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

3 **Running head: TSP1-CD47 Promotes Pulmonary Hypertension in SCD**

4 [1]Enrico M. Novelli, MD, MS, [1]Lynda Little-Ihrig, BS, [2]Heather E. Knupp, MSc, [3]Natasha M.
5 Rogers, MD, PhD, [4]Mingyi Yao, PhD, MS, [1]Jeffrey J. Baust, [5]Daniel Meijles, PhD, [6]Claudette M.
6 St. Croix, PhD, [6]Mark A. Ross, [1]Patrick J. Pagano, PhD, [1]Evan R. DeVallance, PhD, [7]George
7 Miles, MD, PhD, [1,2]Karin P. Potoka, MD, [1]Jeffrey S. Isenberg, MD, MPH and [1]Mark T. Gladwin,
8 MD

9 [1]Heart, Lung, Blood and Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA
10 and UPMC Department of Medicine, Division of Hematology/Oncology, [2]UPMC Children's
11 Hospital of Pittsburgh, Pittsburgh, PA, [3]Department of Medicine, Westmead Clinical School,
12 University of Sydney, Sydney, Australia, [4]Midwestern University, Glendale, AZ, [5]School of
13 Biological Sciences, University of Reading, Reading, UK, [6]Center for Biologic Imaging,
14 University of Pittsburgh, Pittsburgh, PA, [7]Department of Molecular and Human Genetics, Baylor
15 College of Medicine, Houston, TX.
16
17
18
19 [§]To whom correspondence should be addressed:
20 Enrico M. Novelli, MD, MS
21 E1257 Biomedical Science Tower
22 200 Lothrop Street
23 Pittsburgh, PA 15261
24 Phone: (412) 624-2591
25 E-mail: novellie@upmc.edu
26 Fax: (412) 648-9009
27
28
29

32

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 lle)D- Punjab (beta 121Gln)], and display *in vivo* hemoglobin polymerization and erythrocyte sickling, suggested that TSP1 triggered

3

81 erythrocyte microparticle shedding induced endothelial injury and facilitated acute vaso-

82 occlusive events.(14) Further, in patients with SCD, TSP1 plasma levels are elevated at

83 baseline and still further in vaso-occlusive crisis, and high plasma TSP1 levels correlate with an

84 increased risk of vaso-occlusive complications.(55, 56)

85 TSP1 stimulates increased superoxide anion ($O_2^{\bullet-}$) production in vascular endothelial

86 and smooth muscle cells through its cell receptor CD47.(8, 19, 79) Indeed, new studies have

87 found that TSP1, via CD47, limited nitric oxide-mediated vasodilation of distal pulmonary

88 arteries from healthy and end-stage PH lungs.(66) The finding that lungs explanted from

89 patients with PH,(8) including a patient with SCD-associated PH,(23, 68) had upregulated TSP1

90 and CD47 in pulmonary artery smooth muscle cells vessels and parenchyma, also supports the

91 notion that TSP1 may play a pathogenic role in SCD-associated PH.(64) CD47 is also

92 expressed in human and murine RBC, potentially contributing to RBC adhesion to the vascular

93 wall.(59) The role of TSP1-CD47 in SCD has, however, never been established.

94 In the Berkeley (BERK) model of SCD, murine globins are replaced by human α and $β^S$

95 globins.(61) BERK sickle mice mimic the genetic, hematologic and histopathologic features that

96 are found in human subjects afflicted by SCD, as they display irreversibly sickled RBC, anemia,

97 leukocytosis and systemic inflammation, high levels of pulmonary adhesion molecules and

98 multi-organ pathology.(9, 44, 61) As they age, BERK mice also develop PH as measured by

99 increases in pulmonary artery and right ventricular (RV) pressures and RV mass.(32)

100 In the present study, we addressed the hypothesis that increases in circulating plasma

101 levels of TSP1, via binding to the CD47 receptor, stimulate pulmonary ROS production in BERK

102 sickle mice and chimeric animals with sickle erythropoiesis. We further hypothesized that TSP1-

103 dependent activation of CD47, in BERK mice and the chimeric animals and humans with SCD,

104 contributes to vasculopathy and the evolution of PH. Finally, we tested the hypothesis that

105 therapeutic disruption of the TSP1-CD47 ligand-receptor interaction will both prevent and

106 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

5

133 myeloablated (10 Gy) two months old CD47KO and C57BL/6J mice, the background strain of

134 CD47KO mice, by retro-orbital sinus injection. Recipients received recombinant human

135 darbepoetin alfa (Aranesp®, a generous gift by Amgen, Inc. Thousand Oaks, CA) on the day

136 prior to transplantation to promote donor erythropoiesis, and neomycin supplemented water and

137 autoclaved chow for 2 weeks following transplantation. Engraftment was assessed by

138 measurement of HbS percentage by high-performance liquid chromatography (Primus Ultra2,

139 Trinity Biotech, Kansas City, MO) followed by confirmatory capillary zone electrophoresis

140 (Sebia, Évry, France) at the Quest Diagnostics™ laboratories, Chantilly, VA in blood samples

141 obtained at the time of euthanasia, i.e. 4-6 months after transplantation.

142

143 *Open chest cardiac catheterization*

144 Open chest cardiac catheterization of mice under general anesthesia with isoflurane and

145 tracheal intubation for mechanical ventilation was performed prior to euthanasia. The animals

146 were weighed and placed in an anesthesia chamber supplied with 5% isoflurane. Upon

147 induction of anesthesia the animals were then placed on a warming pad and restrained. A nose

148 cone that delivered 2.5% isoflurane was placed over the muzzle. A rectal probe was then placed

149 to monitor body core temperature. A tracheotomy was performed by inserting a 20-gauge

150 intravenous catheter into the trachea and securing it with a length of suture. The anesthesia

151 cone was then removed and the animals were placed on a ventilator set to 150-175 breaths per

152 minute (weight-dependent), and a stroke tidal volume of 200-250ul. A thoracotomy was then

153 performed and the rib cage was retracted to expose the inferior vena cava, the heart and the

154 great vessels. A length of silk suture was loosely placed around the inferior vena cava. A 27-

155 gauge needle was used to pierce the right ventricle (RV). The needle was then removed and a

156 1.2 French catheter (Scisense Inc., London, ON) was then introduced to the apical region of the

157 RV. The catheter was adjusted as needed until the optimal pressure-volume loop was obtained.

158 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

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

186 endothelial activation response.

187

188 *Cell treatment and ROS measurement*

189 Primary human pulmonary artery endothelial cells (hPAEC; Lonza #CC2530, lot#

190 4F3033: 51yo Caucasian male; flow cytometry confirmation of cell line phenotype, Walkersville,

191 MD) were grown in EBM-2 media containing EGM-2 bullet kit components (Lonza). Cells

192 (passages 3-6) were seeded the day before experimentation and synchronized in serum-

193 reduced media (1% FCS) for 4 hours. Synchronized endothelial cells were treated with vehicle

194 or TSP1 (0-10 nmol/L) for 60 minutes and subjected to homogenization in ice-cold disruption

195 buffer (HBSS containing 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 0.1 mM protease inhibitor PMSF).

196 ROS production in endothelial cells was measured using two independent, complementary

197 assays. (1) Superoxide anion ($O_2^{-\cdot}$) production by total cellular homogenates was measured by

198 cytochrome c reduction as described previously.[19] Briefly, $O_2^{-\cdot}$ generation was initiated by the

199 addition of 180 µM NADPH and was calculated from the initial linear rate of SOD (150 U/ml)-

200 inhibitable cytochrome c reduction quantified at 550 nm and using an extinction coefficient of

201 21.1 mM-1 cm-1 (Biotek Synergy 4 Hybrid Multi-Mode Microplate Reader). (2) Endothelial cell

202 homogenate hydrogen peroxide (H_2O_2) production was measured using the Amplex Red

203 (Thermo Fisher Scientific, Waltham, MA) assay as described previously.[1] Briefly, 50 µg/ml

204 protein was added to wells on a 96-well plate containing the assay mixture (25 mM HEPES, pH

205 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).

206 The reaction was initiated by the addition of 36 µM NADPH as published previously.[1]

207

208 *Cell Treatment and Hydrogen Peroxide Production*

209 Coumarin Boronic Acid (CBA, Caymen #14051) probe preparation and methodology

210 was adopted and modified from Zielonka et.al..[80] Primary human pulmonary endothelial cells

8

211   (Promo Cell #C12241, lot# 399Z002: 23yo Caucasian female and Lonza #CC2530, lot# 657513:

212   34yo Caucasian female; both cell lines were phenotypically confirmed by flow cytometry)

213   seeded in a 96 well, clear bottom, black sided plate, were serum starved in 0.2% serum (FBS),

214   phenol red-free Lifeline endothelial cell culture media for 4 hours. Wells were then aspirated,

215   washed with PBS, and assay buffer was added (phenol red-free Lifeline endothelial cell culture

216   media supplemented with 10uM DTPA (Sigma #D6518), 100uM Nω-Nitro-L-arginine methyl

217   ester hydrochloride (L-NAME, Sigma # N5751) and 1mM Taurine (Sigma # T0625) (L-NAME

218   and Taurine were added to inhibit generation of peroxynitrite and scavenge hypochlorous acid,

219   respectively, which could both react with the probe) with the following treatments: vehicle, TSP-

220   1 (10nM, 2.2nM, or 0.2nM), or 2.2nM TSP-1 with 2ug/ml CD47 blocking antibody [clone

221   B6H12.2] (Abcam #ab3283) and select wells received 1 KU/ml bovine liver catalase (Sigma #

222   C1345) to act as a negative control. After the addition of the assay buffer, plates were returned

223   to standard cell culture incubator at 37°C for 60 minutes. During this time the 5X probe solution

224   was prepared by diluting in assay buffer. Plates were removed from the incubator and the probe

225   solution was added to a final concentration of 0.5mM CBA at a final reaction volume of 125uL.

226   Plates were immediately placed in a plate reader preheated to 37°C and read kinetically (every

227   minute for 2 hours) at excitation 350nm, emission 450nm. The average rate of fluorescence

228   generation was determined over the linear portion of the response with the rate of the

229   corresponding catalase control subtracted out. The rate was then normalized to the vehicle

230   control and displayed as fold change in H_2O_2 production.

231

232   *Western blot*

233   Western blot on lung samples was conducted as previously described.[67] Primary

234   antibodies used were rabbit anti-β-actin (Cell Signaling Technology; #4967, dilution 1:5,000),

235   rabbit anti-CD47 (MAIP301, Santa Cruz, Dallas, TX; sc-12731, dilution 1:375), mouse anti-TSP1

236 (Abcam; ab1823, dilution 1:375). The intensity of the bands was quantified using Image J

237 (rsbweb.nih.gov/ij/).

238

239 *Plasma TSP1 measurement*

240   Blood was collected by cardiac aspiration into a BD Microtainer® EDTA microtube (BD

241 Diagnostics, Franklin Lakes, NJ) upon completion of the hemodynamic assessment. An aliquot

242 was set aside for determination of engraftment while the plasma was batch-tested with a murine

243 TSP1 ELISA kit (Cusabio, College Park, MD) as previously described.[56]

244

245 *Immunostaining and histology*

246   For the human lung immunofluorescence staining, formalin-fixed, paraffin-embedded

247 sections of 4-5 μm thickness were deparaffinized and permeabilized using 0.1% Triton X-100

248 (Promega, Madison, WI) in TBS for 10 minutes. Antigen retrieval was performed on paraffin

249 sections by incubation in Heat Induced Epitope Retrieval Citrate Buffer (Thermo Fisher

250 Scientific) for 20 minutes at 97°C or by enzymatic antigen retrieval using 0.05% Gibco Trypsin-

251 EDTA (Thermo Fisher Scientific) for 15 minutes at 37°C. Sections were blocked with 10% horse

252 serum (Sigma Aldrich). The primary antibody of interest CD47 (MAIP 301, Santa Cruz) or TSP1

253 (A6.1, Abcam) and the primary antibody for the endothelial markers (PECAM, von Willebrand

254 Factor) were incubated overnight at 4°C. Inflated mouse lung tissue used for

255 immunofluorescence was fixed in 2% paraformaldehyde, followed by 30% sucrose, OCT

256 embedded and then frozen. Sections cut 6-7 μm thick were brought to room temperature and

257 permeabilized using 0.1% Triton X-100 in PBS for 10 minutes and then blocked with 5% non-

258 specific serum for 45 minutes. The primary antibodies 3-nitrotyrosine (3NT, 39B6, Abcam) and

259 4-hydroxynonenal (4HNE, Abcam) were incubated for 1 hour at room temperature. Following

260 primary antibody binding, all sections were incubated with Alexa Fluor® 594, Alexa Fluor® 480

261 (Thermo Fisher Scientific) or Alexa Fluor® 488 (Life Technologies, Carlsbad, CA) secondary

262 antibodies for 1 hour at room temperature. Sections were counterstained for DAPI (Thermo

263 Fisher Scientific) and coverslipped.

264 Fluorescently labeled human lungs were imaged by confocal with a 40x objective. CD47

265 and TSP1 imaging were done on a FLUOVIEW FV1000 or a Nikon A1 using matched imaging

266 acquisition settings across samples. The CD47 signal/ROI and TSP1 signal/ROI areas were

267 corrected by a normalization factor across samples to account for microscopy differences.

268 PECAM and von Willebrand factor signal were thresholded and used to generate endothelial

269 specific ROI. The average CD47 and TSP1 signals were measured within the ROI of PECAM

270 and von Willebrand factor (respectively) and then divided by the ROI areas. CD47 or TSP1

271 SCD-PH expression was normalized to average control expression and is reported as %

272 control.

273 Fluorescently labeled mouse lungs were imaged by confocal with a 20x objective. 3NT

274 and 4HNE imaging were done on a Nikon A1 using matched imaging acquisition settings across

275 samples. DAPI signal was thresholded and used to generate a cell specific ROI. The average

276 3NT and 4HNE signals were measured within the DAPI ROI. 3NT and 4HNE were reported as

277 intensity per cell (DAPI signal).

278

279 *Statistical analysis*

280 Results are presented as mean ± SEM unless indicated otherwise. Data, including

281 differences in hemodynamic parameters between groups of mice, were analyzed for two group

282 comparisons by Student's *t*-test for ungrouped samples; for multiple group comparisons by

283 ANOVA using Sidak's multiple comparisons test; and for myography using two-way ordinary

284 ANOVA with Sidak's multiple comparisons test. Survival after transplantation was measured by

285 Kaplan-Meyer estimate. Linear regression was used to measure the association between

286 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

290 **Results**

291 *TSP1 and its cognate receptor CD47 are upregulated in the lungs of Sickle mice and patients*

292 *with SCD-associated PH*

293   Similar to humans with SCD, Sickle mice, but not their Hemi control, develop PH as

294 determined by intact chest right heart catheterization.(32) We compared TSP1 and CD47

295 expression in the lungs of age-matched C67BL/6J, Hemi and Sickle female mice (Fig. 1A and

296 1B, Fig. 2) at 4, 9-11 and 13-14 months of age. We found that the TSP1 and CD47 expression

297 levels in the lung increased with age, as compared to both C57BL/6J and Hemi mice (Fig. 2),

298 with significant increases observed at 13-14 months of age. While levels of both proteins were

299 also higher in 4 months old and 9-11 months old Sickle mice, the difference was not significant

300 (Fig. 1A and 1B).

301   Immunofluorescence of lung sections from 6 patients with SCD-associated PH also

302 revealed increased levels of CD47 (red, Fig. 3A) as compared to lung sections from patients

303 without PH or overt lung disease (Fig. 3B). TSP1 levels (red) were elevated in lung tissue

304 sections from PH patients, although the majority of the signal appeared to originate from

305 intraluminal RBC that constitutively express large amounts of TSP1(47) (Fig. 3A), but were

306 undetectable in lung sections from control non-PH lungs (Fig. 3B). In SCD lung samples,

307 immunofluorescent CD47 was widely expressed localizing to the airways, vessels and

308 parenchyma and was also found in some vessel lumens (Fig. 3A).

309

310 *Sickle mice develop PH and vascular dysfunction*

311   We performed comprehensive open chest hemodynamic assessment of male 2-8

312 months old Sickle mice (n=9) and age-matched C57BL/6J mice (n=6) by right heart micro-

313 catheterization. Unlike intact chest catheterization,(32) the open chest technique allows capture

314 of pressure/volume loops and a more thorough assessment of cardiopulmonary pressures and

315 cardiac function. Sickle mice developed PH and RV failure: RV pressures were higher in Sickle

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

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

14

341   C57BL/6J mice (Fig. 4G), although vascular contraction in response to phenylephrine was not

342   different between the groups (Fig. 4H).

343

344   *Absence of activated parenchymal CD47 improves pulmonary hemodynamics and restores*

345   *arterial vasodilator responsiveness in chimeric mice with a sickle erythropoiesis*

346   Transplantation of Sickle mouse bone marrow into wild type mice generates chimeric

347   animals that develop the same sickle phenotype as their donors.(32, 33, 74) We therefore

348   interrogated the TSP1-CD47 axis *in vivo* by generating chimeric animals with a sickle

349   erythropoiesis on a CD47KO background. Sickle bone marrow was transplanted into C57BL/6J

350   (n=24) and CD47KO (n=27) mice in parallel, in seven separate experiments (Fig. 5A).

351   Approximately 80% of the chimeras survived to the day of assessment with no significant

352   difference in survival between the two groups (Fig. 5B). The mice underwent blood sampling for

353   determination of engraftment by electrophoresis (Fig. 5C) and full pulmonary hemodynamic

354   assessment. The chimeras had median 81% (IQ range: 43-98%) HbS, with the engraftment

355   positively correlating with spleen size, thereby showing that splenic extramedullary

356   erythropoiesis was proportional to the degree of hemolytic anemia from HbS (Fig. 5D).(33)

357   Six Sickle-to-CD47KO and three Sickle-to-C57BL/6J died intra-operatively precluding

358   hemodynamic data acquisition. We performed hemodynamic assessment of the remaining

359   Sickle-to-CD47KO and Sickle-to-C57BL/6J chimeras and analyzed the data from those that

360   were fully engrafted (>80% HbS, n=17 and n=10, respectively). RV pressures were lower in

361   Sickle-to-CD47KO chimeras as compared to Sickle-to-C57BL/6J chimeras as shown by the

362   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 ±

363   5.7 mmHg, p=0.020, Fig. 6A). The afterload of the Sickle-to-CD47KO chimeras was decreased

364   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 ±

365   0.9 vs. 2.7 ± 2.6 mmHg/µL, p=0.052, Fig. 6B). The RV systolic function of the Sickle-to-CD47KO

366   chimeras as measured by the contractility index was similar to that of the Sickle-to-C57BL/6J

367   chimeras and overall not as low as that of Sickle mice (112.4 ± 28.3 vs. 108.3 ± 25.2 L/min/m^2,

368   p=0.352, compare Fig. 4C to 6C) while the RV diastolic function as measured by the RV dP/dt$_{min}$

369   was improved in Sickle-to-CD47KO as compared to Sickle-to-C57BL/6J chimeras

370   (-1297.0 ± 318.2 vs. -1604.0 ± 668.2 mmHg/s, p=0.059, Fig. 6D). In summary, Sickle-to-

371   C57BL/6J chimeras developed PH and right ventricular heart failure, similar to their Sickle

372   donors, although the overall magnitude of change in some parameters (i.e. RV max pressure,

373   mPAP) was not as great in Sickle-to-C57BL/6J chimeric mice compared to Sickle mice

374   (compare Fig. 4A-E to Fig. 6A-E). In contrast, Sickle-to-CD47KO chimeras were protected from

375   PH and RV failure and had a cardiopulmonary phenotype that recapitulated that of control

376   C57BL/6J mice (Fig. 6A-E). *In vivo* heart function as shown by pressure-volume relations of the

377   RV of two representative Sickle-to-C57BL/6J and Sickle-to-CD47KO chimeras showed that the

378   Sickle-to-CD47KO chimeras had both a leftward shift of the ESPVR, consistent with decreased

379   hypertrophic remodeling, and increased maximum slope, consistent with improved cardiac

380   function. Specifically, the Sickle-to-CD47KO chimeras had lower EDV, indicative of decreased

381   dilation, ESV and ESP. The slope of the ESPVR was increased, indicating improved RV

382   contractility and systolic function (Fig. 6E). Interestingly, plasma collected from the chimeras

383   after euthanasia revealed lower levels of circulating TSP1 in Sickle-to-CD47KO chimeras (61.45

384   ± 10.11 vs. 76.89 ± 10.84 pg/mL, p=0.012, Fig. 6F).

385   We then compared the vascular responsiveness of isolated aortic segments of Sickle-to-

386   C57BL/6J (n=5) and Sickle-to-CD47KO chimeras (n=13) in the myograph system to determine

387   whether the improved hemodynamic profile associated with alterations in vessel activation. We

388   found that Sickle-to-CD47KO chimeras had improved endothelial-dependent vasorelaxation in

389   response to ACh as compared to Sickle-to-C57BL/6J chimeras (Fig. 6G) while there was no

390   significant difference in the effects of PE between vessel groups (Fig. 6H).

391

392 *TSP1 stimulates increased ROS in human pulmonary endothelial cells, and pulmonary oxidative*

393 *damage and vascular congestion in chimeric mice with a sickle erythropoiesis*

394 Increased ROS generation is pathogenic in SCD,(4, 17, 25, 57) and the pulmonary

395 endothelium is both a source and target of this process.(62) Our group has found that TSP1

396 promotes ROS production in animal models of ischemia-reperfusion injury.(19, 37, 48) We

397 therefore hypothesized that diminution or absence of TSP1-CD47 signaling limits PH by

398 suppressing ROS generation from sickle erythropoiesis. We first sought to demonstrate a direct

399 link between TSP1 and ROS in pulmonary vascular cells. Treatment with exogenous TSP1

400 (2.2×10^{-9} M), a concentration found in the plasma of patients with SCD,(56) for 60 minutes

401 stimulated an increased rate of $O_2^{\bullet-}$ and H_2O_2 generation in human pulmonary arterial

402 endothelial cells as measured by cytochrome c (Fig. 7A) and Amplex Red (Fig. 7B) assays

403 respectively. Importantly, 0.2×10^{-9} M TSP1, a concentration found in plasma from healthy

404 individuals, did not stimulate increased ROS in these cells (Fig. 7A, B), possibly suggesting a

405 requirement for additional hemolytic, inflammatory or vaso-occlusive stress *in vivo* to induce

406 vascular ROS production and oxidative stress. To provide further mechanistic support for the

407 overall hypothesis that TSP1 causes PH via CD47 in sickle mice, we also pretreated human

408 pulmonary arterial endothelial cells with a CD47 blocking antibody (2 µg/mL, clone B6H12.2). As

409 shown in Figure 7C, addition of the CD47 blocking antibody abrogated TSP1-stimulated ROS

410 production.

411 We then corroborated these results with "footprint" assays of TSP1 stimulation of ROS in

412 tissue cross-sections. Mouse lung tissue sections were examined by immunohistochemistry to

413 determine whether TSP1-CD47 signaling promoted ROS-mediated oxidative modifications in

414 SCD lungs. Tissue deposition of 4-hydroxynonenal (4-HNE), a stable aldehyde formed by the

415 degradation of polyunsaturated fatty acids during lipid peroxidation and 3-nitrotyrosine (3-NT), a

416 result of protein nitration by peroxynitrite (byproduct of $O_2^{\bullet-}$ reacting with nitric oxide), were

417 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

19

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 (2.2×10^{-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

506 PH.(50) Development of cell-specific CD47KO mice in tandem with existing specific NOX1

507 inhibitors(18) would likely be important in dissecting the specifics of ROS pathway activation in

508 our chimera SCD model.

509   One intriguing finding is that chimeric CD47KO mice also had decreased RBC

510 pulmonary congestion. RBC congestion has been interpreted as evidence of vaso-occlusion in

511 SCD animal models.(5, 41, 51, 52, 61) TSP1 is itself known to bind SCD RBC.(6, 31) It is

512 possible that disruption of TSP1 binding with endothelial CD47 may have limited RBC adhesion

513 in our model. Regardless, evidence that TSP1 infusion decreases hind limb reperfusion in

514 rats(19) and triggers vaso-occlusion in sickle mice(14) supports a role for circulating TSP1 in

515 acutely altering blood flow.

516   This study has a number of limitations. While Sickle mice, similar to humans with SCD,

517 develop PH, they do not display the hallmark vascular remodeling associated with human

518 disease.(32, 61) In general, there are no PH rodent models that robustly develop plexiform

519 lesions or obliterative vascular hyperplasia. Whether this discrepancy may be attributed to an

520 earlier disease stage or to intrinsically different pathogenesis is unknown. A prior study from our

521 group has pointed to nitric oxide dysfunction as a key mediator of hemolysis-induced PH in the

522 BERK model.(32) In the present study, we have not directly explored the effect of TSP1-CD47

523 on nitric oxide-dependent pathways, thus their relative contribution is unknown. Our group,

524 however, has previously reported that TSP1-CD47 inhibits nitric oxide signaling at several

525 levels.(7, 36, 38, 39) Thus, it is likely that the beneficial effect of TSP1-CD47 disruption on nitric

526 oxide in our model may have compounded effects on other redox pathways. Further, the effect

527 of the open chest procedure on mouse vascular reactivity is not known. Humans with SCD are

528 prone to vaso-occlusion and lung injury after surgical procedures.(76) Thus, it is possible that

529 the open chest catheterization led to exacerbation of disease and acute vascular dysfunction in

530 our Sickle mice and chimeras. If that is the case, at least some of the hemodynamic

531 improvement in the Sickle-to-CD47KO chimeras may have been due to protection from acute

532   injury, rather than long term protection from PH. Countering this hypothesis, it is unlikely that the

533   short surgical procedure we employed may have resulted in immediate, hemodynamically

534   significant lung injury; lending support to this latter hypothesis, acute chest syndrome does not

535   typically develop intraoperatively in humans. The use of C57BL/6J mice as a comparison group

536   for the Sickle mice in the hemodynamic experiments could arguably have been complemented

537   by analysis of Hemi mice (Fig. 4). However, having previously published on the latter mice,[32]

538   we limited our experiments to C57BL/6J controls. Finally, CD47 is not only a receptor for

539   thrombospondin family members, but also a ligand for the transmembrane signaling protein

540   SIRP alpha and a component of a supramolecular complex containing specific integrins,

541   heterotrimeric G proteins and cholesterol. Thus, KO of CD47 could have multiple effects, even

542   without change in TSP levels. For instance, CD47-SIRP alpha interaction could alter

543   inflammatory cell activity and ROS and the interaction with G proteins has been shown to alter

544   cAMP in some cells.[20] Future studies should investigate the relative contribution of these

545   pathways to PH in our model.

546   In summary, we have reported on a novel pathway that mediates SCD-associated PH.

547   TSP1-CD47, by virtue of location at the crossroads of multiple mechanisms of vasculopathy that

548   include nitric oxide dysfunction, vaso-occlusion and oxidative damage, represents a promising

549   therapeutic target in SCD. Pharmacologic inhibitors of CD47 are already clinically available[24]

550   for other diseases and may be useful in the treatment of SCD-associated PH.

551

558

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.

586

587

588

589   **References**

590

591   1.   **Al Ghouleh I, Meijles DN, Mutchler S, Zhang Q, Sahoo S, Gorelova A, Henrich**

592   **Amaral J, Rodriguez AI, Mamonova T, Song GJ, Bisello A, Friedman PA, Cifuentes-**

593   **Pagano ME, and Pagano PJ**. Binding of EBP50 to Nox organizing subunit p47phox is pivotal to

594   cellular reactive species generation and altered vascular phenotype. *Proc Natl Acad Sci U S A*

595   113: E5308-5317, 2016.

596   2.   **Aslan M, and Freeman BA**. Oxidases and oxygenases in regulation of vascular nitric

597   oxide signaling and inflammatory responses. *Immunologic research* 26: 107-118, 2002.

598   3.   **Aslan M, Ryan TM, Adler B, Townes TM, Parks DA, Thompson JA, Tousson A,**

599   **Gladwin MT, Patel RP, Tarpey MM, Batinic-Haberle I, White CR, and Freeman BA**. Oxygen

600   radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. *Proc Natl*

601   *Acad Sci U S A* 98: 15215-15220, 2001.

602   4.   **Aslan M, Thornley-Brown D, and Freeman BA**. Reactive species in sickle cell

603   disease. *Ann N Y Acad Sci* 899: 375-391, 2000.

604   5.   **Bakeer N, James J, Roy S, Wansapura J, Shanmukhappa SK, Lorenz JN, Osinska**

605   **H, Backer K, Huby AC, Shrestha A, Niss O, Fleck R, Quinn CT, Taylor MD, Purevjav E,**

606   **Aronow BJ, Towbin JA, and Malik P**. Sickle cell anemia mice develop a unique

607   cardiomyopathy with restrictive physiology. *Proc Natl Acad Sci U S A* 113: E5182-5191, 2016.

608   6.   **Barabino GA, Wise RJ, Woodbury VA, Zhang B, Bridges KA, Hebbel RP, Lawler J,**

609   **and Ewenstein BM**. Inhibition of sickle erythrocyte adhesion to immobilized thrombospondin by

610   von Willebrand factor under dynamic flow conditions. *Blood* 89: 2560-2567, 1997.

611   7.   **Bauer EM, Qin Y, Miller TW, Bandle RW, Csanyi G, Pagano PJ, Bauer PM,**

612   **Schnermann J, Roberts DD, and Isenberg JS**. Thrombospondin-1 supports blood pressure by

613   limiting eNOS activation and endothelial-dependent vasorelaxation. *Cardiovasc Res* 88: 471-

614   481, 2010.

615   8.   **Bauer PM, Bauer EM, Rogers NM, Yao M, Feijoo-Cuaresma M, Pilewski JM,**

616   **Champion HC, Zuckerbraun BS, Calzada MJ, and Isenberg JS**. Activated CD47 promotes

617   pulmonary arterial hypertension through targeting caveolin-1. *Cardiovasc Res* 2012.

618   9.   **Belcher JD, Bryant CJ, Nguyen J, Bowlin PR, Kielbik MC, Bischof JC, Hebbel RP,**

619   **and Vercellotti GM**. Transgenic sickle mice have vascular inflammation. *Blood* 101: 3953-3959,

620   2003.

621   10.   **Brittain HA, Eckman JR, Swerlick RA, Howard RJ, and Wick TM**. Thrombospondin

622   from activated platelets promotes sickle erythrocyte adherence to human microvascular

623   endothelium under physiologic flow: a potential role for platelet activation in sickle cell vaso-

624   occlusion. *Blood* 81: 2137-2143, 1993.

625   11.   **Brittain JE, Mlinar KJ, Anderson CS, Orringer EP, and Parise LV**. Activation of sickle

626   red blood cell adhesion via integrin-associated protein/CD47-induced signal transduction. *J Clin*

627   *Invest* 107: 1555-1562, 2001.

628   12.   **Brittain JE, Mlinar KJ, Anderson CS, Orringer EP, and Parise LV**. Integrin-

629   associated protein is an adhesion receptor on sickle red blood cells for immobilized

630   thrombospondin. *Blood* 97: 2159-2164, 2001.

631   13.   **Browne PV, Mosher DF, Steinberg MH, and Hebbel RP**. Disturbance of plasma and

632   platelet thrombospondin levels in sickle cell disease. *Am J Hematol* 51: 296-301, 1996.

633   14.   **Camus SM, Gausseres B, Bonnin P, Loufrani L, Grimaud L, Charue D, De Moraes**

634   **JA, Renard JM, Tedgui A, Boulanger CM, Tharaux PL, and Blanc-Brude OP**. Erythrocyte

635   microparticles can induce kidney vaso-occlusions in a murine model of sickle cell disease.

636   *Blood* 120: 5050-5058, 2012.

637   15.   **Castro O**. Systemic fat embolism and pulmonary hypertension in sickle cell disease.

638   *Hematology/oncology clinics of North America* 10: 1289-1303, 1996.

639   16.   **Cevik O, Baykal AT, and Sener A**. Platelets Proteomic Profiles of Acute Ischemic

640   Stroke Patients. *PLoS One* 11: e0158287, 2016.

641   17.   **Chirico EN, and Pialoux V**. Role of oxidative stress in the pathogenesis of sickle cell

642   disease. *IUBMB Life* 64: 72-80, 2012.

643   18.   **Cifuentes-Pagano ME, Meijles DN, and Pagano PJ**. Nox Inhibitors & Therapies:

644   Rational Design of Peptidic and Small Molecule Inhibitors. *Curr Pharm Des* 21: 6023-6035,

645   2015.

646   19.   **Csanyi G, Yao M, Rodriguez AI, Al Ghouleh I, Sharifi-Sanjani M, Frazziano G,**

647   **Huang X, Kelley EE, Isenberg JS, and Pagano PJ**. Thrombospondin-1 regulates blood flow

648   via CD47 receptor-mediated activation of NADPH oxidase 1. *Arterioscler Thromb Vasc Biol* 32:

649   2966-2973, 2012.

650   20.   **Frazier WA, Gao AG, Dimitry J, Chung J, Brown EJ, Lindberg FP, and Linder ME**.

651   The thrombospondin receptor integrin-associated protein (CD47) functionally couples to

652   heterotrimeric Gi. *J Biol Chem* 274: 8554-8560, 1999.

653   21.   **Frenette PS**. Sickle cell vaso-occlusion: multistep and multicellular paradigm. *Current*

654   *opinion in hematology* 9: 101-106, 2002.

655   22.   **George A, Pushkaran S, Konstantinidis DG, Koochaki S, Malik P, Mohandas N,**

656   **Zheng Y, Joiner CH, and Kalfa TA**. Erythrocyte NADPH oxidase activity modulated by Rac

657   GTPases, PKC, and plasma cytokines contributes to oxidative stress in sickle cell disease.

658   *Blood* 121: 2099-2107, 2013.

659   23.   **George MP, Novelli EM, Shigemura N, Simon MA, Feingold B, Krishnamurti L,**

660   **Morrell MR, Gries CG, Haider S, Johnson BA, Crespo MM, Bhama JK, Bermudez C,**

661   **Yousem SA, Toyoda Y, Champion HC, Pilewski JM, and Gladwin MT**. First successful lung

662   transplantation for sickle cell disease with severe pulmonary arterial hypertension and

663   pulmonary veno-occlusive disease. *Pulm Circ* 3: 952-958, 2013.

664   24.   **Gholamin S, Mitra SS, Feroze AH, Liu J, Kahn SA, Zhang M, Esparza R, Richard C,**

665   **Ramaswamy V, Remke M, Volkmer AK, Willingham S, Ponnuswami A, McCarty A,**

666   **Lovelace P, Storm TA, Schubert S, Hutter G, Narayanan C, Chu P, Raabe EH, Harsh Gt,**

667 **Taylor MD, Monje M, Cho YJ, Majeti R, Volkmer JP, Fisher PG, Grant G, Steinberg GK,**

668 **Vogel H, Edwards M, Weissman IL, and Cheshier SH**. Disrupting the CD47-SIRPalpha anti-

669 phagocytic axis by a humanized anti-CD47 antibody is an efficacious treatment for malignant

670 pediatric brain tumors. *Science translational medicine* 9: 2017.

671 25. **Gizi A, Papassotiriou I, Apostolakou F, Lazaropoulou C, Papastamataki M,**

672 **Kanavaki I, Kalotychou V, Goussetis E, Kattamis A, Rombos I, and Kanavakis E**.

673 Assessment of oxidative stress in patients with sickle cell disease: The glutathione system and

674 the oxidant-antioxidant status. *Blood Cells Mol Dis* 46: 220-225, 2011.

675 26. **Gladwin MT, Sachdev V, Jison ML, Shizukuda Y, Plehn JF, Minter K, Brown B,**

676 **Coles WA, Nichols JS, Ernst I, Hunter LA, Blackwelder WC, Schechter AN, Rodgers GP,**

677 **Castro O, and Ognibene FP**. Pulmonary hypertension as a risk factor for death in patients with

678 sickle cell disease. *N Engl J Med* 350: 886-895, 2004.

679 27. **Gupta K, Gupta P, Wild R, Ramakrishnan S, and Hebbel RP**. Binding and

680 displacement of vascular endothelial growth factor (VEGF) by thrombospondin: effect on human

681 microvascular endothelial cell proliferation and angiogenesis. *Angiogenesis* 3: 147-158, 1999.

682 28. **Hebbel RP, Boogaerts MA, Eaton JW, and Steinberg MH**. Erythrocyte adherence to

683 endothelium in sickle-cell anemia. A possible determinant of disease severity. *N Engl J Med*

684 302: 992-995, 1980.

685 29. **Hebbel RP, Boogaerts MA, Koresawa S, Jacob HS, Eaton JW, and Steinberg MH**.

686 Erytrocyte adherence to endothelium as a determinant of vasocclusive severity in sickle cell

687 disease. *Transactions of the Association of American Physicians* 93: 94-99, 1980.

688 30. **Hebbel RP, Morgan WT, Eaton JW, and Hedlund BE**. Accelerated autoxidation and

689 heme loss due to instability of sickle hemoglobin. *Proc Natl Acad Sci U S A* 85: 237-241, 1988.

690 31. **Hillery CA, Scott JP, and Du MC**. The carboxy-terminal cell-binding domain of

691 thrombospondin is essential for sickle red blood cell adhesion. *Blood* 94: 302-309, 1999.

692   32.   **Hsu LL, Champion HC, Campbell-Lee SA, Bivalacqua TJ, Manci EA, Diwan BA,**

693   **Schimel DM, Cochard AE, Wang X, Schechter AN, Noguchi CT, and Gladwin MT**.

694   Hemolysis in sickle cell mice causes pulmonary hypertension due to global impairment in nitric

695   oxide bioavailability. *Blood* 109: 3088-3098, 2007.

696   33.   **Iannone R, Luznik L, Engstrom LW, Tennessee SL, Askin FB, Casella JF, Kickler**

697   **TS, Goodman SN, Hawkins AL, Griffin CA, Noffsinger L, and Fuchs EJ**. Effects of mixed

698   hematopoietic chimerism in a mouse model of bone marrow transplantation for sickle cell

699   anemia. *Blood* 97: 3960-3965, 2001.

700   34.   **Isenberg JS, Frazier WA, and Roberts DD**. Thrombospondin-1: a physiological

701   regulator of nitric oxide signaling. *Cell Mol Life Sci* 65: 728-742, 2008.

702   35.   **Isenberg JS, Hyodo F, Matsumoto K, Romeo MJ, Abu-Asab M, Tsokos M,**

703   **Kuppusamy P, Wink DA, Krishna MC, and Roberts DD**. Thrombospondin-1 limits ischemic

704   tissue survival by inhibiting nitric oxide-mediated vascular smooth muscle relaxation. *Blood* 109:

705   1945-1952, 2007.

706   36.   **Isenberg JS, Jia Y, Fukuyama J, Switzer CH, Wink DA, and Roberts DD**.

707   Thrombospondin-1 inhibits nitric oxide signaling via CD36 by inhibiting myristic acid uptake. *J*

708   *Biol Chem* 282: 15404-15415, 2007.

709   37.   **Isenberg JS, Maxhimer JB, Powers P, Tsokos M, Frazier WA, and Roberts DD**.

710   Treatment of liver ischemia-reperfusion injury by limiting thrombospondin-1/CD47 signaling.

711   *Surgery* 144: 752-761, 2008.

712   38.   **Isenberg JS, Ridnour LA, Perruccio EM, Espey MG, Wink DA, and Roberts DD**.

713   Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent

714   manner. *Proc Natl Acad Sci U S A* 102: 13141-13146, 2005.

715   39.   **Isenberg JS, Wink DA, and Roberts DD**. Thrombospondin-1 antagonizes nitric oxide-

716   stimulated vascular smooth muscle cell responses. *Cardiovasc Res* 71: 785-793, 2006.

717   40.   **Kassa T, Jana S, Strader MB, Meng F, Jia Y, Wilson MT, and Alayash AI**. Sickle Cell

718   Hemoglobin in the Ferryl State Promotes betaCys-93 Oxidation and Mitochondrial Dysfunction

719   in Epithelial Lung Cells (E10). *J Biol Chem* 290: 27939-27958, 2015.

720   41.   **Kauf TL, Coates TD, Huazhi L, Mody-Patel N, and Hartzema AG**. The cost of health

721   care for children and adults with sickle cell disease. *Am J Hematol* 84: 323-327, 2009.

722   42.   **Kaul DK, Fabry ME, and Nagel RL**. Erythrocytic and vascular factors influencing the

723   microcirculatory behavior of blood in sickle cell anemia. *Ann N Y Acad Sci* 565: 316-326, 1989.

724   43.   **Kaur S, Martin-Manso G, Pendrak ML, Garfield SH, Isenberg JS, and Roberts DD**.

725   Thrombospondin-1 inhibits VEGF receptor-2 signaling by disrupting its association with CD47. *J*

726   *Biol Chem* 285: 38923-38932, 2010.

727   44.   **Kohli DR, Li Y, Khasabov SG, Gupta P, Kehl LJ, Ericson ME, Nguyen J, Gupta V,**

728   **Hebbel RP, Simone DA, and Gupta K**. Pain-related behaviors and neurochemical alterations

729   in mice expressing sickle hemoglobin: modulation by cannabinoids. *Blood* 116: 456-465, 2010.

730   45.   **Labrousse-Arias D, Castillo-Gonzalez R, Rogers NM, Torres-Capelli M, Barreira B,**

731   **Aragones J, Cogolludo A, Isenberg JS, and Calzada MJ**. HIF-2alpha-mediated induction of

732   pulmonary thrombospondin-1 contributes to hypoxia-driven vascular remodelling and

733   vasoconstriction. *Cardiovasc Res* 109: 115-130, 2016.

734   46.   **Lindberg FP, Bullard DC, Caver TE, Gresham HD, Beaudet AL, and Brown EJ**.

735   Decreased resistance to bacterial infection and granulocyte defects in IAP-deficient mice.

736   *Science* 274: 795-798, 1996.

737   47.   **Lindberg FP, Lublin DM, Telen MJ, Veile RA, Miller YE, Donis-Keller H, and Brown**

738   **EJ**. Rh-related antigen CD47 is the signal-transducer integrin-associated protein. *J Biol Chem*

739   269: 1567-1570, 1994.

740   48.   **Maxhimer JB, Shih HB, Isenberg JS, Miller TW, and Roberts DD**. Thrombospondin-

741   1/CD47 blockade following ischemia-reperfusion injury is tissue protective. *Plast Reconstr Surg*

742   124: 1880-1889, 2009.

743   49.   **Mehari A, Alam S, Tian X, Cuttica MJ, Barnett CF, Miles G, Xu D, Seamon C, Adams-Graves P, Castro OL, Minniti CP, Sachdev V, Taylor JGt, Kato GJ, and Machado RF**. Hemodynamic predictors of mortality in adults with sickle cell disease. *American journal of respiratory and critical care medicine* 187: 840-847, 2013.

747   50.   **Meijles DN, Sahoo S, Al Ghouleh I, Amaral JH, Bienes-Martinez R, Knupp HE, Attaran S, Sembrat JC, Nouraie SM, Rojas MM, Novelli EM, Gladwin MT, Isenberg JS, Cifuentes-Pagano E, and Pagano PJ**. The matricellular protein TSP1 promotes human and mouse endothelial cell senescence through CD47 and Nox1. *Sci Signal* 10: 2017.

751   51.   **Nath KA, Grande JP, Haggard JJ, Croatt AJ, Katusic ZS, Solovey A, and Hebbel RP**. Oxidative stress and induction of heme oxygenase-1 in the kidney in sickle cell disease. *The American journal of pathology* 158: 893-903, 2001.

754   52.   **Nath KA, Shah V, Haggard JJ, Croatt AJ, Smith LA, Hebbel RP, and Katusic ZS**. Mechanisms of vascular instability in a transgenic mouse model of sickle cell disease. *American journal of physiology* 279: R1949-1955, 2000.

757   53.   **Nevitt C, McKenzie G, Christian K, Austin J, Hencke S, Hoying J, and LeBlanc A**. Physiological levels of thrombospondin-1 decrease NO-dependent vasodilation in coronary microvessels from aged rats. *American journal of physiology* 310: H1842-1850, 2016.

760   54.   **Novelli EM, Cheng L, Yang Y, Leung W, Ramirez M, Tanavde V, Enger C, and Civin CI**. Ex vivo culture of cord blood CD34+ cells expands progenitor cell numbers, preserves engraftment capacity in nonobese diabetic/severe combined immunodeficient mice, and enhances retroviral transduction efficiency. *Hum Gene Ther* 10: 2927-2940, 1999.

764   55.   **Novelli EM, Kato GJ, Hildesheim ME, Barge S, Meyer MP, Lozier J, Hassett AC, Ragni MV, Isenberg JS, and Gladwin MT**. Thrombospondin-1 inhibits ADAMTS13 activity in sickle cell disease. *Haematologica* 98: e132-134, 2013.

767   56.   **Novelli EM, Kato GJ, Ragni MV, Zhang Y, Hildesheim ME, Nouraie M, Barge S, Meyer MP, Hassett AC, Gordeuk VR, Gladwin MT, and Isenberg JS**. Plasma

769 thrombospondin-1 is increased during acute sickle cell vaso-occlusive events and associated
770 with acute chest syndrome, hydroxyurea therapy, and lower hemolytic rates. *Am J Hematol* 87:
771 326-330, 2012.

772 57. **Nur E, Biemond BJ, Otten HM, Brandjes DP, and Schnog JJ**. Oxidative stress in
773 sickle cell disease; pathophysiology and potential implications for disease management. *Am J*
774 *Hematol* 86: 484-489, 2011.

775 58. **Ochoa CD, Yu L, Al-Ansari E, Hales CA, and Quinn DA**. Thrombospondin-1 null mice
776 are resistant to hypoxia-induced pulmonary hypertension. *J Cardiothorac Surg* 5: 32, 2010.

777 59. **Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, and Lindberg**
778 **FP**. Role of CD47 as a marker of self on red blood cells. *Science* 288: 2051-2054, 2000.

779 60. **Parent F, Bachir D, Inamo J, Lionnet F, Driss F, Loko G, Habibi A, Bennani S,**
780 **Savale L, Adnot S, Maitre B, Yaici A, Hajji L, O'Callaghan DS, Clerson P, Girot R,**
781 **Galacteros F, and Simonneau G**. A hemodynamic study of pulmonary hypertension in sickle
782 cell disease. *N Engl J Med* 365: 44-53, 2011.

783 61. **Paszty C, Brion CM, Manci E, Witkowska HE, Stevens ME, Mohandas N, and Rubin**
784 **EM**. Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell
785 disease. *Science* 278: 876-878, 1997.

786 62. **Potoka KP, and Gladwin MT**. Vasculopathy and pulmonary hypertension in sickle cell
787 disease. *Am J Physiol Lung Cell Mol Physiol* 308: L314-324, 2015.

788 63. **Ramirez M, Rottman GA, Shultz LD, and Civin CI**. Mature human hematopoietic cells
789 in donor bone marrow complicate interpretation of stem/progenitor cell assays in xenogeneic
790 hematopoietic chimeras. *Experimental hematology* 26: 332-344, 1998.

791 64. **Rogers NM, Ghimire K, Calzada MJ, and Isenberg JS**. Matricellular Protein
792 Thrombospondin-1 in Pulmonary Hypertension: Multiple Pathways to Disease. *Cardiovasc Res*
793 2017.

794   65.   **Rogers NM, Roberts DD, and Isenberg JS**. Age-associated induction of cell

795   membrane CD47 limits basal and temperature-induced changes in cutaneous blood flow.

796   *Annals of surgery* 258: 184-191, 2013.

797   66.   **Rogers NM, Sharifi-Sanjani M, Yao M, Ghimire K, Bienes-Martinez R, Mutchler SM,**

798   **Knupp HE, Baust J, Novelli EM, Ross M, St Croix C, Kutten JC, Czajka CA, Sembrat JC,**

799   **Rojas M, Labrousse-Arias D, Bachman TN, Vanderpool RR, Zuckerbraun BS, Champion**

800   **HC, Mora AL, Straub AC, Bilonick RA, Calzada MJ, and Isenberg JS**. TSP1-CD47 signaling

801   is upregulated in clinical pulmonary hypertension and contributes to pulmonary arterial

802   vasculopathy and dysfunction. *Cardiovasc Res* 113: 15-29, 2017.

803   67.   **Rogers NM, Thomson AW, and Isenberg JS**. Activation of parenchymal CD47

804   promotes renal ischemia-reperfusion injury. *J Am Soc Nephrol* 23: 1538-1550, 2012.

805   68.   **Rogers NM, Yao M, Sembrat J, George MP, Knupp H, Ross MA, Sharifi-Sanjani M,**

806   **Milosevic J, St. Croix C, Rajkumar R, Frid MG, Hunter KS, Mazzaro L, Novelli EM,**

807   **Stenmark KR, Gladwin MT, Ahmad F, Champion HC, and Isenberg JS**. Cellular,

808   pharmacological, and biophysical evaluation of explanted lungs from a patient with sickle cell

809   disease and severe pulmonary arterial hypertension. *Pulmonary Circulation* 3: 2013.

810   69.   **Shen L, Liao L, Chen C, Guo Y, Song D, Wang Y, Chen Y, Zhang K, Ying M, Li S,**

811   **Liu Q, and Ni J**. Proteomics Analysis of Blood Serums from Alzheimer's Disease Patients Using

812   iTRAQ Labeling Technology. *Journal of Alzheimer's disease : JAD* 56: 361-378, 2017.

813   70.   **Smadja DM, d'Audigier C, Bieche I, Evrard S, Mauge L, Dias JV, Labreuche J,**

814   **Laurendeau I, Marsac B, Dizier B, Wagner-Ballon O, Boisson-Vidal C, Morandi V, Duong-**

815   **Van-Huyen JP, Bruneval P, Dignat-George F, Emmerich J, and Gaussem P**.

816   Thrombospondin-1 is a plasmatic marker of peripheral arterial disease that modulates

817   endothelial progenitor cell angiogenic properties. *Arterioscler Thromb Vasc Biol* 31: 551-559,

818   2011.

819   71.   **Solovey A, Gui L, Key NS, and Hebbel RP**. Tissue factor expression by endothelial

820   cells in sickle cell anemia. *J Clin Invest* 101: 1899-1904, 1998.

821   72.   **Solovey A, Lin Y, Browne P, Choong S, Wayner E, and Hebbel RP**. Circulating

822   activated endothelial cells in sickle cell anemia. *N Engl J Med* 337: 1584-1590, 1997.

823   73.   **Turhan A, Jenab P, Bruhns P, Ravetch JV, Coller BS, and Frenette PS**. Intravenous

824   immune globulin prevents venular vaso-occlusion in sickle cell mice by inhibiting leukocyte

825   adhesion and the interactions between sickle erythrocytes and adherent leukocytes. *Blood* 103:

826   2397-2400, 2004.

827   74.   **Turhan A, Weiss LA, Mohandas N, Coller BS, and Frenette PS**. Primary role for

828   adherent leukocytes in sickle cell vascular occlusion: a new paradigm. *Proc Natl Acad Sci U S A*

829   99: 3047-3051, 2002.

830   75.   **Verresen D, De Backer W, and Vermeire P**. Pulmonary hypertension and sickle

831   hemoglobinopathy. *Chest* 98: 1042, 1990.

832   76.   **Vichinsky EP, Haberkern CM, Neumayr L, Earles AN, Black D, Koshy M, Pegelow**

833   **C, Abboud M, Ohene-Frempong K, and Iyer RV**. A comparison of conservative and

834   aggressive transfusion regimens in the perioperative management of sickle cell disease. The

835   Preoperative Transfusion in Sickle Cell Disease Study Group. *N Engl J Med* 333: 206-213,

836   1995.

837   77.   **Wood KC, Hsu LL, and Gladwin MT**. Sickle cell disease vasculopathy: a state of nitric

838   oxide resistance. *Free Radic Biol Med* 44: 1506-1528, 2008.

839   78.   **Yao M, Roberts DD, and Isenberg JS**. Thrombospondin-1 inhibition of vascular smooth

840   muscle cell responses occurs via modulation of both cAMP and cGMP. *Pharmacological*

841   *research : the official journal of the Italian Pharmacological Society* 63: 13-22, 2011.

842   79.   **Yao M, Rogers NM, Csanyi G, Rodriguez AI, Ross MA, St. Croix C, Knupp H,**

843   **Novelli EM, Thomson AW, Pagano PJ, and Isenberg JS**. Thrombospondin-1 Activation of

844 Signal-Regulatory Protein-a Stimulates Reactive Oxygen Species Production and Promotes

845 Renal Ischemia Reperfusion Injury. *J Am Soc Nephrol* 25: 2014.

846 80.   **Zielonka J, Sikora A, Joseph J, and Kalyanaraman B**. Peroxynitrite is the major

847 species formed from different flux ratios of co-generated nitric oxide and superoxide: direct

848 reaction with boronate-based fluorescent probe. *J Biol Chem* 285: 14210-14216, 2010.

849

Figure legends

851

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

853 (A-B) Lung lysates from female BERK sickling (Sickle), BERK non sickling hemizygous

854 controls (Hemi), C57BL/6J controls (C57BL), CD47KO and TSP1KO mice (n=20) underwent

855 Western Blotting for TSP1 and CD47. CD47KO and TSP1KO mice were used as positive and

856 negative controls for the Western Blot procedure. Each lane represents an individual animal and

857 all data are shown. Densitometry results are shown (normalized to β-actin). Unpaired t-test was

858 applied for comparison between each group. Results are shown as means ± SEM (Standard

859 Error of the Mean).

860

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

862 Lung lysates from 13-14 months old female BERK sickling (Sickle), BERK non sickling

863 hemizygous controls (Hemi), C57BL/6J controls (C57BL), CD47KO and TSP1KO mice (n=26)

864 underwent Western Blotting for TSP1 and CD47. CD47KO and TSP1KO mice were used as

865 positive and negative controls for the Western Blot procedure. Each lane represents an

866 individual animal and all data are shown. The 13-14 months old Sickle mice had a significant

867 increase in pulmonary TSP1 and CD47 as compared to Hemi and C57BL mice. Densitometry

868 results are shown (normalized to β-actin). One way ANOVA with multiple comparisons was

869 applied. Results are shown as means ± SEM. $*P<0.1$, $**P<0.01$, $***P<0.001****P<0.0001$.

870

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

872 *associated PH*

873 TSP1 and CD47 expression was measured by immunofluorescence in the lung sections

874 from 6 patients with SCD-associated PH (SCD-PH) and 6 control patients without PH or overt

875 lung disease. Expression of TSP1 (red) (vWF-green, DAPI-blue) (upper photograph for each

876   patient) was increased in patients with SCD-PH although the signal appeared to originate from

877   intraluminal RBC (A). Increased levels of CD47 (red) (PECAM-green, DAPI-blue) (lower

878   photograph for each patient) were consistently observed in lung sections from all patients as

879   compared to control patients (B). CD47 or TSP1 SCD-PH expression is reported as % control

880   patient. (C). Bar represents 50 µM. Unpaired t-test was applied for comparison between the two

881   groups. Results are shown as means ± SEM. ****P<0.0001.

882

883   *Figure 4: Sickle mice develop PH and vascular dysfunction*

884   Open chest hemodynamic assessment of male 2-8 months old Sickle mice (n=9) and

885   age-matched C57BL mice (n=6) was performed by open chest right heart micro-catheterization.

886   RV pressures were measured by the RV max p and the mPAP (A). Afterload was measured by

887   PVR and RV Ea (B). RV systolic function was measured by the contractility index (C). RV

888   diastolic function was measured by the RV dP/dt_{min}, a measure of RV stiffness (D). Pressure-

889   volume relations of the RV of two representative sickle and C57BL/6J mice (E). Plasma TSP1

890   was measured by ELISA at the time of euthanasia (F). Vascular reactivity of isolated aortic

891   segments of male age-matched Sickle (n=6) and C57BL (n=5) mice was assessed in the

892   myograph system in response to Ach and PE (G-H). Unpaired t-test was applied for comparison

893   between the two groups of mice. Results are shown as means ± SEM. Two-way ordinary

894   ANOVA with Sidak's multiple comparisons test was used for the myograph data. *P<0.05,

895   **P<0.01, ***P<0.001, ****P<0.0001.

896

897   *Figure 5: Generation of chimeras and measurement of engraftment*

898   The TSP1-CD47 axis was interrogated *in vivo* by generating chimeric animals with a

899   sickle erythropoiesis on a CD47KO background. Bone marrow was harvested from flushed

900   femurs and tibias of adult Sickle mice and whole bone marrow (5 X 10^6 cells) were transplanted

901   into age-matched, lethally myeloablated (10 Gy) two months old CD47KO (n=27) and C57BL

902   (n=24) mice, the background strain of CD47KO mice, by retro-orbital sinus injection in 7

903   separate experiments. Schematic representation of the transplantation protocol (A). Kaplan-

904   Meyer survival curve of transplanted chimeras (B). Engraftment was assessed by measurement

905   of HbS percentage by HPLC followed by confirmatory capillary zone electrophoresis (CZE) in

906   blood samples obtained at the time of euthanasia, 4-6 months after transplantation. A CZE gel

907   is shown (upper left quadrant). Human HbA and HbS were readily detectable in a C57BL

908   recipient transplanted with Sickle BM with mixed chimerism (upper right quadrant). CZE of a

909   C57BL recipient (lower left quadrant) and Sickle mouse donor (lower right) are also shown (C).

910   Linear regression of engraftment and spleen size (D). Results are shown as means ± SEM

911   unless otherwise noted. ***$P<0.001$.

912

913   *Figure 6: Absence of tissue-resident TSP1-CD47 signaling improves pulmonary hemodynamics*

914   *and arterial vasodilator responsiveness in chimeric mice with a sickle erythropoiesis.*

915   Full hemodynamic assessment of Sickle-to-CD47KO and Sickle-to-C57BL chimeras was

916   performed by open chest right heart micro-catheterization. RV pressures included RV max p

917   and mPAP (A). The afterload was measured as PVR and RV Ea (B). The RV systolic function

918   was measured by the contractility index (C) while the RV diastolic function was measured by the

919   RV dP/dt$_{min}$ (D). Pressure-volume relations of the RV of two representative Sickle-to-C57BL and

920   Sickle-to-CD47KO chimeras (E). Plasma TSP1 was measured by ELISA at the time of

921   euthanasia (F). Vascular reactivity of isolated aortic segments of Sickle-to-C57BL (n=5) and

922   Sickle-to-CD47KO chimeras (n=13) was assessed in the myograph system in response to Ach

923   and PE (G-H). An unpaired t-test was applied for comparison between the two groups of mice.

924   Results are shown as means ± SEM. Two-way ordinary ANOVA with Sidak's multiple

925   comparisons test was used for the myograph data. *$P<0.05$.

926

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

928   Commercially available hPAEC (n=1 donor) were treated with vehicle or TSP1 (0-10

929   nmol/L) for 60 minutes. ROS production was measured using two independent complementary

930   assays: O_2^- generation by total cellular homogenates measured by cytochrome c reduction (A);

931   and endothelial cell homogenate H_2O_2 production measured using the Amplex Red assay (B).

932   One-way ANOVA followed by Sidak's multiple comparisons test was applied for the analysis.

933   Results are shown as means ± SEM of three experiments.**$P<0.01$

934   Commercially available hPAEC (n=2 donors) were established in a 96 well plate and

935   directly exposed to the following treatments for 60 min: vehicle, TSP-1 (10nM, 2.2nM, or 0.2nM),

936   or 2.2nM TSP-1 with 2µg/ml CD47 blocking antibody [clone B6H12.2], and select wells received

937   1 KU/ml bovine liver catalase to act as a negative control. Coumarin Boronic Acid probe

938   detection of H_2O_2 production in the cells was measured kinetically for 2 hours (C). The average

939   rate of fluorescence generation was normalized to the vehicle control and displayed as fold

940   change in H_2O_2 production. Means ± SEM results are shown of three to four experiments per

941   donor, total n=6-7. *$P<0.05$, **$P<0.01$, ****$P<0.0001$

942

943   *Figure 8. TSP1 augments pulmonary oxidative damage and promotes vascular congestion in*

944   *chimeric mice with a sickle erythropoiesis*

945   Lung tissue sections from chimeras were stained for 4HNE or 3NT and examined by

946   immunofluorescence to determine whether *in vivo* SCD-mediated pulmonary ROS production

947   (4-hydroxynonenal, 4-HNE) and secondary ROS-mediated protein modifications (3-

948   nitrotyrosine, 3-NT) were modified by absence of TSP1-CD47 signaling. 3-NT (green

949   fluorescence, DAPI-blue) staining (A) (n=3 per group, 2 sections per animal). (A) also shows

950   representative 4-HNE deposition by green fluorescent immunohistochemical staining (DAPI-

951   blue) (n=2 mice per group, 3 sections per animal). The fluorescent signal was quantified using

952   ImageJ software and reported as 3-NT or 4-HNE intensity per cell (DAPI signal). Vascular

953   congestion on H&E-stained slides from chimeras (n=3 mice per group) was rated by three

954   blinded, independent readers. The readers used a semi quantitative, relative, 0 to 4 scale where

955   absence of RBC in the lumens of pulmonary blood vessels was rated as 0. Increasing lumen

956   congestion and number of affected vessels were rated 1 to 4 (B). Images were taken using a

957   Nikon A1 confocal microscope or Nikon 90i at 20x. Bars represent 50 µM. Results are shown as

958   representative slides and means ± SEM. *$P<0.05$, ****$P<0.0001$.