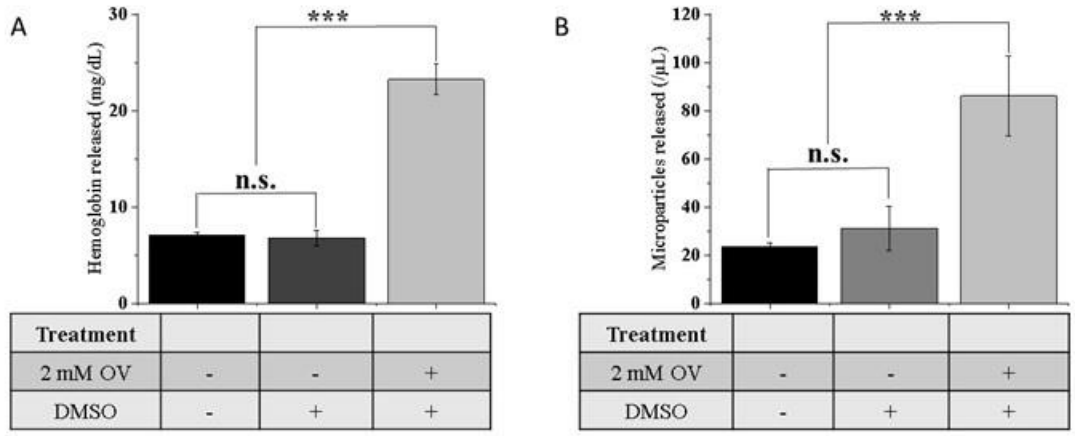
SUPPLEMENTAL INFORMATION

Quantitation of Band 3 Tyrosine Phosphorylation

Washed RBCs were suspended at 30% hematocrit (Hct.) in PBS-G and treated with either 5 μM drug (imatinib, PRT062607 or R406) or vehicle (control) for 4 hours at 37°C under 50 rpm shaking. RBC ghosts were prepared by mixing 150 μL packed RBCs with 1500 μL ice-cold ghost buffer (5 mM Na_2HPO_4, 1 mM EDTA, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride and 1% phosphatase cocktail inhibitors 2 and 3) and incubated on ice for 30 min. Samples were centrifuged at 13000 rpm for 15 min at 4 °C and supernatants were removed. Ghost pellets were washed an additional 3x, after which 10 μL was removed for determination of protein concentration using Pierce™ Rapid Gold BCA Protein Assay (Thermo Fisher Scientific, A53226). Residual ghosts were solubilized in 4x Laemmli buffer (Bio-Rad) at a concentration of 1μg protein/μL buffer and warmed to 45 °C for 15 min prior to storage at -20 °C until use.

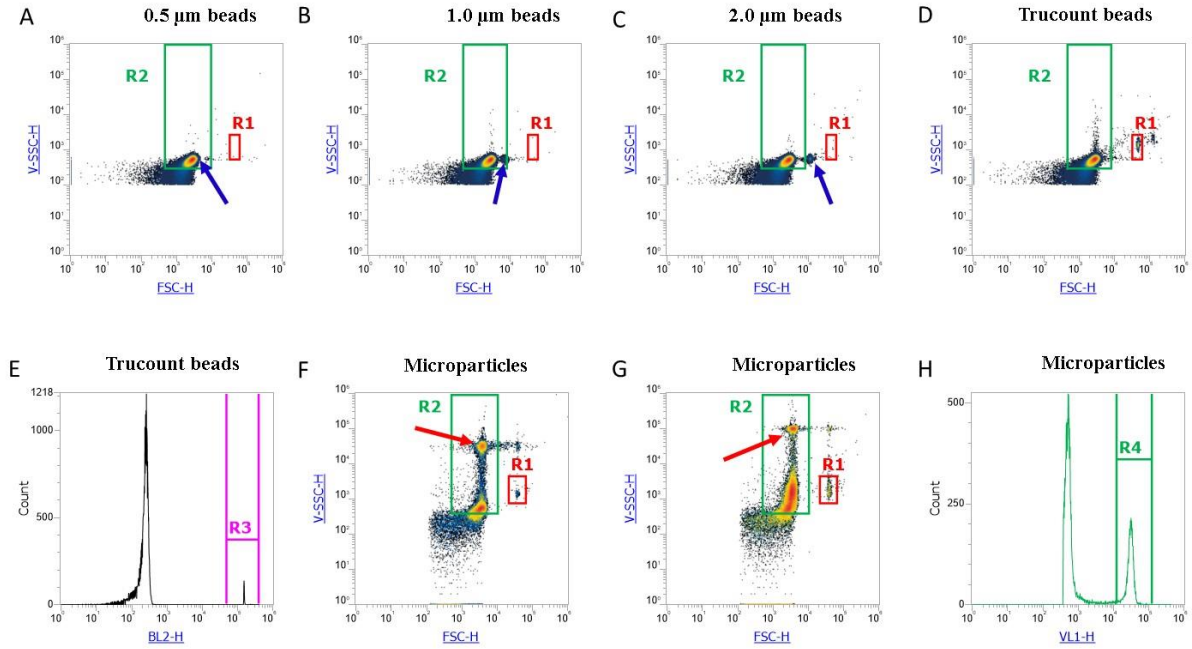
Band 3 tyrosine phosphorylation was quantitated by loading sample onto a 10% SDS-PAGE gel and separating at 90, 120 and 150 V for 30, 45 and 20 minutes, respectively. Proteins were transferred to a nitrocellulose membrane at 100 V for 2 hours, after which membranes were immunostained with anti-phosphotyrosine (1:1000, Cell Signaling Inc., #9411S) or anti-actin (1:20000; Sigma Aldrich #A2103) antibodies dissolved in TBST (25 mM Tris, 140 mM NaCl, 3 mM KCl, 0·05% v/v Tween-20) containing 5% w/v milk. After washing, membranes were incubated in TBST containing anti-mouse-horseradish peroxidase or anti-rabbit-horseradish peroxidase antibody conjugates, then washed 4x in TBST, and incubated with SuperSignal™ West Pico chemiluminescent substrate (Thermo Scientific™) prior to imaging with a Chemidoc XRS+ (BioRad). Band 3 tyrosine phosphorylation intensity was quantitated using image J software.

To induce Band 3 tyrosine phosphorylation and membrane fragmentation in healthy erythrocytes, blood from healthy donors was washed and cells were suspended at 30% Hct in PBS-G prior to incubation with imatinib or dimethyl sulfoxide (DMSO) at 37 °C for 1 h. After addition of 2 mM sodium orthovanadate, cells were incubated for 4 h at 37 °C while shaking at 1400 rpm.To minimize impact of vehicle (DMSO) on the cells, the volume of DMSO added to the cells was kept at ≤0.5% of the total test volume. Figure 1 below illustrates that DMSO doesn't impact hemolysis (Panel A) or microparticle formation (Panel B)

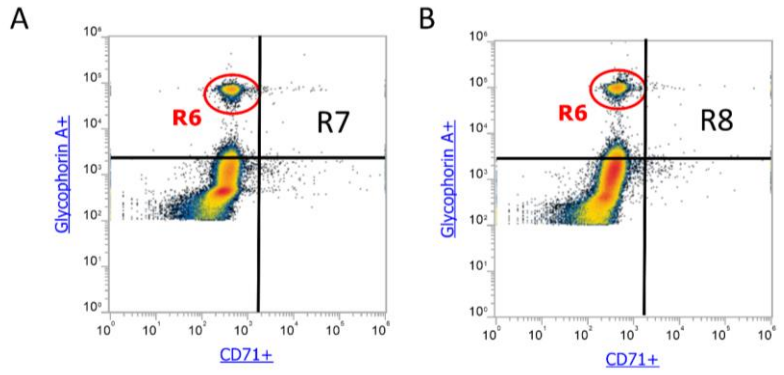


Supplemental Figure 1: Effect of DMSO on release of hemoglobin;Hb (Panel A) and microparticles; MPs. (Panel B).Erythrocytes suspended at 30% hct were treated with0.5% v/v of DMSO and/or Band 3 tyrosine phosphorylation stimulant (sodium orthovanadate; OV) prior to analysis of the amount of Hb and MPs. released.

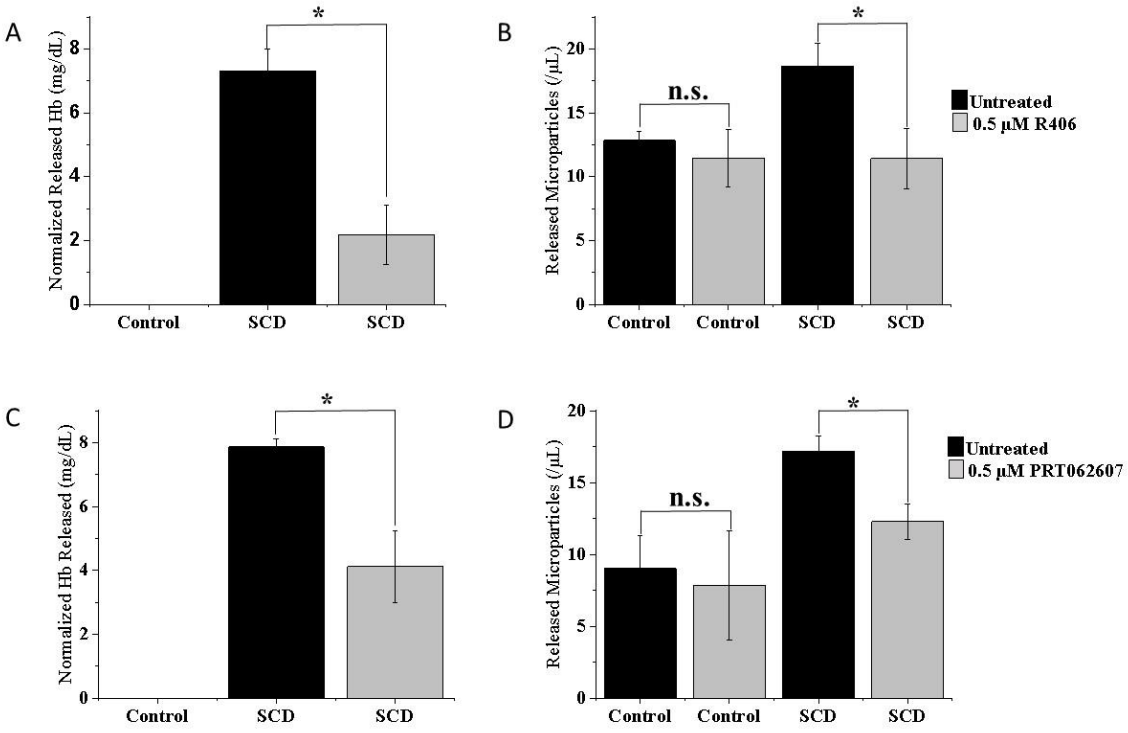
Microparticle quantitation



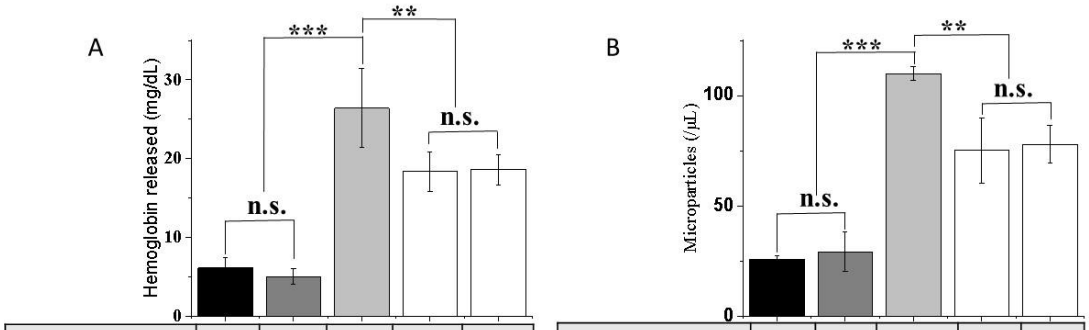
Supplemental Figure 2. Quantitation of erythrocyte-derived microparticles by Attune NxT Flow Cytometer. Microparticles were identified as glycophorin A positive particles in the size range of 0.1-1.0 µm. The size range was determined using flow cytometry sub-micron particle size reference beads (Thermo Fisher # F13839) as illustrated in panels A, B and C for 0.5 µm, 1.0 µm and 2.0 µm diameter beads respectively, on V-SSC-H (violet side scatter; for better small-particle resolution) vs FSC-H (forward scatter) dot plot. Gate R2 (green) was set as the region for microparticles/events smaller than 1.0 µm diameter. Blue arrows in panels A-C indicate the positions of 0.5 µm, 1.0 µm and 2.0 µm diameter beads respectively, while panel D shows Trucount counting beads (~4.2 µm diameter) which are gated in R1. As can be seen in panels C and D, 2.0 µm beads falls outside gate R2 as do the Trucount beads. Trucount beads were detected in the blue fluorescence detector (488 nm) as shown in panel E, gated R3. Panel F and G show glycophorin A positive erythrocyte-derived microparticles (red arrows) in the plasma from healthy and sickle cell patients, respectively (gate R2). Panel H represents events within gate R2 where glycophorin A positive events (erythrocyte-derived microparticles) are gated in R4 (which constitute the number of erythrocyte-derived microparticles reported in this article).



Supplemental Figure 3: Identification of surface markers of erythroid derived Microparticles. Microparticles were stained with 0.5 µL of mouse anti-human glycophorin A antibody and 1.0 µL of CD71 antibody (BD Biosciences #12-0711-82) and incubated for 20 minutes on ice prior to analysis with Attune NxT flow cytometer. Glycophorin A positive microparticles (circled R6) from healthy control (Panel A) and from a non-transfused SCD patients (panel B) turned out to be CD71 negative (quadrant R7 and R8).



Supplemental Figure 4: Inhibition of hemolysis and microparticle formation by well-established Syk inhibitors R406 (panels A and B) and PRT062607 (panels C and D). Washed sickle erythrocytes suspended in PBS-G at 30% Hct were incubated with 0.5 µM of either Syk inhibitor or an equivalent volume of vehicle (DMSO) at 37°C for 4 h under 1400 rpm shaking. (n=3; Error bars are expressed as standard error of the mean (SEM); * denotes p<0.05, and n.s. denotes not statistically significant)



Treatment					
DMSO	+	+	+	-	-
2 mM OV	-	-	+	+	+
R406 0.5μM	-	-	-	+	-
PRT062607 0.5μM	-	-	-	-	+

Treatment					
DMSO	+	+	+	-	-
2 mM OV	-	-	+	+	+
R406 0.5μM	-	-	-	+	-
PRT062607 0.5μM	-	-	-	-	+

Supplemental Figure 5: Effect of treatment of sickle cells with Syk inhibitors on inhibition of: A) release of free Hb, and B) discharge of erythrocyte-derived microparticles. Both Syk inhibitors significantly reduced the amount of Hb and MPs. released following treatment with OV suggesting that Syk is likely the predominant tyrosine kinase that phosphorylates Band 3. (n=3; Error bars are expressed as standard deviation; * denotes p<0.001, ** denotes p<0.01 and n.s. denotes not statistically significant).**

RBC Exit Velocity Methods

Microfluidic capillary devices were fabricated with polydimethylsiloxane (PDMS) using a standard soft photolithographic technique. Capillaries were constructed with a straight channel (w = 75 μm, h = 6.5 μm) containing a constriction (w_c = 5 μm and h = 6.5 μm) in the middle. To control oxygen level inside the channel, the microfluidic device was submerged in a customized glass chamber filled with sodium sulfite solution (0M, 0·01M, 0.1M or 1M) (Sigma-Aldrich). The oxygen level was calibrated using an O_2 indicator (tris(2,2'-bipyridyl) dichlororuthenium(II) hexahydrate (Sigma-Aldrich)), and fluorescence intensity was converted into a pO_2 value using the Stern-Volmer equation, $I^0 / I = 1 + pO_2 \times K_q$, where I^0 is the maximum fluorescence intensity and K_q is the quenching constant.

Sickle cell blood samples were obtained from five patients (n = 5, age 4-9 years old) and RBCs were isolated by washing three times at 500 rcf for 1.5 min in PBS-G and the resuspended to 1% v/v for microfluidic measurements. The RBC suspension was injected into the microfluidic capillary at a constant pressure of 1.6 psi and the velocity of RBCs in the constriction was recorded using a high-speed video camera (Phantom Miro M120, 1900 frames per second) mounted on an inverted microscope (Leica DMI 6000B). Data were analyzed using Phantom Camera Control software. For quantitation of RBC velocity in the presence or absence of imatinib, 10 μL of 0.5 mM imatinib dissolved in DMSO was added to 30 μL packed RBCs in the presence of 960 μL PBS-G. RBC suspension was then incubated for 4 hours at 37˚C and used for RBC capillary velocity measurements.

Microfluidic Endothelial Adhesion Study Methods:

Sample collection

Venous blood samples from 10 subjects with homozygous SCD (HbSS) were obtained from University Hospitals Seidman Cancer Center's Adult Sickle Cell Disease Clinic with standard laboratory procedures approved by the IRB. Upon collection, the blood samples were drawn into vacutainer tubes with anticoagulant ethylenediaminetetraacetic acid (EDTA), and were stored at 4°C. All the experiments were performed within 48 hours of blood collection.

Endothelialization of microfluidic channels

Microfluidic channels were fabricated using a lamination technique, as described in our previous studies.(Alapan *et al*, 2016) Briefly, a top polymethyl methacrylate (PMMA) cap and a double-sided adhesive (DSA) film were laser micro-machined and assembled with a bottom microscope glass slide (Gold Seal, coated with APTES, 3-Aminopropyl Triethoxysilane, Electron Microscopy Sciences, Hatfield, PA), forming a uniform flow domain (Fig. 1A). 25 mg of 4-Maleimidobutyric acid N-hydroxysuccinimide ester (GMBS, ThermoFisher Scientific, Waltham, MA) was dissolved in 0.25 mL of dimethyl sulfoxide (DMSO), and was diluted with pure ethanol to at 0.28 % v/v to obtain the GMBS working solution. The assembled microchannels were rinsed with PBS and pure ethanol, and incubated with GMBS for 15 minutes at room temperature. Thereafter, the microchannels were rinsed again with pure ethanol and PBS before loading the microchannels with a fibronectin (FN) solution at a concentration of 0.2 mg/mL. FN-loaded microchannels were incubated at 37ºC for 1 hour to complete FN-immobilization in the microchannels. To endothelialize the microchannels, HUVECs were seeded into FN-immobilized microchannels at an initial density of 1×10^6 cells/mL and cultured under flow with a flow rate of 100 µL/min at 37ºC and 5% CO_2 for 48-72 hours until a sufficiently confluent monolayer over the surface was achieved, based on our earlier work.(Kucukal *et al*, 2018)

Heme activation of endothelial cells

Prior to the flow adhesion assay, immobilized HUVECs were incubated with 40 µM heme-containing basal medium (Lifeline, Frederick, MD) for 2 hours in 37ºC. The control microchannels were loaded with heme-free basal medium and incubated under the same conditions. Heme stock solution was prepared by dissolving bovine hemin in 0.5 M NaOH solution to obtain a final heme concentration of 20 mM. Next, the stock solution was mixed with 0.5 M HCl solution to adjust pH and further diluted with basal medium to obtain a final concentration of 40 µM.

Imatinib Treatment of sickle RBCs

Imatinib (Sigma-Aldrich, St. Louis, MO) was dissolved into DMSO to obtain a stock solution with the concentration of 2.5 mg/mL and stored at 4ºC. Whole blood samples from subjects with HbSS were centrifuged at 500g for 5 minutes under room temperature to isolate RBCs. Isolated RBCs were washed in PBS for 2 times and re-suspended in PBS-G (PBS containing 5.5 mM glucose). Thereafter, RBC samples were treated with either 5 µM imatinib or vehicle (DMSO) as Control at 30% hematocrit for 4 hours at 37°C under 50 rpm shaking prior to the flow adhesion assay.

Fabrication of the micro-gas exchanger

To assess sickle RBC adhesion to heme-activated endothelium under physiological hypoxia, we fabricated a micro-gas exchanger to facilitate blood deoxygenation. Briefly, the micro-gas exchanger is composed of a gas permeable inner tubing (Silastic Silicone Laboratory Tubing, Dow Corning, Auburn, MI) placed within a gas impermeable outer tubing (Cole-Parmer, Vernon Hills, IL). The blood flow exchanged oxygen through the permeable tubing wall with 5% CO_2 and 95% N_2-controlled gas inside the impermeable tubing by diffusion (Fig. 1B). Simulation results in our previous study showed that the gas exchange results in an SpO_2 of 83%.(Kim *et al*, 2017)

Flow adhesion assay under physiological hypoxia

Treated RBCs were isolated and re-suspended in basal medium supplemented with 10 mM HEPES containing 5 µM imatinib or vehicle (DMSO as Control) at the same hematocrit. A total sample volume of 15 µL was perfused through the microchannel using a syringe pump (NE300, New Era Pump Systems, Farmingdale, NY) at a shear

stress of 1 dyne/cm^2, corresponding to a typical value observed in human post-capillary venules. Non-adherent RBCs were washed of by injecting medium at 1 dyne/cm^2.

Image processing and quantification

Bright field images of microfluidic channels were obtained using an Olympus long working distance objective lens (20×/0.45 ph2) and a commercial software (CellSense Dimension, Olympus Life Science Solutions, Center Valley, PA). Microchannel images were processed using Adobe Photoshop software (San Jose, CA). An area of 32 mm^2 at the center of the microfluidic channel was selected to quantify cell adhesion events.

Statistical analysis

Data acquired in this adhesion assay study were reported as mean ± standard error of the mean (SEM). All statistical analyses were carried out using Minitab 19 Software (Minitab Inc., State College, PA). Data were initially analyzed for normality, which was followed by paired t test. Statistical significance was set at 95% confidence level for all tests ($p<0.05$).

References

Alapan, Y., Kim, C., Adhikari, A., Gray, K.E., Gurkan-Cavusoglu, E., Little, J.A. & Gurkan, U.A. (2016) Sickle cell disease biochip: a functional red blood cell adhesion assay for monitoring sickle cell disease. *Translational research : the journal of laboratory and clinical medicine*, **173,** 74-91.e8 Available at: https://www.ncbi.nlm.nih.gov/pubmed/27063958.

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