The impact of PI3Kα inhibition on hemostasis and thrombosis

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Abstract

Objective - Phosphoinositide 3-kinase α (PI3Kα) is a therapeutic target in oncology but its role in platelets and thrombosis remains ill characterized. In this study we have analyzed the role of PI3Kα *in vitro*, *ex vivo* and *in vivo* in two models of arterial thrombosis.

Approach and results - Using mice selectively-deficient in p110α in the megakaryocyte lineage and isoform-selective inhibitors, we confirm that PI3Kα is not mandatory but participates to thrombus growth over a collagen matrix at arterial shear rate. Our data uncover a role for PI3Kα in low-level activation of the GPVI collagen receptor by contributing to ADP secretion and in turn full activation of PI3Kβ and Akt/PKB. This effect was no longer observed at high level of GPVI agonist concentration. Our study also reveals that over a von Willebrand factor matrix, PI3Kα regulates platelet stationary adhesion contacts under arterial flow through its involvement in the outside-in signaling of von Willebrand factor engaged $\alpha_{IIb}\beta_3$ integrin. *In vivo*, absence or inhibition of PI3Kα resulted in a modest but significant decrease in thrombus size following superficial injuries of mouse mesenteric arteries and an increased time to arterial occlusion following carotid lesion, without modification in the tail bleeding time. Considering the more discrete and non-redundant role of PI3Kα compared to PI3Kβ, selective PI3Kα inhibitors are unlikely to increase the bleeding risk at least in the absence of combination with antiplatelet drugs or thrombopenia.

Conclusions – This study provides mechanistic insight into the role of PI3Kα in platelet activation and arterial thrombosis.

249 words

Abbreviations

ADP: adenosine diphosphate

Akt/PKB: protein kinase B

CRP: collagen-related peptide

GP: glycoprotein

KO: knockout

PI3K: Phosphoinositide 3-kinase

VWF: von Willebrand factor

WT: wild type

Introduction

Platelet adhesion and aggregation at sites of vascular injury are essential for normal hemostasis. However, in diseased vessels, platelet accumulation after erosion or rupture of an atherosclerotic plaque can cause occlusive thrombosis leading to ischemic diseases, a major cause of death and disability worldwide. Von Willebrand factor (VWF) plays a key role in hemostasis by promoting adhesion and spreading of platelets on exposed subendothelial matrix proteins following arterial injury. Under conditions of rapid blood flow, the glycoprotein (GP) Ib/IX/V mediates the initial platelet recruitment through its binding to VWF exposed in the subendothelium.[1] Interaction of GPIb/IX/V with VWF not only mediates platelet rolling and transient adhesion but also initiates a signaling cascade leading to platelet integrin $\alpha_{IIb}\beta_3$ activation (inside-out signaling). Under its high affinity state, $\alpha_{IIb}\beta_3$ can interact with fibrinogen but also with the RGD sequence of the carboxyl-terminal region of VWF, contributing to stable adhesion contact of platelets to VWF under flow conditions. Platelets also interact with collagen through GPVI and $\alpha_2\beta_1$ leading to strong platelet activation, spreading and secretion and in turn thrombus formation. The molecular mechanisms linking GPIb/VWF interaction to platelet activation remain incompletely characterized although several signaling proteins have been implicated, including phosphoinositide 3-kinases (PI3Ks).[1]

Over the last decade, class I PI3Ks have been the subject of intense research in several cells types including platelets.[2,3] Class I PI3Ks (PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ) are important signaling enzymes producing the lipid second messenger phosphatidylinositol(3,4,5)trisphosphate ($PI(3,4,5)P_3$) which, through binding to proteins, organizes functional signal transduction modules.[4] All Class I PI3Ks are expressed in platelets. PI3Kδ has modest role in platelets, PI3Kγ is mainly implicated downstream of the ADP receptor P2Y12 and PI3Kβ has a major role in platelet signaling. Using pharmacological inhibitors and genetic approaches, we and others have demonstrated that PI3Kβ acts downstream of most platelet receptors and integrins [5-9] and is critical to maintain the integrity of the thrombus at high shear rate in contrast to PI3Kα.[3, 10] The absence of PI3Kβ cannot be compensated by PI3Kα, suggesting complementary actions for these two PI3Ks during platelet activation.

PI3Kα has been involved in IGF-1-mediated potentiation of platelet function.[11, 12] Moreover, using pharmacological inhibitors it has been suggested that PI3Kα and β are both required for full platelet activation by collagen [8, 9, 13]. While Gilio et al. [8] could not distinguish differences in the degree of involvement of PI3Kα and β isoforms in platelets activation by GPVI agonist, Kim and al. [9] showed a smaller implication of PI3Kα in GPVI-mediated platelet responses. To precise the role of platelet PI3Kα in hemostasis and thrombosis, we performed a comprehensive study using both, a mouse model in which p110α is selectively inactivated in megakaryocyte lineage [10] and two selective inhibitors, A66 and INK1117 (or MLN1117) [14, 15], the latter being in a phase I clinical trial for oncology indications.[16]

Our data uncover a role for PI3Kα in low-level of GPVI activation by contributing to ADP secretion and in turn full PI3Kβ and Akt/PKB activation. This specific effect was no longer observed at high level of GPVI agonist concentration. Our study also reveals that PI3Kα is implicated in the control of platelet stationary adhesion contact over VWF under shear by acting downstream of VWF-engaged $\alpha_{IIb}\beta_3$ in both, mouse and human. As a consequence, absence or inhibition of PI3Kα in platelets decreased arterial thrombus formation in two models of arterial thrombosis in mouse, without modification of the tail bleeding time.

Materials and Methods

Reagents and Antibodies: Collagen Reagent HORM® (equine) suspension was purchased from Takeda. $DIOC_6$ was purchased from Life Technologies. Antibody against p-Akt was purchased from Cell Signaling and Akt from Sigma Aldrich. Antibody against VWF was purchased from DAKO. The selective PI3Kα inhibitor INK1117 was from Takeda Pharmaceuticals USA, Inc and A66 was from Selleckchem. Ristocetin was from Stago and Integrilin® was provided by Glaxo PEG-400 (Polyethylen glycol–400 grade) was from Fisher Scientific UK. Group Ltd. Serotonin ELISA test was from IBL International. CRP was from Pr. Richard Farndale laboratory (Cambridge, UK). Botrocetin was from Dr. Pierre Mangin laboratory (Strasbourg, France). Wilfactin was purchased from LFB biomedicaments (France). Murine VWF was from Cécile Denis (Paris, France). All other reagents were purchased from Sigma-Aldrich.

Mice: All animal procedures were in accordance with the guidelines of the Ethics Committee on Animal Experimentation and with the French Ministry of Agriculture license (http://anexplo.genotoul.fr). PF4-cre/p110$\alpha^{flox/flox}$ mice (83 males and females) and their p110$\alpha^{flox/flox}$ wild-type (77 males and females) littermates were generated as previously described[10] and are further referred to as p110α^{null} or WT. These animals (C57Bl/6J genetic background; 10 backcrosses) showed normal platelet size and count. Males and females were indifferently used since preliminary analysis revealed no differences between control males and females in the parameters measured in this study.

Preparation of washed mouse platelets and in vitro aggregation studies are described in the supplemental Methods.

***Preparation of washed human platelets*:** Blood from healthy donors was purchased from the Etablissement Français du Sang (Toulouse) and immediately processed for experiments. Blood was collected into ACD anticoagulant and platelets were isolated by successive centrifugation steps essentially as described previously.[17]

Flow assays on collagen, VWF or fibrinogen are described in the supplemental Methods. Briefly, mouse or human blood was drawn into heparin sodium (10 IU/ml). When indicated, human blood was incubated at 37°C with INK1117 (1 µmol/L) or A66 (1 µmol/L) during 10 min. Labeled blood was then perfused through a microcapillary for the indicated time at a wall shear rate of 250 sec^{-1}, 1,500 sec^{-1} or 4,000 sec^{-1} according to the matrix. Platelet adhesion and thrombus formation was visualized with a 40x oil immersion objective.

Serotonin secretion: Serotonin content was measured using IBL Serotonin ELISA test (RE59121, IBL) according to manufacturer instructions.

Static adhesion assay is described in the supplemental Methods. Briefly, glass coverslips were coated with murine VWF [18]. Washed mouse platelet suspension (10^6 platelets) were preincubated or not with Integrilin and botrocetin (2 µg/ml) for 10 min at 37°C, and allowed to adhere to VWF surface. For experiment with ristocetin, human platelet were preincubated with ristocetin and, when indicated, with the selective PI3Kα inhibitors INK1117 (0.5 µmol/L) or A66 (1 µmol/L) for 10 min at 37°C, and allowed to adhere to VWF surface (Wilfactin).

Laser-induced vessel wall injury is described in the supplemental Methods. [19]

Mouse treatment with INK1117: INK1117 was resuspended in a solution of 5% NMP (1-Methyl-2-pyrrolidinone) and 95% PEG-400 (Polyethylen glycol–400 grade) at the concentration of 12 mg/ml. Mice (8 weeks old C57Bl/6J males and females) were treated by

INK1117 at 60 mg/kg bodyweight or vehicle by gavage. Experiments were performed 5 hours after drug intake.

Tail bleeding time is described in the supplemental Methods.

$FeCl_3$ induced carotid artery Thrombosis is described in the supplemental Methods

***Statistical Analysis*:** Data are expressed as mean ± standard error of the mean (SEM). Data were analyzed first by Shapiro-Wilk test and D'Agostino-Pearson test to confirm the normal distribution. Significance of differences was determined using 2-tailed Student *t* test, 1-way ANOVA or 2-way ANOVA, and a Bonferroni post-hoc analysis. P values, $P<0.05$ was considered statistically significant ($*p<0.05$, $**p<0.01$, $***p<0.001$). All statistical analyses were performed using GraphPad Prism 6 software.

Results

PI3Kα contributes to thrombus growth over collagen under shear conditions

To establish the role of platelet p110α in platelet function *in vitro* and *in vivo*, we used our *PF4*-Cre/p110α$^{flox/flox}$ mouse model in which p110α is selectively deficient in the megakaryocyte lineage (hereafter called p110α^{null}). The specific deletion of p110α in platelets had no effect on the expression of the other platelet class I PI3K isoforms or surface glycoproteins GPIb, GPIIb (αIIb) and GPVI.[10]

We first investigated platelet adhesion and thrombus formation on fibrilar type I collagen matrix using a microfluidic system under normal and pathological arterial blood flow. The surface covered by platelets and the thrombus volume were monitored by video microscopy during perfusion of heparinized whole blood from wild type (WT) or p110α^{null} platelet mice. After 2 min of flow at an arterial shear rate of 1,500 sec^{-1}, WT platelets formed stable thrombi while the surface covered by p110α^{null} platelets and the thrombus volume were significantly reduced (Figure 1A). At longer time of perfusion these differences were reduced and even undetectable after 4 min of flow when the thrombi size reached a plateau (Figure 1A). In an another set of experiments, the thrombi formed at a shear rate of 1,500 sec^{-1} during 90 sec were then submitted to a pathological shear rate of 4,000 sec^{-1} (Figure 1B). A significant difference was still observed after 30 sec of flow but then tended to be reduced and was no longer significant (Figure 1B). Of note, under this high shear condition, the stability of the thrombi was comparable in WT and p110α^{null} platelet mice. The specific role of PI3Kα lipid kinase activity in thrombus growth was confirmed in similar experiments using heparinised human whole blood treated with the selective PI3Kα inhibitors A66 (1μM) or INK1117 (1μM)[14,15] (Figure 1C). The two PI3Kα selective inhibitors reproduced the effect observed using blood from p110α^{null} nice.
These results indicate that PI3Kα was not mandatory but significantly contributed to thrombus growth at physiological or pathological shear rate over a collagen matrix and this effect was due to the catalytic activity of PI3Kα.

PI3Kα is involved in aggregation and Akt activation following engagement of GPVI by low agonist concentrations

Washed platelets were then stimulated with different agonists triggering either G-protein-coupled receptors (GPCR) (including TxA2 analogue (U46119), thrombin and ADP) or the collagen receptor GPVI, which uses the ITAM motif of the Fc receptor γ-chain (FcRγ) to stimulate platelets via tyrosine kinases, following addition of collagen-related peptide (CRP) or collagen. A decreased aggregation response to low concentrations of CRP or collagen was observed in p110α^{null} platelets (Figure 2). However, increasing the concentrations of GPVI agonists restored a normal aggregation. Platelet aggregation response was normal in response to U46119, thrombin or ADP, whatever the concentration used (Figure 2).

One of the critical downstream effectors of class I PI3K is the Ser/Thr kinase Akt/PKB. [20] As shown in Figure 3Ai, the lack of PI3Kα did not affect Akt phosphorylation following U46619 or thrombin stimulation, even at low doses of agonists. Conversely, p110α^{null} platelets stimulated by a low dose of CRP (1 μg/ml) showed a strong decrease in Akt phosphorylation. Consistent with the aggregation results, this difference was no longer observed at higher concentrations of CRP (Figure 3Ai). The phosphorylation of both Ser473 and Thr308 of Akt was reduced at low doses of CRP (1 μg/ml) and this difference was observed at any time of stimulation tested (Figure 3Aii). Importantly, invalidation or inhibition of class I PI3Kβ fully inhibited Akt phosphorylation (Figure 3Aiii), indicating that while PI3Kα is important for Akt

activation at low level of GPVI activation, PI3Kβ is mandatory. Since platelet activation and Akt phosphorylation by collagen or CRP are dependent on ADP and $P2Y_{12}$ [21, 22] we investigated the potential implication of PI3Kα on platelet secretion. Serotonin release was measured as a read-out of dense granule secretion. Absence or inhibition of PI3Kα reduced platelet dense granule secretion induced by a low dose of CRP (Figure 3B). Interestingly, addition of low doses of ADP, unable to induce Akt phosphorylation, could rescue Akt phosphorylation induced by 1 µg/ml CRP in the absence of PI3Kα (Figure 3C). As illustrated in Figure 3D, these results revealed a critical role of PI3Kα in initiating the stimulation of platelets exposed to low concentrations of GPVI agonist by contributing to ADP secretion and in turn full activation of PI3Kβ and Akt.

PI3Kα plays an important role in the stationary adhesion contact of platelets over VWF under flow

We then analyzed by microfluidic and videomicroscopy the role of PI3Kα in the interaction of platelets on immobilized murine VWF during perfusion of heparinized whole blood at high shear rate (4,000 sec^{-1}) to challenge GPIb/VWF interactions. Platelet translocation and rolling was recorded continuously (Video 1 and Figure 4). As expected, WT platelets presented reversible contacts leading to platelet translocation and rolling over VWF.[23] Interestingly, $p110\alpha^{null}$ platelets translocated and rolled over the VWF matrix but exhibited much shorter stationary adhesion contact compared to WT as shown by the typical tracking profiles (Figure 4A). After 2 min of flow, quantification indicated that WT platelets spend 76% of their time in arrest *versus* 36% for $p110\alpha^{null}$ platelets (Figure 4B). Consistent with this, real-time analysis revealed that $p110\alpha^{null}$ platelets had a significantly higher velocity compared to WT platelets (12.4 µm/s ± 1.5 for $p110\alpha^{null}$ *vs* 6.2 µm/s ± 1.6 for WT platelets, $p \leq 0.01$, n=6) (Figure 4C). This result correlated with a higher distance covered by $p110\alpha^{null}$ platelets between each arrest, compared to WT platelets (14.5 µm ± 3.2 for $p110\alpha^{null}$ and 5.1 µm ± 1.1 for WT platelets, $p \leq 0.01$, n=6) (Figure 4D). Moreover, when heparinized human blood, treated with INK1117 (1µM) for 10 min, was perfused over immobilized human VWF at 4,000 s^{-1} for 3 min, we observed a significant decrease in platelet stationary adhesion contacts (Supplemental Figure I).

These data indicated that PI3Kα was either involved in the molecular mechanisms linking VWF-GPIb/IX/V complex interaction to integrin $\alpha_{IIb}\beta_3$ inside-out activation or in the outside-in signaling from this integrin engaged by VWF C4 domain to allow stable platelet adhesion.

To investigate whether PI3Kα is involved in the general $\alpha_{IIb}\beta_3$ outside-in signaling, heparinized whole blood from WT or $p110\alpha^{null}$ platelet mice was perfused over a fibrinogen-coated surface at a low shear rate of 250 sec^{-1} for 160 sec and then at 1,500 sec^{-1} for 120 sec (Supplemental Figure IIA). Integrin $\alpha_{IIb}\beta_3$ outside-in signaling is known to stabilize and sustain $\alpha_{IIb}\beta_3$ adhesive bonds necessary for the maintenance of firm adhesion contacts under shear.[24] Absence of PI3Kα did not affect stable platelet adhesion to immobilized fibrinogen whatever the shear applied (250 or 1,500 sec^{-1}). Moreover, the spreading of WT or $p110\alpha^{null}$ platelets on a fibrinogen-coated surface in the presence of 0.3 unit/ml thrombin in static conditions was comparable between $p110\alpha^{null}$ (15.36 ± 1.52 μm^2) and WT (16.45 ± 1.53 μm^2) platelets (Supplemental Figure IIB). These results indicated that PI3Kα was not required for $\alpha_{IIb}\beta_3$ outside-in signaling following engagement of the integrin by immobilized fibrinogen either under flow or static conditions.

PI3Kα mediates platelet spreading on VWF in a $\alpha_{IIb}\beta_3$-dependent manner

We next investigated the ability of $p110\alpha^{null}$ platelets to extend filopodia on immobilized murine VWF matrix under static conditions in the presence of botrocetin (2 µg/ml), a component of the *Bothrops jararaca* venom known to bind to the VWF A1 domain allowing platelet activation through a GPIb-specific mechanism, independently of $\alpha_{IIb}\beta_3$.[25,26] To assess a potential role of PI3Kα specifically downstream of GPIb-IX-V, the interaction of $\alpha_{IIb}\beta_3$ with VWF was blocked by Integrilin. As shown in the scanning electron micrographs (Figure 5A,

left panel), in the presence of Integrilin, VWF was able to support filopodia formation in WT platelets through a GPIb-dependent and $\alpha_{IIb}\beta_3$-independent mechanism. Filopodia formation of p110α^{null} platelets (right panel) was indistinguishable from WT platelets (Figure 5A) as confirmed by observation of the actin cytoskeleton by confocal microscopy (Figure 5B). This result indicated a normal GPIb signaling towards filopodia formation in the absence of PI3Kα.
Another set of experiments was then designed to investigate the role of PI3Kα in lamellipodia formation on immobilized VWF in the presence of ristocetin, a bacterial glycopeptide that binds to the proline-rich region of the A1 domain in VWF. Of note, ristocetin is not recommended for mouse platelets studies.[27], therefore we used human platelets. VWF/ristocetin is known to generate full spreading and robust stress fiber formation through a pathway that is dependent on $\alpha_{IIb}\beta_3$.[25,26] In absence of ristocetin, human platelets were unable to spread on VWF (Supplemental Figure III). Forty minutes after addition of untreated washed human platelets on VWF in the presence of ristocetin, we observed an enrichment of cortical F-actin at the cell periphery and stress fibers in the center of spread platelets (Figure 5C, left panel). Interestingly, two unrelated selective inhibitors of p110α (INK1117 and A66) strongly affected actin reorganization and significantly reduced the mean platelet spreading (Figure 5C).
We then investigated WT and p110α^{null} platelets spreading on VWF matrix in the presence of the secondary agonist ADP. After 60 min, WT murine platelets spread to some extent, formed lamellae-like structure and some filopodia. This was strongly affected by the absence of PI3Kα as shown by quantification of the platelet area (Figure 5D). In both genotypes, spreading was prevented by addition of Integrilin, highlighting the importance of integrin $\alpha_{IIb}\beta_3$ outside-in signaling in this process.
Taken together, these results show that PI3Kα is an important component of $\alpha_{IIb}\beta_3$ outside-in signaling on immobilized VWF but not on immobilized fibrinogen.

Lack of platelet PI3Kα affects thrombus formation *in vivo* without increasing the bleeding time

We next evaluated the consequence of the lack of platelet PI3Kα in primary hemostasis and thrombus formation *in vivo*. The tail bleeding time of the p110α^{null} platelet mice was not significantly different from control mice and the treatment of WT mice with the PI3Kα inhibitor INK1117 (60 mg/kg bodyweight) had no impact (Figure 6A). Moreover, we never observed re-bleeding in PI3Kα^{null} platelet mice. These data suggested that primary hemostasis was spared in the absence of platelet PI3Kα activity. We next monitored platelet thrombus formation by intravital microscopy following laser beam-induced local injury of mesenteric arterioles (Figure 6B). Two types of lesions can be performed by this approach. A deep injury leading to the formation of a thrombus dependent on VWF and thrombin and a superficial lesion that generates a thrombus largely dependent on collagen and VWF.[19] Following deep injury, thrombus formation tended to be slightly reduced in p110α^{null} platelet mice compared to WT mice but the difference was not significance (Figure 6B, lower panel). In contrast, superficial injury of the mesenteric artery induced the formation of a significantly smaller thrombus in p110α^{null} platelet mice as compared to WT mice (364 ± 66 μm^2 in WT *versus* 166 ± 43 μm^2 in p110α^{null} platelet mice at 30 sec, *p = 0.0181) (Figure 6B, upper panel).

Another model of experimental arterial thrombosis triggered by carotid injury with ferric chloride ($FeCl_3$) was tested. While WT mice had a mean occlusion time of 7.3 ± 0.3 min after injury, among eleven p110α^{null} platelet mice, nine needed a significantly longer time to occlude (mean occlusion time: 12.8 ± 1.1 min) and the two other mice did not occlude during the 30 min of analysis (Figure 6C, left panel). Similar results were obtained with mice fed with the PI3Kα inhibitor INK1117 (60 mg/kg) and subjected to $FeCl_3$ lesion 5 hours later (Figure 6C, right panel). These results were consistent with the *ex vivo* data and demonstrated a contribution of PI3Kα catalytic activity to the mechanisms of thrombus formation following arterial injury *in vivo*.

Discussion

A non-redundant contribution of PI3Kα and β in GPVI signaling was suggested by studies using a pharmacological inhibitor *in vitro* [8, 9]. In the current study we confirm that, besides a major role of PI3Kβ, [5] PI3Kα also contributes to GPVI signaling and propose a mechanistic explanation. Indeed, following activation by low doses of GPVI agonists, PI3Kα participate to secretion and that secreted ADP then allows a full activation of PI3Kβ which has the major role in producing PtdIns(3,4,5)P3 downstream of GPVI [28]. Accordingly, addition of low doses of ADP could rescue Akt phosphorylation in PI3Kα-deficient platelets stimulated by low concentration of CRP. Our results also show that PI3Kα was dispensable as soon as the concentrations of GPVI agonists (CRP or collagen) increased. It is noteworthy that while PI3Kβ was mandatory for platelet activation whatever the doses of GPVI agonists used, it was unable to compensate the loss of PI3Kα when GPVI is mildly activated. Consistent with these results we recently observed that the mass of $PI(3,4,5)P_3$ produced was weakly (20%) but significantly decreased in $p110\alpha^{null}$ platelets stimulated by CRP [28]. The identification of this new regulatory loop (Figure 3D) shed light on the complementary and non-redundant role of class I PI3Kα and PI3Kβ in platelets. PI3Kα being activated by binding to tyrosine phosphorylated residues and, in contrast to PI3Kβ, being independent of the β/γ subunits of heterotrimeric Gi-proteins, it can be stimulated independently of ADP secretion and $P2Y_{12}$ activation [22]. Thus, our data together with those from Hers et al. and Kim et al. [11, 12] indicate that PI3Kα is an important lipid kinase in platelet priming or activation by low doses of agonists acting through receptors coupled to tyrosine kinase activation.

Using both $p110\alpha^{null}$ platelet mice and selective inhibitors-treated human or mouse blood we found that PI3Kα was not mandatory but was significantly implicated in *ex vivo* thrombus formation on collagen at a physiological arterial shear rate. Indeed, while $p110\alpha^{null}$ platelets formed smaller thrombi during the early phase of blood perfusion this difference was no longer detectable after a prolonged period of flow when the thrombi size reached a plateau. These data are consistent with those from Gilio et *al.* [8] using PIK-75 inhibitor-treated human or mice blood however the phenotype observed in their study was much stronger than in our study possibly because of PIK-75 off-target effects. In contrast, Blair et *al.*[29] did not observe thrombus formation defect on collagen in the absence of PI3Kα. This discrepancy could be explained by differences in the experimental conditions, particularly by the presence of calcium chelator, since the authors used whole blood anticoagulated with citrate, heparin, and PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone) whereas we only used heparin.
Of note, as we previously observed in $p110\alpha^{null}$ platelets [10], PI3Kα inhibitors (INK1117 and A66) did not affect thrombus stability. These data suggest that amplification of signaling loops within the core of the thrombus *via* low diffusion of released agonists and sustained $\alpha_{IIb}\beta_3$ activation [30] is not dependent on PI3Kα. In contrast, PI3Kβ is critical to control thrombus stability at high shear rate [10] because it is an essential component of the outside-in signaling of various integrins, including $\alpha_{IIb}\beta_3$ engaged by fibrinogen and of ITAM signaling.[31]

Another important new observation is the involvement of PI3Kα in platelet stationary adhesion contact over VWF surface under shear. The interaction of VWF with GPIb allows surface translocation (rolling) that continues until platelets become activated. At that point, a second step mediated by $\alpha_{IIb}\beta_3$, not required for the initial attachment, supports stationary adhesion contacts of individual platelets via interaction of $\alpha_{IIb}\beta_3$ with the C4 domain of VWF, leading to an outside-in signaling and a subsequent cytoskeleton reorganization.[1, 20] The engagement of $\alpha_{IIb}\beta_3$ strengthens the interaction of platelets with the adhesive surface resulting in a marked decrease in velocity and in turn to stationary contact.[23, 32,33] Several signaling molecules have been implicated downstream of GPIb/IX/V, including PI3K. [1] However, the implication of PI3K in GPIb-dependent $\alpha_{IIb}\beta_3$ inside-out [34] or outside-in

signaling, [25, 35] or both, is still a matter of debate and the PI3K isoform(s) involved are unknown. Here, we show that PI3Kα was not required for the GPIb-dependent platelet translocation and rolling over VWF but was important downstream of integrin $\alpha_{IIb}\beta_3$ to allow stationary adhesion contact. Furthermore, no defect in platelet adhesion on immobilized fibrinogen under flow or spreading in static conditions was observed, indicating a requirement of PI3Kα downstream of $\alpha_{IIb}\beta_3$ engaged by binding to immobilized VWF but not in the outside-in signaling of fibrinogen-engaged $\alpha_{IIb}\beta_3$, in contrast to PI3Kβ.[5] This suggests that distinct domains or different affinities for VWF and fibrinogen could be implicated in $\alpha_{IIb}\beta_3$ binding that may elicit different pathways of activation as previously proposed.[36, 37]

Mice in which p110α is selectively deficient in the megakaryocyte lineage had normal platelet count [10] and tail bleeding time, consistent with the fact that platelet rolling on VWF was spared and that they responded normally to TXA2, ADP, thrombin and high concentration of GPVI agonists. Interestingly, these mice exhibited a significant decrease in arterial thrombus formation in two different models of experimental thrombosis. In laser-induced thrombosis in mesenteric arterioles with superficial injury, the thrombus, known to be dependent on GPVI-collagen and GPIb-VWF interactions, was significantly smaller in the absence of platelet PI3Kα. In response to a deep mesenteric artery lesion, where the thrombus formed is thrombin-dependent [19] absence of platelet PI3Kα had no significant effect. By comparison with anti-platelet drugs like clopidogrel or $\alpha_{IIb}\beta_3$ blockers tested in the same model [38] our data suggest a weaker impact of PI3Kα inhibition on thrombus formation.
In the model of arterial thrombosis triggered by carotid injury with $FeCl_3$,[39] the time from injury to complete occlusion of the artery was significantly prolonged in $p110\alpha^{null}$ platelet mice. This model of thrombosis is known to require thrombin and VWF. These data are consistent with the fact that the interaction of platelets to VWF through integrin $\alpha_{IIb}\beta_3$ contributes to thrombus growth.[40, 41]
We also used PI3Kα inhibitors *in vivo* to directly analyze the implication of the lipid kinase activity. As observed in $p110\alpha^{null}$ platelet mice, pharmacological inhibition of p110α with INK1117 had no significant impact on the tail bleeding time and reproduced the defect of thrombus formation following $FeCl_3$ carotid injury. This is important information since selective inhibitors of PI3Kα, including INK1117, are now in clinical trials in the treatment of cancer with gain of function mutations in p110α gene.[16, 42] Our data suggest that PI3Kα inhibitors, on their own, are unlikely to increase the bleeding risk as also observed in mouse treated during 7 days with PIK-75 or with a specific siRNA.[43] Accordingly, a clinical trial with INK1117 did not show particular bleeding.[16] However, associations of these inhibitors with antiplatelet drugs and their use in patients with thrombopenia have not been tested.

In conclusion, using both a genetic approach and pharmacological inhibitors this study, provides new mechanistic insight into the role of PI3Kα in platelets activation and arterial thrombosis.

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Disclosures

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Highlights

- PI3Kα contributes to platelet aggregation at low level of GPVI activation by participating to ADP secretion and in turn PI3Kβ and Akt activation.
- PI3Kα regulates stationary adhesion on VWF under shear by acting downstream of VWF-engaged $\alpha IIb\beta_3$.
- PI3Kα contributes to arterial thrombus growth *in vivo*.
- On their own, PI3Kα inhibitors developed for cancer therapy should not increase the bleeding risk.

Figure legends

Figure 1: ***PI3Kα is implicated in thrombus formation ex vivo under shear rate.*** *(A)* $DIOC_6$-labeled platelets in whole blood from WT or p110α^{null} mice were perfused through a collagen-coated microcapillary at a shear rate of 1,500 sec^{-1} during 240 sec. Representative images of thrombus formation over time are shown. Surface covered by platelet thrombi (%) and thrombi volumes (μm^3) were analyzed using ImageJ software (mean ± SEM; n = 6 independent experiments; ***p* <0.01 and *p<0.05 vs WT according to 2-way ANOVA test). *(B)* Whole blood from WT or p110α^{null} mice was perfused through a collagen-coated microcapillary at a shear rate of 1,500 sec^{-1} during 90 sec and then at a high shear rate of 4,000 sec^{-1}. Thrombi volumes (μm^3) were analyzed using ImageJ software (mean ± SEM; n = 4 to 5 independent experiments; ****p* <0 .001 and ***p* <0.01 vs WT according to 2-way ANOVA test). *(C)* Human blood pre-incubated during 10 min with DMSO, INK1117 (1µM) or A66 (1µM) was perfused through a collagen-coated microcapillary at a shear rate of 1,500 sec^{-1} during 90 sec and then at a high shear rate of 4,000 sec^{-1}. Thrombi volumes (μm^3) were analyzed using ImageJ software (mean ± SEM; n = 3 independent experiments; ****p* <0 .001, ***p* <0.01 and *p<0.05 vs DMSO according to 2-way ANOVA test). Representative images of thrombus formation over time and 3D surface plot of the time point 90 sec are shown.

Figure 2: ***PI3Kα is implicated in platelet aggregation at low concentrations of CRP.*** Washed platelets from WT or p110α^{null} mice were stimulated with collagen, CRP, U46619 or thrombin and aggregation was assessed using a Chrono-log dual-channel aggregometer under stirring for 5 min. For ADP, whole blood from WT or p110α^{null} mice was drawn into heparin (5 000 IU/ml) and was stimulated with ADP and aggregation was assessed during 5 min. The profiles shown are representative of 5 independent experiments. Quantifications of the maximum of aggregation at 5 min are shown on right panels (mean ± SEM; n = 5 independent experiments; ****p* <0 .001 and *p<0.05 vs WT according to 2-tailed Student *t* test).

Figure 3***: PI3Kα is implicated in Akt phosphorylations at low concentrations of CRP.*** *(A) (i)* Platelets from WT or p110α^{null} mice were stimulated by U46619, thrombin or CRP during 5 min at the indicated concentration. Lysates were submitted to immunoblotting with anti-Akt-Ser(P)473 or Akt antibodies (control loading), as indicated. *(ii)* Platelets were stimulated by CRP (1µg/ml) during indicated time. Quantifications by densitometric analysis of the western blots (Akt-Ser(P)473 and -Thr(P)308) are shown (mean ± SEM; n = 4 independent experiments; ***p* <0.01 and *p<0.05 vs WT at independent time point according to 2-tailed Student *t* test). *(iii)* WT or p110β^{null} platelets were preincubated or not with the PI3Kβ inhibitor AZD6482 (1µM) during 10 min and stimulated by CRP (1 µg/ml) during 5 min. Quantifications by densitometric analysis of the Western blots of Akt-Ser(P)473 are mean ± SEM of 3 independent experiments (****p* <0 .001 vs WT according to 1-way ANOVA test). *(B) Role of p110α in promoting dense granule secretion.* WT or p110α^{null} platelets were stimulated by CRP (1 or 3 µg/ml) in presence of fluoxetine (25 µM) to prevent serotonin re-uptake, with or without the PI3Kα inhibitor A66 (1µM). Secreted serotonin was quantified by ELISA assay. Data are expressed as concentration of serotonin secreted from 1.5 10^7 platelets (mean ± SEM; n = 3 independent experiments; *p<0.05 vs WT according to 1-way ANOVA and 2-tailed Student *t* test). *(C) ADP addition rescues Akt phosphorylation in p110α^{null} platelets stimulated by 1 µg/ml CRP*. Platelets from WT or p110α^{null} mice were stimulated or not by CRP (1µg/ml) in the absence or presence of ADP at different concentrations as indicated. Lysates were submitted to immunoblotting with anti-Akt-Ser(P)473 or Akt antibodies (control loading). Quantifications by densitometric analysis of the western blots are shown (mean ± SEM; n = 3 independent experiments; ***p<0.001 and

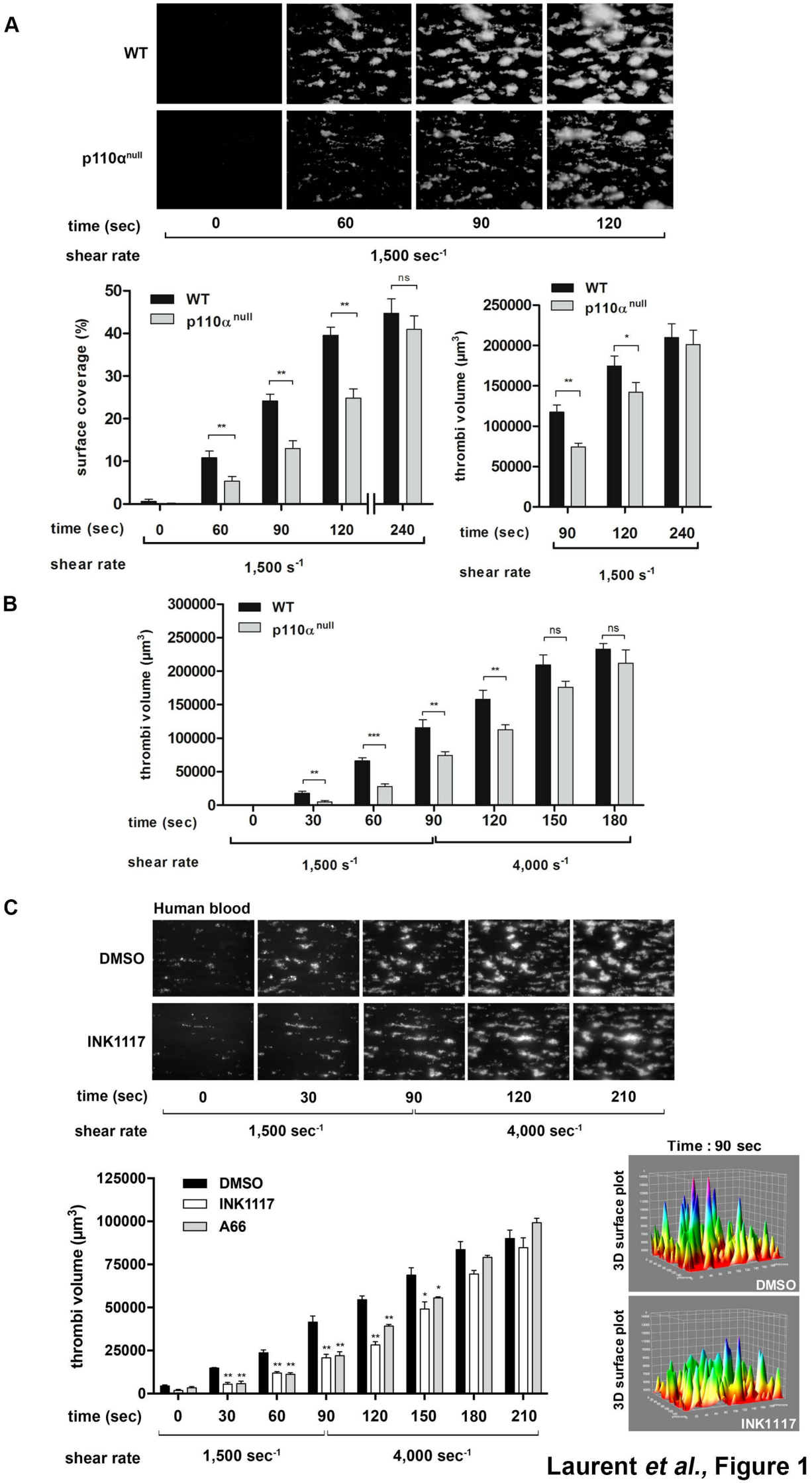
**p<0.01 according to 2-way ANOVA test). *(D) Schematic representation of the role of PI3Kα in platelets stimulated by low doses of GPVI agonist.* Following a weak GPVI activation, PI3Kα contributes to ADP secretion (1) which, via the β/γ subunits of its P2Y12 receptor, allows a full activation of PI3Kβ (2) which has a major role downstream of GPVI. The fact that PI3Kα cannot be replaced by PI3Kβ suggests that, under this condition, these kinases have complementary and non redundant roles.

Figure 4: *PI3Kα is essential for stationary adhesion contact of platelets over VWF matrix under flow.* *(A)* Unlabeled platelets in whole blood from WT or p110α^{null} were perfused through a murine VWF-coated microcapillary at a shear rate of 4,000 sec^{-1} during 3 min (Video 1 and representative images of both genotypes). Representative tracking profiles of platelet arrest or in motion are shown for both genotypes over a period of 30 sec of flow. *(B)* Graph showing the mean time (in %) of platelets stopped or in movement during the flow over VWF for both genotypes. *(C)* The velocity of platelets was evaluated in µm/sec (mean ± SEM; n = 6 independent experiments for each genotype with 30 platelets counted per experiment ***p<0.001 vs WT according to 2-tailed Student *t* test). *(D)* Graph showing the mean distance (µm) covered by platelets between each arrest for both genotypes (mean ± SEM; n = 6 independent experiments for each genotype with 30 platelets counted per experiment; **p<0.01 vs WT according to 2-tailed Student *t* test).

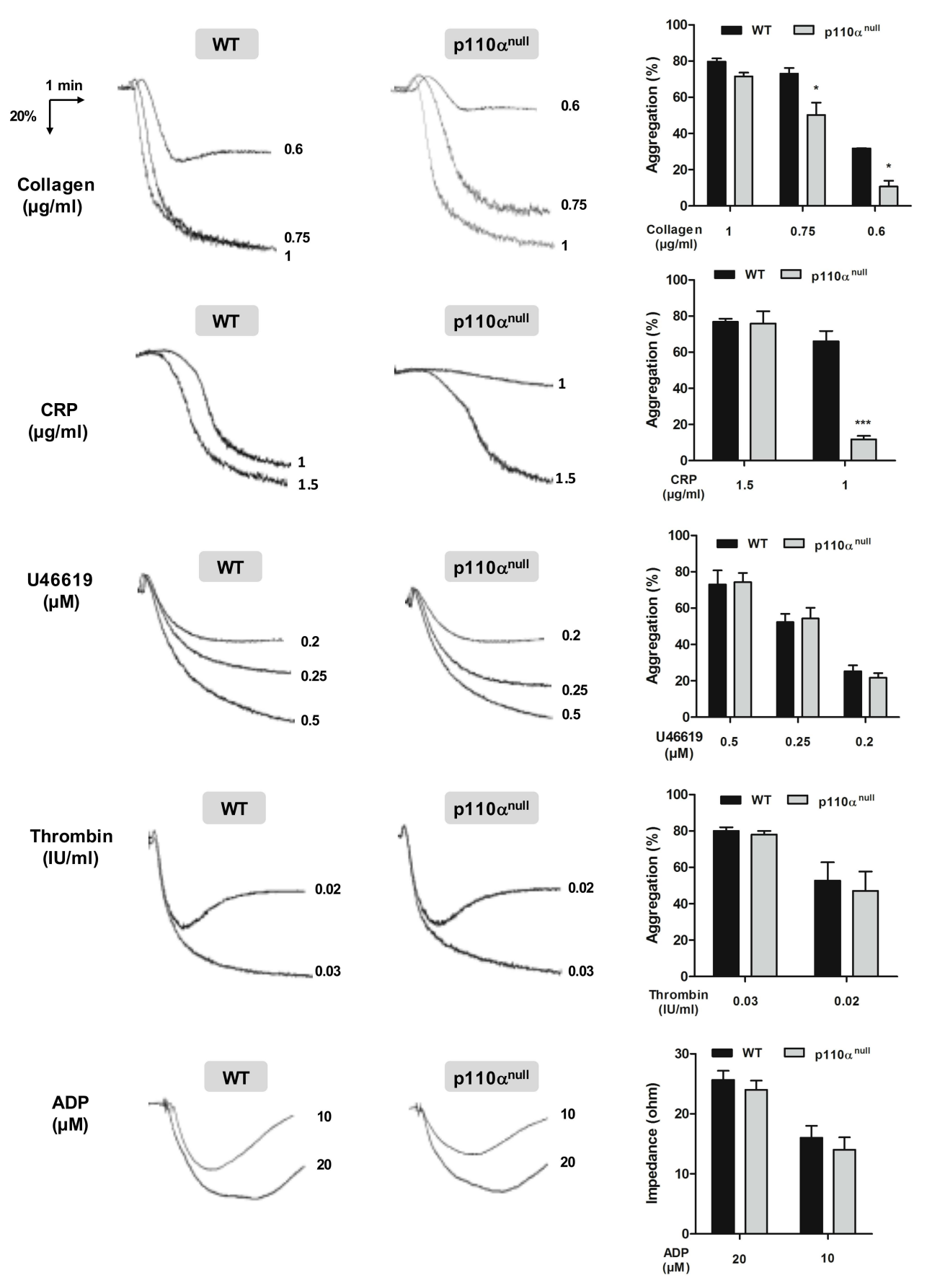
Figure 5: *PI3Kα is not implicated in GPIb/IX/V-mediated filopodia formation on VWF.* Washed platelet suspensions from WT or p110α^{null} mice were incubated with botrocetin in presence of integrilin and adhered to VWF surface for 30 min. *(A*) Platelets were then examined by scanning electron microscopy and representative images are shown, scale bar 10µm, 3 independent experiments. Filopodia per platelet, of both conditions was quantified manually and shown in right panel graphs. (mean ± SEM; n = 3 independent experiments for each genotype with an average of 50 platelets counted per experiment; 2-tailed Student *t* test). *(B)* Platelets were stained with FITC-conjugated phalloidin antibody for confocal microscopy imaging and representative images are shown, scale bar 5 µm, 4 independent experiments. *PI3Kα mediates $\alpha_{IIb}\beta_3$-dependent platelet spreading on VWF. (C)* Washed platelets suspension from human were incubated with ristocetin, in presence or not of the PI3Kα selective inhibitor INK1117 (0.5 µM) or A66 (1 µM), and adhered to VWF surface for 40 min. Platelets were then stained with FITC-conjugated phalloidin antibody for confocal microscopy imaging. Representative images are shown, scale bar 10 µm, 3 independent experiments. Platelets area was analyzed using ImageJ software (mean ± SEM; n = 3 independent experiments with an average of 400 to 500 platelets counted per experiment; ***p<0.001 vs control according to 1-way ANOVA test). *(D)* Washed platelets from WT or p110α^{null} mice were allowed to adhere to a VWF surface for 60 min in the presence of ADP (10 µM) and in the absence or presence of the $\alpha_{IIb}\beta_3$ inhibitor integrilin, and stained with FITC-conjugated phalloidin antibody for confocal microscopy imaging. Representative images are shown, scale bar 5 µm, 3 independent experiments. Platelets area was analyzed using ImageJ software. (mean ± SEM; n = 3 independent experiments with an average of 400 to 500 platelets counted per experiment; ***p<0.001 according to 2-way ANOVA test).

Figure 6: Mice lacking PI3Kα have normal bleeding time, form smaller thrombi *in vivo* following superficial injury and present a decreased occlusion velocity after $FeCl_3$-induced carotid injury. *(A) Absence (left panel) or pharmacological inhibition (right panel) of PI3Kα in platelets has no effect on bleeding time*. Tail bleeding time was performed on WT (n = 6), p110α^{null} (n = 6) or WT mice treated with vehicle (Ve) (n = 12) or PI3Kα inhibitor (INK1117) (n = 14). *(B) PI3Kα is required for thrombus growth in vivo after superficial laser-induced injury of mesenteric arterioles.* Superficial (upper panel) or deep (lower panel) laser injuries of mesenteric arterioles were performed in WT (n = 10) or p110α^{null} (n = 10) mice. Thrombus formation was monitored over time by fluorescence microscopy and typical profiles are shown for each condition. The mean thrombus area was analyzed at 0.3 second intervals, and the shading over the curve represents the SEM at each time point. Bar graphs

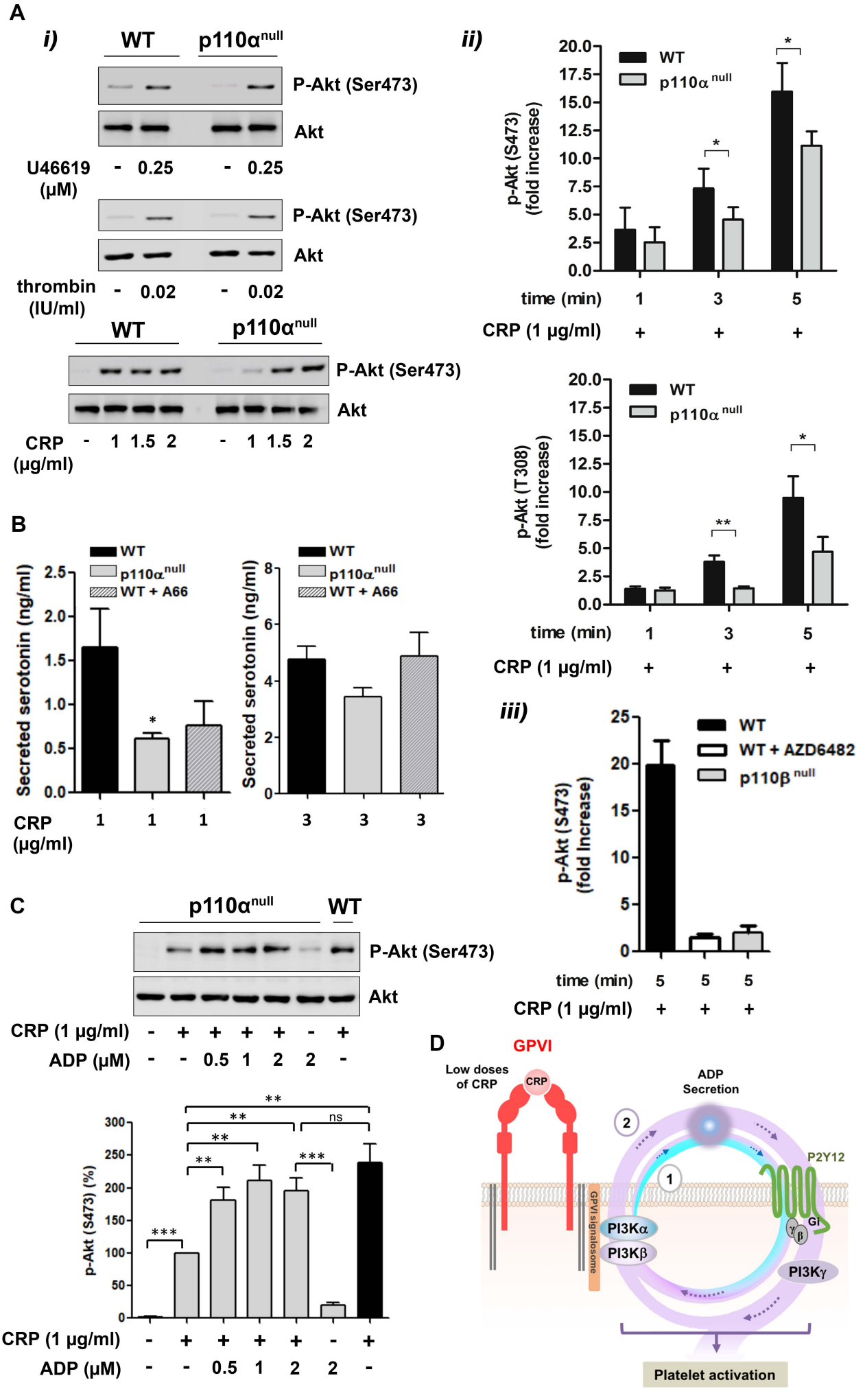
represent the area of the thrombus at t = 30 sec or t = 200 sec after injury and are expressed as the mean ± SEM (*p<0.05). Upper panel: n = 19 vessels in 6 WT mice, n = 18 vessels in 7 p110α-deficient platelet mice. Lower panel: n=4 vessels in 4 WT mice, n=6 vessels in 5 p110α-deficient platelet mice. *(C) Thrombotic response of mice to ferric chloride injury of the carotid artery.* Left panel: Flow rates were measured in the carotid artery from WT or $p110\alpha^{null}$ mice after exposure to 7% $FeCl_3$ for 3 min. The experiment was stopped after 30 min. For both genotype (n = 9), the time necessary to form a stable occlusion is shown for each mouse. Right panel: WT mice were treated with the PI3Kα inhibitor INK1117 (60 mg/kg, n = 12) or vehicle (Ve, n = 8) and subjected to $FeCl_3$ lesion 5 hours later. The experiment was stopped after 30 min. For both conditions, the time necessary to form a stable occlusion is shown for each mouse (***p<0.001 and *p<0.05 vs WT or Ve according to 2-tailed Student *t* test).



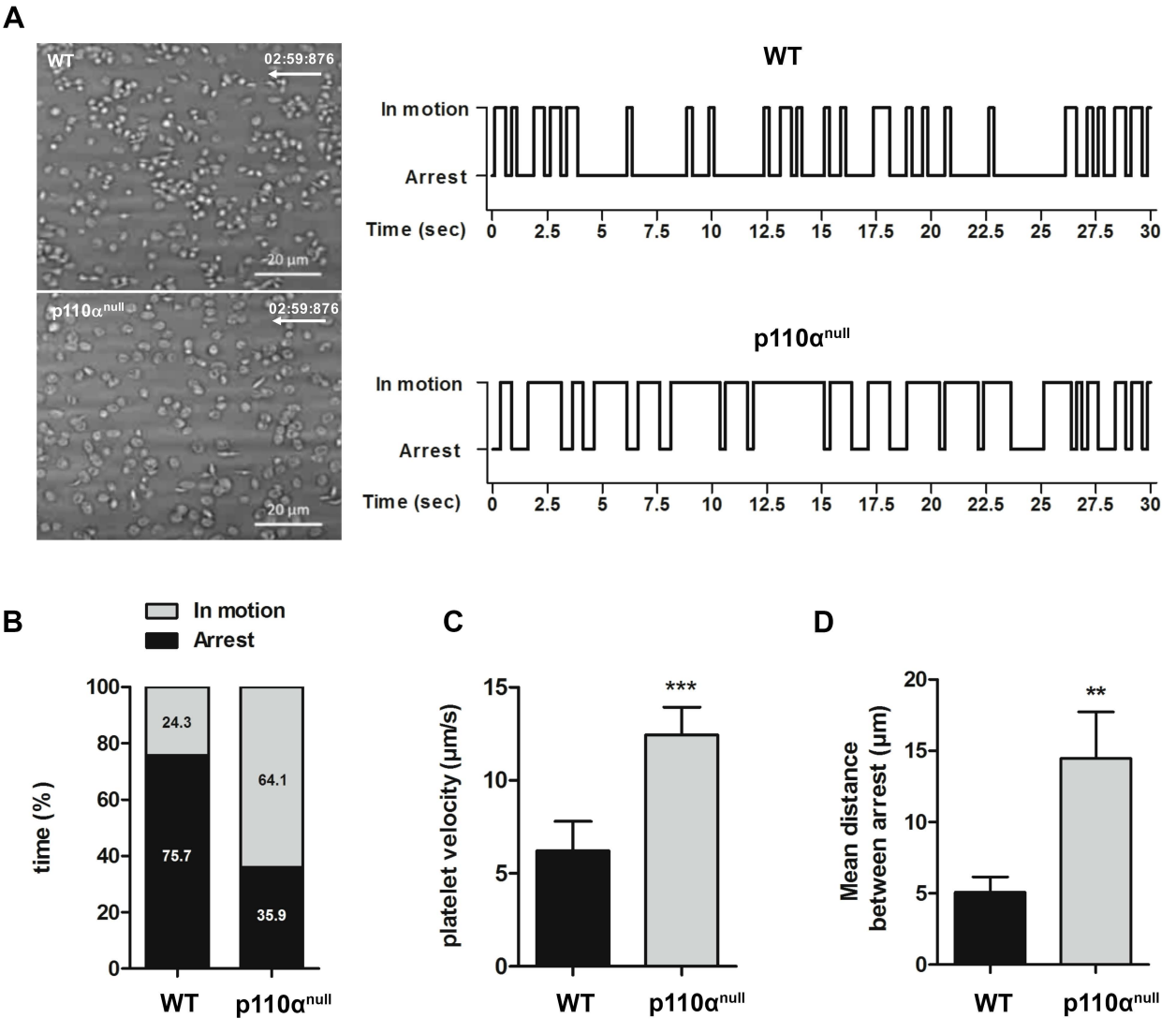
Laurent *et al.*, Figure 1



Laurent *et al.*, Figure 2



Laurent *et al.*, Figure 3



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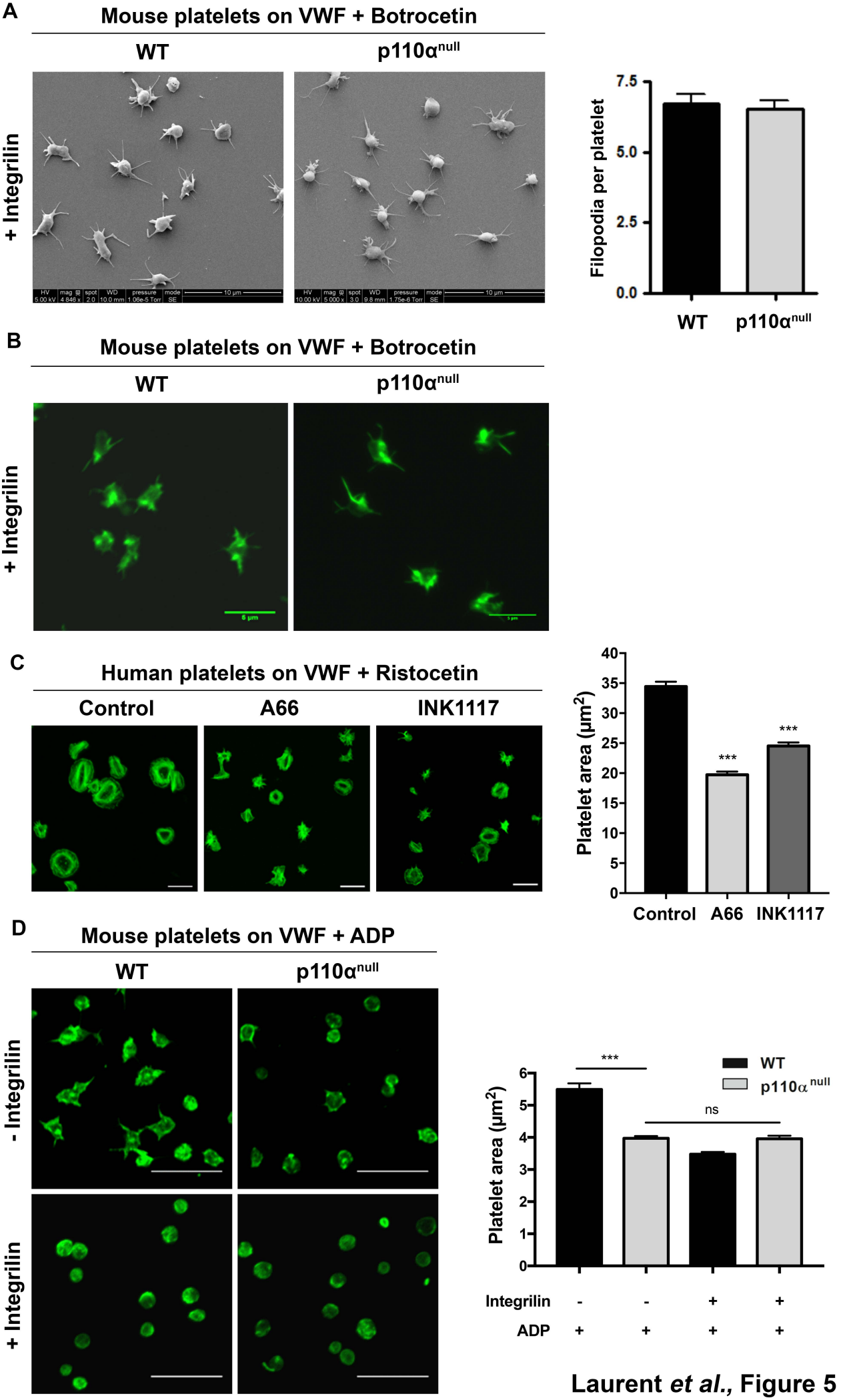
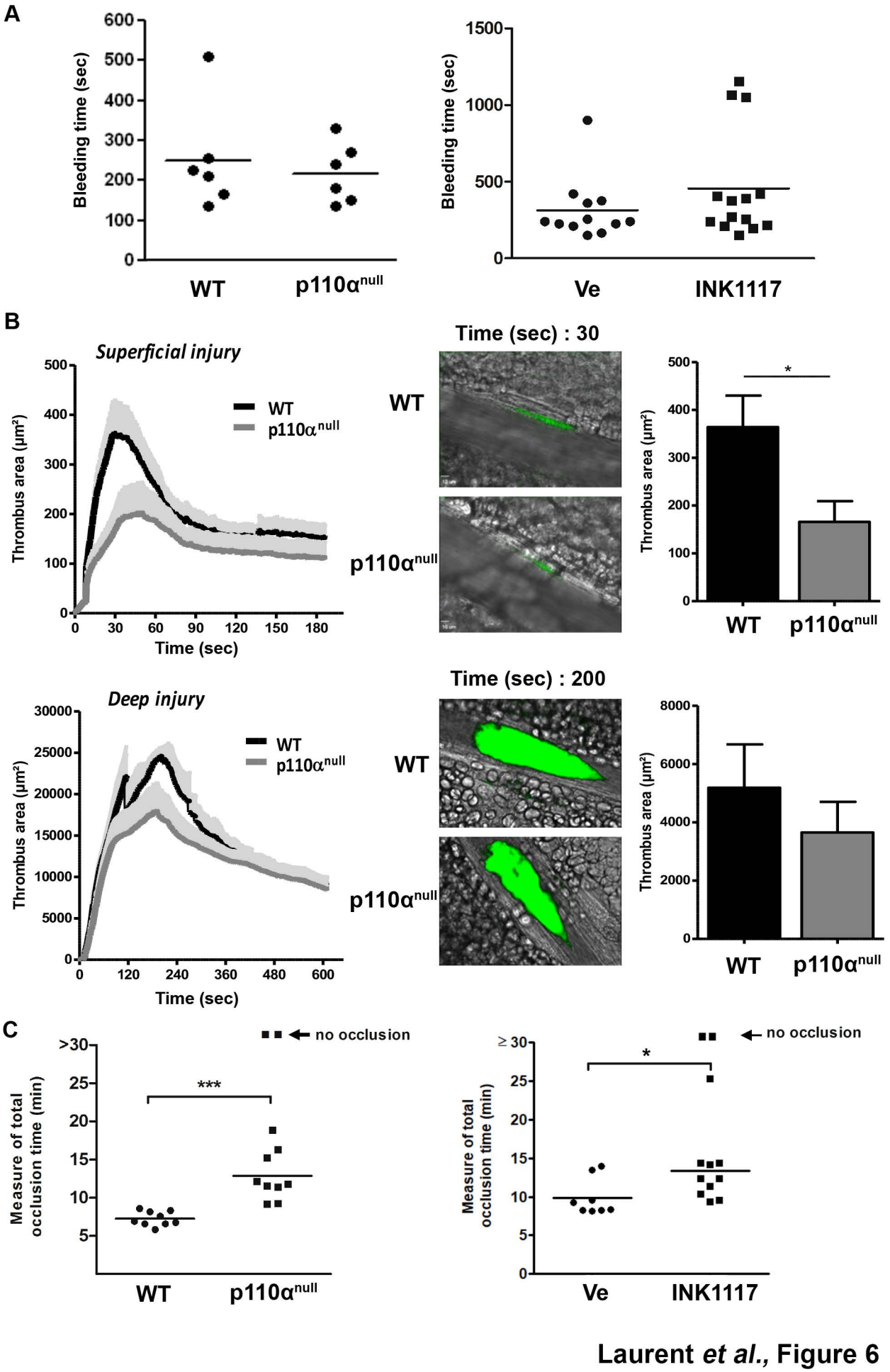


Laurent *et al.*, Figure 5



Laurent *et al.*, Figure 6