HHS Public Access

Author manuscript

J Thromb Haemost. Author manuscript; available in PMC 2021 November 01.

Published in final edited form as:

J Thromb Haemost. 2020 November ; 18(11): 3013–3028. doi:10.1111/jth.15049.

ASK1 Regulates Immune-Mediated Thrombocytopenia, Thrombosis, and Systemic Shock

Pravin Patel[1], **Noor F. Shaik**[1], **Yuhang Zhou**[1,2], **Kalyan Golla**[1,3], **Steven E. McKenzie**[1], **Ulhas P. Naik**[1]

[1]Cardeza Center for Hemostasis, Thrombosis, and Vascular Biology, Cardeza Foundation for Hematologic Research, Department of Medicine, Thomas Jefferson University, Philadelphia, PA

[2]Dell Children's Hospital, University of Texas, Austin, TX

[3]Center for Blood Research, Department of Medicine, University of British Columbia, Vancouver, BC, Canada

Abstract

Background—Immune complexes (ICs) bind to and activate platelets via FcγRIIA causing patients to experience thrombocytopenia, as well as an increased risk of forming occlusive thrombi. While platelets have been shown to mediate IC-induced pathologies, the mechanisms involved have yet to be fully elucidated. We identified that Apoptosis Signal-Regulating Kinase 1 (ASK1) is present in both human and mouse platelets and potentiates many platelet functions.

Objectives—Here we set out to study ASK1's role in regulating IC-mediated platelet functions *in vitro* and IC-induced pathologies using an *in vivo* mouse model.

Methods—Using human platelets treated with an ASK1-specific inhibitor and platelets from *FCGR2A* / *Ask1*$^{-/-}$ transgenic mice, we examined various platelet functions induced by model ICs *in vitro* and *in vivo*.

Results—We found that ASK1 was activated in human platelets following cross-linking of FcγRIIA using either anti-hCD9 or IV.3+GAM. While genetic deletion or inhibition of ASK1 significantly attenuated anti-CD9-induced platelet aggregation, activation of the canonical FcγRIIA signaling targets Syk and PLCγ2 was unaffected. We further found that anti-mCD9-induced $cPla_2$ phosphorylation and TxA_2 generation is delayed in Ask1 null transgenic mouse platelets leading to diminished δ-granule secretion. *In vivo*, absence of *Ask1* protected *FCGR2A* transgenic mice from thrombocytopenia, thrombosis, and systemic shock following injection of anti-mCD9. In whole blood microfluidics, platelet adhesion and thrombus formation on fibrinogen was enhanced by Ask1.

Correspondence: Ulhas P. Naik, Cardeza Center for Hemostasis, Thrombosis, and Vascular Biology, Department of Medicine, Thomas Jefferson University, 1020 Locust st. Rm 394, Philadelphia, PA 19107; ulhas.naik@jefferson.edu; Tel. (215)-955-3824; Fax. (215)-955-8544.

Addendum

Authorship Contributions: P. Patel and K. Golla generated the *FCGR2A*$^{+/+}$/*Ask1*$^{-/-}$ transgenic mice. P. Patel, N. Shaik, Y. Zhou performed experiments. P. Patel made the figures. P. Patel, N. Shaik, S. McKenzie, and U. Naik designed the research, analyzed the data, and wrote the manuscript.

This manuscript has been read and approved for submission to Journal of Thrombosis and Haemostasis by all authors

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Conclusions—These findings suggest that ASK1 inhibition may be a potential target for the treatment of IC-induced shock and other immune-mediated thrombotic disorders.

Keywords

Apoptosis Signal-Regulating Kinase 1; Platelets; Shock; Thrombocytopenia; Thrombosis

Introduction

Immune complexes (ICs) occur when IgG antibodies bind to their antigens and have been implicated in a wide array of pathologies from lupus erythematosus[1] and anti-phospholipid syndrome[2] to bacterial sepsis[3,4] and heparin-induced thrombocytopenia and thrombosis (HIT)[5–8]. The Fc region of ICs can bind, cross-link, and activate FcγRIIA, a low affinity immune receptor expressed on monocytes/macrophages and platelets, among other cells. IC-induced cross-linking of platelet-FcγRIIA activates platelet integrin $\alpha_{IIb}\beta_3$, precipitating immune-induced thrombocytopenia and thrombosis (ITT). Patients who develop HIT, for example, face a 10% mortality risk and significant morbidity.[9]

There has been growing appreciation of platelets in the immune response, promoting activation/recruitment of leukocytes to the site of injury. The immunological role of platelets is distinct from the role of platelets in hemostasis. Previous work identified platelet-FcγRIIA as a key mediator of IC-induced systemic shock/anaphylaxis (henceforth abbreviated as shock), highlighting the role of platelets as effectors of the immune system[10,11]. More recently two independent groups revealed that platelet-FcγRIIA is required for IC-induced serotonin release from platelets to produce symptoms of shock[12,13], thus linking platelet activation and IC-induced pathologies. IC-induced platelet serotonin secretion[14] can induce endothelial hyperpermeability[15] and vasodilation[16], thus causing hypoperfusion and shock[17], while also contributing to feedback activation of platelets[18].

Apoptosis Signal-Regulating Kinase 1 (ASK1) is a Mitogen-Activated Protein 3 Kinase present in human and mouse platelets.[19,20] Previously, we identified that ASK1 is activated in a Ca^{2+} dependent manner following agonist stimulation[21] to potentiate platelet function[19]. To further understand the mechanism of ITT and IC-induced shock, we investigated ASK1's role in IC-induced platelet function, shock, and ITT.

We found that the FcγRIIA agonists anti-hCD9- or IV.3+goat-anti-mouse (GAM) induced ASK1 activation in human platelets. In addition, ASK1 inhibition reduced anti-hCD9- and IV.3+GAM-induced aggregation without affecting activation of the FcγRIIA signaling target Syk. Deletion of *Ask1* in human *FCGR2A*$^{+/+}$*(hFcR)* transgenic mice significantly attenuated anti-mCD9-induced platelet function *ex vivo* and protected *hFcR*/*Ask1*$^{-/-}$ mice from anti-mCD9-induced thrombocytopenia, shock, and pulmonary thromboembolism *in vivo.* Together, our results strongly suggest that ASK1 is a prominent regulator of IC-induced platelet activation and may play a pivotal role in mediating ITT and shock.

Methods

Reagents

Anti-mCD9 [#553758, clone KMC8, rat IgG_{2a}], FITC-conjugated anti-mCD62p [#561923], FITC-conjugated anti-mCD41 [#553848], and FITC-annexin V [#556419] were purchased from BD Pharmingen. Anti-hCD9 [#IM0117, clone Alb6, $mIgG_1$] was purchased from Beckman Coulter. Goat-anti-mouse (GAM) IgG, $F(ab')_2$ [#SAB3701127, lot RI38886] was purchased from Sigma. Anti-mGPIX IgG [#M051–0, clone Xia.B4] was purchased from Emfret analytics. Anti-hFcγRIIA [#60012, clone IV.3] and FITC-conjugated anti-hFcγRIIA [#60012FI, clone IV.3] were purchased from Stemcell technologies. PE-conjugated anti-mGPVI [#FAB6758P] was purchased from R&D Systems. The serotonin ELISA kit [ADI-900–175] was purchased from ENZO. EDTA coated micro capillary tubes [#1–000-1000] were purchased from Drummond Scientific Company. EDTA coated specimen tubes [20.1288.100] were purchased from Sarstedt AG & Co. Selonsertib (GS-4997, NCGC00479176–01) was obtained from the NCATS Pharmaceutical Collection. Acetylsalicylic acid (aspirin, 510016) was obtained from VetOne.

Generation of the Ask1$^{-/-}$/ FCGR2A$^{+/+}$ Transgenic Mice

FCGR2A$^{+/+}$*(hFcR)* transgenic mice[22] and *Ask1*$^{-/-}$ mice[23], both on the C57BL/6J strain background, were crossed to generate the F1 generation (*Ask1*$^{+/-}$*/ FCGR2A*$^{+/-}$*)* transgenic mice. The F1 generation was then intercrossed to generate the F2 generation (*Ask1*$^{-/-}$*/ FCGR2A*$^{+/+}$*)* and (*Ask1*$^{+/+}$*/ FCGR2A*$^{+/+}$) transgenic mice used in this study. Genotypes of all mice used in this study were confirmed, as previously described, by PCR analysis of genomic DNA for *Ask1*[23] and *FCGR2A*[22].

Platelet Preparation

Human—Whole blood was drawn, with written informed consent, by venipuncture from 13 healthy adult volunteers (6 female; 6 White, 6 Black, 1 Asian [self-reported]). Approval for this study was obtained from the Institutional Review Board of Thomas Jefferson University, according to the Declaration of Helsinki. Blood was collected and washed platelets were prepared in Tyrode's buffer as previously described.[24]

Mouse—Male and female 8–12-week-old *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice on the C57BL/6J background were used in these studies. Isolation of mouse platelets was performed as described.[19] Approval for animal experimental studies was received from the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Flow Cytometry

Receptor Surface Expression—~100 μL of mouse whole blood was drawn by retro-orbital bleed using 300 μg of EDTA and 2 U of heparin as anticoagulants. Whole blood was diluted 1:40 with PBS and aliquoted into 50 μL reaction volumes. Samples were then incubated with PE-conjugated anti-mGPVI [1:50 dilution] and either FITC-conjugated anti-mCD41 [1:50 dilution], FITC-conjugated anti-hFcγRIIA [1:50 dilution], or FITC-conjugated IgG isotype control [1:50 dilution] for 20 minutes at room temperature. The

reaction was stopped by the addition of 450 μL of ice-cold PBS (containing 1% paraformaldehyde and 0.2% BSA).

PS Exposure—Surface expression of PS was measured by flow cytometry, as has been previously described.[25] Briefly, 50 μL of washed mouse platelets (0.6×10^8 platelets/mL) were stimulated with various agonists for 10 minutes at room temperature. Next, samples were incubated with FITC-annexin V [1:20 dilution] for 10 minutes. The reaction was stopped by the addition of 450 μL of ice-cold PBS (containing 1% paraformaldehyde and 0.2% BSA).

JON/A and anti-P-selectin binding—Surface binding of PE conjugated JON/A and FITC conjugated Anti-P-selectin (CD62P) IgGs was assessed as previously described.[26] Briefly, 50 μL of washed mouse platelets (0.6×10^8 platelets/mL) were incubated with 2 μL of PE-rat anti-mouse JON/A (Emfret Analytics) for 5 minutes at room temperature after which they were stimulated with anti-mCD9 for 10 minutes at room temperature. Next 1 μg/mL of FITC-rat anti-mouse CD62p was added to each sample and incubated for 20 minutes at 37°C. The reaction was stopped by the addition of 450 μL of ice-cold PBS (containing 1% paraformaldehyde and 0.2% BSA). All samples were held at 4°C until analysis. All antibody-binding activity was measured as mean fluorescent intensity (MFI) or percent positive (% Positive) by a BD Accuri C6 flow cytometer.

Platelet Aggregation

Aggregation was performed with washed platelets (2×10^8 platelets/mL) using a Chrono-Log Lumi-Aggregometer using Aggrolink software (Chrono-Log), as previously described.[26] Lag-time was determined from the time of agonist addition to the start of the downward deflection. Due to known inter-individual variability in the human platelet response to anti-hCD9, each donor was tested with a concentration range (100–1200 ng/mL, 100 ng/mL increments) to determine the intra-donor ‘threshold-dose’, defined as the lowest concentration to induce >60% aggregation within 10 minutes. When IV.3+GAM was used as an agonist pair, IV.3 was added 60 seconds prior to the addition of GAM (recorded as the reaction start time).

Western blotting

100μL aliquots of washed human platelet suspension (4×10^8 platelets/mL) or washed mouse platelet suspension (2×10^8 platelets/mL) in Tyrode’s buffer were placed in an aggregometer and stimulated with various agonists under identical conditions as for platelet aggregation. When IV.3+GAM was used as an agonist pair, GAM was added 60 seconds after the addition of IV.3. Addition of GAM was recorded as the reaction start time. At select time points, samples were lysed by the addition of 25 μL 5X reducing Laemmli-sample buffer. Platelet lysates (30 μL) were then subjected to immunoblotting as described.[27] Membranes were immunoblotted with the following antibodies: Anti-P-p38 (T180/Y182) [#9211, 1:5000 (against-human) 1:4000 (against-mouse)]; Anti-p38 [#9212S, 1:3000 (against-human & mouse)]; Anti-P-Syk (Y525/Y526) [#2711S, 1:1000 (against-human & mouse)]; Anti-Syk [#2712, 1:1000 (against-human & mouse)]. Anti-P-PLCγ2 (Y759) [#3874, 1:1000 (against-mouse)]; Anti-PLCγ2 [#3872S, 1:1000 (against-mouse)]. Anti-P-

cPLA$_2$ (S505) [#2831S, 1:1000 (against-mouse)]; and, Anti-rabbit IgG, HRP conjugated [# 7074, 1:2000–10000] were all obtained from Cell Signaling, Danvers, MA. Anti-ASK1 [sc7931, (H-300), 1:1000 (against-human)] was obtained from Santa Cruz Biotechnology. Additionally, the phosphospecific ASK1 T^{838} polyclonal rabbit antibody [1:2000] was raised against an antigenic synthetic peptide (830-AGINPCTEpTFTGTLQY-845) of human ASK1. The antisera were analyzed by ELISA to determine specificity for ASK1. A polyclonal phospho-specific antibody was affinity-purified from the antisera by Neo Scientific (Cambridge, MA). Blots were incubated with LumiGLO chemiluminescent substrate (Cell Signaling, #7003) and detected using either X-ray film or a ChemiDoc Imaging System (Bio-Rad Laboratories, #17001401). Band intensities were quantified using NIH Image J software.

Serotonin Secretion

100 μL aliquots of washed mouse platelets (2×10^8 platelets/mL) were stimulated in an aggregometer for 10 minutes under identical conditions as for aggregation. Where indicated, platelets were pretreated with aspirin (1mM) for 30 minutes at 37°C. Following 10-minute stimulation, samples were centrifuged at 10,000 g for 1-minute, the supernatant collected and frozen in a dry-ice/ethanol bath, and stored at −80°C. The concentration of serotonin in the releasate was measured using a serotonin ELISA kit following the manufactures instructions. The total serotonin content from platelets for each replicate was determined by lysing 100 μL of resting platelets with 100 μL of a platelet lysis buffer (100 mM Tris-HCl, 150 mM NaCl, 2% (vol./vol.) IGEPAL, pH 7.4). The concentration of secreted serotonin was normalized to their intra-replicate total serotonin level (set as 100%).

Thrombin-Antithrombin Complex Measurement

hFcR/Ask1$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice were injected with various doses of anti-mCD9 (5 to 40 μg/20 g mouse body weight) by retro-orbital injection. One hour after injection of anti-mCD9, whole was drawn by retro-orbital bleeding following the protocol for hematologic analysis. Whole blood was diluted (1:10) with sterile PBS, centrifuged at 2000 g for 10 minutes, and the plasma fraction collected and stored at −80°C until analysis. Plasma concentration of thrombin-antithrombin complexes (TAT) was determined using a TAT Mouse ELISA Kit (Abcam, Ab137994) following the manufactures instructions.

Whole Blood Microfluidics

Microfluidic flow assays were performed in channels with dimensions of 100 μm in height and 500 μm in width over fibrinogen (200 μg/mL) that was adsorbed onto a clean glass surface, as has been described.[28] Briefly, blood from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice was collected using heparin (2 U/mL) and 40 μM of d-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone (PPACK; Calbiochem). Whole blood was diluted 1:2 in calcium-free Tyrode's buffer and incubated with 1mM Aspirin (or vehicle control) for 30 minutes, DiOC6 (1 mg/mL; Sigma) for 10 minutes, and 500 ng/mL anti-mCD9 for 5 minutes at room temperature. Blood was perfused through the flow channel for 3 minutes at a shear rate of 800 s^{-1}. The chamber was rinsed with Tyrode's buffer containing 1 mg/mL DiOC6 for 3 minutes and fixed with 4% paraformaldehyde for 10 minutes. Phase contrast and fluorescent images were taken with a ×20 objective on an EVOS microscope (Thermo Scientific) and

analyzed with Image J (National Institutes of Health). Relative thrombus size was determined from fluorescent intensity. Platelet coverage was calculated from phase contrast images.

In Vivo Models of ITT

ITT was induced using the *hFcR* transgenic mouse model.[10,22,25,29,30] Briefly, ITT was induced by injecting anti-mCD9 (5 to 50 μg/20 g mouse body weight) into the retro-orbital sinus of isoflurane-anesthetized mice. Where indicated, aspirin (2 mg/20 g mouse body weight or DMSO control) was injected i.p. 2 hours before anti-mCD9, a regimen known to inhibit arachidonic acid-induced platelet aggregation.[31]

Whole blood samples (50–75 μL) were collected by retro-orbital bleeding, using EDTA coated micro capillary tubes and placed into a tube containing EDTA to prevent coagulation, at various time points post anti-mCD9 injection for complete blood cell count analysis. The blood cell analysis was performed using a Hemavet 950 (Drew Scientific). Counts were normalized to baseline counts (taken 3–4 days prior).

Symptoms of shock (severe [complete immobility, loss of consciousness], moderate [impaired mobility, irregular respiration], mild [lethargy, shallow respiration], or none) were assessed based on blinded observations of mice from 10–15 minutes following injection of anti-mCD9 as previously described.[10,32] Mouse movements were tracked from 10–15 minutes following injection of anti-mCD9 using Tracker 4 software (Douglas Brown).

Platelets were labeled *in vivo* by retro-orbital injecting mice with anti-mGPIX IgG (2 μg/20 g mouse body weight) conjugated to an 800CW fluorophore using a kit from Licor, as previously described).[32] 5 minutes after injection of anti-GPIX, thrombosis and pulmonary embolism was initiated by retro-orbital injecting anti-mCD9 (50 μg/20 g mouse body weight).[29,30] Mice were sacrificed 30 minutes later, their lungs perfused with PBS and excised. Platelet deposition in the lungs (fluorescence intensity/mm^2) was assessed using a LI-COR Odyssey scanner.

Statistical Analysis

Data (mean ± standard deviation (SD)) were plotted and statistical analysis performed using Prism 8 (GraphPad). Student's t-test, Mann-Whitney test, and One- or Two-way ANOVA were used to determine statistical significance, correcting for multiple hypothesis testing where appropriate. $P \leq 0.05$ was regarded as statistically significant. Experiments were repeated independently at least 3 times using platelets isolated from individual donors/mice.

Results

FcγRIIA signaling activates ASK1 in human platelets

To first investigate if FcγRIIA signaling activates ASK1 in platelets, we stimulated washed human platelets with anti-hCD9 IgG, which activates FcγRIIA via Fc-domain cross-linking. As expected, anti-hCD9 induced activation of Syk (an early and key event in the FcγRIIA signaling cascade) within 1-minute following agonist addition, which steadily increased over time. When assessed for ASK1 activation, we found ASK1 was rapidly activated, peaking

within 3 minutes, as denoted by Thr845[33] autophosphorylation (Figure 1A). Since ASK1 is the sole MAP3K to activate platelet p38, as assessed by phosphorylation at Thr180/Tyr182,[19,20,34] we evaluated the activation of p38. Anti-hCD9 induced a robust and sustained phosphorylation of p38 (Figure 1A). In contrast to anti-hCD9 which induces relatively slow FcγRIIA activation, Fab driven crosslinking of FcγRIIA with the IV.3+GAM induces fast FcγRIIA activation.[30,35] Consistent with this notion, IV.3+GAM stimulated rapid activation of ASK1, Syk, and p38 that peaked within 1-minute following agonist addition. While Syk and p38 phosphorylation was sustained, ASK1 was rapidly dephosphorylated. Together these data show that FcγRIIA/Syk signaling activates ASK1/p38 in platelets.

To further explore ASK1's role as a regulator of FcγRIIA signaling, we investigated the effect of GS-4997, a small molecule inhibitor of ASK1, on FcγRIIA-mediated platelet function. While GS-4997 did not affect anti-hCD9- and IV.3+GAM-induced Syk activation, GS-4997 blocked ASK1 and p38 activation (supplemental Figure 1).

Additionally, when platelets were stimulated with a threshold dose of anti-CD9, we found that 10 μM of GS-4997 reduced platelet aggregation and lag-time response to anti-hCD9. This dose of GS-4997 is one at which ASK1 is effectively inhibited in human platelets[34] and which shows no apparent off target effects [36]. Due to inter-individual variability in the anti-CD9 aggregation response,[30] a threshold dose, defined as the lowest concentration able to induce >60% aggregation within 10 minutes, was used instead of a fixed agonist concentration. When stimulated with IV.3+GAM (which has little if any inter-individual variability) we found that 10 μM of GS-4997 also decreased IV.3+GAM-induced platelet aggregation, suggesting that ASK1 regulates IC-induced platelet activation (Figure 2).

Ask1 regulates anti-mCD9-induced transgenic mouse platelet activation

As mice do not contain an FcγRIIA ortholog, we crossed *Ask1*$^{-/-}$ mice[23] with *hFcR* transgenic mice[22] to generate *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice. PCR analysis confirmed that both strains (*hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$) contain *hFcR* and are WT or KO for *Ask1* (supplemental Figure 2A). Surface expression of FcγRIIA, GPVI, and CD41, as well as hematological parameters, were equivalent for both strains, suggesting that Ask1 does not affect receptor expression or platelet numbers (supplemental Figure 2B–C).

We next stimulated washed platelets, isolated from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice, with anti-mCD9. With the tested doses of anti-mCD9 (440–510 ng/mL), hFcR/Ask1$^{+/+}$ platelets fully (>60%) aggregated, while *hFcR/Ask1*$^{-/-}$ platelets had a significantly attenuated dose response (Figure 3A). When stimulated with doses of anti-mCD9 ≤ 490 ng/mL, *hFcR/Ask1*$^{-/-}$ platelets underwent shape-change and initial aggregation, but failed to fully aggregate within 10 minutes.

The effect on maximal aggregation appears to be a consequence of increased lag-time, as *hFcR/Ask1*$^{-/-}$ platelets aggregated when stimulated with anti-mCD9 doses ≥ 500 ng/mL, albeit with considerable delay. The lag-time in response to 500 ng/mL anti-mCD9 was still significantly increased (~8 min vs. ~5 min) when Ask1 was knocked out. These findings suggest that *hFcR/Ask1*$^{-/-}$ platelets have an overall delayed response to FcγRIIA

stimulation and that the apparent effect on the final extent of aggregation seen at doses < 500 ng/mL could be due to differences in the timing of when platelets started aggregating. (Figure 3Av).

We observed a similar blunted response to anti-mCD9 stimulation when JON/A binding and P-Selectin surface expression on *hFcR/Ask1*$^{-/-}$ platelets was compared to *hFcR/Ask1*$^{+/+}$ platelets (Figure 3B–C). However, due to the fact that low concentrations of platelets were used in flow cytometry assays and that anti-CD9's effect is proportional to platelet concentration[37,38], higher agonist concentrations were required to sufficiently activate platelets.

Since loss of Ask1 reduced the response of platelets to anti-mCD9, we investigated activation of the FcγRIIA signaling pathway. Consistent with our findings in GS-4997-treated human platelets, anti-mCD9-induced activation/phosphorylation of Syk and Plcγ2 was not affected by the loss of Ask1 (Figure 4Bi–Biii). Given that GPVI, a platelet ITAM receptor like FcγRIIA, stimulates ASK1 activation via PLCγ2,[21] and that FcγRIIA signaling induced p38 activation via Syk,[39] our findings suggest that Ask1 may be downstream of FcγRIIA-induced Syk and Plcγ2 activation in platelets.

Ask1 regulates anti-mCD9-induced cPLA$_2$ phosphorylation and TxA$_2$ generation

Anti-CD9-induced Ca^{2+} release and platelet aggregation are dependent on TxA_2 generation, notably at agonist concentrations near threshold.[40–43] As Ask1 regulates TxA_2 generation by other ITAM-receptors,[19] we next investigated ASK1's role in regulating IC-induced TxA_2 generation. Anti-mCD9 (500 ng/mL) induced TxA_2 generation within 2.5 minutes of agonist addition to hFcR/Ask1$^{+/+}$ platelets– with a larger amount of TxA_2 generated by 5 minutes, coinciding with the start of hFcR/Ask1$^{+/+}$ platelet aggregation. TxA_2 generation, however, was delayed past 5 minutes in hFcR/Ask1$^{-/-}$ platelets, only reaching a significant level 5–10 minutes after stimulation, corresponding to the start of hFcR/Ask1$^{-/-}$ aggregation. These data suggest that the increased lag-time of hFcR/Ask1$^{-/-}$ platelets is related to delayed TxA_2 generation (Figure 4A).

cPLA$_2$ is the main phospholipase responsible for releasing arachidonic acid for subsequent TxA_2 generation in platelets.[19,44–46] Platelet stimulation increases cPLA$_2$ phosphorylation at Ser-505, which increases cPLA$_2$ activity.[47–50] In platelets both ERK1/2 and p38 are activated by anti-CD9,[51] are known kinases for cPLA$_2$ Ser-505,[19,34,52–55] and are needed for cPLA$_2$ phosphorylation downstream of FcγRIIA.[56] As ASK1 is a known regulator of p38 in platelets, we examined anti-mCD9-induced cPla$_2$ phosphorylation. While anti-mCD9 induced phosphorylation of both cPla$_2$ and p38 in *hFcR/Ask1*$^{+/+}$ platelets, activation/phosphorylation of p38 was blocked in *hFcR/Ask1*$^{-/-}$ platelets. While early cPla$_2$ phosphorylation (≤ 3 minutes) was reduced, cPla$_2$ phosphorylation occurred at later timepoints in *hFcR/Ask1*$^{-/-}$ platelets, matching the kinetics of TxA_2 generation in *hFcR/Ask1*$^{-/-}$ platelets (Figure 4Biv–v). These data are consistent with our previous findings that ASK1 regulates early, but not late (likely ERK1/2 dependent), cPLA$_2$ phosphorylation in platelets[34].

These findings also suggest that Ask1 regulates anti-CD9-induced platelet activation via regulation of TxA_2 generation. In agreement with this hypothesis, when stimulated directly with arachidonic acid, bypassing any difference in TxA_2 generation, we observed no difference in aggregation or JON/A binding between *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ platelets, suggesting that ASK1 regulates integrin activation and platelet aggregation via TxA_2 generation (supplemental Figure 3).

Ask1 regulates anti-mCD9-induced thrombocytopenia and shock in vivo

To determine ASK1's role in regulating IC-mediated thrombocytopenia, we measured platelet counts in *hFcR* mice treated with anti-mCD9. When treated with a low dose of anti-mCD9 (5 μg/mouse), *hFcR/Ask1*$^{+/+}$ platelet counts fell by ~55% within 1-hour, whereas *hFcR/Ask1*$^{-/-}$ mice only fell by ~25% (Figure 5Ai). However, when the dose of anti-mCD9 was increased to 10 μg, we found no difference in the level of thrombocytopenia, suggesting that Ask1 may not regulate all mechanisms of platelet clearance *in vivo* (Figure 5Aii).

Previous studies showed that ICs concurrently induce thrombocytopenia and shock.[12,13,57] To probe this, we injected *hFcR/Ask1*$^{+/+}$ mice with 5 or 10 μg/mouse anti-mCD9 to elicit a shock phenotype.[10] While *hFcR/Ask1*$^{+/+}$ mice receiving either dose developed shock, characterized by loss of consciousness, *hFcR/Ask1*$^{-/-}$ mice were protected from anti-mCD9-induced shock, even at doses that caused severe thrombocytopenia (Figure 5Bi). To further quantify the shock phenotype, we tracked mouse movements after anti-mCD9 injection. In agreement with our results, *hFcR/Ask1*$^{-/-}$ mice retained a significantly greater degree of mobility compared to *hFcR/Ask1*$^{+/+}$ mice (Figure 5Bii). Combined, these data strongly suggest that Ask1 plays a prominent role in regulating IC-induced shock.

Prior studies identified that platelet serotonin secretion mediates IC-induced shock.[12,13] As TxA_2 potentiates δ-granule secretion[19,58,59], we next asked if Ask1 regulates anti-mCD9-induced serotonin secretion via TxA_2 generation. While there was no difference in total serotonin content between *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ platelets (supplemental Figure 4), *hFcR/Ask1*$^{-/-}$ platelets had significantly attenuated (~40% reduction) serotonin secretion when stimulated with a low (450 ng/mL) dose of anti-mCD9. Importantly, this difference was related to defective TxA_2 generation in *hFcR/Ask1*$^{-/-}$ platelets, as pretreatment with aspirin removed any genotype difference (Figure 5C), in agreement with Ask1's role in regulating δ-granule secretion via TxA_2 generation.[19] When stimulated with a higher dose of anti-mCD9 (500 ng/mL), we found no difference in serotonin secretion between *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ platelets suggesting that TxA_2 generation is not absolutely required for serotonin secretion at higher agonist doses (Figure 5C). Further, when stimulated directly with arachidonic acid, we found no genotype difference in arachidonic acid-induced α-granule secretion as measured by P-selectin exposure (Figure 5D).

As aspirin treatment blocked Ask1-dependent serotonin secretion, we next investigated if aspirin could affect the shock phenotype caused by platelet serotonin secretion *in vivo*. When pretreated with aspirin, *hFcR/Ask1*$^{+/+}$ mice displayed a significantly attenuated shock phenotype (Figure 5E). Combined, these data suggest that Ask1 participates in regulating serotonin secretion and IC-induced shock via regulation of TxA_2 generation.

Ask1 regulates anti-mCD9-induced thrombosis in vivo

To determine if Ask1 regulates IC-induced thrombosis, we measured platelet thrombi in the lungs of *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice treated with anti-mCD9 using fluorescently labeled anti-mGPIX IgG.[29] When injected with 50 μg of anti-mCD9, a dose known to induce pulmonary thrombi[30], lungs of *hFcR/Ask1*$^{+/+}$ mice showed visible evidence of thrombi within 30 minutes. In contrast, platelet deposition was significantly lower in lungs of *hFcR/Ask1*$^{-/-}$ mice, consistent with fewer pulmonary thrombi following anti-mCD9 injection (Figure 6A).

To further explore anti-mCD9 induced thrombosis, we utilized an *ex vivo* thrombosis assay using fibrinogen-coated microfluidic channels. Whole blood isolated from *hFcR/Ask1*$^{+/+}$ mice showed potentiated platelet coverage and thrombus size when preincubated with anti-mCD9, and these were reduced in whole blood isolated from *hFcR/Ask1*$^{-/-}$ mice (Figure 6B). Aspirin pretreatment reduced *hFcR/Ask1*$^{+/+}$ platelet coverage and thrombus size to that of *hFcR/Ask1*$^{-/-}$ whole blood, without affecting these parameters in *hFcR/Ask1*$^{-/-}$ whole blood (Figure 6B). These data suggest that Ask1 regulates IC-induced thrombosis via regulation of TxA_2 generation.

Thrombin generation is a key mediator of IC-induced thrombosis. *In vivo*, efficient thrombin generation requires surface exposure of phosphatidylserine (PS) on activated platelets.[60] To determine Ask1's role in regulating PS exposure, we measured Annexin-V binding to the surface of *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ platelets. There was no difference in Annexin-V binding between *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ platelets when stimulated with either thrombin (0.02–1 U/mL) or a combination of thrombin (0.02 U/mL) and anti-mCD9 (0–10 μg/mL) (supplemental Figure 5). To determine if Ask1 regulates thrombin generation *in vivo* we measured thrombin-antithrombin (TAT) complexes 1-hour after injection of anti-mCD9. While anti-mCD9 dose-dependently increased TAT concentrations, there were no genotype differences (Figure 6C). These results suggest that Ask1 does not regulate platelet procoagulant activation or thrombin generation during ITT. At a higher dose of anti-mCD9 (≥ 20 μg), *hFcR/Ask1*$^{-/-}$ mice displayed the same shock phenotype as *hFcR/Ask1*$^{+/+}$ mice (Figure 6D). These data are consistent with our findings that the effects of Ask1 knockout on serotonin secretion are overcome at high agonist dose (Figure 5C).

Discussion

During the course of IC-induced pathologies, patients may experience thrombocytopenia and increased thrombosis leading to tissue ischemia/necrosis, heart attack or stroke.[61,62] Much work has been done to further our understanding of IC-induced pathologies, including recent studies demonstrating that platelet-FcγRIIA is required for IC-induced platelet activation which mediates shock and thrombus formation in the microvasculature.[12,13] While these studies highlight platelet activation as an attractive target for treating ITT, the current anti-platelet drugs (aspirin, $P2Y_{12}$ inhibitors, dipyridamole) are ineffective in clinical cases of ITT[63,64]. Therefore, elucidation the intracellular mechanisms involved and validation of novel targets that are more robust regulators of platelet activity during ITT is vital to more effectively treat ITT.

One such mechanism is the link between activation of the platelet FcγRIIA receptor and serotonin secretion. ASK1 was recently revealed to be an important regulator of platelet function and a key player in regulating thrombosis *in vivo*.[19,20] However, ASK1's role in mediating IC-induced platelet activities has not been addressed. Since ASK1 can be activated downstream of other platelet ITAM receptors[19,20], we postulated that ASK1 is activated in platelets by FcγRIIA signaling and may mediate IC-dependent platelet activation.

Our findings extend our knowledge of ASK1 and the critical role it plays in IC-induced platelet function and shock. We showed that ASK1 is robustly activated in platelets following FcγRIIA activation. While ASK1 was not needed for activation of the initial steps of the FcγRIIA signaling pathway, inhibition or deletion of ASK1 significantly reduced IC-induced platelet function. As TxA_2 generation[3,4,29,65–67] and ADP secretion[4,29,32,68,69] are known to potentiate FcγRIIA-induced platelet activation, and since TxA_2 signaling potentiates δ-granule secretion[19,58,59], our findings suggest that ASK1 promotes IC-induced platelet activation, in part, by regulating TxA_2 generation. Consistent with this hypothesis, when we used arachidonic acid to bypass any difference in activation of $cPLA_2$, a key enzyme in the pathway of TxA_2 synthesis, we found that arachidonic acid-induced platelet function was not affected by the loss of Ask1. In addition, aspirin treatment removed any genotype difference in δ-granule secretion, suggesting that the defect in *hFcR/Ask1*$^{-/-}$ platelet function was due to defective TxA_2 generation.

IgG ICs induce thrombocytopenia and shock[13,70]. As our results show that Ask1 regulates serotonin secretion via TxA_2 generation, and platelet serotonin release can exacerbate shock following injection of ICs[12,13], it is therefore reasonable to suggest that ASK1 may regulate shock by regulating TxA_2 generation. In fact, we found that loss of Ask1 protected *hFcR/Ask1*$^{-/-}$ mice from IC-induced shock. Furthermore, aspirin treatment protected *hFcR/Ask1*$^{+/+}$ mice from developing severe shock following injection of ICs, suggesting that Ask1 regulates IC-induced shock by regulating TxA_2 dependent serotonin secretion *in vivo*. *hFcR/Ask1*$^{-/-}$ mice were also resistant to IC-induced thrombocytopenia; however, this protection only occurred at low doses of anti-mCD9, indicating that Ask1 may not regulate all mechanisms of platelet clearance *in vivo*, such as clearance mediated by splenic monocytes/macrophages, which can occur independent of platelet activation.[11]

hFcR/Ask1$^{-/-}$ mice were also significantly protected from IC-induced pulmonary thromboembolism. As aforementioned, we and others showed that δ-granule secretion is potentiated by Ask1/ TxA_2 signaling,[19,58,59] and multiple studies demonstrated that δ-granule secretion contributes to IgG-induced thromboembolism *in vivo*.[29,32] Further, Ask1 was recently revealed to phosphorylate and produce sustained $P2Y_{12}$ signaling activity in platelets.[20] Taken together, these findings suggest that ASK1 regulates IC-induced thrombosis, in part, due to ASK1's role as a regulator of TxA_2 generation (and TxA_2-induced granule secretion) as well as Ask1's role in regulating $P2Y_{12}$ signaling. Consistent with this hypothesis, we found that anti-mCD9-induced platelet adhesion and thrombus formation was supported by Ask1-mediated TxA_2 generation.

But why can the loss of Ask1 be overcome at higher doses *in vitro* and *in vivo*? While Ask1 is needed for early $cPla_2$ phosphorylation, it is not absolutely required over longer time periods or at higher agonist doses. Indeed, genotype differences in aggregation, granule secretion, TxA_2 generation, thrombocytopenia, and shock can all be reduced or overcome as both time and/or agonist dose are increased. While the molecular basis for these findings at higher agonist doses will require future examination, it is likely that the observed ASK1/p38 independent $cPLA_2$ phosphorylation and TxA_2 generation is due to the activity of ERK1/2, a $cPLA_2$ kinase[52–54]. This hypothesis is consistent with previous findings that: 1) both ERK1/2 and p38 are activated downstream of FcγRIIA and mediate IV.3-induced $cPLA_2$ phosphorylation in human platelets,[51,56] 2) that inhibition of both ERK1/2 and p38 is needed to block CRP-induced TxA_2 generation in murine platelets,[54] 3) that ERK1/2 is hyperactivated in $Ask1^{-/-}$ platelets, and 4) that ASK1 regulates early, but not late (likely ERK1/2 dependent), $cPLA_2$ phosphorylation in platelets[19,34]. Together, these observations suggest that while ASK1/p38 are needed for $cPLA_2$ phosphorylation and TxA_2 generation immediately after agonist addition, ASK1/p38 are not absolutely required for $cPLA_2$ phosphorylation and TxA_2 generation in platelets.

Our findings, however, do not eliminate the possibility that other, TxA_2-independent mechanisms are at play and could compensate for the loss of Ask1 at higher doses. Indeed, TxA_2 generation is not absolutely required at higher doses as aspirin did not inhibit hFcR/$Ask1^{+/+}$ platelet aggregation or increase lag time at anti-CD9 concentrations ≥ 1ug/mL, but did so at lower concentrations.[29] This TxA_2 independent mechanism also exists in human platelets as aspirin treatment did not completely block anti-CD9 induced Ca^{2+} release, secretion, and aggregation.[40,42,43,71] These findings suggest that anti-CD9 induces platelet activation via TxA_2 dependent and independent mechanisms, favoring TxA_2 dependent mechanisms at low agonist doses. Future examination will be required to fully elucidate the TxA_2 dependent and independent mechanisms and their respective roles *in vitro* and *in vivo*.

On a final note, during the preparation of this manuscript Sledz *et al.*,[54] reported a study on the effect of GS-4997 on human and mouse platelets. While Sledz *et al.*, confirmed our finding that GS-4997 did inhibit agonist-induced ASK1 activation in both human and murine platelets, they found that it only affected mouse TxA_2 generation, and had no effect on TxA_2 generation of human platelets. In contrast, our findings suggest that GS-4997 inhibits human platelet aggregation via TxA_2 generation [36]. Further studies are thus required to determine the precise role of ASK1 in the regulation of TxA_2 generation in human platelets.

As FcγRIIA can also bind to IgG-opsonized bacteria[72], and as Ask1 was shown to mediate bacterial endotoxin- and LPS-induced septic shock and inflammation[73], whether FcγRIIA and ASK1 have any role in regulating platelet-bacteria interactions, and ultimately sepsis, requires additional inquiry. Finally, our *in vivo* studies do not preclude a role for ASK1 in other cell types; for instance, monocytes [74,75] are activated by ICs and express ASK1, so they merit future investigation.

In summary, our findings show that ASK1 is a prominent regulator of IC-induced platelet activation and that ASK1 plays a pivotal role in mediating ITT and shock. These findings place Ask1 at the center of IC-induced secondary agonist generation and highlight ASK1 as a potential target to reduce both TxA_2 generation and $P2Y_{12}$ signaling activity in platelets (Figure 7). Thus, these data suggest that ASK1 may be a potent therapeutic target for preventing excessive/pathological platelet activation seen in autoimmune diseases or systemic infection, that predisposes patients to thrombosis, while avoiding the hemorrhagic risks of other antiplatelet drugs (like aspirin), as residual TxA_2 generation can occur to preserve basal hemostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by; an American Heart Association Predoctoral Fellowship (18PRE33990382) (P. Patel), grants from the National Heart, Lung, and Blood Institute (2R01 HL113188–05) (U. Naik) and (P01HL110860 (S. McKenzie), an American Heart Association Grant-in-Aid Award (#13GRNT16380023) (U. Naik), an American Society of Hematology Bridge Award (U. Naik), and the Cardeza Foundation for Hematologic Research. The sponsors of this study are public or nonprofit organizations that support science in general. They had no role in gathering, analyzing, or interpreting the data. The content of this publication does not necessarily reflect the views or polices of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

Authors would like to thank Hidenori Ichijo (University of Tokyo) for providing $Ask1^{-/-}$ mice. We would also like to thank Michael Whitley and Joanne Vesci (Thomas Jefferson University) for their help in preparing washed human platelets used in some experiments.

References

1. Suurmond J, Zou YR, Kim SJ, Diamond B. Therapeutics to block autoantibody initiation and propagation in systemic lupus erythematosus and rheumatoid arthritis. Science translational medicine. 2015; 7: 280ps5.
2. Cervera R Antiphospholipid syndrome. Thrombosis research. 2017; 151 Suppl 1: S43–S7. [PubMed: 28262233]
3. Arman M, Krauel K, Tilley DO, Weber C, Cox D, Greinacher A, Kerrigan SW, Watson SP. Amplification of bacteria-induced platelet activation is triggered by FcgammaRIIA, integrin alphaIIbbeta3, and platelet factor 4. Blood. 2014; 123: 3166–74. [PubMed: 24642751]
4. Watson CN, Kerrigan SW, Cox D, Henderson IR, Watson SP, Arman M. Human platelet activation by Escherichia coli: roles for FcgammaRIIA and integrin alphaIIbbeta3. Platelets. 2016; 27: 535–40. [PubMed: 27025455]
5. Horne MK 3rd, Alkins BR. Importance of PF4 in heparin-induced thrombocytopenia: confirmation with gray platelets. Blood. 1995; 85: 1408–9. [PubMed: 7858274]
6. Rauova L, Zhai L, Kowalska MA, Arepally GM, Cines DB, Poncz M. Role of platelet surface PF4 antigenic complexes in heparin-induced thrombocytopenia pathogenesis: diagnostic and therapeutic implications. Blood. 2006; 107: 2346–53. [PubMed: 16304054]
7. Cai Z, Yarovoi SV, Zhu Z, Rauova L, Hayes V, Lebedeva T, Liu Q, Poncz M, Arepally G, Cines DB, Greene MI. Atomic description of the immune complex involved in heparin-induced thrombocytopenia. Nat Commun. 2015; 6: 8277. [PubMed: 26391892]
8. Warkentin TE. Clinical picture of heparin-induced thrombocytopenia (HIT) and its differentiation from non-HIT thrombocytopenia. Thrombosis and haemostasis. 2016; 116: 813–22. [PubMed: 27656712]

9. Dhakal B, Kreuziger LB, Rein L, Kleman A, Fraser R, Aster RH, Hari P, Padmanabhan A. Disease burden, complication rates, and health-care costs of heparin-induced thrombocytopenia in the USA: a population-based study. The Lancet Haematology. 2018; 5: e220–e31. [PubMed: 29703336]
10. Taylor SM, Reilly MP, Schreiber AD, Chien P, Tuckosh JR, McKenzie SE. Thrombosis and shock induced by activating antiplatelet antibodies in human Fc gamma RIIA transgenic mice: the interplay among antibody, spleen, and Fc receptor. Blood. 2000; 96: 4254–60. [PubMed: 11110699]
11. Nieswandt B, Bergmeier W, Rackebrandt K, Gessner JE, Zirngibl H. Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice. Blood. 2000; 96: 2520–7. [PubMed: 11001906]
12. Cloutier N, Allaeys I, Marcoux G, Machlus KR, Mailhot B, Zufferey A, Levesque T, Becker Y, Tessandier N, Melki I, Zhi H, Poirier G, Rondina MT, Italiano JE, Flamand L, McKenzie SE, Cote F, Nieswandt B, Khan WI, Flick MJ, Newman PJ, Lacroix S, Fortin PR, Boilard E. Platelets release pathogenic serotonin and return to circulation after immune complex-mediated sequestration. Proceedings of the National Academy of Sciences of the United States of America. 2018; 115: E1550–E9. [PubMed: 29386381]
13. Beutier H, Hechler B, Godon O, Wang Y, Gillis CM, de Chaisemartin L, Gouel-Cheron A, Magnenat S, Macdonald LE, Murphy AJ, group Ns, Chollet-Martin S, Longrois D, Gachet C, Bruhns P, Jonsson F. Platelets expressing IgG receptor FcgammaRIIA/CD32A determine the severity of experimental anaphylaxis. Science immunology. 2018; 3.
14. Reilly MP, Sinha U, Andre P, Taylor SM, Pak Y, Deguzman FR, Nanda N, Pandey A, Stolla M, Bergmeier W, McKenzie SE. PRT-060318, a novel Syk inhibitor, prevents heparin-induced thrombocytopenia and thrombosis in a transgenic mouse model. Blood. 2011; 117: 2241–6. [PubMed: 21088136]
15. Li Y, Hadden C, Cooper A, Ahmed A, Wu H, Lupashin VV, Mayeux PR, Kilic F. Sepsis-induced elevation in plasma serotonin facilitates endothelial hyperpermeability. Scientific reports. 2016; 6: 22747. [PubMed: 26956613]
16. Seitz BM, Orer HS, Krieger-Burke T, Darios ES, Thompson JM, Fink GD, Watts SW. 5-HT causes splanchnic venodilation. Am J Physiol Heart Circ Physiol. 2017; 313: H676–H86. [PubMed: 28626072]
17. Smith N, Lopez RA, Silberman M. Distributive Shock StatPearls. Treasure Island (FL): StatPearls Publishing, 2020.
18. Galan AM, Lopez-Vilchez I, Diaz-Ricart M, Navalon F, Gomez E, Gasto C, Escolar G. Serotonergic mechanisms enhance platelet-mediated thrombogenicity. Thrombosis and haemostasis. 2009; 102: 511–9. [PubMed: 19718472]
19. Naik MU, Patel P, Derstine R, Turaga R, Chen X, Golla K, Neeves KB, Ichijo H, Naik UP. Ask1 regulates murine platelet granule secretion, thromboxane A2 generation, and thrombus formation. Blood. 2017; 129: 1197–209. [PubMed: 28028021]
20. Kamiyama M, Shirai T, Tamura S, Suzuki-Inoue K, Ehata S, Takahashi K, Miyazono K, Hayakawa Y, Sato T, Takeda K, Naguro I, Ichijo H. ASK1 facilitates tumor metastasis through phosphorylation of an ADP receptor P2Y12 in platelets. Cell Death Differ. 2017; 24: 2066–76. [PubMed: 28753204]
21. Patel P, Naik MU, Golla K, Shaik NF, Naik UP. Calcium-induced dissociation of CIB1 from ASK1 regulates agonist-induced activation of the p38 MAPK pathway in platelets. Biochem J. 2019; 476: 2835–50. [PubMed: 31530712]
22. McKenzie SE, Taylor SM, Malladi P, Yuhan H, Cassel DL, Chien P, Schwartz E, Schreiber AD, Surrey S, Reilly MP. The role of the human Fc receptor Fc gamma RIIA in the immune clearance of platelets: a transgenic mouse model. J Immunol. 1999; 162: 4311–8. [PubMed: 10201963]
23. Tobiume K, Matsuzawa A, Takahashi T, Nishitoh H, Morita K, Takeda K, Minowa O, Miyazono K, Noda T, Ichijo H. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO reports. 2001; 2: 222–8. [PubMed: 11266364]
24. Naik UP, Naik MU. Association of CIB with GPIIb/IIIa during outside-in signaling is required for platelet spreading on fibrinogen. Blood. 2003; 102: 1355–62. [PubMed: 12714504]

25. Zhou Y, Abraham S, Renna S, Edelstein LC, Dangelmaier CA, Tsygankov AY, Kunapuli SP, Bray PF, McKenzie SE. TULA-2 (T-Cell Ubiquitin Ligand-2) Inhibits the Platelet Fc Receptor for IgG IIA (FcgammaRIIA) Signaling Pathway and Heparin-Induced Thrombocytopenia in Mice. Arterioscler Thromb Vasc Biol. 2016; 36: 2315–23. [PubMed: 27765766]
26. Naik MU, Nigam A, Manrai P, Millili P, Czymmek K, Sullivan M, Naik UP. CIB1 deficiency results in impaired thrombosis: the potential role of CIB1 in outside-in signaling through integrin alpha IIb beta 3. Journal of thrombosis and haemostasis : JTH. 2009; 7: 1906–14. [PubMed: 19691476]
27. Naik MU, Caplan JL, Naik UP. Junctional adhesion molecule-A suppresses platelet integrin alphaIIbbeta3 signaling by recruiting Csk to the integrin-c-Src complex. Blood. 2014; 123: 1393–402. [PubMed: 24300854]
28. Neeves KB, Onasoga AA, Hansen RR, Lilly JJ, Venckunaite D, Sumner MB, Irish AT, Brodsky G, Manco-Johnson MJ, Di Paola JA. Sources of variability in platelet accumulation on type 1 fibrillar collagen in microfluidic flow assays. PloS one. 2013; 8: e54680. [PubMed: 23355889]
29. Stolla M, Stefanini L, Andre P, Ouellette TD, Reilly MP, McKenzie SE, Bergmeier W. CalDAG-GEFI deficiency protects mice in a novel model of Fcgamma RIIA-mediated thrombosis and thrombocytopenia. Blood. 2011; 118: 1113–20. [PubMed: 21652673]
30. Zhou Y, Abraham S, Andre P, Edelstein LC, Shaw CA, Dangelmaier CA, Tsygankov AY, Kunapuli SP, Bray PF, McKenzie SE. Anti-miR-148a regulates platelet FcgammaRIIA signaling and decreases thrombosis in vivo in mice. Blood. 2015; 126: 2871–81. [PubMed: 26516227]
31. Wang X, Cheng Q, Xu L, Feuerstein GZ, Hsu MY, Smith PL, Seiffert DA, Schumacher WA, Ogletree ML, Gailani D. Effects of factor IX or factor XI deficiency on ferric chloride-induced carotid artery occlusion in mice. Journal of thrombosis and haemostasis : JTH. 2005; 3: 695–702. [PubMed: 15733058]
32. Amirkhosravi A, Boulaftali Y, Robles-Carrillo L, Meyer T, McKenzie SE, Francis JL, Bergmeier W. CalDAG-GEFI deficiency protects mice from FcgammaRIIa-mediated thrombotic thrombocytopenia induced by CD40L and beta2GPI immune complexes. Journal of thrombosis and haemostasis : JTH. 2014; 12: 2113–9. [PubMed: 25287077]
33. Tobiume K, Saitoh M, Ichijo H. Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. J Cell Physiol. 2002; 191: 95–104. [PubMed: 11920685]
34. Patel P, Golla K, Naik UP. PDK1 governs thromboxane generation and thrombosis in platelets by regulating activation of Raf1 in the MAPK pathway: comment. Journal of thrombosis and haemostasis : JTH. 2018; 16: 1901–4. [PubMed: 29983001]
35. Yeung J, Tourdot BE, Fernandez-Perez P, Vesci J, Ren J, Smyrniotis CJ, Luci DK, Jadhav A, Simeonov A, Maloney DJ, Holman TR, McKenzie SE, Holinstat M. Platelet 12-LOX is essential for FcgammaRIIa-mediated platelet activation. Blood. 2014; 124: 2271–9. [PubMed: 25100742]
36. Patel P Role of ASK1 in Platelet Activation and Immune-Induced Thrombocytopenia and Thrombosis (Doctoral dissertation). ETD Collection for Thomas Jefferson University. 2020.
37. Slupsky JR, Cawley JC, Griffith LS, Shaw AR, Zuzel M. Role of Fc gamma RII in platelet activation by monoclonal antibodies. J Immunol. 1992; 148: 3189–94. [PubMed: 1533653]
38. Anderson GP, van de Winkel JG, Anderson CL. Anti-GPIIb/IIIa (CD41) monoclonal antibody-induced platelet activation requires Fc receptor-dependent cell-cell interaction. Br J Haematol. 1991; 79: 75–83. [PubMed: 1832937]
39. Lhermusier T, van Rottem J, Garcia C, Xuereb JM, Ragab A, Martin V, Gratacap MP, Sie P, Payrastre B. The Syk-kinase inhibitor R406 impairs platelet activation and monocyte tissue factor expression triggered by heparin-PF4 complex directed antibodies. J Thromb Haemost. 2011; 9: 2067–76. [PubMed: 21848694]
40. Carroll RC, Worthington RE, Boucheix C. Stimulus-response coupling in human platelets activated by monoclonal antibodies to the CD9 antigen, a 24 kDa surface-membrane glycoprotein. The Biochemical journal. 1990; 266: 527–35. [PubMed: 2317202]
41. Hato T, Ikeda K, Yasukawa M, Watanabe A, Kobayashi Y. Exposure of platelet fibrinogen receptors by a monoclonal antibody to CD9 antigen. Blood. 1988; 72: 224–9. [PubMed: 2455569]

42. Favier R, Lecompte T, Morel MC, Potevin F, Benoit P, Boucheix C, Kaplan C, Samama M. Calcium rise in human platelets elicited by anti-CD9 and -CD41 murine monoclonal antibodies. Thrombosis research. 1989; 55: 591–9. [PubMed: 2510359]
43. Hato T, Sumida M, Yasukawa M, Watanabe A, Okuda H, Kobayashi Y. Induction of platelet Ca2+ influx and mobilization by a monoclonal antibody to CD9 antigen. Blood. 1990; 75: 1087–91. [PubMed: 2306515]
44. Prevost N, Mitsios JV, Kato H, Burke JE, Dennis EA, Shimizu T, Shattil SJ. Group IVA cytosolic phospholipase A2 (cPLA2alpha) and integrin alphaIIbbeta3 reinforce each other's functions during alphaIIbbeta3 signaling in platelets. Blood. 2009; 113: 447–57. [PubMed: 18840708]
45. Wong DA, Kita Y, Uozumi N, Shimizu T. Discrete role for cytosolic phospholipase A(2)alpha in platelets: studies using single and double mutant mice of cytosolic and group IIA secretory phospholipase A(2). J Exp Med. 2002; 196: 349–57. [PubMed: 12163563]
46. Adler DH, Cogan JD, Phillips JA, 3rd, Schnetz-Boutaud N, Milne GL, Iverson T, Stein JA, Brenner DA, Morrow JD, Boutaud O, Oates JA. Inherited human cPLA(2alpha) deficiency is associated with impaired eicosanoid biosynthesis, small intestinal ulceration, and platelet dysfunction. J Clin Invest. 2008; 118: 2121–31. [PubMed: 18451993]
47. Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ. cPLA2 is phosphorylated and activated by MAP kinase. Cell. 1993; 72: 269–78. [PubMed: 8381049]
48. Hefner Y, Borsch-Haubold AG, Murakami M, Wilde JI, Pasquet S, Schieltz D, Ghomashchi F, Yates JR 3rd, Armstrong CG, Paterson A, Cohen P, Fukunaga R, Hunter T, Kudo I, Watson SP, Gelb MH. Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases. J Biol Chem. 2000; 275: 37542–51. [PubMed: 10978317]
49. Gijon MA, Spencer DM, Kaiser AL, Leslie CC. Role of phosphorylation sites and the C2 domain in regulation of cytosolic phospholipase A2. J Cell Biol. 1999; 145: 1219–32. [PubMed: 10366595]
50. Das S, Rafter JD, Kim KP, Gygi SP, Cho W. Mechanism of group IVA cytosolic phospholipase A(2) activation by phosphorylation. J Biol Chem. 2003; 278: 41431–42. [PubMed: 12885780]
51. Saci A, Liu WQ, Vidal M, Garbay C, Rendu F, Bachelot-Loza C. Differential effect of the inhibition of Grb2-SH3 interactions in platelet activation induced by thrombin and by Fc receptor engagement. The Biochemical journal. 2002; 363: 717–25. [PubMed: 11964172]
52. Borsch-Haubold AG, Ghomashchi F, Pasquet S, Goedert M, Cohen P, Gelb MH, Watson SP. Phosphorylation of cytosolic phospholipase A2 in platelets is mediated by multiple stress-activated protein kinase pathways. Eur J Biochem. 1999; 265: 195–203. [PubMed: 10491174]
53. Shankar H, Garcia A, Prabhakar J, Kim S, Kunapuli SP. P2Y12 receptor-mediated potentiation of thrombin-induced thromboxane A2 generation in platelets occurs through regulation of Erk1/2 activation. Journal of thrombosis and haemostasis : JTH. 2006; 4: 638–47. [PubMed: 16460446]
54. Sledz KM, Moore SF, Vijayaragavan V, Mallah S, Goudswaard LJ, Williams CM, Hunter RW, Hers I. Redundant role of ASK1-mediated p38MAPK activation in human platelet function. Cellular signalling. 2020; 68: 109528. [PubMed: 31917191]
55. Shi P, Zhang L, Zhang M, Yang W, Wang K, Zhang J, Otsu K, Huang G, Fan X, Liu J. Platelet-Specific p38alpha Deficiency Improved Cardiac Function After Myocardial Infarction in Mice. Arterioscler Thromb Vasc Biol. 2017; 37: e185–e96. [PubMed: 28982666]
56. Borsch-Haubold AG, Kramer RM, Watson SP. Phosphorylation and activation of cytosolic phospholipase A2 by 38-kDa mitogen-activated protein kinase in collagen-stimulated human platelets. European journal of biochemistry. 1997; 245: 751–9. [PubMed: 9183015]
57. Reilly MP, Taylor SM, Hartman NK, Arepally GM, Sachais BS, Cines DB, Poncz M, McKenzie SE. Heparin-induced thrombocytopenia/thrombosis in a transgenic mouse model requires human platelet factor 4 and platelet activation through FcgammaRIIA. Blood. 2001; 98: 2442–7. [PubMed: 11588041]
58. Cho MJ, Liu J, Pestina TI, Steward SA, Thomas DW, Coffman TM, Wang D, Jackson CW, Gartner TK. The roles of alpha IIb beta 3-mediated outside-in signal transduction, thromboxane A2, and adenosine diphosphate in collagen-induced platelet aggregation. Blood. 2003; 101: 2646–51. [PubMed: 12446460]

59. Falker K, Klarstrom-Engstrom K, Bengtsson T, Lindahl TL, Grenegard M. The toll-like receptor 2/1 (TLR2/1) complex initiates human platelet activation via the src/Syk/LAT/PLCgamma2 signalling cascade. Cell Signal. 2014; 26: 279–86. [PubMed: 24240055]
60. Lentz BR. Exposure of platelet membrane phosphatidylserine regulates blood coagulation. Progress in lipid research. 2003; 42: 423–38. [PubMed: 12814644]
61. Kreidy R, Hatem J. Acute limb ischemia secondary to heparin-induced thrombocytopenia after cardiac surgery. J Med Liban. 2004; 52: 175–81. [PubMed: 16432978]
62. Altoijry A, MacKenzie KS, Corriveau MM, Obrand DI, Abraham CZ, Steinmetz OK. Heparin-induced thrombocytopenia causing graft thrombosis and bowel ischemia postendovascular aneurysm repair. Journal of vascular surgery. 2015; 61: 234–6. [PubMed: 24135622]
63. Ahmad AS, Al-Layla DJ, Dhinakar M. Hats Off to HIT: A Case Report. Oman Med J. 2012; 27: 239–42. [PubMed: 22811776]
64. Selleng K, Selleng S, Raschke R, Schmidt CO, Rosenblood GS, Greinacher A, Warkentin TE. Immune heparin-induced thrombocytopenia can occur in patients receiving clopidogrel and aspirin. Am J Hematol. 2005; 78: 188–92. [PubMed: 15726594]
65. Chong BH, Grace CS, Rozenberg MC. Heparin-induced thrombocytopenia: effect of heparin platelet antibody on platelets. Br J Haematol. 1981; 49: 531–40. [PubMed: 7317283]
66. Anderson GP, Anderson CL. Signal transduction by the platelet Fc receptor. Blood. 1990; 76: 1165–72. [PubMed: 2144775]
67. Takahashi H, Muto S, Nakazawa E, Yanagiba S, Masunaga Y, Miyata Y, Tamba K, Kusano E, Matsuo M, Matsuo T, Asano Y. Combined treatment with nafamostat mesilate and aspirin prevents heparin-induced thrombocytopenia in a hemodialysis patient. Clinical nephrology. 2003; 59: 458–62. [PubMed: 12834179]
68. Polgar J, Eichler P, Greinacher A, Clemetson KJ. Adenosine diphosphate (ADP) and ADP receptor play a major role in platelet activation/aggregation induced by sera from heparin-induced thrombocytopenia patients. Blood. 1998; 91: 549–54. [PubMed: 9427708]
69. Gratacap MP, Herault JP, Viala C, Ragab A, Savi P, Herbert JM, Chap H, Plantavid M, Payrastre B. FcgammaRIIA requires a Gi-dependent pathway for an efficient stimulation of phosphoinositide 3-kinase, calcium mobilization, and platelet aggregation. Blood. 2000; 96: 3439–46. [PubMed: 11071639]
70. Finkelman FD, Khodoun MV, Strait R. Human IgE-independent systemic anaphylaxis. J Allergy Clin Immunol. 2016; 137: 1674–80. [PubMed: 27130857]
71. Kuroda K, Ozaki Y, Qi R, Asazuma N, Yatomi Y, Satoh K, Nomura S, Suzuki M, Kume S. Fc gamma II receptor-mediated platelet activation induced by anti-CD9 monoclonal antibody opens Ca2+ channels which are distinct from those associated with Ca2+ store depletion. Journal of immunology (Baltimore, Md : 1950). 1995; 155: 4427–36.
72. Arman M, Krauel K. Human platelet IgG Fc receptor FcgammaRIIA in immunity and thrombosis. Journal of thrombosis and haemostasis : JTH. 2015; 13: 893–908. [PubMed: 25900780]
73. Matsuzawa A, Saegusa K, Noguchi T, Sadamitsu C, Nishitoh H, Nagai S, Koyasu S, Matsumoto K, Takeda K, Ichijo H. ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4-mediated innate immunity. Nat Immunol. 2005; 6: 587–92. [PubMed: 15864310]
74. Xiao Z, Visentin GP, Dayananda KM, Neelamegham S. Immune complexes formed following the binding of anti-platelet factor 4 (CXCL4) antibodies to CXCL4 stimulate human neutrophil activation and cell adhesion. Blood. 2008; 112: 1091–100. [PubMed: 18539895]
75. Rauova L, Hirsch JD, Greene TK, Zhai L, Hayes VM, Kowalska MA, Cines DB, Poncz M. Monocyte-bound PF4 in the pathogenesis of heparin-induced thrombocytopenia. Blood. 2010; 116: 5021–31. [PubMed: 20724543]

Essentials

- Immune complexes (ICs) bind to and activate platelets via FcγRIIA.
- ICs cause thrombocytopenia, thrombosis, and systemic shock *in vivo*.
- IC-induced platelet activation is positively regulated by ASK1.
- Knockout of Ask1 protected FcγRIIA transgenic mice from IC-induced pathologies *in vivo*.

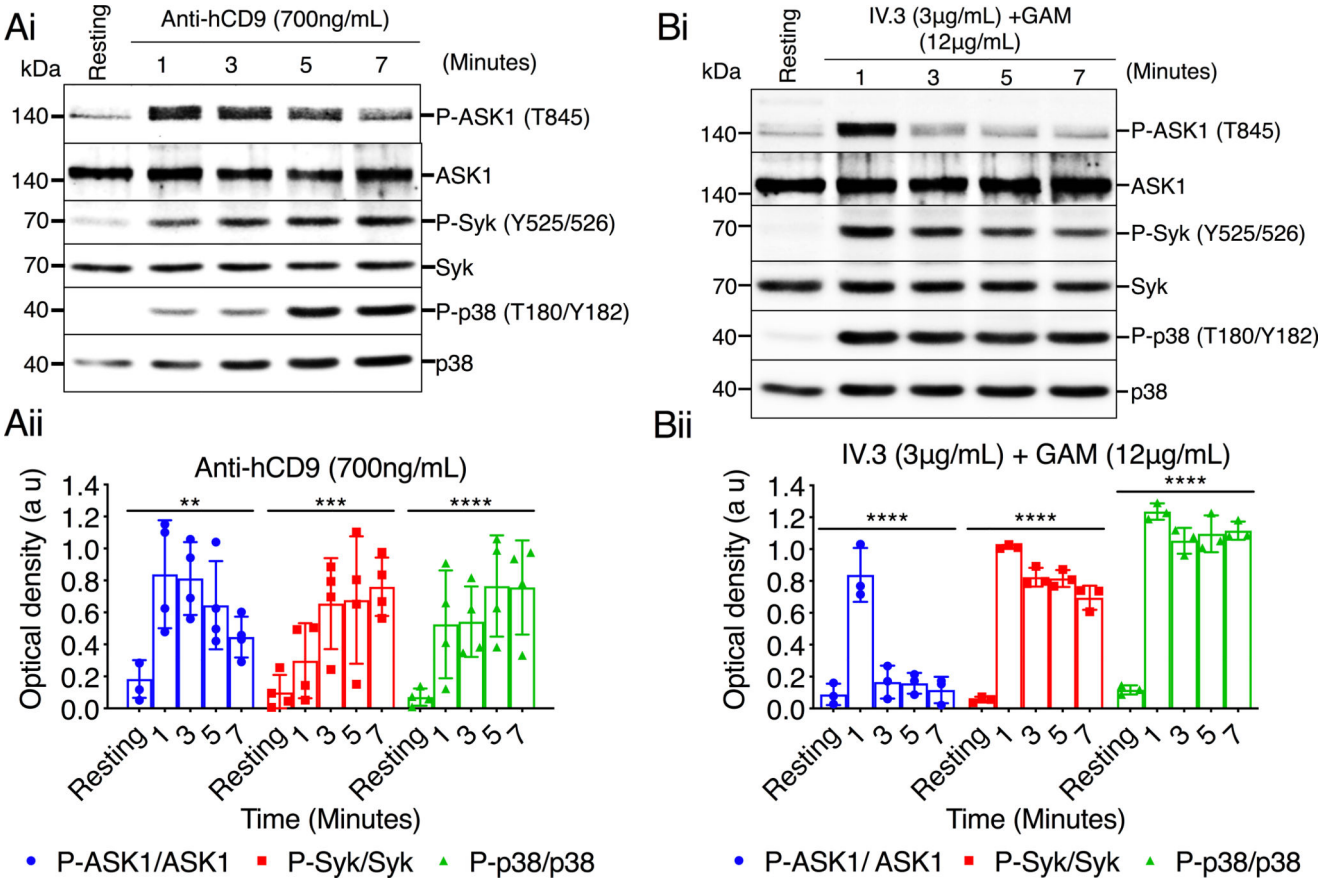

Figure 1. FcγRIIA agonists induce ASK1/p38 activation in human platelets.

(A-B) Western blot analysis of human platelets stimulated with (A) anti-hCD9 (700 ng/mL) or (B) IV.3+GAM (3 and 12 µg/mL respectively) and lysed at the indicated times (or resting control). (Ai & Bi) Images of representative immunoblots of phosphorylation of ASK1 (T845)(●), Syk (Y525/526)(■), and p38 (T180/Y182)(▲) using phospho-specific antibodies. Blots were reprobed with anti-ASK1, anti-Syk, and anti-p38 to ensure equal protein loading. (Aii & Bii) Quantification of band intensities (mean ± SD) from (i). Repeated measures One-way ANOVA. ** P <0.01, *** P <0.001, **** P <0.0001.

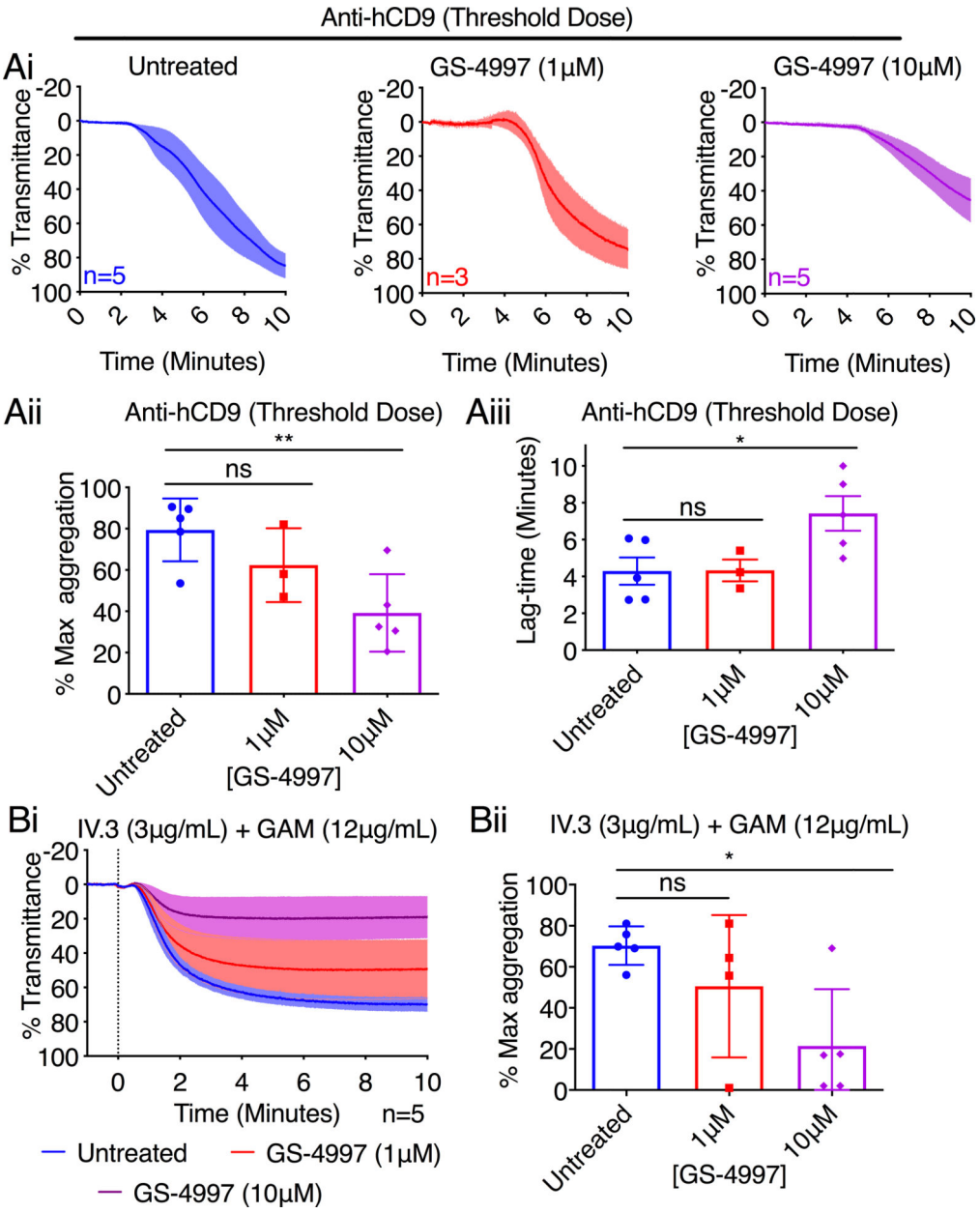

Figure 2. Inhibition of ASK1 attenuates anti-hCD9- and IV.3+GAM-induced platelet aggregation.

(A) Aggregation of human platelets, pretreated with GS-4997 (1–10 μM) (or untreated control), when stimulated with anti–hCD9. The threshold dose was defined as the lowest concentration to induce >60% aggregation within 10 minutes and was calculated for each donor separately. (Ai) Mean (± SEM) aggregation tracings recorded as the percent change in light transmittance. (Aii) Quantification of the maximum light transmittance (% Max Aggregation) of platelets stimulated with anti–hCD9. (Aiii) Quantification of the lag time of platelets stimulated with anti–mCD9. (B) Aggregation of human platelets, pretreated with GS-4997 (1–10 μM) (or untreated control), when stimulated with IV.3+GAM (3 and 12 μg/mL respectively). (Bi) Mean (± SEM) aggregation tracings recorded as the percent

change in light transmittance. (Bii) Quantification of the maximum light transmittance (% Max Aggregation) of platelets stimulated with IV.3+GAM. One-way ANOVA. *P <0.05, **P <0.01. Untreated (●), 1μM (■), 10μM (◆).

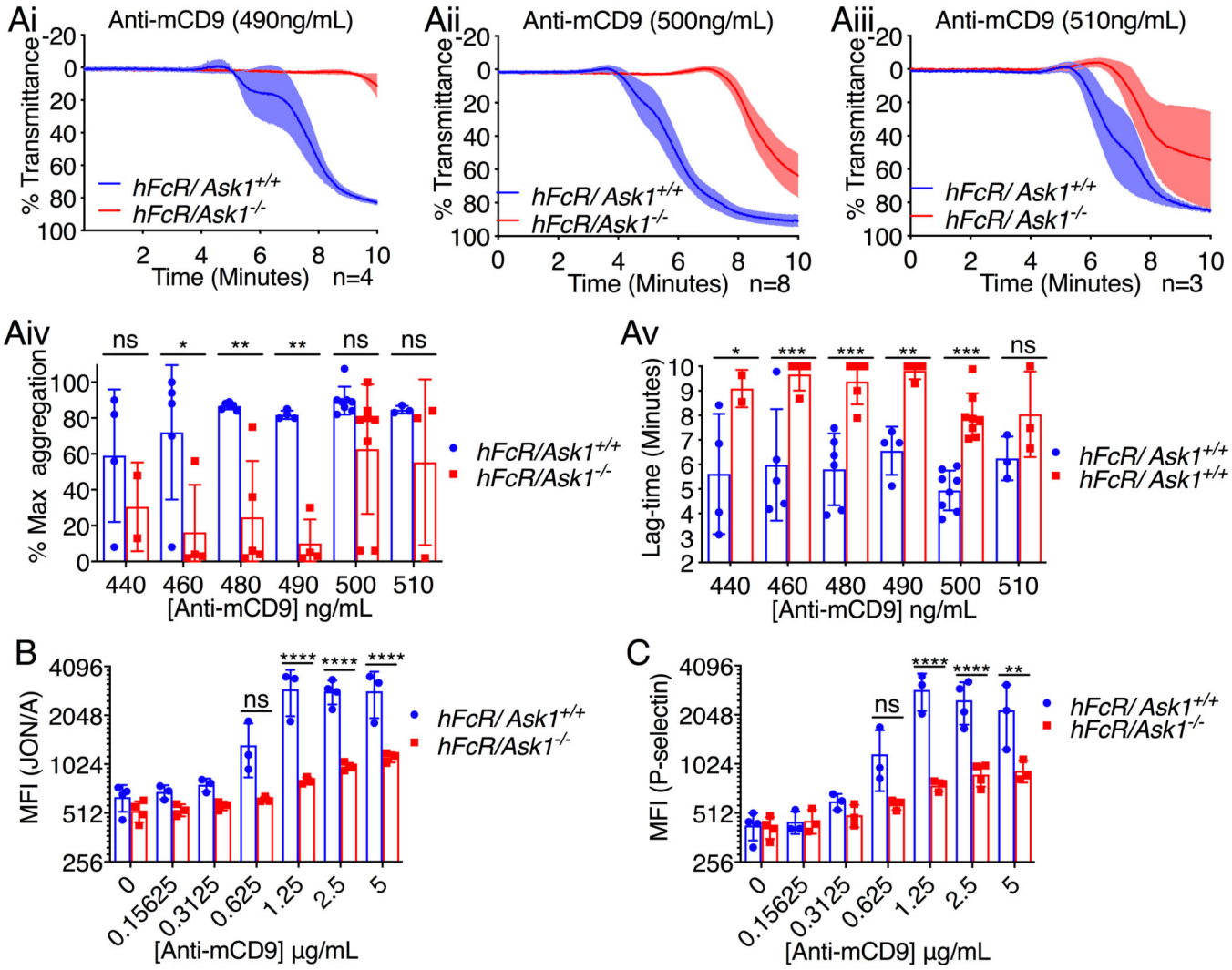

Figure 3. Ask1 regulates anti-mCD9-induced platelet aggregation and lag-time

(A) Aggregation of mouse platelets isolated from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice when stimulated with anti–mCD9. (Ai-Aiii) Mean (± SEM) aggregation tracings recorded as the percent change in light transmittance. (Aiv) Quantification of the maximum light transmittance (% Max Aggregation) of platelets stimulated with anti–mCD9. Two-way ANOVA (with Sidak's multiple comparisons test). (Av) Quantification of the lag time of platelets stimulated with anti–mCD9. Two-way ANOVA (with Sidak's multiple comparisons test). (B) MFI of JON/A binding to mouse platelets isolated from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice when stimulated with anti–mCD9. Two-way ANOVA (with Sidak's multiple comparisons test). (C) MFI of Anti-P-selectin binding to mouse platelets isolated from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice when stimulated with anti–mCD9. Two-way ANOVA (with Sidak's multiple comparisons test). Not significant (ns), **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001. *hFcR/Ask1*$^{+/+}$ (●), *hFcR/Ask1*$^{-/-}$ (■).

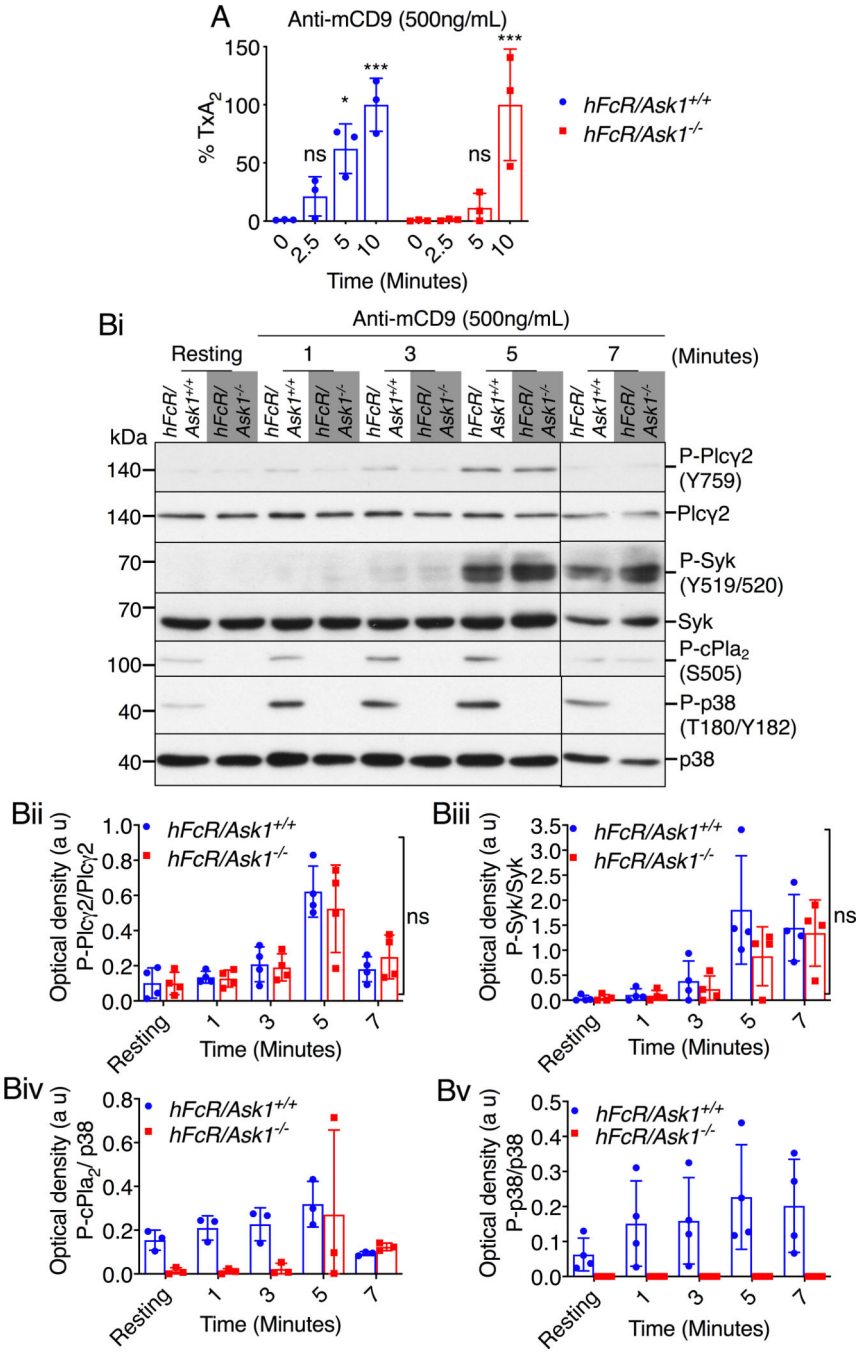

Figure 4. Ask1 regulates cPla$_2$ phosphorylation and TxA$_2$ generation downstream of FcγRIIA.

(A) Quantification of TxA_2 generation from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ platelets stimulated with 500 ng/mL anti-mCD9 and measured by ELISA. Repeated measures Two-way ANOVA (with Dunnett's multiple comparisons test). (B) Western blot analysis of *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ platelets stimulated with 500 ng/mL anti-mCD9 and lysed at the indicated times (or resting control). (Bi) Images of representative immunoblots of phosphorylation of Plcγ2 (Y759), Syk (Y519/520), cPla$_2$ (S505), and p38 (T180/Y182) using phospho-specific antibodies. Blots were reprobed with anti-Plcγ2, anti-Syk, and anti-

p38 to ensure equal protein loading. (Bii-v) Quantification of band intensities from (Bi) for (Bii) P-Plcγ2, (Biii) P-Syk, (Biv) P-cPla$_2$, and (Bv) P-p38. Repeated measures Two-way ANOVA. Not significant (ns), *P<0.05, ***P<0.001. *hFcR/Ask1*$^{+/+}$ (●), *hFcR/Ask1*$^{-/-}$ (■).

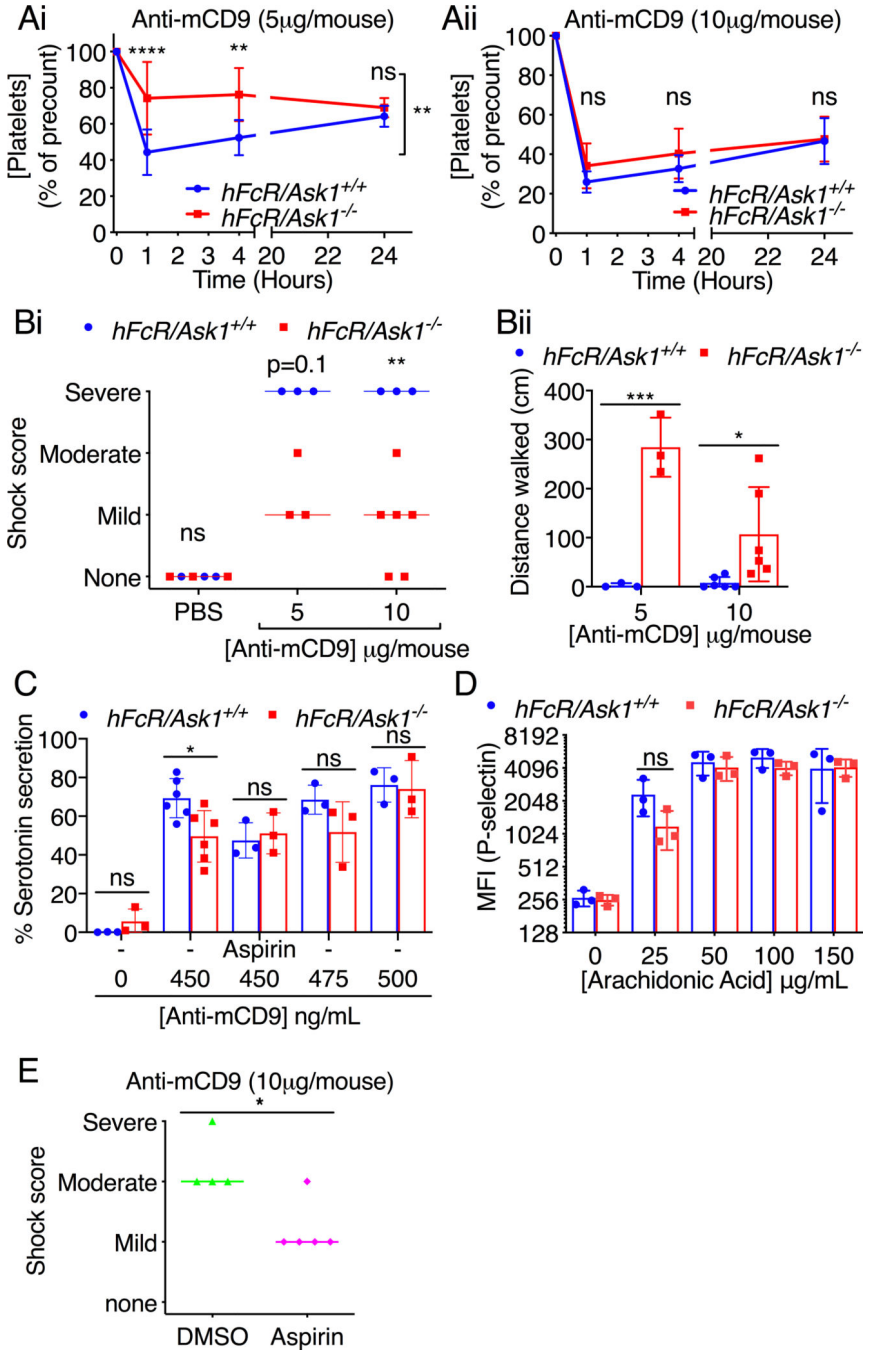

Figure 5. Ask1 regulates anti-mCD9-induced thrombocytopenia and shock in a mouse model of ITT.

(A) The percent change in platelet count, relative to baseline measurements, were measured at the indicated time points from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice that were injected with anti-mCD9 (5 and 10 μg/20 g mouse body weight). Repeated measures Two-way ANOVA (with Sidak's multiple comparisons test). (B) Symptoms of shock following injection of anti-mCD9 (5 and 10 μg/20 g mouse body weight). (Bi) Assessment of shock based on blinded observations of *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice following injection of anti-mCD9. Mann-Whitney test. (Bii) Total distance walked by *hFcR/Ask1*$^{+/+}$ and *hFcR/*

Ask1$^{-/-}$ mice after injection of anti-mCD9. Two-way ANOVA (with Sidak's multiple comparisons test). (C) Quantification of serotonin released from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ platelets, pretreated with 1mM aspirin (or vehicle control), and stimulated with anti-mCD9 (450 and 500 ng/mL), and measured by ELISA. Two-way ANOVA (with Sidak's multiple comparisons test). (D) MFI of Anti-P-selectin binding to mouse platelets isolated from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice when stimulated with arachidonic acid. Two-way ANOVA (with Sidak's multiple comparisons test). (E) Assessment of shock in *hFcR/Ask1*$^{+/+}$ mice pretreated with aspirin (2 mg/20 g mouse body weight or DMSO vehicle control) following injection of anti-mCD9 (10 μg/20 g mouse body weight). Mann-Whitney test. Not significant (ns), *P <0.05, **P <0.01, ***P <0.005, ****P <0.0001. *hFcR/Ask1*$^{+/+}$ (●), *hFcR/Ask1*$^{-/-}$ (■), DMSO (▲), aspirin (◆).

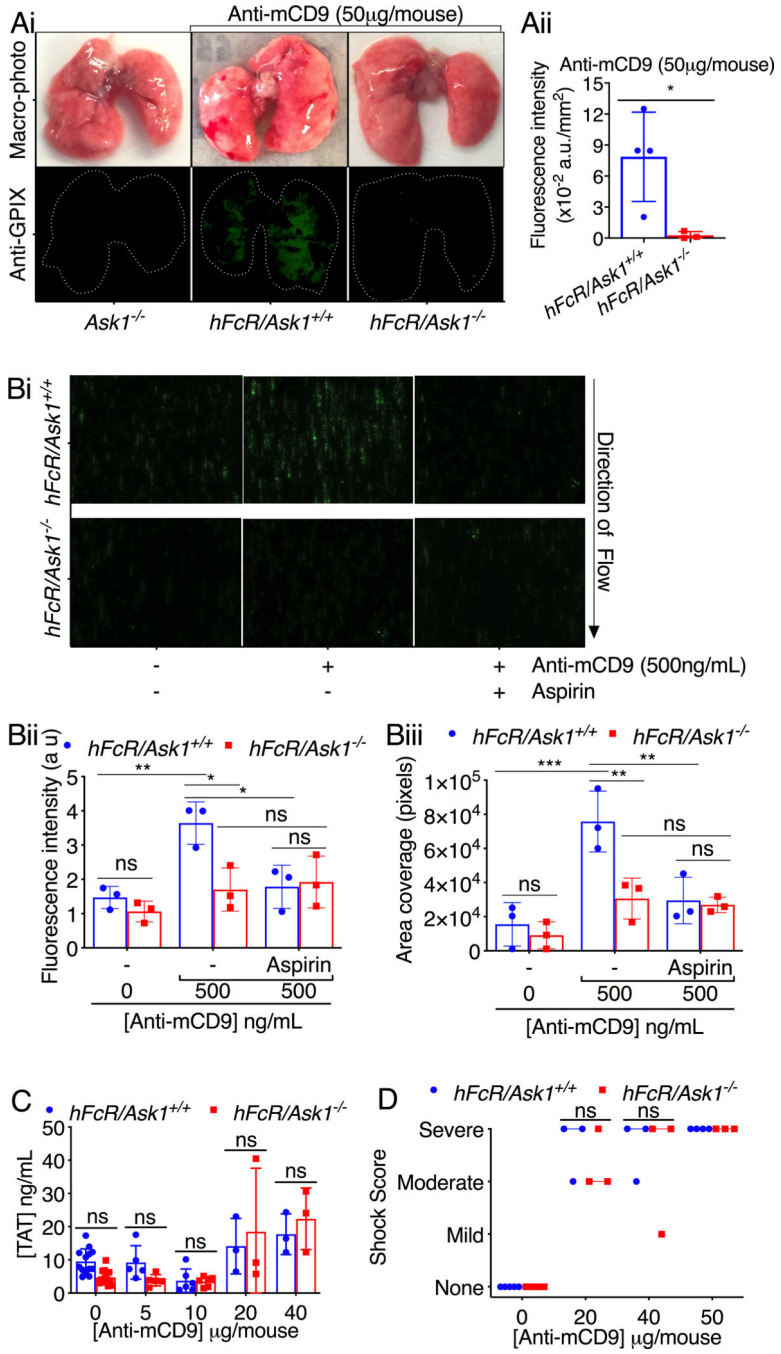

Figure 6. Ask1 regulates thrombosis in a mouse model of ITT.

(A) WT, *hFcR/Ask1*$^{+/+}$, and *hFcR/Ask1*$^{-/-}$ mice were injected with anti-mGPIX-800CW (for *in vivo* labeling of platelets) followed by anti-mCD9 (or PBS vehicle control) to induce thrombosis. (Ai) Representative macroscopic (top) and infrared images (bottom) of mouse lungs extracted 30 minutes after injection of anti-mCD9. Macroscopic images were taken with a using an Apple iPhone 7 at 1x magnification. Infrared images were obtained using a LI-COR Odyssey at a resolution of 21μm and were analyzed using LI-COR Odyssey software. (Aii) Quantification of the fluorescence intensity from (Ai). Unpaired Student's t-

test with Welch's correction for unequal variances. (B) *Ex vivo* microfluidics of anticoagulated (heparin/PPACK) whole blood from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice labeled with DiOC6, pretreated with 1mM aspirin (or vehicle control), 500 ng/mL anti-mCD9, and passed over immobilized fibrinogen at a shear rate of 800 s^{-1} for 3 minutes. (Bi) Representative fluorescent images of channels. Fluorescence and phase contrast images were captured, without imaging medium at room temperature, on an Evos FL-auto microscope running Evos Pearl Scope 64 software at an original magnification of 20X using an Olympus UPlan F1 20x/0.50 objective. Quantification of (Bii) fluorescence intensity (DiOC6, green stained) and (Biii) surface area coverage (taken from phase contrast images, data not shown). Two-way ANOVA (with Tukey's multiple comparisons test). (C) Assessment of *in vivo* TAT complex formation from plasma taken from *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice 1-hour after injection of anti-mCD9 (5–40 μg/20 g mouse body weight) (or PBS vehicle control). Two-way ANOVA (with Sidak's multiple comparisons test). (D) Assessment of shock based on observations of *hFcR/Ask1*$^{+/+}$ and *hFcR/Ask1*$^{-/-}$ mice following injection of anti-mCD9 (20–50 μg/20 g mouse body weight). Mann-Whitney test. Not significant (ns), $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. *hFcR/Ask1*$^{+/+}$ (●), *hFcR/Ask1*$^{-/-}$ (■).

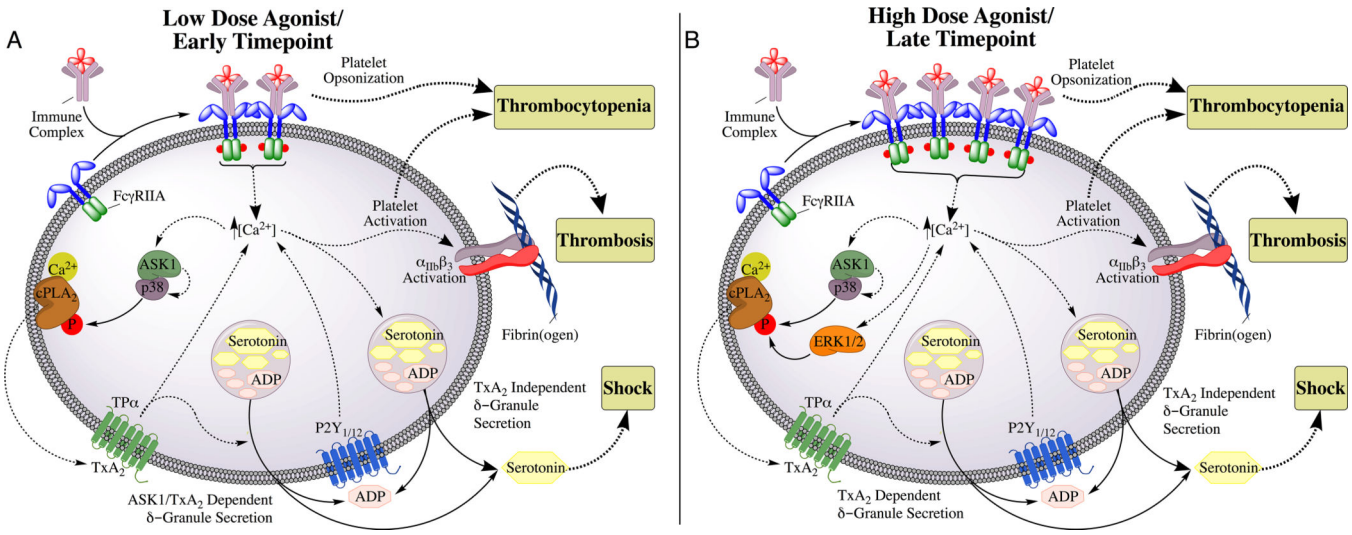

Figure 7. Proposed mechanism of Ask1-mediated platelet activation in the pathogenesis of ITT.

ICs bind and activate platelet-FcγRIIA causing an increase in intracellular Ca^{2+}, which subsequently induces αIIbβ3 activation, δ-granule secretion and TxA_2 generation. αIIbβ3 activation also supports platelet aggregation causing thrombosis, while δ-granule released ADP and serotonin support platelet activation and induce shock, respectively. Binding of ICs also opsonizes platelets, which, along with platelet activation, leads to thrombocytopenia. (A) With low agonist doses (or early time points), only a moderate amount of FcγRIIA is activated. In this state of moderate activity, while pathogenic, ASK1 is the sole regulator of TxA_2 generation. In addition to feedback activation of platelets, ASK1-mediated TxA_2 generation also regulates a second pool of δ-granule secretion. In the absence of ASK1, TxA_2 generation is reduced/delayed, attenuating thrombocytopenia, shock, and thrombosis. Furthermore, limited opsonization of platelets in this condition results in only mild thrombocytopenia. (B) When the dose of agonist increases (or at later time points), ASK1 becomes dispensable for TxA_2 generation, potentially due to a compensatory effect of ERK1/2 activity.