Temporally distinct myeloid cell responses mediate damage and repair after cerebrovascular injury

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Cerebrovascular injuries can cause severe edema and inflammation that adversely affect human health. Here, we observed that recanalization after successful endovascular thrombectomy for acute large vessel occlusion was associated with cerebral edema and poor clinical outcomes in patients who experienced hemorrhagic transformation. To understand this process, we developed a cerebrovascular injury model using transcranial ultrasound that enabled spatiotemporal evaluation of resident and peripheral myeloid cells. We discovered that injurious and reparative responses diverged based on time and cellular origin. Resident microglia initially stabilized damaged vessels in a purinergic receptor–dependent manner, which was followed by an influx of myelomonocytic cells that caused severe edema. Prolonged blockade of myeloid cell recruitment with anti-adhesion molecule therapy prevented severe edema but also promoted neuronal destruction and fibrosis by interfering with vascular repair subsequently orchestrated by proinflammatory monocytes and proangiogenic repair-associated microglia (RAM). These data demonstrate how temporally distinct myeloid cell responses can contain, exacerbate and ultimately repair a cerebrovascular injury.

The cerebral vasculature is sealed by a barrier system that isolates the central nervous system (CNS) from systemic circulation\textsuperscript{1}. The blood–brain barrier (BBB) comprises endothelial cells, basement membrane, pericytes and the glia limitans that control the exchange of cells and substances between the CNS and the circulatory system. The CNS vasculature is susceptible, however, to damage resulting from mechanical forces, degenerative processes and ischemia. Cerebrovascular diseases can have devastating outcomes, which is exemplified by ischemic disorders like stroke, the second leading cause of death and the leading cause of disability in humans worldwide\textsuperscript{2}.

The importance of cerebral vasculature in maintaining CNS homeostasis is demonstrated by the adverse effects associated with other barrier-disruptive disorders, including traumatic brain injury (TBI), primary intracranial hemorrhage (ICH) and hemorrhagic transformation following stroke. For example, TBI can substantially disrupt the cerebrovascular network by widening intercellular junctions between endothelial cells and promoting swelling of perivascular astrocytes as well as by hemorrhage\textsuperscript{3}. Vasogenic edema is another contributor to secondary damage following vascular injury and can induce cerebral herniation, a potentially fatal condition resulting from fluid-induced compression of brain tissue\textsuperscript{4}. Following ischemic stroke, the initial injury is attributed to reduced regional cerebral blood flow\textsuperscript{5,6}. However, secondary damage can occur upon vascular reperfusion, such as BBB breakdown, hemorrhagic transformation and severe brain swelling associated with invasion by peripheral myelomonocytic cells\textsuperscript{7,8}.

Vascular damage is a feature of many CNS pathologies, but most TBI and stroke models evaluate net outcomes of multiple injurious processes and attempt to isolate the contribution of vascular damage to these outcomes. Consequently, the specific contribution of cerebrovascular disruption and subsequent leakage of materials from the blood into the CNS is not well understood. To better understand this process, we developed a model of isolated cerebrovascular injury using a combination of ultrasound and intravenously (i.v.) injected microbubbles (MBs) that allowed us to apply injurious mechanical forces to select beds of brain vasculature. We then used this model to study the complete spatiotemporal progression of the injurious process, from the initial moments following vascular damage to the induction of angiogenic repair programming and subsequent restoration of neurological function.

Results

Intraparenchymal hemorrhage is associated with cerebral edema. Intraparenchymal hemorrhage is associated with TBI, ICH and stroke. Evaluation of these patients by magnetic resonance imaging (MRI) revealed that intraparenchymal hemorrhage promotes T2-weighted (T2) fluid-attenuated inversion recovery (FLAIR) hyperintensities that surround the hemorrhagic lesion, a pattern consistent with the development of edema (Fig. 1a). To gain additional insights into this pathological process, we focused on patients who develop cerebrovascular hemorrhage and edema on a more defined timescale. Specifically, we evaluated 30 patients with large vessel occlusion (LVO) acute ischemic stroke who underwent...
endovascular thrombectomy (EVT), with largely successful recanalization (87% with thrombolysis in cerebral infarction (TICI) 2B or 3) (Supplementary Table 1). In this cohort, 16 patients had no hemorrhage after embolectomy, six had hemorrhagic infarction (petechial blood), and eight had parenchymal hematoma. The presence of a parenchymal hematoma was associated with an increased volume of injured tissue, as evidenced by diffusion-weighted imaging (DWI) hyperintensities and elevated cerebral edema revealed by T2-FLAIR hyperintensities (Fig. 1b–d and Supplementary Table 2). Before EVT, the average volume of DWI hyperintensity did not differ between the groups but increased significantly after EVT in patients who developed a parenchymal hematoma compared to those who did not (75 cm³ versus 15.6 cm³) (Fig. 1c,d). This parameter remained elevated in patients with parenchymal hematoma at 1 d and 5 d post-EVT. The edema volume followed a similar pattern, showing a significant increase in the patients with parenchymal hematoma at both 1 d and 5 d post-EVT (Fig. 1c,d). These data demonstrate that hemorrhagic transformation following EVT is associated with significant brain injury and edema.

**Murine model of cerebrovascular injury.** Because cerebrovascular injury and secondary edema is common in humans, we developed an animal model to study these events, in which cerebrovascular damage could be controlled temporally and spatially. To this end, we applied low-intensity pulse ultrasound (LIPUS) to the thinned skull windows of mice that received an i.v. injection of MBs (Extended Data Fig. 1). This new approach induced reproducible cerebrovascular damage beneath the thinned skull window (Fig. 1e,f). Following treatment with LIPUS and MBs, intraparenchymal hemorrhage was immediately visible through the thinned cranial skull windows of mice that received an i.v. injection of MBs (Extended Data Fig. 1). This new approach induced reproducible cerebrovascular damage beneath the thinned skull window (Fig. 1e,f). Following treatment with LIPUS and MBs, intraparenchymal hemorrhage was immediately visible through the thinned cranial window (Extended Data Fig. 2a) and was evident macroscopically (Extended Data Fig. 2b). Ex vivo MRI confirmed the presence of targeted intraparenchymal hemorrhage that depended on the location of the thinned skull window. We induced damage in either the frontal lobe (anterior model) or in the parietal lobe and underlying midbrain (posterior model) (Fig. 1e). Intravital microscopy following sonication injury revealed the destruction of capillary vessels and Evans blue (EB) leakage within the parenchyma (Fig. 1f and Supplementary Video 1) and rapid cerebral swelling that distorted cortical structures (Supplementary Video 2) as well as injury of glia limitans astrocytes (Fig. 1g and Supplementary Video 3). Cerebrovascular damage in the frontal lobe was not fatal, whereas damage to parietal lobe or midbrain was uniformly fatal (median survival of 2 d; Extended Data Fig. 2c). In both models, water content increased in the injured hemisphere (Extended Data Video 3) and Supplementary Video 6). Because inhibition of P2RY12 receptors, primarily focused on microglia, decreased the total volume of rosette structures by 68.9%, whereas inhibition of connexin 43 hemichannels, required for the release of ATP by astrocytes, reduced rosette formation by 97.3% at 20 min post-injury (Fig. 2c and Supplementary Video 6). Because inhibition of P2RY12 receptors or CX43 hemichannels resulted in blockade of microglia rosette formation, we next evaluated the effect this had on BBB integrity. P2RY12 receptor or CX43 hemichannel inhibition increased extravasation when comparing inhibitor-treated to vehicle control-treated mice as measured by EB (Fig. 2g, Extended Data Fig. 3a,b and Supplementary Video 7). To confirm the role of CX43 hemichannels

**Microglia extend processes that contain the leaky cerebrovascular injury.** To study the innate immune response to cerebrovascular injury, we performed intravital microscopy. We initially focused on microglia by imaging Cx3cr1GFP/−/− mice. We observed that microglia projected their processes toward injured blood vessels immediately after injury (within 20 min), forming tubular structures that completely enveloped vessels. We refer to these structures as ‘rosettes’. Over the ensuing 24 h, microglia further transformed into an ameboid morphology while maintaining their position around damaged vessels (Fig. 2a and Supplementary Video 4). Microglia appeared to contain these leaking blood vessels, a theory supported by confocal images showing microglia processes walling off fibrin clots throughout injured brain tissue within an hour of injury (Fig. 2b). A functional role for microglia in this injury paradigm was established by feeding mice with chow containing the colony-stimulating factor 1 receptor (CSF1R) inhibitor PLX3397 for a month. Intravital imaging studies revealed that microglia-depleted mice had 6.3-fold more extravasation, as shown by EB, 20 min post-injury, relative to untreated controls (Fig. 2c,d and Supplementary Video 5). This result was replicated when a different CSF1R inhibitor, PLX3622, was used for microglia depletion (Extended Data Fig. 3a,b). Microglia depletion also resulted in a loss of lesion containment, with fibrin distributed throughout the injured brain parenchyma instead of being confined to small focal clusters as seen in control mice at 24 h (Extended Data Fig. 3c,d). In addition, enhanced extravasation of lysozyme M-GFP (LysM-GFP) myelomonocytic cells from the blood was observed immediately after injury in microglia-depleted mice (Extended Data Fig. 3e,f), which was associated with elevated expression of Il1m, Vcam1 and Ccl2 in CNS vasculature (Extended Data Fig. 3g,h) and a shift in the expression of inflammatory genes at 24 h (Extended Data Fig. 3i and Supplementary Table 3). Microglia depletion resulted in decreased expression of Il1m, Il1b, Cxcl10, Ccl2, Tlr2, Pigs, Rela, Bdkrb1 and Il6 and increased expression of Il1a, Icam1, Vcam1 and I6 (Extended Data Fig. 3i). To identify the mechanism underlying microglia rosette formation, we applied small molecule inhibitors transcranially to block purine signaling as described previously. Inhibition of P2RY12 receptors, primarily focused on microglia, decreased the total volume of rosette structures by 68.9%, whereas inhibition of connexin 43 (CX43) hemichannels, required for the release of ATP by astrocytes, reduced rosette formation by 97.3% at 20 min post-injury (Fig. 2c,f and Supplementary Video 6). Because inhibition of P2RY12 receptors or CX43 hemichannels resulted in blockade of microglia rosette formation, we next evaluated the effect this had on BBB integrity. P2RY12 receptor or CX43 hemichannel inhibition increased extravasation when comparing inhibitor-treated to vehicle control-treated mice as measured by EB (Fig. 2g, Extended Data Fig. 3a,b and Supplementary Video 7). To confirm the role of CX43 hemichannels.

![Fig. 1](https://example.com/figure1.png) Parenchymal hematoma is associated with the generation of cerebral edema. a, Representative axial T2-FLAIR MRIs of injuries associated with intracerebral hemorrhage (ICH), depicting the associated injury and cerebral edema (white). b, Representative DWI and T2-FLAIR axial MRIs pre- and post-EVT (0–2 h, 1 d, 5 d) show injury and edema evolution after stroke. The white arrow denotes a hemorrhage. c, Dot plot shows the cube root of DWI or FLAIR hyperintensity values (mean ± s.d.) in a cohort of n = 30 patients (Supplementary Tables 1 and 2) with LVO pre- and post-EVT (p < 0.05, ∗∗p < 0.01; one-way ANOVA with Tukey’s test per time point). HI, hemorrhagic infarction; PH, parenchymal hematoma, type I or II. d, Scatterplot of mean ± s.d. shows evolution of injury volume after stroke and 0–2 h, 1 d and 5 d post-EVT (n = 30, ∗p < 0.05, ∗∗p < 0.01; one-way ANOVA with Tukey’s test per time point). e, Ex vivo T2* multiple gradient echo MRI of a mouse cerebrum 1 d after anterior (top) and posterior (bottom) ultrasound injury. White arrows depict bleeding (black lesions), and the surrounding white signal demonstrates edema. Images are representative of five mice per group. f, Representative maximal projections of two-photon z stacks viewed through the thinned skull of a naive B6 mouse 20 min after injury show vascular injury and EB (red) leakage induced by sonication. Images are representative of five mice per group (Supplementary Video 1). g, Representative two-photon images of GFAP-CreER; StopmilTomato mice 20 min post-injury show destruction of the glia limitans superficialis and perivascularis. Astrocytes are shown in reddish orange, and tomato lectin, injected i.v., is shown in green. Images are representative of two independent experiments with three mice per group (Supplementary Video 3).
in rosette formation, we generated glial fibrillary acidic protein (GFAP)-CreER;Cx43<sup>fl/fl</sup> mice and compared them to wild-type (WT) littermate controls. Adult mice were fed tamoxifen for 4 weeks to delete Cx43 from GFAP-expressing astrocytes and then injured. At 1 h post-injury, the mean number of microglia rosettes per mm<sup>2</sup> was significantly reduced in GFAP-CreER-Cx43<sup>fl/fl</sup> mice relative to controls (Fig. 2h and Extended Data Fig. 4c). In addition, EB leakage was increased 8.4-fold in GFAP-CreER-Cx43<sup>fl/fl</sup> mice relative to the control mice (Fig. 2i, Extended Data Fig. 4d and Supplementary Video 8), confirming the results obtained from the CX43 hemichannel inhibitor. These data demonstrate that astrocytic ATP release and subsequent P2RY12 signaling is responsible for microglia rosette formation and containment of BBB leakage following cerebrovascular injury.

**Peripheral myelomonocytic cells cause cerebral edema.** To determine the role of peripheral innate immune cells after cerebrovascular injury, we conducted a series of imaging and flow cytometric
studies. Confocal imaging of injured brain tissue from LysM<sup>GFP/+</sup> mice revealed clusters of parenchymal myelomonocytic cells at 24 h (Fig. 3a). Intravital microscopy demonstrated that these cells rapidly entered the damaged brain from the blood within 1 h of injury (Fig. 3b and Supplementary Video 9). To profile this infiltrate, we performed flow cytometric analyses of injured brain tissue at days 1 and 6 post-injury in Cx3cr1<sup>GFP/+</sup>; Ccr2<sup>ROPE/+</sup> mice, which revealed an increase in inflammatory monocytes (CCR2<sup>Ly6C<sup>+</sup></sup>), engraving or patrolling monocytes (CX3CR1<sup>Ly6C<sup>+</sup>MHCII<sup>+</sup></sup>) and neutrophils (Ly6G<sup>+</sup>Ly6C<sup>+</sup>) at day 1. Both inflammatory monocytes and neutrophils decreased in number by day 6, whereas engraving or patrolling monocytes were similar in number to those on day 1 (Fig. 3c and Extended Data Fig. 5a). Using intravital microscopy, we visualized rapid extravasation and migration of LysM<sup>GFP/+</sup> myelomonocytic cells into the lesion core and perimeter, which was inhibited by one bolus i.v. injection of anti-lymphocyte function-associated antigen (LFA)-1 and anti-very late antigen (VLA)-4 antibodies (αLFA1/VLA4) immediately after injury (Fig. 3d and Supplementary Video 10). At 1 h post-injury, there were, on average, 179 ± 131.4 LysM<sup>MF</sup> cells per field in the lesion core and 139 ± 78.2 cells in the lesion perimeter. Treatment with αLFA1/VLA4 significantly reduced these numbers to 32 ± 7.8 and 9.7 ± 6.2 cells in the lesion core and perimeter, respectively (Fig. 3e). Flow cytometric analyses performed on injured brain tissue at 24 h confirmed effective inhibition of monocyte and neutrophil invasion by αLFA1/VLA4 (Fig. 3f and Extended Data Fig. 5b).

To evaluate whether myelomonocytic cell invasion contributed to cerebral edema after injury, we quantified brain water content as described previously<sup>1</sup>. Depletion of circulating myelomonocytic cells using anti-GR-1 antibodies prevented cerebral edema in the ipsilateral cerebral hemisphere following anterior or posterior (fatal) injury (Extended Data Fig. 6a). Importantly, myelomonocytic cell depletion also promoted survival in all mice after posterior injury, whereas the mice treated with the isotype control succumbed to the injury in 2–3 d. Administration of αLFA1/VLA4 1 d before injury (αLFA1/VLA4 pretreatment) yielded an identical result (Extended Data Fig. 6b). To evaluate the potential therapeutic benefit of αLFA1/VLA4, we administered a single bolus dose at 0 h, 1 h, 6 h or 12 h post-injury (αLFA1/VLA4 bolus treatment following anterior injury). Treatment at 0 h or 6 h after injury promoted 100% survival, whereas treatment at 12 h resulted in a mortality similar to that observed for the group treated with the isotype control (Fig. 3i). Collectively, these data indicate that fatal cerebral edema can be prevented by administering αLFA1/VLA4 up to 6 h post-injury.

To identify the population(s) of myelomonocytic cells responsible for cerebral edema, we evaluated water content in Ccr2<sup>−/−</sup> mice with reduced numbers of proinflammatory monocytes and neutrophils as well as mice pretreated with anti-Ly6G antibody 1 d before injury (Extended Data Fig. 6c,d). Following both anterior and posterior injury, brain water content was reduced in Ccr2<sup>−/−</sup> mice relative to WT controls but not to the same degree as observed in αLFA1/VLA4-treated mice. Moreover, survival was not significantly extended in Ccr2<sup>−/−</sup> mice relative to WT controls (Extended Data Fig. 6c). Similar findings were obtained with anti-Ly6G antibody treatment following depletion of neutrophils 1 d before injury. Neutrophil depletion decreased cerebral edema relative to control mice but not to the same degree as αLFA1/VLA4 treatment, and this had no effect on survival (Extended Data Fig. 6d). These data suggest that the combined extravasation of monocytes and neutrophils, which are both inhibited by αLFA1/VLA4 (Fig. 3i), is responsible for fatal cerebral edema following cerebrovascular injury.

We next evaluated whether anti-LFA1 and anti-VLA4 antibodies could be used alone to achieve a therapeutic benefit. Treatment with the anti-LFA1 or anti-VLA4 antibody had a highly variable effect on brain water content relative to the effect achieved by combined αLFA1/VLA4 treatment. This was observed in the anterior (Extended Data Fig. 7a) and posterior (Extended Data Fig. 7b) injury models. For example, in the anterior paradigm, the ranges of water content following anti-LFA1 or anti-VLA4 antibody treatment were 0.71–0.86 and 0.75–0.87 (wt/wt), respectively, compared to 0.76–0.77 (wt/wt) in the group treated with αLFA1/VLA4 (Extended Data Fig. 7a). Consistent with this variability, administration of anti-αLFA1 or anti-VLA4 antibody alone conferred no survival benefit (Extended Data Fig. 7c).

To determine the reason for intersample variability following single treatment with the anti-LFA1 or anti-VLA4 antibody, we quantified the expression of intercellular adhesion molecule 1 (ICAM-1, LFA1 ligand) and vascular cell adhesion molecule 1 (VCAM-1, VLA4 ligand) on cerebrovascular endothelia after injury. This was achieved in vivo by injecting fluorescently labeled anti-ICAM-1 and anti-VCAM-1 antibodies i.v. at 24 h post-injury and imaging
the expression pattern by intravital microscopy (Extended Data Fig. 7d). Relative to uninjured control mice, injury markedly increased the cerebrovascular expression of ICAM-1 and VCAM-1 (Extended Data Fig. 7d). The expression pattern of these adhesion molecules varied among vessels, with few vessel segments showing coexpression of ICAM-1 and VCAM-1. Sections of cerebrovasculature tended to express ICAM-1 or VCAM-1 but not both. In addition, there was no correlation between ICAM-1 and VCAM-1 expression when quantified from two-photon images (Extended Data Fig. 7f) or by quantitative (q)PCR (Extended Data Fig. 7g). These data indicate that immune cells have at least two potential non-redundant entry mechanisms to access different segments of cerebrovasculature, which provides a potential explanation for why monotherapy with anti-LFA1 or anti-VLA4 antibody alone is ineffective.
Myelomonocytic cells promote angiogenesis and cerebral repair. Myelomonocytic cells contribute to vascular breakdown and edema following cerebrovascular injury (Fig. 3g–i and Extended Data Fig. 6), but we postulated that they would also be involved in repair. To study the reparative process, we used intravital microscopy to image cortical vasculature at 1 d and 10 d after anterior injury. Before imaging, we injected fluorescently tagged tomato lectin i.v. to label vascular endothelia and EB to assess BBB integrity. At 1 d post-injury, imaging revealed a diffuse extravascular EB signal, signifying BBB breakdown (Fig. 4a). In addition, lectin staining was sparse and poorly defined, demonstrating that cerebral blood vessels were injured and often clotted shut at the injury site. In contrast, at 10 d post-injury, we observed a newly formed, flowing network of cerebral blood vessels that stained with tomato lectin and did not leak EB (Fig. 4a). These vessels, however, were irregular in size and distribution. The vascular coverage at 10 d was 8.1 times greater than that observed in uninjured mice, and the area between blood vessels was 0.61 times smaller (Fig. 4b).

To assess the role of peripheral immune cells in this angiogenic process, we treated mice with αLFA1/VLA4 using two different regimens. Mice either received a single dose of αLFA1/VLA4 immediately post-injury (bolus) or multiple doses beginning immediately after injury and then on days 1, 3, 5, 7 and 9 (continuous). Relative to the group treated with the isotype control, a single injection of αLFA1/VLA4 had no effect on the formation of new blood vessels observed at 10 d. By contrast, continuous αLFA1/VLA4 administration completely blocked vascular remodeling (Fig. 4c,d). At 10 d post-injury, the mean volume of vascular coverage in the group with continuous αLFA1/VLA4 treatment was 0.92 times smaller than that from the group treated with the isotype control, and the space between blood vessels was 17.9 times greater (Fig. 4d). Confocal images confirmed the presence of highly gliotic brain tissue without vasculature in mice after continuous αLFA1/VLA4 treatment when compared to mice treated with the isotype control, in which areas of gliosis were filled with new tomato lectin+ blood vessels (Extended Data Fig. 8a,b). Moreover, continuous αLFA1/VLA4 treatment was associated with a substantial reduction of neuronal nuclei (NeuN)+ neurons and an increased number of type 1 collagen+ cells in the lesion area (Fig. 4e,f). These data demonstrate that continuous but not bolus treatment with αLFA1/VLA4 impedes angiogenesis and promotes neuronal loss after cerebrovascular injury.

We next focused on identifying the specific myelomonocytic cell subset responsible for promoting angiogenesis following cerebrovascular injury. We injected C57BL/6J (B6) mice intraperitoneally (i.p.) with anti-GR-1 antibody (to deplete monocytes and neutrophils) or anti-Ly6G antibody to deplete neutrophils 1 d before injury and on days 1, 3, 5, 7 and 9 post-injury. We confirmed persistent depletion of monocytes and neutrophils with anti-GR-1 antibody and depletion of neutrophils with anti-Ly6G antibody at day 6 after injury (Extended Data Fig. 8c,d). Depletion of myelomonocytic cells with anti-GR-1 antibody prevented cerebrovascular repair at 10 d post-injury, whereas neutrophil depletion with anti-Ly6G antibody did not affect the reparative process (Fig. 4g,h). The importance of GR-1+ cells was especially evident upon analysis of angiogenic gene expression in injured brain tissue by real-time qPCR (Fig. 4i, Extended Data Fig. 9 and Supplementary Table 4).

Peripheral immune cells promote generation of proangiogenic microglia. To further understand the mechanism underlying cerebrovascular remodeling after injury, we sought to determine why interfering with the recruitment of peripheral immune cells led to failed angiogenesis. A role for CNS-resident myeloid cells was uncovered in mice treated with the CSF1R inhibitors. Relative to controls, depletion of CNS myeloid cells with PLX3397 (Fig. 5a) or PLX5626 (Extended Data Fig. 10a,b) blocked cerebrovascular repair and angiogenic gene expression (Fig. 5b, Extended Data Fig. 10c,d and Supplementary Table 4) after injury. The gene...
encoding proangiogenic factor vascular endothelial growth factor A (Vegfa) was among the most significantly downregulated genes at 6 d post-injury in PLX3397-treated mice. Confocal microscopy of brain sections from Cx3cr1GFP/+ mice at 6 d post-injury revealed that VEGF-A localized to clusters of CX3CR1+ myeloid cells (Fig. 5c,d). To identify the specific cell source(s) of VEGF-A, we conducted high-parameter flow cytometry on injured brain tissue from Cx3cr1CreER/++; Stop2/+; TdTomato mice at 1 d and 6 d post-injury. These mice allowed us to distinguish brain-resident from peripheral infiltrating myeloid cells, as described previously15. Using this approach, we determined that VEGF-A expression following injury mapped to a unique subset of brain-resident (TdTomato+) CD45loC.
Prolonged inhibition of peripheral immune cells promotes fibrosis and neurological dysfunction. We lastly focused on the functional consequences of impeding immune-mediated cerebrovascular repair after injury. We again evaluated the effects of continuous αLFA1/VLA4 treatment because of its profound negative impact on cerebrovascular repair. Following cerebrovascular injury, mice were treated with αLFA1/VLA4 or isotype control antibodies for 10 d, and gene expression was measured in injured brain tissue by RNA-seq at 20 d post-injury (Supplementary Table 5). Principal component analysis (PCA) revealed that injured control mice clustered near uninjured mice, whereas αLFA1/VLA4-treated mice were distinctly separate from these two groups (Fig. 6a). A Pearson correlation-based clustered heatmap confirmed the separate clustering of αLFA1/VLA4-treated mice from the other two groups (Fig. 6b). Use of Ingenuity Pathway Analysis (IPA) to assess the concordant dysregulated genes in αLFA1/VLA4-treated mice relative to the other two groups uncovered three interconnected networks of upregulated genes, suggesting dysregulation in brain tissue (Fig. 6c). The most significantly enriched function that emerged from these networks was linked to fibrosis (Fig. 6d and Supplementary Table 6; $P = 1.12 \times 10^{-11}$).

The functional impact of this dysregulation in tissue was evident upon evaluation of the different groups of mice using a Y-maze, which measures cognitive–motor function (Fig. 6e,f). Injured mice receiving either continuous or bolus αLFA1/VLA4 were compared to injured mice treated with the isotype control antibody as well as mice that underwent sham surgery (uninjured) at days 10 and 20. The number of Y-maze gates entered (Fig. 6e) and the triplicate ratio (Fig. 6f) were quantified at these two time points. When compared to sham controls, injured mice receiving isotype antibodies demonstrated a reduction in function at day 10 that returned to control levels by day 20, indicating functional recovery. By contrast, mice that received continuous αLFA1/VLA4 treatment also had reduced function at day 10, but this function did not recover by day 20. Mice receiving a single bolus of αLFA1/VLA4 immediately after injury performed the best on this test, showing the lowest functional deterioration at day 10 and complete recovery by day 20. Collectively, these data demonstrate that sustained interference with the peripheral immune system fosters transcriptional dysregulation, fibrosis and an inability to functionally recover after cerebrovascular injury.

Discussion

Cerebrovascular injury and hemorrhage are serious events that can profoundly affect brain homeostasis and neurological function. By studying patients who had strokes and received mechanical recanalization, we observed that hemorrhagic transformation was associated with substantial cerebral edema, a potentially preventable event that accompanies neurological deterioration post-EVT16–18. The presence of cerebrovascular injury in many CNS disorders led us to develop and study a new model. Our data provide a spatiotemporal map of the damage and the subsequent immune reaction that develops following isolated mechanical disruption of cerebrovasculature. We uncovered a complex network of interrelated myeloid cell responses that contributed to divergent outcomes. In the initial minutes following injury, microglia played a crucial role, sealing off the damaged BBB by projecting processes and enveloping vessels. This rapid response depended on ATP release from astrocytes via CX34 hemichannels and subsequent detection by purinergic receptors (P2RY12). This was followed by a massive recruitment of myelomonocytic cells that promoted fatal cerebral edema. Recruitment depended on differential vascular expression of ICAM-1 and VCAM-1, which was inhibited by therapeutic administration of αLFA1/αVLA4. A single injection of these antibodies within 6 h of injury prevented fatal cerebral edema. However, continuous administration of antibodies impeded cerebrovascular repair. Restoration of cerebrovasculature and preservation of neurons depended on the recruitment of CCR2+ monocytes and generation of proangiogenic microglia at later time points. Prolonged interference with peripheral immune cell recruitment induced fibrotic wound healing associated with neuronal loss, collagen deposition and failure to recover neurological function. These findings provide a comprehensive understanding of how microglia and peripheral myeloid cells respond to cerebrovascular injury and offer guidance for when to apply therapeutic immunomodulatory regimens to reduce cerebrovascular edema while still permitting vascular repair.

Microglia provided the first line of protection after cerebrovascular injury by creating tube-like structures (rosettes) that encased damaged and/or clotted vessels, limiting the degree of leakage into the parenchyma. Microglia used their well-described capacity to detect ATP released from astrocytic CX34 hemichannels in a P2RY12-dependent manner. Projection of microglia processes toward purines released from areas of brain damage was initially described using models of focal laser injury19,20. Lou et al. also demonstrated how microglia can extend processes in a...
P2RY12-dependent manner to rapidly close small openings in the BBB induced by laser injury. Our study shows how this conserved response can play out on a much larger scale after extensive cerebrovascular damage. Microglia rapidly reconstructed the BBB following vascular injury and limited the degree of damage. This mechanism may explain why microglia accumulate around cerebral microbleeds and prevent hemorrhage after neonatal focal arterial stroke. We demonstrated that microglia depletion or inhibition of ATP release enhanced cerebrovascular leakage, myelomonocytic cell recruitment, parenchymal fibrin deposition and damage. Microglia depletion also reduced the expression of proinflammatory cytokines at 24 h post-injury. These findings suggest that, in the context of acute hemorrhagic vascular injury, the barrier-sealing properties of microglia are protective and outweigh potential negative functions stemming from their phagocytic state or ability to release proinflammatory molecules.

While microglia played an important early role in BBB stabilization, this was partly undone by massive recruitment of myelomonocytic cells, which induced profound cerebral edema. Massive myelomonocytic cell extravasation similarly promotes brain
swelling and cerebral herniation during viral meningitis. We observed that myelomonocytic cell extravasation following cerebrovascular injury depended almost entirely on LFA1 and VLA4, and therapeutic administration of rLFA1/sVLA4 antibodies within 6 h of injury prevented fatal cerebral edema. These data indicate that there is a relatively short therapeutic window to interfere with this pathogenic event, which closes once myelomonocytic cell extravasation has occurred. Several preclinical studies have inhibited diapedesis as a treatment for ischemic stroke and showed variable effects on stroke lesion volume. Moreover, neutrophil inhibitory factor was used to treat patients with ischemic stroke and had no clinical benefit. This failure could be explained by our results, which demonstrate that both neutrophils and inflammatory monocytes are responsible for edema following cerebrovascular injury. Depletion of neutrophils alone did not improve outcome in our model. The success of therapies designed to interfere with immune cell extravasation and subsequent brain swelling depends critically on timing and entry mechanisms. The ACTION trial found that natalizumab (anti-VLA4 antibody), administered up to 9 h after stroke onset, did not reduce infarct growth but improved secondary functional outcomes. However, our data indicate that myelomonocytic cells can use both LFA1 and VLA4 nonredundantly to extravasate and that there is considerable variation in the vascular expression patterns of ICAM-1 and VCAM-1. Use of the anti-LFA1 or anti-VLA4 antibody alone had little therapeutic benefit in our studies. Both antibodies were required to prevent fatal cerebral edema and improve functional recovery.

Angiogenesis is critical for repair of injured brain tissue and previous studies showed that an increased number of microvessels in infarcted brain tissue after stroke is associated with improved long-term survival. While inflammatory monocytes contribute to cerebral edema in the acute phase of injury, they can also promote angiogenesis. We demonstrated that Ccr2 deficiency or continuous inhibition of diapedesis prevented angiogenesis. The chemokine receptor CCR2 promotes mobilization of inflammatory monocytes from the bone marrow into the blood as well as transmigration into inflamed tissues. A pathogenic role for inflammatory monocytes is supported by studies showing that Ccr2−/− mice have reduced cerebral edema following ischemia–reperfusion injury and improved acute motor function following injection of blood into the brain (a model of ICH). However, circulating inflammatory monocytes were also shown to reduce hemorrhagic transformation, promote new vessel growth and increase functional recovery following stroke. Moreover, following ischemia–reperfusion injury, Ccr2−/− mice were initially shown to have a decreased strike volume but then did not functionally recover and had a higher mortality rate than did WT mice. Our findings clarify these contradictory results by revealing that, in the acute phase following cerebrovascular injury, CCR2+ monocytes contribute to potentially fatal cerebral edema but are later required to promote angiogenesis, neuronal preservation and functional recovery.

We found that angiogenesis following cerebrovascular injury also depended on clusters of proangiogenic VEGF-A-expressing microglia. Studies have begun classifying microglia based on their functional attributes and transcriptional states. We have identified a population of CD45−CX3CR1−P2RY12−CD24+ RAM that clustered around damaged blood vessels and expressed VEGF-A. VEGF-A is a potent inducer of angiogenesis that facilitates endothelial cell proliferation and migration. VEGF expression was observed in activated non-phagocytic microglia or macrophages surrounding injured areas of brain tissue from patients with acute ischemic stroke as well as stroke rodents. A subset of glioma-associated microglia in humans was also shown to express VEGF-A+ and could promote brain tumor angiogenesis in rodents. We observed in microglia-depleted mice that angiogenesis genes were not upregulated and that new blood vessels failed to form following cerebrovascular injury. We postulate that invading monocytes endow microglia with this proangiogenic function, as RAM did not develop when immune cell invasion from the blood was blocked.

While immune cells can induce CNS pathology, their role in promoting tissue repair should not be ignored. Prolonged inhibition of immune function following CNS injury can impede repair and prevent functional recovery. Continuous inhibition of peripheral immune cell invasion in our study not only prevented angiogenesis but also changed the reparative program. In the absence of immune-mediated tissue regeneration, we observed induction of fibrotic wound healing in the injured brain associated with neutonal loss, gliosis, invading type 1 collagen+ cells and failed functional recovery. Genomic analyses revealed that this fibrotic brain tissue was completely distinct at the transcriptional level from healthy or properly repaired tissue. In the periphery, it is known that wound-repair macrophages produce many growth factors and

Fig. 5 | VEGF-expressing microglia are generated during angiogenesis. a. Intravital microscopy of cerebral vasculature 10 d after injury and image-based quantification of vascular coverage and intervascular area in PLX3397− (CSFIR inhibitor) versus vehicle control-treated mice show a lack of angiogenesis after microglia depletion. Blood vessels were labeled with EB (red) and tomato lectin (green). Graphs depict mean ± s.d. of cumulative data from two independent experiments (n = 9 mice per group. **P = 1.4 × 10−6 (left), P = 0.0005 (right), two-tailed Student’s t-test). b. Heatmap depicting qPCR analysis of genes encoding angiogenesis-related proteins 6 d post-injury shows downregulation of the angiogenesis pathway in PLX3397-treated mice relative to the mice treated with the vehicle control. The heatmap includes genes for which the difference between control and PLX3397-treated mice had an FDR of Q < 0.01. Data are representative of two independent experiments with four mice per group per experiment. A comprehensive summary of the results and statistical analysis is shown in Extended Data Table 4. c. Confocal microscopy images of brain sections from Cx3Cr1+/+ (green) mice show VEGF-A (red) expression within myeloid cell clusters 6 d after injury (denoted with white arrows) relative to that in uninjured control mice. d. Graph depicts image-based quantification of VEGF-A+ CX3CR1+ microglial cells. Data show mean ± s.d. and are representative of two independent experiments with control, n = 6 and day 6, n = 5 mice and two high-power fields quantified per injury (**P = 0.012, two-tailed Student’s t-test). e–g. High-parameter flow cytometric analysis of the immune landscape of Cx3Cr1+/+ /Strept × TdTomato mice at days 1 and 6 post-injury relative to uninjured mice. Data are representative of two independent experiments with n = 4 mice per group per experiment. e. Representative uniform manifold approximation and projection (UMAP) plots depict the generation of VEGF-A+ microglia after injury (black arrow). f. Scatterplots depict the absolute numbers of different microglia populations post-injury: microglia (TdTomato+CD45+CD11b+CX3CR1+P2RY12+), activated microglia (TdTomato+CD45+CD11b+CX3CR1+P2RY12+) and VEGF-A+ microglia (TdTomato+CD45+CD11b+CX3CR1+P2RY12+CD24−VEGF-A+) (**P < 0.005, **P < 0.01, one-way ANOVA with Tukey’s test; gating strategy is shown in Extended Data Fig. 1g). g. Fluorescence intensity histograms comparing the expression of TdTomato, CD45, CX3CR1, P2RY12, CD24 and VEGF-A on resting microglia, VEGF-A+ microglia and macrophages (TdTomato−CD45−CD11b−Ly6C−Ly6G−). Microglia were gated as described in f, h, i. Representative flow cytometric dot plots (h) and a corresponding graph (i) show the lack of VEGF-A+ microglia after continuous treatment with rLFA1/VLA4 relative to treatment with isotype control antibodies on day 6 post-injury. A representative flow cytometric plot is also shown for an uninjured mouse, and a fluorescence minus one (FMO) control is provided for VEGF-A staining at day 6. Data are representative of two independent experiments with n = 4 or 5 mice per group per experiment (**P < 0.01; one-way ANOVA with Tukey’s test; gating strategy is shown in Extended Data Fig. 1f and Supplementary Fig. 1c). Graphs depict mean ± s.d.
can orchestrate reparative programs\textsuperscript{48}. In addition, dysregulation of wound healing can result in an irreversible fibrotic response\textsuperscript{49}. For example, Duffield et al. demonstrated in a model of liver injury that early depletion of CD11b\textsuperscript{+} myeloid cells reduced the extent of damage, whereas late depletion resulted in failure to resolve the fibrotic response\textsuperscript{50}. Our studies emphasize that, while the detrimental aspects of myelomonocytic cell invasion after cerebrovascular injury can be prevented with αLFA1/VLA4 antibodies, this treatment...
cannot be given continuously without promoting fibrosis and blocking functional recovery.

In conclusion, we offer new insights into the distinct roles of brain-resident and invading myeloid cells following cerebrovascular injury. These responses diverge based on time, cellular origin and anatomical position. Excepting the early massive extravasation by myelomonocytic cells, the myeloid response to cerebrovascular injury is largely beneficial. The barrier-sealing properties of microglia and their eventual conversion into proangiogenic RAM should be promoted. Peripheral CCR2+ monocytes also play a crucial role.
in brain tissue remodeling and should only be blocked acutely in the initial hours post-injury. Such an acute and transient treatment might help resolve the pathogenic cerebral edema that develops following TBI, ICH and stroke while still permitting angiogenesis and brain repair to occur. We hope that our findings improve patient outcomes by serving as a foundation for the design of future clinical trials to treat cerebrovascular injuries and will discourage approaches that indiscriminately or continuously inhibit proinflammatory myeloid cell responses.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-020-00773-6.

Received: 2 June 2020; Accepted: 8 December 2020; Published online: 18 January 2021

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**Methods**

**Human participants.** The appropriate ethics and institutional review boards (IRBs) (National Institute of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH) IRB for Suburban Hospital, Johns Hopkins Medicine, Bethesda, MD; Mayo Clinic, Washington Hospital Center, Washington, DC) approved the study (NCT00099243). Written informed consent was obtained from all patients. Patients were included in this study if they were screened from April 2015 to July 2018 with multimodal MRI as their baseline scan, and if they received a clinical diagnosis of ischemic stroke involving LVO of the anterior circulation before receiving mechanical embolectomy with or without standard i.v. tissue plasminogen activator. Patients were also required to have follow-up multimodal MRI obtained in the F2 generation after an original cross of GFAP-CreER and Cx3cr126Sortm14/GFP/GFP mice were provided by T. Graf (Albert Einstein College of Medicine). Mice.

Two independent raters measured the hyperintense ischemic areas on DWI sequence at all time points using a validated technique as well as the hyperintense lesion areas on the precontrast FLAIR sequence at 24 h and 5 d, avoiding the inclusion of hyperintense acute reperfusion injury marker (HARM) signal areas.

The volume of the lesion was calculated as the cube root of this value (referred to as the volume index) was used for comparison of different groups. The presence of HARM was qualitatively reviewed by the independent raters, reaching consensus for any discrepancies, using the precontrast FLAIR images at 24 h post-EVT. The presence of HARM was defined as enhancement in the CSF spaces and rated as none (present but not severe) or severe (if occupying ten or more slices). The same two independent raters evaluated the gradient echo sequences at 24h post-EVT compared to the baseline gradient echo sequences, documenting the presence of any hemorrhagic transformation by applying the ECASS-II criteria (applied to MRI) to classify patients with no hemorrhage, hemorrhagic infarction (petechial blood) and parenchymal hematoma. Discrepancies in the HARM and hemorrhagic transformation reads were resolved by consensus. The average volume for all DWI and FLAIR reads at all time points was used in the study analyses. A cubed root transformation producing an effective radius of the lesion (volume index) was used to normalize the distribution of lesion sizes for analysis and display. In addition, qualitative consensus reads for HARM and hemorrhagic transformation were used in the study analyses. Complete recanalization was defined in the interventional radiology suite as TICI scores of 2B or 3.

MRIs from representative cases of TBI, primary ICH and hemorrhagic injury, the skull window was further thinned to 10–15 µm. At this point, 1 µl of Perflutren (2\(\mu\)l per g of Perflutren) was carefully thinned to 20–25 µm. At this point, 1 µl per g of Perflutren Protein-Type A microspheres (5–8 x 10^8) bubbles per ml with a size range of 2–4.5 µm; Optimis, GE Healthcare) was injected iv via re-embolization injection. A drop of CSF was placed over the cranial window. We then applied LIPUS at 1 MHz with a peak negative pressure of ~200 kPa (2.2 W/cm^2), a burst length of 1 ms, 10% duty cycle and a 10-s exposure to the thinned skull using a Sonicator 740x (Mettler Electronics) with a 5-cm^2 dual frequency applicator. For intravitreal imaging after the injury, the skull window was further thinned to 10–15 µm.

**Ex vivo MRI imaging.** Ex vivo MRI of mouse cerebral was performed on a vertical-bore 7T Bruker BioSpin Magnetic using a volume coil (Bruer, internal diameter of 2 cm). Brains were immersed in susceptibility-matching fluid (Fomblin, A-Tek) and imaged with a T2*-weighted three-dimensional Multiple Gradient Echo sequence (repetition time, 100 ms; echo time, 5 ms (first echo)); echo spacing, 5 ms; number of echoes, 10; flip angle, field of view, 30 x 30 x 30 mm; and image resolution, 100 x 100 x 100 µm with number of averages, 6.

**Macroscopic imaging.** Imaging of the skull window was completed using a 1.4 Megapixel INFINITY 2-HRC microscopy camera (Tedeye Lumenera) and INFINITY CAPTURE software 6.5.2. Mice.

Mice. C57BL/6 (B6), B6.129P2-Cx3cr1tm1L就算(FsCreERT2) (F60-8GFP-CreERT2), B6.129P2-Cx43tm1Cgt (CreERT2) knockout), B6.Cg-Tg(GFAP-CreERT2)505Flm1 (GFAP-CreERT2), B6.Cg-Gt(Rosa26)SorSor (GFP reporter), B6.129P2(Cg-CreERT2)7E00 (CreERT2)31knockout). Cx3cr126Sortm14 mice were provided by D. Bergles (Johns Hopkins University) and subsequently backcrossed onto a pure B6 background. B6.LysM^GFP^ mice were provided by T. Graf (Albert Einstein College of Medicine). Cx3cr126Sortm14 mice were generated from an F1 cross of B6 and Cx3cr126Sortm14/GFP/GFP mice by mating with Cx3cr126Sortm14 mice. Double reporter mice were generated from an F1 cross of Cx3cr126Sortm14 mice. Gja1tm1Dlg/J and Cx43tm1Dlg/J mice were purchased from Jackson Laboratory. FVB.Cg-Tg(Cspg4-EGFP*)HDbe/J mice were purchased from Bio X Cell. Cre recombinase was introduced into the Cx3cr126Sortm14/GFP/GFP mice by CreERT2 activity in response to tamoxifen administration. Tamoxifen treatment. For induction of Cre recombinase, 6–8-week-old GFAP-CreERT2; Stop^fl/fl^ and GFAP-CreERT2; Cx3cr126Sortm14/GFP/GFP mice were treated with tamoxifen (80 mg per kg, tamoxifen, Envigo) for 4 weeks. Cx43tm1Cgt/Cx43tm1Cgt knockout mice at 4–6 weeks of age were treated with tamoxifen (500 µg per kg tamoxifen, Envigo) for 4 weeks to activate the Cre recombinase and then switched to regular feed for 4 weeks to allow turn over of circulating CX3CR1^+ cells.

In vivo cell depletions and inhibition of leukocyte extravasation. For microglia depletion, 6–8-week-old B6 mice were treated for 4 weeks with PLX3397 (Adooq), formulated in Chow (Envigo) or treated for 4 d with PLX5562-formulated Chow (Envigo). For temporary myelomonocytic cell depletion, 8–12-week-old mice were given 400 µg of rat anti-mouse Ly6G/Ly6C antibody (GR-1; RB6-8C5) i.p. 1 d before injury (anti-GR-1 antibody bolus), and for persistent myelomonocytic cell depletion, mice were given 400 µg anti-GR-1 antibody (RB6-8C5) i.p. 1 d before injury and on days 1, 3, 5, 7 and 9 post-injury (anti-GR-1 antibody continuous). The B6 mice in the control group of these experiments were given 400 µg of rat anti-mouse IgG2b isotype control (IgG2b) 2.5 µg at the same time points. Similarly, for temporary neutrophil depletion, 8–12-week-old mice were given 500 µg of rat anti-mouse Ly6G antibody (BE0075-1) i.p. 1 d before injury (anti-Ly6G antibody bolus), and for persistent neutrophil depletion, 500 µg of rat anti-mouse IgG2a antibody (BE0075-1) i.p. 1 d before injury and on days 1, 3, 5, 7 and 9 after injury (anti-Ly6G antibody continuous). The B6 mice in the control group of these experiments were given 500 µg of rat anti-mouse IgG2a isotype control (2A3) i.p. at the same time points. To inhibit leukocyte extravasation, 8–12-week-old mice were given 1 µg of rat anti-CD49d (α-LFA1/β2 integrin) antibody (G4/17i, 500 µg of rat anti-mouse/human VLA4 antibody (CD49d; PS2) i.p. 1 d before injury (α-LFA1/VLA4 pretreatment) or one dose immediately post-injury (0h, 1h, 6h or 12h post-injury; α-LFA1/VLA4 bolus) or one dose immediately post-injury and on days 1, 3, 5, 7 and 9 after injury (α-LFA1/VLA4 continuous). In the B6 mice in the control group of these experiments these were given 500 µg of rat anti-mouse IgG2a isotype control (2A3) i.p. and rat anti-mouse IgG2b isotype control (IgG2b) 2.5 µg at the same time points. To evaluate the separate effects of anti-α-LFA1 and α-VLA4 antibodies, we administered 500 µg of rat anti-mouse LFA1α antibody (CD11a; M17/4) i.p. or 500 µg of rat anti-mouse/human VLA4 antibody (CD49d; PS2) i.p. 1 d before injury. All antibodies used for cell depletion and blocking assays were purchased from Bio X Cell.

Intravitreal two-photon microscopy. Following the initial surgical approach described above, a metal bracket was glued on the skull, leaving a circular area of the skull exposed that was centered 2 mm posterior to the coronal suture and 2.5 mm lateral to the sagittal suture (anterior injury). Mouse brains were imaged through the thinned skull window generated for the sonication injury as described above. For uninjured mice, a cranial window (2 x 2 mm) was carefully thinned to 10–15 µm (ref. 1). Injured and control mice were imaged using a Leica SP8 two-photon microscope with an 8,000-Hz resonant scanner, a x25 corrected water-dipping objective (1.0 NA) or a x20 water-dipping objective (1.0 NA), a quad HyD external detector array, a Mai Tai HP DeepSee Laser (Spectra-Physics) tuned to 905 nm for GFP, Alexa Fluor 488 and Dye 488, an Insight DS laser (Spectra-Physics) tuned to 1,050 nm (for EB and TdTomato) or to 1,100 nm (for PE and APC). Acquisition was completed using Leica Application Suite X version 3.5.2.3.976. Three-dimensional time-lapse movies were captured at intervals of 15 m, 30 s, 1 min and 2 min using z stacks of 20–72 planes (2.5- and 3-µm step size).
sizes). The signal contrast was enhanced by averaging 6–8 video frames per plane in resonance scanning mode. For blood vessel visualization, 70 µl of 0.1 mg/ml EB (Sigma) and/or 70 µl tomato lectin DyLight 488 (Vector Labs) was injected i.v. before imaging. To measure the volume of the time-dimension image thinned, EB was injected i.v. immediately after injury and before imaging. All intravital two-photon images obtained were subsequently imported into Imaris version 9.3 software (Bitplane) for further analysis. Adobe Premiere Pro 14.0 (Adobe Systems) was used to create final videos.

Transcranial inhibition of purinergic receptors and hemichannels. Transcranial administration of inhibitors was performed as previously described by our laboratory. Following initial skull thinning to 20–25µm, antagonists diluted in artificial cerebral spinal fluid (Harvard Apparatus) were applied directly to the skull bone as a 3-mm diameter bubble for 2h. Artificial cerebral spinal fluid was applied to the control group. The antagonists used were MeSAMP (P2RY12 inhibitor; 10 mM, Sigma) and carbonoxalone (connexin hemichannel inhibitor; 100 mM, Sigma). The solution was replenished as needed over the 2-h period to prevent drying. This allowed the antagonists to continuously pass through the skull bone. Subsequently, the skull was dried, and an ultrasound injury was induced. The skull was then further thinned to 10–15 µm, and we proceeded with intravitreal microscopy for 60 min. The antagonists were maintained in the submerging solution for the entire imaging experiment.

Imaging of lesion core and perimeter. For these experiments, Lyt3+Gr1− mice were used. To evaluate the lesion core, we followed an injury protocol described above. To evaluate the perimeter of the lesion following the initial sonication through an anterior cranial window (2×2 mm), we created an additional thinned cranial window posteriorly and laterally to the main window. Immediately after injury, a dose of ofLFA1/VLA4 was administered to one group. Three-dimensional time-lapse movies were captured at 30-s intervals using 2 stacks of 20 planes (3-µm step size). Image series were imported into Imaris version 9.0, and, using the volume function, the sum of intensity of EB was measured. To calculate the volume of extravasated EB, We subtracted the initial (t=0) sum volume intensity of intravascular EB from the volume calculated above. We calculated the average extravasated EB from 20 to 40 min per sample.

Rosette quantification assay. Cx3cr1Fucci− mice were imaged for 40–60 min following injury. Three-dimensional time-lapse movies were captured at 2-min intervals using 2 stacks of 30 planes (3-µm step size). Using the surfaces function in Imaris 9.0, we identified the microglia rosettes at time points from 40 to 60 min. We extended the surfaces to the initial time points and quantified the volume of GFP+ microglial processes at time points from 0 to 30 min. We reported the volume of microglia rosettes as an average of the values from time points (18, 20 and 22 µm) per sample.

ICAM-1 (CD54) and VCAM-1 (CD106) expression assay. For quantification of ICAM-1 and VCAM-1 expression, 24 h after injury, mice were i.v. injected with 10mg APC anti-CD106 antibody (429 MVCA4.A, BioLegend) and tomato lectin DyLight 488. One hour after injection, mice were injected using the Leica SP8 two-photon microscope as described above. For the experiment comparing ICAM and VCAM expression in injured animals and injured animals that were fed PLX3397, the Mai Tai HP DeepSee Laser was tuned to 905 nm, and the Insight DS laser was tuned to 905 nm. Both lasers were maintained at 10% power, and the quad HyD external detector array gain was set to ten for both channels. For the experiment comparing ICAM and VCAM expression in uninjured animals and injured animals, the Mai Tai HP DeepSee Laser was tuned to 905 nm, and the Insight DS laser was tuned to 1,050 nm. Lasers were maintained at 15% of power, and the quad HyD external detector array gain was set to 20 for both channels. Images were imported into Imaris version 9.0, and, using the volume function, the sum of intensity for signals from both anti-CD106 APC and anti-CD54 PE antibodies. Quantification of Ly6M+ cells. We imaged Ly6G+/− mice 1 h after injury. The images were imported into Imaris 9.0, and the spots function was used to identify and quantify the number of Ly6M+ cells per image.

Angiogenesis assay and characterization of neovessels. Mice were evaluated 10 d after initial injury. For blood vessel visualization, 70 µl of 0.1 mg/ml EB and/or 70 µl tomato lectin DyLight 488 were injected i.v. before imaging, and the thinned window was rethinned to 20–25 µm. Following intravital imaging, images were imported into Imaris 9.0. The volume of vascular coverage was quantified using the surfaces function to mask EB and tomato lectin DyLight 488-positive vessels and calculate their volume. The total vessel volume was then reported as a fraction of the total volume of the time-dimension image thinned. The intervascular area was obtained by first masking the areas negative for EB and tomato lectin DyLight 488 using the surfaces function. We then separated the surface of each intervascular space in the maximum intensity projection of each image and calculated the area of each intervascular space. We reported the average of all intervascular space surfaces per image. This angiogenesis assay was repeated to quantify angiogenesis following ofLFA1/VLA4 bolus and continuous treatments and oG-1 bolus and continuous treatments, as well as angiogenesis in microglia-depleted mice and Ccr2−/− mice following CD115+ adoptive transfer. Angiogenesis was compared to age-matched untreated B6 mice.

CD115+ adoptive transfer to Ccr2 knockout mice. B6 and Ccr2−/− mice underwent anterior injury. We subsequently pooled blood from B6 mice and incubated it in ACK buffer at 4 °C for 5 min and then washed and centrifuged the samples at 500g for 5 min; the process was repeated until RBGs were lysed and the supernatant was clear. Cells were passed through a 40-µm cell strainer and resuspended in 100 µl PBS 50–100 µm slices. We isolated CD115+ cells using a MACS CD115 MicroBead kit (MACS Miltenyi Biotec). We quantified the number of cells using a hemocytometer. We administered 10° CD115+ cells per mouse immediately after injury and on days 1, 3, 5, 7 and 9 in one group of Ccr2−/− mice. At day 10 following injury, angiogenesis was evaluated by intravitreal microscopy as described above (Angiogenesis assay and characterization of neovessels).

Immunohistochemistry. Brains were collected 1 h, 24 h, 6 d and 10 d after injury. For vascular staining, we used i.v. administration of fluorescence-conjugated tomato lectin DyLight 488 or DyLight 649 (Vector Labs) or anti-CD31 PE antibody (BD Pharmingen). To identify the fibrinogen (5–10 min after the injury) immediately after sonication and 1 h before killing, mice were perfused with 4% paraformaldehyde. The brain was collected and placed in 4% paraformaldehyde overnight. The brain was sectioned axially using a Compressosome Tissue Slicer (Precisionary) to produce 50–100 µm sections. For staining, tissues were initially blocked and permeabilized by incubating them in staining buffer consisting of 0.5% Triton-X in PBS with Background Buster (Innovex Biosciences) as well as FCR block for 30 min at room temperature. Primary antibodies were added directly to the staining buffer and incubated at 4 °C overnight. After primary staining, membranes were washed three times in staining buffer. Secondary antibodies were added and incubated for 4 h at room temperature. After secondary staining, slides were again washed three times in staining buffer. The free-floating slides were carefully mounted on the slide and covered with one drop of FluorSave Reagent (MilliporeSigma), and a coverslip was added. The tissues were stained with the following primary antibodies: rabbit anti-IBA1 (1:500, 019-17947, Wako), chicken anti-GEAP (1:1,000, ab6474, Abcam), guinea pig polyclonal anti-NeuN (1:500, ABN09, Millipore) and mouse VEGF120/164 antibody (1:250, monoclonal rat IgG2b clone 39917, R&D Systems), conjugated to Alexa Fluor 647 using the Alexa Fluor 647 Antibody Labeling kit (Invitrogen). Secondary antibodies (1:1,000 dilution) included donkey anti-rabbit IgG (H&L) Alexa Fluor 488 (A-21206, Thermo Fisher), donkey anti-chicken IgG (H&L) Alexa Fluor 594 (Thermo Fisher), donkey anti-chicken IgG (IgG) (H&L) DyLight 405 (AB_234073, Jackson ImmunoResearch), goat anti-rabbit IgG (H&L) Pacific Blue (P-10994, Thermo Fisher) and donkey Fab anti-chicken IgG (H&L) Rhod-x (Jackson ImmunoResearch). For immunohistochemistry, we evaluated the antibodies used in this study for the degree of nonspecific binding (as measured in samples that do not express the epitope or by using isotype controls for comparison). The anti-CD31 PE antibody was validated by the manufacturer for immunofluorescent staining and was used in previous studies. The rabbit anti-IBA1 antibody was used previously for immunohistochemistry. The chicken anti-GEAP antibody was tested by the manufacturer for immunohistochemistry and was used in previous studies. The guinea pig anti-NeuN antibody was validated by the manufacturer for immunohistochemistry and was used in previous studies. The mouse VEGF120/164 antibody conjugated to Alexa Fluor 647 was evaluated in non-injured controls as shown in Fig. 5c (left). Additionally, the following reporter mice were used to define specific cell types: Cx3cr1Fucci+, LysM+Tomato+, and collagen-GFP. Images were obtained using an Olympus FluorView F10 laser scanning confocal microscope equipped with four detectors, six laser lines (405, 488, 515, 539 and 635 nm) and five objectives (×4, 0.16 NA; ×10, 0.4 NA; ×20, 0.75 NA; ×40, 0.95 NA; and a chromatic aberration-corrected ×60, 1.4-NA objective) with the Olympus FluorView 4.2a software. All confocal images obtained were subsequently imported into Imaris version 9.0 software for further analysis.

Image analysis of immunohistochemistry images. All image analyses were performed by a blinded investigator. To quantify fibrin distribution, Cx3cr1Fucci+ mice were injected i.v. with 100 µl of 2.5 mg/ml fibrinogen Alexa Fluor 647 immediately after sonication and 1 h before collecting the brain. Using the surface function in Imaris 9.0, the volume of fluorescent fibrinogen signal per image.
was measured. Microglia rosettes (defined as tube-like GFP+ structures >50 μm in length) were quantified manually following IBA-1 staining. LysM+ cells were quantified in Ly5.1+ mice using the Imaris spots function. The number of neurons 10 d after injury was evaluated after staining with guinea pig polyclonal anti-NeuN antibody (ABN09P) and also using the spots function in Imaris. The degree of fibrosis was evaluated 10 d after injury in collagen-GFP mice by using the surfaces function in Imaris 9.0 to calculate the volume of collagen-GFP signal per image.

**BBB integrity assay.** To assess the integrity of the BBB following injury, we used mice 1, 2, 4, 7, and 14 d after injury. We administered 100% of [10 wt/vol] sodium fluorescein (NaFl) in PBS (Sigma) intraperitoneally and collected a cube (3 × 3 × 3 mm) of tissue from the injured area or the contralateral frontal lobe as well as 50 μl blood, 10 min after injection. Tissues were homogenized in 7.5% trichloroacetic acid (TCA) in 5 M NaOH and centrifuged at 10,000 × g for 15 min and the supernatant was then collected. We added 25 μl 15% TCA to 25 μl serum, centrifuged the mixture at 10,000 g and 4°C, collected 25 μl of the supernatant and added 125 μl 7.5% TCA in 5 M NaOH. Fluorescence was quantified using a microplate reader (Variouskan Flash; Thermo Fisher) with excitation at 485 nm, emission at 535 nm, and a gain of 53%. Using a standard curve, we calculated the concentrations of NaFl in the tissue and blood serum. We calculated the uptake ratio using the following formula.

\[
\text{Uptake ratio} = \frac{\mu g/\text{ml NaFl in tissue} - \text{weight of tissue} \times \mu g/\text{ml NaFl in serum}}{\text{weight of tissue} \times \mu g/\text{ml NaFl in serum}}
\]

This process was adjusted from our previously published BBB leakage assay.

**Quantification of cerebral water content (edema assay).** To estimate the edema induced by injury, mice were killed 24 h after an anterior injury or when they reached the endpoint in the survival study following posterior injury. Uninjured mice or the contralateral uninjured hemisphere were used as controls. The hemisphere was isolated and weighed using an analytical balance (Mettler Toledo). Subsequently, the hemisphere was incubated overnight at 80°C, and the dry weight was measured.

**Survival studies.** Mice were monitored daily following injury by an investigator blinded to the groups. Mice were monitored twice a day for physical deficiencies and weight loss. Mice were killed when a weight loss >25% of their original weights or sign of distress was observed. The surviving mice were killed 5 d after injury.

**Flow cytometric analysis.** Flow cytometry experiments were performed on day 1 and day 6 after injury. We used B6, Cx3cr1Δtvv/+;Ccr2Δtvv/+ double reporter and Cx3cr1Δtvv/+;Stop++;Tdtomato mice. To obtain brain tissue, mice were anesthetized and perfused with cold PBS. The injured hemisphere or uninjured hemisphere was collected. NaFl was measured. Microglia rosettes (defined as tube-like GFP+ structures >50 μm in length) were quantified manually following IBA-1 staining with anti-NeuN antibody (ABN09P) and also using the spots function in Imaris. The degree of fibrosis was evaluated 10 d after injury in collagen-GFP mice by using the surfaces function in Imaris 9.0 to calculate the volume of collagen-GFP signal per image.

**Real-time PCR analysis.** For real-time PCR analysis (qPCR) mice, a cube of tissue (3 × 3 × 3 mm) was collected and snap frozen using dry ice. Total RNA was extracted with the Qiagen kit following the manufacturer’s protocol, and RNA quantity and integrity were assessed with a spectrophotometer (NanoDrop One, Thermo Scientific). cDNA was generated using a GeneScript cDNA Synthesis kit (Bio-Rad). Premade 96-well PrimePCR plates were used for qPCR experiments (Applied Biosystems). qPCR was also performed using universal SYBR Green Supermix (Bio-Rad) and cDNA template or water (nontemplate negative control) at an annealing temperature of 60°C with a CFX96 Real-Time PCR machine (Bio-Rad). PCR products were subjected to melt analysis to confirm purity after DNA amplification. For each gene, expression values were normalized to the Gapdh housekeeping gene. The resulting relative gene expression was then expressed as a fold change over indicated control samples. More specifically, to evaluate the inflammatory profile following microglia depletion, we obtained tissue 24 h post-injury from the frontal lobe of uninjured B6 mice, injured B6 mice, uninjured mice treated with PLX3397 and injured mice treated with PLX3397. To evaluate the expression of the TgPdh housekeeping gene. The resulting relative gene expression was then expressed as a fold change over indicated control samples. More specifically, to evaluate the inflammatory profile following microglia depletion, we obtained tissue 24 h post-injury from the frontal lobe of uninjured B6 mice, injured B6 mice, uninjured mice treated with PLX3397 and injured mice treated with PLX3397. To evaluate the expression of the TgPdh housekeeping gene.

**Bulk RNA-sequencing.** For RNA-seq, B6 mice were divided into three groups (uninjured B6, injured B6, and injured B6 treated with Four-in-One) at four mice per group. At day 20 following injury, mice were perfused with normal saline, and a cube of injured tissue (3 × 3 × 3 mm) was collected and snap frozen using dry ice. The tissues were stored at −80°C until use. Total RNA was extracted with the Qiagen kit following the manufacturer’s protocol, and RNA quantity and integrity was assessed with the Bioanalyzer (Agilent). Total RNA (500 ng) was used as input for the TrueSeq Stranded Total RNA Library Prep kit (Illumina). The library quality was checked with the Bioanalyzer and quantitated by the Qubit machine (Thermo Fisher Scientific). Equimolar quantities from each sample library were pooled and run on a High-output NextSeq550 kit.

**RNA-seq data analysis.** Paired-end sequence files (.fastq) for each sample were inspected for quality using the FastQC tool 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then adaptor clipped (TruSeq-PE: 2.efa:2.30:10) and trimmed to remove 5’ nucleotide bias (HEADCROP: 11) and low-quality calls (TRAILING:20 SLIDINGWINDOW:4 20). The quality of reads was checked using the Trimmomatic tool 0.39 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and low-quality calls (TRAILING:20 SLIDINGWINDOW:4 20). The quality of reads was checked using the Trimmomatic tool 0.39 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Surviving intact pairs of reads per sample were then imported into the CLCbio Genomics Workbench 11 (https://www.qiagenbioinformatics.com/), downsampled to 40 × 10^6 read pairs per sample and then reference mapped by sample in stranded fashion against the reference instance of the mouse genome (GRCh38) using the ‘RNA-seq Analysis’ tool supported therein under default parameters. Expression per known annotated gene (Mus_musculus.GRCm38.83.gtf) in transcripts per kilobase million (TPM) units was then exported from the Workbench and imported into R (https://cran.r-project.org/). In R, TPM expression per sample was pedestal by two and then log transformed. Genes lacking an expression value >1 post-transformation for at least one sample were discarded. Log2 expression values across samples for undiscovered genes were quantile normalized. To assure the quality of the data post-normalization and the absence of sample-level outliers, an exploratory analysis was performed using a Tukey box plot, a covariance-based PCA scatterplot and a correlation-based heatmap. To remove noise-biased expression values, locally weighted scatterplot smoothing was applied. The resulting gene expression matrix (GRCm38.83.gtf) was then used as input for the CLCbio Genomics Workbench 11 (https://www.qiagenbioinformatics.com/), downsampled to 40 × 10^6 read pairs per sample and then reference mapped by sample in stranded fashion against the reference instance of the mouse genome (GRCh38) using the ‘RNA-seq Analysis’ tool supported therein under default parameters. Expression per known annotated gene (Mus_musculus.GRCm38.83.gtf) in transcripts per kilobase million (TPM) units was then exported from the Workbench and imported into R (https://cran.r-project.org/). In R, TPM expression per sample was pedestal by two and then log transformed. Genes lacking an expression value >1 post-transformation for at least one sample were discarded. Log2 expression values across samples for undiscovered genes were quantile normalized. To assure the quality of the data post-normalization and the absence of sample-level outliers, an exploratory analysis was performed using a Tukey box plot, a covariance-based PCA scatterplot and a correlation-based heatmap. To remove noise-biased expression values, locally weighted scatterplot smoothing was applied.
applied across normalized expression for all genes by sample class (coefficient of variation = mean expression). Locally weighted scatterplot smoothing fits were then overlaid and inspected to identify the common low-end expression value for which the relationship between mean expression (that is, 'signal') and the coefficient of variation (that is, 'noise') grossly deviated from linearity. If less than this value, expression values were then floored to equal this value, while genes for which expression greater than this value was not observed for at least one sample were discarded as noise biased. For undiscarded genes, expression differences across sample classes were tested for significance using the one-factor ANOVA test under the Benjamini–Hochberg FDR multiple comparison correction condition using sample class as the factor. Genes having a type III-corrected P < 0.05 by this test were then subset, and the Tukey HSD post hoc test was used to generate mean differences and P values for each pairwise comparison of classes. Genes having a post hoc P < 0.05 for a specific comparison and a linear difference of means ≥1.5-fold for the same comparison were deemed to have significantly different expression between the compared groups. Post-testing, sample-to-sample relationships were investigated by a covariance-based PCA scatterplot and a Pearson correlation-based clustered heatmap using the unique union of genes deemed to have a significant difference in expression between at least two classes. Enriched pathways, functions and top-scoring networks for the same union set of genes were obtained using the IPA tool (https://www.qiagenbioinformatics.com/).

Cognitive–motor function evaluation. We evaluated the cognitive–motor function of mice following sham surgery as well as mice followed either untreated or following qLFA1/VLA4 bolus or continuous treatment. Evaluation was completed on day 10 and day 20 after injury. A Y maze with three white, opaque plastic arms at 120° angles from one another was used. The mice were introduced in the center of the maze and allowed to freely explore for 5 min per session. An entry was defined when all four limbs were within the arm. We recorded the total number of arm entries to assess the overall movement in the maze. We also recorded the number of times the mouse sequentially entered all three arms (for example, from A to B to C not from A to B to A) as a measure of exploration and cognitive function. The number of triplicate A-to-B-to-C entries was divided by the total number of gates entered to obtain the ‘triplicate ratio’ (Fig. 6I). Each mouse was evaluated at one time point.

Statistical analysis. Statistical analysis and graph design were performed using Prism 8.4 (GraphPad Software), except for bulk RNA-seq analysis, which is described in detail above. Distribution normality was assessed using the Shapiro–Wilk normality test for n > 4 for smaller sample sizes, distribution was considered normal. Experiments containing two groups were analyzed using two-tailed Student's t-test for normally distributed data or the Mann–Whitney U test for non-normally distributed data. Experiments involving more than two groups were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test for normally distributed data or by Kruskal–Wallis test followed by Dunn’s multiple comparison test for non-normally distributed data. Data groups based on more than one nominal variable were analyzed using two-way ANOVA followed by the Holm–Šidák multiple comparisons method. Groups were considered statistically different at P < 0.05. All data are displayed as the mean ± s.d. For the qPCR data analysis, we performed multiple t-tests, using the Benjamini, Krieger and Yekutieli method, to correct for the FDR, with a desired Q value of 1% or 5%. Survival studies were analyzed using the log-rank test. Statistical analyses for each graph are provided in Supplementary Table 7. No statistical methods were used to predetermined sample sizes, but our sample sizes are similar to those reported in the graph are provided in Supplementary Table 7. No statistical methods were used to predetermined sample sizes, but our sample sizes are similar to those reported in previous publications146,153. Animal littermates were randomly assigned to each group, and samples were randomly selected for data acquisition and analysis; no active randomization protocol was used.

Ethics statement. Human studies were approved by the appropriate ethics and IRBs (NINDS, NIH IRB for Suburban Hospital, Johns Hopkins Medicine, Bethesda, MD; Medstar Washington Hospital Center, Washington Hospital Center, Washington, DC) and written consent was obtained from all patients. The animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. The protocol was approved by the NINDS Animal Care and Use Committee (protocol no. 1295–17).

Accession codes. Our bulk RNA-seq data are available in the NCBI Gene Expression Omnibus under accession code GSE161424.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are available from the corresponding author upon request. There are no restrictions on data availability. Bulk RNA-seq data are available in the NCBI Gene Expression Omnibus under accession code GSE161424. Source data are provided with this paper.

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Acknowledgements
This research was supported by the intramural program at the NINDS, NIH. We thank A. Hoofring in the NIH Medical Arts Design Section for generating the illustration shown in Extended Data Fig. 1. We thank A. Elkahloun and W. Wu in the National Human Genome Research Institute Microarray core for their assistance with the RNA-seq experiment.

Author contributions
P.M. and N.M. performed the data acquisition and analysis. P.M., M.L., A.W.H. and L.L. contributed to the design, acquisition and analysis of clinical data. S.R.B., J.W. and J.A.F. contributed to optimization of the ultrasound model and performed the mouse MRI studies. K.J. conducted computation analyses of RNA-seq data. P.M. and D.B.M. wrote and edited the manuscript. D.B.M. supervised and directed the project and participated in data acquisition and analysis.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41593-020-00773-6.
Supplementary information is available for this paper at https://doi.org/10.1038/s41593-020-00773-6.
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Peer review information Nature Neuroscience thanks Thiruma Arumugam, Jonathan Godbout, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.
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Figure S1

Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Model of ultrasound-induced injury. Following surgical generation of a 2 mm x 2 mm x 15 µm thinned skull window, microbubbles were injected intravenously and a drop of aCSF was placed atop the thinned skull bone. Through this aCSF we applied low intensity pulse ultrasound (LIPUS) using a Mettler Sonicator 740x with a 5 cm² planar dual frequency applicator operating at 1 MHz, ~200 KPa peak negative pressure with duty cycle 10% and 1 ms burst. LIPUS induced acoustic cavitation of the microbubbles. Microbubble oscillation, inertial cavitation, and explosion caused internal injury of blood vessel walls, exposing the brain parenchyma to blood contents. This injury creates a relative column of injury in the brain tissue beneath the thinned skull window, as the ultrasound waves are not strong enough to pass through the surrounding intact bone.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Characterization of the cerebrovascular injury model. a, Magnified images of the 2 mm x 2 mm x 15 μm thinned skull window pre- and post-injury depict petechial intraparenchymal hemorrhages at 10 min post-injury. b, Macroscopic depiction of a mouse brain 24 h following posterior sonication injury. c, Kaplan-Meier curve demonstrates a median survival of 2 days after posterior sonication injury. Anterior sonication injury does not result in fatalities. Cumulative data are shown from 2 independent experiments with 10 mice per group (P = 2.96e-10, Log-rank test). d, A graph showing quantification of cerebral water content demonstrates increased edema 24 h after sonication with 7.7% and 7.1% increase in water content after anterior and posterior injury, respectively, relative to uninjured control mice (** P < 0.01, anterior P = 2.9e-7, posterior p = 1.3e-6, One-way ANOVA/Tukey test). Cumulative data are shown from 2 independent experiments with 5 mice per group per experiment. e, A graph showing quantification of fluorescein extravasation into the ipsilateral versus contralateral brain hemisphere at the denoted time points post-injury (** P < 0.01, One-way ANOVA/Tukey test). Data are representative of 2 independent experiments with 4 mice per group per experiment, 2 samples were above the detection limit and not included. f-h, High parameter flow cytometric analysis of brain biopsies from mice at d1 and d6 post-injury relative to uninjured controls. A UMAP plot of concatenated live cells from each group is shown in panel F. A heatmap of Ter-119 signal on a UMAP plot reflecting the concatenated cell populations from a single experiment is shown in panel G. Panel H shows a scatter plot depicting the absolute Ter-119+ RBCs. Cumulative data are shown from 2 independent experiments (Uninjured n = 8, d1 n = 8, d6 n = 9, ** P < 0.01, One-way ANOVA/Tukey test; gating strategy in Supplementary Fig. 1A). Graphs D, E, H show the mean ± SD.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Microglia depletion increases BBB leakage, intraparenchymal hemorrhage, myeloid cell invasion and vascular endothelium activation. **a**, Intravital microscopy of CX3CR1gfp/wt (green) mice 20 min after injury shows extensive intraparenchymal EB (red) extravasation following microglia depletion using an alternate CSF1R inhibitor, PLX5622, to that used in Fig. 2c,d. **b**, EB extravasation assay based on intravital microscopy time lapses depicts increased BBB leakage 20–40 min after microglia depletion using PLX5622. Graph depicts mean ± SD of cumulative data from 2 independent experiments (n = 12 mice per group, **P = 3.9e-9, two-tailed Student’s t-test). **c**, Confocal microscopy images of cortical brain sections from naive and microglia depleted Cx3CR1gfp/wt (green) mice 1 h following injury. Mice received an i.v. injection of fluorescent fibrinogen (white) and tomato-lectin (red). Larger and more diffuse intraparenchymal fibrinogen is observed in mice treated with PLX3397 (CSF1R inhibitor). **d**, Image-based quantification of fibrin burden in the brain parenchyma. Graph depicts mean ± SD of cumulative data from 2 independent experiments (Ctrl n = 10, CSF1R inh n = 13, **P = 0.00017, Mann–Whitney U test). **e**, Two-photon microscopy images captured in the cerebral cortex of injured LysMgfp/wt mice treated with vehicle or PLX3397 show LysM+ myelomonocytic cell (green) invasion at 20 min post-injury. Tomato-lectin is shown in red. **f**, Image-based quantification of myelomonocytic infiltration. Graph depicts mean ± SD of cumulative data from 2 independent experiments (Ctrl n = 8, CSF1R inh n = 12, **P = 0.0002, Mann–Whitney U test). **g**, Intravital microscopy images in the cerebral cortex of vehicle versus PLX3397 treated B6 mice at 24 h post-injury. Prior to imaging, mice received an i.v. injection of APC-anti-CD106 (VCAM-1; red) and PE-anti-CD54 (ICAM-1; green), which revealed increased endothelial expression in PLX3397 treated mice. Representative images are from 2 independent experiments with 3 mice per group. **h**, qPCR analysis of ICAM and VCAM expression in vehicle vs. PLX3397 treated B6 mice at 24 h post-injury. Graph depicts mean ± SD of cumulative data from 2 independent experiments (n = 6 mice per group, Vcam1 P = 3.58e-5, Icam1 P = 0.0072, Two-way ANOVA/Holm-Sidak test). **i**, Heatmap shows qPCR analysis of genes encoding for acute inflammation-related proteins in vehicle vs. PLX3397-treated B6 mice. The fold increase in gene expression was calculated relative to the uninjured contralateral hemisphere for each mouse at 24 h post-injury. Data are representative of 2 independent experiments with 5 mice per group per experiment (*P < 0.05, **P < 0.01, multiple t tests with Holm-Sidak multiple comparisons correction, source data in Supplementary Table S3). CSF1R inh refers to PLX3397.
Extended Data Fig. 4 | See next page for caption.
Representative images establishing a role for P2RY12 receptor and Cx43 hemichannels in microglial rosetting.

Data from these experiments are provided in Fig. 2g.h.i. a, Intravital microscopy images in the cerebral cortex of Cx3CR1gfp/wt (green) mice 20 min post-injury. Mice were treated transcranially with a vehicle or a P2RY12 inhibitor (MeSAMP). Intravenous injection of EB (red) revealed increased intraparenchymal extravasation following pre-treatment with the P2RY12 inhibitor. Images are representative of experimental data graphed in Fig. 2g. b, Intravital microscopy images in the cerebral cortex of Cx3CR1gfp/wt (green) mice 20 min post-injury. Mice were treated transcranially with a vehicle or a Cx43 inhibitor (carbenoxolone). Intravenous injection of EB (red) revealed extensive intraparenchymal extravasation following pre-treatment with the Cx43 inhibitor. Images are representative of experimental data graphed in Fig. 2g. c, Confocal microscopy images of brain sections from littermate control (GFAPCreER-Cx43f/wt and GFAPCreER-Cx43wt/wt) vs. GFAPCreER-Cx43f/f mice 1 h after injury show decreased microglia rosetting in GFAPCreER-Cx43f/f mice. Microglia rosettes were identified with Iba1 staining (green). Tomato-lectin+ blood vessels are shown in white. Images are representative of experimental data graphed in Fig. 2h. d, Intravital microscopy images from the cerebral cortex of control (GFAPCreER-Cx43f/wt, GFAPCreER-Cx43wt/wt) vs. GFAPCreER-Cx43f/f mice 20 min after injury. Intravenous injection of EB (red) revealed extensive intraparenchymal extravasation in GFAPCreER-Cx43f/f mice. Images are representative of experimental data graphed in Fig. 2i.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Immune landscape in the cerebral cortex following cerebrovascular injury in Cx3cr1gfp/wtCcr2rfp/wt mice. Quantification of these flow cytometric experiments is provided in Fig. 3c,f, gating strategy in Supplementary Fig. 1B. The panel used for these experiments includes: Cx3CR1-GFP, Ly6C BB790, MHCII BV480, CD11b BV570, CD115 BV605, CD24 BV650, CD11c BV785, P2RY12 PE, CCR2-RFP, Ter-119 PE/Cy5, CD206 PE/Cy7, CD45 BUV395, CD4 BUV496, Ly6G BUV563, CD19 BUV661, CD44 BUV737, CD8 BUV805, F4/80 APC-R700, TCRb APC/Cy7 and live/dead fixable blue cell staining kit. Plots were pre-gated for CD45 + Ter119- live cells and subsequently analyzed using an unsupervised clustering algorithm to group data into subpopulations (PhenoGraph) and visualized using UMAP. For each experiment, the first row depicts the concatenated samples of 4 independent mice per group, and the legend shows the combined phenograph clusters corresponding to different immune cell populations. a, Immune landscape at 1 d and 6 d post-injury compared to uninjured mice. b, Immune landscape 1 d after injury in mice treated with bolus αLFA1/VLA4 or isotype control antibodies relative to uninjured mice.
Extended Data Fig. 6 | Effect of myelomonocytic cell invasion on cerebral edema and survival after injury. This figure depicts quantification of cerebral water content following anterior and posterior injury as well as survival after posterior injury in four different experimental paradigms. Left column shows quantification of cerebral water content in the ipsilateral hemisphere 1 d after anterior injury in the treatment vs control group compared to the contralateral hemisphere. The middle column shows quantification of cerebral water content after posterior injury when mice reached the survival end point or 5 d post-injury in the treatment vs. control group relative to the contralateral hemisphere (*P < 0.05, **P < 0.01, Two-way ANOVA/Holm-Sidak test). Graphs depict mean ± SD. The right column shows Kaplan-Meier survival curves after posterior injury in treatment vs. control groups (Log-rank test). a, Effect of αGr-1 vs. isotype control bolus administration 24 h prior to injury. b, Effect of αLFA1/VLA4 vs. isotype control bolus administration 24 h prior to injury. c, Water content and survival after injury in CCR2 KO mice compared to B6 mice treated with isotype or αLFA1/VLA4 24 h prior to injury. d, Effect of αLy6G or αLFA1/VLA4 vs. isotype control administration 24 h prior to injury. Data are representative of 2 independent experiments with 4 or 5 mice per group.
Extended Data Fig. 7 | Combined αLFA1 and αVLA4 treatment is required to prevent cerebral edema and death. a–c, Effect of bolus treatment with αLFA1/VLA4, αLFA1, αVLA4 or isotype control on cerebral water content 24 h after anterior (A) or posterior (B, C) injury. Data compilation from 2 independent experiments. The antibodies were administered 24 h before injury. Cerebral water content was determined 1d after anterior injury (A) and 5d or when mice reached the survival endpoint after posterior injury (B). Graphs depict mean ± SD (isotype n = 8, αLV n = 8, αLFA1 n = 4 or 5, αVLA4 n = 5, *P < 0.05, **P < 0.01, Two-way ANOVA/Holm-Sidak test). Panel C demonstrates Kaplan-Meier survival curve following posterior injury (isotype n = 9, αLV n = 10, αLFA1 n = 6, αVLA4 n = 6, Logrank test P = 3.4e-5). d, Intravital microscopy images of the cerebral cortex from uninjured vs. injured B6 mice 1h following i.v. administration of APC-anti-CD106 (VCAM-1; red) and PE-anti-CD54 (ICAM-1; green) show increased endothelial ICAM and VCAM expression 24 h after injury. Two representative images from the injured mice depict inter-sample and inter-vessel variability in VCAM-1 vs. ICAM-1 expression. Representative images are from 2 independent experiments with 3 and 5 mice per group. e, Image based quantification of vascular ICAM and VCAM expression. Graph depicts mean ± SD of cumulative data from 2 independent experiments (uninjured n = 6, d1 n = 10, **P < 0.01, Two-way ANOVA/Holm-Sidak test). f, Scatter plot of sum intensity of ICAM vs VCAM expression depicting the lack of a correlation between the two variables. Each dot represents a single mouse, and the graph is a representation of data points shown in Extended Data Fig. 7e, (R² = 0.09, P = 0.4, Pearson’s product moment correlation test). g, Scatter plot of ICAM vs. VCAM gene expression determined by qPCR in the brain 24 h post-injury. No correlation was observed between the two variables. Each dot represents one mouse. Graphs depict cumulative data from 2 independent experiments with 5 mice per experiment, (R² = 0.000078, P = 0.98, Pearson’s product moment correlation test).
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Effect of myelomonocytic cells on cerebral repair and angiogenesis. 

**a, b**, Confocal microscopy of cerebral cortex 10 days after injury shows areas of gliosis (GFAP, blue) and microglia clustering (Iba-1, red) relative to tomato-lectin+ vasculature. Mice were treated continuously with either isotype control (A) or αLFA1/VLA4 (B) antibodies. Continuous treatment with αLFA1/VLA4 results in large areas of brain tissue with blood vessels. Images are representative of 5 mice per group. 

**c, d**, Flow cytometric analysis and gating strategy of monocyte and neutrophil depletion in blood (C) and brain (D) following continuous treatment with αGR-1 and αLy6G antibodies 6 d post-injury compared to isotype treated controls. The following panel was used: Ly6C FITC, GR-1 BV421, CD11b BV570, Cx3CR1 BV711, P2RY12 PE, CD45 BV395, Ly6G BV563, CD44 BV737, CD115 APC and live/dead fixable blue cell staining kit. Plots were pre-gated for single, live cells and subsequently for CD45+CD11b+ cells. In brain samples gated, microglia were identified as CD45lowCx3Cr1P2RY12+CD44- cells. Monocytes were identified as CD45+CD44+CD115+Ly6G-GR1low and neutrophils as CD45+CD44+CD115-Ly6G+GR1hi. In mice treated with αLy6G, Ly6G was not used to characterize cells flow cytometrically. Moreover, in mice treated with αGR-1, GR-1 was not used to characterize the cells. Graphs show the mean ± SD and are representative of 2 independent experiments with n = 5 (C) and n = 4 (D) mice per group (**P < 0.01, Two-way ANOVA/Holm-Sidak test). 

**e, f**, Intravital microscopy of cerebral vasculature and image-based quantification of vascular coverage in naïve B6 mice, CCR2 KO mice and CCR2 KO mice with CD115+ monocyte adoptive transfer 10 d post-injury. Adoptive transfer of CD115+ monocytes from B6 mice partially reconstitutes the angiogenic process. Graph shows mean ± SD and is representative of two independent experiments with (n = 4 mice per group, **P < 0.01, Kruskal-Wallis/Dunn’s test).
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | qPCR analysis of genes encoding for angiogenesis related proteins. qPCR analysis of genes encoding for angiogenesis related proteins. Data from these experiments are represented in Fig. 4i. a, Volcano plot of angiogenesis related gene expression between uninjured mice and mice d6 after injury. B. Bar graph of gene expression differences between injured and uninjured mice for genes with Q < 5%. c-d, Volcano plot of angiogenesis related gene expression after continuous αGr-1 (C) or αLFA1/VLA4 administration (D). e-f, Bar graphs of gene expression difference for genes with Q < 5%. Data are representative of 2 independent experiments with 4 mice per group. Statistical analysis was performed using multiple t-tests and the Benjamini, Krieger and Yekutieli method to correct for the false discovery rate, with a desired Q value of 5%. Data are representative of 2 independent experiments with 4 mice per group per experiment, source data in Supplementary Table 4.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | VEGF-expressing microglia are involved in angiogenesis. a, b, Intravital microscopy of cerebral vasculature 10 d after injury (A) and image-based quantification of vascular coverage (B) in PLX5622 versus vehicle control treated mice show a lack of angiogenesis after microglia depletion. Blood vessels were labeled with EB (red) and tomato-lectin (green). Graphs depict mean ± SD of cumulative data from 3 independent experiments (n = 16 mice per group, **P = 4e-13, two-tailed Student’s t-test). c, Volcano plot of angiogenesis related gene expression in vehicle vs. PLX3397 treated (CSF1R inhibitor) mice 6 d post-injury. d, Bar graph of gene expression difference for genes with Q < 5%. Data are representative of 2 independent experiments with 4 mice per group. Statistical analysis was performed using multiple t-test and the Benjamini, Krieger and Yekutieli method to correct for the false discovery rate, with a desired Q value of 5%. A heatmap of the data from panels A and B is shown in Fig. 5b, source data in Supplementary Table 4. e, Representative heatmaps used to identify different immune cell populations following high parameter flow cytometric analysis of the immune landscape in the cerebral cortex d1 and d6 following injury in Cx3cr1CreER/+ x Stopf1/+ TdTomato mice. The following panel was used: Ly6C BB790, MHCII BV480, CD11b BV570, CD115 BV605, CD24 BV650, Cx3Crl BV711, CD11c BV785, P2RY12 PE, Ter-119 PE/Cy5, CD206 PE/Cy7, CD45 BUV395, CD4 BUV496, Ly6G BUV563, CD19 BUV661, CD44 BUV737, CD8 BUV805, F4/80 APC-R700, TCRβ APC/Cy7, VEGF-A AF647, TdTomato and live/dead fixable blue cell staining kit. Data were pre-gated on CD45 + Ter119- live cells, subsequently analyzed using an unsupervised clustering algorithm to group data into subpopulations (PhenoGraph) and visualized using UMAP. Quantification of these experiments is provided in Fig. 5f. Gating strategy for flow cytometry experiment demonstrating a lack of VEGF-A + microglia in mice treated with continuous αLFA1/VLA4 after injury. The following panel was used: CD24 FITC, CD11b BV570, P2RY12 PE, TdTomato, CD45 BUV395, VEGF-A AF647 and live/dead fixable blue cell staining kit. Quantification of these experiments is provided in Fig. 5h,i and gating strategy in Supplementary Fig. 1B.
Reporting Summary

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n/a  Confirmed

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☐  A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

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☐  A description of all covariates tested

☐  A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

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☐  For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

   Give P values as exact values whenever suitable.

☐  For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐  For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐  Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
MRI acquisition software: ParaVision 6
Macroscopic imaging: Infinity capture software 6.5.2
Two-photon acquisition: Leica Application Suite X (LAS X) 3.5.5.19976
Confocal imaging acquisition: Olympus Fluoview 4.2a
Flow cytometry acquisition: FACSDiva 8.0.1

Data analysis
MRI analysis: Medical Image Processing, Analysis & Visualization (MIPAV) 8.0.2 and OsiriX 11.
Graphs/Statistics: GraphPad Prism 8.4
Flow cytometry: FlowJo 10.5.3
Confocal/Two-photon image and video analysis: Bitplane Imaris 9.3
Video analysis: Adobe Premiere Pro 14.0
RNA sequencing data analysis: FastQC tool 0.11.7, Trimomatic tool 0.38, CLCbio Genomics Workbench 11, R 3.6.2, Ingenuity Pathway Analysis (IPA) tool

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available from the corresponding author upon request. There are no restrictions in data availability. The following figures have associated source data in the supplementary section: Figures 1C, 1D, 2D, 2G, 2H, 3A, 3B, 3C, 3D, 3E, 4A, 4B, 4C, 4D, 4E, 4F, 4G, 4H, 4I, 5A, 5B, 5C, 5D, 5E, 5F, 6A, 6B, 6C, 6D, 7A, 7B, 7C, 7D, 7E, 7F, 7G, 8A, 8B, 8C, 8D, 8E, 9A, 9B, 9C, 9D, 9E, 9F, 9G, 9H, 10A. Bulk RNA sequencing data are available in the NCBI Gene Expression Omnibus (Access code GSE161424).

Field-specific reporting

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 Livescience

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For all studies, 3-8 animals per experimental group were used and all experiments were repeated 2 or 3 times. The bulk sequencing experiment was performed once. Sample sizes were determined based on our experience in previous studies using similar models and feasibility of surgical procedures. We cite previous manuscripts in which similar group sizes and experimental paradigms were used (PMID: 27540166, 29662169, 24317693)

Data exclusions

No data points were excluded after processing. In Figure 2D, one mouse was not processed due to poor image quality. In Figure 2F, two mice were not processed due to poor image quality. In Figure 3E, three images were not processed due to poor image quality. In Figure 4D, three mice were not processed due to poor image quality.

Replication

All experiments were repeated 2 or 3 times. The bulk sequencing experiment was performed once. Results were reliably reproduced for each line of experimentation. Attempts to replicate were successful once the conditions of the experiment were carefully optimized with pilot experiments.

Randomization

Mice were age matched and randomly assigned to each group. Our study involving patients was a prospective observational study of one cohort over time; therefore, no randomization was required.

Blinding

For survival studies, behavioral studies, imaging analysis and quantification, the investigator was blinded to the groups.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a Involved in the study

- Antibodies
- Eukaryotic cell lines
- Palaeontology and archaeology
- Animals and other organisms
- Human research participants
- Clinical data
- Dual use research of concern

Methods

n/a Involved in the study

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

Antibodies used

Immunohistochemistry:
Primary antibodies: anti-CD31 PE (catalog# 102407, Biolegend, San Diego, CA), Rabbit anti-iba1 (catalog# 019-19741; Wako,
Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

| Laboratory animals | 3C5BL/6J (B6), B6.129P-CtxCR1tm1Litt/J (Cxxcr1gfp/gfp), B6.129(Cg)-Ccr2tm2.1fcl/J (Ccr2rfp/rfp), B6.Cg-Tg(GAF-cre/Ert2)Jo5Fmv/J (GFAP-cre-EER), B6.Cg-Tg(Rosa26)Sortm14(Cag-tdTomato)Hze/J (tdTomato/f), B6.129P2(Cg)-Cx3cr1tm2.1(cre/ERT2)Litt/WganJ (GFAP-cre-ERT2), B6.129(B10.PL)-Ly5.1/J (Ly5.1) | This study did not involve wild animals.

**Wild animals**

Mice were bred and maintained under specific pathogen-free conditions at the National Institute of Health. Housing conditions included temperature 72 F, humidity 50% and light/dark cycle 12h each.
Field-collected samples

This study did not involve samples collected from the field.

Ethics oversight

All mice in this study were handled in accordance with the guidelines set forth by the NIH Animal Care and Use Committee and the recommendations in the AAALAC Guide for the Care and Use of Laboratory Animals. The protocol was approved by the NINDS Animal Care and Use Committee.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

**Human research participants**

**Policy information about studies involving human research participants**

**Population characteristics**

Click on the following weblink to see patient inclusion and exclusion criteria: https://clinicaltrials.gov/ct2/show/NCT00009243?term=Natural+History+of+Stroke+Study&draw=2. The characteristic of our population and analysis of those characteristics by groups is provided in Supplementary Table 1. The characteristics by patient are provided in Supplementary Table 2.

**Recruitment**

Patients were identified at presentation in the emergency room and consented for the study following diagnosis of stroke as part of the NIH Natural History of Stroke Study. In this study we included patients with available MRI 5-days post-endovascular thrombectomy which may self-select the most severe cases that were unable to undergo serial MRIs up to 5 days after the initial ischemic stroke.

**Ethics oversight**

Patients were consented for the NIH Natural History of Stroke Study. The appropriate Ethics and Institutional Review Boards (NINDS/NIH IRB for Suburban Hospital, Johns Hopkins Medicine, Bethesda, MD; and Medstar Washington Hospital Center, Washington Hospital Center, Washington, DC IRB) approved the study (NCT00009243).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

**Clinical data**

**Policy information about clinical studies**

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

| Clinical trial registration | NCT00009243 |
|-----------------------------|-------------|
| Study protocol              | https://clinicaltrials.gov/ct2/show/NCT00009243?term=Natural+History+of+Stroke+Study&draw=2 |
| Data collection             | Subjects were recruited from the collaborative stroke programs between NINDS and affiliated hospitals (Suburban Hospital and MedStar Washington Hospital Center) and were followed for the duration of their hospitalization. Patients were included in this study if they were screened from April 2015 to July 2018 with multimodal magnetic resonance imaging (MRI) as their “baseline” scan, and if they received a clinical diagnosis of ischemic stroke involving large vessel occlusion (LVO) of the anterior circulation prior to receiving mechanical embolectomy with or without standard intravenous tPA (tissue plasminogen activator). Patients were also required to have follow-up multimodal MRI obtained at 2-hours, 24-hours and 5-days post-endovascular thrombectomy (EVT). |
| Outcomes                    | Outcomes included presence of hemorrhage, and the volume of DWI and FLAIR signal as well as presence of HARM in MRI obtained 2-hours, 24-hours and 5-days post-endovascular thrombectomy (EVT). |

**Flow Cytometry**

**Plots**

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

**Sample preparation**

Flow cytometry experiments were performed on day 1 and day 6 after injury. We used B6, CX3CR1gfpo/wt x CCR2rfp/wt double reporter and CX3CR1CreER+/+ x Stopf/wt TdTomato mice. Mice were anesthetized and perfused with cold PBS. The injured hemisphere or uninjured control hemisphere was harvested and placed in a 15 ml dounce homogenizer to create a cell suspension. We then isolated immune cells using a 70% to 30% isotonic Percoll gradient and resuspended in PBS with 2% fetal bovine serum (staining buffer). The cells were passed through a 40 um cell strainer. The cells were resuspended in 50 ul of staining buffer with 1:200 purified rat anti-mouse CD16/CD32 (Fc receptor block; clone 93, Biolegend) and incubated at 4C for 10 min. We then added 50 ul of antibody cocktail mix for 15 min at 4C and then 30 min at room temperature. Subsequently cells were washed 2 to 3 times with staining buffer and resuspended in 4% paraformaldehyde for 10 min and stored in PBS. The following anti-mouse antibodies were used: CD24 FITC (M1/69, Biolegend), Ly6C BB790 (AL21, BD), I-A/I-E BV480 (M5/114.15.2, BD), CD11b BV570 (M1/70, Biolegend), CD115 BV605 (T38-320, BD), CD24 BV650 (M1/69, BD), Cx3Cr1
For MRI brain of human subjects, isotropic diffusion-weighted imaging (DWI) and pre-contrast fluid attenuated MRI imaging was obtained as part of our clinical cohort study and for ex vivo evaluation of mouse brains after cerebrovascular injury. No MRI specific experimental design was used.

**Field strength**
For MRI brain of human subjects, a 3T MRI scanner (Siemens Skyra, Siemens AG, Munich, Germany and Philips Achieva, Philips Healthcare, Best, Netherlands) was used.

For ex vivo MRI imaging of mouse brains, a vertical bore 7T Bruker BioSpin Magnetic (Billerica, MA, USA) with a volume coil (Bruker, internal diameter 2 cm) was used.

**Sequence & imaging parameters**
For MRI brain of human subjects, isotropic diffusion-weighted imaging (DWI) and pre-contrast fluid attenuated inversion recovery (FLAIR) sequences were used.
For ex vivo MRI imaging of mouse brains, T2* weighted 3-D Multiple Gradient Echo (MGE) sequence was used.

For MRI brain of human subjects, a 3T MRI scanner (Siemens Skyra, Siemens AG, Munich, Germany and Philips Achieva, Philips Healthcare, Best, Netherlands) was used.

For ex vivo MRI imaging of mouse brains, a vertical bore 7T Bruker BioSpin Magnetic (Billerica, MA, USA) with a volume coil (Bruker, internal diameter 2 cm) was used.

**Magnetic resonance imaging**

**Experimental design**

| Design type | MRI imaging was obtained as part of our clinical cohort study and for ex vivo evaluation of mouse brains after cerebrovascular injury. No MRI specific experimental design was used. |
|-------------|--------------------------------------------------------------------------------------------------------------------------|
| Design specifications | MRI imaging was obtained as part of our clinical cohort study and for ex vivo evaluation of mouse brains after cerebrovascular injury. No MRI specific experimental design was used. |
| Behavioral performance measures | We did not perform a behavioral or functional MRI study. |

**Acquisition**

| Imaging type(s) | For MRI brain of human subjects, isotropic diffusion-weighted imaging (DWI) and pre-contrast fluid attenuated inversion recovery (FLAIR) sequences were used. For ex vivo MRI imaging of mouse brains, T2* weighted 3-D Multiple Gradient Echo (MGE) sequence was used. |
|-----------------|--------------------------------------------------------------------------------------------------------------------------|
| Field strength | For MRI brain of human subjects, a 3T MRI scanner (Siemens Skyra, Siemens AG, Munich, Germany and Philips Achieva, Philips Healthcare, Best, Netherlands) was used. For ex vivo MRI imaging of mouse brains, a vertical bore 7T Bruker BioSpin Magnetic (Billerica, MA, USA) with a volume coil (Bruker, internal diameter 2 cm) was used. |
| Sequence & imaging parameters | For MRI brain of human subjects, isotropic diffusion-weighted imaging (DWI) and pre-contrast fluid attenuated inversion recovery (FLAIR) sequences were acquired at baseline, 2-hours, 24-hours, and 5-days as part of a standardized imaging protocol including additional sequences. Both DWI and FLAIR were co-localized, sharing the following parameters: 240 mm field of view, 40 axial-oblique contiguous interleaved slices, 3.5 mm thick covering 140 mm inferior to superior, factor of 2 array encoding, flip angle 90. DWI and apparent diffusion coefficient (ADC) maps were generated from an echo-planar diffusion tensor sequence, b=1000 with 15 (Philips) or 12 directions (Siemens), number... |
of averages=2, 2 mm in-plane resolution, TR/TE = 4500/53 ms (Philips) or 7800/81 ms (Siemens). For FLAIR, a TR/TE/TI=9000/120/2600 ms, number of echos=35, 1mm in-plane resolution.

For ex vivo MRI imaging of mouse brains, T2* weighted 3-D Multiple Gradient Echo (MGE) sequence was acquired (repetition time 100 ms, echo time 5 ms (first echo), echo spacing 5ms, number of echos 10, flip angle=30 degree, field of view=30x30x30 mm and image resolution 100x100x100 μm with number of averages=6).

| Area of acquisition | Whole brain was imaged for each sample. |
|---------------------|----------------------------------------|

### Preprocessing

| Preprocessing software | No pre-processing was performed other than during. |
|------------------------|--------------------------------------------------|
| Normalization          | Not applicable - no group averaging was performed. |
| Normalization template  | Not applicable - no normalization template was used. |
| Noise and artifact removal | No noise or artifact removal was performed. |
| Volume censoring        | No volume censoring was used. |

### Statistical modeling & inference

| Model type and settings | Not applicable - no multivariate analysis was performed. |
|-------------------------|--------------------------------------------------------|
| Effect(s) tested        | We did not perform a behavioral or functional MRI study. |
| Specify type of analysis: | ☑ Whole brain   ☐ ROI-based   ☐ Both |
| Statistic type for inference | Not applicable - no voxel-based analysis was performed. |
| Correction               | Not applicable. |

### Models & analysis

| n/a Involved in the study | ☑ Functional and/or effective connectivity |
|---------------------------|-------------------------------------------|
|                           | ☑ Graph analysis                           |
|                           | ☑ Multivariate modeling or predictive analysis |