SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals

All studies were done blinded and performed on adult male mice weighing 25-30g. Wild-type (WT) C57BL/6 mice were purchased from either Jackson Laboratory (Bar Harbor, ME, USA) or Charles River Limited (Margate, Kent, UK). Fpr2/3$^{-/-}$ mice were backcrossed for over six generations on a C57BL/6 background[1] and bred on site. The Fpr2/3$^{-/-}$ mice showed no obvious phenotype and were fertile. Mice were maintained on a 12 hours (h) light-dark cycle during which room temperature was maintained at 21–23°C, and had access to a standard chow pellet diet and tap water *ad libitum*. All animal experiments complied with ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and followed the European Union Directive (2010/63/EU) or approved by the Louisiana State University Health Sciences Center-Shreveport Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

Receptor agonists and drug treatment

Compounds or vehicle (saline, or ethanol plus saline for ATL) were administered (100 μl) intravenously (i.v.) at the start of cerebral reperfusion. Doses chosen were based on previous studies, as follows: AnxA1 mimetic peptide Ac2-26 (AnxA1$_{Ac2–26}$, Ac-AMVSEFLKQAWFIENEEQEYVQTVK, Cambridge Research Biochemicals, Cleveland, UK) was given at a dose of 100 μg per mouse;[2] Boc2 (*N*-*tert*-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe; MPBiomedicals, Cambridge, UK) was given at a dose of 10 μg per mouse,[2] and ATL (stable epimer of LXA$_4$) was given at a dose of 4.0 μg per mouse.[1] In a separate set of experiments, mice were intraperitoneally (i.p.) treated with Aspirin (ASA. 150 mg/kg Sigma-Aldrich, Dorset, UK) 60 minutes (min) prior to I/R.

Middle cerebral artery occlusion and reperfusion (MCAo)

As a cerebral I/R model, MCAo was performed as previously reported.[2] Briefly, mice were anaesthetized with i.p. injection of ketamine (150 mg/kg) and xylazine (7.5 mg/kg) and MCA was occluded for 60 min using a 6-nylon intraluminal filament (Doccol Corporation, Massachusetts, USA), followed by 4 h or 24 h of reperfusion. Sham-operated mice were subject to anesthesia and other surgical procedures without MCA occlusion and analyzed 5 h or 25 h after start of surgery.

Blood sampling and cell count

Mice were either placed under a heat-lamp and the tip of tail (~1mm) was cut with scissors, or they were placed on their back, abdomen opened, and a 21 gauge needle and syringe used to collect blood from the left ventricle. Heparinized capillary tubes were used to collect blood samples. Blood samples obtained from tail-vein bleed were used for measuring differential leukocyte counts (3% citric acid & 10% crystal violet) and platelet (1% buffered ammonium oxalate) counts, and blood samples obtained from the heart were used for whole blood counts. All counts were made using a hemocytometer.

Platelet and leukocyte labeling

Blood from donor mice was withdrawn in syringes prefilled with 85 mM sodium citrate, 62.2 mM citric acid, and 110 mM glucose (ACD buffer, Sigma-Aldrich). Platelet-rich plasma was obtained by centrifugation (118 × *g*, 8 min) before platelet isolation by centrifugation at 735 *g* for 10 min. Platelets were gently resuspended, counted, and labeled by using carboxyfluorescein succinimidyl ester (CFSE, 90 μM, 10 min, Sigma-Aldrich). After confirming absence of aggregates by light microscopy, 100×10^6 platelets in 120 μL saline were injected through the jugular vein of a recipient mouse over 5-minute

infusion as previously described.[3] This was followed by the continuous infusion of 0.02 % rhodamine 6G, which fluorescently labeled circulating leukocytes.

Cerebral intravital fluorescence microscopy (IVM)

IVM was performed as previously described.[2] Briefly, mice were re-anaesthetized with i.p. injection of ketamine (150 mg/kg) and xylazine (7.5 mg/kg), and the jugular artery and vein cannulated to monitor mean arterial blood pressure (MABP), as well as for i.v. administration of rhodamine 6G and *ex vivo* labeled platelets. The head of each mouse was then fixed in a plastic frame in sphinx position. The left parietal bone was exposed by a midline skin incision, followed by a craniectomy (diameter: 2.5 mm) with a drill at 1 mm posterior from the bregma and 4 mm lateral from the midline. The dura mater was not cut because the fluorescent-labeled leukocytes were readily visualized. A 12 mm glass coverslip was placed over the craniectomy and the space between the glass and dura mater was filled with artificial cerebrospinal fluid (aCSF; Na^+ 147.8 mEq/L, K^+ 3.0 mEq/L, Mg^{2+} 2.3 mEq/L, Ca^{2+} 2.3 mEq/L, Cl^- 135.2 mEq/L, HCO_3^- 19.6 mEq/L, $lactate^-$ 1.67 mEq/L, phosphate 1.1 mM, and glucose 3.9 mM; all Sigma-Aldrich). A Zeiss Axioskop microscope (Zeiss, New York, USA) with a mercury lamp was used to observe the pial venules in the cerebral cortex. A Hitachi charge-coupled device color camera (model KPC571; Tokyo, Japan) acquired images that were recorded for offline analysis. One to five randomly selected vessel, 30–70 μm in diameter and 100 μm long, were observed for each mouse after treatment. Adherent leukocytes and platelets were defined as cells remaining stationary within venules for ≥30 seconds (s) or ≥2 s respectively. These parameters were expressed as the number of cells per square millimeter of the vessel surface and calculated from diameter and length, assuming cylindrical shape. Estimates of shear rate or pseudo-shear rate in cerebral vessels were obtained by fluorescence microscopy based on image analysis determinations of the maximal velocity of fluorescently labeled red

blood cells. Such estimates of shear or pseudo-shear rate are obtained using measurements of venular diameter (Dv) and the maximal velocity of flowing red blood cells (V_{cell}) according to the formulation: shear or pseudoshear rate = $(V_{cell}/1.6)/Dv \times 8$.[2]

Infarct volume

After a 24 h reperfusion period, mice were killed and brains were immediately removed, placed into 4°C phosphate-buffered saline (PBS, Sigma-Aldrich) for 15 min; 2 mm coronal sections were then cut with a tissue cutter. The brain sections were stained with 2 % 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer at 37°C for 15 min and fixed by immersion in 10% formaldehyde.[2] The stained sections were photographed and the digitized images of each brain section (and the infarcted area) were quantified using a computerized image analysis program (NIH 1.57 Image Software).

Neurological score

The functional consequences of cerebral I/R injury were evaluated by using a five-point neurological deficit score (0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously)[2] and were assessed in a blinded fashion. A maximal score of 4 could be assigned to each experimental animal.

Blood–Brain Barrier (BBB) Dysfunction

BBB permeability was assessed using the Evans blue (EB) extravasation, as described previously.[4] Briefly, a 2 % solution of EB (Sigma-Aldrich) was injected (4 mL/kg) i.v. Twenty-four hours later, blood was obtained for plasma collection and brains were harvested after transcardial perfusion with PBS for 5 min. The cerebral hemispheres and the plasma were homogenized in 50 % trichloroacetic

acid, sonicated, and then centrifuged. The supernatant was diluted with ethanol and the concentrations of Evans blue in brain tissue and plasma were measured using a fluorescence spectrophotometer (FLUOstar Optima, BMG LABTECH, Inc. Ortenberg, Germany). BBB permeability was normalized by dividing tissue EB concentration (μg/g brain weight) by the plasma concentration (μg/mL).

Myeloperoxidase (MPO) activity

MPO in brain homogenates was measured as a marker for cerebral neutrophil infiltration. Brain homogenates and MPO standards (Sigma-Aldrich) were placed onto a 96-well plate, and 200 μL of *o*-dianisidine (Sigma-Aldrich) solution and 10 μL of 0.1 % H_2O_2 (Sigma-Aldrich) were added. The absorbance was read after 5 min at 405 nm and expressed as units per mg of wet tissue.[2]

Confocal Microscopy

To visualize NPAs, mice were injected with specific antibodies to label: neutrophils (eFluor 660 (green)-labeled anti-Mouse Ly-6G, eBioscience, San Diego, CA. 2μg/mouse) and platelets (Dylight 649 (red)-labeled anti-mouse CD42, (Emfret Analytics, Eibelstadt, Germany. 1μg/mouse), as previously described.[5,6] Mice were placed on an Olympus BX51WI upright microscope (Olympus, Center Valley, PA) with a 20X (LUCPlanFLN) objective and equipped with a 3i LaserStack laser launch (3i, Denver, CO), Yokogawa CSU-X1-A1N-E spinning disk confocal unit (Yokogawa Electric Corporation, Tokyo, Japan) and electron multiplier CCD camera (C9100-13, Hamamatsu, Bridgewater, NJ), in order to visualize NPAs. Slidebook software (3i) was used to drive the confocal system and capture images.[7]

Cytokines in Plasma and Brain Tissue

24 h after reperfusion, blood was collected into heparin-coated syringe, and plasma was obtained. After a 5 min transcardial perfusion with PBS, brain hemispheres were homogenized, sonicated, and centrifuged in PBS containing protease inhibitors (Bio-Rad, California, USA). Levels of the pro- and anti-inflammatory cytokines: interleukin-1β (IL-1β), tumor necrosis factor alpha (TNFα), monocyte chemoattractant protein-1 (MCP-1 or CCL2), IL-10 and IL-6, were measured using standard ELISAs (Quantikine™ immunoassay kits, R&D Systems, Abingdon, UK). ATL and thromboxane B_2 (TXB_2) concentrations in plasma and brain homogenates were determined using ELISA kits from Neogen and GE Healthcare, respectively. We followed the manufacturer's specifications for lipid extraction. Cytokine concentrations were expressed as either pg/ml (plasma) or pg/g brain weight (brain).

Assessment of activated, immature and mature platelets

Murine blood obtained by tail-vein bleed before and after MCAo was mixed with heparin (20U/mL), as described previously.[8] Briefly, platelets were identified by their characteristic light scattering and membrane expression of the specific platelet glycoprotein IIb (CD41) detected with rat anti-mouse CD41-APC antibody (eBiosciences, Inc). Two-color staining of JON/A-PE (Emfret Analytics, Wurzburg, Germany) and thiazole orange (TO, Sigma-Aldrich, St Louis, MO) was used. Platelet activation was assessed by the binding of the JON/A-PE antibody to the activation epitope of GPIIb/IIIa and expressed as mean fluorescent intensity (MFI). Appropriate rat IgGs were used to determine non-specific binding. Immature platelets were identified using TO (1μg/mL dissolved in PBS). Fresh blood was diluted 1:5 and stained for 15 min at 20°C and analyzed with a LSRII flow cytometer (BD Biosciences, San Jose, CA). 20,000-50,000 events were collected, and the data were analyzed using FACSDiva software (BD Biosciences, San Jose, CA). The immature platelet population was identified by setting a TO-high gate that is 5% of the total platelet population, as previously described.[8]

Statistical Analysis

Results from IVM experiments were confirmed to follow a normal distribution using Kolmogorov-Smirnov test of normality with Dallal-Wilkinson-Lillie for corrected *p* value. Data that passed the normality assumption was analyzed using Student's *t*-test (two groups: Figures 3A-E, 5A+B, 5E-H) or ANOVA with Bonferroni post-tests (more than two groups: Figures 1A,B, 2B-D, 3F-J, 4A, 4C+D, 5C+D, supplementary Figure 2A-D), which were performed using GraphPad Prism5 software. Data that failed the normality assumption were analyzed using the non-parametric Mann-Whitney U test (two groups: Table 1 [total WBC]) or Kruskal-Wallis with Dunn's test (more than two groups: Table 1, Figure 2A, Supplementary Figure 1A-D). Data are shown as mean values ± standard error of the mean (SEM), or median with interquartile range (neurological score only). Differences were considered statistically significant at a value of $p < 0.05$.

Supplemental References

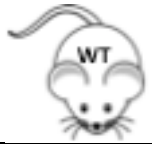
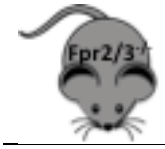
1. Smith HK, Gil CD, Oliani SM, Gavins FN. Targeting formyl peptide receptor 2 reduces leukocyte-endothelial interactions in a murine model of stroke. *FASEB J.* 2015;29:2161-2171.
2. Gavins FNE, Dalli J, Flower RJ, Granger DN, Perretti M. Activation of the annexin 1 counter-regulatory circuit affords protection in the mouse brain microcirculation. *FASEB J* 2007*;*21*(*8*):*1751-1758.
3. Cooper D, Russell J, Chitman KD, Williams MC, Wolf RE, Granger DN. Leukocyte dependence of platelet adhesion in postcapillary venules. *Am J Physiol Heart Circ Physiol.* 2004;286:H1895-900.
4. Vital SA, Terao S, Nagai M, Granger DN. Mechanisms underlying the cerebral microvascular responses to angiotensin II-induced hypertension. *Microcirculation.* 2010;17:641-649.
5. Yipp BG, Kubes P. Antibodies against neutrophil LY6G do not inhibit leukocyte recruitment in mice in vivo. Blood. 2013;121:241-242.
6. Lu SJ, Li F, Yin H, Feng Q, Kimbrel EA, Hahm E, Thon JN, Wang W, Italiano JE, Cho J, Lanza R. Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice. Cell Res. 2011;21:530-545.
7. Yildirim A, Russell J, Yan S, Senchenkova EY, Granger DN. Leukocyte-dependent responses of the microvasculature to chronic angiotensin II exposure. Hypertension. 2012; 60:1503–1509.
8. Yan SL, Russell J, Harris NR, Senchenkova EY, Yildirim A, Granger DN. Platelet abnormalities during colonic inflammation. Inflamm Bowel Dis. 2013;19:1245-1253.

Supplemental Figure Legends

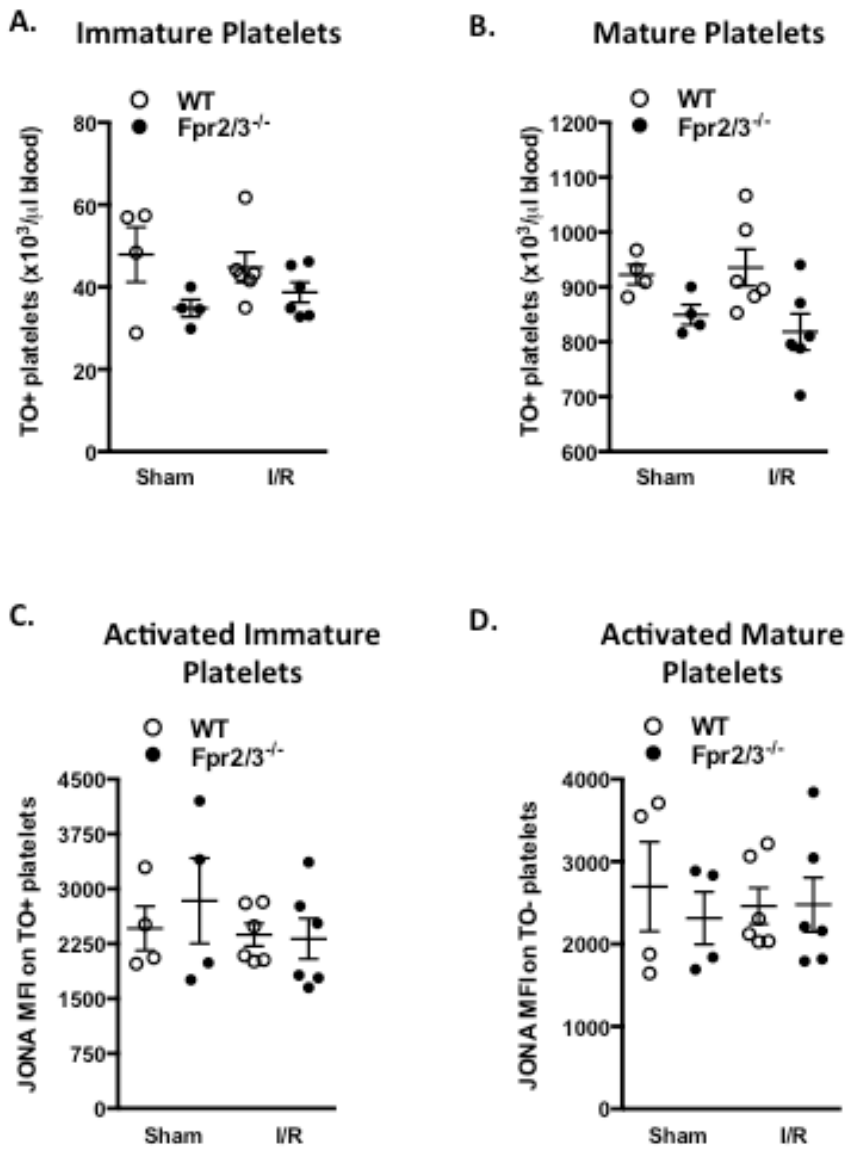
Supplemental Figure 1. Number of peripheral immature and mature platelets and levels of actuvation within these populations in in WT and Fpr2/3 mice, pre and post stroke. Blood samples were taken from wild type (WT, C57BL/6) and Fpr2/3$^{-/-}$ mice, before and after MCAo (60 min, followed by 24 h reperfusion). A) The appearance of immature (identified as TO+ (TO^{high}) platelets) and B) mature platelets (identified as TO- (TO^{low}) platelets) were quantified, and the platelet populations were gated on CD41^{+} events. Circulating mature and immature activated platelets were identified as JONA+ platelets. C) Activated immature platelets (TO+ JONA+) and D) activated mature platelets (JONA+/TO-), expressed as mean fluorescent intensity (MFI). Data are mean ± SEM of 4-6 mice per group.

Supplemental Figure 2. Exogenous Fpr2/3 agonists reduce I/R-induced cerebrovascular injury. Wild type (WT, C57BL/6) and Fpr2/3$^{-/-}$ mice were subjected to MCAo for 60 min, followed by 24 h reperfusion. Vehicle (V. saline) or Agonists: AnxA1$_{Ac2–26}$ (100 μg per mouse) and ATL (stable epimer of LXA_4. 4.0 μg/mouse); antagonist: Boc2 (10 μg per mouse) or a combination (i.e. AnxA1$_{Ac2–26}$ (100 μg per mouse) + Boc2 (10 μg per mouse); ATL (4.0 μg per mouse) + Boc2 (10 μg per mouse)) were given at the start of reperfusion. Leukocyte and platelet recruitment in the cerebral microcirculation was quantified in terms of: C) number of adherent leukocytes (i.e. those cells stationary for 30 sec or longer) and D) number of adherent platelets (i.e. those cells stationary for 2 sec or longer). E) Infarct volume and D) Neurological score were also assessed. Data are mean ± SEM of 6-8 mice per group. $^{*}p < 0.01$ & $^{***}p < 0.001$ vs. vehicle. $^{\#}p < 0.05$ & $^{\#\#\#}p < 0.001$ vs. corresponding Ac2-26 or ATL alone.

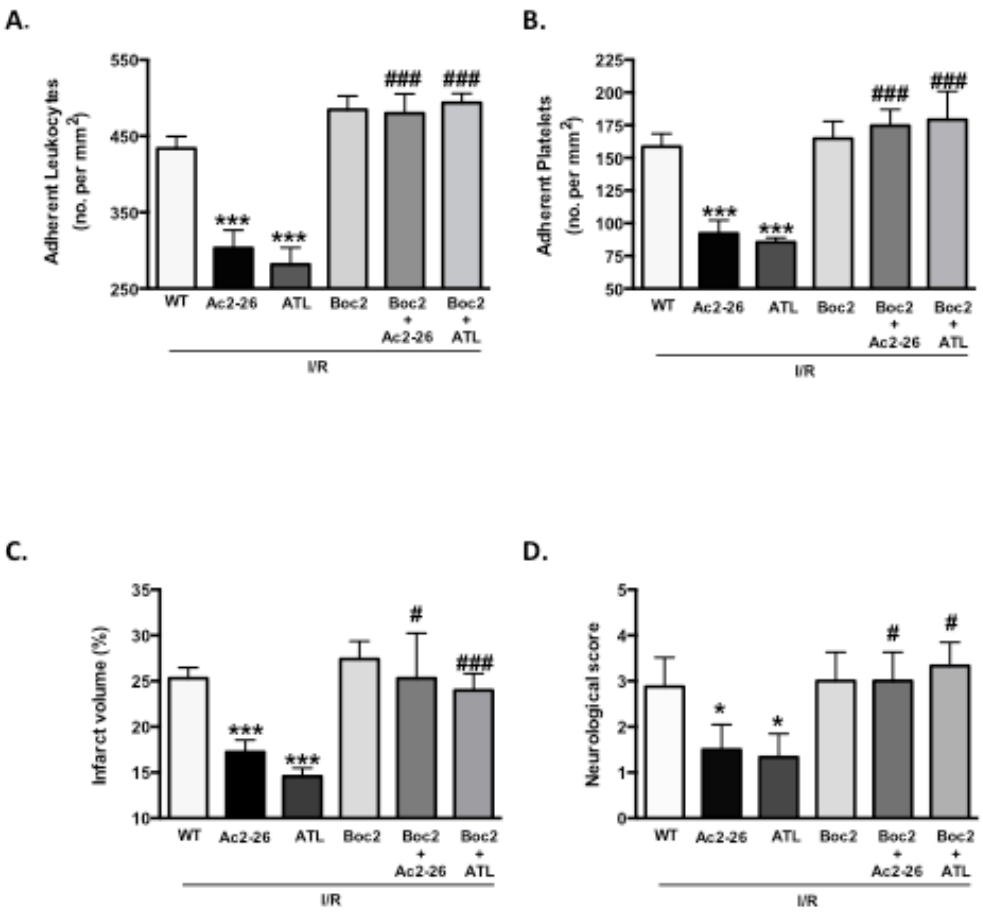
Supplemental Table 1. Hemodynamic parameters of WT and Fpr2/3$^{-/-}$ mice.

Genotype	Body weight (g)	Treatment	Vessel diameter (μm)	pseudo-shear rate (per s)	Mean arterial blood pressure (mm Hg)
	28.5 ± 0.6	Sham	35.8 ± 2.2	599 ± 85	78.3 ± 6.4
	29.5± 0.3	I/R	38.2 ± 1.0	489 ± 59	82.1 ± 3.9
	29.1 ± 0.4	Sham	37.1 ± 1.8	601 ± 72	80.3 ± 4.8
	29.5± 0.5	I/R	36.2 ± 1.4	470 ± 102	83.1 ± 5.4

I/R = 60 min ischemia + 24 h reperfusion.



Supplemental Figure. 1



Supplemental Figure. 2