Vascular TSP1-CD47 Signaling Promotes Sickle Cell-Associated Arterial Vasculopathy and Pulmonary Hypertension in Mice

Running head: TSP1-CD47 Promotes Pulmonary Hypertension in SCD

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Abstract

Pulmonary hypertension (PH) is a leading cause of death in sickle cell disease (SCD). Hemolysis and oxidative stress are contributing factors to SCD-associated PH. We have reported that the protein thrombospondin-1 (TSP1) is elevated in the plasma of patients with SCD and by interacting with its receptor CD47 limits vasodilation of distal pulmonary arteries *ex vivo*. We hypothesized that the TSP1-CD47 interaction may promote PH in SCD. We found that TSP1 and CD47 are upregulated in the lungs of BERK sickle mice and patients with SCD-associated PH. We then generated chimeric animals by transplanting BERK bone marrow into C57BL/6J (n=24) and CD47 knockout (CD47KO, n=27) mice. Fully engrafted Sickle-to-CD47KO chimeras had lower right ventricular (RV) pressures than Sickle-to-C57BL/6J chimeras as shown by the reduced maximum pressure of the RV (p=0.013) and mean pulmonary artery pressure (p=0.020). The afterload of the Sickle-to-CD47KO chimeras was also lower as shown by the diminished pulmonary vascular resistance (p=0.024) and RV effective arterial elastance (p=0.052). On myography, aortic segments from Sickle-to-CD47KO chimeras had improved relaxation to acetylcholine. We hypothesized that in SCD TSP1-CD47 signaling promotes PH, in part, by increasing ROS generation. Treatment with TSP1 stimulated ROS in human pulmonary artery endothelial cells, which was abrogated by CD47 blockade. Explanted lungs of CD47KO chimeras had less vascular congestion and oxidative footprint. Our results show that genetic absence of CD47 ameliorates SCD-associated PH, which may be due to decreased ROS levels. Modulating TSP1-CD47 may provide a new molecular approach to the treatment of SCD-associated PH.

Introduction

Sickle cell disease (SCD) is caused by the inheritance of a mutated hemoglobin (HbS) that polymerizes when sickle red blood cells (RBC) are exposed to hypoxic conditions in the microcirculation. HbS polymerization and brisk hemolysis are compounded by RBC hyper adhesion to the postcapillary venules, leading to end organ ischemia, necrosis and ischemia-reperfusion injury, and vasculopathy.(28, 29, 42, 71, 72) These processes lead to pathologic reactive oxygen species (ROS) production and subsequent oxidative tissue damage. In the pulmonary vasculature, ROS promote endothelial dysfunction and adhesion of sickle RBC.(77)

Chronic pulmonary vasculopathy and pulmonary hypertension (PH) are important pulmonary manifestations of SCD.(15, 75) PH, characterized by elevated pulmonary artery pressure and pulmonary vascular resistance, as determined by right heart catheterization, occurs in 6-10.5% of adult patients with SCD and is associated with high morbidity and mortality.(26, 60) ROS-mediated damage is an increasingly recognized factor in the vascular complications of SCD (for a review see Turhan(73) and Frenette(21)), and has been shown to be related to hemoglobin mediated ROS formation(30, 40) and activation of xanthine(2, 3, 77) and NADPH oxidases.(22) However, the proximate mechanisms for enhanced ROS formation remain to be fully determined.

The secreted matricellular and plasma protein thrombospondin-1 (TSP1) may promote vascular pathology by inhibiting the vasodilatory, anti-adhesive and homeostatic effects of the nitric oxide and vascular endothelial growth factor signaling pathways in the vasculature,(27, 34, 35, 38, 43) thereby adversely affecting tissue perfusion and vascular tone regulation and inciting inflammation. In the pro-inflammatory milieu of SCD, TSP1 is putatively generated by activated platelets and endothelial cells and promotes adhesion of sickle RBC to the endothelium.(10-13) *In vivo* studies in SAD mice, transgenic mice which express a modified sickle hemoglobin, Hb SAD [alpha 2 beta 2S(beta 6val)Antilles (beta 23 Ile)D- Punjab (beta 121Gln)], and display *in vivo* hemoglobin polymerization and erythrocyte sickling, suggested that TSP1 triggered

erythrocyte microparticle shedding induced endothelial injury and facilitated acute vaso-occlusive events.(14) Further, in patients with SCD, TSP1 plasma levels are elevated at baseline and still further in vaso-occlusive crisis, and high plasma TSP1 levels correlate with an increased risk of vaso-occlusive complications.(55, 56)

TSP1 stimulates increased superoxide anion ($O_2^{\bullet -}$) production in vascular endothelial and smooth muscle cells through its cell receptor CD47.(8, 19, 79) Indeed, new studies have found that TSP1, via CD47, limited nitric oxide-mediated vasodilation of distal pulmonary arteries from healthy and end-stage PH lungs.(66) The finding that lungs explanted from patients with PH,(8) including a patient with SCD-associated PH,(23, 68) had upregulated TSP1 and CD47 in pulmonary artery smooth muscle cells vessels and parenchyma, also supports the notion that TSP1 may play a pathogenic role in SCD-associated PH.(64) CD47 is also expressed in human and murine RBC, potentially contributing to RBC adhesion to the vascular wall.(59) The role of TSP1-CD47 in SCD has, however, never been established.

In the Berkeley (BERK) model of SCD, murine globins are replaced by human α and β^S globins.(61) BERK sickle mice mimic the genetic, hematologic and histopathologic features that are found in human subjects afflicted by SCD, as they display irreversibly sickled RBC, anemia, leukocytosis and systemic inflammation, high levels of pulmonary adhesion molecules and multi-organ pathology.(9, 44, 61) As they age, BERK mice also develop PH as measured by increases in pulmonary artery and right ventricular (RV) pressures and RV mass.(32)

In the present study, we addressed the hypothesis that increases in circulating plasma levels of TSP1, via binding to the CD47 receptor, stimulate pulmonary ROS production in BERK sickle mice and chimeric animals with sickle erythropoiesis. We further hypothesized that TSP1-dependent activation of CD47, in BERK mice and the chimeric animals and humans with SCD, contributes to vasculopathy and the evolution of PH. Finally, we tested the hypothesis that therapeutic disruption of the TSP1-CD47 ligand-receptor interaction will both prevent and reverse PH in BERK mice.

Methods

Human lung samples

Human lungs explanted from six patients with SCD-associated PH were obtained through IRB-approved protocols at the National Institutes of Health. Lungs explanted from six control patients without a history of lung disease were obtained through IRB-approved protocols at the University of Pennsylvania. Formalin-fixed, paraffin-embedded lung parenchyma was cut into 4-5 μM thickness sections, mounted onto microscope slides and shipped to the laboratories of the Heart, Lung and Blood Vascular Medicine Institute, Pittsburgh, PA for immunohistochemistry. Clinical and hemodynamic data on the patients with SCD-associated PH have been published.(49) In brief, the patients' age of death was 44.2 ± 9.9 years, two were female and their mean pulmonary artery pressure (mPAP) was 41 ± 9.8 mmHg.

Transgenic mice

CD47KO mice(46) and their background wild type strain C57BL/6J, and BERK mice expressing exclusively human sickle hemoglobin (Homozygous for Hbatm1Paz, Homozygous for Hbbtm1Tow, Hemizygous for Tg(HBA-HBBs)41Paz, Sickle) and their non-sickling hemizygous littermates (Homozygous for Hbatm1Paz, Heterozygous for Hbbtm1Tow, Hemizygous for Tg(HBA-HBBs)41Paz, Hemi),(61) were purchased from Jackson Laboratories, Bar Harbor, ME. All animal experiments were performed under a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee and complying with the federal Animal Welfare Act and all NIH policies regarding vertebrate animals in research. Mice were pathogen free and received routine rodent chow and water unless specified.

Generation of chimeric mice by bone marrow transplantation

Bone marrow was harvested from flushed femurs and tibias of adult Sickle mice as described.(54, 63) Whole bone marrow (5 X 10^6 cells) were transplanted into lethally

myeloablated (10 Gy) two months old CD47KO and C57BL/6J mice, the background strain of CD47KO mice, by retro-orbital sinus injection. Recipients received recombinant human darbepoetin alfa (Aranesp®, a generous gift by Amgen, Inc. Thousand Oaks, CA) on the day prior to transplantation to promote donor erythropoiesis, and neomycin supplemented water and autoclaved chow for 2 weeks following transplantation. Engraftment was assessed by measurement of HbS percentage by high-performance liquid chromatography (Primus Ultra2, Trinity Biotech, Kansas City, MO) followed by confirmatory capillary zone electrophoresis (Sebia, Évry, France) at the Quest Diagnostics™ laboratories, Chantilly, VA in blood samples obtained at the time of euthanasia, i.e. 4-6 months after transplantation.

Open chest cardiac catheterization

Open chest cardiac catheterization of mice under general anesthesia with isoflurane and tracheal intubation for mechanical ventilation was performed prior to euthanasia. The animals were weighed and placed in an anesthesia chamber supplied with 5% isoflurane. Upon induction of anesthesia the animals were then placed on a warming pad and restrained. A nose cone that delivered 2.5% isoflurane was placed over the muzzle. A rectal probe was then placed to monitor body core temperature. A tracheotomy was performed by inserting a 20-gauge intravenous catheter into the trachea and securing it with a length of suture. The anesthesia cone was then removed and the animals were placed on a ventilator set to 150-175 breaths per minute (weight-dependent), and a stroke tidal volume of 200-250ul. A thoracotomy was then performed and the rib cage was retracted to expose the inferior vena cava, the heart and the great vessels. A length of silk suture was loosely placed around the inferior vena cava. A 27-gauge needle was used to pierce the right ventricle (RV). The needle was then removed and a 1.2 French catheter (Scisense Inc., London, ON) was then introduced to the apical region of the RV. The catheter was adjusted as needed until the optimal pressure-volume loop was obtained. The ventilator was turned off and the heart's return blood flow was occluded by gently pulling

159 the suture around the inferior vena cava. Upon collection of the RV occlusion data via pressure-
160 volume loop data-acquisition software (EMKA Technologies, Paris, France), the ventilator was
161 restarted. With the catheter remaining in the RV, Doppler sound readings were collected by first
162 capturing the Doppler image of the pulmonary artery alone and then capturing the image with
163 the catheter advanced into the pulmonary artery. Mice were euthanized by left ventricle terminal
164 blood collection immediately following cardiac catheterization.
165
166 *Organ explant*
167 Following hemodynamic measurements and blood collection via intracardiac puncture,
168 the cardiovascular bed of the mice was flushed with cold normal saline. The left lung was then
169 extracted, snap-frozen in liquid nitrogen and stored at -80°C. A solution of 2% paraformaldehyde
170 was injected into the trachea at a pressure of 27 cm H_2O to inflate the right lung. The right
171 pulmonary hilum was subsequently suture ligated and the lung extracted and placed in 2%
172 paraformaldehyde. The thoracic aorta of mice whose left lung did not undergo fixation was
173 gently cleared of adherent adipose tissue, excised and immediately tested by arterial
174 myography.
175
176 *In vitro arterial myography assays*
177 To assess the arterial responsiveness of the chimeras, we used an *in vitro* dual wire
178 myograph system as described.(7, 78) We elected to test aortas rather than pulmonary arteries
179 due to technical limitations with pulmonary artery sampling and the need to confirm a systemic
180 vascular effect of TSP1-CD47.
181 In brief, murine aortic rings (2 mm in length) were mounted on a dual wire myograph
182 system (Multiple Myograph Model 610 M, DMT, Denmark). Dose-response curves to
183 phenylephrine (PE, Sigma-Aldrich; 10^{-9}-10^{-5} M) were generated for each vascular preparation
184 followed by a single dose of acetylcholine (ACh, Sigma-Aldrich; 10^{-6} M) to confirm endothelial

activity. Ring segments were then treated with a log dose curve of ACh (10^{-9}-10^{-5} M) to test endothelial activation response.

Cell treatment and ROS measurement

Primary human pulmonary artery endothelial cells (hPAEC; Lonza #CC2530, lot# 4F3033: 51yo Caucasian male; flow cytometry confirmation of cell line phenotype, Walkersville, MD) were grown in EBM-2 media containing EGM-2 bullet kit components (Lonza). Cells (passages 3-6) were seeded the day before experimentation and synchronized in serum-reduced media (1% FCS) for 4 hours. Synchronized endothelial cells were treated with vehicle or TSP1 (0-10 nmol/L) for 60 minutes and subjected to homogenization in ice-cold disruption buffer (HBSS containing 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 0.1 mM protease inhibitor PMSF). ROS production in endothelial cells was measured using two independent, complementary assays. (1) Superoxide anion ($O_2^{-\cdot}$) production by total cellular homogenates was measured by cytochrome c reduction as described previously.(19) Briefly, $O_2^{-\cdot}$ generation was initiated by the addition of 180 μM NADPH and was calculated from the initial linear rate of SOD (150 U/ml)-inhibitable cytochrome c reduction quantified at 550 nm and using an extinction coefficient of 21.1 mM-1 cm-1 (Biotek Synergy 4 Hybrid Multi-Mode Microplate Reader). (2) Endothelial cell homogenate hydrogen peroxide (H_2O_2) production was measured using the Amplex Red (Thermo Fisher Scientific, Waltham, MA) assay as described previously.(1) Briefly, 50 μg/ml protein was added to wells on a 96-well plate containing the assay mixture (25 mM HEPES, pH 7.4, containing 0.12 M NaCl, 3 mM KCl, 1 mM $MgCl_2$, 0.1 mM Amplex red, and 0.32 U/ml HRP). The reaction was initiated by the addition of 36 μM NADPH as published previously.(1)

Cell Treatment and Hydrogen Peroxide Production

Coumarin Boronic Acid (CBA, Caymen #14051) probe preparation and methodology was adopted and modified from Zielonka et.al..(80) Primary human pulmonary endothelial cells

(Promo Cell #C12241, lot# 399Z002: 23yo Caucasian female and Lonza #CC2530, lot# 657513: 34yo Caucasian female; both cell lines were phenotypically confirmed by flow cytometry) seeded in a 96 well, clear bottom, black sided plate, were serum starved in 0.2% serum (FBS), phenol red-free Lifeline endothelial cell culture media for 4 hours. Wells were then aspirated, washed with PBS, and assay buffer was added (phenol red-free Lifeline endothelial cell culture media supplemented with 10uM DTPA (Sigma #D6518), 100uM Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma # N5751) and 1mM Taurine (Sigma # T0625) (L-NAME and Taurine were added to inhibit generation of peroxynitrite and scavenge hypochlorous acid, respectively, which could both react with the probe) with the following treatments: vehicle, TSP-1 (10nM, 2.2nM, or 0.2nM), or 2.2nM TSP-1 with 2ug/ml CD47 blocking antibody [clone B6H12.2] (Abcam #ab3283) and select wells received 1 KU/ml bovine liver catalase (Sigma # C1345) to act as a negative control. After the addition of the assay buffer, plates were returned to standard cell culture incubator at 37°C for 60 minutes. During this time the 5X probe solution was prepared by diluting in assay buffer. Plates were removed from the incubator and the probe solution was added to a final concentration of 0.5mM CBA at a final reaction volume of 125uL. Plates were immediately placed in a plate reader preheated to 37°C and read kinetically (every minute for 2 hours) at excitation 350nm, emission 450nm. The average rate of fluorescence generation was determined over the linear portion of the response with the rate of the corresponding catalase control subtracted out. The rate was then normalized to the vehicle control and displayed as fold change in H_2O_2 production.

Western blot

Western blot on lung samples was conducted as previously described.(67) Primary antibodies used were rabbit anti-β-actin (Cell Signaling Technology; #4967, dilution 1:5,000), rabbit anti-CD47 (MAIP301, Santa Cruz, Dallas, TX; sc-12731, dilution 1:375), mouse anti-TSP1

(Abcam; ab1823, dilution 1:375). The intensity of the bands was quantified using Image J (rsbweb.nih.gov/ij/).

Plasma TSP1 measurement

Blood was collected by cardiac aspiration into a BD Microtainer® EDTA microtube (BD Diagnostics, Franklin Lakes, NJ) upon completion of the hemodynamic assessment. An aliquot was set aside for determination of engraftment while the plasma was batch-tested with a murine TSP1 ELISA kit (Cusabio, College Park, MD) as previously described.(56)

Immunostaining and histology

For the human lung immunofluorescence staining, formalin-fixed, paraffin-embedded sections of 4-5 μm thickness were deparaffinized and permeabilized using 0.1% Triton X-100 (Promega, Madison, WI) in TBS for 10 minutes. Antigen retrieval was performed on paraffin sections by incubation in Heat Induced Epitope Retrieval Citrate Buffer (Thermo Fisher Scientific) for 20 minutes at 97°C or by enzymatic antigen retrieval using 0.05% Gibco Trypsin-EDTA (Thermo Fisher Scientific) for 15 minutes at 37°C. Sections were blocked with 10% horse serum (Sigma Aldrich). The primary antibody of interest CD47 (MAIP 301, Santa Cruz) or TSP1 (A6.1, Abcam) and the primary antibody for the endothelial markers (PECAM, von Willebrand Factor) were incubated overnight at 4°C. Inflated mouse lung tissue used for immunofluorescence was fixed in 2% paraformaldehyde, followed by 30% sucrose, OCT embedded and then frozen. Sections cut 6-7 μm thick were brought to room temperature and permeabilized using 0.1% Triton X-100 in PBS for 10 minutes and then blocked with 5% non-specific serum for 45 minutes. The primary antibodies 3-nitrotyrosine (3NT, 39B6, Abcam) and 4-hydroxynonenal (4HNE, Abcam) were incubated for 1 hour at room temperature. Following primary antibody binding, all sections were incubated with Alexa Fluor® 594, Alexa Fluor® 480 (Thermo Fisher Scientific) or Alexa Fluor® 488 (Life Technologies, Carlsbad, CA) secondary

antibodies for 1 hour at room temperature. Sections were counterstained for DAPI (Thermo Fisher Scientific) and coverslipped.

Fluorescently labeled human lungs were imaged by confocal with a 40x objective. CD47 and TSP1 imaging were done on a FLUOVIEW FV1000 or a Nikon A1 using matched imaging acquisition settings across samples. The CD47 signal/ROI and TSP1 signal/ROI areas were corrected by a normalization factor across samples to account for microscopy differences. PECAM and von Willebrand factor signal were thresholded and used to generate endothelial specific ROI. The average CD47 and TSP1 signals were measured within the ROI of PECAM and von Willebrand factor (respectively) and then divided by the ROI areas. CD47 or TSP1 SCD-PH expression was normalized to average control expression and is reported as % control.

Fluorescently labeled mouse lungs were imaged by confocal with a 20x objective. 3NT and 4HNE imaging were done on a Nikon A1 using matched imaging acquisition settings across samples. DAPI signal was thresholded and used to generate a cell specific ROI. The average 3NT and 4HNE signals were measured within the DAPI ROI. 3NT and 4HNE were reported as intensity per cell (DAPI signal).

Statistical analysis

Results are presented as mean ± SEM unless indicated otherwise. Data, including differences in hemodynamic parameters between groups of mice, were analyzed for two group comparisons by Student's *t*-test for ungrouped samples; for multiple group comparisons by ANOVA using Sidak's multiple comparisons test; and for myography using two-way ordinary ANOVA with Sidak's multiple comparisons test. Survival after transplantation was measured by Kaplan-Meyer estimate. Linear regression was used to measure the association between spleen size and engraftment. Graphs and analyses were obtained with GraphPad Prism

287 software (GraphPad Software Inc., La Jolla, CA). $P < 0.05$ was considered statistically
288 significant.
289

Results

TSP1 and its cognate receptor CD47 are upregulated in the lungs of Sickle mice and patients with SCD-associated PH

Similar to humans with SCD, Sickle mice, but not their Hemi control, develop PH as determined by intact chest right heart catheterization.(32) We compared TSP1 and CD47 expression in the lungs of age-matched C67BL/6J, Hemi and Sickle female mice (Fig. 1A and 1B, Fig. 2) at 4, 9-11 and 13-14 months of age. We found that the TSP1 and CD47 expression levels in the lung increased with age, as compared to both C57BL/6J and Hemi mice (Fig. 2), with significant increases observed at 13-14 months of age. While levels of both proteins were also higher in 4 months old and 9-11 months old Sickle mice, the difference was not significant (Fig. 1A and 1B).

Immunofluorescence of lung sections from 6 patients with SCD-associated PH also revealed increased levels of CD47 (red, Fig. 3A) as compared to lung sections from patients without PH or overt lung disease (Fig. 3B). TSP1 levels (red) were elevated in lung tissue sections from PH patients, although the majority of the signal appeared to originate from intraluminal RBC that constitutively express large amounts of TSP1(47) (Fig. 3A), but were undetectable in lung sections from control non-PH lungs (Fig. 3B). In SCD lung samples, immunofluorescent CD47 was widely expressed localizing to the airways, vessels and parenchyma and was also found in some vessel lumens (Fig. 3A).

Sickle mice develop PH and vascular dysfunction

We performed comprehensive open chest hemodynamic assessment of male 2-8 months old Sickle mice (n=9) and age-matched C57BL/6J mice (n=6) by right heart micro-catheterization. Unlike intact chest catheterization,(32) the open chest technique allows capture of pressure/volume loops and a more thorough assessment of cardiopulmonary pressures and cardiac function. Sickle mice developed PH and RV failure: RV pressures were higher in Sickle

316 mice as shown by the maximum pressure of the RV (RV max p, 30.6 ± 3.0 vs. 22.0 ± 1.9
317 mmHg, p<0.001) and the mPAP (20.4 ± 1.9 vs. 14.0 ± 0.6 mmHg, p<0.001, Fig. 4A). The
318 afterload of the Sickle mice was increased as shown by the elevated pulmonary vascular
319 resistance (PVR, 2.18 ± 0.9 vs. 0.7 ± 0.1 mmHg*min/mL, p<0.001) and the RV effective arterial
320 elastance, the ratio of RV end-systolic pressure to stroke volume (RV Ea, 2.5 ± 1.0 vs. 0.9 ± 0.1
321 mmHg/µL, p=0.001, Fig. 4B). In Sickle mice, RV systolic function as measured by the
322 contractility index measured as dP/dt(max)/RVP(max) was decreased (87.2 ± 23.8 vs. 141 ± 7.2
323 L/min/m^2, p<0.001, Fig. 4C). The RV diastolic function, an indicator of RV stiffness, measured
324 by the maximum and minimum rates of pressure rise and decline during the relaxation phase
325 (RV dP/dt_{min}), was also impaired, although the change was not significant (p=0.160, Fig. 4D).
326 The Fulton index (RV/LV+septum) did not differ between the two groups (0.22 ± 0.0 vs. 0.21 ±
327 0.1, p=0.617). *In vivo* heart function as characterized by pressure-volume relations of the RV of
328 two representative sickle and C57BL/6J mice showed that the Sickle mice had a rightward shift
329 of the end-systolic pressure-volume relation (Fig. 4E). The Sickle mice also had higher end
330 diastolic volume (EDV), indicative of increased dilation, end systolic volume (ESV) and end
331 systolic pressure (ESP) compared to control C57BL/6J mice. The slope of the end systolic
332 pressure-volume relationship (ESPVR) was decreased, indicating reduced RV contractility and
333 systolic function in Sickle mice. We also found that, similar to patients with SCD,(56) Sickle mice
334 had elevated plasma levels of TSP1 as compared to both C57BL/6J and CD47KO mice (Fig.
335 4F).

336 As our *in vivo* hemodynamic assessment showed that Sickle mice had increased PVR
337 compared to controls, we tested the vascular responsiveness of isolated aortic segments of
338 male age-matched Sickle (n=6) and C57BL/6J (n=5) mice in a functional myograph bioassay
339 system. We found that arteries from Sickle mice had impaired endothelial-dependent
340 vasorelaxation in response to the endothelial activator acetylcholine (Ach) as compared to

C57BL/6J mice (Fig. 4G), although vascular contraction in response to phenylephrine was not different between the groups (Fig. 4H).

Absence of activated parenchymal CD47 improves pulmonary hemodynamics and restores arterial vasodilator responsiveness in chimeric mice with a sickle erythropoiesis

Transplantation of Sickle mouse bone marrow into wild type mice generates chimeric animals that develop the same sickle phenotype as their donors.(32, 33, 74) We therefore interrogated the TSP1-CD47 axis *in vivo* by generating chimeric animals with a sickle erythropoiesis on a CD47KO background. Sickle bone marrow was transplanted into C57BL/6J (n=24) and CD47KO (n=27) mice in parallel, in seven separate experiments (Fig. 5A). Approximately 80% of the chimeras survived to the day of assessment with no significant difference in survival between the two groups (Fig. 5B). The mice underwent blood sampling for determination of engraftment by electrophoresis (Fig. 5C) and full pulmonary hemodynamic assessment. The chimeras had median 81% (IQ range: 43-98%) HbS, with the engraftment positively correlating with spleen size, thereby showing that splenic extramedullary erythropoiesis was proportional to the degree of hemolytic anemia from HbS (Fig. 5D).(33)

Six Sickle-to-CD47KO and three Sickle-to-C57BL/6J died intra-operatively precluding hemodynamic data acquisition. We performed hemodynamic assessment of the remaining Sickle-to-CD47KO and Sickle-to-C57BL/6J chimeras and analyzed the data from those that were fully engrafted (>80% HbS, n=17 and n=10, respectively). RV pressures were lower in Sickle-to-CD47KO chimeras as compared to Sickle-to-C57BL/6J chimeras as shown by the reduced RV max p (22.1 ± 3.6 vs. 28.1 ± 8.8 mmHg, p=0.013) and mPAP (15.3 ± 2.6 vs. 18.8 ± 5.7 mmHg, p=0.020, Fig. 6A). The afterload of the Sickle-to-CD47KO chimeras was decreased as shown by the decreased PVR (1.2 ± 0.7 vs. 2.4 ± 2.2 Wood units, p=0.024) and RV Ea (1.5 ± 0.9 vs. 2.7 ± 2.6 mmHg/μL, p=0.052, Fig. 6B). The RV systolic function of the Sickle-to-CD47KO chimeras as measured by the contractility index was similar to that of the Sickle-to-C57BL/6J

chimeras and overall not as low as that of Sickle mice (112.4 ± 28.3 vs. 108.3 ± 25.2 L/min/m^2, p=0.352, compare Fig. 4C to 6C) while the RV diastolic function as measured by the RV dP/dt_{min} was improved in Sickle-to-CD47KO as compared to Sickle-to-C57BL/6J chimeras (-1297.0 ± 318.2 vs. -1604.0 ± 668.2 mmHg/s, p=0.059, Fig. 6D). In summary, Sickle-to-C57BL/6J chimeras developed PH and right ventricular heart failure, similar to their Sickle donors, although the overall magnitude of change in some parameters (i.e. RV max pressure, mPAP) was not as great in Sickle-to-C57BL/6J chimeric mice compared to Sickle mice (compare Fig. 4A-E to Fig. 6A-E). In contrast, Sickle-to-CD47KO chimeras were protected from PH and RV failure and had a cardiopulmonary phenotype that recapitulated that of control C57BL/6J mice (Fig. 6A-E). *In vivo* heart function as shown by pressure-volume relations of the RV of two representative Sickle-to-C57BL/6J and Sickle-to-CD47KO chimeras showed that the Sickle-to-CD47KO chimeras had both a leftward shift of the ESPVR, consistent with decreased hypertrophic remodeling, and increased maximum slope, consistent with improved cardiac function. Specifically, the Sickle-to-CD47KO chimeras had lower EDV, indicative of decreased dilation, ESV and ESP. The slope of the ESPVR was increased, indicating improved RV contractility and systolic function (Fig. 6E). Interestingly, plasma collected from the chimeras after euthanasia revealed lower levels of circulating TSP1 in Sickle-to-CD47KO chimeras (61.45 ± 10.11 vs. 76.89 ± 10.84 pg/mL, p=0.012, Fig. 6F).

We then compared the vascular responsiveness of isolated aortic segments of Sickle-to-C57BL/6J (n=5) and Sickle-to-CD47KO chimeras (n=13) in the myograph system to determine whether the improved hemodynamic profile associated with alterations in vessel activation. We found that Sickle-to-CD47KO chimeras had improved endothelial-dependent vasorelaxation in response to ACh as compared to Sickle-to-C57BL/6J chimeras (Fig. 6G) while there was no significant difference in the effects of PE between vessel groups (Fig. 6H).

TSP1 stimulates increased ROS in human pulmonary endothelial cells, and pulmonary oxidative damage and vascular congestion in chimeric mice with a sickle erythropoiesis

Increased ROS generation is pathogenic in SCD,(4, 17, 25, 57) and the pulmonary endothelium is both a source and target of this process.(62) Our group has found that TSP1 promotes ROS production in animal models of ischemia-reperfusion injury.(19, 37, 48) We therefore hypothesized that diminution or absence of TSP1-CD47 signaling limits PH by suppressing ROS generation from sickle erythropoiesis. We first sought to demonstrate a direct link between TSP1 and ROS in pulmonary vascular cells. Treatment with exogenous TSP1 ($2.2x10^{-9}$ M), a concentration found in the plasma of patients with SCD,(56) for 60 minutes stimulated an increased rate of $O_2^{\bullet-}$ and H_2O_2 generation in human pulmonary arterial endothelial cells as measured by cytochrome c (Fig. 7A) and Amplex Red (Fig. 7B) assays respectively. Importantly, $0.2x10^{-9}$ M TSP1, a concentration found in plasma from healthy individuals, did not stimulate increased ROS in these cells (Fig. 7A, B), possibly suggesting a requirement for additional hemolytic, inflammatory or vaso-occlusive stress *in vivo* to induce vascular ROS production and oxidative stress. To provide further mechanistic support for the overall hypothesis that TSP1 causes PH via CD47 in sickle mice, we also pretreated human pulmonary arterial endothelial cells with a CD47 blocking antibody (2 μg/mL, clone B6H12.2). As shown in Figure 7C, addition of the CD47 blocking antibody abrogated TSP1-stimulated ROS production.

We then corroborated these results with “footprint” assays of TSP1 stimulation of ROS in tissue cross-sections. Mouse lung tissue sections were examined by immunohistochemistry to determine whether TSP1-CD47 signaling promoted ROS-mediated oxidative modifications in SCD lungs. Tissue deposition of 4-hydroxynonenal (4-HNE), a stable aldehyde formed by the degradation of polyunsaturated fatty acids during lipid peroxidation and 3-nitrotyrosine (3-NT), a result of protein nitration by peroxynitrite (byproduct of $O_2^{\bullet-}$ reacting with nitric oxide), were modified by absence of TSP1-CD47 signaling. We found that the lungs of Sickle-to-CD47KO

418 chimeras had lower 3-NT (green fluorescence) staining compared to Sickle-to-C57BL/6J
419 chimera lungs (Fig. 8A). We also found 4-HNE (green fluorescence) markedly attenuated in
420 Sickle-to-CD47KO chimeras as compared to Sickle-to-C57BL/6J chimeras (Fig. 8A). The latter
421 results (4-HNE and 3-NT) are consistent with an oxidative modification of proteins and lipids,
422 normally associated with tissue damage in SCD.
423 Vascular congestion in the lungs of sickle mice has been used as a histopathological
424 marker of vaso-occlusion and sickle-related tissue damage.(5, 41, 51, 52, 61) We found that
425 lungs of Sickle-to-CD47KO chimeras had markedly less vascular congestion than those of
426 Sickle-to-C57BL/6J chimeras (Fig. 8B).
427

Discussion

The pathogenesis of SCD-associated PH has not been fully elucidated but vascular dysfunction from increased inflammatory and oxidative stress has been implicated.(62) The matricellular protein TSP1 is at the crossroads of multiple pathways important to the pathogenesis of SCD. Our group has shown that plasma levels of TSP1 are elevated in SCD and that both TSP1 and its receptor CD47 are upregulated in the lungs of a patient with SCD and end-stage PH(68) in specific, and in patients with PH in general.(66) We have also shown that TSP1 induces ROS upon interaction with CD47 in vascular cells and in animal models.(8, 19, 79) The link between this pathway and SCD-associated PH has, however, never been established.

In the experiments presented herein, we employ two distinct strategies to define new mechanisms in the pathogenesis of SCD-associated PH. First, in our genetic-driven studies we show that Sickle mice develop PH and RV hypertrophy as compared to wild type controls (Fig. 4A-F). Second, in the presence of sickle erythropoiesis, absence of tissue-resident TSP1-CD47 signaling improves hemodynamics and mitigates RV dysfunction and hypertrophy (Fig. 6A-F). Together these findings are important for several reasons. They define a novel role for maladaptive TSP1-CD47 signaling to promote disease in a genetic model of PH, and extend previous studies in hypoxic(8, 58) and monocrotaline-treated rodent models of PH.(8) Second, they confirm and extend the characterization of cardiovascular impairment that we first reported in the BERK mouse line.(32) We now also provide an additional pathway for reduced nitric oxide signaling in Sickle mice(32) and posit that upregulation of TSP1 via CD47 in the vasculature and lungs of Sickle mice increases vascular ROS and contributes to endothelial dysfunction. However, it remains to be seen if there is a direct function for TSP1-CD47 signaling to perturb RV homeostasis in SCD.

We found that Sickle mice had elevated pulmonary tissue levels of TSP1 and CD47 protein as compared to Hemi controls and wild type animals (Fig. 1A-B and Fig. 2). The

454 difference reached significance in the oldest mice, aged 13-14 months, and thus complement a
455 previous study that noted age-related increases in TSP1 and CD47 in 18 month old C57BL/6J
456 mice.(65) Results herein suggest an age-mediated component to pulmonary accumulation of
457 TSP1 and CD47 that is accelerated by SCD. They are also in keeping with reports of increased
458 TSP1 levels in older individuals.(16, 69, 70)

459 In addition, Sickle mice had elevated plasma TSP1 levels (Fig. 4F), although the cellular
460 source of the circulating protein was not defined. These results mirror those obtained in humans
461 with SCD, who have elevated TSP1 plasma levels,(13, 56) and those with SCD-associated PH,
462 who have parenchymal overexpression of CD47 (Fig. 3A). Beyond platelets that are a reservoir
463 of pre-formed TSP1, pulmonary endothelial and smooth muscle cells and fibroblasts upregulate
464 TSP1 via hypoxia-inducible factor 2 alpha,(45) and could be a source of plasma TSP1 in SCD-
465 associated PH. Incidentally, we herein found that plasma TSP1 levels were constitutively lower
466 in normoxic CD47KO mice and Sickle-to-CD47KO chimeras compared to controls (Fig. 4F and
467 6F) suggesting cross-talk regulation of TSP1 and CD47.

468 Our hemodynamic data obtained on chimeric mice with open chest cardiac
469 catheterization show that absence of lung or vascular CD47 partially protected the mice from
470 PH, regardless of the presence of circulating TSP1. In contrast, recipient C57BL/J6 mice
471 transplanted with sickle bone marrow, and thus manifesting both circulating and parenchymal
472 CD47 signaling, had increased RV pressure and afterload and decreased RV systolic function,
473 to a degree similar to BERK sickle mice. However, the cardiopulmonary phenotype of Sickle-to-
474 C57BL/J6 chimeras was less severe than that of the Sickle mouse donors and consequently,
475 the differences in hemodynamic data between the two chimera groups were not always
476 significant, particularly because of higher standard deviation (Fig. 6A-E). This could be due to
477 variability introduced by the transplantation protocol, particularly as it concerns whole body
478 radiation, or the time exposure over months of disease needed to develop PH and right heart
479 failure. While we took care to exclude mice that were not fully engrafted, other factors such as

480 the effect of radiation on the lung, heart and other mediastinal structures may have blunted the
481 phenotype or caused variable responses. Myograph studies of aortic segments from chimeric
482 mice showed improved endothelial-dependent vasorelaxation in response to Ach in vessels
483 from Sickle-to-CD47KO mice (Fig. 6G) again pointing to a dominant role for parenchymal, as
484 opposed to circulating, CD47 signaling in suppressing vasodilation in this setting. Further
485 corroborating our findings, in other studies exogenous TSP1 ($2.2x10^{-9}$ M) inhibited murine and
486 rat pulmonary arterial vasodilation,(66) while pulmonary arteries from TSP1 KO mice had
487 preserved endothelial-dependent vascular function under hypoxia (1% O_2) as compared to wild
488 type and normoxic controls.(45) A role for ROS, while inferred in the present work, was not
489 directly tested in myography studies of aortic segments from our chimeras. This could be
490 important as prior studies found that ROS scavenging ameliorated TSP1-mediated inhibition of
491 arterial vasodilation.(53, 79)

492 Research indicates that TSP1 interaction with CD47 is, in part, responsible for ROS
493 generation *in vitro* and *in vivo.*(19) Extending these studies we detected an augmented $O_2^{\bullet-}$ and
494 H_2O_2 after treatment with exogenous TSP1 (Figs. 7A and B). Thus, physiologically relevant
495 concentrations of TSP1 stimulate ROS production in pulmonary vascular endothelial cells.
496 Further, the finding of reduced oxidative modification of lipids and proteins (consistent with
497 damage) in the lungs of chimeric mice without vascular CD47 provide evidence for a role for
498 CD47 signaling in inducing pathologic ROS in SCD (Fig. 8A). Our data on ROS generation by
499 TSP1-CD47 are supported by other investigations from our group that show that ROS
500 production is decreased in endothelial cells from mice lacking CD47 or with knock-down of
501 CD47 in rodent cells.(50) They are also supported by our data obtained in human endothelial
502 cells, where blockade of CD47 by a monoclonal antibody abrogated TSP1-stimulated ROS
503 production (Fig. 7C). While we have not interrogated the source of ROS further, other studies
504 from our group have shown that NADPH oxidase-1 (NOX1) is activated by TSP1-CD47,
505 suggesting that NOX1 activation may be a downstream event in the pathway of SCD-related

PH.(50) Development of cell-specific CD47KO mice in tandem with existing specific NOX1 inhibitors(18) would likely be important in dissecting the specifics of ROS pathway activation in our chimera SCD model.

One intriguing finding is that chimeric CD47KO mice also had decreased RBC pulmonary congestion. RBC congestion has been interpreted as evidence of vaso-occlusion in SCD animal models.(5, 41, 51, 52, 61) TSP1 is itself known to bind SCD RBC.(6, 31) It is possible that disruption of TSP1 binding with endothelial CD47 may have limited RBC adhesion in our model. Regardless, evidence that TSP1 infusion decreases hind limb reperfusion in rats(19) and triggers vaso-occlusion in sickle mice(14) supports a role for circulating TSP1 in acutely altering blood flow.

This study has a number of limitations. While Sickle mice, similar to humans with SCD, develop PH, they do not display the hallmark vascular remodeling associated with human disease.(32, 61) In general, there are no PH rodent models that robustly develop plexiform lesions or obliterative vascular hyperplasia. Whether this discrepancy may be attributed to an earlier disease stage or to intrinsically different pathogenesis is unknown. A prior study from our group has pointed to nitric oxide dysfunction as a key mediator of hemolysis-induced PH in the BERK model.(32) In the present study, we have not directly explored the effect of TSP1-CD47 on nitric oxide-dependent pathways, thus their relative contribution is unknown. Our group, however, has previously reported that TSP1-CD47 inhibits nitric oxide signaling at several levels.(7, 36, 38, 39) Thus, it is likely that the beneficial effect of TSP1-CD47 disruption on nitric oxide in our model may have compounded effects on other redox pathways. Further, the effect of the open chest procedure on mouse vascular reactivity is not known. Humans with SCD are prone to vaso-occlusion and lung injury after surgical procedures.(76) Thus, it is possible that the open chest catheterization led to exacerbation of disease and acute vascular dysfunction in our Sickle mice and chimeras. If that is the case, at least some of the hemodynamic improvement in the Sickle-to-CD47KO chimeras may have been due to protection from acute

injury, rather than long term protection from PH. Countering this hypothesis, it is unlikely that the short surgical procedure we employed may have resulted in immediate, hemodynamically significant lung injury; lending support to this latter hypothesis, acute chest syndrome does not typically develop intraoperatively in humans. The use of C57BL/6J mice as a comparison group for the Sickle mice in the hemodynamic experiments could arguably have been complemented by analysis of Hemi mice (Fig. 4). However, having previously published on the latter mice,(32) we limited our experiments to C57BL/6J controls. Finally, CD47 is not only a receptor for thrombospondin family members, but also a ligand for the transmembrane signaling protein SIRP alpha and a component of a supramolecular complex containing specific integrins, heterotrimeric G proteins and cholesterol. Thus, KO of CD47 could have multiple effects, even without change in TSP levels. For instance, CD47-SIRP alpha interaction could alter inflammatory cell activity and ROS and the interaction with G proteins has been shown to alter cAMP in some cells.(20) Future studies should investigate the relative contribution of these pathways to PH in our model.

In summary, we have reported on a novel pathway that mediates SCD-associated PH. TSP1-CD47, by virtue of location at the crossroads of multiple mechanisms of vasculopathy that include nitric oxide dysfunction, vaso-occlusion and oxidative damage, represents a promising therapeutic target in SCD. Pharmacologic inhibitors of CD47 are already clinically available(24) for other diseases and may be useful in the treatment of SCD-associated PH.

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Authorship and Disclosures

E.M.N. conceived and designed the study, conducted and supervised the experiments, interpreted the data and wrote the manuscript. L.L.I., H.E.K, N.M.R., M.Y., J.J.B., D.M., C.M.S., M.A.R., E.R.DV. and K.P.P. conducted the experiments and edited the manuscript. P.J.P. assisted in interpretation of the data and editing of the manuscript. J.S.I. and M.T.G. designed the study, interpreted the data and edited the manuscript.

J.S.I. serves as Chair of the Scientific Advisory Board of Radiation Control Technologies, Inc. (RCTI, Garden City, NJ) and has equity interest in RCTI and Tioma Therapeutics (St. Louis, MO) that have licensed CD47 technology for development. M.T.G. is a co-inventor of pending patent applications and planned patents directed to the use of recombinant neuroglobin and heme-based molecules as antidotes for CO poisoning, which have recently been licensed by Globin Solutions, Inc. M.T.G. is a shareholder, advisor and director in Globin Solutions, Inc. Additionally, and unrelated to CO poisoning, M.T.G. is a co-inventor on patents directed to the use of nitrite salts in cardiovascular diseases, which have been licensed by United Therapeutics and Hope Pharmaceuticals, and is a co-investigator in a research collaboration with Bayer

584 Pharmaceuticals to evaluate riociguate as a treatment for patients with SCD. The other authors
585 have no COI to report.
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Figure legends

Figure 1: Expression of TSP1 and CD47 in the lungs of 4-11 months old Sickle mice

(A-B) Lung lysates from female BERK sickling (Sickle), BERK non sickling hemizygous controls (Hemi), C57BL/6J controls (C57BL), CD47KO and TSP1KO mice (n=20) underwent Western Blotting for TSP1 and CD47. CD47KO and TSP1KO mice were used as positive and negative controls for the Western Blot procedure. Each lane represents an individual animal and all data are shown. Densitometry results are shown (normalized to β-actin). Unpaired t-test was applied for comparison between each group. Results are shown as means ± SEM (Standard Error of the Mean).

Figure 2: Expression of TSP1 and CD47 in the lungs of 13-14 months old Sickle mice

Lung lysates from 13-14 months old female BERK sickling (Sickle), BERK non sickling hemizygous controls (Hemi), C57BL/6J controls (C57BL), CD47KO and TSP1KO mice (n=26) underwent Western Blotting for TSP1 and CD47. CD47KO and TSP1KO mice were used as positive and negative controls for the Western Blot procedure. Each lane represents an individual animal and all data are shown. The 13-14 months old Sickle mice had a significant increase in pulmonary TSP1 and CD47 as compared to Hemi and C57BL mice. Densitometry results are shown (normalized to β-actin). One way ANOVA with multiple comparisons was applied. Results are shown as means ± SEM. *P<0.1, **P<0.01, ***P<0.001****P<0.0001.

Figure 3 TSP1 and its receptor CD47 are upregulated in the lungs of patients with SCD-associated PH

TSP1 and CD47 expression was measured by immunofluorescence in the lung sections from 6 patients with SCD-associated PH (SCD-PH) and 6 control patients without PH or overt lung disease. Expression of TSP1 (red) (vWF-green, DAPI-blue) (upper photograph for each

patient) was increased in patients with SCD-PH although the signal appeared to originate from intraluminal RBC (A). Increased levels of CD47 (red) (PECAM-green, DAPI-blue) (lower photograph for each patient) were consistently observed in lung sections from all patients as compared to control patients (B). CD47 or TSP1 SCD-PH expression is reported as % control patient. (C). Bar represents 50 μM. Unpaired t-test was applied for comparison between the two groups. Results are shown as means ± SEM. ****P<0.0001.

Figure 4: Sickle mice develop PH and vascular dysfunction

Open chest hemodynamic assessment of male 2-8 months old Sickle mice (n=9) and age-matched C57BL mice (n=6) was performed by open chest right heart micro-catheterization. RV pressures were measured by the RV max p and the mPAP (A). Afterload was measured by PVR and RV Ea (B). RV systolic function was measured by the contractility index (C). RV diastolic function was measured by the RV dP/dt_{min}, a measure of RV stiffness (D). Pressure-volume relations of the RV of two representative sickle and C57BL/6J mice (E). Plasma TSP1 was measured by ELISA at the time of euthanasia (F). Vascular reactivity of isolated aortic segments of male age-matched Sickle (n=6) and C57BL (n=5) mice was assessed in the myograph system in response to Ach and PE (G-H). Unpaired t-test was applied for comparison between the two groups of mice. Results are shown as means ± SEM. Two-way ordinary ANOVA with Sidak's multiple comparisons test was used for the myograph data. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 5: Generation of chimeras and measurement of engraftment

The TSP1-CD47 axis was interrogated *in vivo* by generating chimeric animals with a sickle erythropoiesis on a CD47KO background. Bone marrow was harvested from flushed femurs and tibias of adult Sickle mice and whole bone marrow (5×10^6 cells) were transplanted into age-matched, lethally myeloablated (10 Gy) two months old CD47KO (n=27) and C57BL

(n=24) mice, the background strain of CD47KO mice, by retro-orbital sinus injection in 7 separate experiments. Schematic representation of the transplantation protocol (A). Kaplan-Meyer survival curve of transplanted chimeras (B). Engraftment was assessed by measurement of HbS percentage by HPLC followed by confirmatory capillary zone electrophoresis (CZE) in blood samples obtained at the time of euthanasia, 4-6 months after transplantation. A CZE gel is shown (upper left quadrant). Human HbA and HbS were readily detectable in a C57BL recipient transplanted with Sickle BM with mixed chimerism (upper right quadrant). CZE of a C57BL recipient (lower left quadrant) and Sickle mouse donor (lower right) are also shown (C). Linear regression of engraftment and spleen size (D). Results are shown as means ± SEM unless otherwise noted. ***P<0.001.

Figure 6: Absence of tissue-resident TSP1-CD47 signaling improves pulmonary hemodynamics and arterial vasodilator responsiveness in chimeric mice with a sickle erythropoiesis.

Full hemodynamic assessment of Sickle-to-CD47KO and Sickle-to-C57BL chimeras was performed by open chest right heart micro-catheterization. RV pressures included RV max p and mPAP (A). The afterload was measured as PVR and RV Ea (B). The RV systolic function was measured by the contractility index (C) while the RV diastolic function was measured by the RV dP/dt_{min} (D). Pressure-volume relations of the RV of two representative Sickle-to-C57BL and Sickle-to-CD47KO chimeras (E). Plasma TSP1 was measured by ELISA at the time of euthanasia (F). Vascular reactivity of isolated aortic segments of Sickle-to-C57BL (n=5) and Sickle-to-CD47KO chimeras (n=13) was assessed in the myograph system in response to Ach and PE (G-H). An unpaired t-test was applied for comparison between the two groups of mice. Results are shown as means ± SEM. Two-way ordinary ANOVA with Sidak's multiple comparisons test was used for the myograph data. *P<0.05.

Figure 7. TSP1 augments ROS in human pulmonary endothelial cells via CD47.

Commercially available hPAEC (n=1 donor) were treated with vehicle or TSP1 (0-10 nmol/L) for 60 minutes. ROS production was measured using two independent complementary assays: $O_2^{-\cdot}$ generation by total cellular homogenates measured by cytochrome c reduction (A); and endothelial cell homogenate H_2O_2 production measured using the Amplex Red assay (B). One-way ANOVA followed by Sidak's multiple comparisons test was applied for the analysis. Results are shown as means ± SEM of three experiments.**P<0.01

Commercially available hPAEC (n=2 donors) were established in a 96 well plate and directly exposed to the following treatments for 60 min: vehicle, TSP-1 (10nM, 2.2nM, or 0.2nM), or 2.2nM TSP-1 with 2μg/ml CD47 blocking antibody [clone B6H12.2], and select wells received 1 KU/ml bovine liver catalase to act as a negative control. Coumarin Boronic Acid probe detection of H_2O_2 production in the cells was measured kinetically for 2 hours (C). The average rate of fluorescence generation was normalized to the vehicle control and displayed as fold change in H_2O_2 production. Means ± SEM results are shown of three to four experiments per donor, total n=6-7. *P<0.05, **P<0.01, ****P<0.0001

Figure 8. TSP1 augments pulmonary oxidative damage and promotes vascular congestion in chimeric mice with a sickle erythropoiesis

Lung tissue sections from chimeras were stained for 4HNE or 3NT and examined by immunofluorescence to determine whether *in vivo* SCD-mediated pulmonary ROS production (4-hydroxynonenal, 4-HNE) and secondary ROS-mediated protein modifications (3-nitrotyrosine, 3-NT) were modified by absence of TSP1-CD47 signaling. 3-NT (green fluorescence, DAPI-blue) staining (A) (n=3 per group, 2 sections per animal). (A) also shows representative 4-HNE deposition by green fluorescent immunohistochemical staining (DAPI-blue) (n=2 mice per group, 3 sections per animal). The fluorescent signal was quantified using ImageJ software and reported as 3-NT or 4-HNE intensity per cell (DAPI signal). Vascular

congestion on H&E-stained slides from chimeras (n=3 mice per group) was rated by three blinded, independent readers. The readers used a semi quantitative, relative, 0 to 4 scale where absence of RBC in the lumens of pulmonary blood vessels was rated as 0. Increasing lumen congestion and number of affected vessels were rated 1 to 4 (B). Images were taken using a Nikon A1 confocal microscope or Nikon 90i at 20x. Bars represent 50 μM. Results are shown as representative slides and means ± SEM. *P<0.05, ****P<0.0001.