SUPPLEMENATL MATERIALS

Endogenous SOD2 regulates platelet-dependent thrombin generation and thrombosis during aging

Vijay K Sonkar, PhD,[1] Alicia S Eustes, PhD,[1] Azaj Ahmed, PhD, [1] Melissa Jensen, BS,[1] Mitali V Solanki, MD,[1] Jagadish Swamy, PhD,[1] Rahul Kumar, PhD, [1] Trevor P. Fidler, PhD, [1] Jon C.D. Houtman, PhD,[2] Bryan G. Allen, MD, PhD,[3] Douglas R Spitz, PhD,[3] E. Dale Abel, MD, PhD,[1,4] Sanjana Dayal, PhD,[1,5]

Department of Internal Medicine,[1] Microbiology & Immunology,[2] and Free Radical and Radiation Biology Program, Radiation Oncology, Holden Comprehensive Cancer Center[3] University of Iowa Carver College of Medicine, [4]Current address, David Geffen School of Medicine at UCLA, Los Angeles, CA and Iowa City VA Healthcare System,[5] Iowa City, Iowa

Short Title: Platelet SOD2 associated thrombosis in aging

Corresponding author:
Sanjana Dayal, PhD, FAHA
Department of Internal Medicine
The University of Iowa Carver College of Medicine
100 EMRB
200 Hawkins Drive
Iowa City, IA 52242
Tel: 319-335-7712
Fax: 319-353-8383
Email: sanjana-dayal@uiowa.edu

Supplemental Methods

Complete blood counts

For complete blood cell counts, mice were anesthetized with isoflurane and bled via the retro-orbital plexus using EDTA-coated tubes and samples were analyzed using ADVIA 120 (Siemens, Germany).

Platelet preparation

For platelet flow cytometry, aggregation and adhesion studies, mice were anesthetized with isoflurane and bled *via* the retro-orbital plexus[1, 2] using a heparin-coated capillary tube and blood was collected into 3.2% sodium citrate (9:1). For protein and mRNA expression, adoptive platelet transfer, platelet-dependent thrombin generation, platelet spreading and clot retraction, blood was drawn *via* carotid artery cannulation into sodium citrate. Platelets were prepared as described previously.[3] Briefly, blood was diluted with modified Tyrode's buffer (134 mmol/L NaCl, 2.9 mmol/L KCl, 0.34 mmol/L Na_2HPO_4, 12 mmol/L $NaHCO_3$, 20 mmol/L HEPES, 1.0 mmol/L $MgCl_2$, 5.0 mmol/L glucose, and 0.35% (w/v) bovine serum albumin, pH 7.35) and centrifuged at 100 ***g*** for 15 min at RT. Platelet-rich plasma (PRP) was isolated and processed to prepare washed platelets where PGE1 (1 μM) was added to PRP, and samples were centrifuged at 800 ***g*** for 7 min. to obtain platelet pellets. Pellets were then washed with modified Tyrode's buffer in the presence of PGE1 (1 μM), and finally resuspended in modified Tyrode's buffer. For protein and mRNA expression, platelets were further purified with CD45 and Ter-119 labelled microbeads (Miltenyi Biotec, Auburn, CA) as described by us.[3] For thrombin generation assay,

PRP was prepared without diluting blood and platelet count was adjusted by adding platelet poor plasma (PPP).

Measurement of ROS in platelets using fluorescent dyes

Levels of platelet-derived pro-oxidants were measured with fluorescent dye methods.[3] To measure total cellular changes in pro-oxidants, washed platelets (2 x 10^8/mL) were incubated with 10 μM of the oxidation sensitive dye, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes, USA), or with 5 μM of dihydroethidium (DHE, Molecular probe, USA) for 10 min at 37 °C in the dark. Platelets were then incubated in the presence or absence of thrombin and/or convulxin for 10 min. The sample was diluted 10 times with 1X DPBS and the fluorescent signal of the oxidized adducts were measured by flow cytometry. In some experiments platelets were treated with 50 μM GC4419 (a dismutase mimetic) or vehicle control (Na-bicarbonate buffer) for 15 minutes prior to agonist activation.

Similarly, to probe for mitochondrial pro-oxidants, MitoSox was used as fluorescent probe at a concentration of 10 μM and incubated with platelets in the presence or absence of thrombin and convulxin at given concentrations for 10 min, and the fluorescent signal was measured by flow cytometry. The data on geometric mean fluorescence intensity is presented. In some experiments platelets were treated with 50 μM GC4419 (a dismutase mimetic) or vehicle control (Na-bicarbonate buffer) for 15 minutes prior to agonist activation.

Platelet Aggregation and secretion:

Platelet aggregation and release of ATP from dense granules was measured using Chrono-lume luciferin-luciferase reagent (Chrono-log, stock concentration, 0.2 μM luciferase/luciferin) in Optical/Lumi-Aggregometer (model 700-2) as described previously.[4, 5] Briefly, washed platelets (280 μL of 2 x10^8/mL) containing 1 mM calcium chloride were incubated with 20 μL of Chrono-lume reagents for 2 min at 37 °C. After incubation, thrombin (0.02 U/mL) was added under stirring conditions (1200 rpm) to induce platelet aggregation. Dense granule secretion (ATP release) was monitored in parallel to aggregation. Aggregation and secretion kinetics were calculated as % increase in light transmittance and luminescence respectively.

Microfluidics-based platelet adhesion on collagen matrix under arterial shear

Platelet adhesion and thrombus growth on collagen matrix were measured in a microfluidic BioFlux^{TM} flow chamber (Fluxion Biosciences). High-shear plates were coated with 50 μg collagen (Chrono-log) and blocked with 1% BSA. Platelets (2 x 10^8/mL) labeled with calcein-green (2.5 μg/mL) were perfused over collagen at a physiological arterial shear rate (2000 s^{-1}) for 5 min. Platelet adherence over time was recorded and analyzed using ImageJ (NIH). Thrombus growth over time was measured by accumulation of adhering platelets in a fixed field. Total thrombi area at 5 min was calculated by average accumulation of platelets in 5 representative fields.

Flow cytometric analysis of integrin $\alpha_{IIb}\beta3$ activation, mitochondrial membrane potential and phosphatidylserine exposure

Activation of integrin $\alpha_{IIb}\beta3$ was evaluated by quantifying JON/A binding as previously described,[3] with minor modification. Washed platelets were activated with thrombin for 5 minutes at 37 °C in the presence of 10 μL of PE-conjugated JON/A (1:5, BD Biosciences) antibody, and fixed in 1% paraformaldehyde. To evaluate mitochondrial membrane potential, washed platelets were stained with 500 nM of tetramethylrhodamine methyl ester (TMRM, Invitrogen) for 15 minutes in the presence and absence of Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP),[6] an uncoupler that activates proton conductance. Surface phosphatidylserine exposure was measured by incubating platelets for 15 minutes with annexin V-APC (1:20, Biolegend) followed by activation with thrombin and convulxin for 10 minutes and a binding buffer (100 μL) was added at the end.[7]. All the samples were diluted 10-fold in PBS and analyzed using a LSR Violet flow cytometer (Becton Dickinson, CA). In some experiments, platelets were treated with 50 μM GC4419 or a vehicle control (Na-bicarbonate buffer) for 15 minutes prior to agonist activation. Data for activated integrin $\alpha_{IIb}\beta3$ is presented as mean fluorescence (Geometric mean). The data for TMRM fluorescence is presented as fold change in mean fluorescence from young control mice in the absence of FCCP. Data for annexin V is presented as fold change in mean fluorescence from the young control mice at resting state.

Platelet Spreading

Washed platelets (2×10^7 /mL) were stimulated with 0.05 units of thrombin (Sigma) and adhered to fibrinogen (Millipore) coated glass slides (100 μg/mL) for 30 min at 37°C. Cells were fixed

with an equal volume of 4% paraformaldehyde for 20 min at room temperature. Platelets were stained for actin using phalloidin (Alexa flour 488, Invitrogen) by incubating for 20 min at room temperature. Images were taken with an IX-81 fluorescent microscope with a 60x zoom. Three-five randomly selected non-overlapping areas were imaged for each sample and quantification was performed by measuring the area and fluorescence of each individual cell using automated CellProfiler®. Values from each mouse were averaged and normalized to young control.

Clot Retraction:

Washed platelets were suspended in Tyrode's buffer (2 x 10^8/ mL) and 200 µL of suspension was used to perform clot retraction in the presence of 2 mM CaCl2, 500 µg/mL fibrinogen (Millipore) and 2 µL of red cells (added for clot visualization). Platelets were activated with 1 U/mL of thrombin. Images were taken at baseline and at 5 min intervals for the first half hour and then at 10 min intervals for the next hour. The clot size at each time point was determined with Image J and presented as percent of the initial clot volume.

Platelet-dependent thrombin generation

To measure thrombin generation in PRP, mouse blood was collected in corn trypsin inhibitor (CTI, 50 µg/mL) to inhibit the contact activation pathway, centrifuged at 100 *g* for 15 min, and platelet counts were adjusted to 5 x 10^8 cells/mL with PPP.[8] The Calibrated Automated Thrombogram (CAT, Diagnostica Stago, Inc, Parsippany, NJ) method, including the Thrombinoscope software, Fluoroskan Ascent, and CAT reagents were used to perform the thrombin generation assay.[9] Briefly, 20 µL mouse PRP + 60 µL HBS was incubated for 10 min at 37° C with 20 µL PRP Reagent containing 1 pM tissue factor (final concentration) without

phospholipids, as the phospholipids in the assay to support thrombin generation are provided by the platelets. The FluCa fluorogenic substrate (Z-GGR-AMC) buffer solution containing $CaCl_2$ was added by the instrument, and thrombin generation was measured for 60 min.

Adoptive platelet Transfer

Platelet transfer was performed as described previously.[10] Blood was collected through carotid cannulation and washed platelets were prepared. The host hIL4Tg mice were injected with 0.5 mg/Kg of human CD124 antibody (BD, San Jose, CA) retro-orbitally to deplete endogenous platelets. 20 μL of blood was obtained pre- and 30 min post antibody injection for complete blood cell count (CBC) to quantify circulating platelets. After 2 hrs., mice were infused with donor platelets (2.5×10^6/g body weight) and 15 min later a CBC was repeated to ensure repletion of circulating platelets. This was followed by carotid artery thrombosis.

Expression and activity of SOD2

Total RNA was isolated from washed platelets using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed to prepare cDNA. The levels of mRNA for SOD2 and 18S were measured by quantitative real-time PCR as described previously.[11] About 500 ng cDNA was incubated with TaqMan Universal PCR mix, PCR primers and 6-carboxy fluorescein-labeled probes at 50 °C for 2 minutes and then at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. All the reagents including primers (Mm01313000_m1 [amplicon length 67] for SOD2, and Mm03928990_g1 [amplicon length 61] for 18s rRNA) were purchased from Applied Biosystems. The comparative threshold cycle method was used for

quantification with values normalized to 18S and expressed relative to the levels in young control mice. Platelet purity was confirmed by two methods: 1) complete blood counting; and 2) real-time PCR with primers for CD45, a marker for leukocytes. All samples used for real-time PCR had undetectable levels of CD45 mRNA, and the leukocyte and RBC counts were equivalent to background.

Platelets were lysed in RIPA buffer with protease and phosphatase inhibitors and protein was quantified by bicinchoninic acid (BCA) analysis. Anti-SOD2 antibody (ADI-SOD-110-D: Enzo Biochem Inc) and Anti-β actin (8H10D10: Cell Signaling) were used as primary antibody and blots were incubated with HRP-linked secondary antibody for 1 hour at room temperature. Densitometry was analyzed using ImageJ and the ratio of SOD2/β-actin of each sample is presented. The activity of SOD2 was determined using an assay described previously by our group,[6] using an assay kit purchased from Cayman Chemical (Ann Arbor, MI) and normalized to protein content. SOD activity was determined as inhibition of superoxide anion production over 30 minutes. To specifically determine SOD2 activity, SOD1 activity was inhibited by addition of 3 mM potassium cyanide.

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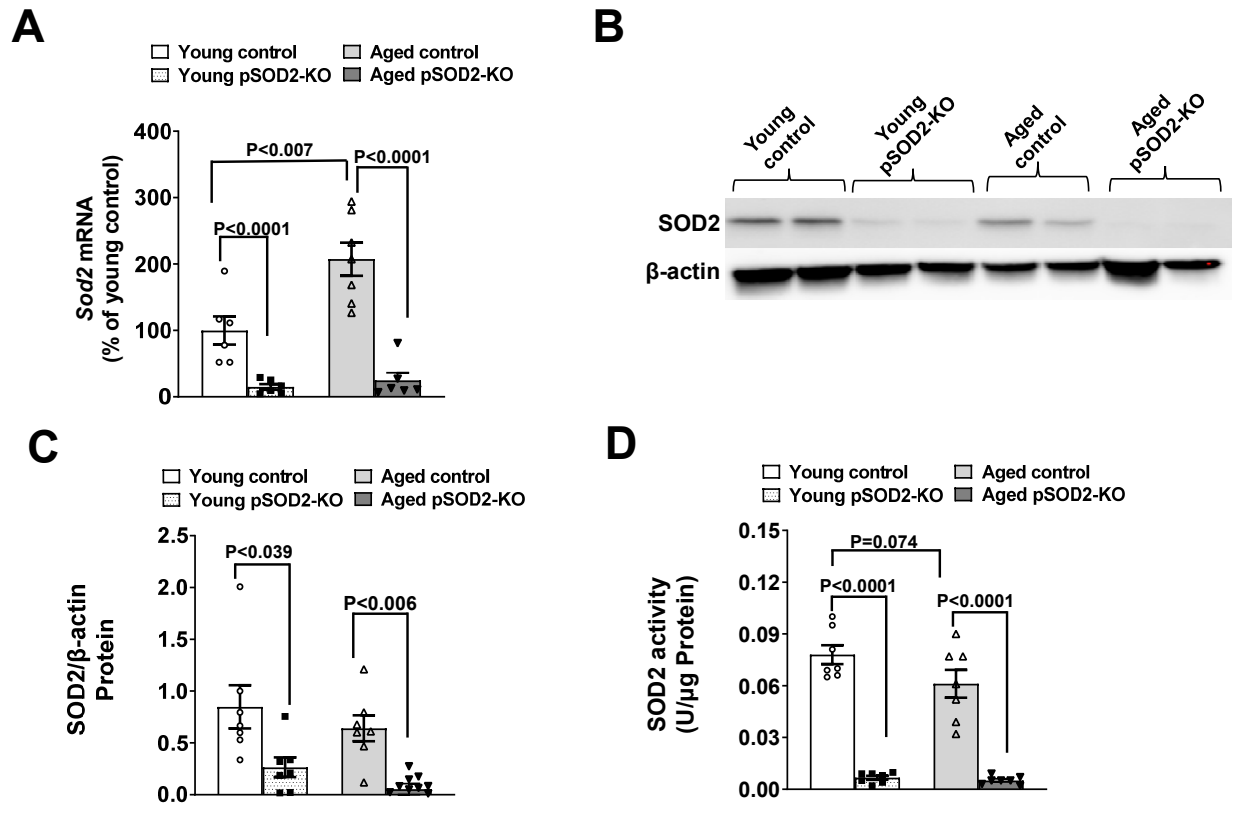
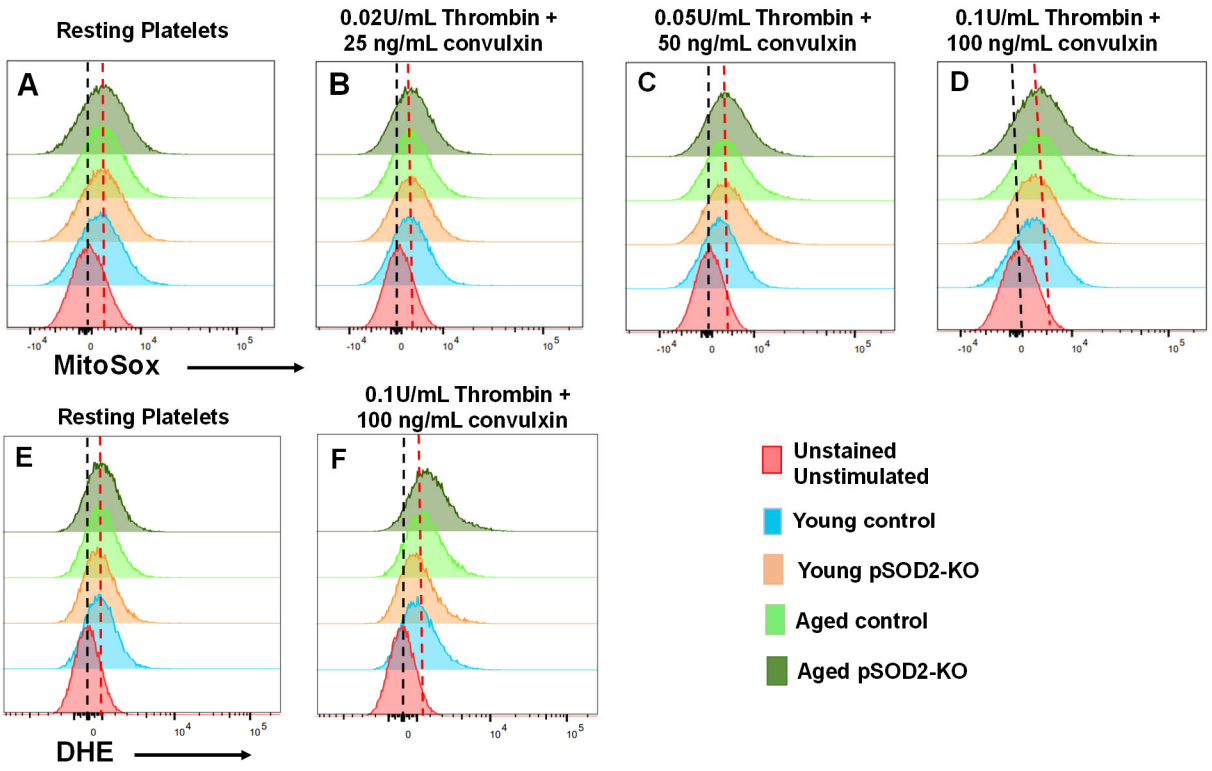
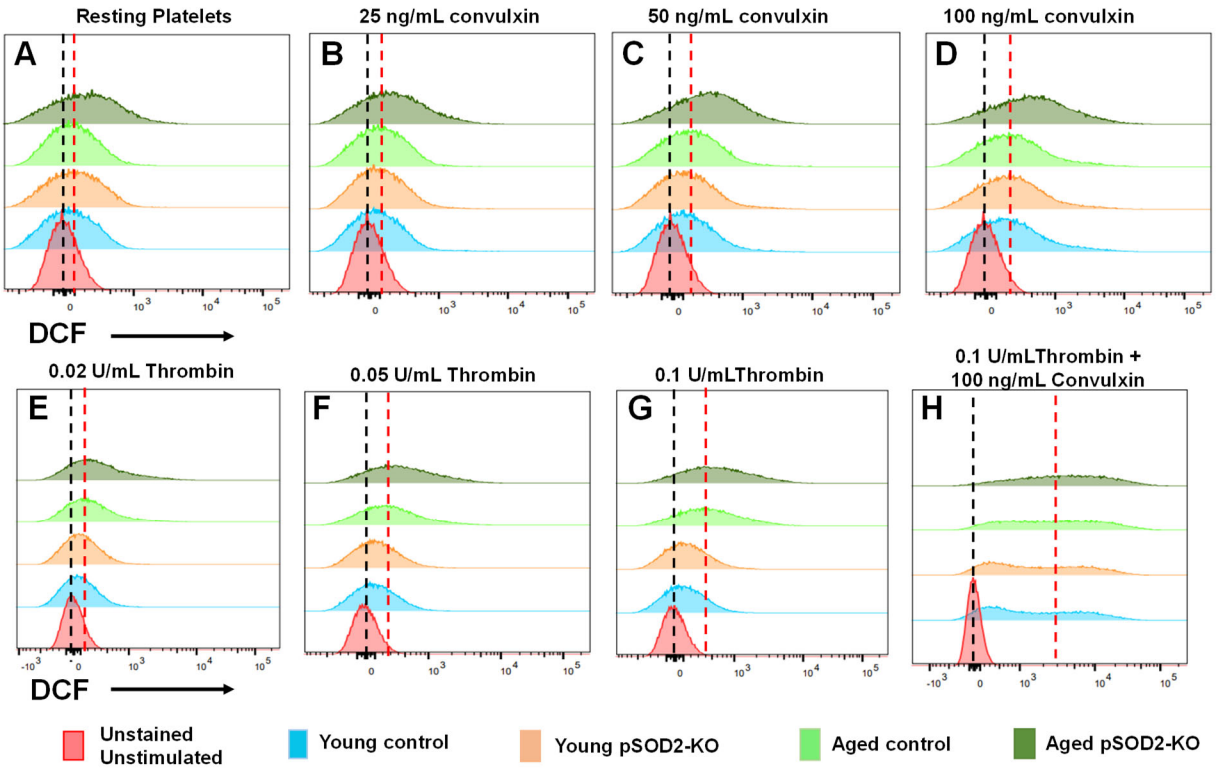
Supplemental Figures



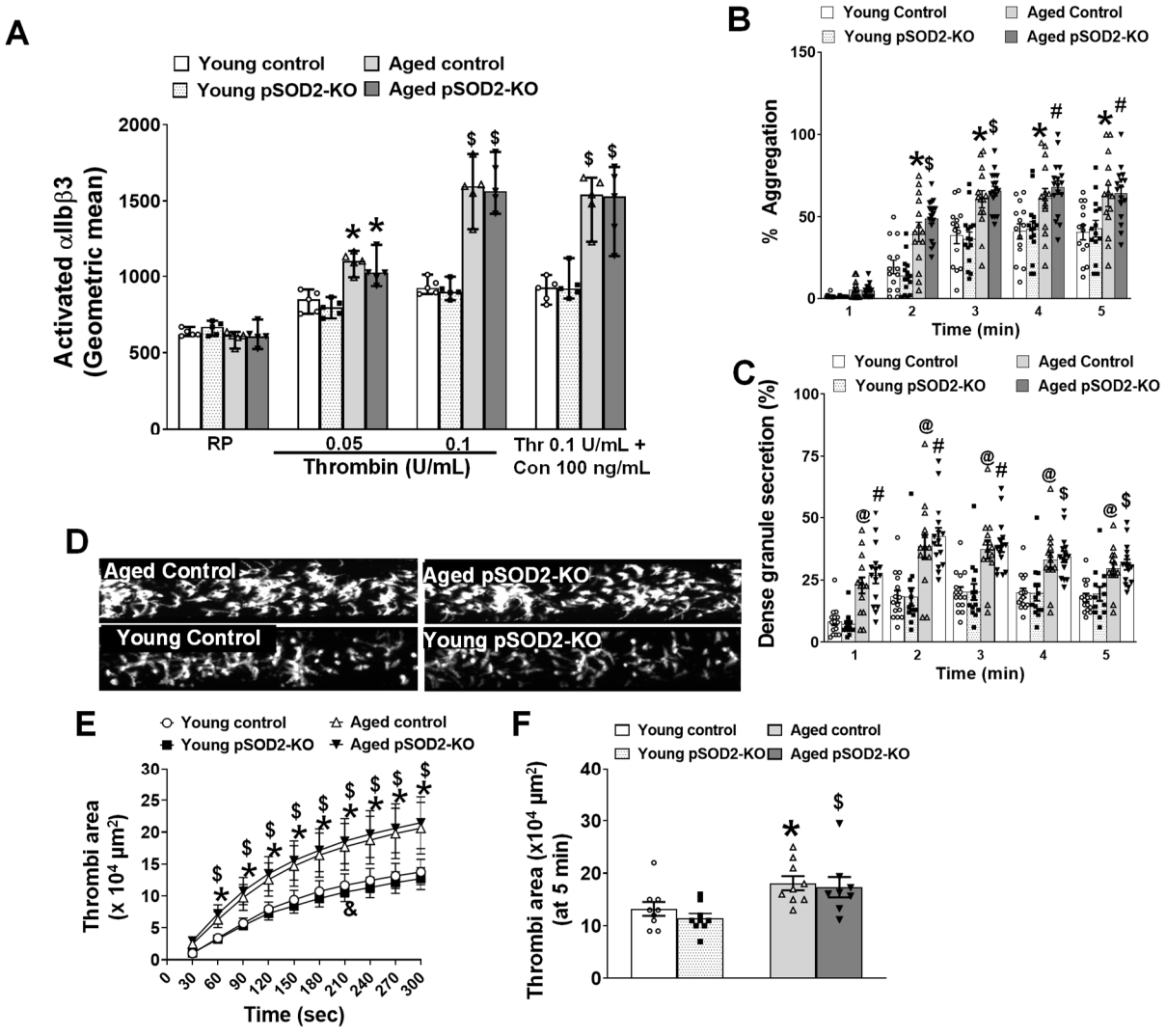
Supplementary Figure S1. SOD2 expression and activity in platelets. Bead purified platelets were prepared from young or aged mice deficient in platelet SOD2 (pSOD2-KO) or littermate control mice. **A.** mRNA. **B.** Representative image of immunoblot of SOD2 protein expression in platelets. **C.** Quantification of SOD2 protein expression using Image J. **D.** SOD2 activity. Data are presented as mean ± SE and analyzed using two-way ANOVA with Tukey's analysis for multiple comparisons. N = 6-9 per group.



Supplementary Figure S2. Platelet mitochondrial and cellular prooxidants are increased in aged pSOD2-deficient mice. Representative histograms of **A-D.** MitoSox and **E & F.** DHE fluorescence in platelets from young or aged mice deficient in platelet SOD2 (pSOD2-KO) or littermate controls, measured by flow cytometry at resting state or following activation with thrombin and convulxin. A black dotted line is added at the mean fluorescence intensity of unstained platelets to show shift from unstained population and a red dotted line is added at the mean fluorescence intensity of aged control mice to highlight the difference between the groups.

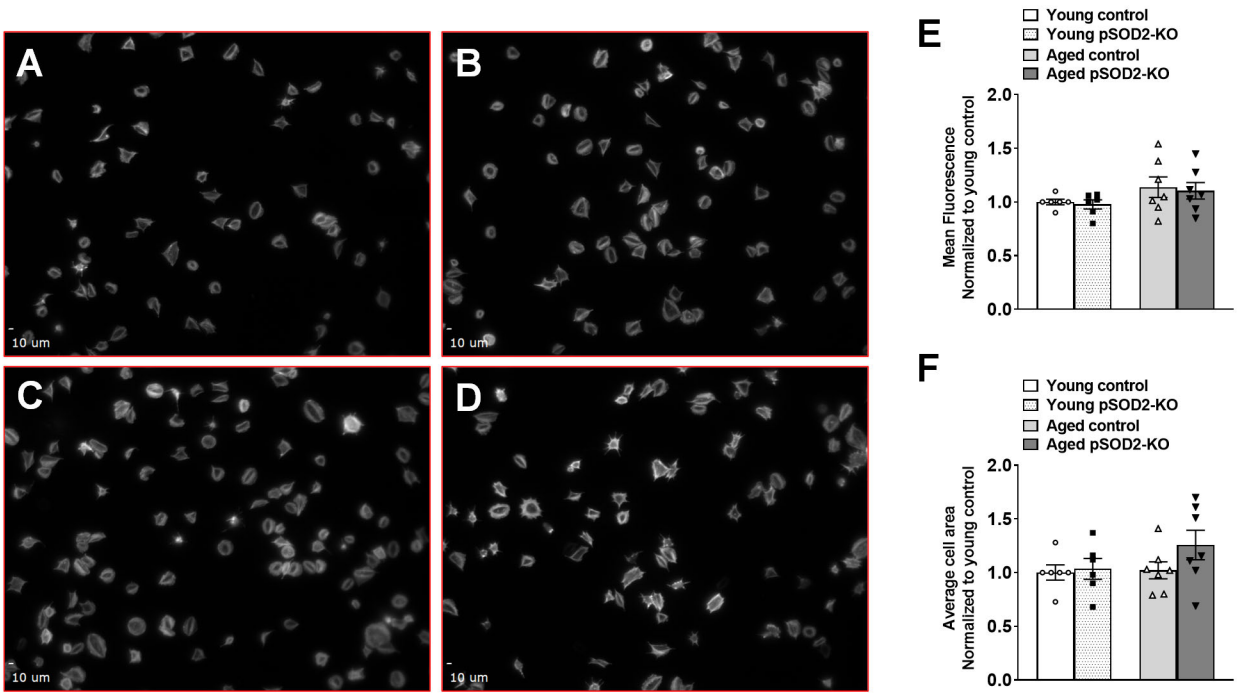


Supplementary Figure S3. Platelet SOD2 deficiency increases total cellular ROS in aged mice. Representative histograms of fluorescence detected by oxidation of CM-H_2DCF in platelets from young or aged mice deficient in platelet SOD2 (pSOD2-KO) or littermate controls, measured by flow cytometry at **A.** resting state or following activation with **B-D.** convulxin, **E-G.** thrombin, or **H.** thrombin and convulxin together. A black dotted line is added at the mean fluorescence intensity of unstained platelets to show shift from unstained population and a red dotted line is added at the mean fluorescence intensity of aged control mice to highlight the difference between the groups.

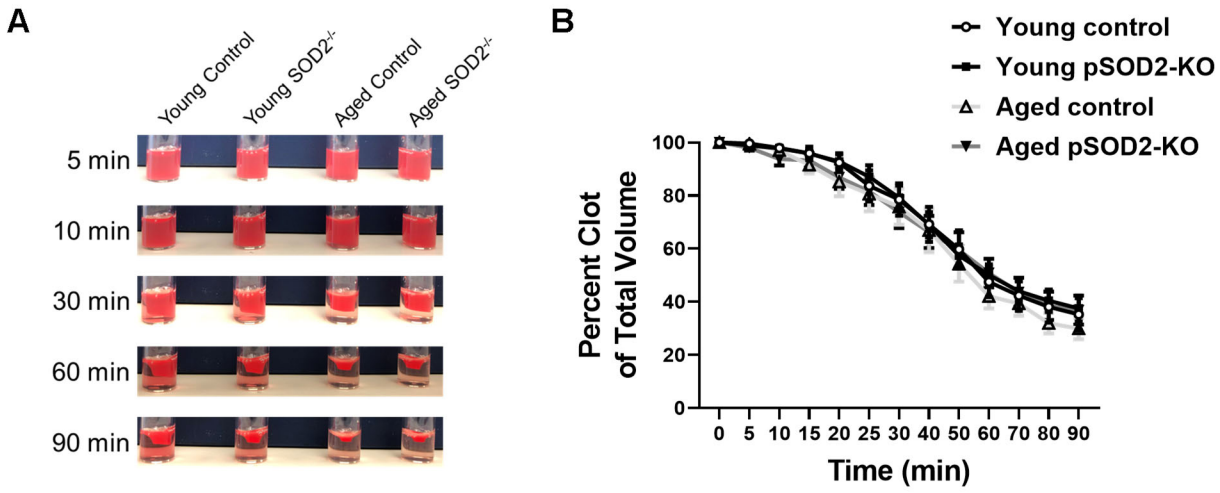


Supplementary Figure S4. Platelets from aged control and aged pSOD2-KO mice show similar increases in $\alpha_{IIb}\beta_3$ activation, aggregation, secretion, and adhesion. Washed platelets were prepared from young or aged mice deficient in platelet SOD2 (pSOD2-KO) or littermate controls. **A.** Activation of $\alpha_{IIb}\beta_3$ in platelets were measured by flow cytometry at resting state (RP) or following activation with thrombin. **B & C.** Percent aggregation and secretion respectively at different time points. **D.** Washed platelets were perfused over a collagen surface

for 5 min in a microfluidic flow chamber at a shear rate of 2000 s^{-1}: representative images of platelet accumulation after 5 minutes (height of the channel is 400 μm), **E.** The time course of accumulation of platelets/thrombi development was calculated as the surface area covered by platelets in a fixed field and **F.** Total thrombi area after 5 min of perfusion was calculated as the average surface area covered by platelets in 5 representative fields. Data for A is presented as median with 95% CI and analyzed using Kruskal Wallis test and Dunn's post hoc test for multiple comparisons. Data for B, C, E & F are presented as mean ± SE. Data for B, C and E are analyzed using Two-way ANOVA with repeated measure and for F is analyzed with two-way ANOVA with Tukey's test for multiple comparisons. *$P < 0.05$ vs young control, $ $P < 0.01$ vs young pSOD2-KO mice, @ $P < 0.01$ vs. young control and # $P < 0.001$ vs young pSOD2-KO mice. N = 5-15 per group.



Supplementary Figure S5. Platelet spreading on fibrinogen. Washed platelets were prepared from young or aged mice deficient in platelet SOD2 (pSOD2-KO) or littermate controls, activated with thrombin and allowed to adhere on slides coated with fibrinogen for 30 minutes. Representative images of phalloidin stained fluorescent platelets from **A.** Young control, **B.** Young pSOD2-KO, **C.** Aged control and **D.** Aged pSOD2-KO mice showing spreading on fibrinogen (scale bar 10 µm). **E.** Mean fluorescence and **F.** Average cell area. Data are normalized to young control and presented as mean ± SE. Data were analyzed with two-way ANOVA with Tukey's test for multiple comparisons. N = 6-7 per group.



Supplementary Figure S6. Clot retraction in young and aged pSOD2-KO is similar to littermate controls. Washed platelets were prepared from young or aged mice deficient in platelet SOD2 (pSOD2-KO) or littermate controls, activated with thrombin in the presence of fibrinogen to generate a clot. The retracting clots were imaged at various intervals until 90 min. **A.** Representative images of clots. **B.** Percent of residual clots from original clot volume. Data are presented as mean ± SE and analyzed with two-way ANOVA with repeated measures using mixed-model analysis and multiple comparisons. N = 5-6 per group.

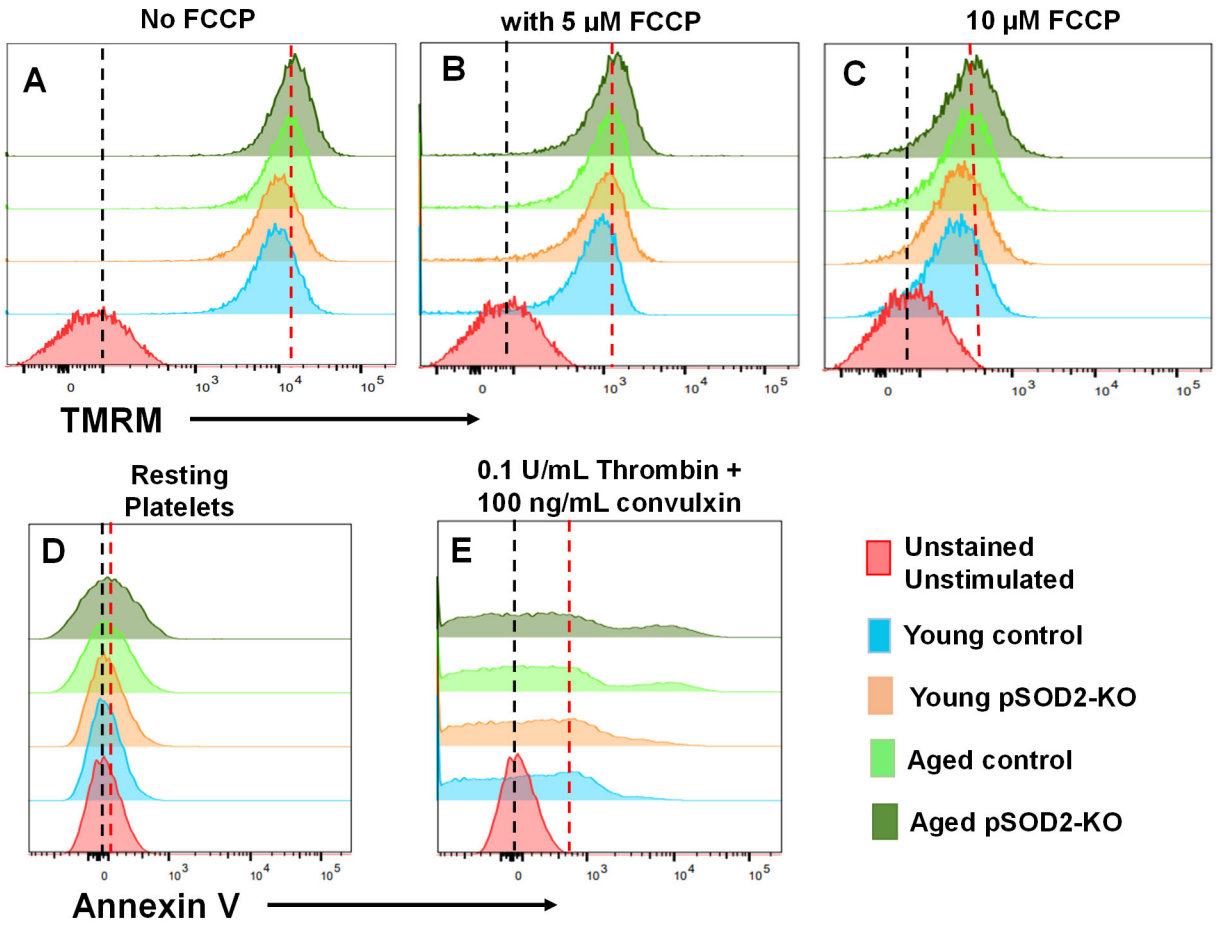
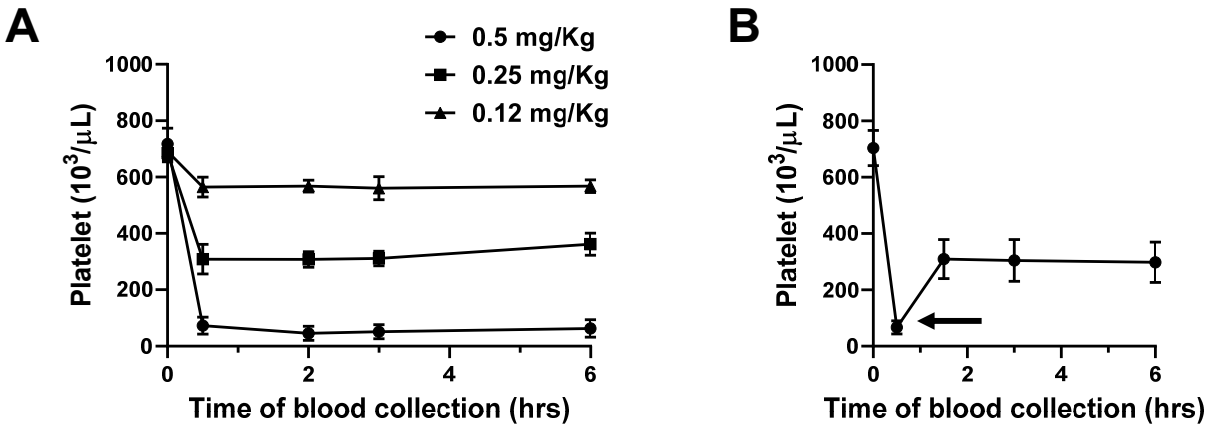
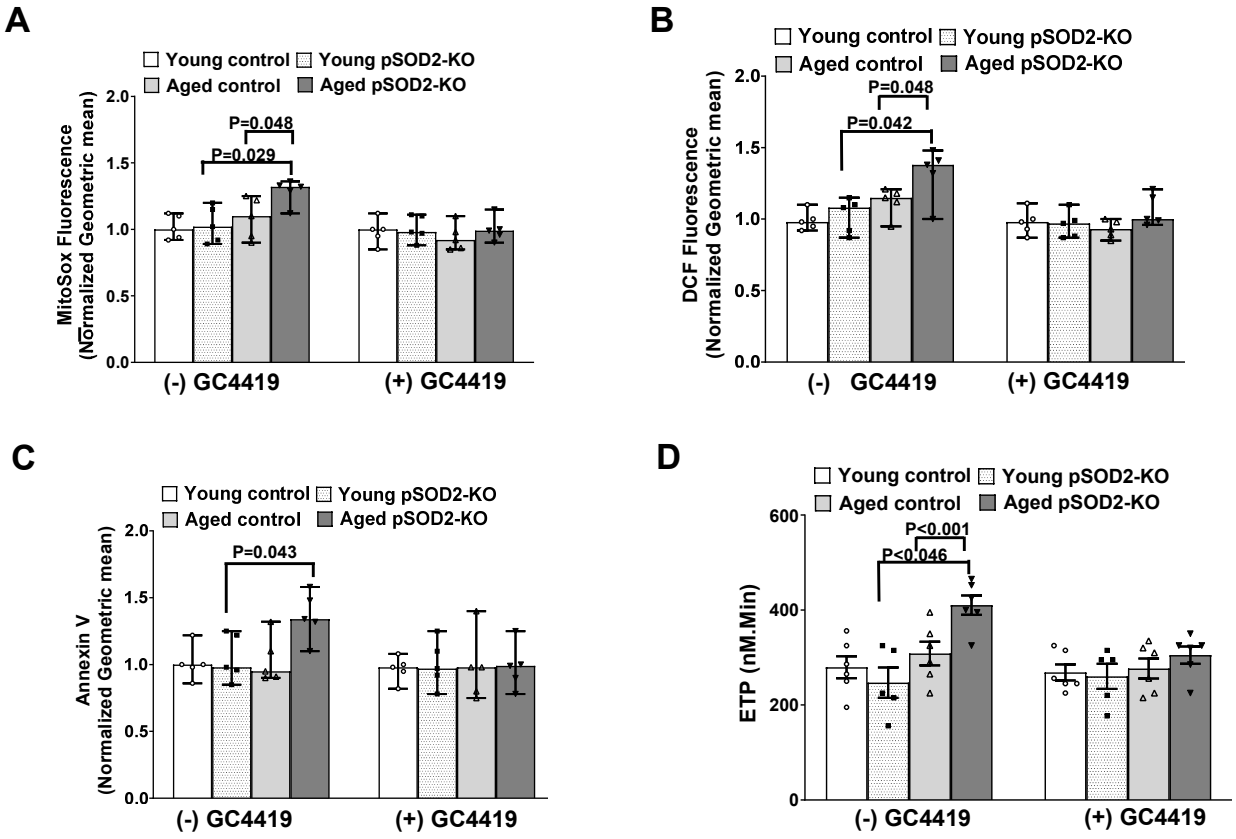


Figure S7. Platelets from aged pSOD2-KO mice show exacerbated mitochondrial hyperpolarization and enhanced annexin V binding. Representative histograms of TMRM staining without and with FCCP (**A-C**) and Annexin V binding **(D & E**) in platelets from young or aged mice deficient in platelet SOD2 (pSOD2-KO) or littermate controls. A black dotted line is added at the mean fluorescence intensity of unstained platelets to show shift from unstained population and a red dotted line is added at the mean fluorescence intensity of aged control mice to highlight the difference between the groups.



Supplementary Figure S8. Circulating platelet counts in host hIL4RTg mice. A. Circulating platelet counts in host hIL4R Tg mice at baseline (0 hr.) and 30 min to 6 hrs. after retro-orbital infusion with anti-hIL4R at various doses. **B.** Circulating platelet counts at baseline, 30 min after platelet depletion with 0.5 mg/Kg anti-hIL4R, and 1 to 6 hrs. after donor platelet infusion in host hIL4R Tg mice. Arrow indicates the time when donor platelets were infused. N = 6 at each dose.



Supplementary Figure S9: Ex vivo treatment with GC4419 protects platelets from aged pSOD2 -KO mice from agonist induced generation of ROS, phosphatidylserine exposure and thrombin generation. Washed platelets were prepared from young or aged mice deficient in platelet SOD2 (pSOD2-KO) or littermate controls. Platelets were activated with 0.1 U/mL thrombin and 100 ng/mL convulxin and analyzed via FACS or were used in thrombin generation assay. Platelets were pre-treated with either GC4419 (+ GC4419) or vehicle buffer (- GC4419) prior to assay. **A.** Mitochondrial superoxide detection using MitoSox. **B.** Levels of intracellular ROS detected by oxidation of CM-H_2DCF (DCF Fluorescence). **C.** Annexin V binding. **D.**

Endogenous thrombin potential measured via Calibrated Automated Thrombogram. The fluorescent signals were normalized to respective young control treated with vehicle buffer. Data for A-C are presented as median with 95% CI and analyzed using Kruskal Wallis test and Dunn's post hoc test for multiple comparisons. Data for D are presented as mean ± SE and analyzed using two-way ANOVA with Tukey's analysis for multiple comparison. N = 5-6 per group.

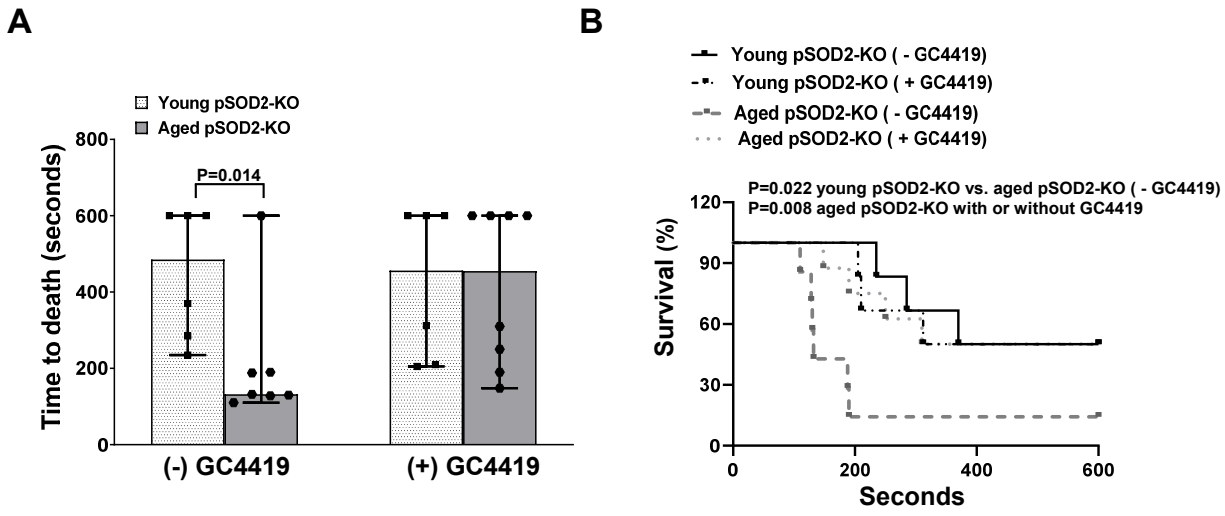
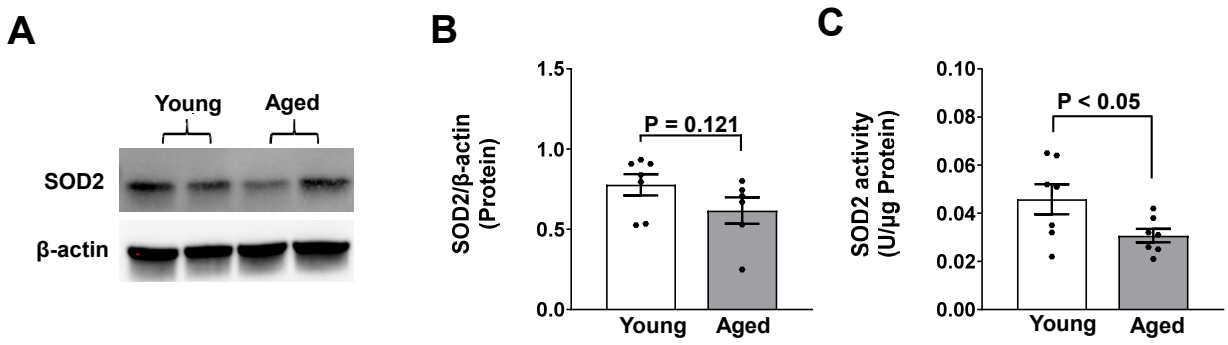


Figure S10: In vivo treatment with GC4419 protects aged pSOD2-KO mice from pulmonary thrombosis. Young and aged platelet-specific pSOD2-KO mice or control littermates were treated with (+) GC4419 (10 mg/Kg daily, IP) or vehicle buffer (- GC4419) for 2 weeks. Time to death after 0.5 µg/g collagen infusion was measured and presented as **A.** bar graph (Median with 95% CI) or **B.** % survival. Data for A is analyzed using Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons and for B is analyzed with Log-rank (Mantel-Cox) test. N = 6-8 per group.



Supplementary Figure S11. SOD2 expression and activity in platelets from C57BL6J mice. Bead purified platelets were prepared from young or aged C57BL6J mice. **A.** Representative image of immunoblot of SOD2 protein expression in platelets. **B.** Quantification of SOD2 protein expression using Image J. **C.** SOD2 activity. Data are presented as mean ± SE and analyzed using Student t-test. N = 6-7 per group.

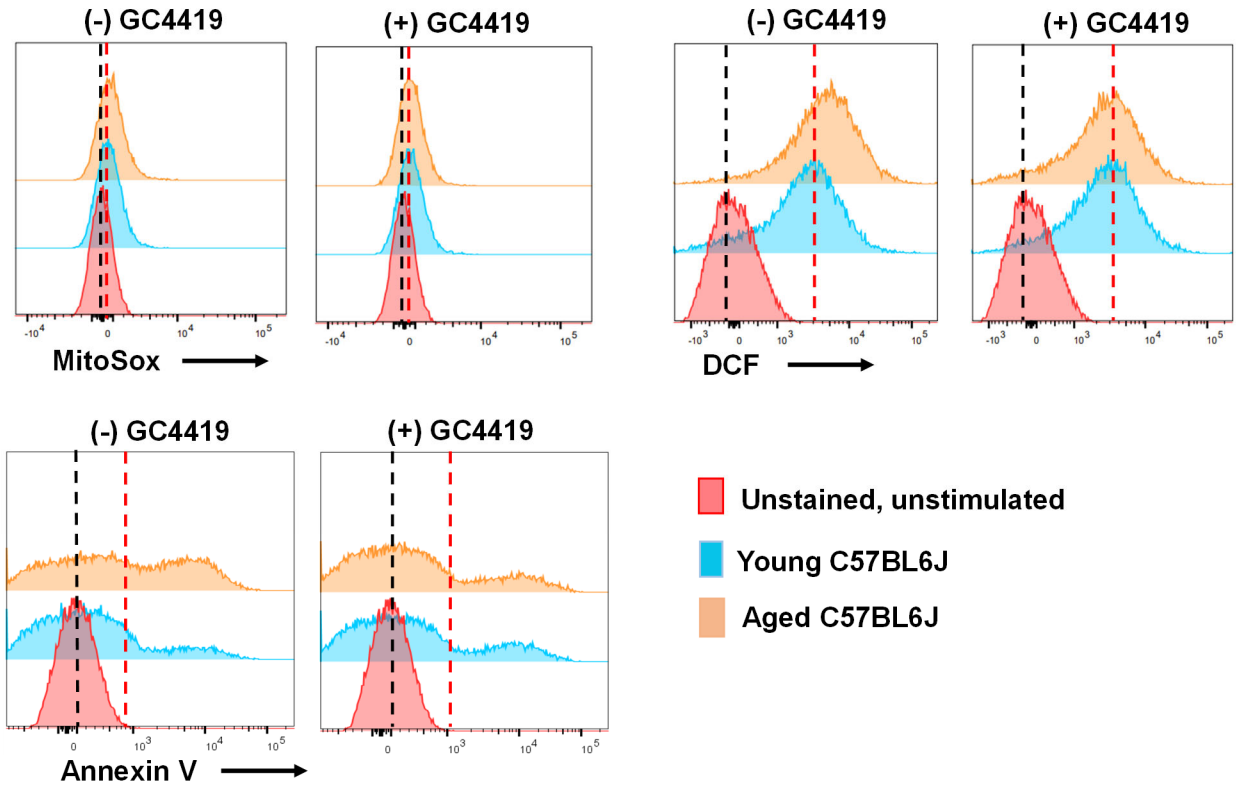


Figure S12. Treatment with GC4419 protects aged C57BL6J mice from increase in mitochondrial and cellular pro-oxidants and phosphatidylserine exposure. Representative histograms of **A.** MitoSox, **B.** DCF staining and **C.** Annexin V binding in platelets upon activation with thrombin and convulxin. Platelets were harvested from young and aged C57BL6J mice treated with GC4419 or vehicle buffer. A black dotted line is added at the mean fluorescence intensity of unstained platelets to show shift from unstained population and a red dotted line is added at the mean fluorescence intensity of aged control mice to highlight the difference between the groups.

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Table S1: Complete blood cell count (CBC).

CBC	Young Control N=6	Young pSOD2-KO N=6	Aged Control N=6	Aged pSOD2-KO N=6
Hemoglobin (g/dL)	11.1±0.4	11.2±0.3	12.1±0.6	11.5±0.8
Hematocrit (%)	41±2.5	38.5±1.7	42.5±2.7	43.5±3.9
WBC ($x10^3/\mu L$)	8.1±0.6	8.2±0.6	8.5±2.1	9.6±2.9
Platelet ($x10^3/\mu L$)	948±128	923±131	1693±245*	1500±152*

*P < 0.01 vs young control or young pSOD2-KO mice. N=5-6 mice in each group.

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
SOD2	Enzo	ADI-SOD-110-D	1/1000	01031960	https://www.enzolifesciences.com/ADI-SOD-110/mn-sod-polyclonal-antibody/
Beta actin	Santa Cruz	Sc-47778	1/1000	H1121	https://www.scbt.com/p/beta-actin-antibody-c4?gclid=Cj0KCQjw1vSZBhDuARIsAKZlijTtJ88XNTLxlkjLU4-KVEH8Fbqz3FzlYUbwF5L0SdYw9RbFiB1Ah7saAj1KEALw_wcB
hIL4R α chain	BD Bioscience	551894	No dilution	NA	
JON/A	Emfret Analytics	M-0232	2 µl/10^8 cells	NA	https://www.labome.com/product/Emfret-Analytics/M023-2.html
PE anti-human CD62P (P-Selectin) Antibody	BioLegends	362806	1 µl/10^8 cells	NA	https://www.biolegend.com/en-us/search-results/alexa-fluor-647-anti-human-cd41-cd61-antibody-12409
Annexin V	Biolegend	640943	2.5 µl/10^8 cells		https://www.biolegend.com/en-us/explore-new-products/alexa-fluor-647-annexin-v-5276

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mus musculus	The Jackson Laboratory	C57BL6/J	Male	
Mus musculus	The Jackson Laboratory	C57BL6/J	Female	

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent - Male	Mus musculus	Provided by Dr. Dale Abel, University of Iowa, Iowa city, USA	C57BL6/J	Male mice harboring homozygous SOD2 floxed alleles were bred to female mice harboring homozygous SOD2 floxed	

				alleles and expressing a Pf4 driven cre recombinase.	
Parent - Female	Mus musculus	Provided by Dr. Dale Abel, University of Iowa, Iowa city, USA	C57BL6/J Or C57BL6/N	Female mice harboring homozygous SOD2 floxed alleles and expressing a Pf4 driven cre recombinase was bred with male mice harboring homozygous SOD2 floxed alleles	
Parent - Male	Mus musculus	Provided by Dr. David Motto, University of Iowa, Iowa city, USA	C57BL6/J	hIL4Rα/GP1bα Tg male were bred to hIL4Rα/GP1bα Tg female mice	
Parent - Female	Mus musculus	Provided by Dr. David Motto, University of Iowa, Iowa city, USA	C57BL6/J	hIL4Rα/GP1bα Tg female mice were bred to male hIL4Rα/GP1bα Tg mice	

Dye	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL
CM-H2DCFDA	Thermofischer	C6827	10 µM	NA	https://www.thermofisher.com/order/catalog/product/C6827?SID=srch-hj-C6827
MitoSOX™ Red	Thermofischer	M36008	5 µM	NA	https://www.thermofisher.com/order/catalog/product/M36008?gclid=Cj0KCQiA_c-OBhDFARIsAIFg3eye6EJL-Srm-OxQHrQHrAN6Se7JIxZ8ZwYAbxUTyoB9uLHaoyH83YYaAmGUEALw_wcB&ef_id=Cj0KCQiA_c-OBhDFARIsAIFg3eye6EJL-SrmOxQHrQHrAN6Se7JIxZ8ZwYAbxUTyoB9uLHaoyH83YYaAmGUEALw_wcB:G:s&s_kwcid=AL!3652!3!447292198772!!!g!!&cid=bid_pca_iva_r01_co_cp1359_pjt0000_bid00000_0se_gaw_dy_pur_con
Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)	Invitrogen	T668		2232873	https://www.thermofisher.com/order/catalog/product/T668
FCCP	Sigma	C2920	250 nM		https://www.sigmaaldrich.com/US/en/product/sigma/c2920 https://www.sigmaaldrich.com/US/en/product/sigma/c2920

Antibodies

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
NA	NA	NA	NA

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
NA	NA	NA	NA

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
NA	NA	NA

Other

Description	Source / Repository	Persistent ID / URL
Alexa Fluor 488 Phalloidin	Invitroten A12379 Lot 2409090	https://www.thermofisher.com/order/catalog/product/A12379?SID=srch-srp-A12379

ARRIVE GUIDELINES

The ARRIVE guidelines (https://arriveguidelines.org/) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
$SOD2^{fl/fl}$	M & F	4-5 months	70	70	Yes	
$SOD2^{fl/fl}$Pf4Cre	M & F	4-5 months	75	75	Yes	
$SOD2^{fl/fl}$	M & F	18-20 months	81	81	Yes	
$SOD2^{fl/fl}$Pf4Cre	M & F	18-20 months	82	82	Yes	
C57BL6/J	M & F	4-5 months	42	42	Yes	
C57BL6/J	M & F	18-20 months	46	46	Yes	
hIL4Rα/GP1bα Tg	M & F	4-5 months	39	39	Yes	
hIL4Rα/GP1bα Tg	M & F	18-20 months	45	45	Yes	

Sample Size: Sample size for this study was based on a previously published study on aging from investigator's lab (Dayal et al Circulation 2013). Following a two-sample t-test for mean ratio, a minimum of 6 mice per age group was required to provide 80.3% power.

Inclusion Criteria: Viable mice able to be genotyped with tail snip were included. Data from all mice undergone experimental procedures were used in the study.

Exclusion Criteria: Mouse not doing well before experimental procedure were excluded from the study.

Randomization: Mice were assigned to experimental groups using a random number generator function.

Blinding: Analysis of Flowcytometry experiments and experimental thrombosis were performed by a blinded investigator.