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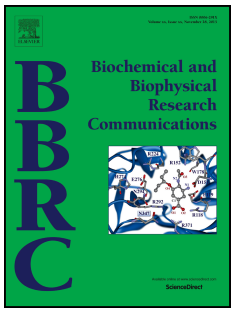

The NLRP3 inflammasome and bruton's tyrosine kinase in platelets co-regulate platelet activation, aggregation, and in vitro thrombus formation

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The NLRP3 inflammasome and bruton's tyrosine kinase in platelets co-regulate platelet activation, aggregation, and in vitro thrombus formation

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Abstract

Cleavage of interleukin-1β (IL-1β) is a key inflammatory event in immune cells and platelets, which is mediated by nucleotide-binding domain leucine rich repeat containing protein (NLRP3)-dependent activation of caspase-1. In immune cells, NLRP3 and caspase-1 form inflammasome complexes with the adaptor proteins apoptosis-associated speck-like protein containing a CARD (ASC) and bruton's tyrosine kinase (BTK). In platelets, however, the regulatory triggers and the functional effects of the NLRP3 inflammasome are unknown. Here, we show in vitro that the platelet NLRP3 inflammasome contributes to platelet activation, aggregation, and thrombus formation. NLRP3 activity, as monitored by caspase-1 activation and cleavage and secretion of IL-1β, was upregulated in activated platelets, which was dependent on platelet BTK. Pharmacological inhibition or genetic ablation of BTK in platelets led to decreased platelet activation, aggregation, and in vitro thrombus formation. Thus, we identify a functionally relevant link between BTK and NLRP3 in platelets, with potential implications in disease states associated with abnormal coagulation and inflammation.

Keywords: platelets; NLRP3; bruton's tyrosine kinase; aggregation; thrombosis

Abbreviations: ASC - apoptosis-associated speck-like protein containing a CARD; BTK - bruton's tyrosine kinase; IL-1β - interleukin-1β; NLR - nucleotide-binding oligomerization domain-like receptor; NLRP3 - nucleotide-binding domain leucine rich repeat containing protein; PRR - pattern recognition receptor; TLR - toll-like receptor; XID - X-linked immunodeficiency

Introduction

Platelets express numerous pattern recognition receptors (PRRs) including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), which enable them to act as circulating cellular sensors that provide a unique link between hemostasis and inflammation [1-6]. We have recently shown that activation of platelet TLR4 by the damage-associated molecular pattern molecule high mobility group box 1 derived from platelets promotes platelet activation, aggregation, and thrombosis [4]. Moreover, nucleotide-binding domain leucine rich repeat containing protein (NLRP3) expressed by platelets may become activated by the dengue virus, which resulted in release of interleukin-1β (IL-1β) and increased vascular permeability [2]. The occurence of cleavage and shedding of pro-IL-1β in its mature form in activated platelets has been reported more than a decade ago [7]. In myeloid immune cells, NLRP3 cooperates with apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, and bruton's tyrosine kinase (BTK) within inflammasome complexes, which typically activate caspase-1 and induce cleavage and secretion of IL-1β [8-11]. Activation of the NLRP3 inflammasome is a critical inflammatory event, which can be targeted by BTK inhibitors [9]. Platelets express BTK, which is known to be involved in the regulation of glycoprotein VI-mediated platelet activation and aggregation [12-13]. The effect of platelet BTK on NLRP3 signaling in platelets, however, and the potential role of the platelet NLRP3 inflammasome in platelet activation and aggregation are unknown. Here, we show that the NLRP3 inflammasome in platelets promotes platelet activation, aggregation, and in vitro thrombus formation, and we identify BTK as a critical NLRP3 inflammasome regulator in platelets.

Materials and Methods

Animals

NLRP3 knockout (KO) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were on a C57BL/6 background [14]. Bruton's tyrosine kinase (BTK) mutant (BTK^{xid}) mice were obtained from the Jackson Laboratory and were on a CBA/CaHN background [15-16]. Mice on a CBA/CaJ background were used as control for BTK^{xid}. For isolation of platelets, mice were anesthetized with isoflurane and blood was drawn via cardiac puncture into anticoagulated tubes. The animal protocol complied with the regulation regarding the care and use of experimental animals published by the US NIH and was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Platelets

Human and murine platelets were isolated as described previously [4]. For human platelet isolation, venous blood from healthy volunteers was used. For certain experiments, isolated platelets were pretreated for 30 minutes with a specific inhibitor against NLRP3 (MCC950; 100 nM, Cayman Chemical, Ann Arbor, MI) [17], caspase-1 (YVAD-CHO; 100 nM, Calbiochem, Darmstadt, Germany) [18] or BTK (CGI1746; 300 nM, Selleckchem, Houston TX) [19]. DMSO (Fisher Scientific, Hampton, NH) was used as control. In other experiments, human platelets or platelets derived from BTK^{xid} mice were treated for 30 minutes with the NLRP3 inflammasome inducer Nigericin [20] (10 µM, Cayman Chemical) in the presence or absence of CGI1746. Platelets were either

kept untreated (resting) or activated by incubation with collagen (2 µg/ml) or thrombin (0.2 U/ml, both from Chrono-Log, Havertown, PA) for 5 or 15 minutes.

Flow cytometry

Platelet surface expression of P-selectin (CD62P) was evaluated by flow cytometry using an APC-conjugated anti-CD62P monoclonal antibody (2 µg/ml, mouse IgG1κ; eBioscience, San Diego, CA) or isotype control antibody (eBioscience). Activation of caspase-1 in platelets was measured using the FAM-FLICA Caspase-1 Assay Kit (Immunochemistry Technologies, Bloomington, MN). Platelets were analyzed by flow cytometry using a BD Accuri C6 Plus (BD Biosciences, San Jose, CA) flow cytometer and FlowJo software (Tree Star, Ashland, OR). Platelets were gated based on their characteristic scatter properties.

Platelet aggregometry

Platelet aggregation was evaluated using whole blood impedance aggregometry (Model 700, ChronoLog) as described previously [4]. Aggregation was measured for 6 minutes at 37℃ with a stir speed of 1,200 rpm. Analysis was performed using the Aggrolink-8 software (ChronoLog).

Flow chamber

Heparinized murine (NLRP3 KO, BTK^{xid}, and respective control mice) or human blood was diluted 2:1 with Tyrodes-HEPES buffer (pH 7.4) and perfused through a parallel-plate flow chamber system (slit depth 50 µm) over a collagen-coated surface

(200 µg/ml) with a shear rate at 1,700 s^{-1} for 5 minutes as described previously [4]. The chamber was then perfused with Tyrodes-HEPES buffer for 5 minutes, and pictures were taken from 3 to 4 different microscopic areas (using ×20 optical objectives). Thrombus area was quantified as mean percentage values with AxioVision software (Carl Zeiss, Jena, Germany). For certain experiments, blood was incubated with the indicated inhibitors for 30 minutes prior to perfusion through the flow chamber.

Immunofluorescence staining of platelets

Resting or collagen-activated (2 µg/ml, 5 minutes) human platelets were fixed with 1% paraformaldehyde, applied to 0.01% poly L-lysine-coated coverslips, and permeabilized with 0.3% Triton X-100. After blocking with 1% BSA-PBS for 1 hour, cells were incubated overnight at 4℃ with anti-NLRP3 mon oclonal antibody (2 µg/ml, mouse IgG2b; AdipoGen, San Diego, CA). Platelets were washed with PBS plus 0.3% Triton X-100 plus 0.1% Tween-20 and incubated with Alexa-Fluor 488-tagged goat anti-mouse IgG (1:100, Invitrogen, San Diego, CA) for 2 hours at room temperature. Following another washing step, platelets were incubated with anti-ASC polyclonal antibody (1 µg/ml, rabbit IgG; Santa Cruz, Heidelberg, Germany), washed, and incubated with Alexa Fluor 568-tagged donkey anti-rabbit IgG (1:100, Invitrogen). The corresponding IgG antibodies (AdipoGen and Santa Cruz) served as control. Confocal microscopic analysis was performed using a LSM510 META confocal laser scanning microscope and ZEN 2012 imaging software (Carl Zeiss).

Detection of IL-1β in platelets and supernatants

Isolated human platelets were kept untreated or treated with MCC950 (100 nM), YVAD (100 nM) or CGI1746 (300 nM) for 30 minutes prior to platelet activation with collagen or thrombin for 5 minutes or 15 minutes. DMSO served as control for these inhibitors. Platelets were lysed with Pierce IP Lysis Buffer (ThermoFisher Scientific; Pittsburgh, PA) and protein concentrations were determined with the Bradford Concentration Assay (ThermoFisher Scientific). 50 ug of protein was resolved by 8.5% SDS-Polyarcylamide gel electrophoresis. Western Blotting onto nitrocellulose membranes (Bio Rad, Hercules, CA) was performed using the Criterion Blotter system (Bio Rad). Membranes were incubated overnight with anti-IL-1β polyclonal antibody (1:2000, rabbit IgG; abcam, Cambridge, MA). Anti-tubulin monoclonal antibody (1:2000, mouse IgM; BD Pharmingen/Biosciences; San Jose, CA) was used as a loading control. Antibody binding was detected with corresponding secondary fluorescence-labeled antibodies, HRP-conjugated Clarity Western Substrate (Bio Rad), and a SRX-101a Film processer (Konica, Cleveland, OH). Densitometry analysis was performed with ImageJ software.

IL-1β concentrations in platelet supernatants were measured using a Tecan Saphire microplate reader and a colorimetric human IL-1β Quantikine ELISA immunoassay (R&D Systems, Minneapolis MN, USA) according to the manufacturer's protocol.

Statistical analysis

All data are presented as mean ± S.D for n ≥ 3 unless stated otherwise. Statistical significance was determined with the 2-tailed Student's t test or 1-way ANOVA with Tukey's post-hoc test using Graph Pad Prism software (GraphPad, San Diego CA, USA).

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Results

The platelet NLRP3 inflammasome mediates platelet activation, aggregation, and in vitro thrombus formation

We studied the role of the platelet NLRP3 inflammasome in activation and aggregation of platelets. NLRP3 was detected by Western blot in human and murine WT platelets, but not in platelets derived from NLRP3 KO mice (data not shown), which was consistent with previous findings [2]. Collagen-induced activation of human platelets, as monitored by expression of P-selectin and flow cytometry, was significantly downregulated in the presence of specific inhibitors for NLRP3 (MCC950) or caspase-1 (YVAD) (Fig. 1A). In addition, ablation of NLRP3 in platelets derived from NLRP3 KO mice resulted in a significantly reduced platelet activation response as compared to WT control platelets (Fig. 1B). Similar results were obtained when thrombin was used as a platelet agonist (data not shown). Moreover, treatment of human platelets with MCC950 or YVAD (Fig. 1C) as well as NLRP3 KO platelets (Fig. 1D) led to significantly suppressed collagen-induced platelet aggregation. In the absence of a platelet agonist, the inhibitors (Fig. 1A, C) or ablation of NLRP3 (Fig. 1B, D) exerted no effect on platelet activation and aggregation.

To test the potential contribution of the NLRP3 inflammasome to thrombus formation in vitro, we perfused human blood through a collagen-coated flow chamber in the presence or absence of MCC950 or YVAD and performed imaging of the forming thrombi on the surface (Fig. 1E). Both inhibitors exerted significant inhibitory effects on thrombus formation. A critical procoagulant role of NLRP3 was confirmed when blood

derived from NLRP3 KO mice was used (Fig. 1F). Thrombus formation in NLRP3 KO blood was significantly suppressed as compared to WT controls.

The NLRP3 inflammasome is upregulated in activated platelets

Next, we sought to define the regulatory triggers of NLRP3 inflammasome activation in platelets. We detected expression of NLRP3 (green) and the adaptor protein ASC (red) in non-activated (resting) and collagen-activated human platelets, as shown with immunofluorescence staining coupled with confocal laser scanning microscopy (Fig. 2A). In activated platelets, ASC changed its distribution and colocalized with NLRP3, which was followed by significant upregulation of platelet caspase-1 activity (Fig. 2B). Caspase-1 activation in collagen-activated platelets was significantly reversed by MCC950, indicating that NLRP3 plays a critical role in the regulation of caspase-1 activity in activated platelets (Fig. 2B). YVAD served as a control for caspase-1 inhibition and significantly downregulated caspase-1 activation. We next investigated cleavage of IL-1β in platelets, which allows monitoring of caspase-1 activity [2, 8], and confirmed with Western Blot that collagen-induced platelet activation substantially upregulated expression of cleaved IL-1β (Fig. 2C). IL-1β cleavage was markedly reduced in the presence of MCC950 and YVAD. Moreover, secretion of cleaved IL-1β from platelets, as quantified by ELISA, was significantly increased upon platelet activation with collagen, which was significantly suppressed by both inhibitors (Fig. 2D). Similar results were obtained when thrombin was used as a platelet agonist (Fig. 2E-G), indicating that activation of platelets by collagen or thrombin

upregulates the platelet NLRP3 inflammasome, followed by activation of caspase-1 and cleavage of IL-1β.

Bruton's tyrosine kinase controls platelet activation, aggregation, and in vitro thrombus formation through the NLRP3 inflammasome in platelets

Bruton's tyrosine kinase (BTK) has recently been identified as an essential regulator of the NLRP3 inflammasome in innate immune cells [9] and is known to be expressed by platelets [12]. Thus, we next investigated whether activation of the NLRP3 inflammasome in activated platelets depends on BTK. Upregulated cleavage (Fig. 3A, B) and secretion of IL-1β (Fig. 3C, D) in collagen-activated (Fig. 3A, C) and thrombin-activated (Fig. 3B, D) human platelets was significantly reversed by the specific BTK inhibitor CGI1746. To investigate the potential role of BTK-dependent regulation of the NLRP3 inflammasome in platelet activation and aggregation, we performed treatments of human platelets with CGI1746 or used platelets derived from BTK mutant mice (BTK^{xid}) in the presence or absence of the NLRP3 activator Nigericin (Fig. 3E-H). Collagen-induced platelet activation (Fig. 3E, F) and aggregation (Fig. 3G, H) were significantly suppressed in the presence of CGI1746 (Fig. 3E, G) or when BTK defective platelets were used (Fig. 3F, H). These inhibitory effects on platelets were significantly restored by Nigericin, indicating a functionally active link between BTK and NLRP3 in platelets. Nigericin did not significantly affect platelet activation and aggregation in the absence of the BTK inhibitor (Fig. 3E, G) or when WT platelets were used (Fig. 3F, H). Similar results were obtained in the flow chamber assay. Treatment of human blood

with CGI1746 (Fig 3I) or blood derived from BTK^{xid} mice (Fig. 3J) exerted significant anti-thrombotic activity, which was significantly reversed by the NLRP3 activator.

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Discussion

In this study, we have shown that the NLRP3 inflammasome in platelets controls platelet activation, aggregation, and in vitro thrombus formation, and we have identified BTK as a critical regulator of these NLRP3-dependent platelet effects. We provide novel insight into the regulatory triggers of activated platelets, reminiscent of key inflammatory events initially discovered in innate immune cells [9-11, 21-22].

The NLRP3 inflammasome recognizes various pathogen-associated molecular patterns, which are predominantly derived from microbes, as well as damage-associated molecular patterns, which are host-derived signals originating from injured or stressed tissues [11]. Upon activation, NLRP3 forms complexes with the proteins ASC, BTK, and caspase-1 that regulate the production of bioactive IL-1β and other proinflammatory cytokines such as IL-18 [9-10, 21-22]. In immune cells, activation of the NLRP3 inflammasome typically requires initial priming signals, such as the exposure to cytokines, which leads to upregulated expression of inflammasome components such as NLRP3 [23]. The platelet NLRP3 inflammasome, however, does not seem to require such a priming signal, since platelets constitutively express functionally active proteins NLRP3, ASC, and BTK and appear to be triggered without prior priming via an additional PRR ligand or cytokine [2, 12]. On the other hand, the NLRP3-dependent effects observed in this study might also be mediated by IL-1β secreted from activated platelets and represent an autocrine and/or paracrine priming loop on IL-1-receptor expressing platelets [24]. In a $FeCl_3$ thrombosis model, IL-1β was detected in significant amounts in platelet-rich clots, which preceded leukocyte accumulation at sites of the lesion, indicating a critical in vivo role of platelet IL-1β early in thrombosis [7]. Moreover,

platelet NLRP3 may exert its effects on platelet activation, aggregation, and thrombus formation through caspase-1-dependent generation of thromboinflammatory microparticles [2]. It will be interesting to now dissect this unique platelet-specific inflammasome pathway further.

NLRP3 is also a critical mediator of a form of caspase-1-dependent cell death called pyroptosis, which is characterized by DNA fragmentation in innate immune cells and results in inflammation [25]. It has recently been shown in bone marrow-derived dendritic cells that NLRP3 controls both pyroptosis and apoptosis through a caspase-8-dependent mechanism [26]. It is tempting to speculate as to whether the platelet NLRP3 inflammasome may be involved in regulating apoptosis- and/or pyroptosis-like changes in platelets.

BTK is a cytoplasmic tyrosine kinase not only expressed by B-cells and myeloid cells, but also platelets [12, 27]. It has recently been shown in innate immune cells that BTK is a critical regulator of the NLRP3 inflammasome, whose inhibition with Ibrutinib, a potent BTK inhibitor approved by the Food and Drug Administration for the treatment of mantle cell lymphoma and chronic lymphocytic leukemia, reversed NLRP3-mediated effects in a murine stroke model [9]. In this study, NLRP3 inflammasome upregulation in activated platelets and NLRP3-dependent platelet effects were controlled by platelet BTK. Our findings provide a functional explanation for the clinical observation that patients experience increased bleeding and reduced platelet aggregation during ibrutinib treatment [28]. Thus, we provide novel evidence for a functionally and clinically relevant link between NLRP3 and BTK in platelets.

In conclusion, we have identified the activation of the NLRP3 inflammasome in activated platelets as a critical event for platelet activation, aggregation, and in vitro thrombus formation, and we have shown that platelet BTK plays a critical regulatory role, with potential therapeutic implications in platelet relevant diseases.

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Figure Legends

Figure 1. The platelet NLRP3 inflammasome mediates platelet activation, aggregation, and in vitro thrombus formation. (A) Collagen-induced platelet activation is reduced in the presence of the NLRP3 inhibitor MCC950 and the caspase-1 inhibitor YVAD. (B) Collagen-induced activation of NLRP3 KO platelets is downregulated as compared to WT control platelets. (C) MCC950 and YVAD exert inhibitory effects on platelet aggregation induced by collagen. (D) Ablation of NLRP3 in platelets results in reduced collagen-induced platelet aggregation. AUC, area under the curve. In vitro thrombus formation, as assessed by flow chamber, is reduced by preincubation of blood with MCC950 or YVAD (E) or when blood derived from NLRP3 KO mice is used (F). Data are presented as mean ± SD for N≥3 and at least three separate experiments in all studies. ** $p<0.01$, *** $p<0.001$ (1-way ANOVA with Tukey's post-hoc test in A, B, C, E; Student's t test in D and F).

Figure 2. The NLRP3 inflammasome is upregulated in activated platelets. (A) In collagen-activated platelets, intracellular ASC (red) translocates towards the platelet plasma membrane and colocalizes with NLRP3 (green), as demonstrated with immunofluorescence stainings and confocal laser scanning microscopy. Scale bar, 1 µm. (B) Caspase-1 activity (measured by FLICA assay) is upregulated in collagen-activated platelets, which is reversed in the presence of the NLRP3 inhibitor MCC950 and the caspase-1 inhibitor YVAD. (C) Collagen-induced cleavage of IL-1β in activated platelets is inhibited by MCC950 and YVAD as assessed by and quantified from

immunoblots. (D) Activation of platelets with collagen induces secretion of IL-1β into supernatants (ELISA), which is downregulated by MCC950 and YVAD. Upregulated caspase-1 activity (E) and cleavage (F) and secretion (G) of IL-1β in thrombin-activated platelets is reduced in the presence of MCC950 and YVAD. Data are presented as mean ± SD for N≥3 and at least three separate experiments in all studies. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (1-way ANOVA with Tukey's post-hoc test in B, D, E, G).

Figure 3. Bruton's tyrosine kinase controls platelet activation, aggregation, and in vitro thrombus formation through the NLRP3 inflammasome in platelets. IL-1β cleavage (A, B) and secretion (C, D) in platelets activated by collagen (A, C) or thrombin (B, D) is reduced in the presence of the BTK inhibitor CGI1746. In the presence of CGI1746 (E, G, I) or in platelets/blood derived from BTK^{xid} mice (F, H, J), platelet activation (E, F), aggregation (G, H), and in vitro thrombus formation (I, J) are reduced, which is restored by the NLRP3 activator Nigericin. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (1-way ANOVA with Tukey's post-hoc test in C-J).

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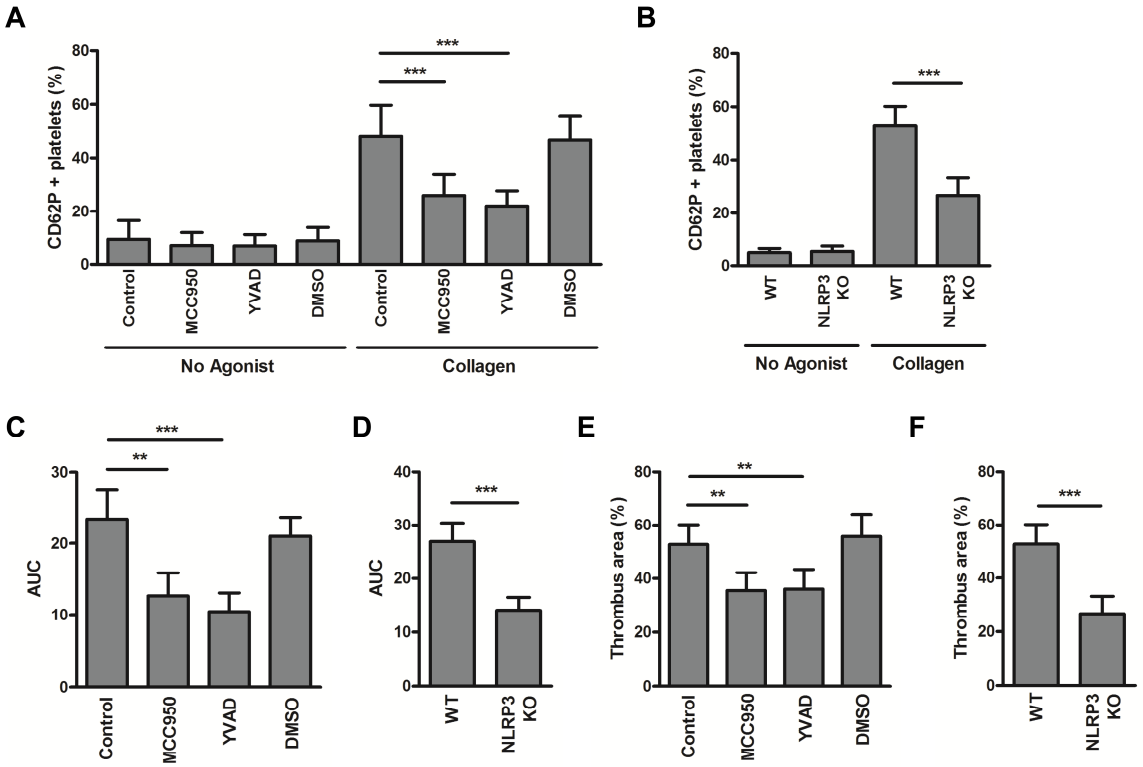

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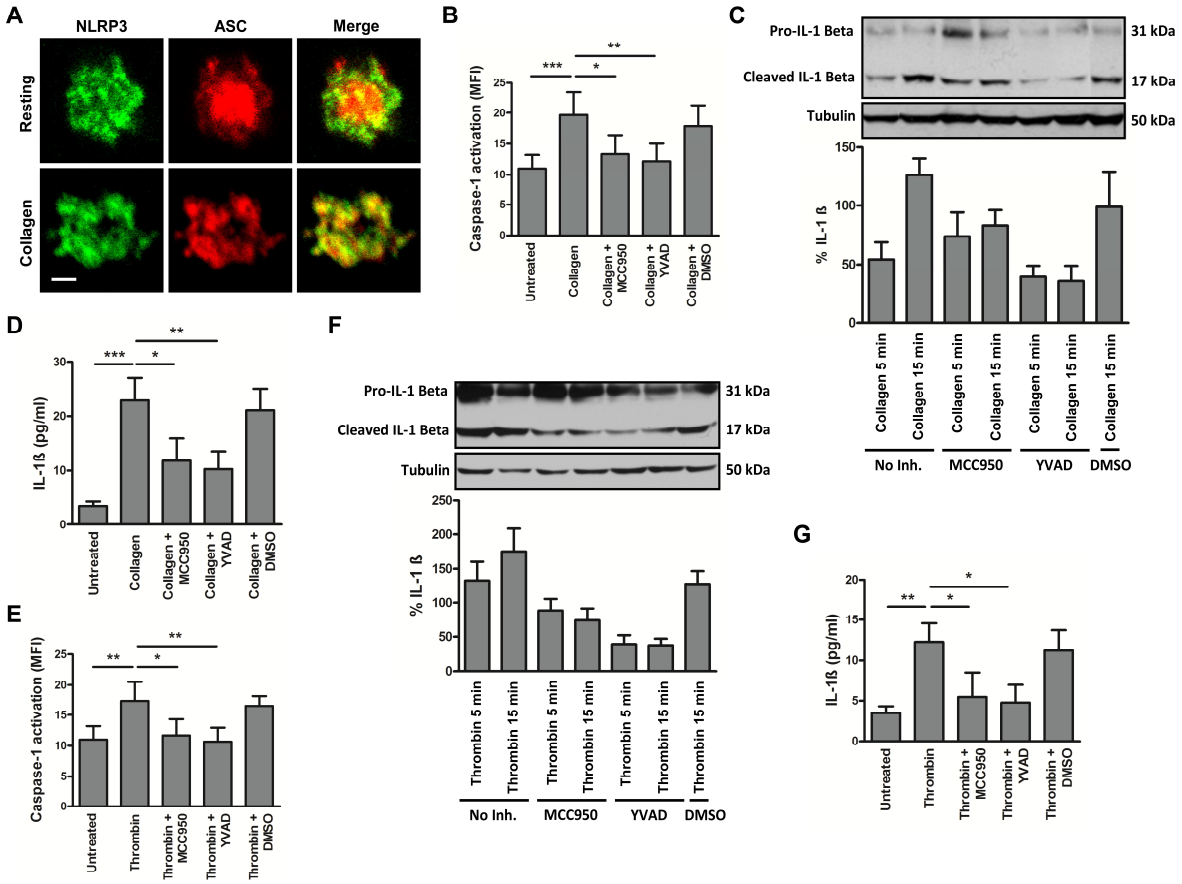

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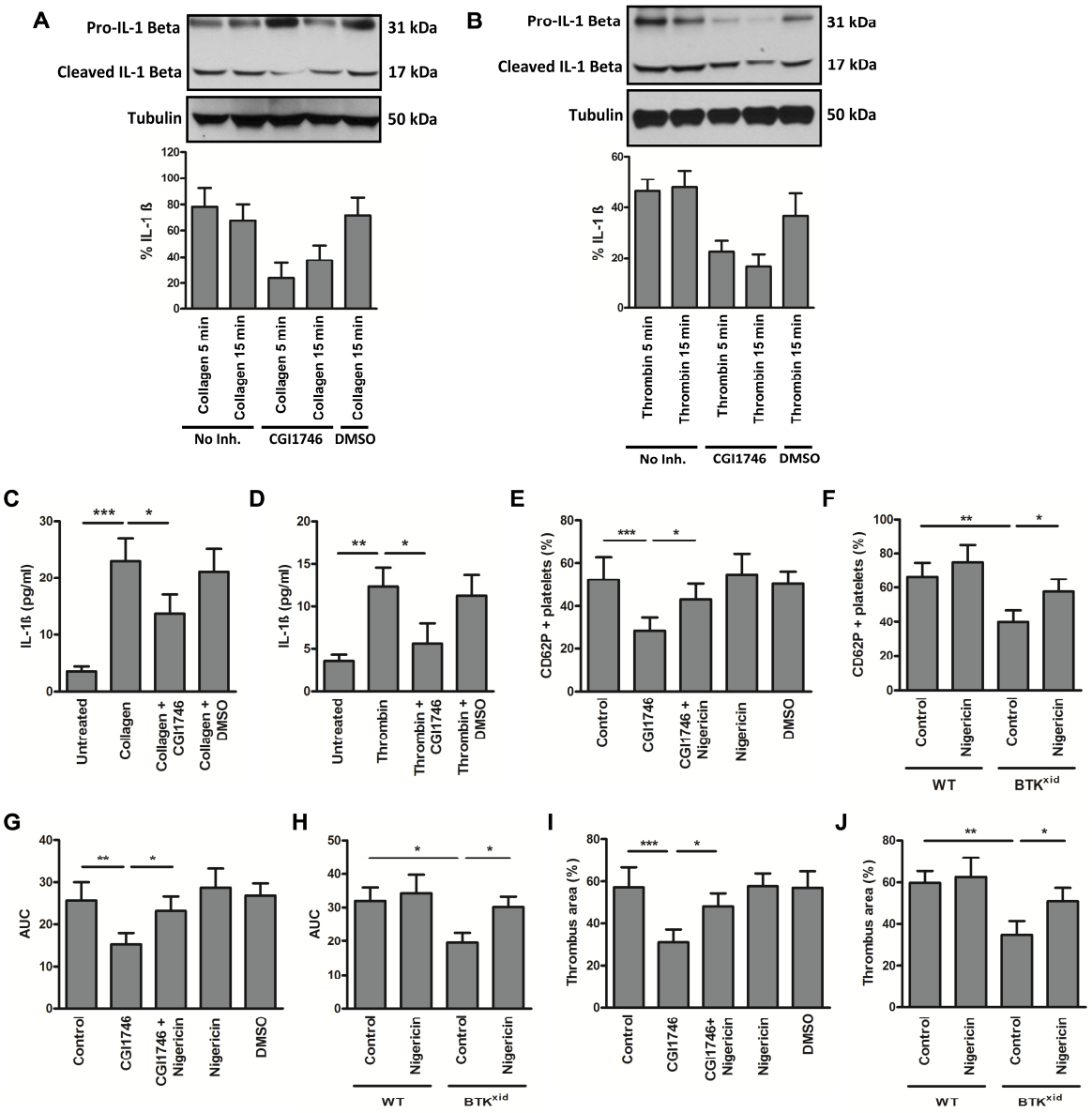
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Pro-IL-1 Beta
Cleaved IL-1 Beta
Tubulin
31 kDa
17 kDa
50 kDa
% IL-1 ß
Collagen 5 min
Collagen 15 min
Collagen 5 min
Collagen 15 min
Collagen 15 min
No Inh.
CGI1746
DMSO
B
Pro-IL-1 Beta
Cleaved IL-1 Beta
Tubulin
31 kDa
17 kDa
50 kDa
% IL-1 ß
Thrombin 5 min
Thrombin 15 min
Thrombin 5 min
Thrombin 15 min
Thrombin 15 min
No Inh.
CGI1746
DMSO
C
IL-1ß (pg/ml)
Untreated
Collagen
Collagen + CGI1746
Collagen + DMSO
D
IL-1ß (pg/ml)
Untreated
Thrombin
Thrombin + CGI1746
Thrombin + DMSO
E
CD62P + platelets (%)
Control
CGI1746
CGI1746 + Nigericin
Nigericin
DMSO
F
CD62P + platelets (%)
Control
Nigericin
Control
Nigericin
WT
BTKxid
G
AUC
Control
CGI1746
CGI1746 + Nigericin
Nigericin
DMSO
H
AUC
Control
Nigericin
Control
Nigericin
WT
BTKxid
I
Thrombus area (%)
Control
CGI1746
CGI1746+ Nigericin
Nigericin
DMSO
J
Thrombus area (%)
Control
Nigericin
Control
Nigericin
WT
BTKxid

Highlights

- Platelet NLRP3 promotes platelet activation, aggregation, and in vitro thrombosis
- The NLRP3 inflammasome is upregulated in activated platelets
- Platelet bruton's tyrosine kinase controls the identified NLRP3-dependent effects

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