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Neutrophil granulocytes promote flow stagnation due to dynamic capillary stalls following experimental stroke

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Highlights

- Permanent ischemic stroke is marked by temporarily and repetitively occurring stalls in the peri-infarct capillary network.
- Neutrophil granulocytes are involved in perfusion derangement following focal ischemia.
- dtTomato expressing neutrophils are a suitable model to analyze vascular dynamics in stroke.

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Author contributions:

Leoni Rolfes: study concept and design, analysis and interpretation of data, writing of the manuscript

Marc Pawlitzki: critical revision of manuscript for intellectual content

Monika Riek-Burchardt: acquisition of data, analysis and interpretation of data

Jens Minnerup: acquisition of data, critical revision of manuscript for intellectual content

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Sven Meuth: critical revision of manuscript for intellectual content

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Abstract

Flow stagnation of peri-ischemic capillaries due to dynamic leukocyte stalls has been described to be a contributor to ongoing penumbral injury in transient brain ischemia, but has not been investigated in permanent experimental stroke so far. Moreover, it is discussed that obstructing neutrophils are involved in this process; however, their contribution has not yet been proven.

Here, we characterize the dynamics of neutrophil granulocytes in two models of permanent stroke (photothrombosis and permanent middle cerebral artery occlusion) using intravital two-photon fluorescence microscopy. Different to previous studies on *LysM-eGFP*$^{+}$ cells we additionally apply a transgenic mouse model with tdTomato-expressing neutrophils to avoid interference from additional immune cell subsets.

We identify repetitively occurring capillary stalls of varying duration promoted by neutrophils in both models of permanent cerebral ischemia, validating the suitability of our new transgenic mouse model in determining neutrophil occlusion formation *in vivo*.

Flow cytometric analysis of peripheral blood (PB) and brain tissue from mice subjected to photothrombosis reveal an increase in the total proportion of neutrophils, with selective upregulation of endothelial adherence markers in the PB.

In conclusion, the dynamic microcirculatory stall phenomenon that is described after transient ischemia followed by reperfusion also occurs after permanent small- or large-vessel stroke and is clearly attributable to neutrophils.

1. Introduction

Thrombolysis and thrombectomy are the only effective treatment options for acute ischemic stroke and a prerequisite for but not warranting beneficial clinical outcomes [1,2]. One reason is non-reperfusion of the ischemic tissue in particular at capillary level despite interventional recanalization. In this context, recanalization of a cerebral artery has been accompanied by downstream microvascular perfusion defects in models of experimental ischemia [3-6] and human stroke [7].

In animal models of stroke [3-6] and other microvascular occlusive diseases [8,9], an underlying mechanism is capillary flow stagnation due to polymorphonuclear leukocyte (PMN) blocking of the capillary lumen. Of note, few studies showed that stall dynamics could be modulated by targeting a neutrophile surface protein (Ly6G) or leukocyte-endothelium adherence [6,10,11]. Thus, treating dynamic capillary stalls might improve the recovery of tissue at risk. Although the above-mentioned studies differ considerably in terms of methodology, timing of analysis and outcome parameters, they are all characterized by using a transient ischemia model with subsequent reperfusion [4-6,9]. No data are available on peri-ischemic perfusion derangements in small-vessel strokes and permanent large-vessel ischemia; however, is of particular importance as a significant proportion of stroke patients suffer from permanent vessel occlusion without interventional or spontaneous reperfusion, respectively [12-14]. Moreover, the term PMN often refers specifically to neutrophil granulocytes. However, a cell-specific contribution to microvascular perfusion abnormalities has not been proven to date. Recently, intravital intracranial two-photon microscopy of LysM-eGFP^{+} cells has been implemented to characterize the dynamic infiltration of neutrophil granulocytes to ischemic tissue [15,16]. However, LysM-eGFP^{+}-mice are known to have not only neutrophils labeled with GFP, but also monocytes and macrophages [17]. A transgenic

animal model in which tdTomato exclusively visualizes neutrophil granulocytes provides an alternative to tackle this shortcoming [15,18].

Here, we aim to address the unresolved issue of capillary flow stagnation following small-vessel stroke and permanent large-vessel ischemia and the unique role of neutrophiles, using high spatio-temporal resolution imaging of capillary circulation in mice. Therefore, mice are subjected either to photothrombosis as a model of occlusion of small cerebral vessels [19] or permanent middle cerebral artery occlusion (pMCAO), as a model of large-vessel ischemia. To unequivocally prove that microvascular perfusion abnormalities are attributed to neutrophils, results from LysM-eGFP^{+} mice are verified in transgenic mice by studying the dynamics of tdTomato-expressing neutrophils with intravital two-photon microscopy. We provide evidence that neutrophil granulocyte entrapment in capillaries contributes to dynamic flow stagnation, different from permanent cellular plugs of no-reflow, likely constituting ischemic tissue injury after permanent brain ischemia.

2. Methods

2.1. Animals

LysM-eGFP^{+} mice were generated as previously described [16,17]. The LysM-promoter is expressed in several myeloid cells. Of all LysM-eGFP-expressing cells the neutrophils are particularly bright (~10×) compared to monocytes or macrophages [16]. To selectively visualize neutrophils, we used the novel genetic mouse model Catchup^{IVM} [18]. Briefly, we created Catchup^{IVM} mice (C57BL/6 Ly6g(tm2621(Cre-tdTomato)Arte) crossed to a tdTomato reporter mouse), using the Cre-lox-system. In Catchup^{IVM} mice the neutrophil-specific locus Ly6G was modulated with a knock-in allele expressing Cre recombinase and the fluorescent protein tdTomato [18]. By transferring bone marrow of five Catchup^{IVM} mice into lethally irradiated (13 gray for 4.5 min) female, 12 to 28-weeks-old C57BL6 recipients animals (purchased from Charles River Laboratories, Sulzfeld, Germany), we generated mice with red fluorescent neutrophils from the donor bone marrow. The transgenic mice strains were bred as heterozygotes. Importantly, PMNs in this mouse line have an entirely physiological function and present in physiological numbers. Mice subjected to focal ischemia were male and 8 to 12 weeks-old. For flow cytometric analysis of leukocyte activation markers, male and 8 to 12-weeks-old C57BL/6J wild-type (WT) mice were purchased from Charles River Laboratories.

Mice were kept in individual ventilated cages. Experiments followed animal welfare regulations. Experimental protocols were approved by the local governmental authorities (Landesverwaltungsamt für Kultur, Bauwesen und Verbraucherschutz, Referat Verbraucherschutz, Veterinärangelegenheiten (DZNE 42502-2-1244)).

2.2. Animal models of focal cerebral ischemia

As previously described, the skull was thinned, polished, and reinforced with a thin layer of glue and cover glass to implement a cranial window before induction of focal ischemia [15,20]. Focal cerebral ischemia was induced either by photothrombosis or pMCAO [16,21]: (i) Briefly, photothrombosis is based on photo-activation of a previously intravenous injected light-sensitive dye resulting in reproducible photo-chemical infarcts. In these experiments, 50 µl of 0.1% Rose Bengal (Sigma-Aldrich, Darmstadt, Germany) were applied by retrobulbar injection and the brain was illuminated for 60 to 90 s with a laser pointer (532 nm wavelength, output <5 mW) through the afore prepared cranial window. Of note, hemorrhagic lesions, which are normally identified by leakage of fluorescence-labeled intravascular dextran, were not observed throughout the experiments; (ii) For pMCAO, the temporalis muscle was removed and a burr hole was drilled into the temporal bone overlying the middle cerebral artery (MCA) above the zygomatic arch. The MCA was electrocoagulated using a cauterizer (Fine Science Tools Inc., Heidelberg, Germany).

In the respective control animals, cranial windows were implemented as described above. However, neither focal ischemia by photoactivation of Rose Bengal nor electrocoagulation of MCA were performed. All stroke experiments adhered to the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

2.3. Two-photon intravital microscopy

Microscopy was performed 24 h after stroke induction, as described before [15,16]. Briefly, Rhodamine/dextran or FITC/dextran was applied intravenously by retrobulbar injection to label blood vessels. For two-photon microscopy, we used an upright Zeiss (Jena, Germany) MP7 microscope, equipped with a Coherent Chameleon laser (Goettingen,

Germany) and a Zeiss 20x water-immersion objective (1.0 NA). Fluorescence was detected at 500–550 nm for GFP and 565–610 nm for tdTomato by non-descanned detectors following excitation at 920 nm. During *in vivo* microscopy, the laser was used with a power of 7–15%, depending on imaging depth. After intravital imaging, mice were sacrificed by an overdose of isoflurane.

2.4. Image processing and quantifications of stalls

Images were processed with ImageJ (NIH). For quantitative analyses of capillary stalls, angiogram time series were used. Stalling capillary segments were identified as a sudden drop in signal intensity by disappearance and reappearance of flowing red blood cells. Segments analyzed were defined as followed: capillaries that do not extend a branching point.

2.5. Cell isolation and flow cytometric analysis

Blood from WT mice subjected to photothrombosis was compared to those from sham animals. The blood was obtained from the heart prior to phosphate-buffered saline (PBS) transcardial perfusion and dissolved in 100µl of citrat. Serum and plasma were separated by centrifugation for 10 min at 2,000 rpm. Serum was subjected to erythrocyte lysis (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA; pH 7.3) and resuspended in assay buffer (PBS with 1% fetal bovine serum (FBS) and 1 mM EDTA). In regard to flow cytometric analysis of central nervous system (CNS) tissue, brains were cut into pieces, homogenized in PBS, layered on a density gradient using Lymphoprep™ (Fresenius, Germany), and separated by centrifugation for 16 min at 2,050 rpm. After isolating cells, they were washed and resuspended in the respective assay buffer. To quantify numbers of cells isolated from the CNS, beads (Beckman Coulter, USA) were added.

Single cell suspensions were stained for 15 min with the appropriate combination of indicated fluorescence-labeled monoclonal antibodies in PBS, containing 0.1% sodium azide and 0.1% bovine serum albumin. The following antibodies were used: Panel 1: FITC – CD11b (Biolegend, Cat: 101206); PE – CD49d (BD Pharmingen, Cat: 557420); APC/Cy7 – CD45 (Biolegend, Cat: 103115); BV510 – live/dead marker (Biolegend Fixable Aqua Stain, Cat: L3965), BV421 – Ly6G (Biolegend, Cat: 127628); BV605 – CXCR4 (Biolegend, Cat: 146519); PE/Cy7 – Cd64 (Biolegend, Cat: 139314); Panel 2: FITC – CD11b (Biolegend, Cat: 101206); PE – MHC II (I-A/I-E; BD Pharmingen, Cat: 562010); APC/Cy7 – CD45 (Biolegend, Cat: 103115); APS – CD162 (BD Pharmingen, Cat: 562806); BV510 - live/dead marker (Biolegend Fixable Aqua Stain, Cat: L3965); BV605 – CD206 (Biolegend, Cat: 141721); BV421 – Ly6G (Biolegend, Cat: 127628); PE/Cy7 CD62L (Biolegend, Cat: 104418). Corresponding isotype controls were used for all stainings. Flow cytometric analysis of stained cells was performed following standard protocols. Cells were analyzed on a BD FACS Calibur Flow Cytometer (BD Biosciences) or a Gallios Flow Cytometer (Beckman Coulter) using Kaluza Analysis Software (Beckman Coulter) and visualized with GraphPad Prism (USA). The gating strategy is shown in supplemental figure 1.

2.6. Statistical analyses

Data analysis was performed in a blinded fashion. Independent groups (stroke vs. control) were compared by Mann-Whitney U test. Volcano plots were constructed by plotting log2 values of the relative difference between the medians (continuous) against the p-values, calculated using the Mann-Whitney U test. Results were expressed as mean ± SEM, unless otherwise indicated. Data were analyzed and plotted with GraphPad Prism. The level of significance was labeled according to the p-values (* $p<0.05$, ** $p<0.01$, or *** $p<0.001$).

2.7. Data availability agreement

Data will be shared with qualified investigators upon request, please contact leoni.rolfes@ukmuenster.de.

3. Results

3.1. LysM-eGFP^{bright} leukocytes induce dynamic obstructions in microvessels after parenchymal damage

In the first set of experiments, we induced parenchymal injury by an intense laser beam without affecting nearby vessels directly. This damage is believed to cause a sterile inflammation, triggering the release of chemokines and cytokines and ultimately upregulating adhesion molecules on endothelial cells and attracting immune cells [15,16]. Following parenchymal injury, we tracked the dynamic behavior of eGFP-expressing leukocytes, expecting to detect leukocyte immigration into the parenchyma. Surprisingly, we observed *LysM-eGFP^{bright}* leukocytes stalling microvascular blood flow of the nearby capillaries, and even leading to dynamic flow stagnation *in vivo* (***figure 1A***). *LysM-eGFP^{bright}* cells did not permanently plug capillaries but slowly moved through them (***figure 1B+C***) to subsequently be released, resulting in a reflow of plasma streams with erythrocytes (***figure 1F, supplemental movie 1***).

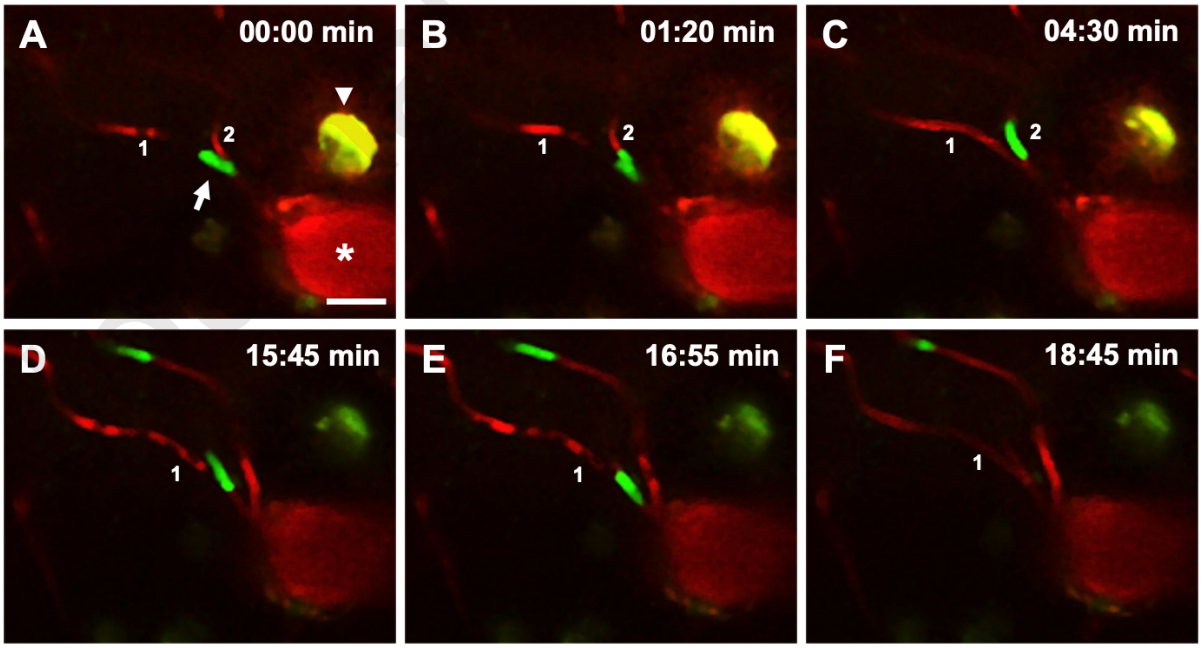

Figure 1: ***Dynamic capillary stalls by LysM-eGFP^{bright} leukocytes after parenchymal damage. A*** Site of damage (arrowhead) next to an ascending venule (asterisk). One leukocyte (*LysM-eGFP^{bright}* cell, arrow) is obstructing capillary 1, stalling the blood flow. The leukocyte crawls over the branch into

capillary 2 against flow direction (***B and C***), clearing the blood flow of capillary 1. Thereafter, the leukocyte moves beyond the branch (***D and E***) and occludes drainage of capillary 1 and 2 into the ascending venule. ***F*** Finally the PMN is washed into the venule, clearing the drainage of both capillaries. Scale bar 20 µm.

3.2. LysM-eGFP^{bright} leukocytes promote capillary flow stagnation after photothrombosis induced focal ischemia

To investigate whether the findings for parenchymal damage also apply to focal ischemia due to permanent occlusion of small cerebral vessels, we induced the latter by performed photothrombosis. This technic is ideally for intravital microscopy because of the well-defined superficial location and predictable sizes of ischemic lesions [15]. In accordance to the above-mentioned experiment, we conducted intravital imaging beyond the core immunological area (***supplemental movie 2***). Indeed, we recorded multiple events of transient (mean duration: about 12 minutes) leukocyte induced capillary flow stagnations, beyond the leukocyte infiltration zone (***figure 2, supplemental movie 3***). We observed two modes of action: either *LysM-eGFP^{bright}* leukocytes remained stuck in the capillaries for some time and were then washed out, resulting in blood flow restoration; or leukocytes crawled back and forth through the capillaries and entered into other segments via branches even against the bloodstream, thereby stalling blood flow (***figure 2, supplemental movie 3***). 15 out of 19 capillary segments (79%) in the field of view were temporarily occluded by a *LysM-eGFP^{bright}* leukocyte during the recording at least once (***supplemental movie 3***). Of note, we observed microvessels that displayed several *LysM-eGFP^{bright}* leukocytes in sequence at the same time (***figure 2, supplemental movie 3***). However, those events were rare with only 2% of capillaries showing more than one leukocyte that stalled microvascular blood flow at the same time and did not include the post-capillary venules. Moreover, approximately 20% (***figure 2A***) and 25% of capillaries (***figure 2B***)

were affected repeatedly over the observation period of 20 minutes (an example is shown in ***figure 2B, supplemental movie 4***).

Furthermore, we extended our analyses of temporary flow interruptions in peri-ischemic capillaries following stroke to include spontaneous stalls in control animals (***figure 2C, supplemental movie 5***). In line with previous results [6,22], we detected lower (3% of capillaries in controls vs. 79% in photothrombosis) and shorter (mean duration: 0:48 minutes in controls vs. 11:48 minutes in photothrombosis) leukocyte induced flow stagnations in control animals over the 20 minutes of observation, making a confounding effect of craniotomy or a systemic phenomenon unlikely for our observations on stalls following photothrombosis (***figure 2A+C-D***). Moreover, no capillaries displayed several *LysM-eGFP*^{bright} leukocytes in sequence at the same time in control animals, contrasting the results following photothrombosis.

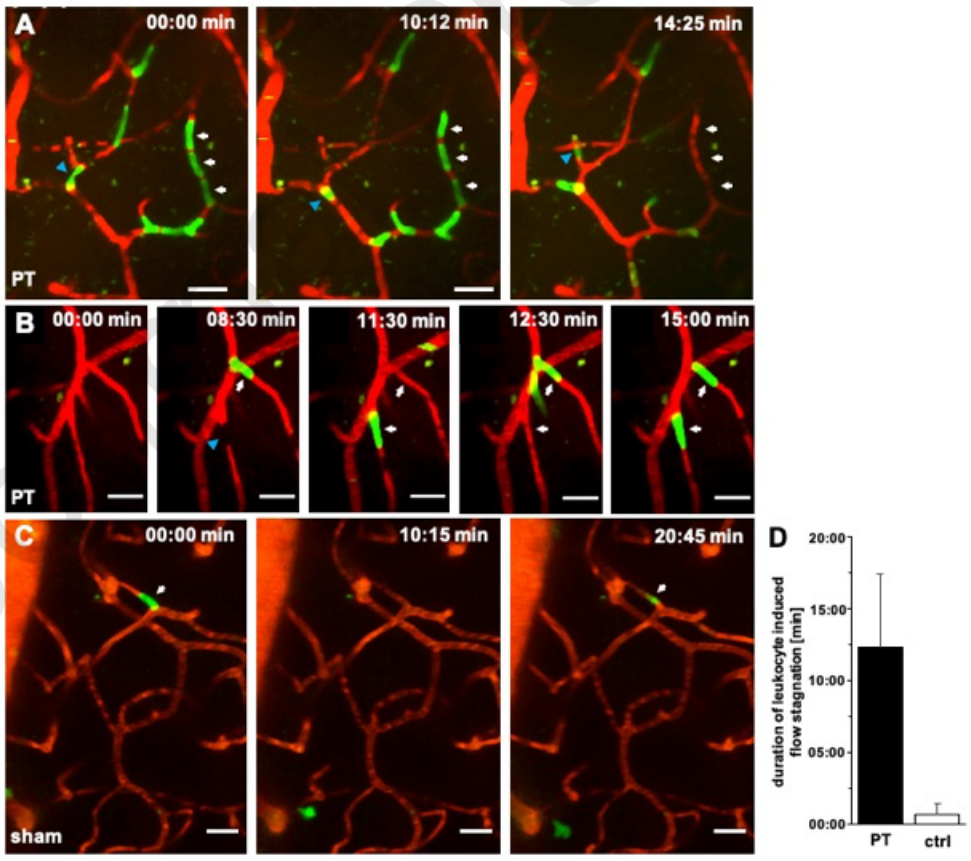

Figure 2: ***Multiple events of leukocyte induced flow stagnation after ischemic injury following photothrombosis. A*** Panel shows z(54µm)-projections of rhodamine-dextran labelled capillaries

(mainly) and *LysM-eGFP*^{bright} leukocytes. Three leukocytes (white arrows) are stuck in the capillary for at least 15 minutes. One leukocyte (blue arrowhead) crawls back and forth, thereby stalling capillary blood flow. Similar patterns were detected for additional leukocytes during that recording ***(supplemental movie 3)***. ***B*** Panel displays sequential sticking of leukocytes at the same capillary spot. The white arrows indicate the spots at which at least two different *LysM-eGFP*^{bright} leukocytes stuck in the capillary during the recording. Note, the blue arrowhead displays a single *LysM-eGFP* negative cell which occludes the capillary temporarily. But 90% of capillary occlusion was associated with *LysM-eGFP*^{bright} leukocytes (***supplemental movie 2-4***). ***C*** Only few and if, short lasting leukocytes induced flow stagnations were observed (white arrow) in sham controls (white arrow) during an observational period of about 20 minutes (***supplemental movie 5)***. ***D*** Duration of leukocyte induced flow stagnation after photothrombosis (PT, mean 11:48 minutes; n=11) vs control (ctrl, mean 0:48 minutes; n=6). Scale bar 20 µm.

In order to dissect whether those microcirculatory abnormalities are actually attributed to leukocyte obstruction rather being a physical event of temporary cerebral blood flow (CBF) impairment (e.g. that blood flow is temporarily halted until collateral supply re-establishes flow, thus mimicking the observed stalling phenomena), we attempted to quantify the time period between leukocyte capillary entrapment and stalling of microvascular blood flow. However, since the event of flow stagnation occurred immediately after *LysM-eGFP*^{bright} leukocytes had entered the capillary segments, no time period was available to calculate. Accordingly, the flow was restored immediately after the blocking leukocytes were washed out. Moreover, we quantified how often stalled blood flow was associated with a *LysM-eGFP*^{bright} leukocyte in the respective microvessel. Indeed, in 90% of capillaries with microvascular perfusion abnormalities, these cells were simultaneously present in the capillary lumen (***supplemental movie 2-4***). Interestingly, blocking leukocytes did not induce upstream perfusion defects in the nearby network in our recordings.

To investigate whether, the activation state of neutrophils could be relevant for the phenomenon of flow stagnation [10,23], we next analyzed several activation and adherence marker of neutrophils by flow cytometry (***supplemental figure 1***). By using the photothrombotic stroke model no mechanical manipulation of blood vessels, e.g. through intra-arterial insertion of a thread or electrocoagulation, took place which could probably affect the results [19]. 24 hours following photothrombosis, peripheral blood (PB) and brain tissue from C57BL/6J WT mice were compared to those obtained from sham animals (***figure 3 and supplemental figure 1-3*).** We detected an increase in the total proportion of neutrophils in regard to all gated cells in the PB and the ipsilateral hemisphere, compared to controls or the contralateral hemisphere (PB: mean [standard deviation, SD], control animals 50.18 [12.62] % vs. stroke animals: 65.63 [15.82] %, *p=0.021*; Brain: 11.18 [3.39] % vs. 35.90 [7.35] %, *p<0.001, **figure 3A+B***). The majority of activation markers in the PB (including CD64, MHC II and CD206) and the cell-adherence marker VLA-4 (CD49d) were decreased in stroke mice (*p<0.001* for all markers; ***figure 3C+D+supplemental figure 2***). However, the proportions of neutrophils expressing the cell-adhesion molecules CD62L and CD162 were significantly increased, compared to controls (CD62L: 56.78 [14.30] % vs. 94.65 [1.74] %, *p<0.001*; CD162: 96.45 [2.88] % vs. 98,88 [0.89] %, *p=0.006*). The engagement of both markers with endothelial-derived ligands initiates neutrophil tethering and rolling behavior along luminal walls of capillaries. In contrast, the proportion of neutrophils expressing CXCR4 – a master regulator of neutrophil trafficking - was slightly increased but without reaching significance (*p=0.09*).

In regard to brain tissue, a higher proportion of neutrophils in the ipsilateral hemisphere showed expression of the activation marker CD64 (30.84 [6.38] % vs. 47.26 [9.87] %, *p=0.015*), while the aforementioned cell-adhesion molecules CD62L and CD162 were significantly decreased (CD62L: 6.45 [1.92] % vs. 4.19 [0.51] %, p=0.008, CD162:

38.75 [9.95] % vs. 16.92 [6.30] %, p=0.008**; *figure 3E+ supplemental figure 2***). Of note, the downregulation of CD62L and CD162 - e.g. through ectodomain shedding - has previously been described during transmigration [24], oxidative stress [25] and by numerous pro-inflammatory stimuli, leading to neutrophile activation [26,27]. It is likely that the same phenomenon took place in the present study, as the level of CD62L per cells (shown as mean fluorescence intensity of CD62L, ***Fig 3F+ supplemental figure 3***) in both PB and CNS decreases following photothrombosis (PB: 17442 [5732] vs. 9761 [1461] %, *p=0.002,* Brain: 145.1 [26.35] vs. 75.42 [26.53], *p=0.016*), probably reflecting an increasing activation state of those cells.

In summary, an increased level of circulating neutrophils is accompanied by an increased proportion of these cells expressing the neutrophil adhesion markers CD62L and CD162 in PB [28]. Those likely stimulate enhanced neutrophil adherence and transmigration. However, with increasing activation - and especially after transmigration into the CNS - these surface markers are down-regulated, most likely to further promote a persistent activation state.

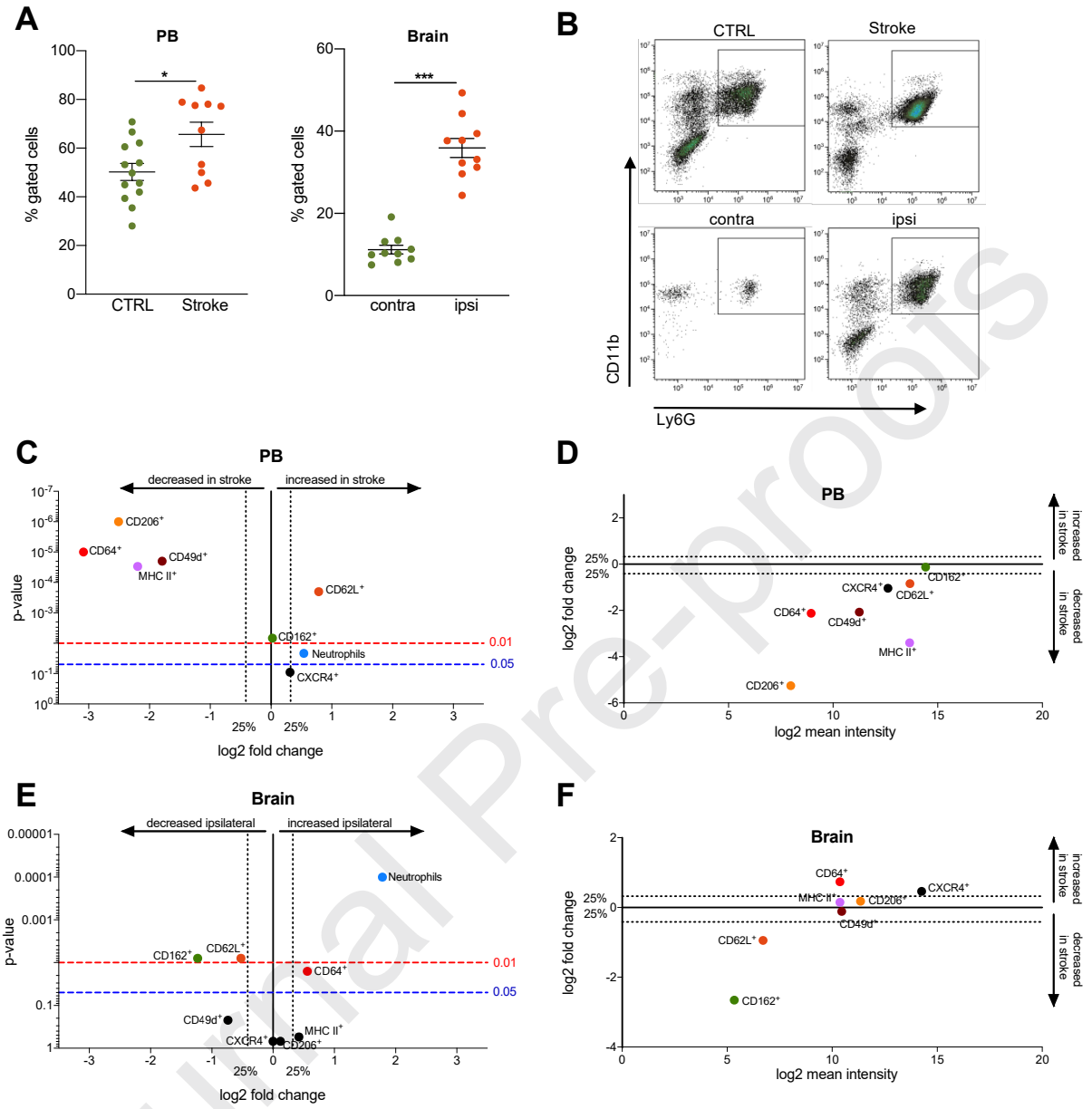

Figure 3: Neutrophils activation marker in the peripheral blood and CNS 24 hours after photothrombosis.

A Proportions of neutrophils in the peripheral blood (PB) of sham animals (CTRL; n = 13, green points) and mice subjected to photothrombosis (stroke; n = 10, orange points), as well as in the contralateral (contra, green points) and ipsilateral (ipsi, orange points) hemisphere of stroke mice, in relation to all gated cells. The P-value was calculated using the Mann-Whitney U test. ***B*** Exemplary flow cytometric staining of PB and brain derived from stroke animals and corresponding CTRL for neutrophils (CD11b+Ly6G+) is outlined. ***C*** Data derived from the flow-cytometric investigation of PB was visualized by volcano plots representing the median fold change in parameters between mice subjected to

photothrombosis (stroke) and sham animals (CTRL). P-values were calculated using the Mann-Whitney U test. ***D*** Median fluorescence intensity of the respective markers in the PB and was visualized by a MA plot, representing the median fold change and the log2 mean intensity. ***E*** Data derived from the flow-cytometric investigation of the brain were visualized by volcano plots representing the median fold change in parameters between the contralateral and the ipsilateral hemisphere of mice subjected to photothrombosis (n = 5-10). P-values were calculated using the Mann-Whitney U test. ***F*** Median fluorescence intensity of the respective markers in the brain was visualized by a MA plot, representing the median fold change and the log2 mean intensity.

3.3. Permanent stroke causes LysM-eGFP^{bright} leukocyte capillary entrapment within the penumbra

A significant proportion of patients suffering a large-vessel stroke does not have access to or is not eligible for iatrogenic recanalization, and the rate of spontaneous recanalization is relatively low (approximately 21%) [12-14]. Consistent with the above-mentioned findings after photothrombosis as a model of occlusion od small cerebral vessels, microvascular perfusion defects might also take place in this patient population and likely influence the extent of neuronal disintegration, particularly regarding the tissue at risk within the penumbra zone [29]. Therefore, we performed an explorative investigation of capillary flow stagnations following experimental long-lasting arterial blockade using the pMCAO model. This model is characterized by a large infarct volume and an ischemic penumbra similar to that of a human stroke [19]. Indeed, 24 hours after pMCAO, *LysM-eGFP^{bright}* leukocytes were enriched in blood vessels in proximity to the ischemic core (***figure 4A+B, supplemental z-stack 1+2***). Two modes of action were observed. Either *LysM-eGFP^{bright}* leukocytes temporary obstructed the capillary segment, resulting in flow maldistribution of varying duration until being washed out (***figure 4C, supplemental movies 6***), or they were not washed out and impaired the local blood flow in the salvageable penumbra during the whole

recording time (***figure 4A+D-E, supplemental movies 7***). In the latter, *LysM-eGFP*^{bright} leukocytes showed slow yet continuous migration or stop-start movements.

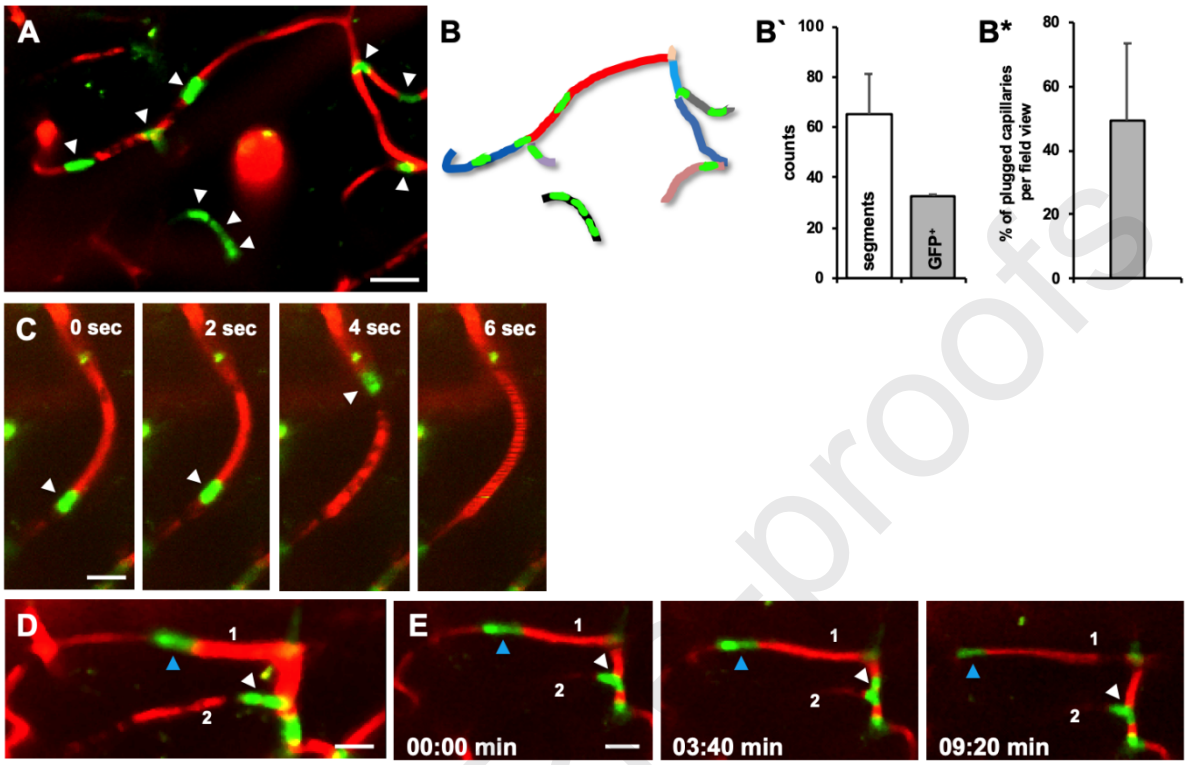

Figure 4: ***Short- and long-lasting leukocyte induced flow stagnation in the vicinity of ischemic injury after permanent middle cerebral artery occlusion (pMCAO). A*** *Z*(12µm)-projection of rhodamine-dextran labeled vessels with multiple *LysM-eGFP*^{bright} leukocytes (white arrowheads) stacking intracapillary next to the site of ischemic injury after pMCAO (***supplemental z-stack 1 and 2***). ***B*** Capillaries are segmented; individual segments are defined between two branches, as shown in different colors (schema refers to ***A***). ***B`*** Capillary segments, and *LysM-eGFP*^{bright} leukocytes within capillaries, are quantified from three different fields per view (455 x 375 µm). ***B**** Percentage of capillary segments stalled by *LysM-eGFP*^{bright} leukocytes. ***C*** Panel shows short-lived leukocyte induced blood flow stalling: The leukocyte (white arrowhead) leads to an occlusion of the capillary and stalls the blood flow, indicated as evenly dextran filled capillary. A few seconds later, the leukocyte is washed out, and blood flow is restored, indicated as black striped capillary *(**supplemental movie 6**)*. ***D*** Long-lived blood flow stalling and intracapillary crawling leukocytes. Z(7,5µm)-projection with leukocytes and capillaries of interest marked with arrowheads and numbers, respectively. ***E*** No blood flow is detected over time, despite leukocytes crawling inside the capillary. One leukocyte (blue arrowhead) crawls steadily from

right to left, another leukocyte (white arrowhead) crawls back and forth from one capillary to another, without restoration of blood flow (***supplemental movie 7***). Scale bar A 20 µm; C, D, E 10 µm.

3.4. Dynamic microvascular perfusion derangements are attributed to neutrophil granulocytes

Having confirmed the obstruction of vessels by *LysM-eGFP*^{bright} cells in two permanent focal ischemia models, we next sought to investigate whether the observed effects are definitively attributed to neutrophil granulocytes. Therefore, we examined tdTomato-expressing neutrophils from Catchup^{IVM} mice [15 18].

Indeed, following photothrombosis induced focal ischemia, we detected tdTomato^{+} neutrophils in the cortical capillary bed of the affected brain parenchyma (***figure 5***), in line with effects observed for *LysM-eGFP*^{bright} cells. These cells were motile, obstructed the capillaries temporarily, and were eventually washed out (***figure 5B, supplemental movie 8***). By implementing the transgentic *Ly6G*^{tdTomato} mouse to evaluate ischemic brain microvascular perfusion defects, we were able to show that the observed effects were caused by neutrophils and no other *LysM-eGFP* positive cells, like monocytes or macrophages.

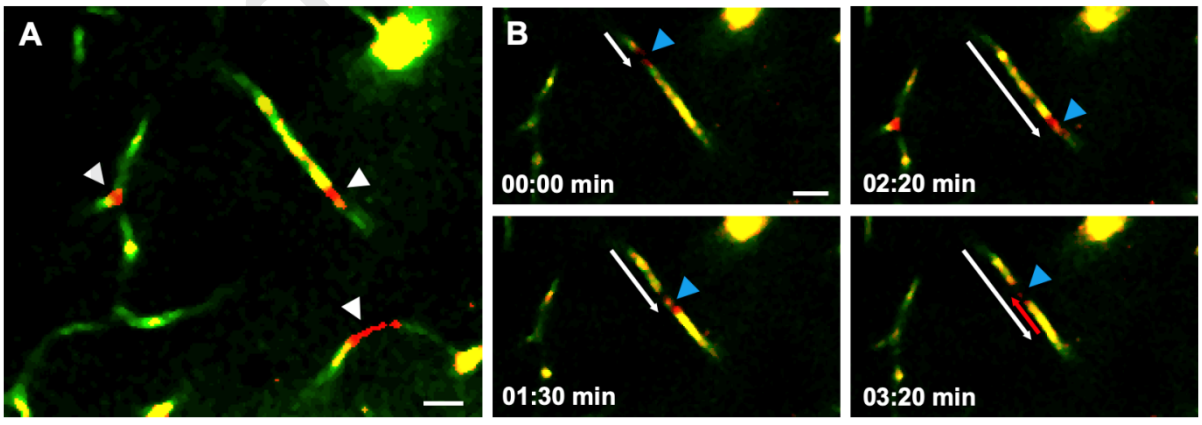

Figure 5: ***Neutrophil granulocyte transiently obstruct vessels after ischemic injury induced by photothrombosis. A*** The image from a movie three hours after photothrombosis displays multiple capillary stalls by neutrophil granulocytes (Catchup^{IVM}) (white arrowheads), labeled with FITC-dextran. ***B*** The panel demonstrates a neutrophil granulocyte (blue arrowhead) that promote capillary flow

stagnation. The neutrophil granulocyte initially crawls in the direction of blood flow (white arrow), then stops and crawls against the direction of blood flow (red arrow), and eventually is washed out (***supplemental movie 8)*.** Scale bar A 10µm; B 20 µm.

4. Discussion

In this study we aimed to uncover if neutrophil granulocytes contribute to ~~no-reflow~~ capillary flow stagnation after permanent ischemic stroke. Employing two-photon intravital microscopy and using Catchup^{IVM} mice with tdTomato-expressing neutrophils, we were able to confirm neutrophil granulocyte involvement in perfusion derangement following focal ischemia. In this context we used two different stroke models - photothrombosis as example for a permanent small-vessel stroke and the pMCAO model, as a commonly used model of permanent large-vessel occlusion.

Ischemic stroke induces a local inflammatory response involving several immune cell types [30]. An impact of neutrophils on stroke development has been controversially discussed [16,31,32]. The initiation and progression of cellular events leading to inflammation and cell death occur within minutes to hours after cessation of blood and oxygen supply. Given the prompt nature of neutrophil-cell responses, these cells likely contribute to infarct formation in early stages [15,16]. This hypothesis is underlined by several preclinical studies demonstrating neutrophils to promote inflammation and neurotoxicity [15,16,33]. The release of oxygen radicals, proteases, and proinflammatory cytokines underlie their detrimental effect [32,34]. Indeed, experimental strategies interfering with neutrophil infiltration into the injured parenchyma have shown to be neuroprotective [16,35]. However, until now it was unknown if neutrophils also contribute to secondary infarct growth by stalling microvascular flow following permanent stroke. Our data reveal dynamic obstruction of individual capillary segments by LysM-eGFP^{+} leukocytes in focal ischemia mouse models, leading to reduced blood flow, which could not be observed under physiological/sham conditions. Within the ischemic penumbra, we observed an extremely irregular capillary flow, with LysM-eGFP^{+} leukocytes repeatedly getting stuck and subsequently being released in the microcirculation. Applying our proof-of-principle experiment with tdTomato-expressing cells from

Catchup^{IVM} mice, we selectively analyzed neutrophil-specific dynamics. Indeed, we found living and motile tdTomato-expressing neutrophils attached to capillary segments, stalling blood flow following photothrombosis. Interestingly, the exit of neutrophil granulocytes from capillaries instantaneously and completely restored blood flow, suggesting that during early ischemia erythrocytes probably do not participate in capillary obstruction. Moreover, postcapillary venules or arterioles were not plugged, suggesting a purely capillary event [5].

Although the influence of perfusion derangement following permanent ischemia on the final neuronal damage is unknown, it is reasonable that in a situation with high metabolic need and a low blood supply, the degree of perfusion is a limiting factor. In previous work, we demonstrated that the zone of neutrophil infiltration typically exceeded the infarcted region [16]. Here, we also confirm this finding for leukocyte induced flow stagnation. Thus, and taking into account that slight changes in stall dynamics can have a profound impact in the overall microvascular blood flow and oxygenation [6,36], neutrophils likely contribute to secondary infarct growth following permanent stroke through microvascular disturbances beyond the infarct core.

Moreover, we demonstrated multiple events of simultaneous blood stalling, which, combined with the rheologic and adhesive properties of these cells, suggests that neutrophils might obstruct a significant portion of postischemic microcirculation. Thus, these dynamic disturbances are likely not only a by-stander phenomenon of the evolving ischemic core but contribute to tissue damage during acute ischemia. Indeed, recent studies indicated that stall dynamics can be modulated by injection of an anti-Ly6G antibody specifically targeting neutrophils [6,11]. Moreover, decreased number and duration of stalls were associated with decreased neuronal damage and improved functional outcome following transient middle cerebral artery occlusion (tMCAO) [6]. Taking into account, that the observed pathological phenomenon of dynamic flow stalls

in cerebral capillaries in the presented study were similar to those described during ischemia/reperfusion injury by Erdener and colleagues [6], it is likely that the beneficial effect of neutrophil ablation would be transferable to permanent stroke models but should be further addressed in future studies. As a limitation it should be noted, that the therapeutic target Ly6G is expressed in mice but not in humans and a promising candidate for humans has not been introduced so far [6].

Interestingly there is an abundance of observations suggesting that PMN contribute to postischemic perfusion derangement following reperfusion in other organs, such as skeletal muscle [9] and heart [8]. If we assume similarities between different organs regarding microcirculatory events occurring under hypoxic conditions, the influence of neutrophils on postischemic flow might reflect a generalized mechanism of microvascular damage.

Of note, this study was not designed to provide in-depth mechanistic insights of the observed process. However, based on previous work [11,37] and our flow cytometric analysis, we speculate receptor-mediated interactions between neutrophils and capillary endothelium to represent a key component here. Indeed, we show a selective increase of peripheral neutrophils expressing cell-adhesion molecules after stroke (CD62L and CD162), which is in line with previous reports [38]. Therefore, endothelial adhesion molecules on neutrophiles are likely upregulated under ischemic conditions for increased leukocytes-endothelium interaction [16]. Therefore, endothelial adhesion molecules on neutrophiles are upregulated under ischemic conditions for increased leukocytes-endothelium interaction [16]. On the other hand, the beta 1 integrin VLA-4 (CD49d), responsible for leukocytes tethering, rolling, and firm adhesion on blood vessels, was decreased in the PB 24 hours after photothrombosis. This finding differs from previous studies that demonstrated no difference of VLA-4 (CD49d) levels on neutrophils following pMCAO compared to healthy controls [16,31]. Furthermore,

pharmacological strategies to induced immune tolerance against the VLA-4/vascular adhesion molecule-1 axis (treatment with anti-VLA-4 antibody) in experimental stroke showed controversial results [16,39]. Also, comparable approaches to modulate the inflammatory response on brain ischemia in several clinical trials, have failed to benefit patients so far (e.g. ACTION, Enlimomab, ASTIN) [40-43]. Interestingly, the Catchup^{IVM} mouse stain used in the present study can successfully be crossed to mice with as floxed allele of VLA-4, which leads to a neutrophil specific deletion of VLA-4 without affecting other cells (e.g. T cells) [44]. Therefore, in the future, this mouse stain likely allows testing the impact of VLA-4 as entry receptor for neutrophils to ischemic brains, or other molecules found to be associated with neutrophil induced ischemic tissue damage (e.g. the abovementioned adherence marker CD62L and CD162).

Also, it is debatable if the extent of capillary flow stagnation depends on the leukocyte activation state [10]. Based on the activation markers included in our flow cytometric analysis, we did not observe an increased activation of peripheral neutrophils 24 hours after photothrombosis. This suggests that neutrophils involved in flow stagnation have not been previously activated while still in free circulation. As a limitation, it should be noted, that our panel was not all-encompassing and markers that we have not included, could obviously still demonstrate a pre-activation. Moreover, our flow cytometric study does not differentiate between (i) intravascular, non-adherent, (ii) intravascular adherent and in the case of brain flow cytometry also (iii) extravascular neutrophils. However, prior to the collection of the brain we performed thorough perfusion with saline in order to eliminate most of the intravascular, non-adherend neutrophils, while adherend and infiltrated brain neutrophils remaining available for analysis. This implies that, although interesting in the context of this study, a comparison of circulating intravascular neutrophils of the brain with adherent non-circulating neutrophils was not

possible in our setting and must be taken into account when interpretating the flow cytometric results.

Moreover, neutrophils interact with other cell types, such as platelets in the ischemic microvasculature [45], mutually activating each other and resulting in a complex scenario of platelet-induced microthrombosis and neutrophil-induced flow stagnation [5]. In histological studies del Zoppo and colleagues observed fibrin deposition and associated degranulated platelet aggregate formations in close proximity to obstructing PMNs [5,46]. They discussed whether platelet activation and subsequent fibrin formation could be due to PMNs (e.g. release of platelet activation factor), implying a vicious cycle of microvascular occlusion. Accordingly, it is debated whether further homeostatic proteins (e.g. plasminogen activators and/or thrombin) contribute to microvessel configuration, probably affecting the physiology and reactivity of the neurovascular unit and in consequence the function of neutrophils during ischemic injury and vica versa [47]. Moreover, it has been discussed that brain pericytes can irreversibly constrict capillaries in cerebral ischemia, impairing capillary reflow despite successful opening of an occluded cerebral artery [48,49]. Whether a therapeutic approach by clearing neutrophils from microcirculation (e.g. by anti-Ly6G antibody as described above) might also affect the final outcome by those alternative mechanisms of microcircular dysfunction (e.g. neutrophile-platelet interaction [5,46,50], activation of the coagulation pathway [47], pericyte constriction [48,49]) is still unclear and requires further investigation.

Finally, the phenomenon of capillary stall might depend on physical contributors, such as local CBF. Consistent with this assumption, Hansell and colleagues described capillary leukostasis to be a pressure-related phenomena rather than receptor-dependent, which was reversible with the early restoration of perfusion pressure [51]. From our study, we cannot deduct that dynamic capillary stalls by neutrophils are

associated with low CBF. However, we consider the slowing of erythrocyte flow is in direct causality with the continuous yet slow migration of neutrophils, rather than to be a rheological phenomenon based on reduced perfusion of collaterals. This assumption is based on the observation that approximately 90% of capillaries with reduced blood flow simultaneously showed *LysM-eGFP*^{bright} leukocytes in their lumen. In contrast, the blood flow of the nearby vessels was maintained, while being stalled in microvessels passed through with neutrophils. Moreover, microvascular flow abnormality occurred promptly after *LysM-eGFP*^{bright} leukocytes entry and flow was immediately and completely re-established after the blocking leukocytes were washed out.

To conclude, the present study provides the first evidence that neutrophils frequently occlude capillaries following permanent focal ischemia thereby stalling capillary flow. In this stagnated plasma, oxygen-transporting erythrocytes are missing, implying a vicious cycle for further exacerbation of parenchymal hypoxia. The phenomenon was different from permanent cellular plugs of no-reflow, with cells showing continuously yet slow migration, until they were washed out and in the majority of cases re-established flow. By selecting transgenic mice with tdTomato expressing neutrophil granulocytes, we identified a suitable mouse model to in-depth characterize neutrophil-mediated vascular dynamics and neuroinflammation in stroke. This improvement in accuracy of analysis probably catalyze more basic and clinical research, leading to novel treatment strategies (e.g. by limiting the number of circulating neutrophils or by targeting of endothelial surface protein expression) and in long term might enhance the clinical outcomes of stroke patients not eligible for interventional recanalization.

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Figure legend

Supplemental figure 1: Gating strategy.

Single cells from the peripheral blood (PB, top) and brain (bottom) were simultaneously analyzed by flow cytometry. Total leukocytes were identified by forward scatter (FSC) and sideward scatter (SSC). Live/dead discrimination was determined using the amine reactive dye Aqua. From these cells, we identified CD45high leukocytes based on SSC signal and CD45 expression. Next, we selected neutrophiles based on CD11b and Ly6G expression. Neutrophiles were further differentiated based on their surface marker expression in CD62L, CD162, CD64, CD49d, CD206, CXCR4, MHC-II positive cells.

Supplemental figure 2: Relative expression levels of neutrophils activation markers in the peripheral blood and CNS 24 hours after photothrombosis.

A Data derived from the flow-cytometric investigation of peripheral blood (PB) was visualized by a MA-plot. The proportions of neutrophils expressing the respective activation and adherence markers in relation to all gated neutrophils are presented as the log fold-change versus the log 2 mean expression level between mice subjected to photothrombosis (stroke; n = 10) and sham animals (controls (CTRL); n = 13). ***B*** Data derived from the flow-cytometric investigation of the brain were visualized by a MA plot. The proportions of neutrophils expressing the respective activation and adherence markera in relation to all gated neutrophils are presented as the log fold-change versus the log 2 mean expression level between the contralateral and the ipsilateral hemisphere of mice subjected to photothrombosis (n = 5-10).

Supplemental figure 3: Median fluorescence intensity of neutrophils surface markers following photothrombosis

Data derived from the flow-cytometric investigation of peripheral blood (***A***) and brain (***B***) is depicted as median fluorescence intensity (MFI) of the respective marker compared between CTRL (green points) and stroke animals (orange points). P-values were calculated by Mann-Whitney U test, *p<0.05, **p<0.01, ***p<0.001. Error bars indicate the mean and standard error of mean.

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Highlights

- Permanent ischemic stroke is marked by temporarily and repetitively occurring stalls in the peri-infarct capillary network.
- Neutrophil granulocytes are involved in perfusion derangement following focal ischemia.
- dtTomato expressing neutrophils are a suitable model to analyze vascular dynamics in stroke.