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

Jianlin Qiao,[1,2,3#] Xiaoqing Wu,[1#] Qi Luo,[1#] Guangyu Wei,[1] Mengdi Xu,[1,2,3] Yulu Wu,[1] Yun Liu,[1] Xiaoqian Li,[2] Jie Zi,[2] Wen Ju,[1,2,3] Lin Fu,[1,2,3] Chong Chen,[1,2,3] Qingyun Wu,[1,2,3] Shengyun Zhu,[1,2,3] Kunming Qi,[2] Depeng Li,[2] Zhenyu Li,[1,2,3] Robert K. Andrews,[4] Lingyu Zeng,[1,3*] Elizabeth E. Gardiner[5*] and Kailin Xu[1,2,3*]

[1]Blood Diseases Institute, Xuzhou Medical University, China; [2]Department of Hematology, the Affiliated Hospital of Xuzhou Medical University, China; [3]Key Laboratory of Bone Marrow Stem Cell, Jiangsu Province, Xuzhou, China; [4]Australian Centre for Blood Diseases, Monash University, Melbourne, Australia and [5]ACRF Department of Cancer Biology and Therapeutics, John Curtin School of Medical Research, Australian National University, Canberra, Australia

#JQ, XW and QL, contributed equally to this study.

**LZ, EEG and KX contributed equally to this study.*

Haematologica 2018
Volume 103(9):1568-1576

Correspondence:
lihmd@163.com

Received: February 20, 2018.
Accepted: May 17, 2018.
Pre-published: 24 May, 2018.

doi:10.3324/haematol.2018.191700

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/103/9/1568

ABSTRACT

In addition to their hemostatic function, platelets play an important role in regulating the inflammatory response. The platelet NLRP3 inflammasome not only promotes interleukin-1β secretion, but was also found to be upregulated during platelet activation and thrombus formation *in vitro*. However, the role of NLRP3 in platelet function and thrombus formation *in vivo* remains unclear. In this study, we aimed to investigate the role of NLRP3 in platelet integrin αIIbβ3 signaling transduction. Using *NLRP3*$^{-/-}$ mice, we showed that NLRP3-deficient platelets do not have significant differences in expression of the platelet-specific adhesive receptors αIIbβ3 integrin, GPIbα or GPVI; however, *NLRP3*$^{-/-}$ platelets transfused into wild-type mice resulted in prolonged tail-bleeding time and delayed arterial thrombus formation, as well as exhibiting impaired spreading on immobilized fibrinogen and defective clot retraction, concomitant with decreased phosphorylation of c-Src, Syk and PLCγ2 in response to thrombin stimulation. Interestingly, addition of exogenous recombinant interleukin-1β reversed the defect in *NLRP3*$^{-/-}$ platelet spreading and clot retraction, and restored thrombin-induced phosphorylation of c-Src/Syk/PLCγ2, whereas an anti-interleukin-1β antibody blocked spreading and clot retraction mediated by wild-type platelets. Using the direct NLRP3 inhibitor, CY-09, we demonstrated significantly reduced human platelet aggregation in response to threshold concentrations of collagen and ADP, as well as impaired clot retraction in CY-09-treated human platelets, supporting a role for NLRP3 also in regulating human platelet αIIbβ3 outside-in signaling. This study identifies a novel role for NLRP3 and interleukin-1β in platelet function, and provides a new potential link between thrombosis and inflammation, suggesting that therapies targeting NLRP3 or interleukin-1β might be beneficial for treating inflammation-associated thrombosis.

Introduction

The primary platelet-specific receptors glycoprotein (GP)VI, which binds collagen and fibrin, and GPIbα, which binds von Willebrand factor, initiate platelet aggregation (thrombus formation) by recognition of exposed von Willebrand factor/collagen in the damaged blood vessel wall.[1,2] Engagement of platelet receptors initiates intra-platelet signaling pathways, which shift platelet integrin αIIbβ3 from

a low- to a high-affinity state (inside-out signaling) and enable platelet aggregation and thrombus formation through binding of soluble fibrinogen and other αIIbβ3 ligands.[3] Ligand binding to αIIbβ3 also triggers αIIbβ3 outside-in signaling,[4] leading to tyrosine phosphorylation of signaling proteins,[5-7] including c-Src, spleen tyrosine kinase (Syk) and phospholipase Cγ2 (PLCγ2), and initiates downstream platelet responses, such as granule secretion, platelet spreading and clot retraction.[8,9]

Platelets also have roles in the inflammatory response and in inflammatory pathology associated with atherosclerosis, malarial or dengue infection and rheumatoid arthritis.[10-13] Inflammasomes are multiprotein complexes that mediate responses to various inflammatory stimuli by controlling secretion of the pro-inflammatory cytokine, interleukin 1β (IL-1β).[14] Upon stimulation, NLRP3 undergoes oligomerization, leading to conversion of pro-caspase-1 to active caspase-1, which then cleaves pro-IL-1β to mature IL-1β.[14] For example, in dengue virus infection, platelet NLPR3 is activated, triggering IL-1β secretion.[13] NLPR3 also contributes to platelet activation, aggregation and thrombus formation *in vitro*, as shown by caspase activity measurements and pharmacological inhibition or genetic ablation of the NLPR3-associated adaptor protein, Bruton tyrosine kinase (BTK).[15] In this study using NLRP3-deficient platelets, we demonstrated a specific contribution of NLRP3 to αIIbβ3 outside-in signaling, and hemostasis and arterial thrombosis *in vivo*.

Methods

Animals

NLRP3$^{-/-}$ C57BL/6 mice[16] were purchased from Jackson Laboratories. All experimental procedures were approved by the Ethics Committee of Xuzhou Medical University.

Platelet preparation

Procedures involving collection of mouse and human blood were approved by the Medical Ethics Committee of Xuzhou Medical University. For mouse platelet studies, blood was collected from the retro-orbital plexus using ACD (85 mM trisodium citrate, 83 mM dextrose, and 21 mM citric acid) as anticoagulant and diluted in modified Tyrode buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 10 mM HEPES, pH 7.4). Platelets were then pelleted by centrifugation at 180 g in the presence of PGE1 (0.1 μg/mL) and apyrase (1 U/mL) (Sigma-Aldrich), washed twice with CGS buffer (120 mM sodium chloride, 12.9 mM trisodium citrate, 30 mM D-glucose, pH 6.5) and re-suspended in modified Tyrode buffer. Isolated platelets were allowed to rest for 1 h at room temperature before use. For human platelet studies, venous human blood was collected and then the platelets were isolated as described previously.[18]

Platelet analyses *in vitro*

Platelet receptor expression, activation, aggregation and immunoblotting were studied as previously described.[17,18] Antibodies against c-Src (anti-Tyr-416, Cell Signaling Technology; pan-c Src, Proteintech), Syk (anti-Tyr-525 and pan-Syk, Bioworld Technology) and PLCγ2 (anti-Tyr-1217 and pan-PLCγ2; Bioworld Technology), IL-1β (Cell Signaling Technology) and Caspase-1 (BioVision) were used.

Detailed methods of the electron microscopy of platelet spreading, and clot retraction are provided in the *Online Supplement.*

Quantitative real-time polymerase chain reaction

The mRNA expression of GPIbα, GPVI and IL-1β was measured by quantitative real-time polymerase chain reaction (PCR) as described previously.[19,20] In brief, RNA was reversely transcribed into cDNA using oligo(dT) and M-MLV Reverse Transcriptase (Thermo Fisher Scientific) and PCR amplification was performed in triplicate on a LightCycler® R480 II (Roche Life Science) with a total volume of 20 μL, consisting of 10 μL SYBR Green qPCR Super Mix, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 5 μL cDNA and 4 μL sterile water. The primers for GPIbα, GPVI and IL-1β were designed as follows: GPIbα forward primer: 5'-AGTTCATACTACCCACTGGAGCC-3', reverse primer: 5'-GTGGGTTTATGAGTTGGAGGC-3'; GPVI forward primer: 5'-AGGAGACCTTCCATCTTACCCA-3', reverse primer: 5'-GAGCAAAACCAAATGGAGGG-3'; IL-1β forward primer: 5'-CCTGAACTCAACTGTGAAATGC-3', reverse primer: 5'-GATGTGCTGCTGCGAGATT-3'. The relative mRNA expression of GPIbα, GPVI and IL-1β was calculated using the $2^{-\Delta\Delta}$Ct method and normalized to an internal control (β-actin).

Detailed methods on RNA extraction are provided in the *Online Supplement.*

Tail bleeding time

Tail bleeding assays were performed as previously described.[18] In brief, a 10-mm segment of tail tip was cut off and the tail was then immersed in pre-warmed sterile saline solution (37°C). Tail bleeding time was calculated as the time taken for bleeding to stop.

$FeCl_3$-induced thrombosis *in vivo*

Platelets isolated from wild-type and *NLRP3*$^{-/-}$ mice were labeled with calcein and infused into wild-type mice via tail vein injection. Injury to mesenteric arterioles was induced by 0.62 M $FeCl_3$ and thrombus formation was monitored by fluorescence microscopy (Olympus BX53).

Statistical analysis

Data are represented as mean ± standard deviation (SD) or standard error (SE) where indicated and analyzed by the Student *t*-test, one-way or two-way ANOVA.

Results

NLRP3 deficiency in platelets impairs *in vivo* hemostasis and thrombosis

To evaluate whether NLRP3 deficiency affects platelet production or clearance, we measured platelet count, mean platelet volume, platelet distribution width and plateletcrit and found similar values in wild-type and *NLRP3*$^{-/-}$ mice (P>0.05) (Figure 1A). Platelet receptors GPIbα, GPVI and integrin αIIbβ3 are critical for platelet function.[21,22] Evaluation of these receptors by flow cytometry and reverse transcriptase PCR revealed equivalent mRNA and protein levels in wild-type and *NLRP3*$^{-/-}$ mice (P>0.05) (Figure 1B). Electron microscopy analysis indicated that NLRP3 deficiency did not affect platelet ultrastructural organization, or the number and size of α- and dense granules (Figure 1C). Together, these data suggest that NLRP3 does not affect platelet production, expression of platelet receptors or granules.

In order to investigate whether NLRP3 influences platelet function *in vivo*, we performed tail bleeding assays and monitored $FeCl_3$-induced mesenteric arteriole thrombus formation. As *NLRP3*$^{-/-}$ mice demonstrated decreased tail bleeding times (mean ± SD) of 33.33 ± 14.43 s (n = 6),

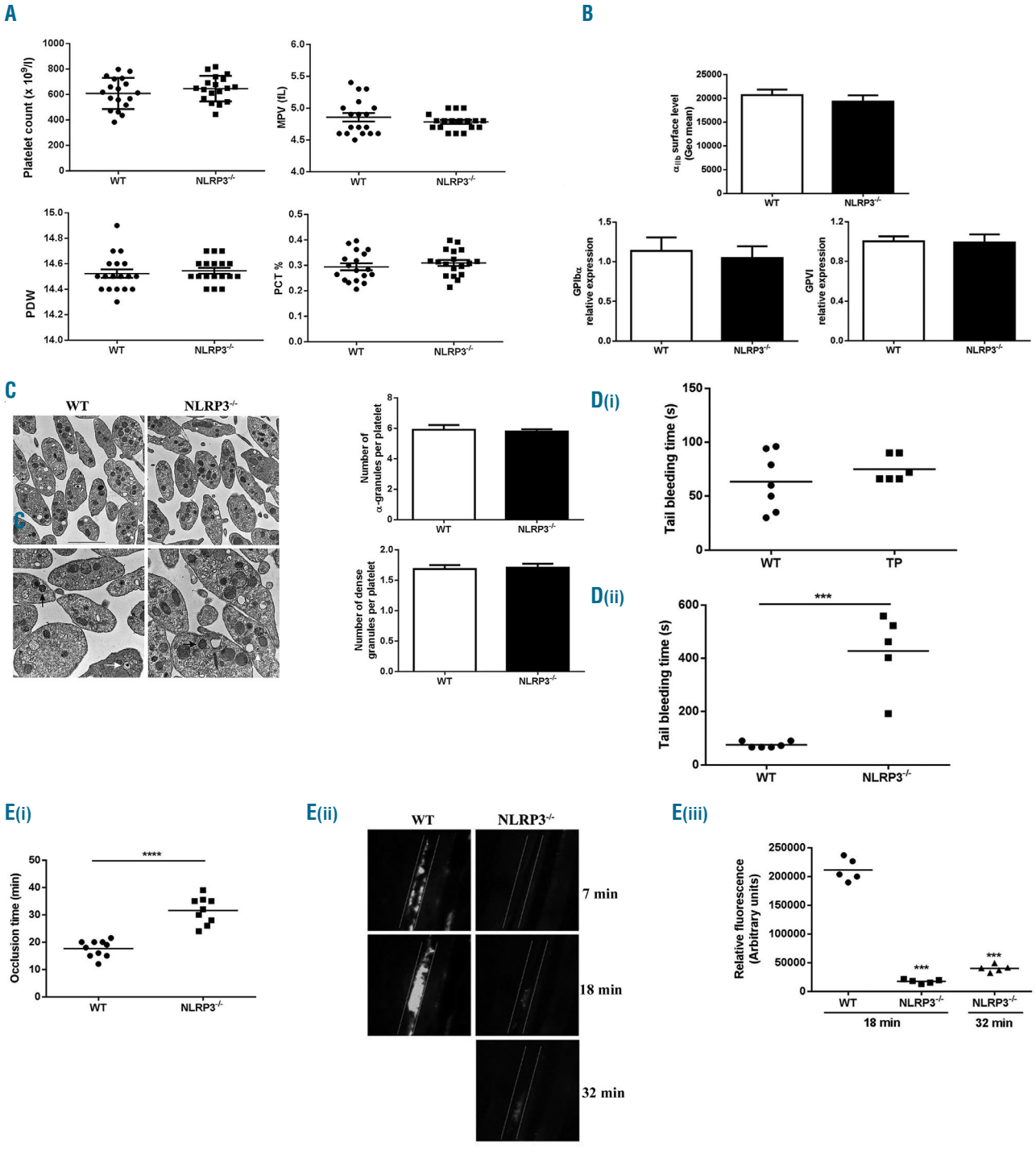

Figure 1. Platelet parameters, adhesion receptor expression, ultrastructure analysis, tail bleeding and arterial occlusion time in wild-type or *NLRP3*$^{-/-}$ mice. (A) Platelet count, mean platelet volume (MPV), platelet distribution width (PDW) and plateletcrit (PCT) determined by an automatic blood analyzer (mean ± SE). (B) Platelet αIIbβ3 surface expression was determined by flow cytometry using FITC-conjugated anti-mouse αIIβ monoclonal antibody; meanwhile total RNA was isolated from washed platelets in order to assess the expression of GPIbα and GPVI by quantitative real-time PCR. Data are represented as a ratio relative to an internal control (β-actin) (mean ± SE, n = 5-7) (Student *t*-test). (C) Analysis of platelet ultrastructure (α-granules and dense granules) by electron microscopy. Scale bar: 2 μm for upper panel (x 15,000 magnification) and 1 μm for lower panel (x 30,000 magnification). Black arrow: α-granule; white arrow: dense granule. The numbers of α-granules and dense granules were counted in 60 wild-type (WT) and 60 NLRP3 knockout platelets (mean ± SE) (Student *t*-test). (D)-i Tail bleeding time analysis of WT and thrombocytopenic (TP) mice after injection of anti-αIIb antibody, followed by infusion of donor platelets (1 x 10^8) from WT mice at 9 h after antibody administration (mean ± SD, n= 6-7) (Student *t*-test). (D)-ii To investigate hemostasis *in vivo*, washed platelets (1 x 10^8) were isolated from WT or *NLRP3*$^{-/-}$ mice and then infused into mice made thrombocytopenic by intraperitoneal injection of 0.1 mg/kg (body weight) rat anti-mouse αIIb antibody (MWReg 30) for 9 h, followed by analysis of tail bleeding time (mean ± SD, n = 5-6) (Student *t*-test). (E) For analysis of thrombosis *in vivo*, $FeCl_3$-induced arterial thrombus formation was initiated after washed platelets from WT or *NLRP3*$^{-/-}$ mice had been infused into WT mice and the time to vessel occlusion was recorded (mean ± SD, n = 9) (Student *t*-test) (i). Representative image of thrombus formation (ii) and the relative fluorescence (mean ± SD, n = 5) (one-way ANOVA) (iii) at different time points are shown. Movies showing real-time platelet adhesion and thrombus formation in $FeCl_3$-induced injured mesenteric arterioles were provided in the supporting information. **P*<0.05;***P*<0.01; ****P*<0.001; *****P*<0.0001. ns: not significant.

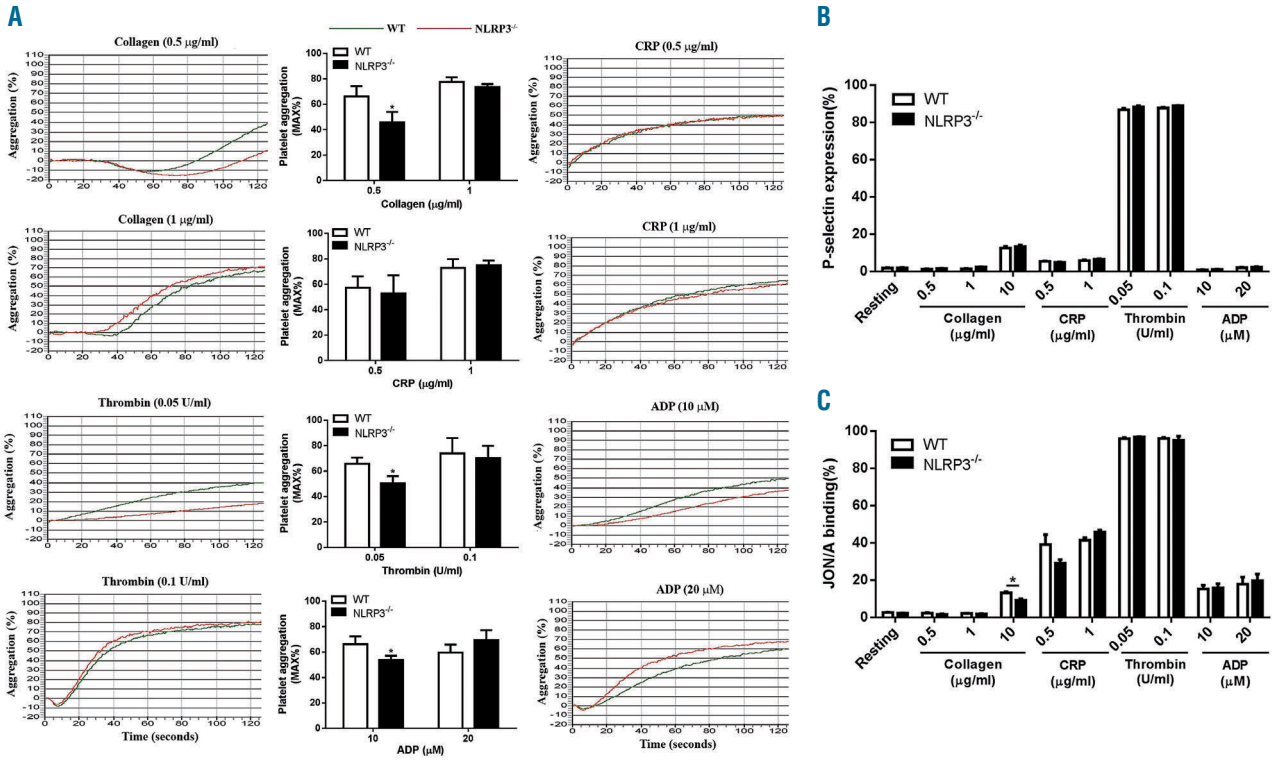

Figure 2. Platelet aggregation and activation. (A) Platelet aggregation was induced by addition of collagen, CRP, thrombin or ADP to platelets from wild-type (WT) or *NLRP3*$^{-/-}$ mice. Representative aggregation traces using platelets from WT (green) or *NLRP3*$^{-/-}$ (red) mice are shown together with combined data (mean ± SE) for three mice (Student *t*-test). (B) Platelet P-selectin expression and (C) activated αIIbβ3 (JON/A binding) were assessed in platelets before and after treatment with the indicated concentrations of collagen, CRP, ADP or thrombin for 10 min by flow cytometry using phycoerythrin-conjugated anti-P-selectin antibody (Ebioscience) or JON/A antibody (emfret ANALYTICS) (mean ± SE, n = 3-6). *P<0.05.

and reduced prothrombin times of 8.54 ± 0.30 s (n = 8) compared with the wild-type control values of 63.43 ± 26.94 s (n = 7) and 9.07 ± 0.37 s (n = 10), respectively, as well as elevated levels of coagulation factors VIII and IX (*data not shown*), platelets from wild-type or *NLRP3*$^{-/-}$ mice were injected into thrombocytopenic or wild-type mice to eliminate non-platelet hemostatic or thrombotic factor variations. The platelet numbers in mice receiving infusion of wild-type or *NLRP3*$^{-/-}$ platelets either before, after injection of anti-αIIb antibody or after infusion were comparable (*Online Supplementary Figure S1*). In addition, injection of anti-αIIb antibody did not affect the function of donor platelets as the tail bleeding time of thrombocytopenic mice receiving donor platelets (9 h after antibody) was similar to that of wild-type mice (Figure 1D-i). A significantly prolonged tail bleeding time (P<0.001) (Figure 1D-ii), delayed arterial thrombus formation (Figure 1E-i) as well as reduced platelet accumulation (relative fluorescence at the site of vascular injury) (P<0.0001) (Figure 1E-ii and iii) were detected in mice with *NLRP3*$^{-/-}$ platelets, suggesting that platelet NLRP3 deficiency significantly impairs hemostasis and arterial thrombus formation *in vivo*.

NLRP3$^{-/-}$ platelets display mildly reduced platelet aggregation and normal degranulation and αIIbβ3 activation

As platelet aggregation plays an important role in platelet function, we further investigated the effect of NLRP3 on platelet aggregation and activation. As shown in Figure 2A, platelet aggregation in response to a low dose of collagen (0.5 μg/mL), thrombin (0.05 U/mL) and ADP (10 μM) was significantly reduced in *NLRP3*$^{-/-}$ platelets compared with that of wide-type platelets (P<0.05), which was consistent with the findings of a previous study showing that NLRP3 deficiency affects platelet aggregation and activation.[15] However, no differences of platelet aggregation were found in response to a relatively high dose of collagen (1 μg/mL), thrombin (0.1 U/mL), ADP (20 μM) as well as collagen-related peptide (CRP: 0.5 and 1 μg/mL) (Figure 2A). As platelet granule secretion induced by agonist stimulation plays critical roles in the amplification of platelet signaling and subsequent activation and aggregation, we also measured platelet degranulation, represented by P-selectin expression and αIIbβ3 activation by flow cytometry. Interestingly, P-selectin upregulation (Figure 2B) and αIIbβ3 activation (Figure 2C) were normal in *NLRP3*$^{-/-}$ platelets after stimulation by collagen, CRP, thrombin or ADP. This is in contrast to the findings of Murthy and colleagues[15] and could be related to subtle differences in platelet preparations and experimental conditions. A direct link between NLRP3 and α granule release has not been described, and differences in rate and extent of P-selectin exposure are consistent with studies that have demonstrated agonist-related differential kinetics of platelet degranulation and secretion.[23-25]

NLRP3 deficiency in platelets affects integrin αIIbβ3 outside-in signaling

Platelet spreading and clot retraction, two processes regulated by early- and late-αIIbβ3 outside-in signaling, respectively,[26] were evaluated. Spreading of NLRP3-deficient platelets (Figure 3A) but not platelet adhesion (Figure

3B) was significantly inhibited on immobilized fibrinogen, together with reduced phosphorylation of c-Src and PLCγ2 (Figure 3C), which mediate platelet spreading.[5,7] Addition of thrombin (2 U/mL) did not reverse the defective spreading of *NLRP3*$^{-/-}$ platelets (Figure 3A), indicating that defective spreading did not result from insufficient activation. Interestingly, impaired platelet spreading was also observed in apyrase (1 U/mL)-treated platelets from wild-type mice, similar to the defective spreading of *NLRP3*$^{-/-}$ platelets. Defective spreading of *NLRP3*$^{-/-}$ platelets was rescued by the addition of ADP (*Online Supplementary Figure S2*), suggesting that ADP secretion might be impaired in *NLRP3*$^{-/-}$ platelets. NLRP3 regulates inflammation through processing pro-IL-1β to IL-1β,[27] and platelets excise introns from IL-1β pre-mRNA, yielding a mature message and translated protein.[28] Wild-type and NLRP3-deficient platelets expressed equivalent levels of IL-1β mRNA (Figure 3D-i) in RNA extracts that contained minimal RNA from contaminating leukocytes (Figure 3D-ii); however, thrombin stimulation triggered release of IL-1β

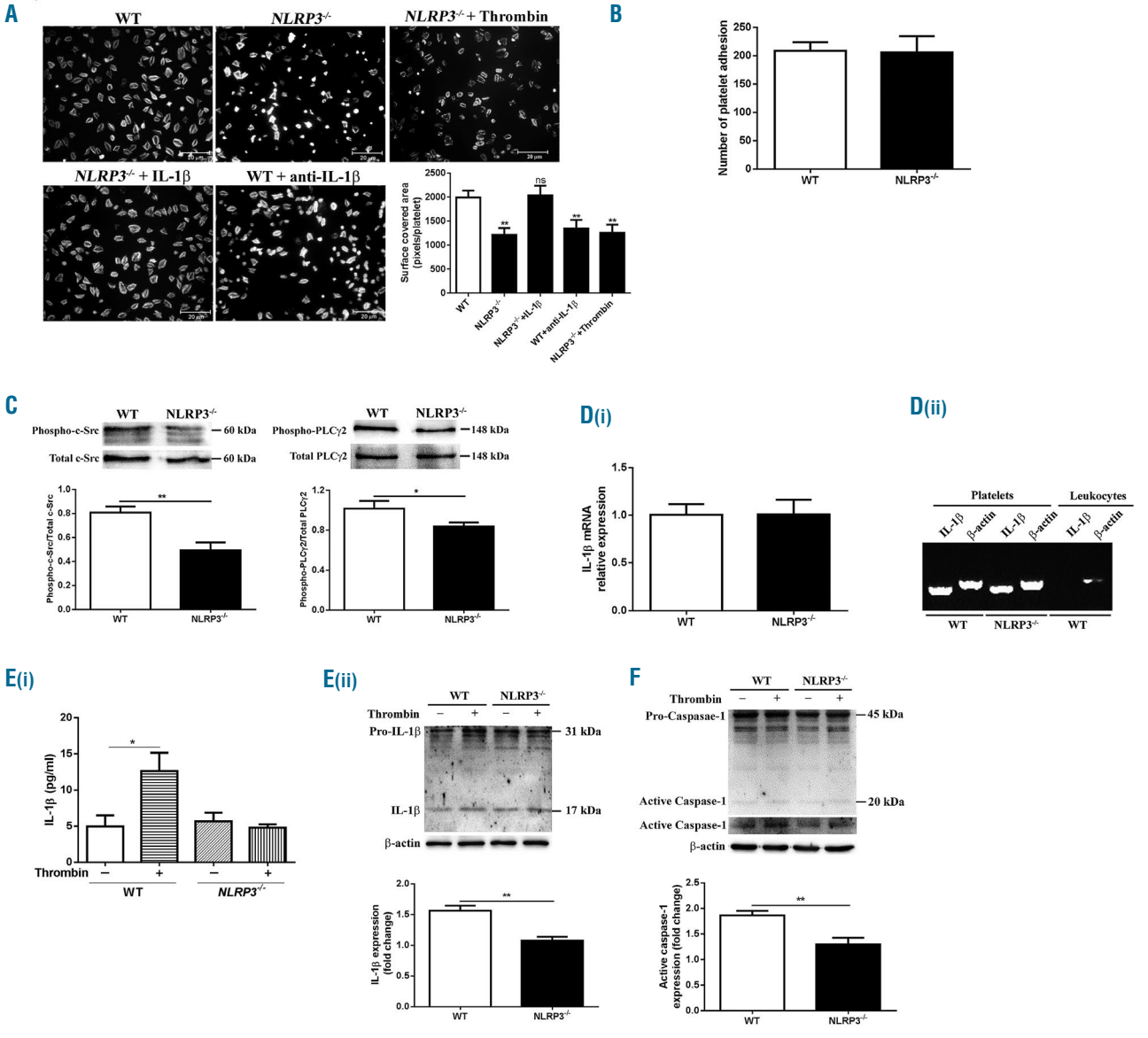

Figure 3. Platelet spreading and interleukin-1β secretion. (A) Platelet spreading on immobilized fibrinogen in the presence or absence of 10 ng/mL IL-1β, 0.5 μg/mL anti-IL-1β or 2 U/mL thrombin. Scale bar = 20 μm. Covered area was quantified by Image J software and analyzed by one-way ANOVA for comparison. Images (X100) are representative of three independent experiments (mean ± SD, n = 3). Compared with wild-type (WT), $^{**}P<0.01$; ns: not significant. (B) Platelet adhesion on fibrinogen for 90 min. The number of platelets that adhered to fibrinogen-coated glass coverslips was calculated using Image J software (mean ± SE, n = 3) (Student *t*-test). (C) Phosphorylation of c-Src and PLCγ2 in platelets after spreading on fibrinogen for 90 min. Data were quantified using Image J software and are represented as a ratio relative to total level (mean ± SD, n = 3) (Student *t*-test). $^{*}P<0.05$, $^{**}P<0.01$. (D) Total RNA was isolated from 5 x 10^8/mL platelets in order to measure IL-1β mRNA expression by RT-PCR (mean ± SD, n = 3) (Student *t*-test) (i); Washed platelet preparations (5 x 10^8/mL) were found to contain approximately 2 x 10^4/mL leukocytes. RNA was isolated from 5 x 10^8/mL platelets or 2 x 10^4/mL leukocytes followed by reverse transcription into cDNA which was used to measure IL-1β mRNA expression by PCR. PCR products were evaluated on 1.5% agarose gel (ii). (E) Washed platelets were stimulated with 0.5 U/mL thrombin before the level of IL-1β in supernatants was measured by enzyme-linked immunosorbent assay (mean ± SE, n = 3-5) (Student *t*-test) (i) and western blot (mean ± SD, n = 3) (ii). (F) Active caspase-1 expression after thrombin stimulation (mean ± SD, n = 3) (Student *t*-test). The western blot analysis of IL-1β or active caspase-1 expression was quantified as fold change to the level without treatment. $^{*}P<0.05$;$^{**}P<0.01$; $^{***}P<0.001$. ns: not significant.

from wild-type but not *NLRP3*$^{-/-}$ platelets (Figure 3E-i). Consistently, western blot analysis showed significantly reduced IL-1β expression after thrombin stimulation (Figure 3E-ii). A preliminary analysis suggested that the expression of active caspase-1, which is responsible for processing IL-1β, was significantly lower in *NLRP3*$^{-/-}$ platelets than in wild-type platelets after thrombin stimulation (Figure 3F). Supporting a role for IL-1β in platelet spreading, anti-IL-1β antibody treatment significantly impaired wild-type platelet spreading (Figure 3A) and when *NLRP3*$^{-/-}$ platelets were pre-treated with recombinant mouse IL-1β, platelet spreading was restored (Figure 3A). Since IL-1β also regulates platelet activation,[29] we hypothesized that defective spreading of *NLRP3*$^{-/-}$ platelets might be due to decreased secretion of IL-1β. To test this, we measured IL-1β release from platelets after spreading for 90 min and found a small but significant decrease in IL-1β secretion from *NLRP3*$^{-/-}$ platelets (59.0 ± 2.4 pg/mL) compared to that from wild-type platelets (52.5 ± 2.5 pg/mL) (mean ± SD; *P*=0.03;), suggesting that efficient spreading requires mature IL-1β. To then determine whether IL-1β exerted its effect on platelet spreading through IL-1 receptor (IL-1R) engagement, we pre-treated platelets with recombinant IL-1R antagonist (IL-1RA) and found defective spreading of wild-type platelets (*Online Supplementary Figure S3*). Furthermore, IL-1RA abolished IL-1β-mediated rescue of spreading of *NLRP3*$^{-/-}$ platelets (*Online Supplementary Figure S3*), suggesting that IL-1β affects platelet spreading via IL-1R.

Consistent with the ablated platelet spreading, *NLRP3*$^{-/-}$ platelets showed a significant impairment of clot retraction (Figure 4A) which was recovered by IL-1β addition, suggesting that NLRP3 regulates clot retraction via an IL-1β-dependent mechanism. Impaired clot retraction was also evident in wild-type platelets treated with anti-IL-1β antibody (Figure 4A). Furthermore, IL-1RA treatment impaired clot retraction of normal platelets and abolished the effect of IL-1β on the recovery of clot retraction in *NLRP3*$^{-/-}$ platelets (*Online Supplementary Figure S4*). As activation of αIIbβ3 outside-in signaling leads to phosphorylation of c-Src, Syk, and PLCγ2, and clot retraction,[5,6] we measured the phosphorylation status of these signaling proteins. Consistent with the defective platelet spreading and clot retraction, *NLRP3*$^{-/-}$ platelets exhibited significantly reduced phosphorylation of c-Src (Figure 4B), Syk (Figure 4C), and PLCγ2 (Figure 4D) following thrombin stimulation compared with that of wild-type platelets. However, treatment with recombinant IL-1β reversed the decreased phosphorylation of signaling proteins (Figure 4B-D). Interestingly, robust phosphorylation of c-Src, Syk and PLCγ2 in response to CRP/GPVI engagement, which does not require αIIbβ3 signaling, was achieved in *NLRP3*$^{-/-}$ platelets (*Online Supplementary Figure S5*). Together, these findings support a specific role for

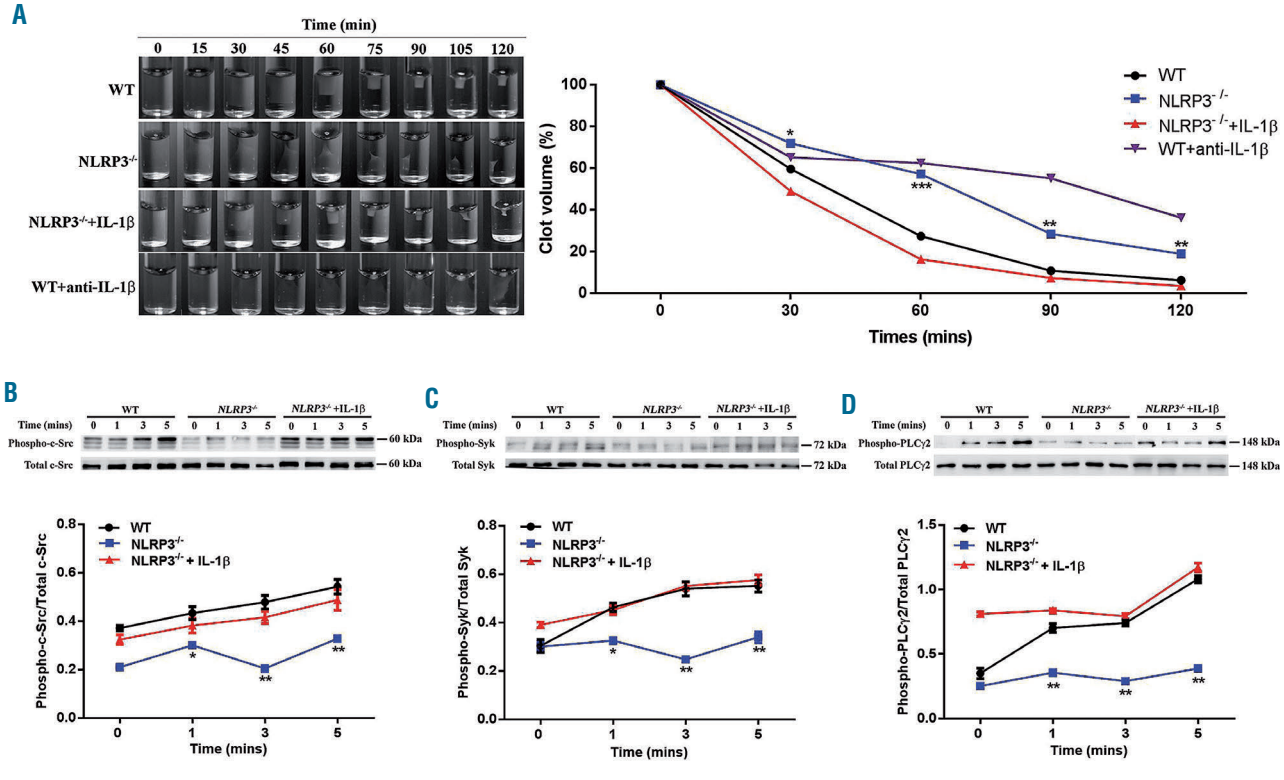

Figure 4. Impaired clot retraction and phosphorylation of c-Src, Syk and PLCγ2 in platelets from *NLRP3*$^{-/-}$ mice after thrombin stimulation. (A) Clot retraction was studied using washed platelets treated with 1 U/mL thrombin in the presence/absence of 10 ng/mL recombinant mouse IL-1β or 0.5 μg/mL anti-IL-1β antibody at 37°C. Representative images at 15, 30, 45, 60, 75, 90, 105 and 120 min from three independent experiments are shown. Data were quantified as the clot volume (%) and are presented as mean values (two-way ANOVA). Western blots of total and phosphorylated (B) c-Src (Tyr-416), (C) Syk (Tyr-525) and (D) PLCγ2 (Tyr-1217) in platelets treated with 1 U/mL thrombin in the presence of 2 mM Ca^{2+} and 0.5 mg/mL fibrinogen with or without 10 ng/mL recombinant mouse IL-1β pre-treatment at different time points were quantified (as a ratio of phosphorylated to total protein level) using Image J software and analyzed by two-way ANOVA for comparison (mean ± SD, n = 3). Images are representative of three independent western blot experiments. Compared with wild-type (WT): **P*<0.05; ***P*<0.01; ****P*<0.001.

NLRP3/IL-1β in the regulation of platelet integrin αIIbβ3 outside-in signaling.

Inhibition of NLRP3 impairs clot retraction in human platelets

Using a selective and direct NLRP3 inhibitor (CY-09),[30] we evaluated the role of NLRP3 in integrin αIIbβ3 signaling transduction in human platelets. We found significantly reduced human platelet aggregation in response to low doses but not high doses of collagen and ADP following treatment with CY-09 (Figure 5A). Interestingly, CY-09 treatment did not affect platelet aggregation in response to CRP stimulation (Figure 5A). Using flow cytometry we found no differences in platelet degranulation (P-selectin

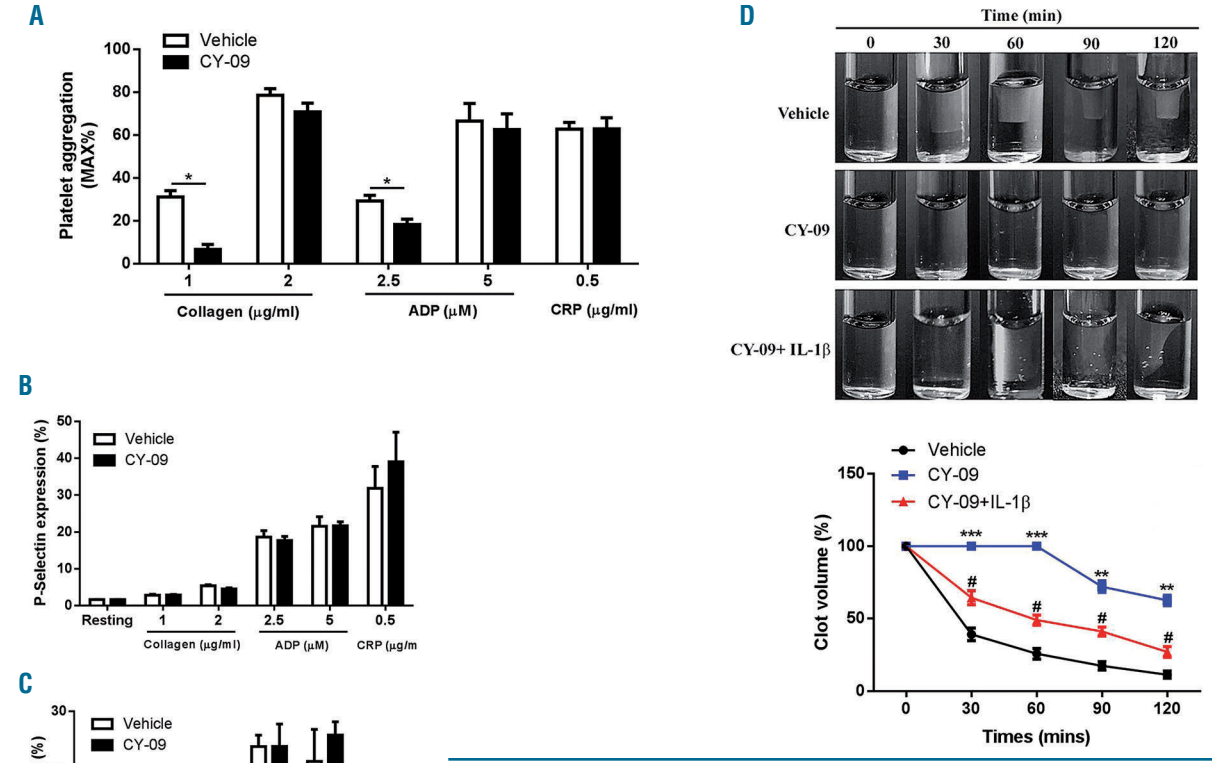

Figure 5. Effect of NLRP3 inhibition on human platelet function. (A) Human platelet-rich plasma was incubated with CY-20 (20 μM) for 30 min at 37 °C and then platelet aggregation in response to collagen (1 and 2 μg/mL), ADP (2.5 and 5 μM) or CRP (0.5 μg/mL) was measured in a light transmittance aggregometry (Helena Aggram, Helena Laboratories, Beaumont, USA). Maximum platelet aggregation (%) was recorded (mean ± SE, n = 3) (two-way ANOVA). (B) Platelet P-selectin expression and (C) αIIbβ3 activation in response to collagen, ADP or CRP stimulation was measured by flow cytometry using phycoerythrin-conjugated anti-P-selectin antibody and FITC-conjugated PAC-1 antibody, respectively, and represented as mean ± SE (n = 3) (one-way ANOVA). (D) Clot retraction was initiated using CY-09-treated washed human platelets stimulated with 1 U/mL thrombin in the presence/absence of recombinant human IL-1β (10 ng/mL). Representative images at 30, 60, 90, and 120 min from three independent experiments are shown and data were quantified as the clot volume (%) (mean ± SD, n = 3) (two-way ANOVA). **P*<0.05. Compared with vehicle, ***P*<0.01; ****P*<0.001. Compared with CY-09, #*P*<0.05.

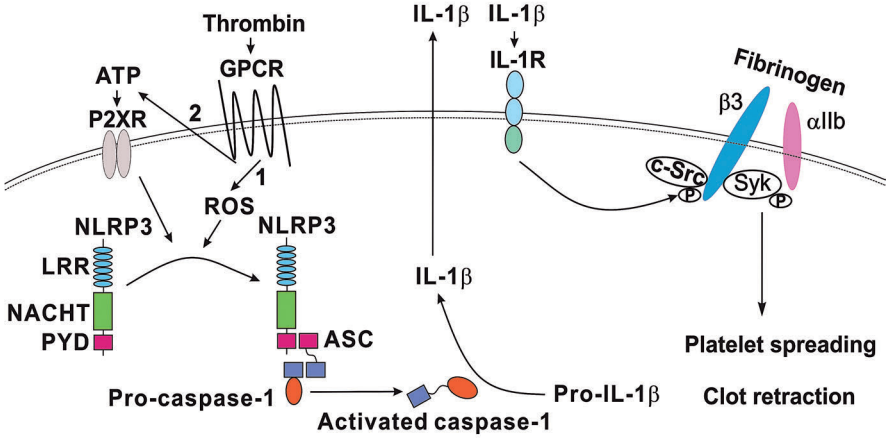

Figure 6. Role of NLRP3 in the regulation of platelet integrin αIIbβ3 outside-in signaling. Engagement of G protein coupled receptors (GPCR) by thrombin induces platelet intracellular reactive oxygen species (ROS) production (1), which activates NLRP3, leading to assembly of the NLRP3 inflammasome and subsequent activation of caspase-1, which processes immature pro-IL-1β into mature IL-1β. Once released, IL-1β binds to IL-1 receptor (IL-1R) and initiates IL-1R intracellular signaling transduction, resulting in phosphorylation of c-Src and Syk, which regulates platelet spreading and clot retraction. Meanwhile, ligation of GPCR also induces ATP release (2), which can activate NLRP3 through binding to P2XR. LRR: Leucine-rich repeat; NACHT: NACHT, NAIP, CIITA, HET-E and TP1; PYD: Pyrin domain; ASC: Apoptosis-associated speck-like protein containing a CARD.

expression) and αIIbβ3 activation (PAC-1 binding) in collagen, ADP or CRP-stimulated human platelets after CY-09 treatment (Figure 5B), consistent with minimal effects of NLRP3 depletion on other platelet activation pathways. Thrombin-initiated clot retraction was significantly impaired in CY-09-treated human platelets (Figure 5C). Addition of recombinant human IL-1β rescued the impaired clot retraction of CY-09-treated platelets, suggesting that NLRP3 might also be involved in the regulation of human platelet integrin αIIbβ3 outside-in signaling. Collectively, our data support a role for NLRP3 in regulating platelet αIIbβ3 outside-in signaling in human and murine platelets.

Discussion

Systemic inflammation has been demonstrated to be a potent prothrombotic stimulus, with mechanisms including upregulation of procoagulant factors, inhibition of natural anticoagulants and fibrinolytic activity as well as increased platelet reactivity.[31,32] However, the relationship between inflammation and platelet function remains poorly understood. In this study, we identified a specific contribution of the NLRP3 inflammasome to αIIbβ3 outside-in signaling, and hemostasis and arterial thrombosis *in vivo*.

Inflammasomes are multiprotein complexes that respond to various inflammatory stimuli by controlling secretion of the pro-inflammatory cytokine, IL-1β.[14] The NLRP3 inflammasome is one of the largest and most studied cytosolic inflammasomes. It undergoes oligomerization upon stimulation, leading to activation of caspase-1, which mediates the maturation of IL-1β.[14] Along with immune cells, platelets express NLRP3 inflammasomes. Platelet NLRP3 has been shown to be involved in the regulation of endothelial permeability after dengue infection by processing pro-IL-1β and releasing mature IL-1β.[13] Additionally, NLRP3 contributes to platelet activation, aggregation and thrombus formation *in vitro*.[15] Consistent with this, we showed reduced platelet aggregation in response to threshold concentrations of collagen, thrombin and ADP in NLRP3-deficient platelets. However, degranulation (P-selectin expression) and αIIbβ3 activation were normal in *NLRP3*$^{-/-}$ platelets. As platelet NLRP3 deficiency significantly impaired hemostasis and arterial thrombosis *in vivo*, we suggest that NLRP3 is a novel molecular link between inflammation and thrombosis.

Platelet spreading and clot retraction, regulated by αIIbβ3 outside-in signaling, play important roles in stabilizing thrombus formation.[26] To investigate the role of NLRP3 in αIIbβ3 outside-in signaling, we measured platelet spreading and clot retraction and showed that NLRP3 deficiency significantly decreased platelet spreading on immobilized fibrinogen and impaired clot retraction. As IL-1β has been demonstrated to increase platelet adhesion to collagen and fibrinogen,[29] we hypothesized that the effect of NLRP3 on αIIbβ3 signaling might be through IL-1β. To test that, we measured IL-1β secretion from thrombin-stimulated platelets and found significantly reduced IL-1β release from NLRP3-deficient platelets, indicating that NLRP3 is responsible for processing IL-1β in platelets. Moreover, addition of IL-1β rescued impaired platelet spreading and clot retraction, supporting the role of NLRP3/IL-1β in platelet integrin αIIbβ3 signaling. Through the use of a selective and direct NLRP3 inhibitor, CY-09,[30] we also evaluated whether NLRP3 exerts the same functional effects on human platelets and showed reduced platelet aggregation in response to threshold doses of collagen and ADP without differences in degranulation and αIIbβ3 activation. However, inhibition of NLRP3 by CY-09 significantly ablated clot retraction in human platelets. Importantly, addition of recombinant human IL-1β rescued impaired clot retraction of CY-09-treated human platelets, indicating that NLRP3 might also participate in the regulation of αIIbβ3 outside-in signaling in human platelets, which is consistent with findings in mouse platelets.

A potential limitation of our findings is that we cannot exclude the interesting possibility that NLRP3 depletion in mice affects platelet hemostatic/thrombotic function through as yet unknown indirect pathways. Further studies are required to explore the exact mechanisms of NLRP3/IL-1β signaling and the specific role of this inflammasome complex in other thrombotic models and human disease.

In conclusion, our results demonstrate that NLRP3 regulates platelet spreading and clot retraction by a mechanism involving IL-1β. Moreover, impaired hemostasis and arterial thrombosis were observed *in vivo* in mice with *NLRP3*$^{-/-}$ platelets. Furthermore, inhibition of NLRP3 impairs clot retraction in human platelets. These data identify a unique role for NLRP3 in the regulation of platelet function and thrombus formation (Figure 6), and provide a novel molecular link between thrombosis and inflammation.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (grant n. 81400082, 81370602, 81570096, 81671584, 81641151 and 81700178), the Natural Science Foundation of Jiangsu Province (grant n. BK20141138 BK20140219), funding for the Distinguished Professorship Program of Jiangsu Province, the Shuangchuang Project of Jiangsu Province, the National Health and Medical Research Council of Australia, the Six Talent Peaks Project of Jiangsu Province (WSN-133), the 333 projects of Jiangsu Province (BRA2017542), the Key University Science Research Project of Jiangsu Province (17KJA320008), Jiangsu Province's Key Provincial Talents Program (ZDRCA2016054), the Colleges' Science Foundation of Jiangsu Province (16KJB320013), Postgraduate Research Innovation Project of Jiangsu Province (KYCX18_2186), and Key University Science Research Project of Jiangsu Province (18KJA320010).

We thank Prof. Rongbin Zhou (University of Science and Technology of China, Hefei, China) for kindly providing the NLRP3 inhibitor CY-09.

References

1. Qiao JL, Shen Y, Gardiner EE, Andrews RK. Proteolysis of platelet receptors in humans and other species. Biol Chem. 2010;391(8):893-900.
2. Qiao J, Arthur JF, Gardiner EE, Andrews RK, Zeng L, Xu K. Regulation of platelet activation and thrombus formation by reactive oxygen species. Redox Biol. 2017;14:126-130.
3. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell. 1992;69(1):11-25.
4. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol. 2010;30(12):2341-2349.
5. Clark EA, Shattil SJ, Ginsberg MH, Bolen J, Brugge JS. Regulation of the protein tyrosine kinase pp72syk by platelet agonists and the

integrin alpha IIb beta 3. J Biol Chem. 1994;269(46):28859-28864.
6. Suzuki-Inoue K, Hughes CE, Inoue O, et al. Involvement of Src kinases and PLCgamma2 in clot retraction. Thromb Res. 2007;120(2):251-258.
7. Wonerow P, Pearce AC, Vaux DJ, Watson SP. A critical role for phospholipase Cgamma2 in alphaIIbbeta3-mediated platelet spreading. J Biol Chem. 2003;278 (39):37520-37529.
8. Phillips DR, Jennings LK, Edwards HH. Identification of membrane proteins mediating the interaction of human platelets. J Cell Biol. 1980;86(1):77-86.
9. Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling: the platelet paradigm. Blood. 1998;91(8):2645-2657.
10. Morrell CN, Aggrey AA, Chapman LM, Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. Blood. 2014;123(18):2759-2767.
11. Kapur R, Semple JW. The nonhemostatic immune functions of platelets. Semin Hematol. 2016;53 (Suppl 1):S2-6.
12. Duerschmied D, Bode C, Ahrens I. Immune functions of platelets. Thromb Haemost. 2014;112(4):678-691.
13. Hottz ED, Lopes JF, Freitas C, et al. Platelets mediate increased endothelium permeability in dengue through NLRP3-inflammasome activation. Blood. 2013;122(20):3405-3414.
14. Haneklaus M, O'Neill LA. NLRP3 at the interface of metabolism and inflammation. Immunol Rev. 2015;265(1):53-62.
15. Murthy P, Durco F, Miller-Ocuin JL, et al. The NLRP3 inflammasome and Bruton's tyrosine kinase in platelets co-regulate platelet activation, aggregation, and in vitro thrombus formation. Biochem Biophys Res Commun. 2017;483(1):230-236.
16. Kovarova M, Hesker PR, Jania L, et al. NLRP1-dependent pyroptosis leads to acute lung injury and morbidity in mice. J Immunol. 2012;189(4):2006-2016.
17. Qiao J, Wu Y, Liu Y, et al. Busulfan triggers intrinsic mitochondrial-dependent platelet apoptosis independent of platelet activation. Biol Blood Marrow Transplant. 2016;22(9): 1565-1572.
18. Qiao J, Wu Y, Wu X, et al. An absence of platelet activation following thalidomide treatment in vitro or in vivo. Oncotarget. 2017;8(22):35776-35782.
19. Qiao J, Liu Y, Li D, et al. Imbalanced expression of Bcl-xL and Bax in platelets treated with plasma from immune thrombocytopenia. Immunol Res. 2016;64(2):604-609.
20. Qiao J, Huang Y, Xia Y, et al. Busulfan and cyclosphamide induce liver inflammation through NLRP3 activation in mice after hematopoietic stem cell transplantation. Sci Rep. 2015;5:17828.
21. Rivera J, Lozano ML, Navarro-Nunez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica. 2009;94(5):700-711.
22. Swieringa F, Kuijpers MJ, Heemskerk JW, van der Meijden PE. Targeting platelet receptor function in thrombus formation: the risk of bleeding. Blood Rev. 2014;28(1):9-21.
23. Pagel O, Walter E, Jurk K, Zahedi RP. Taking the stock of granule cargo: platelet releasate proteomics. Platelets. 2017;28(2):119-128.
24. Jonnalagadda D, Izu LT, Whiteheart SW. Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. Blood. 2012;120(26):5209-5216.
25. Italiano JE, Richardson JL, Patel-Hett S, et al. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood. 2008;111(3):1227-1233.
26. Flevaris P, Stojanovic A, Gong H, Chishti A, Welch E, Du X. A molecular switch that controls cell spreading and retraction. J Cell Biol. 2007;179(3):553-565.
27. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. Annu Rev Immunol. 2011;29:707-735.
28. Denis MM, Tolley ND, Bunting M, et al. Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. Cell. 2005;122(3):379-391.
29. Beaulieu LM, Lin E, Mick E, et al. Interleukin 1 receptor 1 and interleukin 1beta regulate megakaryocyte maturation, platelet activation, and transcript profile during inflammation in mice and humans. Arterioscler Thromb Vasc Biol. 2014;34(3): 552-564.
30. Jiang H, He H, Chen Y, et al. Identification of a selective and direct NLRP3 inhibitor to treat inflammatory disorders. J Exp Med. 2017;214(11):3219-3238.
31. Aksu K, Donmez A, Keser G. Inflammation-induced thrombosis: mechanisms, disease associations and management. Curr Pharm Des. 2012;18(11):1478-1493.
32. Esmon CT. Inflammation and thrombosis. J Thromb Haemost. 2003;1(7):1343-1348.