ASK1 inhibition triggers platelet apoptosis *via* p38-MAPK-mediated mitochondrial dysfunction

Apoptosis signal-regulating kinase 1 (ASK1) is a well-defined, universally expressed and highly appreciated member of the mitogen-activated protein kinase (MAPK) family. Several cell stress responses like reactive oxygen species (ROS)-mediated oxidative damage, endoplasmic reticulum (ER) stress, endotoxins, and inflammatory cytokines can activate ASK1 by dissociating it from thioredoxin.[1,2] The activation of ASK1 in nucleated cells stimulates a signaling cascade involving phosphorylation of MAP2K (MKK3/MKK6) leading to the activation of p38 and JNK MAPK to mediate apoptosis, inflammation and fibrosis.[1,2] MAPK are a family of evolutionarily con-

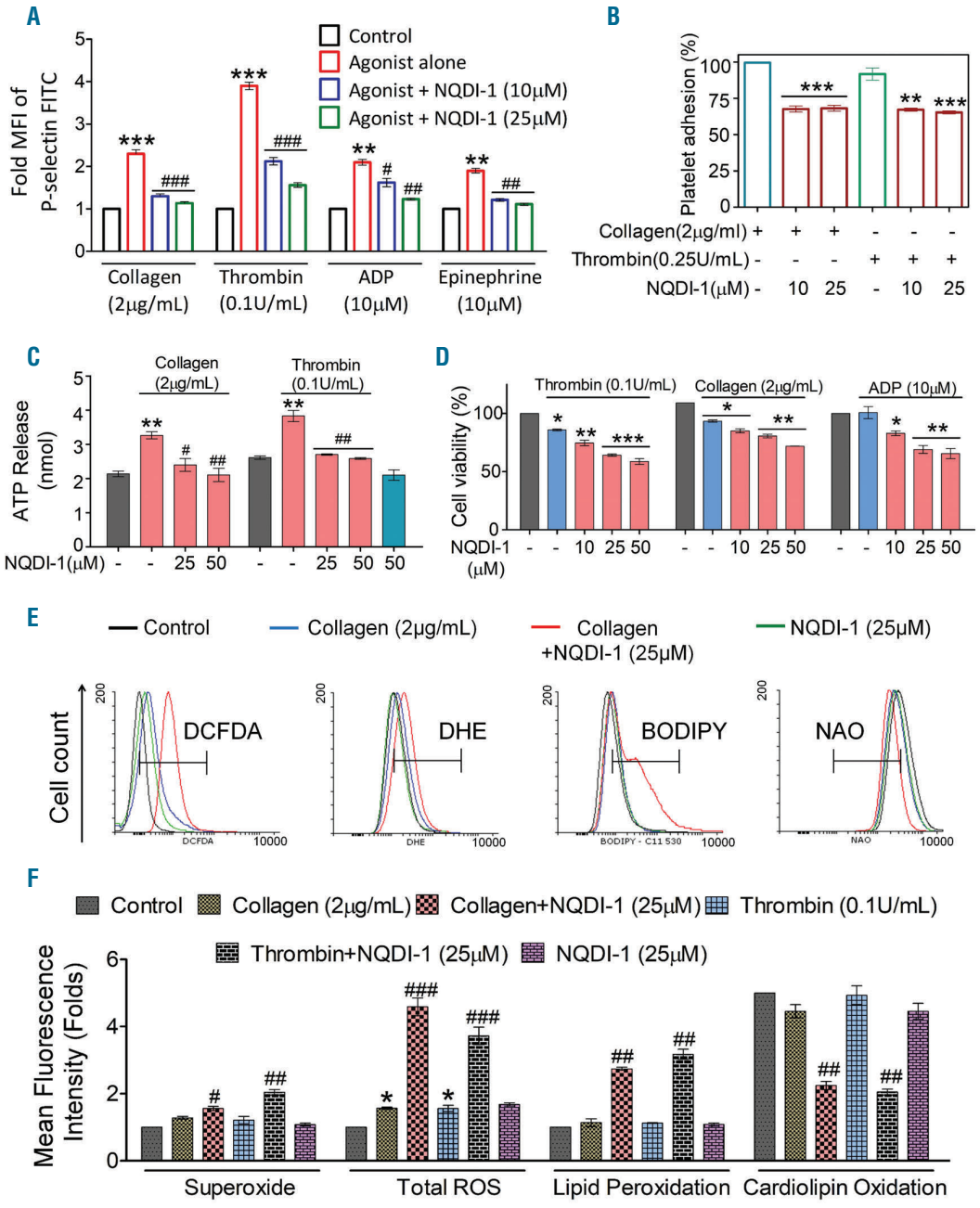

Figure 1. ASK1 inhibition reduces platelet aggregation and activation, but induces reactive oxygen species-mediated platelet death. (A) Fluorescence-activated cell sorting analysis of surface p-selectin expression in platelets treatment with various agonists (collagen, ADP, epinephrine and thrombin) pre-treated with various concentration of NQDI-1. (B) Fibrinogen binding analysis of platelets treated with thrombin (0.25 U/mL) or collagen (2 μg/mL) in the presence or absence of NQDI-1 (10 and 25 μM). (C) Quantification of secretory ATP levels using luciferin-luciferase kit method in collagen and thrombin induced platelet pre-treated with NQDI-1 (25 and 50 μM). (D) Evaluation of platelet viability in collagen and thrombin treated platelets pre-treated with various concentration of NQDI-1. (E and F) Flow cytometric analysis of different reactive oxygen species (ROS) levels in platelet treated with various agonists [collagen and thrombin] in the presence of NQDI-1 and its bar representation. Data represented are presented as mean ± standard error of the mean (SEM) (n=5). One-way ANOVA followed by Bonferroni *post hoc* multiple comparisons was used for statistical analysis; *P**/#<0.05, *P***/##<0.01, *P****/###<0.001; [*] significant compared to untreated control sample; [#] significant compared to platelet agonist-treated samples.

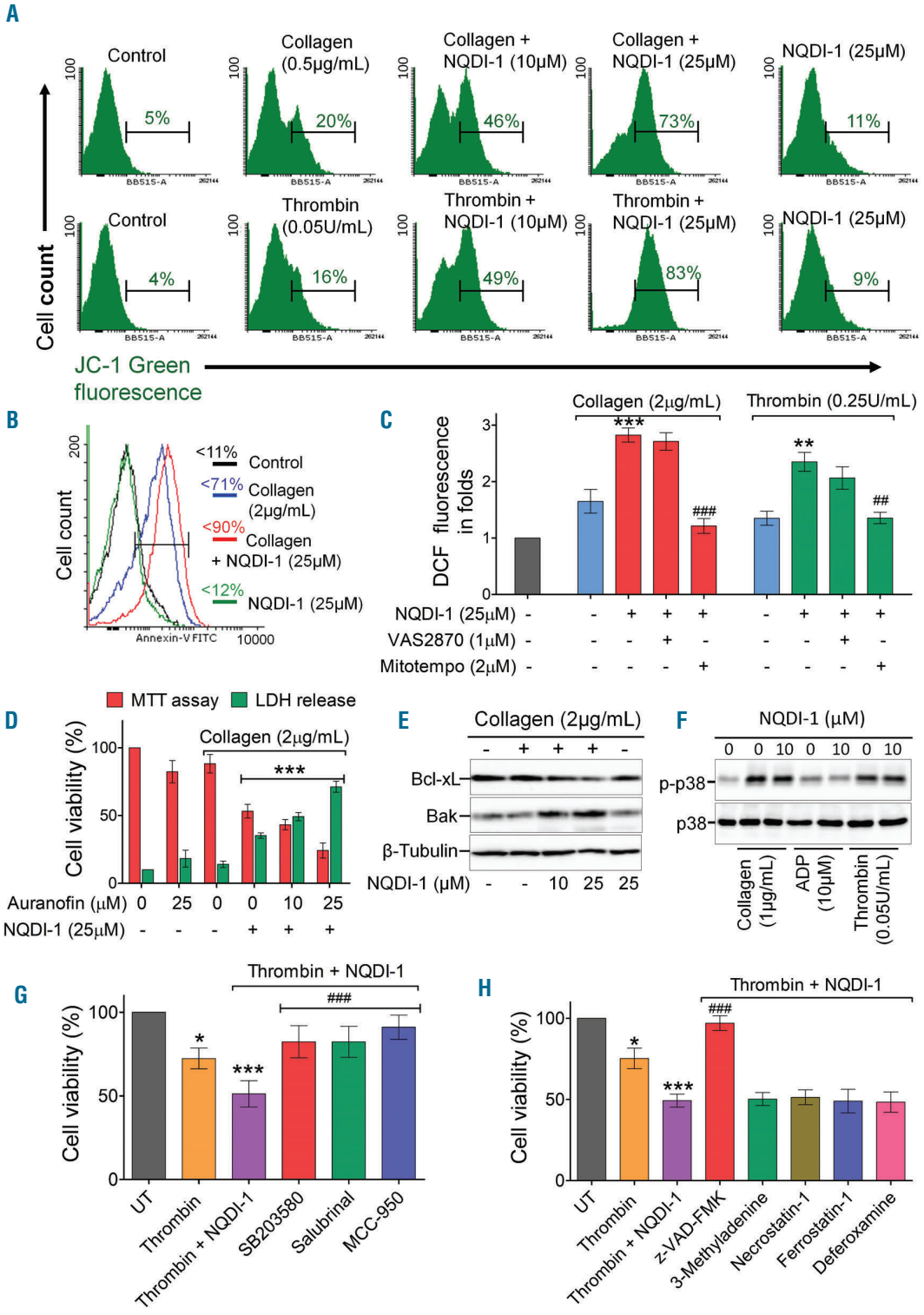

Figure 2. ASK1 inhibition induces p38 MAPK-mediated mitochondrial dysfunction and NLRP3 activation in platelets. (A) Flow cytometric analysis of mitochondrial membrane potential using JC-1 in collagen (0.5 µg/mL) and thrombin (0.05 U/mL) treated platelets, pre-treated with NQDI-1. (B) Fluorescence-activated cell sorting analysis of phosphatidylserine (PS) externalization in collagen and NQDI-1 treated platelets using annexin-V-FITC. (C) Quantification of endogenous reactive oxygen species (ROS) in collagen/thrombin treated platelets, pre-treated with NQDI-1 along with VAS2870 (NADPH oxidase inhibitor) and Mito-TEMPO (mitochondrial ROS scavenger). (D) Evaluation of cell viability in the presence or absence of auranofin (thioredoxin reductase inhibitor) in collagen and NQDI-1 treated platelets. (E) Immunoblotting analysis of BCL-2 and BAK levels in NQDI-1 pre-treated platelets followed by collagen treatment. (F) Immunoblot analysis of Phospho-p38 expression in various agonist treated platelets, pre-treated with NQDI-1. (G) Evaluation of cell viability using various cell signaling inhibitors: SB203580 (5 µM) the p38 MAPK inhibitor, Salubrinal (50 µM) a specific inhibitor of endoplasmic reticulum (ER) stress, and MCC950 (5 µM) a selective NLRP3 inhibitor) in thrombin and NQDI-1 treated platelets. (H) Evaluation of platelet viability in thrombin and NQDI-1 treatment pre-treated with various specific cell death inhibitors: z-VAD-FMK (10 µM) the pan caspase inhibitor, 3-Methyladenine (1 mM) the autophagy inhibitor; Necrostatin-1 (10 µM) an inhibitor of necroptosis, Ferrostatin-1 (3 µM) and Deferoxamine (100 µM) ferroptosis specific inhibitors. Data represented are presented as mean ± standard error of the mean (SEM) (n=5). One-way ANOVA followed by Bonferroni *post hoc* multiple comparisons was used for statistical analysis; *P**/#<0.05, *P***/##<0.01, *P****/###<0.001; [*] is significant compared to untreated control sample; [#] is significant compared to platelet agonist-treated samples.

served and ubiquitously expressed kinases, which regulate a wide range of responses to various cues in eukaryotic cells.[3] In anuclear platelets, MAPK are essential to navigate their activation and aggregation, and thrombus formation. In addition, ASK1 is critical for platelet activation and granule secretion.[4] Platelet agonists initiate ASK1 signaling to induce p38 MAPK-mediated Integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation, in addition to inducing thromboxane A2 (TxA2) generation, thus significantly impacting thrombus formation.[4]

Lately, mounting interest in ASK1 as a disease modifying therapeutic target has paved way to the discovery of potent and selective ASK1-inhibitors.[5] The selective ASK1 inhibitors like NQDI-1 and GS-444217 are demonstrated to regulate various diseases such as cardiac and renal ischemia-reperfusion injury, multiple sclerosis, tumor growth, contact hypersensitivity, hypertension and diabetic nephropathy *in vivo*, which was further confirmed in *Ask1* knockout mice (*Ask1*$^{-/-}$).[2,5] Recently, Naik *et al*. demonstrated that *Ask1*$^{-/-}$ platelets exhibit impaired activation and granule secretion. Besides, *Ask1*$^{-/-}$ mice display defective thrombosis due to dysfunctional platelets. These studies confirmed the critical role of ASK1 in both hemostasis and thrombosis.[4]

In the present study, we demonstrate that ASK1-p38 MAPK signaling axis is a molecular switch to regulate platelet activation and apoptosis. Further, we also delineate the mechanism of ASK1-p38 MAPK axis-mediated platelet apoptosis for the first time. It has been shown previously that *Ask1*$^{-/-}$ platelets exhibit functional defects like impaired integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation in response to agonist-induced stimuli. Although in *Ask1*$^{-/-}$ platelets the thrombin-induced $Ca2^{+}$ release was not affected, thrombin-induced granule secretion was significantly alleviated *via* p38-MAPK and cPLA2 phosphorylation-dependent TxA2 generation.[4] Here, we confirm that the inhibition of ASK1 by NQDI-1 in platelets significantly diminishes both collagen and thrombin-induced platelet aggregation and activation (*Online Supplementary Figure S1-4* and Figure 1A-C) further confirming the previously reported results. Generally, ASK1 arbitrates apoptosis *via* mitochondria-mediated release of cytochrome c, which in turn activates caspase-3 in nucleated cells.[6] In human ovarian carcinoma (OVCAR-3) and human kidney (293T) cells, the ASK1-MKK3/MKK4-JNK/p38 signaling axis is shown to induce caspase activation to mediate genotoxic stress-induced apoptosis.[7] However, the role of ASK1 in platelet death is obscure. Therefore, in this study we aimed to delineate the role of ASK1 in platelet death. We demonstrate that ASK1 inhibition significantly reduced platelet viability upon agonist treatment, however, the ASK1 inhibitor by itself did not alter platelet viability (Figure 1D and *Online Supplementary Figure S5*). We have previously demonstrated that elevated intracellular ROS levels in platelets drive them into either caspase-dependent (apoptosis) or caspase-independent (ferroptosis) death.[8,9] ASK1 inhibition significantly generates agonist-induced ROS including superoxides and lipid peroxides along with cardiolipin peroxidation in platelets (Figure 1E-F). Cardiolipin is a mitochondria-specific phospholipid that constitutes 15-20% of the inner mitochondrial membrane, which is critical for maintaining the integrity of the mitochondrial membrane.[10] Further, ASK1-inhibition significantly altered the mitochondrial membrane potential and subsequent phosphatidylserine externalization upon agonist stimulation (Figure 2A-B). This confirms that the ASK1 inhibition drives ROS-mediated mitochondrial dysfunction causing apoptosis in platelets upon stimulation by the agonists.

Next, we aimed to investigate the mechanism and type of cell death induced by agonists in ASK1-inhibited platelets. Generally, mitochondria and NADPH oxidase are the major sources of ROS that induce cell death. Here, we show that inhibition of ASK1 specifically induces mitochondrial ROS and not NADPH oxidase upon stimulation with platelet agonists (Figure 2C). In addition, release of thioredoxin (Trx) is critical to activate ASK1; reduced Trx binds to the N-terminal domain of ASK1 thereby inactivating it and subsequently initiating ubiquitination and degradation.[11] Our results show that Trx reductase inhibitor reduced the platelet viability upon ASK1 inhibition in the presence of collagen, again confirming the role of ASK1-Trx interaction in platelet apoptosis (Figure 2D). Furthermore, we examined the molecular events associated with ASK1 signaling and platelet death. Cardiolipin oxidation regulates mitochondria-mediated apoptosis by engaging cytochrome c, tBID and other BCL-2 proteins.[10] ASK1 inhibition by NQDI-1 significantly collapsed both collagen and thrombin-induced mitochondrial membrane potential in platelets, resulting in the release of cytochrome c and alteration in the levels of BCL-2 family proteins like BCL-xL and BAK orchestrating apoptosis as shown in Figure 2E (*Online Supplementary Figure S6A-B*). Further, the p38-MAPK signaling plays a central role in oxidative stress-induced apoptosis upstream of ASK1. The autophosphorylation of ASK1 at Thr845 consequently activates its downstream targets MAPKK (MKK3/6) and p38-MAPK.[1,2] However, in this study we demonstrate that the p38-MAPK plays a differential role in platelets. It acts as a molecular switch to regulate platelet activation and apoptosis exhibiting a biphasic response to maintain the homeostatic balance between platelet activation and apoptosis.[4,12] In platelets, ASK1 inhibition did not inhibit collagen and thrombin-induced p38-MAPK phosphorylation, rather it further triggers it (Figure 2F and *Online Supplementary Figure S6C*). This might be due to the fact that p38-MAPK is also involved in ROS-mediated platelet apoptosis as published previously.[12,13] Further, ASK1 inhibition-mediated ROS generation in platelets in response to thrombin resulted in NLRP3 inflammasome activation, which influences platelet apoptosis but not platelet aggregation. To evaluate the activation of NLRP3 in thrombin-treated platelets under ASK1 inhibition, we evaluated caspase-1 activity and observed a significant increase in the activity (*Online Supplementary Figure S7*). By inhibiting NLRP3 inflammasome activation, thrombin-mediated cell death is restored in ASK1-inhibited platelets (Figure 2G and *Online Supplementary Figure S8*). The mitochondrial ROS can directly stimulate NLRP3 inflammasome activation, eventually causing mitochondria-mediated, caspase-dependent apoptosis and the release of mitochondrial DNA into the cytosol.[9,14] Although, NLRP3 inflammasome activation has been shown to be associated with platelet activation and aggregation, we demonstrate its involvement in platelet apoptosis as well. Besides, we also confirm that ASK1 inhibition drives platelets to caspase-dependent apoptosis and not necrosis or ferroptosis (Figure 2H).

Next, we validated the above observations *in vivo* using a murine model. The *in vivo* studies showed that ASK1 inhibition marginally increased the survival rates of collagen-epinephrine-induced acute pulmonary thromboembolic death of mice. The majority of the collagen-epinephrine-treated mice succumb to death within five minutes of treatment due to acute pulmonary thromboembolism. The injection of ASK1 inhibitor prolongs the sur-

vival time of mice by fifteen minutes and prevents platelet thrombi accumulation in lungs, however, it failed to prevent the death of mice (Figure 3A). The hematoxylin and eosin stained lung tissue sections of collagen and epinephrine mixture-administered mice exhibited a significantly high number of lung vessels occluded by platelet thrombi, however, ASK1 inhibitor administration significantly diminished the occlusion of vessels in lungs (Figure 3B-C). In addition, ASK1 inhibitor-treatment significantly decreased the levels of soluble P-selectin (Figure 3D). Platelet function plays a crucial role in arterial thrombosis. In response to endothelial cell injury, acti-

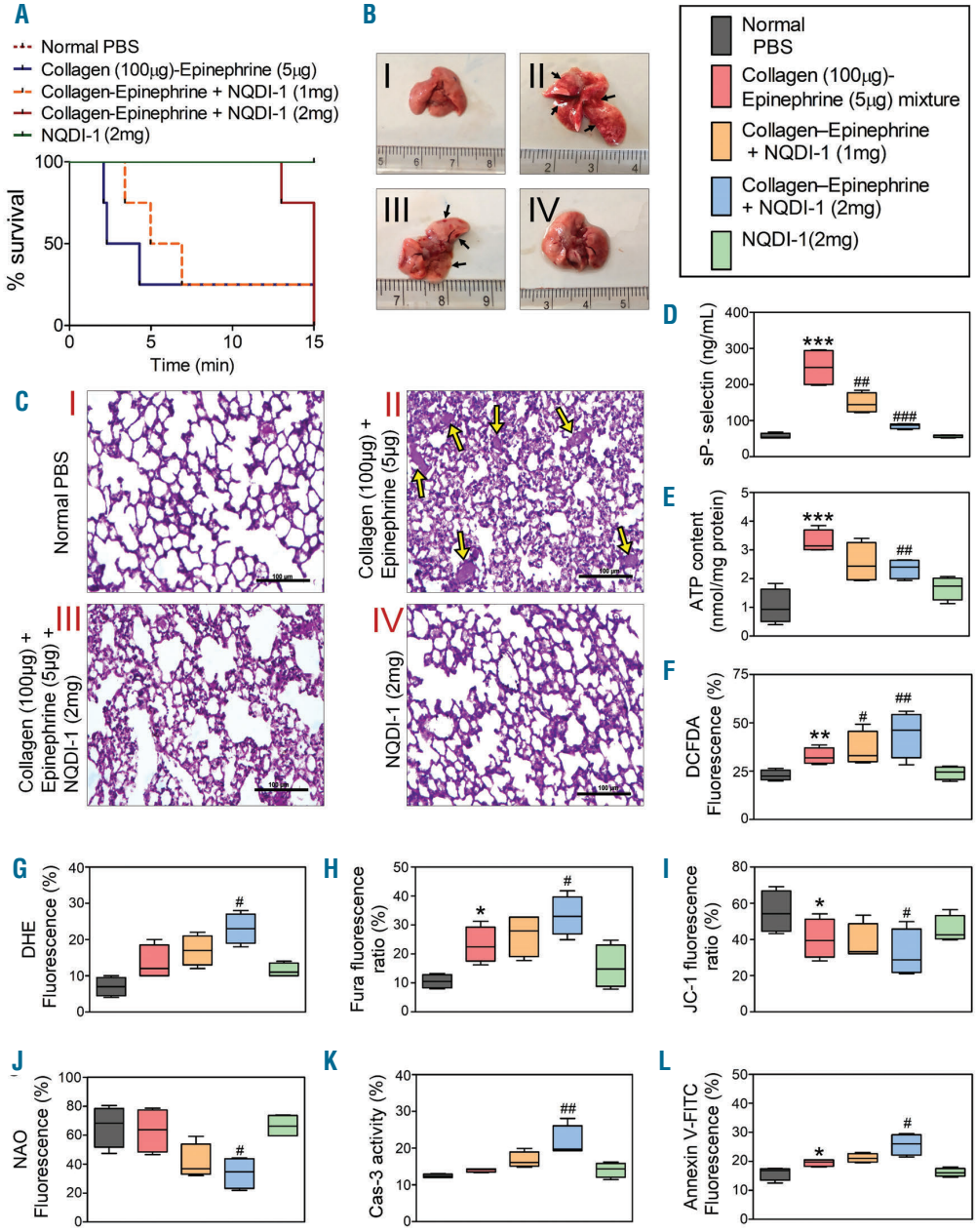

Figure 3. ASK1 inhibition enhances platelet death *in vivo*. (A) Kaplan-Meier survival curves of normal PBS and NQDI-1 (1 and 2 mg/kg body weight) intravenously-treated mice after tail vein injection of a mixture of collagen (100 µg/mL) and epinephrine (5 µg/mL) in 100 µL of PBS. (B) Photographic image of lungs harvested from the treated mice; arrows indicate the formation of visible thrombosis clots. (C) Representative hematoxylin and eosin (H&E)-stained lung tissue sections after intravenous injection of mice with (I) normal PBS, (II) mixture of collagen and epinephrine, (III) collagen-epinephrine and NQDI-1 (2 mg/kg body weight), and (IV) NQDI-1 alone (2 mg/kg body weight). Arrows indicate platelet-rich thrombi obstructing in lung vessels. (D) Enzyme-linked immunosorbent assay (ELISA) based quantification of soluble P-selectin (sP-selectin) levels in plasma obtained from blood of mice treated with collagen and epinephrine, pre-treated with various doses of NQDI-1. (E) Estimation of platelet ATP level in treated mice using bioluminescence detection kit according to the manufacturer's protocol. Fluorometric analysis of platelets isolated from treated mice blood stained with (F) DCFDA for reactive oxygen species (ROS) generation, (G) DHE for superoxide detection, (H) Fura-2AM for intracellular calcium estimation, (I) JC-1 for mitochondrial membrane potential, (J) NAO for cardiolipin oxidation, (K) fluorogenic substrate for caspase-3 activity, and (L) Annexin V-FITC for externalized phosphatidylserine (PS). Data represented are presented as mean ± standard error of mean (SEM) (n=5). One-way analysis of variance, followed by Dunnett's *post hoc* test was used for statistical analysis; *P**/#<0.05, *P***/##<0.01, *P****/###< 0.001; [*] is significant compared to control PBS group; [#] is significant compared to collagen and epinephrine treated group.

vated platelets swiftly adhere to the sub-endothelial matrix and aggregate to produce hemostatic thrombi to stop bleeding and preserve vascular integrity. However, dysregulated thrombus formation triggers blood vessel blockage causing ischemia.[15] Besides, plasma DNA is prominent during active vasculitis, a high thrombotic risk condition. High plasma DNA levels and ATP content are reported in patients with thrombotic microangiopathies, thrombotic thrombocytopenic purpura, and coronary artery disease. It is considered as one of the prominent clinical biomarkers for thrombosis. The NET formation releases cell-free DNA into the blood and tissue, which can be measured in the plasma/serum.[16] Our study demonstrates that collagen-epinephrine-treated mice significantly induced the release of extracellular DNA and ATP content, however, inhibition of ASK1 remarkably reduced both extracellular DNA and ATP content in mice plasma (*Online Supplementary Figure S9A* and Figure 3E). These results suggest that although ASK1 inhibition mitigates the platelet activation and thrombi formation, it did not completely stop death in mice, which might be due to persistent thrombocytopenia.

Previously, we have demonstrated proliferation of ROS-mediated thrombocytopenia *in vivo* reporting the key events of thrombocytopenia in a murine model.[13] Further investigation into thrombocytopenia in collagen-epinephrine-treated ASK1-inhibited mice confirmed elevated levels of total ROS, superoxides and calcium levels along with depleted glutathione in platelets (Figure 3F-H and *Online Supplementary Figure S9B*). In addition, significantly altered mitochondrial membrane potential and elevated cardiolipin peroxidation was evident from the observed results (Figure 3I-J). Finally, highly upregulated caspase-3 and phosphatidylserine levels confirmed platelet apoptosis in collagen-epinephrine-treated ASK1-inhibited mice, established the enduring thrombocytopenia (Figure 3K-L).

In summary, this study is the first report to demonstrate the differential role of p38-MAPK in ASK1-inhibited platelets. The p38-MAPK acts as a molecular switch to regulate platelet activation and apoptosis, exhibiting a biphasic response to maintain the homeostatic balance between platelet activation and apoptosis. We also demonstrate for the first time that ASK1 inhibition in platelets triggers mitochondrial superoxide production causing inflammasome-mediated caspase activation leading to cell death. The selective ASK1 inhibitors like NQDI-1 and GS-444217 are shown to regulate various diseases such as cardiac and renal ischemia-reperfusion injury, multiple sclerosis, tumor growth, contact hypersensitivity, hypertension and diabetic nephropathy *in vivo*, which was further confirmed in *Ask1*$^{-/-}$ mice. The increased interest in ASK1-p38 MAPK axis as a disease modifying therapeutic target has paved the way to the discovery of potent and selective ASK1 inhibitors. However, our study clearly demonstrates that targeting ASK1 in platelets results in cell death and may lead to thrombocytopenia (*Online Supplementary Figure S10*). Therefore, moving forward, a vigilant assessment needs to be considered when using ASK1 inhibitor to regulate human diseases because such a process may result in fatal thrombocytopenia.

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