SUPPLEMENTARY APPENDIX

NLRP3 regulates platelet integrin αIIbβ3 outside-in signaling, hemostasis and arterial thrombosis

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Detailed methods

Electron microscopy

Washed platelets were fixed in 3% glutaraldehyde, dehydrated, immersion and embedded using Epon812 followed by ultrathin sectioned using LKB-V ultramicrotome and subsequent stained with lead citrate and uranyl acetate. Samples were analyzed in a transmission electron microscope (JEOL-1200EX) and images were acquired with a Morada G2 digital camera.

Platelet spreading and adhesion

Platelets (2 x 10^7/ml) were placed on fibrinogen-coated glass coverslips (10 μg/ml fibrinogen, 4℃ overnight) at 37℃ for 90 min. After washing with PBS, platelets were fixed, permeabilized, stained with Alexa Fluor-546-labelled phalloidin and viewed by fluorescence microscopy (Nikon-80i) using an X100 oil objective. Surface coverage and the number of platelet adhesion on fibrinogen was quantified using Image J software. For some experiments, platelets were pre-treated with recombinant mouse IL-1β (Bioworld Technology), anti-IL-1β antibody (R&D Systems) or IL-1 receptor antagonist (IL-1RA, Sigma-Aldrich).

Clot retraction

Platelets (3 x 10^8/ml) were supplemented with 2 mM Ca^{2+} and 0.5 mg/ml fibrinogen and clot retraction was initiated by thrombin (1 U/ml) stimulation at 37℃. Images were captured every 15 min.

RNA isolation

Total RNA was isolated from 5 x 10^8/ml using Trizol reagent. Briefly, platelets were resuspended in 1 ml Trizol reagent and transferred into diethylpyrocarbonate (DEPC)-treated tubes followed by serial purification through addition of chloroform and precipitation by isopropyl alcohol. Total RNA was harvested by centrifugation at 15,000 x *g* for 10 min at 4℃ and then washed with 75% (v/v) ethanol. Isolated total RNA was resuspended in 50 μl DEPC-treated sterilized water. RNA was quantified by measuring the absorbance of RNA at 260 nm on a spectrophotometer.

Supporting Information

Movie S1: Platelet adhesion/thrombus formation in response to arterial injury in wild-type mice receiving infusion of wild-type platelets.

Movie S2: Platelet adhesion/thrombus formation in response to arterial injury in wild-type mice receiving infusion of *NLRP3*$^{-/-}$ platelets.

Supplementary Figures

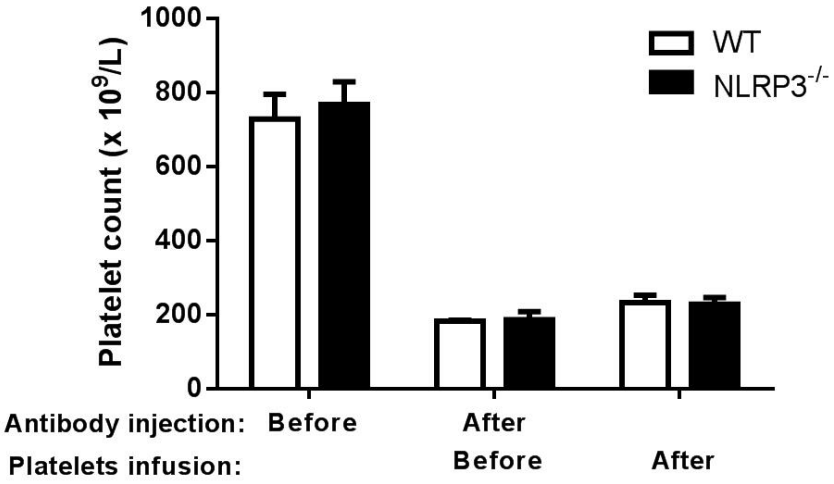

Figure S1. Platelet count. Platelet number was assessed before and after anti-GPIIb antibody injection, and after platelet infusion (mean ± SE, n = 3).

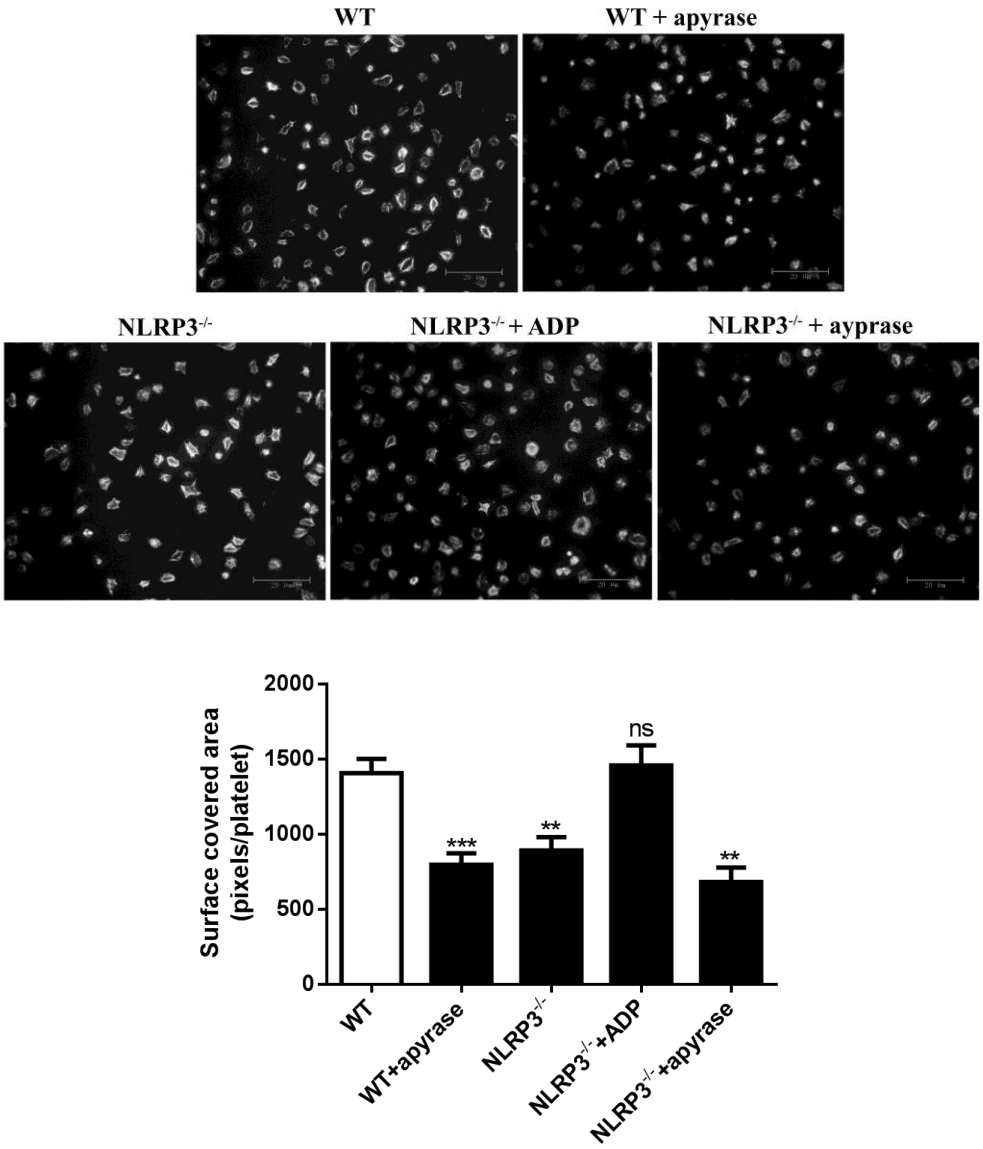

Figure S2. Platelet spreading on immobilized fibrinogen. Platelets were isolated from wild-type (WT) or *NLRP3*$^{-/-}$ mice and allowed to adhere and spread on immobilized fibrinogen in the absence or presence of apyrase (1 U/ml) or ADP (10 μM) for 90 min. Representative of three independent experiments were shown (mean ± SD, n = 3). Compared with WT, **P < 0.01, ns: not significant.

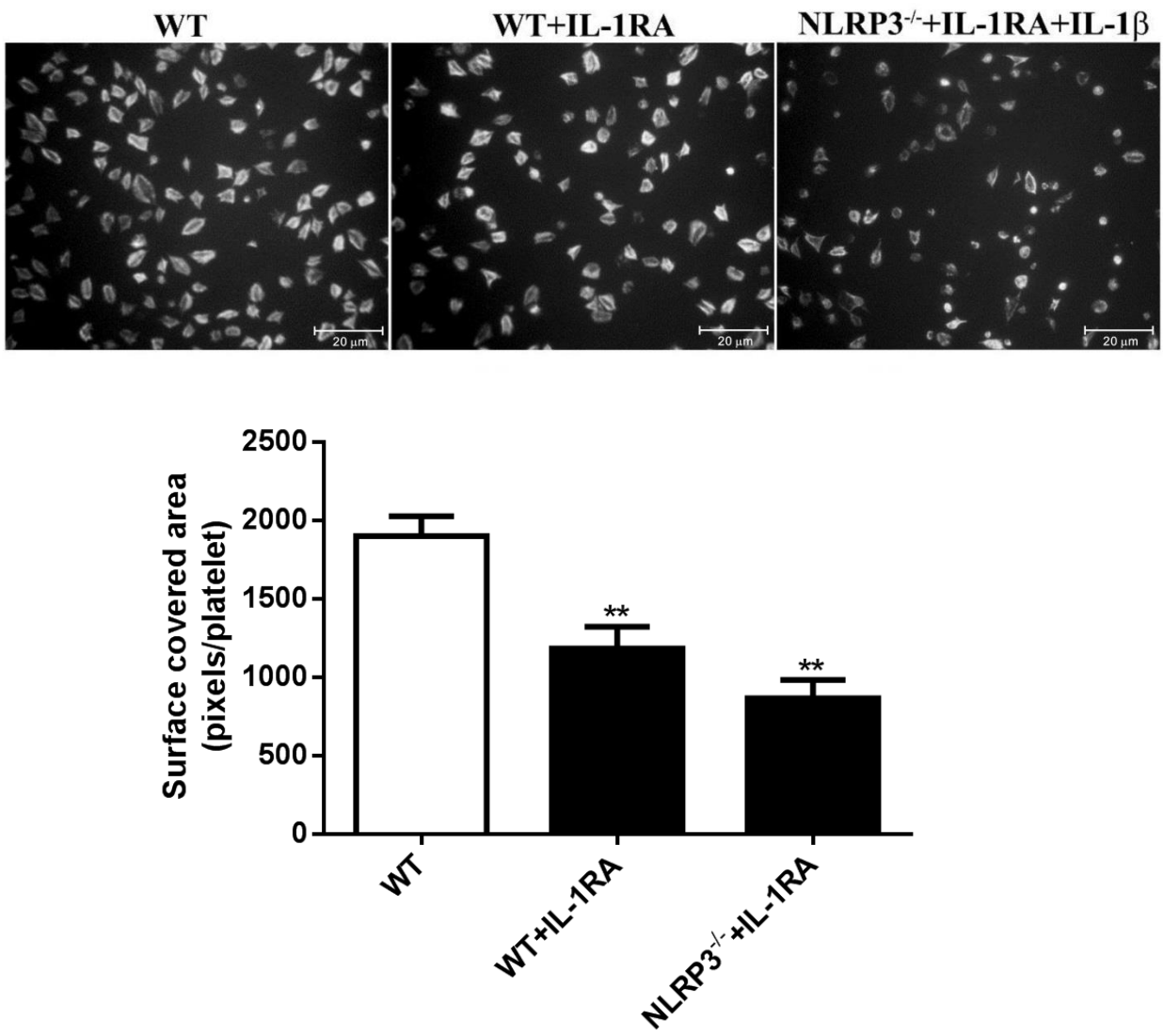

Figure S3. Defective platelet spreading after IL-1RA treatment. Platelets were isolated from WT or *NLRP3*$^{-/-}$ mice and pretreated with recombinant IL-1R antagonist (IL-1RA) (100 ng/ml) for 5 min followed by spreading on immobilized fibrinogen in the presence or absence of 10 ng/ml IL-1β. Images (X100) are representative of three independent experiments. Compared with WT, ***P < 0.001. (Scale bar = 20 μm)

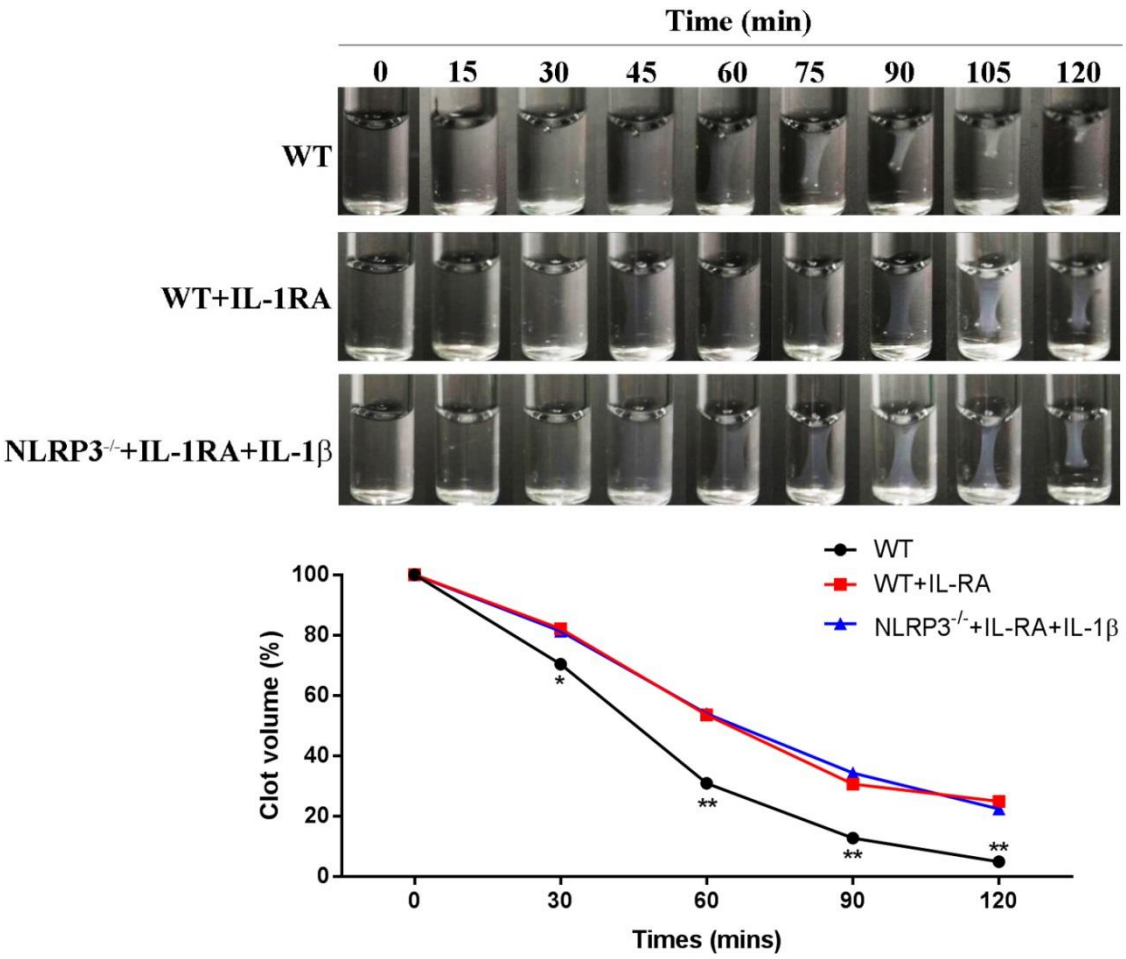

Figure S4. Impaired clot retraction of platelets treated with IL-1RA. Washed platelets from WT or *NLRP3*$^{-/-}$ mice were treated with IL-1RA (100 ng/ml) for 5 min followed by initiation of clot retraction after stimulated by 1 U/ml thrombin in the presence or absence of IL-1β (10 ng/ml). Images were take across 120 min and are represented as means from three independent experiments (n = 3). Compared with WT+IL-1RA or NLRP3$^{-/-}$+IL-1RA+IL-1β, *P < 0.05, **P < 0.01.

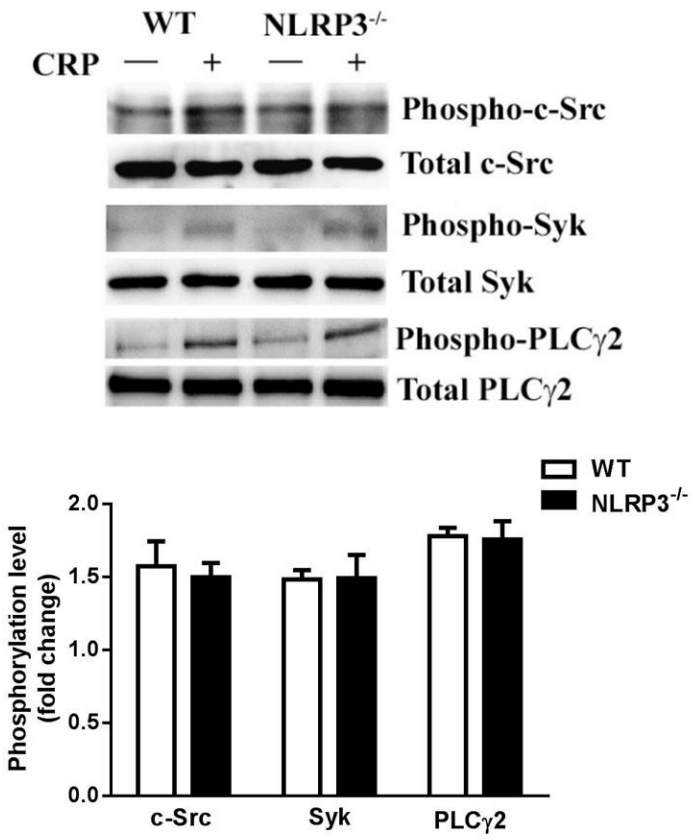

Figure S5. Phosphorylation of c-Src, Syk and PLCγ2 in platelets after GPVI engagement. Washed mouse platelets were treated with the GPVI-specific agonist, CRP at 10 μg/ml for 10 min (mean ± SD, n = 3) followed by western blot analysis of the phosphorylation of c-Src, Syk and PLCγ2. The phosphorylation level was quantified using Image J software and presented as fold change relative to phosphorylation level without stimulation.