DYNAMIC INTRAVITAL IMAGING OF IMMUNE CELLS
DURING THE INITIATING EVENTS OF
EXPERIMENTAL AUTOIMMUNE
ENCEPHALOMYELITIS

by

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We also certify that written approval has been obtained for any proprietary material contained therein.
DEDICATION

I dedicate this work to my father, Boon Kheng, who because of his own experience of opportunities gained through education, ultimately made life sacrifices along with my mother, Lucy, to ensure that my siblings and I were able to receive a higher education. I hope I have fulfilled his vision of empowerment through education. I also dedicate this work to my siblings, for their love, support and who have influenced my life in so many ways. Agnes for being the calming voice within me when I am stressed; Elizabeth for being my rock and the best role model for women in engineering; David for being consistently concerned for me and so supportive; Sandra, who in my mind was my first teacher and who has continued to teach me throughout life; and Timothy who not only showed me the rigor to excel but helped me make sense of life. Most of all, I dedicated this work to Kestutis, the love of my life, who not only helped me analyze hours of imaging data with Matlab, helped me construct microscope environment chambers but provided me with delicious food, many laughs and most of all love and support, I could not have done this without you.
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Dynamic Intravital Imaging of Immune Cells During the Initiating
Events of Experimental Autoimmune Encephalomyelitis

ABSTRACT

By

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Experimental autoimmune encephalomyelitis (EAE) is a murine model of central nervous system (CNS) inflammatory disease resulting in immune cell accumulation, neuronal tissue destruction and paralysis.

With this work, we endeavor to reconcile the literature on EAE, from the action of pertussis toxin (PTx) in the initiation of inflammation through to infiltration of CD4⁺ T cells. We show for the first time that localized, transient vessel leak initiates an inflammatory state within the CNS that results in microglia activation, and recruitment of dendritic cells (DCs). We also show for the first time that microglia are able to project extensions into the blood vessel with an intact blood brain barrier under non-inflammatory conditions and that the number of projections increase during the first 12 days of EAE induction. These resident microglia and peripheral DCs accumulate near vessel leaks and recruit myelin-specific T cells to cause subsequent tissue damage and plaque formation. This inflammatory process, serial immune cell infiltration and tissue destruction can be dampened by the use of anti-histamines to inhibit the activation of microglia at the onset of disease initiation.
1 Introduction

EAE is driven by a population of myelin-specific, auto-reactive T cell and aspects resemble multiple sclerosis (MS). EAE presents as a disease of the subpial spinal cord in the lumbar region in areas in contact with the CSF, whereas MS lesions are found in the brain with demyelination of the cerebral and cerebellar cortex\(^1\). Involvement of the cortex in EAE has been difficult to prove given standard techniques and therefore it has not been well studied. Via bioluminescence imaging of these myelin-specific T cells, it was shown that in EAE, T cells do enter the brain by day 3 followed by infiltration into the spine by day 7 and both of these events happen before clinical disease onset around day 10\(^2\).\(^3\), Microglia are the dominant surveying immune cell population in the CNS parenchyma\(^4\). DCs are bone marrow derived professional antigen presenting cells (APCs) and are virtually non-existent in the CNS parenchyma under non-inflammatory conditions\(^3\).\(^5\). In EAE, peripherally activated or transferred effector T cells cross from blood circulation through either the choroid plexus into the cerebrospinal fluid (CSF) or at Virchow-Robin Spaces (VRS) to a perivascular location. The re-activation of T cells by macrophages or DCs in the CSF or VRS are postulated to play an important role during the induction of EAE\(^6\).\(^7\).\(^8\). However, antigen surveillance and presentation in the CNS is unique from other tissues of the body due to: (1) suppression of microglia responses by neurons\(^9\), and; (2) the blood-brain barrier (BBB) which poses a structural impediment for peripheral antigen presenting cells (APCs)\(^10\).\(^11\).\(^12\). It has been shown that parenchyma infiltrating DC are required for induction and progression of disease in EAE\(^5\).\(^13\).\(^14\) although the route of entry of these cells into the CNS have not
been identified. Thus taken together, current dogma suggests that the presentation of CNS tissue-derived myelin peptides by APCs and subsequent re-activation of myelin-specific T cells can occur randomly in the CNS and are not restricted to specific locations during inflammation.

Histologic studies of static brain tissues provide evidence that perivascular CD11c+ APCs help to orchestrate immune responses by extending dendritic extensions into CNS parenchyma, presumably to sample and present brain-derived antigens to other immune cells in the VRS\textsuperscript{15}. The observation that APCs can insert dendritic extensions for antigen sampling in a separate anatomic compartment has been shown in many organs of the body in contact with the external environment\textsuperscript{16, 17, 18, 19, 20, 21, 22, 23}. This new phenomenon provides a location-specific mechanism by which T cells could enter the CNS. We hypothesize that microglia can sample directly into the blood vessel lumen without breaching vessel integrity, where circulating antigen-specific lymphocytes could potentially directly recognize antigens on the intravascular dendrites to result in intraluminal activation and subsequent extravasation.

Post mitotic neurons in the CNS are protected from peripheral pathogens and immune reactions by physical barriers and a host of immunosuppressive cytokines including TGF-β\textsuperscript{24, 25}. Intravital imaging has shown that in areas of microglia-macrophage clusters, there are vessel leaks during EAE\textsuperscript{25, 26, 27, 28, 29, 30}.  


Factors found in blood such as platelets, fibrinogen, and complement factors, have been shown to activate microglia within the CNS and lead to neuronal destruction whereas erythropoietin and von Willebrand factor have been shown to be beneficial for remyelination. There is growing evidence of the role of vascular leakiness in the etiology of neurodegenerative diseases, in particular MS, vascular dementia, epilepsy, Alzheimer’s, Parkinson’s and most obviously stroke. We propose that vascular dysfunction induced by paracrine factors and toxins within circulation allow solutes to enter the CNS parenchyma, initiating an inflammatory environment that leads to microglia activation, infiltration of APCs and T cells, resulting in glia and neuronal destruction (Fig 1.)

Together, these two hypotheses study the communication of the immune system from both sides of the solute and cellular gate keeper of the CNS, the BBB. First, we explore during a peripheral immune response against myelin antigen that is largely found on the other side of the BBB, how and when the circulating immune cells gain access to the CNS. Second, we investigate how microglia behind a tightly regulated wall are able to either survey peripheral systemic pathogens or recruit peripheral immune cells into the CNS during disease. Are vascular leaks a cause or response to microglia extensions, or is there no correlation?
We propose that vascular dysfunction induced by paracrine factors within circulation allow solutes and toxins to enter the CNS parenchyma, initiating an inflammatory environment that leads to microglia activation, infiltration of peripheral APCs and T cells, resulting in glia and neuronal destruction.
Models for Studying Multiple Sclerosis in Rodents

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS). MS is a progressive neurodegenerative disease initiated by CNS inflammation that is characterized by extensive immune cell infiltration, which results in myelin and in some cases axonal destruction. The exact cause for the inflammation remains unclear and the heterogeneity of demyelination patterns coupled with differences in surrounding oligodendrocytes and microglia suggests that the initiating pathology may also be heterogeneous but is thought to be an inflammatory T cell initiated and mediated disease. Experimental autoimmune encephalomyelitis (EAE) is a murine model of CNS inflammatory disease resulting in peripheral immune cell accumulation, neuronal tissue destruction and ascending paralysis which resembles clinical MS. Even though induction of the disease is reproducible, by combining myelin peptides with an adjuvant to activate T cells in peripheral lymph nodes (LN) in conjunction with a systemic inflammatory stimulus of pertussis toxin (PTx), the exact initiation events remain unknown.

From the first attempts at producing an animal model for MS, CNS homogenates were used in xenograft immunization protocols to induce paralysis. Iterations from these first animal models have been small and today there are four main myelin peptide based immunization protocols used to induce EAE:
(a) myelin basic protein (MBP) in Lewis rats induces a monophasic, self-remitting form of paralysis\textsuperscript{47,48}

(b) myelin oligodendrocyte glycoprotein 35-55 (MOG) in C57BL/6J mice induces a chronic-progressive form\textsuperscript{49}

(c) proteolipid protein (PLP) in SJL/J mice induces a relapsing-remitting form of disease\textsuperscript{50}

(d) myelin-associated oligodendrocytic basic protein and 20,30-cyclic nucleotide 30-phosphodiesterase for Balb/C and SJL/J mice\textsuperscript{51}

Survey of methodology in the literature shows that there is a wide range of induction variables with differing amounts of peptide, complete Freuds adjuvant, amount and dosing schedule of pertussis toxin, and some include irradiation of the recipient animal. The interpretation of results becomes more problematic when it was discovered that primed, activated myelin specific T cells could be isolated from a donor rat, in vitro expanded and transferred into a recipient rat to cause disease\textsuperscript{52}. Thus more variables were introduced including in vitro expansion conditions, the number of transferred T cells and the inflammatory conditions of the recipient mouse. Key features common to all models include:

(a) Ascending paralysis
(b) Peripherally activated or transferred effector myelin-specific, auto-reactive T cells cross from blood circulation through the choroid plexus and Virchow-Robin space into the parenchyma or CSF.

(c) Peripherally activated dendritic cells are required for the crossing of these peripherally activated or transferred effector T cells into the parenchyma or CSF during the effector phase of disease induction.

(d) Some toxic agent or irradiation is required to cause dysfunction of the BBB.

There are viral models that induce CNS demyelinating lesions even though there is weak evidence of viral persistence as a cause of MS\(^1\). Theiler's encephalomyelitis virus is the most common\(^53\) model used to study some aspects of CNS inflammation.

**Anatomy of the Blood Brain Barrier**

The pathway for antigen surveillance and presentation in the CNS is unique from other tissues of the body due to the BBB, which poses a structural impediment for APCs as well as antigens.
The BBB is a functional unit comprised of: endothelia; astrocyte end feet and microglia that together, highly regulate the solutes and cells passing from the blood vessel into the parenchyma\textsuperscript{54} (Fig. 2a). The endothelia of the CNS have complex tight junctions, low endocytic activity and an absence of fenestrations, that prevent the passage of most polar and hydrophilic solutes from the blood into the brain\textsuperscript{55}. Astrocytes have two main roles within the CNS: they regulate ions and uptake excess neurotransmitters at the synapse\textsuperscript{56} they regulate blood flow\textsuperscript{57} and the passage of cells and solutes into the parenchyma\textsuperscript{58}. The basal lamina surrounding the endothelial cells is contiguous with the plasma membrane of astrocyte end-feet. Overlapping astrocytic end-feet have been shown by ultrastructure 3D reconstruction to cover the entire length of endothelial basal lamina, with exception of areas with pericytes, and where microglia connect with the basal lamina\textsuperscript{59}. The meninges surrounding the CNS consists of three distinct layers: (1) the outermost dura mater; (2) the arachnoid mater; and (3) the pia mater; collectively termed the leptomeninges. Astrocyte foot processes are found up against the collagen rich pia mater, forming a barrier termed the glia limitans, and the astrocytic end-feet ensheath blood vessels as they penetrate the parenchyma from the sub arachnoid space (SAS) in the leptomeninges\textsuperscript{60} (Fig. 2b).

Whereas peripherally activated APCs and soluble antigens can drain directly to the closest LN, in the CNS the cerebral spinal fluid (CSF) acts as the lymphatic fluid. Clearance of soluble protein waste products occurs through the removal of
(a) The BBB is comprised of endothelial cells, astrocyte end feet and microglia. Pericytes and peripheral immune cells such as macrophages can be found distributed along the blood vessel between the endothelium and basal lamina\textsuperscript{61}. (b) The blood-CSF (BCSF) barrier is different from the BBB in that astrocytes in the CNS parenchyma ensheath vessels with foot processes as they penetrate the CNS from the meninges, through the pial layer. The vessel on the subarachnoid side of the pial layer does not have the same barrier regulation\textsuperscript{6}. 

Figure 2: Cellular components of the blood brain barrier. Adapted from Guilleman\textsuperscript{61} and Ransohoff\textsuperscript{6}.
interstitial fluid termed the glymphatic system. Para-arterial influx of CSF was shown to enter the parenchyma, and wash through extracellular solutes from the interstitial space to drain into the para-venous space where it is routed to cervical lymph nodes and to the CSF at the glia limitans. Up to 50% of the CSF soluble contents exit via the cribriform plate into deep cervical LNs, and the rest that contains solutes and cells, drains at the spinal roots or the venous sinus directly into venous circulation. It was shown that activated T cells can be found in the cervical lymph node in the early days of EAE induction and that removing the cervical lymph nodes result in delayed onset and decrease in disease severity but how APCs in the parenchyma relate to the APCs in the cervical lymph node and their role in licensing and activation of T cells in the parenchyma is still unknown. Vessels that carry CSF along the cribriform plate are only 100-150 nm wide which is too small for dendritic cells, macrophage or microglia to migrate to the deep cervical lymph node.

**Antigen Presenting Cells of the CNS**

The antigen presenting cells (APCs) that have access to the brain parenchyma are microglia, macrophages and dendritic cells. Microglia, defined as Ly6Clo/CD45lo/Iba-1+/CX3CR1+, constitute ~10% of the cells in the CNS and are the main immune cell population in the parenchyma under non-inflamed conditions. Microglia are derived from the yolk sac before embryonic day 8, once they have migrated to the CNS they are walled off from the blood vessel
and peripheral system by astrocytes. Microglia are self-renewing, replaced by local proliferation throughout life. Microglia are evenly distributed throughout the CNS and have a ramified morphology to survey all of the CNS. There is heterogeneity in the protein expression and functional responses of microglia based on location within the CNS. Microglia are also the first cells to respond to injury in the CNS and are thought to play a role in antigen presentation in the CNS, even though they have been shown to not be as effective at presenting antigen as macrophages and dendritic cells. CX3CR1 is a fractalkine receptor almost exclusively expressed in microglia in the CNS, while NK cells, activated CD8 T cells, dendritic cells, and a subset of monocytes also express the GFP marker in the peripheral tissues.

Perivascular and meningeal macrophages are derived from circulating Ly6Chi/CD45hi/CCR2+ monocytes and can sample CSF contents in the arachnoid and Virchow-Robin space. Monocyte development occurs initially in the yolk sac like microglia but later in development, monocyte production moves to the fetal liver, before finally residing in the bone marrow before birth. In the adult, monocytes arise from hematopoietic stem cells in the bone marrow. Interestingly, resident macrophages in peripheral tissue are primarily maintained by local proliferation, similar to microglia. Tissue resident macrophages are exquisitely tuned immune cells that provide balanced homeostatic and regulatory function (dead cell phagocytosis, antigen presentation, and soluble factor regulation) within the organ systems they survey. However, subpopulations of
macrophages display a discrepancy in their antigen presentation capabilities based on the tissue microenvironments in which they reside\textsuperscript{77}. For example, lung resident macrophages are exposed to the external environment and express high amounts of esterase, TNF-α and MIP-1α; however, microglia do not express these cytokines in high amounts nor present antigen as efficiently because the majority of the available antigen under homeostatic conditions are self-derived, neurons regulate microglia function, and by-stander damage to neurons could result in severe deficits for the whole body.

Until recently, there were 2 known markers that differentiated microglia from peripheral macrophages because these cells express many of the same surface receptors, including CD11b, CX3CR1, CD68 and Iba-1\textsuperscript{4, 78}. Distinction via morphology has been generally accepted however could be problematic as activated microglia have been reported to retract processes and either take on a spindle shape or balled morphology\textsuperscript{61} similar to the morphology of peripheral infiltrating macrophages. CD45 commonly assayed by FACS, is a membrane-bound protein tyrosine phosphatase highly expressed on the surface of monocytes and macrophages and in lower levels on microglia\textsuperscript{79, 80}. However, it has been shown that levels of CD45 can increase on microglia during activation and inflammation\textsuperscript{81}. The second marker is not widely used due to human toxicity issues, mistletoe lectin is used in IHC and stains microglia but not macrophages\textsuperscript{82}. An extensive gene and microRNA array analysis of microglia isolated from brain versus resident macrophages from various other organs,
identified P2ry12, Fcrls, Tmem119, Olfml3, Hexb and Tgfbr1 as unique microglial genes and 74 proteins that were uniquely expressed in microglia\textsuperscript{83}. P2ry12 and FCRLS were stringently analyzed for protein expression and FACS analysis revealed that antibodies to FCRLS and P2ry12 stained isolated adult microglia but did not stain CD11b-gated myeloid cells from murine spleen, bone marrow and peripheral blood. Via IHC, the P2ry12 signal co-localized with CX3CR1GFP/+ microglia.

CD11c+ dendritic cells (DCs) are the most potent and effective antigen presenting cell\textsuperscript{84}, defined as Ly6Chi/CD45hi/Iba-1-. DCs are bone marrow derived and are virtually non-existent in the CNS parenchyma under non-inflamed conditions\textsuperscript{3, 85}. It has also been shown that MHCII restricted to infiltrating dendritic cells is required for induction and progression of disease in EAE\textsuperscript{13, 86}. There are 2 critical times within the induction of EAE where DCs play a pivotal role: during the activation of T cells in the periphery, and; during re-activation of T cells right before entry into the parenchyma. To date, the trafficking to and specific interactions of these APCs and T cells within the CNS have not been elucidated. In other tissues during inflammation, T cells interact with selectins, adhesion molecules and chemokines expressed on endothelial cells to home to the site of inflammation. In the CNS, it is thought that T cells require interaction with an APC before entering the parenchyma. The idea of an APC needing to license the T cell is born from the observation that in a chimera model where peripheral cells lack MHCII and microglia have functional MHCII, transferred cells
are unable to infiltrate the CNS, whereas in a chimera where microglia lack MHCII and peripheral cells have functional MHCII, these mice develop EAE normally. In addition, transferred activated T cells still require 3 or more days to gain access to the parenchyma even when the recipient is irradiated to cause openings in the BBB.

The role of APCs in EAE is still controversial. In contrast to the role of DCs described above, other studies have shown that infiltrating macrophages using the CCL2-CCR2 axis are required for disease\(^\text{41, 75}\). Once in the CSF the re-activation of T cells occurs in locations in contact with CSF during inflammation, where the presentation of CNS myelin peptides by meningeal macrophages is postulated to play an important role during the induction of EAE\(^\text{87}\). Additionally, it has been shown that increase in circulating inflammatory monocytes correlates with relapses in EAE\(^\text{88, 89}\). Finally, activation of resident microglia have been shown to play a critical role in the destruction of axons in EAE\(^\text{25}\). Historically, chimeric mouse models have been used to differentiate the APC contribution of microglia, which are radio resistant, from peripheral bone marrow derived cells that are more readily susceptible to irradiation or drug treatments such as macrophages and dendritic cells. The flaw in this methodology is that irradiation causes alterations in the BBB endothelium\(^\text{90}\), and in astrocyte function thus allowing previously inhibited substances and cells to move from the blood stream into the CNS parenchyma\(^\text{91}\). This method can result in small numbers of donor cells that take on microglia-like morphology in the parenchyma of the brain\(^\text{92}\).
Two methods that avoid irradiation are in utero chimeras and parabiosis. In utero chimerism is achieved by transferring liver cells of a donor fetus into the recipient fetus on E13 while hematopoiesis is transferring from the liver to the bone marrow after the formation of microglia on E8\textsuperscript{93, 94} but this method often results in low efficiency. In parabiosis, two animals are surgically joined by the skin at the side so they fuse circulation, eliminating the injection of donor hematopoetic stem cells and allowing the naturally circulating hematopoetic stem cells to populate both animals. In this method lower levels of chimerism are achieved unless one of the animals is irradiated\textsuperscript{68, 91}.

Exploiting the differential expression of CCR2 and CX3CR1 on macrophages and microglia lead to the development of a double transgenic mice in which CCR2 is replaced by RFP and CX3CR1 is replaced by GFP\textsuperscript{72, 95}. In these animals, microglia express GFP and are RFP negative and newly infiltrating macrophages express RFP but emerging evidence shows CX3CR1 GFP expression increases over time while RFP expression decreases the longer the cell stays in the tissue\textsuperscript{96}. The limitation of this mouse model is there may be a differential disease dependency on heterozygote versus homozygote expression of the receptor. For example, in some inflammatory models all infiltrating cells have been found to the CCR2 positive, but others have shown a combination of CCR2+ and CCR2- monocytes\textsuperscript{88}. 
To circumvent artifacts created by ex-vivo manipulation and irradiation we will use APC fluorescent reporter mice, CX3CR1 GFP/+ for microglia and macrophage or CD11c-GFP for dendritic cell. We will use the transgenic T cell 2D2 mouse where T cells have a TCR specific for myelin oligodendrocyte glycoprotein (MOG35-55) and cross it to a mouse that express cyan fluorescent protein under the beta actin promoter.

**Trafficking of Peripheral Immune Cells into the CNS**

Neuroscientists have spent hundreds of years dissecting and defining neuronal regions and pathways within the CNS, while assuming that the accessory cells function uniformly throughout the tissue. It is emerging that there is a diverse response from microglia and astrocytes in different regions of the CNS and it follows that during inflammatory conditions, varied outcomes can be observed\(^97, \)\(^4, \)\(^24\). Many immune cell subsets have been studied for relative contribution to EAE and MS during the late phases of active CNS tissue destruction in an attempt to identify the culprit and develop a therapeutic target. T cells have been most intensely researched because: (1) a new category of autoimmune T helper cells that express IL-17 were discovered using the EAE system\(^98, \)\(^99\) and (2) these Th17 cells can be transferred to other animals to drive disease but it is still uncertain exactly how these cells traffic from the peripheral location of activation into the CNS\(^8, \)\(^100\).
Research has focused on the acute rather than induction phase of EAE, thus there are still many unanswered questions about the immune cells that play a role in initiating the disease. One current theory of immune cell trafficking during inflammation induced by EAE depicted in Fig. 3, is that T cells are activated in lymph nodes as described above and travel to the blood stream where they traffic to the CNS, through the choroid plexus into the cerebrospinal fluid, once within the CSF, they can encounter meningeal macrophages. Alternatively, T cells can move from the blood stream into perivascular spaces between the endothelial cell and the basal lamina, where they can encounter antigen presenting cells such as macrophages, dendritic cells and even microglia. Once the T cell has made contact with the APC, the T cell is allowed or licensed to move into the CNS parenchyma.

The chemokines produced during inflammation in the CNS and chemokine receptors expressed on circulating immune cells is essential to fully understand trafficking into the parenchyma. With the availability of many chemokine receptor knockout mice in the genetic background in which EAE is induced, many enlightening studies have been performed. It is important to know that inducing EAE in CXCR3\textsuperscript{101}, and CX3CR1\textsuperscript{102} knockout mice results in exacerbated disease. However, when CXCR3 antibody therapy was explored, there was exacerbation of disease when applied before onset of disease but conversely, there was a decrease in disease when antibodies were applied during the acute phase, thus the timing of use of this therapy would be critically important\textsuperscript{103}. 
Peripheral and CNS activation of myelin-specific CD4+ T cells. CD4+ T cells are primed in the periphery by DCs presenting myelin antigen. (1) CD4+ T cells enter the subarachnoid space by crossing the blood–cerebrospinal fluid (CSF) barrier either in the choroid plexus or the meningeal venules. (2) the T cells are re-activated within the subarachnoid space by MHC class II-expressing macrophages and DCs expressing myelin epitopes. (3) Reactivated T cells activate microglial cells in the subpial region, triggering activation of distal microglial cells and blood vessels. (4) Activated T cells adhere to and cross the activated blood–brain barrier, enter the perivascular space and are reactivated by perivascular macrophages and DCs. (5) T cells enter the parenchyma and, together with activated macrophages and microglial cells, secrete soluble mediators that trigger demyelination.
CCL21 has been shown to be constitutively expressed in the mouse CNS regardless of inflammation\textsuperscript{105}, and infiltrating T cells express CCR7, but CCR7-deficient mice have comparable disease to WT control animals\textsuperscript{106} thus it is not an important chemokine receptor in trafficking.

CCR6 deficient\textsuperscript{107} and CCR2 deficient mice\textsuperscript{108} are resistant to EAE but each chemokine receptor are expressed by different cell types. Activated WT MOG specific T cells transferred into a CCR6-/- mouse can enter the CNS and recruit large numbers of effector CCR6-/- T cells into the parenchyma, therefore it is the expression of CCR6 on T cells that mediate disease. Activated macrophages and dendritic cells cross into the parenchyma using CCR2\textsuperscript{66, 74, 75, 76}. In EAE, it has been shown that CCR2 expression is during the initiation and during the spontaneous relapse of chronic relapsing increased EAE\textsuperscript{109} and CCL2 is expressed by injured cells including neurons, astrocytes and endothelial cells\textsuperscript{76, 110, 111, 112} and correlates with disease severity in active EAE\textsuperscript{109}. Taken together, the infiltration of DCs and macrophages are critical to the induction of EAE and they use CCR2 to traffic to the CNS at the critical times to activate T cells. In addition, EAE disease is attenuated in CXCL13 deficient mice with fewer infiltrating mononuclear cells into the CNS\textsuperscript{105}. Finally, CCL3 and CCL5 were shown to be highly expressed in the CNS during inflammation\textsuperscript{109}, disease is attenuated in CCR1-deficient mice\textsuperscript{113} and a specific CCR1 antagonist was able to ameliorate EAE\textsuperscript{114}.
Vessel Permeability is implicated in CNS Pathologies

The cellular components of the BBB are redundant mechanisms to control the passage of solutes and cells into the parenchyma. This neurovascular unit is formed early in development such that the cells trapped behind this wall are never exposed to most of the components comprising the blood, including microglia. The outcome is a hyper response to blood vessel leaks that does not occur in other tissues. It is becoming clear there is a role of blood vessel leaks in the pathogenesis of neurodegenerative diseases\textsuperscript{39} including multiple sclerosis (MS), vascular dementia, epilepsy, Alzheimer's, and most obviously stroke\textsuperscript{26, 27, 28, 29, 30, 40}.

Neuron depolarization occurs in patients following stroke, subarachnoid hemorrhage and traumatic brain injury\textsuperscript{115, 116, 117}. Indeed it has been shown that breakdown of the BBB and astrocytic based dysregulation of extracellular electrolytes causes neuron depolarizations\textsuperscript{116}.

Inability of astrocytes to maintain BBB ion homeostasis and problems with edema has been linked with epilepsy\textsuperscript{118}. The same mechanism of breakdown of the extracellular ionic gradients responsible for stroke and hemorrhage are also responsible for epileptic seizures. MMP9 and albumin was increased in the CSF of patients after epileptic seizures demonstrating the link between BBB leak and
seizures\textsuperscript{119, 120}. It is assumed but not clear whether the BBB leak caused the seizure or vice versa.

In patients with Alzheimer’s, asymptomatic BBB leakage and diffusion abnormality was detected via MRI\textsuperscript{121, 122}. Transgenic mice that express human amyloid precursor protein had increased permeability of brain microvessels compared to WT controls, along with reduced tight junction proteins and increased MMP expression\textsuperscript{121, 123, 124}.

MRI of MS patients have shown abnormal BBB permeability in normal appearing white matter\textsuperscript{125, 126}. Gene chip analysis derived from the MOG EAE model in NOD mice show that on day 12, non-specific neutrophil-related BBB impairment was secondary to immunization with CFA\textsuperscript{127}. It is possible to reverse the effect of BBB breakdown on disease progression. Tetracycline induced expression of claudin-1 in BBB tight junctions resulted in amelioration of chronic but not acute EAE\textsuperscript{128}. Others have extracted small molecules from Tuscan black kale and shown it stops permeability of BBB in EAE\textsuperscript{129}.

It is evident that MMPs BBB leaks and dysregulation can be identified and correlated to multiple neurodegenerative diseases. It is encouraging that
therapies that target the endothelial cells rather than immune cells can have an effect on disease outcome.

**Role of Toxins in EAE Induction**

During education of T cells in the thymus, generally self-reactive T cells are eliminated. It is possible that in a normal mouse repertoire, MOG-reactive T cells escape negative selection in the thymus and egress to the periphery, where they then patrol both the secondary lymphoid tissues and the CNS. However, T cells that are self-reactive generally have weak affinity to the peptide to have escaped this selection process\(^{130, 131, 132}\). As discussed previously in the “Animal Models” section, early attempts at inducing a MS like paralysis in animal models started with multiple injections of whole brain homogenates. Upon injection of homogenates with complete Freund’s adjuvant (CFA), disease induction was achieved in several animal species\(^ {133}\). This addition was borne from decades of research that showed immunization with unrelated antigen could boosted the immune response the pathogen\(^ {134}\). When mycobacteria was resuspended in a butter or paraffin oil emulsion, production of antigen-specific antibodies was significantly increased. The actions of CFA are: 1) prolonged presence of antigens at the site of injection; 2) enhanced antigen uptake of antigens and promoting DC maturation; 3) increased antibody production; 4) PAMP receptors recognize the mycobacteria and skew to a TH1 type response resulting in increased IL-2 and IFN cytokine production\(^ {135}\).
Pertussis toxin (PTX) is a major virulence factor of Bordetella pertussis, the causative agent of whooping cough. The use of PTX in immunology was traditionally to inhibit G protein signaling via the ability of PTX to ADP-ribosylate G proteins. It is interesting that this toxin is used to induce EAE because PTX would affect all chemokine receptors on immune cells, thus inhibiting the migration of cells within the lymph node and trafficking of immune cells into the CNS. PTX does affect lymphocyte accumulation in the lymph nodes but not the spleen however, in the spleen T cells and B cells are unable to migrate into the white pulp possibly contributing to activation of auto-reactive T cells\(^\text{136}\). However, PTX has pleiotropic effects that last days after administration. First, ADP-ribosylation results in a loss of inhibition of adenylate cyclase activity and causes an increase of the intracellular concentration of cAMP. This accumulation of cAMP disturbs cellular metabolic processes, including moderate endothelial hypersensitivity to histamine, pancreatic islet activation and lymphocytosis\(^\text{137}\). It is generally accepted that mice injected with PTX have changes in brain endothelial permeability, but upon inspection of published results, the brain permeability measured by radioactively labelled BSA is subtle, approximately 1.3 fold increase compared to PBS control injection\(^\text{138}\). Second, PTX is able to activate both MyD88-dependent and independent signalling pathways in DCs\(^\text{139}\) leading to activation promotes the maturation of this antigen presenting cell, with up-regulation of co-stimulatory molecules and the production of pro-inflammatory cytokines such as IL-6\(^\text{140}\). Third, apart from the enzymatic action, the B moiety of the PTX is preferentially mitogenic for T cells and activates TCR signaling similar
to that of anti-CD3, confirmed by signaling through Lck and ZAP-70. The B moiety of the PTx causes internalization of CXCR4 and down-regulates signaling through the TCR signaling path.

Role of Histamine in the CNS

Histamine is used by both the nervous system and the immune system. Histamine has pleiotropic effects including neurotransmission, secretion of pituitary hormones, and other brain functions such as sleep cycles, it regulates gastrointestinal and circulatory functions. Histamine is a potent mediator of inflammation and a regulator of innate and adaptive immune responses. Mast cells and basophils are the major sources of stored histamine and it is rapidly released from these cells on demand.

All other immune cells, including lymphocytes, which lack histamine vesicles are able to synthesize "inducible" histamine.

Histamine has four receptors that are named H1, H2, H3, and H4. H1R and H2R are expressed on: mammalian brain; gastrointestinal tract; genitourinary system; cardiovascular system; adrenal medulla; hepatocytes; nerve cells; airway and vascular smooth muscle cells; endothelial cells; eosinophils;
monocytes; neutrophils; dendritic cells; and lymphocytes\textsuperscript{143, 154}. H3R expression is restricted to neuronal cells in the brain and some peripheral tissues while H4R is expressed exclusively on hematopoietic cells\textsuperscript{143}.

H1R couples to G\textsubscript{αq/11} proteins\textsuperscript{143} and generally, activation of H1R leads to stimulation of phospholipase C, resulting in calcium mobilization from intracellular stores and activation of protein kinase C (PKC)\textsuperscript{155}. However, H1R ligation causes signaling in other pathways: the production of phospholipase A2 and arachidonic acid\textsuperscript{156}, cGMP and nitric oxide\textsuperscript{155, 157, 158}, the activation of NF-κB\textsuperscript{159}, and STAT1\textsuperscript{160}. H1R-mediated PKC\textsubscript{α} stimulation activates MAP kinase pathways\textsuperscript{161, 162, 163}. H2R couples to G\textsubscript{αs} proteins that leads to increased cAMP production and calcium mobilization\textsuperscript{156, 164, 165, 166}. H3R and H4R couples G\textsubscript{αi/o} proteins that leads to inhibition of cAMP and mobilization of calcium\textsuperscript{167, 168, 169, 170}.

In endothelial cells, H1R-mediated signals lead to disassembly of VE-cadherin complexes that regulate endothelial barrier function\textsuperscript{171}, calcium mobilization and PKC activation results in cytoskeletal and shape change\textsuperscript{172}. These effects result in increased vascular permeability. H1R signaling increases expression of adhesion molecules ICAM-1, VCAM-1 and P-selectin on endothelial cells\textsuperscript{173, 174, 175, 176}. 
In dendritic cells, H1R ligation upregulates co-stimulatory molecules by increasing the production of proinflammatory cytokines such as IL-1, IL-6, IL-8, MCP-1 and MIP-1α. It causes chemotaxis of immature dendritic cells by inducing intracellular calcium flux and actin polymerization. In B cells, H1R signaling enhances anti-IgM mediated proliferation and antibody production. In CD4+ T cells, H1R signaling regulates IFN-γ and IL-4 production.

Mast cells accumulate at the site of inflammatory demyelination in the brain and spinal cord both in animal models and in MS patients. Histamine is significantly higher in CSF of MS patients. However, histidine decarboxylase deficient mice which lack histamine develop a severe disease than WT mice. Thus histamine plays an immune regulatory role in the development of EAE and thus histamine receptors are a better therapeutic target. H1RKO mice develop an attenuated disease than WT mice. Additionally, H1H2RKO mice develop an attenuated disease while H3H4RKO mice develop an exaggerated form of EAE when compared to WT mice. Correspondingly, the BBB permeability index of H1H2RKO mice is significantly lower than H3H4RKO or WT mice. With the many locations H1R is expressed, it was interesting to find that mice with endothelial cell expressing H1R under control of the von Willebrand factor promoter exhibited decreased BBB permeability and enhanced protection from EAE compared with H1RKO mice suggesting that endothelial H1R signaling may be important in the maintenance of cerebrovascular integrity.
Anti-histamine studies in EAE generally use H1R blockers, and show a reduction of EAE disease\textsuperscript{138, 184, 192, 193, 194, 195}. In humans, administration of anti-H1R agents either reduced the risk of MS\textsuperscript{196} or improved the neurological symptoms\textsuperscript{197}. Mast cell-stabilizing drugs have been shown to improve disease symptoms in EAE\textsuperscript{184, 198, 199}. C-kit knockout were thought to be mast cell-deficient mice and exhibited delayed onset and reduced disease severity. However, characterization showed that c-kit was expressed by many immune cells and although the results are impressive, it cannot be attributed solely to mast cells.

Anti-histamine has proven to be a simple and effective way to treat EAE and MS, however, it does not ameliorate disease and the multiple tissues that express H1R makes it hard to determine the exact mechanism by which anti-histamine reduces disease burden. We show that the anti-histamine, hydroxyzine, reduces microglia phagocytosis and activation resulting in the reduction of peripherally infiltrating APCs and T cells.
Summary

Studies using EAE have clarified our understanding of trafficking of immune cells from the periphery into the CNS in response to inflammation, the important cellular populations and their spatiotemporal role in the late stages of the disease\textsuperscript{200}. However, the earliest initiating cellular and tissue events that lead to massive immune cell recruitment prior to disease manifestation have not been described. For these reasons, we focus the current research on defining the earliest events of EAE with the intent to identify potential new therapeutic targets that can be applied to MS patients at diagnosis and recurrence in relapsing remitting MS. The goal is to reduce long term recurring inflammation and thus reduce neurologic deficits.

Through careful surgery and experimental design, we have confirmed a series of events that lead to demyelination (Fig. 4):

1) Vascular dysfunction induced by PTx leads to localized, transient vessel leaks
2) Leak of vessel contents causes microglia phagocytosis and activation
3) The phagocytosis overload possibly causes microglia to recruit peripheral DCs and macrophages that are more proficient at phagocytosis
4) The inflammatory environment encountered by APCs causes recruitment of T cells
5) Peripherally activated, myelin specific T cells and activated APCs in the CNS are required for T cell licensing and motility within the parenchyma.
6) Microglia are able to extend processes across an intact BBB and that the number of these projections found along the blood vessel doubles by days 9-12 of EAE induction.
Figure 4: Sequence of events in the initiating phase of EAE

(a) Vascular dysfunction induced by PTx leads to histamine release and localized, transient vessel leaks. (b) Leak of vessel contents causes microglia phagocytosis and activation (c) Recruitment of peripheral DCs and macrophages. (d) The inflammatory environment encountered by infiltrating APCs results in recruitment of T cells (e) Microglia are able to extend processes across an intact BBB and the number of these projections doubles by days 9-12 of EAE induction.
2 Focal Intermittent CNS Vascular Leak Contributes to Sequential Immune Cell Infiltration during the Asymptomatic Induction Phase of Experimental Autoimmune Encephalomyelitis

2.1 Abstract
Immune cell accumulation, neuronal destruction and paralysis are hallmarks of experimental autoimmune encephalomyelitis, a central nervous system inflammatory disease. Due to the spatial-temporal constrains of traditional methods used to study this disease, debate continues as to whether vessel disruption represents a cause or a byproduct in the neuroinflammatory pathophysiology. Here, we provide dynamic, intravital, time-resolved single-cell information on the sequential structural and cellular events within the mouse cortex during the asymptomatic first 12 days of disease induction. Transient focal vessel disruptions preceded microglia activation, followed by infiltration of and interaction between circulating APC and T cells. Histamine H1 receptor blockade prevents microglia activation, resulting in reduced immune cell accumulation, disease incidence and clinical severity.

2.2 Introduction
Mounting evidence suggests that peripheral immune cells are central to the pathogenesis of multiple sclerosis (MS) and other neurodegenerative and neuroinflammatory diseases\textsuperscript{80, 201, 202, 203, 204}. Past studies have elucidated many
of the key features of experimental autoimmune encephalomyelitis (EAE), a widely studied animal model of neuroinflammation with features that mimic certain aspects of MS\textsuperscript{41, 205}. However, the precise sequence of cellular events in EAE pathogenesis during the early asymptomatic induction phase of the disease remains poorly defined.

Current theory posits that disease induction is a two-step paradigm consisting of an initiation stage and an infiltration stage for pathogenic T cells\textsuperscript{73, 206, 207}. In the initiation stage, naïve myelin-specific CD4\textsuperscript{+} T cells that have escaped thymic negative selection are induced to undergo proliferation and differentiation in lymph nodes (LNs) by activated myelin peptide-containing antigen presenting cells (APCs) are subsequently released into the systemic circulation. The blood-CNS barrier, which normally protects the CNS from peripheral inflammatory cell infiltration, is rendered “leaky” by irradiation or pertussis toxin administration that involves local histamine-mediated regulation of vessel integrity\textsuperscript{138}. This breach in the blood-CNS barrier then allows myelin-reactive T cells to traverse through the blood-brain barrier (BBB) and/or the blood cerebrospinal fluid barrier (B-CSFB) via chemokine guidance, including the action of CCR6\textsuperscript{208}. These T cells encounter another population of APCs presenting myelin peptides in the perivascular Virchow-Robin Space (VRS) and/or the subarachnoid space (SAS) and are then licensed for access to the CNS parenchyma\textsuperscript{6, 104}. This model supports an essential role for infiltrating DCs in the re-activation of myelin-specific T cells within the CNS, although it is unclear what perivascular niche
factors drive circulating monocytes to become DCs and macrophages in the CNS. Using chimeric models where the BBB has been compromised, it has been shown that microglia are activated during the asymptomatic induction phase\textsuperscript{209}, but MHCII expression by microglia is not required for infiltration of cells\textsuperscript{210}. This raises the question of whether other functions of microglia resulting in activation could play a role in disease progression, in particular the phagocytosis of myelin\textsuperscript{211} or neuron\textsuperscript{212} debris and potentially blood vessel contents\textsuperscript{25}. Upon exerting their effector function, the pathogenic CD4\textsuperscript{+} T cells recruit other effector immune cells including additional CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells, neutrophils and B cells that gain access through compromised CNS vessels, with ensuing inflammation, focal neuronal demyelination and cell destruction resulting in paralysis\textsuperscript{41, 213, 214}.

The identity of initiating T cells in EAE has been widely studied\textsuperscript{63, 215}. Current evidence strongly suggests that IL-17-producing, myelin-specific CD4\textsuperscript{+} Th17 cells play a critical role both in EAE and in MS\textsuperscript{132, 216} and that adoptive transfer of these T cells into irradiated recipient mice induces neuronal destruction\textsuperscript{41, 132}. In this passive transfer model that mimics the later infiltration stage of EAE, disease occurs either in the context of toll-like receptor (TLR) signaling in the CNS\textsuperscript{217, 218} and in recipients with vessel-CNS barrier dysfunction induced by pertussis toxin (PTx) and/or irradiation\textsuperscript{219, 220}. Recent studies have addressed the recruitment and migration behavior of activated Th17 cells directly \textit{in situ} by applying intravital two-photon microscopy (TPM) to either the brain or spinal cord tissues
in rodents. While these studies shed new light on how immune cells were recruited from the blood vessels into EAE lesions, these observations were generally made in the passive transfer model and during the onset of peak clinical symptoms. As such, they do not address how peripherally activated, myelin-specific T cells first access the CNS during the critical transition from the initiation stage to the infiltration stage of EAE pathogenesis, before the arrival of late-stage effector immune cells which amplify local CNS inflammation, tissue destruction and further vessel disruption.

CNS vessel integrity disruption is a hallmark of MS; yet, it is not clear whether vascular compromise is the initiator or the result of EAE pathology. Furthermore, although the breakdown of the blood-CNS barrier has been implicated, its contribution during the early, asymptomatic phase of active EAE induction has not been directly characterized in situ. Current available data regarding vessel integrity in neuroinflammation does not adequately describe the nature and dynamics of the vessel “leakiness”. We hypothesize the vessel leak is the initiating event of microglia activation and subsequent neuroinflammation.

In the current study, we use sequential intravital TPM through a cranial observation window to examine cellular events at the tissue interface between SAS and CNS parenchyma in the cortex beginning on day 0 of active EAE induction to include the entire asymptomatic phase (first 12 days). We describe
early focal, intermittent and transient disruptions of post-capillary junctional blood venules associated with PTx administration, which precede subsequent microglia activation, immune cell accumulation and inflammation. The transient pial vessel leaks initiate an inflammatory response in the parenchyma within hours to days as evidenced first by microglial uptake of blood contents, followed by an influx of both CD11c\(^+\) and CD11b\(^+\) APCs. The increasing numbers of activated CNS-resident and blood-derived APCs on days 3-6 then facilitate the ingress and retention of activated T cells in a myelin antigen-specific manner into the perivascular space and the brain parenchyma on days 6-12. These immune cellular infiltrations are found in close proximity to each other and to sites of early blood vessel leaks, forming cellular plaques similar to characterized cortical lesions in MS. In addition, we show that histamine H1 receptor antagonist acts to curtail the frequency of PTx-induced focal vessel leakage, reduce the degree of microglia activation, diminish subsequent recruitment of blood-derived immune cells, thus protecting mice from EAE incidence and disease severity.

2.3 RESULTS

2.3.1 Transient focal leaks in leptomeningeal vessels occur early during EAE induction
Review of the literature reveals EAE induction efficiency and clinical presentation can often be variable. To reliably capture rare cellular events in the CNS by TPM, we introduced fluorescent-reporter naïve myelin-specific 2D2 transgenic CD4\(^+\) T
cells into recipient animals in order to increase the precursor frequency of myelin-specific T cells prior to disease induction. To induce disease while preventing mortality, a low dose of pertussis toxin (PTx) was administered daily on the first 3 days of induction without irradiation\textsuperscript{230}. This resulted in 100% disease incidence compared to mice without transferred 2D2 CD4\textsuperscript{+} T cells, with hind limb paralysis achieved in all mice by day 21 (Fig. 5a). Previously, a non-invasive bioluminescence study indicated that T cell infiltration first occurs in the brain around day 7 after EAE induction, 3 days before spinal cord infiltration and 5 to 7 days before onset of clinical symptoms\textsuperscript{2}. Therefore, we focused our intravital observation in the cortex during the asymptomatic first 12 days of EAE induction. A cranial observation window was surgically implanted 4 to 10 days prior to 2D2 cell transfer, ensuring limited residual inflammatory changes to underlying leptomingeal structures and cellular response\textsuperscript{231}. We have previously demonstrated that, when carefully performed, cranial window implantation yields similar observations as those obtained through a thinned-skull approach, and can provide an experimental platform for longitudinal serial observation of meningeal and parenchymal changes in the cortex of EAE mice\textsuperscript{231}.

The role of PTx in EAE induction has been attributed, in part, to its ability to disrupt the BBB integrity\textsuperscript{138}, although the exact location, timing and dynamics of such BBB breach have not been carefully characterized. Using TPM imaging and fluorescently labeled vessel dye, we were able to directly observe focal transient
Figure 5: PTx administration causes transient and focal CNS vessel leak.
Figure 5 (continued): PTx administration causes transient and focal CNS vessel leak.

(a) Reliable and consistent clinical scores were achieved in mice pre-transferred with $3 \times 10^6$ naïve 2D2 T cells (filled squares) as compared to WT (non-transferred) controls (open squares) prior to EAE induction. (b) TPM imaging of pial vessels (red) on day 3 after EAE induction revealed localized transient vessel leaks at post-capillary venule junctions that resolved over a few minutes (region of interest (ROI) 1, 2), while other vessels within the imaging field remain intact (ROI 3, 4). Scale bar = 50μm. (c) Dynamic mean fluorescent intensity (MFI) measurements of the indicated ROI’s in (b) over time revealed the transient and focal nature of the vessel leaks in ROI 1 and 2. (d) Frequency of cerebral vessel leaks was quantitated sequentially in a cohort of mice by TPM during the first 12 days of EAE induction. An increased frequency of vessel leaks was associated with PTx administration on day 0 to 2. A second wave of vessel leaks was observed on days 7 to 9 at a time when effector immune cells were shown to infiltrate the CNS$^{232}$. Arrows: PTx injections. (e) The duration of individual vessel leaks in (d) was measured dynamically by TPM. Arrows: PTx injections. (f) ROI 5, a cross-sectional view of ROI 1 in (b) revealed a slower clearance of vessel dye under the pia surface in the parenchyma (P) as compared to the meninges (M). Scale bar = 25μm (g) The presence of vessel dyes (red) can be detected in the perivascular areas of the CNS parenchyma in fixed CX3CR1$^{+}$GFP brain tissue by IHC, both near the superficial vessels and the deep vessels away from the meningeal-parenchyma interface (*). Dash line: Imaging depth achievable by TPM. Scale bar = 100μm.
leakage of CNS vascular contents hours to days after PTx injections on days 0 to 2. These transient BBB breaches were visualized as blooms of dye leaking from the blood vessels (Fig. 5b; Supplemental movie 1). The effect of PTx on CNS vessel integrity disruption was neither uniform nor permanent, as certain post-capillary venule junctions were prone to intermittent vessel leaks while other vessels of similar caliber within the same imaging volume remained completely intact. Some vessels exhibited multiple leaks, while others exhibited the leak only once during the hour-long imaging session (Fig. 5c). As a control, mice with implanted cranial windows and 2D2 cell transfer without PTx or EAE induction exhibited little to no vessel leaks, indicating that these leaks were directly related to EAE induction and not damages associated with TPM or prior surgical procedures (Fig. 6). In addition, we also observed vessel leaks that originated from venule junctions that were outside of the imaging field, arguing against this phenomenon as a result of artifacts of intravital TPM imaging (Supplemental movie 2).

When we characterized these vessel leaks in the same mouse cohort serially through the first 12 days of EAE induction, we observed that the highest frequency of vessel leaks occurred during the first 3 days, coincident with PTx administration (Fig. 5d). The duration of individual leaks during this early period was variable, with some lasting ~30 minutes before resolving (Fig. 5e). Interestingly, the frequency and duration of vessel leaks subsided gradually on
Control mice undergoing cranial window implantation but not full EAE induction exhibited a small number of vessel leaks only in the first 3 days following the surgical procedure. In addition, the duration of the leaks was smaller. There were no flares detected after day 3, and none of these mice, observed up to 21 days, showed any signs of clinical disease. Data obtained from 4 control mice.
subsequent days before increasing again on days 8-10 at a time when effector T cells were known to traffic to and accumulate in the CNS.\textsuperscript{232}

**Blood solutes from vessel breach persist within the CNS parenchyma**

While signal from the vessel dye at the pial surface dissipated within a few minutes where bulk CSF flow in the meningeal space can explain the clearance of the dye, blood solutes released into the parenchyma took a longer time to clear after the BBB breach had resolved (Fig. 5f). Evidence of dye persistence can be found deep in the parenchyma, as fluorescently labeled dextran can be found on day 3 of induction in fixed brain tissue sections (Fig. 5g). We hypothesized that the presence of the blood contents may initiate CNS-resident microglia activation\textsuperscript{201, 233}, thereby starting a cellular cascade that results in the subsequent infiltration and accumulation of immune cells from circulation.

**Intermittent vessel leaks result in localized phagocytic uptake of blood vessel contents by microglia**

The observed focal nature of the BBB breach led us to develop an assay to evaluate the overall BBB integrity by measuring the location and phagocytic activity of APCs that have taken up vessel dye throughout the course of EAE induction. We introduced TRITC-labeled dextran intravenously into recipient mice and measured the activity of TRITC-labeled phagocytic cells as an indirect
readout of the vessel integrity in the meningeal and parenchymal compartments hours to days later. Using this method, we observed patchy distribution of TRITC-labeled phagocytes in the brain parenchyma 3 days after PTx and TRITC-dextran administration, while other areas within the TPM scanning field have little to no detectable phagocytic cells (Fig. 7a). The phagocyte distribution in the parenchyma is consistent with the distribution pattern of transient vessel leaks observed during dynamic imaging (Fig. 5).

Toxin dependent phagocytic activation

To assess how the phagocyte distribution may be affected by neuroinflammatory stimuli, we compared TRITC$^+$ phagocyte distribution and numbers in the CNS following systemic exposure to PTx and the TLR4 agonist, lipopolysaccharide (LPS). Second harmonic generation (SHG) signals were used to precisely locate the collagen-rich pia and to differentiate parenchyma from meninges (data not shown). Interestingly, we observed a distinct distribution pattern of parenchymal TRITC-positive phagocytic cells between the two stimuli. Systemic LPS treatment resulted in an even distribution of phagocytic cells throughout the entire parenchyma and meninges, whereas PTx exposure resulted in a patchy phagocyte distribution (Fig. 7b) as seen with EAE induction (Fig. 7a). Both PTx and LPS administration resulted in a significant increase in phagocytic cells in both the meninges and parenchyma as compared to PBS control, with slightly higher number of cells with phagocytic uptake in the meninges of mice exposed
Figure 7: Focal vessel leaks induce localized activation of endogenous CNS immune cells and increased DC accumulation.
Figure 7 (continued): Focal vessel leaks induce localized activation of endogenous CNS immune cells and increased DC accumulation.

(a) TPM vessel imaging of cortex on day 3 following PTx administration revealed patchy activation of CNS phagocytes (red) while other image areas were devoid of phagocytic activities. Numbers shown are phagocytic cell counts within the boxed parenchymal volume. Scale bar = 100μm. (b) PTx and LPS resulted in distinct distribution of phagocytic cells (yellow) in the CNS; LPS caused diffuse phagocytic activity while PTx caused patchy phagocytic activity around vessels (red). Scale = 100μm. (c) Quantification of phagocytes in the meninges and parenchyma following systemic exposure to LPS and PTx. * p<0.01, ** p<0.001, *** p<0.0001. (d) Sequential TPM CNS imaging of the CD11c-GFP mice over the first 12 days of EAE induction showed progressive changes in the accumulation of CD11c− (green) and phagocytic CD11c+ cells (purple) in left panel and phagocytic cells (red) in right panel over time. Scale bar = 60μm. (e) Quantification of CD11c− (green), phagocytic CD11c+ cells (purple) and phagocytic (red) cells in the meninges and CNS parenchyma over a 12-day longitudinal imaging sequence as shown in (d).
to LPS (Figure 7c). This in vivo phagocytic assay allows us to sequentially discern the patterns of vessel leaks in the same hosts with finer time and space resolution than previously reported\textsuperscript{234, 235, 236, 237, 238}.

**TRITC\textsuperscript{+} phagocytes are CX\textsubscript{3}CR1\textsuperscript{+} microglia and macrophages in early EAE induction**

CX\textsubscript{3}CR1 is a chemokine receptor that is constitutively expressed by microglia and a sub-population of macrophages in the CNS\textsuperscript{4}. To identify whether the TRITC\textsuperscript{+} phagocytes include CX\textsubscript{3}CR1\textsuperscript{+} microglia / macrophages, we induced EAE in \textit{Cx3cr1}\textsuperscript{+/GFP} mice and observed phagocytic activity through a cranial window following TRITC-dextran injection. 5 hours after PTx administration on day 0, we found that the only TRITC-containing cells in the meninges and parenchyma were exclusively CX\textsubscript{3}CR1\textsuperscript{+} cells, as we did not detect any CX3CR1\textsuperscript{-} TRITC\textsuperscript{+} phagocytes (Supplemental movie 3).

**CD11c\textsuperscript{+} DCs infiltrate the CNS following phagocyte activation**

Prior studies have shown that CD11c\textsuperscript{+} DCs are required in EAE disease induction, although the exact timing of CD11c\textsuperscript{+} infiltration in the CNS during the disease process is not clearly understood\textsuperscript{13, 85, 239}. To investigate this, we imaged CD11c-GFP\textsuperscript{+} mice daily in the first 12 days of active EAE induction to determine the time course of infiltrating DCs and their phagocytic activities during disease
progression. We quantified the number of CD11c-GFP+ cells, TRITC+ phagocytic cells, or both in the meninges and parenchyma over 12 days (Fig. 7e). We observed an increased number of CD11c+TRITC− cells in the meninges starting on days 3 through 12, with increasing phagocytic activities from days 6 to 12. The entry of CD11c+TRITC− DCs into CNS parenchyma was much lower compared to those in the meninges, with increasing numbers of DCs starting on day 6. Their phagocytic capacity increases over the subsequent 6 days. In contrast, the number of CD11c− TRITC+ phagocytes decreased from days 3 to 12 in the meninges, even as parenchymal CD11c− TRITC+ phagocytes remain constant throughout this period.

Presentation of myelin antigens by CNS APCs enhances activated myelin-specific T cell entry and retention

Following the characterization of CD11c+ and CD11c− APCs in the EAE brain, we investigated the timing and antigen requirement of 2D2 cell recruitment and behavior within the CNS. We co-injected equal number of differentially labeled naïve OTII and 2D2 cells into recipient mice followed by immunization with both OVA323-339 and MOG35-55 peptides in the presence of CFA and PTx (Fig. 8a). Significantly more 2D2 cells began to infiltrate the brain parenchyma as early as day 3 and day 6, even though both T cell populations were found in equivalent numbers within the peripheral lymph nodes (Fig. 9c). The majority of the OTII cells found within the CNS remain in the perivascular location near
Figure 8: Peripherally activated T cells accumulate and migrate near sites of vessel leak and DC aggregate in an antigen-dependent manner.
Figure 8 (continued): Peripherally activated T cells accumulate and migrate near sites of vessel leak and DC aggregate in an antigen-dependent manner.

(a) Equal numbers of naïve OTII (open circle) and 2D2 (closed circle) CD4+ T cells were transferred into WT recipients 24 hours before s.c. immunization with OVA323-339 and MOG35-55 peptides in the presence of CFA and PTx. Direct longitudinal TPM observation of the same recipient mice revealed that 2D2 cells migrated to the CNS in greater numbers than the OTII cells at each time point in the first 12 days following immunization. (b) Similar experiments were conducted as in (a) except the recipient mice were immunized only against the OVA323-339 peptide. Relatively low numbers of both T cell populations were found in the CNS on day 3 during the first wave of vessel leak but not seen at all on subsequent days. (c) Sites of focal vessel leaks early (day 4) in EAE induction resulted in phagocyte (red) and CD11c-GFP+ (green) cell accumulation (days 6-9), and localized 2D2 cell (blue) migration and accumulation (day 9). Colored tracks on day 9 highlight paths taken by individual 2D2 cells (blue) near areas of CD11c-GFP+ cell clusters (green) in the parenchyma (P) compared to the meninges (M). (d) Distribution of contact duration with CD11c-GFP+ cells by 2D2 T cells on day 9 showed predominantly short interactions (<5 mins) with the CD11c-GFP+ cells. (e) 2D2 cells with short interactions (<2 mins) with the CD11c-GFP+ cells exhibited a higher overall velocity than 2D2 cells that maintain a longer interaction with DCs (average velocity: 14.8 ± 4.2 μm/min versus 12.5 ± 3.6 μm/min, respectively). (f) Total number of unique 2D2 cells migrating towards CD11c+ rich regions (GFP hi) are significantly higher than those migrating towards low CD11c-GFP (GFP lo) regions on days 9 and 12. (g) 2D2 cells exhibited similar overall contact time with phagocytic (TRITC+GFP+), non-phagocytic CD11c+ (TRITC+GFP-) cells and phagocytic non-CD11c+ (TRITC+GFP+) cells on days 9 and 12. (h) More 2D2 cells were observed to egress (average 0.67%) from the blood vessels into the parenchyma than those 2D2 cells entering (average 0.30%) into the blood vessels from the CNS on days 9 and 12. ** p<0.001. n.s.: not significant.
Figure 9: Characterization of OTII and 2D2 cells when co-activated during induction.

(a) Distribution of OTII T cells (green) and 2D2 T cells (yellow) in the brain parenchyma on day 9 revealed that a few OTII cells are restricted to the perivascular spaces. Scale = 100µm  (b) Quantification of OTII and 2D2 cells revealed a greater accumulation of 2D2 cells in the parenchyma. (c) OTII and 2D2 cells proliferate to similar numbers in the peripheral draining lymph nodes following injections of MOG35-55 / OVA323-339 / CFA emulsions. (d) Comparison of the average speeds of OTII and 2D2 cells found in the brain revealed that 2D2 cells are more motile than OTII cells in the brain parenchyma.
sites of previous vessel leaks, migrated with a reduced speed as compared with 2D2 cells (Fig. 9b), and remain in the CNS only transiently (data not shown). However, when mice infused with naïve OTII and 2D2 cells were immunized only with OVA323-339, we saw a near complete absence of infiltrating 2D2 or OTII cells in the meninges or the CNS parenchyma (Fig. 8b).

This absence of activated myelin-specific T cells (Fig. 8b) disrupts this intricate cellular and structural collaboration, resulting in a complete absence of effector cell infiltration and disease induction (data not shown), even though vessel leaks, phagocytic APC accumulation and robust activation of non-CNS specific T cells were all present within the host. Taken together, our data imply that the cooperation between PTx-induced leaky vessels, resident and infiltrating APCs, the availability of CNS-derived antigens and activated myelin-specific T cells are preconditions for MOG-specific T cell entry into the CNS, which in turn amplifies other cellular infiltration later in the disease progression.

Localized blood vessel leak precedes phagocyte accumulation and the infiltration of DCs and T cells

To further examine the cellular and structural cooperation during successful EAE induction, we performed multiplex sequential imaging of the vessel leak and cellular interaction among TRITC⁺ phagocytic microglia, CD11c-GFP⁺ DCs and
CFP⁺ 2D2 cells during the first 9 days of EAE induction. Following transient vessel leaks (Fig. 8c, day 4), the vessel content was cleared with a concomitant increase in the number of TRITC⁺ phagocytes and the arrival of CD11c⁺ DCs by day 6 (Fig. 8c, days 6-9). This was followed on day 9 by a large number of infiltrating 2D2 cells that congregated around locations of DC accumulation near the sites of previous vessel leaks (Fig. 8c, day 9), eventually leading to extensive inflammatory changes and tail and limb weakness beginning on days 10 to 12 (data not shown). When we analyzed the behavior of 2D2 cells found on day 9, we observed that the activated 2D2 cells surveyed a large area of the CNS parenchyma, with a particular focal concentration of movements surrounding previous vessel injury sites where clusters of CD11c⁺ cells were found (Fig. 8c, day 9; Supplemental movie 4). This surveillance behavior toward CD11c⁺ cells by 2D2 cells was reminiscent of chemotactic guidance behavior of T cells by activated DCs in an inflamed LN²⁴⁰. The 2D2 cells exhibited both transient (< 2 min) and prolonged (≥ 2 min) contacts with CD11c⁺ DCs (Fig. 8d). Those 2D2 cells that interacted briefly with CD11c⁺ DCs (<2 mins) migrated at a faster velocities (~16 μm/min), while those that exhibited prolonged interaction with CD11c⁺ DCs traveled with reduced velocities (~12 μm/min) (Fig. 8e). In all, 17.7 ± 11.2% of total 2D2 cells were stationary versus 83% motile and 6.1 ± 5.1% of 2D2 cells were found to be perivascular with limited motility.

2D2 cells preferentially congregate to CD11c⁺ DC-rich regions
To further assess whether 2D2 cells were preferentially drawn to sites of DC aggregates in the CNS parenchyma, we analyzed the number of 2D2 cells that enter a $50 \mu m$ radius of a CD11c$^+$ cellular aggregate. Our analysis revealed a significant increase in the number of unique 2D2 cells moving towards an area high in CD11c-GFP signal as compared to areas with low CD11c-GFP signal within the same imaging field (Fig. 8f), suggesting that indeed the T cells were attracted preferentially to CD11c$^+$ cells. Interestingly, interaction duration analysis of 2D2 cells with CD11c$^+$ and CD11c$^-$ phagocytes showed a trend toward higher duration with CD11c$^+$ cells, although the difference was not statistically significant (Fig. 8g). When the number of 2D2 cells crossing the BBB was analyzed, we found a greater number of 2D2 cells egressing from nearby blood vessels to enter the CD11c$^+$ rich regions as compared to 2D2 cells entering those vessels from the parenchyma (Fig. 8h).

**Histamine H1 receptor blockade reduces microglia activation, DC recruitment, disease incidence and severity**

As histamine release by mast cells has been postulated to mediate PTx-induced vessel leak$^{138}$, we investigated whether blockade of the histamine H1 receptor (H1R) may ameliorate observed vessel leak and subsequent phagocytic cell activation, T cell recruitment and disease progression. We administered hydroxyzine (HXYZ), a first-generation antagonist of H1R that readily passes across the BBB, in the drinking water of mice undergoing EAE induction. In
contrast to 100% disease incidence in conventional EAE induction in naïve 2D2-transferred recipients (Fig. 5a), we observed a 60% overall reduction of EAE incidence in mice receiving HXYZ treatment, with 40% of mice developing delayed disease onset and reduced clinical scores, and another 20% of the mice never showed clinical symptoms (Figure 10a). Note that vessel leaks after PTx administration were observed despite HXYZ treatment via gavage (Figs. 10b, 10c), suggesting the regulation of CNS vessel integrity under this stimulation condition was partially controlled via a non-histamine H1R mediated mechanism. Interestingly, however, HXYZ gavage did result in a reduction in the number of TRITC+ phagocytes in the parenchyma following EAE induction (Figs. 10b; Supplementary movie 5). This is not entirely unexpected, as microglia has been shown to express H1R, and engagement of the receptor induces activation of microglia241. Similarly, there was a significant reduction in the number of both CD11b+CD11c+ DCs and CD11b+CD11c- macrophages on days 6-9 as assessed by whole brain flow cytometry (Figs. 10d, 10e). These data were further corroborated by direct TPM observation, showing reduction of phagocytic DCs and microglia and well as non-phagocytic DCs in the brain as late as day 12 post EAE induction (Fig. 10f). There was also an absence of detectable 2D2 cells by TPM on day 12 of HXYZ-treated EAE mouse (data not shown). Flow analysis of T cell infiltration revealed a similar reduction in the number of infiltrating CD4+ and CD8+ T cells in HXYZ-treated groups early in disease induction, with
Figure 10: Histamine receptor blockade inhibits early activation of CNS phagocytes, diminishes DC and 2D2 accumulation, and blunts EAE severity.
Figure 10 (continued): Histamine receptor blockade inhibits early activation of CNS phagocytes, diminishes DC and 2D2 accumulation, and blunts EAE severity.

(a) 60% of mice undergoing EAE induction displayed either absent or reduced disease severity when exposed to hydroxyzine (HXYZ) via drinking water during EAE induction, with 20% disease-free incidence and 40% with a delayed onset and reduced overall clinical severity. (b) Mice treated with PTx for 3 days and exposed to HXYZ showed a dramatic reduction in phagocytic cell number. Scale bar = 100um. (c) Longitudinal TPM imaging revealed no statistical difference of vessel leaks in HXYZ-gavaged mice compared to control mice during EAE induction, except on day 6 when 2/3 of HXYZ-treated mice exhibited more vessel leaks compared with controls. (d) HXYZ was administered to mice via gavage and EAE was induced. After isolation, flow cytometry analysis revealed a significant decrease in the number of CD11b+CD11c- cells in the CNS of HXYZ-treated mice on days 3 and 6 following EAE induction (e) The number of CD11b+CD11c+ cells in the CNS of HXYZ-treated mice was also significantly lower as compared to control on day 6 following EAE induction. (f) Longitudinal sequential TPM imaging analysis of HXYZ-treated and control mice confirmed the findings in (e) and (f).
Figure 11: Additional effects of HXYZ on immune cells during EAE induction.
Figure 11 (continued): Additional effects of HXYZ on immune cells during EAE induction.

HXYZ was administered via gavage from day -1 to day 12 while EAE was being induced. Whole-brain percoll isolations of individual cells from HXYZ-treated and control mice were performed. Flow cytometry analysis of the isolated cells showed, (a) a significant decrease in the number of CD8$^+$ cells in the CNS with HXYZ treatment on Day 3; (b) a significant decrease in the number of CD4$^+$ cells in the CNS with HXYZ treatment on Day 3; (c) the total number of CD4$^+$ T cells expressing IFN-γ, IL-17 and FoxP3 were not significantly different with HXYZ treatment in the spleen on day 9; (d) even though the total number of CD4$^+$ cells in the CNS was similar between treatment and control groups, there was a significant decrease in the number of IFN-γ and IL-17 producing cells with the CNS on day 9; and (e) there is a significant increase in phagocytic cell number in both meninges and parenchyma with PTx treatment as compared to PBS controls, whereas the parenchymal phagocyte numbers in PTx + HXYZ treated animals were comparable to PBS controls.
reduced number of IFN-γ and IL-17 producing CD4$^+$ T cells at later time points (days 9; Fig. 11).

2.4 Discussion
CNS blood vessel leak has been implicated as a trigger in neurodegenerative disorders including epilepsy, Alzheimer, and Parkinson’s disease$^{29, 54, 242}$. Although changes in CNS vessel permeability have been documented previously in vivo, the timing and kinetics of how this occurs at the tissue level during neuroinflammation has not been described$^{25, 32, 225, 234, 243}$. In this study, we provided dynamic evidence of the transient pulsatile nature of altered CNS vascular permeability, supporting the hypothesis that BBB leakage is an initiating event rather than a byproduct of effector immune cell aggregation and tissue destruction near the perivascular EAE lesions. These leaks were focal, transient, and they occurred during PTx treatment well in advance of observable EAE symptoms. Our observations are in accordance with histologic characteristics found in sub-pial demyelinating gray matter lesions$^{6, 244}$ such as the accumulation of macrophages$^{91}$ and CD4$^+$ T cells$^{87, 245}$. The location of these clinical sub-pial gray matter lesions further highlights the importance of our imaging at the meninges-parenchyma interface to understand sequential structural and cellular changes at this location.
Vessel leaks were found both in the meninges and throughout the parenchyma. Fluorescent dextran released from vessels in the SAS was cleared quickly due to CSF flow and phagocytes, whereas fluorescent dextran released into the CNS parenchyma was not cleared as efficiently. Evidence of vessel leaks can be detected by IHC beyond the TPM imaging depth away from leptomeningeal areas. During the first 3 days of EAE induction, blood contents including plasma proteins, PTx and fibrinogen\textsuperscript{25} are released into the parenchyma and initiate a parenchymal inflammatory response, including activating the phagocytic capacity of resident CX\textsubscript{3}CR\textsubscript{1}+ microglia\textsuperscript{246}. During the initial transient vessel leaks following PTx administration, we did not visualize any influx of circulating monocytes or T cells migrating across the blood vessel wall in the cortex, suggesting that the opening of the BBB at this stage does not permit an influx of circulating cells into the CNS. At later time point (days 4-6) when the vessel leakage subsides and perivascular microglia had phagocytosed vessel contents, we began to observe increasing immune cell infiltrate coincident with a second wave of vessel leak (days 7-9), an observation which is consistent with the two-step paradigm of EAE induction\textsuperscript{73}.

Microglia act as neuronal support cells, continuously monitoring, phagocytizing or endocytosing contents and interacting with their local tissue microenvironment\textsuperscript{4,247}. Not only are they important in pruning neuronal synapses\textsuperscript{248}, they also contribute to membrane turnover in oligodendrocytes\textsuperscript{249} and are the CNS resident immune cells capable of reacting to potential danger from internal and
external sources\textsuperscript{4}. Microglial phagocytosis has been shown to be essential for effective remyelination in injury models\textsuperscript{211}. Upon exposure to toll-like receptor stimuli, microglia respond as tissue-resident APCs, retracting dendritic processes to assume an ameboid shape while upregulating MHCII and co-stimulatory molecules to present potentially harmful antigens to T cells\textsuperscript{4, 89, 201, 250, 251}. Mice with functionally deficient microglia exhibited delayed onset and reduced severity of EAE, suggesting the important contribution of microglia in EAE pathogenesis\textsuperscript{210, 250}. Using the CX\textsubscript{3}CR\textsubscript{1}+/GFP reporter mouse, we showed that microglia and macrophages constitute the main phagocyte populations that absorb vessel contents in the first 3 days of EAE induction. These phagocytes can internalize vessel contents in response to TLR agonist stimulation by one of two ways. First, microglia and macrophage act as scavengers, removing spilled blood solutes in the parenchyma as a direct result of transient vessel leakage. Second, perivascular CX\textsubscript{3}CR\textsubscript{1}\textsuperscript{+} cells can sample blood contents directly within intact blood vessels through their intravascular dendritic processes, as has recently been reported\textsuperscript{231}.

Bone marrow derived myeloid cells are important in the regulation of inflammatory response in the CNS. In particular, infiltrating CD11c\textsuperscript{+} DCs have shown to be required for the induction of EAE\textsuperscript{13, 239, 252, 253, 254}. In the adoptive effector T cell transfer model, blood derived APCs with functional MHCII are required for T cell infiltration and for potentiating EAE\textsuperscript{61, 210, 250, 255}. Depletion of plasmacytoid DCs in the late inflammatory phase resulted in reduced disease\textsuperscript{86}. 
To demonstrate that the infiltration and retention of activated 2D2 cells require APCs to actively present myelin antigen in the CNS and not simply through disrupted BBB, we either activated OTII cells alone or co-activated both 2D2 and OTII cells in the periphery and showed that activated OTII cells were able to access the CNS only in the presence of co-activated 2D2 cells. On the other hand, activated OTII cells with naïve 2D2 cells were incapable of accessing CNS parenchyma, despite the presence of activated microglia and myeloid-derived APCs. Our data confirmed the notion that APCs presenting endogenous CNS peptides are critical to license T cell recruitment and retention in the EAE brain. Our sequential imaging and FACS data are in good agreement with reports showing that 5-10% of CD11b+ cells present myelin basic protein as early as day 1 after EAE induction, whereas CD11c+ DCs are not detected till day 5 and only present myelin basic protein from day 7 onwards.

T cell infiltration into the brain has traditionally thought to occur at the choroid plexus, although recent data suggest an alternate route via post-capillary venules and the pial vessels from the SAS. Our intravital TPM demonstrated T cells migrating directly into the parenchyma from post-capillary venules. We also detected T cells that directly enter these vessels from the brain parenchyma (Fig. 8). Upon entering the brain, the T cells migrated in a pattern and exhibited APC scanning behavior that were in contrast to published data showing non-directed T cell behavior during peak EAE. A novel finding in our current study is the focal nature of T cell accumulation and their repeated preferential
interaction with DCs during the asymptomatic phase of EAE progression, suggesting that there is intricate communication between newly arrived 2D2 cells and parenchymal DCs. We speculate that through these interactions, 2D2 cells are re-activated through myelin-derived peptide presentation by the DCs before the T cells exert their pathogenic effector function that eventually leads to the development of clinical symptoms.

PTx has been shown to increase the permeability of vessels ex vivo, with direct effects on the choroid plexus and cerebral endothelial cells. In the brain, PTx is postulated to also act through the release of histamine, which causes vasodilation and changes adhesion molecule expression, and alters both endothelial tight junctions and astrocyte end feet barrier integrity. Histamine receptor 1 and 2 double knock-out mice have a reduced EAE severity and BBB permeability. One study has shown that the CSF of MS patients contains elevated levels of histamine, and H1R antagonists have shown clinical benefits in MS. In our study, we used the first generation histamine H1 receptor antagonist, hydroxyzine, which readily passes into the CNS, to decrease vessel permeability. In a clinical trial, hydroxyzine was shown to stabilize or improve clinical symptoms in 75% of MS patients. Here, we showed that hydroxyzine treatment reduced EAE disease incidence or severity in 60% of the mice. Hydroxyzine also decreased the total number of T cells found within the CNS on days during PTx administration. Furthermore, hydroxyzine reduced the extent of meningeal macrophage and microglia phagocytosis.
Second, it decreased the myeloid-derived APC infiltration on days 3-9. Third, hydroxyzine decreased all APC subtypes and functional T cells found in the CNS on day 12 (Fig. 10; Fig. 11). Although these phenomena were observed in the brain, the number of IFN-γ, IL-17 and FoxP3 producing T cells in the spleen were similar to controls (Fig. 11). Preventing the early activation of microglia with hydroxyzine resulted in a reduction of both non-specific T cell infiltration on day 3 and antigen-specific T cell infiltration on day 12. These data provide further evidence supporting the view that microglia play a critical role in early EAE induction, and that blocking microglia activation during the inflammatory phase can lessen myelin and neuronal destruction\textsuperscript{217, 255, 264}. Future work remains to fully understand the regulation of focal and intermittent vessel disruption that was only partially blocked by H1R blockade.

The exact kinetics of cellular inflammation in the brain has been hard to study, especially during the very early stages of EAE induction, due to the low number of relevant cells detected using conventional bulk cellular assays. Here, we developed an approach to reproducibly induce EAE with low variability. This method did not require irradiation or the use of chimeras that may disrupt BBB integrity, and we employed different fluorescent reporter mice to provide dynamic cellular data in an \textit{in situ} disease model. Our study revealed the localized sequential events involving early intermittent BBB disruption, local activation of microglia, the recruitment of DCs, and the retention and licensing of activated T cells in the CNS prior to disease manifestation.
Supplemental movie 1: Illustration of a transient vessel leak with vessel content persistence in parenchyma and of multiple transient vessel leaks from the same point of origin.

Region 1: At time 00:31:30, a focal transient leakage of TRITC vessel dye (red) starts and the fluorescence persists in the parenchyma until the end of the movie. Region 2: Starting at time 00:16:00, a focal transient leakage of TRITC vessel dye (red) initiates and resolves, then initiates again at time 00:38:00 and resolves with no residual fluorescence persisting in the parenchyma. Note: other vessels of similar caliber within the same imaging volume remained completely intact. Total time: 52 min 30 sec. Playback speed: 300X. Scale bar = 60 um. Time hh:mm:ss.

Supplemental movie 2: Transient vessel leaks originate from outside the dynamic field of imaging.

Maps of the whole cranial window were used to create a composite image on which a movie was overlaid. Overlaid movie on the static composite image enables an approximation of the vessels that gave rise to vessel leaks seen in the dynamic movie. Total time: 60 min. Playback speed: 300X.

Supplemental movie 3: Only CX3CR1+/GFP cells phagocyte TRITC vessel dye.

Imaging on day 0 after EAE induction shows only CX3CR1+/GFP (green) cells take in 150kD TRITC vessel dye (red). Shown first is the TRITC-only channel where we observed the cells that take up vessel dye over time. At the end of the video is a two-channel overlay, showing TRITC+ cells were all within the CX3CR1+/GFP population. Note: no TRITC+ GFP- cells were observed. Playback speed: 300X.

Supplemental movie 4: Infiltrating myelin specific T cells traffic to locations of previous vessel leak and CD11c+ accumulations.

Imaging on day 9 after EAE induction shows 2D2 T cells (blue) surveying the parenchyma concentrating around CD11c-GFP+ rich region. TRITC vessel dye also highlights phagocytic cells (red). Colored tracks
show the paths taken by 2D2 cells during the duration of the video, revealing high traffic around CD11c-GFP+ rich region. Total time: 90 min. Playback speed: 300X. Scale bar = 100um. Time stamp: hh:mm:sec.

Supplemental movie 5: HXYZ treatment of EAE induced mice does not prevent vessel leak but does prevent phagocytosis.

Mice were given HXYZ via gavage over the course of EAE induction. The left panel shows the dynamic imaging of an full EAE-induced mouse while the right panel shows the dynamic imaging of a HXYZ-treated EAE-induced mouse on day 3. At time 00:18:00 the EAE mouse exhibited a vessel leak (arrow) and phagocytic cells can be seen throughout the whole field. The HXYZ treatment mouse showed 2 locations of vessel leak (arrow) and no phagocytic cells. From time 00:01:00 – 00:14:00 one leak was observed. At a second location, the same vessel leaked 3 times during the imaging session, at times 00:03:30, 00:08:00 and 00:28:30. Total time: 59 min. Playback speed: 300X. Time hh:mm:ss.
3 Extravascular CX3CR1⁺ Cells Extend Intravascular Dendritic Processes into Intact CNS Vessel Lumen

3.1 Abstract
Within the CNS, APCs play a critical role in orchestrating inflammatory responses where they present CNS-derived antigens to immune cells that are recruited from circulation to the CSF and SAS. Available data indicate that APCs do so indirectly from outside of CNS blood vessels without direct contact with blood luminal contents. Here, we applied high-resolution, dynamic intravital two-photon laser scanning microscopy (2P-LSM) to directly visualize extravascular CX3CR1⁺ APC behavior deep within undisrupted CNS tissues in two distinct anatomical sites under three different inflammatory stimuli. Surprisingly, we observed that CNS-resident APCs dynamically extend their cellular processes across intact vessel wall into the vascular lumen with preservation of vessel integrity. While only a small number of APCs displayed intravascular extensions in intact, non-inflamed vessels in the brain and the spinal cord, the frequency of projections increased over days in experimental autoimmune encephalomyelitis (EAE), whereas the number of projections remained stable compared to baseline days after tissue injury such as CNS tumor infiltration and aseptic spinal cord trauma. Our observation of this unique behavior by parenchyma CX3CR1⁺ cells in the CNS argues for further exploration into their functional role in antigen sampling and immune cell recruitment.
3.2 Introduction

In non-central nervous system (CNS) tissues, activation of antigen-presenting cells (APCs) by pathogen- or damage-associated molecular patterns (PAMP or DAMP) results in APC homing to sentinel lymph nodes (LNs) where they process and present antigens to lymphocytes\textsuperscript{265}. On the other hand, the pathway for antigen surveillance and presentation in the CNS is unique for a variety of reasons. First, the blood-brain barrier (BBB) poses a structural impediment for circulating immune cells to freely move into and out of the CNS under steady-state conditions. Therefore circulating lymphocytes have limited direct access to CNS-resident APCs and associated antigens from inside the vessel lumen. Second, whereas peripherally activated APCs and soluble antigens can drain directly to the closest LN, in the CNS the cerebral spinal fluid (CSF) acts as the lymphatic fluid that drains both soluble contents and myeloid-derived cells via the cribriform plate into deep cervical LNs where antigen presentation can occur\textsuperscript{6, 8, 266, 267, 268, 269}. Third, CNS APCs are both blood-derived and tissue-resident, with each subset having distinct surveillance locations and functional roles\textsuperscript{61, 270, 271}. CX3CR1$^{+}$ microglia, defined as Ly6C$^{lo}$/CD45$^{lo}$/Iba-1$^{+}$, are tissue-resident and constitute the main immune cell population in the CNS parenchyma under non-inflamed conditions\textsuperscript{61, 64, 95}. Microglia are present in both grey and white matter and, although controversial, presumably possess different activation states and numbers at different anatomic sites\textsuperscript{272, 273}. For example, grey matter lesions in multiple sclerosis (MS) display less microglia activation compared to that found in white matter lesions\textsuperscript{274}. Microglia function as tissue APCs in all anatomic sites.
within the CNS, and infiltrating monocytes have been shown to migrate to the cervical LNs through the cribriform plate\textsuperscript{89, 268, 275}.

On the other hand, CX3CR1\textsuperscript{+} perivascular and meningeal macrophages are derived from circulating Ly6C\textsuperscript{hi}/CD45\textsuperscript{hi}/CCR2\textsuperscript{+} monocytes and can sample CSF contents in the arachnoid and Virchow-Robin space\textsuperscript{3, 85}. For these reasons, perivascular macrophages have been postulated to play an important role in the presentation of myelin-derived peptides to and re-activation of myelin-specific T cells residing in the perivascular space during the induction of experimental autoimmune encephalomyelitis (EAE). Activated effector T cells cross from blood circulation into the CSF through the choroid plexus and Virchow-Robin space in a CCR6-CCL20 dependent mechanism\textsuperscript{6, 65, 73, 221}. Once in the CSF, primed myelin-specific T cells encounter meningeal macrophages that present CNS tissue-derived myelin peptides. The re-activation of T cells results in a local inflammatory response, effector immune cell recruitment, tissue destruction and BBB damage, eventually leading to the pathological hallmarks of EAE: immune cell accumulation, neuronal damage and conduction loss\textsuperscript{6, 200, 276}.

Recently, histologic studies of static brain tissues suggest that parenchymal CD11c\textsuperscript{+} APCs help to orchestrate immune responses by inserting dendritic extensions into the glial limitans, presumably to sample and present brain-derived antigens to passing immune cells in the Virchow-Robin space\textsuperscript{277}. The observation that APCs can insert dendritic extensions through dense intact
tissues for antigen sampling in a separate anatomic compartment is not unique. For example, dendritic cells have been shown to extend cellular processes across intact epithelial and endothelial tight junctions in the Peyer’s patches\textsuperscript{16}, mucosa of the small intestine\textsuperscript{17}, the lungs\textsuperscript{18}, the nasal mucosa\textsuperscript{19}, cardiac valves\textsuperscript{20}, cornea\textsuperscript{21} and the lymphatic conduits in the LNs\textsuperscript{22,23}. In all these cases, APCs extend their dendrites across the host-environment interface or another tissue compartment and scan for potential foreign antigens for subsequent presentation to immune cells on the same side of the tissue compartment as the APCs.

In the current study, we utilized high-definition intravital two-photon laser scanning microscopy (2P-LSM) to observe CX3CR1\textsuperscript{+} extravascular APC behavior in the mouse brain and spinal cord under a variety of inflammatory conditions. Using several surgical procedures to expose tissues for intravital imaging including acute and chronic cranial window\textsuperscript{278}, thinned-skull\textsuperscript{279,280} and open laminectomy\textsuperscript{281}, we observed in a dynamic fashion the ability of extravascular APCs to display dendritic extensions into intact CNS vessel lumen. Furthermore, we showed that the frequency of such intravascular extensions increased during the progression of EAE and was greater within the cortical grey matter as compared with dorsal column white matter in the spinal cord. The frequency of extensions was unchanged during CNS tumor infiltration, and decreased immediately following spinal trauma before recovering within the first week following injury. In contrast with previous observations of APC extensions across intact tissues or the host-environment interface, we showed tissue-
resident APCs displaying dendrites directly into the blood vessel lumen without breaching vessel integrity. Hence, our novel observation adds another dimension to the ever-evolving understanding of immune cell behavior in the CNS.

3.1 Results
In order to visualize the behavior of perivascular and parenchymal CX3CR1+ cells in the CNS parenchyma, we applied 2P-LSM to the parietal lobe of a Cx3cr1+/GFP mouse through implanted cranial glass windows covering either an open craniotomy or a thinned-skull preparation (Figs 12A-E). The CNS parenchyma was visualized below the collagen-rich meningeal layers and the pial surface was outlined by second-harmonic signal generation under 2-photon excitation (Figs 13A-B). Under steady-state conditions, the majority of the CX3CR1+ cells in the mouse CNS parenchyma consisted of Ly6Clo/CD45lo/Iba-1+ microglia, with very few Ly6Chi/CD45hi/iBa-1hi blood-derived monocytes/macrophages. Indeed, dynamic intravital images revealed GFP+ cells with extensive ramified processes are evenly distributed throughout the CNS parenchyma with a cellular morphology consistent with that of ramified non-activated microglia (Fig. 14A; Figs 12A-C). Sequential imaging showed that the cell bodies of these CX3CR1+ cells were stationary, with extensive dynamic dendritic processes surveying surrounding tissues (Supplemental movie 6). Upon closer inspection, some of the ramified GFP+ CX3CR1+ cells were located near CNS vessels, with their processes wrapped around the outer vessel wall (Figs
While the larger-sized extravascular GFP$^+$ cells had morphology consistent with that of microglia and macrophages, other smaller
Figure 12: Comparison of CX3CR1+ cellular morphology and baseline projection frequency using different tissue preparations.

The morphology of CX3CR1+ cells (green) in control Cx3cr1+/GFP mice are shown using: (A) thinned-skull approach, where the images were obtained with an intact bone thickness of 25–50 mm (D, E; c: cranial bone); (B) cranial window implanted 4 days prior to imaging; and (C) acute open craniotomy through a glass window on the day of imaging. CX3CR1+ cellular morphology was similar in all cases, with some differences in the overall imaging properties as imaging depth and structural resolution was greatest with open craniotomy and least with thinned-skull preparation. Parenchymal vessels were labeled with TRITC-dextran (red). Scale bar = 100 mm. (F) Baseline number of intravascular projections was quantified from four individual Cx3cr1+/GFP mice using 4-day implantation window and six individual Cx3cr1+/GFP mice using thinned-skull preparations, showing an average of 172.5 ± 96.8 projections/mm² and 171.5 ± 82.3 projections/mm², respectively. n.s. = not significant. Only CX3CR1+ cells in the parenchyma were analyzed (Fig. 3).
spherical and highly mobile GFP$^+$ cells could also be seen crawling within the vessel lumen, which most likely represent other CX3CR1$^+$ cell populations including NK cells and monocytes in the systemic circulation$^{102}$ (Supplemental movie 8). High-resolution fluorescence and 3-dimensional reconstruction images revealed a surprising finding that on occasion, extravascular, stationary GFP$^+$ CX3CR1$^+$ cells were capable of extending their cellular projections into intact CNS vessel lumen (Fig. 14B). These intraluminal cellular processes persisted throughout a 45-minute imaging session, with the intravascular processes of two nearby extravascular CX3CR1$^+$ cells making contact with each other inside the vessel lumen (Fig. 14C; Supplemental movie 9). Furthermore, these cells seemed capable of inserting their cellular processes into the vessel lumen without breaching the vascular integrity, as evidenced by the lack of intravascular fluorescent dye leakage into CNS parenchyma.

To interrogate whether insertion of intravascular dendritic processes by extravascular CX3CR1$^+$ cells is only a behavior exhibited by extravascular CNS APCs under steady state or a more general behavior found in CX3CR1$^+$ cells near CNS vessels under inflammatory conditions, we induced a Cx3cr1$^{+/GFP}$ mouse to undergo experimental autoimmune encephalomyelitis (EAE). Consistent with our previously published work$^{282}$, Cx3cr1$^{+/GFP}$ mice developed worsening clinical scores starting around day 12 following induction of disease. Using similar intravital imaging techniques, we were able to easily visualize the presence of intraluminal dendritic processes extending from the main bodies of
Figure 13: Visualization, identification, and analysis of parenchymal CX3CR1+ cells with Intravital imaging methods.

Three-dimensional (xy, xz, yz) display views of the maximum intensity projection images of the spinal cord (A) and the brain (B) of a control Thy-1-YFP-H+ Cx3cr1+GFP mouse acquired through an open laminectomy (A) and a cranial window (B), showing the relative positions of individual Thy-1-YFP-H axons (yellow), CX3CR1+ cells (green), intact TRITC-dextran labeled blood vessels (red), and dura (blue, second harmonic signals). All of our quantitative analyses took place in the layers where Thy-1-YFP is present below the meninges and pial surface (dotted line). (C) Immunofluorescence histology of fixed tissue sections from Fig. 13 confirmed that the majority of CX3CR1+GFP (green) cells in the visualized field also co-stained (E) for Iba-1 (D, red), further identifying them as belonging to the activated monocytic lineage.283, 284
Figure 14: Intravital microscopy reveals persistent intravascular CX3CR1+ dendritic projections into intact CNS vessels.
Figure 14 (continued): Intravital microscopy reveals persistent intravascular CX3CR1+ dendritic projections into intact CNS vessels.

(A-C) A low-power snapshot from Supplemental movie 6 is shown in (A), demonstrating the overall distribution and ramified cellular morphology of CX3CR1+ cells (green) in the CNS parenchyma of a Cx3cr1+/GFP mouse through a cranial window. Scale bar = 100 um. A few CX3CR1+ cells are found in close proximity to the blood vessel (red) (B-D; Supplemental movie 7). (B) Fluorescence and 3-D surface rendering of a snapshot from inset in (A) at time = 0 min of Supplemental movie 7, demonstrating intravascular dendritic insertion (white arrows) of extravascular CX3CR1+ cells (green) through an intact CNS vessel (red). Scale bar = 15 um. (C) Fluorescence and 3-D surface rendering of the same cells as in (B) at time = 45 min, showing persistence of intraluminal dendritic insertions (white arrows) over the duration of the imaging session. Projections from two extravascular CX3CR1 cells are seen touching each other inside the blood lumen. Scale bar = 15 um. (D) Extravascular CX3CR1+ cells with ramified morphology displayed projections (white arrows) into the vessels (red) on Day 12 after EAE induction, while a non-ramified, elongated perivascular CX3CR1+ cell nearby did not (asterisk) and were not included in the final analysis. Scale bar = 10 um. (E) The number of intraluminal projections (white arrows) by extracellular CX3CR1+ cells increases in the CNS of Cx3cr1+/GFP mouse on day 5 after EAE induction. Scale bar = 10 um. Only CX3CR1+ cells in the parenchyma were analyzed in A-E (Fig. 13).
extravascular CX3CR1+ cells near intact blood vessels on day 5 during EAE induction (Fig. 14E), demonstrating that the ability of the extravascular CX3CR1+ cell to protrude intravascular dendritic processes was not unique to steady-state cells. To provide corroborating evidence for how the parenchymal CX3CR1+ cells managed to display their dendritic processes through multicellular perivascular structures of CNS vessels, we analyzed naïve, nonmanipulated brain (Figs. 15A, 15B) as well as EAE-induced tissue sections (Figs. 15C, 15D) by immunohistochemical (IHC) analysis. We confirmed that the intravascular dendritic processes within a vessel lumen in Fig. 14 were derived from the extravascular CX3CR1+ cells with dendritic morphology and were not a result of imaging artifact or blood-derived CX3CR1+ cells in the process of transmigration across the vessel lumen. We also confirmed the juxtaposition of extravascular CX3CR1+ cells and the extent of the reconstructed blood vessel lumen by two different intravital labeling methods (Fig. 16). In addition, electron micrographs (EM) of fixed brain sections from naïve, unmanipulated mice confirmed the presence of intravascular dendritic processes displayed by a CX3CR1+ cell through the basement membrane and endothelium, with a cell body beyond astrocyte end feet and in close proximity to surrounding neurons in the CNS parenchyma (Figs. 15E, 15F). Within one thin section, we found 176 vessels in 38,556 mm³ of tissue and one projection.
Figure 15: Immuno-histology and TEM confirm intravascular CX3CR1+ dendritic projections into intact CNS vessels.

(A-B) Immunofluorescence microscopy of fixed tissue sections on day 30 after EAE induction confirmed the presence of dendritic extensions (arrows) by extracellular parenchyma (P) CX3CR1+ cells (green) into the vessel lumen (L) as outlined by anti-CD31 (F; red) and tomato-lectin (G; red) staining. (C-D) Immunofluorescence microscopy of fixed tissue sections of a non-inflamed Cx3cr1+/GFP mouse confirmed the presence of dendritic extensions (green) flanking by GFAP+ astrocytic end-feet (pink) next to vessel lumen as outlined by laminin (blue). (E) TEM of fixed tissue sections of a non-inflamed Cx3cr1+/GFP mouse confirmed the presence of dendritic processes (blue) with intact endothelial cells and basement membrane. Astrocytic processes (pink) and axons (green) are visualized next to the dendritic extension. Magnification = 10 000X.
We then assessed changes in the frequency of intravascular dendritic processes during the course of EAE induction. To accomplish this, we conducted sequential imaging of CNS parenchymal CX3CR1\(^+\) cells on the same animal cohort for 12 days through either acute imaging of different mice or chronic cranial implantation windows in the same animal (Fig. 17A-C). To further facilitate the visualization of brain parenchyma versus meninges for subsequent analysis, we utilized double transgenic mice in which YFP is expressed under a neuron-restricted Thy1 promoter while one of the Cx3cr1 alleles is replaced by GFP (Fig. 13). Thy-1 YFP marker allowed us to identify the parenchyma within the imaging field. Under non-inflamed conditions, CX3CR1\(^+\) cells were uniformly present throughout the parenchyma with a ramified morphology in both white and grey matter (Fig. 13A-B). CX3CR1\(^+\) cells within the cortical grey matter had a more ramified and delicate pattern than those in the spinal cord white matter. Intravascular dendritic processes from extravascular CX3CR1\(^+\) cells were again detected (Fig. 17D-E). As EAE developed, parenchymal CX3CR1\(^+\) cell density increased with concomitant increasing number of extravascular GFP\(^+\) cells positioned next to CNS vessels (Fig. 17C). Next, we enumerated the number of intraluminal dendritic processes (“projections”) by extravascular stationary CX3CR1\(^+\) cells throughout the first 12 days of EAE induction (Fig. 17F). In order to ensure that we did not count as intraluminal dendritic processes GFP\(^+\) cells that were within the vascular lumen caught in the process of extravasation, we excluded cells with migration speed of > 3 \(\mu\)m/min in the dynamic imaging dataset, as well as small, spherical and motile GFP\(^+\) cells or elongated,
Figure 16: Vessel boundary outlined by different in vivo labeling techniques.

CNS vessel lumen boundary measured similarly during intravital imaging of the same region by sequentially injecting (A) TRITC-dextran (700 ug / mouse); and (B) tomato-lectin (16 ug / mouse) i.v. on two consecutive imaging days. Scale bar = 10 um.
Figure 17: Increasing number of extravascular CX3CR1+ cells with intravascular dendritic projections in the brain during early EAE induction.

(A) A snapshot of vessel (red) within the CNS of a Thy-1-YFP-H x Cx3cr1+/GFP mouse taken 4 days after cranial window implantation shows the steady-state distribution of CX3CR1+ cells (green) and intact neurons (yellow) within the CNS parenchyma, with the CX3CR1+ cells remaining in a ramified state. Scale bar = 50 um. (B, C) Snapshots of the mouse brain on days 0 (B) and 12 (C) after EAE induction show morphologic changes and an increase in the number of CX3CR1+ microglia (green). The blood vessels are outlined by the TRITC-dextran dye (red). Intraluminal portions of the CX3CR1+ cells are highlighted in grey. Projections (arrows) occur in both the large and small vessels, but are more common in the smaller vessels. YFP axon signal is removed for ease of visualizing microglia Scale bar = 50 um. (D, E) Coronal fluorescence (D) and surface rendering (E) view of a blood vessel (red) in (C) demonstrates an intraluminal dendritic projection (white arrows) by an extravascular CX3CR1+ cell (green). Scale bar = 15um. (F) The number of intraluminal projections are quantified and normalized to total vessel surface area (#Projections / mm²) over the course of EAE induction, showing a 2-fold increase in projection frequency over the first 12 days. Only CX3CR1+ cells in the parenchyma were analyzed (Fig. 13).
perivascular GFP$^+$ cells that had $>30\%$ of the cell volume inside the vessel lumen. At baseline, we found that CX3CR1$^+$ cells exhibited $172.50 \pm 24.2$ projections per mm$^2$ of vessel wall surface (Fig. 17F). The number of dendritic projections more than doubled (not statistically significant, $p>0.05$) that found in the non-inflamed control over the first 9 days following EAE induction, with the frequency of projections plateauing between days 9 and 12 (Fig. 17F).

To compare number and characteristics of intravascular dendritic projections at different anatomical sites containing grey matter or white matter, we also examined the behavior of extravascular CX3CR1$^+$ cells in the dorsal column white matter next to spinal vessels during EAE. Under steady-state conditions, CX3CR1$^+$ cells in the spine were uniformly distributed throughout the parenchyma of the spinal cord, with some cells closely associated with both large and small caliber vessels (Fig. 18A-B). Similar to the brain, GFP$^+$ dendritic projections were again visualized to insert into both large and smaller spinal vessels (Fig. 18D-E). Again, parenchymal CX3CR1$^+$ cells in the spine appeared in a ramified, non-activated state (Fig. 18A-B), with an average of $74.6 \pm 11.03$ projections per mm$^2$ of visualized vessel surfaces (Fig. 18F). Upon EAE induction, the number of intravascular projections doubled (not statistically significant, $p>0.05$) to an average of $159.97 \pm 27.26$ projections per mm$^2$ of vessel surface by day 12, with cells appearing in an activated, less ramified morphology (Fig. 18C). Although intravascular projections could be seen in the large central venous vessel, they were more commonly observed in close association with the smaller venous vessels (Fig. 18C).
Figure 18: Increasing number of extravascular CX3CR1+ cells with intravascular dendritic projections in the spine during early EAE induction.

(A) A snapshot of the central dorsal spinal vein of a Thy-1-YFP-H x Cx3cr1+/GFP mouse taken immediately after T10 laminectomy shows the steady-state distribution of CX3CR1+ cells (green) and intact axons in the spinal parenchyma (yellow), with the CX3CR1+ cells remaining in a ramified state. Scale bar = 50 um. (B, C) Snapshots of the spine on days 0 (B) and 12 (C) after EAE induction show morphologic changes and an increase in the number of CX3CR1+ cells (green). The blood vessels are outlined by TRITC-dextran dye (red). Intraluminal portions of the CX3CR1+ cells are highlighted in grey. Projections (arrows) occur in both the large and small vessels, but are more common in the smaller vessels. YFP axon signal is removed for ease of visualizing microglia. Scale bar = 50 um. (D, E) Coronal fluorescence (D) and surface rendering (E) view of a blood vessel (red) in (C) demonstrates intravascular dendritic projections (white arrows) by extravascular CX3CR1+ cells (green). Scale bar = 15um. (F) The number of intraluminal projections are quantified and normalized to total vessel surface area (# projections / mm²) over the course of EAE induction, showing a 2-fold increase in projection frequency over the first 12 days. Only CX3CR1+ cells in the parenchyma were analyzed (Fig. 13).
To test whether the increase in intravascular dendritic processes was simply due to activation of brain and spinal CX3CR1\(^+\) cells in response to non-specific tissue injury, we examined intravascular dendritic projections in other models of CNS inflammation. First, we investigated an aseptic traumatic spinal cord injury model in which a traumatic crush injury was created in the dorsal column without breaking the major blood vessels. We observed that CX3CR1\(^+\) cells accumulated at the injury site over a course of 8 days post injury while the vessel integrity to large molecular weight solutes remained intact throughout the healing process, as evidenced by the lack of vessel dye uptake by surrounding tissue phagocytes (Fig. 19A-B). Similar to the findings in EAE, extravascular CX3CR1\(^+\) cells can insert their GFP\(^+\) dendritic processes through intact vessel walls into the blood lumen (Fig. 19C-D). Contrary to the findings in EAE, however, CX3CR1\(^+\) cells accumulated around both the large and small vessels at the spinal injury site (Fig. 18C, 19B). We observed an average of 46.59 ± 10.1 intravascular projections per mm\(^2\) of intact vessel wall at baseline (Fig. 19E). Upon enumeration of intravascular dendritic projections during an 8-day span of tissue recovery, we observed an immediate 40.5 % reduction (p = 0.048) in projections at the site of injury, and then a slow recovery to an average intravascular projection frequency of 39.46 ± 7.91 per mm\(^2\) in the lesion (not statistically significant, p>0.05), a frequency that was 84.7 % of a healthy spinal cord (Fig. 18F, 19E).
Figure 19: Intravascular projections decrease and then recover in aseptic traumatic spinal cord injury and do not increase in the CNS tumor microenvironment.
Figure 19 (continued): Intravascular projections decrease and then recover in aseptic traumatic spinal cord injury and do not increase in the CNS tumor microenvironment.

(A, B) Snapshots from tile scan of the spinal cord dorsal columns at T10 on days 0 (A) and 8 (B) after crush injury (dashed box) shows intact dorsal vein (red) and CX3CR1+ cell (green) distribution. Intraluminal portions of the CX3CR1+ cells are highlighted in grey. The morphology of CX3CR1+ cells on Day 8 appeared to be more rounded and less ramified. (C, D) Coronal fluorescence (C) and surface rendering (D) view of a blood vessel (red) in (B) demonstrates intravascular dendritic projections (white arrows) by extravascular CX3CR1+ cell (green). (E) Quantification of intravascular projections post crush injury showing a near-full recovery in projection numbers in 8 days. (F) A snapshot of the CNS tumor microenvironment 7 days after inoculation of MM1-DsRed into a Cx3cr1+/GFP mouse shows local accumulation of CX3CR1+ cells and development of neo-vasculature. Intraluminal portions of the CX3CR1+ cells are highlighted in grey. MM1-DsRed signals are removed in F-H for ease of visualizing CX3CR1+ cells. (G, H) Fluorescence (G) and surface rendering (H) view of a blood vessel (red) in (F) demonstrates intravascular dendritic projections (white arrows) by extravascular CX3CR1+ cells (green). (I) The number of intravascular projections in the CNS tumor microenvironment are quantified from 3 tumor-bearing mice and normalized to total vessel surface area, showing an average number of 72.9 ± 6.3 projections / mm2 (same as baseline in Figs 12F and 13F). Only CX3CR1+ cells in the parenchyma were analyzed (Fig. 13).
A second tissue injury model was a syngeneic CNS tumor model. To determine if intraluminal projections by extravascular CX3CR1\(^+\) cells could also be detected in the vessels within a CNS tumor microenvironment, we inoculated \textit{i.c.} MM1-DsRed2, a fluorescent syngeneic mouse medulloblastoma cell line derived from \textit{Patch}\(^{+/+}/p53^{+-}\) mice, into three \textit{Cx3cr1}\(^{+/GFP}\) mice and recorded the responses of CX3CR1\(^+\) cells in the CNS tumor-associated neo-vasculature. We observed local accumulation of CX3CR1\(^+\) cells in the CNS tumor microenvironment with extravascular CX3CR1\(^+\) cells extending intravascular dendritic projections into the tumor neo-vasculature (Fig. 19F-H). This was similar to that found in the spine and brain parenchyma in EAE and spinal trauma. Distinct from the EAE model, the average frequency of intraluminal dendritic processes per mm\(^2\) of intact tumor-associated vessel wall was only 72.9 ± 6.3, a number that was consistent across tumor lesions in three different experimental animals and in good agreement with that observed in the control brain and spinal cord (Fig. 17F, 18F, 19E). Our findings from EAE, aseptic traumatic spinal cord injury and CNS tumor models suggest that, while there is a baseline frequency of intravascular dendritic projections by extravascular CX3CR1\(^+\) cells in vessels of the brain and the spine, there exists discernable differences in regulating the number of intravascular processes by CX3CR1\(^+\) cells in response to the inflammatory signals associated with EAE induction compared to that in response to the inflammatory signals in association with tissue injury and CNS tumor growth.
3.2 Discussion
At baseline, we observed an average of 60 and 173 intravascular projections by extravascular parenchymal CX3CR1⁺ cells per mm² of vessels found within the white matter of the dorsal columns of the spinal cord and the grey matter in the cortex, respectively (Fig. 18F, 17F). The number of projections doubled in the spine and the brain on day 12 following EAE induction, at a time when mice first began to exhibit neurologic deterioration. The differences in projection frequencies suggest that there may be differences in these two tissue types with respect to CX3CR1⁺ cellular response that inversely correlate with the abundance of myelin found at each site. Interestingly, our observation of projection frequencies during steady state and inflammatory conditions mirrored that seen on peri-epithelial APCs in the lamina propria of Cx3cr1⁺/GFP mice. The consistent observation of CX3CR1⁺ APC behavior in multiple tissue types under steady state as well as distinct inflammatory inducers highly suggest that the extra-compartmental dendritic protrusions through intact endothelia and epithelia tight junctions are a fundamental feature of the immune surveillance strategy employed by CX3CR1⁺ APCs. Such important cellular features may have previously been under-appreciated, in the current study, however, we have been able to successfully preserve these processes by transcardial PFA tissue fixation procedures (Fig. 15).

All three CNS inflammatory models examined in the current report entail different states of vascular activation. In EAE there is an acute induction of systemic
inflammation by Toll-like receptor (TLR) signals and pertussis toxin used in the immunization cocktail. In aseptic spinal cord injury or CNS tumor model, the vessel endothelium and CNS APCs are spared from exposure to such signals from bacterial products as contained in the EAE immunization cocktails. How differential states of endothelial activation contribute to the observed frequency of intravascular dendritic projections will be a subject of future investigation, specifically with respect to potential signaling crosstalk between the endothelium and extravascular CX3CR1\(^+\) APCs.

It has been argued that, in contrast to thin skull procedures, the cranial window technique engenders too much local trauma and inflammation to the imaged brain tissue such that the observed APC populations in the meninges and parenchyma could be artificially activated\(^ {225, 285, 286} \). However, the thin-skull approach, while avoiding direct contact between the surgical instruments with the meninges, requires the breach of bone marrow integrity and therefore could elicit systemic inflammation affecting underlying meningeal and parenchymal tissues. We performed acute and chronic craniotomy windows as well as thinned-skull surgical approach in the current study. In our hands, we found that the relative frequencies of intravascular extensions are comparable when these procedures were executed carefully (Fig. 12). In all of our intravital experiments, we paid special attention during intravital surgical procedures and post-operatively, and excluded mice from imaging analyses that had undergone a sub-optimal surgery procedure or endured visible vessel trauma. Both cranial window and thinned-
skull approaches revealed ramified parenchymal CX3CR1\(^+\) cells at baseline, consistent with CNS-resident APCs in a non-activated state (Figs. 14, 15A-B, 17A-B; Fig. 12). It is possible that both surgical methodologies designed to visualize the resident brain parenchymal cells induce some degree of injury. Regardless of the basal level of inflammation due to surgical trauma, the frequency of intravascular extension by parenchymal CX3CR1\(^+\) cells was in good agreement with that seen in the gut\(^\text{16, 17}\), and increased with additional tissue inflammation in EAE but not in spinal trauma or tumor inoculation (Figs 17F, 18F, 19E, 19I). More importantly, we were able to capture intraluminal extension of CX3CR1\(^+\) cells in naïve, noninflamed brain tissues by IHC and EM (Fig. 15). Using these methods, we observed the entrance of CX3CR1\(^+\) cells into the vessel lumen through the basement membrane and endothelial layer of the CNS vessel. Furthermore, the astrocyte end feet were on either side of the dendrite entry point, indicating that they have shifted positions to allow for the CX3CR1\(^+\) projections into the vessel lumen.

Intravenous administration of both fluorescent dextran and tomato-lectin provided clear fluorescent signals to consistently delineate the interface between the blood content and the vessel wall throughout the prolonged duration of intravital imaging (Fig. 16). We used a combination of 3-dimensional static images and time-resolved sequential datasets in our analysis of dendritic projection to verify that the identified intraluminal GFP signals came from stationary CX3CR1\(^+\) cells whose cell body was largely outside of the vessel wall, and not from smaller,
spherical mobile CX3CR1⁺ cells such as NK cells and T cell subsets that attached transiently to the luminal wall or from Ly6C⁻/CX3CR1⁺ vessel-patrolling monocytes that were in the process of transmigrating from the blood lumen to the perivascular space. Other than by morphology and Iba-1 staining (Fig. 13C), the Cx3cr1⁺/GFP mice used in our current study do not allow for precise identification of the specific CX3CR1⁺ cell populations in the captured 2P-LSM images (i.e. microglia versus perivascular monocyte/macrophages or dendritic cells). This shortcoming is especially relevant in later stages of CNS inflammatory processes where both tissue-resident and blood-derived APCs are present and express CX3CR1. To overcome this shortfall, future experiments will require the use of bi-phenotypic fluorescent reporter mice such as the Cx3cr1⁺/GFP / Ccr2⁺/RFP mice or crossing CD11c-mCherry mice to Cx3cr1⁺/GFP mice. Furthermore, the extent of intravascular dendritic projections in the brain of Cx3cr1⁺/GFP mice can be compared with that in Cx3cr1⁺/GFP mice to further delineate whether functional CX3CR1 is required for the dendritic protrusion.

Recent years have seen a paradigm shift in our understanding of the immune compartment in the CNS. While at steady state few circulating immune cells are found in the meninges and brain parenchyma, the CNS allows orchestrated infiltration of multiple immune cell subtypes in conditions such as infections, trauma and autoimmune diseases. These observations suggest that the BBB is a barrier that can be manipulated to regulate traffic of immune cells in-and-out of the CNS. Work in models of viral infection and EAE have thus far
focused on the role of inflamed CNS vascular endothelium in recruiting circulating immune cells into the CNS via adhesion molecules and inflammatory chemokines\textsuperscript{289, 290, 291}. In these models, both blood-derived (i.e. monocytes / macrophages / dendritic cells) and CNS-resident (microglia) APCs are implicated in the re-activation of infiltrated lymphocytes in the perivascular CSF space\textsuperscript{65, 73}. This suggests APCs play an ancillary role in the recruitment of initial immune cell invasion since cognate antigen recognition is postulated to only occur after the immune cells have been actively recruited to the CNS tissue with the aid of the inflamed endothelium. In this light, our current observation that CX3CR1\textsuperscript{+} APCs extend intravascular dendritic projections directly into intact CNS vessel lumen is highly significant, as it suggests an opportunity for: 1) antigen surveillance directly in the blood lumen by CNS APCs through intact CNS vessels; and 2) direct CNS antigen presentation to and recruitment of circulating immune cells by CNS APCs. Further explorations will be required to test these possibilities. If true, this hypothesis could explain how circulating lymphocytes were able to extravasate to CNS parenchyma in an antigen specific manner in early stages of pathological conditions such as EAE, where the BBB is presumably intact.

In summary, we used dynamic high-resolution optical fluorescent microscopy with 2-photon excitation, coupled with cell lineage-specific fluorescent reporter mice and multiple surgical techniques that allowed for longitudinal monitoring to study cellular events in the CNS. We observed that extravascular CX3CR1\textsuperscript{+} cells possess the capacity of inserting part of their cellular extensions through intact
CNS endothelium. How these cells accomplish this through multiple cell layers that comprise the BBB on a cellular and molecular level remains to be explored. Furthermore, the functional role such biological process may have in regulating immune responses in the CNS needs to be carefully tested. Our discovery highlights the important role that high-definition intravital dynamic optical fluorescence imaging plays in uncovering this novel cellular process in the CNS. In addition, our observations may offer opportunities for potential targeted therapeutic strategies in CNS-related diseases including infection, cancer and autoimmunity by interfering with the regulation of intravascular dendritic extensions by extravascular CNS APCs through intact CNS vascular endothelium.
Supplemental movie 6: Microglia morphology and distribution in non-inflamed CNS parenchyma of a Cx3cr1+/GFP mouse.

Sequential imaging of the parietal lobe of a Cx3cr1+/GFP mouse was captured through a cranial window implanted 4 days prior to imaging. CNS vessels are highlighted by TRITC-dextran. Total imaging time: 60 min. Playback speed: 300X.

Supplemental movie 7: Dynamic dendritic motility of stationary extravascular CX3CR1+ cells.

A zoomed-in view of a GFP+ CX3CR1+ cell (green) next to an intact CNS blood vessel (red) illustrates the highly dynamic motility of dendritic extensions probing the extravascular space. Other smaller, spherical CX3CR1+ cells can be seen crawling in the blood vessel lumen, which most likely represents circulating CX3CR1+ NK cells or monocytes. Total imaging time: 45 min. Playback speed: 300X.

Supplemental movie 8: Extravascular CX3CR1+ cells project dendrites into CNS vessels.

Dendritic projections of extravascular CX3CR1+ cells are vividly visualized within the CNS vessel lumen. The extravascular microglia body projects stably into the vessel lumen for at least 30 minutes. Dendritic projections from two CX3CR1+ cells can be seen contacting each other within the vessel lumen. Note the absence of TRITC-dextran dye in the surrounding parenchyma at the site of intravascular dendritic insertions. Total time: 45 min. Playback speed = 300X.

Supplemental movie 9: Three-dimensional view of the intravascular dendritic extension by extravascular CX3CR1+ cells.

A snapshot from Supplemental movie 7 (at time stamp = 36 min 30 sec) is shown in a 3-dimensional rendering view, demonstrating the relative position of the green dendritic body with respect to intact CNS blood vessel wall. Total time: 22 min. Playback speed: 450X. Scale bar = 15 um.
4 Image Analysis of Dynamic Intravital Multi-photon Imaging.

4.1 Introduction
With the advent of two-photon laser scanning microscopy it became possible to perform dynamic, intravital, immuno-imaging at the cellular level\textsuperscript{292, 293, 294, 295}. Cell-cell contact has been shown to be important in adaptive immunity\textsuperscript{296, 297} for information sharing in the partnership of antigen presenting cell and T cell within the lymph node\textsuperscript{298, 299}. High resolution, real-time fluorescence imaging has permitted visualization of single cell interactions and therefore insights into immune cell interaction, the immunologic synapse and interactions with the environment, rather than bulk population behavior\textsuperscript{300}. First generation analyses of T cells in lymph nodes and their interactions with other APC populations such as dendritic cells, macrophages and B cells, have shown that there are a limited set of motility variables that can be examined\textsuperscript{301, 302, 303} (Fig. 20) and from these measurements, general interpretations can be made about cellular behaviors. In our process of image analysis, we utilize a software (Imaris, Bitplane Inc.) that can segment fluorescent images and determine the tracks of cells. From there, we import the fluorescent information, cell and track position data into another software (Matlab, Mathworks Inc.) for further interaction analysis. In this chapter, we will describe analysis strategies and the algorithms used in these programs.
Figure 20: From Matheu. Two-photon multidimensional data analysis parameters.
Path length in μm is the entire distance the cell moved. Greater path length generally signifies cell scanning behavior. However, cells engaged in productive interactions can scan the surface of the target cell generating long path lengths within a confined region.

Displacement in μm is the distance from the first point to the last point the cell moved and is mainly used to determine whether a cell is stationary or engaged in a productive interaction. When used in conjunction with distance, it can indicate whether the cell took an efficient path. This measure is termed chemotactic index which is defined as distance / displacement. The closer the chemotactic index is to 1, the straighter the path to the target and thus the chemoattractant signal is strong. To understand the implications of chemotactic index one needs to understand the concept of chemokines and chemokine gradients. The other parameters of cell motility in Fig. 20 can be clearly described within the context of chemokine signaling.

4.2 Chemokine Influence on Motile Cells
In contrast to the neural system that is tightly packed with neurons and accessory cells fixed in place, the immune system is comprised of multiple subsets of highly mobile cells. This constant motion and surveillance of all tissues in the body enables rapid deployment to sites of pathogen challenge. Complex T cell, B cell and DC cell-cell interactions rely on motility in order to communicate and
coordinate responses for tolerance to self and effector responses to various pathogens. The pathogen threat that we face is very diverse, thus our adaptive immune system has evolved the ability to recognize the entire gamut of the antigenic universe. They do this by undergoing a form of genetic rearrangement called somatic hypermutation. By recombining the genes that encodes the alpha and beta chains of the T cell receptor, T cells are able to generate a trillion different unique sequences capable of identifying 25 million different specificities. Because of the different specificities, only 1 in $10^5 – 10^6$ T cell recognize cognate antigen in a primary immune response$^{304}$. Despite these incredible odds, our immune system has no problem mounting a timely and efficient response under normal circumstances. This is largely due to chemoattractants directing immune cells via chemokine receptors on their surface.

Interestingly, the first experiments to understand chemokine-mediated signaling showed that treatment with PTx inhibited the mobilization of neutrophils by CXCL8. It was determined from this that chemokine receptors had to be a G-coupled protein$^{305}$. It is now known that chemokine receptors are seven-transmembrane G protein–coupled receptors that can have multiple ligand binding partners and can signal through multiple pathways to induce differential responses. The single signaling pathway common to all chemokine receptors is cellular migration.
Chemokines are cytokines that cause the directed movement of immune cells. Even though chemokines are secreted they are not free floating but are immobilized on cells or extracellular matrix surrounding the cell producing the chemokine, thus effectively reducing the total amount of chemokine produced by the cell while maintaining a gradient. A population assessment of chemotactic index, especially if large number cells in a region are close to 1, coupled with this knowledge of the extracellular matrix can also be an indicator of the structures within the microenvironment allowing this movement. For example, it has been shown that fibroblastic reticular cells within the lymph node create an immune cell highway network to allow cells to efficiently traverse and encounter APCs with a cognate antigen.

Motility coefficient was devised to characterize Brownian motion, where the closer the mean displacement / square root of time is to 1, the closer the cell is to random Brownian motion. Initial two-photon microscopy reports of immune cell motility within the lymph node used naïve T cells under homeostatic conditions which resulted in no discerning pattern of movement. Under these conditions and in the context of a burgeoning chemokine field, it was logical to use this measure to describe a population of cells within an imaging field and concluded that movement within a lymph node had a motility coefficient close to 1 and was therefore described random motion. This measure ignores the direction vector of these cells, and subsequently parameters have been devised to more fully describe the motion of a cell within the context of a chemokine gradient.
Recently, motility coefficient has been used to compare across different conditions, such as the effect of a pharmaceutical therapy or motility within a chemokine knockout mouse. It has been determined the closer the motility coefficient is to 0 there is confined migration, if it is greater than 1 there is directed migration.

### 4.3 Point of Reference
Before the idea of chemokine directed motility, the first attempts to describe cell movement was based on the assumption of Brownian motion and that the movement of cells were random, resulting in the motility coefficient. Graphing of this process resulted in taking track data and referencing the beginning of the track to 0,0 of an XY-plot. We programed a plot generator to plot each track beginning of the track to 0,0, and as depicted in literature, the result looks like there is no directional trend and can be determined as a random walk. Each track gets assigned a different line style and color, where the initial position is removed from all samples along the path. This latter step ‘resets” the origin of each track, where this allows different path lengths and shapes to be visually discriminated.

However, in the case of chemokine directed cell movement, the interesting part of the cell path is the end, whether the cell ends at the target of interest. A related plot generator as described above was created, however, the final position is reassigned to 0,0 of an XY-plot by removing this position from all samples along
the path. This ‘resets” the destination of each track, where cells that are being
influenced by a chemokine gradient will take a directed path as opposed to non-
interacting cells that should follow a Brownian path to their final destination
during the imaging experiment. The resulting graph often shows a directional
trend towards the target of interest. This data is also represented in a 3D plot
(Note: the net output of this code resembles the appearance of the Deathstar
from the movie “Star Wars.”)

4.4 Angle of Approach
For T cells, a change in direction towards a target in the vicinity of an APC is an
important indicator of chemokines creating a path towards a productive
interaction. Turning angle is the angle of a portion of the path relative to the
previous portion as a measure of a directional change made by a cell. A wider
turning angle implies that the cell has deviated greatly from initial course and has
changed course to follow a chemotactic signal. Of the hundreds to thousands of
tracks in the position record of a typical experiment, most will start far away from
the user-defined target location. Thus, the analysis of turning angle has caveats.
First, the diameter for the volume of influence about an APC has a substantial
impact on the reported value: a diameter that is too large will lead to many “false
positive” tracks, whereas a diameter that is too small does not accurately reflect
the physical action of chemokines at a distance from the APC. Second, there is a
presumption that the spatial distribution and gradient for the chemokine signal
follows unrestricted diffusion, but the physical tissue microstructure that is not
visualized, might alter the cues for the T cell to change direction. Third, the angle of approach and the time at which the cell detects the chemokine gradient impacts the measurement. For example, a cell travelling tangentially to the volume of influence will deviate from the initial path and produce a wide angle to move towards a target, but a cell already travelling directly towards a target from a distance, will not deviate much from the initial path when it enters the volume of influence even if it engages in a productive interaction with the target. From this example, it can be seen that population averages of turning angle generally do not result in a representative measure. The complementary measurement of turning angle is directional persistence and is dictated by intracellular actin rearrangement. Directional persistence is the length of time a cell moves in a particular direction with small angular deviations from the mean and is also an indication of a chemokine gradient.

Some strong chemoattractants expressed by a single cell or a cohort of cells can have far reaching effect, and is visually astounding in dynamic data. A program was developed to identify the flux of T cells to a cell of interest. The code first centers the volume of interest (VOI) at a user-defined location and then excludes tracks that are never inside the VOI throughout the duration of the experiment. Also, tracks that originate within the VOI, or have less than three timeframes before or after entering the VOI are also excluded because these tracks are usually at the beginning or the end of the imaging session and conclusions cannot be made about these tracks. For the remaining set of tracks, a linear
model of motion based on location in three timeframes prior to crossing the defined threshold for interaction is used to construct an equation for a line in 3D. Similarly, a linear model is constructed using the three points after the track crosses the threshold of the VOI. The angle between these two lines is calculated and compared against the 3D angle to the target at the timeframe just prior to entering the VOI. For example, if the cell in tangential to the VOI and the required angle change to go straight to the target is +90º and the actual change is +80º, the scenario indicates a strong response to the cues for the T cell to move towards the target APC. Likewise, an actual change of -5º would correspond to a weak negative response to the chemotactic cues for moving towards the APC. Thus, there needs to be a categorization of tracks based on the angle of approach to the VOI. As shown in Fig. 21, a representative selection of track paths from an actual experiment demonstrate a subset of features that can be quickly inferred from this analysis framework.

4.5 Other Motility Parameters
Average velocity in μm/min is the total distance / total time and instantaneous velocity change in distance / change in time. Average velocity can be used to describe a population of cells and the state of the microenvironment, for example the average speed of a T cell in an inflamed lymph node is 10-12μm/min whereas microglia within a non-inflamed CNS will have an average speed of
Figure 21: Types of T Cell tracks
Figure 21 (continued): Types of T Cell tracks.

Only tracks that cross into a spherical volume of influence (VOI) with diameter 50um (gray circle) about an APC (gray dot) during the imaging experiment are considered for analysis. Paths in real data have indicated: (a) no interaction, (b) a direct approach with minimal interaction while inside the VOI, (c) a tangential approach followed by a direction change towards the target and some surveying before escaping the VOI (i.e. path of black dots), (d) a productive interaction at reduced velocity followed by escape from the VOI, or (e) a productive interaction with a substantial number of interaction points. This only shows the top-view of the vicinity of the target (i.e. the focal plane runs into and out of the page), where linear extrapolation of position three timeframes before (blue line) and after (red line) were used to compare changes in the T cell track direction.
0.5\mu m/min. Instantaneous velocity of a cell can be linked to chemokine gradients where a decrease implies interaction with chemokine gradient and presumably the cell is close to the chemoattractant source.

Contact time is the total time two cells are in contact, min. A longer contact time implies a more productive cell-cell interaction. Hit rate analysis was developed to quantify the frequency of a T cell touching a target DC and to show differences in T cell-DC interactions under different inflammatory chemokine guidance\textsuperscript{312}. Kon is the calculated hit rate (mm\textsuperscript{6} hr\textsuperscript{-1}) of the T cell touching a DC, but could be generalized to any cell type of interest.

Kon = \frac{A}{[T \text{ Cells}] [DC] \cdot t}.

A is the total observed number of DC-T cell contacts, [T cells] and [DC] are densities of T cells and DC in a data collection volume, while t represents the duration of imaging session. Hit rate ratio is a way to normalize a population of interest to a control and is determined as the ratio between the Kon for T cells interacting with activate DCs and the Kon for interactions involving a control set of DCs within the same imaging region.

The interesting finding from the analysis of Kon is that stable productive interactions result in better T cell activation and memory generation\textsuperscript{312}. As an extension of that work it was determined that T cells encounter many DCs within
the lymph node and that at different inflammatory phases, T cells interact
differently with DCs\textsuperscript{313, 314} (Fig. 22). It was subsequently determined that the
information or signals from sequential T cell-DC encounters are integrated by T
cells in antigen specific activation\textsuperscript{315}. These types of cellular behavior findings
can only be accomplished by intravital two-photon microscopy.

The use of high resolution confocal imaging of cell motility \textit{in vitro} discovered
many key aspects of the effects of chemokines, the most important being that
chemokine receptors polarize on the leading edge of the cell and this localization
is essential to signaling for motility and cell activation\textsuperscript{316, 317}. This localization of
chemokine receptors also plays a role in the actin cytoskeleton remodeling and
thus the shape of the cell as it moves either across endothelium or through the
extracellular matrix. Shape index is a measure of the longest axis / the shortest
axis of a cell. Stationary cells are usually spherical and have a shape index close
to 1. However, motile cells have a larger shape index due to elongation as a
result of taxis. In addition, cell activation generally results in enlargement of the
cell thus cell volume can be used as a measurement of cell activation and in
some instances proliferation, however there are more reliable ways than imaging
to determine activation and proliferation.
During the first 8 h after entering from the blood, T cells underwent multiple short encounters with DCs, progressively decreased their motility, and upregulated activation markers. During the subsequent 12 h T cells formed long-lasting stable conjugates with DCs and began to secrete interleukin-2 and interferon-γ. On the second day, coinciding with the onset of proliferation, T cells resumed their rapid migration and short DC contacts. Thus, T-cell priming by DCs occurs in three successive stages: transient serial encounters during the first activation phase are followed by a second phase of stable contacts culminating in cytokine production, which makes a transition into a third phase of high motility and rapid proliferation.
Recently there have been developments in functional reporter mice\textsuperscript{319} and functional reporter constructs that can be introduced into the cell \textit{ex vivo}. \textit{Ex vivo} calcium flux dyes have been used for years as a functional indicator of T cell activation\textsuperscript{320} however, the applications were mainly \textit{in vitro} as these dyes exhausted within hours. \textit{In vivo} functional imaging based on a calcium flux fluorescent reporter construct where the functional response of T cells within the CNS during EAE infiltration has recently been described and T cells in the CNS were shown to have very different kinetics than those seen during the T cell activation phase in the lymph node\textsuperscript{226}. \textit{In vivo} calcium flux fluorescence imaging cannot be normalized to a known concentration of calcium thus a ratio was developed to report increases in calcium within a cell\textsuperscript{321}.

\[ \Delta F/F = (F-F_0)/F_0 \]

where F is the maximum fluorescent signal detected in the cell whereas, F\textsubscript{0} is the baseline fluorescent signal detected during the imaging session. This can be correlated to events such as cell-cell interactions and recently a productive interaction was defined as one where the velocity was low and the fluorescent ratio was high\textsuperscript{226}.

\section*{4.6 Conclusion}

TPM technology allows sequential high-sensitivity and high-resolution detection of individual fluorescently labeled cells and structures within the undisturbed microenvironment of a live, anesthetized animal. Our unique imaging approach
offers serial high-definition dynamic real-time visualization of developing inflammation and invading cells within the structural context of the CNS in an anesthetized, live mouse during the entire inflammatory process. These parameters help us understand the migration and interaction behavior of immune cells within different microenvironments such as inflammation. Intravital imaging of reporter mice also serves as a potentially powerful experimental platform for future in vivo therapeutic efficacy monitoring.
5. Conclusion and Future Direction

5.1. Conclusion

The literature on the role of resident immune cells in autoimmunity and infection within the CNS has conflicting evidence partly because it has been difficult to extract cells from the tissue and probe individual function due to the intimate connection microglia have with other glia accessory cells, microglia in different parts of the brain have different responses, and until recently, the integral inhibitory relationship of microglia and neurons had not been fully realized. Preservation of an organ critical for high level mammalian functions has led to fine regulation by immune cells that localize the inflammation so as to minimize detrimental effects on the CNS. This work elucidates localized mechanisms that account for the patchy, immune cell filled lesions seen in EAE and MS, while complementing and confirming previous seminal works in the field.

We showed that during inflammation, there are transient blood leaks that result in activation of the microglia surrounding the blood vessel. This activation of microglia from the first day of neurotoxic stimuli up to day 4 is followed by infiltration of peripheral DCs on day 6, and subsequently, T cell infiltration beginning on day 9. The activation of microglia can be affected by the administration of histamine receptor antagonist, hydroxyzine thus affecting the successive infiltration of peripheral immune cells.
We were also the first to find that during this inflammation, an increased number of microglia are able to interface with other cells of the BBB to project extensions into the blood vessel through an intact BBB. This work advanced our knowledge about the initiating events of EAE and deepened our understanding of microglia behavior with the blood vessel under inflammatory conditions. Here I will set forth a line of investigation that will further our knowledge of the interface between the resident immune cells of the CNS and the peripheral immune cells.

5.2. Imaging to Detect Vessel Leaks in a Relapsing-Remitting Model

The model used in this thesis took advantage of C57BL/6J mice that allows the use of syngeneic myelin-specific 2D2 transgenic T cells and syngeneic APC fluorescent reporter mice for dendritic cells, microglia and macrophages to dissect the temporal role of each cell. The use of MOG 35-55 to induce EAE results in a chronic-progressive form of disease\textsuperscript{49}. More akin to human disease, proteolipid protein used in the induction of SJL/J mice results in a relapsing-remitting form of disease\textsuperscript{50} but there are no T cell transgenic mice nor fluorescent reporter mice due to the genetic strain of the SJL/J mice. However, the leakiness of the blood vessel and phagocytic uptake of cells can still be studied. In fact, repeating the blood vessel integrity studies in this mouse model would provide superior insight into whether the vessel leak is initiating inflammation because the relapsing portion of the disease does not require further induction or manipulation, and has fairly consistent timing of recurrence.
5.3. Imaging of CX3CR1 Knock-out Mice

Chapter 2 focused on the role and timing of CD11c-GFP+ cells infiltrating the brain parenchyma and eluded to the role of microglia in the first 4 days of induction using phagocytosis as a marker of resident cells. Chapter 3 showed data from the CX3CR1\textsuperscript{GFP/+} mouse model where microglia and its interaction with the blood vessel could be directly visualize during EAE induction. In future studies, it would be advantageous to use a report mouse where CX3CR1\textsuperscript{GFP/+} was crossed with a CD11c-mCherry mouse into which 2D2-CFP cells were transferred resulting in the ability to visualize GFP microglia, mCherry red CD11c and CFP MOG T cells simultaneously during induction. This would illuminate the important interplay between these cells within the CNS using TPM.

In addition, it has been shown that CX3CR1\textsuperscript{GFP/GFP} mice induce with EAE show increased disease severity\textsuperscript{9}. It would be helpful to understand whether CX3CR1 expression on microglia plays a role in the recruitment or retention of cells in the CNS. Thus I would create a CX3CR1\textsuperscript{GFP/GFP} CD11c-mCherry reporter mouse and transfer 2D2-CFP cells for TPM analysis.

5.4. Signaling of Vessel Projections

The novel finding of microglia projecting across the intact BBB into the blood stream invokes intriguing, lingering questions: why do we see extensions in
steady state? Can the projections during non-inflammed conditions be the gateways that allow vessel leak and/or recruit immune cells to that particular location? Is there a distribution difference of microglia projections throughout the CNS? What is on the surface of the microglia extension in the blood vessel? Could it be upregulating adhesion molecules on the endothelial cells, releasing protein fragments, micelles, cytokines into the blood stream? What is the activation state of microglia when they are projecting processes across the endothelium? Could microglia extension be plugging a vessel leak?

To be able to probe these questions about microglia projections, one would have to start with the correct mouse model. Identifying the cell that is projecting across into the blood vessel expressing the peptide of interest would be important in assessing function. This functional reporter would be advantageous to quickly identify cells, and to identify similarly behaving cells so that molecular analysis is not diluted by a cohort. In the liMOG transgenic mouse\textsuperscript{322}, MOG35-55 peptide is expressed as a CLIP replacement peptide upon tamoxifen injection. Similar to published experiments\textsuperscript{323}, one could cross the liMOG mouse to a tamoxifen inducible CX3CR1-CreERT mouse to have microglia that express MOG in the context of MHCII. The efficiency of the liMOG when crossed to a CD11c-CreERT resulted in only 5% of the total CD11c population expressing the correct peptide\textsuperscript{323}. We also know that CX3CR1 is expressed on both microglia and macrophages and this distinction is particularly important in the EAE model. So we would develop a P2ry12-CreERT mouse because it has been determined that
P2ry12 is expressed on microglia but not macrophages. In addition, we want a way to identify cells only when the MOG35-55 peptide is presented on the MHCII on the microglia. To do this, we would need to create a liMOG-iRES-mCherry-lox inversion mouse, thus when this mouse is crossed with the P2ry12-CreERT mouse, the mCherry fluorescent reporter protein would only get translated when tamoxifen is administered. Then when a microglia cell is projecting into the blood vessel and expressing mCherry the researcher would be reassured that microglia is presenting MOG35-55 and thus interactions with fluorescently labelled 2D2 T cells would be an antigen specific interaction. It may be important to still cross this mouse to the CX3CR1GFP/+ mouse because it would also be interesting if none of the projections into the blood vessel ever expressed the peptide.

Once the P2ry12-CreERT liMOG-mCherry-Lox CX3CR1GFP/+ mouse has been created, create a cranial window observation to see if microglia extensions can be detected in the blood stream, with and without PTx administration, with naïve or activated fluorescently labelled 2D2 T cells. It would be important to know the expression level of MHCII in the blood vessel projection and this could be answered via IHC. If these preliminary experiments prove to be positive, one would perform a full induction by creating a cranial window observation, introducing fluorescently labelled 2D2 T cells and inducing EAE with an emulsion of peptide and PTx. To definitively determine if the interaction with the projection is productive, we could retrovirally transduce the 2D2 T cells with TN-XXLCDΔneoR, a real-time calcium flux indicator. Thus if a T cell contacts a
microglia expressing MOG peptide and if the T cell produces a calcium flux, this is a functional read-out of a productive interaction.

The most comprehensive assay after developing this mouse would be to use laser microdissection to isolate the specific cell that projecting into the vessel, presenting antigen, followed by RNA chip sequencing and possibly microRNA analysis. To adequately determine the activation state of microglia with active vessel projections, we would also isolate microglia that are not near the vessel, in both non-inflamed and inflamed models. It would be useful in this system to capture the astrocytes adjacent to the projection site, oligodendrocytes, and any infiltrating immune cells. Often research is focused on the status of the immune cells infiltrating or microglia without determining the status of other accessory cells and neurons adjacent to the cells of interest, but by collecting this information concurrently we can better determine the environmental status around this phenomenon.

The questions of what is being expressed on the surface can be pursued via IHC of most likely upregulated candidates from the RNA analysis. Upregulation of adhesion molecules on endothelial cells around projections can also be addressed by IHC.
The recruitment of immune cells into the brain parenchyma could only be effectively proven by dynamic intravital microscopy, using methods previously described in this manuscript. Detecting the origin of proteins and cytokines in the blood stream would be impossible to determine from the rare events of microglia projecting into the blood stream. It would be possible to determine blood cytokine levels by ELISA. Even a reporter mouse that expressed fluorescent protein driven by the promoter of a particular cytokine would only report that the cell produces that cytokine, not that the cytokine is released from the projection into the blood.

Activated macrophages release microvesicles containing polarized M1 or M2 mRNAs\textsuperscript{324}. It has been recently published that a common antigen from many mouse tumor types can be detected with high sensitivity from blood samples by using a nested RT-PCR method\textsuperscript{325}. This could be a method by which we could detect possible protein fragments and micelles released from microglia extensions into the blood stream.

5.5. Determining the Role of Microglia in the Balance of T cell Regulation versus Autoimmune Destruction of the CNS.

Our work elucidated a set of cellular events that showed that microglia are activated in the first 3 days and that this activation state causes recruitment of
peripheral APCs. With the treatment of HXYZ, the activation of microglia is decreased which in turn reduces the recruitment of peripheral APCs. We had postulated that microglia become overwhelmed during the initial stages PTx administration, with activation and phagocytosis functions thus recruiting peripheral cells to help resolve the inflammation, and it was published that in fact, TREM2 signaling in microglia is initiated by apoptotic neurons to help with phagocytosis and overexpression of TREM2 on myeloid precursor cells can help clear debris that microglia were unable to eliminate and resulted in recovery in EAE. In addition, it has been shown that CX3CR1 mice develop more severe disease. We also know that CX3CL1 is expressed on neurons to keep microglia in a less inflammatory state. When this regulatory signaling is removed in the CX3CR1 mice, microglia become hyperactivated and I propose it is this activation state of microglia that detrimental to tissue integrity.

Histamine vesicle degranulation is used all over the body to cause the blood vessel to dilate and allow cells to get into the target tissue during and inflammatory event. Efforts to alleviate vessel leaks in our model via this histamine mechanism did not work at doses indicated in literature, and warrants a dose curve study. An intriguing finding not reported in literature before was the non-specific infiltration of T cells on day 3 of EAE induction as determined by FACs which we presume is due to the action of PTx. The administration of HXYZ did have an effect on the non-specific infiltration of T cells on day 3 and phagocytic capacity of microglia on day 6, thus further investigation on the
activation state of these cells in response to HXYZ would be informative. Lastly,
EAE induced mast cell-deficient mice exhibited a lower frequency of IFN-γ positive cells in the draining lymph node compared to induced WT mice which is consistent with our findings of cells isolated from brains on day 9\(^{328, 329}\).

Regulatory T cells (Treg) are CD4\(^+\)CD25\(^+\)FoxP3\(^+\) cells that have immune suppressive effects and an association of T cell plasticity in MS and EAE has been made due to the commonality of inducing cytokines\(^{330}\). It has been documented that Treg cells can provide some protection during induction of relapsing-remitting disease and that Treg cells administered after onset of disease could reduce disease burden\(^{331, 332}\). It would be interesting if the timed use of anti-histamine to reduce the number of IFN-γ positive cells followed by transfer of myelin-specific Treg cells could further reduce CNS inflammation and provide a better therapeutic outcome after the onset of clinical disease.
APPENDIX I Material and Methods

Mice

Six- to 12-week old syngeneic female C57BL/6, B6.129P $\text{Cx3cr1}^{\text{GFP/GFP}}$ (stock #005582) and B6.Cg-Tg Thy-1-YFP-H (stock #003782) mice (H-2$^b$), B6.FVB-Tg CD11c-DTR/GFP mice (stock #004509), C57BL/6-Tg 2D2 mice (stock #006912), B6.129 (ICR)-Tg actin beta CFP mice (stock #004218), and C57BL/6-Tg ubiquitin GFP mice (#004353) were obtained from the Jackson Laboratory (Bar Harbor, ME). B6.129S6 OTII mice (stock #1896) were obtained from Taconic. 2D2 mice expressing the TCR transgene specific for MOG35-55 peptide / I-Ab were crossed with actin beta CFP mice to derive mice expressing 2D2-CFP cells for in vivo tracking by TPM. Similarly, OTII mice expressing the TCR transgene specific for OVA323–339 peptide / I-Ab were crossed with ubiquitin GFP mice to derive OTII-GFP cells for in vivo TPM tracking. $\text{Cx3cr1}^{\text{+/GFP}}$ reporter mice have one Cx3cr1 allele replaced with the gene encoding GFP, and are derived by crossing Cx3cr1$^{\text{GFP/GFP}}$ with C57BL/6$^{72}$. CX3CR1 (fractalkine receptor) is almost exclusively expressed in the microglia population in the CNS of these mice, while NK cells, activated CD8$^+$ T cells, dendritic cells and a subset of monocytes also express the GFP marker in the peripheral tissues$^{72}$. Thy-1-YFP-H mice harbor yellow fluorescent protein (YFP) expression in a subset of neurons in the dorsal root ganglion and cortical layers$^{333}$. Thy-1-YFP-H mice were crossed with Cx3cr1$^{\text{GFP/GFP}}$ mice to obtain double transgenic mice, Thy-1-YFP-H x Cx3cr1$^{\text{+/GFP}}$. 
Animals were housed, bred and handled in the Animal Resource Center facilities at Case Western Reserve University according to approved protocols. Similarly, all animal experiments were executed with strict adherence to active experimental animal protocols approved by Case Western Reserve University Institutional Animal Care and Use Committee.

**EAE Induction**

Mice were induced to develop EAE using established protocols\(^{191, 192}\). Briefly, 3\( \times 10^6 \) naïve splenic 2D2 CFP CD4 T cells, isolated by negative depletion using Dynal beads (Life Technologies, Grand Island, NY, USA), were injected into recipient mice. 24 hours later, an emulsion with 200 µg myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (MOG35–55; Anaspec, San Jose, CA, USA) with 8 mg/ml H37RA and incomplete Freuds adjuvant was injected subcutaneously (s.c.) bilaterally on the lower back of the recipient mouse (Hooke Laboratories, Lawrence, MA, USA). Control mice were injected s.c. bilaterally with an emulsion of PBS, 8 mg/ml H37RA and incomplete Freund’s adjuvant. 100 ng Pertussis toxin was administered intraperitoneal (i.p.) on Days 0, 1 and 2. Clinical scores were assigned as follows\(^{334, 335}\): 0, no motor deficits; 1, tail weakness; 2, hind limb weakness; 3, hind limb hemiplegia; 4, total hind limb paralysis; 5, moribund. This induction protocol results in limp tail, hind leg paralysis and progressive neurological dysfunction in recipient mice beginning around days 12-14 post induction\(^{191}\).
Primbing of Antigen-specific T cells
To determine whether myelin specific T cells require antigen-specific APCs for recruitment to the CNS, EAE was actively induced using a combination of two antigenic peptides in 8-12 week old female recipient mice. $3 \times 10^6$ naïve 2D2-CFP and $3 \times 10^6$ naïve OTII-GFP CD4$^+$ T cells were isolated separately by negative depletion using Dynal beads and co-injected into naïve recipient mice. 24 hours later, an emulsion of 200 µg MOG35-55 only, 10 µg OVA323-339 only, or 200 µg MOG35-55 and 10 µg OVA323-339 combined (Anaspec, San Jose, CA, USA) with 8 mg/mL H37RA (Difco Laboratories, Detroit, MI, USA) and incomplete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA) was injected s.c. bilaterally on the lower back of the recipient mice. Control mice were injected s.c. bilaterally with an emulsion of PBS, 8 mg/mL H37RA and incomplete Freund’s adjuvant. 100 ng Pertussis toxin (List Biological Laboratories, Inc, Campbell, CA, USA) was administered i.p. on days 0, 1 and 2 to induce EAE.

Spinal Trauma Model
We modified published protocols to create a dorsal column crush injury$^{281}$. Briefly, mice were anesthetized with inhaled 1-2% isoflurane and a laminectomy was performed aseptically to expose a spinal cord segment at the T10 level, allowing for microscopic examination in both EAE and spinal cord trauma
models. To create the spinal trauma model, small dural openings were made at 0.5 mm lateral to the midline with a 30 gauge needle, and a dorsal column crush lesion was made by inserting and squeezing a pair of Dumont # 4 forceps 1 mm into the dorsal spinal cord and holding pressure for 10 seconds three times. The laminectomy site was covered with saline-soaked Gelfoam (Pfizer), and the para-spinal muscles and skin were closed with sutures. For post-operative pain control, mice received a single dose of Marcaine (1.0 mg/kg) s.c. at the incision site and buprenorphine (0.1 mg/kg) intramuscularly (i.m.) daily for 3 days.

**Tumor Cell Preparation and Injection**
Mouse medulloblastoma (MB) cell line, MM1, was derived from Patch+/p53−/− mice (H-2b) as a spontaneous tumor, and was a generous gift from Dr. Gregory Plautz at the Cleveland Clinic Foundation. MM1 was transfected with a plasmid carrying the red fluorescent reporter, DsRed2 (pDsRed2-N1), with a neomycin selection marker using JetPEI (Polyplus Transfection). Transfected cells (MM1-DsRed2) were selected with G418 (300 μg/ml) and enriched for red fluorescence expression by fluorescence-activated cell sorting (FACS). Female Cx3cr1+/GFP mice were anesthetized with inhaled 1-2% isoflurane (Aerrane; Baxter) and placed in a stereotactictic holder. After removing a circular scalp flap, 3x10⁴ MM1-DsRed2 cells were injected intracranially (i.c.) at a depth of 1.5 mm into the center of the left parietal cortex with a Hamilton syringe. The left parietal region of the skull was then removed and a cranial observation window was installed as previously
described\textsuperscript{187}. Intracranial MM1-DsRed2 tumor development was then imaged serially with 2P-LSM on days 7, 10, and 14 after tumor inoculation.

**Mouse Surgery and Preparation for Intravital Imaging**

In all intravital experiments involving imaging the mouse brain, mice were implanted with cranial windows according to published protocol\textsuperscript{187} and imaging sessions were carried out immediately for acute analysis or at least 4 days following the implantation procedure. Briefly, 5 mg/kg carprofen (Butler-Schein, Dublin, OH, USA) was used given s.c. as an analgesic, and 0.2 mg/kg dexamethasone (Butler-Schein, Dublin, OH, USA) was used given s.c. as a general anti-inflammatory. The scalp was removed to expose the skull. A craniotomy was performed using a dental drill and the bone was replaced with a sterile 5 mm #1 circular glass coverslip. The glass coverslip was glued in place with Vetbond, and dental acrylic (Butler-Schein, Dublin, OH, USA) was used to create a permanent well for imaging. Alternatively, thinned-skull preparations were utilized according to published protocol\textsuperscript{188, 189}, leaving an intact skull of 25-50 um thickness (Fig. 12). Mice were anesthetized with nebulized isoflurane (2\% induction, 1.5\% maintenance) in 30\% O2 / 70\% air, with body temperatures maintained at 37°C via a temperature-controlled environmental chamber and heating pads throughout the entire mouse preparation and imaging session. Breath rate and animal responsiveness were used to monitor adequate levels of anesthesia, with breath rate maintained at ~60-100 breaths per minute and animal responsiveness assessed by foot and tail pinch. Mice were placed onto a
stereotactic holder, and the entire assembly was placed in a temperature-controlled environmental chamber. The body temperature was monitored and maintained between 36.5 to 38°C using a combination of an environmental temperature probe and a rectal probe. Ten to 30 minutes prior to imaging, fluorescent dye markers were injected intravenously (i.v.) to allow blood vessel visualization. The vessel dyes used in the experiments included 700 μg of TRITC-Dextran (150KD; Sigma, Inc.) or 100 ul of 0.1 μM non-targeted QTracker-655 (Invitrogen, Inc.). Tomato-lectin (Vector Laboratories; 16 ug/mouse) was also used to visualize the endothelial cells. Large molecular weight dextran markers are selected to study BBB integrity as previously described.

**Spinal Imaging**

For sequential spinal cord imaging of traumatic injury, skin and muscle flaps covering the previous T10 laminectomy site were re-opened aseptically with the animal under anesthesia. The site of injury was exposed by carefully dissecting the muscles and previously implanted Gelfoam (Pfizer) with the aid of a dissection scope. In preparation for spinal imaging in EAE mice, a laminectomy was performed at the S1 level. In both cases, para-spinal muscle was removed from the lateral part of the vertebrae one level above and one level below the site of laminectomy to allow for attachment of Narshinge STS-A spinal clamps mounted on a custom aluminum base. The clamps were covered with Parafilm and a well for immersion fluid (Dulbecco’s aCSF) was created around the spinal clamps with OrthoJet dental acrylic (Lang Dental).
Two-Photon Imaging Equipment, Data Acquisition
Upon completion of tissue preparation for intravital imaging, the entire mouse imaging assembly, including the stereotactic holder, was placed on the microscope stage enclosed within a custom-made temperature-controlled environmental chamber. The tissues were imaged using a Leica SP5 fitted with a DM6000 stage, a 20X water immersion lens (N.A. 1.0; Leica HCX-APO-L), and a 16W Ti/Sapphire IR laser (Chameleon, Coherent) tuned to excitation wavelengths between 800nm and 880nm. Imaging planes (760 x 760 μm) collected at 1-5 μm z-intervals were repeated at 20-60 second intervals for up to 6 hours to yield xyzt data sets collected through a four channel non-descanned external detector using a filter set separating 400-455, 467-499, 500-550, 565-605 for brain and spine imaging, and, a filter set separating 467-499, 500-550, 565-605, 625-675 for tumor imaging. This raw data set was then used for processing and analysis. The imaging platform also included a motorized stage with Tile Scan capabilities to allow for broad-field survey and high-resolution voxel (0.75 x 0.75 x 1 μm) image collection of the tissues (see below).

Image Analysis
High-resolution fluorescent 4D imaging data sets collected from intravital 2P-LSM experiments were analyzed using Imaris (BitPlane, Inc). Mosaic broad-field survey images were compiled using Xuv Stitch software (Xuv Tools). Volumes of 1550x1550x150 um^3 up to 1550x2025x150 um^3 were analyzed for EAE imaging in
the brain. For imaging of the spine during EAE, spinal cord crush injury and CNS tumor models, a volume of $775 \times 775 \times 150 - 300 \, \mu m^3$ was analyzed. For spinal crush models, only areas within the lesion site containing damaged axons were analyzed. Projection numbers were normalized to surface area of vessels found within the lesion. Image processing included Gaussian smoothing and creating a surface rendering of vessel walls as defined by the extent of the intravenous dyes. GFP signals within the vessel lumen were then segmented from the total images, and surface rendering of GFP$^+$ projections were created. Only cells residing in the parenchyma were chosen for projection frequency analysis, excluding those in the meninges (Fig. 12). When choosing relevant extravascular GFP$^+$ APC populations to analyze for intravascular dendrites, and to avoid including GFP$^+$ cells that may be perivascular or circulating cells in the process of extravasation from vessels, we applied the following three criteria to exclude GFP$^+$ cells from analyses: (1) Cells with migration speed $>3 \, \mu m/min$ or were visualized to be in an active process of transmigration across the vessel wall from vascular lumen in the 4D dynamic imaging data sets; (2) Surfaces with a sphericity of 0.9-1 as possible rolling or circulating cells within the vessel; (3) Surfaces that only extended within the perivascular space and did not cross the vessel wall surface; and (4) Surfaces that had more than 30% of the cell volume within the vessel as possible perivascular cells extending processes into the parenchyma. The total number of dendritic projections that met the above criteria was then normalized to the calculated surface area of the vessel wall in order to derive a frequency of dendritic protrusions per unit vessel surface area.
Based on the dynamic 4D data in which the direction of vascular flow can be determined, the projections were from CX3CR1+ cells outside of post-capillary venules and larger size veins.

For Chapter 3 and cranial window imaging, second harmonic generation (SHG) signal produced by high collagen content structures was used to identify the pial layer and to discriminate parenchyma from meninges. Relative fluorescent intensity region of interest (ROI) measurements of dextran leaking into the parenchyma were made using the LAS-AF software (Leica Microsystems Inc, Buffalo Grove, IL, USA). Timing and duration of vessel leaks were manually processed. Phagocytic cells and CD11c-GFP were identified by fluorescent intensity. Co-localization of TRITC and GFP signals and volume data were calculated using the Imaris software (BitPlane Inc, Zurich, Switzerland). T cell motility data was also analyzed using the Imaris software, which allows cell identification, as well as 3D tracking over time by producing information on individual positions, time and track length. T cell interactions with APCs were grouped into short contacts (< 2 min) and prolonged interactions (≥ 2 min). Individual T cell migration tracking was further analyzed for track length, velocity, mean fluorescent intensity and cell flux using MATLAB software (Mathworks Inc, Natick, MA, USA). Cell flux was calculated by determining the vector between the beginning of the T cell track and the point at which the T cell came within a 50 μm radius from the center of an APC cluster. Contact time between APC and T cells, and T cell movement to and from the blood vessel were analyzed manually.
by visual inspection of individual image slices over time using the Imaris software.

**Electron Microscopy**

Normal, non-manipulated CX3CR1 GFP/+ mice were fixed by transcardial perfusion with 0.1% glutaraldehyde and 4% PFA and postfixed overnight. Samples were stained en bloc with anti-GFP primary antibody (Life Technologies) and goat anti-rabbit 2nm gold conjugated secondary (Ted Pella, Inc., Redding, CA, USA). Gold particles were then enhanced using the silver enhancer kit (Sigma) until darkening of the sample was seen. Osmication with 1% osmium (EM Sciences), dehydration in ethanol (Sigma) and then propylene oxide (EM Sciences) and eponate-12 resin (Ted Pella) infiltration were performed using the corresponding Pelco BioWave Pro microwave protocols. Samples were embedded in resin and polymerized at 60 for 48 hours in BEEM capsules in a traditional polymerization oven. Blocks were trimmed and 85nm ultrathin sections cut on an ultramicrotome and placed on copper formvar support grids (EM Sciences). Tissue was negative stained with lead acetate (EM Sciences) and uranyl acetate (EM Sciences). Grids were imaged using the STEM mode on a FEI Helios Nanolab 650 for rapid scanning of large areas (Swagelock Center, Case Western Reserve University). EM micrographs were captured at 10,000X.

**Blood brain barrier leakiness analysis**
To compare the pattern of vessel leakiness induced by different inflammatory stimulants, 100 ng LPS (Sigma-Aldrich, St. Louis, MO, USA), 100 ng Pertussis toxin or PBS were injected i.p. into C57BL/6 mice on days 0, 1 and 2. 700 ng of 150 kDa TRITC dextran (Sigma-Aldrich, St. Louis, MO, USA) vessel dye was injected i.v. on day 1 and day 2. On day 3, QTracker-655 (Life Technologies, Grand Island, NY, USA) were injected to highlight the vessels. The mice were sacrificed and whole brain tissues were imaged immediately by TPM.

**Immunofluorescence Histology**

Naive or inflamed Cx3cr1^+/GFP mice were sacrificed and subjected to transcardial perfusion with 4% PFA. The brain and spinal tissues were harvested, and 12um cryosections were stained with DyLight 594 tomato-lectin (Vector Laboratories) or antibodies against CD31 (MEC13.3; BD Biosciences), laminin (Biotrend, Germany), Iba-1 (Wako Chemicals, USA), or GFAP (Z0334; Dako, Glostrup, Denmark) followed by appropriate secondary antibodies conjugated to Alexa fluor (Life Technologies). The samples were then subjected to confocal microscopy to obtain fluorescent micrographs.

**Flow cytometry analysis**

For single-cell APC and T cell characterization, brain tissues were harvested on days 0 through 12 of EAE induction. Tissues were dissociated by collagenase D (1 mg/mL) and DNAse (250 units/mL) (Sigma-Aldrich, St. Louis, MO, USA) to
form a single cell suspension after gentle dissociation with a dounce homogenizer and subjected to a 70%/37%/30% Percoll gradient\textsuperscript{339}. Isolated cells were stained for CD45, CD11c, CD11b, CD4, CD8, IL-17, IFN-γ and FoxP3. Absolute numbers of cells per brain were determined by multiplying the number of events on FACS per collection volume by the total volume of cell samples. Spleen and LN were isolated from mice at the end of imaging on Day 12. Organs were crushed with the ends of 1 ml syringes through a 40 µm cell strainer into single cell suspensions and were stained for Vα2 (OTII TCRα chain), Vβ11 (2D2 TCRβ chain), CD4, CD25, CD44, and CD69. Samples were collected and analyzed using the Accuri flow cytometer (BD Biosciences Co, San Jose, CA, USA).

**Statistical consideration**

In Chapter 2, the number of mice and projections quantified in the imaging experiments are as follows: Fig. 15F – 3-4 mice per group, 7092 total projection events; Fig. 17F - 3 mice per group, 503 total projection events; Fig. 18E - 3 mice per group, 216 total projection events; Fig. 18I - 3 mice total, 196 total projection events; Fig. 16F - 4-6 mice per group, 2213 total projection events.

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc, San Diego, CA). To test for statistical significance the Student's T-test and two-way analysis of variance was used to compare between treatment groups. For all analyses P < 0.05 was considered significant.
Results are collective data from 2 to 6 repeat experiments with minimum of 3 mice per experiment.
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