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SUPPLEMENTARY INFORMATION

Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation

Dimitrios Davalos, Jae Kyu Ryu, Mario Merlini, Kim M. Baeten, Natacha Le Moan, Mark A. Petersen, Thomas J. Deerinck, Dimitri S. Smirnoff, Catherine Bedard, Hiroyuki Hakozaki, Sara Gonias Murray, Jennie B. Ling, Hans Lassmann, Jay L. Degen, Mark H. Ellisman, Katerina Akassoglou

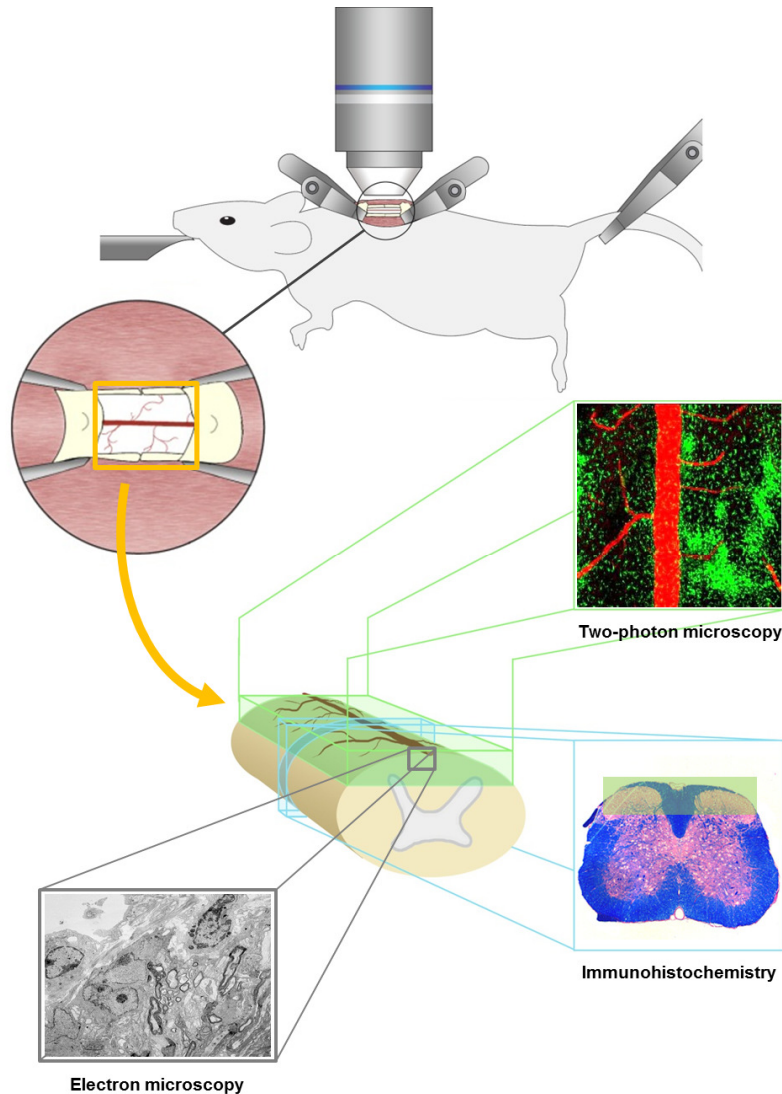
CORRESPONDENCE: Katerina Akassoglou, Ph.D., Gladstone Institute of Neurological Disease, University of California, San Francisco, San Francisco, CA, 94158, Tel. 415-734-2512; Fax. 415-355-0827; Email: kakassoglou@gladstone.ucsf.edu

This file includes:

Supplementary Figures S1-S4

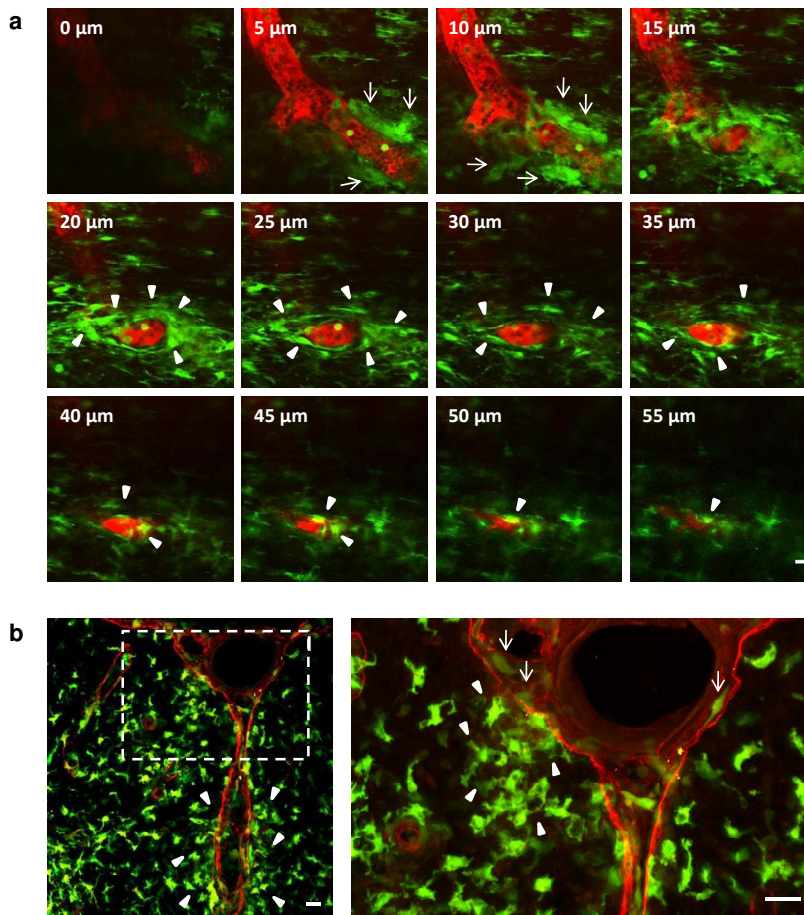
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Supplementary Figure S1. Schematic representation of the correlated analysis approach of the same spinal cord segments by different imaging and tissue processing modalities.



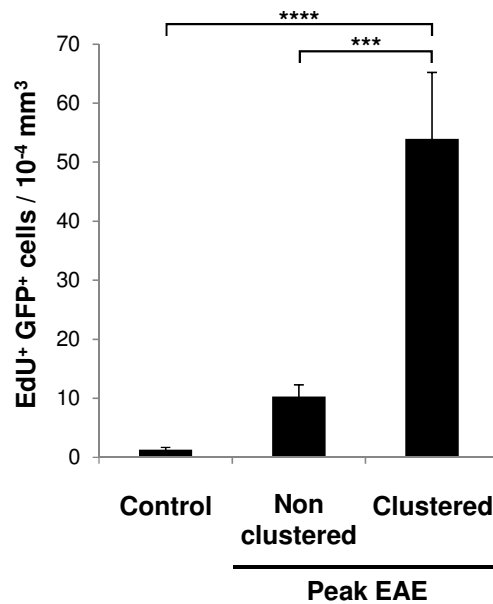
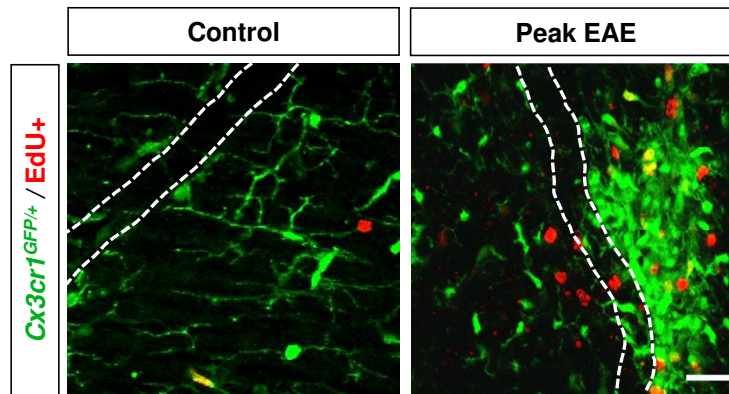
Supplementary Figure S1. Healthy controls and mice at different stages of EAE were imaged in vivo using two-photon microscopy and an imaging method that we previously developed. The block of spinal cord that was imaged in vivo (orange box in round inset) was excised after perfusion and processed for immunohistochemistry or electron microscopy. All the correlated analyses in this study were performed exclusively in corresponding spinal cord areas previously imaged in vivo (green volume in spinal cord explant). The top part of the schematic was adapted from ref. 12 (reproduce with permission from Elsevier, copyright 2008).

Supplementary Figure S2. Spatial relationship between GFP-positive cells in *Cx3cr1*^{GFP/+} mice and the vasculature at the peak of EAE.



Supplementary Figure S2. (a) Single-plane analysis of perivascular clusters in the spinal cord of *Cx3cr1*^{GFP/+} mice at the peak of EAE. Serial xy-planes from a representative z stack showing the spatial relationship between the spinal cord vasculature (red) and a cluster (green) imaged in vivo at different depths. The sequence starts at the surface of a pial blood vessel (0 μm). The pial blood vessel appears in longitudinal orientation at depths of 5–10 μm . As the depth increases, the pial vessel branches inward and becomes a penetrating vessel that appears in cross-section in the spinal cord parenchyma (15–45 μm), where it further branches into smaller diameter vessels and capillaries (50–55 μm). Based on the anatomy of the spinal vasculature, the perivascular cluster consists of pial macrophages (arrows) next to the longitudinal pial vessel and parenchymal microglia (arrowheads) surrounding the vascular branches that penetrate the spinal cord parenchyma (10–55 μm). Scale bar, 10 μm . (b) Immunohistochemical staining of the basal membranes with a pan-laminin antibody demonstrates the perivascular clustering of microglia and pial macrophages outside the parenchymal basement membrane (arrowheads). Inset also shows some macrophages expressing low levels of GFP in the Virchow-Robin space (arrows). Scale bars, 50 μm .

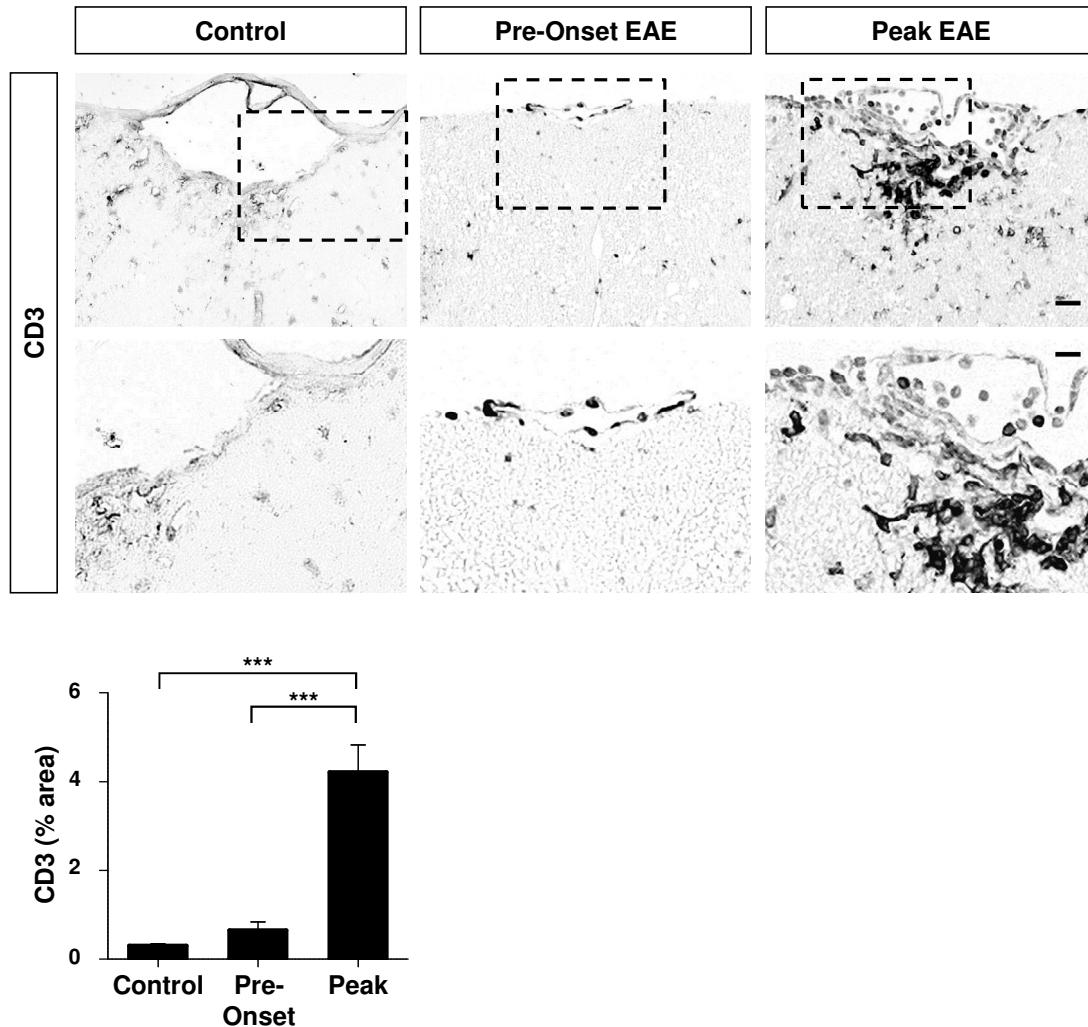
Supplementary Figure S3. Local cell proliferation within the GFP⁺ clusters.



Supplementary Figure S3. Cell proliferation progressively increased within the clusters at peak of EAE. A small number of GFP⁺ EdU⁺ cells was detected in healthy spinal cords. Values are mean \pm SEM for n= 4 control, n=8 peak EAE mice. ***P < 0.001, ****P < 0.0001 (one-way ANOVA). Scale bar, 20 μ m.

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Supplementary Figure S4. Microglial perivascular clustering precedes T cell infiltration in the EAE spinal cord parenchyma.



Supplementary Figure S4. Correlated immunohistochemical analysis in spinal cord sections from areas previously imaged in vivo. Minimal CD3 immunoreactivity is detected in healthy controls. In mice before the onset of EAE while microglial clusters start forming, T cells appear mostly in the lumen of some blood vessels while at the peak of disease they show extensive infiltration of the spinal cord parenchyma. Values are mean \pm SEM for n= 4 control, n=6 pre-onset EAE and n=8 peak EAE mice. ***P < 0.001 (one-way ANOVA). Scale bars, top: 20 μ m, bottom: 10 μ m.