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Extravascular CX3CR1⁺ Cells Extend Intravascular Dendritic Processes into Intact Central Nervous System Vessel Lumen

Deborah S. Barkauskas^{1,†}, Teresa A. Evans^{2,†}, Jay Myers¹, Agne Petrosiute¹, Jerry Silver², and Alex Y. Huang^{1,*}

¹Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

²Department of Neurosciences, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

Abstract

Within the central nervous system (CNS), antigen-presenting cells (APCs) play a critical role in orchestrating inflammatory responses where they present CNS-derived antigens to immune cells that are recruited from the circulation to the cerebrospinal fluid, parenchyma, and perivascular space. Available data indicate that APCs do so indirectly from outside of CNS vessels without direct access to luminal contents. Here, we applied high-resolution, dynamic intravital two-photon laser scanning microscopy 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 an intact vessel wall into the vascular lumen with preservation of vessel integrity. While only a small number of APCs displayed intravascular extensions in intact, noninflamed vessels in the brain and the spinal cord, the frequency of projections increased over days in an experimental autoimmune encephalomyelitis model, 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.

Keywords

two-photon microscopy; intravital; antigen-presenting cells; blood-brain barrier; central nervous system; microglia; spinal cord injury; EAE; CNS vessels

Introduction

In non-central nervous system (non-CNS) tissues, activation of antigen-presenting cells (APCs) by pathogen- or damage-associated molecular patterns results in APC homing to sentinel lymph nodes (LNs) where they process and present antigens to lymphocytes (Itano & Jenkins, 2003). On the other hand, the pathway for antigen surveillance and presentation

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*Corresponding author. alex.y.huang@case.edu.

†These authors contributed equally to this work.

Supplementary Material

To view supplementary material for this article (including Supplementary Figs. 1–3 and Supplementary Movies 1–4), please visit <http://dx.doi.org/10.1017/S1431927613000482>.

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 within the vessel lumen have limited direct access to CNS-resident APCs and associated antigens. 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 (de Vos et al., 2002; Engelhardt & Ransohoff, 2012; Iliff et al., 2012; Kaminski et al., 2012; Locatelli et al., 2012; Ransohoff & Engelhardt, 2012). Third, CNS APCs are both blood-derived and tissue-resident, with each subset having distinct surveillance locations and functional roles (Stoll & Jander, 1999; Guillemin & Brew, 2004; Ransohoff & Cardona, 2010). 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 noninflamed conditions (Guillemin & Brew, 2004; Nimmerjahn et al., 2005; Saederup et al., 2010). Microglia are present in both gray and white matter and, although controversial, presumably possess different activation states and numbers at different anatomic sites (Lawson et al., 1990; Mittelbronn et al., 2001). For example, gray matter lesions in multiple sclerosis (MS) display less microglia activation compared to that found in white matter lesions (Clarner et al., 2012). Microglia function as tissue APCs in all anatomic sites within the CNS, while infiltrating monocytes have been shown to migrate to the cervical LNs through the cribriform plate (Aloisi, 2001; Almolda et al., 2011; Kaminski et al., 2012).

On the other hand, CX3CR1⁺ perivascular and meningeal macrophages are derived from circulating Ly6C^{hi}/CD45^{hi}/CCR2⁺ monocytes and can sample CSF contents in the arachnoid and Virchow-Robin space (McMahon et al., 2006; D'Agostino et al., 2012). For these reasons, perivascular macrophages have been postulated to play an important role in the presentation of myelin-derived peptides to and reactivation 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 (Bartholomaeus et al., 2009; Goverman, 2009; Ransohoff & Engelhardt, 2012; Sallusto et al., 2012). Once in the CSF, primed myelin-specific T cells encounter meningeal macrophages that present CNS tissue-derived myelin peptides. The reactivation 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 (Lassmann, 2011; Pachner, 2011; Ransohoff, 2012).

Recently, histologic studies of static brain tissues suggest that parenchymal CD11c⁺ 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 (Prodinger et al., 2011). 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 (Lelouard et al., 2012), mucosa of the small intestine (Niess et al., 2005), the lungs (Thornton et al., 2012), the nasal mucosa (Takano et al., 2005), cardiac valves (Choi et al., 2009), cornea (Lee et al., 2010), and the lymphatic conduits in the LNs (Roosendaal et al., 2009; Girard et al., 2012). 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⁺ 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 (Mostany & Portera-Cailliau, 2008b), thinned-skull (Marker et al., 2010; Yang et al., 2010), and open laminectomy (Shen et al., 2009), we observed in a dynamic fashion the ability of extravascular APCs to display dendritic extensions into an 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 gray 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.

Materials and Methods

Mice

Six- to 12-week old syngeneic female C57BL/6, *Cx3cr1^{GFP/GFP}* (stock #5582) and Thy-1-YFP-H (stock #3782) mice (H-2^b) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). *Cx3cr1^{+/GFP}* reporter mice have one *Cx3cr1* allele replaced with the gene encoding the green fluorescent protein (GFP) and are derived by crossing *Cx3cr1^{GFP/GFP}* with C57BL/6 (Jung et al., 2000). 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 (Jung et al., 2000). Thy-1-YFP-H mice harbor yellow fluorescent protein (YFP) expression in a subset of neurons in the dorsal root ganglion and cortical layers (Feng et al., 2000). Thy-1-YFP-H mice were crossed with *Cx3cr1^{GFP/GFP}* mice to obtain double transgenic mice, Thy-1-YFP-H × *Cx3cr1^{+/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 (Stromnes & Goverman, 2006; Mi et al., 2007). Briefly, synthetic myelin oligodendrocyte glycoprotein peptide (MOG 35–55; 200 µg) was emulsified with H37RA (8 mg/mL) and complete Freund's adjuvant in premixed solutions (Hooke Laboratory, Lawrence, MA, USA), and mice were injected with the mixture in 100 µL subcutaneously (*s.c.*) on the lower back bilaterally on day 0. On days 0, 1, and 2, the mice were injected with Pertussis toxin (100 ng) *i.p.* This induction protocol results in limp tail, hind leg paralysis, and progressive neurological dysfunction in recipient mice beginning around days 12–14 postinduction (Stromnes & Goverman, 2006).

Spinal Trauma Model

We modified published protocols to create a dorsal column crush injury (Shen et al., 2009). Briefly, mice were anesthetized with inhaled 1–2% isoflurane (Aerrane; Baxter, Deerfield, IL, USA), 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 s three times. The laminectomy site was covered with saline-soaked Gelfoam (Pfizer, New York, NY, USA), and the para-spinal muscles and skin were closed with sutures. For postoperative 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-2^b) 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, Illkirch, France). 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 and placed in a stereotactic holder. After removing a circular scalp flap, 3×10^4 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 (Mostany & Portera-Cailliau, 2008b). 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 protocols (Mostany & Portera-Cailliau, 2008a, 2008b), and imaging sessions were carried out immediately for acute analysis or at least 4 days following the implantation procedure. Alternatively, thinned-skull preparations were utilized according to published protocols (Marker et al., 2010; Yang et al., 2010), leaving an intact skull of 25–50 µm thickness (Supplementary Fig. 1). 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 min 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-Aldrich, St. Louis, MO, USA) or 100 µL of 0.1 µM nontargeted QTracker-655 (Invitrogen Corporation, Carlsbad, CA, USA). Tomato-lectin (Vector Laboratories, Burlingame, CA, USA; 16 µg/mouse) (Huang et al., 2003) was also used to visualize the endothelial cells (Jahrling et al., 2009). Large molecular weight dextran markers were selected to study BBB integrity as previously described (Ishii et al., 2010).

Spinal Imaging

For sequential spinal cord imaging of traumatic injury, skin and muscle flaps covering the previous T10 laminectomy site were reopened 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, Wheeling, IL, USA).

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 (Leica Microsystems, Wetzlar, Germany), a 20× water immersion lens (NA. 1.0; Leica HCX-APO-L), and a 16W Ti/Sapphire IR laser (Chameleon, Coherent, Inc., Santa Clara, CA, USA) tuned to excitation wavelengths between 800 and 880 nm. Imaging planes ($760 \times 760 \mu\text{m}$) collected at 1–5 μm z-intervals were repeated at 20–60 s intervals for up to 6 h to yield *xyz*t datasets collected through a four channel nondescanned external detector using a filter set separating <455, 467–499, 500–550, 565–605 for brain and spine imaging, and a filter set separating <495, 500–550, 565–605, 625–675 for tumor imaging. This raw dataset 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 ($0.75 \times 0.75 \times 1 \mu\text{m}$) image collection of the tissues (see below).

Image and Statistical Analysis

High-resolution fluorescent four-dimensional (4D) imaging datasets collected from intravital 2P-LSM experiments were analyzed using Imaris (BitPlane, Inc., South Windsor, CT, USA). Mosaic broad-field survey images were compiled using *Xuv Stitch* software (XuvTools; Emmenlauer et al., 2009). Volumes of $1550 \times 1550 \times 150 \mu\text{m}^3$ up to $1550 \times 2025 \times 150 \mu\text{m}^3$ 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 $775 \times 775 \times 150\text{--}300 \mu\text{m}^3$ 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 (Supplementary Fig. 1). 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 $>3 \mu\text{m}/\text{min}$ or were visualized to be in an active process of transmigration across the vessel wall from vascular lumen in the 4D dynamic imaging datasets; (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 (Prodinger et al., 2011). 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. The number of mice and projections quantified in the imaging experiments are as follows: Figure 3F: 3–4 mice per group, 7092 total projection events; Figure 4F: 3 mice per group, 503 total projection events; Figure 5E: 3 mice per group, 216 total projection events; Figure 5I: 3 mice total, 196 total projection events; Supplementary Figure 1F: 4–6 mice per group, 2213 total projection events. Paired experimental data were tested for statistical significance by two-tailed type 2 *t*-test. For multiple comparisons, two way ANOVAS were used.

Immunofluorescence Histology

Naive or inflamed *Cx3cr1*^{+/GFP} mice were sacrificed and subjected to transcardial perfusion with 4% paraformaldehyde (PFA). The brain and spinal tissues were harvested, and 12 μ m cryosections were stained with DyLight 594 tomato-lectin (Vector Laboratories) (Jahrling et al., 2009) or antibodies against CD31 (MEC13.3; BD Biosciences, San Jose, CA, USA), Iba-1 (#019–19741; Wako Chemicals, Richmond, VA, USA), or GFAP (Z0334; Dako, Glostrup, Denmark) followed by appropriate secondary antibodies conjugated to Alexa Fluor (Life Technologies, Carlsbad, CA, USA). The samples were then subjected to confocal microscopy to obtain fluorescent micrographs.

Electron Microscopy

Naive, nonmanipulated *Cx3cr1*^{+/GFP} mice were subjected to transcardial perfusion with 0.1% glutaraldehyde and 4% PFA, and tissues were fixed overnight. Samples were stained en bloc with anti-GFP primary antibody (Life Technologies) and goat anti-rabbit 2 nm gold conjugated secondary antibody (Ted Pella, Inc., Redding, CA, USA). Gold particles were then enhanced using the silver enhancer kit (Sigma). The tissues then underwent osmication with 1% osmium (Electron Microscopy Sciences, Hatfield, PA, USA) and dehydration in ethanol, followed by infiltration with propylene oxide (Electron Microscopy Sciences) and eponate-12 resin (Ted Pella, Inc.). Samples were embedded in resin and polymerized at 60° for 48 h in BEEM capsules in a polymerization oven. Blocks were trimmed and cut into 85 nm ultrathin sections using an ultramicrotome, and placed on 150 square mesh copper formvar support grids (Electron Microscopy Sciences). The tissue was stained with lead citrate and uranyl acetate (Electron Microscopy Sciences), and the grids were imaged using the scanning transmission electron microscopy mode for rapid scanning of large areas on a FEI (FEI Company, Hillsboro, OR, USA) Helios Nanolab 650 (Swagelok Center, Case Western Reserve University) and high-resolution transmission electron microscopy micrographs were acquired on a FEI Tecnai G2 Spirit (Imaging Core, Cleveland Clinic Foundation) at 18,500 $\times$ magnification.

Results

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 thinned-skull preparations (Supplementary Figs. 1A–1E). The CNS parenchyma was visualized below the collagen-rich meningeal layers, and the pial surface was outlined by second-harmonic signal generation under two-photon excitation (Supplementary Figs. 2A, 2B). Under steady-state conditions, the majority of the CX3CR1⁺ cells in the mouse CNS parenchyma consists of Ly6C^{lo}/CD45^{lo}/Iba-1⁺ microglia, with very few Ly6C^{hi}/CD45^{hi}/CCR2^{hi} blood-derived monocytes/macrophages (Saederup et al., 2010). Indeed, dynamic intravital images revealed that GFP⁺ cells with extensive ramified processes are evenly distributed throughout the CNS parenchyma with a cellular morphology consistent with that of ramified nonactivated microglia (Fig. 1A; Supplementary Figs. 1A–1C). Sequential imaging showed that the cell

bodies of these CX3CR1⁺ cells were stationary, with extensive dynamic dendritic processes surveying surrounding tissues (Supplementary Movie 1). 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. 1B–1D; Supplementary Movie 2). While the larger-sized extravascular GFP⁺ cells had morphology consistent with that of microglia and macrophages, other smaller, 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 (Huang et al., 2006) (Supplementary Movie 2). High-resolution fluorescence and three-dimensional (3D) reconstruction images revealed a surprising finding that on occasion extravascular, stationary GFP⁺ CX3CR1⁺ cells were capable of extending their cellular projections into an intact CNS vessel lumen (Fig. 1B). These intraluminal cellular processes persisted throughout a 45-min imaging session, with the intravascular processes of two nearby extravascular CX3CR1⁺ cells making contact with each other inside the vessel lumen (Fig. 1C; Supplementary Movie 2). 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 EAE. Consistent with our previously published work (Pareek et al., 2010), *Cx3cr1*^{+/GFP} mice developed worsening clinical scores starting around day 12 following induction of disease. Using similar intravital imaging techniques, we were able to visualize the presence of intraluminal dendritic processes extending from the main bodies of extravascular CX3CR1⁺ cells near intact blood vessels on day 5 during EAE induction (Fig. 1E), 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. 2A, 2B) as well as EAE-induced tissue sections (Figs. 2C, 2D) by immunohistochemical (IHC) analysis. We confirmed that the intravascular dendritic processes within a vessel lumen in Figure 1 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 (Supplementary Fig. 3). 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. 2E, 2F). Within one thin section, we found 176 vessels in 38,556 μm^3 of tissue and one projection.

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 (Figs. 3A–3C). 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 Thyl promoter while one of the *Cx3cr1* alleles is replaced by GFP

(Supplementary Fig. 2). Thy-1 YFP marker allowed us to identify the parenchyma within the imaging field. Under noninflamed conditions, CX3CR1⁺ cells were uniformly present throughout the parenchyma with a ramified morphology in both white and gray matter (Figs. 3A, 3B). CX3CR1⁺ cells within the cortical gray 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 (Figs. 3D, 3E). As EAE developed, parenchymal CX3CR1⁺ cell density increased with concomitant increasing number of extravascular GFP⁺ cells positioned next to CNS vessels (Fig. 3C). Next, we enumerated the number of intraluminal dendritic processes (“projections”) by extravascular stationary CX3CR1⁺ cells throughout the first 12 days of EAE induction (Fig. 3F). 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 $\mu\text{m}/\text{min}$ in the dynamic imaging dataset, as well as small, spherical, and motile GFP⁺ cells or elongated, perivascular GFP⁺ cells that had >30% of the cell volume inside the vessel lumen. At baseline, we found that CX3CR1⁺ cells exhibited 172.50 ± 24.2 projections per mm^2 of vessel wall surface (Fig. 3F). The number of dendritic projections more than doubled that found in the noninflamed control over the first 9 days following EAE induction, with the frequency of projections plateauing between days 9 and 12 (Fig. 3F).

To compare number and characteristics of intravascular dendritic projections at different anatomical sites containing gray 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 (Figs. 4A, 4B). Similar to the brain, GFP⁺ dendritic projections were again visualized to insert into both large and smaller spinal vessels (Figs. 4D, 4E). Again, parenchymal CX3CR1⁺ cells in the spine appeared in a ramified, nonactivated state (Figs. 4A, 4B), with an average of 74.6 ± 11.03 projections per mm^2 of visualized vessel surfaces (Fig. 4F). Upon EAE induction, the number of intravascular projections doubled to an average of 159.97 ± 27.26 projections per mm^2 of vessel surface by day 12, with cells appearing in an activated, less ramified morphology (Fig. 4C). 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. 4C). When compared to the frequency of intravascular projections in the brain parenchyma during peak of EAE induction (day 12; Fig. 3F), the frequency of intravascular projections in the EAE spine was not statistically different (day 12; Fig. 4F).

To test whether the increase in intravascular dendritic processes was simply due to activation of brain and spinal CX3CR1⁺ cells in response to nonspecific 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 (Figs. 5A, 5B). Similar to the findings in EAE, extravascular CX3CR1⁺ cells can insert their GFP⁺ dendritic processes through intact vessel walls into the blood vessel (Figs. 5C, 5D). Contrary to the findings in EAE, however, CX3CR1⁺ cells accumulated around both the large and small vessels at the spinal injury site (Figs. 4C, 5B). We observed an average of 46.59 ± 10.1 intravascular projections per mm^2 of intact vessel wall at baseline (Fig. 5E). 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 $39.46 \pm$

7.91 per mm² in the lesion, a frequency that was 84.7% of a healthy spinal cord ($p > 0.05$; Figs. 4F, 5E).

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 *i.c.* MMI-DsRed2, a fluorescent syngeneic mouse medulloblastoma cell line derived from *Patch*^{+/-}/*p53*^{-/-} mice, into three *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 (Figs. 5F–5H). 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 mm² 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 (Figs. 3F, 4F, 5E). 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.

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 gray matter in the cortex, respectively (Figs. 3F, 4F). 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 periepithelial APCs in the lamina propria of *Cx3cr1*^{+GFP} mice (Lelouard et al., 2012). The consistent observation of CX3CR1⁺ APC behavior in multiple tissue types under 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 underappreciated, as these processes may be too fragile to sustain tissue manipulations that are required for traditional histological analysis techniques (Chieppa et al., 2006; Lelouard et al., 2012). In the current study, however, we have been able to successfully preserve these processes by transcardial PFA tissue fixation procedure either with or without glutaraldehyde and confirmed their presence by IHC and EM (Fig. 2).

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 models, 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 thinned-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 (Xu et al., 2007; Drew et al., 2010; McGavern & Kang, 2011). However, the thinned-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 the 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 (Supplementary Fig. 1). 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 suboptimal surgery procedure or endured visible vessel trauma. Although both imaging methodologies can induce some degree of tissue injury, both cranial window and thinned-skull approaches revealed ramified parenchymal CX3CR1⁺ cells at baseline, consistent with CNS-resident APCs in a nonactivated state (Figs. 1, 3A, 3B, 4A, 4B; Supplementary Fig. 1). Regardless of the basal level of inflammation due to surgical trauma, the frequency of intravascular extension by parenchyma CX3CR1⁺ cells was in good agreement with that seen in the gut (Niess et al., 2005; Lelouard et al., 2012) and increased with additional tissue inflammation in EAE but not in spinal trauma or tumor inoculation (Figs. 3F, 4F, 5E, 5I). More importantly, we were able to capture intraluminal extension of CX3CR1⁺ cells in naive, noninflamed brain tissues by IHC and EM (Fig. 2). 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 (Huang et al., 2003) provided clear fluorescent signals to consistently delineate the interface between the blood content and the vessel wall throughout the prolonged duration of intravital imaging (Supplementary Fig. 3). We used a combination of 3D 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 (Jung et al., 2000; Huang et al., 2006) or from Ly6C^{lo}/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 (Supplementary Fig. 2C) (Imai & Kohsaka, 2002; Kanazawa et al., 2002), 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 (Saederup et al., 2010) or crossing *CD11c-mCherry* mice (Khanna et al., 2010) 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/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 (Carson et al., 2006; Galea et al., 2007a). 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 (Galea et al., 2007b; McGavern & Kang, 2011; Sallusto et al., 2012). In these models, both blood-derived (i.e., monocytes/ macrophages/dendritic cells) and CNS-resident (microglia) APCs are implicated in the reactivation of infiltrated lymphocytes in the perivascular CSF space (Goverman, 2009; Sallusto et al., 2012). This implies APCs play an ancillary role in the recruitment of initial immune cell invasion because 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⁺ APCs extend intravascular dendritic projections directly into an intact CNS vessel lumen is highly significant, as it suggests an opportunity for (1) antigen surveillance directly in the blood vessel 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 fluorescence microscopy with two-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⁺ cells possess the capacity of inserting part of their cellular extensions through an intact CNS blood vessel 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 that such a 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A.Y.H. conceived the experiments. D.S.B., T.A.E., J.M., and A.R. executed the experiment and analyzed the data. D.S.B., T.A.E., J.M., A.R., J.S., and A.Y.H. contributed to writing the manuscript.

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Figure 1.

Intravital microscopy reveals persistent intravascular CX3CR1⁺ dendritic projections into intact CNS vessels. **A–C:** A low-power snapshot from Supplementary Movie 1 is shown in image **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 μ m. A few CX3CR1⁺ cells are found in close proximity to the blood vessel (red) (**B–D**, Supplementary Movie 2). **B:** Fluorescence and 3D surface rendering of a snapshot from inset in image **A** at time = 0 min of Supplementary Movie 2, demonstrating intravascular dendritic insertion (white arrows) of extravascular CX3CR1⁺ cells (green) through an intact CNS vessel (red). Scale bar = 15 μ m. **C:** Fluorescence and 3D

surface rendering of the same cells as in image **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 μ m. **D**: Extravascular CX3CR1⁺ cells with ramified morphology displayed projections (white arrows) into the vessels (red) on day 12 after EAE induction, while a nonramified, elongated perivascular CX3CR1⁺ cell nearby did not (asterisk) and were not included in the final analysis. Scale bar = 10 μ m. **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 μ m. Only CX3CR1⁺ cells in the parenchyma were analyzed in images **A–E** (Supplementary Fig. 2).

Figure 2.

IHC and EM confirmation of intravascular dendritic projections by CX3CR1⁺ cells into intact CNS vessels. **A, B:** IHC of fixed, noninflamed naive *Cx3cr1^{+/GFP}* brain tissues confirmed the presence of dendritic extensions (green) flanked by GFAP⁺ astrocyte end feet (pink) next to vessel lumen as outlined by laminin (blue). Scale bar = 5 μm. **C, D:** IHC of fixed, EAE-induced *Cx3cr1^{+/GFP}* tissues confirmed the presence of dendritic extensions (arrows) by extracellular parenchyma (P) CX3CR1⁺ cells (green) into the vessel lumen (L) as outlined by anti-CD31 (**C**, red) and tomato-lectin (**D**, red) staining. Scale bar = 10 μm. **E, F:** EM of fixed tissue sections of a noninflamed, naive brain from a *Cx3cr1^{+/GFP}* mouse

confirmed the presence of CX3CR1⁺ dendritic processes (green, P) with intact endothelial endothelium (red, En) and basement membrane (yellow, B). The CX3CR1⁺ cell is in close contact with axons (purple, Ax) and astrocyte end feet (blue, A), demonstrating its parenchymal location in the brain. Gold particle: immuno-gold labeling for GFP (arrows, G). Magnification = 18,500 $\times$.

Figure 3.

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 $\times$ *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 μ m. **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 gray. 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 μ m. **D, E:** Coronal fluorescence (**D**) and surface rendering (**E**) view of a blood vessel (red) in image **C** demonstrates an intraluminal dendritic projection (white arrows) by an extravascular CX3CR1⁺ cell (green). Scale bar = 15 μ m. **F:** The number of intraluminal projections is quantified and normalized to total vessel surface area (#Projections/mm²) over the course of EAE induction, showing a twofold increase in projection frequency over the first 12 days. Only CX3CR1⁺ cells in the parenchyma were analyzed (Supplementary Fig. 2). n.s. = not significant.

Figure 4.

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 × 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 μm. **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 gray. 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 μm. **D, E:** Coronal fluorescence (**D**) and surface rendering (**E**) view of a blood vessel (red) in image **C** demonstrates intravascular dendritic projections (white arrows) by extravascular CX3CR1⁺ cells (green). Scale bar = 15 μm. **F:** The number of intraluminal projections is quantified and normalized to total vessel surface area (# projections/mm²) over the course of EAE induction, showing a twofold increase in projection frequency over the first 12 days. Only CX3CR1⁺ cells in the parenchyma were analyzed (Supplementary Fig. 2).

Figure 5.

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 gray. 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 image **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. n.s. = not significant. **F:** A snapshot of the CNS tumor microenvironment 7 days after inoculation of MMI-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 gray. MMI-DsRed signals are removed in images **F–H** for ease of visualizing CX3CR1⁺ cells. **G, H:** Fluorescence (**G**) and surface rendering (**H**) view of a blood vessel (red) in image **F** demonstrates intravascular dendritic projections (white arrows) by extravascular CX3CR1⁺ cells (green). **I:** The number of intravascular projections in the CNS tumor microenvironment is quantified from three 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. 3F and 4F). Only CX3CR1⁺ cells in the parenchyma were analyzed (Supplementary Fig. 2).