Research paper

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

Jiyun V. Kim a,⁎, Ning Jiang a,1, Carlos E. Tadokoro a,2, Liping Liub, Richard M. Ransohoffb, Juan J. Lafaillea, Michael L. Dustin a,⁎

a Helen L. and Martin S. Kimmel Center for Biology and Medicine of the Skirball Institute of Biomolecular Medicine, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA
b Neuroinflammation Research Center, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA

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The mouse spinal cord is an important site for autoimmune and injury models. Skull thinning surgery provides a minimally invasive window for microscopy of the mouse cerebral cortex, but there are no parallel methods for the spinal cord. We introduce a novel, facile and inexpensive method for two-photon laser scanning microscopy of the intact spinal cord in the mouse by taking advantage of the naturally accessible intervertebral space. These are powerful methods when combined with gene-targeted mice in which endogenous immune cells are labeled with green fluorescent protein (GFP). We first demonstrate that generation of the intervertebral window does not elicit a reaction of GFP+ microglial cells in CX3CR1gfp/+ mice. We next demonstrate a distinct rostrocaudal migration of GFP+ immune cells in the spinal cord of CXCR6 gfp/+ mice during active experimental autoimmune encephalomyelitis (EAE). Interestingly, infiltration of the cerebral cortex by GFP+ cells in these mice required three conditions: EAE induction, cortical injury and expression of CXCR6 on immune cells.

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1. Introduction

1.1. Two photon laser scanning microscopy and CNS intravital surgeries

Two-photon laser scanning microscopy (TPLSM) has revolutionized the study of the nervous system and the immune system by providing dynamic views of sub-cellular structures and cells in a tissue context in vivo (Denk et al., 1994; Miller et al., 2003). These methods generally require surgical exposure of the tissue of interest with resulting potential for induction of inflammation, which may contaminate observations. Imaging of the intact CNS has been accomplished in vivo using thinned skull methods, in which imaging is performed through a thin plate of bone without the need for a craniotomy (Grunzleider et al., 2002; Xu et al., 2007). Microscopy of the living mouse spinal cord reported to date requires a laminectomy, the removal of the vertebral bone, with potential disturbance of the underlying dura and the surface of the spinal cord (Engelhardt et al., 2003; Odoardi et al., 2007; Davalos et al., 2008). The exposed spinal cord in this system is vulnerable to sterile injury and foreign materials that may contain innate immune activators such as endotoxin. Such injury has been reported in intravital microscopy observations through a craniotomy, but not with the thinned skull method (Xu et al.,...
The heterozygous mice (CXCR6gfp/+; 2000) in which CXCR6 expressing cells can be tracked have further examined the role of CXCR6 in EAE here. CXCL16 receptor (Matloubian et al., 2000; Shimaoka et al., 2000), CXCR6 (also called BONZO, STRL33 and TYM-STR), the serine and oxidized lipoprotein (SR-PSOX) (Shimaoka et al., 2004). Although CXCL16 also functions as a scavenger receptor cells and myeloid cells via parenchymal vessels. Antibodies to through the choroid plexus (Reboldi et al., 2009). These cells involve the CCR6 dependent entry of Th17 type CD4+ T cells in the spinal cord and the cerebellum (Bettelli et al., 2003; Wensky et al., 2005) and evaluated based on an ascending paralysis, starting with tail weakness, progressing to hindlimb weakness and in most severe cases involving forelimb paralysis. Paralysis is associated with white matter inflammatory lesions in the spinal cord that include CD4+ T cells generally without substantial T cell accumulation in the gray matter. The dynamics of these cells in the intact spinal cord during EAE is not known.

1.2. Role of CXCR6 in EAE

Three chemokine receptors have been implicated in guidance of T cells into the central nervous system. In the steady state, a population of memory T cells enters the cerebrospinal fluid (CSF) via the choroid plexus (Kivisakk et al., 2003, 2004). These cells are highly enriched for expression of CCR7 and active PSGL-1, which suggests these molecules are involved in CNS entry. Recent reports also indicate that the induction of EAE involves the CCR6 dependent entry of Th17 type CD4+ T cells through the choroid plexus (Reboldi et al., 2009). These cells then trigger the CCR6 independent recruitment of additional T cells and myeloid cells via parenchymal vessels. Antibodies to CXCL16 (ligand to CXCR6) reduce EAE severity (Fukumoto et al., 2004). Although CXCL16 also functions as a scavenger receptor referred to as the scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (SR-PSOX) (Shimaoka et al., 2000), CXCR6 (also called BONZO, STRL33 and TYM-STR), the CXCL16 receptor (Matloubian et al., 2000; Shimaoka et al., 2000) could be important in T cell infiltration in EAE. CXCL16 is expressed on potential antigen-presenting cells including dendritic cells (Matloubian et al., 2000), macrophages (Shimaoka et al., 2003) and astrocytes (Ludwig et al., 2005) and, is a chemokine receptor that is expressed in activated CD4+ T cell subsets (Loetscher et al., 1997; Matloubian et al., 2000), NKT cells (Matloubian et al., 2000), and activated CD8+ T cells (Matloubian et al., 2000; Sato et al., 2005). CXCR6 is known to be associated with antigen-specific interferon-γ producing cells in multiple sclerosis (MS) (Calabresi et al., 2002). Therefore, we have further examined the role of CXCR6 in EAE here.

We used mice in which the major coding exon of CXCR6 has been replaced with GFP in gene-targeted mice (Unutmaz et al., 2000) in which CXCR6 expressing cells can be tracked in vivo (Geissmann et al., 2005). The heterozygous mice (CXCR6gfp/+gfp) have half the wild type levels of CXCR6 and express cytoplasmic GFP, while the homozygous mice (CXCR6gfp/gfp or CXCR6−/−) lack CXCR6 expression, but instead express only cytoplasmic GFP (Geissmann et al., 2005). We utilized this mouse strain on the B6 background to study the dynamics of GFP+ cells in the living mouse spinal cord and brain during EAE. CXCR6+ cells migrated rapidly in the spinal cord with a rostrocaudal bias in a CXCR6 independent manner. Unexpectedly, our studies also addressed the molecular basis of T cell infiltration into cortical injury sites in the context of EAE.

1.3. An inflammation model for initiating cortical MS lesions

MS is also characterized by cortical lesions with inflammation which may contribute to inducing direct damage to neurons and neurites (Bo et al., 2003; Geurts and Barkhof, 2008). Cortical demyelinating lesions can be either leukocortical, involving the cortex and the adjacent subcortical white matter, or subpial, involving the first three layers of cortex without associated white matter demyelination (Moll et al., 2008). In very late stage chronic progressive MS cortical lesions contain relatively few T cells. However, recent studies of subpial cortical lesions in relapsing-remitting MS show abundant T cell and macrophage inflammation in the affected gray matter (Moll, N., R.M.R., B. Kelley, J.E. Parisi, and C. Lucchinetti, personal communication). Gray matter lesions could precede cognitive deficits and increase the morbidity of MS (Geurts and Barkhof, 2008). Perry and colleagues developed a rodent model for cortical inflammation based on stereotactic injection of heat killed bacillus Calmette-Guérin (BCG) in the parenchyma and subcutaneous inoculation with complete Freund’s adjuvant, which contains heat killed Mycobacterium tuberculosis (MTB) (Matyszak and Perry, 1998). Either stimulus alone was insufficient to induce demyelination (Matyszak and Perry, 1995). In mice, one can image sterile cortical injury through a thinned skull window (Grutzendler et al., 2002), but this appears to be a local reaction, much like that to injection of BCG into the parenchyma. Injury in the context of inflammation elsewhere in the tissue may lower the threshold of the blood brain barrier through effects of cytokines (Schnell et al., 1999; Sun et al., 2004).

Therefore, since classical EAE models in the mouse do not demonstrate cortical lesions, we examined recruitment of T cells in response to the combination of EAE and laser injury in layer 1 barrel cortex (Davalos et al., 2005).

2. Materials and methods

2.1. Transgenic mice

CXCR6+/−/fl, CXCR6gfp/gfp and CX3CR1−/−/fl, and LysMgfp/+ mice were a gift of D.R. Littman (NYU School of Medicine, New York, NY) (Jung et al., 2000; Geissmann et al., 2005) and Thomas Graf (AECOM, Bronx, NY) (Faust et al., 2000), respectively. All strains were backcrossed onto C57BL/6 for at least 12 generations and housed in specific pathogen-free conditions in accordance with Institutional Animal Care and Use Committee protocols of New York University School of Medicine.

2.2. Peptides

The Dana-Farber Cancer Institute Molecular Biology Core Facility (Boston, MA) synthesized peptides.
2.4.3. Intravital spinal cord (See Fig. 1 and Results (3.1) for details of the method.)

We anesthetized mice and exposed the thoracic spine by dissecting the overlying muscle and connective tissue. We bent a thin stainless steel plate with an open central slot to separate the inner edges of the plate to engage two adjacent vertebrae in a funnel clamp and then immobilized the plate by attaching it to two posts using screws. We then acquired Images through the thinned intervertebral connective tissue sealed above with 2% low melting agarose (Sigma, St. Louis, MO) and a cover glass over the area.

2.4.2. Thinned skull and laser injury

In all the intravital imaging described below, we maintained anesthetized mice on warming plates and the intravital window through an objective heater to maintain core temperature at 37 °C. We performed thinned skull intravital window surgeries and laser ablation as previously described (Grutzendler et al., 2002; Davalos et al., 2005). The size of injury induced was typically 15 µm in diameter. We hydrated mice with scores >2 daily and sacrificed for score ≥4.

2.4.1. EAE

We induced classical EAE with MOG peptide in CFA and pertussis toxin (Mendel et al., 1995). Briefly, we injected 100 µg MOG (35–55) peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA with heat killed mycobacterium tuberculosis (4 mg/ml) at the base of the tail on day 0 and pertussis toxin (100 ng in sterile saline) into the tail vein on days 0 and 2. We scored mice for EAE as follows: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, fore limb weakness; 5, moribund. We hydrated mice with scores >2 daily and sacrificed for score ≥4.

2.3. Quantitative PCR

We perfused anesthetized mice with PBS containing 2 mM EDTA. We isolated brain and spinal cord and snap froze in liquid nitrogen and stored in −80 °C until further processing. We isolated RNA from tissue with TRIzol (Invitrogen, Carlsbad, CA), and treated with DNase I from which we generated cDNA using Superscript III enzyme system (Invitrogen) and amplified with ABI 7900 cycler (Applied Biosystems, Foster City, CA). The primer and probe oligonucleotides used for CXCL16 were, cxcl16-forward, ggaagccagacaccttggt, cxcl16-reverse, ttggtgtgaacacttcccc and cxcl16 probe, [6-fam]ttgagcgcaaa-gagttgaacctgtg[5 TAMRA]. We used HPRT and G6PD for internal control housekeeper genes, HPRT-forward, aagttgcaagcttggt, HPRT-reverse, tgaagtacctataagtggaagg, and HPRT probe, [5TET]tgggataccgacagctgtttgat[TAMRA], and G6PD-forward, tgaagctccctgatgcctat, G6PD-reverse, caagcttgctggt, HPRT-reverse, tgaagtactcattatagtcaagggc, and G6PD probe [5TET]gaagctccctgatgcctat, and G6PD probe [5TET]gaagctccctgatgcctat, and G6PD probe [5TET]gaagctccctgatgcctat, and G6PD probe [5TET]gaagctccctgatgcctat. Cycle temperatures were 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s. We performed melting curve analyses at the genomics core facility of the NYU Cancer Institute.

2.4. Surgery and experimental procedures

2.4.3. Intravital spinal cord (See Fig. 1 and Results (3.1) for details of the method.)

We anesthetized mice and exposed the thoracic spine by dissecting the overlying muscle and connective tissue. We bent a thin stainless steel plate with an open central slot to separate the inner edges of the plate to engage two adjacent vertebrae in a funnel clamp and then immobilized the plate by attaching it to two posts using screws. We then acquired Images through the thinned intervertebral connective tissue sealed above with 2% low melting agarose (Sigma, St. Louis, MO) and a cover glass over the area.

2.3.1. Intervertebral spinal cord microscopy preparation

We first devised an inexpensive apparatus to immobilize and create an intervertebral window to image the intact spinal cord in a live anesthetized mouse (Fig. 1). We manipulated standard double edge razor blades to generate a spinal cord clamp and mounting system with correct dimensions for an adult mouse. We assembled the apparatus in three stages: in the first stage, a small stack of 2–3 cemented blades served as a spring clamp to hold the vertebral column; in the second stage, we immobilized this bottom plate by cementing to a transverse blade that was firmly attached to two posts to a position resulting in a stabilized mouse; and in the third stage, a small amount of low melt agarose further stabilized the intact dura and the underlying spinal cord for high-resolution imaging. Bending the inner tabs at 45° below the plane generated a spring clamp to hold the spinal cord (Fig. 1A). A midline dorsal incision exposed the thoracic muscles over T3 to T7, which are dissected free from the sides of the vertebral bone in an anesthetized mouse. The bent bottom plate separated the tabs, which in turn can grip either side of the vertebral bone T3–T7 vertebrae (Fig. 1B). We then cemented a second plate composed of two razor blades transversely to the bottom plate such that the large central opening is aligned over the intervertebral space. We then secured the top plate to two magnetic posts on either side of the mouse that are positioned to partly lift the thoracic region off the underlying heating plate to reduce effects of breathing movements on the stability of the spinal cord (Fig. 1C). We can then carefully peeled the intervertebral fascia leaving the intact dura (~50 µm) to protect the spinal cord and further dampen the
spinal cord motions by sealing with a drop of low melt agarose on the dura and a coverglass (Fig. 1D). The curvature of the mouse thoracic spine resulted in a very natural posture of the anesthetized mouse in the apparatus (Fig. 1E). The white matter to either side of the central vein was visible through the intervertebral window after removal of the fascia and application of the agarose and coverslip (Fig. 1F and G). We verified that microglial cells in CX3CR1+/gfp mice did not show evidence of surface injury (Supplementary Fig. 1) and that neutrophils from LysMgfp/+ mice did not arrest inside the vessels or extravasate spontaneously over a 2-hour observation period (data not shown). With this method we isolated the inflammation of our disease model, EAE, with minimal artifacts induced by surgical trauma.

3.2. CXCR6 is not required for EAE-associated ascending paralysis

To study the behavior of inflammatory lymphocytes within the intact spinal cord and cerebral cortex we characterized reporter mice in which GFP replaces CXCR6. First, we determined the course of EAE in CXCR6gfp/+ and CXCR6gfp/gfp C57BL/6 mice compared to the wild type (WT) by inducing EAE with MOG (35–55) peptide in CFA. EAE disease course was similar between all three groups up to day 21 (Fig. 2A), with mean onset of disease at day 9.7±1.25 (WT), 8.9±1.5 days (CXCR6gfp/+) and 9.0±1.6 (CXCR6 gfp/gfp) (Fig. 2C), mean maximal score of 2.75±0.75 (WT), 2.7±0.6 (CXCR6gfp/+) and 2.7±0.9 (CXCR6gfp/gfp) (Fig. 2D), and proportion responding of 10/10 (WT), 29/29 (CXCR6gfp/) and 20/22 (CXCR6gfp/gfp) (Fig. 2E), respectively. We further followed EAE disease up to 50 days between CXCR6gfp/+ and CXCR6gfp/gfp mice and found no statistical difference (data not shown). Immunohistochemistry and flow cytometry of cells isolated from the spinal cord indicated that GFP+ T cells were CD4+ (Supplementary Figs. 2–5) and were pro-inflammatory since less than 5% expressed FOXP3 (data not shown). Although 2/22 animals in the CXCR6gfp/gfp group remained completely healthy, this was not statistically significant when compared to

Fig. 1. Intervertebral intravital window. (A) 2–3 dulled razor blades are cemented together to produce a more stable steel plate. Inner wings of the blade are bent to serve as funnel clamps. (B) This bent plate is then inserted on either side of the thoracic spinal vertebral bone in an anesthetized mouse after cutting the thoracic muscles on either side of the spine. (C) A second flat plate is glued perpendicularly to the first to immobilize the mouse to the magnetic posts on either side of the holding plate. (D) The resultant anatomy and intravital window imaged through the objective is illustrated in this schematic. The intervertebral space is dissected down to leave the dura layer of about 50 µm and 2% agarose in aCSF is overlayed to further dampen any organ movement without inducing pressure. A microscope coverglass then seals the intravital window for which water or oil immersion objectives can be used. (E) The anesthetized mouse is placed under the objective on a 37 °C surface with low flow oxygenation mask. The objective heater also provides a close temperature control to the intravital window. (F and G) The intervertebral space with the spinal cord and the posterior spinal vein are translucently visible under the coversglass.
the heterozygous mice. These healthy mice were not included in the analysis or imaging experiments due to concerns that there may have been problems with the inoculation given that the other 20 animals had a very similar disease course to the CXCR6gfp/+ . We concluded that CXCR6 is not required for ascending paralysis in this model of EAE and thus these mice can be used to study the migratory behavior of inflammatory T cells in the spinal cord.

### 3.3. T cell movement is biased in the spinal cord

The GFP expression by the CXCR6gfp/+ and CXCR6gfp/gfp mice enabled imaging the behavior of inflammatory T cells in the spinal cord of living mice with EAE and to examine whether CXCR6 contributes to migration patterns. We imaged the spinal cord through the intervertebral space in the thoracic spine area by TPLSM in CXCR6gfp/+ (n = 10 animals) and CXCR6gfp/gfp (n = 5) mice with EAE (Fig. 3A and B; Movie 1). GFP+ cells were mostly confined to the spinal cord white matter based immunofluorescence histology (Supplementary Fig. 2). Two-photon imaging in perfused thick sections demonstrated that GFP+ cells also rarely penetrated into the underlying gray matter, which can be distinguished from white matter based upon differential autofluorescence (Supplementary Fig. 3). Furthermore, the GFP+ cells were positive for CD4 but negative for CD8, NK1.1, CD11b and CD11c (Supplementary Figs. 2, 4 and 5). There were no significant differences in numbers of GFP+ cells or their movement between CXCR6gfp/+ and CXCR6gfp/gfp mice: the mean speeds were 4.9 μm/min and 4.7 μm/min (Fig. 3C), the mean arrested fractions were 22.2% and 26.2% (Fig. 3D), the mean con

### 3.4. Gray matter infiltration in EAE requires CXCR6

In contrast to MS, the monophasic mouse EAE model does not display gray matter lesions (Zamvil and Steinman, 1990). Therefore, it is not surprising that GFP+ cells were not detected in the cortical gray matter while imaging through thinned skull windows of CXCR6gfp/+ or CXCR6gfp/gfp mice with EAE. One reason for the lack of subpial cortical lesions in EAE could be the relatively shorter time frame of the EAE process compared to that of MS. We hypothesized that in humans the neuroinflammation associated with MS may be combined in a long time-frame of small, sterile injuries in the cerebral cortex, which may in combination serve as a nidus for cortical lesions in MS. In order to model the effects of small sterile injury in the context of EAE, we applied a previously established injury model in which we induced acute gray matter injury using a brief exposure to radiation from the femtosecond pulsed laser (Davalos et al., 2005). We reported that such microscopic injuries do not disrupt the blood–brain barrier or induce leukocyte infiltration in healthy mice (Kim and Dustin, 2006). Consistent to this previous finding, there was no T cell infiltration to gray matter parenchymal injury focus in healthy mice based on the absence of GFP+ cells detected through thinned skull windows by TPLSM in injured CXCR6gfp–/– mice over 7 days observation period (data not shown). We chose a stable period of EAE (days 16–20) to monitor the injured brain intravitally through the thinned skull window using TPLSM in anesthetized mice after inducing a single sterile focal laser injury (∼15 μm in diameter). We found that in mice with EAE, injury caused a significant infiltration by GFP+ cells around the injury site in the cortex of CXCR6gfp–/– mice, but not, in CXCR6gfp/gfp mice. 3D rendering of such an injury site

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**Fig. 2.** CXCR6 and EAE course. (A) CXCR6 did not affect the course of EAE at the onset, active (days 0–15) and remission phases (days 20–52) (scores ± s.d.) for C57BL/6 WT (n = 10) CXCR6gfp/+ (n = 29) and CXCR6gfp/gfp (n = 22) aged 9–12 weeks. (B) Day of onset defined by the day on which the score ≥ 1 (day ± s.d.). (C) The mean maximum score for each genotype (score ± s.d.). (D) The fraction of animals developing EAE, which was 10/10 WT, 29/29 CXCR6gfp/+ and 20/22 of the CXCR6gfp/gfp (the results here are not statistically significant by Pearson’s Chi-square test).
revealed subpial infiltration of GFP+ cells around the laser injury (arrow) (Fig. 4A). We excluded that meningeal inflammation induced by repeated thin skull procedures contributed to the GFP+ cell infiltration by quantifying cells that are farther than 60 μm from the skull (i.e. not in the meninges) (Fig. 4B,D) (Kim and Dustin, 2006). GFP+ cell recruitment began as early as...
24 h after injury, peaked between days 4 and 10 in 4 different CXCR6gfp/+ mice with EAE score 1–2, and then waned to basal levels by day 21 after injury (Fig. 4D). In contrast, the same type of injury did not recruit GFP+ T cells into the parenchyma of CXCR6gfp/gfp mice with a similar EAE disease score to the CXCR6gfp/+ mice (Fig. 4C,D). The increased infiltration of GFP+ cells into cortical injury sites in mice with EAE compared to healthy mice may be due to increased abundance of GFP+ effector cells in circulation in addition to increased permeability of the blood brain barrier induced by circulating cytokines from the vaccination site and draining lymph nodes. To test this possibility, we vaccinated CXCR6gfp/+ mice (n=5) with ovalbumin in CFA with pertussis toxin (on days 0 and 2). We then induced laser injury into the barrel cortex on day 7 after vaccination, the peak of this response, and found no infiltration of GFP+ cells to the injured focus under these circumstances. Therefore, the accumulation of GFP+ cells in the gray matter after laser injury requires both CXCR6 and CNS inflammation. This result is consistent with work by Perry and colleagues (Matyszak and Perry, 1995, 1996a,b, 1998), in which BCG implanted in the CNS did not elicit a T cell response unless mice were vaccinated with CFA in the periphery.

3.5. EAE induces CXCL16 expression in the brain

Although the small size of the subpial laser lesion precluded characterization of the GFP+ cells in the injury focus by histology, global CXCL16 expression in the brain was evaluated by qPCR (Fig. 4E). CXCL16 mRNA from whole brain tissue at various days after EAE induction and injury in CXCR6gfp/+ and CXCR6gfp/gfp mice (n=3 mice per bar graph). Controls are mice without EAE on days 15 and 26.
dramatically during EAE in both CXCR6<sup>gfp</sup>/+ and CXCR6<sup>gfp/gfp</sup> mice with or without laser injury. Therefore, CXCL16 mRNA is increased in EAE brain tissue, but was not sufficient on its own to induce CXCR6<sup>+</sup> immune cell infiltration. T cell accumulation in the cortex required an additional event, provided in our study by the sterile laser injury.

### 3.6. CXCR6<sup>+</sup> cells decelerate and become confined close to the injury focus

The dynamics of the accumulated GFP<sup>+</sup> cells in the gray matter was analyzed by 3D tracking of cell movements by TPLSM through the thinned skull. Inspection of the movies revealed that the GFP<sup>+</sup> cells formed a highly dynamic swarm around the injury site with cells farther from the injury moving faster than cells close to the injury, which were sometimes arrested (Fig. 5). The average speed of the distant cells was statistically indistinguishable from that of the cells in the spinal cord at 4.7±2.5 (day 4 post injury) and 5.4±1.6 (day 10 post injury) μm/min (mean ± s.d.) (Fig. 5C) but with less confinement than in the spinal cord with a confinement index of 0.69±0.21 (day 4 post injury) and 0.67±0.26 (day 10 post injury) (Fig. 5D). In contrast, the cells within 50 μm of the injury site decelerated to 2.2±1.1 (day 4 post injury) and 3.1±1.9 (day 10 post injury) μm/min (Fig. 5C) and were more restricted in movement than both the cells outside the 50 μm radius of injury and those in the spinal cord with a confinement index of 0.42±0.26 (day 4 post injury) and 0.38±0.28 (Fig. 5D).

### 4. Discussion

We report a new intravital apparatus and methodology, both simple and inexpensive, for TPLSM of the intact mouse spinal cord. We then used this apparatus and the related thinned skull methods for intact cerebral cortex to examine the role of CXCR6 in the MOG (35–55) EAE-B6 mouse model. There was no difference in disease course or intravital GFP<sup>+</sup> cell dynamics in the spinal cord between CXCR6<sup>gfp</sup>/+ and CXCR6<sup>gfp/gfp</sup> mice. However, we report for the first time a preference for rostrocaudal movement by the infiltrating T cells within the living spinal cord in both genotypes. Surprisingly, a reversible recruitment of CXCR6<sup>gfp</sup>+, but not CXCR6<sup>gfp/gfp</sup> T cells, was induced in response to a focal sterile injury inside the gray matter of the brain parenchyma during EAE. Finally, we demonstrate that CXCL16 expression is upregulated in the brain during EAE, but that this signal is not sufficient to induce gray matter infiltration by GFP<sup>+</sup> immune cells in CXCR6<sup>gfp/+</sup> mice without additional conditions established by a small sterile injury.

#### 4.1. Spinal cord intravital window

Our surgical strategy was practical for visualizing the intact spinal cord. The thoracic spine curvature is naturally amenable for maximizing the size of the intervertebral space. This method is both accessible and adoptable in any laboratory; it is inexpensive and does not require prolonged surgical training. A minor disadvantage compared to laminectomy is that the small field of view that limits the tracking distance (maximum ~300 μm). However, we have successfully extended the rostrocaudal field of view on either end of the intervertebral space for an additional ~300 μm by thinning adjacent laminar bones in lieu of laminectomy to produce an intravital window through the bone similar to the thinned skull procedure (Grunzendorf et al., 2002) (JVK, unpublished findings). The quality of imaging is similar through the thinned bone and the

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**Fig. 5.** Dynamics of GFP<sup>+</sup> T cells in the gray matter. (A and B) TPLSM 40 μm z-stack through thin skull revealed a laser injury of about 15 μm in diameter at 50 μm below the brain surface induced on day 17 of EAE in a CXCR6<sup>gfp/+</sup> mouse. GFP<sup>+</sup> cells are recruited to the gray matter injury focus on day 10 after injury: GFP<sup>+</sup> cells in green, vasculature in red, bone in blue, and the center of the autofluorescent injury site marked with a yellow asterisk in A. A and B are the same images with tracks superimposed for injury proximal and distal cells (corresponds to Fig. 4A and Movie 2). C, D — Plots of cell speed and confinement coefficient as a function of distance from the injury site (n = 3 mice, each category). Close and far indicate within and greater than 50 μm from injury focus.
intervertebral space. A second limitation of this method is that due to the extensive dissection of most of the dorsal muscles to expose the sides of the vertebral bone we can only recommend it as a terminal procedure not amenable to longitudinal studies. An apparatus that can hold the same points in the vertebral bone, but not require extensive dissection of the muscle tissue could allow longitudinal studies in the mouse spinal cord. A third limitation is that the intravital window examines the spinal cord under thoracic rather than lumbar spine, where the most highly disease-correlated lesions are found (Mendel et al., 1995). Therefore, our findings of T cell dynamics may not reflect T cells in the disease-correlated EAE foci in the lumbar spine. However, on staining for myelin basic protein (MBP) in the EAE afflicted mice used in our studies, we found diminished MBP staining in the white matter extending into the thoracic spinal cord regions (data not shown). Thus, our imaging does reflect areas that are infiltrated to an extent that leads to demyelination, although not as severe as in the lumbar region. By imaging thoracic rather than lumbar areas we may observe T cell dynamics at the advancing edge of lesions in EAE rather than the core of demyelinated foci. A fourth limitation is that the gray matter of the spinal cord cannot be imaged as they lie deeper than 1 mm below the surface.

4.2. Rostrocaudal migrational bias

We report for the first time that the effector T cells migrate preferentially along the rostrocaudal spinal cord axis during EAE. There may be several mechanisms for this phenomenon. One possibility is that T cells may be migrating along myelinated axons or their extracellular matrix sheaths, which are oriented along the rostrocaudal axis and this anatomical environment of the spinal cord may play a similar role in guiding T cell migration in the spinal cord as the stromal cell network in lymph nodes (Bajenoff et al., 2006). Alternatively, rather than be guided, barriers to lateral movement in the white matter may confine T cells. It is difficult to distinguish these possibilities in the absence of direct visualization of guiding or confining elements. The stromal elements in the spinal cord white matter are oriented with the fiber tracts paralleling the predominantly rostrocaudal migration pattern we observe for GFP+ cells (Peters et al., 1991; Huang et al., 2007). T cells may then preferentially encounter APCs that are positioned along these tracks. This biased migration guided or confined by oriented fiber tracks provides a mechanism for spread of inflammation within the spinal cord during EAE (Peters et al., 1991; Huang et al., 2007). We note that previous intravital TPLSM publications using laminectomy to expose the rat spinal cord did not report this striking T cell behavior thus far (Kawakami et al., 2005; Odoardi et al., 2007). There are at least three possibilities for this difference. First, we are not using a T cell transgenic system and the observed infiltrating T cells in the spinal cord are presumably polyclonal with a spectrum of affinities for locally expressed mMHC. Therefore, we may be seeing a higher percentage of lower affinity T cells and bystander T cells, which do not arrest significantly on antigen presenting cells and thus emphasize scanning behavior. Second, the laminectomy performed in the rat model may disrupt this pattern of movement. Consistent with this, Engelhardt and colleagues reported that their laminectomy intravital spinal cord preparation was stable for only ~30 min (Engelhardt et al., 2003), suggesting inflammatory changes from the procedure at longer times that may increase T cell arrest. Third, we may be visualizing this axial motion more readily because the thoracic spinal cord is infiltrated by T cells, but suffers less severe damage than the lumbar region, in which the rostrocaudal tracks may be destroyed by advanced demyelination.

Another possibility for the axial bias in T cell migration may be due to newly formed extracellular matrix (ECM) structures and cells in inflamed spinal cord. For example, during LCMV meningoitis ER-TR7+ cells on pial surface are extensively infected and also appear to serve as a substrate for T cell migration (Kim et al., 2009). In other examples, in both human MS tissue (Mohan et al., 2008) and mouse CNS toxoplasmosis brain (Wilson et al., 2009), T cells adhere to inflammation-induced ECM. Such ECM and cellular networks induced in inflamed CNS tissue could affect the biased T cell migration we observed in this study and constitute an interesting body of future research to explore in terms of examining sequestrations of specific adhesion and chemokine molecules to these structures (Bajenoff et al., 2006).

4.3. CXCR6 is not required for EAE

We have shown that CXCR6 deficient mice were equally susceptible to EAE as CXCR6 positive mice and displayed normal levels of T cell infiltration into the spinal cord. In this study we have not examined maintenance of EAE in relapsing and remitting mouse models of EAE and therefore these should be examined for future studies. In contrast to our results with CXCR6, anti-CXCL16 antibodies reduce EAE severity (Fukumoto et al., 2004). Taken together, this suggests that the effects of anti-CXCL16 are not mediated by blocking interaction with CXCR6. Therefore, the effect of CXCL16 may be mediated by its other function as a scavenger receptor for phosphatidylserine on the surface of apoptotic cells, as well as, for oxidized lipids, which are increased in injured tissue (Shimaoka et al., 2000). Antigen presenting cells may utilize CXCL16 in uptake and processing of myelin-associated antigens for MHC class II-mediated presentation, which may be critical steps for disease progression and epitope spreading (Vanderlugt et al., 1998). CXCL16 expression is 10-fold higher in spinal cord than brain during EAE. This may be attributed to accumulation of inflammatory cells in the spinal cord, including CD11b+ macrophages, which are known to have high levels of CXCL16 (Matloubian et al., 2000; Fukumoto et al., 2004; Ludwig et al., 2005). However, inflamed endogenous cells such as astrocytes (Ludwig et al., 2005) and endothelial cells (Hofnagel et al., 2002) may also contribute to this difference. Under inflammatory conditions, astrocytes can to a lesser extent than microglia transform into active antigen presenting cells in vitro (Aloisi et al., 2000) and are certainly capable of innate immune activation (Suh et al., 2007). The elevated CXCL16 levels in the EAE may help to recruit CXCR6+ cells across the blood brain barrier into injured gray matter foci but additional unknown signals provided by injury are required for grey matter infiltration. Our imaging study provides an interesting cellular homing behavior to new injury foci in EAE. Based on our findings and the current body of research literature we have discussed many possibilities of future studies to explore the exact mechanism for both the infiltration and the slower and
confined T cell dynamics adjacent to the gray matter injury in EAE mice.

4.4. CXCR6 is required for T cell entry into injured the gray matter during EAE

A limitation of most mouse EAE models is that gray matter lesions are not observed. In MS, gray matter involvement correlates with more severe disease. MRI and pathology findings demonstrate that gray matter infiltration can be significant in patients with relapsing-remitting disease (Audoin et al., 2007; Lassmann et al., 2007) and dendritic cells are located in the gray matter in chronic active disease (Cudrici et al., 2007). Cortical pathology in early relapsing MS has been demonstrated both by neuropathological evaluation and by magnetic resonance imaging studies showing gray matter atrophy at this stage of disease (Bo et al., 2006; Summers et al., 2008). Recent results demonstrate that subpial cortical lesions display marked T cell accumulation early in MS (Moll, N., R.M.R., B. Kelley, J.E. Parisi, and C. Lucchinetti, personal communication) — this is often carried out by comparing human MS patients MRI atrophy levels that follow previously infiltrated areas (Rudick et al., 2009).

To determine if sterile injury can occur to promote gray matter infiltration, we have extended the EAE mouse model by inducing small focal injury followed by monitoring of CXCR6-GFP$^+$ cells. Our study suggests that CXCR6 could have a potential role in gray matter infiltration in the context of MS.

CXCR6 was required for the accumulation of activated T cells in the injured cerebral cortex in the presence of EAE. It is not known how T cells enter these injury sites since we did not directly observe the entry process. However, although it is also possible that the T cells migrated from the nearby meninges or over a greater distance from the underlying white matter, it is most likely that recruitment was through parenchymal microvessels (A. Flügel, N. Kawakami, personal communications). CXCR6-dependent accumulation may arise from increased entry, decreased egress, or could also arise from increased survival as previously shown for NKT cells (Geissmann et al., 2005). This response in the cortex was not sustained and resolved over a period of weeks. This suggests that the immune cell infiltration at the cortical injury site does trigger a sustainable cycle of tissue injury and recruitment.

An ongoing controversy surrounds the role of trauma and its relationship to triggering or worsening of EAE and MS (Phillips et al., 1995; Jung et al., 2000; Marcondes et al., 2005; Ling et al., 2006). Weller and colleagues reported exacerbated disease following cortical cryolesions. However, these large injuries appear to disrupt the blood brain barrier since activated CD8$^+$ cells are capable of entering cryolesioned brain parenchyma independent of local antigen presence (Ling et al., 2006). It is established at least in two rodent models that injury trigger disease. In a MBP based mouse EAE model, spinal cord injury initiates disease (Marcondes et al., 2005). Antigen-specific T cell infiltration occurs along Wallerian degeneration sites where microglia express class II molecule I-A (Konno et al., 1990; Mollleston et al., 1993). In the latter model, the blood brain barrier is not affected by the injury similarly to our model. Although injury has less effect in provoking inflammation in the brain than in the spinal cord and the brain (Schnell et al., 1999), systemic inflammation can alter immunological barrier and therefore the effect of CNS injury in part mediated by elaborations of in situ cytokine productions (Sun et al., 2004; Teeling and Perry, 2009). The small laser injury in the layer 1 barrel cortex in our hands is the smallest injury reported to date in EAE rodent models and demonstrated recruitment of CXCR6$^+$ cells to the gray matter of mice afflicted with EAE, although not surprisingly given the size of injury, with no apparent side effects in the mouse or statistically significant changes in EAE scores (data not shown). We note, however that the injury site in the barrel cortex might lead to sensory changes near the whiskers, which is difficult to assess.

5. Conclusions

Our new intravital spinal cord method revealed a biased T cell migration in EAE spinal cord. The mechanisms remain to be further examined. Our results on CXCR6 suggest a two hit model for vulnerability of the cerebral cortex to T cell infiltration. CXCL16 expression pattern in the CNS suggests that spinal cord infiltration spills over into the brain. In fact, early T cell recruitment takes place through the choroid plexus, which supplies the CSF to the brain and spinal cord (Reboldi et al., 2009). CXCL16 expression in the EAE brain is not itself sufficient to mediate T cell infiltration. Similarly, laser injury alone, which sets up a cascade of astrocyte and microglial responses (Davalos et al., 2005; Kim and Dustin, 2006) is also insufficient to recruit T cells into the parenchyma. However, when both of these hits are present, robust CXCR6 dependent T cell infiltration is observed. This may be due to a lowered threshold for traversing the blood brain barrier. The prevalence of CXCR6 and CXCL16 expression in human clinical specimens should be further evaluated as a first step to assess the potential for anti-CXCR6 based therapies in treating and/or preventing gray matter involvement in MS and promoting recovery of function by reducing inflammatory T cell infiltration (De Jager and Hafler, 2007). Laser injury is but a simple maneuver that was convenient in our mouse model, but we emphasize that its relationship to cortical lesions in MS is not established and therefore the clinical significance of our results in terms of therapeutic targeting of CXCR6 in treating or preventing cortical inflammation in MS will need further investigation.

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Author contribution
JVK and MLD designed and analyzed most of the experiments and JVK performed most of the experiments. Blinded scoring in all EAE mice for Fig. 2 was performed by NJ. JVK designed the intervertebral space surgery. CET designed the spinal cord clamp and performed immunohistochemistry experiments in Supplementary Figs. 2, 4 and 5. RMR, JJL, LL and MLD advised, analyzed and helped to write the manuscript. JVK and MLD wrote the manuscript.

Appendix A. Supplementary data
Supplementary data and movies associated with this article can be found, in the online version, at doi:10.1016/j.jim.2009.09.007.

References


