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The *Gp1ba-Cre* transgenic mouse: a new model to delineate platelet and leukocyte functions

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Key Points

1. The *Gp1ba-Cre* mouse allows highly specific deletion of *floxed* genes in the megakaryocyte lineage.
2. The *Gp1ba-Cre* mouse enables differentiation of platelet and leukocyte functions.

Abstract

Conditional knockout (KO) mouse models are invaluable for elucidating the physiological roles of platelets. The *Pf4-Cre* transgenic mouse is the current model of choice for generating megakaryocyte/platelet-specific KO mice. Platelets and leukocytes work closely together in a wide range of disease settings, yet the specific contribution of platelets to these processes remains unclear. This is partially due to the *Pf4-Cre* transgene being expressed in a variety of leukocyte populations. To overcome this issue, we developed a *Gp1ba-Cre* transgenic mouse strain in which *Cre* expression in driven by the endogenous *Gp1ba* locus. By crossing *Gp1ba-Cre* and *Pf4-Cre* mice to the *mT/mG* dual-fluorescence reporter mouse and performing a head-to-head comparison, we demonstrate more stringent megakaryocyte lineage-specific expression of the *Gp1ba-Cre* transgene. Broader tissue expression was observed with the *Pf4-Cre* transgene, leading to recombination in many hematopoietic lineages, including monocytes, macrophages, granulocytes, dendritic, B and T cells. Direct comparison of phenotypes of *Csk, Shp1* or *CD148* conditional KO mice generated using either the *Gp1ba-Cre* or *Pf4-Cre* strains revealed similar platelet phenotypes. However, additional inflammatory and immunological anomalies were observed in *Pf4-Cre*-generated KO mice due to non-specific deletion in other hematopoietic lineages. By excluding leukocyte contributions to phenotypes, the *Gp1ba-Cre* mouse will advance our understanding of the role of platelets in inflammation and other pathophysiological processes where platelet-leukocyte interactions are involved.

Introduction

Platelets and leukocytes have closely interlinked functions. Platelets are increasingly recognized to contribute to inflammation,[1] immunity,[2,3] atherogenesis,[4] cancer metastasis[5] and the separation of blood and lymphatic vasculature.[6] Concomitantly, leukocytes play a critical role in thrombo-inflammatory diseases, including ischemic stroke, myocardial infarction and deep vein thrombosis.[7] Megakaryocyte (MK)/platelet-specific conditional knockout (KO) mouse models have become invaluable means of determining the molecular mechanisms regulating platelet production and pathophysiological functions in which they are involved. The current model of choice for generating MK/platelet-specific conditional KO mice is the *Platelet factor 4-Cre recombinase* (*Pf4-Cre*) transgenic mouse, developed by Skoda and co-workers.[8] The *Pf4-Cre* mouse has been used in over 160 studies to date to generate a variety of MK/platelet-specific conditional KO mice (Supplemental Table 1), providing numerous novel insights into platelet production and function.

However, the utility of the *Pf4-Cre* strain to delineate the role of platelets from leukocytes is limited. Recent studies demonstrate transgene expression outside the MK lineage, including hematopoietic stem cells, a sub-population of circulating leukocytes and macrophages.[9-12] Recombination in these cells can result in phenotypes unrelated to deletion of proteins in platelets, a case in point being reduced plasma levels of FXIII-A in *F13a1*$^{fl/fl}$*;Pf4-Cre* mice being due to factor (F)XIII-A ablation in Pf4-expressing macrophages.[13] A particularly confounding issue with *Pf4-Cre* transgene is that a considerable proportion of leukocytes undergo recombination upon inflammatory stimulation,[9,12] complicating *in vivo* experiments.

Importantly, findings on broader *Pf4-Cre* expression are in agreement with reports on endogenous Pf4 (also referred to as C-X-C motif chemokine 4; Cxcl4) expression pattern. Although this chemokine was for many years thought to be expressed exclusively in MKs

and platelets;[14,15] there is now unequivocal evidence that endogenous Pf4 is also expressed in a variety of immune cells, including monocytes,[16-20] macrophages,[13,16,18-24] microglia,[25,26] dendritic cells,[20,24,27-30] granulocytes,[31] mast cells,[32] T cells[12,17] and B cells,[20,24] either constitutively or upon stimulation. Moreover, broader endogenous Pf4 expression is also supported by gene expression databases.[33-35] Unexpectedly, endogenous Pf4 expression has also been described outside the hematopoietic lineage in a population of intestinal epithelial cells.[36] Moreover, *Pf4-Cre*-mediated recombination was also observed in a subset of epithelial cells in the colon, resulting in the development of colon cancer in *Adenomatous polyposis coli*$^{fl/fl}$*;Pf4-Cre* mice.[9] Thus, caution must be taken when interpreting phenotypes arising from *Pf4-Cre*-generated conditional KO mouse models. Notably, there are currently no alternative Cre deleter strains available that are specific for the MK lineage.

To overcome these limitations of the *Pf4-Cre* deleter strain, we developed a *Gp1ba-Cre* transgenic mouse model, utilizing the MK/platelet-specific endogenous *Gp1ba* locus to drive Cre expression. *Gp1ba* encodes the glycoprotein (GP)Ibα subunit of the GPIb-IX-V complex, the receptor for von Willebrand factor (vWF).[37] Our results demonstrate that the *Gp1ba-Cre* deleter mouse enables highly efficient and specific ablation of *floxed* genes in the MK lineage. We provide a new tool for generating MK/platelet-specific KO mice that allows researchers to differentiate the functional roles of platelets and leukocytes in any pathophysiological condition.

Materials and methods

Mouse models

All mice used were on a C57BL/6 background. The *Gp1ba-Cre* transgenic mouse was generated as described below. Membrane-targeted tandem dimer tomato/membrane-targeted green fluorescent protein (*mT/mG*) double fluorescent Cre reporter mice were obtained from Jackson Laboratory.[38] *Pf4-Cre*$^{+/KI}$, *Csk*$^{fl/fl}$, *CD148*$^{fl/fl}$, and *Shp1*$^{fl/fl}$ mice were generated as previously described.[8,39-41] *mT/mG*$^{+/fl}$*;Pf4-Cre*$^{+/KI}$, *Csk*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$, *CD148*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$, and *Shp1*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice were generated by crossing *Pf4-Cre*$^{+/KI}$ male mice with *mT/mG*$^{+/fl}$, *Csk*$^{fl/fl}$, *CD148*$^{fl/fl}$ or *Shp1*$^{fl/fl}$ female mice, respectively.[42,43] Similarly, *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$, *Csk*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$, *CD148*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$, and *Shp1*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice were generated by crossing *Gp1ba-Cre*$^{+/KI}$ male mice with *mT/mG*$^{+/fl}$, *Csk*$^{fl/fl}$, *CD148*$^{fl/fl}$ or *Shp1*$^{fl/fl}$ female mice. All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986.

Generation of *Gp1ba-Cre* transgenic mouse

The targeting strategy enabled the generation of a constitutive knock-in (KI) of a *T2A-improved-Cre (iCre)* in the endogenous *Gp1ba* gene (Taconic Biosciences, Hudson, NY, USA) (Figure 1). Exon 2 of *Gp1ba* gene contains the complete open reading frame. The sequences for the *T2A* and the open reading frame of *iCre* have been inserted between the last amino acid and the translation termination codon in exon 2 of *Gp1ba* (NCBI Reference Sequence: NM_010326_2). The positive selection marker (Puromycin resistance) was flanked by *FRT* sites and inserted downstream of the mouse 3' untranslated region. The targeting vector was transfected into the Taconic Biosciences C57BL/6N Tac ES cell line and homologous recombinant clones were isolated using positive (puromycin resistance) and

negative (thymidine kinase) selections. The constitutive KI allele expresses a chimeric transcript harboring the *Gp1ba* gene fused to the *T2A* and the *iCre* sequences and was obtained after *in vivo* Flp-mediated removal of the selection marker, which was confirmed by PCR on genomic DNA. To detect the constitutive KI allele (372 bp fragment), as well as the wild-type (WT) allele (297 bp fragment) by PCR, the following primers were used: 5'-GAACACAACTCTCCTTGCTGG-3'– forward, 5'-GAAGAGTTAATGGCAGGAAAGAG-3'–reverse.

All other materials and methods along with the statistical analysis are described in supplemental Materials and methods.

Results

Generation of the *Gp1ba-Cre* transgenic mouse strain

The *Gp1ba* gene was selected to drive Cre expression, because it is well established that GPIbα is highly and specifically expressed in MKs and platelets.[44] The *T2A* sequence was employed in the targeted insertion strategy, which allows multiple proteins to be expressed from a single multi-cistronic transcript.[45] To enable the expression of the recombinase under control of the endogenous *Gp1ba* locus, *T2A* was inserted in-frame with *improved-Cre* (*iCre*) between the last amino acid and the translation termination codon in *Gp1ba* (Figure 1). Upon translation of the chimeric *Gp1ba-T2A-iCre* mRNA, the *T2A* sequence will lead to 'ribosome skipping', resulting in the co-expression of GPIbα and Cre as discrete proteins in cells where GPIbα is endogenously expressed. As a result of the T2A technology, the GPIbα protein is expected to possess an additional 17 amino acids (EGRGSLLTCGDVEENPG) at its C-terminus and a proline on the N-terminus of iCre. *Gp1ba-Cre* mice are viable, fertile, displayed no overt developmental or behavioral defects and are born at expected Mendelian frequencies (Supplemental Table 2).

Normal platelet count and minor changes in receptor expression

The *Gp1ba-Cre* mice used in the present study were heterozygous for the knock-in (KI) allele *(Gp1ba-Cre$^{+/KI}$)*. Platelet count and other hematological parameters were normal in these mice, except platelet volume, which was increased by 18% (Figure 2A and Supplemental Table 3). To test whether expression of the GPIb-IX-V complex was affected, we measured surface receptor expression levels of each subunit of the complex, namely GPIbα, GPIbβ, GPIX and GPV, as well as GPVI, CLEC-2 and the integrins αIIbβ3 and α2 by flow cytometry (Figure 2B). GPIbα expression was reduced by 33%, likely accounting for the increase in platelet volume (Figure 2B). GPIbβ and GPIX were concomitantly

reduced by 18% and 19%, respectively, whereas GPV expression was normal. In contrast, surface expression of the integrin αIIbβ3 was increased by 18%, likely reflecting the increase in platelet volume. No significant differences were detected in surface expression of integrin α2, GPVI and CLEC-2 (Figure 2B).

To better understand the consequences of the *Gp1ba-Cre* targeted insertion strategy on GPIbα expression and other platelet parameters, we analyzed mice homozygous for the KI allele *(Gp1ba-Cre$^{KI/KI}$)*. These mice had a 49% reduction in platelet count, 71% increase in platelet volume, and reduced surface expression of GPIbα (72%), GPIbβ (51%), GPIX (51%), GPV (50%) compared with WT mice (Supplemental Figure 1). The relationship between GPIbα levels and platelet volume and count is well established both in humans and mice,[46,47] and suggests that the targeted insertion might interfere with mRNA stability, translation or GPIbα protein stability or trafficking.

Normal platelet function and hemostasis

To study whether the reduction in GPIb-IX complex levels translate to aberrant platelet function in the *Gp1ba-Cre$^{+/KI}$* mice, platelet aggregation and ATP secretion were measured in response to a range of platelet agonists. No differences were found in aggregation or secretion in response to collagen (3 and 10 μg/ml), the synthetic GPVI-specific agonist collagen-related peptide (CRP, 3 and 10 μg/ml), thrombin (0.06 and 0.1 U/ml), the thromboxane A2 analog U46619 (3 and 10 μM) or ADP (10 and 30 μM) (Figure 3A and Supplemental Figure 2). Recombinant botrocetin-2 (rBot2; 1 μg/ml) and mouse vWF (10 μg/ml) were used in combination to specifically test GPIb-IX-V-mediated platelet agglutination.[48] Despite the reduced expression of GPIb-IX complex on the platelet surface,

no differences were observed in the function of GPIbα in platelets from *Gp1ba-Cre*$^{+/KI}$ mice (Figure 3B).

The ability of *Gp1ba-Cre* platelets to adhere and spread on a fibrinogen-coated surface under static conditions was subsequently assessed. Non-stimulated and thrombin pre-activated *Gp1ba-Cre* platelets spread to the same extent as WT platelets on fibrinogen (Supplemental Figure 3), demonstrating normal integrin αIIbβ3-mediated functional responses.

To investigate whether minor defects described above translated into hemostatic complications in *Gp1ba-Cre*$^{+/KI}$ mice, we assessed tail bleeding following excision of a 5 mm portion of the tail tip. No significant differences in blood loss were observed in *Gp1ba-Cre*$^{+/KI}$ mice compared with WT mice (Supplemental Figure 4). Collectively, these findings demonstrate that platelet function is normal in *Gp1ba-Cre*$^{+/KI}$ mice. *Gp1ba-Cre*$^{+/KI}$ mice were used to generate conditional KO mice and as controls in all subsequent experiments.

Highly specific recombination in the MK lineage

The expression pattern of Cre is expected to mirror that of endogenous GPIbα in the *Gp1ba-Cre*$^{+/KI}$ mouse. To test whether Cre is specifically expressed in the MK lineage, we crossed the *Gp1ba-Cre*$^{+/KI}$ mouse with the *mT/mG* double-fluorescent Cre reporter mouse.[38] Cells in the *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ mouse express membrane-targeted tandem dimer Tomato (mT; tdTomato) prior to Cre-mediated recombination and membrane-targeted enhanced green fluorescent protein (mG; EGFP) following Cre-mediated excision. Although, the EGFP signal appears rapidly, within 24 hours after Cre expression, the tdTomato fluorescence disappears slowly, due to gradually declining tdTomato mRNA and protein levels.[38] Monitoring both EGFP and tdTomato fluorescence can provide unique insights on the onset of Cre expression, which is especially useful for studying megakaryopoiesis. For direct

comparison, we also crossed the *mT/mG* reporter strain with the *Pf4-Cre* mouse to generate *mT/mG*$^{+/fl}$*;Pf4-Cre*$^{+/KI}$ mice and analyzed them in parallel with the *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice.

We measured recombination in platelets from peripheral blood using flow cytometry, by counting EGFP^{+} events in the CD41^{+} (integrin αIIb^{+}) population. We found that 98.7% and 99.9% of platelets were EGFP^{+}, thus undergoing recombination in *Gp1ba-Cre*- and *Pf4-Cre*-generated mice, respectively (Figure 4A). Interestingly, 64.1% and 2.4% of *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ and *mT/mG*$^{+/fl}$*;Pf4-Cre*$^{+/KI}$ were EGFP^{+}tdTomato^{+}, respectively, suggesting recombination takes place later in *Gp1ba-Cre*-generated mice (Supplemental Figure 5). We next determined EGFP^{+} fractions of TER-119^{+} (erythroid marker) cells and found that 0.1% and 0.2% of red blood cells expressed EGFP in *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ and *mT/mG*$^{+/fl}$*;Pf4-Cre*$^{+/KI}$ mice, respectively (Figure 4A). Subsequently, we quantified EGFP expression in CD45^{+} (pan-leukocyte marker) blood cells and found 0.3% EGFP^{+}tdTomato^{-} and 2.3% EGFP^{+}tdTomato^{+} cells in *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice. Whereas the former population likely represents *bona fide* recombined cells, we cannot exclude the possibility that the latter population includes EGFP^{+} platelet-leukocyte aggregates. Notably, we found that 25.6% of CD45^{+} cells were EGFP^{+}tdTomato^{-} in *mT/mG*$^{+/fl}$*;Pf4-Cre*$^{+/KI}$ mice, suggesting recombination in a large population of circulating leukocytes.

We next quantified EGFP-expressing bone marrow (BM) cells. We observed 0.7% EGFP^{+}tdTomato^{-}, 54.6% EGFP^{+}tdTomato^{+} and 42.9% EGFP^{-}tdTomato^{+} BM cells within the CD41 high (CD41^{hi}) population in *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice (Figure 4B). When we gated on mature GPIbα^{hi} MKs, we found 1.9% EGFP^{+}tdTomato^{-}, 72% EGFP^{+}tdTomato^{+} and 23% EGFP^{-}tdTomato^{+} cells in the same mouse model (Figure 4B). In contrast, in *mT/mG*$^{+/fl}$*;Pf4-Cre*$^{+/KI}$ mice, the CD41^{hi} population contained 14.9% EGFP^{+}tdTomato^{-},

73.9% EGFP$^+$tdTomato$^+$ and 3.6% EGFP$^-$tdTomato$^+$ cells, whereas the GPIbα^{hi} population contained 26.8% EGFP$^+$tdTomato$^-$, 68.4% EGFP$^+$tdTomato$^+$ and 2.3% EGFP$^-$tdTomato$^+$ cells (Figure 4B). These results indicate that *Pf4-Cre* is expressed earlier and most (95.2%) of GPIbα^{hi} MKs are EGFP$^+$, whereas because *Gp1ba-Cre* is expressed relatively later fewer (73.9%) of GPIbα^{hi} MKs are EGFP$^+$. This is most likely due to the 24 hour delay in generating detectable levels of EGFP following recombination.**[ref]** Subsequently, we focused on the TER-119$^+$ BM cell population representing erythroid lineage cells. We observed 6.2% EGFP$^+$tdTomato$^-$ cells in the *mT/mG$^{+/fl}$;Gp1ba-Cre$^{+/KI}$* mice, likely representing a common MK/erythroid progenitor population (Figure 4B). In contrast, 24.4% of TER-119$^+$ BM cells were EGFP$^+$tdTomato$^-$ in the *mT/mG$^{+/fl}$;Pf4-Cre$^{+/KI}$* mice, suggesting recombination in maturing erythroid lineage cells in these mice. The lack of EGFP$^+$ or tdTomato$^+$ erythrocytes in either mouse model is most likely due to protein degradation during the long lifespan of these cells (Figure 4A).

We then investigated EGFP expression in different subsets of spleen cells. We found less than 0.3% EGFP$^+$tdTomato$^-$ myeloid cells (CD11b$^+$Gr-1$^-$), granulocytes (CD11b$^+$Gr-1^{hi}), conventional dendritic cells (CD11c^{hi}B220$^-$), B cells (B220$^+$CD11c$^-$) and T cells (CD3ε$^+$F4/80$^-$) in *mT/mG$^{+/fl}$;Gp1ba-Cre$^{+/KI}$* mice (Figure 4C). In addition, we observed 4.7% EGFP$^+$tdTomato$^+$ macrophages (F4/80$^+$CD11b$^+$) in these mice, which either represent *bona fide* recombined macrophages or cells which have phagocytosed EGFP$^+$ platelets.

Strikingly, we detected high proportions of EGFP$^+$tdTomato$^-$ myeloid and lymphoid cell subsets in *mT/mG$^{+/fl}$;Pf4-Cre$^{+/KI}$* spleens, including 23.2% myeloid cells (CD11b$^+$Gr-1$^-$), 25.2% granulocytes (CD11b$^+$Gr-1^{hi}), 23.3% conventional dendritic cells (CD11c^{hi}B220$^-$), 13.8% B cells (B220$^+$CD11c$^-$) and 14.1% T cells (CD3ε$^+$F4/80$^-$) (Figure 4C). Furthermore, we identified 48% EGFP$^+$tdTomato$^+$ macrophages (F4/80$^+$CD11b$^+$) in these mice, suggesting recombination in immature macrophages.

Collectively, these findings demonstrate Cre-mediated recombination in a large proportion of platelets and MKs in both *Gp1ba-Cre*$^{+/KI}$ and *Pf4-Cre*$^{+/KI}$ mice. However, the earlier onset of *Pf4-Cre* also results in recombination in ~15-50% of erythroid, myeloid and lymphoid cells, not observed in *Gp1ba-Cre* mice.

Similar platelet phenotypes

After establishing the lineage-specificity of Cre expression in hematopoietic cells, we sought to compare protein ablation efficiency and phenotypes of conditional KO mice generated with either *Gp1ba-Cre* or *Pf4-Cre* mouse. To achieve this, we crossed both deleter strains to *Csk*-[39], *CD148*-,[40] or *Shp1-floxed* mice[41], respectively for direct comparison. Csk, CD148 and Shp1 are widely expressed in hematopoietic cells and have been implicated in regulating immune cell development and function.[39-41,49]

Protein levels of Csk from *Csk*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ and *Csk*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ platelets were analyzed by quantitative capillary-based immunoassay (ProteinSimple Wes) and compared to their corresponding controls, *Csk*$^{+/+}$*;Gp1ba-Cre*$^{+/KI}$ and *Csk*$^{+/+}$*;Pf4-Cre*$^{+/KI}$ platelet lysates, respectively. We found efficient protein ablation in *Csk*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ platelets revealing a 93% reduction of Csk levels, whereas *Csk*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ platelets showed a 99% reduction (Figure 5A). *Csk*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice showed a 71% reduction in platelet count, a 35% increase in platelet volume, an 85% reduction in GPVI and a 42% increase in G6b-B levels (Figure 5B-C). Importantly, *Csk*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice exhibited a similar, but less severe platelet phenotype, including a 32% reduction in platelet count, a 15% increase in platelet volume, a 62% decrease in GPVI and a 37% increase in G6b-B levels (Figure 5B-C). Differences between the two models likely reflect incomplete Csk ablation in *Csk*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ platelets and can be explained by the later onset of *Gp1ba-Cre* transgene expression (Figure 4B). Notably, we found that white blood cell and lymphocyte counts were

significantly elevated in the *Csk*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice, but unaltered in the *Csk*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice (Figure 5D), suggesting Csk deletion in lymphocytes in *Csk*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice.

Surface receptor levels of CD148 on *CD148*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ and *CD148*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ platelets displayed 95.1% and 93.6% reduction, respectively, as determined by flow cytometry (Figure 6A). Surface levels of GPVI were reduced by 58% and 57% in the two mouse models, respectively (Figure 6B). *CD148*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ platelets showed remarkably similar aggregation responses to *CD148*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ platelets, including the previously described GPVI-specific aggregation defect in response to CRP and normal aggregation in response to thrombin (Figure 6C).[42,50] Highly similar aggregation responses were observed to a range of CRP concentrations (Figure 6D), demonstrating that *CD148*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ platelets phenocopy their *Pf4-Cre*-generated counterparts.

No associated inflammatory complications

Shp1 levels were reduced by 90% and 96% in platelets from *Shp1*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ and *Shp1*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$, respectively (Figure 7A). Intriguingly, 75% of *Shp1*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice developed a *motheaten*-like inflammatory phenotype by 8 weeks of age culminating in the inflammation of the paws, ears or nose (Figure 7B-C). The *motheaten* phenotype arises from a spontaneous mutation in *Shp1*, leading to loss of Shp1 expression.[51] It is characterized by hyper-inflammatory and autoimmune responses whereby different hematopoietic cell subsets contribute to different aspects of the phenotype.[52] In contrast, none of the *Shp1*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice showed signs of the *motheaten*-like phenotype (Figure 7B), suggesting that it is unlikely to be driven by the lack of platelet Shp1. To better understand the etiology of the inflammatory phenotype, we analyzed leukocyte counts in peripheral blood and spleen of these mice. *Shp1*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice showed significantly elevated monocyte, atypical lymphocyte (ALY) and large immature cell (LIC) counts, suggesting aberrant development of

multiple leukocyte subsets. This was not the case in *Shp1*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice, which had normal blood counts (Figure 7D). *Shp1*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice displayed significantly increased spleen/body weight ratio, total splenocyte, granulocyte (Gr-1^{hi}F4/80^{-}CD11c^{-}B220^{-}) and conventional dendritic cell (cDC; CD11c^{hi}B220^{-}) counts, and activated cDCs (CD86^{+}) (Figure 7E). All of these parameters were normal in *Shp1*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice (Figure 7E), highlighting the lack of transgene expression in these other lineages. These results strongly suggest that Shp1 is ablated outside of the MK lineage in the *Shp1*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice, supporting findings from *mT/mG* $^{fl/+}$*;Pf4-Cre*$^{+/KI}$ mice (Figure 4A-C).

Taken together, these findings provide evidence that the novel *Gp1ba-Cre* mouse strain is highly efficient at ablating *floxed* genes in MKs, leading to an almost complete deletion of corresponding proteins in platelets. Reporter mouse experiments together with the lack of leukocyte-related phenotypes in *Gp1ba-Cre*-generated mice support MK lineage-specific transgene expression, which offers a powerful alternative strategy to generate MK/platelet-specific conditional KO mice.

Discussion

In this study, we report a new Cre deleter mouse strain that allows MK/platelet lineage-specific recombination of *floxed* genes. We demonstrate that 98.7% of platelets undergo recombination in *Gp1ba-Cre*$^{+/KI}$ mice. We show that this transgene is highly efficient at deleting proteins in platelets and results in similar platelet phenotypes to *Pf4-Cre*-generated KO mice. To test the utility of this new model, we have directly compared it to the *Pf4-Cre* mouse strain in fluorescent reporter mouse experiments revealing highly specific expression in the MK lineage with the *Gp1ba-Cre* transgene and wider expression with *Pf4-Cre*. To highlight the advantage of the *Gp1ba-Cre* strain, we show that the *motheaten*-like inflammatory phenotype exhibited by *Pf4-Cre*-generated *Shp1* conditional KO mice is not observed in *Gp1ba-Cre*-generated KO mice. These results, along with widespread recombination of *mT/mG*$^{+/fl}$*;Pf4-Cre*$^{+/KI}$ leukocytes strongly support that the inflammatory phenotype is driven by ablation of *Shp1* in multiple leukocyte lineages. Platelets and leukocytes are often involved in the same processes; and recombined leukocytes can contribute towards the overall phenotype of *Pf4-Cre*-generated KO mice as demonstrated in the present study. The *Gp1ba-Cre* mouse will advance the platelet and immunology fields by enabling the separation of platelet and leukocyte contributions to *in vivo* phenotypes in an array of pathological conditions.

Gp1ba was selected to drive Cre expression, because it is highly specific and drives high expression of GPIbα from an early stage during megakaryopoiesis.[44,53] Although its specificity to the MK lineage is well established, a limited number of early studies reported GPIbα expression in cultured endothelial and smooth muscle cells.[54,55] However, these findings were never validated by other groups or *in vivo*.[44] In our targeting strategy, we utilized the endogenous *Gp1ba* locus, thus *Cre* is controlled by the native promoter with its intact enhancer and repressor elements. In contrast, the *Pf4-Cre* mouse contains four

additional chemokine genes as a consequence of the bacterial artificial chromosome construct utilized for its generation, namely *Cxcl3*, *Cxcl5*, *Cxcl7* and *Cxcl15*.[8] Additional copies of these genes may explain the significantly lower platelet counts in the *Pf4-Cre* mouse,[9] but can also confound inflammatory and immune responses, as they are involved in modulating leukocyte recruitment and activation, [56-58] which is not an issue with our targeting strategy.

The *T2A* sequence used to couple Cre to GPIbα expression allows the equimolar expression of proteins from a single multi-cistronic transcript.[45] The *T2A*-based insertion results in addition of a short peptide sequence to the C-terminus of GPIbα, which may explain the observed reduction in GPIbα surface expression levels in *Gp1ba-Cre* mice. Alternatively, reduced GPIbα expression might arise from less efficient chimeric mRNA transcription or reduced translation. The modest decrease in GPIbα likely underlies the marginal increase in platelet volume in *Gp1ba-Cre*$^{KI/+}$ mice, which is well established in humans and mice with reduced GPIbα expression.[46,47,59] Although minor changes in platelet volume and receptor levels did not translate into defects in platelet function or hemostatic complications, we advocate using *Gp1ba-Cre*$^{+/KI}$ mice as controls for conditional KO mice generated with this strain, as is the case with *Pf4-Cre*-generated or any other conditional KO mouse models.

We demonstrate that 73.9% GPIbα^{hi} MKs undergo recombination in the *mT/mG;Gp1ba-Cre* mice, pointing to high Cre expression in mature MKs. A possible reason for incomplete EGFP labeling of MKs is the short lag between GPIbα and Cre expression, recombination and EGFP accumulation.[38] GPIbα was recently reported in unipotent MK progenitors further supporting the lineage specificity of *Gp1ba*.[53] In contrast, CD41 is not exclusively MK-specific, but is also expressed on hematopoietic progenitors,[60-62] providing an explanation for the observed lower recombination of 55.3% of CD41^{hi} BM cells compared

to GPIbα^{hi} MKs. As a consequence, *CD41-Cre* mice are not suitable for generating MK/plt-specific KO mice.[60]

A limitation of the *Gp1ba-Cre* mouse is that the onset of recombination occurs later than *Pf4-Cre* during megakaryopoiesis, resulting in later gene deletion, and a shorter timeframe for remaining mRNA and protein to be degraded. This is demonstrated by higher tdTomato signal in *mT/mG$^{+/fl}$;Gp1ba-Cre$^{+/KI}$* MKs and platelets compared with *mT/mG$^{+/fl}$;Pf4-Cre$^{+/KI}$*. Consequently, we found that 5-10% of targeted proteins persisted in *Gp1ba-Cre*-generated platelets, whereas only 1-6% protein levels remaining in *Pf4-Cre*-generated platelets. Several factors contribute to these differences in protein expression, including the onset of transgene and *floxed* gene expression, mRNA and protein stability. This is exemplified by *Shp1* and *Csk* conditional KO models, which have more residual protein in platelets than *CD148* conditional KO mice, due to earlier onset of gene expression. An advantage of slightly higher residual protein levels is less severe developmental defects in MKs and platelets, providing more opportunity to investigate functional consequences of reduced protein expression. Dosing studies can also be designed by knocking out the same gene using the *Pf4-Cre* and *Gp1ba-Cre* transgenes, and comparing phenotypes of heterozygous and homozygous KO models in parallel. This can be particularly useful in studying megakaryopoiesis. In addition, *Gp1ba-Cre*-generated mouse models may more closely model hemizygosity in human patients. Incomplete protein ablation is not uncommon with other hematopoietic deleter strains. For example, the lifespan of circulating neutrophils in mice is less than a day,[63] and the protein ablation efficiency of the neutrophil lineage-specific Cre model *Ly6G-Cre* is approximately 55%.[64] With two complementary MK/platelet-specific deleter strains now at our disposal, we can address the issues of leakiness and dosing effects of gene expression in the MK lineage.

Importantly, we provide further evidence of the wide tissue expression of the *Pf4-Cre* transgene by analyzing *mT/mG*$^{+/fl}$*;Pf4-Cre*$^{+/KI}$ splenocytes.[9-11] Findings were corroborated with the *Shp1*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mouse model. We observed recombination of ~15-50% of erythroid, myeloid and lymphoid cells, suggesting that *Pf4* is expressed before MK lineage commitment. Of note, we detected 6.2% TER-119^{+} BM cell population in the *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice which underwent recombination, suggesting expression of GPIbα in a common MK-erythroid progenitor. Furthermore, we observed 4.7% EGFP^{+}tdTomato^{+} red pulp macrophages in these mice, which may represent recombined cells or macrophages that have phagocytosed EGFP^{+} platelets. We are currently actively engaged in developing a more detailed expression pattern of *Gp1ba-Cre*, covering a range of cell and tissue types under normal and inflammatory conditions. This will be further advanced as the *Gp1ba-Cre* mouse becomes more widely used, as was the case of *Pf4-Cre*.[9-11]

In summary, the *Gp1ba-Cre* mouse exhibits improved specificity towards the MK lineage compared with the existing *Pf4-Cre* mouse commonly used by platelet and leukocyte biologists. As proof-of-concept, we demonstrate that the *motheaten*-like hyper-inflammatory phenotype observed upon ablation of Shp1 with the *Pf4-Cre* transgene is absent in the *Shp1* conditional KO mice generated with *Gp1ba-Cre*. These results discount the potential role of platelets in this phenotype and highlight the anomalies that arise from non-specific ablation in *Pf4-Cre*-generated KO mice. The more lineage-specific gene ablation provided by the *Gp1ba-Cre* mouse will make it possible to delineate the relative contribution of platelets to a variety of pathophysiological processes, and refine the involvement of leukocytes in thrombo-inflammatory conditions.

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Authorship

Contribution: Conceptualization, YAS; Methodology, ZN, MJG, TV, RG, GED, AM and YAS; Investigation, ZN, TV, MJG, JM, SH, GDN, GED, AM and YAS; Resources, YAS; Mouse models and reagents, AT, AW, BGN and YAS; Writing – Original Draft, ZN and YAS; Writing – Review & Editing, ZN, TV, AM and YAS; Supervision, YAS; Funding Acquisition, YAS.

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Figure Legends

Figure 1. Targeting strategy to generate the *Gp1ba-Cre* mouse.

The mouse *Gp1ba* gene consists of a 5' untranslated exon (1) followed by a short intron and an exon (2) containing the open reading frame encoding GPIbα protein.[59] The targeting strategy enables the generation of a constitutive knock-in (KI) of a *T2A-improved-Cre (iCre)* in the endogenous *Gp1ba* gene. The sequences for the *T2A* and the open reading frame of *iCre* have been inserted between the last amino acid and the translation termination codon in exon 2 of *Gp1ba*. Puromycin resistance (positive selection marker) is flanked by *FRT* sites and inserted downstream of the mouse 3' untranslated region. The constitutive KI allele is obtained after *in vivo* Flp-mediated removal of the selection marker and expresses a chimeric transcript harboring the *iCre* gene fused to the *Gp1ba* gene via the *T2A* sequence.

Figure 2. **Platelet parameters of the *Gp1ba-Cre* mouse.**

(A) Platelet counts and platelet volumes, n = 40-42 mice/genotype. (B) Platelet surface receptor expression of GPIbα, GPIbβ, GPIX and GPV; and integrin αIIbβ3, integrin α2, GPVI and CLEC-2 were measured by flow cytometry and shown as median fluorescence intensity, n = 5-6 mice/genotype. $^{**}P < 0.01$, $^{***}P < 0.001$, unpaired, two-tailed t-test, mean ± SD.

Figure 3. Aggregation and agglutination of *Gp1ba-Cre* platelets.

(A) Mean platelet aggregation and secretion traces in response to the indicated agonists, n = 4-8 mice/condition/genotype. (B) Mean platelet agglutination traces in response to 10 µg/ml vWF (for 2 minutes) followed by 1 µg/ml rBot2[48] stimulation, n = 3 mice/condition/genotype.

Figure 4. Tail bleeding of the *Gp1ba-Cre* mouse.

Hemostatic response was measured in tail bleeding assays by an excision of a 5-mm portion of the tail tip followed by the determination of lost blood/body weight (normalized blood loss), n = 18 mice/genotype. Tail bleeding assays were conducted in a blinded manner. Unpaired, two-tailed t-test.

Figure 5. *Gp1ba-Cre*-mediated recombination of blood, bone marrow and spleen cells.

(A) Flow cytometry analysis of whole blood showing the proportions of $EGFP^+$ events of platelets, n = 13 mice/genotype, leukocytes, n = 8 mice/genotype and erythrocytes, n = 6 mice/genotype from *mT/mG*$^{+/fl}$ and *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice. (B) Flow cytometry analysis of bone marrow cells showing the proportions of $EGFP^+$ events of all isolated bone marrow cells, n = 7 mice/genotype, $CD41^{hi}$ bone marrow cells, n = 7 mice/genotype, $CD45^+CD41^-$ bone marrow cells, n = 3 mice/genotype, and $TER\text{-}119^+$ bone marrow cells, n = 6 mice/genotype from *mT/mG*$^{+/fl}$ and *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice. (C) Flow cytometry analysis showing the proportions of $EGFP^+$ events of spleen cells, n = 4-5 mice/genotype from *mT/mG*$^{+/fl}$ and *mT/mG*$^{+/fl}$*;Gp1ba-Cre*$^{+/KI}$ mice. Mean ± SD.

Figure 6. Efficient protein ablation, similar platelet phenotypes but no altered leukocyte counts in *Csk;Gp1ba-Cre* mice.

(A) Capillary-based immunoassays on platelet lysates with Csk (upper panel) and GAPDH (lower panel) antibodies and quantification of the ratio of Csk/GAPDH peak areas, n = 3-6 mice/genotype. (B) Platelet counts and platelet volumes, n = 7-10 mice/genotype. (C) Platelet surface receptor expression of GPVI and G6b-B were measured by flow cytometry and shown as median fluorescence intensity, n = 6-10 mice/genotype. (D) White blood cell and lymphocyte counts, n = 9-33 mice/genotype. ***$P < 0.001$, unpaired, two-tailed t-test,

mean ± SD. *Csk*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ and *Csk*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice were compared to *Csk*$^{+/+}$*;Gp1ba-Cre*$^{+/KI}$ and *Csk*$^{+/+}$*;Pf4-Cre*$^{+/KI}$ mice, respectively.

Figure 7. Hyper-inflammatory phenotype and altered leukocyte counts are absent in *Ptpn6;Gp1ba-Cre* mice.

(A) Capillary-based immunoassays on platelet lysates with Shp1 (upper panel) and GAPDH (lower panel) antibodies and quantification of the ratio of Shp1/GAPDH peak areas, n = 3 mice/genotype. (B) Mice were monitored every week for signs of *motheaten*-like phenotype (paw, nose or ear inflammation). Percentage of disease-free mice for the indicated time period was calculated using Kaplan-Meier survival analysis with a log-rank (Mantel-Cox) test. ***$P < 0.001$, n values are indicated in the Figure. (C) White blood cell, monocyte, atypical lymphocyte and large immature cell counts, n= 33-40 mice/genotype. (A and C) **$P < 0.01$, ***$P < 0.001$, unpaired, two-tailed t-test, mean ± SD. *Ptpn6*$^{fl/fl}$*;Gp1ba-Cre*$^{+/KI}$ and *Ptpn6*$^{fl/fl}$*;Pf4-Cre*$^{+/KI}$ mice were compared to *Ptpn6*$^{+/+}$*;Gp1ba-Cre*$^{+/KI}$ and *Ptpn6*$^{+/+}$*;Pf4-Cre*$^{+/KI}$ mice, respectively.