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Fluorescent labeling of endogenous platelets for intravital microscopy: effects on platelet function

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Abstract

Objective—Monitoring endogenous platelets during intravital microscopy often involves two approaches: fluorescently-labeled antibodies or genetic models of platelet-specific fluorescent protein expression. Due to limited data available on platelet functional changes induced by these methods, we compared functional effects of these methods on platelets.

Methods—Platelet aggregation to collagen and thrombin, and collagen matrix-mediated platelet adhesion/aggregation under flow were tested. We assessed platelets from mice expressing EYFP on platelets (Cre(+)), littermate controls (Cre(–)), C57BL/6 mice, and platelets from vehicle control and DyLight 488-conjugated anti-mouse GP Ib β antibody (x-488) treatment. We utilized intravital microscopy to monitor platelets *in vivo* using Cre(+) mice and x-488 treatment.

Results—Both genetic and antibody-based approaches yielded substantial platelet-specific fluorescence. Platelets from Cre(+) and Cre(–) mice behaved similarly in aggregation and adhesion/aggregation under flow. However, they exhibited significantly enhanced aggregation and higher adhesion/aggregation as compared to platelets from C57BL/6 mice. Compared to vehicle control, x-488 platelet labeling did not induce significant functional changes *in vitro*. Both methods of platelet labeling provided satisfactory platelet detectability *in vivo*.

Conclusions—x-488 antibody labeling of platelets induced less alteration of platelet function than genetic approaches under our experimental conditions, and seems more suitable for monitoring of endogenous platelets.

Keywords

platelet; fluorescent labeling; intravital microscopy; aggregation and adhesion; shear stress

Introduction

An increasing number of laboratories utilize intravital microscopy to monitor platelet function *in vivo*. [1–3] Various non-specific fluorescent techniques, such as rhodamine 6G or acridine red, have been utilized for this purpose. [4,5] Whereas these approaches are effective for labeling endogenous platelets, the lack of specificity leads to labeling of other

cells such as leukocytes, which often results in a stronger fluorescent signal than the intended platelets.[6] Other methods involve obtaining blood from a donor animal, platelet isolation, *ex vivo* labeling with a fluorescent dye (e.g., carboxyfluorescein succinimidyl ester), followed by re-infusion into a recipient animal. The platelet isolation process is labor intensive, may activate platelets,[7,8] and is not suitable for monitoring endogenous platelets. Recent studies aimed at labeling endogenous platelets in a specific manner often use either of two approaches. One approach involves fluorescently-labeled platelet-specific antibodies, such as the commonly used ones against mouse GP Ib β (CD42c), GP IIb (CD41), and integrin α 2 (CD49b).[1,2,9] The second approach involves genetic manipulation to generate models with platelet-specific fluorescent protein expression.[10–12]

One critical assumption made in experiments involving tracking fluorescent platelets with intravital microscopy is that labeled platelets have a comparable function to that of unlabeled platelets from control animals. Although some publications include limited data to address this question,[10,11] there has been insufficient attention paid in the literature devoted to the critical evaluation of this assumption. Therefore, we compared the effects of antibody- and genetic-based approaches for labeling platelets on two important measures of platelet function: aggregation and adhesion/aggregation under flow.

Materials and methods

Animals

All strains originated from the Jackson Laboratory (Bar Harbor, ME). R26R-EYFP^{F/F}/PF4-Cre mice were generated by crossing floxed R26R-EYFP^{F/F} mice (B6.129X1-Gt(ROSA)26Sor^{tm1(EYFP)Cos/J}) with Platelet Factor 4 (PF4)-Cre^{W/T} transgenic mice (C57BL/6-Tg(PF4-icre)Q3Rsko/J). The resultant genotype is R26R-EYFP^{F/F}:PF4-Cre^{W/T}, referred to as Cre(+), which is homozygous for the floxed R26R-EYFP loci and heterozygous for the PF4-Cre transgene. The corresponding littermate control R26R-EYFP^{F/F}:PF4-Cre^{W/W}, referred to as Cre(-), is homozygous for the floxed R26R-EYFP loci and lacks the PF4-Cre transgene. The inserted R26R-EYFP loci and presence/absence of Cre recombinase in the transgenic mice were genotyped using standard genomic PCR techniques of tail clippings. As both R26R-EYFP^{F/F} and PF4-Cre^{W/T} strains were on a C57BL/6 background, we also used C57BL/6 mice as an additional control strain. Both male and female mice were used for experiments at age of 10-30 weeks. Preliminary data from our laboratory demonstrate that platelet aggregation in response to low-dose thrombin is comparable between mice aged 10-20 weeks and mice aged 21-30 weeks (50 $\pm$ 23%, N=15; 44 $\pm$ 17%, N=14; N.S.). Studies were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and by the Michael E. DeBakey Veterans Affairs Medical Center and follow all local, state and federal guidelines for the humane care and use of animals.

Reagents

DyLight 488-conjugated anti-mouse GP Ib β antibody (a rat-monoclonal IgG2a derivative, x-488, Cat. X488) was purchased from Emfret Analytics (Eibelstadt, Germany).

Allophycocyanin (APC)-conjugated anti-mouse CD45 antibody (Cat. 559864) and BD FACS lysing solution (Cat. 349202) were from BD Bioscience (San Jose, CA). APC-conjugated anti-mouse CD41 antibody (Cat. 133914) was from Biolegend (San Diego, CA). The Sepharose CL-4B matrix (Cat. 17015001) was purchased from GE Healthcare Life Sciences. Ultrapure 0.5 M Ethylenediaminetetraacetic acid (EDTA, Cat. 15575020) and $1 \times$ Dulbecco's Phosphate-Buffered Saline (DPBS, Cat. 14190094) were from Thermo Fisher Scientific (Waltham, MA). Paraformaldehyde 32% solution (Cat. 15714) was from Electron Microscopy Sciences (Hartfield, PA). Citrate-phosphate-dextrose solution (Cat. C7165), thrombin (Cat. T6884), calcium chloride (Cat. C1016), magnesium chloride (Cat. M8266), LPS from *E. coli* (Cat. L3024), sodium chloride (Cat. 71376), sodium citrate dihydrate (Cat. W302600), Tris base (Cat. T1503), ϵ -amino-caproic acid (Cat. A2504), Aquacide II (Cat. 17851), bovine serum albumin fraction V (BSA, Cat. 126593) were from Millipore Sigma (St. Louis, MO). D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK, Cat. 15160) and prostaglandin I₂ (Cat. 18220) were purchased from Cayman Chemicals (Ann Arbor, MI). Type I collagen (Cat. 5368) for platelet aggregometry was purchased from Helena Laboratories (Beaumont, TX). Type III collagen (Cat. 5021-10MG) for platelet adhesion under flow experiments was purchased from Advanced Biomatrix (Carlsbad, CA). All reagents were used as received without further purification.

Imaging flow cytometry

To assay enhanced yellow fluorescence protein (EYFP) expression level in mouse platelets and leukocytes, blood collected in citrate-phosphate-dextrose solution was incubated with either 2 $\mu\text{g/mL}$ APC-conjugated anti-CD41 antibody or APC-conjugated anti-CD45 antibody to identify platelet and leukocyte populations. The blood was then diluted 20 times with DPBS and 5mM EDTA and analyzed with an imaging flow cytometer (Imagestream MKII, Millipore Sigma) within 4 hours of isolation. The leukocyte population was gated based on CD45 staining and cell size on a Bright Field (BF)-Area versus APC-CD45 plot (Fig. 1A). The platelet population was identified using a BF-Area versus APC-CD41 plot (Fig. 1B). EYFP fluorescence intensity of leukocyte and platelet populations in C57BL/6 mice was measured, averaged, and normalized to 1. EYFP fluorescence intensity from R26R-EYFP^{F/F}/PF4-Cre mice was transformed relative to that of C57BL/6 mice. To assay the percentage of platelets that were labeled with x-488 *in vitro*, whole blood following labeling with x-488 antibody (1.3 $\mu\text{g/mL}$ or 5 $\mu\text{g/mL}$) and APC conjugated anti-CD41 antibody (2 $\mu\text{g/mL}$) was lysed and fixed with BD FACS lysing solution and subjected to imaging flow cytometry analysis. The platelet population was identified using a BF-Area Versus APC-CD41 plot. The fluorescence intensity of x-488 labeling and the percentage of platelets labeled were assayed within the platelet population.

Platelet isolation and aggregation

Mouse blood was collected from inferior vena cava into citrate-phosphate-dextrose solution in a 9:1 ratio. To test the effect of antibody labeling on platelet aggregation, blood collected from C57BL/6 mice was incubated with 5 $\mu\text{g/mL}$ x-488 at 37 °C for 20 min before platelet isolation. Platelet isolation was performed as described previously.[13] Briefly, blood was diluted with an equal volume of DPBS and then subjected to $80 \times g$ centrifugation at room temperature (RT). Platelet-rich plasma was collected and pelleted with $500 \times g$

centrifugation. Platelet pellets were suspended with DPBS and then rested for 1h at RT before experiments. To prevent platelet activation, 500 nM prostacyclin was added throughout the whole procedure. Isolated platelets supplemented with 1.8 mM CaCl_2 and 1 mM MgCl_2 were challenged with collagen (0.1 $\mu\text{g}/\text{ml}$ or 0.2 $\mu\text{g}/\text{mL}$) or thrombin (0.01 U/mL or 0.02 U/mL) and platelet aggregation was measured under stirring conditions using an eight-channel Bio/Data PAP-8C aggregometer (Bio/Data Corporation, Horsham, PA) at 37 °C.

Platelet adhesion/aggregation under flow

We used a high-throughput microfluidics flow system (BioFlux 1000z, Fluxion Biosciences, CA) to monitor platelet adhesion/aggregation under flow.[14] Micro-channels from 48-well high-shear plates were coated with 50 $\mu\text{g}/\text{ml}$ of type III collagen at RT for 2 h, and then were washed with DPBS before perfusion. Mouse blood was collected using 80 μM final concentration of PPACK as anticoagulant. Whole blood was treated with vehicle control (0.2% BSA in DPBS) or x-488 for 20 min at 37 °C before perfusing over type III collagen-coated channels at a shear rate of 1,500 s^{-1} (shear stress of 60 dyne/cm^2) for 3 min. The channel was then washed and fixed with 1% paraformaldehyde in DPBS for another 1 h. Platelet adhesion/aggregate formation was visualized under bright-field illumination and quantified with FIJI ImageJ software.[15] To evaluate the effect of x-488 on platelet adhesion to von Willebrand factor (VWF), we first isolated murine VWF using the same techniques we reported previously to isolate human VWF.[14] Briefly, mouse blood was anticoagulated with 5mM of EDTA and then centrifuged at $1,500 \times g$ for 15 min to isolate plasma. Plasma was centrifuged at $14,000 \times g$ for 15 min to clear debris. The plasma was then subjected to size-exclusion chromatography using a XK16/70 column (GE Healthcare Life Sciences), with Sepharose CL-4B matrix as solid phase and purification buffer (20 mM Tris, 20 mM sodium citrate, 20 mM ϵ -amino-caproic acid, 150 mM sodium chloride, pH 6.5) as fluid phase. VWF was eluted in the first peak and the collected fraction was subjected to Aquacide II-mediated concentration at 4 °C. Micro-channels from 48-well high-shear plates were coated with 50 $\mu\text{g}/\text{mL}$ of isolated murine VWF at RT for 1h. Channels were washed with DPBS before perfusion. Mouse blood was collected into 3.8% sodium citrate with a ratio of 9:1. Whole blood was incubated with 2 $\mu\text{g}/\text{mL}$ APC conjugated anti-CD41 antibody and vehicle control (0.2% BSA in DPBS) or 5 $\mu\text{g}/\text{mL}$ x-488 for 20 min at 37 °C before perfusing over murine VWF-coated channels at a shear rate of 1,000 s^{-1} . Platelets adherent to the surface were visualized using APC channel and quantified with Bioflux Montage software (Fluxion Biosciences, CA).

LPS induced platelet adhesion in cremaster venules

Male mice (12-24 weeks old; ~30 g) were used for the experiment. The detailed surgical preparation of the cremaster venules for imaging and microscope setup were described in our previous publication.[16] Briefly, mice received intraperitoneal injections of either pyrogen-free isotonic saline or 5 mg LPS/kg body weight (diluted in isotonic saline) 4 hours prior to the experiment. Mice were anesthetized with pentobarbital sodium salt and underwent jugular vein catheterization, tracheostomy, and the cremaster venue preparation for imaging. Then, 0.33 μg per gram body weight of DyLight 488-conjugated anti-GPIIb/IIIa antibody was injected intravenously via the jugular vein. Adhered platelets (stationary for at least 5

seconds) and the corresponding blood vessels were visualized and video-recorded under epifluorescence.

The platelet aggregation, platelet adhesion/aggregation under flow, and platelet adhesion in venules experiments were performed and quantified in a blinded fashion, where the investigator was unaware of the genotype of the mice, the presence of α -488 antibody, or the LPS challenge status until the quantification was complete.

Platelet tracking in the liver

12-24 week old mice of either sex (20-30 g) were used for liver imaging. Detailed surgical preparation of mice was followed [17] with minor modifications. Briefly, mice were anesthetized with pentobarbital and underwent jugular vein-catheterization followed by liver externalization. Mice were placed on a custom-made stage on an inverted microscope with the liver centered over a glass coverslip and superfused with physiologic buffer at 37°C. Mice were kept at 37°C using a radiant warmer and monitored with a rectal probe (Kent Scientific; Torrington, CT). For tracking platelet movement using α -488, C57BL/6 mice were injected with 0.2 μ g per gram body weight of antibody 15 min prior to imaging. For R26R-EYFP^{F/F}:PF4-Cre^{W/T} mice, no other manipulation was needed. Platelet movement was observed using an Olympus FV3000RS super-resolution laser confocal scanning microscope. Time-lapse imaging was taken with a 20 $\times$ /NA0.7 dry objective with 5 $\times$ optical zoom at 30 fps using the resonance scanner operating in two-way mode.

Statistics

Data were expressed as mean $\pm$ standard deviation. Unpaired data were analyzed either by unpaired Student's t-test or by one-way ANOVA with Tukey's post-hoc test. Paired data were analyzed with a paired Student's t-test. $P < 0.05$ was considered statistically significant.

Results

EYFP expression profile in transgenic R26R-EYFP^{F/F}:PF4-Cre mice

Although PF4-Cre^{W/T} mice are used frequently to generate megakaryocyte- and platelet-specific mutants, [18] the specificity for this cell lineage is not absolute, as there are reports of low levels of expression in other blood cells like monocytes and macrophages. [19,20] Therefore, we used imaging flow cytometry to assess the specificity of platelet EYFP expression, as well as percentage of EYFP-expressing platelets. To avoid fluorescent spectral overlap, we used APC-conjugated anti-CD45 and APC-conjugated anti-CD41 antibodies to identify leukocytes and platelets. Leukocytes were gated based on both size and CD45 staining (Fig. 1A) and platelets were gated based on both size and CD41 staining (Fig. 1B). Fluorescence intensity of EYFP in platelets (Fig. 1C) or leukocytes was assayed and compared between samples derived from Cre(-), Cre(+) and C57BL/6 mice. Figure 1D shows representative images of leukocytes and platelets from these three strains of mice. After quantification, EYFP expression in the leukocyte population from Cre(-) and Cre(+) mice was comparable to that of C57BL/6 mice (Fig. 1E), while significant EYFP expression was evident in the platelet population from Cre(+) mice as compared to that of Cre(-) and

C57BL/6 mice (Fig. 1E). Nearly 100% of platelets in the Cre(+) mice were EYFP positive (Fig. 1F).

Platelets from R26R-EYFP^{F/F}:PF4-Cre mice were hyperactive independent of Cre recombinase expression

Fluorescent reporter proteins such as enhanced yellow, green, and red fluorescent proteins (EYFP, EGFP, RFP) are widely used to track cells *in vitro* or *in vivo*; however, introducing fluorescent proteins into cells may potentially alter cellular function. [21] To determine whether platelet function changed as a result of EYFP expression, we first tested platelet aggregation. Platelets from Cre(-), Cre(+) and C57BL/6 mice were isolated and stimulated with collagen or thrombin to monitor platelet aggregation. We performed pilot experiments to determine the optimal doses of the agonists for these experiments (data not shown). At lower dose of collagen (0.5 µg/mL), platelets from Cre(-) and Cre(+) mice responded similarly (Fig. 2A), indicating that expression of EYFP, per se, does not increase the sensitivity of platelets to collagen. However, aggregation of platelets from both Cre(-) and Cre(+) mice was more pronounced than that of platelets from the control strain C57BL/6 in response to low doses of collagen, suggesting that platelets are more active in both strains of transgenic mice as compared to controls. When stimulated with thrombin, platelets from Cre(-) and Cre(+) mice were also more sensitive to low doses of thrombin compared to that of C57BL/6 mice (Fig. 2B). The difference in sensitivity to collagen or thrombin disappeared when the platelets were subjected to higher doses of the agonists (Fig. 2).

While the platelet aggregation assay tested the sensitivity of platelets to agonists, we also used a microfluidics flow system (Bioflux 1000z) to monitor platelet adhesion/aggregation under flow conditions.[14] Murine blood was anticoagulated with PPACK to avoid disturbance of Ca²⁺ levels. Whole blood was perfused over a type III collagen-coated surface and platelet adhesion/aggregation was imaged by bright-field microscopy. As shown in Figure 3A, thrombus formation, which indicates platelet adhesion/aggregation under flow, of blood from Cre(-) and Cre(+) mice was greater as compared to that of C57BL/6 mice. Semi-quantitative analysis conducted with ImageJ also confirmed visual inspection, suggesting that platelets from Cre(-) and Cre(+) mice are prone to adhesion and aggregation under flow condition (Fig 3B).

Platelets labeled with x-488 functioned similarly to that of unlabeled platelets

A variety of antibodies have been used to track endogenous platelets *in vivo*, directed at various platelet ligands including GP IIb (CD41), GP Ibβ (CD42c), and integrin α2 (CD49b).[1,2,9] We used a commercially available antibody for *in vivo* platelet labeling, DyLight 488-conjugated anti-mouse GP Ibβ, to assess the effects of antibody labeling on platelet function. Blood taken from C57BL/6 mice was incubated with either 1.6 µg/ml or 5 µg/ml final concentration of x-488 or vehicle control (0.2% BSA in DPBS) for 20 min at 37 °C before being subjected to either platelet isolation or adhesion/aggregation under flow experiment. We selected the lower concentration of antibody labeling based on published studies using this antibody to track platelets *in vivo*, which typically administered 0.1-0.15 µg intravenously per gram body weight.[22,23] We selected the higher antibody concentration based on preliminary *in vivo* observations demonstrating optimal signal-to-

noise ratio in our fluorescent microscope setup at a dose of 0.33 μg x-488 per gram body weight. Assuming the blood volume in mice is approximately 0.08 mL per gram body weight,[24] the predicted antibody concentration in the published studies would be 1.25-1.875 $\mu\text{g}/\text{ml}$ and 4.125 $\mu\text{g}/\text{ml}$ in our experiments.

After labeling, blood was sampled to determine the efficiency of platelet labeling. The platelet population was identified based on size and APC-CD41 staining (Fig. 4A). Both 1.6 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$ x-488 labeling concentration resulted in nearly 100% labeling efficiency (Fig. 4B, C), while the higher x-488 concentration yielded higher DyLight 488 fluorescence intensity in the platelet population (Fig. 4B, D).

Platelets isolated from higher antibody concentration labeling (5 $\mu\text{g}/\text{ml}$) showed similar aggregation to different doses of collagen and thrombin as compared to vehicle controls (Fig. 5A, B). Further, by using microfluidics to assess platelet adhesion/aggregation under flow, the extent of thrombus formation from the labeled blood was indistinguishable from that of control blood (Fig. 5 C, D). The lower antibody concentration (1.6 $\mu\text{g}/\text{mL}$) showed similar results (data not shown). We also assessed the effect of x-488 labeling on platelet-VWF interaction by quantifying the number of adherent platelets (labeled with APC conjugated anti-CD41 antibody) on murine VWF-immobilized surface at a shear rate of 1000 s^{-1} (Fig. 5E). The number of platelets adherent per field in x-488-treated samples (212 ± 48) was similar to that of control treatment (195 ± 47 ; $N=4$, $p=0.66$, paired Student's t-test; Fig. 5F). These data suggest that antibody labeling of platelets with x-488, even at a concentration that is three times higher than that recommended by the manufacturer (0.1 μg per gram body weight), did not affect platelet aggregation or adhesion/aggregation under flow *in vitro*.

In vivo behavior of Cre(+) mice platelets or x-488 labeled platelets

In addition to the *in vitro* experiments, we performed *in vivo* tracking of platelets using these two models of endogenous platelets labeling. Cre(+) mice or C57BL/6 mice infused with x-488 were subjected to liver imaging using a laser scanning confocal microscope. Both methods resulted in satisfactory tracking of platelets *in vivo* in liver sinusoids (supporting videos S2–S5). In addition, we also employed x-488 labeling to monitor platelet behavior in cremaster venules with or without LPS challenge. More platelets adhered to vessel walls in LPS treated mice compared to saline control mice (Fig 6 and supporting videos S6, S7), indicating that labeling of platelets with x-488 does is suitable to detect increased platelet adhesion *in vivo*.

Discussion

The most common approaches to label endogenous platelets *in vivo* involve either platelet-specific antibodies or genetically introduced fluorescent proteins. However, data on the possible functional alterations induced by these two approaches are limited. We used platelet aggregation and platelet adhesion/aggregation under flow as functional endpoints to evaluate these two approaches. By crossing R26R-EYFP^{F/F} with PF4-Cre^{W/T} mice, we introduced platelet-specific EYFP expression. EYFP expressing platelets from Cre(+) mice behaved similarly to non-EYFP-expressing platelets from Cre(–) in both platelet aggregation and

platelet adhesion/aggregation under flow. However, the generation of Cre(+) and Cre(-) mice seemed to render the platelets more active when compared with platelets from C57BL/6 mice. Meanwhile, labeling platelets by DyLight 488-conjugated anti-GP Ib β antibody, at the concentrations used in our experiments, did not affect platelet function as tested by either platelet aggregation or adhesion/aggregation under flow.

As shown in supporting videos 2–7, both methods of platelet labeling resulted in satisfactory detectability of platelets *in vivo*, and as shown in Figure 6, the adhesive responses *in vivo* of x-488-labeled platelets following LPS stimulation were similar to those reported previously with other techniques [16]. Despite these limited *in vivo* data, our manuscript demonstrates significant differences in platelet function *ex vivo* between antibody and genetic approaches of platelet labeling.

There are several strategies to genetically label platelets using fluorescent proteins. Zhang et al. integrated YFP protein gene just after the CD41 promoter using homologous recombination and generated a mouse strain that expresses membrane-bound YFP in platelets.[10] Similarly, CD41 promoter-controlled GFP or zsGreen1 fluorescent protein expressing plasmids were used to label platelets in zebrafish or rat.[11,12] In the above-mentioned models, the expression of fluorescent protein in platelets was heterogeneous [10,11] and some functional changes of YFP-positive platelets were observed. [10] *In vivo* recruitment of platelets from CD41-YFP mice was reported by Jenne et al. and appeared comparable to that of platelets labeled with anti-CD49b antibodies; [9] However, a thorough comparison of platelet function between the genetic- and antibody-based approaches was not reported. CD41 expression has been reported in cells other than megakaryocytes/platelets, [25] thus using CD41 as a promoter may result in fluorescent protein expression in other cell types.[10][10] By crossing R26R-EYFP^{F/F} and PF4-Cre^{W/T} mice, we employed a different strategy to generate mice expressing EYFP in platelets. Expression of EYFP in R26R-EYFP mice is tightly regulated by the presence of Cre recombinase in specific tissues [26] and EYFP expression is relatively stable and homogenous as a result of reduced susceptibility to epigenetic modification in the ROSA26 locus, [27] which is supported by our data showing that nearly 100% of platelets were EYFP positive in Cre(+) mice (Fig. 1F). The specificity of EYFP expression is determined by PF4 expression in our transgenic model. As PF4 expression is not limited to megakaryocytes/platelets, [19,20,28] we cannot exclude EYFP expression in other cell types and tissues although mean fluorescence intensity of EYFP in leukocytes from Cre(+) mice was comparable to that from Cre(-) and C57BL/6 mice (Fig. 1E).

Several publications have provided evidence showing that GFP/EGFP introduction may disrupt normal cellular process, [29] increase ROS production [30] and lead to labeled cell clearance through immune mechanisms.[31] Although structurally similar to GFP,[32] YFP/EYFP proteins have been reported to result in lower cytotoxicity compared to GFP.[21,33] In the mouse model created by Zhang et al., a higher percentage of YFP positive platelets exposed P-selectin in response to thrombin stimulation as compared to YFP negative platelets, suggesting that YFP expression may increase platelet activity.[10] When we assayed platelet function in our studies, surprisingly we found out that EYFP expression, per se, did not induce any detectable functional changes as compared to the littermate controls.

Rather, platelets from both Cre(-) (R26R-EYFP^{F/F}:PF4-Cre^{W/W}) and Cre(+) (R26R-EYFP^{F/F}:PF4-Cre^{W/T}) mice exhibited enhanced platelet aggregation and adhesion/aggregation under flow than those of C57BL/6 mice. Two genetic factors might explain the heightened response of platelets from Cre(+) and Cre(-) mice. At the ROSA26 site where the EYFP sequence was inserted to construct the R26R-EYFP^{F/F} strain, insertion of targeting vector potentially could disrupt two non-coding sequences and one coding sequence[34] that are conserved between mice and humans.[35] Additionally, the original R26R-EYFP strain was on a mixed background of 129X1/SvJ and C57BL/6J[26] and was backcrossed onto a C57BL/6J for at least 6 generations. The remaining differences in genetic background of Cre(+) and Cre(-) mice as compared to that of C57BL/6 could conceivably contribute to the heightened response. Even though it is difficult to pinpoint what caused the enhanced reactivity of the platelets from Cre(+) and Cre(-) mice, our findings demonstrate that the function of fluorescent platelets in these R26R-EYFP^{F/F}:PF4-Cre^{W/T} mice did not reflect the function of unlabeled platelets derived from control C57BL/6 mice.

The previously constructed fluorescent platelet models were mainly used to track fluorescent megakaryocytes or platelets.[10–12] However, the platelet functional assays carried out by these groups were relatively limited and did not pertain to the hemostatic/thrombotic role platelets played.[10,12] Since our lab focuses on monitoring the incorporation of platelets into thrombi during *in vivo* thrombosis, we used platelet aggregation to test the innate ability of platelets in response to different physiological stimuli. To further examine how platelets would behave under physiologic flow, we used the parallel flow system to test platelets' ability to adhere and aggregate under shear stress. Under the shear rate we used for the assay ($>1,000\text{ s}^{-1}$), initial platelet adhesion is known to be mediated by binding of platelet GP Iba receptor to VWF adsorbed to the immobilized collagen surface. [36] Since we found no effect of x-488 labeling on platelet adhesion/aggregation under flow (Fig. 5C, D), this suggests that x-488 (DyLight 488 conjugated anti-GP Ib β antibody) did not substantially affect platelets' ability to bind VWF under our experimental conditions. Furthermore, platelets from vehicle control and x-488 treatment group showed similar adhesion to murine VWF at a shear rate of $1,000\text{ s}^{-1}$. The lack of effect of x-488 on von Willebrand-mediated platelet adhesion differs from reported effects of a different clone of anti-GP Ib β antibody (clone RAM.1), which inhibited platelet adhesion to VWF under shear.[37] Clearly *in vitro* assays of platelet function do not fully reflect platelet behavior *in vivo* conditions, particularly under conditions such as thrombosis or inflammation. However, our results serve as a useful guide in selecting a method that may be less likely to interfere with platelet function. Although our intravital microscopy data demonstrate that both methods are suitable to detect platelets *in vivo*, the fact that EYFP expressing platelets were more active than control platelets may limit the usefulness of this genetic model in our experimental setups. In contrast, antibody labeling using DyLight488 conjugated anti-mouse GP Ib β at the concentrations used in our experiments, did not induce perceptible changes in the hemostatic/thrombotic function of platelets, which suggests it may represent a more suitable alternative than this specific genetic model in monitoring endogenous platelets for intravital microscopy-based experiments.

Perspectives

We compared the functional effects of a transgenic model (*R26R-EYFP^{F/F}:PF4-Cre*) and a commercially available antibody mediated fluorescent labeling of platelets on two measures of platelet function: platelet aggregation and platelet adhesion/aggregation under flow. The transgenic approach altered platelet function, resulting in enhanced activity as compared to C57BL/6 controls. In contrast, the anti- GP Ib β antibody-based labeling approach did not induce detectable changes in platelet function under our experimental conditions, and thus might be better suited to monitor platelet function *in vivo*. Careful attention to functional effects of platelets is critical for intravital microscopy research involving fluorescent labeling of endogenous platelets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

ADP	adenosine diphosphate
APC	allophycocyanin
BF	Bright Field
CFSE	carboxyfluorescein succinimidyl ester
Cre(–)	R26R-EYFP ^{F/F} :PF4-Cre ^{W/W}
Cre(+)	R26R-EYFP ^{F/F} :PF4-Cre ^{W/T}
DPBS	Dulbecco's Phosphate-Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EYFP	Enhanced Yellow Fluorescent Protein
GFP	Green Fluorescent Protein
LPS	lipopolysaccharide

RFP	Red Fluorescent Protein
RT	Room Temperature
VWF	von Willebrand factor
x-488	DyLight 488 conjugated anti-mouse GP Ib β antibody
YFP	Yellow Fluorescent Protein

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Figure 1.

EYFP expression profile in leukocyte and platelet populations from Cre(-), Cre(+) and C57BL/6 mice. **(A)** Leukocytes were gated based on size and CD45 staining. The leukocyte population was difficult to see due to the limited size of the graph as well as the small number of cells. A higher resolution figure is provided in the Supporting Information (S1). **(B)** Platelets were identified by size and CD41 staining. **(C)** Representative EYFP histogram of platelets from C57BL/6, Cre(-) and Cre(+) mice. **(D)** Representative images of leukocytes and platelets from Cre(-), Cre(+), and C57BL/6 mice in the BF, EYFP, CD45 and CD41 channels. **(E)** Mean fluorescence intensity of leukocytes and platelets in individual mice were normalized to the averaged mean fluorescence intensity of C57BL/6

mice and expressed as EYFP fold change. **(F)** Percentage of EYFP positive platelets in individual Cre(-), Cre(+), and C57BL/6 mice. * indicates $p < 0.05$ using one-way ANOVA with Tukey's post-hoc test.

Figure 2.

Aggregation responses of platelets from Cre(-), Cre(+) and C57BL/6 mice to different doses of agonists. Washed platelets were stimulated with two doses of collagen (**A**) or thrombin (**B**) and platelet aggregation was measured. * indicates $p < 0.05$ using one-way ANOVA with Tukey's post-hoc test; N.S., not significant.

Figure 3.

Platelet adhesion/aggregation under flow assay from Cre(-), Cre(+), and C57BL/6 mice. Murine blood from each strain was perfused over a collagen surface and platelet adhesion/aggregation under flow (shear rate $1,500 \text{ s}^{-1}$) was evaluated. Representative images are shown in (A) and semi-quantification of thrombi formation from each group is shown in (B). * indicates $p < 0.05$ using one-way ANOVA with Tukey's post-hoc test; N.S., not significant; Scale bar=20 μm . Arrows indicate large thrombi.

Figure 4.

In vitro labeling efficiency of x-488. Blood samples labeled *in vitro* with x-488 were analyzed with imaging flow cytometry. (A) Platelets were gated based on size and CD41 staining. (B) Representative x-488 histogram showed vehicle control, 1.6 µg/mL, and 5 µg/mL labeled platelet population. Percentage of (C) x-488 positive platelets and (D) x-488 fluorescence intensity in different treatment groups were measured and plotted. * indicates $p < 0.05$ using one-way ANOVA with Tukey's post-hoc test.

Figure 5.

Effect of x-488 labeling on platelet aggregation and adhesion/aggregation under flow. Platelets isolated from whole blood treated with vehicle control (Ctrl) or 5 µg/mL x-488 antibody (x-488) were subjected to collagen (A) and thrombin (B) induced aggregation. Whole blood labeled with either vehicle control or x-488 antibody were perfused over type I collagen coated surface under shear stress of 60 dyne/cm². Representative images of platelet adhesion/aggregation were shown (C) with corresponding semi-quantification of thrombi formed (D). Whole blood was labeled with vehicle control plus APC conjugated anti-CD41 antibody or x-488 plus APC conjugated anti-CD41 and perfused over a murine VWF-coated surface under shear stress of 40 dyne/cm². Platelet adhesion was monitored using the APC channel. Representative images of platelet adhesion were shown with white background and

black platelets to enhance visibility (**E**) and corresponding quantification of platelet adhesion (**F**). Unpaired Student's t-test was used to analyze (A) and (B) whereas paired Student's t-test was used to analyze (D) and (F). N.S., not significant; Scale bar=20 μm .

Figure 6.

Platelet adhesion to cremaster venules visualized with x-488 labeling. Mice were challenged with Saline or LPS 4 hours prior to imaging. Endogenous platelets were monitored by intravenously injected x-488 antibody. Number of platelets adhered on the vessel wall per mm² vessel wall surface area in saline or LPS challenged mice was counted. Data were analyzed using unpaired Student's t-test. * indicates p<0.05