Microglia are integral functional elements of the central nervous system, but the contribution of these cells to the structural integrity of the neurovascular unit has not hitherto been assessed. We show here that following blood-brain barrier (BBB) breakdown, P2RY12 (purinergic receptor P2Y, G-protein coupled, 12)-mediated chemotaxis of microglia processes is required for the rapid closure of the BBB. Mice treated with the P2RY12 inhibitor clopidogrel, as well as those in which P2RY12 was genetically ablated, exhibited significantly diminished movement of juxtavascular microglial processes and failed to close laser-induced openings of the BBB. Thus, microglial cells play a previously unrecognized protective role in the maintenance of BBB integrity following cerebrovascular damage. Because clopidogrel antagonizes the platelet P2Y12 receptor, it is widely prescribed for patients with coronary artery and cerebrovascular disease. As such, these observations suggest the need for caution in the postincident continuation of P2RY12-targeted platelet inhibition.

The purinergic receptor P2RY12 is a clinical target in both cardiovascular and cerebrovascular disease in that inhibition of platelet P2RY12 prevents ADP-induced platelet aggregation and thereby reduces the risk of thrombosis (14, 20). Since its approval in 1997 by the Food and Drug Administration, clopidogrel has been prescribed to over 52 million patients worldwide (21). Clopidogrel is a thienopyridine prodrug, whose active metabolite acts as an irreversible inhibitor of P2RY12 (22). Systemic expression of P2RY12 is limited to platelets, so that clopidogrel exhibits few side effects in addition to the prolongation of bleeding time and hemorrhagic risk ascribable to its anti-platelet actions. Other organ systems are essentially devoid of P2RY12, with the exception of CNS microglial cells.

Although clopidogrel reduces the risk of stroke, a large number of treated patients nonetheless experience cerebral ischemic events; in one study, 9% of patients taking clopidogrel suffered an ischemic event on clopidogrel over a 2.4-year observation period (23). Because BBB compromise is a hallmark of stroke, clopidogrel and its active metabolite may thereby gain entry into the affected CNS, resulting in the local suppression of P2RY12-mediated microglial activation within ischemic tissue.

### Results

**Systemic Clopidogrel Suppresses Juxtavascular Microglial Cell Activation After BBB Breakdown.** The active metabolite of clopidogrel [molecular weight (MW) 353 Da] has low-BBB permeability (24). Clopidogrel would thus not be expected to interfere with the movement of microglia or their ramified processes under control conditions. To test this supposition, we evoked small focal lesions within the cerebral parenchyma using two-photon focused laser

<ref>Significance</ref>

<ref>Microglia</ref>

<ref>Antagonists</ref>
We observed a rapid chemotactic response of nearby microglial cell processes in CX3CR1−/EGFP mice (Fig. 1 A and B and Movie S1). Earlier studies have shown that P2RY12 drives microglial cell process movement toward focal lesions (18). We confirmed that mice with deletion of P2RY12 (P2RY12−/−) exhibited significantly less process accumulation around focal lesions (Fig. 1 A and B and Movie S2). In contrast, pretreatment of P2RY12−/− mice with 20 mg/kg clopidogrel for 3 d before the experiment did not suppress microglia process motility, suggesting that clopidogrel does not inhibit microglial P2RY12 in the normal mouse brain in the absence of vascular injury (Fig. 1 A and B and Movie S3). We next asked whether clopidogrel could inhibit microglial process motility in the setting of vascular injury. The focal laser injury was targeted to induce injury in single capillaries, located 80–150 μm below the pial surface. The capillary injury was calibrated to cause minimal, nonhemorrhagic damage, evaluated by the lack of an extravascular leakage of 70 kDa of Texas Red-dextran (Fig. 1C). Similarly to brain parenchyma, juxtavascular microglial processes in control mice were immediately attracted to the site of the capillary lesion; within 20–30 min, they formed a dense sheet of EGFP+ processes plastered around the vessel wall (Fig. 1 C and D and Movie S4), which was significantly reduced in CX3CR1/P2RY12−/− mice (P < 0.05, Tukey–Kramer test) (Fig. 1 C and D and Movie S5). Moreover, mice pretreated with clopidogrel exhibited a significant suppression of movement of EGFP+ juxtavascular microglial processes toward laser-injured capillaries (P < 0.01, Tukey–Kramer test) (Fig. 1 C and D and Movie S6). Of note, we chose a dose of 20 mg/kg clopidogrel, which increased the bleeding time by 84.8% and reduced platelet aggregation by 35.5% (Fig. 1E); patients receiving 75 mg of clopidogrel daily experienced a mean increase in bleeding time of ~140% (25) and an increase in platelet aggregation time of 35% (26). Clopidogrel’s only targets in the adult CNS were confirmed to be microglial cells (14, 15, 18), because the immunohistochemical labeling of P2RY12 colocalized with CX3CR1-EGFP (Fig. 1D), whereas blood-borne platelets also accumulated around the capillary inside CX3CR1−/− mice (27, 28). However, suppression of platelet activity in blood by clopidogrel is unlikely to be the cause of juxtavascular microglial motility reduction, because non-P2RY12−/− dependent platelet antagonists—acetylsalicylic acid (10 mg/kg per day for 3 d) and heparin (200 IU/kg i.v.)—did not reduce the motility of juxtavascular microglial processes (P > 0.05, Tukey–Kramer test) (Fig. 1D), even though both agents completely suppressed hemostasis of tail bleeding for over 20 min (n = 3–7). In addition, the same laser injury failed to initiate platelet accumulation inside the capillary at the injured site (P > 0.05 with vs. without injury, Tukey–Kramer), whereas collagen injection induced the accumulation of platelets in random positions in capillaries (Fig. 1 G and H). Taken together, whereas P2RY12 deletion reduced juxtavascular microglial chemotaxis in response to both vascular and nonvascular injury, clopidogrel suppressed only microglial motility when the injury was targeted to the local vascular bed, the presumed entry site of clopidogrel and its metabolites.

Motility of Juxtavascular Microglial Cells Contributes to the Rapid Closure of the BBB. Our data suggest that at sites of vascular injury opening of the BBB may lead to influx of low-molecular-weight compounds, including clopidogrel (MW 353 Da), which in turn suppress the P2RY12-dependent movement of juxtavascular microglial processes to sites of vascular injury (Fig. 2A–D). To establish whether the laser injury indeed triggered opening of the BBB, we developed a technique by which BBB permeability could be serially assessed (Fig. 2A). Alexa Fluor 488 (MW 640 Da, 10 μL) was injected into the internal carotid artery every 10 min after laser injury. Leakage across the BBB was calculated as the peak fluorescence signal outside the capillary, divided by the fluorescence signal inside the vessel lumen within the same frame (Fig. 2B and Movies S7 and S8). Using this approach, we noted that the efflux of Alexa Fluor 488 gradually decreased after laser injury and that the BBB defect was resealed at 39.6 ± 8.6 min in P2RY12−/− mice. Similarly, neither acetylsalicylic acid nor heparin significantly slowed the closure of BBB leakage after injury (P > 0.05, Tukey–Kramer test) (Fig. 2 C and D). In contrast, both P2RY12−/− and clopidogrel-treated mice exhibited much slower rates of BBB resealing (P < 0.01, Tukey–Kramer test) (Fig. 2 C and D). Because microglia are the only cells of the neurovascular unit that express detectable levels of P2RY12 (14, 15, 18) (Fig. 1F), these observations suggest that juxtavascular...
microglia are critical to the rapid closure of BBB defects. Ultrastructural analysis based on electron microscopy (EM) of the laser injury revealed, as expected, aggregation of densely packed processes, which completely ensheathed the site of injury. Adjacent processes exhibited closely apposed membrane (Fig. 3A). Immunolabeling revealed that the juxtavascular microglial cell processes extending toward the site of laser injury exhibited very high P2RY12 expression (Fig. 3B), as well as polarized expression of the adherens junction molecule E-cadherin. In contrast, a tight junction protein occludin was not detected (Fig. 3B). These observations showed that microglial cell processes were in direct contact with each other after they aggregated around the site of injury. It is possible that the E-cadherin-positive membrane appositions will restrict diffusion between the lesion and surrounding tissue and thus reseal acute BBB openings. An alternative possibility—that juxtavascular microglial process-mediated restriction of the injured capillary wall reduced capillary perfusion—was not supported because capillary diameter, erythrocyte flow velocity, and flux did not differ among P2RY12−/− mice receiving clopidogrel (20 mg/kg, red), acetylsalicylic acid (10 mg/kg, blue), or heparin (200 IU/kg, turquoise). Different color gradients indicate an individual set of capillaries. The lines indicate the average of linear regression curves, obtained by averaging the slopes and Y-intercept of each regression line from a single capillary. The average regression lines were used to obtain BBB closure time (X-intercept). (D) Summary histogram of BBB closure time. n = 4–7 capillaries from four to seven animals; ns, P > 0.05; **P < 0.01; one-way ANOVA with Tukey–Kramer test.

Discussion

The experiments in this study show that P2RY12-mediated activation of juxtavascular microglial cells contributes to the rapid closure of small openings in the BBB, and that the movement of microglial cells processes toward the site of vascular injury is a key determinant of how rapidly those leaks are closed (Figs. 1 and 2). Our in vivo analysis suggested that juxtavascular microglial cell processes aggregate to form a physical barrier, consisting of E-cadherin–expressing membrane appositions, and that the microglial cuff around the vessel wall temporally assumes the functions of the BBB lost in the setting of acute vascular injury (Fig. 3). In contrast, laser-targeted elimination of juxtavascular microglial cells (Fig. 4), as well as both pharmacological inhibition and genetic deletion of P2RY12 receptors, delayed resealing of small openings in the BBB. Although our EM analysis suggests that juxtavascular microglial processes formed a physical barrier that temporarily sealed the BBB, our data also permit the possibility that these microglial processes released trophic factors that accelerated endothelial cell closure of the BBB opening. As such, this study, to our knowledge, is the first to identify a key role for microglial cells as integral to the structural integrity of the neurovascular unit, and paramount in the acute closure of injury–associated BBB leaks. Juxtavascular microglia thus join pericytes and astrocytes as critical contributors to the unique barrier functions of brain endothelial cells (33, 34).

Although most relevant studies have highlighted the relative specificity of brain P2RY12 expression to microglial cells, some controversy on the point exists, in that studies have variably reported that P2RY12 might also be expressed in brain endothelial

Fig. 2. P2RY12 is required for rapid closure of the BBB, and juxtavascular microglia processes may temporarily seal BBB openings. (A) Experimental setup. The large MW weight tracer, Texas Red-dextran, was injected i.v. to outline the vasculature, and the small molecular Alexa Fluor 488 (10 μL, 80 μM) was repeatedly delivered by a catheter inserted into the internal carotid artery every 10 min to map the duration of closure of BBB openings induced by laser injury of single capillaries. (B, Upper panels) Time lapse of Alexa Fluor 488 (green) passage through a control, noninjured capillary. (Lower panels) Similar time lapse of Alexa Fluor 488 (green) passage through a capillary exposed to laser injury. The capillary is outlined by Texas Red-dextran (red). The dotted white square indicates the region used for quantification of Alexa Fluor 488 leakage. Alexa Fluor 488 leakage was defined as “peak fluorescence signal intensity inside the vessel divided by fluorescence signal intensity inside the vessel.” (Scale bar, 10 μm.) (C) Scatter histograms of Alexa Fluor 488 leakage in P2RY12−/− mice (black), P2RY12−/− mice receiving clopidogrel (20 mg/kg, red), acetylsalicylic acid (10 mg/kg, blue), or heparin (200 IU/kg, turquoise). Different color gradients indicate an individual set of capillaries. The lines indicate the average of linear regression curves, obtained by averaging the slopes and Y-intercept of each regression line from a single capillary. The average regression lines were used to obtain BBB closure time (X-intercept). (D) Summary histogram of BBB closure time. n = 4–7 capillaries from four to seven animals; ns, P > 0.05; **P < 0.01; one-way ANOVA with Tukey–Kramer test.

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Fig. 3. Laser injury induces accumulation of juxtavascular microglia processes and does not affect capillary perfusion. (A) Electron microscopic image of laser injury in cerebral cortex. Yellow dotted line with a yellow star indicates the site of the focal injury. Green dotted line indicates the accumulated juxtavascular microglial processes, with arrows indicating the close apposition of adjacent microglial processes extended toward the injury site. (B) Immunohistochemical analysis of focal laser injury site in cerebral cortex of CX3CR1+/EGFP mice. P2RY12 and E-cadherin (red) colocalized with EGFP (green) and are highly expressed in microglial cell processes encircling the injury site. In contrast, occludin (red) was detected only in vascular endothelial cells, but not in microglial cells. (Scale bars, 20 μm.) (C) Time lapse of a laser-injured capillary (red) with microglia (gray) in CX3CR1+/EGFP/P2RY12+/−, CX3CR1+/EGFP/P2RY12+/+, and CX3CR1−/EGFP/P2RY12+/+ mice treated with 20 mg/kg clopidogrel. (Scale bars, 10 μm.) (D) Plots of capillary diameter at the site of laser injury plotted as a function of time in CX3CR1+/EGFP/P2RY12+/−, CX3CR1+/EGFP/P2RY12+/+, and CX3CR1−/EGFP/P2RY12+/+ mice treated with 20 mg/kg clopidogrel. n = 3–5 capillaries from three to five animals. (E, Left) Strategy for collecting time series of XT line-scan images in capillaries filled with Texas Red-dextran (red). (Right) Line scans were collected at 0–64 min after laser injury. (F) Plots of RBC velocity and flux of capillary exposed to laser injury in P2RY12+/−, P2RY12+/+, and P2RY12−/− mice treated with 20 mg/kg clopidogrel. n = 5–12 capillaries from three animals.

Fig. 4. Juxtavascular microglia ablation attenuates vascular closure. (A) Ablation of juxtavascular microglial cell. Propidium iodide (30 μM) was applied after a juxtavascular microglia was ablated with focused laser radiation. Only the ablated microglial cell (blue circle) that lost EGFP fluorescence was located within a radius of 40 μM from the target capillary (red, at the crosshair) in a CX3CR1−/EGFP/P2RY12+/+ mouse. (Scale bars, 20 μm.) (B) Projection images (55 μm in z direction) and orthogonal views (XZ and YZ planes at yellow dotted lines) of a field before (Left) and after (Right) laser ablation of six juxtavascular microglial cells (white with blue circles) located within a radius of 40 μM around the target capillary, (red, at the crosshair) in a CX3CR1−/EGFP/P2RY12+/+ mouse. (Scale bars, 20 μm.) (C) Time series of experiment with ablation of juxtavascular microglial cells shows that the region around the injured capillary remained free of microglial cell processes for the duration of the experiment. No juxtavascular microglial cell processes were in contact with the injured capillary at 70 min. (Scale bar, 20 μm.) (D) Scatter histogram of Alexa Fluor 488 leakage in microglia-ablated CX3CR1−/EGFP/P2RY12+/+ mice. Different color gradients indicate an individual set of capillaries. The line indicates the average of linear regression curves (r = 0.0144±0.125.84), obtained by averaging slopes and Y-intercept of each regression line from each capillary. Rate of BBB closure was 1.44 ± 0.87%/min (n = 5 capillaries), indicating that the leak worsened over time rather than gradually closing. (Inset) Scatter histogram of Alexa Fluor 488 leakage following juxtavascular microglial cell ablation but without laser injury to the capillary. The line indicates the average of linear regression curves (r = 0.0002±0.01092; n = 5 capillaries).

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Juxtavascular microglia ablation attenuates vascular closure. (A) Ablation of juxtavascular microglial cell. Propidium iodide (30 μM) was applied after a juxtavascular microglia was ablated with focused laser radiation. Only the ablated microglial cell (blue circle) that lost EGFP fluorescence was located within a radius of 40 μM from the target capillary (red, at the crosshair) in a CX3CR1−/EGFP/P2RY12+/+ mouse. (Scale bars, 20 μm.) (B) Projection images (55 μm in z direction) and orthogonal views (XZ and YZ planes at yellow dotted lines) of a field before (Left) and after (Right) laser ablation of six juxtavascular microglial cells (white with blue circles) located within a radius of 40 μM around the target capillary, (red, at the crosshair) in a CX3CR1−/EGFP/P2RY12+/+ mouse. (Scale bars, 20 μm.) (C) Time series of experiment with ablation of juxtavascular microglial cells shows that the region around the injured capillary remained free of microglial cell processes for the duration of the experiment. No juxtavascular microglial cell processes were in contact with the injured capillary at 70 min. (Scale bar, 20 μm.) (D) Scatter histogram of Alexa Fluor 488 leakage in microglia-ablated CX3CR1−/EGFP/P2RY12+/+ mice. Different color gradients indicate an individual set of capillaries. The line indicates the average of linear regression curves (r = 0.0144±0.125.84), obtained by averaging slopes and Y-intercept of each regression line from each capillary. Rate of BBB closure was 1.44 ± 0.87%/min (n = 5 capillaries), indicating that the leak worsened over time rather than gradually closing. (Inset) Scatter histogram of Alexa Fluor 488 leakage following juxtavascular microglial cell ablation but without laser injury to the capillary. The line indicates the average of linear regression curves (r = 0.0002±0.01092; n = 5 capillaries).
followed by injection of ticagrelor (a reversible P2RY12 antagon-ist) 10 min to 36 h later (50). Both studies ascribed the apparent neuroprotective effect of P2RY12 inhibition to a suppression of the inflammatory response to ischemic cellular injury. How then can these observations be reconciled with our findings that clopidogrel potently inhibited the repair of vascular injury, thus potentially aggravating edema and ischemic injury? Clinically, clopidogrel must be administered for several consecutive days to achieve a therapeutic effect, as the active metabolite of clopidogrel is produced in the liver (51). The typical patient takes clopidogrel for months or years before an ischemic event. By administering clopidogrel after the ischemic event, the two aforementioned studies (49, 50) would not have been expected to achieve functional P2RY12 inhibition until several days after injury, long after the acute role of microglia in rean-nealing the BBB would have been accomplished. As such, the designs of these studies would have effectively eliminated any deleterious effects of clopidogrel at the time of acute injury. In contrast, our study was designed to model a more clinically relevant scenario, by administering clopidogrel over 3 consecutive days before the ischemic event, so that functional P2RY12 in-hibition would be in effect at the time of vascular occlusion, as might be expected of a patient on clopidogrel prophylaxis who proceeds to nonetheless have a cerebral ischemic event. Also, we selected a dose of clopidogrel that induced a clinically relevant prolongation of bleeding time and platelet aggregation, which was verified to be so at the time of the ischemic event. Using this design, we found that clopidogrel indeed prolongs the opening of the BBB at the time of experimental vaso-injury and thereby may aggravate ischemic injury in those patients who proceed to have an ischemic event while on clopidogrel as a prophylactic. To-gether, these data suggest the hitherto unappreciated impor-tance of juxtavascular microglial cells in the structural integrity and functional maintenance of the gliovascular unit and BBB, while highlighting the need for further studies modeling the potential risks of inadvertent microglial inhibition when targeting P2RY12 for purposes of platelet inhibition.

Materials and Methods

Mouse Strains. CX3CR1EGFP+ mice were purchased from Jackson Labs (strain name B6.129P-CX3CR1tm1Litt/J, stock no. 005582), P2RY12 within normal limits. The normal limits for pCO2 were set at 35–45 mm Hg; pO2, 80–105 mm Hg; and for arterial blood pH, 7.35–7.45 (52).

In Vivo Two-Photon Laser Scanning Microscopy. A custom-built microscope attached to a Ti:Sapphire laser (Mai Tai, SpectraPhysics), a scanning box (FV300, Olympus) operated by Fluoview software (Olympus), and a 20× water-immersion objective lens (0.95 N.A., Olympus) was used for imaging. Experiments were performed on C57BL/6J mice that were i.p. injected with 10 mg/kg Acetylsalicylic acid was prepared as 10 mg/mL in saline and administered p.o. 30 min before the ischemic event. Also, we used a custom-built microscope attached to a Ti:Sapphire laser (Mai Tai, SpectraPhysics), a scanning box (FV300, Olympus) operated by Fluoview software (Olympus), and a 20× water-immersion objective lens (0.95 N.A., Olympus) was used for imaging. Experiments were performed on C57BL/6J mice that were i.p. injected with 10 mg/kg Acetylsalicylic acid was prepared as 10 mg/mL in saline and administered p.o. 30 min before the ischemic event.


Movie S1. Accumulation of microglial cell processes (EGFP, green) after focal laser injury at cerebral tissue away from capillaries (Texas Red-dextran; red) in CX3CR1<sup>EGFP</sup>/p2RY12<sup>−/−</sup> mice. Cross sign indicates the point of the injury, devoid of blood vessels and microglial processes. The movie was taken immediately after the injury with 12 s per frame for about 20 min in cortical layer 2. Scale (μm) and time (min:s) are indicated in the movie.

Movie S1
Movie S2. Accumulation of microglial cell processes (EGFP; green) after focal laser injury at cerebral tissue away from capillaries (Texas Red-dextran; red) in CX3CR1<sup>−/−</sup>P2RY12<sup>−/−</sup> mice. Cross sign indicates the point of the injury, devoid of blood vessels and microglial processes. The movie was taken immediately after the injury with 10 s per frame for about 20 min in cortical layer 2. Scale (μm) and time (min:s) are indicated in the movie.

Movie S2

Movie S3. Accumulation of microglial cell processes (EGFP; green) after focal laser injury at cerebral tissue away from capillaries (Texas Red-dextran; red) in CX3CR1<sup>−/−</sup>P2RY12<sup>−/−</sup> mice treated with clopidogrel. Cross sign indicates the point of the injury, devoid of blood vessels and microglial processes. The movie was taken immediately after the injury with 12 s per frame for about 20 min in cortical layer 2. Scale (μm) and time (min:s) are indicated in the movie.

Movie S3
Movie S4. Accumulation of microglial cell processes (EGFP; green) after a focal laser injury at a capillary (Texas Red-dextran; red) in cerebral cortex of CX3CR1^+/EGFP^−/P2RY12^+/− mice. Cross sign indicates the point of the injury. The movie was taken immediately after the injury with 10 s per frame for about 30 min in cortical layer 2. Scale (μm) and time (min:s) are indicated in the movie.

Movie S5. Accumulation of microglial cell processes (EGFP; green) after a focal laser injury at a capillary (Texas Red-dextran; red) in cerebral cortex of CX3CR1^+/EGFP^−/P2RY12^+/− mice. Cross sign indicates the point of the injury. The movie was taken immediately after the injury with 10 s per frame for about 30 min in cortical layer 2. Scale (μm) and time (min:s) are indicated in the movie.
Movie S6. Accumulation of microglial cell processes (EGFP; green) after a focal laser injury at a capillary (Texas Red-dextran; red) in cerebral cortex of CX3CR1<sup>−/−</sup>EGFP<sup>−/−</sup>P2RY12<sup>−/−</sup> mice treated with clopidogrel. Cross sign indicates the point of the injury. The movie was taken immediately after the injury with 10 s per frame for about 30 min in cortical layer 2. Scale (μm) and time (min:s) are indicated in the movie.

Movie S7. Leak of small molecular dye Alexa Fluor 488 (green) from a capillary, visualized by Texas Red-dextran (red) in blood plasma without focal laser injury. After a short pulse of Alexa Fluor 488 (10 μL, administered through internal carotid artery) passes through the capillary, no diffusion of the dye to the surrounding tissue is visible outside the capillary. Scale (μm) and time (s) are indicated in the movie.
Movie S8. Leak of small molecular dye Alexa Fluor 488 (green) from a capillary, visualized by Texas Red-dextran (red) in blood plasma after focal laser injury. After a short pulse of Alexa Fluor 488 (10 μL, administered through internal carotid artery) passes through the capillary, the diffusion of the dye to the surrounding tissue is visible around the injury site. Scale (μm) and time (s) are indicated in the movie.