by revisiting the false assumption that equilibration of T cells between the circulations of partner mice is an instantaneous process. Whole blood fluorescence analysis has placed the mean exchange flow rate at 0.66% of the circulating blood volume per hour[337]. Indeed, there is virtually no chimerism in the first three days post-surgery, as blood vessels have not yet re-vascularized[338]. By substituting an instantaneous blood "mixing" rate with blood chimerism data fitted to the more natural curves of Fick's law of diffusion (Figure 12.1 and 12.2), we are able to more accurately place the intra-meningeal turnover rate at 50%/7.8 days, nearly twice the turnover rate under the assumption of instantaneous blood chimerism. While the diffusive system of Fick's Law may not entirely recapitulate a system under conditions of flow, it is vastly more natural than a model of instantaneous blood chimerism, and does well to illustrate that the original turnover rate of approximately 50% within 15 days is a significant underestimation.

Even when taking into account the gradual homogenization of T cells in the blood of partner mice, we may still be grossly underestimating the intra-tissue T cell turnover. So far we have based these idealized models on the fundamental assumption that lymphocytes flow freely and indiscriminately between the circulations of partner mice. However, one of the unexpected outcomes of our parabiotic studies was the observation that CD44 expressing T cells cross into the circulation of partner mice much less efficiently than naïve T cells (Figure 10.5). While we discuss this phenomenon in detail later, if we consider the impeded crossover of $CD44^{hi}$ T cells in light of the finding that, like other peripheral tissues, the meninges is highly selective for CD44 expressing T cells, then we can appreciate that the theoretical 50%/7.8 day intra-meningeal turnover rate may still be an underestimation.

Collectively the outcomes of the Ly5.2-Ly5.1 parabiotic pairing suggest that T cells in the meningeal space are not fixed but turn over readily from the blood. The class of "fixed" T cells which reside in the peripheral tissues long-term and with limited turnover from the blood, have been broadly categorized as tissue-resident memory cells (T_{RM}s). To illustrate the comparatively fixed nature of T_{RM}s: flushed gut biopsies show <3% turnover of $CD8^+$ intraepithelial lymphocytes of the small intestines 15 days following parabiotic surgery[236]. Similarly, lung tropic hemagglutinin antigen-specific memory CD4 T cells showed virtually no egress from the host lung 21 days after parabiotic conjunction[235], VACV-ova specific CD8 T_{RM}s in the skin were still detectable 28 weeks post challenge[215], and VSV-specific CD8T_{RM}s continued to persisted in the brain 120 post infection[216]. The Ly5.1-Ly5.2 parabiotic system would suggest that T cells of the meninges are not of this variety. In contrast to truly long-lived tissue-resident cells, T cells of the meninges turn over comparatively rapidly. Nevertheless, the turnover rate observed in the Ly5.1-Ly5.2 parabiotic pairing does not definitively rule out the T_{RM}s, as the meninges might contain recirculating subsets along with a non-circulating T_{RM} subset in an undefined anatomical subcompartment. Indeed this is the case in the gut in which T cells of the Peyer's patch and lamina propria of the small intestines recirculate readily while intraepithelial lymphocytes appear to be persistent T_{RM} cells[236].

However in comparison with the lymphoid organs as well as with the naïve lung (Figure 10.1) and liver[237], CD4 T cell chimerism appears to be comparatively delayed in the meninges. The delayed chimerism of both the meninges and lung relative to the lymphoid compartments is expected. Naïve blood-borne T cells enter the secondary lymphoid organs via HEVs in a process of rolling, activation, and arrest, egress through efferent lymphatic vessels, and return to the circulation via the thoracic duct, all within a span of 10-20 hours[213, 339]. The rapid recirculation rate of naïve T cells, coupled with the approximately 5:1 ratio of naïve:memory blood-born T cells (Figure 10.5) is likely sufficient to account

for the faster chimerism in the secondary lymphoid vs. non-lymphoid tissues of the Ly5.1-Ly5.2 parabiotic pairing (Figure 10.1 and 10.2).

Whereas the delayed chimerism in the non-lymphoid relative to the lymphoid compartment is easily appreciated, the differential chimerism within the non-lymphoid tissues (e.g. meninges vs. lung) is more difficult to reconcile. The differential could arise from one, or a combination of numerous factors that are known to regulate lymphocyte entry and egress in peripheral tissues. With regards to a differential restriction on T cell entry, the vascular endothelium of the lung and meninges may differ in the forms of chemokine and adhesion molecules they express. While the precise combinations of chemokine receptor, integrins and selectins needed for trafficking into the gut[231, 232] and skin[228-230] are well appreciated; whether similar imprinting patterns exist for other tissues including the lung and meninges remains unknown.

The differential in T cell chimerism between the meninges and lung may also exist at the level of migration *within* the tissue. T cells are known to move at different velocities inside tissues, and even within the same tissue the rate of migration can be subcompartmentalized, as T cells in the dermis have been shown to have a higher velocity than those of the epidermis[340]. While the factors that govern the rate of T cell migration through the tissues are not entirely understood, the structure of tissue including extracellular matrixes such as the basement membrane beneath the epidermis, and networks of tissue stromal cells may be contributing factors[213].

Finally, the differential between meninges and lung T cell chimerism in the Ly5.1-Ly5.2 parabiotic system may arise as a consequence of restrictions on egress. T cells exit non-lymphoid tissues via the lymphatics in a CCR7 dependent mechanism[341, 342]. While the differential could conceivably reflect altered CCR7 expression on meningeal vs. lung T cells, it may also be a direct consequence of lymphatic anatomy. Unlike the conventional lymphatic drainage of the lung, lymphatic

drainage of the meninges (reviewed in Chapter 1) is indirect. In the meninges, both cells and soluble antigen exit the meninges via the cribriform plate where the route of drainage transitions from the meningeal subarachnoid space surrounding the olfactory nerve to the perineurial space of the nerve[20, 21] CSF and its cellular and soluble constituents disperses into the connective tissue at the nasal mucosa and only then drain into adjacent lymphatic vessels towards the deep cervical lymph node. It is conceivable that this atypical route of lymphatic drainage may pose a bottle-neck on lymphocyte egress, and account for the slower turnover observed in the meninges relative to the more conventionally drained lung.

The same restrictions on entry, intra-tissue migration, and egress that dictate the differential in T cell chimerism between the lung and meninges in Ly5.1-Ly5.2 parabiotic partners, apply to other experimental paradigms of Chapter 10. In apparent reversal of the delayed meningeal turnover observed in the Ly5.1-Ly5.2 parabiotic pairings, when wild-type Thy1.1 T cells were adoptively transferred into OTII recipients they preferentially seeded the host meninges (Figure 10.3). Indeed, it appears that the pattern of relative chimerism was reversed across all of the common tissues examined. Whereas the Ly5.1 chimerism in the Ly5.1-Ly5.2 pairing from greatest to least was iLN>cLN>meninges (Figure 10.1), the order of T cell chimerism after adoptive transfer of wild-type Thy1.1 splenocytes into OTII hosts was meninges>cLN>iLN (Figure 10.3). While this preferential chimerism in the meninges is seemingly incongruent with the delayed chimerism observed in the Ly5.1-Ly5.2 parabiosis, it must be considered in the context of ongoing homeostatic expansion.

In both the adoptive transfer of wild-type splenocytes into OTII hosts (Figure 10.3), as well as the Ly5.1-OTII pairing (Figure 10.4), the same homeostatic proliferation process is taking place. The homeostatic expansion of T cells in lymphopenic hosts has been described repeatedly[152, 154, 156, 157, 160, 161, 163-166]. Interestingly this same expansion process occurs in TCR transgenic host mice with clonally limited T cell repertoires[155, 180], indicative of a homeostatic driving force that seeks not only maximal T

cell numbers, but also maximal clonal diversity. Indeed, this observation has led to consideration of TCR transgenic mice to be "functionally lymphopenic" [180].

The homeostatic expansion of wild-type T cells in TCR transgenic mice is evident in our own studies as well. Figure 10.3 shows a dramatic expansion of Thy1.1 T cells between 3 and 8 weeks after adoptive transfer into OTII hosts. The phenomenon is also observable in naïve OTII animals, as the proportion of postrevisional, endogenous TCR bearing T cells in OTII mice continues to expand between 10 and 35 weeks (again with the meninges showing a marked preference for these expanded $CD44^{hi}$ cells) (Figure 10.10). This is also the case in the unmanipulated heterozygous 2D2 TCR transgenic mouse, in which the small proportion of naturally occurring endogenous T cells expand with age (once again with preferential meningeal accumulation) (Figure 12.3). Importantly, in all of these cases, expanding T cells express CD44, consistent with the memory phenotype that has been ascribed to homeostatically expanding T cells in the literature[153, 158, 159, 162].

While it is clear that homeostatic expansion leads to the preferential accumulation of T cell in the meningeal spaces, it is unclear what restriction (e.g. entry, intra-tissue migration, or egress) is being lifted. Homeostatic expansion is accompanied by changes in expression of molecules important for T cell homing including VLA-4[343] and CD44[158], which in combination with unique chemokine and adhesion molecule expression on the meningeal vascular endothelium may contribute to increased lymphocyte entry. Intra-tissue migration may be similarly effected, with increased retention of proliferating T cells in the meninges. Homeostatic proliferation has been shown to occur preferentially in the bone marrow[344], and the large numbers of homeostatically proliferating T cells observed in the meninges may be a consequence of its anatomical position beneath the cranial bone marrow.

The deep cervical lymph node

Both the uniquely reduced T cell chimerism in the meninges of Ly5.1-L5.2 parabiotic pairings (Figure 10.1-10.2), as well as the increased seeding of the meninges of OTII mice by wild-type T cells (Figures 10.3-10.4) was also apparent in the deep cervical lymph nodes (dCLN). The same correlation was observed in the distribution of postrevisional T cells in naïve OTII mice, where like the meninges, the dCLN showed a preferential accumulation of postrevisional cells (twice that of the ILN at 10 weeks) (Figure 10.10).

To explore possible coupling of T cells in the meninges and dCLNs, and to find out how interruption of this coupling might affect the T-cell composition of the meninges, we surgically resected dCLNs of wild-type mice. Two weeks after surgery, the numbers of CD4 T cells in the meninges of operated mice were substantially increased relative to sham controls (Figure 10.7a).

Given that T cell entry into the lymph nodes occurs through lymphatic vessels and the blood[345], the pattern of delayed chimerism in the dCLN of Ly5.1-Ly5.2 pairings, as well as the increased chimerism of Thy1.1 T cells in the dCLN following adoptive transfer into OTII hosts, may suggest a unique preference of the dCLN for the lymphatic route over the blood route of T cell entry. Indeed, we entertained the idea that dCLNs may lack high-endothelial venules (HEVs) altogether, however this hypothesis was rebuked by immunohistochemistry of the lymph nodes showing comparable staining for the HEV marker MECA-79 between the dCLNs, ILNs and mesenteric LNs (data not shown). Nevertheless, there may still be unique functional and structural properties of the dCLN HEVs that would account for apparent disfavor for blood route of T cell entry including a host of HEV associated chemokines[345], and dendritic cells which have been shown to be key regulators of HEV function[346].

We drew on the observations of coupling between the meninges and deep cervical lymph nodes to ask how surgical resection of the lymph node might impact behavioral outcomes in the

MWM. From this experiment we conclude that the perturbation of the deep cervical lymph node affects the upstream meningeal space, and that resection of the lymph node results in impaired cognitive outcome in the MWM (Figure 10.7).

While the decline in MWM performance of dCLN-resected mice is an interesting phenomenon, it is an observation that is difficult to interpret, and assign causality. One possible rational for the impaired MWM outcome draws from the observation that when EAE was induced in mice, deep cervical lymph node resected groups had lower EAE disease scores than their sham operated controls (Figure 12.4). As previously shown[321, 347], by removing the dCLNs we effectively removed the route of antigen drainage from the CNS (Chapter 1), impeding initiation of the T cell response to CNS antigens, and ultimately ameliorating disease severity. It may be that a similar adaptive immune response is generated in the deep cervical lymph node following MWM stress, however efforts at capturing the early activation marker CD69 expression in the dCLNs following MWM training have been unsuccessful. We also cannot rule out a T cell independent effect of dCLN resection on higher brain functions, as the lymph nodes carry out immune-independent homeostatic functions. The cervical lymph nodes in particular have been implicated in maintaining normal intracranial pressure via drainage of CSF[348-350]. While the study does raise the interesting possibility that higher brain functions can be perturbed by disruption of meningeal T cell egress, more careful experiments are needed to substantiate this claim.

Implication for immune surveillance

Collectively, the aforementioned parabiotic and adoptive transfer studies show that CD4 T cells persist in the meninges relatively longer than in the lung and lymphoid tissue. However, their delayed turnover is nothing like that observed in T_{RM}s, which persist in tissues for weeks to months, with only minimal loss and turnover. While we cannot rule out the presence of T_{RM}s in the meninges

owing to the potential for anatomic subcompartmentalization discussed earlier, it is clear that the meninges is continually visited by circulating $CD44^{hi}$ memory T cells. The immunological protection conferred by having antigen experienced memory T cells patrolling the borders of the brain is self-evident.

Interestingly, the slow T cell turnover rate in the meninges may also have important implications for immune surveillance. When OTII.$Rag1^{-/-}$ mice were immunized with OVA/IFA, we observed a preferential accumulation of $CD44^{hi}$ memory CD4 T cells in the meninges of immunized mice 15 days later (Figure 10.12 and 10.13). Interestingly, even the few $CD44^{hi}$ T cells that were stochastically activated by IFA alone were uniquely observable in the meninges (Figure 10.13). It may be that following an infection, this bottle-necking effect would lead to preferential memory T cell accumulation in the meninges and consequently to enhanced and preferential immune surveillance at the borders of the brain.

Indeed there may be a great selective advantage in making the brain an immune surveillance priority. Not only is the brain supremely important to body homeostasis, but neural tissue of the CNS is effectively incapable of regeneration, making a failure of immunoprotection far more costly here than in any other tissue. Additionally, the absence of lymphocytes and lymphatics in the parenchyma leave the brain especially vulnerable to pathogenic infiltration.

While the benefit of close immune surveillance of the CNS is clear, indiscriminant access of inflammatory $CD44^{hi}$ T cells to the borders of the brain can also be a liability. Interestingly, immunization of mice with ova-expressing adenovirus resulted in systemic virus specific T cell expansion. Eight days after infection, $CD44^{+}$ T cells infiltrated the liver, lung and kidney but not the meninges (Figure 12.5)—suggesting that regulation of immune surveillance at the meninges is understandably more nuanced than CD44 expression alone. The discrepancy between meningeal

homing of CD44^{+} T cells in ova/IFA immunized OTII.Rag1$^{-/-}$ mice, and the apparent absence of meningeal homing of CD44^{+} adenovirus activated T cells, may be a matter of timecourse, as meningeal T cell composition was assessed at day 15 post-immunization in the former system, and at the day 7 peak of T cell response in the adenovirus immunization model.

A brief note on the parabiotic model.

One of the unexpected findings from this work unmasks an important caveat of the parabiotic system, which to our knowledge has so far been overlooked (a recent 2013 study has since addressed this caveat in monocytes). The parabiotic systems employed in this study conclusively show that while naïve T and B cells equilibrate normally between partner mice, CD44^{hi} T cells (Figure 10.5) as well as CD11b^{+} monocytes (Figure 10.6) pass into partner mice with poor relative efficiency. This is not a trivial conclusion, as what we show undermines one of the basic assumptions of a fundamental model of immunology. The parabiotic system rests on the assumption that cells that circulate in the blood of one mouse will be equally present in the blood of their partner, which we show not be the case with CD44^{hi} T cells or monocytes. Indeed, a recent study has affirmed the lack of chimerism of monocytes and macrophages between the blood of parabiotic partners — a phenomenon that was used to prove that tissue-resident macrophages self-maintain throughout life, without contribution from circulating monocytes[322].

While we have not explored the mechanisms behind the incomplete chimerism between some blood cell types and not others, the findings may be reconciled by a model in which a monocyte or activated T cell extravasates at the site of parabiotic surgery, egresses to the skin draining lymph nodes and is either eliminated or recirculated back into the blood, thereby never crossing into the circulation of the partner mouse.

It would be overreaching to state that CD44^{hi} T cells categorically do not cross the parabiotic vasculature, as they may do so in other contexts such as infection. It was shown that antigen experienced CD8 T cells reactive to vesicular stomatitis virus were able to cross from one mouse into the other parabiotic partner[351]. Therefore the inability of circulating wild-type CD44^{+} T cells in OTII mice to circulate back into the wild-type partner may have to be viewed in the context of their specific activation as homeostatically expanding T cells in clonally restricted hosts. This distinction (if some activated T cells can pass, while others cannot) could indeed be teased out in experiments that assess the transfer of activated T cells into parabiotically connected partners in different T cell activating paradigms (e.g. infection, immunization, and autoimmunity).

Antigen specificity of T cells in the meningeal space:

The limitations of techniques to determine the antigen specificity of a CD4 T cell population were nicely illustrated in a 2013 study by Baruch et al[319]. As sequencing of the TCR cannot reveal its antigen specificity, they developed a novel approach to assess the clonotypic enrichment of CNS-specific T cells in the choroid plexus. They show that the sequence of splenic TCRs of mice immunized with spinal cord-homogenates more closely resembles the TCR sequences of T cells of the choroid plexus, than do TCRs of naïve T cells or splenic TCRs derived from mice immunized with ovalbumin. Even if the controls of the experiment had been more carefully selected, the study would still do more to illustrate the inability to identify T cell antigen specificity in a given population than actually show CNS-specific reactivity of T cells in the choroid plexus. Nevertheless, the model was at least correlatively consistent with previous work showing that transgenic mice with TCR directed against the CNS self-antigen myelin basic protein (MBP) are competent in the MWM, whereas D011.10 mice with TCR directed against irrelevant ovalbumin antigen are not[4]. While there are several major caveats to both sets of findings (discussed in Chapter 9) they do raise the intriguing possibility that resident T cells of the meninges and choroid plexus are specific to CNS self-antigen.

We address the question of meningeal T cell antigen specificity independently of learning and memory in several studies of Chapter 10.

The CD44 memory requirement of meningeal T cells that has been addressed in this chapter does not necessarily obviate a role for antigen specificity. Indeed, at least in the pronounced chimerism of wild-type T cells in the OTII host meninges of the adoptive transfer and parabiotic schemes, it could be argued that the T cells observed in the meninges are specific to CNS self-antigens. A similar argument could be made for the observations in Figure 10.10. While overrepresentation of postrevisional T cells in the meninges was associated with CD44 memory status, those memory cells may again be specific for CNS self-antigens.

To directly test for TCR dependent preferential trafficking of T cells to the meninges, we compared meningeal T cell chimerism between Ly5.1-Ly5.2. Ly5.1-OTII, and Ly5.1-2D2 parabiotic pairings (Figure 10.8). In the normal Ly5.1-Ly5.2 pairing, 15 days after parabiotic surgery, approximately 25% of the meningeal T cells in Ly5.1 animals came from the partner mouse. We speculated that if CNS antigen-specific T cells preferentially trafficked to the meninges, then the observed 25% chimerism in the Ly5.1-Ly5.2 pairing may have been limited by the number of CNS specific T cells that were circulating in the unified vasculature. By parabiotically joining wild-type Ly5.1 to 2D2 mice (TCR transgenic for the myelin oligodendrocyte glycoprotein), we sought to vastly increase the number of CNS reactive T cells entering the circulation of wild-type partners. Specifically, we hypothesized that if there were a preference for CNS-reactive T cells, then the meninges of wild-type mice would become inundated with Tmog reactive T cells. Conversely, we hypothesized that the percentage would be depressed in Ly5.1-OTII pairings, as OTII mice presumably contain a lower abundance of CNS reactive T cells in the blood than wild-type Ly5.2 animals. However, neither supportive outcome was realized. Fifteen days after parabiotic surgery, the Ly5.1 meninges contained roughly the same 25% chimerism, whether from wild-type, 2D2, or OTII T cells (Figure 10.8).

The notion of preferential meningeal trafficking of CNS antigen-specific T cells was further undermined in two subsequent experimental systems. If antigen specificity was a requirement for meningeal trafficking, it would inherently imply the expression of CNS derived antigens on MHCII in the meningeal space. To test if CD4 T cell-MHCII recognition was necessary to bring T cells into the meninges, we carried out parabiotic joining of wild-type and MHCII$^{-/-}$ mice. As anticipated, wild-type mice in this pairing received virtually no T cells from MHCII$^{-/-}$ partners, as MHCII deficient animals have very few T cells in the blood to begin with owing to a failure of positive selection in the thymus. However, the meninges of MHCII$^{-/-}$ mice received approximately the same total number of T cells that wild type meninges received in Ly5.1-Ly5.2 pairings, suggesting that the entry of T cells into the meningeal space was independent of MHCII function in the meninges, and thereby independent of antigen specificity (Figure 10.9). This point was reiterated when OTII.Rag1$^{-/-}$ were immunized subcutaneously with ovalbumin/IFA (Figure 10.12 and 10.13). Fifteen days post-immunization the meninges of these mice, which normally lack any CD4 T cells, showed a robust population of CD44^{+} OTII.Rag1$^{-/-}$ T cells, demonstrating that CNS-antigen irrelevant T cells do in fact gain access to the meningeal space.

Collectively, the outcome of each of these three experiments, meant to directly scrutinize the hypothesis that antigen specific T cells preferentially traffic to the meninges, did not support the model, lending credence instead to a model in which T cells traffic to the meninges on the basis of their memory phenotype. Importantly, while the generation of T cell memory is very much an antigen dependent phenomenon, traffic to the meninges appears to be independent of TCR specificity. Indeed, while accumulation of CNS self-antigen specific T cells may be an attractive idea in the context of learning and memory, it is selectively counterproductive. Not only would a population of CNS-reactive T cells in the meningeal space be a major liability for encephalitogenic autoimmunity, but it may also impede normal immune surveillance, as the slow migration of *pathogen*-specific T cells in the

meninges has obvious benefits for immune protection, whereas *CNS*-specific T cell migration would confer no such immunoprotection.

Importantly, these findings should not be misconstrued to suggest that CNS specific T cells do not traffic into the meninges. To the contrary, these findings argue that as long as the CNS-specific T cell in question is of the sufficient memory phenotype, it will similarly traffic through the meningeal space. Indeed, MBP and MOG specific T cells do in fact traffic to the meninges in the context of EAE[320, 335]. While these cells may not preferentially access the meninges relative to memory cells of non-CNS antigenic specificity, they might still be the specific subset that mediates higher brain functions.

In acknowledgement of this idea, we show in chapter 11 that MOG specific T cells can rescue the MWM impairment of OTII mice (Figure 11.10). Remarkably, while Tmog cells were not recoverable from the meninges 2 weeks following adoptive transfer, they left a meninges-specific cell population in their wake, suggesting that at some point in the two weeks prior, Tmog cells did in fact visit the meninges. While more immediate time-points post-transfer would be needed to validate the meningeal trafficking of Tmog cells, the absence of Tmog cells in the meninges 2 weeks post-transfer would be consistent with the theorized >50%/7.8 days meningeal T cell turnover rate calculated in this study (Figure 12.1 and 12.2).

T cells in learning and memory

Largely independent of meningeal immunity, the second overall aim of this work was to determine the lymphocyte subset necessary for normal learning and memory, and to elucidate what antigenic specificity requirement (if any) participates in the phenomenon. While meningeal immunity was not a precondition of this aim, the final studies of chapter 11 connect back to those of chapter 10,

raising the possibility that the composition and phenotype of the meningeal compartment can significantly affect higher brain function.

T cell deficient mice have persistently been observed to demonstrate impaired spatial learning and memory (reviewed in Chapter 9). While reconstitution of SCID and nude mice with wild-type splenocytes significantly rescues MWM measured learning and memory deficits, several fundamental questions about both the nature of the baseline behavioral impairment of lymphopenic mice, as well as their rescue by adoptive transfer, remain unanswered. Specifically, when this research was initiated, it was unclear what splenocyte population (i.e. CD4 *vs.* CD8) was necessary for normal cognitive task performance, and for the rescue of aberrant task performance in lymphopenic animals.

The first experiments in Chapter 11 essentially validated in $Rag1^{-/-}$ and $Rag2^{-/-}$ animals what had previously been shown in SCID and nude mice (Figure 11.1). Nevertheless affirmation of a robust and significant MWM impairment, particularly in $Rag2^{-/-}$ animals was worthwhile, as unlike Rag1[76] and Prkdc[352], Rag2 does not appear to be expressed at any point in the developing CNS.

It had been suspected that CD4 T cells were the lymphocyte subset necessary for normal learning and memory function, yet this hypothesis had not been directly addressed. While the MWM performance of nude mice was significantly improved by the adoptive transfer of wild-type splenocytes[81], it was unclear whether this represented a rescue of impaired learning or an enhancement of normal, unimpaired learning, as hairless nude mice cannot be fairly compared to wild-type animals in a water maze paradigm due to differences in thermal insulation and buoyancy. Indeed, the rampant homeostatic expansion that occurs when T cells are adoptively transferred into lymphopenic animals may have an intrinsic pro-cognitive effect that is unobservable in wild-type animals in which homeostatic proliferation does not take place (the possibility of a pro-cognitive effect associated with homeostatic proliferation is addressed later in this chapter). Therefore, while transfer of whole

splenocytes improved the MWM performance of nude mice, due to the inability to compare wild-type to nude mice in the MWM, it is not clear from this study whether the T cell deficient nude mouse has an intrinsic spatial learning and memory impairment. Nevertheless, it is clear from previous observations in SCID mice[81, 82, 100, 294] that naive lymphopenic mice manifest with impaired spatial learning and memory. Having recapitulated the SCID MWM impairment in Rag deficient animals (Figure 11.1), we set out to identify the relevant lymphocyte subset (i.e B cells, CD4s or CD8s) without the confounding consequence of a possible pro-cognitive effect of homeostatic proliferation (i.e. without adoptive transfer). Having ruled out an effect of B cell deficiency in MWM performance (B cell deficient uMT mice had normal MWM curves (Figure 11.2)), we next employed the MHCII knockout mouse to distinguish between the contribution of CD4 and CD8 T cells in MWM task performance.

Importantly the MHCII knockout model allowed us to assess the role of CD4/MHCII in a model in which CD8/MHCI interactions remained intact (Figure 11.4). While an MHCI knockout model presents itself as an obvious control to the MHCII knockout, MHCI plays a crucial role in the remodeling of synapses in development (Chapter 3) and cannot serve as an appropriate control to address the role of CD8 T cells in learning and memory. While the MHCII knockout is not a perfect system, owing to the possibility of a T cell independent function of MHCII in learning and memory, we viewed this possibility as remote, and conclude that the significant MWM impairment of MHCII$^{-/-}$ mice is attributable to the near total ablation of CD4 T cells in the animal (Figure 11.5).

While the aim of this study was to arrive at the subset and antigenic specificity of the lymphocytes involved in learning behavior, and not the function of these lymphocytes per-se, the possible mechanism by which CD4 T cells function in normal learning behavior merits discussion.

Admittedly, outside of phenomenological observations, we know virtually nothing about the mechanistic role of T cells in higher brain function. Nevertheless, the first step towards arriving at the role of T cells in learning and memory is drawing a distinction between two categories of T cell function: an active pro-cognitive function of T cells vs. an indirect function in cognitive impairment as a result of homeostatic dysregulation that occurs in the absence of T cells. In other words, do T cells actively participate in enhancing learning and memory formation, or does their absence cause a subclinical pathology that we measure in the MWM? While the former hypothesis is undeniably attractive, there is no evidence so far that this is taking place in a naïve animal; (a possible pro-cognitive effect of homeostatically expanding T cells is discussed later). On the other hand, T cell deficient mice have been repeatedly shown to have a systemic pro-inflammatory cytokine milieu, which in turn has been associated with impaired learning behavior.

In support of the model in which T cell insufficiency leads to homeostatic dysregulation, Derecki et al have shown a pro-inflammatory signature in the myeloid compartment of SCID mice following MWM training characterized by TNF and IL12[100, 241]. Similarly, Kim et al have shown dramatically enhanced serum TNF, IFNg, MCP1 and IL-6 expression upon PolyIC treatment in both $Rag1^{-/-}$ and SCID animals, and demonstrate that the death of SCID and $Rag1^{-/-}$ mice following viral infection stemmed not from an inability to control virus, but from unchecked innate inflammation in the absence of T cells[243]. Indeed, a similar (albeit more subtle) inflammatory response may be taking place as a consequence of the stress of MWM training, and it would be worthwhile to characterize any changes in the cytokine milieu that result from MWM training. Importantly, both Derecki and Kim reported diminutions of inflammatory cytokines, and a shift back to normalcy, upon adoptive transfer of wild-type splenocytes. While the Kim studies show that tempering of cytokine production could be achieved in vitro by both Tregs and non-Tregs, Derecki demonstrated that wild-

type but not IL-4 deficient pan T cells could temper intracellular proinflammatory cytokine production in meningeal myeloid cells[100].

While our studies did not address intracellular cytokines of meningeal myeloid cells, in light of the pro-inflammatory signature of lymphopenic mice, and in particular the demonstration by Derecki et al that pro-inflammatory cytokine production could be mitigated by pan T cells, it is likely that the antigen specific CD4 T cells, which we show to be necessary for normal learning and memory, are the subset that is mitigating the pro-inflammatory skew of lymphopenic mice. Future characterization of the myeloid compartment of lymphopenic mice before and after MWM training as well as determination of the relative contribution of CD4 regulatory T cells vs. non-Tregs in tempering the skewed myeloid phenotype of these animals should prove immensely informative.

Rescue of MWM impairment by adoptive transfer

There have been two general experimental observations in the study of T cell influence on higher brain function. One, that lymphopenic mice have impaired spatial learning and memory, and two, that this impairment can be rescued by the transfer of wild-type splenocyte and pan T cell populations, which we further refined to the CD4 compartment. Understandably, both observations have been viewed as manifestations of a single phenomenon, that is, the specific function of T cells in learning and memory is lost in lymphopenic mice, and the same function is restored following adoptive transfer. However, this chapter presents data that challenge this basic assumption, raising the possibility that T cells may rescue MWM impairment by a different mechanism than they use to achieve baseline MWM competency. Specifically, we show here that the common denominator in each MWM rescue paradigm is not the presence of CD4 T cells in the meninges, but rather the emergence of a new $CD11b^{+}$ $GR1^{+}$ MDSC population in the meningeal space (MDSCs are reviewed in chapter 5).

Importantly, each of the adoptive transfers attempted in this study (with the exception of Tmog transfer into OTII hosts) induces prolific homeostatic expansion. While the subject of T cell homeostatic expansion in the lymphopenic host is complex (we dedicate the entirety of chapter 5 to this process), one thing is very clear — that transfer of lymphocytes into a lymphopenic host does not yield a wild-type animal. The reconstituted mouse differs from its wild-type counterpart in the narrowing of its clonal diversity, in the disproportionate representation of T cells directed against self-antigens, in its heavy Th2 and memory phenotype skew, in the dramatic expansion of tissue infiltrating MDSCs and its totally unique cytokine milieu (Chapter 5). Therefore, the notion that T cells have unique consequences in the reconstituted lymphopenic mouse is not inconceivable.

The emergence of purported MDSCs warrants particular consideration, as it is the sole common feature in each of three successful schemes of impaired MWM rescue by adoptive transfer. The transfer of wild-type T cells into either OTII or $Rag1^{-/-}$ hosts resulted in not only the accumulation of meningeal T cells, but also in the unexpected emergence of a $GR1^{hi}$ population of cells, which was only minimally present in the naïve $Rag1^{-/-}$, OTII or WT mouse (Figure 11.12 and 11.14). The sheer abundance of these $GR1^{hi}$ cells, particularly in the meninges, was remarkable, and perhaps even reconcilable with the CD44 stringency model as these cells express high levels of CD44.

With one critical difference, the same dramatic induction of $GR1^{hi}$ cells was observed following adoptive transfer of Tmog cells into OTII hosts (Figure 11.12). Whereas the transfer of polyclonal wild-type T cells into OTII and $Rag1^{-/-}$ hosts resulted in the systemic emergence of $GR1^{hi}$ cells, the transfer of a monoclonal Tmog population resulted in a meninges specific accumulation. This striking outcome is easily appreciated (if not predicted) by the fact that MDSCs follow in the wake of autoreactive T cells.

While self-reactive Tmog cells are exactly what is transferred in the Tmog to OTII host scheme, the presence of autoreactive T cells resulting from transfer of wild-type T cells into either OTII or Rag1$^{-/-}$ hosts is more nuanced. The self-peptide MHCII-TCR interaction-dependent expansion of T cells in the lymphopenic host is an intrinsically autoreactive process, and the homeostatically expanding T cell repertoire has been shown to successively narrow in the direction of self-reactive TCR composition[164-166, 169, 170]. Indeed, the fact that host mice do not readily succumb to systemic autoimmune disease may be directly attributable to a compensatory induction of MDSC.

MDSCs are a heterogeneous cell population loosely defined as GR-1^{+} and CD11b^{+}. Analysis of GR1^{hi} cells in the meninges shows them to be CD11b^{hi} and Ly6C^{mid}, consistent with the phenotype of granulocytic MDSCs (Figure 11.13). However, the phenotypic designation of MDSCs is extremely murky, as neutrophils express identical surface markers. Indeed granulocytic MDSCs and neutrophils share many common features, and plasticity between the two cell types has been considered in several reviews[353-355]. Nevertheless, the possibility that these GR1^{hi} CD11b^{+} cells are classical inflammatory neutrophils is extremely unlikely. To start, these cells persist in the tissue for a very long time after adoptive transfer. Even in the transfer of Tmog cells into OTII hosts where Tmog cells are not found in the meninges two weeks post-transfer (Figure 11.11), these GR1^{hi} CD11b^{+} cells persist in the meninges for up to 11 weeks post-transfer, (Figure 11.12), which would not be consistent with the short-lived nature of inflammatory neutrophils[356, 357]. Furthermore, homeostatic expansion is an intrinsically autoreactive phenomenon, in which Tregs appear to be in abeyance to allow for homeostatic reconstitution (Chapter 5). Long-term neutrophil activity under these conditions would almost certainly result in full-scale systemic tissue destruction and autoimmunity, which is not observed. Finally, MDSCs have been described before in the context of homeostatic T cell expansion[193], autoimmunity[188, 189], and specifically in EAE induction via Tmog[331].

Importantly, induction of MDSCs by autoreactive T cells is a tissue-specific phenomenon; that is, MDSCs arise in the tissues that autoreactive T cells infiltrate[332]. In the case of homeostatic expansion in the lymphopenic host, autoreactive T cells are systemic, which accounts for the systemic induction of MDSCs (Figure 11.12), whereas in the Tmog transfer, autoimmune T cells are CNS specific, which explains their presence exclusively in the meninges (Figure 11.14).

Admittedly, the discovery of MDSCs in these systems was made entirely in retrospect from a serendipitous inclusion of GR1 antibody in FACS staining cocktails designed with other objectives in mind. However, given that these MDSCs are the common feature of all MWM rescues achieved by adoptive transfer, that they are potently anti-inflammatory, and that they are present in such astonishing numbers, it easily conceivable that these cells could temper the intrinsic pro-inflammatory skew of lymphopenic mice, leading to normalized (and possibly even enhanced), learning and memory function.

While MDSCs are probably not participating in the naïve wild-type animal, their role in mediating cognitive task performance may not be an entirely experimental phenomenon. Indeed, transient lymphopenia and homeostatic proliferation is a common feature of many viral infections (Chapter 5), and if MDSC induction is a feature of these acute bouts of homeostatic proliferation, then it is conceivable that this process may have natural relevance. The phenomenon may also be germane clinically in light of the homeostatic expansion that arises in response to radio/chemotherapy.

In spite of the natural relevance of the phenomenon, the sheer magnitude of MDSC induction after rescue of MWM impairment by adoptive transfer means that it cannot be ignored. Subsequent studies will need to explore a possible causal effect of MDSCs on spatial learning and memory outcomes. To this end, MDSCs can be isolated from the tissues and their functional capacity assessed in vitro, and GR-1 depleting antibody can be employed to selectively block MDSCs

following adoptive transfer. Additionally, the lack of homeostatic expansion of OTII T cells in lymphopenic hosts is the exception and not the norm for a monoclonal T cell (Chapter 5), and it will be valuable to see if MDSCs are induced after transfer of the many monoclonal T cell populations that do in fact expand homeostatically in lymphopenic hosts, and to determine whether these monoclonal adoptive transfers lead to tissue specific MDSC accumulation and improved MWM performance.

Concluding summary:

T cell influence on higher brain functions is not the product of one or two cytokines and cell types, but represents the homeostatic confluence of two entire ecosystems. In spite of the intrinsic complexity and interconnectedness of this phenomenon, these studies have made significant advances in defining the subset, antigenic specificity and localization of the T cells involved.

While the data generated in Chapter 10 did not support my initial idea of resident, CNS antigen-specific T cells in the meninges, the experiments that invalidate this hypothesis have shed light on more fundamental neuroimmunological processes. Specifically we show a tight T cell connectivity between the meninges and deep cervical lymph node, and reveal a preferentially robust program of immune surveillance at the meninges–a finding with an elegant selective advantage and one that may have significant consequences for neuroimmune etiology.

These studies have for the first time firmly defined antigen specific CD4 T cells as necessary for both normal MWM behavior, and sufficient for rescue of impaired behavior in lymphopenic mice. Importantly, the work presented here raises the possibility that T cells may rescue MWM impairment by a different mechanism than they use to achieve baseline MWM competency. We submit that future experimentation will have to tackle the role of T cells in maintaining learning and memory function as a separate phenomenon from the ability of adoptively transferred T cells to rescue

behavioral impairment in lymphopenic animals–a distinction that has so far eluded the field and confounded interpretation from many model systems.

In pursuit of the first phenomenon–the necessity of antigen specific T cells in basal MWM competency–the leading mechanistic contender is cognitive impairment brought on by a pro-inflammatory skew in the meningeal myeloid compartment in the absence of antigen specific CD4 T cells. Future efforts on this front should include thorough characterization of the meningeal inflammatory milieu across lymphopenic and TCR transgenic mouse models, and whether or not the pro-inflammatory skew is precipitated by the MWM task or whether it exists in spite of it. Nevertheless, at the mechanistic heart of the matter is the question of why the meningeal myeloid phenotype is so severely skewed in the absence of T cells. Insight into this fundamental question may come from studies of exaggerated cytokine responses in lymphopenic mice to infection and PolyIC treatment[243, 358]. Those studies have implicated a role for T cells in suppression of the inflammasomal pathway[244], and it will be gainful to determine if the same mechanism is at play in this system.

While T cells are *sufficient* to rescue MWM impairment in lymphopenic (or functionally lymphopenic) mice, they are not *necessary* (model presented in Figure 12.6). In other words, the MWM impairment of lymphopenic mice may be rescued by more than a single means, or specifically, by any route that mitigates the intrinsic inflammatory skew of meningeal myeloid cells in these animals. Our studies reveal a potential for induced meningeal MDSCs to counterbalance the pro-inflammatory skew of lymphopenic animals and rescue MWM impairment. However other routes of rescue likely exist, including transfer of M2 skewed macrophages[238]. While these cell types warrant further investigation based on their sufficiency to rescue cognitive impairments in mice, they are not immediately practical targets for clinical therapy. Finding pharmacological modes of suppressing the chronic meningeal inflammation that may underlie cognitive impairments from chemobrain to age-

and HIV-associated dementia, would also be a relevant line of future investigation encouraged by this body of work.

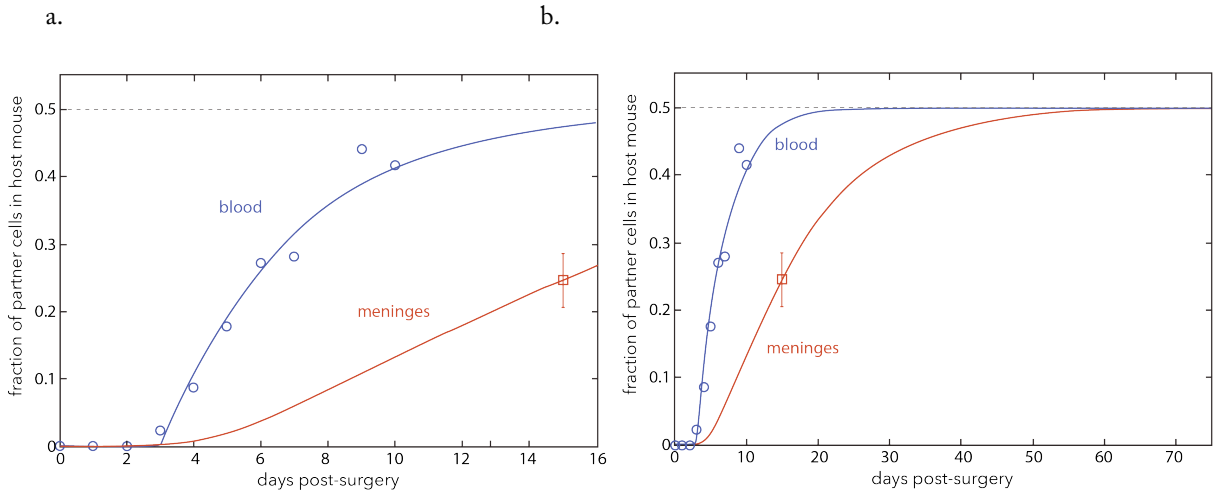


Figure 12.1: Theoretical kinetic determination of blood and meningeal T cell chimerism in Ly5.1-Ly5.2 parabiotic pairings. a. blood and meningeal chimerism from 0→16 days post-surgery. b. blood and meningeal chimerism from 0→70 days post-surgery. Graphs illustrate the delayed chimerism kinetics of T cells in the meninges relative to the blood in Ly5.1-Ly5.2 parabiotic pairings. Values for blood chimerism derived from % CD45 chimerism in blood of parabiotic mice assessed daily from 0→10 days post-parabiotic surgery in Wright, D.E., Wagers, A.J., Gulati, A.P., Johnson, F.L. & Weissman, I.L. Physiological migration of hematopoietic stem and progenitor cells. *Science* 294, 1933-1936 (2001).

Figure 12.2: **Theoretical calculation of the meningeal T cell turnover rate.** Calculations for the theoretical turnover rate of meningeal T cells assuming non-instantaneous blood chimerism were the kind courtesy of Dr. Brian Skinner who was provided with values of meningeal T cell chimerism at day 15 post-surgery, and values of blood chimerism derived from a published study in which the percent blood chimerism was determined daily from 0->10 days post-surgery[338]. The calculations are provided in his correspondence:

Figure 12.2 continued from previous page:

Below is everything I understand about your problem. Since I'm an impatient person with no appreciation of nuance, I'll talk about the "green mouse" and the "white mouse", and "green blood" and "normal blood." I'll also just say "brain" instead of "meninges".

All of the results I work out below are for the fraction of green blood in the white mouse. Assuming the mice are weight-matched, everything is symmetric, so it should be the same as the fraction of white blood in the green mouse. If you want to get the result as a percent, multiply everything by 100.

Let me call c(t) the concentration of green blood (as a fraction of the total) in the body of the white mouse. After the white and green mouse are stitched together, the green blood starts to flow flow from the green mouse to the white mouse. This means that c(t) increases from zero and eventually levels off at c(t) = 1/2. The question is how c(t) increases with time.

There is a very general law in physics for situations like this, called Fick's law. It says that the flow rate of something (here, green blood) is proportional to the gradient of concentration (here, the difference in green blood concentration between the green mouse and the white mouse). The concentration of green blood in the white mouse is c(t). The concentration of green blood in the white mouse is 1 - c(t). So the difference is concentration between the two mice is 1 - 2c(t). This difference is proportional to the flow of blood from one mouse to the other, which in turn is proportional to the rate at which c(t) is increasing. As an equation, this is

$$\frac{dc(t)}{dt} \propto 1 - 2c(t)$$

You can turn this into an equation with an equals sign by writing

$$\frac{dc(t)}{dt} = \frac{1 - 2c(t)}{\tau_c}$$

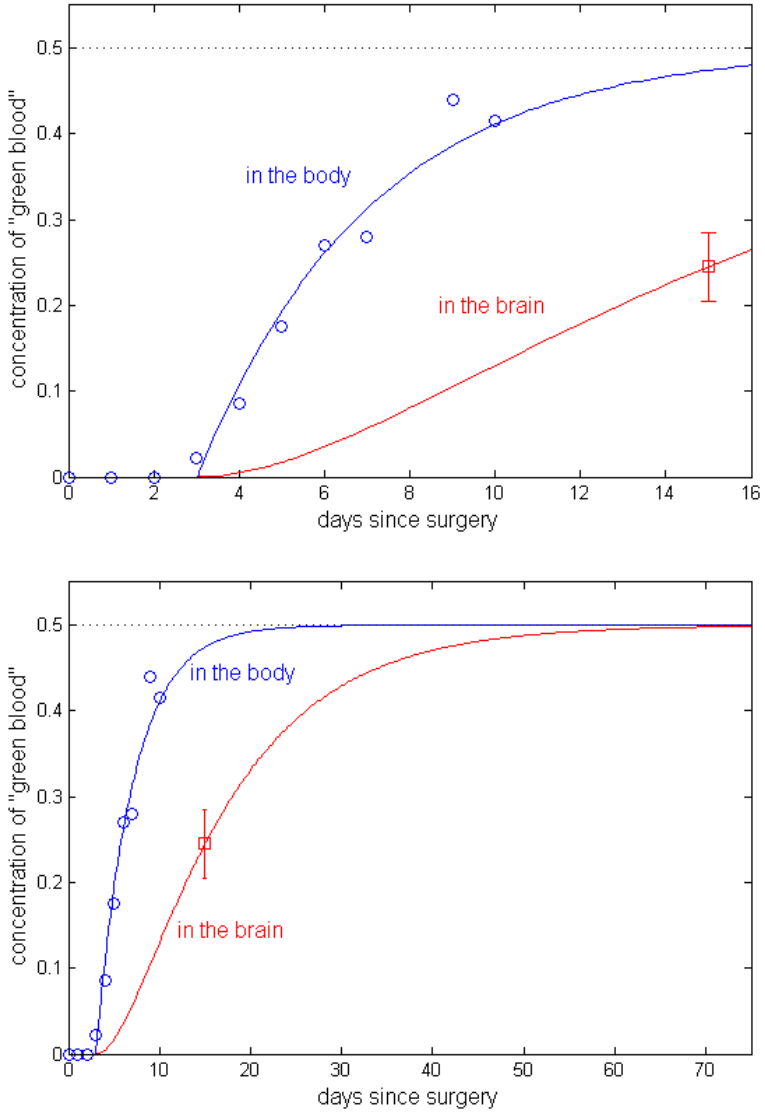
where the variable "tau_c" is some constant that has units of days. Roughly speaking, tau_c defines how long it takes blood to move from one mouse to the other. The solution to this equation is

$$c(t) = \frac{1}{2}(1 - e^{-t/\tau_c})$$

[If you remember how to take derivatives, you can plug it into the equation above and check that everything works out.]

In order to figure out the value of tau_c, you can compare the equation for c(t) to the data from the published figure you sent me. Making a fit to the data tells you two things:
1) Blood really doesn't start exchanging between the two mice until day 3. I'm guessing that's because the blood vessels haven't grown together yet. This means that "t" in the equations above should be measured from day 3 after the surgery, not day 1.
2) tau_c = 4 days.
You can check out how this equation looks compared to the data in the first attached figure. The

Figure 12.2 continued from previous page:



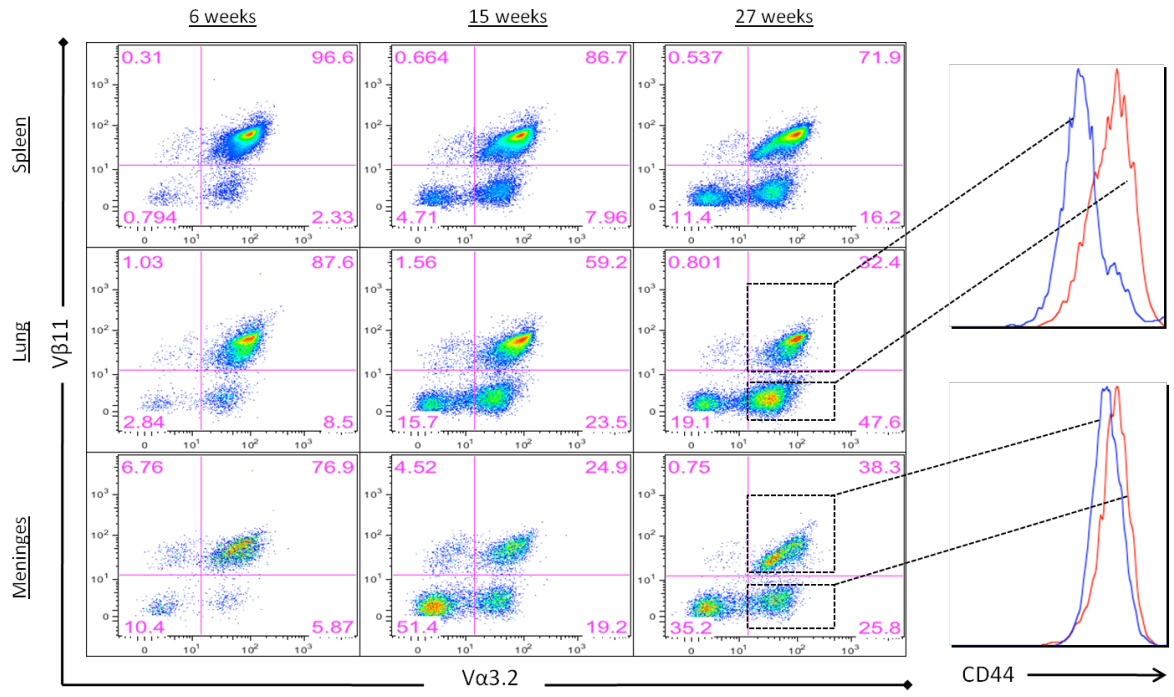


Figure 12.3: Preferential and age-dependent accumulation of the non-transgenic T cell pool in the meninges of 2D2 mice. FACS analysis of CD4⁺ T cells of 2D2 mice heterozygous for MOG reactive TCR (Vβ11⁺/vα3.2⁺) sacrificed at the indicated ages, showing overrepresentation of non-transgenic T cells in the meninges relative to lung and spleen. In the right histograms, CD44 expression is compared on MOG reactive TCR transgene baring T-cells (VB11⁺, Va3.2⁺) with T cells bearing the transgenic vα3.2 and an endogenous Vβ.

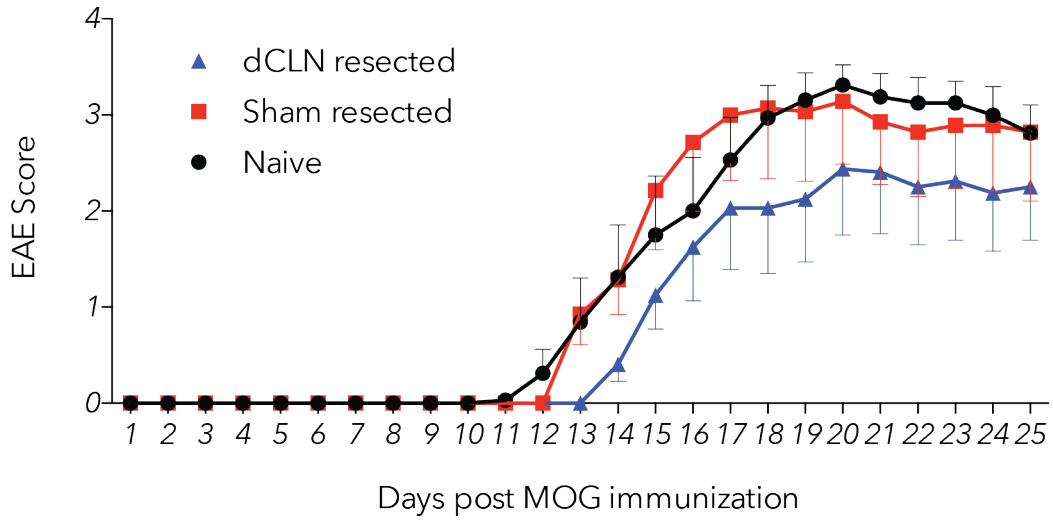


Figure 12.4: **Reduced EAE severity in dCLN resected mice.** MOG induced EAE disease severity scores in naïve, dCLN resected and Sham resected mice, showing reduced disease severity scores in dCLN resected mice relative to sham and naïve groups. n=8 mice/group.

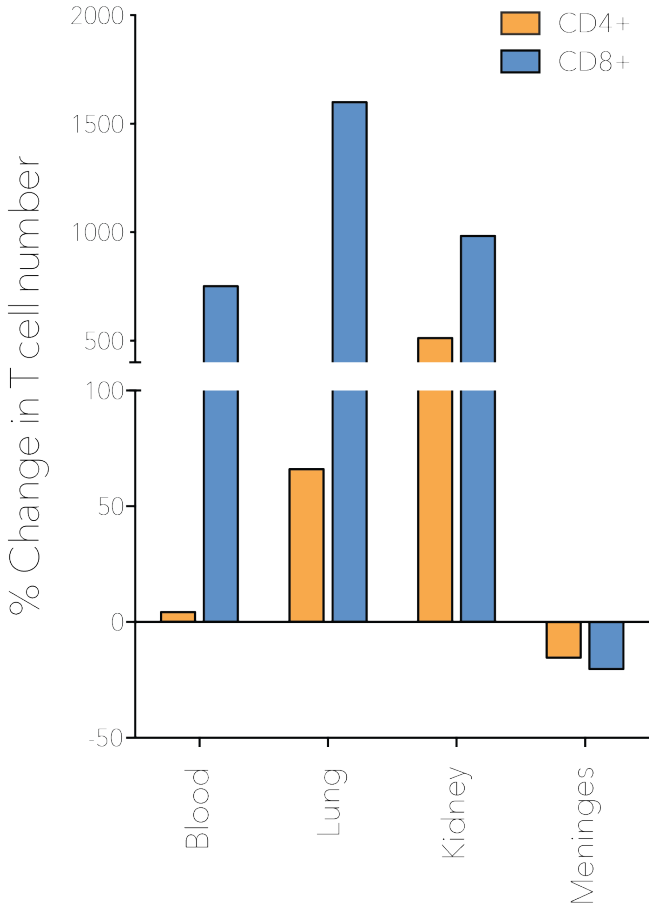


Figure 12.5: Change in Total T cell numbers in the indicated tissues of mice immunized with non-replicating adenovirus. Wild-type mice were immunized i.v. with non-replicating ova-expressing adenovirus and the indicated tissues were examined for changes in total CD4 and CD8 T cell numbers eight days post-infection. Data shows that not all expanding/activated T cells gain access to the meningeal space. n=2 mice/group.

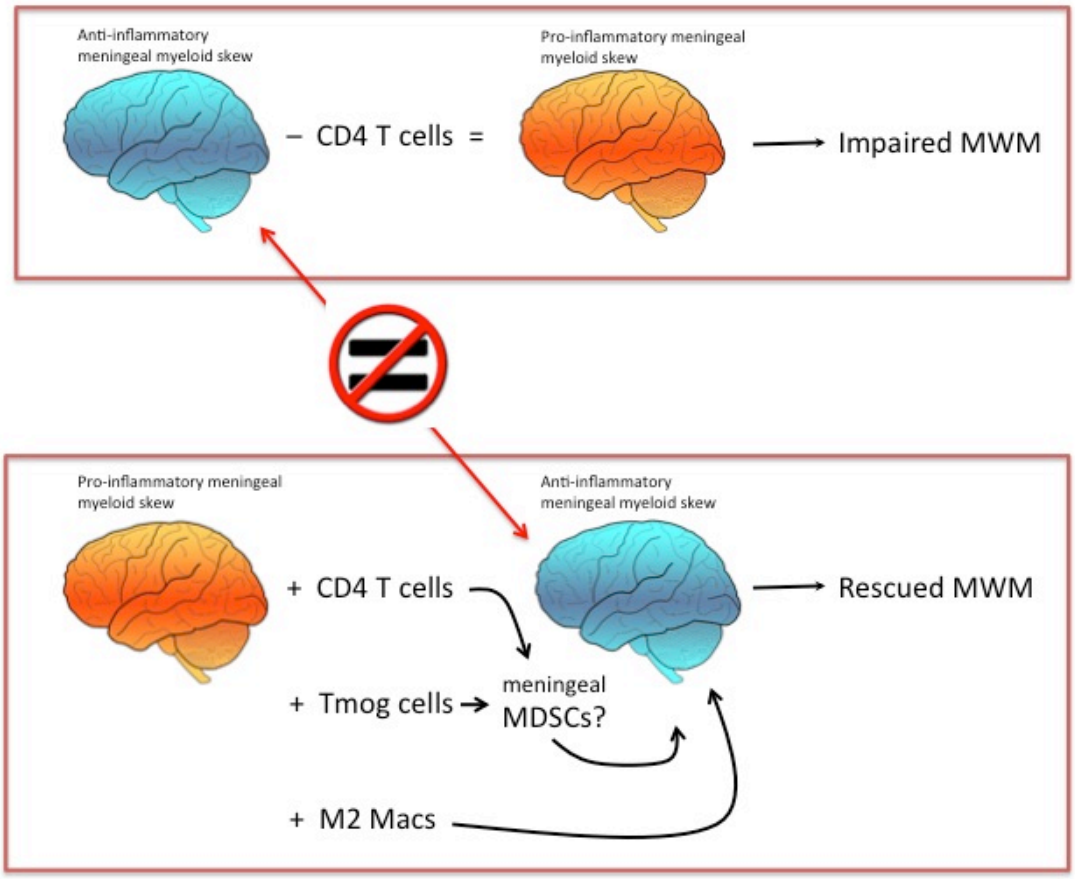


Figure 12.6: A model proposing distinct T cell functions in maintaining normal learning and memory, and rescuing learning and memory impairment in lymphopenic mice. The upper box summarizes the generalized model in which loss of CD4 T cells results in the pro-inflammatory skew of meningeal myeloid cells, which in turn results in impaired MWM function. The lower box summarizes the models of MWM rescue in lymphopenic mice. Adoptive transfer of polyclonal CD4 T cells, or CNS-reactive Tmog cells into lymphopenic hosts results in rescue of MWM performance, possibly through the induction of MDSCS. T cells are sufficient but not necessary to rescue MWM impairment in lymphopenic mice, as transfer of M2 skewed macrophages also achieves MWM rescue. The arrows connecting the two boxes illustrates that the immune system of the T cell reconstituted mouse is not the same as that of the wild-type animal.

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