



# Characterization of the Role of Integrin $\alpha 5\beta 1$ in Platelet Function, Hemostasis, and Experimental Thrombosis

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## Abstract

**Objective** Integrins are key regulators of various platelet functions. The pathophysiological importance of most platelet integrins has been investigated, with the exception of  $\alpha 5\beta 1$ , a receptor for fibronectin. The aim of this study was to characterize the role of  $\alpha 5\beta 1$  in megakaryopoiesis, platelet function, and to determine its importance in hemostasis and arterial thrombosis.

**Approach and Results** We generated a mouse strain deficient for integrin  $\alpha 5\beta 1$  on megakaryocytes and platelets (PF4Cre- $\alpha 5^{-/-}$ ). PF4Cre- $\alpha 5^{-/-}$  mice were viable, fertile, and presented no apparent signs of abnormality. Megakaryopoiesis appears unaltered as evidence by a normal megakaryocyte morphology and development, which is in agreement with a normal platelet count. Expression of the main platelet receptors and the response of PF4Cre- $\alpha 5^{-/-}$  platelets to a series of agonists were all completely normal. Adhesion and aggregation of PF4Cre- $\alpha 5^{-/-}$  platelets under shear flow on fibrinogen, laminin, or von Willebrand factor were unimpaired. In contrast, PF4Cre- $\alpha 5^{-/-}$  platelets displayed a marked decrease in adhesion, activation, and aggregation on fibrillar cellular fibronectin and collagen. PF4Cre- $\alpha 5^{-/-}$  mice presented no defect in a tail-bleeding time assay and no increase in inflammatory bleeding in a reverse passive Arthus model and a lipopolysaccharide pulmonary inflammation model. Finally, no defects were observed in three distinct experimental models of arterial thrombosis based on ferric chloride-induced injury of the carotid artery, mechanical injury of the abdominal aorta, or laser-induced injury of mesenteric vessels.

**Conclusion** In summary, this study shows that platelet integrin  $\alpha 5\beta 1$  is a key receptor for fibrillar cellular fibronectin but is dispensable in hemostasis and arterial thrombosis.

## Keywords

- ▶ platelets
- ▶ arterial thrombosis
- ▶ integrin  $\alpha 5\beta 1$
- ▶ fibronectin

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## Introduction

Platelets adhere, become activated, and aggregate at a site of vessel injury to form a hemostatic plug which stops bleeding. They are also involved in maintaining vascular integrity and in the arrest of inflammatory bleeding in various organs.<sup>1</sup> On the other hand, platelets play an instrumental role in arterial thrombosis by inducing the formation of an occlusive thrombus in a diseased artery, which results in life-threatening ischemic pathologies such as myocardial infarction or ischemic stroke.<sup>2</sup> The molecular mechanisms involved in the interactions of platelets with an injured vessel wall have been extensively investigated. The initial step of attachment of circulating platelets is ensured by binding of the glycoprotein (GP) Ib-IX complex to subendothelial von Willebrand factor (vWF) at elevated shear rates.<sup>3,4</sup> When the flow is slower,  $\beta 1$  and  $\beta 3$  integrins assist the GPIb-IX complex to allow further platelet recruitment as well as stable adhesion to various extracellular matrix proteins.<sup>3,4</sup> This enables the interaction of GPVI with its ligands, including collagen, which initiates platelet activation.<sup>5</sup> Aggregation results through interactions of integrin  $\alpha IIb\beta 3$  with plasma fibrinogen, forming a plug that seals the breach in healthy vessels<sup>6</sup> or a pathological thrombus in diseased arteries.<sup>2</sup>

Platelets express at their surface five different integrins of the  $\beta 1$  and  $\beta 3$  families, namely  $\alpha 2\beta 1$ ,  $\alpha 5\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha v\beta 3$ , and  $\alpha IIb\beta 3$  whose main ligands are collagen, fibronectin, laminins, vitronectin, and fibrinogen, respectively.  $\alpha IIb\beta 3$ , the most abundant integrin at the platelet surface,<sup>7</sup> enables platelet adhesion and aggregation through its binding to fibrinogen. This receptor plays a major role in hemostasis as evidenced by the hemorrhagic disorder known as Glanzmann's thrombasthenia, where  $\alpha IIb\beta 3$  is absent or nonfunctional.<sup>8</sup> It is also the target of a class of potent antiplatelet agents, illustrating its key involvement in arterial thrombosis.<sup>9</sup> The role of the other integrins, notably the  $\beta 1$  integrins, appears to be limited to the initial step of platelet adhesion and activation through interactions with extracellular matrix proteins. Concerning their importance, it has been shown that the absence of either  $\alpha 2\beta 1$  or  $\alpha 6\beta 1$  has no major impact on the tail-bleeding time in mice,<sup>10,11</sup> but reduces thrombosis in several experimental models.<sup>11-13</sup> In contrast, the importance of  $\alpha 5\beta 1$  in hemostasis and arterial thrombosis has never been studied.

Integrin  $\alpha 5\beta 1$  is a well-known receptor for fibronectin, which is broadly expressed on various cell types and plays an important role in migration and differentiation, especially during fetal development. As a consequence, knocking out the  $\alpha 5$  gene results in death at the embryonic stage due to a defect in the mesoderm.<sup>14</sup> Concerning platelets, it has been shown that  $\alpha 5\beta 1$  together with  $\alpha IIb\beta 3$  plays a central role in platelet adhesion to fibronectin under shear flow.<sup>15,16</sup> Plasma fibronectin is very weak in supporting platelet adhesion and activation when compared to cellular fibronectin, which is probably explained by the presence of additional binding domains in the latter. In addition, both forms of fibronectin increase markedly their reactivity after polymerization and fiber formation, especially for cellular fibronectin.<sup>17</sup> Howev-

er, although the role of  $\alpha 5\beta 1$  as a platelet receptor for fibronectin is recognized, its importance in hemostasis and arterial thrombosis remains unknown.

To study the role of integrin  $\alpha 5\beta 1$  in hemostasis and arterial thrombosis, we generated a new mouse strain which does not express this integrin on megakaryocytes or platelets (PF4Cre- $\alpha 5^{-/-}$ ) by crossing PF4Cre<sup>+</sup> mice with animals expressing the  $\alpha 5$  gene flanked by loxP sites. The PF4Cre- $\alpha 5^{-/-}$  megakaryocyte ultrastructure and maturation were characterized using transmission electron microscopy (TEM). The functions of platelets from PF4Cre- $\alpha 5^{-/-}$  mice were characterized using flow cytometry, aggregometry, and flow-based in vitro assays. We also employed a tail-bleeding time assay, a reverse passive Arthus (rpA) model, a lipopolysaccharide (LPS) pulmonary inflammation model, and experimental thrombosis models to evaluate the participation of integrin  $\alpha 5\beta 1$  in hemostasis and arterial thrombosis.

## Methods

### Materials

Materials and antibodies are described in the **Supplementary Material** (available in the online version).

### Mice

Mice lacking integrin  $\alpha 5$  in platelets were generated by crossing mice having a pure C57BL/6J background containing the *Itga5* gene flanked by loxP sites ( $\alpha 5^{fl/fl}$ ) with pure C57BL/6J transgenic animals selectively expressing Cre recombinase in the megakaryocyte lineage under control of the platelet factor 4 (PF4) gene promoter (PF4-Cre<sup>+</sup>; Jackson Laboratories, Bar Harbor, United States). The offspring were intercrossed to produce littermate animals homozygous for the floxed allele (PF4-Cre<sup>+</sup>/ $\alpha 5^{fl/fl}$ , hereafter called PF4Cre- $\alpha 5^{-/-}$ ). C57BL/6J PF4-Cre<sup>+</sup> mice served as controls (Ctrl), unless specified otherwise. Male and female mice were used.

### Megakaryocyte Ultrastructure

Bone marrow samples were fixed in 2.5% glutaraldehyde and prepared for TEM as described previously.<sup>18</sup> Transversal thin sections of the entire bone marrow were cut, stained with uranyl acetate and lead citrate, and examined under a Jeol JEM 2100-Plus (Japan). The number of cells was expressed as the density per unit area (defined as one square of the grid, i.e., 16,000  $\mu m^2$ ). Megakaryocytes at stages I, II, and III were identified according to Eckly et al using distinct ultrastructural characteristics.<sup>18</sup> Stage I corresponded to a cell 10 to 15  $\mu m$  in diameter with a large nucleus; stage II, to a cell 15 to 20  $\mu m$  in diameter containing platelet-specific granules; and stage III, to mature megakaryocytes having a well-developed demarcation membrane system with clearly defined platelet territories and a peripheral zone. Megakaryocytes from three different mice were analyzed for both the control and the PF4Cre- $\alpha 5^{-/-}$  strain.

### Platelet Count, Volume, and Glycoprotein Expression

Whole blood was collected into ethylenediaminetetraacetic acid (EDTA) (6 mM) after severing the tail of anesthetized

mice. Platelet count and volume were analyzed in an automatic cell counter (Scil Animal Care Company, Altorf, France) and surface GP expression was determined by flow cytometry. Expression of the  $\alpha 5$  subunit was quantified in platelet lysates using automated capillary-based immunoassay (ProteinSimple Wes, San Jose, United States), as previously described.<sup>19</sup>

### Platelet-Rich Plasma Aggregations

Mouse platelet-rich plasma (PRP) was prepared by centrifugation of blood collected on hirudin (100 U/mL) and adjusted to 300,000 platelets/ $\mu$ L with platelet-poor plasma from the same mouse. Platelet aggregation was measured as reported previously.<sup>20</sup>

### Preparation and Properties of Washed Mouse Platelets

Washed mouse platelets were prepared as reported previously.<sup>20</sup> Agonist-induced binding of soluble fibrinogen and exposure of P-selectin were determined as previously described,<sup>11</sup> while phosphatidylserine exposure was quantified by Alexa Fluor 488-annexin V binding.

### In Vitro Flow-Based Adhesion Assay

PDMS flow chambers (0.1  $\times$  1 mm) were coated with vWF-binding protein (DDR2, 100  $\mu$ g/mL), fibrinogen (100  $\mu$ g/mL), laminins (100  $\mu$ g/mL), collagen (200  $\mu$ g/mL), or soluble cellular fibronectin (300  $\mu$ g/mL) overnight at 4°C. Mechanical stretching of soluble cellular fibronectin was performed to form fibrillar cellular fibronectin as described previously.<sup>17</sup> To prevent nonspecific adhesion, the channels were blocked with phosphate-buffered saline containing human serum albumin (10 mg/mL) for 30 minutes at room temperature. Hirudinized (100 U/mL) whole blood was drawn from the abdominal aorta of anesthetized mice. The hirudinized blood was perfused through the chambers at the indicated wall shear rates and platelet adhesion was observed in real time and analyzed as detailed elsewhere.<sup>11</sup> Thrombus formation on fibrillar cellular fibronectin was monitored as previously reported.<sup>17</sup>

### In Vivo Thrombosis Models

Platelets were labeled by administering 3,3'-dihexyloxacarbocyanine iodide to anesthetized mice. Ferric chloride ( $\text{FeCl}_3$ )-mediated thrombosis was induced by applying a 3  $\times$  3 mm Whatman filter paper saturated with 7.5%  $\text{FeCl}_3$  laterally to the carotid artery for 2.5 minutes. Thrombosis was initiated mechanically by pinching the abdominal aorta with forceps for 15 seconds. Thrombus formation was monitored in real time with a fluorescence microscope (Leica Microsystems, San Westlar, Germany) and a CCD (charge-coupled device) camera (CoolSNAP HQ2, Photometrics, Roper Scientific). Laser-induced thrombosis was triggered in mesenteric arterioles using a high-intensity 440-nm pulsed nitrogen dye laser applied with a Micropoint system (Photonic Instruments, Andor Technology, Belfast, United Kingdom) causing a deep injury. Thrombus formation was

monitored in real time by bright field and fluorescence microscopy (Leica DM IRB) using a CMOS ORCA Flash V2 camera (Hamamatsu Photonics, Massy, France).

### Bleeding Time

The bleeding time and volume of blood lost were determined by transversally severing a 3-mm segment from mouse tails, as reported previously.<sup>21</sup>

### Cutaneous and Pulmonary Inflammation Models

An rpA reaction was elicited in anesthetized mice by intradermal injection of an antbovine serum albumin antibody (60  $\mu$ g/spot), followed by retro-orbital injection of bovine serum albumin (75 mg/kg), as previously described.<sup>1</sup>

A lung inflammation model was induced in anesthetized mice by intranasal inoculation of *Pseudomonas aeruginosa* LPS (1  $\mu$ g/mouse) in 60  $\mu$ L of saline, as previously described.<sup>1</sup>

### Statistical Analyses

Statistical analyses were performed with GraphPad Prism software (see figure legends).

## Results

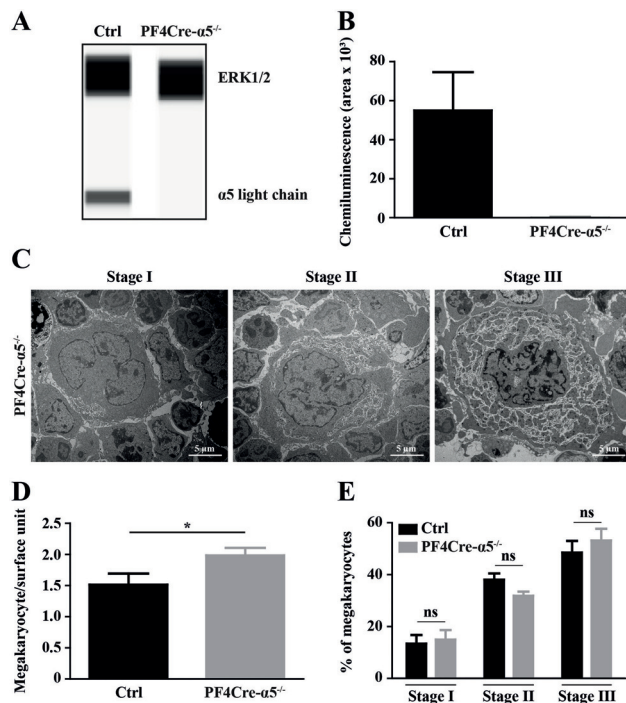
### Characterization and Megakaryopoiesis of PF4Cre- $\alpha 5$ -Deficient Mice

To study the role of platelet integrin  $\alpha 5\beta 1$ , we crossed a mouse strain floxed for the  $\alpha 5$  gene with a strain expressing Cre recombinase under control of the PF4 promoter (PF4Cre- $\alpha 5^{-/-}$ ). We used a quantitative biochemical approach to provide evidence that platelets from these mice expressed almost no integrin  $\alpha 5$  anymore as compared to PF4-Cre mice (**Fig. 1A, B**). No obvious abnormalities were detected in PF4Cre- $\alpha 5^{-/-}$  mice that were bred and developed normally. These mice have no increase in embryonic lethality, nor variation of the litter size and they present no abnormality in survival. Physical appearance and behavior are also unchanged.

PF4Cre- $\alpha 5^{-/-}$  mice presented a normal maturation and morphology of their megakaryocytes as assessed by their observation of ultrastructure on TEM images (**Fig. 1C**). While the number of megakaryocytes in the bone marrow appears slightly increased in PF4Cre- $\alpha 5^{-/-}$  mice, the distribution of the different maturation stages was unchanged in PF4Cre- $\alpha 5^{-/-}$  as compared to control mice, suggesting no major impact of integrin  $\alpha 5$  in megakaryopoiesis (**Fig. 1D, E**). In agreement, we observed that deletion of  $\alpha 5$  had no impact on the platelet count or volume (**Table 1**). Thus, PF4Cre- $\alpha 5$ -deficient mice appeared to be normal and PF4Cre- $\alpha 5$ -deficient megakaryocytes displayed no difference in development and maturation.

### Characterization of PF4Cre- $\alpha 5$ -Deficient Platelets

The surface expression of the major GPs was normal (**Table 1**), except for that of integrin  $\alpha 5$  (**Fig. 1A, B**). Using light-transmission aggregometry, we found that platelets



**Fig. 1** Absence of platelet  $\alpha 5$  expression and normal megakaryopoiesis in PF4Cre- $\alpha 5^{-/-}$  mice. (A) Representative immunoblots of the  $\alpha 5$  subunit and ERK1/2 (loading control). (B) Quantification of chemiluminescence peak areas of electropherograms obtained by capillary-based immunoassay of platelet lysates with anti- $\alpha 5$  antibody. Data expressed as mean  $\pm$  standard error of the mean (SEM) from three separate experiments. (C) Transmission electron microscopy (TEM) images illustrating a typical ultrastructure of PF4Cre- $\alpha 5^{-/-}$  megakaryocytes at their different maturation stages. (D) In situ quantification of megakaryocytes in the bone marrow by TEM. Values are expressed as the mean  $\pm$  SEM for three mice. (E) The distribution in the different maturation stages was established according to megakaryocyte morphology (see the Materials and Methods section) on TEM images. Values are expressed as the mean  $\pm$  SEM for three mice. Data were compared by Student unpaired *t*-test (D) or Chi-square test (E); \**p* < 0.05.

from PF4Cre- $\alpha 5^{-/-}$  mice aggregated normally in response to a series of agonists including adenosine diphosphate (ADP) (5  $\mu\text{mol/L}$ ), collagen (2.5  $\mu\text{g/mL}$ ), and U46619 (2  $\mu\text{mol/L}$ ) in PRP, presenting the advantage to contain plasma fibronectin (**►Fig. 2A, B**). Similar results were obtained with washed platelet aggregation to various agonists (**►Supplementary Fig. S1**, available in the online version). In addition, no differences in P-selectin exposure, fibrinogen binding or annexin V binding in response to ADP, thrombin, proteinase-activated receptor 4 (PAR-4) peptide or convulxin were observed by flow cytometry in PF4Cre- $\alpha 5^{-/-}$  platelets as compared to controls (**►Fig. 2C–E**). This confirmed that platelets deficient in integrin  $\alpha 5$  respond normally to a GPIIb/IIIa ligand and a series of soluble agonists. These results indicate that PF4Cre- $\alpha 5$ -deficient platelets presented no defect in response to soluble agonists.

### Characterization of PF4Cre- $\alpha 5$ -Deficient Platelet Adhesion under Shear Flow

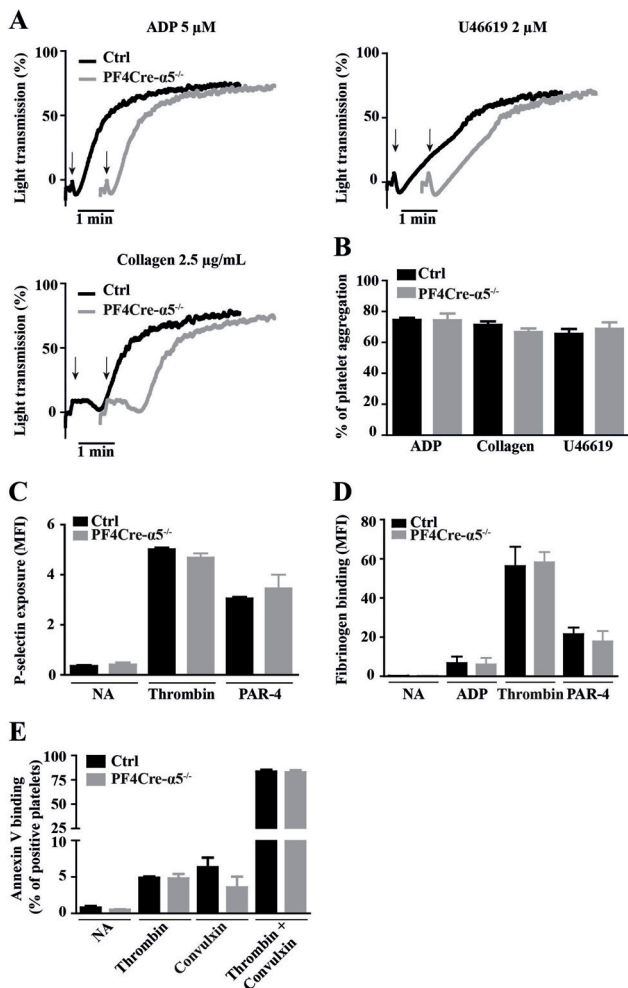
We next evaluated the ability of  $\alpha 5\beta 1$  to support platelet adhesion to various surfaces under flow by perfusing hirudinized whole blood over immobilized proteins. We observed normal adhesion and rolling of PF4Cre- $\alpha 5^{-/-}$  platelets recruited to vWF bound to DDR2 at 1,500  $\text{s}^{-1}$  (**►Fig. 3A**) and normal adhesion of these platelets to fibrinogen and laminin at 300  $\text{s}^{-1}$  (**►Fig. 3B**). In contrast, adhesion of PF4Cre- $\alpha 5^{-/-}$  platelets to immobilized fibrillar cellular fibronectin displayed a major defect as compared to controls, with a 78% reduction in the number of adherent platelets at 8 minutes (Ctrl:  $7.1 \pm 0.7 \times 10^3/\text{mm}^2$ ; PF4Cre- $\alpha 5^{-/-}$ :  $1.6 \pm 0.05 \times 10^3/\text{mm}^2$ ) (**►Fig. 3C, D**). A detailed analysis indicated that  $\alpha 5\beta 1$  was important to establish the initial bond with fibronectin, as the recruitment of PF4Cre- $\alpha 5^{-/-}$  platelets to the surface was decreased by 55% as compared to controls (**►Fig. 3E**). Moreover, study of the adhesive behavior

**Table 1** Platelet counts, volumes, and expression of major surface glycoproteins in Ctrl and PFC4re- $\alpha 5^{-/-}$  mice

	Ctrl	PF4Cre- $\alpha 5^{-/-}$	Number of mice
Platelet count	$1,095 \pm 48 \times 10^3/\mu\text{L}$	$1,119 \pm 29 \times 10^3/\mu\text{L}$	13
Mean platelet volume	$4.98 \pm 0.07 \mu\text{m}^3$	$4.68 \pm 0.02 \mu\text{m}^3$	13
$\alpha\text{IIb}\beta 3$	$5.28 \pm 0.98$	$4.87 \pm 1.20$	6
$\alpha 2$	$10.50 \pm 0.07$	$11.03 \pm 0.19$	6
$\alpha 5$	$1.23 \pm 0.04$	$0.32 \pm 0.04$	9
$\alpha 6$	$13.57 \pm 0.22$	$13.27 \pm 0.13$	6
$\beta 1$	$4.69 \pm 0.92$	$4.55 \pm 1.01$	6
GPIIb $\alpha$	$5.01 \pm 0.85$	$5.04 \pm 0.15$	6
GPV	$0.99 \pm 0.06$	$0.97 \pm 0.03$	6
GPIX	$2.04 \pm 0.12$	$2.06 \pm 0.03$	6
GPIIb	$1.66 \pm 0.05$	$1.46 \pm 0.04$	6

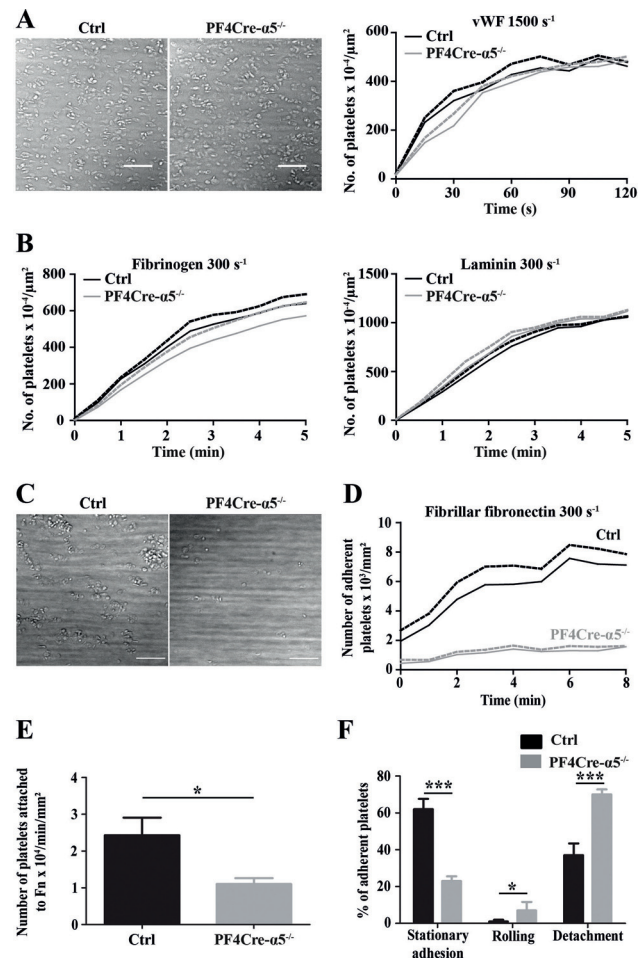
Abbreviation: GP, glycoprotein.

Note: Platelet counts and volumes in Ctrl and PF4Cre- $\alpha 5^{-/-}$  mice were analyzed with an automatic cell counter; values represent the mean  $\pm$  standard error of the mean (SEM). The surface expression of various glycoproteins on platelets in whole blood from Ctrl and PF4Cre- $\alpha 5^{-/-}$  mice was evaluated using selective antibodies and flow cytometry; results are expressed as the mean fluorescence intensity (MFI)  $\pm$  SEM.



**Fig. 2** Characterization of the functional properties of PF4Cre- $\alpha 5^{-/-}$  platelets. (A, B) Plasma-rich platelets (PRPs) ( $3.0 \times 10^5$  platelet/ $\mu\text{L}$ ) from Ctrl and PF4Cre- $\alpha 5^{-/-}$  mice were stimulated with adenosine diphosphate (ADP) (5  $\mu\text{mol/L}$ ), U46619 (2  $\mu\text{mol/L}$ ), or collagen (2.5  $\mu\text{g/mL}$ ). Arrows indicate the point of agonist addition and aggregation profiles are representative of three separate experiments (A). The bar graph represents the percentage of platelet aggregation at 5 minutes ( $n = 3$ ) (B). (C, D) Washed platelets ( $5.0 \times 10^4/\mu\text{L}$ ) from Ctrl and PF4Cre- $\alpha 5^{-/-}$  mice were stimulated for 10 minutes with ADP (2  $\mu\text{mol/L}$ ), thrombin (0.25 U/ml) or protease-activated receptor 4 (PAR-4) (1 mmol/L) and the binding of a fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin antibody (C) or FITC-fibrinogen (D) was detected by flow cytometry. Results represent the mean fluorescence intensity (MFI)  $\pm$  standard error of the mean (SEM) in three separate experiments performed in duplicate and were compared by the Mann-Whitney test. (E) Washed platelets ( $3.0 \times 10^5/\mu\text{L}$ ) from Ctrl and PF4Cre- $\alpha 5^{-/-}$  mice were stimulated with thrombin (0.25 U/ml), convulxin (15 nmol/L), or both for 15 minutes, incubated with Alexa Fluor 488-annexin V for 20 minutes and analyzed by flow cytometry. The forward light scatter and fluorescence intensity of 10,000 cells were collected with a logarithmic gain and the percentage of annexin V-positive platelets was determined in the upper quadrant of the plot. Data are expressed as the mean  $\pm$  SEM in three separate experiments performed in duplicate.

of the recruited platelets showed a marked increase in numbers of PF4Cre- $\alpha 5^{-/-}$  platelets detaching from the surface and a clear decrease in stationary adhesion, highlighting the importance of  $\alpha 5\beta 1$  in stabilizing the bonds between platelets and fibronectin ( $\rightarrow$  Fig. 3F). These results

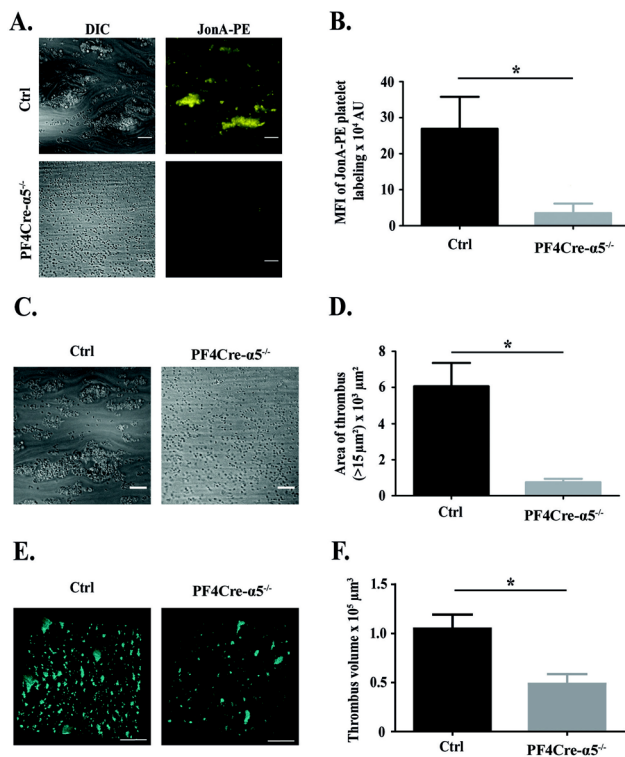


**Fig. 3** PF4Cre- $\alpha 5^{-/-}$  platelets adhere normally to von Willebrand factor (vWF), fibrinogen, and laminin but not to fibronectin. (A) Whole blood from Ctrl ( $n = 3$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 3$ ) was perfused at  $1,500 \text{ s}^{-1}$  through PDMS flow chambers coated with vWF binding protein (DDR2, 100  $\mu\text{g/mL}$ ). Platelet adhesion was visualized in random fields by differential interference contrast (DIC) microscopy, scale bar: 20  $\mu\text{m}$ . The number of adherent platelets was determined over 2 minutes. (B) Whole blood from Ctrl ( $n = 3$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 3$ ) was perfused at  $300 \text{ s}^{-1}$  through PDMS flow chambers coated with fibrinogen (100  $\mu\text{g/mL}$ ) or laminin 411 (100  $\mu\text{g/mL}$ ). Platelet adhesion was visualized in random fields by DIC microscopy and the number of adherent platelets was quantified over 5 minutes. (C, D) Whole blood from Ctrl ( $n = 5$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 4$ ) was perfused at  $300 \text{ s}^{-1}$  through PDMS flow chambers coated with cellular fibrillar fibronectin (300  $\mu\text{g/mL}$ ). Platelet adhesion was visualized in random fields by DIC microscopy, scale bar: 20  $\mu\text{m}$  (C). The number of adherent platelets was determined over 8 minutes (D). (E, F) Whole blood from Ctrl ( $n = 5$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 5$ ) was perfused at  $300 \text{ s}^{-1}$  through PDMS flow chambers coated with fibronectin and platelet attachment was quantified over 60 seconds (E). The behavior of platelets on the fibronectin surface was recorded for 20 platelets per perfusion over a period of 90 seconds in 5 different movies (F). Results are expressed as the mean  $\pm$  standard error of the mean (SEM) and in the curved graphs (A, B, and D) the solid line represents the mean and dashed line the SEM. Results were compared by two-way ANOVA (D), the Mann-Whitney test (E), or Chi-square test (F); \* $p < 0.05$ .

indicated that platelet integrin  $\alpha 5\beta 1$  is a major receptor for fibronectin supporting platelet attachment and maintaining the bonds to ensure stable adhesion.

### A Major Defect of PF4Cre- $\alpha 5^{-/-}$ Platelet Activation and Aggregation on Fibrillar Cellular Fibronectin and Collagen

Platelet activation is a key step in thrombus formation. Microscopic fluorescence images showed a clear JonA-PE signal for control platelets accumulating on fibrillar cellular fibronectin under flow at  $300\text{ s}^{-1}$ , while only a weak signal was detected for PF4Cre- $\alpha 5^{-/-}$  platelets, indicating that  $\alpha 5\beta 1$  is important to promote  $\alpha \text{IIb}\beta 3$  activation on fibronectin (Ctrl:  $26.9 \pm 8.8 \times 10^4$  AU; PF4Cre- $\alpha 5^{-/-}$ :  $3.5 \pm 2.7 \times 10^4$  AU) (►Fig. 4A, B). We observed that thrombi formed in control blood but not in PF4Cre- $\alpha 5^{-/-}$  blood, highlighting a key role of  $\alpha 5\beta 1$  in platelet aggregation on fibrillar cellular fibronectin (►Fig. 4C, D). Surprisingly, a defect in thrombus formation was also observed when PF4Cre- $\alpha 5^{-/-}$  blood was perfused over fibrillar collagen, a surface which does not



**Fig. 4** Defective activation and aggregation of PF4Cre- $\alpha 5^{-/-}$  mouse platelets on fibronectin and collagen. (A, B) Whole blood from Ctrl ( $n = 5$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 5$ ) was perfused at  $300\text{ s}^{-1}$  through PDMS flow chambers coated with cellular fibrillar fibronectin ( $300\ \mu\text{g}/\text{mL}$ ) for 15 minutes. Platelet adhesion was visualized in random fields by differential interference contrast (DIC) microscopy and JonA-PE fluorescence microscopy, scale bar:  $20\ \mu\text{m}$  (A). The mean fluorescence intensity of JonA-PE platelet labeling was quantified (B). (C, D) Whole blood from Ctrl ( $n = 4$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 4$ ) was perfused at  $300\text{ s}^{-1}$  through PDMS flow chambers coated with fibronectin for 15 minutes. Thrombus formation was visualized in random fields by DIC microscopy, scale bar:  $20\ \mu\text{m}$  (C). The area of thrombi of more than  $15\ \mu\text{m}^2$  was quantified (D). (E, F) Whole blood from PF4Cre- $\alpha 5^{-/-}$  ( $n = 7$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 7$ ) was perfused at  $300\text{ s}^{-1}$  through PDMS flow chambers coated with collagen ( $200\ \mu\text{g}/\text{mL}$ ) for 10 minutes. Platelet aggregation was visualized in random fields by confocal microscopy, scale bar:  $50\ \mu\text{m}$  (E). The volume of the thrombi was quantified (F). Results are expressed as the mean  $\pm$  standard error of the mean (SEM) and were compared by the Mann-Whitney test (B, D, and F);  $*p < 0.05$ .

directly activate  $\alpha 5\beta 1$ , suggesting that this integrin participates in thrombus build-up, probably through interactions with plasma fibronectin (►Fig. 4E, F). This role of mouse  $\alpha 5\beta 1$  in thrombus growth over fibrillar collagen was however not observed when human blood was perfused with a blocking anti- $\alpha 5$  antibody, suggesting a species difference (data not shown). Altogether, these findings indicated that integrin  $\alpha 5\beta 1$  plays an important role in thrombus growth on fibronectin.

### $\alpha 5\beta 1$ Does Not Act as a Major Platelet Receptor in Experimental Thrombosis

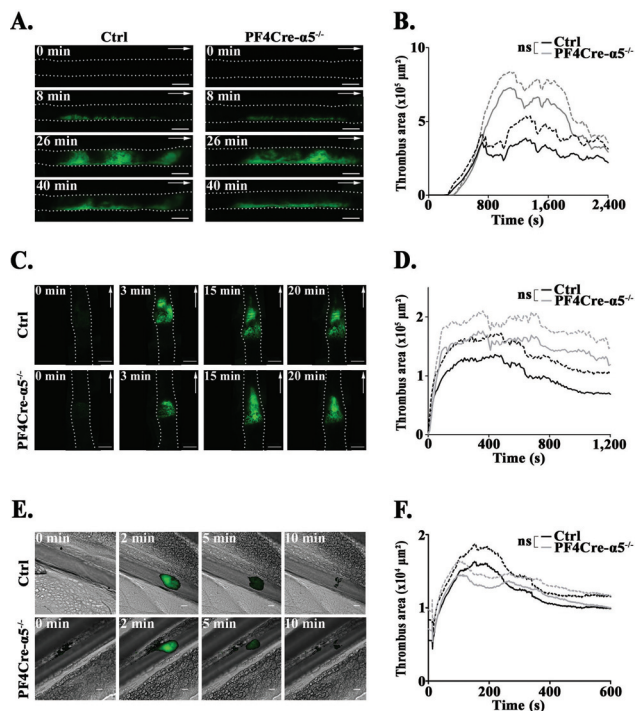
Mice deficient in platelet integrin  $\alpha 5\beta 1$  were studied in three distinct models of localized vascular injury to expose the subendothelium matrix, which is known to contain fibrillar cellular fibronectin. When the common carotid artery was injured with a 7.5% solution of  $\text{FeCl}_3$ , PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 9$ ) presented a similar profile of thrombus formation and disaggregation as compared to controls ( $n = 6$ ) (area under the curve, Ctrl:  $6 \pm 2 \times 10^8\ \mu\text{m}^2$ ; PF4Cre- $\alpha 5^{-/-}$ :  $10 \pm 2 \times 10^8\ \mu\text{m}^2$ ;  $p > 0.05$ ; ►Fig. 5A, B; ►Supplementary Fig. S2A, available in the online version). A comparable result was obtained after mechanical injury of the abdominal aorta with forceps, the thrombus area in PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 8$ ) being not significantly different from that in control mice ( $n = 8$ ) (area under the curve, Ctrl:  $12 \pm 4 \times 10^7\ \mu\text{m}^2$ ; PF4Cre- $\alpha 5^{-/-}$ :  $17 \pm 4 \times 10^7\ \mu\text{m}^2$ ;  $p > 0.05$ ; ►Fig. 5C, D; ►Supplementary Fig. S2B, available in the online version). Finally, following laser-induced injury of mesenteric arteries, PF4Cre- $\alpha 5^{-/-}$  ( $n = 5$  vessels on three mice) also formed thrombi in a similar manner to control mice ( $n = 6$  vessels in three mice) (area under the curve, Ctrl:  $7 \pm 1 \times 10^6\ \mu\text{m}^2$ ; PF4Cre- $\alpha 5^{-/-}$ :  $7 \pm 1 \times 10^6\ \mu\text{m}^2$ ;  $p > 0.05$ ), (►Fig. 5E, F; ►Supplementary Fig. S2C, available in the online version). Hence mouse platelet  $\alpha 5\beta 1$  did not appear to be a major receptor for arterial thrombosis.

### Platelets from PF4Cre- $\alpha 5^{-/-}$ Mice Display No Important Hemostatic Defect

PF4Cre- $\alpha 5^{-/-}$  mice did not present any spontaneous bleeding. In addition, PF4Cre- $\alpha 5^{-/-}$  mice showed no signs of excessive bleeding during surgery as compared to control animals, suggesting that the lack of this integrin on platelets does not critically affect hemostasis. This was further supported by a normal bleeding time ( $n = 6$ ) (Ctrl:  $302 \pm 119\text{ s}$ ; PF4Cre- $\alpha 5^{-/-}$ :  $121 \pm 17\text{ s}$ ) and normal volume of blood lost ( $n = 6$ ) (Ctrl:  $259 \pm 130\ \mu\text{L}$ ; PF4Cre- $\alpha 5^{-/-}$ :  $130 \pm 84\ \mu\text{L}$ ) in a tail-bleeding time assay (►Fig. 6A, B). Finally, we did not observe any effect on inflammatory bleeding in PF4Cre- $\alpha 5^{-/-}$  mice as compared to control mice in an rPA model of skin inflammation (►Fig. 6C, D) and in a LPS pulmonary inflammation model (►Fig. 6E, F). These results do not favor a major role of integrin  $\alpha 5\beta 1$  in the murine hemostatic system.

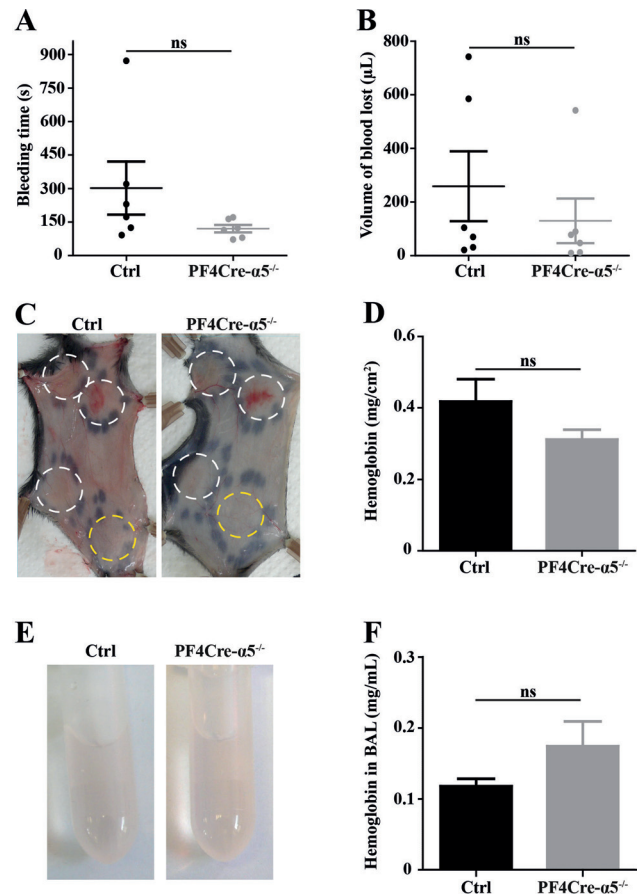
### Discussion

Platelets express numerous adhesion receptors, notably integrins, to sense the proteins exposed on the



**Fig. 5** Platelet integrin  $\alpha 5\beta 1$  does not play a major role in experimental thrombosis in mice. (A, B) Thrombosis was triggered in Ctrl ( $n = 6$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 9$ ) by application of a filter paper saturated with 7.5% FeCl<sub>3</sub> to the common carotid artery. Representative fluorescence images of the thrombus (green) at the indicated time points after injury, scale bar: 500  $\mu\text{m}$  (A). (C, D) thrombosis was triggered in Ctrl ( $n = 8$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 8$ ) by compression of the abdominal aorta with forceps. Representative fluorescence images of the thrombus (green) at the indicated time points after injury, scale bar: 500  $\mu\text{m}$  (C). (E, F) thrombosis was triggered in 6 vessels in 3 control mice and in 5 vessels in 3 PF4Cre- $\alpha 5^{-/-}$  mice by superficial laser injury of a mesenteric arteriole. Representative composite images of the thrombus (green) using bright field and fluorescence microscopy at the indicated time points after injury, scale bar: 50  $\mu\text{m}$  (E). Arrows indicate the direction of blood flow and the dotted lines the borders of the vessels. The time course of thrombus growth is represented by its surface area (mean in solid line  $\pm$  standard error of the mean (SEM) in dashed line) (B, D and F). Results were compared by two-way ANOVA.

subendothelium of an injured vessel and to ensure their efficient recruitment to the site of injury. We show in this study, using tissue-specific knock-out combined with in vitro flow-based assays, that integrin  $\alpha 5\beta 1$  is a functionally key receptor for efficient platelet adhesion, activation, and aggregation on immobilized fibrillar cellular fibronectin, which are found in the vessel wall. Interestingly, in the absence of  $\alpha 5\beta 1$ , the other main platelet receptor for fibronectin,  $\alpha \text{IIb}\beta 3$ , was inefficient in promoting normal platelet adhesion, activation, and aggregation, highlighting the importance of  $\alpha 5\beta 1$  in these processes. We also identified an unexpected role of  $\alpha 5\beta 1$  in platelet aggregation on collagen. Despite these important functional roles identified in vitro, we found that platelet  $\alpha 5\beta 1$  did not play an essential role in the arrest of bleeding after trauma or under inflammatory conditions. Moreover, three distinct experimental models of thrombosis revealed no impact on thrombus formation in



**Fig. 6** PF4Cre- $\alpha 5^{-/-}$  mice display a normal bleeding time and do not present cutaneous or pulmonary bleeding under inflammatory conditions. (A, B) The tail of Ctrl ( $n = 6$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 6$ ) was sectioned. The time required for the first arrest of bleeding was recorded (A) and the volume of blood lost was measured over 30 minutes (B). The symbols correspond to individual mice. (C) Representative images of skin lesions 4 hours after induction of a reverse passive Arthus (rpA) reaction in Ctrl and PF4Cre- $\alpha 5^{-/-}$  mice. White circles represent the points of injection of an antibody and yellow circles the points of injection of control IgG. (D) Quantification of the hemoglobin content (mg/cm<sup>2</sup>) in skin biopsies from Ctrl ( $n = 4$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 4$ ). (E) Representative images of bronchoalveolar liquid (BAL) 24 hours after LPS intranasal inoculation in Ctrl and PF4Cre- $\alpha 5^{-/-}$  mice. (F) Quantification of the hemoglobin content (mg/mL) in the BAL from Ctrl ( $n = 8$ ) and PF4Cre- $\alpha 5^{-/-}$  mice ( $n = 8$ ). Values are the mean  $\pm$  standard error of the mean (SEM) and results were compared by the Mann-Whitney test. IgG, immunoglobulin G; LPS, lipopolysaccharide.

$\alpha 5\beta 1$ -deficient mice as compared to controls, suggesting that this receptor is unlikely to represent a major factor in arterial thrombosis.

We observed that  $\alpha 5\beta 1$ -deficient platelets aggregated normally in response to a series of soluble agonists and to collagen. In addition, fibrinogen binding and P-selectin and annexin V exposure in response to soluble agonists were completely normal, indicating no role of  $\alpha 5\beta 1$  in the amplification step of platelet activation driven by these agonists. Adhesion under flow of  $\alpha 5\beta 1$ -deficient platelets to many adhesive surfaces including fibrinogen, laminins, and vWF

was likewise unchanged. In contrast, we observed a marked impairment of the adhesion of these platelets to immobilized fibrillar cellular fibronectin and collagen. These results suggest that  $\alpha 5\beta 1$  is a highly specialized platelet receptor for probably only one adhesive protein, fibronectin. This is not a unique case, as platelets express many receptors to sense a single adhesive protein, notably the GPIb-IX complex and integrins  $\alpha 2\beta 1$ ,  $\alpha 6\beta 1$ , and  $\alpha v\beta 3$  which bind respectively to vWF, collagen, laminins, and vitronectin.<sup>6</sup> The reason why platelets have maintained distinct specific receptors is probably to ensure efficient adhesion to a wide range of matrices which can be exposed by different types of vessels and at different depths of injury.<sup>22</sup>

It is well established that platelets express two main receptors for fibronectin,  $\alpha 5\beta 1$  and  $\alpha IIb\beta 3$ . Our results support a major role for  $\alpha 5\beta 1$  as a receptor for fibronectin, since in its absence, we observed a dramatic reduction in platelet adhesion and activation on fibrillar cellular fibronectin with almost no aggregation at all. As a consequence, our observations also indicate that  $\alpha IIb\beta 3$  alone is not sufficient to compensate for the absence of  $\alpha 5\beta 1$  in the case of adhesion to a fibronectin surface. We further found that  $\alpha 5\beta 1$ , in addition to its role in initiating platelet adhesion and activation on fibronectin,<sup>15,16</sup> contributed to thrombus growth on collagen, a process known to be mainly ensured by  $\alpha IIb\beta 3$ .<sup>23-25</sup> However, the impairment we observed when perfusing PF4Cre- $\alpha 5^{-/-}$  blood over collagen was modest as compared to what has been reported when  $\alpha IIb\beta 3$  is blocked or absent, where no thrombus growth occurs and only a platelet monolayer forms.<sup>4</sup> One remaining question is the mechanism by which  $\alpha 5\beta 1$  participates in thrombus build-up, as this receptor does not interact with fibrinogen or vWF, two adhesive proteins found in a growing thrombus. Ni et al reported many years ago that mice deficient in both vWF and fibrinogen were still able to form thrombi at late stages and proposed a role of fibronectin in this process.<sup>26</sup> It is therefore reasonable to propose that fibronectin exposed after platelet activation or plasma fibronectin, through interaction with  $\alpha 5\beta 1$ , participates in platelet aggregation. This is consistent with the role of platelet  $\alpha 5\beta 1$  in facilitating the assembly of soluble plasma fibronectin into fibrils on the surface of activated platelets within a growing thrombus.<sup>27</sup> Such a multimerization of fibronectin would increase its reactivity and prothrombotic potential. Moreover, it has been proposed that the CD40 ligand (CD40L) can interact with  $\alpha 5\beta 1$ <sup>28</sup> and that it can support thrombus formation under flow over fibrillar collagen.<sup>29</sup> It is therefore possible that CD40L secreted from granules upon platelet activation can interact with platelet  $\alpha 5\beta 1$  integrin and participate in thrombus growth over collagen.

Petzold et al reported that the absence of all three platelet  $\beta 1$  integrins resulted in an increased tail-bleeding time, suggesting that one or more of these integrins play an important part in normal hemostasis.<sup>30</sup> Using a similar tail-bleeding time assay, we found that  $\alpha 5\beta 1$ -deficient mice did not bleed longer or lose more blood as compared to controls, indicating that  $\alpha 5\beta 1$  is not a key platelet receptor

for hemostasis in mice. This result might appear surprising, especially since the absence of either  $\alpha 2$  or  $\alpha 6$  likewise had no impact on the tail-bleeding time.<sup>10,11</sup> A possible explanation is that  $\beta 1$  integrins play redundant roles in hemostasis, where the absence of one integrin can be compensated by the others to ensure the interaction of platelets with the extracellular matrix components.

Platelets not only arrest bleeding after trauma, but also maintain vascular integrity and stop inflammatory bleeding.<sup>1</sup> The molecular mechanism relies on the immunoreceptor tyrosine-based activation motif, GPVI, and C-type lectin-like receptor II, which promote platelet activation.<sup>31,32</sup> An unresolved question is which receptors support platelet adhesion at sites of inflammatory bleeding, since the process appears to be independent of two major adhesion receptors, the GPIb-IX complex and integrin  $\alpha IIb\beta 3$ .<sup>1,33</sup> We have recently identified a role of  $\beta 1$  integrins in this process (Janus-Bell et al, submitted manuscript). However, our results exclude a key role of  $\alpha 5\beta 1$ , since no bleeding phenotype was observed in PF4Cre- $\alpha 5^{-/-}$  mice using a cutaneous rpA model and a LPS pulmonary inflammation model. While further studies will be required to identify the adhesion receptors important to stop inflammatory bleeding, it is tempting to speculate that compensatory mechanisms between such receptors could explain why no major bleeding occurs when only one of them is absent.

Using three distinct models of arterial thrombosis, in different vessels and under distinct rheological conditions, we did not observe any significant difference in any experimental model of thrombosis. These results indicate that  $\alpha 5\beta 1$  alone does not play a central role in experimental thrombosis. Moreover,  $\alpha 5\beta 1$  appears to be less important than the two other  $\beta 1$  integrins,  $\alpha 2\beta 1$  and  $\alpha 6\beta 1$ , which have both been shown to contribute to experimental thrombosis in at least one of the models used in this study.<sup>11,12,34</sup> The apparent discrepancy we observed between an important role of  $\alpha 5\beta 1$  in platelet aggregation on fibronectin *in vitro* and the absence of a role in experimental models of thrombosis could be linked to the nature of the surface exposed to the flowing blood *in vivo*, which might not contain enough fibrillar cellular fibronectin. Another explanation could be the ability of other platelet adhesion receptors to compensate for the lack of  $\alpha 5\beta 1$  on platelets. As it has been reported that an evolved atherosclerotic plaque is particularly rich in fibrillar cellular fibronectin,<sup>35,36</sup> it might be interesting to test the impact of  $\alpha 5\beta 1$  blockade in apolipoprotein E-deficient models of atherosclerotic plaque rupture. To date, there is no information available about the role of human  $\alpha 5\beta 1$  integrin in arterial thrombosis in humans.

In conclusion, *in vitro* experiments performed with  $\alpha 5\beta 1$ -deficient mouse platelets indicated that this integrin plays a very important role in supporting platelet adhesion, activation, and aggregation on fibrillar fibronectin and participates in thrombus growth. However, integrin  $\alpha 5\beta 1$  appears to be dispensable for hemostasis under normal and inflammatory conditions and is also not essential in *in vivo* models of experimental thrombosis.



### What is known about this topic?

- Platelets express  $\beta 1$  and  $\beta 3$  integrins to allow adhesion, activation, and aggregation.
- $\alpha 5\beta 1$  and  $\alpha IIb\beta 3$  are the main platelet receptors for fibronectin supporting platelet adhesion to fibronectin under flow condition.

### What does this paper add?

- This study highlights the important role of  $\alpha 5\beta 1$  in supporting platelet adhesion, activation, and aggregation on fibrillar cellular fibronectin using PF4Cre- $\alpha 5$ -deficient mice.
- We provide evidence that in the absence of  $\alpha 5\beta 1$ , the other platelet receptor for fibronectin,  $\alpha IIb\beta 3$ , is unable to support normal platelet adhesion to immobilized fibronectin with no platelet aggregation at all.
- Platelet  $\alpha 5\beta 1$  is dispensable for hemostasis under normal and inflammatory conditions and does not play a key role in experimental thrombosis.

#### Author Contributions

A.Y., C.S., E.J.B., and N.R. acquired, analyzed, and interpreted the data and wrote the manuscript; C.B., C.L., C.M., and U.M.A. acquired and analyzed the data; A.E., M.A.P., and Y.A.S. designed the research, interpreted the data, and wrote the manuscript; C.G. contributed to writing of the manuscript; P.H.M. conceived and designed the research, interpreted the data, wrote the manuscript, and handled funding and supervision.

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#### Conflict of Interest

None declared.

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