Title

CNS resident macrophages enhance dysfunctional angiogenesis and circulating monocytes infiltration in brain arteriovenous malformation

Authors

Ma, Li
Zhu, Xiaonan
Tang, Chaoliang
et al.

Publication Date

2023-05-12

DOI

10.21203/rs.3.rs-2899768/v1

Original Article

JCBFM

Journal of Cerebral Blood Flow & Metabolism
0(0) 1–13

DOI: 10.1177/0271678X241236008
journals.sagepub.com/home/jcbfm
Sage

CNS resident macrophages enhance dysfunctional angiogenesis and circulating monocytes infiltration in brain arteriovenous malformation

Li Ma[1,2], Xiaonan Zhu[1,2], Chaoliang Tang[1,2], Peipei Pan[1,2], Alka Yadav[1,2], Rich Liang[1,2], Kelly Press[1,2], Jeffrey Nelson[1,2] and Hua Su[1,2]

Abstract

Myeloid immune cells are abundant in both ruptured and unruptured brain arteriovenous malformations (bAVMs). The role of central nervous system (CNS) resident and circulating monocyte-derived macrophages in bAVM pathogenesis has not been fully understood. We hypothesize that CNS resident macrophages enhance bAVM development and hemorrhage. RNA sequencing using cultured endothelial cells (ECs) and mouse bAVM samples revealed that down-regulation of two bAVM causative genes, activin-like kinase 1 *(ALK1)* or endoglin, increased inflammation and innate immune signaling. To understand the role of CNS resident macrophages in bAVM development and hemorrhage, we administrated a colony-stimulating factor 1 receptor inhibitor to bAVM mice with brain focal *Alk1* deletion. Transient depletion of CNS resident macrophages at an early stage of bAVM development mitigated the phenotype severity of bAVM, including a prolonged inhibition of angiogenesis, dysplastic vasculature formation, and infiltration of CNS resident and circulating monocyte-derived macrophages during bAVM development. Transient depletion of CNS resident macrophages increased EC tight junction protein expression, reduced the number of dysplasia vessels and severe hemorrhage in established bAVMs. Thus, EC AVM causative gene mutation can activate CNS resident macrophages promoting bAVM progression. CNS resident macrophage could be a therapeutic target to mitigate the development and severity of bAVMs.

Keywords

Arteriovenous malformation, angiogenesis, CNS resident macrophages, colony-stimulating factor 1 receptor inhibitor, myeloid derived macrophages

Received 18 September 2023; Revised 8 December 2023; Accepted 6 February 2024

Introduction

Arteriovenous malformations (AVMs) are active angiogenic lesions consisting of tangles of abnormal vessels which shunt blood directly from arteries to veins without a true capillary bed.[1] Innate immune response in bAVM is characterized by macrophage infiltration. An abnormally high number of macrophages are present in and around vascular walls in human brain AVM (bAVM) specimens, regardless of hemorrhage, which suggests that macrophage accumulation is not simply a response to hemorrhage.[2–4] Polymorphisms in inflammatory cytokine genes and elevated expressions of inflammation-related genes in bAVM patients suggest active roles of inflammation in bAVM pathogenesis.[5] We found increased macrophage burden in mouse bAVMs generated through conditional deletion of endoglin (*Eng*) or activin-like kinase 1 (*Alk1,* also known as *ACVLR1*), in combination with

[1]Center for Cerebrovascular Research, University of California, San Francisco, California, USA
[2]Department of Anesthesia and Perioperative Care, University of California, San Francisco, California, USA

Corresponding author:
Hua Su, Department of Anesthesia and Perioperative Care, University of California, San Francisco, Box 1363, 2540 23rd St. Floor 5, San Francisco, CA 94143, USA.
Email: hua.su@ucsf.edu

focal angiogenic stimulation.[6–9] *ENG* and *ALK1* are causative genes of hereditary hemorrhagic telangiectasia (HHT), a familial disorder with high prevalence of bAVMs.[1] The activated macrophages were also found abundantly in mouse bAVM with endothelial overexpression of $KRAS^{G12V}$,[10] a somatic mutation identified in sporadic bAVMs.[10] Macrophage accumulation in bAVM represents unresolved inflammation, which can enhance abnormal vascular remodeling and the severity of the bAVM phenotype. It is unclear whether these known gene mutations directly contribute to neuroinflammation in bAVMs. Understanding the mechanism of macrophage accumulation may afford opportunities to identify therapeutic targets for developing new therapies that improve outcomes of bAVM patients.[4]

In addition, persistent proinflammatory differentiation of macrophages have been found to be critical in bAVM progression. We identified both the central nervous system (CNS) resident and circulating myeloid cells as potential precursors of proinflammatory macrophages in bAVMs.[11] Recent studies on human bAVM tissues unraveled the crosstalk between immune cells and vascular components.[12] More importantly, a heterogenous spectrum of myeloid cells with typical molecular signature and spatial distribution were clustered, which were distinct in ruptured bAVMs and associated with instability of AVM vasculature. Resident myeloid cells were the major immune cells infiltrating along the perivascular space and deeper brain tissue, while some specific monocytes were over-represented in ruptured AVMs. It is undetermined which cell clusters act as initiators and which ones respond subsequently. Moreover, potential interplay between CNS endogenous and circulating myeloid cells has yet to be determined. Further exploration is needed to elucidate the distinct role of myeloid cell subsets and to catalog more specific therapeutic targets in bAVM.

Circulating monocytes can infiltrate the CNS in brain vascular disorders and differentiate into macrophages.[13,14] The recruitment of monocytes into tissues, including CNS tissues, depends on monocyte chemoattractant protein CCL2 and its receptor, CCR2. We found a delayed but persistent accumulation of $Ccr2^{+}$ cells in mouse bAVMs.[11] In contrast, CNS resident macrophage populations are established in the CNS during embryonic development, which are maintained independently of circulating monocytes. There are two major clusters of CNS resident macrophages based on anatomical location, morphology, and molecular signatures: parenchymal specific macrophages (microglia) and non-parenchymal macrophages (further classified as perivascular, subdural meninges and choroid plexus macrophages.[15–17] Ionized calcium-binding adaptor molecule 1 (Iba1), C-X3-C motif chemokine receptor 1 (CX3CR1), and colony stimulating factor 1 receptor (CSF1R) markers are expressed across all CNS resident populations.[15,18] The stimulation from CSF1R is critical for the development and maintenance of CNS resident macrophages.[19] Pharmacological inhibition of CSF1R depletes $Iba1^{+}$ and $CX3CR1^{+}$ cells by 94% to 98% in the parenchymal and ~70% in perivascular space.[20] The CSF1R inhibitor PLX5622 depletes CNS resident myeloid cells without interfering the infiltration of circulating $CCR2^{+}$ monocytes.[13,21] Therefore, use of PLX5622 will allow us to understand the role of CNS resident macrophages.

In this study, we demonstrate that mutation of AVM causative genes in endothelial cells (ECs) can upregulate neuroinflammatory signaling pathways, and CNS resident macrophages actively involved in bAVM pathogenesis.

Material and methods

Animals

The protocol and experimental procedures for using laboratory animals were approved by the Institution of Animal Care and Use Committee (IACUC) at the University of California, San Francisco and were conducted in accordance with the guide for the Care and use of Laboratory animals. The experiment results are reported according to ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). Animal husbandry was provided by the Animal Core Facility and the IACUC under the guidance and supervision of certified Animal Technologists. Veterinary care was provided by IACUC faculty and veterinary residents located on the Zuckerberg San Francisco General Hospital campus.

Three mouse models were used: 1) $Alk1^{f/f}$ mice in which two alleles of *Alk1* gene (exons 4 to 6) are flanked by LoxP sites,[22] 2) $Alk1^{f/f}$;$Ccr2^{RFP/+}$;$Cx3cr1^{GFP/+}$ mice with red fluorescent protein (RFP) gene knocked into one allele of *Ccr2* gene and green fluorescent protein gene (GFP) knocked into one allele of *Cx3cr1* gene,[23] and 3) *Pdgfbi*CreER;$Eng^{f/f}$ mice that have Pdgfb promoter driven cre recombinase expression in ECs and two alleles of *Eng* gene (exons 4 to 5) flanked by loxP sites.[24] Equal numbers of male and female mice were included. All mice were 8- to 10-week-old and had C57BL backgrounds.

Induction of bAVM through stereotactic injection of viral vector

Brain AVMs were induced in $Alk1^{f/f}$ and $Alk1^{f/f}$;$Ccr2^{RFP/+}$;$Cx3cr1^{GFP/+}$ mice through stereotactic

intracerebral injection of viral vectors as described in our previous paper (Supplementary Fig. 1).[6] Mice were anesthetized through inhalation of 4% isoflurane and placed in a stereotactic frame with a holder (David Kopf Instruments, Tujunga, CA). A burr hole was drilled in the pericranium 2 mm lateral to the sagittal suture and 1 mm posterior to the coronal suture. A total of 2 μL viral vector suspension containing 2×10^9 genome copies (gcs) of AAV-VEGF (an adeno-associated viral vector carrying CMV promoter driving human vascular endothelial growth factor) and 2×10^7 plaque-forming units (PFU) of Ad-Cre (an adenoviral vector carrying CMV promoter driving Cre recombinase expression) were injected into the basal ganglia 3 mm beneath the brain surface. AAV-LacZ and Ad-GFP were used as controls for AAV-VEGF and Ad-Cre, respectively.

Brain AVMs were induced in *Pdgfb*iCreER;*Eng*$^{f/f}$ mice through intra-peritoneal injection of tamoxifen (TM, 3 doses of 2.5 mg/25 g of body weight) in 3 consecutive days and intra-brain injection of AAV-VEGF (2×10^9 gcs) at the first day of TM injection.[9] Control mice were treated with 3 doses of corn oil and intra-brain injection of AAV-VEGF. Brain AVM tissues were collected 8 weeks after model induction. Total RNAs were isolated from bAVM lesions and brain angiogenic regions (controls) for sequencing.

ALK1 *knockdown in ECs and RNA sequencing (RNAseq)*

Human umbilical cord ECs [HUVECs, American Type Culture Collection (ATCC), Manassas, VA)] were cultured in Vascular Cell Basal Medium supplemented with EC growth factors in Endothelial Cell Growth Kits provided by ATCC. Cells within 6 passages were used. To knockdown *ALK1*, 180 pmol of *ALK1* siRNA (Sequence: 5′-CCCUCUACGACUUUCUGCA-3′)[25] custom synthesized by Thermo Fisher Scientific (South San Francisco, CA) were transfected into HUVECs using FlexiTube siRNA (QIAGEN, Hilden, Germany) in RNAiMax (Invitrogen, Waltham MA), according to manufacturer instructions. Scrambled siRNA transfected cells were used as controls. Total RNAs were extracted from the cells 48 hours after the transfection using RNAzol RT (Molecular Research Center, Cincinnati, OH).

To check the knockdown efficiency, the RNAs were reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA) with gene-specific primers and probes (Applied Biosystems): *ALK1* (Hs00953798_m1), and *GAPDH* (Hs02758991_g1). The relative gene expression was calculated using the comparative threshold cycle (CT) and normalized to *GAPDH* (ΔCT).

Total RNAs isolated from HUVECs with *ALK1* being downregulated by more than 80% and control cell, as well as total RNAs isolated from bAVMs and brain angiogenic region of control mice were sent to Novogene Co (Davis, CA) for sequencing using the company's standard protocol (Supplemental material 1). The outcome data were also analyzed by Novogene Co.

Administration of inhibitor of CSF1R (PLX5622)

Alk1$^{f/f}$ and *Alk1*$^{f/f}$;*Ccr2*$^{RFP/+}$;*Cx3cr1*$^{GFP/+}$ mice were used in testing the effect of PLX5662. Mice were randomly assigned by generating random numbers to 4 experimental groups. PLX5622 (180 mg/kg of body weight/day, Plexxikon Biotech Company, South San Francisco, CA) was incorporated into chow and orally administered for 7 days starting at 1 week or 8 weeks after model induction (Supplementary Fig. 1). The placebo chow was administrated in the same pattern to the control group.

Experimental groups are:

1. AVM + V: bAVM mice treated with vehicle starting at 1 or 8 weeks after model induction. This group serves as a control for PLX5622 treatment.
2. AVM + P: bAVM mice treated with PLX5622. The effect of PLX5622 on bAVM was tested in this group.
3. Ang + P: Mice with brain focal angiogenesis treated with PLX5622. This group is for testing the effect of PLX5622 on normal brain angiogenesis.
4. WT + P: Mice received intra-brain injection of control vectors and PLX5622 treatment starting at 1 or 8 weeks after model induction. This group is for testing the effect of PLX5622 on normal brain.

Quantitative assessment of vessel morphology and macrophages

Brain samples were collected 8 weeks or 9 weeks after model induction. After being anesthetized with isoflurane inhalation, Cy5-fluorescein-conjugated lycopersicon esculentum lectin (Vector Laboratories, Burlingame, CA) was injected via jugular vein to stain endothelial cells. Mice were then perfused with heparinized PBS through the left cardiac ventricle to clear blood from vessels, followed by 4% paraformaldehyde. Brain samples were collected and incubated in 4% paraformaldehyde containing 20% sucrose until they sunk to the bottom of the solution. Brain samples were then snap-frozen in dry ice for section.

Coronal sections (20-μm-thick) were cut using a cryostat (Leica, CM1900, Germany). Two coronal

sections, 0.5 mm anterior and 0.5 mm posterior to the needle track, from each mouse were cover slipped with Vectashield mounting medium containing 4′-6-diamidino-2- phenylinidole (DAPI, Vector Laboratories, Newark, CA) to label cell nuclei for quantification of vascular density, dysplasia, RFP^+ and GFP^+ cells.

For analyzing claudin 5 expression, brains were frozen in dry ice and cut into 20-μm-thick coronal sections using a Leica CM1950 Cryostat (Leica Microsystems). Claudin 5 expression on vessels was visualized by double immunostaining using rabbit anti mouse claudin 5 antibody (1:100, Thermo Fisher Scientific, Waltham, MA) and goat anti-mouse CD31 antibody (1:250, R&D Systems, Minneapolis, MN). Alexa Fluor 594-conjugated (1:250) and Alexa Fluor 488-conjugated IgG (1:250, Invitrogen, Carlsbad, CA) were used as secondary antibodies.

Three images were taken under 20 X objective field of each section (left, right, and below the injection site, Supplementary Fig. 1) using a fluorescent microscope (Keyence BZ-9000, Itasca, IL). Vascular density (number of vessels per mm^2), RFP^+ and GFP^+ cells (per mm^2) were quantified in using NIH Image 1.63 software. For claudin 5 quantification, claudin 5 positive areas and CD31 positive areas were quantified using NIH Image 1.63 software. The claudin 5 levels were expressed as ratio of claudin 5 positive areas and CD31 positive areas. Dysplasia vessels were counted manually and expressed by Dysplasia index (the number of vessels with lumen diameter larger than 15 $\mu m/mm^2$).

Prussian blue staining

Iron Stain Kit (Sigma-Aldrich, St. Louis, MO) was used to detect iron deposition. Two sections within the injection site were chosen for staining for each brain. Data were presented as percentage of Prussian blue positive area in the hemisphere.

Power analysis

Based on the testing study on normal mouse brains (Supplementary Fig. 2), we chose a sample size of 6 animals per group to detect a 30% difference with 80% changes in CNS macrophage number.

Statistics

For quantification of vessels density, dysplasia index, RFP^+ and GFP^+ cell numbers, vascular claudin 5 coverage, and Prussian blue positive areas, section numbers were scrambled. The quantification was done independently by at least two researchers who were blinded to the treatment groups. Inter-observer discrepancy was controlled within one standard deviation, and the means were used for further analysis. Data are presented as mean ± standard deviation (SD). Histogram was used to test if the data were normally distributed. All data except Prussian blue staining were analyzed through t test for two sample-comparation, or one-way ANOVA for multiple sample-comparation followed by Tukey's multiple comparisons using GraphPad Prism 9 software. The non-parametric Kruskal-Wallis test method was used to analyze differences of the percentages of Prussian blue positive area versus the total hemisphere areas between groups using stata 18.0 MP (StataCorp. 2023. Stata Statistical Software: Release 18. College Station, TX: StataCorp LLC.) with the aid of the dunntest command (Dinno A. 2014. dunntest: Dunn's test of multiple comparisons using rank sums. Stata software package. URL: https://alexisdinno.com/stata/dunntest.html), since the data were not normally distributed and had value of zeros. The percentages of Prussian blue positive area versus total hemisphere area were also analyzed by frequency distribution using GraphPad Prism 9 software. A P value <0.05 was considered to be significant. Sample sizes were indicated in figures.

Results

Downregulation of ALK1 or Eng in ECs upregulated pro-inflammatory and innate immune signaling

To understand the roles of *ALk1* and *ENG* genes in endogenous angiogenesis and inflammation, *ALK1* was knocked down in HUVECs and *Eng* was knocked out in mouse ECs. The transcriptional profiles of *ALK1* deficient HUVECs, bAVMs of *Eng* deficient mice, and angiogenic region in control mice (corn oil treated mice) were analyzed via RNAseq. We found that down regulation of *ALK1* in HUVECs upregulated the expression of 507 genes and downregulated the expression of 563 genes compared to scrambled siRNA treated HUVECs. Gene Ontology (GO) enrichment analyses showed that knockdown of *ALK1* in HUVEC increased the transcription of genes regulating immune response and cell migration, including cellular response to interferon-gamma (INF γ, adjust $P = 0.005$), positive regulation of response to cytokine stimulus (adjust $P = 0.015$) and positive regulation of cell migration (adjust $P = 0.03$). Top 10 upregulated biological pathways are shown in Figure 1(a).

We have also analyzed the changes of gene transcription in bAVMs of mice with *Eng* deleted in ECs. We found 1243 genes were upregulated, and 1830 genes were downregulated in bAVM, compared to brain angiogenic of control mice. GO enrichment analyses showed that knockout of *Eng* in ECs increased the transcription of genes upregulating angiogenesis (adjust $P = 2.1 \times 10^{-17}$), vascular development

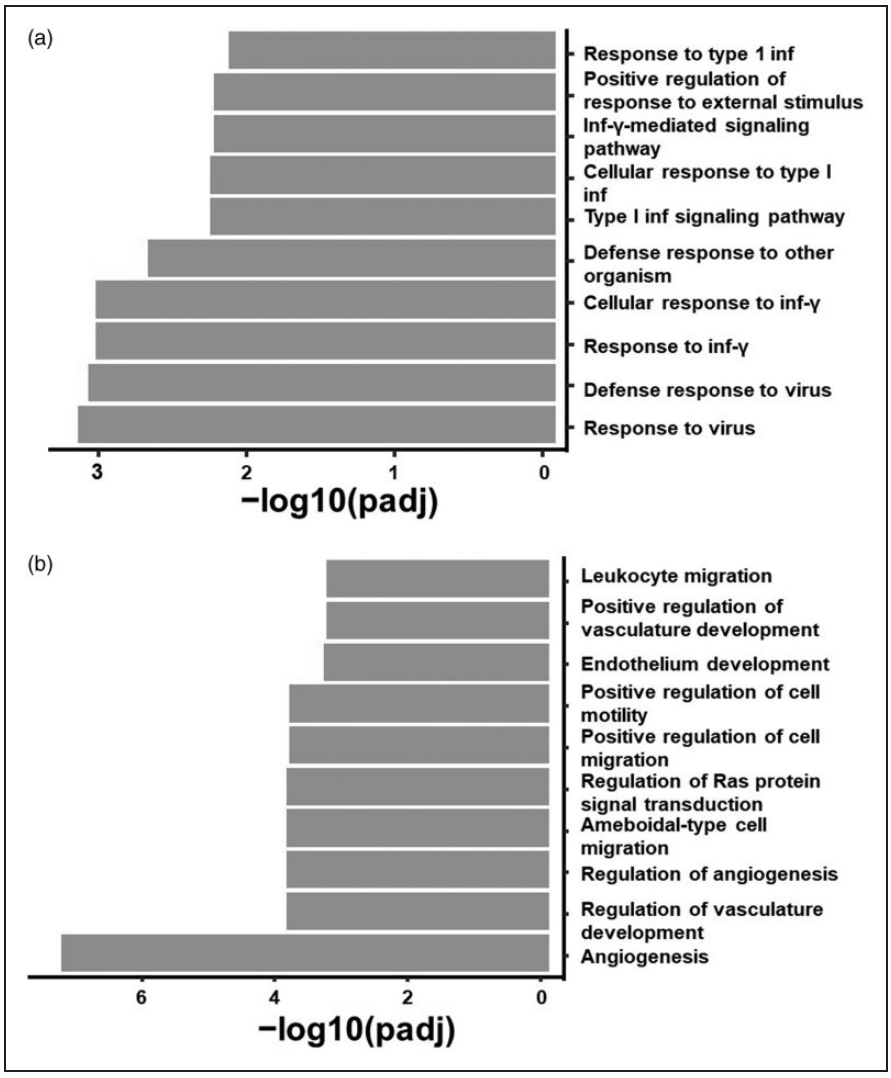

Figure 1. Down regulation of *ALK1* in HUVECs or *Eng* in mouse ECs increased pro-inflammation signaling in HUVECs and mouse bAVM lesion. (a) Bar graph showing the top 10 upregulated biological pathways in *ALK1* siRNA treated HUVECs compared to scrambled siRNA treated HUVECs and (b) Bar graph showing the top 10 upregulated biological pathways in Eng deficiency bAVMs compared to normal brain angiogenic regions.

(adjust $p = 2.5 \times 10^{-08}$), leukocyte migration (adjust $P = 2.2 \times 10^{-07}$), and myeloid cell differentiation (adjust $P = 1.5 \times 10^{-05}$). Top 10 upregulated biological pathways are shown in Figure 1(b). GO enrichment analyses also show that *Eng* deficiency ECs increased the transcription of gene sets related to myeloid leukocyte differentiation and migration, myeloid leukocyte activation, phagocytosis, activation and regulation of innate immune response, neutrophil chemotaxis, myeloid cell development, positive regulation of chemotaxis, and glial cell proliferation and migration (Supplementary Table 1). Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses shown that EC *Eng* deficiency increased PI3K-Akt (adjust $p = 0.04$) and chemokine (adjust $p = 0.04$) pathways, and cell adhesion molecules (CAMs, adjust $p = 0.05$). Differential analysis shown that the transcription of *Cx3cr1* (adjust $P < 0.001$) and *Csf1* gene (adjusted $P = 0.003$) were increased significantly in the bAVM of EC *Eng* deficient mice.

These data indicate that down regulation of *ALK1* or *Eng* expression in ECs increases EC inflammation, immune and inflammatory response, and angiogenic activity.

Transient depletion of CNS resident macrophages reduced the burden of Cx3cr1$^+$ and Ccr2$^+$ macrophages in bAVM

To investigate the specific function of CNS resident macrophages in bAVM inflammation, Cx3cr1$^+$ CNS resident macrophages were transiently depleted through

administration of PLX5622. We first tested the efficiency of PLX5622 on depletion of CNS resident macrophages by administration of PLX5622 (180 mg/kg of body weight/day) in chow to *Ccr2*$^{RFP/+}$; *Cx3cr1*$^{GFP/+}$ mice for 7 days. We found 93% of Cx3cr1^{+} were depleted in the brains of treated mice (Supplementary Fig. 2).

We next administrated same dose of PLX5622 in chow to mice with bAVMs generated by focal deletion of *Alk1* gene plus angiogenic stimulation[6] to test the effect of transient depletion of CNS resident macrophage on bAVM pathogenesis. PLX5622 treatment was performed for 7 days, starting either 1 week after model induction, when bAVM development begins ($P = 0.004$), or 8 weeks after model induction, when bAVMs have established ($P < 0.001$), reduced *Cx3cr1*$^{+}$ resident macrophages in bAVM lesions (Figure 2). In the disease condition, some Ccr2^{+} peripheral macrophages can adopt characters of the CNS resident macrophages and express both Ccr2 and Cx3cr1, showing yellow in Figure 2.[23] To avoid double counting Cx3cr1^{+} cells, we subtracted double positive cells (yellow) from Cx3cr1^{+} cell counts.

Administration of PLX5622 starting at 1 week after model induction, during the beginning of bAVM development, also reduced Ccr2^{+} circulating monocyte-derived macrophages in bAVMs ($P < 0.001$, Figure 2), however PLX5622 treatment failed to reduce the number of circulating derived Ccr2^{+} macrophages in already established bAVMs. This could be due to more severe inflammation in the established bAVMs (Figure 2).

Transient depletion of CNS resident macrophages did not reduce angiogenic activity in bAVMs significantly

To explore the role of CNS resident macrophages on bAVM angiogenesis, we assessed the blood vessel

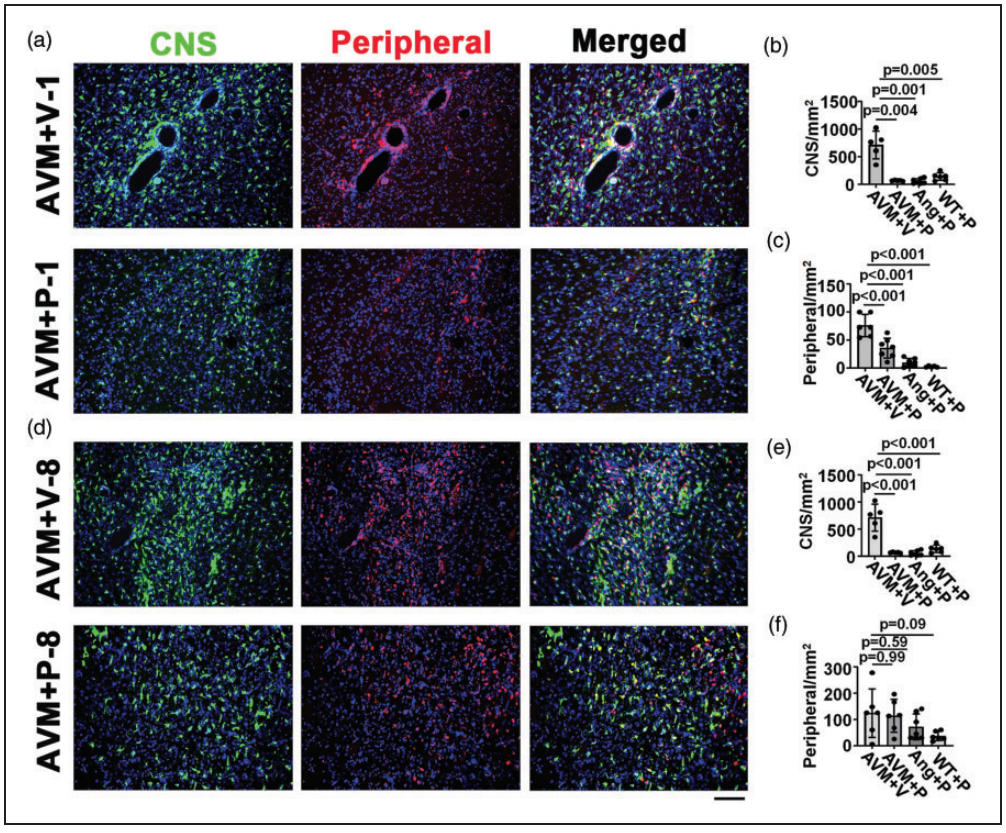

Figure 2. PLX5622 treatment reduced CNS resident and circulating monocyte-derived macrophages in mouse bAVMs. (a) & (d). Representative images of bAVMs collected from mice that received PLX5622 starting 1 week (a) or 8 weeks (d) after model induction. CNS resident macrophages (CNS) are Cx3cr1^{+} (green) and peripheral monocyte-derived macrophages (Peripheral) are CCR2^{+} (red). Merged images show some cells expressing both Cx3cr1^{+} and CCR2^{+} (yellow). Nuclei were counterstained with DAPI (blue). Scale bar= 50 μm. (b) and (c). Quantifications of CNS resident macrophages (b) and peripheral monocyte-derived macrophages (c) in bAVMs of mice that received PLX5622 starting 1 week after model induction. (e) and (f). Quantifications of CNS resident macrophages (e) and peripheral monocyte-derived macrophages (f) in bAVMs of mice that received PLX5622 8 weeks following model induction. AVM + V: AVM mice treated with vehicle; AVM + P: AVM mice treated with PLX5622; Ang + P: mice with brain angiogenesis and PLX5622 treatment; WT + P: WT mice treated with PLX5622 treatment. N = 5–7.

densities of bAVMs and brain angiogenic regions in control mice (Supplementary Fig. 1), then compared to treated mice. The vessel densities of bAVMs in mice treated with PLX5622 at the beginning of bAVM development (434.1 ± 128.9 vessels/mm^2) were trend toward lower than that in vehicle-treated mice (567.5 ± 110 vessels/mm^2, $P = 0.079$) and were similar to that in normal brain angiogenic region of angiogenic control mice (419.3 ± 63.3 vessels/mm^2, $p = 0.99$, Figure 3). The vessel densities in the bAVMs of mice treated with PLX5622 at 8 weeks after model induction (542.5 ± 104.5 vessels/mm^2) were also trend toward lower than that in vehicle treatment (466.5 ± 80.0 vessels/mm^2, $p = 0.070$) and were similar to that in angiogenic control mice (361.3 ± 13.24, $p = 0.55$, Figure 3). Therefore, transient depletion of CNS resident macrophages did not inhibit angiogenic activity effectively.

Transient depletion of CNS resident macrophages alleviated bAVM severity

We next evaluated the effect of PLX5622 treatment on bAVM severity by examining abnormal vessels with dilated and irregular lumens. PLX5622 treatment starting at 1 week after the model induction inhibited bAVM development. Even though PLX5622 treatment had stopped for 6 weeks, there were still fewer dysplasia vessels in the treated group (15.2 ± 2.6 vessels/mm^2) than the control group (23.1 ± 2.4 vessels/mm^2, $P = 0.002$). However, the PLX5622 treated bAVM group still had more dysplasia than the angiogenic

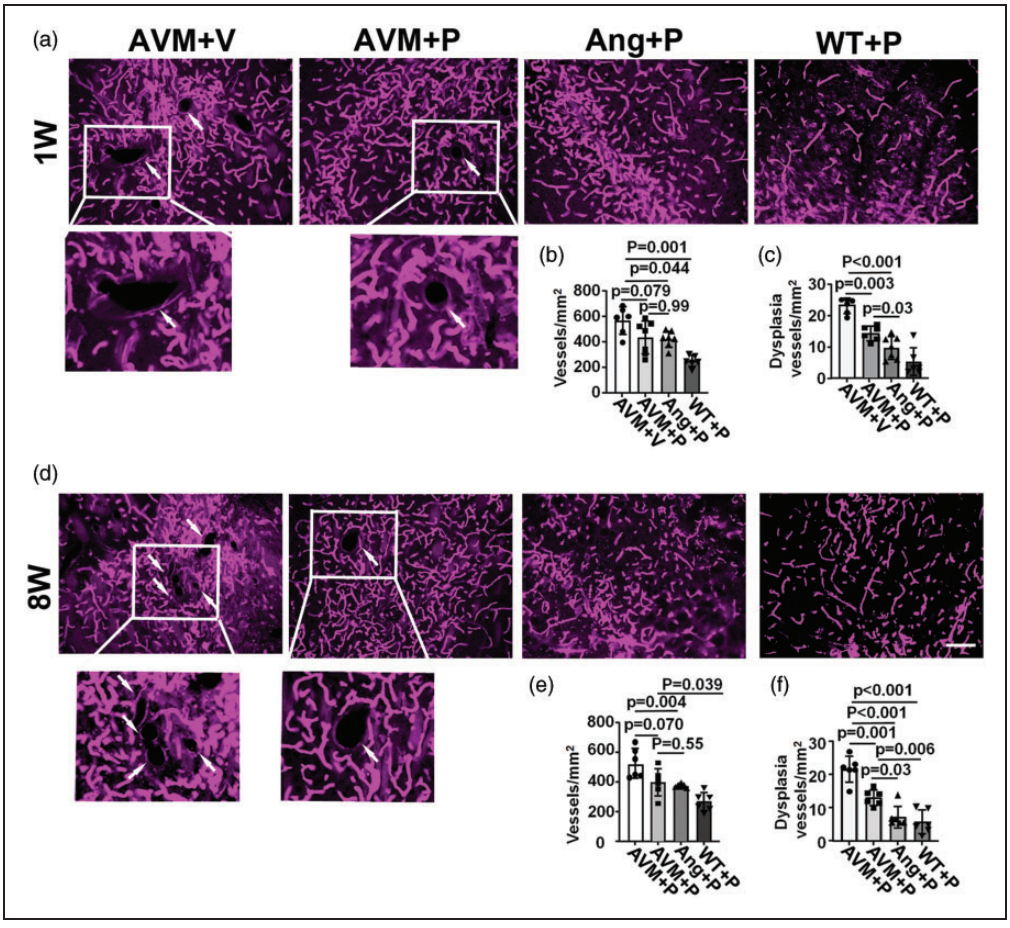

Figure 3. PLX5622 treatment reduced dysplastic vessels in mouse bAVMs. (a) and (d) Representative images of brain sections collected from lectin-perfused mice. Vessels are labeled by Cy5 fluorescence labeled lectin (violet). The pictures under 1 W and 8 W AVM + V and AVM + P are enlarged pictures of the squared areas in the pictures above them showing dysplasia vessels. White arrows indicate dysplastic vessels. Scale bars = 50 μm. (b) and (c) Quantifications of vessel densities (b) and dysplasia index (number of vessels with lumen diameter >15 mm/mm^2) (c) for mice treated with PLX5622 starting at 1 week after model induction. (e) and (f) Quantifications of vessel densities (e) and dysplasia index (f) for mice treated with PLX5622 starting at 8 week after model induction. AVM + V: AVM mice treated with vehicle; AVM + P: AVM mice treated with PLX5622; Ang + P: mice with brain angiogenesis and PLX5622 treatment; WT + P: WT mice treated with PLX5622 treatment. 1 W and 8 W: treatment started at 1 and 8 weeks after model induction. N = 6 or 7.

region of PLX5622 treated control group (9.6 ± 3.6 vessels/mm^2, $P < 0.001$, Figure 3). Administration of PLX5622 starting at 8 weeks after the model induction when bAVMs have already established[6] also reduced the number of abnormal vessels (treated: 13.3 ± 2.4 vessels/mm^2 vs. control: 21.8 ± 3.9 vessels/mm^2, $P = 0.001$) (Figure 3). Again, the PLX5662 treated bAVMs still have more dysplasia than PLX5662 treated angiogenic controls (7.3 ± 3.2, $p = 0.03$). Notably, the number of Cx3cr1$^+$ CNS resident macrophages was positively correlated with the number of abnormal vessels in the bAVMs ($r^2 = 0.63$, $P < 0.01$, Figure 4). Taken together, these data show that transient depletion of CNS resident macrophages not only reduced bAVM development, but also alleviated the severity of established bAVMs.

Transient depletion of CNS resident macrophages increased blood brain barrier (BBB) tight junction protein expression and attenuated severe hemorrhage in bAVMs

Our previous study revealed impairment of vascular integrity in bAVMs.[7] To explore the impact of CNS resident macrophages on BBB integrity of established bAVM, the expression of tight junction protein, claudin 5, was quantified. *Alk1*$^{f/f}$ mice received intra-brain injections of Ad-Cre and AAV-VEGF were randomly assigned to PLX5622 and vehicle groups. The treatments started at 8 weeks after model induction and the brains were collected 9 weeks after model induction (Supplementary Fig. 1). We found PLX5622 treatment increased the expression of claudin 5 in established bAVMs (Figure 5), suggesting that transient depletion of CNS resident macrophages can reduce BBB impairment independently of circulating monocyte infiltration.

We further tested whether transient depletion of CNS resident macrophages reduces or prevents bAVM hemorrhage. Hemorrhage in bAVMs were detected by analyzing iron deposition using Prussian blue staining. Although significant differences among groups were identified by Kruskal-Wallis test for mice treated at 1 week ($P = 0.007$) and at 8 weeks ($P = 0.004$) after model induction, the differences were driven by more hemorrhage in bAVMs than control brains (CP). There was no difference between bAVM mice treated with vehicle (AVM + V) and PLX5622 (AVM + P, $P = 0.19$ for 1 week group and $P = 0.22$ for 8-week group). Frequency distribution analyses shown that large hemorrhagic areas were only present in vehicle treated bAVM mice. PLX5622 treatment starting at 1 or 8 weeks after model induction prevented severe hemorrhage (Figure 6). No mouse in treated groups had hemorrhage area larger than 1% of total hemisphere, the average hemorrhage areas were 0.32% for mice that received the treatment starting at 1 week and 0.29% for mice that received treatment starting at 8 weeks after model induction. In contrast, the hemorrhage was more severe in vehicle treated bAVM mice. Two out of six mice in 1-week vehicle group and two out of seven mice in 8-week vehicle group had hemorrhage areas over 1% of the total hemisphere (Figure 6).

Discussion

Our findings demonstrated that the genetic dysfunction of *ENG* and *ALK1* in ECs promotes EC inflammation, immune and inflammatory response, and angiogenic activity. Transient depletion of CNS resident macrophages either at the beginning of bAVM development or at the time bAVMs have been fully established reduced the infiltration of both resident and circulating inflammatory cells and dysplasia vessels in the bAVMs. In the initial stage of bAVM development, a transient depletion of CNS resident macrophages reduced the subsequent recruitment of Ccr2$^+$ circulating cells and mitigated the development of bAVM. The effect extended 6 weeks after cessation of PLX5622 treatment. These findings suggest that CNS resident

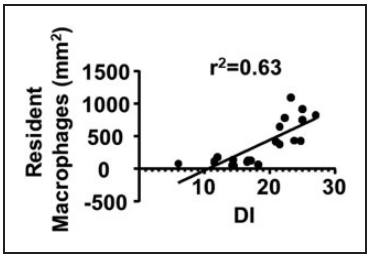

Figure 4. The number of CNS resident macrophages is positively corrected with the number of dysplasia vessels in bAVM. DI: dysplasia index.

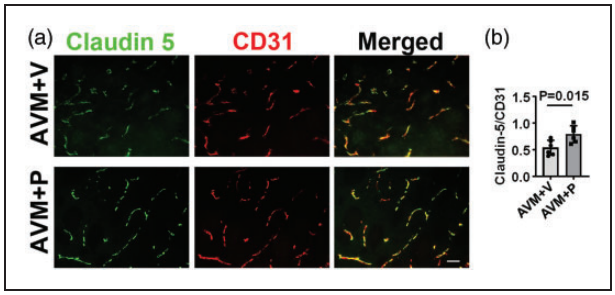

Figure 5. PLX5622 treatment increased claudin 5 expressions in bAVM vessels. (a) Representative images of sections stained with anti-claudin 5 (green) and CD31 (endothelial cells, red) antibodies. Scale bar = 50 μm and (b) Quantification of the ratio of claudin-5 and CD31. AVM + V: AVM mice treated with vehicle; AVM + P: AVM mice treated with PLX5622. N = 6.

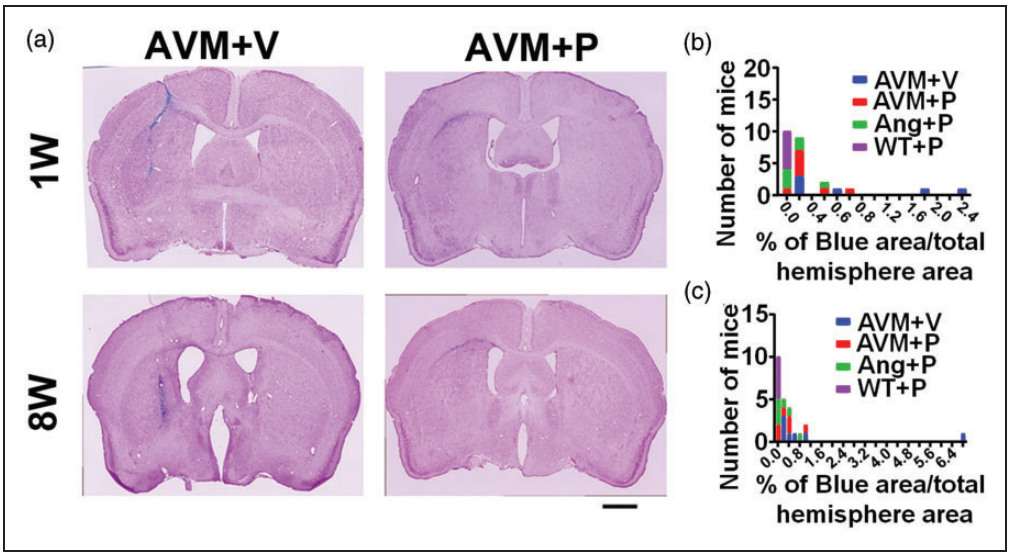

Figure 6. PLX5622 treatment reduced the incidence of severe hemorrhage in bAVMs. (a) Representative images of Prussian blue stained sections. Iron depositions (blue) in bAVMs indicates hemorrhage. Nuclei were counterstained with Fast Red. Scale bar = 1 mm. (b) and (c) Frequencies and degrees of hemorrhage in bAVMs treated with PLX5622 starting at 1 week (b) and 8 weeks (c) after bAVM model induction. 1 W and 8 W: treatment started at 1 and 8 weeks after model induction. AVM + V: AVM mice treated with vehicle; AVM + P: AVM mice treated with PLX5622; Ang + P: mice with brain angiogenesis and PLX5622 treatment; WT + P: WT mice treated with PLX5622 treatment. N = 5–6.

macrophages may serve as prerequisites for abnormal angiogenesis and recruitment of blood-borne monocytes into bAVM foci.

In established bAVMs with abundant dysplastic vessels and inflammatory cells, CNS resident macrophages continued to play a critical role in inducing vascular impairment and hemorrhage. Short-term treatment with PLX5622 in mice with established bAVMs, which are more approximate to the clinical scenario, had restored dysplastic vessels to normal phenotype and improved vascular integrity, which ultimately led to a reduction of severe hemorrhage.

Iba1 expressing myeloid immune cells were identified as major inflammatory components in human bAVMs. The increased Iba1$^+$ cells were composed of two clusters: the P2RY12$^-$ perivascular macrophage and the P2RY12$^+$ microglia predominantly in brains adjacent to bAVMs.[12] Previous data have demonstrated a good correlation between the expression of Iba1 and Cx3cr1 in cells located within the perivascular space and brain parenchyma,[20,26] indicating that Cx3cr1 served as an optimal marker for these CNS resident macrophages. The fate mapping analysis of brain Cx3cr1$^+$ cells confirmed their common prenatal origin and their continuous residence in the CNS since embryonic development.[15,26] These cells subsequently differentiate into a range of CNS resident macrophages in specific niches: microglia, perivascular, meningeal, and choroid plexus macrophages.[15,26] However, inflammation in bAVM involves more than just resident immune cells. More CD34$^+$ peripheral blood cells of patient with *ALK1* or *ENG* gene mutation than those from normal controls differentiated into activated myeloid immune cells in an *in vitro* culture system.[11] Our previous study revealed that both Ccr2$^+$ circulating monocyte-derived macrophages and Cx3cr1$^+$ resident microglia are present and persistently in bAVMs.[11] The increased levels of monocyte-derived macrophages were even more prominent than that of resident microglia. However, it is not clear which cell-population causes the unresolved inflammation, CNS resident macrophages or the circulating monocytes. In the present study, we revealed that the CNS resident macrophages play a major role in recruiting more resident and monocyte-derived macrophages into bAVMs and promoting bAVM progression. The Csf1r inhibitor PLX5622 has been shown to be effective in eliminating Cx3cr1$^+$ cells present in both the parenchyma and perivascular space of CNS, which represent microglia and perivascular resident macrophages respectively, but not impede the influx of circulating monocytes into the brain.[20] In this study, we showed that transient PLX5622 treatment reduced the recruitment of Ccr2$^+$ circulating monocytes at early stage bAVM development. Transient PLX5622 treatment after bAVM formation could still reduce hemorrhage and vascular dysplasia, although it did not prevent the influx of Ccr2$^+$ cell into the bAVM. Thus, transient depletion of CNS resident macrophage could be developed into therapies to reduce bAVM progression and hemorrhage.

The cause of increased inflammatory cell infiltrates in bAVMs is not fully understood. We believe that alterations to the ECs via gene mutations or damage play a primary role in orchestrating the infiltration of inflammatory cells in bAVMs. This may include a loss of the barrier properties of the ECs and an up-regulation of adhesion molecules and other inflammatory mediators in mutant ECs. Indeed, in this study we found that knockdown of *ALK1* in HUVECs increased the transcription of genes regulating immune response and cell migration, including positive regulation of response to cytokine stimulus (adjusted $p = 0.015$) and positive regulation of cell migration (adjusted $p = 0.03$); and knockout of *Eng* in ECs in mouse also increased cell adhesion molecules (CAMs, adjusted $p = 0.05$) expression in bAVMs. In addition, altered communication between ECs and surrounding perivascular cells may establish an inflammatory microenvironment that renders vessels susceptible to rupture. Recent studies show that vascular-associated macrophages (VAM) promote highly localized ICAM1 expression in ECs initiating EC-neutrophil adhesive interactions.[27] Perivascular microglia could also physically contact with ECs and modulate the expression of tight junction protein claudin-5.[28] In this study, we showed an improvement of claudin-5 expression after a transient reduction of resident macrophages in bAVMs, which further indicates that the resident macrophages play roles in maintaining BBB integrity and priming inflammation in disease conditions. More studies are needed to determine the exact interaction of mutant ECs and microglia in future.

Activated resident macrophages can recruit more microglia and peripheral circulating macrophages and promote extravasation of peripheral immune cells via producing a variety of cytokines and chemokines including CCL2.[29] Our RNAseq data showed that knockout of *Eng* in ECs increased positive regulation of chemotaxis. Of note, the present findings underscored the potential trajectory of communications among different cell clusters. Transient depletion of resident macrophages could interrupt the recruitment of both resident and circulating macrophages, while may not be able to reverse the recruited circulating immune cells. Corresponding changes in communicating mediators could be examined and modulated in future studies to understand the intercellular action during this process.

CSF1R inhibitors have served as effective tools for investigating the interplay of peripheral and CNS resident myeloid populations. These orally administrated small-molecule inhibitors achieve robust but reversible brain-wide resident macrophage elimination, without reactive inflammatory response or cytokine storm.[30,31] Remarkably, pexidartinib (PLX3397) has been approved by FDA for the treatment of tenosynovial giant cell tumors in 2019 and is already in clinical trials for CNS tumors.[32] PLX5622 used in this study, has the same potency as its predecessor in CSF1R inhibition, but is 10-fold more selective in receptor binding and with better brain penetrance.[31] This highly selective brain penetrant CSF1R inhibitor can consistently deplete over 90% of microglia after a 7-day treatment.[21] A sustained treatment of PLX5622 for 24 weeks is well-tolerated in rodents.[31] Upon cessation of PLX5622 treatment, the repopulation of microglia occurred within 24 hours and returned to normal level in 36 hours.[30] The present study suggests a prolonged impact of PLX5622 in preventing bAVM progression, which lasts after microglia repopulation. Remarkably, the efficacy of PLX5622 in elimination of resident macrophages in bAVM was not as effective in normal brain area, suggesting a consistent infiltration of macrophages in bAVMs. Macrophage recruitment is predisposed by *ALK1* or *Eng* gene dysfunction, indicated by our RNAseq data. It is important to determine whether an extended treatment period could further improve therapeutic effects of PLX5622 in bAVMs, and if the long-term outcome after treatment persists in further study.

Notably, our present findings were not based on the assumption that CSF1R inhibitor has minimal effects on peripheral immune cells. There are disputes that the population of peripheral circulating and bone-marrow derived myeloid cells could also be affected by PLX5622 treatment.[33] The peripheral circulating and bone-marrow derived myeloid cells rebound at 7 days after PLX5622 cessation.[33] Monocyte-derived macrophages in CNS lesions have shown a competitive increase after resident population elimination with CSF1R inhibitor.[13] However, in a murine model of severe neuroinflammation induced by West Nile virus Encephalitis, PLX5622 effectively depleted CNS macrophages and mature $Ly6C^{hi}$ monocytes in the bone marrow and inhibited their proliferation and recruitment into the infected brain.[34] In the present study, infiltration of $Ccr2^{+}$ cells within established bAVM lesion were not affected by PLX5622 treatment. Moreover, we did not find a rebound or competitive increase of $Ccr2^{+}$ cells after cessation of PLX5622 treatment. The mice treated transiently with PLX5622 at the early stage of bAVM development reduced $Ccr2^{+}$ cells in bAVMs analyzed 6 weeks after PLX5622 treatment stopped. The improvement of vascular phenotypes was comparable between mice with PLX5622 treatment at the early stage of bAVM development and after bAVMs had established. Therefore, the beneficial effect of CSF1R inhibition in bAVM severity appears to be independent of $Ccr2^{+}$ cell infiltration. We also showed that the number of CNS

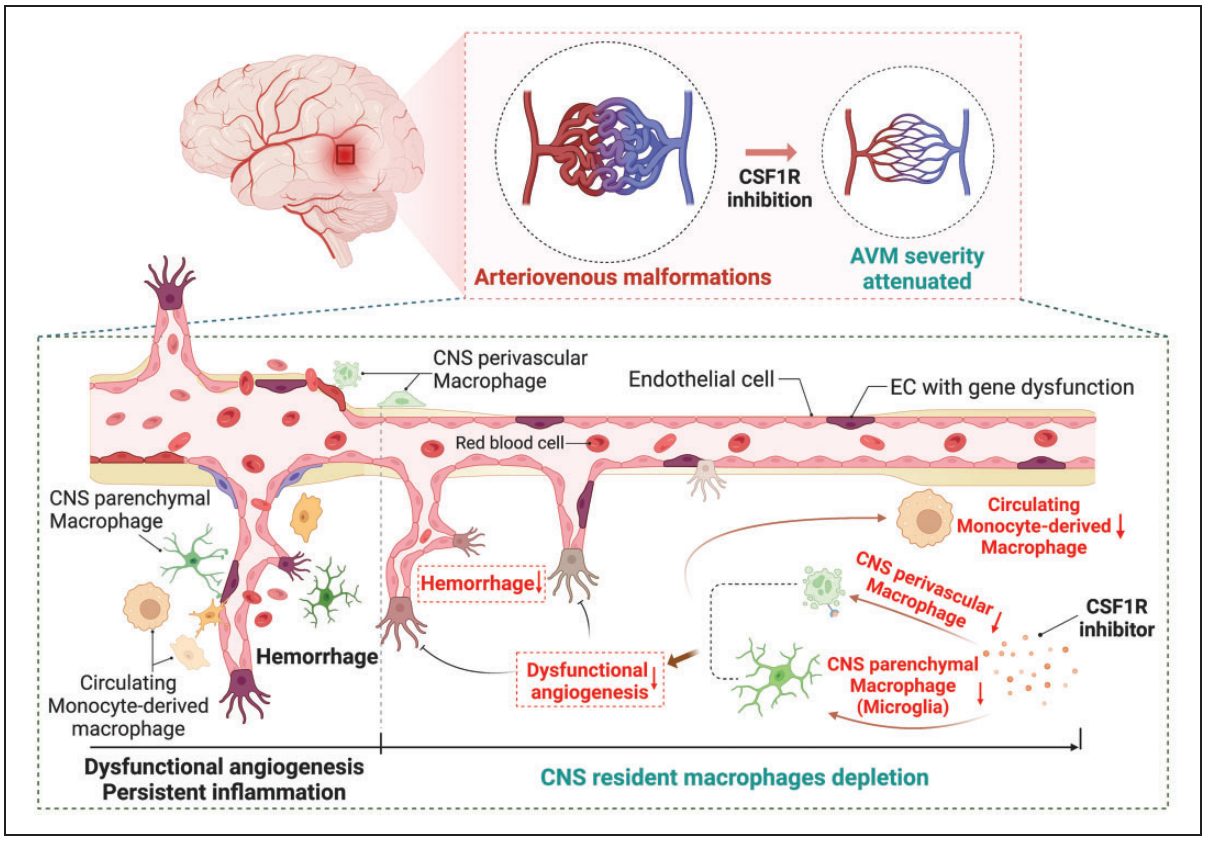

Figure 7. Summary of the influences of CNS resident and circulating monocyte derived macrophages in bAVM development and hemorrhage. Accumulation of CNS resident and circulating macrophages in bAVM cause dysfunctional angiogenesis and persistent inflammation, which increase the risk of hemorrhage. Transient depletion of CNS resident macrophages through inhibition of CSFR1 reduced dysfunctional angiogenesis, burden of CNS resident macrophages, including perivascular macrophages, microglia, and circulating monocyte-derived macrophages, as well as incidence of severe hemorrhage.

resident macrophages was positively correlated with the number of dysplasia vessels in bAVMs. Taken together, our data showed that CNS resident macrophages may assist bAVM progression more than circulating monocytes.

There are several limitations in this study. (1) The RNAseq study compared differential gene expression in bAVMs of TM treated and normal brain angiogenic regions of corn oil treated *Pdgfb*iCreER;$Eng^{f/f}$ mice. The potential influence of TM was not analyzed. (2) Csf1r inhibitor also alters the lymphoid compartment of bone marrow by suppressing T cells ($CD3^+$, $CD4^+$, and $CD8^+$) and up-regulating $CD19^+$ cells.[33] (3) The direct communications among ECs, resident macrophages and circulating monocytes and the potential mediators were not tested. (4) The sample size used in this study is not large enough to test the potential influence of sex or age. (5) Only one bAVM model was used to test PLX effect in this study. (6) Claudin 5 was quantified on immunostaining images, which is less reliable than western blot. The therapeutic effect of PLX should be validated in more bAVM models. More studies are needed to address these limitations.

Conclusions

The present study revealed that genetic dysfunction of *ALK1* or *Eng* predisposed a pro-inflammatory and pro-angiogenic microenvironment for abnormal vascular development. The CNS resident macrophages, including microglia and other non-parenchymal macrophages, play fundamental roles in orchestrating dysfunctional angiogenesis and persistent recruitment of both circulating monocytes and more resident macrophages into bAVMs, promoting bAVM progression. A modulation of CNS resident macrophage could reduce the vascular aberrancy, restore vascular integrity, and prevent severe hemorrhage of bAVMs (Figure 7).

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this

article: This study was supported by the National Institutes of Health (R01 HL122774, NS027713 and NS112819) and from the Michael Ryan Zodda Foundation to H.S.

Acknowledgements

We thank Calvin Wang for proofreading.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

HS and LM conceived the hypothesis. LM, XZ, CT, PP, AY, RL and KP performed the experiments. LM, XZ, CT, PP, AY, RL, KP and HS analyzed or interpreted the data. JN performed statistical analysis. LM and HS prepared the manuscript.

Availability of data and material

The authors declare that all data supporting the findings of this study are available in the paper and its Supplementary Information.

Supplementary material

Supplemental material for this article is available online.

ORCID iDs

Li Ma https://orcid.org/0000-0003-0894-7867
Hua Su https://orcid.org/0000-0003-1566-9877

References

1. Kim H, Su H, Weinsheimer S, et al. Brain arteriovenous malformation pathogenesis: a response-to-injury paradigm. *Acta Neurochir Suppl* 2011; 111: 83–92.
2. Chen Y, Zhu W, Bollen AW, et al. Evidence of inflammatory cell involvement in brain arteriovenous malformations. *Neurosurgery* 2008; 62: 1340–1349. discussion 1349–1350.
3. Guo Y, Tihan T, Kim H, et al. Distinctive distribution of lymphocytes in unruptured and previously untreated brain arteriovenous malformation. *Neuroimmunol Neuroinflamm* 2014; 1: 147–152.
4. Ma L, Guo Y, Zhao YL, et al. The role of macrophage in the pathogenesis of brain arteriovenous malformation. *Int J Hematol Res* 2015; 1: 52–56.
5. Pawlikowska L, Tran MN, Achrol AS, UCSF BAVM Study Project, et al. Polymorphisms in genes involved in inflammatory and angiogenic pathways and the risk of hemorrhagic presentation of brain arteriovenous malformations. *Stroke* 2004; 35: 2294–2300.
6. Walker EJ, Su H, Shen F, et al. Arteriovenous malformation in the adult mouse brain resembling the human disease. *Ann Neurol* 2011; 69: 954–962.
7. Chen W, Guo Y, Walker EJ, et al. Reduced mural cell coverage and impaired vessel integrity after angiogenic stimulation in the *Alk1*-deficient brain. *Arterioscler Thromb Vasc Biol* 2013; 33: 305–310.
8. Chen W, Sun Z, Han Z, et al. De novo cerebrovascular malformation in the adult mouse after endothelial Alk1 deletion and angiogenic stimulation. *Stroke* 2014; 45: 900–902.
9. Choi EJ, Chen W, Jun K, et al. Novel brain arteriovenous malformation mouse models for type 1 hereditary hemorrhagic telangiectasia. *PLoS One* 2014; 9: e88511.
10. Park ES, Kim S, Huang S, et al. Selective endothelial hyperactivation of oncogenic KRAS induces brain arteriovenous malformations in mice. *Ann Neurol* 2021; 89: 926–941.
11. Zhang R, Han Z, Degos V, et al. Persistent infiltration and pro-inflammatory differentiation of monocytes cause unresolved inflammation in brain arteriovenous malformation. *Angiogenesis* 2016; 19: 451–461.
12. Winkler EA, Kim CN, Ross JM, et al. A single-cell atlas of the normal and malformed human brain vasculature. *Science* 375: eabi7377.
13. Pombo Antunes AR, Scheyltjens I, Lodi F, et al. Single-cell profiling of myeloid cells in glioblastoma across species and disease stage reveals macrophage competition and specialization. *Nat Neurosci* 2021; 24: 595–610.
14. Ye F, Yang J, Holste KG, et al. Characteristics of activation of monocyte-derived macrophages versus microglia after mouse experimental intracerebral hemorrhage. *J Cereb Blood Flow Metab* 2023; 43: 1475–1489.
15. Goldmann T, Wieghofer P, Jordao MJ, et al. Origin, fate and dynamics of macrophages at central nervous system interfaces. *Nat Immunol* 2016; 17: 797–805.
16. Li Q and Barres BA. Microglia and macrophages in brain homeostasis and disease. *Nat Rev Immunol* 2018; 18: 225–242.
17. McKinsey GL, Lizama CO, Keown-Lang AE, et al. A new genetic strategy for targeting microglia in development and disease. *Elife* 2020; 9: e54590.
18. Wen W, Cheng J and Tang Y. Brain perivascular macrophages: current understanding and future prospects. *Brain* 2023; 147: 39–55.
19. Han J, Chitu V, Stanley ER, et al. Inhibition of colony stimulating factor-1 receptor (CSF-1R) as a potential therapeutic strategy for neurodegenerative diseases: opportunities and challenges. *Cell Mol Life Sci* 2022; 79: 219.
20. Kerkhofs D, van Hagen BT, Milanova IV, et al. Pharmacological depletion of microglia and perivascular macrophages prevents vascular cognitive impairment in ang II-induced hypertension. *Theranostics* 2020; 10: 9512–9527.
21. Feng X, Valdearcos M, Uchida Y, et al. Microglia mediate postoperative hippocampal inflammation and cognitive decline in mice. *JCI Insight* 2017; 2: e91229.
22. Park SO, Lee YJ, Seki T, et al. ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2 (HHT2). *Blood* 2008; 111: 633–642.
23. Saederup N, Cardona AE, Croft K, et al. Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. *PLoS One* 2010; 5: e13693.

24. Allinson KR, Carvalho RL, van den Brink S, et al. Generation of a floxed allele of the mouse endoglin gene. *Genesis* 2007; 45: 391–395.
25. Kraehling JR, Chidlow JH, Rajagopal C, et al. Genome-wide RNAi screen reveals ALK1 mediates LDL uptake and transcytosis in endothelial cells. *Nat Commun* 2016; 7: 13516.
26. Masuda T, Amann L, Monaco G, et al. Specification of CNS macrophage subsets occurs postnatally in defined niches. *Nature* 2022; 604: 740–748.
27. Ren X, Manzanares LD, Piccolo EB, et al. Macrophage–endothelial cell crosstalk orchestrates neutrophil recruitment in inflamed mucosa. *Journal of Clinical Investigation* 2023; 133: e170733.
28. Haruwaka K, Ikegami A, Tachibana Y, et al. Dual microglia effects on blood brain barrier permeability induced by systemic inflammation. *Nat Commun* 2019; 10: 5816.
29. Zhang Y, Lian L, Fu R, et al. Microglia: the hub of intercellular communication in ischemic stroke. *Front Cell Neurosci* 2022; 16: 889442.
30. Elmore MR, Najafi AR, Koike MA, et al. Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron* 2014; 82: 380–397.
31. Spangenberg E, Severson PL, Hohsfield LA, et al. Sustained microglial depletion with CSF1R inhibitor impairs parenchymal plaque development in an alzheimer's disease model. *Nat Commun* 2019; 10: 3758.
32. Butowski N, Colman H, De Groot JF, et al. Orally administered colony stimulating factor 1 receptor inhibitor PLX3397 in recurrent glioblastoma: an ivy foundation early phase clinical trials consortium phase II study. *Neuro Oncol* 2016; 18: 557–564.
33. Lei F, Cui N, Zhou C, et al. CSF1R inhibition by a small-molecule inhibitor is not microglia specific; affecting hematopoiesis and the function of macrophages. *Proc Natl Acad Sci U S A* 2020; 117: 23336–23338.
34. Spiteri AG, Ni D, Ling ZL, et al. PLX5622 reduces disease severity in lethal CNS infection by off-Target inhibition of peripheral inflammatory monocyte production. *Front Immunol* 2022; 13: 851556.