

THROMBOSIS AND HEMOSTASIS

Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease

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Key Points

- Inhibition of FXa or thrombin might be considered to reduce thrombotic complications and vascular inflammation in sickle cell patients.
- PAR-2 could be a potential target to inhibit vascular pathology associated with sickle cell disease.

Activation of coagulation and vascular inflammation are prominent features of sickle cell disease (SCD). Previously, we have shown that inhibition of tissue factor (TF) attenuates activation of coagulation and vascular inflammation in mouse models of SCD. In this study, we examined the mechanism by which coagulation proteases enhance vascular inflammation in sickle BERK mice. To specifically investigate the contribution of FXa and thrombin, mice were fed chow containing either rivaroxaban or dabigatran, respectively. In addition, we used bone marrow transplantation to generate sickle mice deficient in either protease activated receptor-1 (PAR-1) or protease activated receptor-2 (PAR-2) on nonhematopoietic cells. FXa inhibition and PAR-2 deficiency in nonhematopoietic cells attenuated systemic inflammation, measured by plasma levels of interleukin-6 (IL-6). In contrast, neither thrombin inhibition nor PAR-1 deficiency in nonhematopoietic cells affected plasma levels of IL-6 in sickle mice. However, thrombin did contribute to neutrophil infiltration in the lung, independently of PAR-1 expressed by nonhematopoietic cells. Furthermore, the TF-dependent increase in plasma levels of soluble vascular cell adhesion molecule-1 in sickle mice was not mediated by FXa or thrombin. Our data indicate that TF, FXa, and thrombin differentially contribute to vascular inflammation in a mouse model of SCD. (*Blood*. 2014;123(11):1747-1756)

Introduction

Sickle cell disease (SCD) is a hematologic disorder caused by a single nucleotide mutation in the β -globin gene. Under hypoxic conditions, the mutation leads to abnormal polymerization of hemoglobin tetramers. Aggregation of abnormally large hemoglobin polymers results in the formation of sickle red blood cells that are susceptible to hemolysis, adhere to the endothelium, and form aggregates with leukocytes and platelets.¹ These processes result in the 2 primary pathologies of SCD: hemolytic anemia and vaso-occlusive crises.^{2,3} Furthermore, SCD is also associated with a chronic activation of coagulation and vascular inflammation.

Despite the well-documented hypercoagulable state of SCD, little is known about the contribution of coagulation to the pathology of the disease. Clinical studies investigating the use of warfarin,⁴ acenocoumarol,⁵ and heparin⁶ in acute pain crises in sickle cell patients were inconclusive; however, these studies were performed on a small number of patients, lacked placebo controls, and used pain crises as the only clinical end point.⁷ Notably, the only adequately powered and placebo-controlled study to examine the effect of the low-molecular-weight heparin tinzaparin demonstrated a significant reduction in the duration of a painful crisis and

hospital stay,⁸ suggesting that anticoagulants might be beneficial in SCD.

In SCD, increased expression of tissue factor (TF) has been demonstrated in both leukocytes and endothelial cells (ECs).^{9,10} We and others recently demonstrated that inhibition of TF inhibited activation of coagulation and attenuated the enhanced thrombosis in cerebral microvessels in sickle mice.^{11,12} Importantly, we demonstrated that inhibition of TF also reduced plasma levels of interleukin-6 (IL-6) and soluble vascular cell adhesion molecule-1 (sVCAM-1) as well as reduced local lung inflammation measured by tissue levels of myeloperoxidase (MPO). These data indicate that TF not only promotes activation of coagulation but also contributes to chronic vascular inflammation in a mouse model of SCD.¹¹ Moreover, we have shown that EC-specific deletion of the TF gene significantly reduced plasma levels of IL-6 without affecting activation of coagulation,¹¹ suggesting that EC TF contributes to the expression of IL-6 independent of thrombin generation.

Coagulation proteases, such as FXa and thrombin, can induce multiple cellular effects via activation of protease activated receptors (PARs). PARs are the family of G-protein-coupled receptors that are

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Table 1. Hematologic parameters of BERK^{AA} and BERK^{SS} mice treated with dabigatran (10 mg/g chow) or placebo

Variable	AA/Con	AA/Dab	SS/Con	SS/Dab
RBC (10 ⁶ /μL)	8.9 ± 0.1	9.2 ± 0.3	6.0 ± 0.3***	6.2 ± 0.5***
Hematocrit (%)	37.0 ± 0.6	37.1 ± 1.1	27.7 ± 1.5***	28.5 ± 2.1***
RDW (%)	18.6 ± 0.5	18.1 ± 0.3	28.1 ± 0.8*	27.9 ± 1.4*
WBC (10 ³ /μL)	2.9 ± 0.4	3.5 ± 0.7	28.3 ± 5.5***	23.6 ± 4.4***
Neutrophil (10 ³ /μL)	0.8 ± 0.1	0.7 ± 0.1	2.75 ± 0.5***	2.3 ± 0.4**
Lymphocyte (10 ³ /μL)	2.1 ± 0.2	2.7 ± 0.6	24.0 ± 4.9***	20.3 ± 3.9**
Monocyte (10 ³ /μL)	0.1 ± 0.01	0.1 ± 0.04	1.4 ± 0.3***	1.0 ± 0.2*
Platelet (10 ³ /μL)	564 ± 19	554 ± 17	568 ± 34	537 ± 43

Asterisks indicate statistical significance compared with BERK^{AA} mice within the same treatment (**P* < .05, ***P* < .01, and ****P* < .001). There were no statistically significant differences in any parameters between dabigatran and control within BERK^{AA} or BERK^{SS} mice.

Con, control placebo; Dab, dabigatran; RDW, red cell distribution width; WBC, white blood cell.

activated by proteolytic cleavage. PAR-1^{AA} is activated by various proteases, including FXa and thrombin, whereas PAR-2 is activated by the TF:FVIIa complex, FXa, and other proteases.¹³⁻¹⁶ It has been demonstrated that coagulation protease-dependent activation of PARs contributes to inflammation in many vascular disorders.¹⁷⁻¹⁹

In this study, we investigated the mechanism by which 2 coagulation proteases, FXa and thrombin, contribute to the vascular inflammation in sickle cell mice. Mice were treated with either the direct FXa inhibitor rivaroxaban or the direct thrombin inhibitor dabigatran. We also determined the role of nonhematopoietic PAR-1 and PAR-2 by transplanting bone marrow (BM) from sickle mice into PAR-1- or PAR-2-deficient mice. Our results demonstrate that FXa and thrombin differentially contribute to the vascular inflammation in a mouse model of SCD.

Methods

Mice

We used the Berkeley (BERK) mouse model of SCD.²⁰ BERK mice have a transgene (Tg) containing normal human α-, γ-, δ-globins and sickle β-globin and targeted deletion of murine α- and β-globins (α^{-/-}, β^{-/-}, Tg). We generated these mice by intercrossing α^{-/-}, β^{-/-}, Tg males with α^{-/-}, β^{-/-}, Tg females. As a control, we used wild-type (WT) mice that have no human transgenes (α^{+/+}, β^{+/+}) on the similar mixed genetic background. These mice were used as a BM donor for experiments investigating the role of PAR-1 and PAR-2. Generation of PAR-1- and PAR-2-deficient mice was previously described.^{21,22} They were backcrossed to a C57Bl/6J background for 10 generations, and experimental animals were generated by intercrossing PAR-1^{+/-} or PAR-2^{+/-} mice. For dabigatran and rivaroxaban experiments, we used BERK^{AA} and BERK^{SS} mice containing double copies of the transgenes (provided by Dr Robert Hebbel). All experiments were approved by the University of North Carolina Animal Care and Use Committees and complied with National Institutes of Health guidelines.

BM transplantation

PAR-1^{+/+}, PAR-1^{-/-}, PAR-2^{+/+}, and PAR-2^{-/-} mice (8 weeks old) were lethally irradiated with 14 Gy (2 doses of 7 Gy 4 hours apart) with a ¹³⁷Cs irradiator (Mark I Irradiator, J. L. Shepherd & Associates). Recipient mice were injected via the retro-orbital sinus with BM cells (4-5 × 10⁶ nucleated cells) from BERK or WT control mice that do not express any human transgenes on the same mixed genetic background.¹¹ These mice are referred to as BERK^{BM} or WT^{BM}. Mice were used for experiment 4 months later and samples were collected as described below. Reconstitution of BM was confirmed by electrophoretic analysis of the different forms of hemoglobin.¹¹

Optimizing the doses of dabigatran and rivaroxaban

NMRI mice were fed with chow containing dabigatran (Boehringer Ingelheim, Biberach, Germany) at various concentrations (0.75, 5, 10, and 15 mg/g chow) or matching placebo for 4 days. Blood was collected in the morning, and the concentration of dabigatran (the active compound) was assessed by liquid chromatography mass spectrometry.²³ In similar studies, C57Bl/6J mice were fed with placebo or rivaroxaban (Janssen Pharmaceuticals, Bayer AG) at 0.0125, 0.2, or 0.4 mg/g chow (Dyets, Bethlehem, PA) for 10 days. Blood was collected in the morning, and prothrombin time (PT) was assessed in plasma as described below. To measure the plasma concentration of rivaroxaban in mice, the PT was compared with a standard curve generated by measuring the PT of normal mouse plasma containing known concentrations of rivaroxaban. We assumed that plasma concentrations of dabigatran and rivaroxaban would be at the peak in the morning because mice are nocturnal.

Saphenous vein bleeding model

Eight-week-old male C57Bl/6J mice were fed chow containing placebo, dabigatran (10 mg/g chow), or rivaroxaban (0.4 mg/g chow) for 5 days. On the morning of the last day of treatment, the level of anticoagulation was determined using the saphenous vein bleeding model as previously described.²⁴

Dabigatran and rivaroxaban treatment and sample collection

Once the optimal doses of dabigatran and rivaroxaban were determined, 4-month-old BERK^{AA} and BERK^{SS} mice were fed chow containing placebo, dabigatran (10 mg/g chow), or rivaroxaban (0.4 mg/g chow) ad libitum for 10 days. Four months after BM transplantation or 10 days after feeding with chow containing anticoagulants, mice were anesthetized with isoflurane. Blood was drawn from the inferior vena cava into 3.4% sodium citrate (final ratio of 9:1) and plasma was stored at -80°C. The entire left lung was collected for histologic analysis and remaining tissue was snap frozen in liquid nitrogen and stored at -80°C.

Analysis of hematologic profile, PT, aPTT, TAT, sVCAM-1, and IL-6

Blood cell count and hematologic profile were determined using the automatic HEMAVET system (Drew Scientific, Inc.). PT was determined by a 1-stage clotting assay with a Start 4 Clotting Machine (Diagnostics Stago, Parsippany, NJ) using Thromboplastin D reagent (Thermo Scientific). Activated partial thromboplastin time (aPTT) of plasma samples was analyzed with TriniClot aPTT reagents (Diagnostics Stago). The plasma level of thrombin-antithrombin complex (TAT; Siemens Healthcare Diagnostics), IL-6, and sVCAM-1 (R&D Systems) was determined by enzyme-linked immunosorbent assay.

Table 2. Hematologic parameters from BERK^{AA} and BERK^{SS} mice treated with rivaroxaban (0.4 mg/g chow) or placebo chow

Variable	AA/Con	AA/Riv	SS/Con	SS/Riv
RBC (10 ⁶ /μL)	8.0 ± 0.7	8.2 ± 0.9	5.1 ± 0.7***	4.7 ± 0.8***
Hematocrit (%)	32.7 ± 3.3	33.1 ± 3.7	22.5 ± 3.8***	22.0 ± 3.7***
RDW (%)	18.3 ± 1.0	18.7 ± 2.1	28.6 ± 2.1***	29.1 ± 2.8***
WBC (10 ³ /μL)	4.1 ± 2.1	3.92 ± 1.1	39.1 ± 10.7***	39.3 ± 13.1***
Neutrophil (10 ³ /μL)	0.9 ± 0.7	0.9 ± 0.4	3.9 ± 2.1***	3.5 ± 2.3***
Lymphocyte (10 ³ /μL)	3.0 ± 1.8	2.9 ± 0.9	33.1 ± 8.6***	33.6 ± 10.1***
Monocyte (10 ³ /μL)	0.2 ± 0.1	0.2 ± 0.1	2.0 ± 0.7***	2.2 ± 1.2***
Platelet (10 ³ /μL)	652 ± 85	626 ± 117	709 ± 144	688 ± 85

Asterisks indicate significant difference compared with BERK^{AA} mice within the same treatment (****P* < .001). There were no statistically significant differences in any parameters between Riv and Con within BERK^{AA} or BERK^{SS} mice.

Con, control placebo; RDW, red cell distribution width; Riv, rivaroxaban; WBC, white blood cell.

Table 3. Hematologic parameters from PAR-1^{+/+} or PAR-1^{-/-} mice transplanted with bone marrow from WT or sickle BERK (SS) mice

Variable	WT/PAR-1 ^{+/+}	WT/PAR-1 ^{-/-}	SS/PAR-1 ^{+/+}	SS/PAR-1 ^{-/-}
RBC (10 ⁶ /μL)	7.4 ± 0.1	8.1 ± 0.3	4.9 ± 0.3**	4.0 ± 0.3***
Hematocrit (%)	35.3 ± 0.3	37.0 ± 0.6	26.4 ± 0.6***	24.6 ± 0.7***
RDW (%)	17.1 ± 0.8	16.2 ± 0.1	30.4 ± 0.5***	29.2 ± 0.4***
WBC (10 ³ /μL)	7.4 ± 1.9	10.9 ± 1.4	18.7 ± 1.5***	18.8 ± 1.9***
Neutrophil (10 ³ /μL)	1.4 ± 0.2	1.5 ± 0.2	3.9 ± 0.4*	4.6 ± 0.9**
Lymphocyte (10 ³ /μL)	5.7 ± 1.8	8.9 ± 1.4	13.5 ± 1.0***	12.9 ± 1.0
Monocyte (10 ³ /μL)	0.3 ± 0.04	0.5 ± 0.08	1.3 ± 0.2***	1.2 ± 0.1***
Platelet (10 ³ /μL)	699 ± 23	687 ± 21	690 ± 29	687 ± 43

Asterisks indicate statistical significance compared with WT^{BM} mice within the same treatment (**P* < .05, ***P* < .01, and ****P* < .001). There were no statistically significant differences between PAR1^{+/+} and PAR1^{-/-} mice within WT^{BM} or SS^{BM}.

Analysis of MPO in lung tissue

Protein was isolated from lung as previously described,¹¹ and total protein concentration was determined by the bicinchoninic acid protein assay (Bio-Rad, Hercules, CA). MPO levels were detected by enzyme-linked immunosorbent assay (Hycult Biotech, Plymouth Meeting, PA).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (4 μm) of the entire left lobe were stained for neutrophils as previously described.¹¹ Images were acquired with DX51W1 microscope (Olympus America) equipped with a DP70 digital camera and DP Controller Software V 02.03 (Olympus America). Neutrophils were quantified by counting positive cells in 10 high-powered fields (original magnification ×400) per section. All pictures were identically adjusted for contrast, brightness, and color saturation.

Statistical analysis

Data are presented as a mean ± standard error of the mean. One- and 2-way ANOVA were performed where appropriate, and Bonferroni post-test analyses were applied for multiple comparisons. The criterion for significance was *P* < .05. Statistical analysis was performed with GraphPad Prism Version 5 software.

Results

BM transplantation model

Reconstitution of BM in WT^{BM} or BERK^{BM} mice was determined by cellulose acetate electrophoretic analysis of the different forms of hemoglobin. Using this method, we did not observe the presence of any normal mouse hemoglobin in BERK^{BM} mice (supplemental Figure 1, available on the *Blood* Web site). However, we cannot exclude the possibility that very small amounts of normal hemoglobin, which are below the detection level of the assay, are still present in BERK^{BM} mice. Even small amounts of WT hemoglobin have been

demonstrated to partially attenuate the sickle phenotype in chimeric mice.²⁵ This may explain why the phenotype observed in BERK^{BM} mice is less pronounced than that observed in BERK^{SS} mice, especially regarding plasma levels of TAT and sVCAM-1. We also observed that the lymphocytosis in BERK^{SS} mice (Tables 1 and 2) was not as severe in BERK^{BM} mice (Tables 3 and 4) and that WT^{BM} controls had higher levels of circulating lymphocytes than BERK^{AA} controls. Less severe leukocytosis may also contribute to the milder phenotype observed in BERK^{BM} mice compared with BERK^{SS} mice.

Anticoagulating mice with dabigatran and rivaroxaban

Treatment of WT mice with various concentrations of dabigatran for 4 days caused a dose-dependent prolongation of the aPTT compared with untreated control mice (Figure 1A). The plasma concentration of dabigatran was significantly increased in mice fed with chow containing 5, 10, and 15 mg of dabigatran per gram (Figure 1B), and the 10 mg/g chow dose was used for all subsequent studies. The PT assay is considered more sensitive than the aPTT to determine the level of anticoagulation by rivaroxaban treatment, so we measured the PT in WT mice treated with rivaroxaban for 10 days. The PT was significantly prolonged with 0.2 and 0.4 mg/g rivaroxaban compared with mice treated with placebo or 0.0125 mg/g chow (Figure 1C). Rivaroxaban was detectable in the plasma of mice treated with the 0.2-mg/g and 0.4-mg/g doses (Figure 1D). The 0.4-mg/g chow dose was used for subsequent studies. We observed no spontaneous bleeding in mice treated with 10 mg/g dabigatran or 0.4 mg/g rivaroxaban. To directly compare the levels of anticoagulation after dabigatran and rivaroxaban treatment, WT mice were treated with dabigatran or rivaroxaban ad libitum for 5 days and hemostasis time was determined using a saphenous vein bleeding model.²⁴ Because mice are nocturnal, all measurements were performed in the morning to minimize the variability in the plasma drug levels due to differences in chow consumption. Both rivaroxaban and dabigatran significantly prolonged hemostasis time compared with untreated

Table 4. Hematologic parameters from PAR-2^{+/+} or PAR-2^{-/-} mice transplanted with bone marrow from WT or sickle BERK (SS) mice

Variable	WT/PAR-2 ^{+/+}	WT/PAR-2 ^{-/-}	SS/PAR-2 ^{+/+}	SS/PAR-2 ^{-/-}
RBC (10 ⁶ /μL)	8.2 ± 0.7	8.6 ± 0.36	5.4 ± 0.7***	5.4 ± 0.6***
Hematocrit (%)	38.3 ± 3.4	40.5 ± 1.6	27.3 ± 5.2***	25.4 ± 3.6***
RDW (%)	17.0 ± 0.7	16.6 ± 0.3	31.7 ± 2.4***	31.9 ± 4.4***
Wbc (10 ³ /μL)	12.9 ± 4.7	15.5 ± 4.7	19.4 ± 5.4*	19.1 ± 4.3
Neutrophil (10 ³ /μL)	2.2 ± 1.3	1.9 ± 0.8	5.38 ± 4.6*	1.96 ± 0.5#
Lymphocyte (10 ³ /μL)	10.5 ± 3.6	13.3 ± 3.9	13.0 ± 3.7	16.4 ± 4.0
Monocyte (10 ³ /μL)	0.31 ± 0.16	0.04 ± 0.05	0.96 ± 0.3***	0.92 ± 0.3***
Platelet (10 ³ /μL)	788 ± 84	734 ± 29	894 ± 161	914 ± 141*

Asterisks indicate statistical difference compared with WT^{BM} within the same genotype (**P* < .05 and ****P* < .001). Hash sign indicates statistical difference between SS/PAR-2^{+/+} and SS/PAR-2^{-/-} (#*P* < .05).

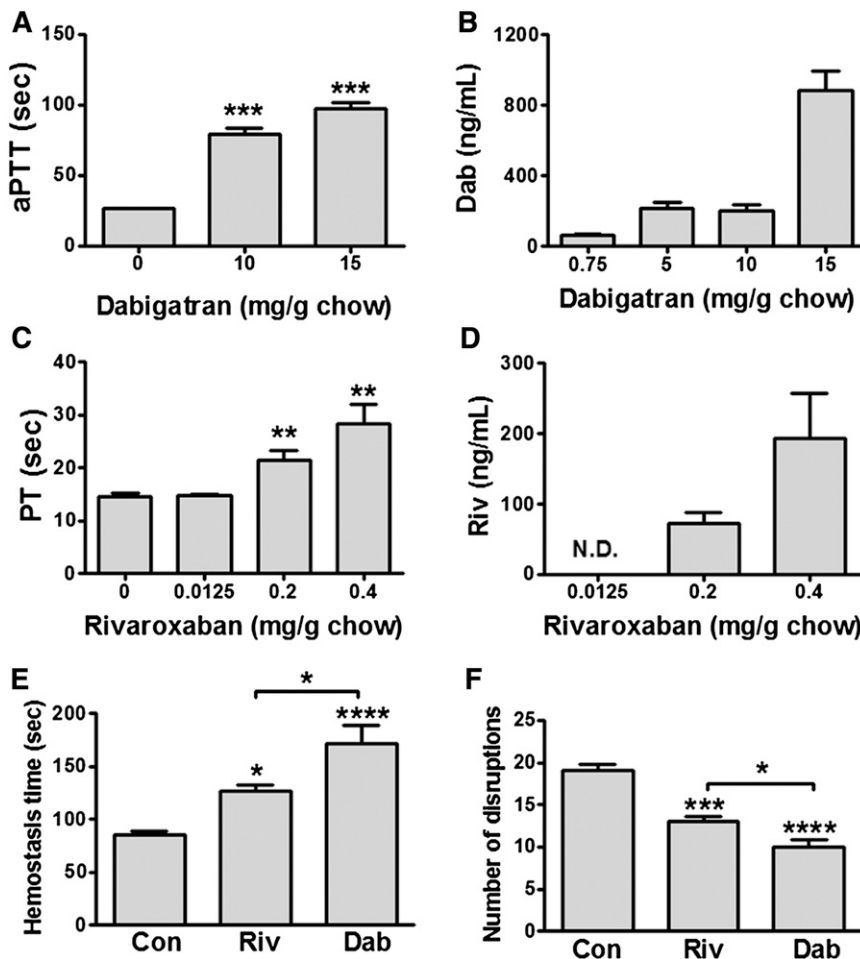


Figure 1. Characterization of anticoagulation by dabigatran and rivaroxaban. (A-B) aPTT (A) and plasma concentration (B) of dabigatran (active compound) in mice fed for 4 days with chow containing placebo or different amounts of inactive dabigatran ($n = 3-5$ per group). The overall P value from a 1-way ANOVA is $P < .0001$. (C-D) PT (seconds) (C) and plasma concentration (D) of rivaroxaban in mice fed for 10 days with chow containing placebo or different amounts of rivaroxaban ($n = 5$ per group). The overall P value from a 1-way ANOVA is $P = .0134$. (E-F) Hemostasis time (E) and the number of disruptions (F) in WT mice fed with chow containing placebo ($n = 6$), rivaroxaban (0.4 mg/g chow; $n = 6$), or dabigatran (10 mg/g chow; $n = 5$) for 5 days. Bleeding was observed for 30 minutes, and any formed clots were disrupted. Average bleeding time was calculated from the individual bleeding times determined during the 30-minute period; the number of clots formed is also reported (disruption number). Asterisks directly above the bars indicate the statistical significance of dabigatran- or rivaroxaban-treated mice compared with placebo-treated mice (* $P < .05$, ** $P < .01$, and *** $P < .001$). Dab, dabigatran; Con, control placebo; Riv, rivaroxaban.

mice; dabigatran-treated mice had a significantly longer mean hemostasis time than rivaroxaban-treated mice (Figure 1E). In addition, the number of clots formed during 30 minutes was significantly reduced in mice fed with chow containing rivaroxaban and dabigatran compared with mice fed with control chow, and dabigatran treatment had a significantly stronger effect compared with rivaroxaban treatment (Figure 1F). These data indicate that the dose of dabigatran used for the study had a more potent anticoagulant effect compared with the dose of rivaroxaban.

Effect of thrombin inhibition on vascular inflammation in SCD

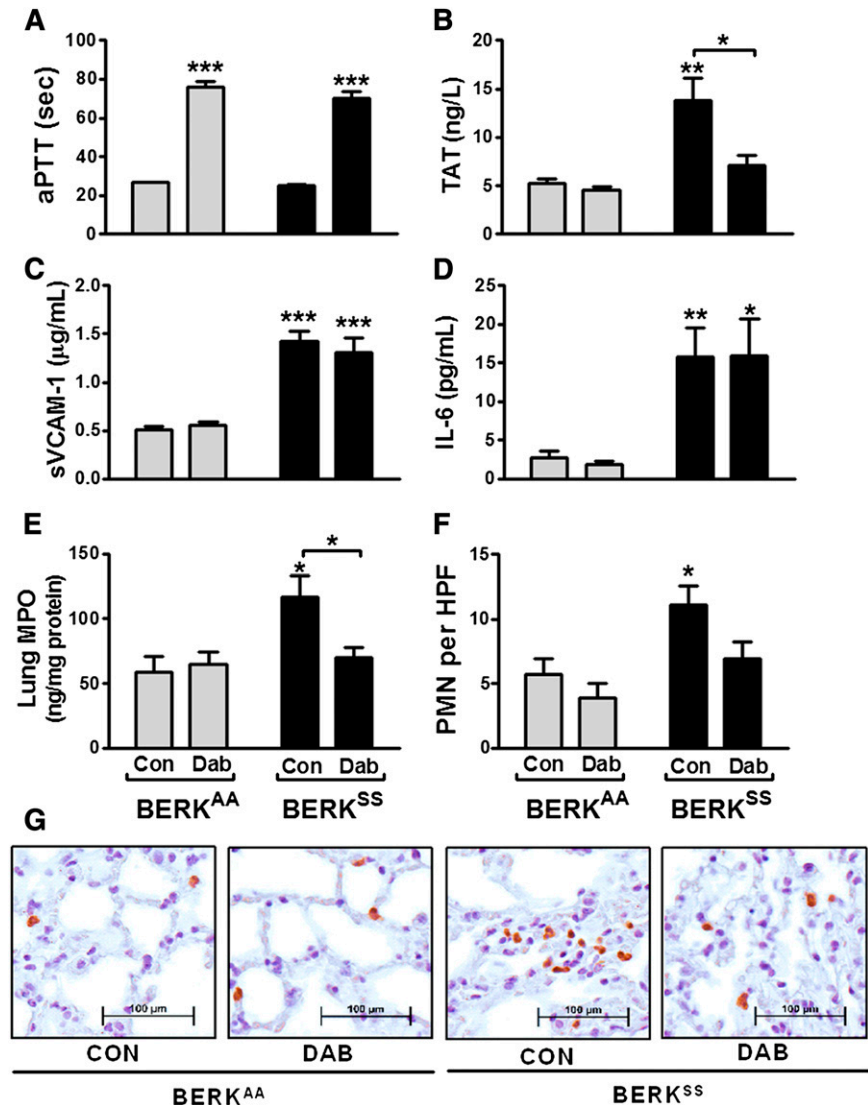
BERK^{SS} mice had significantly lower red blood cell (RBC) counts, hemoglobin levels, and hematocrits compared with BERK^{AA} controls; thrombin inhibition with dabigatran had no effect on these parameters, indicating that thrombin inhibition does not affect hemolysis of sickle RBCs (Table 1). There was also a significant increase in white blood cell counts in BERK^{SS} mice that was not affected by thrombin inhibition (Table 1). Dabigatran prolonged aPTT in both BERK^{AA} and BERK^{SS} mice compared with mice on placebo chow (Figure 2A). There was a significant increase in plasma TAT levels in BERK^{SS} mice compared with BERK^{AA} controls that was attenuated with dabigatran treatment (Figure 2B). BERK^{SS} mice had elevated plasma levels of IL-6 and sVCAM-1 compared with BERK^{AA} controls; however, thrombin inhibition had no effect on these parameters (Figure 2C-D). Importantly, thrombin inhibition significantly attenuated the increase in MPO levels (Figure 2E) and the number of neutrophils observed in

the lungs of BERK^{SS} mice (Figure 2F-G). These data indicate that TF-dependent thrombin generation has no effect on systemic inflammation but does contribute to local inflammation in the lung via a mechanism involving neutrophil recruitment.

Role of nonhematopoietic cell PAR-1 on vascular inflammation in SCD

To determine if the thrombin-mediated effect in the lung was PAR-1 dependent, we generated sickle cell mice lacking PAR-1 expression on all nonhematopoietic cells. PAR-1^{+/+} and PAR-1^{-/-} mice transplanted with BERK^{BM} had significantly decreased RBC counts and hematocrits compared with mice transplanted with WT^{BM}, indicating that deletion of PAR-1 from nonhematopoietic cells has no effect on anemia in sickle mice (Table 3). We also observed an increase in the total number of white blood cells in BERK^{BM} mice; deletion of PAR-1 from nonhematopoietic cells had no effect on these parameters (Table 3). PAR-1^{+/+} mice transplanted with BM from BERK mice had significantly elevated plasma levels of TAT, sVCAM-1, and IL-6 (Figure 3A-C), indicating that transplantation with sickle cell BM caused coagulation activation, vascular inflammation, and EC activation. PAR-1 deletion from all nonhematopoietic cells did not attenuate any of these parameters (Figure 3A-C). Furthermore, PAR-1^{+/+} mice transplanted with sickle BM had significantly elevated MPO levels and the number of neutrophils in the lungs; however, these parameters were not affected by deletion of PAR-1 from nonhematopoietic cells (Figure 3D-E). These data

Figure 2. Role of thrombin in vascular inflammation in SCD. (A-E) aPTT (A) and plasma levels of TAT (B), sVCAM-1 (C), IL-6 (D), and MPO (E) in the lungs of BERK^{AA} (n = 9-10) and BERK^{SS} (n = 10-12) mice that received dabigatran (Dab) or control placebo (Con) for 10 days. Formalin-fixed lungs were stained for neutrophils, and neutrophils were counted in 10 high-power fields (HPF; original magnification $\times 400$) for each mouse. (F) Average number of neutrophils in 10 HPFs ($\times 400$) of lungs. (G) Representative lung sections demonstrating neutrophil infiltration in four different groups of mice. Neutrophils stain as brown. Asterisks directly above the bars indicate statistically significant difference between BERK^{SS} compared with BERK^{AA} mice within the same treatment group (Dab or Con) (* $P < .05$, ** $P < .01$, and *** $P < .001$).



suggest that thrombin contributes to local lung inflammation in sickle cell mice independently of PAR-1 activation on nonhematopoietic cells.

Effect of FXa inhibition on vascular inflammation in SCD

FXa inhibition had no effect on markers of anemia in BERK^{SS} mice, nor did it affect leukocytosis (Table 2). Rivaroxaban significantly prolonged PT in both BERK^{AA} and BERK^{SS} mice compared with mice on placebo chow (Figure 4A). Furthermore, the increase in plasma TAT levels observed in BERK^{SS} mice was attenuated by rivaroxaban (Figure 4B), whereas increased levels of sVCAM-1 were not affected (Figure 4C). Interestingly, FXa inhibition abolished the increase in plasma IL-6 in BERK^{SS} mice (Figure 4D). We also observed a significant increase in MPO levels in the lungs of BERK^{SS} mice compared with BERK^{AA} mice fed with control chow (Figure 4E). In contrast, we did not observe a significant increase in MPO levels in the lung of BERK^{SS} mice fed with chow containing rivaroxaban. Consistent with this, there were significantly more neutrophils in the lungs of BERK^{SS} mice compared with BERK^{AA} controls, and rivaroxaban treatment significantly reduced these numbers in BERK^{SS} mice (Figure 4F).

Role of nonhematopoietic cell PAR-2 on vascular inflammation in SCD

Next, we determined if the proinflammatory effect of FXa in sickle cell mice is due to activation of PAR-2 on nonhematopoietic cells. PAR-2^{+/+} and PAR-2^{-/-} mice were lethally irradiated and transplanted with WT^{BM} or BERK^{BM}. As expected, reconstitution with BM from BERK mice significantly decreased RBC counts, hemoglobin, and hematocrit in PAR-2^{+/+} mice, and deletion of PAR-2 from nonhematopoietic cells had no effect on these parameters (Table 4). Interestingly, we observed a significant decrease in neutrophil numbers in PAR-2^{-/-} sickle mice compared with sickle PAR-2^{+/+} controls (Table 4).

PAR-2^{+/+} mice transplanted with BERK^{BM} had significantly increased plasma TAT, sVCAM-1, and IL-6 levels (Figure 5A-C). Deletion of PAR-2 from nonhematopoietic cells had no effect on TAT or sVCAM-1 but dramatically reduced the increase in plasma levels of IL-6 in sickle mice (Figure 5A-C). These data indicate that PAR-2 contributes to systemic inflammation. Furthermore, PAR-2 deletion from nonhematopoietic cells attenuated the increase in MPO levels (Figure 5D) and the number of neutrophils in the lungs of BERK^{BM} mice (Figure 5E).

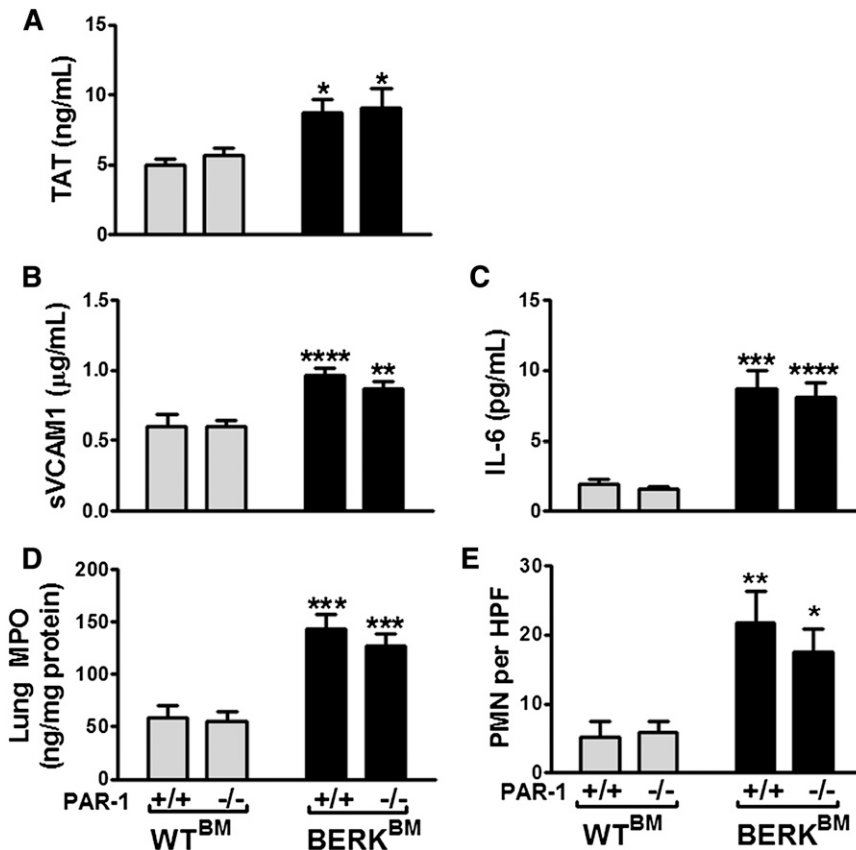


Figure 3. Role of PAR-1 in vascular inflammation in SCD. (A-D) Plasma levels of TAT (A), sVCAM-1 (B), IL-6 (C), and MPO (D) in the lung of PAR-1^{+/+} or PAR-1^{-/-} mice transplanted with bone marrow from WT (n = 9-11) or BERK (n = 15-16) mice. Formalin-fixed lungs were stained for neutrophils, and neutrophils were counted in 10 HPF (×400) for each mouse. (E) Average number of neutrophils in lungs. Neutrophils stain brown. Asterisks directly above the bars indicate statistically significant difference between BERK^{BM} mice compared with WT^{BM} mice within the same PAR-1 genotype (**P* < .05, ***P* < .01, and ****P* < .001). There were no differences between PAR-1^{+/+} and PAR-1^{-/-} mice.

Discussion

The vascular pathology of SCD is characterized by chronic activation of coagulation and inflammation. In our previous study, we demonstrated that inhibition of TF reduced plasma levels of sVCAM-1 and IL-6, as well as MPO, in the lungs of sickle mice, indicating a crosstalk between coagulation and inflammation.¹¹ TF-dependent coagulation activation results in the generation of the downstream coagulation proteases FXa and thrombin, which increase inflammation, in part, by activating PARs.¹⁸ In this study, we investigated the role of FXa, thrombin, PAR-1, and PAR-2 in vascular inflammation in a mouse model of SCD.

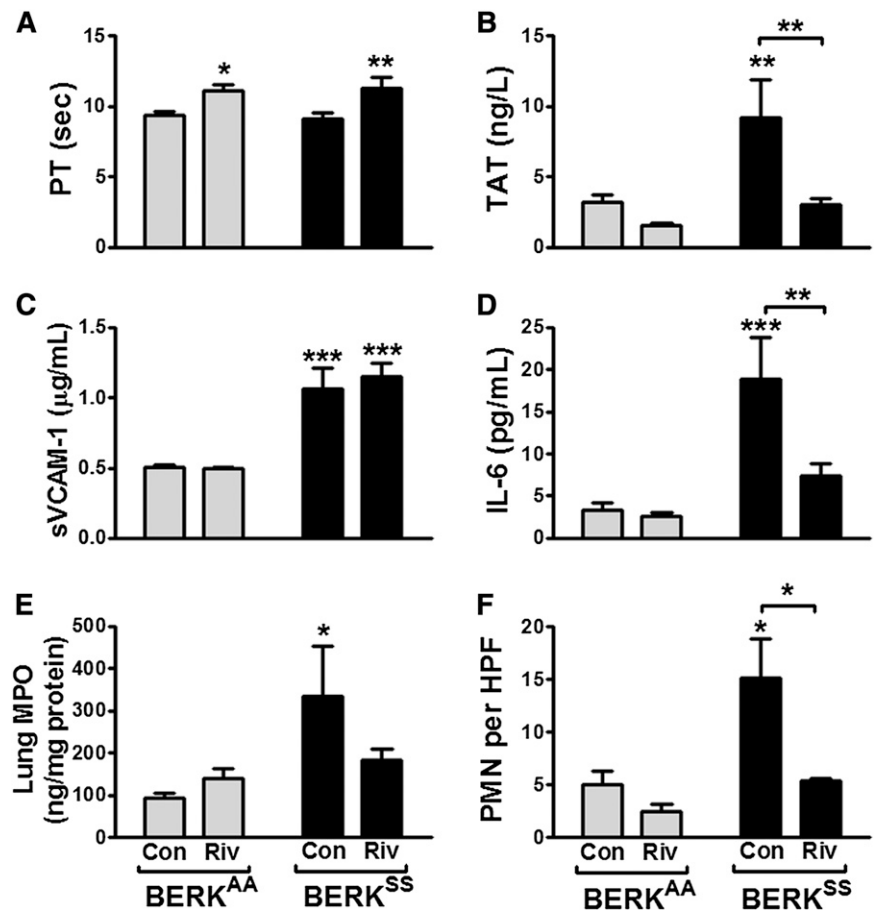
The hypercoagulable state of SCD is well characterized. Rivaroxaban and dabigatran are new direct inhibitors of FXa and thrombin, respectively, which were recently approved to prevent stroke and venous thromboembolism.²⁶⁻²⁹ We found that feeding mice dabigatran (10 mg/g chow) or rivaroxaban (0.4 mg/g chow) resulted in plasma levels of the active drugs similar to those seen in human patients receiving 150 mg of dabigatran twice daily³⁰ or rivaroxaban 10 mg once daily.³¹ Dabigatran and rivaroxaban significantly prolonged the aPTT and PT, respectively, in both BERK^{AA} and BERK^{SS} mice and normalized plasma TAT levels in BERK^{SS} mice without causing spontaneous bleeding. These data indicate that both FXa and thrombin inhibition can anticoagulate sickle cell mice and that treatment with the new oral anticoagulants might reduce thrombotic complications in SCD.

Importantly, inhibition of either FXa or thrombin resulted in a reduction in the elevated MPO levels in the lungs of sickle cell mice similar to that observed in sickle mice treated with anti-TF antibody.¹¹ These results suggest that TF contributes to local lung inflammation

in a thrombin-dependent manner. Importantly, dabigatran-treated BERK^{SS} mice also demonstrated reduced number of neutrophils in the lung tissue, indicating that increased levels of MPO observed in the lung of BERK^{SS} mice results from thrombin-mediated neutrophil infiltration rather than from thrombin-mediated neutrophil activation. Surprisingly, deletion of PAR-1 from nonhematopoietic cells did not attenuate neutrophil numbers or lung MPO levels in sickle mice, suggesting that PAR-1 activation on nonhematopoietic cells is not essential for thrombin-mediated neutrophil recruitment to the lung. One possibility is that thrombin contributes to neutrophil recruitment via a fibrin- or fibrin degradation product-dependent mechanism. For instance, the E1 fibrin degradation fragment has been shown to facilitate neutrophil-EC interactions and mediates infiltration of neutrophils into inflamed tissue.³² Inhibition of thrombin would reduce formation of the E1 fragment and may explain, in part, the anti-inflammatory effects of dabigatran in the lung. Inhibition of this pathway may also reduce neutrophil infiltration in BERK^{SS} mice treated with rivaroxaban.

Our previous study showed that EC-specific deletion of the TF gene selectively reduced plasma levels of IL-6 without affecting any other parameters, including TAT.¹¹ These results strongly suggested that in sickle mice, EC TF contributes to the increased expression of IL-6 independently of thrombin generation. Consistent with this notion, our current study demonstrated that neither thrombin inhibition nor deletion of PAR-1 from nonhematopoietic cells reduced plasma levels of IL-6. In contrast, FXa inhibition abolished the increase in plasma levels of IL-6 in BERK^{SS} mice, similar to that observed in sickle mice treated with anti-TF antibody or lacking the TF gene in ECs.¹¹ Because prolongation of the saphenous vein bleeding time was significantly longer in dabigatran-fed mice than in rivaroxaban-fed mice, the absence of an effect on IL-6 levels in

Figure 4. Role of FXa in vascular inflammation in SCD. (A-F) PT (A) and plasma levels of TAT (B), sVCAM-1 (C), IL-6 (D), and MPO (E) in the lungs and average number of neutrophils (F) in lungs of BERK^{AA} (n = 16) and BERK^{SS} (n = 13-15) mice fed with chow containing rivaroxaban (Riv) or placebo control (Con) for 10 days. Asterisks directly above bars indicate statistical significance between BERK^{SS} mice compared with BERK^{AA} mice within the same treatment group (Con or Riv) (**P* < .05, ***P* < .01, ****P* < .001).



BERK^{SS} fed with dabigatran cannot be attributed to insufficient anticoagulation. Therefore, we proposed that EC TF contributes to IL-6 expression via a mechanism involving FXa-dependent activation of PAR-2 in nonhematopoietic cells. Whether PAR-2 activation is mediated by the ternary TF:FVIIa:FXa complex assembled on the EC surface³³ or by locally generated FXa remains to be determined.

PAR-2 deletion from nonhematopoietic cells not only reduced plasma levels of IL-6 but also completely attenuated lung MPO levels and the number of neutrophils in the lungs, indicating that activation of PAR-2 in nonhematopoietic cells plays a crucial role in the recruitment/activation of neutrophils in SCD. At this point, we do not know if this PAR-2-mediated effect results from FXa-dependent activation of the receptor. It is possible that other PAR-2 agonists, such as mast cell tryptase,¹⁹ can also contribute to this response. Indeed, elevated levels of mast cell tryptase have been reported in BERK^{SS} mice compared with BERK^{AA} controls.³⁴ Furthermore, PAR-2 contributed to the neutrophilia, which is commonly observed in both sickle cell patients and animal models of SCD.^{11,35} Sickle cell mice lacking PAR-2 in all nonhematopoietic cells had a significantly reduced number of circulating neutrophils compared with PAR-2-proficient sickle cell mice. Interestingly, neutrophilia was not affected by inhibition of TF^{F11} or by rivaroxaban or dabigatran treatment, further supporting the concept that in sickle cell mice, PAR-2 can be activated by ligands other than coagulation proteases. In support of this, neutrophilia is attenuated in BERK^{SS} mice lacking mast cells,³⁴ suggesting that mast cell tryptase-dependent PAR-2 activation might play a role in neutrophilia in SCD.

Deletion of PAR-1 from nonhematopoietic cells had no effect on coagulation, systemic inflammation, or EC activation in BERK^{BM} mice. Although thrombin is the main physiologic ligand for PAR-1, a recent study demonstrated that PAR-1 can be also activated by activated protein C (APC).³⁶ In contrast to thrombin-mediated activation of PAR-1, APC-dependent cleavage of PAR-1 requires the presence of endothelial protein C receptor and results in antiapoptotic and anti-inflammatory effects.³⁷⁻³⁹ The APC pathway is activated in sickle cell patients, most likely due to the prothrombotic state associated with SCD.^{12,40} Our results from BERK^{BM}, PAR-1^{-/-} mice suggest that endogenous APC:ECPR-PAR-1 signaling pathway does not play a major role in vascular inflammation in SCD. However, both administration of exogenous APC and genetic overexpression of the endothelial protein C receptor-protected sickle mice from cerebral microvascular thrombosis,¹² which warrants further studies to investigate both anticoagulant and possibly anti-inflammatory effects of exogenous APC administration.

Pulmonary hypertension (PH) is a common clinical complication of SCD, but the etiology has yet to be defined.^{41,42} Proposed mechanisms include vasoconstriction and endothelial dysfunction due to nitric oxide depletion^{40,41} or the hypercoagulable state and chronic inflammation.^{3,43-45} Indeed, SCD patients with PH have higher levels of coagulation and inflammation biomarkers compared with the sickle cell patients without PH.⁴⁶ There is an emerging concept that inflammation is an important proximal mediator in the generation of PH in the general population.⁴⁷ Recent studies demonstrated that rivaroxaban treatment, as well as PAR-2 or IL-6 deficiency, attenuates PH in animal models.⁴⁸⁻⁵⁰ Also, there is at least 1 case report in which PH

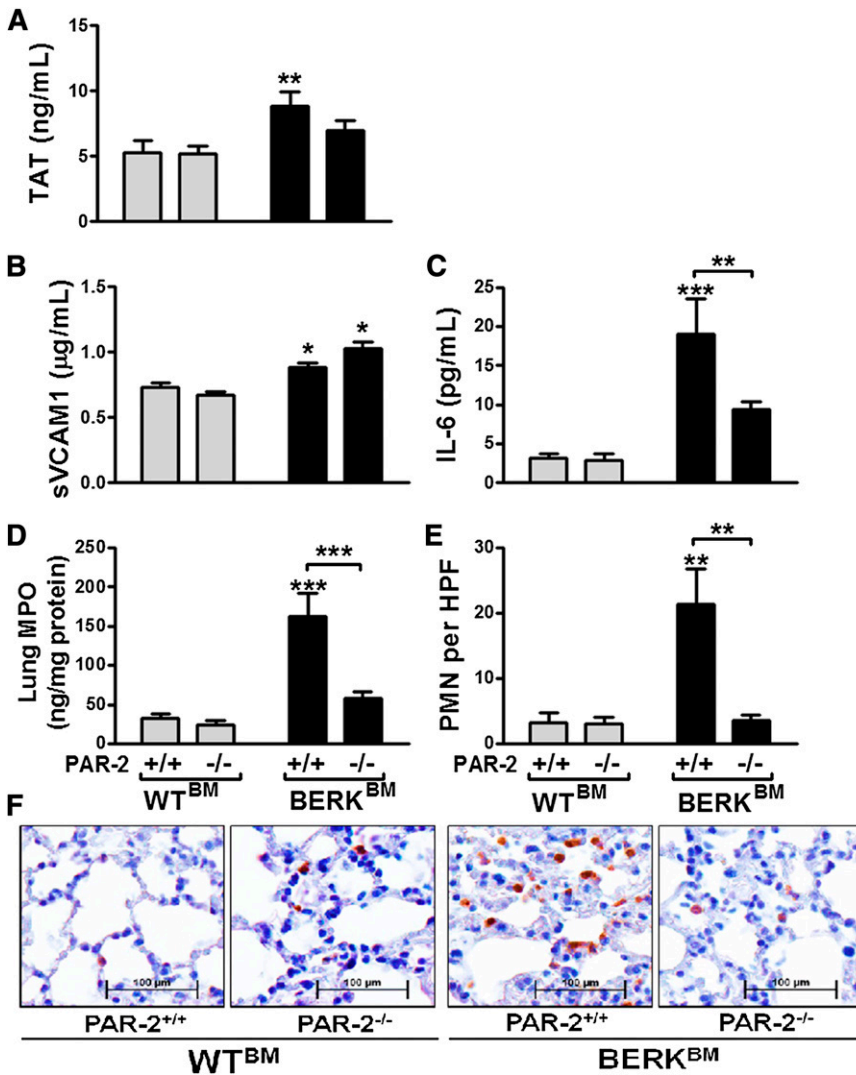


Figure 5. Role of PAR-2 in vascular inflammation in SCD. (A-E) Plasma levels of TAT (A), sVCAM-1 (B), IL-6 (C), and MPO (D) and average number of neutrophils (E) in the lung of PAR-2^{+/+} or PAR-2^{-/-} mice transplanted with bone marrow from WT (n = 8-9) or BERK (n = 8-12) mice. (F) Representative lung sections demonstrating neutrophil infiltration in four different groups of mice. Neutrophils stain as brown. Asterisks directly above the bars indicate statistically significant difference between BERK^{BM} mice compared with WT^{BM} mice within the same PAR-2 genotype (**P* < .05, ***P* < .01, and ****P* < .001).

improved in a patient treated with an IL-6 receptor-blocking antibody.⁵¹ Therefore, our data from PAR-2-deficient mice and rivaroxaban experiments imply that targeting FXa-mediated PAR-2 activation may reduce IL-6 expression and potentially attenuate PH in SCD.

ECs are chronically activated in SCD, in part due to increased adherence of sickle RBCs and activated leukocytes, as well as high levels of cell-free hemoglobin and heme in the blood.⁴⁰ We

previously found that inhibition of TF with a rat monoclonal antibody called 1H1 attenuates EC activation in sickle cell mice, as measured by plasma levels of sVCAM-1¹¹; however, deletion of TF from ECs¹¹ or low levels of TF in all nonhematopoietic cells (data not shown) had no effect on plasma levels of sVCAM-1. In the present study, neither FXa nor thrombin inhibition had any effect on this marker of EC activation in BERK^{SS} mice. Moreover, nonhematopoietic PAR-1 and PAR-2 also did not affect plasma

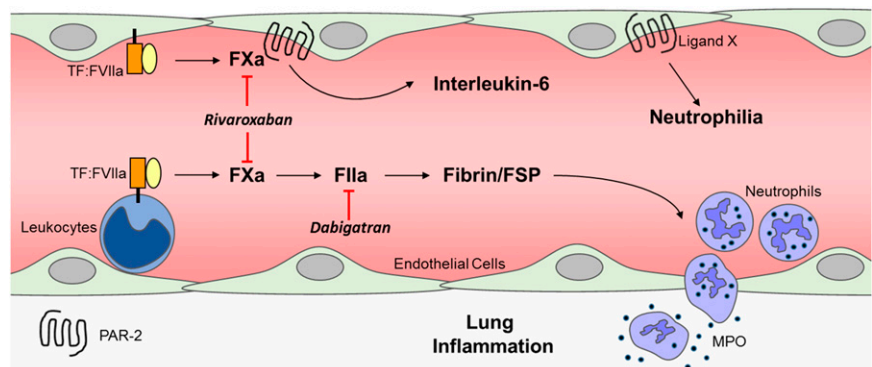


Figure 6. Proposed role of FXa and thrombin in vascular inflammation in SCD. FSP, fibrin split product.

levels of sVCAM-1 in sickle cell mice. These data suggest that the TF-dependent increase in plasma levels of sVCAM-1 is mediated by TF:FVIIa signaling in hematopoietic cells, which then indirectly results in EC activation. An alternative hypothesis is that alternatively spliced TF (asTF), a truncated, soluble form of TF, might mediate EC activation.⁵² It was recently demonstrated that interaction between human asTF and β 1 integrin increases the expression of adhesion molecules, including VCAM-1, on microvascular ECs and modulates monocyte-EC interactions.⁵³ However, future studies will have to determine if 1H1 antibody can disrupt asTF- β 1 integrin interactions and the possible contribution of asTF to vascular inflammation in SCD.

SCD is one of a few pathological conditions in which TF expression is upregulated not only on leukocytes but also on ECs. Our data indicate that TF expressed by different cell types has specific roles in the pathology of the disease (Figure 6). We propose that EC TF does not contribute to the activation of coagulation in SCD but instead contributes to systemic inflammation mediated by FXa-dependent activation of PAR-2 on ECs. The fact that deletion of TF from ECs¹¹ or low levels of TF in all nonhematopoietic cells (data not shown) does not affect coagulation activation in sickle cell mice suggests that hematopoietic cells are the major source of TF that is responsible for the activation of coagulation. Indeed, increased TF expression by monocytes strongly correlates with markers of coagulation activation in sickle cell patients.⁵⁴

In summary, we have demonstrated that FXa and thrombin differentially contribute to the vascular inflammation in a mouse model of SCD. We identified PAR-2 as a potential target to inhibit vascular pathology associated with SCD. We also demonstrated that the recently developed oral anticoagulants dabigatran and rivaroxaban might be useful in treating sickle cell patients by reducing

thrombotic complications as well as reducing both systemic and local inflammatory responses.

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Authorship

Contribution: E.M.S. and P.C. designed the experiments, performed the experiments, and interpreted the data; N.M., D.M.M., and N.S.K. aided in data interpretation and editing the manuscript; R.P.H. and J.v.R. provided valuable reagents and critically read the manuscript; D.M.M. designed and J.M. performed the hemostasis experiment; and R.P. designed the experiments, interpreted the data, wrote the manuscript, and contributed to the overall design of the study.

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