ONLINE SUPPLEMENT

Neutrophil recruitment to the brain in mouse and human ischemic stroke

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1) Supplementary Methods

Experimental brain ischemia

Focal brain ischemia was induced by permanent occlusion of the right middle cerebral artery (MCA) in mice, using two different techniques: either by cauterization of the distal portion of the MCA (c-MCAo) (n=43), or by filament intraluminal MCA occlusion (il-MCAo) (n=28). Anesthesia was induced with 4 % isoflurane in a mixture of 30% O_2 and 70% N_2O and it was maintained with 1-1.5 % isoflurane in the same mixture by the aid of a facial mask. The cauterization technique was carried out as previously reported [43]. Briefly, after drilling a small hole in the cranium at the level of the distal portion of the MCA, the artery elevated with a hook, was occluded by cauterization and was cut to ensure permanent blood flow interruption. Flow obstruction was visually verified. Animals showing subdural hemorrhages or signs of incorrect surgery were immediately excluded from the study (n=2 mice). After surgery, animals were allowed to recover from the anesthesia and were studied at different time points ranging from 10 min to 15 days following c-MCAo. None of the mice died during the study. For sham-operation (n=7), all surgical procedures were carried out but the MCA was not cauterized and the mice were killed at 24 hours. To exclude any effects due to cauterization, in a small group of mice (n=3) we carried out ligature of the MCA (8/0 black-braided silk suture) instead of cauterization, and the mice were studied 24 hours later.

Permanent il-MCAo was induced as previously reported [13], with modifications. Anesthesia was induced with 4 % isoflurane in a mixture of 30 % O_2 and 70 % N_2O and maintained with 1–1.5 % isoflurane in the same a mixture with a facial mask. A longitudinal cut was made in the ventral middle line of the neck to expose the right carotid territory. Next, the submaxillary glands and the omohyoid and sternohyoid muscles were separated, exposing the carotid vessels. The thyroid artery and occipital arteries were thermocoagulated. The right common carotid artery was firmly tied with a 6/0 silk suture and a 7/0 nylon

monofilament blunted at the tip (Suturas Arago, Spain) was introduced through the internal carotid artery to the level where the MCA branches out. The drop of cerebral blood flow was assessed with laser Doppler flowmetry (Perimed, Sweden). After surgery, the mice were kept under a heating lamp for approximately 30 min until they recovered from the anesthetic and they were killed 6, 15 or 24 hours after the occlusion. Three mice died before 24 hours after il-MCAo. Sham-operated mice (n=4) were subjected to surgery but the filament was only briefly introduced into the external carotid artery and was immediately removed, and mice were killed after 24 hours. Control mice (n=4) were naïve mice not subjected to surgery or anesthesia.

MRI was carried out in some of the animals to assess the infarction at 24h. MRI was performed in a 7.0 T BioSpec 70/30 horizontal animal scanner (Bruker BioSpin, Ettlingen, Germany), equipped with a 12-cm inner diameter actively shielded gradient system (400 mT/m). The receiver coil was a phased array surface coil for mouse brain. Mice were placed in a supine position in a Plexiglas holder with a nose cone for anesthesia administration (isoflurane in a mixture of 30% O_2 and 70% N_2O), fixed with a tooth and ear bars, and adhesive tape and maintained under controlled temperature during the acquisition period. Tripilot scans were carried out for accurate positioning of the animal's head in the isocenter of the magnet. T2 relaxometry maps were acquired with a multi-slice multi-echo acquisition sequence with 16 effective echo times from 11 to 176 ms, TR=4764 ms, FOV=20x20x6 mm^3, matrix size 256x256x18 pixels and spatial resolution 0.078x0.078x0.5 mm^3/pixel. Data were processed using Paravision 5.0 software (Bruker).

Isolation of cells from tissues

Mice were anesthetized and transcardially perfused with 40 mL saline. The ischemic brain tissue (ipsilateral to the MCAo) and the corresponding mirror regions of the non-ischemic hemisphere (contralateral) were dissected out and analyzed separately. For the c-MCAo

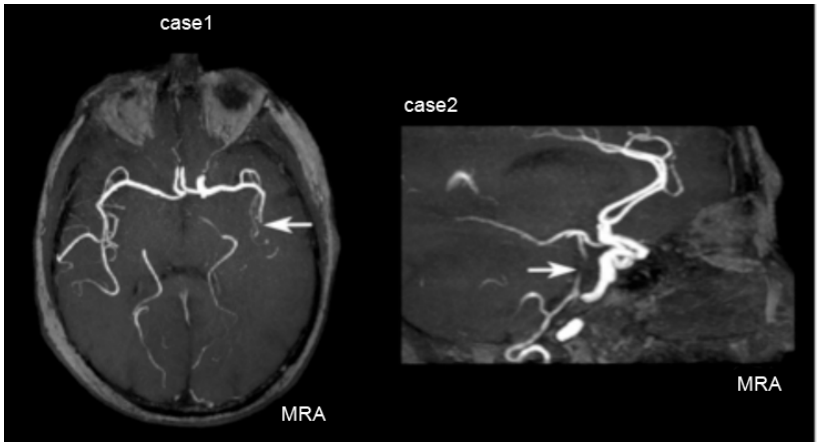
model the cortex was dissected out, whereas for the il-MCAo model the dissected tissue included the cortex and the striatum. The tissue was incubated for 30 minutes at 37°C in 2 mL of RPMI 1640 medium (Life Technologies S.A., Alcobendas, Madrid, Spain) containing 100 U/mL collagenase IV and 50 U/mL DNAse I. Brain tissue was passed through a tissue grinder and cells were recovered after centrifugation at 400 x*g* for 10 min at RT and separated from myelin and debris in 70 % and 30 % isotonic Percoll gradient (GE Healthcare) prepared in Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Samples were centrifuged at 1,000 x *g* for 30 min without acceleration or brake. Cells were collected from the interface, washed once with HBSS, and processed for flow cytometry.

Flow cytometry

Isolated brain cells were washed with fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline, 2 mM EDTA, 2% FBS), incubated at 4 ºC for 10 min with FcBlock (1/200; Clone 2.4G2; BD Pharmingen), and incubated with primary antibodies in FACS buffer for 30 min at 4ºC. The antibodies used were rat anti-mouse CD11b (clone M1/70, Alexa Fluor 647, BD Pharmingen), CD45 (clone 30-F11, FITC, BD Pharmingen), and Ly6G (clone 1A8, PE-Cy7, BD Pharmingen). Isotype controls were rat IgG2bκ (clone A95-1, Alexa Fluor 647 or FITC, BD Pharmingen), rat IgG2a (FITC, Hycult Biotech), and rat IgG2aκ (clone R35-95, PE-Cy7, BD Pharmingen). Data acquisition was carried out in a BD FacsCantoII cytometer (BD Biosciences) using the FacsDiva software (BD Biosciences). Cells were morphologically identified by linear forward scatter (FSC-A) and side scatter (SSC-A) parameters. Data analysis was carried out with FlowJo software (version 7.6.5, TreeStar Inc., Ashland, OR, USA). Again, cells were plotted on forward versus side scatter and single cells were gated on FSC-A versus FSC-H linearity. Flow-Count Fluorospheres (Beckman-Coulter) were used for absolute cell number quantification.

2) Supplementary Figures

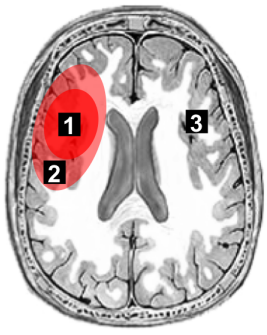
Supplementary Figure 1. Imaging of the intracranial occlusion site of patients (arrows).



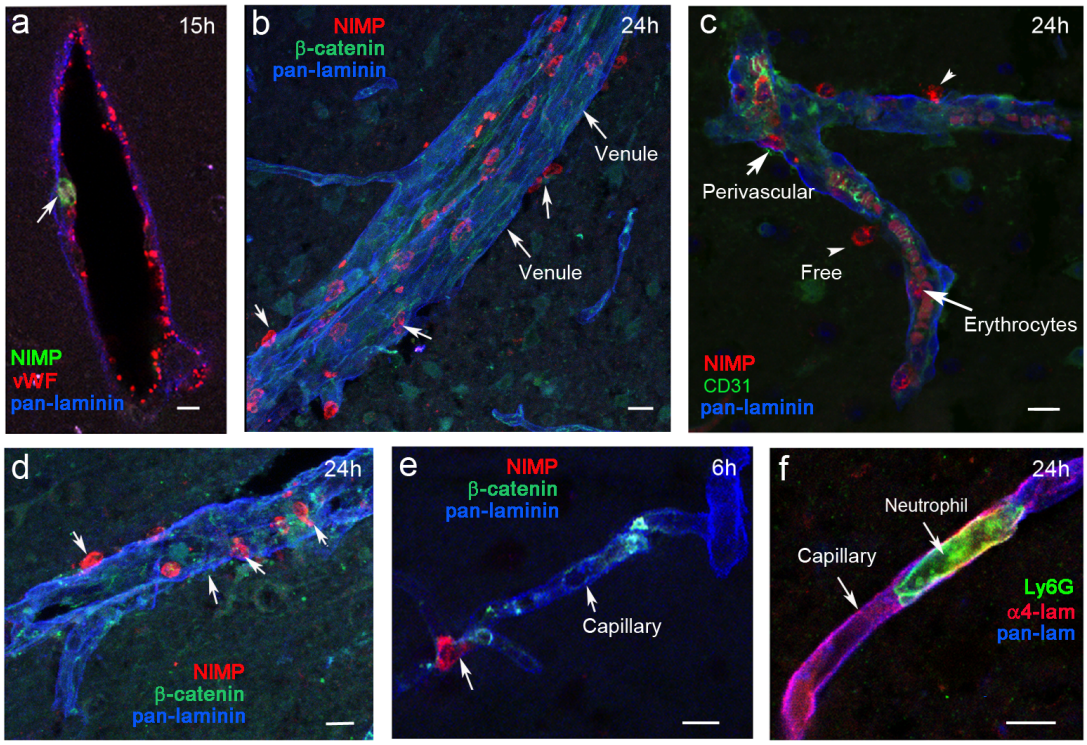
Images were obtained within the first day after stroke onset. Case 1 had a branch occlusion (M2) in the left middle cerebral artery. Case 2 had an occlusion of the middle segment of the basilar artery. MRA: magnetic resonance angiography.

Supplementary Figure 2. Schematic representation of the human brain regions that where dissected out.

The exact anatomic regional location varies from patient to patient depending on infarct localization. (1) Is the core of infarction, (2) is the periphery, and (3) is a non-ischemic region distant from the affected core.

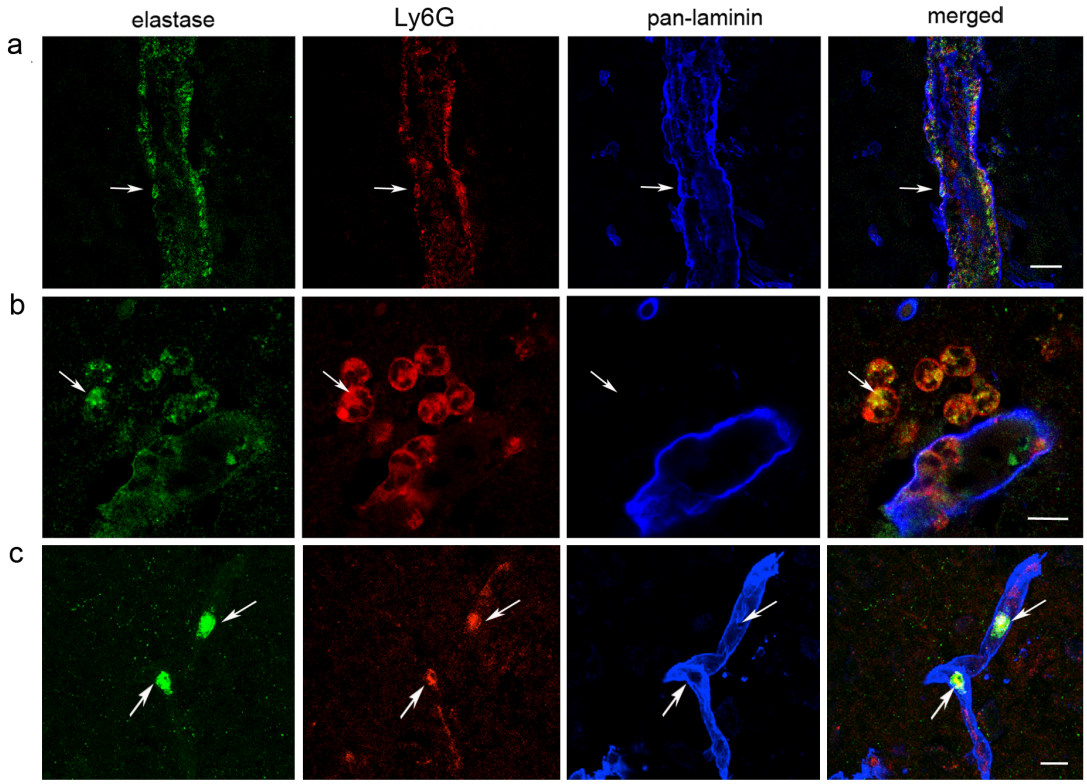


Supplementary Figure 3. Neutrophils in post-capillary venules and in the lumen of capillaries.



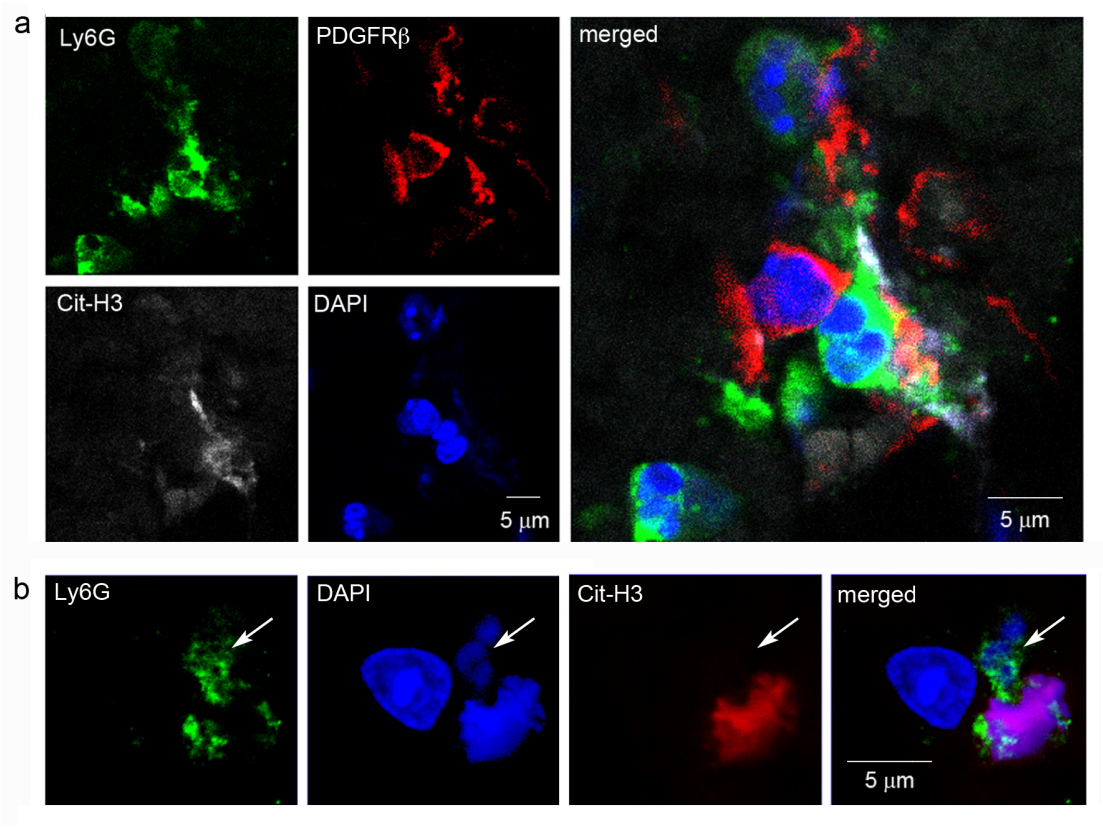
(a-e) Cauterization c-MCAo model and (f) intraluminal il-MCAo model. NIMP-R14+ or Ly6G+ cells, as indicated in each panel, are located in venules (a-d) or in the capillary lumen (e,f). Images are representative of n=9 mice for the c-MCAo model (n=3 at 15 hours and n=6 at 24 hours), and n=8 for the il-MCAo model. Bar scale: 10 μm.

Supplementary Figure 4. Ly6G+ cells express elastase**.**



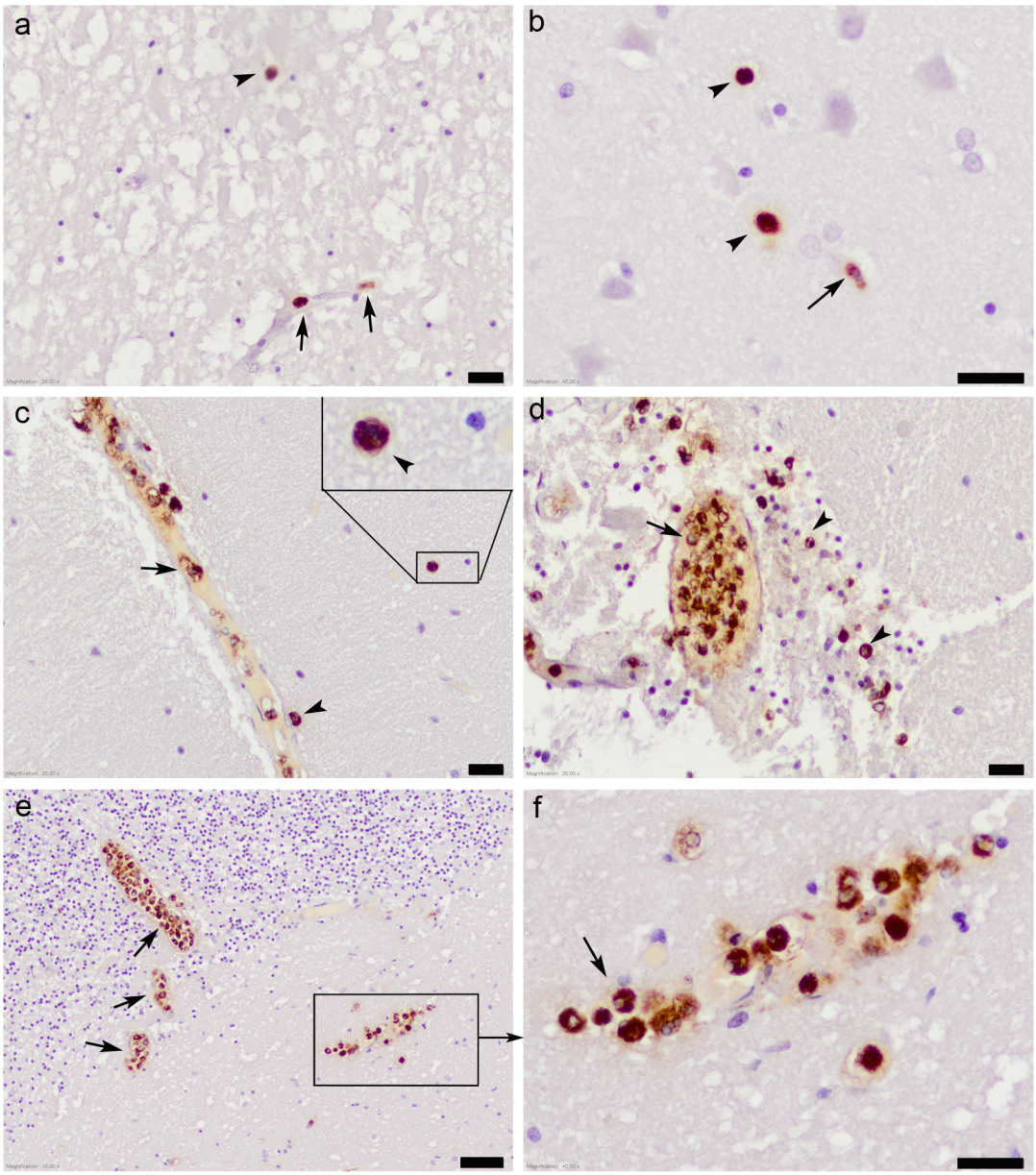
(a-b) Images are representative of mice for the cauterization c-MCAo model (n=6) and (c) intraluminal il-MCAo model (n=8). Ly6G+ cells are elastase+. Double stained cells are seen in perivascular spaces (a), extravasated in the parenchyma (b), and intravascularly (c). Bar scale: a, 25 μm; b-c, 10 μm.

Supplementary Figure 5. Signs of NET formation 24 hours after c-MCAo.



a) Perivascular neutrophils (green, Ly6G) nearby a pericyte (red, PDGFRβ) positive for Cit-H3 (white). DNA is shown in blue (DAPI). b) Neutrophil (green, Ly6G) showing Cit-H3 (red) in the nucleus (blue, DAPI) and expulsion of DNA (arrow). b) Images show two cells, one of them is Ly6G+ (green), shows a Cit-H3+ reaction, and expels cellular material (arrow) and DNA (blue, DAPI). Images are representative for n=6 mice 24h after c-MCAo. Bar scale: 5 μm.

Supplementary Fig. 6. Myeloperoxidase staining in human stroke tissue.



Myeloperoxidase (MPO) positive cells (dark brown) in paraffin sections of infarcted regions counterstained with hematoxylin (blue). Arrows indicate vascular or perivascular MPO+ cells and arrowheads point to MPO+ cells apparently free in the ischemic parenchyma. a, b) show the ischemic cortex in the temporal lobe region of patient number 1. c-f) show the ischemic cerebellum of patient number 2, illustrating MPO+ cells in a large vessel and in the parenchyma (c), in the leptomeninges proximal to infarction (d), and perivascularly in vessels of the infarcted cerebellar parenchyma (e, f). Inset in c corresponds to x3 magnification. Inset in e is shown at higher magnification in f illustrating a vessel in the infarcted cerebellar white matter. Bar scale: a-d, f: 20 μm; e: 50 μm.