Online Supplementary Figures and Tables

For

Elevated phingosine-1-phosphate promotes sickling and sickle cell disease progression

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Running Title: Sphingosine-1-phosphate in sickle cell disease

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Supplementary methods

Mature erythrocytes and reticulocytes isolation from SCD mice. Purification of mature erythrocytes and young reticulocytes was performed as described[1]. First, 100% percoll was made by mixing 90ml of percoll plus (GE Healthcare) with 10 mL 1.5M sodium chloride. Then, 70% or 75% percoll was prepared by dilution of 100% percoll with different amount of 0.15M sodium chloride. To make a percoll gradient, 4mL of 70% percoll was very gently layered on the top of 75% percoll in a 15 mL-conical centrifuge tube. 200 µL of mouse EDTA anti-coagulated blood was loaded on the top of 70% percoll. The sample was centrifuged at 1000 ×g for 20 minutes at room temperature in a centrifuge with swing tube holder. After centrifugation, the top layer (platelets and white blood cells) was removed and the middle layer (reticulocytes and granulocytes) and bottom layer (mature erythrocytes) were collected into different 1.5-mL tubes and washed twice by PBS. The reticulocyte fraction was resuspended in 0.5mL PBS with addition of 20 µg anti-granulocyte antibody (BD Pharmingen) and incubated at room temperature for 30 minutes. The cell suspension was washed once by PBS, followed by addition of anti-rat IgG-magnetic beads and incubation at room temperature for 30 minutes. Antibody-bound cells (granulocytes) were pulled down by magnetic bar (Biorad). The supernatant (reticulocytes) was centrifuged at 1000 ×g for 5 minutes at room temperature. Cell pellets were washed twice with PBS, frozen in liquid nitrogen and stored at -80 ^{0}C for Sphk1 activity assay.

Effects of SK1-I, PF-543, S1P and S1P receptor antagonists on hypoxia-induced sickling in cultured human and mouse sickle erythrocytes, and SPHK1 activity measurement. Human and mouse blood collected with heparin as an anti-coagulant was centrifuged at 240 xg for 10 minutes at room temperature, followed by aspiration of plasma and white interface. Cells were washed and resuspended in culture media (F-10 Ham's with 1% penicillin/streptomycin) to 4% hematocrit (HCT) and 1 mL of cells was placed into each well of a 12-well plate. The cells were exposed to 4% O_2 concentration for 4 hours with shaking at 37 ^{o}C to induce sickling for Sphk1 inhibitor experiments. Some of the cells were treated with SK1-I or PF-543[2] at different concentrations. SK1-I was dissolved in saline, thus saline treatment was used as a control for SK1-1. Because PF-543 was dissolved in DMSO, DMSO was used as a control for PF-543 treatment. Moreover, some of the cells were exposed to different O_2 tension ranging from 5 mmHg to 40 mmHg to determine the relationship of hypoxia to sickling with or without drug treatment. For S1P induced sickling, erythrocytes were treated with S1P (100-500nM) or S1P receptor antagonists (0.5 and 5µM respectively) (JET 013: $S1P_2$ receptor antagonist, VPC 23019: $S1P_1$ and $S1P_3$ receptor antagonist from R &D Systems) under different oxygen pressure for 4 hours at 37^{0}C with shaking. At the end of experiment, cells were fixed by 1% glutaraldehyde in PBS for 30 minutes. Blood smears were prepared using 5 µl of fixed cell suspension. The rest of cells were

centrifuged for 5 minutes at 1000 xg. The cell pellets were collected and stored at -80 ^{o}C for S1P measurements and SPHK1 activity assay (Echelon Bioscience). Supernatant was collected for hemolysis analysis by measuring Hb concentration (BioAssay Systems, Hayword, CA).

Morphology study of erythrocytes including sickle cell and reticulocyte count. Blood smears were made using 1% glutaradehyde fixed cultured human or tail blood from bone marrow transplanted mice. Blood smears were stained by WG16-500ml kit (Sigma-Aldrich) for sickle cell. Blood smears stained by these procedures were observed using the 100x oil immersion objective of an Olympus BX60 microscope. Areas where red blood cells do not overlap were randomly picked, at least 10 fields were observed and 1000 red blood cells including sickle cells were counted. The percentages of sickle cells in red blood cells were calculated. Reticulocytes was labeled by Retic-COUNT Reagent (Beckton Dickinson) and quantified by flow cytometry[3].

Hemolytic analysis. The hemoglobin, haptoglobin and total bilirubin in mouse plasma were quantified by ELISA kits following instructions provided by the vendor (BioAssay Systems, Hayword, CA).

Survival Rates of SCD mice in response to hypoxic condition. SCD chimeras with SphK1 specific knockdown and the controls or SCD mice with or without PF-543 treatment were exposed to 8% oxygen after 4 weeks PF-543 treatment or 12-16 week following bone marrow transplantation. After 360 minutes of hypoxia (8% oxygen), the survival rates were estimated by a percentage of survived mice.

ELISA for measurement of multiple cytokines in the mouse lung homogenates and circulation Multiple pro-inflammatory cytokine levels in mouse lung homogenates and circulation were determined using ELISA kits[4] (QIAGEN).

Measurement of life span of erythrocytes in SCD Tg mice. Erythrocytes were labeled *in vivo by* using N-hydroxysuccinimide (NHS) biotin and the life span of circulating red blood cells was measured as described[3]. Specifically, 50 mg/kg of NHS biotin was injected into the retro-orbital plexus of SCD mice (prepared in 100 μL sterile saline just prior to injection; initially dissolved at 50 mg/mL in dimethyl sulfoxide. Blood samples (only 5 μL) were collected the first day after biotin-injection from tail vein by venipuncture to determine the percentage of erythrocytes labeled with biotin. Subsequently, 5 μl of blood were obtained by tail vein venipuncture on day 1, 2, 3, 5 and 8 for measurement of biotinylated erythrocytes. The percentage of biotinylated erythrocytes was calculated by determining the fraction of peripheral blood cells labeled with Ter-119 (to identify erythrocytes) that were also labeled with a streptavidin-conjugated fluorochrome by flow cytometry[4].

Mouse organ isolation and histological analysis. Mice were anesthetized and spleen, livers and kidneys were isolated. Half of each organ was quickly frozen in liquid nitrogen and then stored at -80 ^{0}C for later use in the assay for inflammatory cytokines. The remaining half of each organ was fixed with 10% paraformaldehyde in PBS overnight at 4 °C. Fixed tissues were rinsed in PBS, dehydrated through graded ethanol washes, and embedded in paraffin. 5 µm sections were collected on slides and stained with hematoxylin and eosin (H&E). The semi-quantitative analysis of histological changes was conducted as previously described using a computerized program[4, 5]. Ten digital images were taken from each H&E stained mouse tissue section at 20X magnification from different areas. The congestion, necrosis or cysts on sections were identified according to their structure and color. Briefly, the dark red color was chosen for quantification of congestion and it was performed on 10 fields/mouse tissue sections at 20 x magnification using software analysis (Image Pro Plus 4.0; Media Cybernetics, Bethesda, MD, USA). Additionally, the areas of necrosis in the livers and cysts in the renal cortex were first manually marked by a magical pen tool available in Adobe Photoshop Program. Then the quantification was conducted on 10 fields/mouse tissue sections at 20 x using software analysis (Image Pro Plus 4.0; Media Cybernetics, Bethesda, MD, USA). The whole areas of each image were considered as 100 %. The percentage of pathological areas to whole area of image was recorded.

Measurement of vascular permeability of lungs. Mice were injected intraperitoneally with 200 µL 0.5% Evans blue. Four hours later, the injected mice were anesthetized with Avertin, the neck opened and trachea exposed. The trachea was partially cut and a catheter inserted. The chest was opened and the heart was perfused with 10 mL saline. The lung was inflated by gentle infusion of PBS at a 25 mmHg H_2O pressure. After inflation, the trachea was tied, the lung was removed and photographed. The lung was stored at -20 ^{0}C for Evens blue measurement. The measurement was as previously described[6].

Measurement of albumin in bronchoalveolar lavage fluid. Mice were anesthetized by avertin and placed on plate with face up. A small incision in the mouse skin at the abdomen was made, the skin was cut to expose thoracic cage and neck. A small incision was made in the trachea and gauge lavage needle was inserted to trachea. The needle was stabilized by surgical suture. 1 mL PBS was drawn into the syringe and the syringe was connected to lavage needle. Lung was washed 3 times by gently pushing and pulling the barrel of syringe. The lavage fluid was injected to 1.5 mL tube and stored at -80 ^{0}C for albumin measurement which was done by using a kit from Exocell [6].

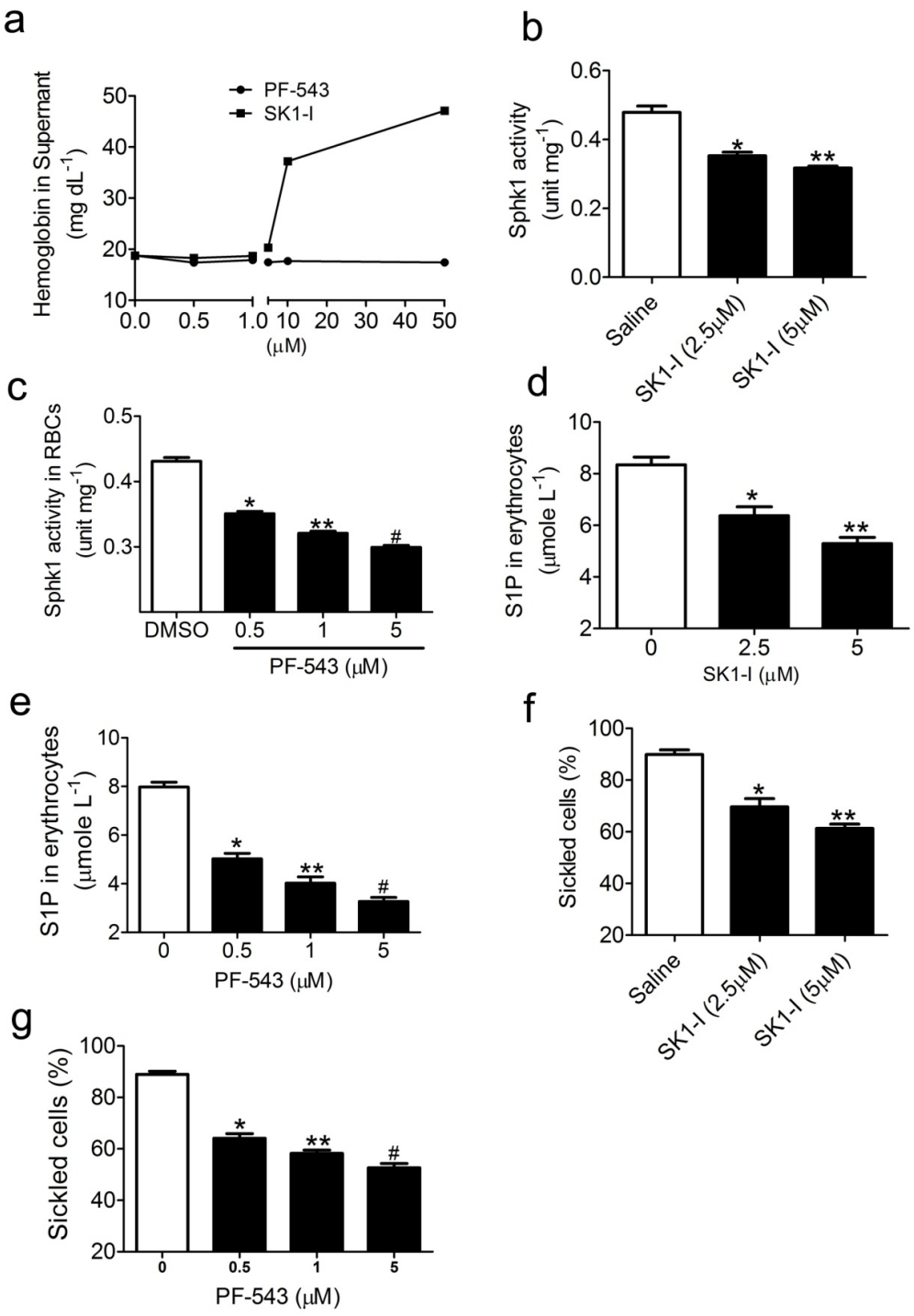
Analysis of hemoglobins by electrophoresis in SCD chimeras.

Hemoglobin preparation: one volume of red blood cells is mixed with nine volumes of 5mM EDTA. The sample was centrifuge at 10000 ×g for 5 minutes at 40 °C, the supernatant is collected and hemoglobin concentration measured (BioAssay System). 5 µg/10 µl of hemoglobin was mixed with 10 µl of 2X running buffer (1X running buffer: Tris (hydroxymethyl) aminomethane 2.51g, EDTA 0.325g, boric acid 0.192g in one liter of water pH 8.7) with 20 % glycerol. Hemoglobin A or S was separated by 5 % of acrylamide gel. The gel was stained with safestain (Invitrogen) for one hour at room temperature and destained with water[7].

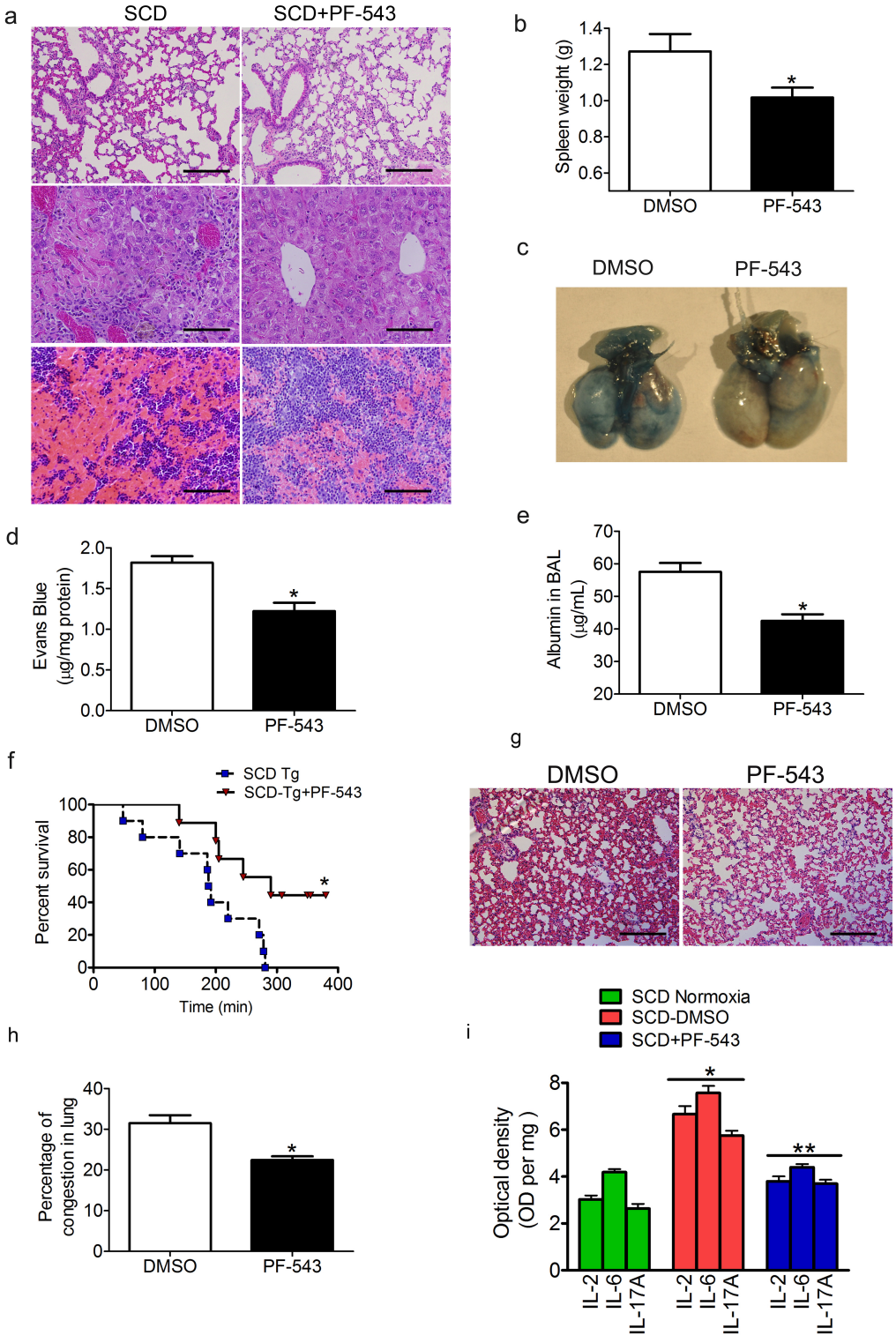
Analysis of hemoglobins by High-Performance Liquid Chromatography (HPLC) in SCD chimeras. Analysis of different hemoglobin variants was performed by HPLC using Agilent 1100 series HPLC system (Agilent Technologies, CA) and PolyCAT A^{TM} weak cation-exchange column (100 x 4.6-mm, 3 µM, 1500 A; catalog #104CT0315, PolyLC inc., Columbia, MD) as previously described[8]. ***Chemicals:*** Bis-Tris, potassium cyanide, and hemoglobin S and HbA were obtained from Sigma-Aldrich. ***Sample preparation***: Isolated red blood cells were frozen, fully lysed by addition of three volumes of water, vortexed, and centrifuged at 12,000 ×g for 2 min using Eppendorf bench-top centrifuge. A 20 µl sample of hemolysate was added to 180 µl of mobile phase B buffer, and from this stock, a 25 µl aliquot was injected into HPLC and monitored at 415 nm. Obtained peak areas were used for the quantitation of individual hemoglobin peaks. For determination of peak positions in HPLC profile, purified hemoglobin A and/or hemoglobin S were analyzed. ***HPLC assay:*** The chromatographic separation was achieved at 24 °C by a gradient elution of the following mobile phases: mobile phase A contained 40 mM Bis-Tris, 2 mM KCN, pH 6.5; mobile phase B contained 40mM Bis-Tris, 2 mM KCN, 0.2 M NaCl, pH 6.8. Using a flow rate of 1 ml/min, HPLC column was pre-incubated for 5 min with 18 % mobile phase B before sample application. Elution of sample was performed by increasing the mobile phase B to 45 % at 8 min, and to 100 % at 12 min, then decreasing it back to 18 % at 13 min. The column was ready for next sample after re-equilibrating with 18 % mobile phase B for 5 min.

Survival Rates of SCD mice in response to hypoxic condition.

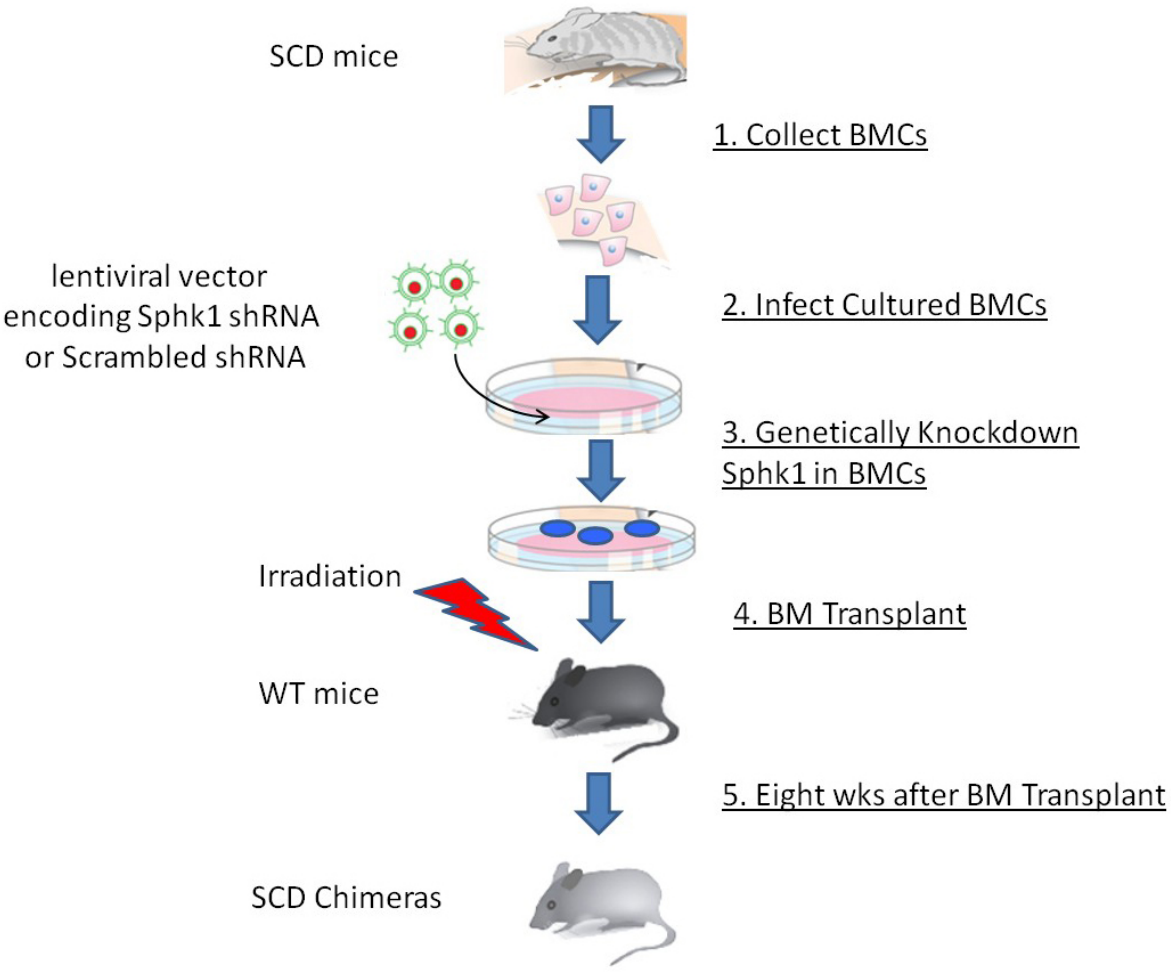
SCD chimeras with SphK1 specific knockdown and the controls or SCD mice with or without PF-543 treatment were exposed to 8% oxygen after 4 weeks PF-543 treatment or 12-16 week following bone marrow transplantation. After 360 minutes of hypoxia (8% oxygen), the survival rates were estimated by a percentage of survived mice.



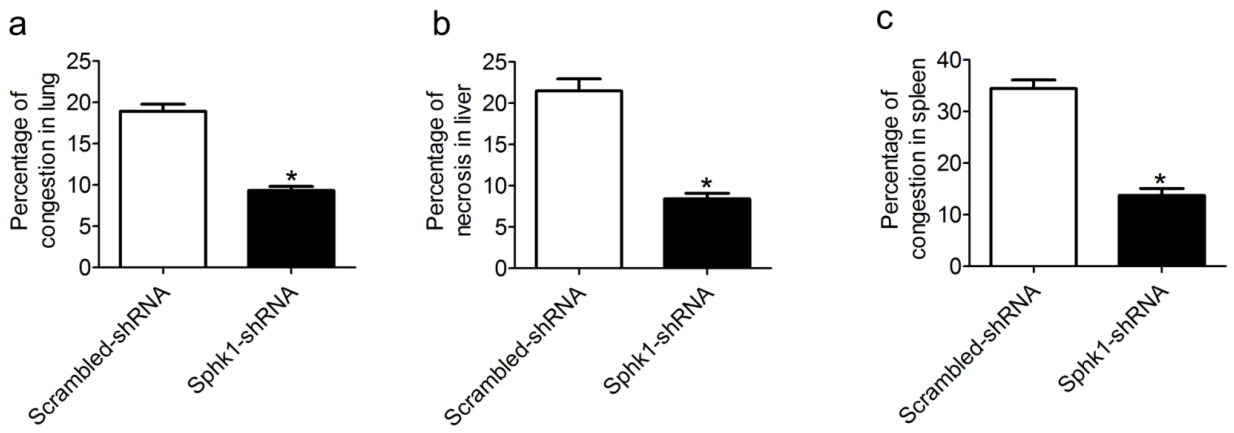
Supplementary Figure 1. Effects of SK1-I and PF-543 on SphK1 activity and sickling in cultured mouse SCD erythrocytes. (a) SK1-I induces hemolysis at 5μM, while PF-543 does not induce hemolysis even at 50μM in cultured mouse SCD erythrocytes under normoxic conditions. (b-c) SK1-I and PF-543 inhibit Sphk1 activity in a dosage-dependent manner in cultured mouse SCD erythrocytes under hypoxic conditions (4% oxygen). (d-e) SK1-I and PF-543 reduce S1P levels in cultured mouse SCD erythrocytes under hypoxic conditions. (f-g) SK1-I and PF-543 treatment reduces the percentage of sickled cells in a dosage-dependent manner in cultured mouse SCD erythrocytes under hypoxic conditions. Values shown represent the mean ± SEM. n=6. **P*<0.05 versus solvent treatment; ***P*<0.05 versus lower concentrations.



Supplementary Figure 2. The therapeutic effects of SphK1 inhibitor, PF-543, on SCD mice. (a-e) PF-543 Treatment decreased organ damage (original amplification, X20), splenomegaly, lung vascular leakage in SCD mice**.** (f-i). PF-543 treatment increased survival rate, decreased lung congestion (original amplification, X20) and pulmonary inflammatory cytokines under hypoxia (8% oxygen) in SCD mice. Data are presented as the mean ± SEM. n=6. **P*<0.05 versus solvent treatment or normoxia; ***P*<0.05 versus DMSO treatment. Scale bar: 200uM.



Supplementary Figure 3. Schematic drawing of protocol using lentiviral vectors encoding specific shRNA to knockdown SphK1 in bone marrow cells of SCD mice. This approach allows us to genetically, specifically and efficiently knockdown SphK1 in the hematopoietic stem cells (HSCs) of SCD mice.



Supplementary Figure 4. Semi-quantitative analysis of H&E stained sections showed SphK1 knockdown in hematopoietic stem cells (HSCs) attenuated organs injury. (a) SphK1 knockdown in HSCs decreased lung congestion in SCD chimeras. (b) SphK1 knockdown in HSCs decreased liver necrosis in SCD chimeras. (c) SphK1 knockdown in HSCs decreased spleen congestion in SCD chimeras. $^{*}P < 0.05$, versus SCD chimeras without SphK1 knockdown. n = 6-9.

Supplementary table 1. Hematological parameters of control and PF-543 treated SCD mice

	RBC (M/µl)	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)	WBC (k/µl)
SCD +DMSO (n=8)	4.66±0.71	5.63±0.97	21.93±2.85	51.02±7.10	12.48±1.27	24.95±1.34	33.09±3.28	22.99±6.31
SCD +PF-543 (n=6)	6.18±1.06*	7.85±0.97*	31.40±2.68*	51.47±6.48	12.32±1.45	25.31±1.13	27.40±2.15*	6.45±2.71*

SCD: sickle cell disease transgenic mice; RBC: red blood cells; Hb: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; WBC: white blood cell. * $P<0.05$ vs. SCD Tg mice without PF-543 treatment.

Supplementary table 2: **Hematological parameters of the SCD chimeras with or without prior SphK1 knockdown in donor bone marrow cells**

	RBC (M/µl)	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	RDW (%)	WBC (K/µl)
Scrambled-shRNA (n=8)	5.51±0.22	6.42±0.26	28.59±1.14	52.07±1.14	12.08±0.70	23.32±1.44	25.95±2.16	12.62±3.41
Sphk1-shRNA (n=9)	6.60±0.23*	9.36±0.38*	35.65±0.76*	54.26±1.46	14.14±0.35*	25.86±0.77	18.23±0.96*	4.97±1.12*

Scrambled-shRNA: wild type mice were transplanted with SCD bone marrow cells transduced with recombinant lentiviral vectors encoding scrambled shRNA sequence; Sphk1-shRNA: wild type mice were transplanted with SCD mouse bone marrow transduced with recombinant lentiviral vectors encoding shRNA specifically for Sphk1 mRNA.

RBC: red blood cells; Hb: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; WBC: white blood cells. * $P<0.05$ Sphk1-shRNA group comparing with Scrambled-shRNA group.

Supplementary Table 3: Clinical information for control individuals and SCD patients

	Control	SCD patients
Number	14	30
Gender	M=4, F=10	M=13, F=17
Age (years)	42.43±11.63	34.43±11.73
RBCs (10^6/μl)	4.65±0.44	2.85±0.85*
Hb (g/dl)	13.24±1.19	8.95±1.96*
HCT (%)	40.56±3.93	26.59±6.21*
WBC (10^3/μl)	5.59±1.47	10.52±3.80*
HbS	ND	74.61±16.69
HbA	ND	6.56±11.4
HbA2	ND	4.17±0.69
HbF	ND	12.75±12.11
Hydroxyurea	No	N=18

Control: Healthy volunteers; SCD: sickle cell disease patient; RBC: red blood cells; Hb: hemoglobin; HCT: hematocrit; WBC: white blood cell; HbS: hemoglobin S; HbA: hemoglobin A; HBA2: hemoglobin A2; HbF: hemoglobin F; ND: not tested; No: no treatment. *P≤0.05 vs. control. All subjects are of African-American decent.

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