Phosphatidylinositol transfer protein-α in platelets is inconsequential for thrombosis yet is utilized for tumor metastasis

Liang Zhao, Chelsea L. Thorsheim, Aae Suzuki, Timothy J. Stalker, Sang H. Min, Lurong Lian, Gregory D. Fairn, Shamshad Cockcroft, Amy Durham, Sriram Krishnaswamy & Charles S. Abrams

Platelets are increasingly recognized for their contributions to tumor metastasis. Here, we show that the phosphoinositide signaling modulated by phosphatidylinositol transfer protein type α (PITPα), a protein which shuttles phosphatidylinositol between organelles, is essential for platelet-mediated tumor metastasis. PITPα-deficient platelets have reduced intracellular pools of phosphoinositides and an 80% reduction in IP₃ generation upon platelet activation. Unexpectedly, mice lacking platelet PITPα form thrombi normally at sites of intravascular injuries. However, following intravenous injection of tumor cells, mice lacking PITPα develop fewer lung metastases due to a reduction of fibrin formation surrounding the tumor cells, rendering the metastases susceptible to mucosal immunity. These findings demonstrate that platelet PITPα-mediated phosphoinositide signaling is inconsequential for in vivo hemostasis, yet is critical for in vivo dissemination. Moreover, this demonstrates that signaling pathways within platelets may be segregated into pathways that are essential for thrombosis formation and pathways that are important for non-hemostatic functions.

DOI: 10.1038/s41467-017-01181-4
Platelets are best known for their contribution to hemostasis, but several lines of evidence indicate that they also contribute to tumor metastasis. For example, elevated platelet counts are associated with a poor prognosis in patients with cancer, whereas low platelet counts are associated with reduced metastatic burden. Additionally, studies suggest that platelets assist tumor cell adhesion to the vasculature and enhance tumor cell growth. Aberrant platelet activation and aggregation are frequently found in the vasculature of cancer patients, especially in patients with metastatic tumors. Furthermore, studies that use genetically engineered mice with conditional targeting strategy. Exons 8, 9, and 10 were targeted by the insertion of loxP recombination sites to generate PtdIns synthesis in thrombin-stimulated platelets derived from expressed in platelet lysates using isoform-specific antibodies. Shown is the relative abundance of individual PITP isoforms in platelets from wild-type mice when normalized to recombinant PITP protein standards (*p < 0.05, unpaired Student’s t-test). Since PtdIns contain hydrophobic acyl side chains that render them insoluble in the aqueous cytoplasm, they are assumed to always be associated within a lipid bilayer. Curiously, the sites of synthesis of different PtdIns are on the surfaces of distinct cellular organelles. For example, phosphatidylinositol is synthesized in the endoplasmic reticulum (ER), while PtdIns(4)P is synthesized on the surface of internal granules, such as on endosomes, and PtdIns(4,5)P2 is synthesized on the cell membrane. This implies that the sequential synthesis of polyphosphorylated phosphatidylinositols, such as PtdIns(4,5)P2, begins with...
phosphatidylinositol production in the ER, and the intermediate isoforms are trafficked between the organelles before final production in the plasma membrane. We and others have shown that the sequential enzymatic steps required for higher-order phosphoinositide synthesis occur within seconds14-28. Consequently, a mechanism for rapidly transferring phosphatidylinositol between membranes is required.

Phosphatidylinositol transfer proteins (PITP) facilitate the transfer of insoluble phosphatidylinositol from one membrane to another in vitro. They achieve this by binding and encompassing the fatty acid side chains of phospholipids and transporting the phospholipids throughout the cytoplasm. Mammalian cells contain both PITPα and PITPβ isoforms, which are small, highly conserved, and ubiquitously expressed single PITP domain soluble proteins. Ablation or knock down of PITPα impairs viability, membrane trafficking, and cytokinesis22-25. Because the phosphatidylinositol transfer activity of PITPα is required for the synthesis of phosphoinositides and for the regulation of PtdIns kinases, it has been assumed that PITPα is essential for all of the signaling events that utilize phosphoinositides, including platelets26, 27.

In this study, we investigate whether platelet-specific disruption of phosphatidylinositol metabolism mediated by PITPα impacts hemostatic and non-hemostatic functions of platelets in tumor dissemination. Our study unexpectedly shows that PITPα, the major PITP isoform in murine platelets, is inconsequential for hemostasis in vivo. However, PITPα-mediated PtdIns(4,5)P2 synthesis and IP3 production in platelets are critical to promote platelet-mediated lung metastasis of intravenous injected tumor cells. We demonstrate that signaling pathways required for thrombosis formation may be independent from those pathways required for non-hemostatic platelet functions, such as those pathways that regulate interactions with immune cells.

Results

Conditional deletion of the PITPα in murine platelets. Platelets, like other mammalian cells, contain both the PITPα and PITPβ isoforms. We observed that the PITPα isoform is approximately six-fold more abundant than PITPβ in murine platelets (Fig. 1a and Supplementary Fig. 1). To determine the role of PITPα in platelets, we genetically deleted PITPα from murine platelets and megakaryocytes (Fig. 1b). To accomplish this, the PITPα gene was conditionally targeted at the domain previously demonstrated to be essential for the phosphatidylinositol transfer function of PITPα22, 28 and the resultant mice were crossed with transgenic mice expressing the CRE recombinase, whose expression was controlled by the megakaryocyte-specific platelet-factor-4 promoter (Pf4-Cre)29. We found no detectable full-length or truncated forms of PITPα protein in the lysates of platelets derived from Pf4-Cre-positive PITPα floxed mice (Pitpαfl/fl/Pf4-Cre+ mice) by immunoblotting. The PITPβ protein expression remained unchanged in the Pitpαfl/fl/Pf4-Cre+ mice, with similar expression levels to those in the control Pitpα+/fl/Pf4-Cre+ mice (Fig. 1c).

The Pitpαfl/fl/Pf4-Cre+ mice were grossly normal, with body weight, organ morphology, leukocyte counts, and hemoglobin levels that were indistinguishable from the littermate controls (Pitpα+/fl/Pf4-Cre+ mice) (Supplementary Fig. 2). Although there was a 15% decrease in platelet counts in the Pitpαfl/fl/Pf4-Cre+ mice (Fig. 1d), it is still within normal range. The mice grew to adulthood without any observable increase in spontaneous hemorrhage, thrombosis, or mortality.

PITPα is essential for platelet phosphoinositide signaling. To study the role of PITPα in the synthesis of phosphoinositides in platelets, we analyzed the concentration of specific phosphoinositides within platelets from Pitpαfl/fl/Pf4-Cre+ mice by using thin layer chromatography (TLC). These platelets had a 34.7% reduction of PtdIns(4,5)P2 and a 41.2% reduction in PtdIns(4,5)P3 at resting conditions (Fig. 1e). When stimulated with thrombin, the deficiency remained in PITPα-null platelets (30.5% reduction of PtdIns(4)P levels and 34.1% reduction of PtdIns(4,5)P2 levels), demonstrating that PITPα is required for the synthesis of higher-order phosphoinositides in platelets.

Following thrombin stimulation, PtdIns(4,5)P2 in platelets is hydrolyzed by phospholipase C to generate second messengers, such as IP3. We analyzed IP3 synthesis in thrombin-stimulated platelets that lack PITPα, in order to test whether phosphoinositide transfer is required for rapid IP3 production. Although the baseline IP3 level in platelets derived from Pitpαfl/fl/Pf4-Cre+ mice was not different from the control mice, the IP3 generation following thrombin stimulation was blunted by approximately 80%, an effect that was apparent as early as 5 s after agonist stimulation (Fig. 1f). Furthermore, the intracellular calcium levels were lower in Pitpαfl/fl/Pf4-Cre+ platelets following thrombin stimulation (Supplementary Fig. 3). These data indicate that PITPα-mediated synthesis of PtdIns(4,5)P2 contributes to IP3 formation, which occurs within seconds after agonist stimulation. It is noteworthy that although PITPα is only required for the synthesis of approximately 40% of platelet PtdIns(4,5)P2 (Fig. 1e), it is required for a much larger percentage of the IP3, that is synthesized after agonist stimulation (Fig. 1f). Since IP3 is derived entirely from PtdIns(4,5)P3, these results suggest that PITPα may be required for the production of the specific pool of PtdIns(4,5)P3 that is utilized for IP3 formation.

PITPα is inconsequential for platelet aggregation. To determine whether the deletion of PITPα impacts platelet function, we compared the ex vivo aggregation of PITPα-null platelets with those of the littermate controls using light transmission aggregometry. PITPα-null platelets aggregated normally in response to the platelet agonists that include thrombin, collagen, ADP, PMA, and U46619, at all dosages tested (Fig. 2a and Supplementary Table 1). Ex vivo platelet dIbβ integrin activation (JON/A) and secretion (P-selectin) measured by flow cytometry were also normal (Supplementary Table 2). These results were unexpected given the defects in phosphoinositide synthesis and second messenger formation observed in PITPα-null platelets.

To determine whether these results also pertained to platelet function in vivo, we investigated whether platelets lacking PITPα formed thrombi normally by using three established murine models. First, we analyzed whether the loss of PITPα in platelets affected tail bleeding times. By analyzing a large number of animals (90 Pitpαfl/fl/Pf4-Cre+ mice and 40 Pitpα+/fl/Pf4-Cre+ mice), we could demonstrate that the bleeding time was longer in the Pitpαfl/fl/Pf4-Cre+ mice (median time 58.5 s) when compared with the Pitpα+/fl/Pf4-Cre+ mice (median time = 29.0 s, p < 0.005, unequal Student’s t-test; Fig. 2b). However, the average blood volume lost was comparable between the Pitpαfl/fl/Pf4-Cre+ and Pitpα+/fl/Pf4-Cre+ mice (Supplementary Fig. 4). Since the absolute differences in bleeding time were small, there was a large amount of variability in this assay even within mice of the same genotype, and the results may be influenced by platelet-independent factors such as coagulation and vascular muscle tone, we analyzed the ability of these mice to form intravascular thrombi using several alternative methods. Platelet-mediated in vivo thrombosis were directly tested using a FeCl3 carotid injury model by analyzing thrombus formation in response to a chemical injury. Using this method, we could not identify any difference in the rate of thrombus formation between
Platelet PITpα is essential for melanoma cell metastasis. In addition to hemostasis, platelets have been implicated in other processes, such as the formation of tumor metastases [31]. To determine whether PITPα-mediated phosphoinositide

Taken together, our studies demonstrate that the predominant platelet PITP isoform, PITPα, does not contribute significantly to platelet-mediated hemostasis ex vivo or in vivo.

Fig. 2 Platelet PITPα is inconsequential for hemostasis. a Platelets lacking PITPα were analyzed after agonist stimulation in a Lumi-Dual aggregometer. Platelets derived from Pitpα knockout mice exhibited essentially no defect in aggregation in response to all analyzed agonists. b Tail bleeding times in mice lacking PITPα. Pitpαfl/flPf4-Cre− mice (n = 90) and Pitpαfl/flPf4-Cre+ mice (n = 40) were analyzed for the duration of their tail bleeding. The median difference was 29.5 s (p < 0.005, unpaired Student’s t-test). c Carotid arteries of Pitpαfl/flPf4-Cre+ mice (n = 5) and Pitpαfl/flPf4-Cre− mice (n = 7) were subjected to a ferric chloride induced injury, and blood flow was monitored as a measure of thrombus formation. The time-to-form occlusions (TTFO) of the blood flow was not found to be significantly different between mice lacking PITPα in their platelets and the control mice. Statistical analysis was performed using an unpaired Student’s t-test. Error bars are s.d. (d, e). Laser-induced injury model demonstrates normal in vivo thrombosis and platelet secretion in Pitpαfl/flPf4-Cre+ mice. Shown is platelet accumulation (d) and P-selectin exposure (e) in response to a laser-induced injury to mouse cremaster arteries. The area under the area/time curve and peak area are shown. The graphs show the means for n = 15 injuries in three Pitpαfl/flPf4-Cre+ mice and n = 25 injuries in three Pitpαfl/flPf4-Cre− mice. Statistical analysis was performed using an unpaired Student’s t-test. Shown are the means ± s.d. for all figure panels.

Pitpαfl/flPf4-Cre+ mice and Pitpαfl/flPf4-Cre− mice (Fig. 2c). We also analyzed thrombosis formation and platelet α-granule secretion in response to a laser-induced vascular injury [30]. As shown in Fig. 2d, the loss of platelet PITPα did not significantly decrease platelet accumulation at the injured vessel wall. Similarly, PITPα-null platelets translocated normal amounts of P-selectin onto their surface (Fig. 2e), indicating that these genetically modified platelets have no obvious defect in α-granule secretion in vivo, confirming the previous ex vivo secretion data.
Fig. 3 Loss of PITPα in platelets impairs tumor metastasis formation. a The lungs derived from Pitpα−/−P4-Cre− and Pitpαfl/flP4-Cre− mice harvested 2 weeks after tail vein injection with B16F10 melanoma cells. Shown are representative lungs. The number of tumor nodules on the lung surface was reduced in Pitpα−/−P4-Cre− mice when compared with Pitpαfl/flP4-Cre− controls. Scale bar is 10 mm. *p < 0.0001, unpaired Student’s t-test. b Similarly, 3 weeks after tumor injection, the lungs of the Pitpα−/−P4-Cre− mice contained less metastasis than the Pitpαfl/flP4-Cre− mice as indicated by their weights. Scale bar is 10 mm. **p < 0.0001, unpaired Student’s t-test. c H&E staining of tumors in lung tissue sections at 3 weeks after tumor injection show no significant fibrosis or hemorrhage. Black scale bars represent 100 μm. d Shown are serial platelet counts analyzed after tumor injection. Control mice have an abrupt decrease in their platelet counts 3 h after tumor injection. This reduction was markedly blunted in the Pitpα−/−P4-Cre− mice (n = 6 for each genotype, ***p < 0.05, unpaired Student’s t-test). Shown are the means ± s.d. e H&E staining for lung tissues at 3 h after tumor cell injection demonstrated tumor-induced thrombi formation within lung tissue. The stars indicate the tumor-induced thrombi. Black scale bars represent 200 μm. f Quantification of CD41-positive thrombi formation observed within lung tissue of the Pitpα−/−P4-Cre− mice (n = 4) and the Pitpαfl/flP4-Cre− control mice (n = 5). ***p = 0.02, unpaired Student’s t-test. Error bars are s.d. g Immunohistochemistry analysis of tumor-induced thrombi in lung tissues at 3 h after tumor injection staining for CD41 (platelet marker) and TRP1 (B16F10 tumor cells). The arrows indicate the tumor cells. This demonstrates that the tumor cells are coated with large clusters of platelets in the Pitpα−/−P4-Cre− mice. This phenomenon was less frequently found in the Pitpαfl/flP4-Cre− mice. Black scale bars represent 100 μm. h Ex vivo adhesions of platelets on tumor cell layers indicate impaired interactions between Pitpα−/−P4-Cre− platelets and tumor cells. N = 3 mice per group. Shown are the mean ± s.d. for all figure panels.
metabolism in platelets is involved in tumor dissemination, we utilized a well-characterized B16F10 melanoma model of tumor metastasis. In this model, the quantity of tumor foci were measured following intravenous injection of B16F10 melanoma cells in mice. Two weeks after tumor cell injection, Pitpα/β/Pf4-Cre+/− mice developed 50% fewer tumor foci on the surface of their lungs as compared to the littermate controls (p < 0.0001, unpaired Student’s t-test, Fig. 3a). Furthermore, 3 weeks after tumor cell injection, the lung weights of freshly dissected Pitpα/β/Pf4-Cre+/− mice were 50% lower than those harvested from Pitpα/β/Pf4-Cre−/− mice (p < 0.0001, unpaired Student’s t-test, Fig. 3b). The histology showed that compared to the Pitpα/β/Pf4-Cre−/− mice, Pitpα/β/Pf4-Cre+/− mice did not develop more hemorrhages or fibrosis in their lungs at 3 weeks after tumor injection (Fig. 3c). This suggested that the increased lung weight in the control mice is due to the metastatic burden of the tumors. Thus, these data indicate that platelet PITPα contributes to the dissemination of intravenously injected B16F10 melanoma cells.

To determine whether this observation was limited to B16F10 melanoma cells, Lewis lung carcinoma (LLC) cells also were injected intravenously, and metastasis formation was evaluated at 2 weeks after tumor injection by counting the number of tumor foci on the lung surface (Supplementary Fig. 5). Again, Pitpα/β/Pf4-Cre+/− mice developed significantly less lung metastasis when compared to their littermate controls, demonstrating that the relationship between Pitpα/β/Pf4-Cre+/− platelets and metastasis formation is not unique to a specific tumor cell line.

One possible mechanism by which platelet PITPα contributes to the dissemination of tumor cells was revealed as control mice, but not Pitpα/β/Pf4-Cre−/− mice, quickly become thrombocytopenic just 3 h after injecting the tumor cells (Fig. 3d). Platelet counts in the control mice dropped 70%, while Pitpα/β/Pf4-Cre−/− mice had relatively stable platelet counts. Histologic analyses of the lung tissue at the 3 h time point after tumor cell injection revealed that Pitpα/β/Pf4-Cre−/− mice have significantly fewer (Fig. 3e, f) and smaller (Fig. 3g) thrombi in their lungs and pulmonary vasculature than those found in the control mice. These data suggest that the formation of thrombi mediated by platelet PITPα contributes to the thrombocytopenia induced by the tumor cells.

Previous studies have supported the hypothesis that a direct in vivo interaction (adhesion) between platelets and tumor cells can facilitate the implantation of the metastasis and promote tumor cell invasion and propagation. To determine whether PITPα facilitates platelet interactions with B16F10 tumor cells, we characterized the pulmonary thrombi in more detail by immunohistochemical staining at 3 h after tumor injection. We observed that at the core of these thrombi, there were a small number of tumor cells that were enveloped by platelets. This shroud of platelet-rich thrombi was much larger in the control mice than that found in the Pitpα/β/Pf4-Cre−/− mice (Fig. 3h). Correspondingly, PITPα-null platelets have significantly reduced ex vivo adhesion to tissue-cultured tumor cells than PITPα wild-type platelets (Fig. 3h). Together, these findings suggest that PITPα in platelets promotes platelet–tumor interaction and platelet-rich thrombi around tumors.

Tumor-induced thrombin and fibrin generation requires PITPα. Since hemostatic plugs are often composed of aggregated platelets that are woven together by fibrin, we analyzed the tumor-induced thrombi for fibrin by immunohistochemistry. We observed that the area of fibrin staining was about six-fold higher in the lung tissues of the control mice when compared to the Pitpα/β/Pf4-Cre−/− mice (Fig. 4a, b). Platelets support fibrin formation by providing a negatively charged surface for the prothrombinase components (FVα and FXa) to assemble and activate thrombin. This occurs once...
platelets flip the negatively charged phospholipid phosphatidylserine from the inner leaflet of their cell membrane to the outer leaflet. Using the Annexin V-binding assay to detect phosphatidylserine exposure on the outer membrane leaflet of platelets, we observed that Annexin V-binding was significantly reduced in PITPα-null platelets after activation by agonists (thrombin at 0.1 U/ml and collagen at 5 µg/ml) when compared to the control platelets (Fig. 4c). This indicates that PITPα-null platelets have an impaired ability to externalize negatively charged lipids on their surface, which in turn causes defects in prothrombinase complex assembly, prothrombin activation, and ultimately fibrin generation.

B16F10 melanoma cells have been shown to express high levels of tissue factor on their surface, thereby allowing these tumor cells to initiate the coagulation cascade. To analyze the contribution of platelet PITPα to B16F10 melanoma cell-induced prothrombin activation, we utilized a well-described fluorometric thrombin generation assay (TGA) to investigate the impact of platelet PITPα on thrombin generation in platelet-rich plasma (PRP). When tumor cells were added to PRP that was derived from the Pitpαfl/−Pf4-Cre+ mice, we observed 80% less thrombin generation when compared to PRP derived from the controls (Fig. 4d, e). This effect was platelet-dependent, since thrombin generation in platelet poor plasma (PPP) was identical between the two genotypes. We also analyzed the ability of Pitpαfl/−Pf4-Cre+ mouse-derived PRP to support thrombin generation when stimulated by standard tissue factors, and we found similar, albeit smaller, effects (Supplementary Fig. 6). A peculiar finding is that thrombin generation is further suppressed in PRP from Pitpαfl/−Pf4-Cre+ mice in comparison to that seen in PPP (Fig. 4d). Alternatively, the explanation for this observation is unclear, it suggests that platelets lacking PITPα suppress the ability of tumor cell tissue factor to initiate coagulation.

Under normal circumstances, generation of IP3 within platelets stimulates a rise in cytoplasmic calcium, which in turn exposes phosphatidylserine on the surface of platelets to support thrombin generation. We hypothesized that the defect in thrombin generation found in Pitpαfl/−Pf4-Cre+ plasma was due to impaired IP3 production, as shown previously, which caused low cytoplasmic calcium concentrations. To test this hypothesis, we artiﬁcially raised the intracellular calcium concentration and analyzed thrombin generation in PRP. We used ionophore A23187, which transports extracellular calcium into the platelet cytoplasm, as well as thapsigargin (TG), which can mobilize intracellular calcium by an IP3-independent mechanism. Both reagents have been demonstrated to activate platelets by increasing cytoplasmic free calcium levels. Both Pitpαfl/−Pf4-Cre+ and Pitpαfl/−Pf4-Cre− PRP generated a similar level of thrombin in the presence of the ionophore (10 µM) or TG (2 µM) (Fig. 4f, g). This demonstrates that increasing the intracellular calcium completely reverts the thrombin generation defect induced by the loss of PITPα. Thus, PITPα within platelets contributes to tumor metastasis through a signaling pathway that involves IP3 formation, calcium inﬂux, phosphatidylserine externalization, and thrombin generation. Together, these data demonstrate that platelet PITPα is required for tumor-induced thrombin generation in vitro and thrombus formation in vivo.

Platelet PITPα impairs the development of bronchus-associated lymphoid tissue (BALT) hyperplasia. Although Pitpαfl/−Pf4-Cre+ mice developed less platelet–tumor adhesions than control mice during the first few hours after tumor injection (Fig. 3e–g), these results were not sufficient to explain the long-term effect of platelet PITPα on metastasis formation. To address this issue, we analyzed the histology of tumor metastasis over time. Two days after injection of the tumor cells, Pitpαfl/−Pf4-Cre+ mice developed strikingly hyperplastic BALT, a reaction that was essentially absent in the controls (Fig. 5a–d). This hyperplasia primarily consisted of hematopoietic-derived CD45R+ cells, including T cells (CD3+, neutrophils (Ly-6G/C+), and natural killer cells (NKP46+) (Fig. 5e–h). Thus, the initial stages of tumor implantation in mice lacking platelet PITPα are associated with inflammatory reactions that involve multiple types of leukocytes. These data suggest that signals generated by PITPα in platelets protect tumor cells from immune-mediated cytotoxicity, and thereby enable tumor cell survival and propagation in both the vasculature and in the tissues.
Anticoagulation limits metastasis in Pitpαfl/flPf4-Cre− mice.

Tissue factor-initiated coagulation has been demonstrated to protect circulating tumor cells from elimination by immune surveillance, which facilitates tumor metastasis, and B16F10 melanoma cells express high levels of tissue factor35. To understand whether tumor cell-induced thrombin generation and fibrin formation mediated by PITPα in platelets contributes to metastasis, we investigated the effects of a coagulation FVIIa inhibitor, nematode anticoagulant protein c2 (NAPc2)41 injection on the lung metastasis formation of B16F10 melanoma cells in vivo.

NAPc2 treatment in vivo inhibited lung metastasis when analyzed 2 weeks after injection in both genotypes. Anticoagulation reduced metastasis formation and thrombocytopenia in both genotypes (Fig. 6a, b). Furthermore, the effect of the PITPα mutation in platelets was completely reverted by anticoagulation. Histological analyses on lung tissue sections showed no significant thrombi formation at 3 h after injection, and no BALT hyperplasia developed 48 h after injection (Supplementary Fig. 7). Together, these data suggest that tumor-initiated coagulation contributes to tumor metastasis in normal mice, and that this effect can be impaired by either anticoagulation or by the PITPα mutation in platelets.

To confirm that the hemostatic process mediated by PITPα in platelets contributes to the lung metastasis of intravenous injected B16F10 melanoma cells, we investigated the impact of platelet PITPα on the growth of tumor cells injected subcutaneously into the flanks of mice. The data show that the flank tumors that developed in Pitpαfl/flPf4-Cre+ mice are similar to those that developed in the control mice (Supplementary Fig. 8), demonstrating that tumor cells grow at equivalent rates within the two groups. Stated alternatively, since this model does not depend on coagulation or mucosal immunity, there is no influence on tumor formation by the loss of PITPα in platelets.

Discussion

Clinical observations and experimental animal models have demonstrated that platelets support tumor metastasis formation.
Our work confirms these findings and expands on these previous studies by establishing three additional and surprising observations: (1) Loss of PITPα in platelets causes only a partial deficit in phosphoinositide synthesis, yet it results in a major defect in second messenger formation; (2) In spite of a major biochemical defect in PITPα-null platelets, there is no obvious hemostatic defect; and (3) The loss of PITPα in platelets leads to a defect in tumor-induced thrombosis formation that affects anti-tumor immunity. The data shown in this study demonstrate that PITPα-mediated phospholipid signaling in platelets contributes to lung metastasis of intravenously injected tumor cells, but has no significant impact on hemostasis. Together, this demonstrates that signaling pathways in platelets that are important for hemostasis can be distinct from those pathways involved in non-hemostatic functions.

Our data demonstrates that the loss of PITPα leads to the moderate loss of PtdIns(4,5)P₂, but curiously leads to a far greater loss of its product, IP₃. This suggests that PITPα is generating a particular pool of PtdIns(4,5)P₂ that is critical for the formation of second messengers, such as IP₃. Our previous work on PIP5KI (the enzyme required for the final step of PtdIns(4,5)P₂ synthesis) demonstrated that second messengers, including IP₃, are generated exclusively from a pool of recently synthesized PtdIns(4,5)P₂. Together, these data show that in order to make second messengers, platelets require a cooperative effect between PITPα and PIP5KI to rapidly synthesize PtdIns(4,5)P₂ in discrete microdomains.

Since PITPα is required to generate normal amounts of phosphoinositides and second messengers within platelets, we anticipated finding defects in platelet-mediated hemostasis. However, we were surprised to see that the substantial amount of phosphoinositide synthesis mediated by PITPα does not contribute to pathways required for in vivo or ex vivo hemostasis. Our previous work has demonstrated that the loss of specific pools of PtdIns(4,5)P₂ produced by PIP5KI within microdomains of platelets led to discrete platelet defects. Similarly, we speculate that microdomains of PtdIns(4,5)P₂ production, that are regulated by PITPα, are tightly coupled to calcium levels in proximity to PS scramblases. In this scenario, the loss of PITPα would only diminish the pool of phosphoinositides required for PS exposure, and with the remaining pools of phosphoinositides, calcium and PS exposure would be sufficient for normal hemostatic function. Alternatively, stated, we hypothesize that PITPα contributes to the synthesis of discrete pools of phosphoinositides within specific microdomains in platelets, and these pools are not required for hemostasis.

We observed that platelets lacking PITPα do not efficiently adhere to tumor cells in vivo and ex vivo by a mechanism that is related to a deficiency in thrombin generation and fibrin formation. As shown in model (Fig. 6c), PITPα-null platelets cannot form platelet–fibrin complexes that usually envelop tumor cells. This prevents thrombocytopenia in Pitpα/fl PitP4-Cre+ mice following tumor cell injection. Without the platelet–fibrin shroud surrounding these tumors, these malignant cells are more vulnerable to mucosal immune responses that block tumor cell implantation. The evidence shown in this study demonstrates that impaired IP₃-induced calcium release is the critical mechanism that prevents metastasis in Pitpα/fl PitP4-Cre+ mice. Influx of intracellular calcium activates membrane scramblase activity and exposes phosphatidylserine on the platelet surface. This facilitates tenase and prothrombinase complexes that lead to thrombin generation and fibrin polymerization around the tumor cells. Together, these findings demonstrate that PITPα-mediated signaling in platelets indirectly augments the early stages of tumor dissemination.

While dysfunction of PITPα-null platelets seems to be the major contributor for decreased metastasis, it is important to note that there was a 15% decrease in platelet count for these mice. Both clinically and experimentally, this small degree of thrombocytopenia was not found to be critical for hemostasis. In addition, the data in this study demonstrate that the mild thrombocytopenia in Pitpα/fl PitP4-Cre+ mice also did not contribute to the dissemination of this tumors. This is based on the observation of the pronounced acute thrombocytopenia in control mice after tumor injection that was not evident in the Pitpα/fl PitP4-Cre+ mice (Fig. 3d). Consequently, when compared to the control mice, there were actually more platelets in the circulation of the Pitpα/fl PitP4-Cre+ mice during the critical early hours after tumor injection even though the metastasis defect was occurring. Thus, the inability of the tumors to metastasize could not be due to mild basal thrombocytopenia in the Pitpα/fl PitP4-Cre+ mice, but rather due to an intrinsic defect of platelet function.

In 1985, Dr. Armand Trouseau first described the association between cancer and thrombosis. 10. Ironically, years later, Dr. Trouseau himself tragically succumbed to widespread thrombosis caused by his pancreatic cancer. Tumor cells are known to activate platelets, which in turn facilitates tumor implantation, adhesion, propagation and invasion of the tumor mass. The study demonstrates the vital role of platelet phosphoinositide signaling in the dissemination of cancer. Furthermore, this work also clearly distinguishes the platelet signaling processes required for hemostasis from those processes that augment metastasis formation. Additional studies will be required to determine whether targeting specific non-hemostatic platelet signaling pathways could be exploited in the development of clinical therapeutics.

Methods

Mice. Animals were maintained on standard chow and tap water. Animal procedures and experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Pitpα conditional mice, Pitpα+ mice, and PitP4-Cre transgenic mice were used in this study. The genetic background of all mice used was C57BL/6, and both sexes of mice were used for all experiments unless noted in the methods. The ages of mice used were between 8 and 20 weeks. To produce mice with conditional targeting of the murine Pitpα gene, a 10.8 kb genomic fragment was identified in a C57BL/6 (RPCI23: 105D24) BAC library and then cloned into the pSP72 vector (Promega). The “long homology arm” was 7.42 kb in length, and the “short homology arm” was 1.83 kb in length. The PGK-gb2 loxP/FRT Neo cassette was inserted into a region that corresponded to the 3′ end of exon 10, and an additional loxP site was inserted into a region that corresponded to the 5′ end of exon 8. Therefore, this targeting design removed a 1.55 kb region of Pitpα genomic DNA that included exons 8, 9, and 10. The targeting vector was confirmed by PCR, restriction analysis, and sequencing after each modification step. Additional confirmation of the final clones was performed by Southern blot analysis.

The neomycin cassette flanked by FRT sites was removed by crossing with flippase (FLP) transgenic mice (Jackson Lab). Transgenic mice that contain Cre recombinase driven by platelet factor-4 promoter (PitP4-Cre) (obtained as a generous gift from Radek Skoda, of the University of Basel, Switzerland) were further crossed with Pitpα conditional mice to induce the deletion of PITPα specifically in platelets and in megakaryocytes.

Genomic DNA isolated from tail biopsies was used for the genotyping of the conditional alleles of the Pitpα gene by PCR, using forward primer (5′-GAACAGAACATATCCACAGACAGAC-3′) and reverse primer (5′-CTTCCTCGCCTGTGTAATCCTAGG-3′).

Antibodies and reagents. Antibodies used in this study were as follows: mouse anti-human PITPα (5F12) (Santa Cruz Biotechnology, sc-13569; 1:400); rabbit anti-human PITPγ (Abcam, ab88795; 1:1000); rabbit anti-β-Actin (Cell Signalling, #4970; 1:2500); goat anti-mouse TRP1 (A-20) (Santa Cruz Biotechnology, sc-10446; 1:150); rat anti-mouse CD41 (MWReg30) (Santa Cruz Biotechnology, sc-19963; 1:50); rat anti-CD41 F(ab)2 fragments (MWReg30) (BD Biosciences, custom order; 0.12 µg/g body weight); anti-P-selectin (RB40.34) (BD Bioscience, custom order; 0.2 µg/g body weight); rat anti-mouse neutrophils (Ly-6G/C) (Santa Cruz Biotechnology, sc-10446; 1:150); rat anti-mouse CD3 (17A2) (BioLegend, 1:1000; rat anti-mouse CD5 (L5/85) (BioLegend, 1:150); rat anti-mouse CD35 (NP46) (BioLegend, 1:120); anti-mouse fibrin (clone 59D8, gift from Dr. Rodney M. Camire of Children’s Hospital of Philadelphia, 1:500); PE-anti-mouse/rat CD62P P-selectin (RMP-1) (BioLegend, 1:1000; 1:150); PE-labeled rat anti-mouse integrin αIIbβ3 antibody (ION/A)
CaCl₂. The aggregation was measured by the turbidometric method at 37 °C in the

Washed platelet preparation and aggregation. Washed platelets were prepared from blood collected from the inferior vena cava of anesthetized mice in the presence of 6× acid citrate dextrose buffer (ACD, pH 4.4, 85 mM sodium citrate, 12 mM NaH₂PO₄, 12 mM NaCl, 5 mM MgCl₂, 5 mM Heps, 5 mM glucose, and 0.35% BSA). For the aggregation assay, washed platelets were adjusted to 2 x 10⁷ platelets/ml by using Hepes-Tyrode’s buffer and supplemented with 1 mM CaCl₂. The aggregation was measured by the turbidometric method at 37 °C in the presence of CHRONO-LUME luciferase and Luciferin (CHRONO-LOG, #393; Vector Laboratories); and ImmPRESS reagent anti-Goat Ig (MP-7405, Vector Laboratories). Quantitation of thrombus formation. Tumor injection-induced thrombi formations in lung tissue were quantified on paraffin-embedded tissue sections. Three slides across 300 µm were collected in each sample at 100 µm intervals. Platelet-rich thrombi were identified by immunohistochemistry staining of CD41, and the numbers either in the vasculature or within the pulmonary tissue were counted under the microscope at 10X magnification from three randomly selected optical fields.

Immunohistochemistry. The slides were de-paraminized with xylene, dehydrated in ethanol, and then treated with 20 µg/ml protease K in TE buffer, pH 8.0 containing 0.5% triton X-100 for 15 min at 37 °C. The slides were then treated with 3% H₂O₂ for 15 min to block endogenous peroxidase, and then treated with 2.5% normal serum for 60 min to block non-specific antibody binding. The tissue sections were incubated with primary antibodies and peroxidase secondary antibody as instructed by the ImmPRESS reagent kit (Vector Laboratories). The signals were visualized by developing with DAB as indicated by the ImmPact DAB peroxidase substrate kit (Vector Laboratories).

Immunoblotting. Washed platelets were prepared as described above. After final centrifugation, the platelet pellet was resuspended in ice-cold RIPA buffer (Sigma-Aldrich, B0278) supplemented with Chrometri protease inhibitor (Roche). After homogenization, 50–100 µg of proteins from each sample aliquot were loaded on NuPage 4–12% Bis-Tris protein gel (Invitrogen, NP0321) and run in reducing conditions with a NuPage MOPS SDS running buffer (Invitrogen, NP0001). Gels were then blotted onto polyvinylidene fluoride membrane (Invitrogen, LC2002). After blotting, membranes were blocked with 5% Blotting Grade Blocker Non-Fat Dry Milk (BioRad #1706435XTU) in 0.1% Tween/TBS (BioRad #1706435; #1662404) for 1 h. Blots were incubated with primary antibody in blocking buffer overnight at 4 °C. Afterwards, blots were incubated with secondary antibody conjugated with HRP (anti-rabbit or anti-mouse; Cell Signaling Technology, #7074, #7076) for 1 h at room temperature. Blots were washed several times with 0.1% TWEEN/TBS between each step. Blots were developed with ECL Prime Western Blotting detection reagent (GE Healthcare Life Sciences, RPN2232), exposed on autoradiographic film (Denville Scientific, E3018), and digitized on a scanner. Uncropped immunoblots are shown in Supplementary Fig. 9.

Annexin V binding on agonist-activated platelets. The binding of Annexin V on platelets was quantified by flow cytometry47. Whole blood was collected from the tail vein in the final rest mass (Sigma, #563009) and diluted in the microtiter objective solution (Sigma, #88551; 14.34 mM NaCl, 3 mM KCl, 0.3 mM NaH₂PO₄, 12 mM NaHCO₃, 2 mM MgCl₂, 5 mM Heps, 5 mM glucose, and 0.35% BSA) that contained 2 mM CaCl₂. GPRP (Sigma,
Ca²⁺ G1895-25mg) at a final concentration of 1 nM was supplemented to prevent fibrin polymerization before the stimulation of agonists. Diluted platelets in whole blood were stimulated with an agonist combination that contained 5 μg/ml collagen and 0.1 U/ml thrombin at 37°C for 10 min. The samples were then labeled with 50 nM Annexin V-Alexa-FITC antibody (BD Biosciences, #56420; 1:20) and CD41a-PE antibody (BD Biosciences, #558040; 1:100) to identify platelets at room temperature for 30 min. The binding was analyzed by flow cytometry (BD FACSCanto II Flow Cytometry System) gating on the CD41a-positive cell population.

Flow cytometry. After collecting platelets, as described above, cells were diluted to 10³ cells/ml. Platelets (100 μl) were stained with PE-labeled rat anti-mouse integrin αIIbβ3 antibody (JON/A) (Emfret Analytics, #M023-2; 1:20) for 30 min. The tubes were then washed 2 times with PBS and resuspended with 0.1% BSA in PBS before analyzing cells on FACSCanto II Flow Cytometry System (BD Bioscience). Flow cytometry was assisted by the Flow Cytometry Core Laboratory at Children’s Hospital of Philadelphia core facility.

Thrombin generation assay. Thrombin generation in platelets was measured in the automated analyzer CEVERON alpha with a TGA module (TECHNO-THROMBIN TGA, DiaPharma) based on monitoring the formation of thrombin by means of a fluorogenic substrate upon activation of the coagulation cascade by tissue factor. Blood was withdrawn in the presence of 6x ACD, and PRP was collected after centrifuging at 200 x g for 10 min. The samples were further centrifuged for 15 min at 1500 x g to collect the PPP as controls. Platelets in PRP were countersotted, and the concentration was adjusted to 5 x 10⁵/ml. For each measurement, 40 μl of each sample, 10 μl of TGA trigger B16F10 tumor cells (1 x 10⁷/ml), and agonists were mixed in 96-well plates in duplicate. The samples were maintained at 33°C. Once 50 μl of TGA substrate was added into each well, measurements were obtained at 30 s intervals for 90 min. Thrombin generation was calculated by the manufacturer’s software utilizing a standard thrombin calibration curve that was derived separately.

Quantification of BALT hyperplasia. The BALT hyperplasia on each section was manually counted under microscope at 4x magnification after H&E staining. One section per 100 μm tissue was used for the quantification, and a total of three slides across 300 μm were collected.

Platelet counts. Peripheral blood was collected through retro-orbital puncture of Pitpα−/−/Pitpα−/−Cre mice for matching age and gender. Complete blood counts (CBC) and mean platelet volumes were analyzed using a Drew Hemavet Hemacytometer (HV1700).

Intracellular calcium flux measurement. PRP was collected and platelets were suspended to 5 x 10⁵/ml in Tyrode’s buffer without calcium. Apyrase and PGE-1 were added to prevent aggregation. Platelets were loaded with fura-2/AM (5 μM) in the presence of Fluronic F-127 (0.2 μg/ml) for 15 min at 37°C, then washed and resuspended in Tyrode’s buffer without calcium. Stirred platelets were activated with thrombin, and fluorescence was measured with an Aminco Bowman Series 2 Luminescence spectrophotometer. The excitation wavelengths alternated between 340 and 380 nm, and emission was measured at 510 nm. Each measurement was calibrated using Triton X-100 and EGTA. Fura-2 fluorescence change was determined by subtracting baseline before stimulus from peak.

Fibrin staining. Fibrin production in thrombi was stained by rapid microwave phosphotungstic acid hematoxylin (P.T.A.H) (American MasterTech) on 6 μm paraffin-embedded sections of lung tissue. De-paraffinized slides were incubated in microwave heated 10% zinc chloride solution for 15 min, washed in running tap water, and followed by incubation in microwave heated 5% ferric ammonium sulfate solution for 2 min. After washing with water, the slides were further stained for 30 min in P.T.A.H that had been pre-heated in a microwave for 10–20 s. The slides were dehydrated in 95% alcohol and then covered with a mounting slip prior to microscope analysis.

Lung metastatic analysis of intravenous injected LLC. LLC cell line was obtained from the American Type Culture Collection (Manassas, VA, USA, #CRL-1642) and cultured in Dulbecco’s Modified Eagle’s Medium that was supplemented with 10% v/v fetal calf serum (Sigma Aldrich), and 300 μg/ml L-glutamine (Invitrogen). Tumor cells from a mid-log phase culture were collected by brief exposure to 0.05% trypsin/EDTA solution, washed twice with PBS, and then resuspended in PBS at a density of 5 x 10⁵/ml. A 200 μl aliquot of tumor cells (1 x 10⁵ cells) was injected through the tail vein of the mice. The mice were euthanized at 3 weeks after injection, and perfused with 10 ml ice-cold PBS that was supplemented with 4% formalin. The lungs were carefully removed, dissected free of other connective tissues, and then fixed in 10% formalin. The tumor nodules on the lung surface were counted under microscopy. Some of the lungs were paraffin-embedded and sectioned at 6 μm for H&E staining. The tumors on tissue sections were counted under microscope at 10x magnification across 300 μm of each lung and presented as average number of tumors per section.

Flank tumors. B16F10 melanoma cells were utilized for flank tumor growth measurement. Tumor cells at 1 x 10⁶/ml in PBS were subcutaneously injected. Two weeks after injection, mice were euthanized and the tumors were dissected, weighed, and fixed in 10% formalin.

Statistics. Two-tailed unpaired or paired Student’s t-tests were applied for the comparison of two means for all analyses, except for the tail bleeding times and intravitral assay, which utilized the Mann-Whitney test. P-values of less than 0.05 were considered statistically significant.

Data availability. All relevant data are available from the authors upon reasonable request.

References

1. Sierko, E. & Wojtuskiewicz, M. Z. Platelets and angiogenesis in malignancy. *Semin. Thromb. Hemost.* 30, 95–108 (2004).
2. Costantini, V., Zacharski, L. R., Moritz, T. E. & Edwards, R. L. The platelet count in carcinoma of the lung and colon. *Thromb. Haemost.* 64, 501–505 (1990).
3. Ayhan, A. et al. The value of preoperative platelet count in the prediction of cervical involvement and poor prognostic variables in patients with endometrial carcinoma. *Gynecol. Oncol.* 103, 902–905 (2006).
4. Taucher, S. et al. Impact of pretreatment thrombocytosis on survival in primary breast cancer. *Thromb. Haemost.* 89, 1098–1106 (2003).
5. Brown, K. M., Domm, C., Aranha, G. V., Yong, S. & Shoup, M. Increased preoperative platelet count is associated with decreased survival after resection for adenocarcinoma of the pancreas. *Am. J. Surg.* 189, 278–282 (2005).
6. Kaushansky, K. Historical review: megalakaryopoiesis and thrombopoiesis. *Blood* 111, 981–986 (2008).
7. Boucharaba, A. et al. Platelet-derived lysophosphatic acid supports the progression of osteolytic bone metastases in breast cancer. *J. Clin. Invest.* 114, 1714–1725 (2004).
8. Camerer, E. el al. Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. *Blood* 104, 397–401 (2004).
9. Bakewell, S. J. et al. Platelet and osteoclast beta3 integrins are critical for bone metastasis. *Proc. Natl Acad. Sci. USA* 100, 14205–14210 (2003).
10. Jain, S. et al. Platelet glycoprotein Ib alpha supports experimental lung metastasis. *Proc. Natl Acad. Sci. USA* 104, 9024–9028 (2007).
11. Palumbo, J. S. et al. Platelets and fibrinogen increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. *Blood* 105, 178–185 (2005).
12. Ho-Tin-Noé, B. et al. Innate immune cells induce hemorrhage in tumors during thrombocytopenia. *Am. J. Pathol.* 175, 1699–1708 (2009).
13. Min, S. H. & Abrams, C. S. Regulation of platelet plug formation by phosphoinositide metabolism. *Blood* 122, 1358–1365 (2013).
14. Wang, Y. et al. Loss of PIP5KβIIbeta demonstrates that PIP5KI isoform-specific PIP2 synthesis is required for IP3 formation. *Proc. Natl Acad. Sci. USA* 105, 14064–14069 (2008).
15. Wang, Y. et al. Platelets lacking PIP5KβII have normal integrin activation but impaired cytoskeletal-membrane integrity and adhesion. *Blood* 121, 2743–2752 (2013).
16. Hartwig, J. H. et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* 82, 643–653 (1995).
17. Yang, S. A., Carpenter, C. L. & Abrams, C. S. Rho and Rho-kinase mediate thrombin-induced phosphatidylinositol 4-phosphate 5-kinase trafficking in platelets. *J. Biol. Chem.* 279, 42331–42336 (2004).
18. De Matteis, M. A. & Godi, A. PI-lotting membrane traffic. *Nat. Cell Biol.* 6, 1492 (2004).
19. Di Paolo, G. & De Camilli, P. Phosphoinositides in cell regulation and cell signaling. *EMBO Rep.* 8, 241–246 (2007).
22. Alb, J. G. Jr et al. Mice lacking phosphatidylinositol transfer protein-alpha exhibit spinocerebellar degeneration, intestinal and hepatic steatosis, and hypoglycemia. J. Biol. Chem. 278, 33501–33518 (2003).

23. Jones, S. M., Alb, J. G. Jr, Phillips, S. E., Bankaitis, V. A. & Howell, K. E. A phosphatidylinositol 3-kinase and phosphatidylinositol transfer protein act synergistically in formation of constitutive transport vesicles from the trans-Golgi network. J. Biol. Chem. 273, 10349–10354 (1998).

24. Giannanti, M. G. et al. The class I PITP giotto is required for Drosophila cytokinesis. Curr. Biol. 16, 195–201 (2006).

25. Xie, Y. et al. Phosphatidylinositol transfer protein-alpha in netrin-1-induced PLC signaling and neurite outgrowth. Nat. Cell Biol. 7, 1124–1132 (2005).

26. Cunningham, E., Thomas, G. M., Ball, A., Hiles, I. & Cockcroft, S. Phosphatidylinositol transfer protein dictates the rate of inositol trisphosphate production by promoting the synthesis of PIP2. Curr. Biol. 5, 775–783 (1995).

27. Panaretou, C., Domín, J., Cockcroft, S. & Waterfield, M. D. Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150. PtdIns 3-kinase complex. J. Biol. Chem. 272, 2477–2483 (1997).

28. Alb, J. G. Jr et al. Genetic ablation of phosphatidylinositol transfer protein function in murine embryonic stem cells. Mol. Biol. Cell 13, 739–754 (2002).

29. Tiedt, R., Schomber, T., Hao-Shen, H. & Skoda, R. C. P4f-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. Blood 109, 1503–1506 (2007).

30. Stalker, T. J. et al. Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. Blood 121, 1875–1885 (2013).

31. Gay, L. J. & Felding-Habermann, B. Contribution of platelets to tumor metastasis. Nat. Rev. Cancer 11, 123–134 (2011).

32. Eccles, S. A. Basic principles for the study of metastasis using animal models. Methods Mol. Biol. 58, 161–171 (2001).

33. Labelle, M. & Hynes, R. O. The initial hours of metastasis: the importance of cooperative host-tumor cell interactions during hematogenous dissemination. Cancer Discov. 2, 1091–1099 (2012).

34. Schumacher, D., Strlic, B., Sivaraj, K. K., Wettschureck, N. & Offermanns, S. Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. Cancer Cell 24, 130–137 (2013).

35. Kirszberg, C. et al. Simultaneous tissue factor expression and phosphatidylinositol transfer protein-alpha in neutrophils enhances at the plasma membrane. J. Biol. Chem. 264, 12266–12271 (1989).

36. Jackson, T. R., Patterson, S. I., Thastrup, O. & Hanley, M. R. A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca2+ without generation of inositol phosphates in NG115–401I neuronal cells. Biochem. J. 253, 81–86 (1988).

37. Dobryndeva, Y., Williams, R. L. & Blackmore, P. F. Trans-resveratrol inhibits calcium influx in thrombin-stimulated human platelets. Br. J. Pharmacol. 128, 149–157 (1999).

38. White, J. G., Rao, G. H. & Gerrard, J. M. Effects of the ionophore A23187 on blood platelets I. Influence on aggregation and secretion. Am. J. Pathol. 77, 135–149 (1974).

39. Gerrard, J. M., White, J. G. & Rao, G. H. Effects of the ionophore A23187 on the blood platelets II. Influence on ultrastructure. Am. J. Pathol. 77, 151–166 (1974).

40. Stassens, P. et al. Anticoagulant repertoire of the hookworm Ancylostoma caninum. Proc. Natl Acad. Sci. USA 93, 2149–2154 (1996).

41. Liu, Y., Jennings, N. L., Dart, A. M. & Du, X. J. Standardizing a simpler, more sensitive and accurate tail bleeding assay in mice. World J. Exp. Med. 2, 30–36 (2012).

42. Kimmelstiel, C. et al. Bivalirudin is a dual inhibitor of thrombin and collagen-dependent platelet activation in patients undergoing percutaneous coronary intervention. Circ. Cardiovasc. Interv. 4, 171–179 (2011).

43. Gupta, S. et al. CLP36 is a negative regulator of glycoprotein VI signaling in platelets. Circ. Res. 111, 1410–1420 (2012).

Acknowledgements

This study was supported by US Public Health Service Grants from the NIH: PO1 HL120846 and PO1 HL40387 to C.S.A.

Author contributions

L.Z., C.L.T., A.S., T.J.S., S.H.M., L.L., G.D.F., S.C., A.D., S.K., and C.S.A. designed and performed the experiments. L.Z., C.L.T., T.J.S., S.H.M., L.L., G.D.F., S.C., A.D., S.K., and C.S.A. analyzed the data. L.Z., C.L.T., A.S., T.J.S., S.H.M., and C.S.A. wrote the manuscript. L.Z., C.L.T., S.H.M., L.L., G.D.F., S.C., A.D., S.K., and C.S.A. reviewed and approved the manuscript.

Additional information

Supplementary Information accompanies this paper at doi:10.1038/s41467-017-01181-4.

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.