Experimental Neurology 322 (2019) 113046

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Review Article

Imaging the dynamic interactions between immune cells and the neurovascular interface in the spinal cord

Nozha Borjini[a], Evi Paouri[a], Reshmi Tognatta[b], Katerina Akassoglou[b,c], Dimitrios Davalos[a,d,*]

[a] Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA
[b] Gladstone Institutes, San Francisco, CA, USA
[c] Department of Neurology, University of California, San Francisco, San Francisco, CA, USA
[d] Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, Case Western Reserve University, Cleveland, OH, USA

A B S T R A C T

Imaging the dynamic interactions between immune cells, glia, neurons and the vasculature in living rodents has revolutionized our understanding of physiological and pathological mechanisms of the CNS. Emerging microscopy and imaging technologies have enabled longitudinal tracking of structural and functional changes in a plethora of different cell types in the brain. The development of novel methods also allowed stable and longitudinal optical access to the spinal cord with minimum tissue perturbation. These important advances facilitated the application of *in vivo* imaging using two-photon microscopy for studies of the healthy, diseased, or injured spinal cord. Indeed, decoding the interactions between peripheral and resident cells with the spinal cord vasculature has shed new light on neuroimmune and vascular mechanisms regulating the onset and progression of neurological diseases. This review focuses on imaging studies of the interactions between the vasculature and peripheral immune cells or microglia, with emphasis on their contribution to neuroinflammation. We also discuss *in vivo* imaging studies highlighting the importance of neurovascular changes following spinal cord injury. Real-time imaging of blood-brain barrier (BBB) permeability and other vascular changes, perivascular glial responses, and immune cell entry has revealed unanticipated cellular mechanisms and novel molecular pathways that can be targeted to protect the injured or diseased CNS. Imaging the cell-cell interactions between the vasculature, immune cells, and neurons as they occur in real time, is a powerful tool both for testing the efficacy of existing therapeutic approaches, and for identifying new targets for limiting damage or enhancing the potential for repair of the affected spinal cord tissue.

1. Introduction

The central nervous system (CNS) is considered an immunologically privileged site in the mammalian anatomy, with structurally unique compartments regulating both its physical separation from the rest of the body and its immune surveillance and communication with the peripheral immune system. In contrast to the rest of the body, where access of immune cells to tissues through the vascular endothelium and drainage of tissue antigens to proximal lymph nodes are unrestricted, the intact CNS tightly regulates these processes. Functional lymphatic vessels exist in the meninges that surround the brain's outer surfaces, in the cribriform plate, and along cranial and spinal nerve roots (Aspelund et al., 2015; Engelhardt et al., 2016; Louveau et al., 2015). Since they do not penetrate the brain parenchyma, these lymphatic vessels offer limited yet canonical drainage of brain antigens to deep cervical lymph nodes. In contrast, an extensive network of blood vessels do penetrate and span the entire CNS parenchyma, thereby comprising the main interface between periphery and CNS. However, due to the elaborate cellular architecture of all CNS blood vessels, a physical and functional barrier –the blood-brain barrier (BBB)– tightly controls the molecular exchange and cell trafficking between blood circulation and CNS. During neuroinflammatory conditions changes at the BBB allow the entry of both blood components and peripheral immune cells into the CNS. Depending on the initial challenge, these cells participate in the resolution of insults or in the development of autoimmune responses. Irrespective of the type of challenge though, changes in BBB integrity, chemo-attraction, and eventually infiltration of peripheral immune cells into the CNS occur in a highly regulated multi-step process.

On the parenchymal side of the BBB, microglia –the CNS-resident immune cells, constantly survey the CNS by process extension and retraction, and constitute the first-line of defense against injury or disease in brain or spinal cord (Davalos et al., 2005; Davalos et al., 2008; Nimmerjahn et al., 2005). Microglial activation and BBB disruption occur early in neuroinflammatory disease, before peripheral immune cells start migrating into the CNS parenchyma (Davalos et al., 2012). Disruption of the BBB and infiltration of peripheral immune cells trigger detrimental changes within the CNS. In the case of autoimmune disease these involve activation of microglia and astrocytes, and eventually damage to myelin and neurons, which are the main events responsible for observed neurological deficits. Unraveling the cellular and

* Corresponding author at: Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA.
E-mail address: davalod@ccf.org (D. Davalos).

https://doi.org/10.1016/j.expneurol.2019.113046
Received 9 July 2019; Received in revised form 16 August 2019; Accepted 27 August 2019
Available online 28 August 2019
0014-4886/

molecular mechanisms that orchestrate the onset and progression of BBB disruption and peripheral immune cell infiltration of the CNS is fundamental for our understanding of CNS pathophysiology and the development of effective therapeutic strategies against CNS disease and injury.

The spinal cord is an integral part of the CNS with major functional significance. It comprises the main conduit through which most of the neuronal tracts connecting the brain and peripheral organs travel. These neuronal pathways facilitate posture, dexterity, and movement, and allow brain access to most of the sensory input from the entire body. Its anatomical proximity to vital organs such as the heart and lungs also allow spinal cord neurons to facilitate most of the autonomic functions in the body. At the same time, this proximity to continuously moving organs make the spinal cord a far more challenging CNS compartment to steadily image than the brain, which is held mostly steady inside the skull. Imaging the living brain in rodents revealed a wealth of information about the abilities, functional properties, and potential responses of cells that reside within or interact with parts of the brain. However, a number of physiological properties and pathological mechanisms are unique for the spinal cord, which is also subject to neurological injuries and diseases that have limited or no presentation in the brain. For these reasons, conquering the spinal cord as a site for *in vivo* imaging with its own unique challenges and opportunities was unavoidable.

In 2008, Davalos et al., developed the first comprehensive method for imaging the spinal cord in living mice using two-photon microscopy (2 PM). The strong movement artifacts generated by the breathing and heartbeat of anesthetized animals were overcome by devising a novel spinal stabilization approach (Fig. 1) (Davalos and Akassoglou, 2012; Davalos et al., 2008). This method allowed for stable and repetitive imaging of axons, microglia and blood vessels in the spinal cord of transgenic mice, and quickly became the reference point for numerous studies that benefited from the advantages of 2 PM to image the living spinal cord. Since then, several groups have published modifications and improvements to this method to address specific experimental needs. For example, there are different ways to expose the spinal cord for imaging either through the intervertebral space (Kim et al., 2010), or through one or serial laminectomies, and with or without implanting permanent spinal cord windows (Farrar et al., 2012; Fenrich et al., 2013; Haghayegh Jahromi et al., 2017; Sekiguchi et al., 2016). This special issue contains articles that describe in more detail all currently available spinal cord imaging protocols and mouse lines that have made longitudinal imaging of spinal cord biology possible (Cheng et al., 2019; Evans et al., 2019). In addition, others in this issue discuss different aspects of spinal cord injury (Denecke et al., 2019; Schaffran et al., 2019; Zheng et al., 2019) and findings regarding the execution phase of neuroinflammatory disease (Schumacher et al., 2019), as they were derived from spinal cord imaging studies in living animals. In this article, we will discuss spinal cord imaging studies that have revealed novel interactions among peripheral or CNS-resident immune cells and the spinal cord vasculature. In doing so, we will specifically highlight how these studies at the BBB –the main interface between periphery and CNS– have expanded the scientific understanding of the cellular and molecular mechanisms underlying neuro-inflammatory disease and spinal cord injury. Although many studies differentiate between the blood-brain and blood-spinal cord barrier, since they share structural similarities we will refer to them both as the BBB in this article.

2. *In vivo* imaging studies of the interactions between CNS-resident immune cells and the spinal cord vasculature in neuroinflammatory disease

In vivo imaging using 2 PM is particularly informative for studying dynamic cellular behaviors and cell-cell interactions. In addition, when performed longitudinally, it can decipher the sequence of events taking place during evolving biological processes such as the development of pathologies. This kind of real time information offers unique insight in underlying biological mechanisms than can often be overlooked with time course analyses using traditional histology. Davalos et al., took advantage of this technology to characterize the sequence of events leading to neuroinflammation and neuronal dysfunction in experimental autoimmune encephalomyelitis (EAE), an established animal model for multiple sclerosis (MS) (Davalos et al., 2012). In EAE, CNS inflammation is more prominent in the spinal cord, and it involves both CNS-resident immune cells (microglia and meningeal and perivascular macrophages) and peripheral immune cells infiltrating the vascular walls in a step-wise process that involves disruption of the BBB. Using mice expressing EGFP under the *Cx3cr1* promoter (Jung et al., 2000) the dynamic interactions between the vasculature and EGFP-labeled microglia and pial macrophages were recorded for the first time in real-time in the spinal cord of mice as they began developing EAE. Interestingly, CNS-resident microglia and pial macrophages began clustering on blood vessels as early as 7 days post-immunization, before the onset of clinical manifestations or other tissue pathology or peripheral immune cell infiltration (Fig. 2A and (Davalos et al., 2012)). At this time point most EGFP-positive cells responding to the vasculature were indeed ramified microglia or CNS resident perivascular macrophages, consistent with studies that identified microglia as the first cells to get activated in EAE prior to peripheral macrophage infiltration (Ajami et al., 2011; Saederup et al., 2010). Interestingly, real-time imaging of clusters beginning to form showed that microglia or perivascular macrophages that were located in close proximity to the vasculature maintained their association with it, while cells located further away from the blood vessels extended their processes or migrated always toward the vasculature (Fig. 3A). Repetitive *in vivo* imaging at different days after immunization showed that clusters grew in size and number as disease progressed, both by way of cell migration and by local proliferation (Fig. 2A and (Davalos et al., 2012)). The massive clusters of EGFP-positive cells detected at disease peak consisted of a mixed population of CNS-resident and infiltrating monocytes, since peripheral macrophages also upregulate CX3CR1 in the CNS microenvironment as EAE pathology progresses to the paralytic phase (Saederup et al., 2010).

By using fluorescently labeled vascular tracers while imaging, microglial clusters were identified to form around vessels with compromised vascular integrity. Disruption of the BBB allowed localized leakage of the blood coagulation factor fibrinogen and the local initiation of the coagulation cascade, leading to the formation of fibrin deposits in the perivascular space around these leaky vessels (Davalos et al., 2012). Fibrinogen is a potent activator of microglia and its depletion suppresses EAE pathogenesis (Adams et al., 2007; Petersen et al., 2018). Indeed, fibrinogen in the CNS induced rapid and sustained microglial responses, and resulted in their inflammatory activation as demonstrated by *in vivo* detection of reactive oxygen species (Davalos et al., 2012). This was in accordance with results from human MS studies showing early signs of microglial activation in association with deposits of fibrinogen in pre-active lesions without demyelination or leukocyte infiltration (Marik et al., 2007; van der Valk and Amor, 2009; Vos et al., 2005). Studies in marmoset EAE using longitudinal gadolinium MRI imaging co-registered with histopathology also identified early fibrinogen deposits correlating with microglial activation even prior to gadolinium enhancement (Lee et al., 2018).

Moreover, simultaneous *in vivo* imaging of EGFP-labeled CX3CR1 + cells, CFP-labeled axons, and Alexa594-labeled fibrinogen injected intravenously, showed that these perivascular CX3CR1 + cell clusters around disrupted blood vessels become the sites of new lesions where living axons become physically damaged, and their fragments can be up-taken by CNS-resident microglia or macrophages (Davalos et al., 2012). Importantly, pharmacological treatment with anticoagulants targeting fibrin formation, or genetically-induced disruption of the binding of fibrinogen to its CD11b/CD18 microglial receptor decreased clinical signs, perivascular microglial clustering and axonal damage in EAE (Fig. 4A) (Adams et al., 2007; Davalos et al., 2012; Petersen et al.,

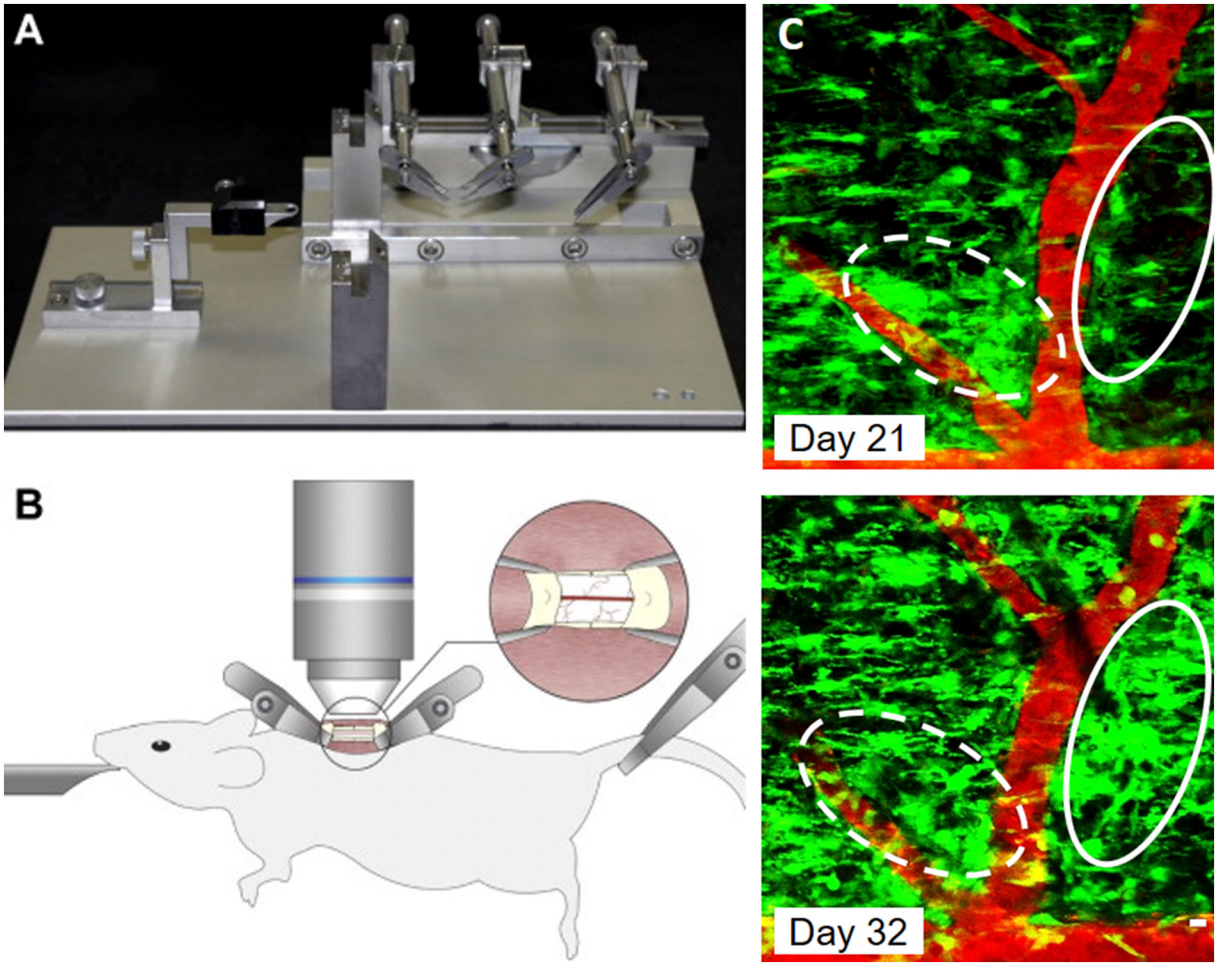


Fig. 1. Development of spinal stabilization methods allows for stable and longitudinal *in vivo* imaging of the rodent spinal cord.
A- A customized spinal column stabilization device compatible with the 2 PM was built by Davalos et al., 2008, using a steel base plate to support and align compact spinal column clamps and a head holding adaptor. B- Schematic showing how adult transgenic mice are positioned on the spinal stabilization device for *in vivo* imaging. A small retraction of the paravertebral muscles allows the insertion of the fine tips of the clamping device and a laminectomy exposes the spinal cord. The use of the device allows for adequate breathing space underneath the animal's body, thus limiting movement artifacts associated with breathing and heartbeat during *in vivo* imaging of the spinal cord and allowing for the acquisition of stable high-resolution images. (Adapted from (Davalos and Akassoglou, 2012; Davalos et al., 2008)). C– This method facilitates stable and repetitive *in vivo* imaging, by allowing to revisit the same spinal cord area of the same animal using the vasculature (red) as a map. In the depicted example, microglial cell (green) responses are observed 21 and 32 days after EAE induction. Longitudinal *in vivo* imaging allowed for the detection of a newly formed perivascular microglial cluster (solid oval) on one side of a blood vessel and a resolving cluster (dotted oval) on the opposite side of the same vessel. Scale bar: 10 μm. (Adapted from (Davalos et al., 2012).

2018). These results showed that fibrinogen is sufficient for triggering early microglial activation and is required for the development of axonal degeneration. Overall, this imaging study provided robust *in vivo* support for a biological mechanism that placed innate immune activation upstream of autoimmune responses, and linked a blood factor with the onset and progression of neuroinflammatory disease (Davalos et al., 2012; Petersen et al., 2018). Indeed, fibrinogen induces a unique transcriptional signature in CD11b + microglia cells, thereby promoting the recruitment and local CNS activation of myelin antigen-specific Th1 cells (Ryu et al., 2015). In addition to the induction of a proinflammatory pathway, fibrinogen-induced activation of microglia activates a pro-oxidant pathway and induces NADPH activation and ROS release leading to neurodegeneration and cognitive decline (Merlini et al., 2019; Ryu et al., 2018). Furthermore, specific targeting of the inflammatory activation of CD11b-expressing monocytes by fibrin-targeting immunotherapy confers robust protection from oxidative stress-driven neurotoxicity and from paralytic disease, without interfering with fibrinogen's blood clotting properties (Ryu et al., 2018). The biological mechanism linking coagulation factors and inflammatory processes was further exploited to provide proof-of-concept for a possible imaging biomarker of BBB disruption and early disease activity in the CNS. Imaging thrombin activity *in vivo* with a molecular probe showed that increased coagulation activity comprises one of the earliest events in EAE that correlated not only with fibrin deposition and microglial clustering but also with sites of demyelination and axonal damage (Davalos et al., 2014). Overall, these studies exemplify the value of developing and integrating *in vivo* imaging using 2 PM into the study of neuroinflammatory and other multifactorial and multi-step disease mechanisms. This approach provides a powerful tool to determine the cascade of pathological events in diseases with a neuroimmune or neurovascular component, thus allowing the identification of novel therapeutic targets and the discovery of imaging biomarkers for neuroinflammation (Bain et al., 2018).

3. *In vivo* imaging studies of peripheral immune cell infiltration in EAE

In addition to resident immune cells, the recruitment of peripheral immune cells into the CNS is a critical step in the pathogenesis of neuroinflammatory disease such as MS and its animal model, EAE.

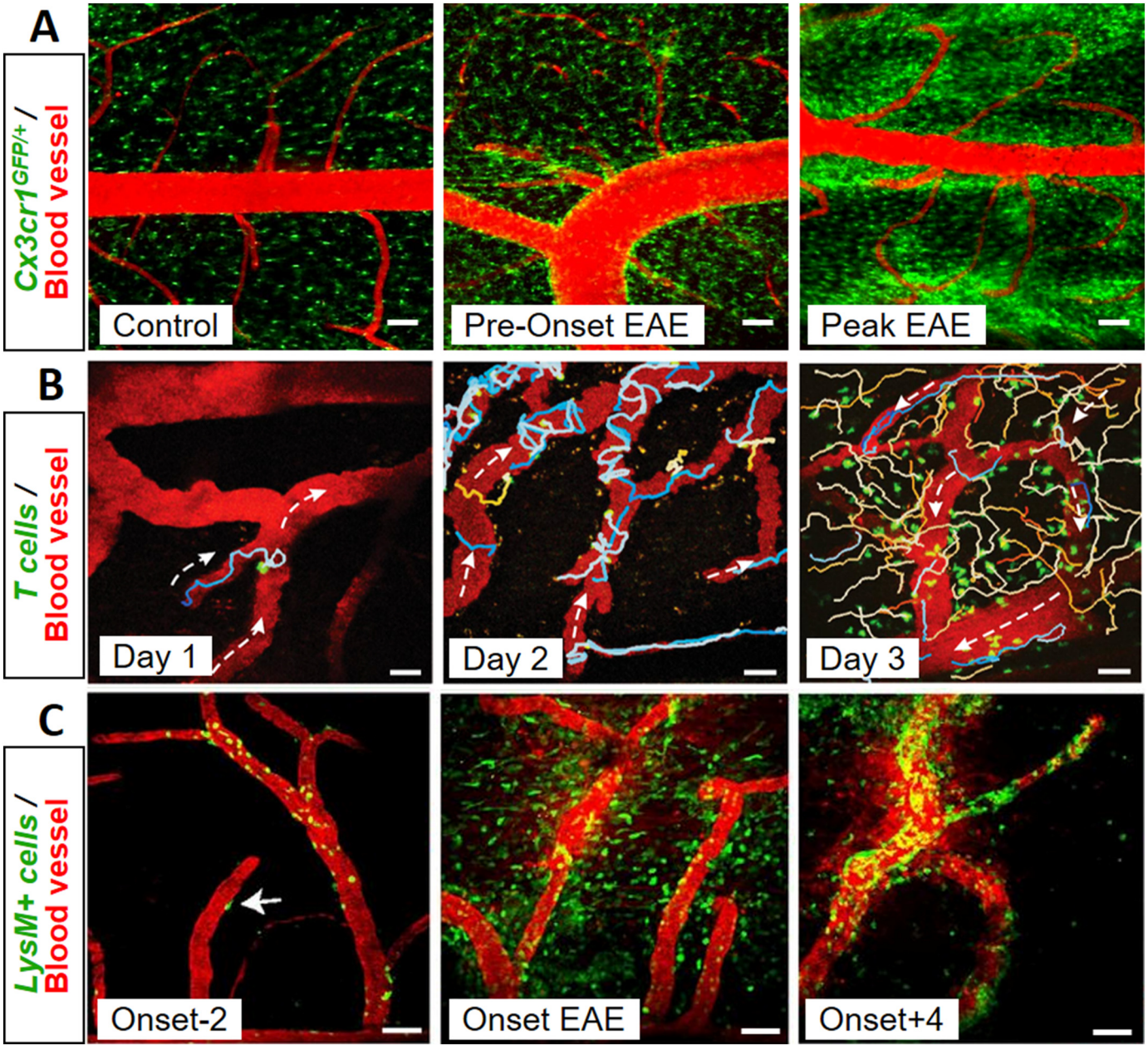


Fig. 2. *In vivo* imaging of developing immune cell interactions with the vasculature over the course of neuroinflammatory disease.
A- *In vivo* imaging of microglia and pial macrophages (green) and the vasculature (red) in healthy CX3CR1$^{GFP/+}$ mice (control), pre-symptomatic EAE (pre-onset EAE), and at the peak of clinical signs (peak EAE). In healthy conditions, microglia are evenly distributed around spinal cord blood vessels, while increased cell density and gradual cluster formation is observed in close proximity to the vasculature over the course of the disease. Scale bars: 100 µm. (Adapted from (Davalos et al., 2012)). B- *In vivo* recording of the escalating CNS invasion of MBP-specific GFP-labeled T cells in 3 consecutive days after adoptive T cell transfer in rats. Migratory paths of intraluminal (blue lines) or extravasated (yellow lines) $T_{MBP–GFP}$ cells (green) in the spinal cord are shown. Color intensities increase with time. Arrows indicate the blood flow. Blood vessels are labeled in red. Scale bars: 50 µm. (Adapted from (Bartholomäus et al., 2009)). C– *In vivo* imaging revealed that BBB disruption shortly precedes EAE onset and coincides with the infiltration of LysM+ neutrophils in mice. Permeability of the BBB to Alexa-594 fluorescent tracers (red) was followed *in vivo* in *lys*-eGFP-*ki* mice at different time points throughout disease course. BBB disruption correlates with neutrophil infiltration, starting at the EAE onset and peaking 4 days after EAE onset. Scale bars: 50 µm. (Adapted from (Aubé et al., 2014)).

Peripheral immune cells can migrate across several distinct routes in order to reach the CNS parenchyma. These include a vascular route through the BBB, the blood-cerebrospinal fluid route through an epithelial barrier present in the choroid plexus, and the meningeal lymphatic route on the surface of the brain (Daneman and Engelhardt, 2017; Louveau et al., 2017). Early in the disease process, encephalitogenic immune cells arrive at CNS blood vessels and begin interacting with CNS endothelial cells, which then become activated, and express cell adhesion and signaling molecules that are essential for leukocytes to cross the BBB in a multi-step process. This process involves the initial arresting of circulating cells on the luminal side of the vascular endothelium, then the rolling of cells, followed by firm adhesion on the endothelial cells, and after specific molecular interactions between activated endothelial cells and the arrested lymphocytes, the trans-cellular or para-cellular transmigration of these immune cells into the perivascular space (reviewed in (Erickson and Banks, 2018; Takeshita and Ransohoff, 2012)). Crossing the BBB requires penetration through two basement membranes, and reactivation by local antigen

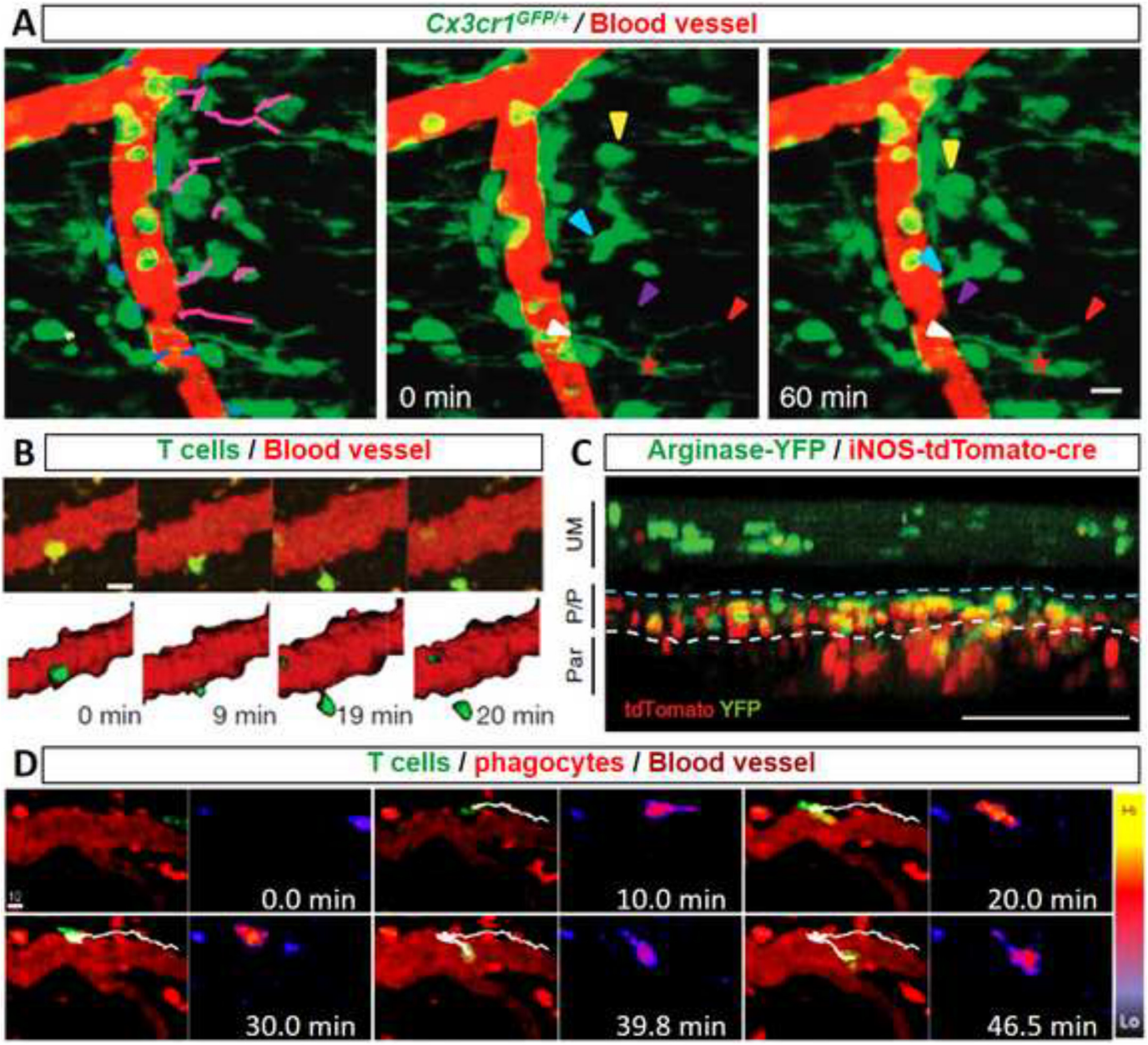
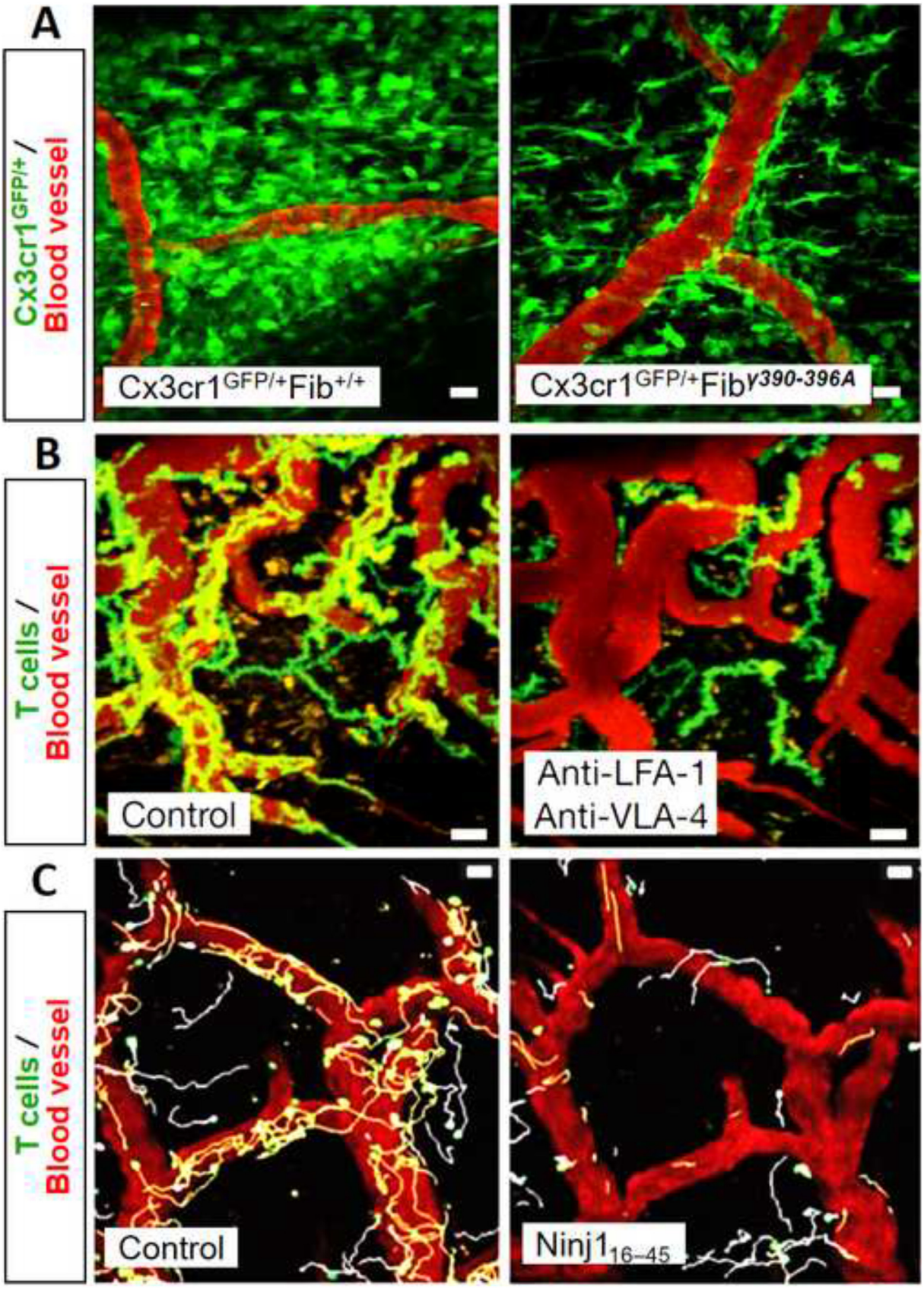


Fig. 3. Real time *in vivo* tracking of immune cell interactions with blood vessels and changes in their functional states as they migrate through separate spinal cord compartments.

A- Time-lapse *in vivo* imaging showing pial macrophages and microglia rapidly associating with the vasculature at the peak of EAE. Left: The leading edge of individual cell bodies or processes closest to the vasculature was tracked over a period of 60 min. Blue tracks identify cells that maintained their proximity to the vasculature and magenta tracks identify cells that either moved closer to the vasculature or extended processes toward it. Middle and right: Yellow arrowheads show a cell approaching the blood vessel and associating with pre-existing perivascular cells over time. Blue arrowheads show a cell with processes already in contact with the blood vessel that migrates toward the vessel and eventually extends a new process toward it. Red star identifies a cell that maintains its point of contact with the vessel wall throughout the 60-min time course (white arrowheads), while it extends a new process toward the same blood vessel (magenta arrowheads), and retracts one of its processes that was directed away from the vessel (red arrowheads). Scale bar: 10 µm. (Adapted from (Davalos et al., 2012)). B- *In vivo* tracking of $T_{MBP-GFP}$ cells (green) infiltrating the spinal cord through a leptomeningeal vessel (red) at day 2 after adoptive T cell transfer in rats over the course of 20 min. Scale bar: 10 µm. (Adapted from (Bartholomäus et al., 2009)). C– *In vivo* imaging of the spinal cord of iNOS-Cre;tdTomato × Arginase-YFP mice at EAE onset, tracking the polarization of phagocytic cells invading the CNS parenchyma. In the upper meninges (UM) most of these cells display an $M^{Arginase}$ phenotype. At the pia–parenchyma border (P/P) they display mixed $M^{iNOS/Arginase}$ phenotype. Having crossed the spinal cord parenchyma (Par) they acquire M^{iNOS} polarized phenotype. Dashed lines indicate upper (cyan) and lower (white) P/P limits. Scale bar: 90 µm. (Adapted from (Locatelli et al., 2018)). D- Real time *in vivo* imaging of T-cell activation and translocation in EAE mice over the course of 46.5 min. *In vivo* calcium imaging of the meninges on day 2 after adoptive T cell transfer showed an increase of calcium activity in $T_{MBP-Twitch1}$ cells (pseudocolor ratio image on the right). As shown in the left images, T cell calcium activity increases after interaction with phagocytes (light red, visualized by intrathecally injected fluorescent dextran) in close proximity to the blood vessels (Dark red, visualized by i.v. infusion of fluorescent dextran). A trajectory line (white) is overlaid and represents a single T cell track. Scale bar: 10 µm. (Adapted from (Kyratsous et al., 2017)).



(caption on next page)

Fig. 4. *In vivo* imaging of the effects of pharmacological or genetic manipulations on immune cells responses in neuroinflammatory disease.
A- *In vivo* imaging of *Cx3cr1*$^{GFP/+}$*Fib*$^{\gamma 390\text{-}396A}$ mice at the peak of EAE shows fewer perivascular clusters (left) than in *Cx3cr1*$^{GFP/+}$*Fib*$^{+/+}$ controls. Scale bar: 10 μm. (Adapted from (Davalos et al., 2012)). B- Detachment of intraluminally crawling $T_{MBP–GFP}$ cells after co-application of anti-LFA-1 and anti-VLA-4 antibodies 48 h after transfer. Migratory tracks (green, 30 min duration) of $T_{MBP–GFP}$ cells before (control) or 5 min after intravenous antibodies infusion are shown. Scale bars, 50 μm. (Adapted from (Bartholomäus et al., 2009)). C– $T_{migratory}$ cells 60 h after T-cell transfer were recorded by 2 PM before or 90 min after intravenous injection of Ninj1 blocking peptide ($Ninj1_{16–45}$, amino acids 16–45). Motility tracks (30 min recording time) of $T_{MBP–GFP}$ cells crawling inside the leptomeningeal vessels (yellow tracks). Scale bars, 20 μm. (Adapted from (Odoardi et al., 2012)).

presenting cells (APCs) which participate in the recruitment of inflammatory effector cells to the CNS, resulting in demyelination and axonal damage (Engelhardt, 2010; Engelhardt and Ransohoff, 2012; Kawakami, 2016; Kawakami and Flugel, 2010).

The earliest studies used fluorescence video-microscopy to perform *in vivo* imaging of the interactions between circulating encephalitogenic T cells and the microvasculature in the spinal cord white matter of healthy mice. Vajkoczy et al., offered the first *in vivo* evidence that adoptively transferred CD4+ T cells localize to and migrate through the CNS microvasculature in mice. While 50% of these cells squeeze through the capillary segment and re-enter the blood stream within 4–7 s, the other 50% are promptly captured at the endothelium of postcapillary venules (Vajkoczy et al., 2001). In a follow up study, the same group focused on the potential involvement of the leukocyte function-associated antigen-1 (LFA-1; also known as αLβ2 integrin) in the multi-step interaction of T lymphoblasts with the BBB endothelium. By pre-incubating the T lymphoblasts with an anti-LFA-1 antibody, the study showed that LFA-1 is required for the firm adhesion of encephalitogenic T cells on the spinal cord microvasculature, but not for their initial adhesion/arrest or their capture on specific vascular locations. Interestingly, blocking of LFA-1 resulted not only in significantly reduced T cell numbers adhering within spinal cord vessels within only 2 h after anti-LFA-1 antibody administration, but also in significantly decreased T cell numbers subsequently penetrating the spinal cord parenchyma. These results showed that encephalitogenic T cells use LFA-1 for trans-endothelial migration but not for capture and initial adhesion in the lumen of spinal cord blood vessels (Laschinger et al., 2002).

Several imaging studies have since then used 2 PM to detail the interactions between T cells and the spinal cord vasculature in rodent models of neuroinflammation. Bartholomäus et al., used an adoptive transfer EAE model in rats and tracked the same spinal cord segments by imaging them *in vivo* over time as the animals started developing disease. They elegantly showed that the first T cells appeared in the CNS before onset of clinical EAE, on days 1–2.5 after transfer and were found to be restricted to the subarachnoid spaces (Fig. 3B). The numbers of intraluminal T cells then increased steeply, and passed to the outer side of the pial vessels. Most of the extravasated T cells initially remained associated with the abluminal surface of the vascular wall before detaching and crawling on the surface of the neuropil with distinct velocities (Fig. 2B). Using timelapse imaging they calculated that the average velocity of intraluminal T cells was 12.5 μm/min, when crawling either with or against the blood flow, however the extravascular T cells were slightly slower (11.3 μm/min). In addition, they showed that T cells migrated in all directions, but intraluminal cells moved preferentially against the blood flow (42%, *versus* 19% along the blood flow) (Bartholomäus et al., 2009). This is in contrast to another study that showed after arresting on the vascular wall, CD4+ T cells started to crawl both with and against the direction of blood flow in cervical spinal cord post-capillary venules (Haghayegh Jahromi et al., 2017). On average, most T cells spent ~10 min crawling, 5–10% of them continued their hike on the vascular bed over more than 30 min, while some crawling T cells detached and were washed away by the blood stream (Bartholomäus et al., 2009).

The infiltration of circulating cells across the inflamed BBB is mediated by the sequential interaction of different adhesion and signaling molecules on T cells and the vascular endothelium (Engelhardt, 2010; Engelhardt and Ransohoff, 2012; Kawakami, 2016; Kawakami and Flugel, 2010; Kim et al., 2010; Rossi and Constantin, 2016; Zenaro et al., 2013). The multi-step cascade starts with the initial contact of T cells with adhesion molecules of the selectin family (*E*- and P-selectin) and their glycoprotein ligand-1 (PSGL-1) or alternatively by α4-integrins and their ligands, vascular cellular adhesion molecule (VCAM-1) and intracellular adhesion molecule −1 (ICAM-1). Sathiyanadan et al., performed real time epifluorescence microscopy, to study the interaction of adoptively transferred wild-type or PSGL-1$^{-/-}$ proteolipid protein-specific T cells with the inflamed spinal cord micro-vessels of mice deficient for both E- and P-selectin, during EAE. They demonstrated that T-cell rolling but not capture was completely abrogated in the absence of either PSGL-1 or E- and P-selectin. They also recorded rapid T-cell rolling on the vascular wall at an average speed of 18 μm/s in cervical spinal cord micro-vessels (Sathiyanadan et al., 2014). Interestingly, a strong reduction of the numbers of T cells crawling on the intraluminal side of the leptomeningeal vessels, was also recorded by 2 PM in rats undergoing EAE, by using intravenous infusion of a Ninjurin1-blocking peptide that blocks homophilic Ninj1 binding; another transmembrane adhesion molecule (Fig. 4C) (Odoardi et al., 2012). In addition to the early *in vivo* imaging studies described above, others demonstrated that encephalitogenic T cells rely also on their VLA4 (known as α4β1 integrin) to arrest and crawl, whereas LFA-1 is required for their firm adhesion on the vessel wall of the CNS white matter microvasculature (Bauer et al., 2009; Coisne et al., 2013; Coisne et al., 2009; Engelhardt et al., 2003; Vajkoczy et al., 2001). Interestingly monoclonal antibodies against VLA-4 and LFA-1 were very effective in detaching crawling T cells from the luminal vascular walls almost instantaneously as was shown by 2 PM in rats undergoing EAE (Fig. 4B) (Bartholomäus et al., 2009). Therapeutic targeting of α4-integrins has been translated to the clinic, where the humanized monoclonal anti-α4-integrin antibody natalizumab has proven one of the most effective therapeutics in the treatment of relapsing remitting MS (Coisne et al., 2009; Engelhardt and Kappos, 2008; Yednock et al., 1992).

The next step in the process of lymphocyte entry into the CNS involves the extravasation of circulating cells across the inflamed endothelium. This can occur through either the tight junctions (TJs) connecting endothelial cells with each other (paracellular migration) or though individual endothelial cells, by engulfment of migrating cells inside vesicles and releasing them on the abluminal side (transcellular migration) (Martinelli et al., 2014; Wolburg et al., 2005). Several studies have focused on the dynamic remodeling of BBB tight junction proteins such as Claudin1, Claudin5, Occludin and ZO-1. These proteins are indeed disrupted in inflammatory diseases, including MS and EAE (Bennett et al., 2010; Kirk et al., 2003) enabling inflammatory proteins and leukocytes to enter the CNS. In the CNS endothelium, most endocytic vesicles are non-clathrin-coated caveolae containing Caveolin1 (Cav1) (Kovtun et al., 2015; Nag, 2003). It was reported several decades ago that the expression of Cav1 and the density of endocytotic vesicles in CNS endothelium increase during acute MS and EAE (Brown, 1978; Claudio and Brosnan, 1992). Endothelial caveolae were thus believed to provide a migratory route for myelin-specific T cells into the CNS (Raine et al., 1990), and thereby regulate cytokine-induced tight junction turnover in endothelial cells (Stamatovic et al., 2009; Sun et al., 2009), and enhance leukocyte migration across the meninges (Santizo et al., 2002). Among the various immune cell subtypes, T

helper 1 (Th1) and T helper 17 (Th17) lymphocytes are distinguished by unique effector cytokines that cause CNS damage. It is known that Th17 cells enter the CNS before Th1 cells in EAE (Murphy et al., 2010; Rothhammer et al., 2011). By using 2 PM in transgenic mice expressing a fusion of eGFP with Claudin 5 in CNS endothelial cells, Lutz et al. demonstrated that TJ remodeling facilitates Th17 migration across the BBB in a paracellular manner; on the other hand, Cav1 promotes Th1 entry into the CNS through the transcellular migration path along the walls of the spinal cord vasculature (Lutz et al., 2017).

Once extravasated through the pial vascular wall, T cells make contact with perivascular/meningeal phagocytes or cells with the ability to function as APCs. These cells are located around vessels and within the pial membrane, monitoring their microenvironment, and their interactions with infiltrating cells is essential for their reactivation, leading to their penetration into the CNS parenchyma. For imaging purposes these APCs have been labeled either by intrathecally infused Texas-red-tagged dextran (Walther et al., 2001) or, by using transgenically labeled bone-marrow chimeric rats (Hickey et al., 1992). Odoardi et al., investigated the mechanisms mediating this interaction between infiltrating T cells and APCs by *in vivo* 2 PM in a rat model of adoptive transfer EAE. Four or five days after the adoptive transfer of MBP-specific GFP-expressing T cells in these animals (a stage when the majority of T cells have left the peripheral immune organs and are entering the CNS) the animals were intravenously injected with soluble MBP, the myelin basic protein peptide used for T cell priming and EAE induction in the first place. Interestingly, the additional exogenous autoantigen dramatically changed the motility and function of autoreactive effector T cells in the CNS lesions of these animals. T cells were arrested around putative APCs and re-activated to massively produce pro-inflammatory mediators, further exacerbating clinical disease. Interestingly, ovalbumin-specific T cells (OVA, a non-encephalitogenic control peptide) were also recruited to EAE lesions, but did not interact with APCs. However, these non-CNS specific T cells were similarly activated within the CNS parenchyma only after i.v. OVA infusion and then also aggravated CNS inflammation and disease severity (Odoardi et al., 2007). Furthermore, T cells are known to form diverse contacts with APCs through adhesion molecules and cognate antigen recognition (Kawakami et al., 2005; Pesic et al., 2013). By imaging these contacts as they took place in the living spinal cord, Bartholomäus et al., found some contacts to be short lasting (less than 10 min), whereas others lasted for longer periods of time. The duration of these contacts also increased with disease progression (Bartholomäus et al., 2009). These results highlight the importance of T cell reactivation by local APCs for the development of inflammatory lesions and neuroinflammatory disease.

Although the field has mostly focused on studying T cell responses, there is now a number of studies that have also employed *in vivo* spinal cord imaging for the investigation of behaviors of other immune cells with the vasculature in the context of EAE. For example some studies focused on the behavior of circulating monocytes in the spinal cord at various time points after EAE induction, and found increased crawling behavior early after immunization (Chrobok et al., 2017). In another study monocytes and neutrophils were shown to invade the spinal cord parenchyma through the meninges rather than by extravasation. Once in the parenchyma, monocytes were shown to mature into monocyte-derived dendritic cells (DCs) (Caravagna et al., 2018). Jain et al., provided evidence of DC migration through the BBB, and α4-integrins as crucial players in the multistep cascade of DCs trafficking into the CNS. However, because blocking of α4-integrin did not affect the initial rolling of DCs, it is possible that two distinct mechanisms are responsible for the initial recruitment of DCs to the vascular endothelium and their eventual migration across the vascular walls (Jain et al., 2010). By using 2 PM, Aubé et al., showed that neutrophils are recruited in the lumbar spinal cord early in the inflammatory process, before disease onset, and their infiltration into the CNS directly parallels a loss of BBB integrity (Fig. 2C) (Aubé et al., 2014).

In recent years, *in vivo* spinal cord imaging of immune cell interactions with resident cells and components of the BBB has expanded to include functional imaging assays as real time indicators of their activation status. For example, the use of calcium reporters has been used as an indicator for T-cell signaling and activation. Using such reporters revealed that along the luminal surface of leptomeningeal vessels, T cells roll and crawl without showing calcium activity (Kyratsous et al., 2017). However, after crossing the BBB, T cells sustained contacts with APCs promote autoreactive T-cell activation and result in long-lasting calcium activity and nuclear factor of activated T-cells (NFAT) translocation from cytosol to nucleus (Fig. 3D) (Kyratsous et al., 2017; Lodygin et al., 2013; Pesic et al., 2013). In another study, Locatelli et al., showed that proinflammatory polarization of blood-derived phagocytes is only induced after CNS entry. They established a novel phenotype imaging approach based on reporter mice that translate the pro- or anti-inflammatory polarization of phagocytes (M^{iNOS} or $M^{Arginase}$ respectively) into distinct fluorescent signals, using iNOS-Cre-driven tdTomato expressing mice and arginase-YFP mice. By tracking the phagocyte cells at different time points over the course of EAE, they observed that in the upper meninges (dura and arachnoid mater) most of these cells displayed the $M^{Arginase}$ phenotype. At the pia–parenchyma border they displayed a mixed $M^{iNOS/Arginase}$ phenotype. Only after crossing the spinal cord parenchyma they acquired the proinflammatory M^{iNOS} polarization (Fig. 3C). In this study, they also showed that although initial lesions are dominated by M^{iNOS} phagocytes, over time these are replaced by $M^{iNOS/Arginase}$ and ultimately by $M^{Arginase}$ phagocytes, indicating that a shift of phenotype toward an anti-inflammatory state in these cells might be important for the resolution of inflammation and the tissue repair processes underlying disease remission (Locatelli et al., 2018).

All of these studies have significantly contributed to our understanding of immune cell responses and their interactions with the vascular compartments within and around the CNS in the context of neuroinflammatory disease. As we are beginning to decipher the key cellular responses and the molecular mechanisms required for immune cell trafficking into the CNS, it is important to be alert to the distinct anatomical characteristics and BBB properties of the blood vessels in the meninges, on the pial surface and in the CNS parenchyma. Here we highlighted findings describing immune cell – vascular interactions in each of these compartments as they were described in the original publications. Given that most vessels in the meninges and those on the pial surface run along the surfaces of the brain or spinal cord, they are distinguishable from vessels penetrating inward into the CNS parenchyma that often appear as cross sections in *in vivo* imaging figures or movie files. Future *in vivo* imaging studies should carefully differentiate responses recorded within entirely meningeal, pial surface, or CNS parenchymal vessels, as the differences among them could better profile immune cell responses at the distinct interfaces between periphery and CNS.

4. *In vivo* imaging studies of the effects of vascular changes and immune cell-vascular interactions on neuronal health following spinal cord injury

Spinal cord injury (SCI) causes significant vascular damage at the site of injury and activation of resident immune cells and infiltration of peripheral immune cells at the lesion site. Vascular pathology is evident immediately after SCI and endothelial cells appear to be the first to undergo cellular death following SCI. Early vascular disruption after SCI, including increased BBB permeability and hemorrhagic events, are followed by secondary pathologies like inflammation, cell apoptosis and demyelination, which further exacerbate clinical manifestations. *In vivo* spinal cord imaging has been used to better characterize the sequence of post-injury pathophysiological events and better understand the underlying mechanisms causing primary and secondary tissue damage, and possibly affecting the potential for tissue repair.

Dray et al., developed a noninvasive *in vivo* imaging protocol to follow the dynamics of degeneration/regeneration in populations of injured spinal cord axons while simultaneously monitoring the fate of the vascular network in the same animals. The lesion produced localized destruction of blood vessels which was soon compensated for by a phase of spontaneous angiogenesis, which resulted in increased plasticity and remodeling of the local vascular network during the second week after SCI. By monitoring the interactions between vessels and regrowing axons during the first 2 weeks post-injury, they found that regenerating axons tend to grow in close contact with blood vessels. Time-lapse analysis showed that neovessels exerted a potent growth stimulating action, but no guidance effect on neighboring sprouts, suggesting that stimulation of angiogenesis would probably be beneficial for axonal regeneration following injury (Dray et al., 2009).

Several studies support that BBB integrity correlates with neuronal structural changes and behavioral performance after SCI, highlighting the importance of tracking the fate of the vascular network as an integral part of tissue repair processes. For example a two-compartment pharmacokinetic model was established to measure BBB permeability in spinal cord injured rats. The authors determined the distribution of gadopentetate-dimeglumine (Gd) concentration in injured spinal cord tissue using serial *in vivo* dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) on living rats on post-injury days 0, 10, 20 and 30. The rates of the Gd transport between plasma and injured cord tissue were determined as a measure of BBB permeability. The results indicated that Gd transport rates decrease steadily and correlate with motor function improvement of the rats following injury, suggesting for a significant link between the neurobehavioral function and the restoration of BBB integrity (Bilgen et al., 2002). DCE-MRI and diffusion tensor imaging (DTI) was later used to simultaneously examine vascular permeability and microstructural alterations in mice with SCI. At the injury sites, the area and volume of damaged BBB regions were reduced from day 1 to day 3 after SCI, suggesting for spatio-temporal remodeling of the microvasculature and its architecture in the injured spinal cord. Comparison of permeability and diffusion measurements indicated that regions with dysfunctional BBB display structural changes in the form of greater axonal loss and demyelination, as also supported by histopathology. Although these studies used imaging modalities with decreased cellular resolution, they demonstrated the importance of characterizing the neurovascular alterations and reorganization following SCI in longitudinal preclinical experiments, with potential clinical implications (Tatar et al., 2009).

Furthermore, *in vivo* spinal cord imaging has proven a valuable tool for preclinical evaluation of current and candidate drugs as well as for testing how they affect their cellular targets. For example, it has been shown by *in vivo* 2 PM that methylprednisolone treatment, the current standard therapy for SCI, increases microvascular blood flow and decreases microvessel loss along with inhibition of microglia/macrophage accumulation and prevention of axonal damage in mice after SCI (Tang et al., 2015). Neutrophil recruitment to the spinal cord microvasculature has also been reported in the context of SCI using real-time *in vivo* imaging. Neutrophils start tethering and rolling onto endothelial cells as early as 15 min post-injury, with endothelial cell adhesion being evident at 6-12 h and continuing up to 48 h after SCI. *In vivo* imaging revealed that depletion of neutrophils by an anti-Ly6G/Gr-1 antibody decreases leukocyte rolling and firm adhesion at 6 and 12 h and reduces neutrophil numbers within the spinal cord at 24 and 48 h after SCI. Neutrophil reduction does not affect microglia/macrophage density and correlates with worsened neurological outcome, suggesting that neutrophils hold a protective role and promote wound healing after SCI (Stirling et al., 2009).

5. Discussion and conclusions

In the past 10 years, *in vivo* imaging in the spinal cord has proven essential for studying the interactions of peripheral and CNS immune cells with the spinal cord blood vessels under physiological conditions and in the context of neuroinflammation or injury. This became possible with the advent of 2 PM and the development of technologies that allowed the generation of reporter mice for both CNS-resident neuronal and glial cells, and peripheral immune cells that enter the CNS following injury or disease. Indeed these methodologies allowed the use of 2 PM in the spinal cord of numerous mouse lines and under a broad range of experimental challenges, and offered unprecedented insights into cellular mechanisms occurring at the neurovascular interface. Understanding the sequence of events linking BBB disruption to immune cell responses, and neuronal dysfunction in neuroinflammatory disease remains critical for the development of effective therapies and the identification of novel biomarkers for early diagnosis. Recent advances for repetitive imaging over the disease course *via* a stable optical window, has made *in vivo* imaging an efficient translational conduit for therapeutic interventions. Direct application of drugs through a spinal cord laminectomy or small cranial windows and real time drug assessment can lead to developing acute pharmacodynamic models to accelerate drug discovery (Davalos et al., 2005). The development of tools for real-time tracking of multiple cell types including glia, peripheral immune cells, neurons and vasculature has led to the identification of novel cellular interactions within the neurovascular unit, and has the potential to reveal new upstream targets for therapeutic intervention in neuroinflammation and neurodegeneration.

Although intravital imaging is an excellent tool to study the neurovascular unit, it is beset by the limitation of having a relatively small field of view during image acquisition. While the small optical window allows for high-resolution longitudinal imaging of cellular processes in real time, combining *in vivo* imaging with imaging of large tissue volumes *ex vivo* may add critical information on the scale and pattern of neuroinflammatory and neurodegenerative pathologies. Multiform imaging modalities that combine immunolabeling-enabled 3D imaging of solvent-cleared organs (iDISCO) and acute *in vivo* imaging are ideal to characterize the spatiotemporal distribution of BBB disruption in large CNS volumes (Merlini et al., 2019). Co-registration of *in vivo* 2 PM with electron microscopy imaging data represents a powerful analytical approach to explore sub-cellular correlates of immune cell activation, axonal damage and blood protein distribution in the CNS (Davalos et al., 2012; Romanelli et al., 2016).

In vivo imaging using 2 PM has shed ample light on fundamental cellular and structural responses in the spinal cord; however, there is a need for developing robust molecular approaches to reveal disease mechanisms. A cross-functional approach involving highly specific imaging probes can identify novel molecular targets which are crucial to our understanding of disease onset and progression. Examples of such molecular probes include those that detect enzymatic activity or protein-binding interactions in the CNS to further provide functional measures of neurovascular perturbations and to be used as early disease biomarkers (Bain et al., 2018; Davalos et al., 2014). Thus, *in vivo* imaging of the spinal cord can be complemented by molecular probes and emerging volume imaging technologies to further the discovery of new mechanisms of disease pathogenesis and the identification of novel therapeutic targets for neurological diseases.

Acknowledgements

This work was supported by a postdoctoral fellowship by the Bodossaki Foundation to E.P., grants from the NIH/NINDS (R35 NS097976), NIH/NIA (R01 AG064926), DoD (MS160082), and the Conrad N. Hilton Foundation to K.A., and grants from the NIH/NINDS (R01 NS112526), the National Multiple Sclerosis Society (RG-1807-32113), VeloSano, and the Lerner Research Institute of the Cleveland Clinic to D.D.

Declaration of Competing Interest

K.A. is a co-founder of MedaRed, Inc., and named inventor on patents and patent applications related to fibrinogen. Her interests are managed by the Gladstone Institutes in accordance with its conflict of interest policy.

References

Adams, R.A., Bauer, Flick, M.J., Sikorski, S.L., Nuriel, T., Lassmann, H., Degen, J.L., Akassoglou, K., 2007. The fibrin-derived gamma377-395 peptide inhibits microglia activation and suppresses relapsing paralysis in central nervous system autoimmune disease. J. Exp. Med. 204, 571–582.

Ajami, B., Bennett, J.L., Krieger, C., McNagny, K.M., Rossi, F.M., 2011. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat. Neurosci. 14, 1142–1149.

Aspelund, A., Antila, S., Proulx, S.T., Karlsen, T.V., Karaman, S., Detmar, M., Wiig, H., Alitalo, K., 2015. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. J. Exp. Med. 212, 991–999.

Aubé, B., Levesque, S.A., Pare, A., Chamma, E., Kebir, H., Gorina, R., Lecuyer, M.A., Alvarez, J.I., De Koninck, Y., Engelhardt, B., Prat, A., Cote, D., Lacroix, S., 2014. Neutrophils mediate blood-spinal cord barrier disruption in demyelinating neuroinflammatory diseases. J. Immunol. 193, 2438–2454.

Bain, L., Keren, N.I., Norris Posey, S.M., 2018. Biomarkers of Neuroinflammation: Proceedings of a Workshop. The National Academies Press, Washington, DC.

Bartholomäus, I., Kawakami, N., Odoardi, F., Schläger, C., Miljkovic, D., Ellwart, J.W., Klinkert, W.E.F., Flügel-Koch, C., Issekutz, T.B., Wekerle, H., Flügel, A., 2009. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. Nature 462, 94–98.

Bauer, M., Brakebusch, C., Coisne, C., Sixt, M., Wekerle, H., Engelhardt, B., Fassler, R., 2009. Beta1 integrins differentially control extravasation of inflammatory cell subsets into the CNS during autoimmunity. Proc. Natl. Acad. Sci. U. S. A. 106, 1920–1925.

Bennett, J., Basivireddy, J., Kollar, A., Biron, K.E., Reickmann, P., Jefferies, W.A., McQuaid, S., 2010. Blood-brain barrier disruption and enhanced vascular permeability in the multiple sclerosis model EAE. J. Neuroimmunol. 229, 180–191.

Bilgen, M., Dogan, B., Narayana, P.A., 2002. In vivo assessment of blood-spinal cord barrier permeability: serial dynamic contrast enhanced MRI of spinal cord injury. Magn. Reson. Imaging 20, 337–341.

Brown, W.J., 1978. The capillaries in acute and subacute multiple sclerosis plaques: a morphometric analysis. Neurology 28, 84–92.

Caravagna, C., Jaouen, A., Desplat-Jego, S., Fenrich, K.K., Bergot, E., Luche, H., Grenot, P., Rougon, G., Malissen, M., Debarbieux, F., 2018. Diversity of innate immune cell subsets across spatial and temporal scales in an EAE mouse model. Sci. Rep. 8, 5146.

Cheng, Y.-T., Lett, K.M., Schaffer, C.B., 2019. Surgical preparations, labeling strategies, and optical techniques for cell-resolved, in vivo imaging in the mouse spinal cord. Exp. Neurol. 318, 192–204.

Chrobok, N.L., Jaouen, A., Fenrich, K.K., Bol, J.G.J.M., Wilhelmus, M.M.M., Drukarch, B., Debarbieux, F., van Dam, A.-M., 2017. Monocyte behaviour and tissue transglutaminase expression during experimental autoimmune encephalomyelitis in transgenic CX3CR1gfp/gfp mice. Amino Acids 49, 643–658.

Claudio, L., Brosnan, C.F., 1992. Effects of prazosin on the blood-brain barrier during experimental autoimmune encephalomyelitis. Brain Res. 594, 233–243.

Coisne, C., Mao, W., Engelhardt, B., 2009. Cutting edge: Natalizumab blocks adhesion but not initial contact of human T cells to the blood-brain barrier in vivo in an animal model of multiple sclerosis. J. Immunol. 182, 5909–5913.

Coisne, C., Lyck, R., Engelhardt, B., 2013. Live cell imaging techniques to study T cell trafficking across the blood-brain barrier in vitro and in vivo. Fluids Barriers CNS 10, 7.

Daneman, R., Engelhardt, B., 2017. Brain barriers in health and disease. Neurobiol. Dis. 107, 1–3.

Davalos, D., Akassoglou, K., 2012. In vivo imaging of the mouse spinal cord using two-photon microscopy. J. Vis. Exp., e2760 Jan 5, PMID: 22258623; PMCID: PMC3369767.

Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L., Gan, W.B., 2005. ATP mediates rapid microglial response to local brain injury in vivo. Nat. Neurosci. 8, 752–758.

Davalos, D., Lee, J.K., Smith, W.B., Brinkman, B., Ellisman, M.H., Zheng, B., Akassoglou, K., 2008. Stable in vivo imaging of densely populated glia, axons and blood vessels in the mouse spinal cord using two-photon microscopy. J. Neurosci. Methods 169, 1–7.

Davalos, D., Ryu, J.K., Merlini, M., Baeten, K.M., Le Moan, N., Petersen, M.A., Deerinck, T.J., Smirnoff, D.S., Bedard, C., Hakozaki, H., Gonias Murray, S., Ling, J.B., Lassmann, H., Degen, J.L., Ellisman, M.H., Akassoglou, K., 2012. Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation. Nat. Commun. 3, 1227.

Davalos, D., Baeten, K.M., Whitney, M.A., Mullins, E.S., Friedman, B., Olson, E.S., Ryu, J.K., Smirnoff, D.S., Petersen, M.A., Bedard, C., Degen, J.L., Tsien, R.Y., Akassoglou, K., 2014. Early detection of thrombin activity in neuroinflammatory disease. Ann. Neurol. 75, 303–308.

Denecke, C.K., Aljović, A., Bareyre, F.M., 2019. Combining molecular intervention with in vivo imaging to untangle mechanisms of axon pathology and outgrowth following spinal cord injury. Exp. Neurol. 318, 1–11.

Dray, C., Rougon, G., Debarbieux, F., 2009. Quantitative analysis by in vivo imaging of the dynamics of vascular and axonal networks in injured mouse spinal cord. Proc. Natl. Acad. Sci. U. S. A. 106, 9459–9464.

Engelhardt, B., 2010. T cell migration into the central nervous system during health and disease: different molecular keys allow access to different central nervous system compartments. Clin. Exp. Neuroimmunol. 1, 79–93.

Engelhardt, B., Kappos, L., 2008. Natalizumab: targeting alpha4-integrins in multiple sclerosis. Neurodegener. Dis. 5, 16–22.

Engelhardt, B., Ransohoff, R.M., 2012. Capture, crawl, cross: the T cell code to breach the blood-brain barriers. Trends Immunol. 33, 579–589.

Engelhardt, B., Vajkoczy, P., Laschinger, M., 2003. Detection of endothelial/lymphocyte interaction in spinal cord microvasculature by intravital videomicroscopy. Methods Mol. Med. 89, 83–93.

Engelhardt, B., Carare, R.O., Bechmann, I., Flugel, A., Laman, J.D., Weller, R.O., 2016. Vascular, glial, and lymphatic immune gateways of the central nervous system. Acta Neuropathol. 132, 317–338.

Erickson, M.A., Banks, W.A., 2018. Neuroimmune axes of the blood-brain barriers and blood-brain interfaces: bases for physiological regulation, disease states, and pharmacological interventions. Pharmacol. Rev. 70, 278–314.

Evans, T.A., Barkauskas, D.S., Silver, J., 2019. Intravital imaging of immune cells and their interactions with other cell types in the spinal cord: experiments with multicolored moving cells. Exp. Neurol. 320, 112972.

Farrar, M.J., Bernstein, I.M., Schlafer, D.H., Cleland, T.A., Fetcho, J.R., Schaffer, C.B., 2012. Chronic in vivo imaging in the mouse spinal cord using an implanted chamber. Nat. Methods 9, 297–302.

Fenrich, K.K., Weber, P., Rougon, G., Debarbieux, F., 2013 Dec 21. Implanting glass spinal cord windows in adult mice with experimental autoimmune encephalomyelitis. J. Vis. Exp.(82), e50826. https://doi.org/10.3791/50826.

Haghayegh Jahromi, N., Tardent, H., Enzmann, G., Deutsch, U., Kawakami, N., Bittner, S., Vestweber, D., Zipp, F., Stein, J.V., Engelhardt, B., 2017. A novel cervical spinal cord window preparation allows for two-photon imaging of T-cell interactions with the cervical spinal cord microvasculature during experimental autoimmune encephalomyelitis. Front. Immunol. 8, 406.

Hickey, W.F., Vass, K., Lassmann, H., 1992. Bone marrow-derived elements in the central nervous system: an immunohistochemical and ultrastructural survey of rat chimeras. J. Neuropathol. Exp. Neurol. 51, 246–256.

Jain, P., Coisne, C., Enzmann, G., Rottapel, R., Engelhardt, B., 2010. Alpha4beta1 integrin mediates the recruitment of immature dendritic cells across the blood-brain barrier during experimental autoimmune encephalomyelitis. J. Immunol. 184, 7196–7206.

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., Sher, A., Littman, D.R., 2000. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol. Cell. Biol. 20, 4106–4114.

Kawakami, N., 2016. In vivo imaging in autoimmune diseases in the central nervous system. Allergol. Int. 65, 235–242.

Kawakami, N., Flugel, A., 2010. Knocking at the brain's door: intravital two-photon imaging of autoreactive T cell interactions with CNS structures. Semin. Immunopathol. 32, 275–287.

Kawakami, N., Nagerl, U.V., Odoardi, F., Bonhoeffer, T., Wekerle, H., Flugel, A., 2005. Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. J. Exp. Med. 201, 1805–1814.

Kim, J.V., Jiang, N., Tadokoro, C.E., Liu, L., Ransohoff, R.M., Lafaille, J.J., Dustin, M.L., 2010. Two-photon laser scanning microscopy imaging of intact spinal cord and cerebral cortex reveals requirement for CXCR6 and neuroinflammation in immune cell infiltration of cortical injury sites. J. Immunol. Methods 352, 89–100.

Kirk, J., Plumb, J., Mirakhur, M., McQuaid, S., 2003. Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood-brain barrier leakage and active demyelination. J. Pathol. 201, 319–327.

Kovtun, O., Tillu, V.A., Ariotti, N., Parton, R.G., Collins, B.M., 2015. Cavin family proteins and the assembly of caveolae. J. Cell Sci. 128, 1269–1278.

Kyratsous, N.I., Bauer, I.J., Zhang, G., Pesic, M., Bartholomaus, I., Mues, M., Fang, P., Worner, M., Everts, S., Ellwart, J.W., Watt, J.M., Potter, B.V.L., Hohlfeld, R., Wekerle, H., Kawakami, N., 2017. Visualizing context-dependent calcium signaling in encephalitogenic T cells in vivo by two-photon microscopy. Proc. Natl. Acad. Sci. U. S. A. 114, E6381–e6389.

Laschinger, M., Vajkoczy, P., Engelhardt, B., 2002. Encephalitogenic T cells use LFA-1 for transendothelial migration but not during capture and initial adhesion strengthening in healthy spinal cord microvessels in vivo. Eur. J. Immunol. 32, 3598–3606.

Lee, N.J., Ha, S.K., Sati, P., Absinta, M., Luciano, N.J., Lefeuvre, J.A., Schindler, M.K., Leibovitch, E.C., Ryu, J.K., Petersen, M.A., Silva, A.C., Jacobson, S., Akassoglou, K., Reich, D.S., 2018. Spatiotemporal distribution of fibrinogen in marmoset and human inflammatory demyelination. Brain 141, 1637–1649.

Locatelli, G., Theodorou, D., Kendirli, A., Jordao, M.J.C., Staszewski, O., Phulphagar, K., Cantuti-Castelvetri, L., Dagkalis, A., Bessis, A., Simons, M., Meissner, F., Prinz, M., Kerschensteiner, M., 2018. Mononuclear phagocytes locally specify and adapt their phenotype in a multiple sclerosis model. Nat. Neurosci. 21, 1196–1208.

Lodygin, D., Odoardi, F., Schlager, C., Korner, H., Kitz, A., Nosov, M., van den Brandt, J., Reichardt, H.M., Haberl, M., Flugel, A., 2013. A combination of fluorescent NFAT and H2B sensors uncovers dynamics of T cell activation in real time during CNS autoimmunity. Nat. Med. 19, 784–790.

Louveau, A., Smirnov, I., Keyes, T.J., Eccles, J.D., Rouhani, S.J., Peske, J.D., Derecki, N.C., Castle, D., Mandell, J.W., Lee, K.S., Harris, T.H., Kipnis, J., 2015. Structural and functional features of central nervous system lymphatic vessels. Nature 523, 337–341.

Louveau, A., Plog, B.A., Antila, S., Alitalo, K., Nedergaard, M., Kipnis, J., 2017. Understanding the functions and relationships of the glymphatic system and meningeal lymphatics. J. Clin. Invest. 127, 3210–3219.

Lutz, S.E., Smith, J.R., Kim, D.H., Olson, C.V.L., Ellefsen, K., Bates, J.M., Gandhi, S.P., Agalliu, D., 2017. Caveolin1 is required for Th1 cell infiltration, but not tight junction

remodeling, at the blood-brain barrier in autoimmune neuroinflammation. Cell Rep. 21, 2104–2117.
Marik, C., Felts, P.A., Bauer, J., Lassmann, H., Smith, K.J., 2007. Lesion genesis in a subset of patients with multiple sclerosis: a role for innate immunity? Brain 130, 2800–2815.
Martinelli, R., Zeiger, A.S., Whitfield, M., Sciuto, T.E., Dvorak, A., Van Vliet, K.J., Greenwood, J., Carman, C.V., 2014. Probing the biomechanical contribution of the endothelium to lymphocyte migration: diapedesis by the path of least resistance. J. Cell Sci. 127, 3720–3734.
Merlini, M., Rafalski, V.A., Rios Coronado, P.E., Gill, T.M., Ellisman, M., Muthukumar, G., Subramanian, K.S., Ryu, J.K., Syme, C.A., Davalos, D., Seeley, W.W., Mucke, L., Nelson, R.B., Akassoglou, K., 2019. Fibrinogen induces microglia-mediated spine elimination and cognitive impairment in an Alzheimer's disease model. Neuron 101 (1099–1108), e1096.
Murphy, A.C., Lalor, S.J., Lynch, M.A., Mills, K.H., 2010. Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. Brain Behav. Immun. 24, 641–651.
Nag, S., 2003. Ultracytochemical studies of the compromised blood-brain barrier. Methods Mol. Med. 89, 145–160.
Nimmerjahn, A., Kirchhoff, F., Helmchen, F., 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science (New York, N.Y.) 308, 1314–1318.
Odoardi, F., Kawakami, N., Klinkert, W.E., Wekerle, H., Flugel, A., 2007. Blood-borne soluble protein antigen intensifies T cell activation in autoimmune CNS lesions and exacerbates clinical disease. Proc. Natl. Acad. Sci. U. S. A. 104, 18625–18630.
Odoardi, F., Sie, C., Streyl, K., Ulaganathan, V.K., Schlager, C., Lodygin, D., Heckelsmiller, K., Nietfeld, W., Ellwart, J., Klinkert, W.E., Lottaz, C., Nosov, M., Brinkmann, V., Spang, R., Lehrach, H., Vingron, M., Wekerle, H., Flugel-Koch, C., Flugel, A., 2012. T cells become licensed in the lung to enter the central nervous system. Nature 488, 675–679.
Pesic, M., Bartholomaus, I., Kyratsous, N.I., Heissmeyer, V., Wekerle, H., Kawakami, N., 2013. 2-photon imaging of phagocyte-mediated T cell activation in the CNS. J. Clin. Invest. 123, 1192–1201.
Petersen, M.A., Ryu, J.K., Akassoglou, K., 2018. Fibrinogen in neurological diseases: mechanisms, imaging and therapeutics. Nat. Rev. Neurosci. 19, 283–301.
Raine, C.S., Cannella, B., Duijvestijn, A.M., Cross, A.H., 1990. Homing to central nervous system vasculature by antigen-specific lymphocytes. II. Lymphocyte/endothelial cell adhesion during the initial stages of autoimmune demyelination. Lab. Investig. 63, 476–489.
Romanelli, E., Merkler, D., Mezydlo, A., Weil, M.T., Weber, M.S., Nikic, I., Potz, S., Meinl, E., Matznick, F.E., Kreutzfeldt, M., Ghanem, A., Conzelmann, K.K., Metz, I., Bruck, W., Routh, M., Simons, M., Bishop, D., Misgeld, T., Kerschensteiner, M., 2016. Myelinosome formation represents an early stage of oligodendrocyte damage in multiple sclerosis and its animal model. Nat. Commun. 7, 13275.
Rossi, B., Constantin, G., 2016. Live imaging of immune responses in experimental models of multiple sclerosis. Front. Immunol. 7, 506.
Rothhammer, V., Heink, S., Petermann, F., Srivastava, R., Claussen, M.C., Hemmer, B., Korn, T., 2011. Th17 lymphocytes traffic to the central nervous system independently of alpha4 integrin expression during EAE. J. Exp. Med. 208, 2465–2476.
Ryu, J.K., Petersen, M.A., Murray, S.G., Baeten, K.M., Meyer-Franke, A., Chan, J.P., Vagena, E., Bedard, C., Machado, M.R., Rios Coronado, P.E., Prod'homme, T., Charo, I.F., Lassmann, H., Degen, J.L., Zamvil, S.S., Akassoglou, K., 2015. Blood coagulation protein fibrinogen promotes autoimmunity and demyelination via chemokine release and antigen presentation. Nat. Commun. 6, 8164.
Ryu, J.K., Rafalski, V.A., Meyer-Franke, A., Adams, R.A., Poda, S.B., Rios Coronado, P.E., Pedersen, L.O., Menon, V., Baeten, K.M., Sikorski, S.L., Bedard, C., Hanspers, K., Bardehle, S., Mendiola, A.S., Davalos, D., Machado, M.R., Chan, J.P., Plastira, I., Petersen, M.A., Pfaff, S.J., Ang, K.K., Hallenbeck, K.K., Syme, C., Hakozaki, H., Ellisman, M.H., Swanson, R.A., Zamvil, S.S., Arkin, M.R., Zorn, S.H., Pico, A.R., Mucke, L., Freedman, S.B., Stavenhagen, J.B., Nelson, R.B., Akassoglou, K., 2018. Fibrin-targeting immunotherapy protects against neuroinflammation and neurodegeneration. Nat. Immunol. 19, 1212–1223.
Saederup, N., Cardona, A.E., Croft, K., Mizutani, M., Cotleur, A.C., Tsou, C.-L., Ransohoff, R.M., Charo, I.F., 2010. Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein Knock-in mice. PLoS One 5, e13693.
Santizo, R.A., Xu, H.L., Galea, E., Muyskens, S., Baughman, V.L., Pelligrino, D.A., 2002. Combined endothelial nitric oxide synthase upregulation and caveolin-1 down-regulation decrease leukocyte adhesion in pial venules of ovariectomized female rats. Stroke 33, 613–616.
Sathiyanadan, K., Coisne, C., Enzmann, G., Deutsch, U., Engelhardt, B., 2014. PSGL-1 and E/P-selectins are essential for T-cell rolling in inflamed CNS microvessels but dispensable for initiation of EAE. Eur. J. Immunol. 44, 2287–2294.
Schaffran, B., Hilton, B.J., Bradke, F., 2019. Imaging in vivo dynamics of sensory axon responses to CNS injury. Exp. Neurol. 317, 110–118.
Schumacher, A.-M., Misgeld, T., Kerschensteiner, M., Snaidero, N., 2019. Imaging the execution phase of neuroinflammatory disease models. Exp. Neurol. 320, 112968.
Sekiguchi, K.J., Shekhtmeyster, P., Merten, K., Arena, A., Cook, D., Hoffman, E., Ngo, A., Nimmerjahn, A., 2016. Imaging large-scale cellular activity in spinal cord of freely behaving mice. Nat. Commun. 7, 11450.
Stamatovic, S.M., Keep, R.F., Wang, M.M., Jankovic, I., Andjelkovic, A.V., 2009. Caveolae-mediated internalization of occludin and claudin-5 during CCL2-induced tight junction remodeling in brain endothelial cells. J. Biol. Chem. 284, 19053–19066.
Stirling, D.P., Liu, S., Kubes, P., Yong, V.W., 2009. Depletion of Ly6G/Gr-1 leukocytes after spinal cord injury in mice alters wound healing and worsens neurological outcome. J. Neurosci. 29, 753–764.
Sun, Y., Hu, G., Zhang, X., Minshall, R.D., 2009. Phosphorylation of caveolin-1 regulates oxidant-induced pulmonary vascular permeability via paracellular and transcellular pathways. Circ. Res. 105, 676–685 (615 p following 685).
Takeshita, Y., Ransohoff, R.M., 2012. Inflammatory cell trafficking across the blood-brain barrier: chemokine regulation and in vitro models. Immunol. Rev. 248, 228–239.
Tang, P., Zhang, Y., Chen, C., Ji, X., Ju, F., Liu, X., Gan, W.B., He, Z., Zhang, S., Li, W., Zhang, L., 2015. In vivo two-photon imaging of axonal dieback, blood flow, and calcium influx with methylprednisolone therapy after spinal cord injury. Sci. Rep. 5, 9691.
Tatar, I., Chou, P.C., Desouki, M.M., El Sayed, H., Bilgen, M., 2009. Evaluating regional blood spinal cord barrier dysfunction following spinal cord injury using longitudinal dynamic contrast-enhanced MRI. BMC Med. Imaging 9, 10.
Vajkoczy, P., Laschinger, M., Engelhardt, B., 2001. α4-integrin-VCAM-1 binding mediates G protein–independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. J. Clin. Invest. 108, 557–565.
van der Valk, P., Amor, S., 2009. Preactive lesions in multiple sclerosis. Curr. Opin. Neurol. 22, 207–213.
Vos, C.M., Geurts, J.J., Montagne, L., van Haastert, E.S., Bo, L., van der Valk, P., Barkhof, F., de Vries, H.E., 2005. Blood-brain barrier alterations in both focal and diffuse abnormalities on postmortem MRI in multiple sclerosis. Neurobiol. Dis. 20, 953–960.
Walther, M., Popratiloff, A., Lachnit, N., Hofmann, N., Streppel, M., Guntinas-Lichius, O., Neiss, W.F., Angelov, D.N., 2001. Exogenous antigen containing perivascular phagocytes induce a non-encephalitogenic extravasation of primed lymphocytes. J. Neuroimmunol. 117, 30–42.
Wolburg, H., Wolburg-Buchholz, K., Engelhardt, B., 2005. Diapedesis of mononuclear cells across cerebral venules during experimental autoimmune encephalomyelitis leaves tight junctions intact. Acta Neuropathol. 109, 181–190.
Yednock, T.A., Cannon, C., Fritz, L.C., Sanchez-Madrid, F., Steinman, L., Karin, N., 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. Nature 356, 63–66.
Zenaro, E., Rossi, B., Angiari, S., Constantin, G., 2013. Use of imaging to study leukocyte trafficking in the central nervous system. Immunol. Cell Biol. 91, 271–280.
Zheng, B., Lorenzana, A.O., Ma, L., 2019. Understanding the axonal response to injury by in vivo imaging in the mouse spinal cord: a tale of two branches. Exp. Neurol. 318, 277–285.