Published in final edited form as:

J Thromb Haemost. 2010 October ; 8(10): 2294–2304. doi:10.1111/j.1538-7836.2010.03998.x.

What Is Vinculin Needed for in Platelets?

John V. Mitsios^{*}, Nicolas Prévost^{*}, Ana Kasirer-Friede^{*}, Edgar Gutierrez[†], Alex Groisman[†], Charles S. Abrams[§], Yanfeng Wang[§], Rustem I. Litvinov[¶], Alice Zemljic-Harpe^{*,††}, Robert S. Ross^{*,††}, and Sanford J. Shattil^{*}

^{*} Department of Medicine, University of California, San Diego, La Jolla, CA 92093

[†] Department of Physics, University of California, San Diego, La Jolla, CA 92093

^{††} VA Healthcare San Diego, San Diego, CA 92161

[§] Department of Medicine and Cell, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

[¶] Department of Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Summary

Background—Vinculin links integrins to the cell cytoskeleton by virtue of its binding to proteins such as talin and F-actin. It has been implicated in the transmission of mechanical forces from the extracellular matrix to the cytoskeleton of migrating cells. Vinculin's function in platelets is unknown.

Objective—To determine whether vinculin is required for the functions of platelets and their major integrin, $\alpha\text{IIb}\beta 3$.

Methods—The murine vinculin gene (*Vcl*) was deleted in the megakaryocyte/platelet lineage by breeding *Vcl*^{fl/fl} mice with *Pf4-Cre* mice. Platelet and integrin functions were studied *in vivo* and *ex vivo*.

Results—Vinculin was undetectable in platelets from *Vcl*^{fl/fl} Cre⁺ mice, as determined by immunoblotting and fluorescence microscopy. Vinculin-deficient megakaryocytes exhibited increased membrane tethers in response to mechanical pulling on $\alpha\text{IIb}\beta 3$ with laser tweezers, suggesting that vinculin helps to maintain membrane cytoskeleton integrity. Surprisingly, vinculin-deficient platelets displayed normal agonist-induced fibrinogen binding to $\alpha\text{IIb}\beta 3$, aggregation, spreading, actin polymerization/organization, clot retraction and the ability to form a procoagulant surface. Furthermore, vinculin-deficient platelets adhered to immobilized fibrinogen or collagen normally, both under static and flow conditions. Tail bleeding times were prolonged in 59% of vinculin-deficient mice. However, these mice exhibited no spontaneous bleeding and they formed occlusive platelet thrombi comparable to wild-type littermates in response to carotid artery injury with FeCl₃.

Conclusion—Despite promoting membrane cytoskeleton integrity when mechanical force is applied to $\alpha\text{IIb}\beta 3$, vinculin is not required for the traditional functions of $\alpha\text{IIb}\beta 3$ or the platelet actin cytoskeleton.

Corresponding Author: Dr. Sanford J. Shattil, Department of Medicine, University of California, San Diego, Leichtag Biomedical Research Bldg, Room 180, 9500 Gilman Dr. La Jolla, CA. 92093-0726, sshattil@ucsd.edu.

Addendum. J.V. Mitsios- designed and performed research, analyzed data and wrote paper; N. Prevost- designed research and analyzed data; A. Kasirer-Friede- performed research and analyzed data; E. Gutierrez and A. Groisman- designed research and analyzed data; C.S. Abrams, Y. Wang and R.I. Litvinov- performed research and analyzed data, A. Zemljic-Harpe and R.S. Ross- supplied essential mice; and S.J. Shattil- designed research, analyzed data and wrote paper.

Keywords

actin; cytoskeleton; integrin; platelet; vinculin

Introduction

Vinculin is a 117 kDa protein that plays a role in cellular responses to mechanical forces [1–3]. A less abundant splice variant, metavinculin, is present in certain cells, including platelets [4]. Both proteins contain N-terminal head (residues 1–835 in vinculin) and C-terminal tail (residues 896–1066) domains connected by a flexible hinge region (residues 836–895) [5]. Vinculin is thought to be in a “closed” head-to-tail conformation in the cytoplasm of unstimulated cells. Upon cell activation, the head and tail domains dissociate, exposing binding sites for a variety of ligands implicated in cytoskeletal rearrangements, including talin, VASP, paxillin, F-actin and PIP₂ [1,5–7]. A recurrent theme in the literature is the relationship between vinculin, integrins and actin within adhesion complexes, mediated in part by interactions of the vinculin head and tail domains with talin and F-actin, respectively [7,8].

Vinculin has been studied primarily in nucleated cells. Vinculin (*Vcl*) knockout F9 embryonic carcinoma cells exhibit a round shape with fewer, less stable lamellipodia and smaller focal adhesions than wild-type F9 cells [9,10]. The knockout cells also show decreased adhesion and increased motility on matrix ligands, such as fibronectin [11]. In contrast, vinculin overexpression in mouse 3T3 fibroblasts leads to stronger adhesion and less motility [9]. Since global deletion of murine *Vcl* causes embryonic lethality by E10 due to heart and brain defects [12], investigators have begun to study the effects of vinculin depletion in specific cell types using Cre-loxP technology. For example, *Vcl* deletion in cardiac myocytes disrupts adherens junctions in the myocardium, causing death by three months of age by either sudden death or dilated cardiomyopathy with progressive heart failure [13].

In contrast to most cell types, platelets are anucleate and non-migratory, and their main physiological function is to promote hemostasis by forming thrombi at sites of vessel injury. When platelets are allowed to spread on extracellular matrices via integrin α IIb β 3, vinculin can be found at the cell periphery and within patches at the termini of F-actin cables [14,15]. Since the precise role of vinculin in platelets is unknown, we deleted vinculin selectively in the megakaryocyte/platelet lineage by mating vinculin-floxed (*Vcl fl/fl*) mice [13] with *Platelet factor 4* (*Pf4*)-*Cre* mice [16]. *Vcl fl/fl* *Cre*⁺ mice were viable and healthy and exhibited normal platelet counts, but their platelets were devoid of vinculin, affording us an unprecedented opportunity to determine what functions vinculin might be required for in these cells. Contrary to our expectations, we demonstrate here that vinculin is not required for most platelet responses in hemostasis and thrombosis, including those dependent on α IIb β 3 and the actin cytoskeleton.

Material and Methods

Generation and genotyping of mice conditionally deficient in vinculin

Mice homozygous for the vinculin floxed allele on a mixed C57BL/6-Sv129 genetic background were crossed with *Pf4-Cre* mice on a C57BL/6 background. The construction of the *Vcl* floxed mice has been detailed previously [13]. Genotypes were determined by PCR with primers VinF (5'-AAATGGGTTTGGCTCACCTG-3') and VinR (5'-TGGAGTCATCTCTCCAGCCT-3') that amplified 562-bp from wild-type allele and 679-bp of from the floxed allele [13,16]. Primers specific for the *Pf4* promoter (5'-

CCCATACAGCACACCTTTTG-3') and Cre-cDNA (5'-TGCACAGTCAGCAGGTT-3') produced a 450-bp Cre recombinase product. All mouse protocols were approved by the Animal Care Program of the University of California.

Platelet function studies in vivo

Tail bleeding assays were performed by cutting 2 to 3 mm off the tail tip and immersing the tail in isotonic saline at 37°C [17]. Bleeding times were taken as the time required for bleeding to cease for at least 60 seconds. *Platelet thrombus formation* was studied using a carotid artery FeCl₃ injury model [18]. Each side of the adventitia of the left carotid artery was treated with 5% FeCl₃-soaked filter paper for 3 minutes. Blood flow was monitored with a miniature ultrasound flow probe (T402 flowmeter, Transonic Systems, Ithaca, NY) for at least 2 minutes before and 30 minutes after application of FeCl₃. The operator was blinded to the genotype of the mice, and each experiment included vinculin-deficient mice and littermate controls.

Platelet function studies ex vivo

Blood was collected from tail veins in ethylenediaminetetraacetic acid (EDTA) for complete blood counts (Hemavet 850FS Multi Species Hematology System, Drew Scientific, Waterbury, CT). All experiments with mouse platelets included age- and sex-matched controls. To obtain platelets for functional studies, blood was collected by cardiac puncture, anti-coagulated with acid-citrate-dextrose anticoagulant, washed and finally resuspended in Walsh buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂•6H₂O, 3.3 mM NaH₂PO₄•H₂O, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4, 0.1% glucose, 0.1% bovine serum albumin (BSA)) as described [19]. Cells were lysed in NP-40-containing buffer for western blotting of proteins [18].

Platelet adhesion to immobilized fibrinogen under static conditions was determined by acid phosphatase assay and specific adhesion expressed as a percent of total platelets added to each microtiter well [19]. Platelet adhesion to immobilized fibrinogen (20 µg mL⁻¹) or collagen (50 µg mL⁻¹) in laminar flow was determined over a range of shear stresses using a microfluidic device as described [20,21]. Briefly, mouse blood was drawn by cardiac puncture into heparin-containing syringes (20 U mL⁻¹) and platelet dense granules were labeled with 2 µM mepacrine [22]. After mounting the microfluidic device on the mechanical stage of a Nikon Diaphot inverted fluorescence microscope, platelets were imaged using a GFP filter set (Ex470/Em525), a 63× (NA=1.4) oil immersion objective lens, a 0.42× video adapter, and a Sony SX900 IEEE1394 camera with a 1/2" (1280×960 pixel) CCD array.

Fibrinogen binding to agonist-stimulated platelets was determined by flow cytometry using 150 µg mL⁻¹ of Alexa-fluor 647-labeled fibrinogen (Invitrogen, Carlsbad, CA) [19]. Non-specific binding was determined in the presence of 5 mM EDTA and specific binding was taken as total minus non-specific binding. In some experiments, F-actin-dependent, irreversible fibrinogen binding to agonist-stimulated platelets was determined as described [23]. αIIbβ3 expression was measured with a FITC-conjugated anti-mouse αIIb antibody (anti-CD41; BD Biosciences). Platelet surface *P-selectin expression* was measured with a biotin-conjugated anti-mouse P-selectin antibody (BD Biosciences) and phycoerythrin-conjugated streptavidin (Invitrogen, Carlsbad, CA) [24]. For *platelet aggregation and fibrin clot retraction* [25] assays, blood was anticoagulated 9:1 with 3.8% sodium citrate and diluted 1:1 or 1:2.3 with Walsh buffer, respectively. After pooling blood from four mice of each genotype, aggregation was measured in a Chrono-log aggregometer (Chrono-log, Havertown, PA). The relative F-actin content of unstimulated platelets and platelets stimulated with a Protease-activated Receptor 4 (PAR4) agonist peptide (AYPGKF) was

determined by flow cytometry using BODIPY-phalloidin (Invitrogen, Carlsbad, CA) [26]. The ability of platelets to develop a procoagulant surface was determined by stimulating washed platelets with 1 μ M A23187 Ca^{2+} -ionophore for 15 min at 37° C, followed by flow cytometric measurement of factor Va light chain binding to platelets and platelet-derived microparticles [27].

Platelet spreading on fibrinogen-coated coverslips and actin nodule formation during platelet spreading [28] were assessed by deconvolution microscopy [19]. The extent of spreading was quantified by measuring individual platelet surface areas using Image-Pro plus software (Media Cybernetics, Inc. Silver Springs, MD, USA).

Vinculin and membrane-cytoskeletal interactions in megakaryocytes

Megakaryocytes were harvested from the femurs of adult mice and expanded and separated as described [29]. Large, mature megakaryocytes were isolated by passing the culture through a discontinuous BSA density gradient. For *immunofluorescence microscopy*, megakaryocytes were fixed and stained with rhodamine-phalloidin to label F-actin, an antibody to label vinculin (Sigma-Aldrich) and a nuclear label (Hoechst, Molecular Probes).

The ability of the plasma membranes of mature megakaryocytes to resist external mechanical pulling was assessed with fibrinogen-coated beads and laser tweezers [30]. Briefly, mouse fibrinogen (Sigma-Aldrich) was covalently coupled to 1.87- μ m carboxylate-modified latex beads (Bangs Laboratories). A mixture of megakaryocytes and beads in culture medium containing 1 mM MnCl_2 (to activate integrins) was incubated for 5–10 min in a poly-L-lysine-coated flow microscope chamber. Then beads were laser-trapped near adherent megakaryocytes, and the microscope focus was adjusted so that bead and cell centers were approximately the same distance from the bottom surface. The position of the laser trap was then oscillated in a triangular waveform with a frequency of 1 Hz and constant peak-to-peak amplitude of 2 μ m (0.2 pN nm^{-1} trap stiffness; loading rate, 800 pN s^{-1}). Bead position was sensed and digitized at a rate of 2,000 scans per second [30]. In this system, the less megakaryocyte plasma membranes are able to resist force, the greater their tendency to form long *membrane tethers* connected to the beads. Tether formation was calculated by pooling data from three independent experiments performed with cells of the same genotype.

Statistical analyses

Analyses of variance were conducted by Student's t-test except where noted. All error bars in figures represent the mean $\pm$ SEM.

Results

Selective depletion of vinculin from megakaryocytes and platelets

PCR analyses of genomic DNA isolated from ear biopsies showed that mice homozygous for the floxed vinculin allele (*Vcl fl/fl*) could be distinguished from those with a wild-type allele (*Vcl +/+*) (Fig. 1A), facilitating genetic analysis of mice mated with *Pf4-Cre* mice to generate *Vcl fl/fl Cre⁺* mice in which *Vcl* exon 3 flanked by loxP sites had been excised from mature megakaryocytes and platelets. Cre-mediated excision should result in deletion of the *Vcl* gene, which produces both vinculin and metavinculin in platelets. Western blotting of various mouse tissues confirmed depletion of vinculin from *Vcl fl/fl Cre⁺* platelets, but not from *Vcl fl/fl Cre⁺* heart, kidney, spleen or liver (Fig. 1B,C). Western blots of megakaryocytes isolated from primary bone marrow cultures of *Vcl fl/fl Cre⁺* mice showed a small amount of residual vinculin (Fig. 1C), likely the result of contamination with non-megakaryocytic cells and/or to the presence of immature megakaryocytes not yet expressing

Cre recombinase [16]. Vinculin was not detected in mature, large polyploid megakaryocytes by immunofluorescence microscopy (Fig. 1D). Although metavinculin should also be deleted in *Vcl fl/fl* Cre⁺ platelets, we could not detect it even in control *Vcl fl/fl* Cre⁻ platelets using two different antibodies. Several proteins other than vinculin were expressed at normal levels in *Vcl fl/fl* Cre⁺ platelets, including FAK, paxillin, α -actinin, kindlin-3, Erk1/2, β -actin (Fig. 1E) and α IIB β 3 (flow cytometry data not shown). Thus, *Vcl fl/fl* Cre⁺ platelets provided us with an opportunity to study platelet function in the absence of vinculin.

Platelet functions in vivo

Vinculin-deficient, *Vcl fl/fl* Cre⁺ mice were healthy, did not exhibit spontaneous bleeding and their blood counts were equivalent to those of *Vcl fl/fl* Cre⁻ littermates (Table 1). Tail-bleeding times, which are dependent in part on platelet function [31], averaged 132 ± 10 sec in control *Vcl fl/fl* Cre⁻ mice and 334 ± 26 sec in *Vcl fl/fl* Cre⁺. Fifty-nine % of the platelet vinculin-deficient mice had tail bleeding times that exceeded the highest value observed in *Vcl fl/fl* Cre⁻ littermate controls, and their mean bleeding time was significantly greater than that of the controls ($P < 0.0001$), Fig. 2A). Thus, deficiency of platelet vinculin may cause a mild defect in hemostasis.

Another way to test platelet function in mice is to monitor platelet thrombus formation in response to carotid artery injury with FeCl₃ [18]. In normal mice this typically leads to complete carotid artery occlusion within 5–10 min of injury, a response that can be monitored with a flow probe [32]. In some mice with defects in α IIB β 3 signaling, occlusions may be transient or not occur at all [18]. The average time to carotid artery occlusion in vinculin-deficient mice was within the expected normal limits, similar to that of *Vcl fl/fl* Cre⁻ controls (Fig. 2B–C). Consequently, any dysfunction of vinculin-deficient platelets is not severe enough to protect mice from this type or degree of arterial injury.

Platelet functions ex vivo

Since vinculin plays a role in integrin-cytoskeletal connections that enable nucleated cells to resist mechanical forces including laminar shear stress [33–36], the adhesion of vinculin-deficient platelets to immobilized integrin ligands was examined under static and flow conditions. The adhesion of *Vcl fl/fl* Cre⁺ platelets to fibrinogen was similar to that of *Vcl fl/fl* Cre⁻ platelets under static conditions whether or not platelets had been activated by ADP (Fig. 3A). Moreover, vinculin-deficient platelets adhered normally to fibrinogen (Fig. 3B) and collagen (Fig. 3C) under flow over a range of biologically relevant shear stresses. Thus contrary to expectations based on studies in nucleated cells, vinculin appears dispensable for platelet adhesion to fibrinogen via α IIB β 3 [20,21] or to collagen via α 2 β 1 [37].

Inside out signals initiated by platelet agonists convert α IIB β 3 from a low- to a high-affinity state, enabling soluble fibrinogen binding and platelet aggregation [38]. When platelets were stimulated with ADP or a PAR4 thrombin receptor agonist peptide, AYPGFK, specific fibrinogen binding to *Vcl fl/fl* Cre⁺ platelets was not significantly different from binding to *Vcl fl/fl* Cre⁻ control platelets (Fig. 4A). Furthermore under stirring conditions, *Vcl fl/fl* Cre⁺ platelets aggregated normally in response to ADP (0.5, 1, 5 μ M), AYPGKF (0.05, 0.1 mM) or collagen (10 μ g mL⁻¹) (Fig. 4B).

When platelets attach to immobilized fibrinogen, they undergo outside-in α IIB β 3 signaling and a spreading response over 30–60 min that is characterized by the formation of F-actin nodules early in the process [28], progressive actin polymerization/reorganization, filopodial protrusion and lamellipodial extension [14]. Full platelet spreading requires co-signaling from α IIB β 3 and agonist receptors [26]. The spreading of *Vcl fl/fl* Cre⁺ platelets on

fibrinogen was determined by quantification of individual platelet surface areas. The spreading of vinculin-deficient platelets was similar to that of *Vcl fl/fl Cre⁻* control platelets, both in the absence and presence of ADP or AYPGKF (Fig. 5A). Moreover, actin nodules could be observed in both sets of platelets (Fig. 5B), and fully spread platelets displayed a rim distribution of F-actin and prominent F-actin cables (Fig. 5C). When stained for the focal adhesion protein, Hic-5 [39], vinculin-deficient platelets even exhibited characteristic rim staining and patches at the termini of actin cables (Fig. 5C).

The above results do not exclude a role for vinculin in other PAR4-mediated and actin-dependent platelet responses. These include actin polymerization [40], fibrin clot retraction [41], and irreversible fibrinogen binding, a process that is blocked by cytochalasins and resistant to EDTA [23,42]. However when F-actin content was monitored with BODIPY-phalloidin, vinculin-deficient platelets stimulated with AYPGFK exhibited an increase in F-actin comparable to control platelets (Figure 6A). Furthermore, vinculin-deficient platelets exhibited normal fibrin clot retraction (Figure 6B) and irreversible fibrinogen binding (Figure 6C).

Two additional platelet responses that may be partially dependent on α IIB β 3 and/or actin are granule secretion and the development of a procoagulant surface [43]. The former can be monitored indirectly by surface expression of P-selectin in response to the PAR4 thrombin receptor-activating peptide AYPGFK, and the latter by the binding of factor Va light chain to the platelet procoagulant surface in response to Ca^{2+} -ionophore A23187 [27]. When measured by flow cytometry, vinculin deficiency had no effect on either of these platelet responses (Fig. 7A,B). Taken together, this set of studies indicates that vinculin is dispensable for *ex vivo* platelet responses that involve α IIB β 3, F-actin or both proteins.

Vinculin and membrane-cytoskeletal interactions in megakaryocytes

Vinculin is required for plasma membrane cytoskeleton integrity, as determined by measurements of membrane “stiffness” of mouse embryonic fibroblasts [44]. Recently, primary murine megakaryocytes were used in conjunction with laser tweezers and fibrinogen-coated beads to generate an external pulling force on α IIB β 3, leading to increased plasma membrane tethers in mice deficient in phosphatidylinositol-4-phosphate-5-kinase 1 γ [45]. To determine if loss of vinculin would result in a similar increase in membrane tethers, mature megakaryocytes from *Vcl fl/fl Cre⁺* and *Vcl fl/fl Cre⁻* mice were generated in culture and laser tweezers were used to pull on fibrinogen-coated beads adherent to the cells. Under these conditions, megakaryocytes from *Vcl fl/fl Cre⁺* mice displayed significantly more membrane tethers than megakaryocytes from the control mice ($P < 0.0003$, ANOVA, Fig. 8), suggesting that vinculin is required to maintain megakaryocyte membrane cytoskeleton integrity.

Discussion

Integrins, including those expressed in platelets, are linked to the actin cytoskeleton both structurally and functionally [37,38,46]. With binding sites for actin and for integrin-associated proteins such as talin, vinculin is thought to regulate integrin-actin linkages, thereby influencing the adhesion and motility of nucleated cells [5,7,33,34,47]. Here we sought to determine the function of vinculin in platelets by selectively deleting the murine vinculin gene in the megakaryocyte/platelet lineage. *Vcl fl/fl Cre⁺* mice were viable and their platelets and mature megakaryocytes were deficient in vinculin as demonstrated by western blotting and fluorescence microscopy. Studies of these mice and their platelets enabled us to conclude that: 1) Mice deficient in megakaryocyte/platelet vinculin display megakaryocytes with reduced membrane cytoskeleton integrity, as demonstrated by increased membrane tether formation in response to external force imposed on α IIB β 3. 2) Mice with vinculin-

deficient megakaryocytes and platelets have normal platelet counts and they do not bleed spontaneously. However, a majority of them exhibit prolonged tail bleeding times, suggesting a mild platelet function defect. 3) On the other hand, platelet vinculin is not essential for the formation of occlusive thrombi in response to a carotid artery injury, nor is it required for a variety of *ex vivo* platelet responses under static or flow conditions that involve bidirectional α IIb β 3 signaling. 4) Most surprisingly, vinculin is not required for agonist-induced platelet functions that are dependent in part on α IIb β 3/actin linkages. These include actin polymerization and reorganization, fibrin clot retraction, irreversible fibrinogen binding, α -granule secretion, and the development of a procoagulant surface. Collectively, these results have forced us to reexamine assumptions about vinculin function in platelets that were based largely on studies in migratory nucleated cells. They suggest either that vinculin is not required for most platelet functions involving α IIb β 3 and F-actin, or that other proteins can substitute or compensate for vinculin in its absence.

In nucleated cells, vinculin is often localized at sites of cell contact with the extracellular matrix, where it functions as a scaffolding protein to couple adhesion receptors to the actin cytoskeleton [7,8,10]. Biophysical studies comparing vinculin-deficient and wild-type nucleated cells in culture have concluded that vinculin helps to stabilize the plasma membrane cytoskeleton [44,48]. Our experiments with laser tweezers demonstrated an increase in membrane tethers in vinculin-deficient megakaryocytes when a pulling force was applied to fibrinogen-coated beads adherent to these cells (Fig. 8). Similar results with megakaryocytes from phosphatidylinositol-4-phosphate-5-kinase 1 γ knockout mice have implicated this enzyme in maintaining the integrity of the membrane cytoskeleton [45]. Interestingly, platelets can form long membrane tethers at very high shear stresses during von Willebrand factor-dependent platelet aggregation [49–51]. The role of vinculin in the generation of these types of membrane tethers and the relationship of these tethers to the ones we have studied in megakaryocytes warrant further study.

Platelet counts were normal in *Vin fl/fl Cre⁺* mice, but tail-bleeding times were prolonged in a majority of them when compared to *Vin fl/fl Cre⁻* littermates (Fig. 2A). This suggests that a deficiency of vinculin may cause a subtle defect in platelet function during hemostasis in a tail wound [31]. To further evaluate platelet function *in vivo*, FeCl₃ was used to injure the carotid artery and to generate occlusive thrombi, one of several mouse arterial thrombosis models that are dependent on platelets and α IIb β 3 [52,53]. However, occlusive thrombi formed normally in this model in the absence of vinculin (Figure 2B,C). It is always possible that a different form of arterial injury might have yielded a different result. Nonetheless, we can conclude that vinculin is not necessarily required for the formation of an occlusive platelet thrombus *in vivo*. There are several possible explanations for why tail-bleeding times were affected by vinculin deficiency but carotid artery thrombus formation was not. These include differences in rheological conditions as well potential differences in the repertoire of platelet agonists generated after tail transection vs. chemical carotid artery injury [52,53]. The importance of environmental conditions in determining the requirements for vinculin in cellular responses is underscored by a recent study with nucleated cells where vinculin deficiency enhanced cell motility on a two-dimensional collagen matrix but retarded cell invasion in a three-dimensional matrix [3]. Thus, the functions of platelet vinculin may be context-dependent.

We find it remarkable that vinculin-deficient platelets functioned normally in a wide variety of assays of platelet function *ex vivo*. Inside-out α IIb β 3 signaling, as measured by the specific binding of soluble fibrinogen to activated platelets, was similar in *Vin fl/fl Cre⁺* and *Vin fl/fl Cre⁻* backgrounds. Under normal circumstances, fibrinogen binding to platelets is initially reversible in that bound fibrinogen can be chased with EDTA. However, fibrinogen binding becomes irreversible over time, a process variously ascribed to an increase in α IIb β 3

bond strength, α IIB β 3 clustering, and/or α IIB β 3 internalization. Significantly, irreversible fibrinogen binding can be prevented by pretreatment of platelets with cytochalasins, which inhibit actin polymerization [23,42]. However, we found that irreversible fibrinogen binding was unaffected by vinculin depletion (Fig. 6C), leading us to conclude that vinculin is not required for either reversible or irreversible fibrinogen binding to α IIB β 3.

Following fibrinogen binding to α IIB β 3, outside-in signals are triggered by enzymes that associate with the cytoplasmic tails of the integrin, and these signals collaborate with those generated through occupancy of various agonist receptors to generate a number of platelet responses, many involving actin polymerization and reorganization [38]. We found that vinculin-deficient platelets underwent actin polymerization normally in response to PAR4 thrombin receptor activation (Fig. 6A). This result is consistent with the observation in mouse F9 embryonic carcinoma cells that F-actin and vinculin contents are not correlated [54]. Also, platelet vinculin deficiency failed to impair platelet spreading, F-actin cable formation or fibrin clot retraction (Fig. 5 and 6). Even platelet adhesion to fibrinogen and collagen under flow conditions was normal in vinculin-deficient platelets (Fig. 3). It is possible that other cytoskeletal proteins, such as talin [47], α -actinin [55] or kindlin-3 [56] may substitute for vinculin in promoting α IIB β 3- and actin-dependent responses in platelets. Alternatively, expression of some cytoskeletal proteins may be upregulated in *Vin fl/fl Cre*⁺ platelets in order to compensate for the loss of vinculin, although we did not find this to be the case for several proteins examined (Figure 1E). Our flow studies were conducted under conditions of low-to-intermediate levels of shear stress. We cannot rule out the possibility that the absence of vinculin may affect platelet responses under conditions of extremely high shear stresses, such as those that may be operative in highly stenotic blood vessels [50].

Finally, we speculate that the function of the actin cytoskeleton, and vinculin in particular, is subtly, if not fundamentally, different in platelets compared to many types of nucleated cells. For example, certain nucleated cells migrate extensively during development or following injury, and this migration appears dependent on vinculin [10,57,58]. In contrast, whereas platelets roll, adhere, spread and aggregate following vascular injury, they are not known to engage in haptokinetic or amoeboid migration. To understand better what vinculin is needed for in platelets, it may be informative to mate *Vin fl/fl Cre*⁺ mice with mice harboring heterozygous or homozygous deletions of other cytoskeletal proteins to expose phenotypes in the offspring that are not obvious in the parents.

Acknowledgments

We thank Radek Skoda (Basel University Hospital, Switzerland) for providing *Pf4*-Cre mice, Barry Collier (Rockefeller University, New York, NY) for the 1B5 antibody, Keith Burrige (University of North Carolina, Chapel Hill, NC) for the α -actinin antibody and Peter J. Sims (University of Rochester Medical Center, Rochester, NY) for factor Va and the V237 antibody.

Research was supported by NIH grants HL56595, HL57900 and T32 HL086344-02 (to SJS) and HL046345 (to RSR).

References

1. Ziegler WH, Liddington RC, Critchley DR. The structure and regulation of vinculin. *Trends Cell Biol.* 2006; 16:453–60. [PubMed: 16893648]
2. Mierke CT, Kollmannsberger P, Zitterbart DP, Smith J, Fabry B, Goldmann WH. Mechano-coupling and regulation of contractility by the vinculin tail domain. *Biophys J.* 2008; 94:661–70. [PubMed: 17890382]
3. Mierke CT, Kollmannsberger P, Zitterbart DP, Diez G, Koch TM, Marg S, Ziegler WH, Goldmann WH, Fabry B. Vinculin facilitates cell invasion into 3D collagen matrices. *J Biol Chem.* 2010; 285:13121–30. [PubMed: 20181946]

4. Turner CE, Burridge K. Detection of metavinculin in human platelets using a modified talin overlay assay. *Eur J Cell Biol.* 1989; 49:202–6. [PubMed: 2503380]
5. Bakolitsa C, Cohen DM, Bankston LA, Bobkov AA, Cadwell GW, Jennings L, Critchley DR, Craig SW, Liddington RC. Structural basis for vinculin activation at sites of cell adhesion. *Nature.* 2004; 430:583–6. [PubMed: 15195105]
6. Chen H, Choudhury DM, Craig SW. Coincidence of actin filaments and talin is required to activate vinculin. *J Biol Chem.* 2006; 281:40389–98. [PubMed: 17074767]
7. Humphries JD, Wang P, Streuli C, Geiger B, Humphries MJ, Ballestrem C. Vinculin controls focal adhesion formation by direct interactions with talin and actin. *J Cell Biol.* 2007; 179:1043–57. [PubMed: 18056416]
8. Geiger B, Bershadsky A. Exploring the neighborhood: adhesion-coupled cell mechanosensors. *Cell.* 2002; 110:139–42. [PubMed: 12150922]
9. Rodriguez Fernandez JL, Geiger B, Salomon D, Ben-Ze'ev A. Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility, and anchorage-dependent growth of 3T3 cells. *J Cell Biol.* 1993; 122:1285–94. [PubMed: 8376463]
10. Goldmann WH, Schindl M, Cardozo TJ, Ezzell RM. Motility of vinculin-deficient F9 embryonic carcinoma cells analyzed by video, laser confocal, and reflection interference contrast microscopy. *Exp Cell Res.* 1995; 221:311–9. [PubMed: 7493629]
11. Xu W, Coll JL, Adamson ED. Rescue of the mutant phenotype by reexpression of full-length vinculin in null F9 cells; effects on cell locomotion by domain deleted vinculin. *J Cell Sci.* 1998; 111 (Pt 11):1535–44. [PubMed: 9580561]
12. Xu W, Baribault H, Adamson ED. Vinculin knockout results in heart and brain defects during embryonic development. *Development.* 1998; 125:327–37. [PubMed: 9486805]
13. Zemljic-Harpf AE, Miller JC, Henderson SA, Wright AT, Manso AM, Elsherif L, Dalton ND, Thor AK, Perkins GA, McCulloch AD, Ross RS. Cardiac-myocyte-specific excision of the vinculin gene disrupts cellular junctions, causing sudden death or dilated cardiomyopathy. *Mol Cell Biol.* 2007; 27:7522–37. [PubMed: 17785437]
14. Leng L, Kashiwagi H, Ren XD, Shattil SJ. RhoA and the function of platelet integrin $\alpha\text{IIb}\beta\text{3}$. *Blood.* 1998; 91:4206–15. [PubMed: 9596668]
15. Watanabe N, Bodin L, Pandey M, Krause M, Coughlin S, Boussiotis VA, Ginsberg MH, Shattil SJ. Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin $\alpha\text{IIb}\beta\text{3}$. *J Cell Biol.* 2008; 181:1211–22. [PubMed: 18573917]
16. Tiedt R, Schomber T, Hao-Shen H, Skoda RC. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood.* 2007; 109:1503–6. [PubMed: 17032923]
17. Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Collier BS, Teitelbaum S, Hynes RO. β3 -integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest.* 1999; 103:229–38. [PubMed: 9916135]
18. Ablooglu AJ, Kang J, Petrich BG, Ginsberg MH, Shattil SJ. Antithrombotic effects of targeting $\alpha\text{IIb}\beta\text{3}$ signaling in platelets. *Blood.* 2009; 113:3585–92. [PubMed: 19005179]
19. Prevost N, Kato H, Bodin L, Shattil SJ. Platelet integrin adhesive functions and signaling. *Methods Enzymol.* 2007; 426:103–15. [PubMed: 17697882]
20. Gutierrez E, Petrich BG, Shattil SJ, Ginsberg MH, Groisman A, Kasirer-Friede A. Microfluidic devices for studies of shear-dependent platelet adhesion. *Lab Chip.* 2008; 8:1486–95. [PubMed: 18818803]
21. Tkachenko E, Gutierrez E, Ginsberg MH, Groisman A. An easy to assemble microfluidic perfusion device with a magnetic clamp. *Lab Chip.* 2009; 9:1085–95. [PubMed: 19350090]
22. Savage B, Saldivar E, Ruggeri ZM. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell.* 1996; 84:289–97. [PubMed: 8565074]
23. Fox JE, Shattil SJ, Kinlough-Rathbone RL, Richardson M, Packham MA, Sanan DA. The platelet cytoskeleton stabilizes the interaction between $\alpha\text{IIb}\beta\text{3}$ and its ligand and induces selective movements of ligand-occupied integrin. *J Biol Chem.* 1996; 271:7004–11. [PubMed: 8636130]

24. Judd BA, Myung PS, Obergfell A, Myers EE, Cheng AM, Watson SP, Pear WS, Allman D, Shattil SJ, Koretzky GA. Differential requirement for LAT and SLP-76 in GPVI versus T cell receptor signaling. *J Exp Med.* 2002; 195:705–17. [PubMed: 11901197]
25. Arias-Salgado EG, Haj F, Dubois C, Moran B, Kasirer-Friede A, Furie BC, Furie B, Neel BG, Shattil SJ. PTP-1B is an essential positive regulator of platelet integrin signaling. *J Cell Biol.* 2005; 170:837–45. [PubMed: 16115959]
26. Shattil SJ, Haimovich B, Cunningham M, Lipfert L, Parsons JT, Ginsberg MH, Brugge JS. Tyrosine phosphorylation of pp125FAK in platelets requires coordinated signaling through integrin and agonist receptors. *J Biol Chem.* 1994; 269:14738–45. [PubMed: 7514181]
27. Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J Biol Chem.* 1989; 264:17049–57. [PubMed: 2793843]
28. Calaminus SD, Thomas S, McCarty OJ, Machesky LM, Watson SP. Identification of a novel, actin-rich structure, the actin nodule, in the early stages of platelet spreading. *J Thromb Haemost.* 2008; 6:1944–52. [PubMed: 18761725]
29. Geddis AE, Kaushansky K. Megakaryocytes express functional Aurora-B kinase in endomitosis. *Blood.* 2004; 104:1017–24. [PubMed: 15130946]
30. Litvinov RI, Bennett JS, Weisel JW, Shuman H. Multi-step fibrinogen binding to the integrin (alpha)IIb(beta)3 detected using force spectroscopy. *Biophys J.* 2005; 89:2824–34. [PubMed: 16040750]
31. Tsakiris DA, Scudder L, Hodiola-Dilke K, Hynes RO, Collier BS. Hemostasis in the mouse (*Mus musculus*): a review. *Thromb Haemost.* 1999; 81:177–88. [PubMed: 10063988]
32. Westrick RJ, Winn ME, Eitzman DT. Murine models of vascular thrombosis (Eitzman series). *Arterioscler Thromb Vasc Biol.* 2007; 27:2079–93. [PubMed: 17600224]
33. Puklin-Faucher E, Sheetz MP. The mechanical integrin cycle. *J Cell Sci.* 2009; 122:179–86. [PubMed: 19118210]
34. Mierke CT. The role of vinculin in the regulation of the mechanical properties of cells. *Cell Biochem Biophys.* 2009; 53:115–26. [PubMed: 19350419]
35. Albuquerque ML, Flozak AS. Lamellipodial motility in wounded endothelial cells exposed to physiologic flow is associated with different patterns of beta1-integrin and vinculin localization. *J Cell Physiol.* 2003; 195:50–60. [PubMed: 12599208]
36. Roberts GC, Critchley DR. Structural and biophysical properties of the integrin-associated cytoskeletal protein talin. *Biophys Rev.* 2009; 1:61–9. [PubMed: 19655048]
37. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin alphaIIb beta3 signaling in platelets. *J Thromb Haemost.* 2005; 3:1752–62. [PubMed: 16102042]
38. Collier BS, Shattil SJ. The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood.* 2008; 112:3011–25. [PubMed: 18840725]
39. Osada M, Ohmori T, Yatomi Y, Satoh K, Hosogaya S, Ozaki Y. Involvement of Hic-5 in platelet activation: integrin alphaIIbbeta3-dependent tyrosine phosphorylation and association with proline-rich tyrosine kinase 2. *Biochem J.* 2001; 355:691–7. [PubMed: 11311131]
40. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol.* 1992; 118:1421–42. [PubMed: 1325975]
41. Chen YP, O'Toole TE, Leong L, Liu BQ, Diaz-Gonzalez F, Ginsberg MH. Beta 3 integrin-mediated fibrin clot retraction by nucleated cells: differing behavior of alpha IIb beta 3 and alpha v beta 3. *Blood.* 1995; 86:2606–15. [PubMed: 7545462]
42. Peerschke EI. Stabilization of platelet-fibrinogen interactions is an integral property of the glycoprotein IIb-IIIa complex. *J Lab Clin Med.* 1994; 124:439–46. [PubMed: 8083588]
43. Gemmell CH, Sefton MV, Yeo EL. Platelet-derived microparticle formation involves glycoprotein IIb-IIIa. Inhibition by RGDS and a Glanzmann's thrombasthenia defect. *J Biol Chem.* 1993; 268:14586–9. [PubMed: 8325838]
44. Klemm AH, Diez G, Alonso JL, Goldmann WH. Comparing the mechanical influence of vinculin, focal adhesion kinase and p53 in mouse embryonic fibroblasts. *Biochem Biophys Res Commun.* 2009; 379:799–801. [PubMed: 19126403]

45. Wang Y, Litvinov RI, Chen X, Bach TL, Lian L, Petrich BG, Monkley SJ, Kanaho Y, Critchley DR, Sasaki T, Birnbaum MJ, Weisel JW, Hartwig J, Abrams CS. Loss of PIP5KI γ , unlike other PIP5KI isoforms, impairs the integrity of the membrane cytoskeleton in murine megakaryocytes. *J Clin Invest.* 2008; 118:812–9. [PubMed: 18188447]
46. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell.* 2002; 110:673–87. [PubMed: 12297042]
47. Ziegler WH, Gingras AR, Critchley DR, Emsley J. Integrin connections to the cytoskeleton through talin and vinculin. *Biochem Soc Trans.* 2008; 36:235–9. [PubMed: 18363566]
48. Alenghat FJ, Fabry B, Tsai KY, Goldmann WH, Ingber DE. Analysis of cell mechanics in single vinculin-deficient cells using a magnetic tweezer. *Biochem Biophys Res Commun.* 2000; 277:93–9. [PubMed: 11027646]
49. Maxwell MJ, Westein E, Nesbitt WS, Giuliano S, Dopheide SM, Jackson SP. Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation. *Blood.* 2007; 109:566–76. [PubMed: 16990596]
50. Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med.* 2009; 15:665–73. [PubMed: 19465929]
51. Ruggeri ZM, Orje JN, Habermann R, Federici AB, Reininger AJ. Activation-independent platelet adhesion and aggregation under elevated shear stress. *Blood.* 2006; 108:1903–10. [PubMed: 16772609]
52. Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest.* 2005; 115:3355–62. [PubMed: 16322780]
53. Dubois C, Panicot-Dubois L, Merrill-Skoloff G, Furie B, Furie BC. Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo. *Blood.* 2006; 107:3902–6. [PubMed: 16455953]
54. Ezzell RM, Goldmann WH, Wang N, Parashurama N, Ingber DE. Vinculin promotes cell spreading by mechanically coupling integrins to the cytoskeleton. *Exp Cell Res.* 1997; 231:14–26. [PubMed: 9056408]
55. Greenwood JA, Theibert AB, Prestwich GD, Murphy-Ullrich JE. Restructuring of focal adhesion plaques by PI 3-kinase. Regulation by PtdIns (3,4,5)-p(3) binding to alpha-actinin. *J Cell Biol.* 2000; 150:627–42. [PubMed: 10931873]
56. Moser M, Nieswandt B, Ussar S, Pozgajova M, Fassler R. Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med.* 2008; 14:325–30. [PubMed: 18278053]
57. Subauste MC, Pertz O, Adamson ED, Turner CE, Junger S, Hahn KM. Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. *J Cell Biol.* 2004; 165:371–81. [PubMed: 15138291]
58. Hong T, Grabel LB. Migration of F9 parietal endoderm cells is regulated by the ERK pathway. *J Cell Biochem.* 2006; 97:1339–49. [PubMed: 16329137]

Fig. 1.

Genotyping and vinculin expression. A) Genomic DNA isolated from ear biopsies of *Vcl* +/+ , *Vcl* fl/+ and *Vcl* fl/fl mice were analyzed by PCR as described in Materials and Methods. B) Vinculin expression in 20 μ g of platelet lysates from two mice of each genotype was analyzed on immunoblots (IB). Note the absence of a detectable vinculin band in lysates from *Vcl* fl/fl Cre⁺ platelets. C) Vinculin expression in 20 μ g lysates from various cells and tissues. D) Vinculin and F-actin staining of mature, polyploid megakaryocytes. Cells were plated on fibrinogen for 45 min, then fixed, permeabilized and stained as indicated. The merged images included staining for vinculin (green) and F-actin (rhodamine-phalloidin, red). Note the absence of detectable vinculin in *Vcl* fl/fl Cre⁺ mice megakaryocytes. (40 $\times$ objective; size bar = 15 μ m). E) Immunoblots showing the expression of various proteins in 20 μ g of lysates from platelets of the indicated genotypes.

Fig. 2. Platelet function *in vivo*. A) Tail bleeding times. Each triangle represents one mouse (*Vcl fl/fl Cre⁻*, n=30; *Vcl fl/fl Cre⁺*, n=46). Time to cessation of bleeding after tail resection was recorded for up to 10 min, at which time bleeding was stopped by cauterization. Horizontal lines indicate the means. B) Times to complete occlusion of carotid arteries after FeCl_3 injury. Three mice of each genotype were studied. C) Carotid artery blood flow profiles from representative mice. Blood flow was measured for at least 25 minutes.

Fig. 3. Platelet adhesion *ex vivo*. A) Adhesion to fibrinogen under static conditions $\pm$ 20 μM ADP as described in Materials and Methods. Data represent means $\pm$ SEM of five independent experiments, each performed in triplicate. B) Adhesion to fibrinogen under flow conditions, as described in Materials and Methods. The fibrinogen coating concentration was 20 $\mu\text{g mL}^{-1}$ and adhesion was quantified after 1 min of flow at the indicated shear rates ($n = 3$). C) Adhesion to collagen under flow conditions. The collagen coating concentration was 50 $\mu\text{g mL}^{-1}$ and adhesion was quantified after 1 min of flow ($n = 3$).

Fig. 4. Fibrinogen binding and platelet aggregation. A) Washed platelets were incubated for 20 min at room temperature with Alexa-fluor 647-labeled fibrinogen ± ADP or AYPGKF. Specific fibrinogen binding was determined by flow cytometry and depicted as mean fluorescence intensity (MFI) in arbitrary fluorescence units (n = 3). B) Representative platelet aggregation tracings from *Vcl fl/fl Cre⁻* and *Vcl fl/fl Cre⁺* mice. Results are representative of at least 3 experiments.

Fig. 5.

Platelet spreading and cytoskeletal organization. A) Platelet spreading. Washed platelets were plated onto fibrinogen-coated coverslips ($100 \mu\text{g mL}^{-1}$) for 45 min at $37^\circ\text{C} \pm \text{MnCl}_2$, ADP or ADPGKF. Cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.2% Triton X-100 and stained for $\alpha\text{IIb}\beta_3$ with antibody 1B5. Platelet spreading was assessed by deconvolution microscopy and expressed as the mean platelet surface area. Data represent 500–800 platelets analyzed for each genotype. B) Representative platelet images showing actin nodules. Washed platelets were plated on fibrinogen ($100 \mu\text{g mL}^{-1}$) in the presence of 2 U mL^{-1} apyrase and $10 \mu\text{M}$ indomethacin for 45 mins at 37°C [28]. After fixation, permeabilization and staining with rhodamine-phalloidin to label F-actin, images were captured by deconvolution microscopy (scale bar = $10 \mu\text{m}$). The area of interest in a single platelet (bordered in white) is expanded for better visualization of actin nodules, denoted by the white arrows. C) Representative deconvolution images of platelets stained for vinculin (green) or Hic-5 (green) and F-actin (red) (scale bar = $10 \mu\text{m}$).

Fig. 6.

Analyses of platelet F-actin. A) F-actin content. Washed platelets were incubated for 3 min $\pm$ 1 mM AYPGKF. After fixation and permeabilization, platelets were stained with BODIPY-Phalloidin $\pm$ unlabeled excess phalloidin, and specific BODIPY-Phalloidin was quantified by flow cytometry and taken as the relative platelet F-actin content ($n = 3$) [26]. B) Fibrin clot retraction. Clotting proceeded for 2 h at 37° C in platelet-rich plasma after addition of 9.4 U mL⁻¹ thrombin and 1.9 mM CaCl₂ ($n = 3$, each performed in duplicate). C) Irreversible fibrinogen binding was analyzed by flow cytometry as described [23]. Note that irreversible binding (defined as binding not chased by EDTA) increased with time after platelet stimulation with AYPGKF but did not occur in the presence of cytochalasin E (cyto E) ($n = 3$).

Fig. 7.

Platelet α -granule secretion and procoagulant surface. Washed platelets were stimulated with the indicated agonists for 15 min at 37° C. A) α -granule secretion was monitored by surface expression of P-selectin and detected by flow cytometry (n = 3). B) After platelet stimulation with 1 μ M A23187 + 2 μ g mL⁻¹ factor Va light chain, the development of a procoagulant surface was assessed by measuring factor Va light chain binding to platelets and platelet-derived microparticles with antibody FITC-V237 [27]. These data represent the binding to platelets and microparticles combined (n=3).

Fig. 8.

Membrane tether formation in response to a mechanical pulling force applied to α IIB β 3. Megakaryocytes were generated in culture from bone marrows of megakaryocyte/platelet vinculin-deficient mice (*Vcl fl/fl Cre⁺*) and two sets of control mice (C57/BL6 and *Vcl fl/fl Cre⁻*). Experiments were performed by pulling with laser tweezers on adherent fibrinogen-coated beads, as described in Material and Methods. The number of fibrinogen-coated bead/membrane interactions analyzed for tether formation was 343 (C57/BL6), 473 (*Vcl fl/fl Cre⁻*) and 663 (*Vcl fl/fl Cre⁺*). Data are expressed as the percent of interactions resulting in membrane tethers [45]. The statistical analysis was performed using the χ^2 test.

Table 1

Blood cell parameters

	Vcl fl/fl Cre ⁻	Vcl fl/fl Cre ⁺	Normal range
Leukocytes			
WBC ($\times 10^3$)	9.9 $\pm$ 0.1	13.4 $\pm$ 0.6	3.0–16.1
Ne ($\times 10^3$)	1.2 $\pm$ 0.1	1.6 $\pm$ 0.1	0.3–3.3
Ly ($\times 10^3$)	8.1 $\pm$ 0.2	11.2 $\pm$ 0.5	2.2–12.9
Mo ($\times 10^3$)	0.5 $\pm$ 0.0	0.4 $\pm$ 0.0	0.1–0.9
Eo ($\times 10^3$)	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.0–0.3
Ba ($\times 10^3$)	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0–0.2
Erythrocytes			
RBC ($\times 10^6$)	9.2 $\pm$ 0.1	9.8 $\pm$ 0.2	8.0–12.0
Hemoglobin (g/dL)	13.3 $\pm$ 0.1	13.9 $\pm$ 0.2	12.8–16.4
Hematocrit (%)	48.0 $\pm$ 0.5	52.0 $\pm$ 0.7	38.0–59.3
MCV (fL)	52.2 $\pm$ 0.3	52.4 $\pm$ 0.5	43.0–54.3
Thrombocytes			
Platelet ($\times 10^3$)	602 $\pm$ 29	578 $\pm$ 11	473–1220
MPV (fL)	4.7 $\pm$ 0.0	4.8 $\pm$ 0.1	4.3–5.9

Values are expressed as the mean $\pm$ SEM, n=3 for both groups. WBC indicates white blood cells; Ne, neutrophils; Ly, Lymphocytes; Mo, Monocytes; Eo, eosinophils; Ba, basophils; RBC, red blood cells; MCV, mean cell volume; MPV, mean platelet volume.