ARQ 092, an orally-available, selective AKT inhibitor, attenuates neutrophil-platelet interactions in sickle cell disease

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Supplementary Videos

Video 1

Video 2

Video 3

Video 4

Video 5

SUPPLEMENTAL METHODS

Reagents

Hydroxyurea, N-formyl-methionyl-leucyl-phenylalanine (fMLP), 2',7'-dichlorofluorescein diacetate (DCFH-DA), human thrombin, and PGE1 were purchased from Sigma (St. Louis, MO). D-Phe-Pro-Arg-chloromethyl ketone (PPACK) and a mouse anti-human CD42b (Glycoprotein Ibα (GPIbα), MM2/174) antibody were from EMD Millipore (Billerica, MA). Collagen-related peptide (CRP) was obtained from Dr. Richard Farndale (Department of Biochemistry, University of Cambridge, UK). Antibodies against p-PI3K p85α/β at Tyr458/p55α/γ at Tyr199, p-Src at Tyr416, p-AKT at Ser473, and AKT were from Cell Signaling (Danvers, MA). Recombinant mouse TNF-α, an Alexa Fluor 647-conjugated antibody against mouse Ly-6G, a PE-conjugated antibody against mouse αMβ2 (M1/70), an APC-conjugated antibody against mouse PECAM-1, an antibody against mouse ICAM-1, a Dylight 488-conjugated antibody against rat IgG, PE-conjugated monoclonal antibodies against total (ICRF44) or activated human αMβ2 (CBRM1/5), a Dylight 488-conjugated antibody against mouse IgG, and isotype control IgGs were purchased from BioLegend (San Diego, CA). Unlabeled or FITC-conjugated control IgG or anti-human L-selectin antibodies (Dreg56) were obtained from eBioscience (San Diego, CA). DyLight 488-conjugated anti-mouse CD42c (GPIbβ) and FITC-conjugated anti-mouse P-selectin (Wug.E9) or anti-CD42b (GPIbα, Xia.B2) antibodies were purchased from Emfret Analytics (Eibelstadt, Germany). Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit, CellTracker™ Red CMTPX, Calcium AM, and DyLight 488 dye were obtained from Life Technologies (Grand Island, NY). Human fibrinogen and human von Willebrand Factor (vWF) was purchased from Haematologic Technologies

(Essex Junction, VT). Human fibrinogen was conjugated with DyLight 488. Polyclonal antibodies against β-actin were obtained from Novus Biologicals (Littleton, CO). Unlabeled or APC-labeled control IgG or monoclonal anti-human CD41a (HIP8) antibodies were purchased from eBioscience (San Diego, CA). Monoclonal anti-mouse E-selectin (10E9.6) antibodies were from BD Biosciences (San Diego, CA). Ristocetin was obtained from American Biochemical & Pharmaceuticals Ltd (Marlton, NJ). Botrocetin was purified as described previously.[1] Ca^{2+} dye (FLIPR Calcium Assay kit) was from Molecular Devices (Sunnyvale, CA). Carboxy-PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (potassium salt)) was obtained from Santa Cruz Biotechnology (Dallas, TX). A Nitrate/Nitrite Colorimetric Assay kit was purchased from Cayman Chemical (Ann Arbor, MI).

Generation of chimeric Berkeley mice. Chimeric Berkeley mice were generated by transplantation of bone marrow cells isolated from Berkeley mice into lethally irradiated WT mice. Briefly, bone marrow cells of Berkeley mice (8-12 weeks old) were harvested from both femurs and tibias as described previously.[2] After lysing red blood cells, marrow cells were washed with ice-cold RPMI 1640 containing 10 U/mL heparin and 20 mM HEPES, pH 7.4. Recipient WT mice (6 weeks old, male) were fed with sterile food and acidic water, pH 3.5, containing 0.1 mg/mL neomycin 1 week before irradiation. The recipient mice underwent radiation (950 rad) one day prior to receiving 5×10^6 bone marrow cells in 250 µl RPMI 1640 by tail-vein injection. Recipient mice were fed with sterile food and acidic water containing neomycin until the experiment. Fourteen weeks

after transplantation, age-matched (20- to 24-week old) chimeric Berkeley mice were used in our studies.

Genotyping and chimerism. PCR and electrophoresis analyses were performed using blood from SCD mice. For PCR analysis, 200 µl of blood was treated with 1 ml red blood cell lysis buffer (8.3 mg/mL NH4Cl, 1 mg/mL NaHCO3, 0.01 mg/mL EDTA) at room temperature for 5 minutes. After spinning at 750 g for 5 minutes, the supernatant containing Hb was saved for electrophoresis. The cell pellet was washed once with PBS and resuspended in 100 µl Tris buffer (0.67 mM Tris-HCl, 0.07 mM EDTA pH 7.5). Cells were frozen and then thawed, followed by centrifugation at 13,000 g for 10 minutes. The supernatant was collected, and DNA was purified using the Isolate II PCR and gel kit (Bioline, Taunton, MA), followed by PCR analysis using 100 ng DNA. Primers for the region of the deleted mouse Hb β allele (398 base pairs) are: forward, 5'-TTAGGTGGTCTTAAAACTTTTGTGG-3', and reverse, 5'-ACTGGCACAGAGCATTGT TATG-3'. Primers for detection of mouse Hb β (291 base pairs) are: forward, 5'-AGATGTTTTTTTCACATTCTTGAGC-3', and reverse, 5'-AATGCCTGCTCTTTACTGAA GG-3'. Primers for detection of human sickle transgene of Hb β (480 base pairs): forward, 5'-GTATGGGAGAGGCTCCAACTC-3', and reverse, 5'-TCTGCCCAAATCTTAGACAAA AC-3'.[3]

For detection of human and mouse Hb, electrophoresis in a 15% acidic polyacrylamide gel containing urea was carried out by a modified method of Alter and Goff.[4] The gel solution consisted of 15% poly acrylamide (37.5:1, acrylamide: bis-acrylamide), 5% glacial acetic acid, 4.25 M Urea which was freshly deionized with mixed bed resin

(Sigma), 0.5% ammonium persulfate, and 3% TEMED. The electrophoresis buffer was 5% acetic acid. The gel was pre-electrophoresed twice with fresh running buffer for 60 minutes at 200 V with polarity reversed. The current fell from 30 mA to 10 mA during pre-electrophoresis, which indicated completion. Hb, 2μg, obtained from WT, Hbb+/-, and SCD (Hbb-/-) mice was mixed with 20 μl sample buffer (8 M freshly deionized urea containing 5% acetic acid), followed by electrophoresis for 4 hours 15 minutes and stained with Coomassie blue. The degree of chimerism was determined by comparison of the band density of mouse and human Hbα/β.

Flow cytometry. In the *in vitro* studies, neutrophils or platelets were pretreated with vehicle (0.1% DMSO) or different concentrations of ARQ 092 for 30 minutes at 37°C. For the *ex vivo* studies, vehicle (0.01 M phosphoric acid) or ARQ 092 (100 mg/kg/10 ml) was given by oral gavage to Berkeley mice that were fasted overnight. Blood and femur/tibia were collected at 30 minutes after treatment. In other experiments, Berkeley mice were fasted overnight and pretreated with or without PTIO (1 mg/kg/5 ml saline, an NO scavenger) 30 minutes before TNF-α treatment. The mice were then treated with saline or 250 mg/kg of HU (50 mg/ml) by oral gavage and subsequently with ip injection of TNF-α (500 ng). Phosphoric acid (0.01 M) or ARQ 092 (100 mg/kg) was administered orally at 2.5 hours after TNF-α treatment. Blood and femur/tibia were collected at 3 hours after TNF-α treatment to isolate platelets and neutrophils, respectively. Neutrophils were treated with or without fMLP (0.5 μM for human and 10 μM for mouse) or 20 ng/ml TNF-α for 10 minutes at 37°C, followed by incubation with PE-conjugated isotype control IgG or antibodies against human αMβ2 (ICRF44), activated human αMβ2 (CBRM1/5), or

mouse αMβ2, or Alexa Fluor 488-conjugated FG. Platelets were incubated with or without 0.025 U/ml thrombin for 5 minutes at 37°C, followed by incubation with PE-conjugated control IgG or antibodies against human P-selectin for 15 minutes. Cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry (Cyan ADP, Beckman Coulter). The geometric mean fluorescence intensity of antibodies was normalized to that of control IgG, and data are presented as fold increase compared with vehicle-treated, unstimulated cells.

AKT phosphorylation in neutrophils and platelets *in vitro* and *ex vivo*. Isolated neutrophils and platelets were pretreated with or without 0.1% DMSO or ARQ 092, followed by stimulation with an agonist. Cell lysates were immunoblotted with antibodies against total or phospho-AKT (p-AKT). For *ex vivo* studies, Berkeley mice were fasted overnight and treated with oral injection of vehicle or 100 mg/kg body weight (BW) of ARQ 092 (10 mg/ml in 0.01 M phosphoric acid) at 0.5 hours before collecting the blood and femur/tibia. Isolated neutrophils or platelets were incubated with fMLP or thrombin, respectively, for 2 minutes at 37°C. Equal amounts of proteins (50 µg) in cell lysates were immunoblotted with antibodies against p-AKT at Ser473, AKT, p-phosphoinositide 3-kinase (PI3K) p85α/β at Tyr458/p55α/γ at Tyr199, or p-Src at Tyr416, followed by densitometry.

Neutrophil-platelet aggregation assay. The *in vitro* cell-cell aggregation assay was performed under stirring conditions mimicking venous shear as we described.[5] Neutrophils and/or platelets isolated from SCD patients were incubated with 500 nM ARQ

092 for 30 minutes at 37°C and washed, followed by labeling with FITC-conjugated anti-L-selectin (2 μg/ml) and APC-conjugated anti-human CD41a antibodies (15 ng/ml), respectively. Platelets were then treated with 0.025 U/ml thrombin at 37°C for 5 minutes. After quenching with 50 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), activated platelets were mixed with neutrophils for 5 minutes under a stirring condition of 1,000 rpm in an aggregometer. Cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry as described above.

Platelet aggregation and agglutination assay. Washed patient platelets were pre-treated with vehicle (0.01% DMSO) or 50 and 500 nM ARQ 092 for 30 minutes at 37°C. Platelet aggregation was induced by stimulation with different concentrations of thrombin or CRP. For *ex vivo* studies, vehicle, HU, ARQ 092, or both were given orally to non-challenged or TNF-α-challenged Berkeley mice as described in flow cytometry. Isolated platelets were stimulated with thrombin or CRP. Platelet aggregation was measured in a platelet lumi-aggregometer (Chronolog Corp, Havertown, PA) at 37°C with stirring (1,000 rpm). To measure platelet agglutination,[5] patient platelets or Berkeley mouse 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 $CaCl_2$ and BSA. Human platelets were incubated with 0.5 μg/ml human vWF and then 0.3 mg/ml ristocetin, and mouse platelets were treated with 10 μg/ml human vWF and then 10 μg/ml botrocetin, followed by measurement of agglutination in an aggregometer.

Survival times. During or after intravital microscopic studies, survival times for each mouse were recorded. Each time point began at TNF-α injection and ended when the mouse died or at 6 hours after TNF-α injection. Each time point was plotted and analyzed in GraphPad Prism 6.

Immunohistochemistry. Following *in vivo* imaging, the cremaster muscle was carefully removed from each mouse and mounted on dental wax. The stretched muscle was then fixed in formalin and immersed in 70% ethanol. The tissues were embedded in paraffin, sectioned with a thickness of 5 µm, and mounted to slides. Each slide was sequentially rehydrated with xylene, decreasing concentrations of ethanol (100%, 95%, and 70%), and water. Antigen retrieval was carried out by immersing the slides into boiling citrate buffer (pH 6.2) for 5 seconds, and cooling for 30 minutes. Slides were washed with water, and endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol, followed by washing with 20 mM Tris-buffered saline with 0.1% tween 20 (TBS-T). The slides were incubated with 1 µg/ml control IgG, or rat anti-mouse ICAM-1 or anti-mouse E-selectin antibodies, followed by 1 µg/ml Dylight 488-conjugated goat anti-rat IgG antibodies. Then, slides were incubated with 1 µg/ml Alexa 647-labeled rat anti-mouse PECAM-1 antibodies, followed by mounting with Vectashield containing DAPI. Images were taken using a Nikon microscope (ECLIPSE Ti) equipped with a Plan Fluor ×100/1.30NA oil objective lens and recorded with a digital camera (CoolSNAP ES2). The data were analyzed using NIS-Elements (AR 3.2). Mean fluorescence intensity (MFI) was calculated using Image J by outlining the ECs, allowing the background signaling to be removed. The average MFI from the IgG samples was subtracted from each sample.

Histochemistry of lung sections. Following intravital microscopy, mouse lungs were removed, washed with ice-cold PBS, and fixed in 10% formalin for 48 hours. The lungs were then placed in 70% ethanol, embedded in paraffin, sectioned, and mounted on slides. Slides were rehydrated by 5 minute subsequent incubations in xylene (twice), 100% ethanol (twice), 95% ethanol, 70% ethanol, and water. Neutrophils were stained with a Naphthol AS-D Chloroacetate (specific esterase) kit (Sigma). The slides were then stained with hematoxylin and mounted with Vectashield containing DAPI. Images were taken using a Zeiss Axioplan 2 microscope equipped with a Plan-Neofluar ×40/1.3 NA oil objective lens and recorded with a digital camera. Neutrophils were manually counted in the field of view (110 mm^2) in 10 different areas per sample.

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, 40 μl, was used for a Nitrate/Nitrite Colorimetric Assay kit and the absorbance of each sample was measured at 540 nm using a microplate reader (PHERAstar, BMG Labtech).

Measurement of ROS production. Intracellular ROS generation was measured as described previously.[5] Vehicle, HU, ARQ 092, or both were given orally to TNF-α-challenged Berkeley mice as described in flow cytometry. Mouse neutrophils (2.5 x 10^5) and platelets (3 x 10^6) were incubated with 5 μM DCFH-DA for 10 minutes at 37ºC. After stimulation with 10 μM fMLP or 0.025 U/ml thrombin, the DCF signal was measured by flow cytometry. The extracellular H_2O_2 level was measured using the Amplex® Red H_2O_2

assay kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, neutrophils, 5×10^5 in 50 μl HBSS, and platelets, 5×10^7 in 50 μl HEPES-Tyrode buffer containing 1 mM $CaCl_2$. Neutrophils and platelets were treated with or without fMLP and thrombin, respectively, and then mixed with 50 μl of Amplex red reagent. The Amplex red signal was measured immediately using a PHERAstar FS (BMG Labtech, Cary, NC) with an absorbance of 560 nm for 30 minutes.

Ca^{2+} mobilization. Vehicle, HU, ARQ 092, or both were administered orally to TNF-α-challenged Berkeley mice as described in flow cytometry. Mouse neutrophils (2×10^6) and platelets (3×10^7) were suspended in HBSS and HEPES buffer, pH 7.3, respectively, without extracellular Ca^{2+}. Cells were incubated with Ca^{2+} dye for 30 minutes at 37°C in the dark, and neutrophils and platelets were then treated with 10 μM fMLP or 0.025 U/ml thrombin, respectively. To sequentially detect Ca^{2+} influx, 2 mM $CaCl_2$ was added at 200-300 seconds after treatment with an agonist. The Ca^{2+} signal was measured using a FlexStation spectrofluorometer (Molecular Devices) with an excitation wavelength of 485 nm and an emission wavelength of 525 nm. The cytosolic Ca^{2+} level was expressed as relative fluorescence unit and quantified by the area under the curve.

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Figure S1

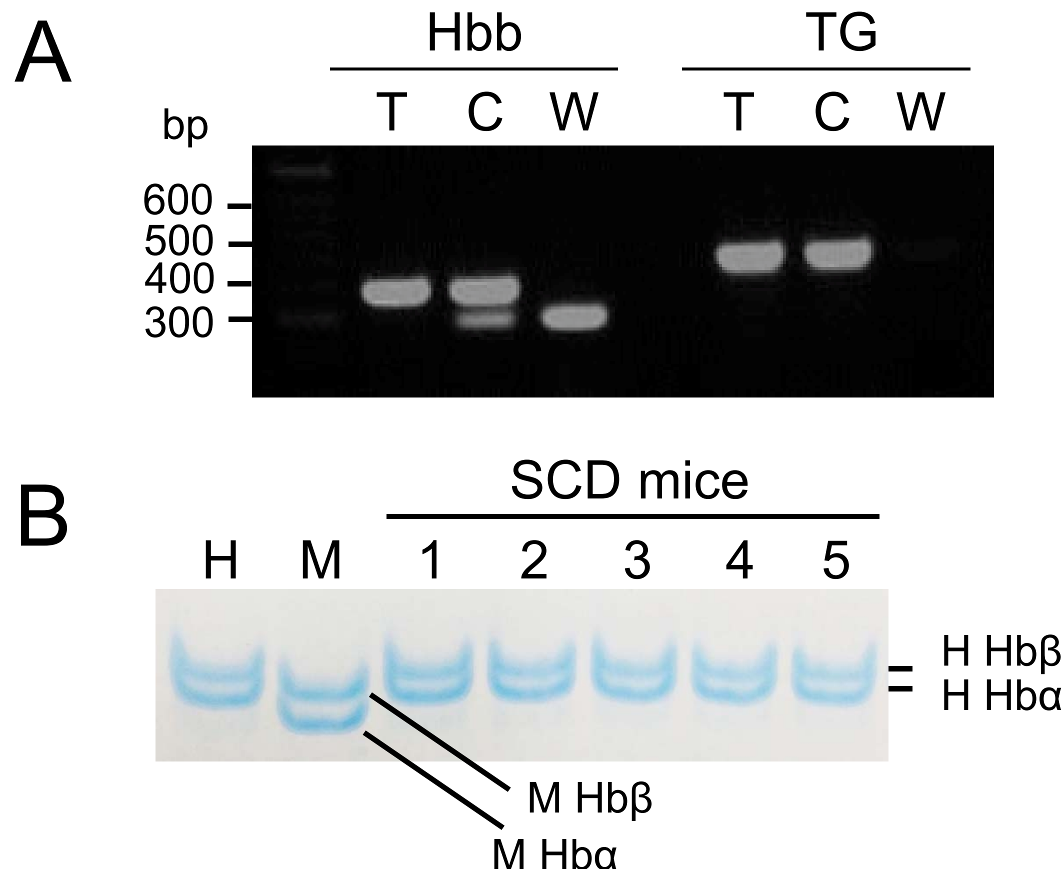
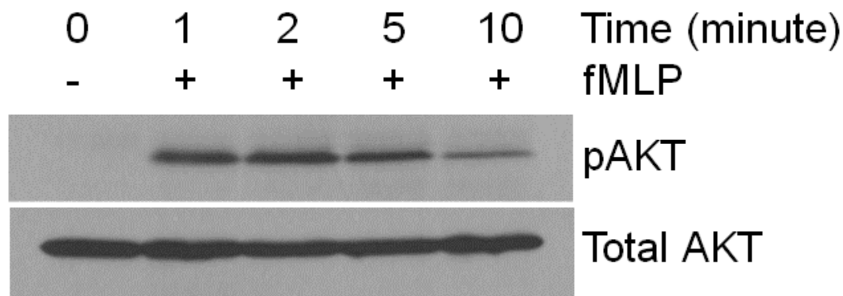


Figure S1. Chimerism of SCD mice. (A) Genotyping of genomic DNA isolated from mouse blood. T: SCD (Hbβ-/-) mice, C: control hemozygous (Hbβ+/-) mice, and W: C57BL/6 mice. (B) Chimerism of SCD mice by Hb electrophoresis. Hb lysates, 2μg, were electrophoresed on an acidic polyacrylamide gel containing urea. Human Hb (H), WT mouse (C57BL/6, M), and different SCD mice Hb. No mouse Hbα was detected in SCD mice tested.

Figure S2

A



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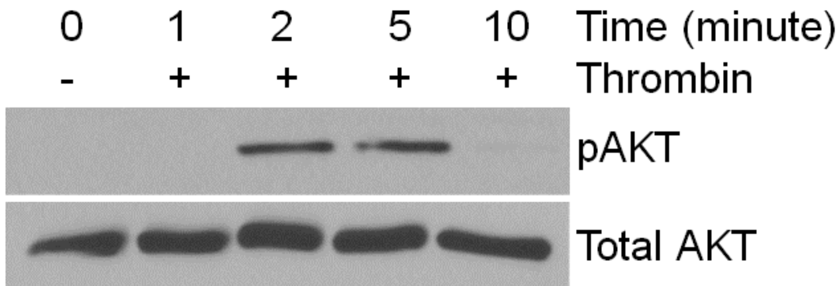

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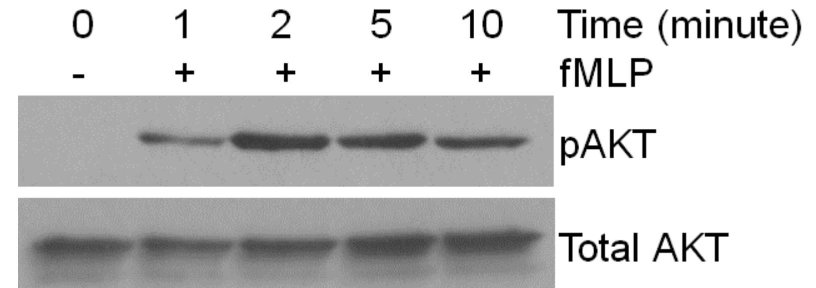

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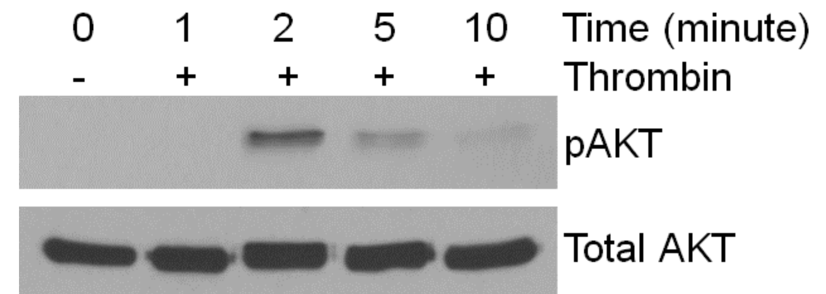
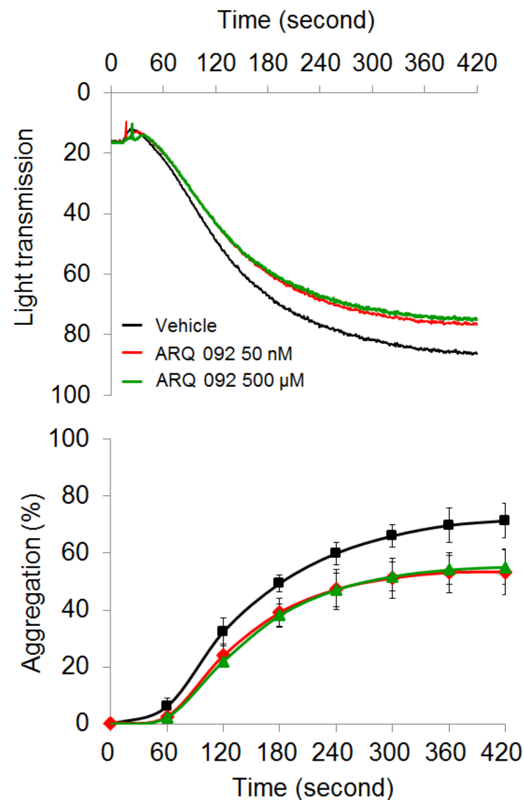


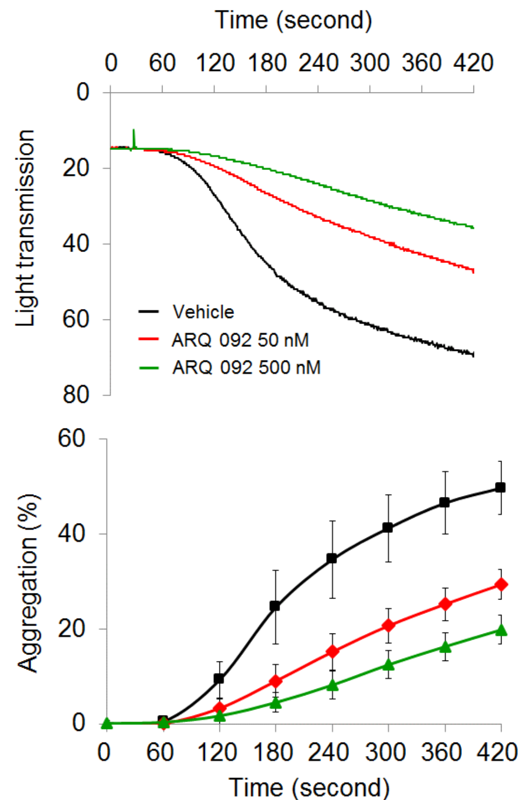
Figure S2. Kinetics of AKT phosphorylation in neutrophils and platelets following agonist stimulation. Neutrophils (A and C) and platelets (B and D) were isolated from SCD patients (A and B) and mice (C and D). Neutrophils or platelets were treated with or without fMLP (0.5 μM for human or 10 μM for mouse) or 0.025 U/ml thrombin, respectively, for 1, 2, 5, or 10 minutes. Equal amounts (50 μg) of cell lysate protein were immunoblotted. Representative blots were obtained from three independent experiments.

Figure S3

A 0.05 U/ml thrombin



B 0.25 µg/ml CRP



C 0.5 µg/ml CRP

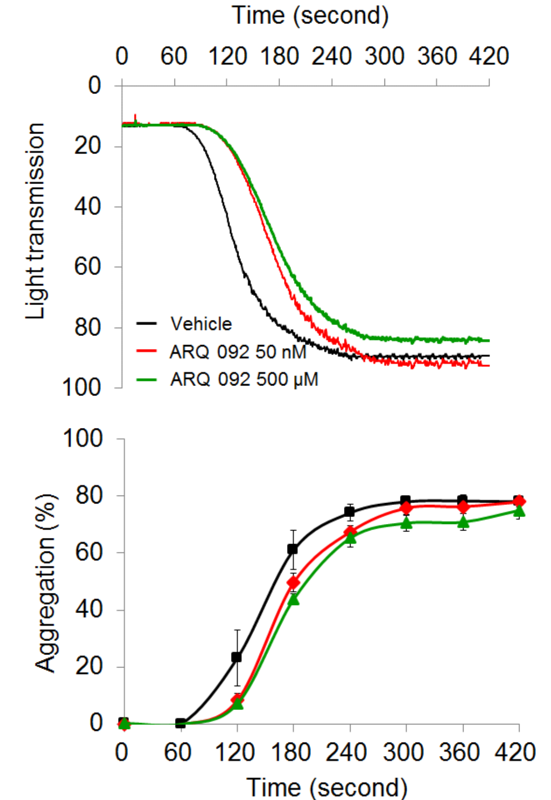


Figure S3. Inhibitory effects of ARQ 092 on aggregation of platelets isolated from SCD patients. Platelets were pre-treated with vehicle (0.1 % DMSO), or 50 or 500 nM ARQ 092, and aggregation was induced with 0.05 U/ml thrombin (A) or 0.25-0.5 µg/ml CRP (B-C). Data represent the mean ± SD (n = 3).

Figure S4

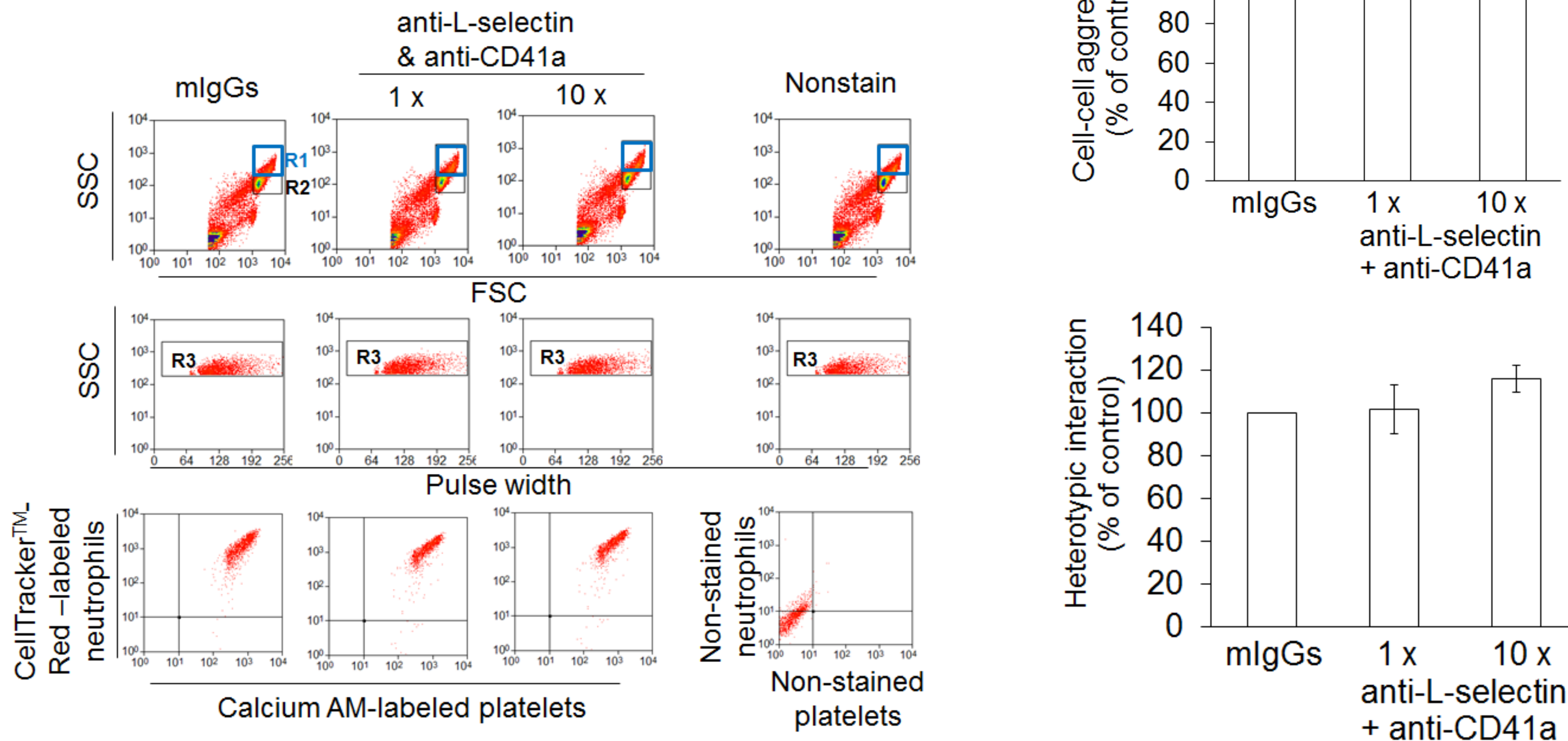
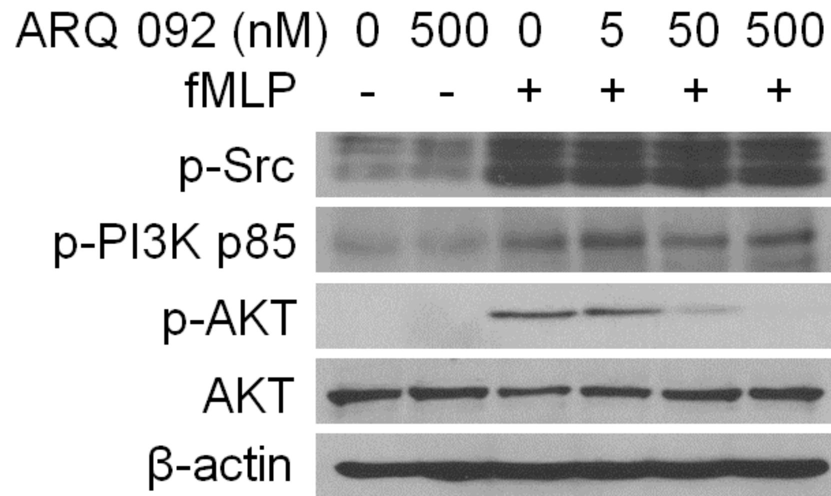


Figure S4. Effect of anti-L-selectin and anti-CD41a antibodies on neutrophil-platelet aggregation. Neutrophils and platelets isolated from SCD patients were labeled with CellTracker™ Red and calcein-AM, respectively, and incubated with anti-L-selectin (2 µg/ml) and anti-CD41a antibodies (15 ng/ml) at the concentration used for Figure 2 (1 x). Additionally, a 10-fold higher (10 x) concentration (20 µg/ml and 150 ng/ml) of the antibodies were also used. After thrombin stimulation, platelets were mixed with neutrophils under shear conditions. Flow cytometry was performed as described in Figure 2. Data represent the mean ± SD (n = 3).

Figure S5



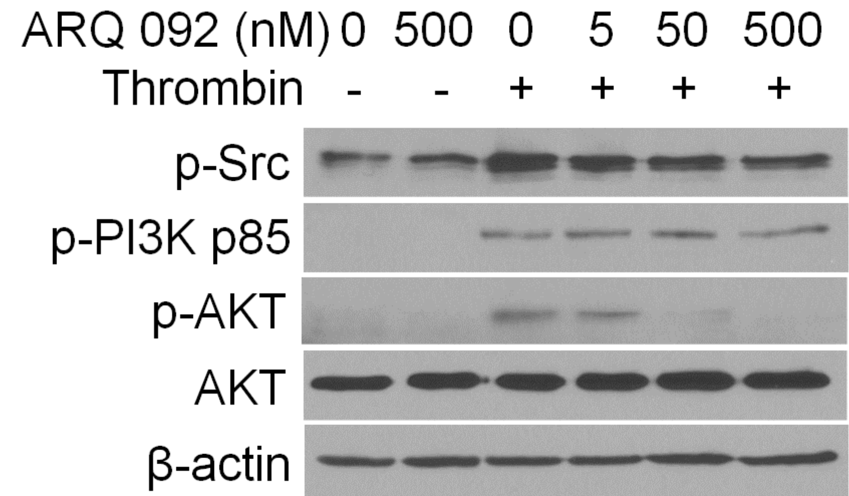


Figure S5. Inhibitory effects of ARQ 092 on AKT phosphorylation in SCD mouse neutrophils and platelets *in vitro*. Neutrophils (A) and platelets (B) isolated from SCD mice were pre-treated with 0.1% DMSO (0), or 5, 50, or 500 nM ARQ 092, followed by stimulation with 10 μM fMLP or 0.025 U/ml thrombin. Equal amounts (50 μg) of cell lysate protein were immunoblotted. Representative blots were obtained from three independent experiments.

Figure S6

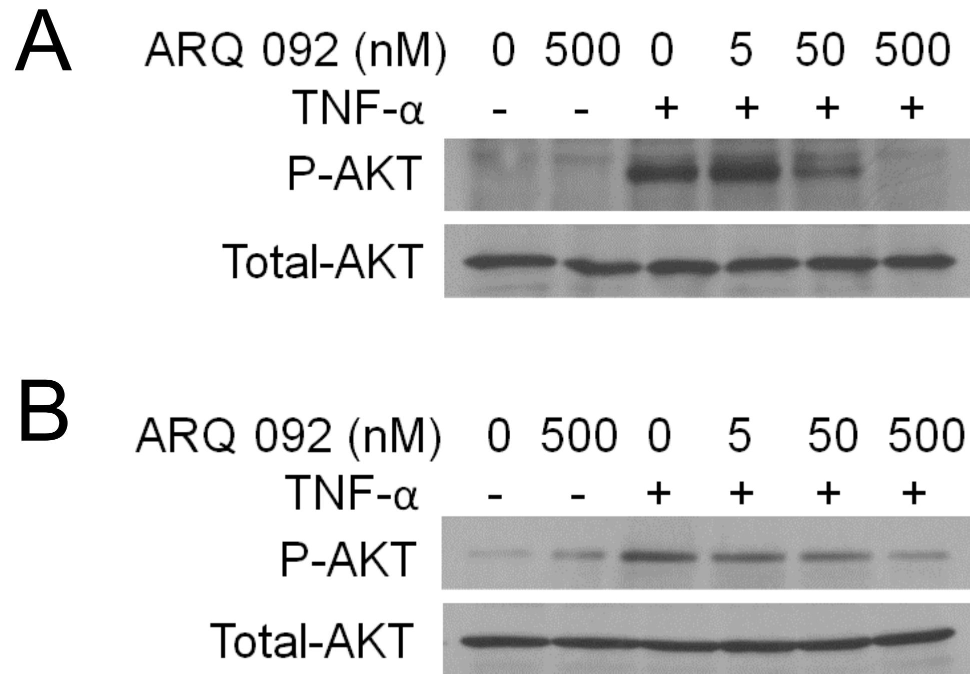


Figure S6. Inhibitory effects of ARQ 092 on AKT phosphorylation in TNF-α-stimulated neutrophils *in vitro*. Neutrophils isolated from SCD patients (A) and mice (B) were pre-treated with 0.1% DMSO (0), or 5, 50, or 500 nM ARQ 092, followed by stimulation with 20 ng/ml TNF-α. Equal amounts (50 μg) of cell lysate protein were immunoblotted. Representative blots were obtained from three independent experiments.

Figure S7

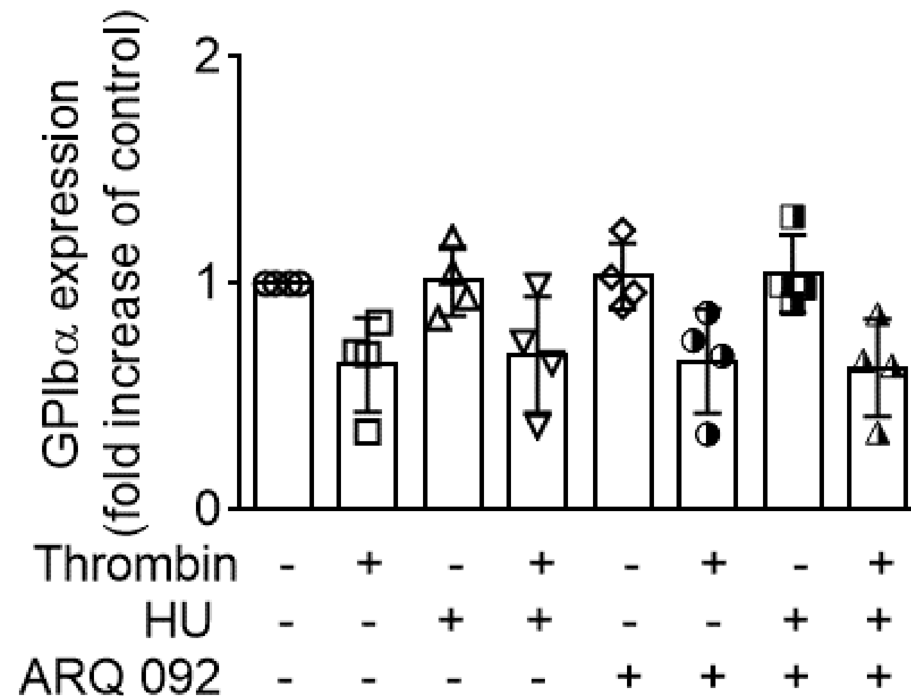


Figure S7. The surface expression of GPIbα on platelets isolated from TNF-α-challenged SCD mice. Platelets were isolated from TNF-α-challenged SCD mice after oral administration of vehicle (-, 0.01 M phosphoric acid), HU (250 mg/kg), ARQ 092 (100 mg/kg), or both inhibitors as described in Methods. Platelets were treated with or without 0.025 U/ml thrombin. The surface expression of GPIbα was measured by flow cytometry using anti-mouse GPIbα antibodies (Xia.B2). Data represent the mean ± SD (n = 3).