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Phosphoinositide 3-kinase p110α negatively regulates thrombopoietin-mediated platelet activation and thrombus formation.

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Abstract

Phosphoinositide 3-kinase (PI3K) plays an important role in platelet function and contributes to platelet hyperreactivity induced by elevated levels of circulating peptide hormones, including thrombopoietin (TPO). Previous work established an important role for the PI3K isoform; p110β in platelet function, however the role of p110α is still largely unexplored. Here we sought to investigate the role of p110α in TPO-mediated hyperactivity by using a conditional p110α knockout (KO) murine model in conjunction with platelet functional assays. We found that TPO-mediated enhancement of collagen-related peptide (CRP-XL)-induced platelet aggregation and adenosine triphosphate (ATP) secretion were significantly increased in p110α KO platelets. Furthermore, TPO-mediated enhancement of thrombus formation by p110α KO platelets was elevated over wild-type (WT) platelets, suggesting that p110α negatively regulates TPO-mediated priming of platelet function. The enhancements were not due to increased flow through the PI3K pathway as phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) formation and phosphorylation of Akt and glycogen synthase kinase 3 (GSK3) were comparable between WT and p110α KO platelets. In contrast, extracellular responsive kinase (ERK) phosphorylation and thromboxane (TxA_2) formation were significantly enhanced in p110α KO platelets, both of which were blocked by the MEK inhibitor PD184352, whereas the p38 MAPK inhibitor VX-702 and p110α inhibitor PIK-75 had no effect. Acetylsalicylic acid (ASA) blocked the enhancement of thrombus formation by TPO in both WT and p110α KO mice. Together, these results demonstrate that p110α negatively regulates TPO-mediated enhancement of platelet function by restricting ERK phosphorylation and TxA_2 synthesis in a manner independent of its kinase activity.

Keywords: Phosphoinositide 3-kinase, Platelets, Knockout Mice, Thrombopoietin, Thrombosis.

1.Introduction

The production of the second messenger molecule PtdIns(3,4,5)P_3 (PIP_3) in response to agonist stimulation is well established in playing a vital role supporting platelet activation and thrombus formation. This second messenger molecule is the product of the class I phosphoinositide 3-kinases (PI3K) phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP_2). All four catalytic isoforms of PI3K (p110α, p110β, p110δ, and p110γ) are expressed in platelets with evidence using pharmacological agents and genetic models determining that the p110β isoform plays a dominant role in regulating platelet function[1-5] , whereas the other isoforms (p110α, p110δ, and p110γ) are thought to play important ancillary roles in promoting platelet activation[6-10].

Initial work examining the role of the p110α isoform using the isoform selective inhibitor PIK-75 suggested that it was involved in supporting GPVI-induced platelet activation[1, 11] and thrombus formation. However, these findings could be attributed to PIK-75's inhibitory action on multiple PI3K isoforms at higher concentrations[6, 12, 13]. Use of genetic models and appropriate pharmacological inhibition has in fact determined that loss/inhibition of p110α does not dampen platelet activation in response to platelet agonists or result in reductions in thrombus formation[5, 6, 14].

However, p110α does appear to play a role in regulating the ability of primers, which do not induce platelet aggregation by themselves, but potentiate platelet activation induced by low agonist doses and can increase platelet adhesion and thrombus growth on collagen. The priming abilities of both IGF-1 and antiphospholipid antibodies were found to be altered using isoform selective inhibitors and mice with megakaryocyte/platelet lineage-specific inactivation of p110α, with p110β also playing a supporting role[6, 14].

Here, we extended these studies to the haematopoietic cytokine thrombopoietin (TPO). The primary physiological function of this cytokine is to regulate megakaryocyte (MK) differentiation/maturation and platelet production by binding to the cellular homologue of the myeloproliferative leukaemia virus oncogene (c-Mpl) receptor[15]. Platelets also express c-Mpl and binding of TPO enhances platelet activation by platelet agonists via JAK2 and PI3K[16] and increasing TxA_2 production via ERK1/2[17, 18] . We hypothesised that deletion of p110α would result in a reduction in the ability of TPO ability to enhance platelet activation and thrombus formation. However, in contrast we demonstrate that deletion of p110α results in significant enhancements of GPVI-mediated platelet activation and thrombus formation in the presence TPO. This is the first study to demonstrate a negative role for the PI3K isoform p110α in regulating platelet function and thrombosis.

2. Materials and Methods

2.1 Materials

Cross-linked collagen-related peptide (CRP-XL) was from R. Farndale (Department of Biochemistry, University of Cambridge, UK). Fibrillar HORM collagen (type I) derived from equine tendon from Takeda (Linz, Austria). Recombinant murine thrombopoietin (TPO) was from PeproTech (London, UK). D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem (Merck Chemicals, Watford, UK). 3,3'-Dihexyloxacarbocyanine iodide ($DiOC_6$) and TxB_2 ELISA kit were from Enzo Life Sciences (Exeter, UK). CHRONO-LUME® reagent was from CHRONO-LOG (Labmedics, Abingdon, UK). Akt^{S473} (#4060), Akt^{T308} (#13038), Akt (#9272), $ERK1/2^{T202/Y204}$ (#9101), GSK-3α/$\beta^{S21/9}$ (#9331), $JAK2^{Y1007/1008}$ (#3771), JAK2 (#3230), p110α (#4249), Phospho-(Ser) PKC Substrate (#2261), STAT5α/β^{Y694} (#4322), and STAT5α/β (#9358) were from Cell Signaling Technology (New England Biolabs, Hitchin, UK). GAPDH (#sc-25778) antibody was from Santa Cruz Biotechnology (Insight Biotechnology, Middlesex, UK). PD184352, VX-702 and TGX-221 were from Bio-Techne (Abingdon, UK). PIK-75 and A66 were from Cayman Chemicals (Cambridge Bioscience, Cambridge, UK). Secondary antibodies for immunoblotting were from Jackson Immunoresearch (Stratech Scientific, Ely, UK). All other reagents were from Sigma (Poole, UK), unless otherwise indicated.

2.2 Mice

All animal studies were approved by the local research ethics committee at the University of Bristol, UK and mice were bred and maintained for this purpose under the United Kingdom Home Office project license PPL30/3445. p110α flox/flox:Pf4-Cre mice are as previously described[6]. p110αflox/flox:Pf4-Cre- mice are henceforth referred to as wild-type (WT) and p110α flox/flox:Pf4-Cre+ mice as knockout (KO).

2.3 Murine platelet preparation

Age and sex-matched mice, 8-24 weeks of age were sacrificed by rising CO_2 inhalation, in accordance with Schedule 1 of the Animals (Scientific Procedures) Act (ASPA), 1986. Blood was drawn from the inferior vena cava into a syringe containing 4% trisodium citrate (1:10 v/v). Washed platelets were then prepared as previously described[6, 19].

2.4 Platelet aggregation

Platelet aggregation was monitored as previously described[20]. Briefly, washed platelets (2×10^8/mL) were stimulated with agonist and aggregation monitored using a CHRONO-LOG 490-4D aggregometer at 37°C under stirring conditions.

2.5 ATP secretion

ATP secretion as a measure of platelet δ-granule secretion was monitored in a 96-well plate using the luciferin-luciferase reagent; CHRONO-LUME® [52-54]. Luminescence was monitored using a Tecan Infinite M200 PRO plate reader (Tecan Group Ltd, Switzerland). ATP standard (0.8 nM) was employed to calibrate the readings and results are expressed as area under the curve.

2.6 *In vitro* thrombus formation

In vitro thrombus formation assays were performed under non-coagulating conditions, as previously described[6, 21, 22]. Briefly, anticoagulated blood labelled with $DiOC_6$ (10 min) was perfused at 1000 s^{-1} for 2 min over either collagen-coated (50 μg/mL) Ibidi μ-Slide VI 0.1 (Thistle Scientific Ltd, UK) flow-chambers or Vena8 glass-bottomed biochips (Cellix Ltd Microfluidic Solutions, Ireland). Vena8 glass-bottomed biochips (Cellix Ltd Microfluidic Solutions, Ireland) were used for analysing the effect of ASA on thrombus formation to improve resolution of platelet deposition as a monolayer. Platelets were fixed by perfusing 4% paraformaldehyde solution across each of the test channels for 2 min. Non-adherent cells and excess fixative were removed by flushing channels. Data was imaged using a Leica SP5-II confocal laser scanning microscope attached to a Leica DMI 6000 inverted epifluorescence microscope (Leica Microsystems, UK). Confocal z-stacks (512 x 512 pixels, stack distance 1 μm) from five randomly chosen fields of view per channel were captured with a 40X oil-immersion objective. Quantification of surface coverage was performed with Image J 1.46 (NIH, USA) and Volocity 6.1.1 quantitation software (Perkin Elmer Inc, USA).

2.7 Platelet phosphoinositide measurement by lipidomic mass spectrometry

PtdIns(3,4,5)P_3 generation was measured as previously described[23]. Briefly washed platelets were treated as indicated and reactions terminated by the addition of ice-cold 1 M hydrochloric acid. Platelets were pelleted (12, 000 xg, 10 min, 4^{o}C) and snap-frozen. Mass spectrometry was used to measure inositol lipid levels essentially as previously described [24], using a QTRAP 4000 mass spectrometer (SCIEX, AB Sciex UK Ltd, Warrington, UK) and employing the lipid extraction and derivatization method described for cells in suspension, with the modification that 10 ng C17:0/C16:0 PtdIns(3,4,5)P3 internal standard (ISD) and 100 ng C17:0/C16:0 PtdIns ISD were added to primary extracts, and that final samples were dried in a speed-vac concentrator rather than under N2.

2.8 Protein extraction and immunoblotting:

Washed platelets ($4x10^8$/mL) were treated as indicated and lysed directly in 4X NuPAGE sample buffer containing 0.5 M DTT. Lysates were analysed by SDS-PAGE/western-blotting using 7% (w/v) bis-tris gels as previously described[25]. Proteins were visualised by ECL or near-infrared detection using a LI-COR® Odyssey imaging system. Densitometry was conducted using Image J or LI-COR® Image Studio.

2.9 Measurement of TxA_2 production

To assess TxA_2 levels, the stable metabolite TxB_2 was analysed using a commercial ELISA kit (Enzo Life Sciences, Exeter, UK) as previously described [21]. Briefly, platelets ($4×10^8$/mL) in the absence of indomethacin were stimulated under non-stirring conditions (37^{o}C) before addition of EDTA (5 mM) and indomethacin (200 μM). Platelets were pelleted (4 min, 12,000 ×g) and supernatant collected. TxB_2 levels were assessed according to manufacturer's protocol.

2.10 Data Analysis

Data were analysed using GraphPad Prism 7 software. All data are presented as the mean ± s.e.m of at least three independent observations. Data presented with statistical analysis were tested using one-way/two-way ANOVA as appropriate with Sidak's multiple comparison post-hoc test.

3. Results

3.1 Deletion of p110α results in amplification of TPO's ability to enhance GPVI-induced platelet activation and thrombus formation.

TPO is unable to induce platelet functional responses (aggregation, granule secretion and thrombus formation) by itself, but is known to potentiate GPVI-mediated platelet function, δ-granule secretion and Ca^{2+} mobilization via a PI3K-dependent mechanism[26-28]. In line with these findings, we found that TPO alone did not evoke platelet aggregation or ATP secretion. However, TPO did enhanced platelet aggregation induced by a sub-threshold concentration (0.2 μg/mL) of CRP-XL (Fig.1Ai), as well as ATP release elicited by a range of CRP-XL concentrations (Fig.1B). Platelet responses to CRP-XL or TPO were unaltered in platelets where p110α was knocked-out, this agrees with our previous findings[6]. In contrast, p110α KO platelets exhibited increased responsiveness to the combined CRP-XL and TPO stimulation. This was illustrated by an increase in maximum aggregation from ~10% in the WT samples to ~70% in KO platelets (Fig. 1A), and a significant increase in the area under the aggregation curve (Fig. 1Aii). ATP secretion in response to co-stimulation was also enhanced (Fig.1B). TPO was also able to enhance protease-activated receptor (PAR)-mediated platelet functional responses in both WT and KO platelets, however we did not observe any enhancement in responsiveness to combined PAR-peptide (AYPGKF) and TPO stimulation in KO platelets (data not shown). When we examined thrombus formation on collagen at arterial shear-rates (1000 s^{-1}), we found that deletion of p110α from platelets did not alter thrombus formation on collagen (Fig. 2A, B), which is in agreement with previous studies[5, 6, 14]. Pre-treatment of whole blood with TPO (100 ng/mL) enhanced platelet deposition on collagen in both WT and KO samples (Fig.2Ai,ii). However, the amount of platelet deposition induced by TPO was greater in the KO samples compared to WT (Fig.2Ai,ii). We found that despite the area covered by thrombi (%) (Fig.2A, B) at the thrombus base (0 μm) being comparable between WT and KO samples (Fig.2A, B), there was an increase in area covered at the +4, +8 and +12 μm z-planes in KO samples, which correlated with a significant increase in total thrombus volume (Fig.2C). This increase cannot be accounted for by alterations in haematological parameters as platelet counts and mean-platelet volume were comparable between WT and KO samples (data not shown, [6]). Together these data indicate that p110α negatively regulates the ability of TPO to enhance GPVI-induced platelet activation.

3.2 Signalling directly downstream of c-MPL is unaltered in p110α KO platelets.

One potential explanation for the enhancement in TPO-mediated increases in platelet aggregation, secretion and thrombus formation in p110α KO platelets is that signalling directly downstream of the TPO receptor; c-MPL is enhanced. c-MPL doesn't possess intrinsic

tyrosine kinase activity itself and instead relies on transactivation of associated JAK2. The active/phosphorylated JAK2 will subsequently phosphorylate tyrosine residues on the receptor leading to the recruitment and phosphorylation of SH2 domain containing proteins such as STAT5α/β. As expected stimulation of platelets with TPO but not CRP-XL induced tyrosine phosphorylation of JAK2 at Tyr1007/1008 and STAT5α/β at Tyr694 (Fig.3A). Combined treatment of platelets with CRP-XL and TPO did not induce any further increases in either JAK2 Tyr1007/1008 or STAT5α/β Tyr694 phosphorylation. The deletion of p110α did not significantly alter phosphorylation of JAK2 or STAT5α/β compared to WT platelets (Fig.3Ai-iii). Together, these results demonstrate that enhancements in TPO priming of platelet function are not due to increased signalling through c-MPL/JAK2.

3.3 Enhancement of TPO-mediated priming is not due to increased flow through the PI3K pathway.

To further explore the enhancement in TPO-mediated priming observed in p110α KO platelets we examined whether there was an up-regulation in PI3K signalling. The p110α isoform belongs to the class I PI3K family of kinases which predominantly regulates cell function by preferentially phosphorylating $PI(4,5)P_2$ to the second messenger $PI(3,4,5)P_3$[29]. Platelets have an extensive $PI(3,4,5)P_3$ interactome comprising a vast array of PI3K effectors known to play important roles in platelet activation[23]. Consequently, the effect of TPO administration on GPVI-induced $PI(3,4,5)P_3$ formation was explored. CRP-XL, TPO and a combined treatment of CRP-XL and TPO were all observed to increase $PI(3,4,5)P_3$ levels in platelets (Fig.3B). The amount of $PI(3,4,5)P_3$ formation induced by these treatments was however not significantly altered in p110α deficient platelets (Fig.3B). In agreement with these findings, phosphorylation of the major PI3K effector Akt at Thr308 and of the Akt substrate GSK3 at Ser21/9 in response to CRP-XL, TPO or CRP-XL+TPO were unaltered in KO platelets compared to WT platelets (Fig.3Ci, ii, iv). Interestingly, phosphorylation of Akt at Ser473 induced by CRP-XL alone was significantly elevated in p110α KO platelets (Fig.3Ciii), supporting previous studies that suggest that the more reliable marker of Akt activity is phosphorylation of the Thr308 site[30]. Taken together, these results demonstrate that enhanced TPO-mediated priming of GPVI platelet activation in p110α-deficient platelets is not due to increased flow through the PI3K pathway.

3.4 Blockade of TxA_2 production ablates the enhancement in TPO-mediated priming in WT and p110α KO platelets.

TxA_2 is known to play an important role in the amplification of platelet responses to physiological stimuli and has been reported to contribute to TPO-mediated enhancement of platelet function [17, 18, 31]. Considering this, we were interested to investigate whether enhanced TxA_2 production may underlie the p110α KO hyperreactive platelet phenotype. Indeed, we found that pre-treatment of p110α KO platelets with TPO (100 ng/mL) resulted in a significantly greater increase in TxA_2 generation than in WT platelets (Fig. 4A). Increases in TxA_2 generation induced by TPO were concentration-dependent (3 – 100 ng/mL) with a trend for enhancement in TxA_2 in the KO platelets occurring across the concentration range (S.Fig1A). To test whether the elevation in TxA_2 production was driving the hyperreactive phenotype of p110α KO platelets, whole blood samples were pre-treated with the COX

inhibitor, ASA and platelet deposition on collagen under the influence of TPO treatment was monitored. Interestingly, ASA not only ablated the elevation in TPO-mediated priming of thrombus formation in p110α KO samples, but also blocked TPO's priming effect on WT platelets (Fig. 4B, C). Taken together, these findings suggest that (i) TxA_2 production plays a critical role in TPO-mediated priming of platelet function and thrombus formation and that (ii) enhanced TxA_2 production is a critical driver of the hyperreactive phenotype observed in p110α KO platelets.

3.5 Increased TxA_2 production is blocked by MEK1/2 inhibition and correlates with increased activation of ERK.

We were interested to explore the underlying mechanism by which p110α deletion could regulate an enhancement in TxA_2 production. Agonist-induced p38 MAPK/ERK signalling has been implicated in the activation of cytosolic phospholipase A2 (cPLA2), an enzyme that drives arachidonic acid (AA) production and subsequently triggers the formation of TxA_2 in platelets[18, 32-34]. Here we show that pre-treatment of platelets with the MEK1/2 inhibitor PD184352 resulted in a significant reduction in TxA_2 generation elicited by CRP-XL and TPO (Fig.5A). Furthermore, in the presence of PD184352 the level of TxA_2 generation in p110α KO samples was comparable to WT. In contrast, optimal concentrations of the p38 inhibitor VX-702 and the p110α inhibitor PIK-75 did not alter TxA2 generation (Fig.5A), whereas the p110β inhibitor TGX-221 blocked responses under all conditions. The findings with PIK-75 suggest that the p110α KO platelet phenotype is not due to an absence of p110α kinase activity; similar findings were observed with the p110α inhibitor A66 (S.Fig1B). The results with PD184352 and VX-702 highlight that ERK but not p38 plays an important role in regulating the enhancement in TxA_2 synthesis observed in p110α KO platelets. In support of these findings, we found that phosphorylation of ERK1/2 at Thr202/Tyr204 was significantly enhanced in p110α KO platelets (Fig.5Bi, ii), whereas the phosphorylation of p38 at Thr180/Tyr182 and of the PKC substrate pleckstrin were unaltered (Fig.5Bi, iii, iv). Together, these results demonstrate an important role for ERK in TPO-mediated enhanced TxA_2 synthesis in p110α KO platelets in a manner that is independent of the loss of p110α kinase activity.

4. Discussion

TPO is unable to independently induce platelet aggregation and granule secretion, but in combination with an array of physiological stimuli can enhance platelet functional responses[16, 26, 31, 35, 36]. Platelets play a pivotal role in thrombosis, a process that contributes to the pathogenesis of cardiovascular disease[37, 38]. Elevated levels of TPO brought about by various clinical conditions or chronic cigarette smoking may contribute to platelet hyperreactivity and consequently cardiovascular disease[39-45]. PI3K p110α has previously been shown to be involved in primer-mediated enhancement of platelet function, therefore we explored the contribution of p110α to TPO-mediated enhancement of platelet function. Surprisingly, we found that p110α negatively regulates TPO's ability to enhance GPVI-induced platelet activation and thrombus formation, in a process dependent on alterations in ERK signalling and TxA_2 production.

This is the first study to demonstrate that exogenous addition of TPO can potentiate platelet deposition and thrombus formation on a collagen-coated surface under conditions of arterial shear ($1000s^{-1}$). TPO treatment not only significantly increased the collagen area covered with platelets, but also significantly enhanced the total thrombus volume compared to vehicle-treated platelets. In contrast, van Os and colleagues[46] found that TPO did not enhance platelet thrombus formation on collagen type III under conditions of shear. The difference may be explained by the use of collagen type I in our study, which comprises of large fibres that have higher thrombogenic activity and induce more potent activation of GPVI in platelets than collagen type III[47, 48].

In this study, we observed that genetic deletion of p110α from platelets heightened the ability of TPO to enhance CRP-XL-mediated platelet aggregation and thrombus formation on collagen. This suggests a role for p110α as a negative regulator of TPO-induced increases of GPVI-mediated platelet function. This enhancement was not due to upregulation of other PI3K isoforms as genetic deletion of p110α does not cause alterations in the expression levels of other class I PI3K isoforms in this specific p110α murine model[6]. Although this is the first time that PI3K p110α has been demonstrated to negatively regulate platelet function, similar negative regulatory roles of p110α have previously been reported in BON (human endocrine cell line) cells, whereby the isoform negatively regulates secretion; while, in cardiomyocytes p110α negatively controls GPCR (G protein-coupled receptor)-induced ERK and Akt activation[49, 50]. In platelets however, p110α's capability to negatively regulate TPO's priming effect on platelet function is likely to occur via a PI(3,4,5)P_3-independent mechanism, as PI(3,4,5)P_3 generation in WT and KO samples was comparable. Additionally, TPO did not significantly enhance GPVI-induced phosphorylation of the PI3K effector; Akt at Thr308 or Akt's substrate; GSK3, in p110α KO platelets. Consequently, the amplification of TPO-mediated enhancements may be due to negative regulation of a pathway downstream of and/or parallel to PI3K. Alternatively, it may suggest that p110α acts as a negative regulator via a kinase-independent mechanism. Indeed, we found that the platelet p110α phenotype could not be mimicked by the presence of the p110α inhibitor PIK-75. Kinase-independent functions of p110α have recently been described in cells with gain-of-function mutations in the p110α gene (PIK3CA; hot spot mutations in E545K/H1047R)[51]. Lipid kinase-independent functions have also been demonstrated for the Class I PI3K isoforms p110β and p110δ, which contribute to insulin signalling and cell survival respectively[52, 53]. Considering the other reported lipid kinase-independent functions of the Class I PI3K isoforms in other cells, it is plausible that p110α can negatively regulate TPO-mediated enhancement of platelet function via a similar mechanism.

TPO's priming effect on GPVI-mediated ATP release (δ-granule secretion), ERK phosphorylation and TxA_2 production were also elevated in p110α KO platelets. Pasquet and colleagues have previously demonstrated that TPO can enhance GPVI-induced Ca^{2+} mobilization[26], which in turn results in PKC activation and subsequent ERK activation[54]. ERK has been shown to regulate $cPLA_2$ and subsequently TxA_2 generation in platelets[18]. Interestingly, we found that the COX inhibitor, ASA, which inhibits TxA_2 synthesis, not only (i) ablated the elevated TPO-mediated enhancement of thrombus formation on collagen

demonstrated by p110α KO platelets but (ii) blocked TPO-induced enhancements in WT samples also. Enhanced TPO-mediated increases in ATP secretion in the p110α KO platelets did not appear to compensate for the loss of TxA_2 production following ASA treatment. Indeed, TPO-mediated potentiation of GPVI-induced platelet aggregation can occur in the presence of the ADP scavenger, apyrase, suggesting that TPO-induced functional enhancement is at least partially ADP-independent[26]. Studies have also demonstrated that ASA inhibits TPO-mediated enhancement of ADP-induced secondary aggregation, possibly explaining the inability of ADP to compensate for the loss of TxA_2 production in whole blood samples treated with ASA[17]. Taken together these data demonstrate that TPO-mediated enhancement of GPVI-induced platelet function is driven by TxA_2 generation and this process is negatively regulated by PI3K p110α.

In conclusion, we have demonstrated (i) the ability of TPO to enhance thrombus formation on collagen and (ii) that the PI3K isoform p110α negatively regulates TPO-mediated enhancement of TxA_2 generation and consequently platelet function. This is the first study to show a kinase- independent negative regulatory role for PI3K p110α in platelet function.

5. Author Contributions

T. A. Blair designed and performed experiments, analysed data, contributed to discussion and wrote the manuscript. S. F. Moore designed and performed experiments, analysed data, contributed to discussion and wrote the manuscript. T.G. Walsh, J. L. Hutchinson and T.N. Durrant performed research and analysed data. K. E. Anderson performed lipidomic analysis and analysed data. A. W. Poole contributed to discussion. I. Hers conceived the experiments, supervised the project, contributed to discussion and wrote the manuscript.

6.Acknowledgements

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Figure Legends

Figure 1. Deletion of p110α enhances TPO-mediated priming of CRP-XL-induced platelet aggregation and δ-granule secretion. **A)** Representative aggregation traces (i) and area under the curve analysis of platelet aggregation (ii) induced by sub-maximal CRP-XL (0.2 μg/mL) in WT and KO platelets ($2x10^8$/mL) in the absence or presence of TPO (100 ng/mL, 5 min). Aggregation was monitored (5 min, 37°C) under continuous stirring conditions (1200 rpm) using a CHRONO-LOG 490-4D aggregometer. Data demonstrates that deletion of p110α from platelets results in a significant enhancement in TPO-mediated priming of CRP-XL-induced platelet aggregation. n =3 ± s.e.mean. **B)** Analysis of ATP release as a measure of δ-granule secretion from WT and KO platelets stimulated with CRP-XL in the absence or presence of TPO (100 ng/mL, 5 min). ATP release was monitored using CHRONO-LUME® reagent on a Tecan Infinite M200 PRO plate reader. Data are expressed as area under the curve (AU), n = 4 ± s.e.mean. Statistical analysis was performed using a two-way ANOVA followed by Sidak's multiple comparison post-hoc test. **; $p < 0.01$.

Figure 2. Deletion of p110α results in amplification of TPO-mediated priming of thrombus formation on collagen. A) Representative images of platelet deposition on collagen acquired from the collagen base (z-position = 0 μm) at 4 μm increments to a max thrombus height of 12 μm as indicated. Fluorescently labelled anti-coagulated whole blood from WT **(i)** and p110α KO mice **(ii)**, pre-treated with either vehicle (HEPES-Tyrode's) or TPO (100 ng/mL, 5 min) was perfused at an arterial shear rate of 1000s-1 over collagen-coated (50 μg/mL, 2 min) Ibidi μ-Slide VI 0.1 (Thistle Scientific Ltd, UK) flow-chambers, before fixation with 4% PFA. Images were acquired using a Leica SP5-II confocal LSM. **B)** Analysis of area coverage and **C)** total thrombus volume performed using Image J 1.46 (NIH, USA) and Volocity 6.1.1 (Perkin Elmer Inc, USA) revealed that deletion of p110α results in an amplification of TPO's ability to enhance the area covered by platelets (%) across the various z-positions and the total thrombus volume (μm^3). Data represent the average results taken from 5 random microscopic fields per experimental condition, n = 7 ± s.e.mean. Statistical analysis was performed using a two-way ANOVA followed by Sidak's multiple comparison post-hoc test. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

Figure 3. Deletion of p110α does not increase flow through the PI3K pathway. **A)** Representative immunoblot examining the activation of JAK2/STAT5 induced by CRP-XL (1 μg/mL), TPO (100 ng/mL) and CRP-XL+TPO. Histograms demonstrate phosphorylation of JAK2 at Tyr1007/1008 and STAT5α/β at Tyr694 are comparable between WT and KO platelets, n = 4 ± s.e.mean . **B)** Histograms of C38:4 PI(3,4,5)P3 levels in WT and KO platelets stimulated with CRP-XL (1μg/mL, 5 min) in the absence or presence of TPO (100 ng/mL). PI(3,4,5)P3 levels were determined using phosphate methylation in conjunction with HPLC/MS. Data are peak area measurements, n = 3 ± s.e.mean. **C)** Representative immunoblot examining activation of PI3K signalling induced by CRP-XL, TPO and CRP-XL+TPO. Histograms demonstrate that phosphorylation of Akt at Thr308 and GSK3α/β at Ser21/9 was not significantly altered in KO platelets. In contrast phosphorylation of Akt at Ser473 was significantly enhanced in response

to CRP-XL in KO platelets compared to WT. n = 4/5 ± s.e.mean. Statistical analysis was performed using a two-way ANOVA followed by Sidak's multiple comparison post-hoc test. **; $p < 0.01$.

Figure 4. Amplification of TPO-mediated priming of thrombus formation is driven by enhanced TxA_2 production. **A)** Histogram of TxB_2 in supernatants collected from platelets stimulated with CRP-XL (0.5 μg/mL), TPO (100 ng/mL) and CRP-XL+TPO. TxB_2 levels were quantified by ELISA (Enzo Life Sciences, Exeter, UK). The data demonstrate that TxA_2 production induced by CRP-XL+TPO is enhanced in KO platelets, n = 5 ± s.e.mean. **B)** Representative images of platelet deposition on collagen acquired from the collagen base (z-position = 0 μm) at 4 μm increments to a max thrombus height of 12 μm as indicated. Fluorescently labelled anti-coagulated whole blood from WT and p110α KO mice, pre-treated with TPO (100 ng/mL, 5 min) in the absence **(i)** or presence **(ii)** of acetylsalicylic acid (ASA, aspirin; 30 μM) was perfused at an arterial shear rate of 1000s-1 over collagen-coated (50 μg/mL) Vena8 glass-bottomed biochips (Cellix Ltd Microfluidic Solutions, Ireland), before fixation with 4% PFA. Images were acquired using a Leica SP5-II confocal LSM. **C)** Analysis of total thrombus volume performed using Image J 1.46 (NIH, USA) and Volocity 6.1.1 (Perkin Elmer Inc, USA) revealed that ASA ablated TPO's ability to amplify thrombus formation. Data represent the average results taken from 5 random microscopic fields per experimental condition, n = 6 ± s.e.mean. Statistical analysis was performed using a two-way ANOVA followed by Sidak's multiple comparison post-hoc test. **; $p < 0.01$.

Figure 5. MEK/ERK pathway is implicated in driving enhanced TxA2 production in p110α KO platelets. **A)** Histogram of TxB_2 concentration in supernatants collected from platelets pre-treated with 0.2% DMSO, PD184352 (300 nM), VX-702 (300 nM), PIK-75 (50 nM) or TGX221 (200 nM) for 15 min before stimulation with CRP-XL (0.5 μg/mL, 5 min) in the absence or presence of TPO (100 ng/mL, 5 min pre-treatment). TxB_2 levels were quantified by ELISA (Enzo Life Sciences, Exeter, UK). The data demonstrate that TxA_2 production induced by CRP-XL+TPO was enhanced in KO platelets and that this enhancement was reduced in the presence of PD184352. TGX221 was found to ablate TxA2 generation induced by CRP-XL alone and the combined treatment. n = 4 ± s.e.mean. **B)** Representative immunoblot examining the activation of MAPK and PKC pathways in WT and p110α KO platelets. Histogram demonstrates that CRP-XL and CRP-XL+TPO-mediated phosphorylation of ERK1/2 at Thr202/Tyr204 was significantly enhanced in KO platelets, n = 7 ± s.e.mean. In contrast phosphorylation of p38 at Thr180/Tyr182 and of the PKC substrate; pleckstrin was unaltered in KO platelets, n = 4 ± s.e.mean. Statistical analysis was performed using a two-way ANOVA followed by Sidak's multiple comparison post-hoc test. **; $p < 0.01$, ***; $p < 0.001$.

Figure 1

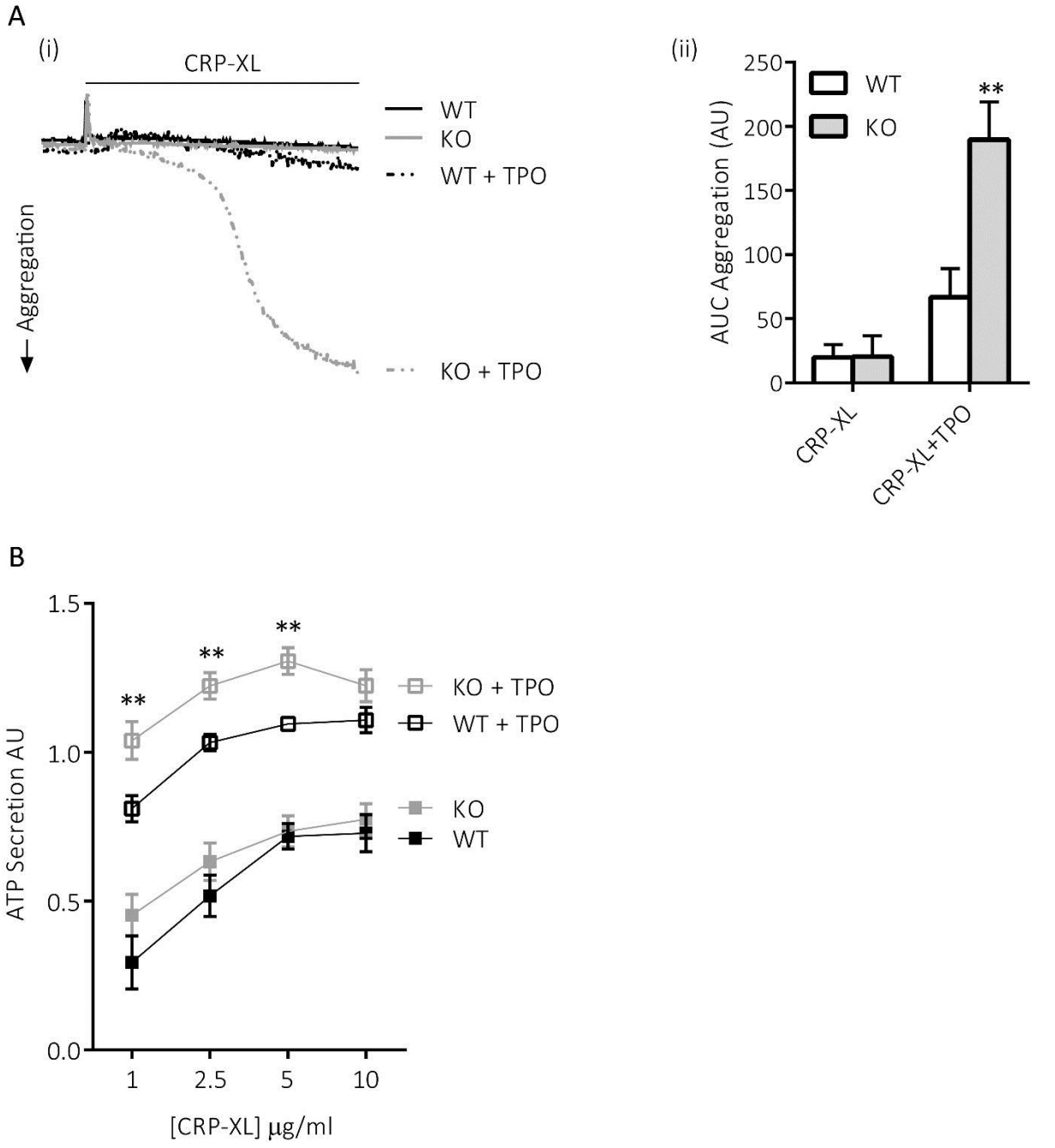
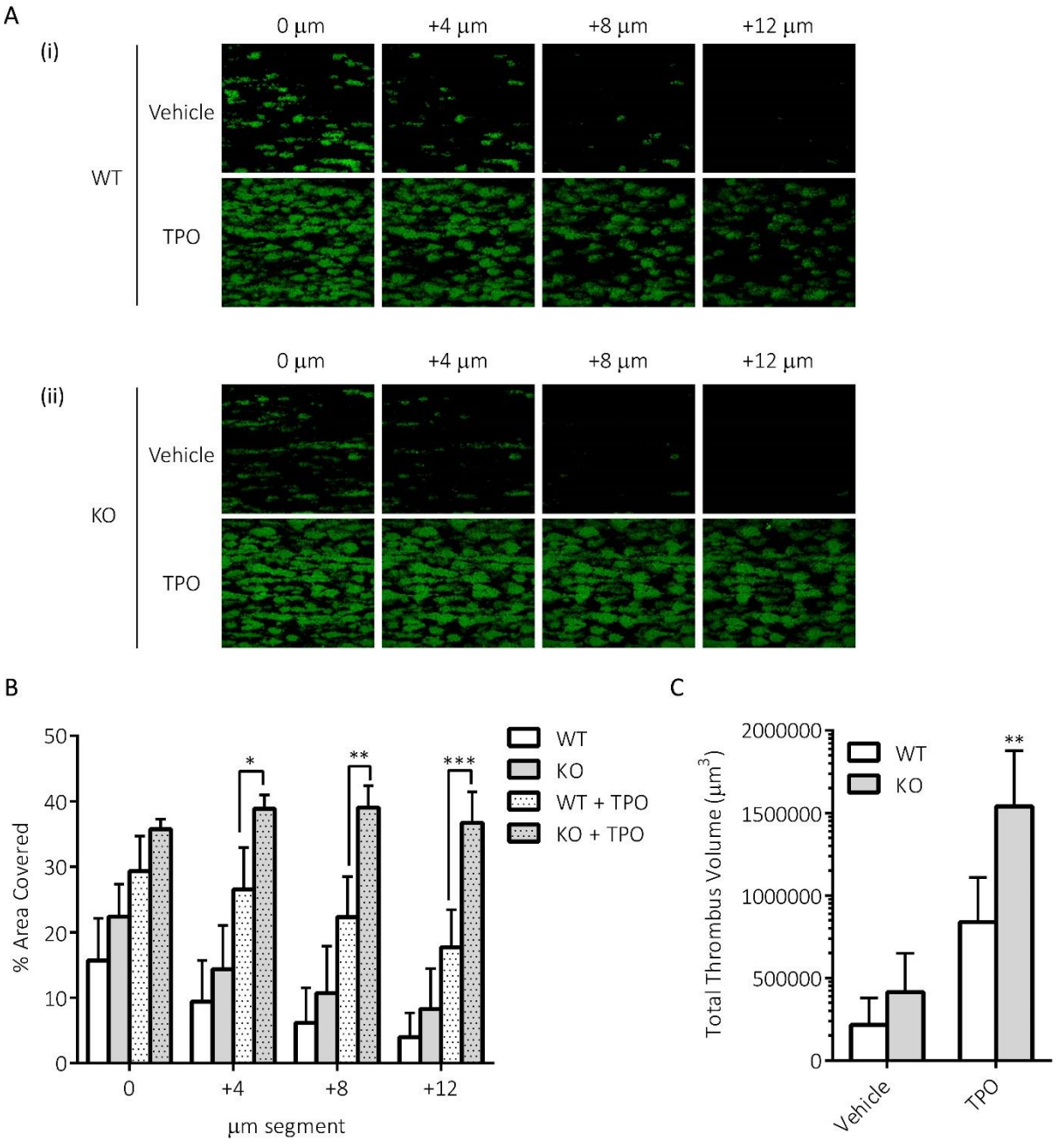

A
(i)
CRP-XL
WT
KO
WT + TPO
KO + TPO
Aggregation
(ii)
AUC Aggregation (AU)
250
200
150
100
50
0
WT
KO
**
CRP-XL
CRP-XL+TPO
B
ATP Secretion AU
1.5
1.0
0.5
0.0
**
**
**
KO + TPO
WT + TPO
KO
WT
1
2.5
5
10
[CRP-XL] µg/ml

Figure 2


A
(i)
0 µm
+4 µm
+8 µm
+12 µm
Vehicle
WT
TPO
(ii)
0 µm
+4 µm
+8 µm
+12 µm
Vehicle
KO
TPO
B
% Area Covered
50
40
30
20
10
0
*
**
***
WT
KO
WT + TPO
KO + TPO
0
+4
+8
+12
µm segment
C
Total Thrombus Volume (µm3)
2000000
1500000
1000000
500000
0
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WT
KO
Vehicle
TPO

Figure 3

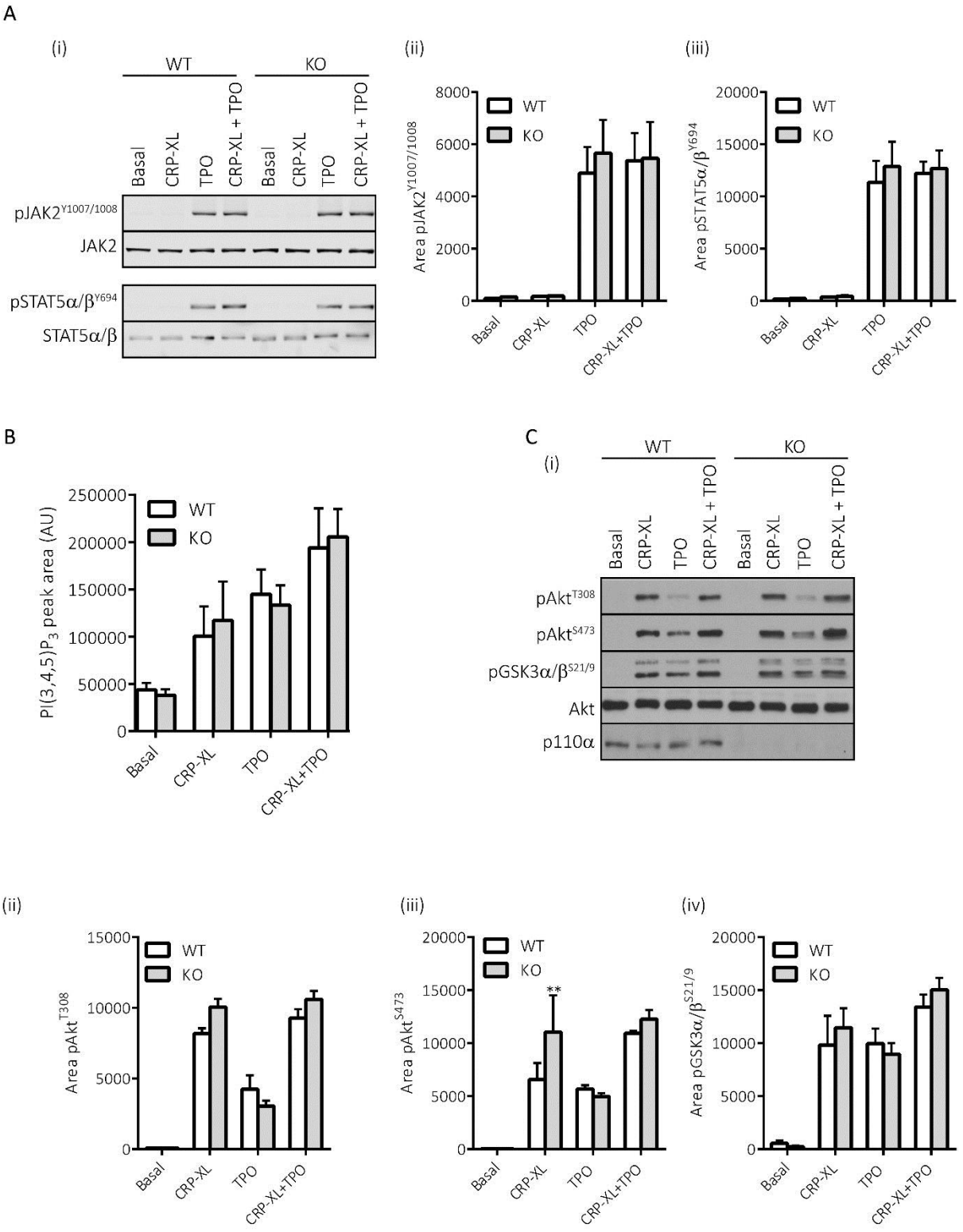
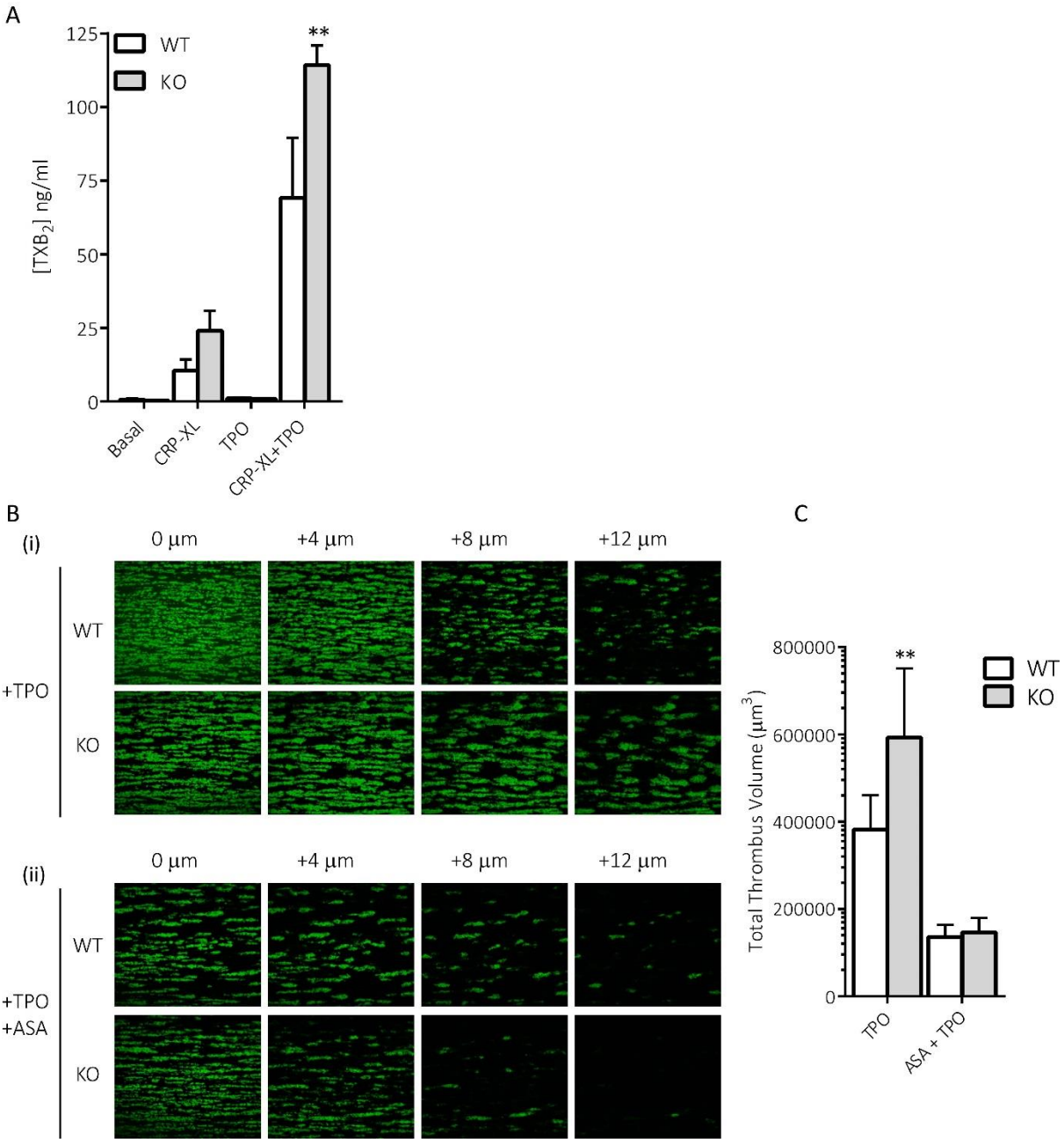

A
(i)
WT
KO
Basal
CRP-XL
TPO
CRP-XL + TPO
pJAK2Y1007/1008
JAK2
pSTAT5α/βY694
STAT5α/β
(ii)
Area pJAK2Y1007/1008
WT
KO
Basal
CRP-XL
TPO
CRP-XL+TPO
(iii)
Area pSTAT5α/βY694
B
PI(3,4,5)P3 peak area (AU)
C
(i)
pAktT308
pAktS473
pGSK3α/βS21/9
Akt
p110α
(ii)
Area pAktT308
(iii)
Area pAktS473
**
(iv)
Area pGSK3α/βS21/9

Figure 4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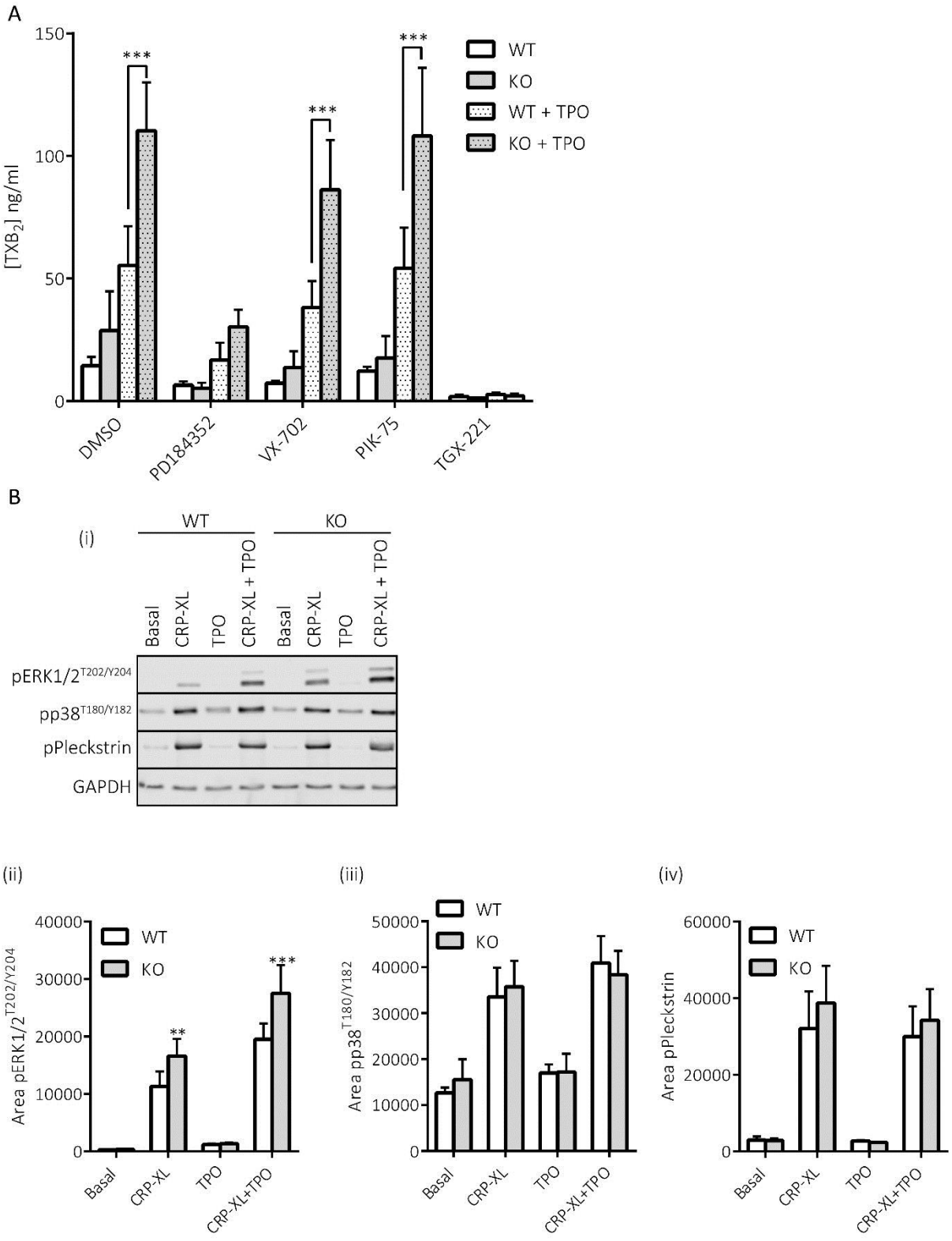
125
100
75
50
25
0
WT
KO
**
[TXB2] ng/ml
Basal
CRP-XL
TPO
CRP-XL+TPO
B
(i)
0 µm
+4 µm
+8 µm
+12 µm
+TPO
WT
KO
(ii)
+TPO
+ASA
C
800000
600000
400000
200000
0
Total Thrombus Volume (µm3)
TPO
ASA + TPO

Figure 5


A
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50
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[TXB2] ng/ml
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WT
KO
WT + TPO
KO + TPO
DMSO
PD184352
VX-702
PIK-75
TGX-221
B
(i)
WT
KO
Basal
CRP-XL
TPO
CRP-XL + TPO
pERK1/2T202/Y204
pp38T180/Y182
pPleckstrin
GAPDH
(ii)
Area pERK1/2T202/Y204
40000
30000
20000
10000
0
**
***
WT
KO
Basal
CRP-XL
TPO
CRP-XL+TPO
(iii)
Area pp38T180/Y182
50000
40000
30000
20000
10000
0
WT
KO
Basal
CRP-XL
TPO
CRP-XL+TPO
(iv)
Area pPleckstrin
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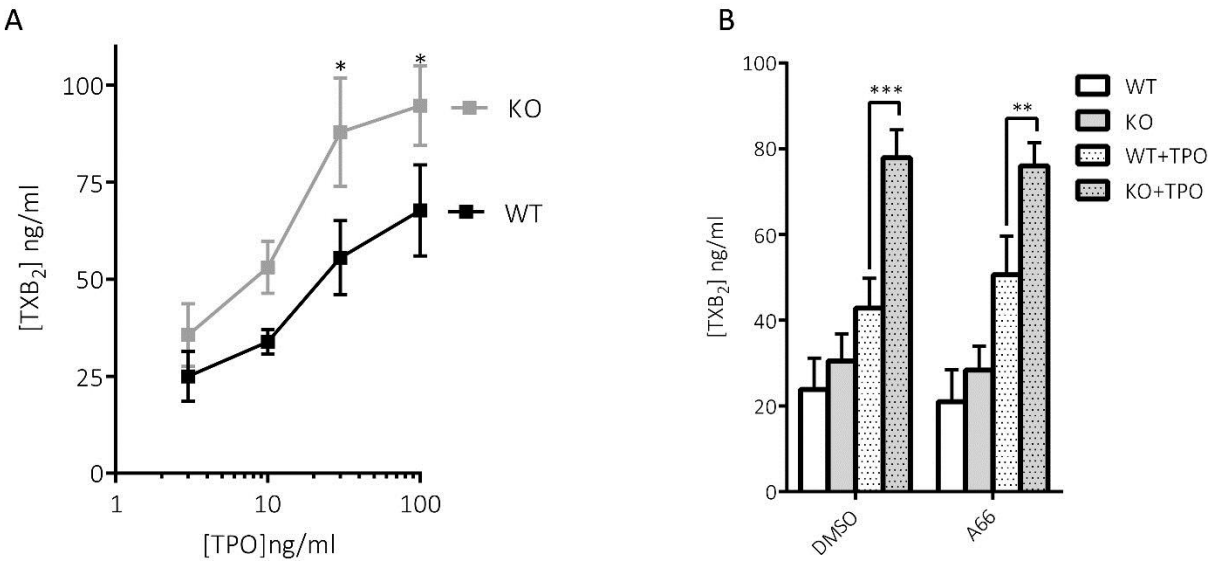
CRP-XL
TPO
CRP-XL+TPO

Data Supplement

S.Figure1



Supplementary Figure 1. A) Pre-treatment of platelets with TPO at indicated concentrations can enhance CRP-XL mediated TxB_2 generation, furthermore these increases in TxB_2 generation are greater in p110α deficient (KO) platelets compared to WT control. n = 3 ± s.e.mean. **B)** Histogram of TxB_2 concentration in supernatants collected from platelets pre-treated with 0.2% DMSO or the p110α inhibitor; A66 (300 nM) for 15 min before stimulation with CRP-XL in the absence or presence of TPO. n = 5 ± s.e.mean. TxB_2 levels were quantified by ELISA (Enzo Life Sciences, Exeter, UK). Statistical analysis was performed using a two-way ANOVA followed by Sidak's multiple comparison post-hoc test. *; $p<0.05$, **; $p< 0.01$, ***; $p< 0.001$.