SUPPLEMENTAL DATA

Materials and Methods

Reagents. N-formyl-methionyl-leucyl-phenylalanine (fMLF), human thrombin, anti-human talin1, and other chemicals were purchased from Sigma (St. Louis, MO). Anti-phosphoAkt-Ser473 antibodies and antibodies sampler kit for Akt isoforms were obtained from Cell Signaling (Danvers, MA). Recombinant mouse TNF-α, control IgGs, mouse monoclonal blocking antibodies against total (ICRF44) and activated (CBRM1/5) human αMβ2, rat monoclonal antibodies against mouse β2 (M18/2) or αM (M1/70), PE-conjugated control IgGs or antibodies against mouse PSGL-1 (2PH1) or αM, FITC-conjugated control IgGs or anti-mouse αLβ2 (M17/4) antibodies, and an Alexa Fluor 647-conjugated anti-mouse Gr-1 antibody were from BioLegend (San Diego, CA). FITC-conjugated control IgG or anti-human L-selectin antibodies (Dreg56) were purchased from eBioscience (San Diego, CA). Anti-mouse GPIbα (Xia.B2), anti-mouse P-selectin (Wug.E9), and Dylight 488-conjugated anti-mouse CD42c antibodies were obtained from Emfret analytics (Germany). PE-conjugated control IgG or anti-human P-selectin antibodies, and APC-conjugated anti-human CD41a antibodies were from BD Pharmingen (Franklin Lakes, NJ). Polyclonal anti-mouse talin1, anti-mouse β2, and anti-actin antibodies were purchased from Santa Cruz (Santa Cruz, CA). Polyclonal anti-human β2 and anti-mouse P-selectin antibodies were obtained from R&D Systems (Minneapolis, MN). PPACK (D-Phe-Pro-Arg-chloromethylketone) and Akt inhibitors (Akti X and Akti XII) were from EMD Millipore (Billerica, MA). Protein G agarose beads were purchased from Thermo Scientific (Hanover Park, IL). Calcein red, calcein AM, and yellow-green fluorescent microspheres (0.1 μm) were from Invitrogen (Grand Island, NY).

SCD patients. Twenty homozygous (hemoglobin SS) patients aged 21-55 years (13 men and 7 women) were included in our studies. Among them, ten patients were treated with hydroxyurea prior to blood donation. No significant difference was observed in platelet P-selectin exposure and neutrophil $\alpha M\beta 2$ integrin activation in patients with or without the treatment. As for blood count, all patients were anemic (low numbers of red blood cells and low levels of hemoglobin and hematocrit), three patients had leukocytosis, and four patients had thrombocytosis. Blood of all patients was drawn at routine clinic visit without a pain crisis.

Isolation of human and mouse platelets. Human platelet-rich plasma (PRP) was obtained by centrifugation of sodium citrate-treated human blood at 200 g for 25 minutes. PRP was further centrifuged at 700 g for 10 minutes in the presence of 0.5 μM PGE1 and 10% citrate buffer. The pellet was resuspended with HEPES-Tyrode buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 0.42 mM $NaHPO_4$, 10 mM HEPES, 1 mM $MgCl_2$) containing 0.15 μM PGE1, and centrifuged at 800 g for 5 minutes. As for mouse platelets, sodium citrate-treated mouse blood (0.9 ml) was diluted 50% with HEPES-Tyrode buffer (20 mM HEPES, pH 7.3, 136 mM NaCl, 2.7 mM KCl, 3.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM glucose, and 0.1% BSA) before centrifugation at 300 g for 20 minutes. The PRP was collected and centrifuged at 700 g for 5 minutes in the presence of 0.5 μM PGE1. The platelet pellet was suspended in HEPES-Tyrode buffer containing 10% sodium citrate solution, and centrifuged at 700 g for 5 minutes. The pellet was re-suspended in HEPES-Tyrode buffer containing 1 mM $CaCl_2$. Final suspensions of

washed human and mouse platelets were adjusted to 2 × 10^8 and 3 x 10^8 platelets/ml, respectively.

Bone marrow transplant. Bone marrow cells of WT and Akt2 KO mice (6-8 weeks old) were harvested from both femurs and tibias of donor mice. After lysing red blood cells, marrow cells were washed with ice-cold RPMI 1640, 2.1mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10 U/mL heparin, and 20 mM HEPES, pH 7.4. Recipient WT and Akt2 KO mice 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 1 radiation (950 rad) before receiving 5 x 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 for 2 weeks. After that, the mice were provided with acidic water without the antibiotic until experiments. Eight weeks after transplantation, blood was counted and genotype studies were performed with blood of the transplanted mice.

Flow cytometric analysis. Human neutrophils treated with or without fMLF were incubated with 10 μg/ml of control IgG or anti-αM (ICRF44 and CBRM1/5) and then with 10 μg/ml of Alexa Fluor 488-labeled F(ab)'2 rabbit anti-mouse IgG. In some experiments, neutrophils were pretreated with or without various concentrations of Akt inhibitors for 45 minutes at 37ºC prior to stimulation with fMLF. Mouse neutrophils were stimulated with or without fMLF and incubated with 10 μg/ml of PE- or FITC-conjugated control IgGs, anti-αM, anti-αL or anti-PSGL-1 antibodies. Human and mouse platelets were activated with 0.25 U/ml thrombin for 2 minute at RT and incubated with anti-P-selectin or anti-GPIbα

antibodies. Cells were fixed and analyzed by flow cytometry (Cyan ADP, Beckman Coulter).

Immunoprecipitation and immunoblotting. Neutrophils (2 x 10^7 cells/ml) or platelets (3 x 10^8 cells/ml) were treated with or without Akt inhibitors and activated with fMLF and thrombin, respectively. Cells were lysed by lysis buffer (20 mM HEPES, pH 7.4, containing 1% NP-40, 1% CHAPS, 0.5% sodium dexoycholate, 150 mM NaCl, proteinase inhibitor cocktail, 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM NaF) on ice. Lysates were incubated with protein G beads coupled with control IgGs or anti-phosphoAkt-Ser473 antibodies. The bound fractions were analyzed by SDS-PAGE under reduced conditions and immunoblotted. To determine the specific effect of Akti XII on Akt2 phosphorylation *ex vivo*, saline or Akti XII, 10-30 μg/g BW, was infused into WT mice. Thirty minutes after Akti XII treatment, mouse platelets and neutrophils were isolated and immunoprecipitation was performed as described above. In some experiments, neutrophils and platelets were lysed 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). Lysates were electrophoresed under reduced conditions and immunoblotted. The band density was measured by densitometry using Scion Image (v4.0). To examine the β2-talin1 interaction, human neutrophils treated with 5 μM Akti XII or neutrophils isolated from WT and Akt KO mice were stimulated with fMLF for 1 minute. Lysates were immunoprecipitated with protein G beads coupled with control IgGs or anti-β2 antibodies, followed by immunoblotting.

Ca^{2+} mobilization. Human and mouse neutrophils (2 x 10^6) were pre-treated with or without Akti XII and re-suspended in HBSS buffer without Ca^{2+}. Cells were incubated with calcium dye (FLIPR Calcium Assay kit, Molecular Devices) for 30 minutes at 37°C in the dark and then treated with fMLF or 1 μM thapsigargin. 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 intracellular Ca^{2+} level was expressed as relative fluorescence unit and quantified by area under the curve.

Supplemental Video 1-4. Neutrophil recruitment and neutrophil-platelet interactions on TNF-α-inflamed cremaster venular endothelium in WT (Movie 1), Akt1 KO (Movie 2), Akt2 KO, (Movie 3), and Akt3 KO (Movie 4) mice. Neutrophils and platelets were monitored by Alexa Fluor 647-conjugated anti-mouse Gr-1 and Dylight 488-conjugated anti-CD42c antibodies, respectively.

Supplemental Figure 1. Akti XII specifically inhibits Akt2 phosphorylation in mouse neutrophils and platelets. Mouse neutrophils (A) and platelets (B) were pretreated with 5-15 μM Akti XII and then stimulated with 10 μM fMLF and 0.25 U/ml thrombin, respectively. Lysates were immunoprecipitated with anti-phosphoAkt-Ser473 antibodies and immunoblotted. The band density represents the mean ± SD (n = 3). **: $P < 0.01$ vs vehicle control by ANOVA and Dunnett's test.

Supplemental Figure 2. Akti X inhibits phosphorylation of all Akt isoforms and heterotypic neutrophil-platelet aggregation *in vitro*. Human neutrophils (A) and platelets (B) were pretreated with 10-30 μM Akti X and then stimulated with 0.5 μM fMLF and 0.25 U/ml thrombin, respectively. Immunoprecipitation was performed as described in Figure S1. **: $P < 0.01$ and ***: $P < 0.001$ vs vehicle control by ANOVA and Dunnett's test (n = 3). (C) Human neutrophils and platelets were pretreated with 10-30 μM Akti X and used for the heterotypic aggregation assay as described in Methods. Heterotypic interactions were measured by cell-cell aggregation. Data represent the mean ± SD (n = 3). #: $P < 0.05$ by ANOVA.

Supplemental Figure 3. Effect of inhibition or gene deletion of αM, αL, P-selectin, and αIIbβ3 integrin on neutrophil-platelet aggregation. Neutrophils and platelets isolated from WT or KO mice were incubated with or without an inhibitor (10 μg/ml). *In vitro* aggregation was performed as described in Methods. Heterotypic interactions were measured by cell-cell aggregation. INT: integrilin. **: $P < 0.01$ and ***: $P < 0.001$ vs vehicle or IgG control by ANOVA and Dunnett's test, and #: $P < 0.05$ by Student's *t*-test (n = 5).

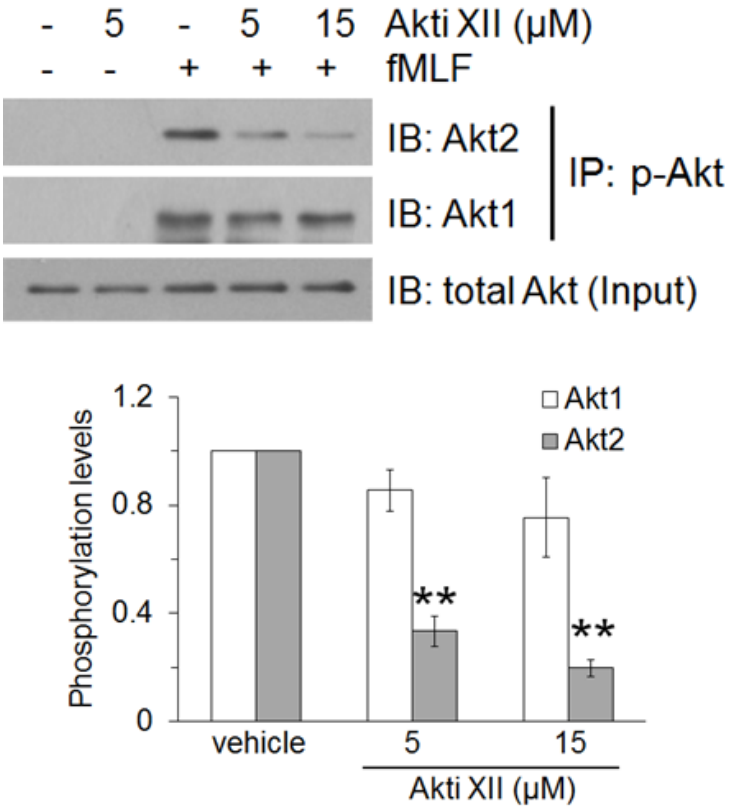
Supplemental Figure 4. Effect of Akti XII on neutrophil recruitment and neutrophil-platelet interactions during TNF-α-induced venular inflammation in WT mice and on Akt phosphorylation *ex vivo*. (A-C) Vascular inflammation was induced by intrascrotal injection of TNF-α into WT (C57BL/6) mice. Three hours after TNF-α injection, the mouse was treated with Akti XII, 10 or 30 μg/g BW, and intravital microscopy was performed as described in Methods. (A) The number of adherent neutrophils. (B) Ratio of crawling/adherent neutrophils (%). (C) The median integrated fluorescence intensities of anti-CD42c antibodies (F platelets). Data represent the mean ± SEM (n = 30-32 venules in 4 mice per group). *: $P < 0.05$ and **: $P < 0.01$ vs vehicle control by ANOVA and Dunnett's test. (D-E) Vehicle or Akti XII, 10-30 μg/g BW, was injected into WT mice through a jugular vein. Thirty minutes after Akti XII injection, blood was drawn and bone marrow was isolated. Platelets and neutrophils were treated with or without thrombin or fMLF, respectively. Immunoprecipitation was performed as described in Supplemental Figure 1. *: $P < 0.05$ and **: $P < 0.01$ vs vehicle control by ANOVA and Dunnett's test (mean ± SD, n = 4 mice per group).

Supplemental Figure 5. Effect of Akti XII on neutrophil adhesion and neutrophil-platelet interactions in venules of TNF-α-challenged Berkeley mice. Inflammation was induced by intraperitoneal injection of TNF-α (0.5 μg) into Berkeley mice. Three hours after TNF-α injection, the mice were treated with vehicle or Akti XII (10 or 30 μg/g BW). Intravital microscopy was performed as described in Methods. (A-B) The number of rolling and adherent neutrophils is shown. (C) The median integrated fluorescence intensities of anti-CD42c antibodies (F platelets). Data represent the mean ± SEM (n = 30-32 venules in 4 mice per group). **: $P < 0.01$ and ***: $P < 0.001$ vs vehicle control by ANOVA and Dunnett's test.

Supplemental Figure 6. Surface expression of activated αMβ2 integrin and P-selectin on SCD patients' cells. Flow cytometric analysis was performed to determine the surface expression of activated αMβ2 integrin and P-selectin exposure on SCD patients' neutrophils and platelets following fMLF and thrombin stimulation, respectively, as described in Methods. Data represent the mean ± SD (n = 6 patients per group). *: $P < 0.05$ vs healthy donors' cells by Student's *t*-test.

Supplemental Figure 1

A



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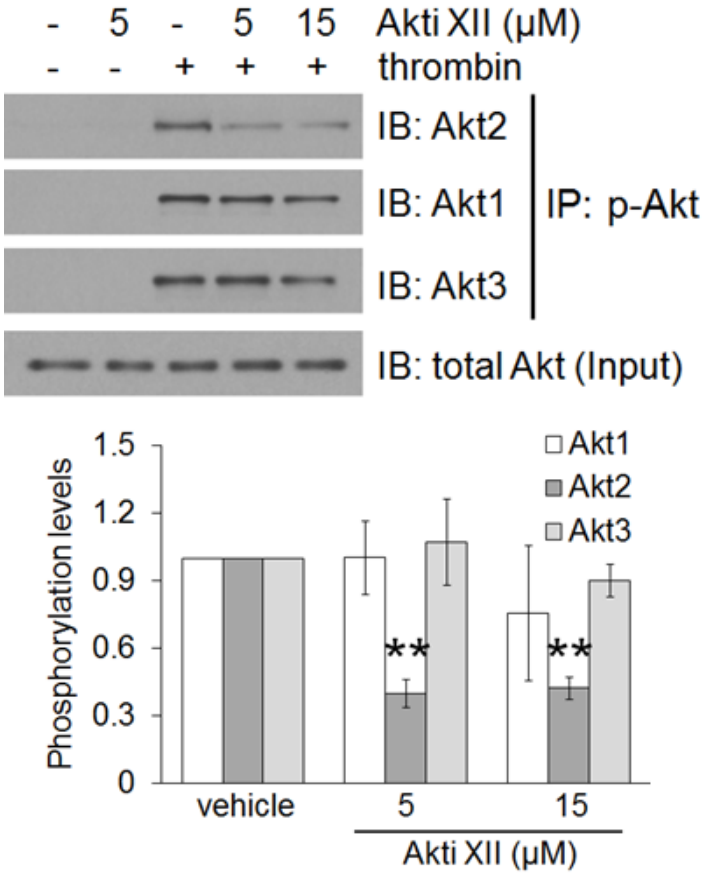

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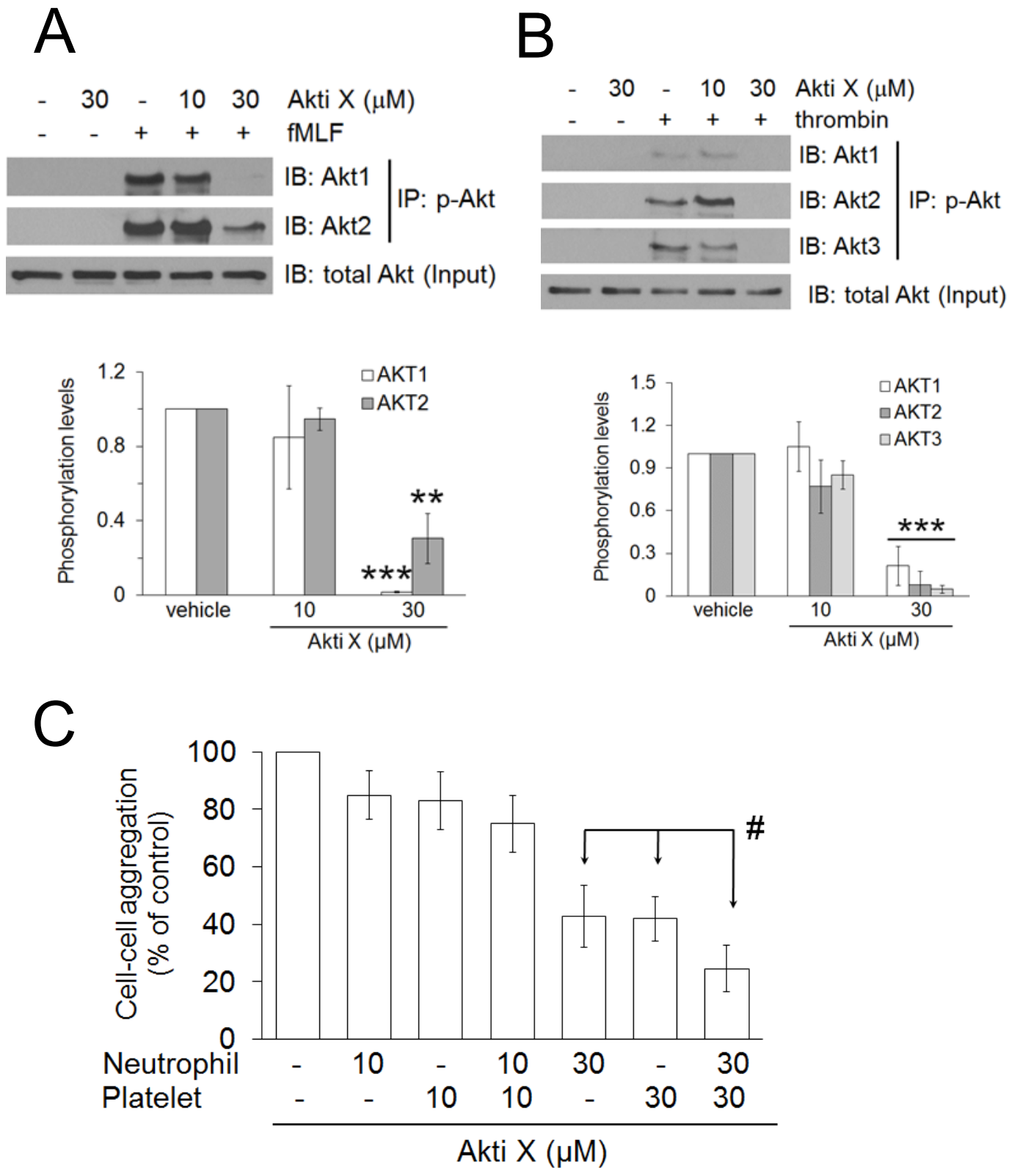

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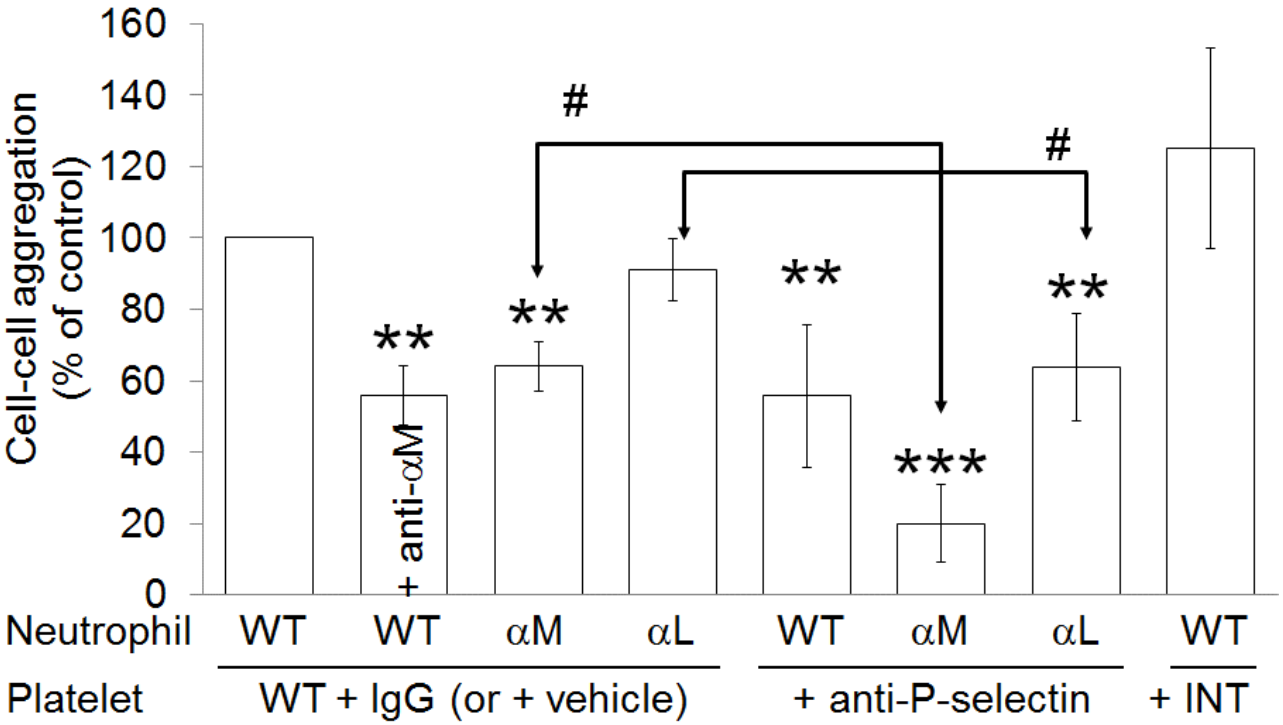
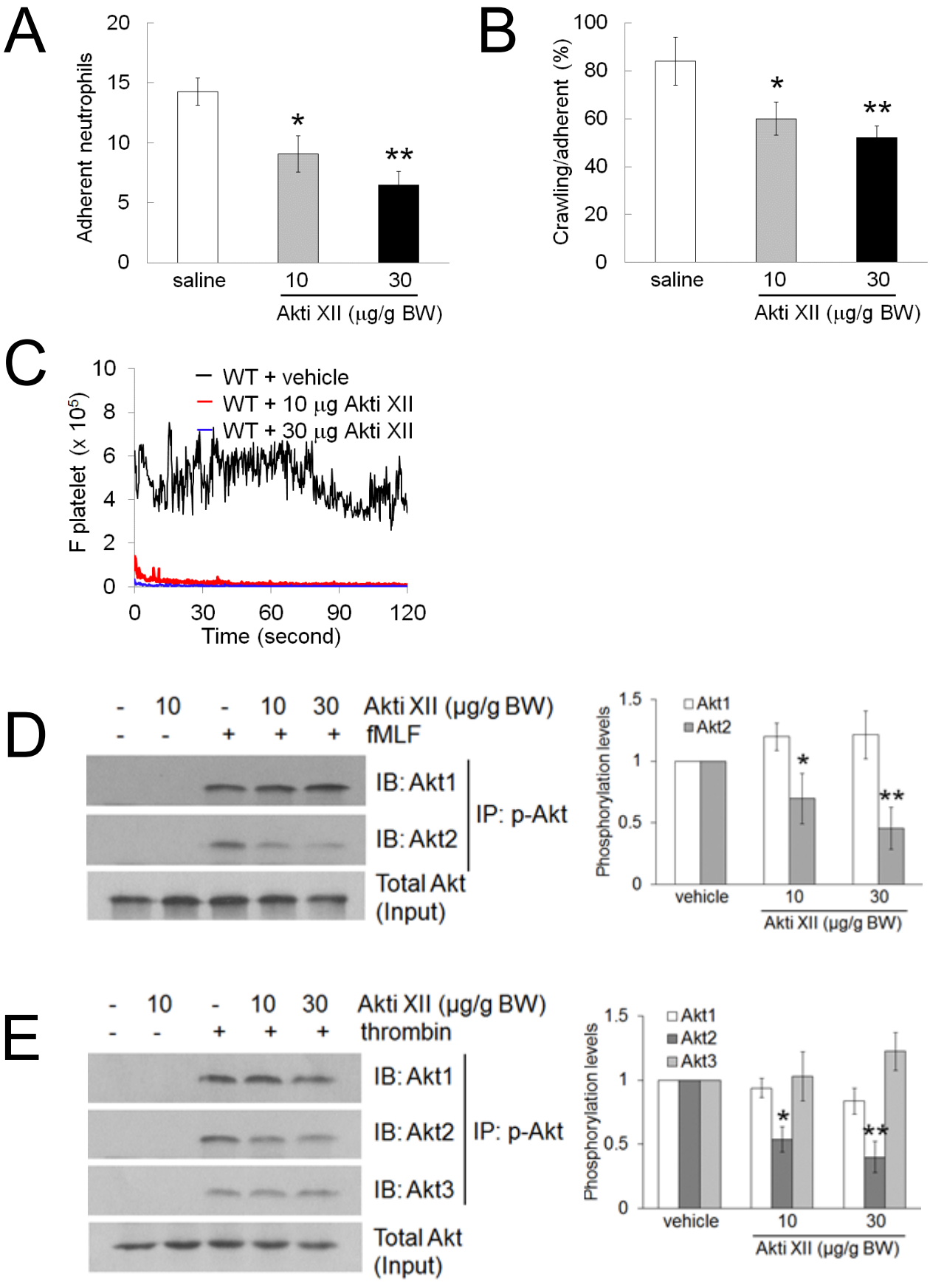
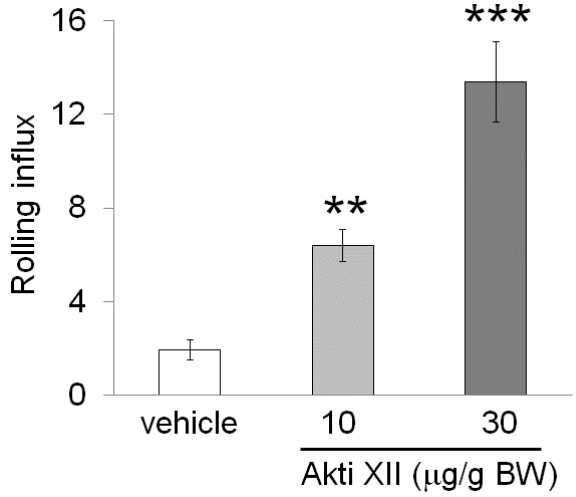


Supplemental Figure 4



Supplemental Figure 5

A



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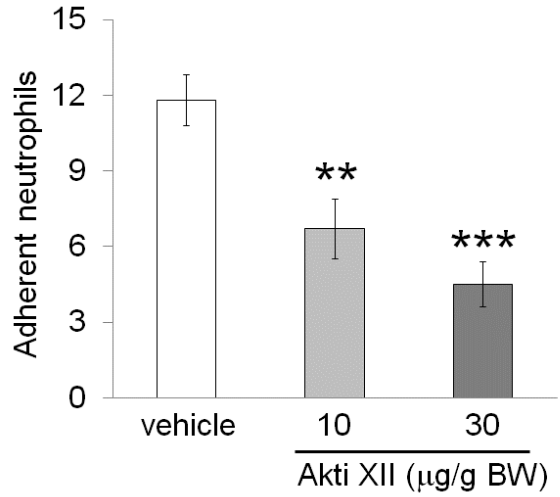

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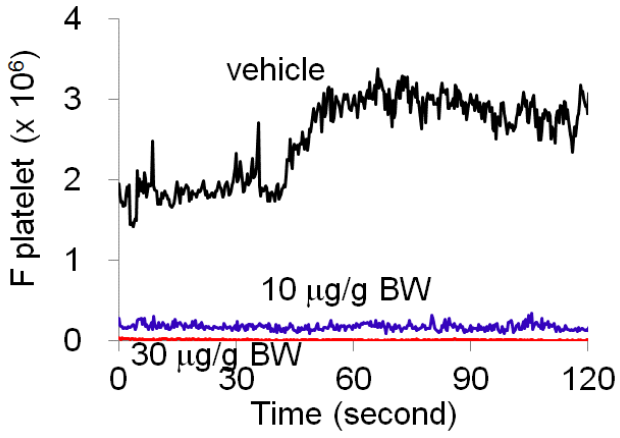

Supplemental Figure 6