Thrombin-mediated activation of PAR-1 contributes to microvascular stasis in mouse models of sickle cell disease. Sparkenbaugh et al.

Supplemental Materials and Methods

Bone marrow transplantation procedure

PAR-$1^{+/+}$ and PAR-$1^{-/-}$ mice (8 weeks old) were lethally irradiated with 14 Gy (2 doses of 7 Gy 4 hours apart) to ablate bone marrow (BM) using a Cesium-137 irradiator (Mark I Irradiator; J. L. Shepherd & Associates), injected via the retro-orbital sinus with 2×10^6 nucleated BM cells from HbSS-Townes, and used for experiments 4 months after BM transplantation. We have previously shown that at this time point after BM transplantation, PAR-1 deficiency had no effect on leukocyte count, hematocrit, or the properties of sickle red blood cells[1].

Heme-induced microvascular stasis

Dorsal skinfold chambers were implanted in sickle mice 3 days before the experiments as previously described[2]. This invasive procedure is not well tolerated by HbSS-Townes mice, therefore we used NY1DD sickle and BM transplanted mice for these studies. Microvascular stasis was determined in 20–25 pre-selected flowing post-capillary venules in response to intravenous infusion of 1.6 µmol/kg stroma-free hemoglobin (SFH, a gift from Sangart, San Diego, CA) and was expressed as percent non-flowing vessels one hour after hemoglobin infusion. Infusion of SFH results in microvascular stasis that is inhibited by hemopexin in NY1DD mice, indicating that hemoglobin releases heme into the circulation. Furthermore, we have previously shown that the injection of SFH (0.32 µmols/kg) resulted in the occlusion of 10% of pre-selected micro-vessels in HbAA-Townes whereas injection of high doses of SFH (32 µmols/kg) resulted in 5.9% stasis in C57BL/6 mice[2]. It is also important to note that 1 hour after infusion of saline, stasis was observed in 6.6% and 5.5% of preselected vessels in NY1DD and C57Bl/6 mice, respectively, most likely caused by the surgical procedures[2].

Rat anti–mouse TF[3] (1H1, generous gift from Dr. Daniel Kirchhofer, Genentech, South San Francisco, CA) or control rat IgG antibodies (Cedarlane Labs, Burlington, Ontario Canada) were injected (25 mg/kg, i.p.) 30 minutes before induction of stasis. Mice were fed with control chow or chow containing either the factor Xa (FXa) inhibitor, rivaroxaban (0.4 mg/g chow; Xarelto 20 mg tabs, Janssen Pharmaceuticals Inc, Belguim), or the thrombin inhibitor dabigatran etexilate (10 mg/g chow; Pradaxa 150 mg tabs, Boehringer Ingelheim, Germany) ad libitum for 4 days prior to stasis experiments. These treatments efficiently anticoagulated sickle mice without

bleeding complications[1,4]. NY1DD mice received saline or vorapaxar (150 µg/kg, AOBIOUS, Gloucester, MA) via oral gavage once daily for 3 days prior to stasis experiments.

We previously characterized the level of anticoagulation achieved by dabigatran (10 mg/g chow) and rivaroxaban (0.4 mg/g chow) after 4 days of treatment[1]. We found that dabigatran significantly prolonged aPTT from 25 seconds to 80 seconds, and reached a plasma concentration of 200 ng/mL. Rivaroxaban prolonged PT from 15 seconds to 25 seconds, and reached a plasma concentration of 20 ng/mL. Athough we did not observe any spontaneous bleeding complications, we also found that these doses of dabigatran and rivaroxaban significantly impaired hemostatic responses in a femoral vein bleeding model[1]. Furthermore, neither drug had any effect on leukocyte counts, hematocrit, or the properties of sickle red blood cells in either control or sickle mice[1].

Lung Immunohistochemistry

Lungs were excised from SS/PAR-$1^{+/+}$ and SS/PAR-$1^{-/-}$ mice 4 hours after SFH infusion, embedded in Optimal Cutting Temperature compound (Fisher Scientific, Pittsburgh PA), snap frozen, and stored at -80°C. Thirty-micrometer sections of lung were mounted on glass slides and fixed in 4% paraformaldehyde. Slides were stained with goat anti-pSel (Santa Cruz Biotechnology), sheep anti-VWF IgG (Cedarlane, Burlington, Ontario, Canada), and hamster anti-CD31 (Abcam), and visualized with appropriate secondary IgG labelled with Cy2 (pSel, green), Cy3 (VWF, red) and Cy5 (CD31, blue). Images were acquired using a FluoView FV1000 BX2 upright confocal microscope (Olympus, Center Valley, PA) with a 40X objective, and processed with FluoView (Olympus, Center Valley, PA). Images were analyzed with Adobe Photoshop to quantify pSel and VWF pixels as described previously[5].

Experimental design of quantitative fluorescent intravital lung microscopy (qFILM) studies

HbSS-Townes mice were treated with anti-TF 1H1 antibody (5 mg/kg, i.v. generous gift from Dr. Daniel Kirchhofer, Genentech) 30 minutes prior to infusion with LPS (0.1 µg/kg; *E. coli 011:B4;* Sigma-Aldrich, St. Louis, MO), and real time platelet-neutrophil aggregate-mediated pulmonary vaso-occlusion was quantified as previously described[6]. To visualize the pulmonary circulation, HbSS-Townes mice were infused with FITC dextran (MW 70,000) (Molecular Probes Inc., San Diego, CA). Neutrophils and platelets were labelled via intravenous infusion with fluorescent antibodies including Alexa Fluor 546-conjugated rat anti-mouse Ly6G monocolonal antibody (clone 1A8, Biolegend, San Diego, CA) and Violet 450-conjugated rat anti-mouse CD49b

monoclonal antibody (clone DX5, BD Biosciences, San Jose, CA). The microscopic setup used in qFILM has been described in detail previously[7,8].

Statistical analysis

Unless otherwise specified, a two-tailed unpaired Student's t-test was used for statistical analysis, and a $p<0.05$ was considered statistically significant. Statistics were calculated with Prism (V8, GraphPad Software, San Diego, CA).

Supplemental Figure Legends

Supplemental Video 1: Representative video of pulmonary vaso-occlusion from a sickle mouse 2 hours after LPS infusion. The video is a composite of circulation (FITC-dextran, purple), neutrophils (red), and platelets (green).

Supplemental Video 2: Representative video of pulmonary vaso-occlusion from a sickle mouse 2 hours after LPS infusion. The video is a composite of circulation (FITC-dextran, purple), neutrophils (red), and platelets (green).

Supplemental Video 3: Representative video of pulmonary vaso-occlusion from a sickle mouse 2 hours after LPS infusion. 1H1 antibody was administer 30 minutes before LPS infusion. The video is a composite of circulation (FITC-dextran, purple), neutrophils (red), and platelets (green).

Supplemental Video 4: Representative video of pulmonary vaso-occlusion from a sickle mouse 2 hours after LPS infusion. 1H1 antibody was administer 30 minutes before LPS infusion. The video is a composite of circulation (FITC-dextran, purple), neutrophils (red), and platelets (green).

1. Sparkenbaugh EM, Chantrathammachart P, Mickelson J, et al. Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease. *Blood*. 2014;123(11):1747-1756.
2. Belcher JD, Chen C, Nguyen J, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. 2014;123(3):377-390.
3. Kirchhofer D, Moran P, Bullens S, Peale F, Bunting S. A monoclonal antibody that inhibits mouse tissue factor function. *J Thromb Haemost*. 2005;3(5):1098-1099.
4. Chantrathammachart P, Mackman N, Sparkenbaugh E, et al. Tissue factor promotes activation of coagulation and inflammation in a mouse model of sickle cell disease. *Blood*. 2012;120(3):636-646.
5. Vercellotti GM, Dalmasso AP, Schaid TR, Jr., et al. Critical role of C5a in sickle cell disease. *Am J Hematol*. 2019;94(3):327-337.
6. Bennewitz MF, Jimenez MA, Vats R, et al. Lung vaso-occlusion in sickle cell disease mediated by arteriolar neutrophil-platelet microemboli. *JCI Insight*. 2017;2(1):e89761.

7. Bennewitz MF, Watkins SC, Sundd P. Quantitative intravital two-photon excitation microscopy reveals absence of pulmonary vaso-occlusion in unchallenged Sickle Cell Disease mice. *Intravital*. 2014;3(2):e29748.
8. Vats R, Brzoska T, Bennewitz MF, et al. Platelet Extracellular Vesicles Drive Inflammasome-IL1beta-dependent Lung Injury in Sickle Cell Disease. *Am J Respir Crit Care Med*. 2019.