

ORIGINAL ARTICLE

Integrin-linked kinase regulates the rate of platelet activation and is essential for the formation of stable thrombi

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Summary. *Background:* Integrin-linked kinase (ILK) and its associated complex of proteins are involved in many cellular activation processes, including cell adhesion and integrin signaling. We have previously demonstrated that mice with induced platelet ILK deficiency show reduced platelet activation and aggregation, but only a minor bleeding defect. Here, we explore this apparent disparity between the cellular and hemostatic phenotypes. *Methods:* The impact of ILK inhibition on integrin $\alpha_{IIb}\beta_3$ activation and degranulation was assessed with the ILK-specific inhibitor QLT0267, and a conditional ILK-deficient mouse model was used to assess the impact of ILK deficiency on *in vivo* platelet aggregation and thrombus formation. *Results:* Inhibition of ILK reduced the rate of both fibrinogen binding and α -granule secretion, but was accompanied by only a moderate reduction in the maximum extent of platelet activation or aggregation *in vitro*. The reduction in the rate of fibrinogen binding occurred prior to degranulation or translocation of $\alpha_{IIb}\beta_3$ to the platelet surface. The change in the rate of platelet activation in the absence of functional ILK led to a reduction in platelet aggregation *in vivo*, but did not change the size of thrombi formed following laser injury of the cremaster arteriole wall in ILK-deficient mice. It did, however, result in a marked decrease in the stability of thrombi formed in ILK-deficient mice. *Conclusion:* Taken together,

the findings of this study indicate that, although ILK is not essential for platelet activation, it plays a critical role in facilitating rapid platelet activation, which is essential for stable thrombus formation.

Keywords: embolism; integrin α -IIb β -3; integrin-linked kinase; platelets; thrombus.

Introduction

Integrin-linked kinase (ILK) was identified for its interaction with the cytoplasmic tail of β -integrin subunits and its serine/threonine kinase activity, which is upregulated by the stimulation of platelets [1,2]. ILK functions as an adaptor protein, binding a number of focal adhesion (FA) proteins [3,4], including, but not restricted to, PINCH [5,6] and parvin [7,8], to form the ILK–PINCH–parvin (IPP) complex [9]. The IPP complex promotes integrin clustering and the generation of FA complexes, providing a structural link between the extracellular matrix and the cytoskeleton, and an array of signaling events [2,10–13]. ILK is important in a large range of tissue types and cellular responses that depend on integrin clustering and the formation of FAs [8,11,14–17]. In platelets, where integrins have a vital role in hemostasis, by facilitating adhesion to sites of damage and platelet–platelet interactions, ILK interacts with, and regulates the function of, β_1 and β_3 integrin subunits [18,19].

Mice with induced ILK deficiency show reduced platelet activation and aggregation, but only a minor bleeding defect [14]. This disparity between the cellular and gross hemostatic phenotypes may result from a number of factors. Our previous work showed that ILK deficiency reduced platelet aggregation but that this effect had a temporal component, with the inhibition being greater at 90 s than at 300 s poststimulation [14]. Given the dynamic nature of thrombus formation, it seems intuitive that the rate of platelet activation will have a critical role

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in regulating thrombus development; however, given the lack of bleeding in these mice, it is possible that this subtle cellular phenotype is overcome *in vivo* and does not greatly alter thrombus formation.

Here, we explored this apparent disparity between the cellular and hemostatic phenotypes by using an ILK-deficient mouse model and the ILK inhibitor QLT0267 to investigate ILK-mediated regulation of the platelet response to stimuli and the impact of this on thrombus formation *in vivo*. We show that ILK plays a critical role in regulating the rate of platelet activation, and that this is essential for stable thrombus formation.

Materials and methods

Platelet aggregation

Systemically inducible ILK-deficient mice were generated as previously reported [14,20]. LoxP-flanked ILK transgenic mice containing a Cre-transgene under control of the Mx1 promoter were generated and, in all cases, littermate ILK(+ / +): MxCre were used as the control (Ctrl). Mice were given an intraperitoneal injection of 300 mg of poly(I)-poly(C) 8 days prior to experiments, after which ILK could not be detected in knockout (KO) mouse platelets [14]. Platelets were prepared from mouse whole blood by differential centrifugation, and aggregation was measured in an optical aggregometer following stimulation with collagen ($100 \mu\text{g mL}^{-1}$) at 37°C [14].

For aggregation studies on human blood, platelets were prepared from healthy donors by differential centrifugation [21], and stimulated with collagen ($10 \mu\text{g mL}^{-1}$), in the presence of QLT0267 ($1 \mu\text{M}$) (QLT, Vancouver, BC, Canada) or dimethylsulfoxide (DMSO) (0.025% v/v) vehicle control. Aggregation was measured in an optical aggregometer at 37°C .

Clot retraction

Clot retraction was measured by mixing human platelet-rich plasma (PRP) ($200 \mu\text{L}$), red blood cells ($5 \mu\text{L}$) and QLT0267 ($1 \mu\text{M}$) or DMSO (0.025% v/v) vehicle control with modified Tyrodes–Hepes buffer to a final volume of 1 mL [22]. Clotting was initiated with thrombin (1 U mL^{-1}), and a sealed glass capillary was placed at the center of the glass test tube, around which the clot formed. Clots were removed from the tube after 1.5 h and 3 h, and superficial liquid was removed from the surface before the clot was pushed from the glass capillary liquid and weighed.

Flow cytometry

Fibrinogen binding to platelets from KO or Ctrl mice in the presence of QLT0267 was measured by diluting PRP in Hepes-buffered saline (HBS) containing fluorescein

isothiocyanate (FITC)–anti-fibrinogen antibodies (Dako, Ely, UK) and cross-linked collagen-related peptide (CRP-XL; monomeric sequence GCI[GPO]₁₀GCOG), prepared as previously described [23] ($0.5 \mu\text{g mL}^{-1}$, $1 \mu\text{g mL}^{-1}$, and $2 \mu\text{g mL}^{-1}$). Reactions were stopped after 45 s, 90 s and 300 s by 100-fold dilution in 0.2% formyl saline, and analyzed on an Accuri C6 flow cytometer (BD, Oxford, UK).

For time-resolved flow cytometry experiments measuring fibrinogen binding, P-selectin exposure, and calcium flux, human PRP was diluted in HBS containing either FITC–anti-fibrinogen antibodies (Dako) and phycoerythrin–anti-P-selectin antibodies (BD), or Fluo-4 NW dye (Invitrogen, Paisley, UK), together with QLT0267 ($1 \mu\text{M}$) or DMSO (0.025% v/v) vehicle control. All reagents were dialyzed prior to use to remove azide. Samples were transferred to a 96-well plate on an Accuri C6 flow cytometer (BD) modified to maintain the plate at 37°C , and stimulated during data acquisition with CRP-XL ($1 \mu\text{g mL}^{-1}$), ADP ($1 \times 10^{-6} \text{ M}$) or thrombin (0.3 U mL^{-1}) in the presence of Gly-Pro-Arg-Pro peptide (Sigma, Poole, UK). Data were acquired for 300 s at approximately one platelet per 1 ms, and analyzed with R (www.r-project.org) by calculating median fluorescence intensity (MFI) every 100 ms and fitting a LOESS curve to this, from which the maximum platelet response and the rate of platelet response were derived.

For time-resolved flow cytometry experiments measuring surface expression of integrin $\alpha_{\text{IIb}}\beta_3$, human PRP was diluted in HBS, maintained at 37°C , stimulated with CRP-XL ($1 \mu\text{g mL}^{-1}$) for 15–300 s, and then fixed with an equal volume of 4% formyl saline. Fixed platelets were washed twice with HBS, and then incubated with anti- β_3 antibodies (Dako) for 20 min, and for a further 20 min with Alexa Fluor 647-labeled goat anti-mouse secondary antibodies (Life Technologies, Paisley, UK).

In vivo platelet aggregation

In vivo aggregation of radiolabeled platelets was assessed by measuring the percentage change in radioactive counts in the pulmonary vascular bed following the injection of collagen into the femoral vein of Ctrl/KO mice [24]. Blood was collected into acidified citrate–dextrose solution from terminally anesthetized (urethane [25% w/v] at $10 \mu\text{L g}^{-1}$ intraperitoneal) donor mice by cardiac puncture. Platelets were isolated by differential centrifugation, incubated with 1.8 MBq of indium-111 oxine for 10 min, and then repelleted, washed, and resuspended. Anesthetized (urethane [25% w/v] at $10 \mu\text{L g}^{-1}$ intraperitoneal) recipient mice were infused with radiolabeled platelets, and allowed to equilibrate for 15 min before collagen ($25\text{--}100 \mu\text{g kg}^{-1}$ intravenous; Nycomed, Konstanz, Germany) was injected via a femoral vein. A single point extended area radiation detector (eV Products, Saxonburg, PA, USA) was fixed over the pulmonary vascular

bed, and counts were recorded on a UCS-20 spectrometer (Spectrum Techniques, Oak Ridge, CA, USA) with custom-made software (Mumed Systems, London, UK). Platelet responses were then determined as an increase in the counts in the pulmonary vascular bed associated with the platelet agonists.

In vivo thrombus formation

In vivo thrombus formation following laser injury of the cremaster arteriole wall was performed and analyzed as described previously [21]. Mice were anesthetized by intraperitoneal injection of ketamine (125 mg kg^{-1}), xylazine (12.5 mg kg^{-1}), and atropine (0.25 mg kg^{-1}), and anesthesia was maintained with 5 mg kg^{-1} pentobarbital as required through a jugular vein cannula. The cremaster muscle was exteriorized, connective tissue was removed, and the muscle was fixed as a single sheet over a glass slide. Throughout this procedure, the muscle preparation was hydrated with buffer (135 mM NaCl , 4.7 mM KCl , 2.7 mM CaCl_2 , 18 mM NaHCO_3 , pH 7.4). Platelets were labeled with Alexa Fluor 488-conjugated anti-mouse glycoprotein (GP)Ib β antibody ($0.2 \mu\text{g g}^{-1}$ of mouse weight) (Emfret Analytics, Eibelstadt, Germany). Injury of the cremaster arteriole wall was induced with a Micro-point Ablation Laser Unit (Andor Technology, Belfast, UK). Thrombus formation was observed with an Olympus BX61W1 microscope (Olympus Imaging, Southend-on-Sea, UK) and a Hamamatsu C9300 digital camera (Hamamatsu Photonics, Welwyn Garden City, UK), and analyzed with SLIDEBOOK5 software (Intelligent Imaging Innovations, Denver, CO, USA).

Statistical analysis

Data are presented as mean \pm standard deviation of the mean. Statistical analyses were performed with PRISM 5 GRAPHPAD software (GraphPad Software, La Jolla, CA, USA). Data were compared by use of a Student's *t*-test or two-way ANOVA and Bonferroni *post hoc* test analysis as appropriate.

Results

Reanalysis of aggregation in platelets from KO mice demonstrated that aggregation in response to a high concentration of collagen ($100 \mu\text{g mL}^{-1}$) was almost entirely abolished in KO mice as compared with Ctrl mice ($P < 0.0001$) during the first 60 s, and then recovered by 300 s (Fig. 1A,B).

We have previously shown that platelets from these mice have significantly reduced levels of both PINCH and α -parvin [14]. To confirm that inhibition of ILK alone causes a reduction in platelet function, and to confirm our previous findings in humans, we used QLT0267, a small-molecule inhibitor of the kinase-like domain of ILK

[25,26]. Although this inhibitor has been shown to effectively inhibit ILK at doses ranging from $1 \mu\text{M}$ to $25 \mu\text{M}$, non-specific effects have been reported at high, but not lower, concentrations [25,27]. To confirm the selectivity of this inhibitor at a concentration previously reported to inhibit ILK but without non-specific effects, fibrinogen binding to CRP-XL-stimulated platelets from KO and Ctrl mice was measured in the presence or absence of QLT0267 ($1 \mu\text{M}$). In Ctrl mice, QLT0267 inhibited fibrinogen binding at all three concentrations of CRP-XL and across all time points (Fig. 1C). Platelets from KO mice showed reduced fibrinogen binding as compared with Ctrl mice, but showed no further inhibition in the presence of QLT0267 (Fig. 1D), indicating that, at this concentration, it is specific for ILK. Collagen-stimulated human platelets incubated with QLT0267 showed similar inhibition of aggregation ($P = 0.0008$), again characterized by complete inhibition of aggregation in the first 45 s, accompanied by only a moderate impairment of maximal aggregation (Fig. 1E).

ILK inhibition also reduced the rate of clot retraction. In the presence of QLT0267, clot retraction was inhibited at 1.5 h but not at 3 h (Fig. 1F), consistent with reduced 'outside-in' signaling through $\alpha_{\text{IIb}}\beta_3$.

We have previously shown that, although the levels of $\alpha_{\text{IIb}}\beta_3$ on the surfaces of unstimulated platelets from Ctrl and KO mice are indistinguishable, following stimulation, which leads to translocation of $\alpha_{\text{IIb}}\beta_3$ to the platelet surface, the surface expression of $\alpha_{\text{IIb}}\beta_3$ is significantly lower in KO than in Ctrl mice [14]. To test whether the observed decrease in the rate of aggregation and clot retraction following ILK inhibition or deficiency is attributable to reduced $\alpha_{\text{IIb}}\beta_3$ surface expression or reduced $\alpha_{\text{IIb}}\beta_3$ activation as such, which may, in turn, lead to reduced secretion, we assessed the activation of $\alpha_{\text{IIb}}\beta_3$ and surface exposure of $\alpha_{\text{IIb}}\beta_3$ and P-selectin (a marker of α -granule secretion) over time. Real-time flow cytometric analysis of the rates of fibrinogen binding and P-selectin exposure confirmed a reduction in response to the GPVI-specific agonist CRP-XL in the presence of QLT0267. The rate at which platelets bound fibrinogen (rate of change in MFI) peaked at $50.8 \pm 18.3 \text{ s}$ of stimulation, and this rate was reduced significantly in the presence of QLT0267 (Fig. 2A; dashed line). The rate of α -granule release, as measured by P-selectin exposure, peaked later than fibrinogen binding, at $103.3 \pm 19.5 \text{ s}$, but was similarly reduced (Fig. 2B; dashed line). Although there was a trend towards a decrease in maximum fibrinogen binding or P-selectin exposure following treatment with QLT0267, there was no significant difference in either (Fig. 2A,B; solid line). As CRP-XL is a GPVI-specific agonist, this suggests that the reduction in platelet activation is not attributable to the involvement of ILK in $\alpha_2\beta_1$ signaling. This effect of ILK appears to be restricted to stimulation via GPVI, as there was no observable difference in either the rate of or maximum fibrinogen binding or P-selectin

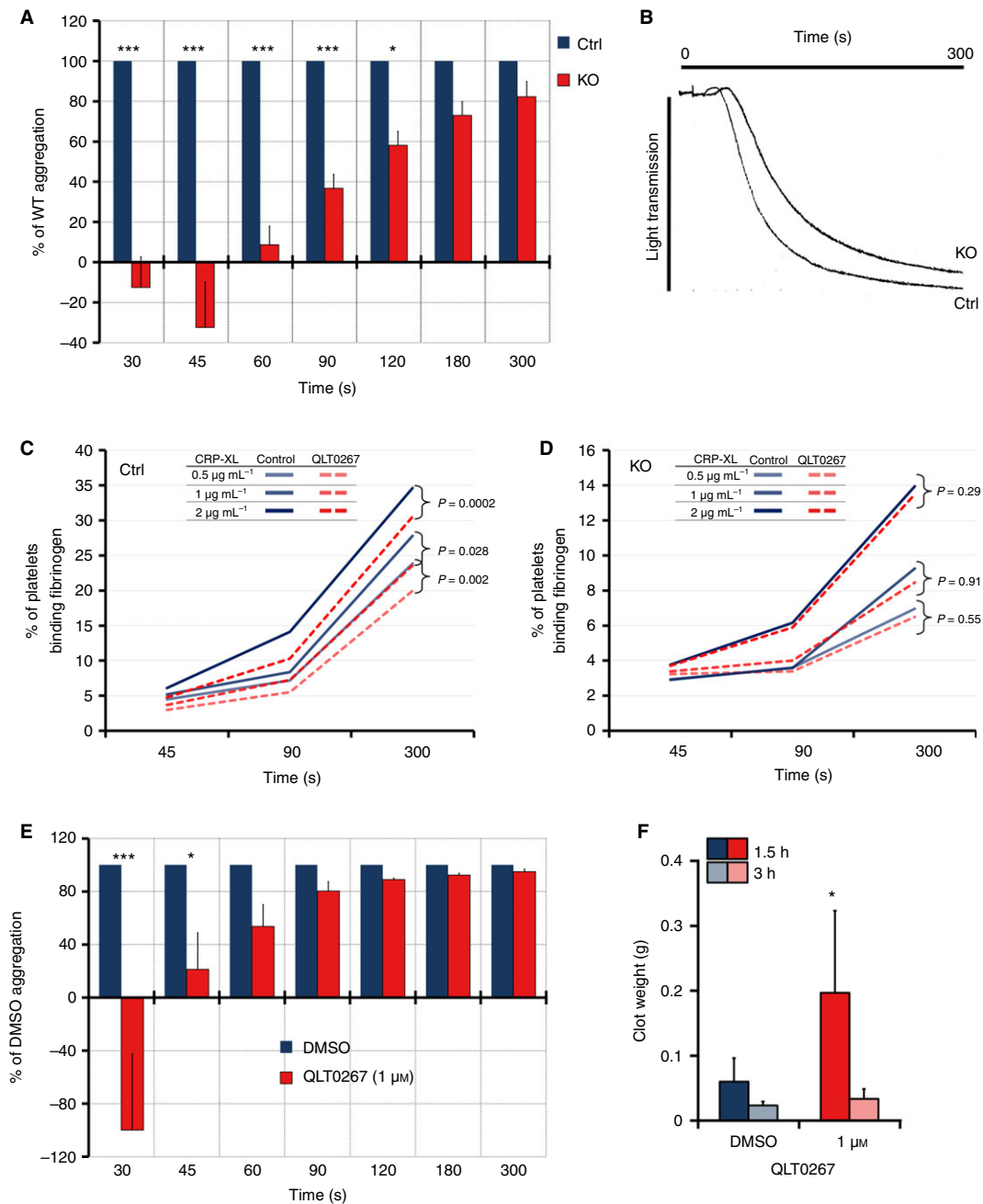


Fig. 1. Deficiency or inhibition of integrin-linked kinase (ILK) reduces the rate of platelet activation. (A) Platelet aggregation in response to collagen ($100 \mu\text{g mL}^{-1}$) was measured in mice with an induced ILK deficiency (KO) as compared with sibling matched controls (Ctrl) [14]. All data are expressed as a percentage of Ctrl aggregation at each time point. (B) A representative trace is shown. (C, D) The binding of anti-fibrinogen antibodies to cross-linked collagen-related peptide (CRP-XL)-stimulated platelets from (C) Ctrl and (D) KO mice was measured by flow cytometry in the presence (red dashed line) or absence (blue line) of QLT0267 (1 μM). (E) Aggregation of collagen-stimulated ($10 \mu\text{g mL}^{-1}$) human washed platelets was measured in the presence of the ILK inhibitor QLT0267 (1 μM) or dimethylsulfoxide (DMSO) control. All data are expressed as a percentage of aggregation in the DMSO control samples at each time point. (F) Clot retraction was measured in the presence of QLT0267 (1 μM) or DMSO (0.025% v/v). The clotting was initiated with thrombin (1 U mL^{-1}), and a sealed glass capillary was placed at the center of the glass test tube, around which the clot formed. Clots were removed, gently blotted to remove superficial liquid, and then weighed after 1.5 and 3 h. In all cases: * $P \leq 0.05$, *** $P \leq 0.001$, $n = 3$. All data are presented as mean \pm standard deviation, and were analyzed by two-way ANOVA with *post hoc* Bonferroni correction for multiple comparisons to compare treated and control samples at individual time points. WT, wild-type.

exposure following treatment with QLT0267, when platelets were stimulated with either ADP (Fig. 2C,D) or thrombin (Fig. 2E,F). It is worth pointing out that ILK

may affect other aspects of thrombin-mediated platelet activation, as QLT0267 clearly retards thrombin-induced clot retraction (Fig. 1F), and, although KO mice did not

show an aggregation defect following thrombin stimulation (Fig. S1), we have previously reported a reduction in other aspects of thrombin-induced platelet activation in these mice [14].

Importantly, the inhibitory effect of QLT0267 on the rate of fibrinogen binding following CRP-XL stimulation occurred before any measurable degranulation was observed (Fig. 2A,B). Furthermore, there was no significant difference between treated and untreated platelets in the surface expression of $\alpha_{IIb}\beta_3$ at the early time points (0–50 s), when the rate of fibrinogen binding to activated $\alpha_{IIb}\beta_3$ is reduced (Fig. 2G). This demonstrates that the reduction in the rate of fibrinogen binding to platelets is not attributable to reduced $\alpha_{IIb}\beta_3$ surface expression, but to a reduction in early activation of $\alpha_{IIb}\beta_3$. In line with our previous data on KO mice, inhibition of ILK did reduce the surface expression of $\alpha_{IIb}\beta_3$ at later time points (Fig. 2G).

To establish whether the inhibition of $\alpha_{IIb}\beta_3$ activation at early time points was attributable to inhibition of signaling events leading to 'inside-out' activation of $\alpha_{IIb}\beta_3$, we measured calcium flux in the presence or absence of QLT0267. Surprisingly, the inhibition of ILK with QLT0267 led to a significant reduction in the rate of calcium flux in response to CRP-XL, but had no detectable effect on peak calcium flux (Fig. 3A). It seems unlikely that ILK has a direct effect on signaling immediately downstream of GPVI; instead, we hypothesized that this inhibitory effect may be the result of reduced $\alpha_{IIb}\beta_3$ signaling in the very earliest stages of platelet activation. To test this, we measured calcium flux in response to CRP-XL with or without QLT0267 in the presence of EDTA to prevent $\alpha_{IIb}\beta_3$ activation and isolate the GPVI-dependent mobilization of calcium. Under these conditions, ILK inhibition did not affect the rate of calcium flux (Fig. 3B), indicating that the inhibitory effect of QLT0267 on platelet calcium mobilization is not attributable to augmentation of signaling downstream of GPVI, but to signaling downstream of $\alpha_{IIb}\beta_3$. The observation that inhibition of ILK does not affect the initial rate of platelet activation following ADP and thrombin stimulation may stem from the different mechanisms by which these agonists control the activation of RAP1 leading to $\alpha_{IIb}\beta_3$ activation [28,29].

Collectively, the aggregation, flow cytometry and clot retraction data show that deficiency or inhibition of ILK impairs the rate at which platelets can respond to stimulation while only modestly inhibiting the maximal extent to which platelets can become activated. Given the dynamic nature of thrombus formation, it may be that a reduction in the rate, rather than in the maximum extent, of platelet activation is critical in regulating thrombus development. It is, however, also conceivable that this apparently subtle phenotype is overwhelmed and rendered inconsequential *in vivo* by vascular endothelial-derived mediators (e.g. nitric oxide or prostanooids) resulting in the limited bleed-

ing phenotype. It is worth pointing out that expression of the Mx1 promoter is not specific to megakaryocytes, and may therefore have unknown effects on other cell types that account for the disparity between our previous *in vivo* and *in vitro* data. To test these competing hypotheses, we assessed platelet aggregation *in vivo* in Ctrl and KO mice. Platelet aggregation *in vivo* was reduced significantly in KO mice (Fig. 4). Over the range of collagen concentrations used, both the maximum percentage change in counts and the area under the curve were reduced in KO mice ($P < 0.0001$ and $P = 0.0001$, respectively). This demonstrates the importance of a change in the rate of platelet activation in this more physiologic setting. It also demonstrates that this platelet phenotype is still functionally relevant *in vivo*, but raises a number of questions. Is the reduced level of accumulation of thrombi in the lungs attributable to a reduced platelet response to collagen or reduced stability of the thrombi that form? Are the effects of ILK deficiency overcome by the high concentration of collagen at the site of injury? The latter question may be important, given that the reduction in platelet aggregation in KO mice was most pronounced at intermediate concentrations of collagen, and largely overcome at higher concentrations (Fig. 4B,C, E,F). This may help to explain the disparity between platelet and hemostatic responses in these mice.

To test whether thrombus stability was altered in KO mice, and whether local collagen concentrations at the site of injury overcome the reduction in platelet function observed in KO mice, thereby reducing the impact of ILK on thrombus formation, we measured thrombus formation following laser injury of the cremaster arteriole wall in Ctrl and KO mice. Thrombus formation was altered significantly in KO mice (Fig. 5A–D; Fig. S2). Although there was no statistical difference between Ctrl and KO mice in the sum fluorescence intensity of thrombi (a measure of the total platelet mass recruited to the site of injury) or the thrombus area (a measure of thrombus size) at any time point, or between the maxima of these measures (Fig. 5C,D), there were noticeable differences between their thrombi. Notably, the thrombi in KO mice were unstable, forming, embolizing and reforming continuously (Fig. 5A, Fig. S2). It is this instability that, at later time points, leads to the apparent greater thrombus size in KO mice, which is the result of multiple unstable thrombi forming and embolizing out of phase with each other (Fig. 5C,D; Fig. S2B). Methods to quantify thrombus stability in this system have not been described. To capture, in a quantifiable manner, this important readout of thrombus stability, we developed a new method that allowed us to assess the stability of thrombi. The thrombus instability index (TII) measures the standard deviation of data immediately preceding each time point to quantify changes (growth, retraction, or embolization) in thrombus size. Ctrl mice had a characteristic double-peaked TII trace. TII increased as thrombi grew in

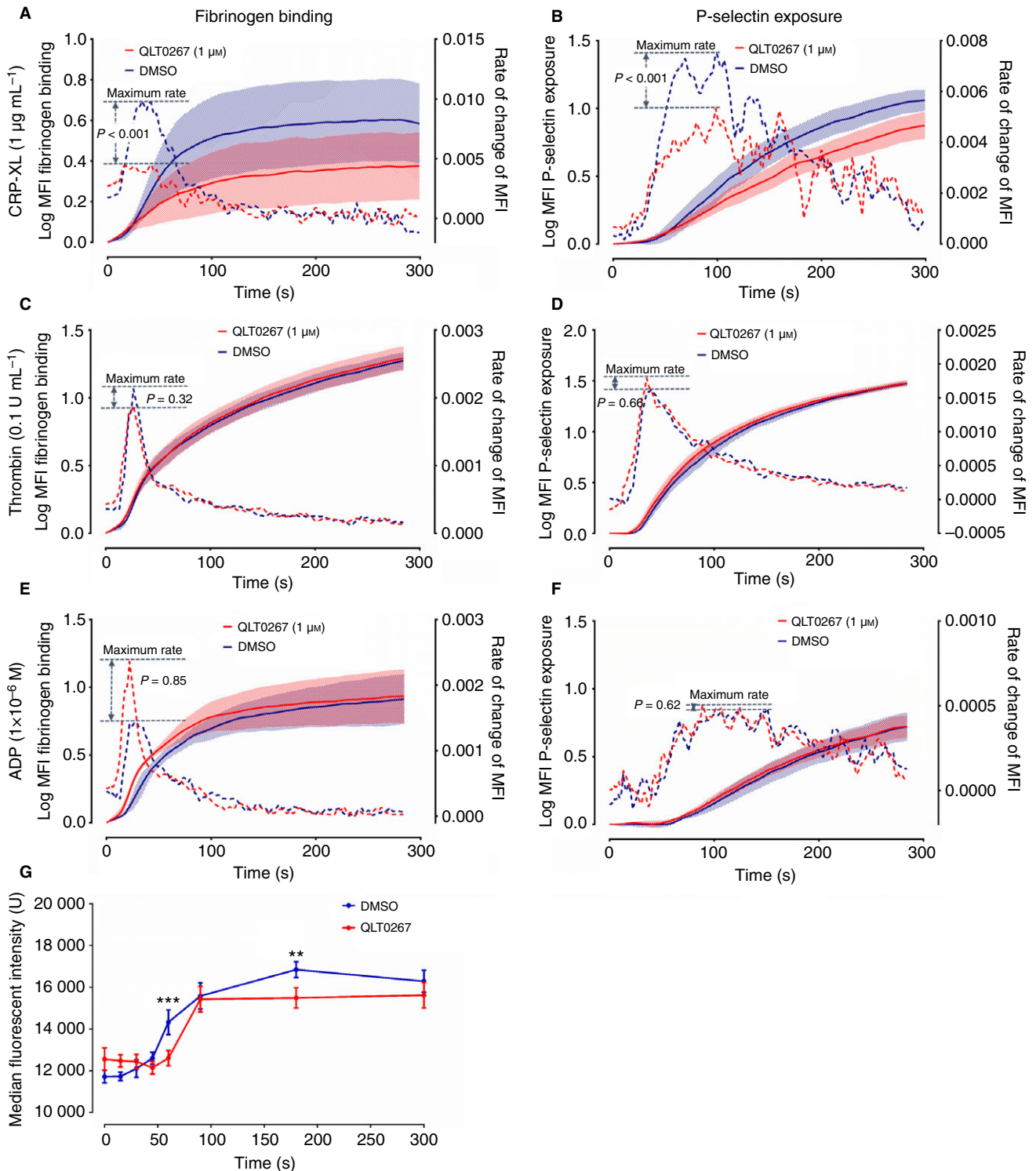


Fig. 2. Reduction in the rate of $\alpha_{\text{IIb}}\beta_3$ activation is not attributable to reduced $\alpha_{\text{IIb}}\beta_3$ surface expression. (A–F) Real-time measurements of fibrinogen binding (A, C, E) and P-selectin exposure (B, D, F) in response to cross-linked collagen-related peptide (CRP-XL) ($1 \mu\text{g mL}^{-1}$) (A, B), thrombin (C, D) and ADP (E, F) were made by flow cytometry following incubation of human platelet-rich plasma with QLT0267 ($1 \mu\text{M}$) or dimethylsulfoxide (DMSO) control. In both cases, solid lines with shaded areas show the LOESS curve of the median fluorescence intensity (MFI) and standard deviation (plotted on the left-hand axis), and the dashed lines show the rate of change of the LOESS curve (plotted on the right-hand axis). (G) Surface expression of $\alpha_{\text{IIb}}\beta_3$ on human platelets stimulated with CRP-XL ($1 \mu\text{g mL}^{-1}$) in the presence (red line) or absence (blue line) of QLT0267 ($1 \mu\text{M}$). $**P \leq 0.01$, $***P \leq 0.0001$, $n = 3$ or $n = 4$. All data are presented as mean \pm standard deviation, and were analyzed by two-way ANOVA with *post hoc* Bonferroni correction for multiple comparisons to compare treated and control samples at individual time points.

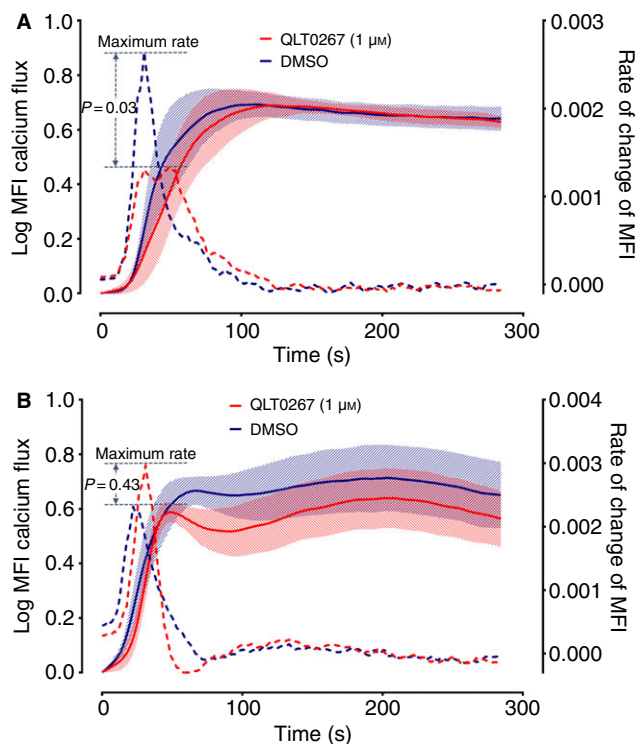


Fig. 3. Reduction in the rate of calcium mobilization resulting from reduced $\alpha_{IIb}\beta_3$ activation. Real-time measurements of calcium flux in response to cross-linked collagen-related peptide ($1 \mu\text{g mL}^{-1}$) in the (A) absence or (B) presence of EDTA were made by flow cytometry following incubation of human platelet-rich plasma with QLT0267 ($1 \mu\text{M}$) or dimethylsulfoxide (DMSO) control. In both cases, solid lines with shaded areas show the LOESS curve of the median fluorescence intensity (MFI) and standard deviation (plotted on the left-hand axis), and the dashed lines show the rate of change of the LOESS curve (plotted on the right-hand axis). $n = 4$. All data are presented as mean \pm standard deviation.

response to injury, decreased as thrombus size reached its maximum and plateaued, increased again during retraction, and then showed a sustained period of stability (low TII) resulting from the formation of a stable hemostatic thrombus (Fig. 5E, top panel). In contrast, thrombi forming in KO mice showed greater instability, growing to a similar size as those of Ctrl mice but then embolizing, growing again, and embolizing repeatedly. This was clearly observed by the extension of peaks in the TII trace from KO mice into the third and fourth minute after laser injury (Fig. 5E, bottom panel). Thus, in KO mice, TII remained high over the 4-min measurement period, and was significantly higher than in Ctrl mice after 2 min (Fig. 5F). Clearly, ILK deficiency and the associated reduction in the rate of platelet activation does not prevent thrombus formation, but it does significantly alter the development and stability of thrombus formation.

Discussion

We set out to examine the role of ILK in regulating the rate of platelet activation and thrombus formation, in an

effort to understand the previously reported disparity between the reduced platelet function and the only modest increase in bleeding in mice with an induced ILK deficiency. We confirmed that inhibition of ILK reduced the rates of both fibrinogen binding and α -granule secretion in response to CRP-XL but not ADP or thrombin, and demonstrated that the reduced rate of fibrinogen binding was a consequence of reduced $\alpha_{IIb}\beta_3$ activation rather than reduced translocation of $\alpha_{IIb}\beta_3$ to the platelet surface. Inhibition of ILK resulted in a reduced rate of clot retraction (indicating reduced 'outside-in' signaling) and a reduction in the rate of calcium flux that was driven by signaling downstream of $\alpha_{IIb}\beta_3$, not GPVI. The reduced rate of platelet activation was accompanied by only moderate reduction of the maximum extent of platelet activation or aggregation *in vitro*. However, absence of ILK led to a reduction in platelet aggregation *in vivo*, and a marked increase in the instability of thrombi. Taken together, these findings indicate that, although ILK is not essential for platelet activation, it plays a critical role in facilitating timely platelet activation, which is essential for stable thrombus formation. This deeper understanding of the role of ILK provides an explanation for previous findings. Mice with systemically induced ILK deficiency do have a defect in thrombus development, as would be expected, given the impairment of platelet function, but they still form thrombi and hence prevent excessive bleeding.

Thrombus formation is a highly dynamic, sequential process, and it is therefore intuitive that the rate at which platelets become activated during this process will have a significant impact on thrombus size and structure. The results for KO mice clearly demonstrate this effect. It is particularly interesting that ILK deficiency does not prevent thrombus formation, as can be seen in CalDEG-GEFI-deficient mice, in which the rate of platelet activation is also impaired [28–30], but instead alters the stability of thrombi that form. In interpreting this difference, it is important to note that: (i) although the maximum rate of activation is reduced in Figs 2 and 3, the very earliest responses to CRP-XL (the time at which the first detectable response can be seen and the very earliest rate of response) are the same with or without ILK inhibition; and (ii) the rate of fibrinogen binding and P-selectin expression in response to ADP and thrombin are unaffected by ILK inhibition.

Together, these data suggest a scenario in which, in the absence of ILK or following ILK inhibition, platelets adhere to the site of injury and become activated by collagen. This is supported by our previous findings that the surface exposure of $\alpha_2\beta_1$ or GPVI is not altered in ILK-deficient platelets, and neither is the initial adhesion of platelet to collagen [14]. Full activation of these initially adherent platelets is, however, delayed, as shown by reductions in the rate of calcium flux, $\alpha_{IIb}\beta_3$ activation, degranulation, and clot retraction. Subsequent platelets binding to these partially activated platelets will react normally to stimulation by thrombin and ADP, leading

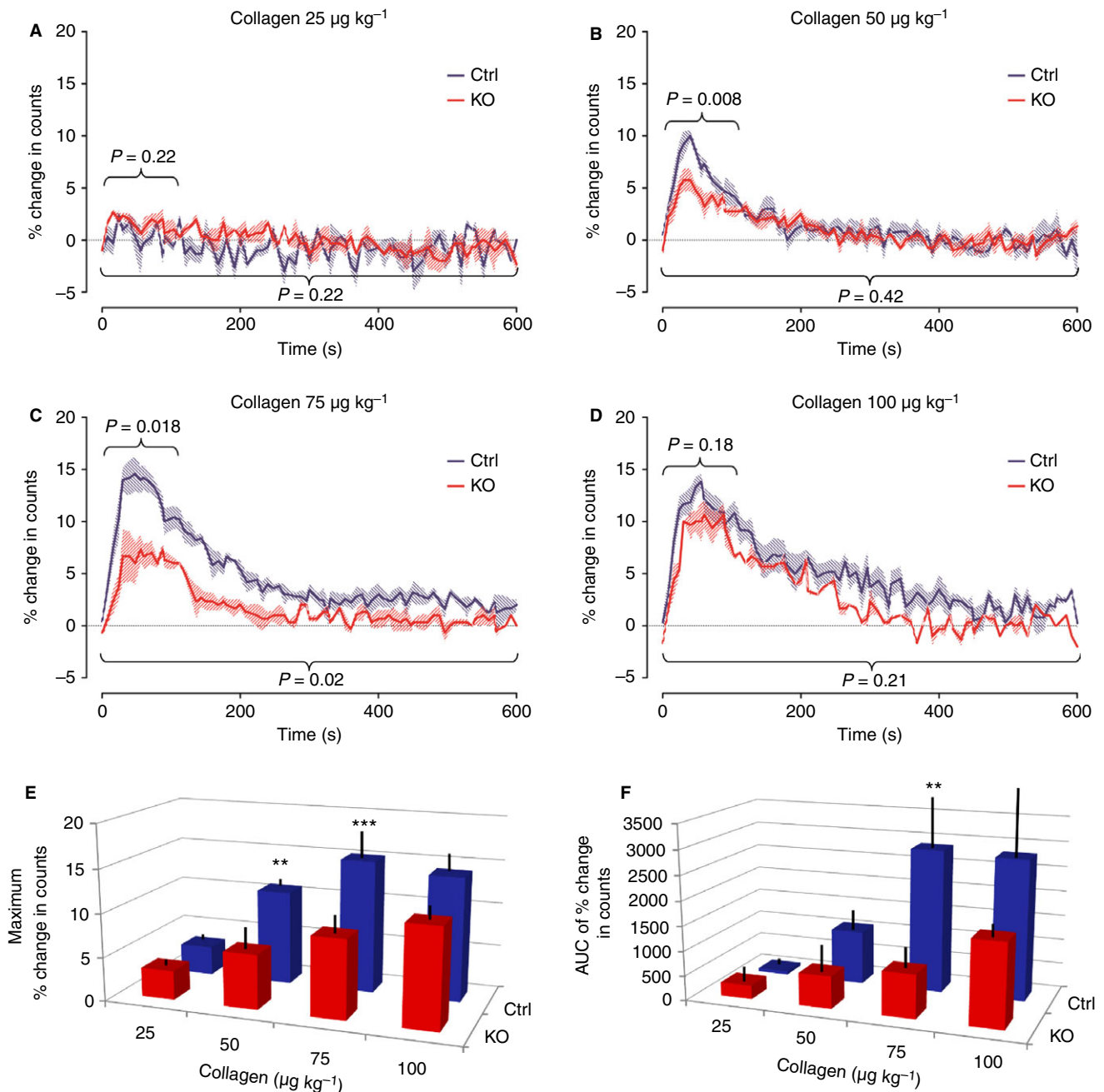


Fig. 4. *In vivo* platelet aggregation is reduced in mice with induced integrin-linked kinase (ILK) deficiency. (A–D) Platelet aggregation *in vivo* was measured over a period of 10 min in mice with induced ILK deficiency (KO) and sibling matched controls (Ctrl) in response to collagen at (A) 25 $\mu\text{g kg}^{-1}$ (KO, $n = 3$; Ctrl, $n = 3$), (B) 50 $\mu\text{g kg}^{-1}$ (KO, $n = 4$; Ctrl, $n = 6$), (C) 75 $\mu\text{g kg}^{-1}$ (KO, $n = 3$; Ctrl, $n = 5$), and (D) 100 $\mu\text{g kg}^{-1}$ (KO, $n = 3$; Ctrl, $n = 6$). The shaded area shows the standard deviation about the mean, and P -values are given for the statistical difference, assessed by two-way ANOVA, between Ctrl and KO mice over the first 2 min postinjection or over the entire 10-min measurement period. (E, F) Maximum response, as measured by the maximum percentage change in radioactive counts between consecutive counting windows, was significantly reduced in KO mice ($P < 0.0001$) (E), as was the area under the curve (AUC) ($P = 0.0001$) (F). All data are presented as mean \pm standard deviation, and were analyzed by two-way ANOVA with *post hoc* Bonferroni correction for multiple comparisons to compare treated and control samples at individual collagen concentrations. ** $P \leq 0.01$, *** $P \leq 0.001$.

to thrombus formation. We hypothesize that, as the thrombus grows, the force exerted on the initial, collagen-activated platelets at the base of the thrombus will increase. The delay in full activation of these platelets renders the thrombus unstable, as the force exerted on

these platelets becomes greater than the strength of the bonds between the platelets, leading to embolization.

It is clear that relatively subtle changes in the dynamics of platelet activation can have a profound effect on thrombus formation, and it is perhaps worth speculating

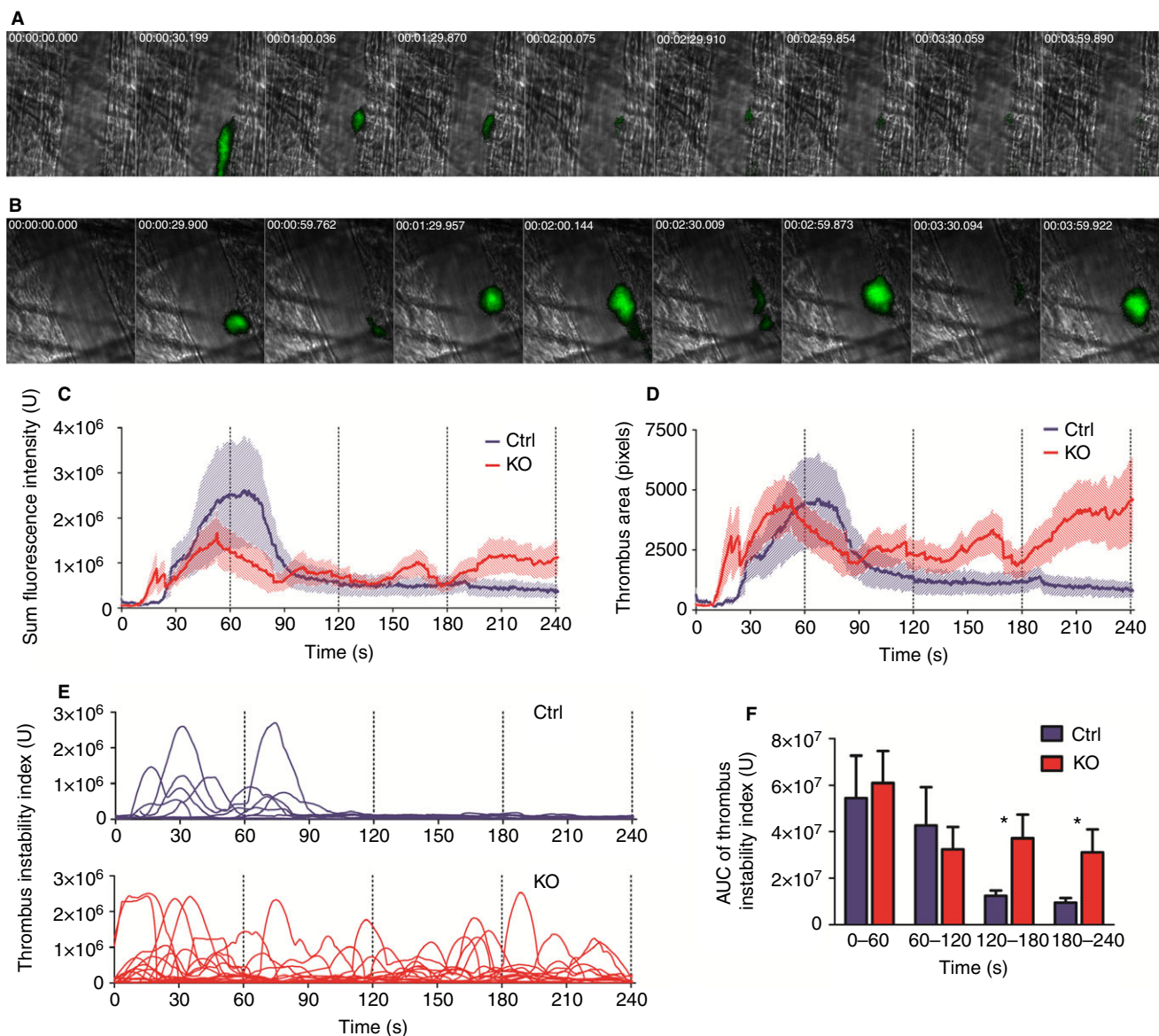


Fig. 5. Induced integrin-linked kinase (ILK) deficiency increases thrombus instability. (A, B) Representative images of thrombus formation following laser injury of the cremaster arteriole wall of (A) control (Ctrl) and (B) knockout (KO) mice measured over a period of 4 min. (C, D) There was no significant difference in (C) the sum fluorescence intensity or (D) the area of thrombi formed in KO and Ctrl mice. The shaded area shows the standard deviation about the mean. KO, $n = 4$; Ctrl, $n = 3$; an average of 4.6 thrombi were generated per mouse. (E, F) Quantification of the stability of thrombi that formed in these mice by use of a thrombus instability index (standard deviation of data immediately preceding each time point) (E) identified prolonged instability in the thrombi of KO mice as compared with Ctrl mice (F). In all cases: $*P \leq 0.05$. All data are presented as mean \pm standard deviation, and were analyzed by t -test. AUC, area under the curve.

that components of signaling pathways that regulate the rates of different aspects of platelet activation or alter the rates of activation to differing degrees may have profoundly different effects on thrombus formation.

Previous studies have shown that ILK effects both integrin activation and downstream signaling [2,31]. The data presented here suggest that in platelets these two facets of ILK are linked. Thus, that upon collagen receptor stimulation, the effect of ILK in enhancing 'outside-in' signaling at early (< 60 s) time points acts as a positive feedback loop that enhances the rate of calcium flux and

further 'inside-out' $\alpha_{IIb}\beta_3$ activation. Modulation of the rate of $\alpha_{IIb}\beta_3$ activation in the presence of QLT0267, which is a selective inhibitor of the serine/threonine kinase domain of ILK, suggests that this domain is important. The role of ILK as a kinase is controversial [32]; however, it is through the kinase domain that ILK binds to parvin, paxillin, c-Src, and others [33–35]. It is therefore possible that QLT0267 disrupts the ability of ILK to act as an adaptor protein, possibly altering the role of the IPP complex in regulating Ras and Rho family GTPases, modulation of which has been shown to alter

the rate of platelet activation and thrombus stability [30,36]. Another potential consequence of ILK inhibition or deficiency may be reduced kindlin activation. The interaction of ILK with kindlin has been shown to be an important step in regulating the conformational change of kindlin that precipitates its binding to β -integrins [37], which, in concert with the binding of talin, leads to activation of $\alpha_{IIb}\beta_3$. Whichever of these mechanisms is responsible for ILK-mediated regulation of $\alpha_{IIb}\beta_3$, it is interesting to note that the effects of inhibiting ILK could only be observed following stimulation with collagen or CRP-XL, and not in response to ADP or thrombin. This difference may relate to the mechanisms by which GPVI and the G-protein-coupled receptors regulate the activation of talin and hence 'inside-out' signaling, and their relative reliance on feedback mechanisms (such as 'outside-in' signaling) to fully activate the large numbers of $\alpha_{IIb}\beta_3$ molecules on the platelet surface. Teasing apart the relative importance of these mechanisms and their impact on different agonist pathways will be the focus of future work.

We conclude that ILK has a role in regulating the rate of the platelet response to collagen via GPVI. Inhibition or deficiency of ILK reduces the rate at which platelets activate and form stable aggregates. The physiologic consequences of this are a reduction in thrombus stability and an inability to form tight hemostatic thrombi in a timely manner.

Addendum

C. I. Jones designed, performed and analyzed the research, and wrote the paper. K. L. Tucker designed, performed and analyzed the research, and wrote the paper. P. Sasikumar performed the research. T. Sage performed the research. W. J. Kaiser performed the research. C. Moore performed the research. M. Emerson designed the research. J. M. Gibbins designed the research and wrote the paper.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Deficiency of ILK does not reduce the rate of platelet aggregation in response to thrombin.

Fig. S2. Induced ILK deficiency increases thrombus instability.

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