SUPPLEMENTARY APPENDIX

Repurposing pyridoxamine for therapeutic intervention of intravascular cell-cell interactions in mouse models of sickle cell disease

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Supplemental Methods

Materials. Pyridoxamine, Vitamin B6 (pyridoxine), HU, N-formyl-methionyl-leucyl-phenylalanine (fMLP), 2',7'-dichlorofluorescein diacetate (DCFH-DA), Percoll, donkey serum, human thrombin, prostaglandin E1 (PGE1), red blood cell lysis buffer, hematoxylin and eosin solution, and acid citrate dextrose were purchased from Sigma (St. Louis, MO). D-Phe-Pro-Arg-chloromethyl ketone (PPACK) was from EMD Millipore (Billerica, MA). The luciferin/luciferase reagent for measurement of ATP release was purchased from Chrono-log Corporation (Havertown, PA). Collagen-related peptide (CRP) was obtained from Dr. Richard Farndale (Department of Biochemistry, University of Cambridge, UK). Recombinant mouse TNF-α, a monoclonal anti-mouse E-selectin (10E9.6) antibody, and ELISA kits for soluble ICAM-1, E-selectin, IL-1β and IL-6 were obtained from R&D Systems (Minneapolis, MN). A polyclonal antibody against β-actin was purchased from Novus Biologicals (Littleton, CO). Isotype control IgGs, Alexa Fluor 647-conjugated anti-Ly-6G, Alexa Fluor 647-conjugated anti-mouse CXCR4 (L276F12), PE-conjugated anti-mouse αMβ2 (M1/70), APC-conjugated anti-mouse platelet-endothelial cell adhesion molecules-1 (PECAM-1) (MEC13.3), monoclonal anti-mouse intercellular adhesion molecule-1 (ICAM-1) (YN1/1.74), PE-conjugated anti-mouse L-selectin (MEL-14), PE-conjugated anti-human L-selectin (DREG-56), and FITC-conjugated anti-human activated αIIbβ3 (PAC-1) antibodies were obtained from BioLegend (San Diego, CA). DyLight 488-conjugated anti-mouse CD42c, PE-conjugated anti-mouse P-selectin (Wug.E9), and PE-conjugated anti-activated αIIbβ3 (JON/A) antibodies were from Emfret Analytics (Eibelstadt, Germany). A PE-conjugated anti-human P-selectin antibody (AC1.2) was from BD Biosciences (San Jose, CA). Antibodies against phosphorylated

phosphoinositide 3-kinase (p-PI3K) p85 (Tyr458)/p55 (Tyr199), phosphorylated Akt (p-Akt Ser473), total Akt, or β-actin were obtained from Cell Signaling (Danvers, MA). A nitrate/nitrite colorimetric assay kit was purchased from Cayman Chemical (Ann Arbor, MI). An immunotag mouse AGEs ELISA kit was obtained from G-Biosciences (St. Louis, MO). Vectashield containing DAPI was from Vector Laboratories (Burlingame, California). Tissue-Tek® O.C.T. Compound was purchased from Sakura Finetek USA (Torrance, CA). Urine osmometer controls were obtained from Advanced Instruments (Norwood, MA). A DyLight 488-conjugated goat anti-rat IgG antibody was from Thermo Fisher Scientific (Carlsbad, CA).

Mice. WT (C57BL/6, 6-7 weeks old male), Berkeley (SCD) (Tg(Hu-miniLCRα1 $^{G}\gamma^{A}\gamma\delta\beta^{S}$) *Hba*$^{-/-}$ *Hbb*$^{-/-}$), and hemizygous control (Tg(Hu-miniLCRα1 $^{G}\gamma^{A}\gamma\delta\beta^{S}$) *Hba*$^{-/-}$ *Hbb*$^{+/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Chimeric Berkeley mice were generated by transplantation of bone marrow cells isolated from Berkeley mice into lethally irradiated WT mice as we reported.[1] Three months after transplantation, PCR analysis showed that chimeric mice expressed the transgene (human HbS) as described previously.[2] These chimeric Berkeley mice are hereafter referred to as SCD mice and were used at 20-24 and 34-38 weeks old for short- and long-term treatment with pyridoxamine, respectively. The University of Illinois Institutional Animal Care and Use Committee approved all animal care and experimental procedures.

SCD patients. Six homozygous (HbSS) adult patients (18-60 years, 3 men and 3 women) were included in our studies. All patients were under HU therapy prior to blood donation.

Blood was drawn at routine clinic visits without a pain crisis. Multiple experiments were performed using one patient blood sample and each experiment was repeated with blood from 3-4 different patients. All patients enrolled in this study provided informed consent. The collection and use of blood samples for laboratory analysis were approved by the Institutional Review Board of the University of Illinois at Chicago.

Isolation of platelets and neutrophils. Platelets were isolated from SCD mice and patients as we described previously.[2] Platelets were suspended in HEPES-Tyrode buffer (20 mM HEPES, pH 7.3, 136 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, and 5.5 mM glucose without $CaCl_2$) at a concentration of 3 x 10^8 platelets/ml. Neutrophils were isolated from the bone marrow of SCD mice as described previously.[3] Human neutrophils were isolated using an EasySep™ Direct Human Neutrophil Isolation kit according to the manufacturer's instructions. Human and mouse neutrophils were adjusted to 2 x 10^6 and 1 x 10^7 neutrophils/ml, respectively, in RPMI1640 media, unless otherwise stated.

Platelet aggregation. Washed platelets in HEPES-Tyrode buffer were pre-treated with vehicle, vitamin B6, or pyridoxamine for 10 minutes at 37°C, followed by stimulation with various concentrations of thrombin or CRP. ATP secretion (dense granule secretion) was monitored as ADP/ATP release by addition of luciferin/luciferase reagent to the platelet suspension. Platelet aggregation and ATP secretion were measured in a platelet lumi-aggregometer (Chrono-log Corporation) at 37°C with stirring (1,000 rpm).

Flow cytometry. Neutrophils were treated with vehicle, vitamin B6, or pyridoxamine for

30 minutes at 37°C, followed by treatment with or without fMLP for 10 minutes at 37°C. The cells were labeled with PE-conjugated anti-αMβ2 or anti-L-selectin, or Alexa Fluor 647-conjugated anti-Ly-6G antibodies, or an isotype control IgG for 20 minutes at room temperature. Platelets were treated with vehicle, vitamin B6, or pyridoxamine for 10 minutes at 37°C, followed by incubation with or without thrombin for 5 minutes at 37°C. After the addition of 50 μM PPACK, the cells were labeled with FITC- or PE-conjugated anti-P-selectin, anti-activated αIIbβ3, or control IgG for 30 minutes. In some experiments, neutrophils or platelets were incubated with 20 μM DCFH-DA for 15 minutes at 37°C and then treated with vehicle, vitamin B6, or pyridoxamine for 15 minutes at 37°C, followed by stimulation with an agonist. After fixation with 1% paraformaldehyde, flow cytometry was performed using a CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, IN) and analyzed by the mean fluorescence intensity values.

Neutrophil aging. SCD mice were housed in a 14-hour light (5 AM to 7 PM):10-hour dark (7 PM to 5 AM) cycle. After fasting for 12 hours, mice were bled from the tail, and 10 μl of blood was collected into a tube containing 20 μl ACD. Pyridoxamine (100 mg/kg BW) was given orally to mice, followed by blood drawing at 11 AM, 3 PM, and 7 PM. After lysis of red blood cells, blood cells were resuspended with Hank's Balanced Salt Solution and stained with Alexa Fluor 647-conjugated anti-Ly-6G, or with both Alexa Fluor 647-conjugated anti-mouse CXCR4 and PE-conjugated anti-mouse L-selectin. After fixation, cells were analyzed by flow cytometry (CytoFLEX, Beckman Coulter). The numbers of total and aged neutrophils were counted, and the percentage of aged neutrophils was calculated.

Measurement of urine osmolality. Mice were deprived of drinking water for 16 hours before the collection of urine. Urine samples were collected every other week and diluted 10-fold with distilled water. Urine osmolality was measured by an osmometer (Model 3320, Advance Instrument, Norwood, MA) according to the manufacturer's instructions.

Measurement of plasma nitrate/nitrite levels. After recording survival times, blood was immediately drawn, and the plasma was isolated and stored at -80 °C until use. Plasma (150 µl) was filtered using an Amicon Ultra centrifugal filter (cut-off: 10K, Sigma) and 40 µl plasma was used for the measurement of nitrate/nitrite levels. The absorbance of each sample was measured at 540 nm using a microplate reader (PHERAstar, BMG Labtech).

Measurement of plasma AGE, ICAM-1, E-selectin, IL-1β and IL-6 levels. Plasma (50 µl) or diluted plasma was used for each ELISA kit and the absorbance of each sample was measured at 450 nm using a microplate reader.

Immunohistochemistry. Following intravital microscopy, the cremaster muscle from each mouse was carefully removed, washed with phosphate buffered saline (PBS), and cryopreserved in optimal cutting temperature compound. The frozen blocks were sectioned at 5 µm thickness per slice and mounted to slides. The slides were rehydrated in PBS for 10 minutes at room temperature and blocked with donkey serum for 30 minutes. The tissue sections were then incubated with 1 µg/ml isotype control IgG or rat anti-mouse ICAM-1 or anti-mouse E-selectin antibodies for 1 hour, followed by incubation with 1 µg/ml

DyLight 488-conjugated goat anti-rat IgG antibodies for 1 hour. The sections were further labeled with 1 μg/ml APC-labeled rat anti-mouse PECAM-1 antibodies and mounted with Vectashield containing DAPI. Imaging was performed using a Nikon microscope (ECLIPSE Ti) equipped with a Plan Fluor ×40/1.30NA oil objective lens and recorded with a digital camera (CoolSNAP ES2). Care was taken to image a given fluorochrome at the same settings for all experimental permutations using NIS Elements (AR 3.2). Mean fluorescence intensity (MFI) values were calculated using Image J by outlining the endothelium. The average MFI of the IgG samples was subtracted from that of each sample. Multiple vessels (3-7 vessels/mouse) were analyzed from 3-6 mice per group.

Histopathology analyses of organ samples. After long-term treatment with pyridoxamine, SCD mice were challenged with ip injection of TNF-α (500 ng) and used for intravital microscopy (see ***In vivo*** **intravital microscopy**). After recording a survival time, mice were euthanized. As a control, unchallenged SCD mice were also used. Kidney, spleen, and liver were taken out and fixed in 10% neutral buffered formalin solution for 48 hours at room temperature. Tissues were processed for paraffin embedding, sectioned to a thickness of 5 μm, and mounted on slides. Slides were rehydrated with xylene, decreasing concentrations of ethanol (100%, 95%, and 70%), and water and stained with hematoxylin and eosin. Images were obtained with Olympus BX51 microscope equipped with the Olympus Plan N 20x/0.40 (for spleen and liver) and UPlanFL N 40x/0.75 (for kidney) objective lenses and a digital camera (Olympus DP71). Adherent neutrophils on the hepatic vessel wall (10-15 vessels per mouse) were counted. The glomerular size (10 glomeruli per mouse) was calculated using image J.

Immunoblotting Platelets and neutrophils from SCD mice were treated with 1 mM pyridoxamine at 37℃ for 10 and 30 minutes, respectively. Cells were then stimulated with an agonist and lysed at different time points using RIPA buffer (Tris-HCl, pH 7.4 containing 1% Triton-X100, 0.05% SDS, proteinase inhibitor cocktail, 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM NaF). Equal amounts of proteins (30 μg) in cell lysates were immunoblotted with antibodies against p-Akt at Ser473, total Akt, or p-PI3K p85α/β at Tyr458/p55α/γ at Tyr199, followed by densitometry.

References

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Supplemental Figure legends

Figure S1. One oral administration of pyridoxamine does not affect neutrophil aging in SCD mice. SCD mice were fasted for 12 hours, and blood (10 µl) was collected at 7 AM. Mice were immedaitely treated with one oral administration of pyridoxamine (100 mg/kg BW), followed by blood drawings at 11 AM, 3 PM, and 7 PM. After red blood cell lysis, cells were stained with Alexa Fluor 647-conjugated anti-CXCR4 and PE-conjugated anti-L-selectin antibodies. Flow cytometric analysis was performed to measure the percentage of aged neutrophils. (A) Representative dot plots of mouse blood after lysis of red blood cells. Neutrophils (P1) are Ly-6G positive. (B) Representative dot plots depicting aged neutrophils at different time points in vehicle- or pyridoxamine-treated mice. (C) The percentage of aged neutrophils at different times points in two groups. Data represent the mean ± SD (n = 3).

Figure S2. Meausrement of body weight and complete blood count during long-term treatment with pyridoxamine in SCD mice. Acidic water (water) or pyridoxamine-containing water (Pyri) for 5.5 months in male and female SCD mice. Body weight was measured every other week. Blood was also drawn and counted using an automated hematology analyzer (Hemavet 950, Drew Scientific). Data represent the mean ± SD (n = 8-10 mice per group). **P < 0.01, ***P < 0.001, and ****P < 0.0001 versus control at each time point, Student's *t*-test.

Figure S3. Urine osmolality during long-term treatment with pyridoxamine in SCD mice. SCD mice were treated with acidic water (water) or pyridoxamine-containing water

(Pyri) for 5.5 months. Urine was collected and the osmolality was measured every other week as described in Methods.

Figure S4. Long-term treatment with pyridoxamine reduces neutrophil-EC interactions in venules of H/R-challenged SCD mice. SCD mice were given acidic water or pyridoxamine (2 g/L) in the drinking water for 5.5 months. Intravital microscopy was performed as described in Figure 1. (A) The rolling influx of neutrophils. (B) The number of adherent neutrophils. (C) The number of platelets adhered to neutrophils and ECs was counted. (D) After intravital microscopy, blood was drawn from each mouse, diluted in PBS, and smeared for microscopy. Representative images and quantitative graphs (% of sickle RBC). Bar = 10 μm. Data represent the mean ± SD (n = 39-42 venules in 6 mice per group). *P < 0.05 and **P < 0.01, Student's *t*-test.

Figure S5. Treatment of WT mouse platelets with pyridoxamine, but not vitamin B6, inhibits platelet aggregation in response to thrombin or CRP. WT mouse platelets were pretreated with 0.3 or 1 mM pyridoxamine or vitamin B6, followed by stimulation with thrombin or CRP. (A-D) Representative aggregation traces. Data were obtained from three independent experiments.

Figure S6. Treatment of WT mouse platelets with pyridoxamine, but not vitamin B6, inhibits the activation state of mouse platelets. (A-C) WT mouse platelets were pretreated with 1 mM pyridoxamine, followed by stimulation with 0.05 U/ml thrombin. (A-C) Flow cytometry was performed to assess P-selectin exposure, αIIbβ3 activation and

ROS generation. Data represent the mean ± SD (n = 4). *P < 0.05 versus vehicle control, Student's *t*-test.

Figure S7. Treatment with pyridoxamine reduces phosphorylation of kinases in platelets and neutrophils from SCD mice. Platelets and neutrophils were isolated from SCD mice and treated with 1 mM pyridoxamine. Cells were then stimulated with an agonist and lysed at different time points. The lysates were immunoblotted using the indicated antibodies. (A and C) Representative blots. (B and D) Quantitative graphs of densitometry. Data represent the mean ± SEM (n = 3). **P < 0.01 and ****P<0.0001, Student's *t*-test.

Video 1. Intravital microscopy in H/R-challenged SCD mice treated with vehicle.

Video 2. Intravital microscopy in H/R-challenged SCD mice treated with pyridoxamine (10 mg/kg mouse).

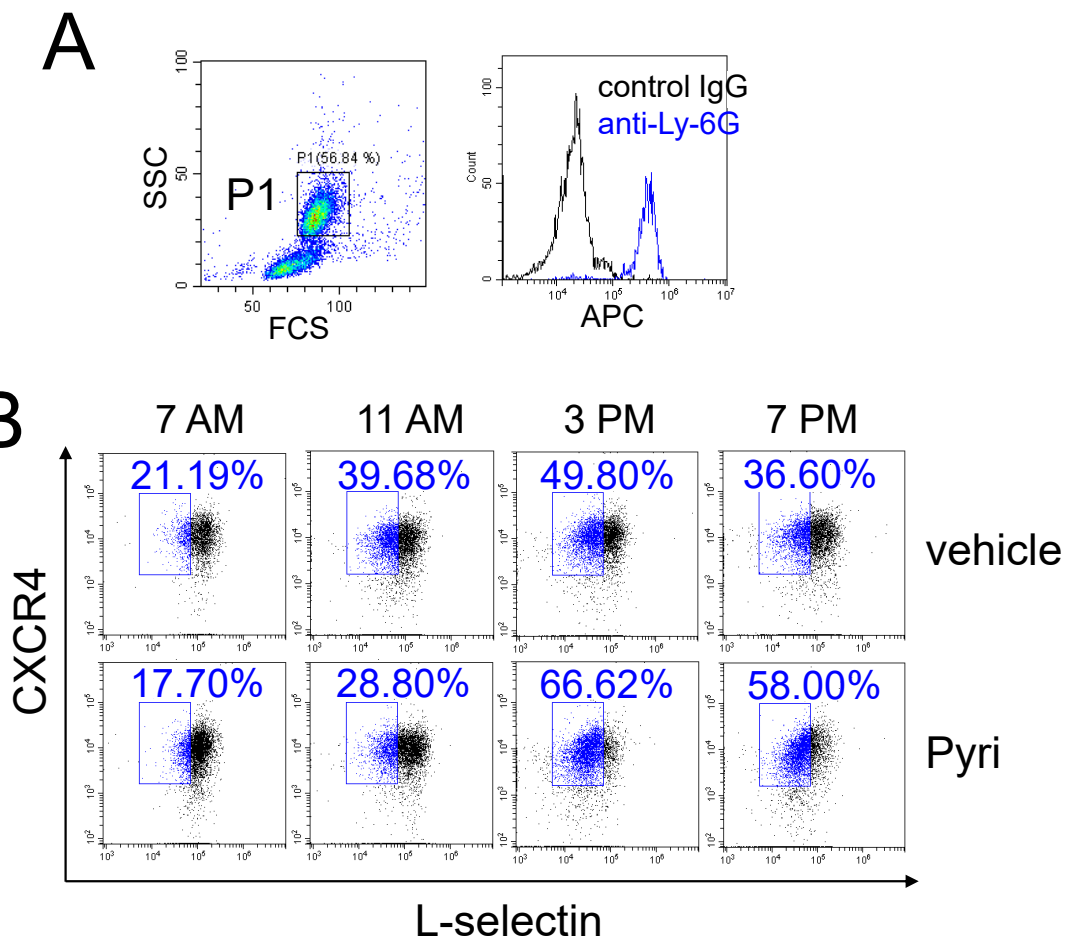
Video 3. Intravital microscopy in H/R-challenged SCD mice treated with pyridoxamine (30 mg/kg mouse).

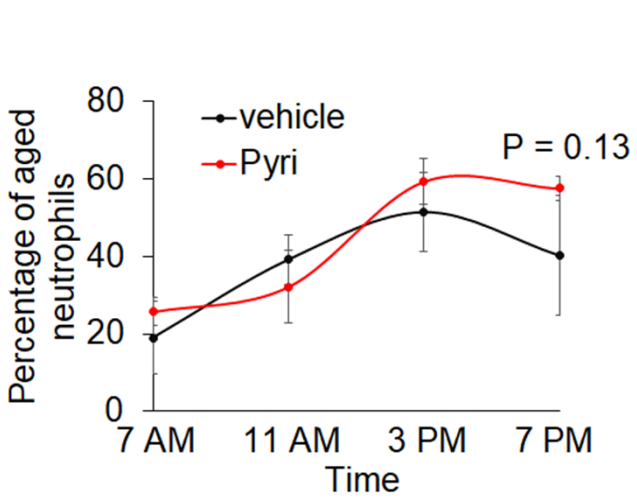
Video 4. Intravital microscopy in H/R-challenged SCD mice treated with pyridoxamine (100 mg/kg mouse).

Video 5. Intravital microscopy in TNF-α-challenged SCD mice treated with saline.

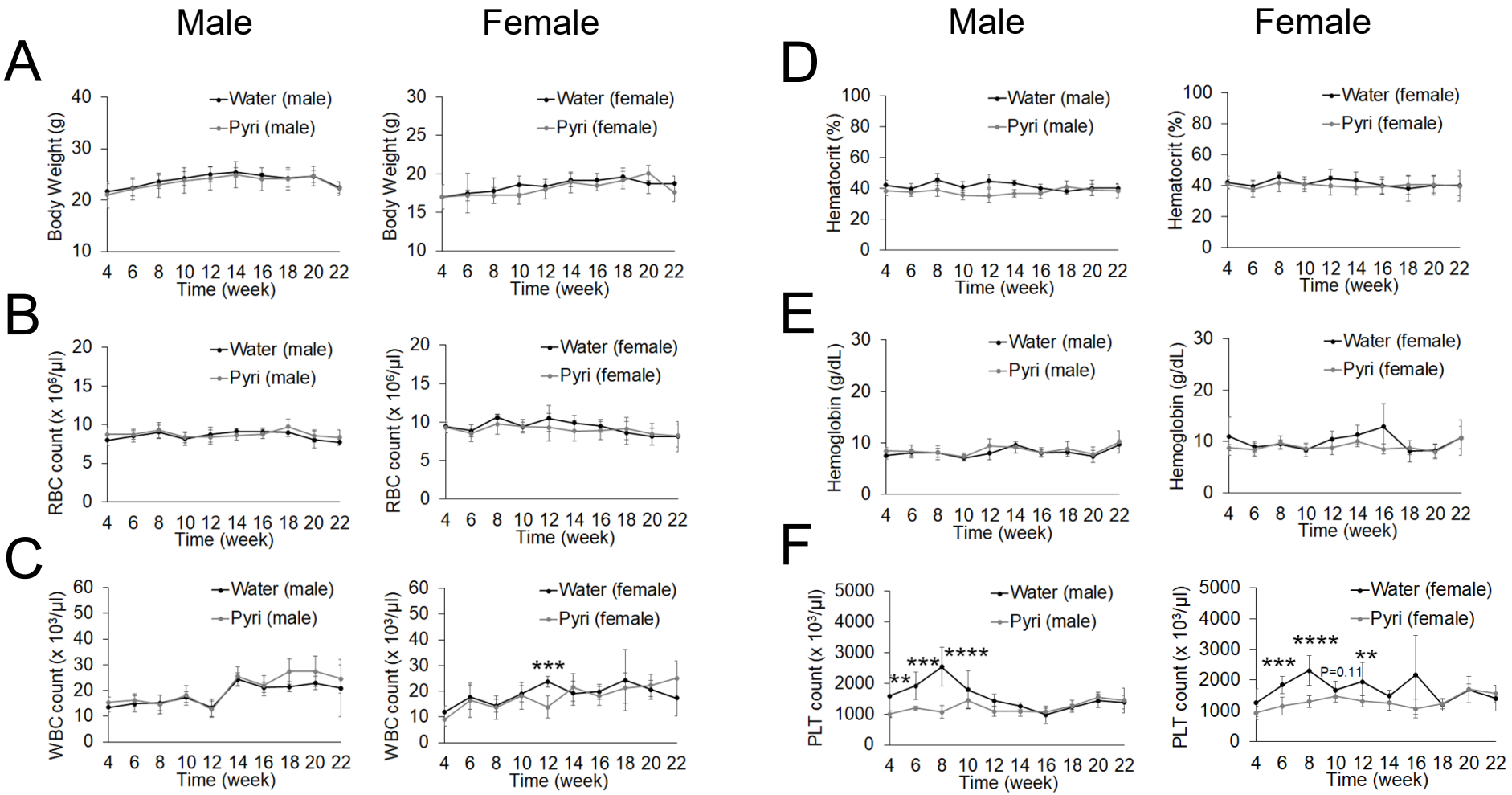
Video 6. Intravital microscopy in TNF-α-challenged SCD mice treated with pyridoxamine (100 mg/kg mouse).

Supplemental Figure S1





Supplemental Figure S2



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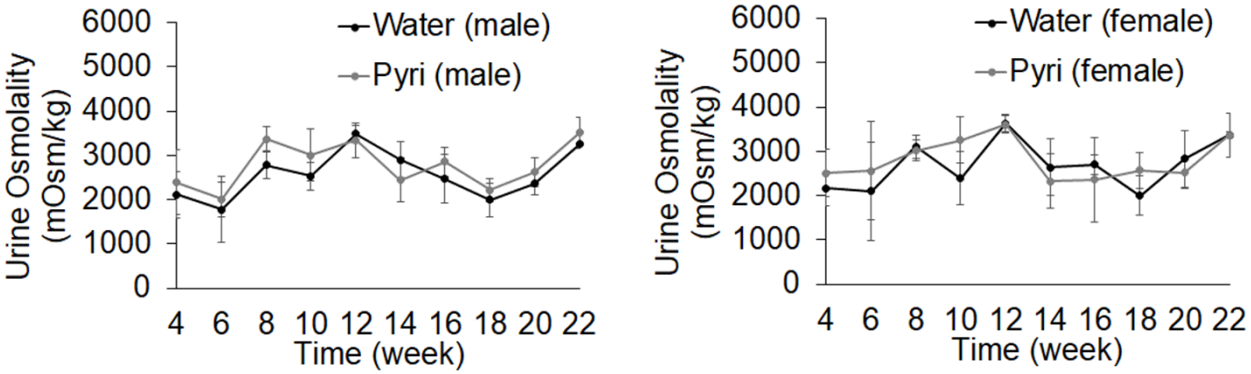

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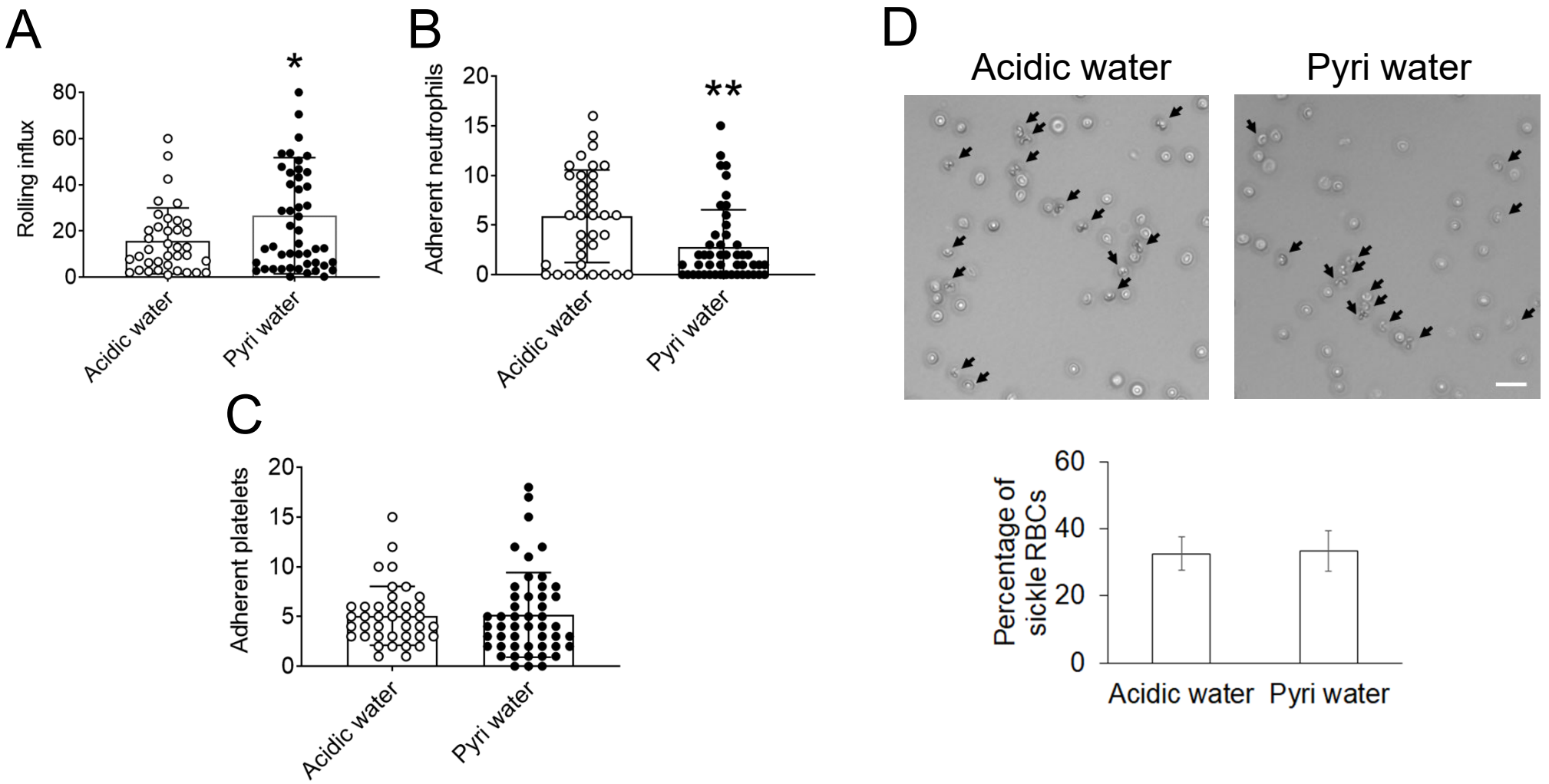

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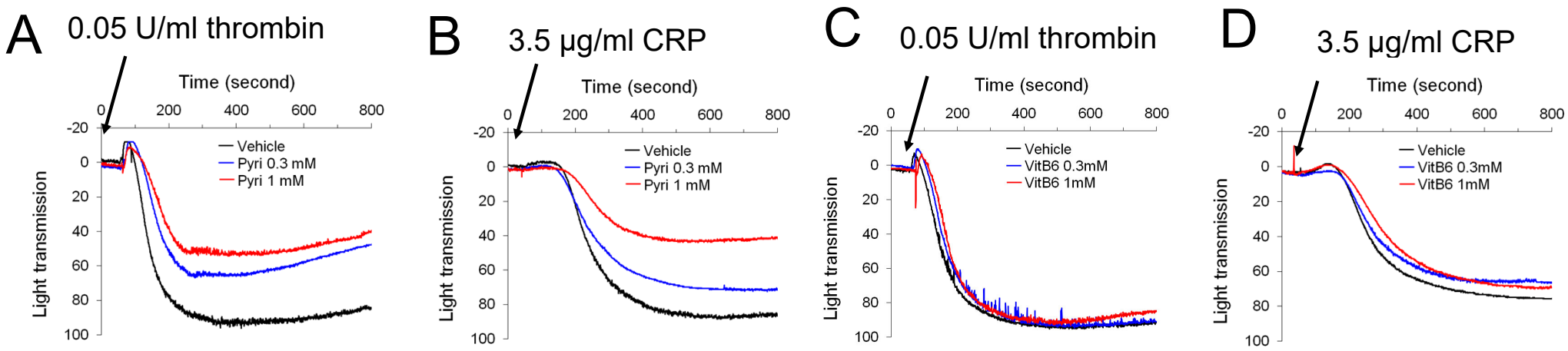

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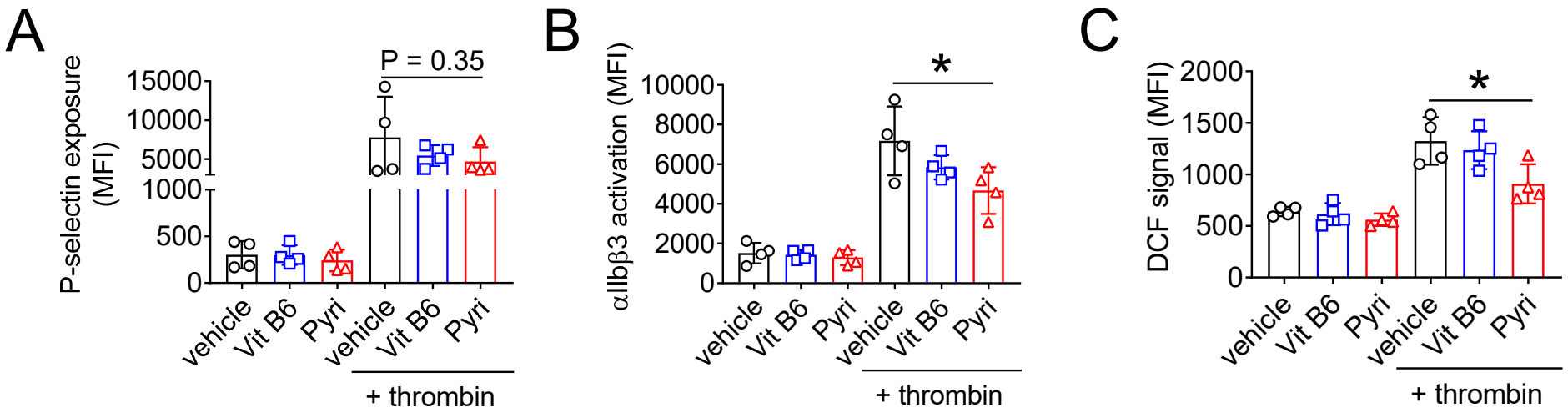

Supplemental Figure S7