

Murine platelet production is suppressed by S1P release in the hematopoietic niche, not facilitated by blood S1P sensing

Hira Niazi,^{1,2,*} Nesrine Zoghiani,^{1,2,*} Ludovic Couty,^{1,2} Alexandre Leuci,^{2,3} Anja Nitzsche,^{1,2} Maria L. Allende,⁴ Boubacar Mariko,^{1,2,5} Rameez Ishaq,^{6,7} Yetki Aslan,⁸ Pierre Hadrien Becker,^{9,10} Salomé L. Gazit,^{1,2} Sonia Poirault-Chassac,^{2,3} Benoit Decouture,^{2,3} Veronique Baudrie,^{1,2} Erica De Candia,¹¹ Mari Kono,⁴ Ammar Benarab,^{1,2} Pascale Gaussem,^{2,3} Pierre-Louis Tharaux,^{1,2} Jerold Chun,¹² Sylvain Provot,⁸ Najet Debili,⁶ Patrice Therond,^{9,10} Richard L. Proia,⁴ Christilla Bachelot-Loza,^{2,3} and Eric Camerer^{1,2}

¹INSERM U970, Paris Cardiovascular Research Centre, Paris, France; ²Department of Medicine, Université Paris-Descartes, Sorbonne Paris Cité, Paris, France; ³INSERM U1140, Faculté de Pharmacie, Paris, France; ⁴Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD; ⁵College of Agronomy and Veterinary Science, University of Ségou, Ségou, Mali; ⁶INSERM Unité Mixte de Recherche 1170, Université Paris-Saclay & Gustave Roussy, Villejuif, France; ⁷Gustave Roussy Cancer Campus, Université Paris Diderot, Paris, France; ⁸INSERM, Hôpital Lariboisière–Centre Viggo Petersen, Paris, France; ⁹Assistance Publique–Hôpitaux de Paris, Hôpital Bicêtre, Service de Biochimie, Le Kremlin Bicêtre, France; ¹⁰Université Paris-Sud, Équipes d'Accueil 7357 Lipides: systèmes analytiques et biologiques, Unité de Formation et de Recherche de Pharmacie, Châtenay-Malabry, France; ¹¹Servizio Malattie Emorragiche e Trombotiche, Polo Oncologia ed Ematologia, Istituto di Medicina Interna, Università Cattolica del Sacro Cuore, Rome, Italy; and ¹²Neuroscience Drug Discovery, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA

Key Points

- The vascular S1P gradient is dispensable for platelet formation in mice.
- Instead, local S1P production restrains megakaryopoiesis via S1P₁ and can further suppress platelet production via S1P₂ when deregulated.

The bioactive lipid mediator sphingosine 1-phosphate (S1P) was recently assigned critical roles in platelet biology: whereas S1P₁ receptor-mediated S1P gradient sensing was reported to be essential for directing proplatelet extensions from megakaryocytes (MKs) toward bone marrow sinusoids, MK sphingosine kinase 2 (Sphk2)–derived S1P was reported to further promote platelet shedding through receptor-independent intracellular actions, and platelet aggregation through S1P₁. Yet clinical use of S1P pathway modulators including fingolimod has not been associated with risk of bleeding or thrombosis. We therefore revisited the role of S1P in platelet biology in mice. Surprisingly, no reduction in platelet counts was observed when the vascular S1P gradient was ablated by impairing S1P provision to plasma or S1P degradation in interstitial fluids, nor when gradient sensing was impaired by *S1pr1* deletion selectively in MKs. Moreover, S1P₁ expression and signaling were both undetectable in mature MKs in situ, and MK *S1pr1* deletion did not affect platelet aggregation or spreading. When *S1pr1* deletion was induced in hematopoietic progenitor cells, platelet counts were instead significantly elevated. Isolated global Sphk2 deficiency was associated with thrombocytopenia, but this was not replicated by MK-restricted *Sphk2* deletion and was reversed by compound deletion of either *Sphk1* or *S1pr2*, suggesting that this phenotype arises from increased S1P export and S1P₂ activation secondary to redistribution of sphingosine to Sphk1. Consistent with clinical observations, we thus observe no essential role for S1P₁ in facilitating platelet production or activation. Instead, S1P restricts megakaryopoiesis through S1P₁, and can further suppress thrombopoiesis through S1P₂ when aberrantly secreted in the hematopoietic niche.

Introduction

Sphingosine-1-phosphate (S1P) is a lipid mediator that plays critical roles in the homeostasis of vascular and immune systems.¹ Although most of its functions are mediated by 5 cognate G protein-coupled receptors (S1P₁₋₅; encoded by *S1pr1-5*), S1P also plays intracellular receptor-independent roles.¹

Submitted 21 January 2019; accepted 17 April 2019. DOI 10.1182/bloodadvances.2019031948.

*H.N. and N.Z. contributed equally to this study.

For original data, please contact eric.camerer@inserm.fr.

The full-text version of this article contains a data supplement.

Sphingosine is phosphorylated to S1P by sphingosine kinases (Sphk) 1&2.^{2,3} Compound deletion of *Sphk1&2* results in a tissue-wide loss of S1P, isolated *Sphk1* deletion in a ~50% reduction in plasma S1P and complete loss of red blood cell (RBC) S1P, and *Sphk2* deletion in a paradoxical doubling of plasma S1P and near-complete loss of megakaryocyte (MK)/platelet S1P.⁴⁻⁹ While RBCs and endothelial cells continuously export S1P to plasma and lymph through transporters Mfsd2b and spinster 2, respectively, S1P is actively broken down and removed from interstitial fluids by S1P lyase (encoded by *Sgpl1*) and lipid phosphatases.¹⁰⁻¹⁵ Platelets store abundant S1P, roughly equivalent to the plasma pool, that can be exported by Mfsd2b on activation.^{6,11,14}

Combined with a short half-life, tight control of export and degradation thus allows the maintenance of a steep gradient of S1P between blood and interstitial fluids that is used by hematopoietic cells to gauge their proximity to blood and lymph.^{10,16} When sensed by S1P₁, S1P drives lymphocytes into circulation by activation of G α i and Rac; when sensed by S1P₂, it confines lymphocytes within germinal centers through G α _{12/13} and RhoA.¹⁷⁻¹⁹ Plasma membrane receptor expression thereby dictates how a cell responds to S1P. S1P₁ is internalized once cells reach the circulation and are exposed to receptor-saturating S1P levels, and reexpressed after cells are attracted back to S1P-poor environments by other chemokines.¹⁰ The clinically approved multiple sclerosis drug fingolimod (AKA FTY720, Gilenya) induces immunosuppression by disruption of S1P₁-mediated gradient sensing. Once phosphorylated by Sphk2, fingolimod acts as a functional antagonist of S1P₁, first activating and then rapidly desensitizing the receptor.^{10,16} Fingolimod also targets S1P₃₋₅.¹

MKs were recently proposed to use S1P₁ to direct proplatelet (PP) extensions along the S1P gradient toward bone marrow sinusoids, and for subsequent platelet shedding, as schematically presented in Figure 1A.^{20,21} A parallel, MK-intrinsic receptor-independent role for S1P was proposed in platelet shedding (Figure 1A),²² and platelet-derived S1P and S1P₁ have been further implicated in the amplification of platelet aggregation during arterial thrombosis.⁷ S1P₄ is also involved in terminal differentiation of MKs, although S1P₄-deficient animals have normal platelet counts.²³ Together, these studies position S1P as a key player in platelet production and function. Fingolimod and S1P₁-selective modulators are being explored for the treatment of a range of disease conditions, some of which are associated with risk of hemorrhage and thrombosis.^{1,24} Sphks and S1P lyase are also emerging as potential drug targets for treatment of cancer, pulmonary hypertension, bone loss, and sickle cell disease.²⁴⁻²⁶ Should it be a concern that these drugs also target S1P production and signaling in MKs and platelets? In this regard, it is reassuring that case reports of thrombocytopenia and bleeding in patients with MS receiving fingolimod are rare, and that pilot trials with fingolimod for ischemic and hemorrhagic stroke have not revealed an increase in bleeding propensity.^{27,28} Whether this reflects on the complex mechanisms of action of fingolimod or if experimental studies have overestimated the role of S1P₁ in platelet production and function is unclear.

The role for S1P gradient sensing in lymphocyte trafficking was demonstrated by rendering either interstitial fluids S1P-rich by

inhibiting S1P lyase, or blood and lymph S1P-poor by tissue-specific *Sphk1&2* deletion.¹⁰ A key role for S1P₁ was suggested using S1P₁-modulating drugs and established by hematopoietic or lymphocyte selective deletion of *S1pr1*.^{10,29,30} Using similar tools to interrogate the role of S1P in platelet biology, we fail to confirm a critical role for the S1P gradient and S1P₁ in platelet production or function, consistent with clinical observations. We instead reveal that S1P₁ signaling in the hematopoietic compartment restrains megakaryopoiesis, and that aberrant S1P₂ activation can further suppress platelet production when S1P is released in the hematopoietic niche.

Materials and methods

Generation and validation of conditional (f) and global (-) knock-outs of *Sphk1*, *Sphk2*, *S1pr1*, and *Sgpl1* (encoding Sphk1, Sphk2, S1P₁, and S1P lyase, respectively), plasma S1Pless mice (*Sphk1^{f/f}:2^{f/f}:Mx1Cre⁺*), platelet S1Pless mice (*Sphk1^{f/f}:2^{f/f}:Pf4Cre*), S1P₁ ECKO mice (*S1pr1^{f/f}:PdgbfCre⁺*), and S1P₁ signaling reporter mice (*S1pr1^{GFP}*) has been described.^{6,11,31-34} Mice deficient in *S1pr1* or *Sphk2* in MKs and platelets (*S1pr1^{f/f}:Pf4Cre⁺*; *Sphk2^{f/f}:Pf4Cre⁺*) were generated with Cre recombinase driven by the platelet factor 4 promoter.³⁵ Experiments were littermate controlled. Complete blood cell counts were obtained with a HemaVet (Drew Scientific). Scanning and transmission electron microscopy was performed as previously described.^{6,36} S1P and sphingosine concentrations in plasma and cell lysates were quantified by liquid chromatography/tandem mass spectrometry and high-performance liquid chromatography, as described.⁶ Bone marrow (BM) hematopoietic stem cells (Lin⁻:Sca-1⁺:c-Kit⁺), common MK/erythrocyte progenitor cells (Lin⁻:Sca-1⁻:c-Kit⁺:CD34⁻:CD16/32⁻), and MK progenitor cells (MKPs; lineage⁻:Sca-1⁻:c-Kit⁺:CD34⁻:CD16/32⁻:CD41⁺) were quantified by flow cytometry. Reagents and methods for platelet isolation and functional analyses, platelet half-life, microcomputed tomography imaging and analyses, further immunohistochemical analyses, quantitative polymerase chain reaction, MK culture, and platelet formation are detailed in supplemental Information. Experimental procedures involving animals were approved by the Paris Descartes Ethical Committee and the French Ministry of Education. Statistical significance was assessed using GraphPad Prism software; details of tests used are provided in the figure legends. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

Results

The S1P gradient is dispensable for platelet production

We and others have reported that compound deletion of *Sphk1&2* in hematopoietic and other Mx1Cre-sensitive cells greatly reduces RBC, platelet, and plasma S1P levels, and that S1P lyase deficiency increases serum S1P levels more than threefold and tissue S1P greatly, both resulting in profound lymphopenia resulting from ablation of the S1P gradient.^{6,11,32,33,37} Current literature predicts that combined loss of the S1P gradient and MK S1P production should result in equally profound thrombocytopenia from additive effects of defective PP formation and fragmentation (Figure 1A).^{1,20-22} We were therefore surprised to observe that peripheral blood platelet counts in mice lacking S1P in both plasma and MKs (*Sphk1^{f/f}:2^{f/f}:Mx1Cre⁺*) were instead higher than in littermate controls, whereas alternative S1P gradient disruption with S1P lyase deficiency (*Sgpl1^{-/-}*) had no

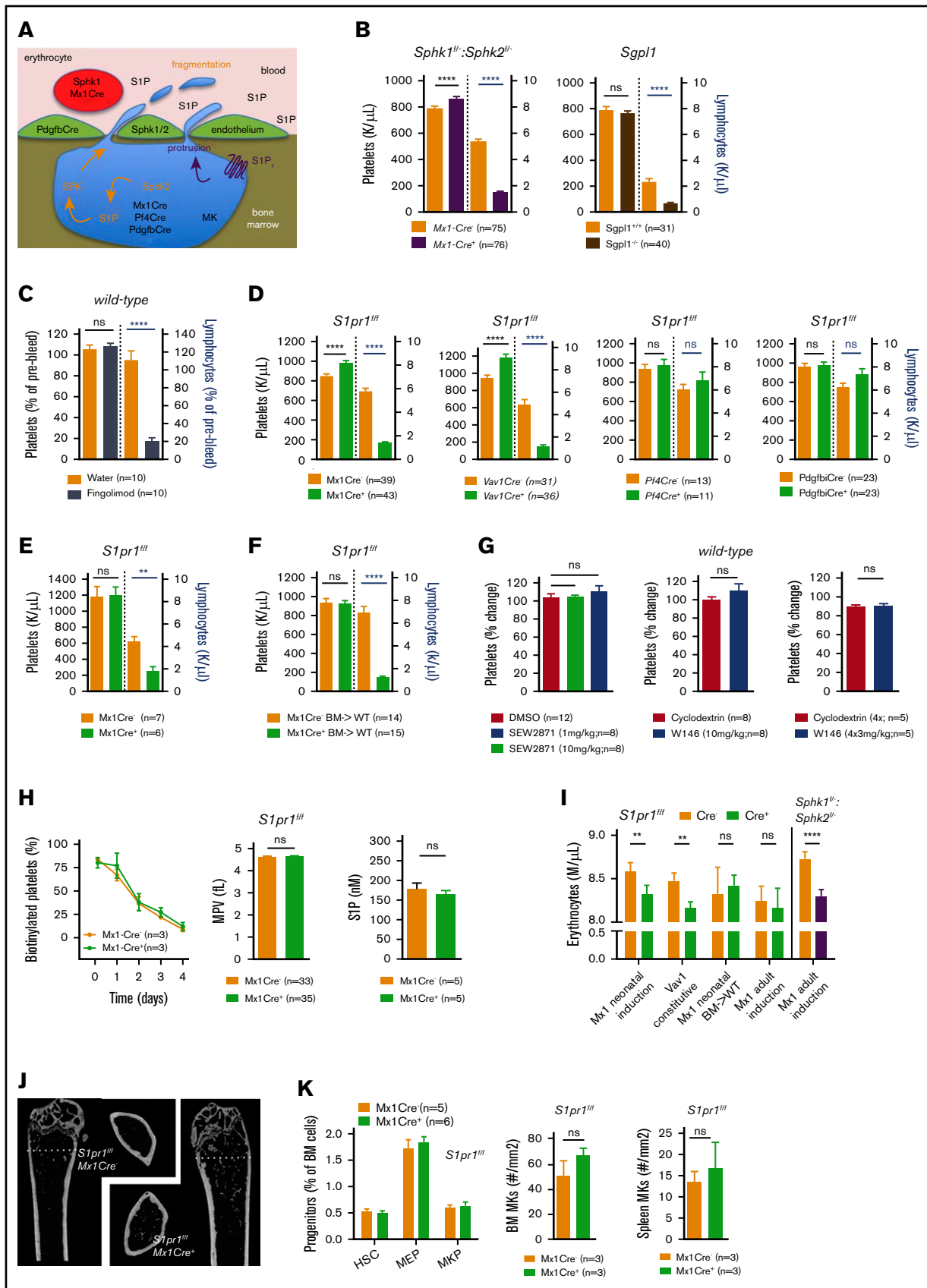


Figure 1.

effect on platelet counts, despite expected lymphopenia in both models (Figure 1B). Murine thrombopoiesis is therefore critically dependent neither on the S1P gradient²⁰ nor on MK S1P production.²²

S1P₁ suppresses platelet production by a non-cell-autonomous mechanism

We next asked if S1P₁ promotes platelet production independent of gradient sensing. Fingolimod both activates and desensitizes S1P₁, thus disrupting S1P₁-mediated S1P sensing. Administration of fingolimod (2 mg/L) to the drinking water of wild-type mice for 1 week impaired lymphocyte trafficking, but had no effect on platelet counts (Figure 1C). Postnatal deletion of *S1pr1* in hematopoietic and other Mx1Cre-sensitive cells also yielded profound lymphopenia, but unexpectedly, a significant increase rather than a decrease in platelet counts (Figure 1D). This was replicated by constitutive pan-hematopoietic deletion of *S1pr1* with Vav1Cre, whereas restricted deletion in MKs with Pf4Cre³⁵ or endothelial cells and a subset of MKs with PdgfbCre³⁸ had no effect on platelet or lymphocyte counts (Figure 1D). When Mx1Cre-mediated *S1pr1* excision was induced in adulthood or when *S1pr1^{fl/fl}Mx1Cre⁺* BM cells were transplanted into lethally irradiated wild-type recipients, we observed lymphopenia but not thrombocytosis (Figure 1E-F). This argued that hematopoietic S1P₁ signaling has an indirect and delayed negative effect on platelet production. Concordantly, 24-hour platelet counts were not altered by selective S1P₁ agonism (SEW2871; 1 or 10 mg/kg) or antagonism (W146; 10 mg/kg³⁹ or 4 × 3 mg/kg at 6-hour intervals²⁰; Figure 1G), although SEW2871 did induce transient lymphopenia and W146 vascular leak, as has been reported (supplemental Figure 1A-C).^{40,41} Mx1Cre-mediated *S1pr1* deletion did not affect platelet half-life or size, nor plasma S1P levels, consistent with a role in megakaryopoiesis (Figure 1H). Neonatal and constitutive hematopoietic *S1pr1* deletion also yielded a significant reduction in RBC counts that was neither conferred by BM transplantation nor induced with Mx1Cre-mediated *S1pr1* deletion in adulthood, but was also observed with neonatal *Sphk1&2* deletion (Figure 1I). This may suggest that S1P₁ signaling influences fate decisions of a common progenitor, although delayed effects could also reflect

indirect roles of hematopoietic S1P₁ signaling in bone development or tissue distribution of progenitors.⁴²⁻⁴⁵ As Mx1Cre is active in osteoclasts and deletion of *S1pr1* in osteoclasts has been demonstrated to increase their attachment to the bone surface, triggering osteoporosis,⁴⁶ we evaluated bone density in neonatally induced *S1pr1^{fl/fl}Mx1Cre⁺* mice. If anything, microcomputed tomography analysis suggested a modest increase in bone density in young adults (Figure 1J; supplemental Figure 2), unlikely to directly affect megakaryopoiesis.⁴⁷ Although we did not observe a significant effect of S1P₁ deficiency on the number of hematopoietic stem cells, MK-erythroid progenitors, or MKPs in BM, a modest increase in the abundance of MKs in BM and spleen was nevertheless suggestive of an increase in megakaryopoiesis sufficient to explain the increase in platelet counts (Figure 1K; supplemental Figure 3). Collectively, these observations argue that not only is S1P₁ dispensable for thrombopoiesis, but also that it is a net negative regulator of platelet production.

S1P₁ is not expressed in murine MKs

As our findings directly contradict the critical role reported for S1P₁ in MKs,²⁰ we next addressed the efficiency of our genetic approaches. S1P₁ was undetectable on *S1pr1^{fl/fl}Pf4Cre⁺* and *S1pr1^{fl/fl}Mx1Cre⁺* splenic and BM MKs (Figure 2A). Surprisingly, we also did not observe MK S1P₁ in controls, despite readily detectable *S1pr1*-dependent endothelial immunostaining (Figure 2A; supplemental Figure 4). To address if S1P₁ was functionally expressed below the detection limit of our immunostaining, we visualized S1P₁ signaling in BM, spleen, and liver of naive and S1P₁ agonist-treated S1P₁ signaling reporter mice.³⁴ Nuclear GFP accumulation reflected S1P₁ signaling in a subset of endothelial cells and hematopoietic cells in BM and spleen that appeared more abundant after treatment with fingolimod and the S1P₁ selective agonists RP001, both of which also induced marked GFP accumulation in hepatocytes (Figure 2B; supplemental Figure 5). In contrast, MKs were consistently GFP negative, independent of treatment. Moreover, consistent with reported downregulation of S1P₁ transcription with progenitor cell commitment to the MK lineage,²³ BM-derived MKs expressed S1P₂ and S1P₄, but no detectable S1P₁ (Figure 2C). The same primers readily amplified S1P₁ from total lung cDNA (not

Figure 1. MK S1P, S1P, S1P, and the S1P gradient are dispensable for platelet production in mice. (A) Current literature suggests that S1P supports platelet production by 2 independent mechanisms: S1P₁ senses the S1P gradient to promote PP extensions toward blood sinusoids (purple) and further supports fragmentation, and S1P supports platelet fragmentation by receptor-independent promotion of Src family kinase (SFK) expression and activation in MKs (orange). Removal of the S1P gradient and MK S1P production would thus be predicted to have cumulative effects on platelet production. Target cells of Cre alleles used in this study are indicated. (B) Peripheral blood platelet and lymphocyte counts in mice with combined loss of lymphatic endothelium and hematopoietic S1P production (*Sphk1^{fl/fl}:2^{fl/fl}:Mx1Cre⁺*) and with alternative gradient ablation by impaired S1P breakdown (*Sgpl1^{-/-}*). (C) Relative changes in the same cell populations after supplying the functional S1P₁ antagonist fingolimod (2 mg/L) in the drinking water of wild-type mice for 1 week. (D) Platelet and lymphocyte counts in mice after deletion of *S1pr1* in hematopoietic and other cells (postnatal induction, *Mx1Cre⁺*), in all hematopoietic cells (constitutive deletion, *Vav1 Cre⁺*), in MKs (constitutive deletion; *Pf4Cre⁺*), or endothelial cells and MKs (postnatal induction, *PdgfbCreERT2⁺*). (E-F) Platelet and lymphocyte counts in adult *S1pr1^{fl/fl}:Mx1Cre^{+/+}* mice 1 month after 3 consecutive injections of Poly IC (E) or in lethally irradiated wild-type mice 1 month after transfusion of *S1pr1^{fl/fl}:Mx1Cre^{+/+}* BM cells (F). (G) Relative change in platelet counts 24 hours after injections of the S1P₁ agonist SEW2871 or antagonist W146 or respective vehicle controls, as indicated (W146 was injected either as a single bolus [middle] or at 0, 6, 12, and 18 hours [right]). Lymphocyte counts and acute effects of drug treatment in supplemental Figure 1. (H) Platelet half-life, mean platelet volume (MPV), and plasma S1P levels in *S1pr1^{fl/fl}:Mx1Cre⁺* mice. (I) RBC counts in mice with hematopoietic deletion of *S1pr1* or *Sphk1&2* (the same mice as in Figure 1D, 1F, 1E, and 1B, respectively). (J) Representative microcomputed tomography images of femurs from *S1pr1^{fl/fl}:Mx1Cre⁺* mice and littermate controls. Representative coronal and transverse sections (approximate area indicated) are shown, quantification in supplemental Figure 2. (K) BM progenitors as percentage of total bone marrow cells and MK density in BM and spleen of *S1pr1^{fl/fl}:Mx1Cre⁺* mice. Representative images in supplemental Figure 3. All animals are compared with their respective littermate controls, n indicates the number of animals from which samples were obtained, mean + standard error of the mean shown. Statistical analyses by Mann-Whitney *U* test. ns, not significant.

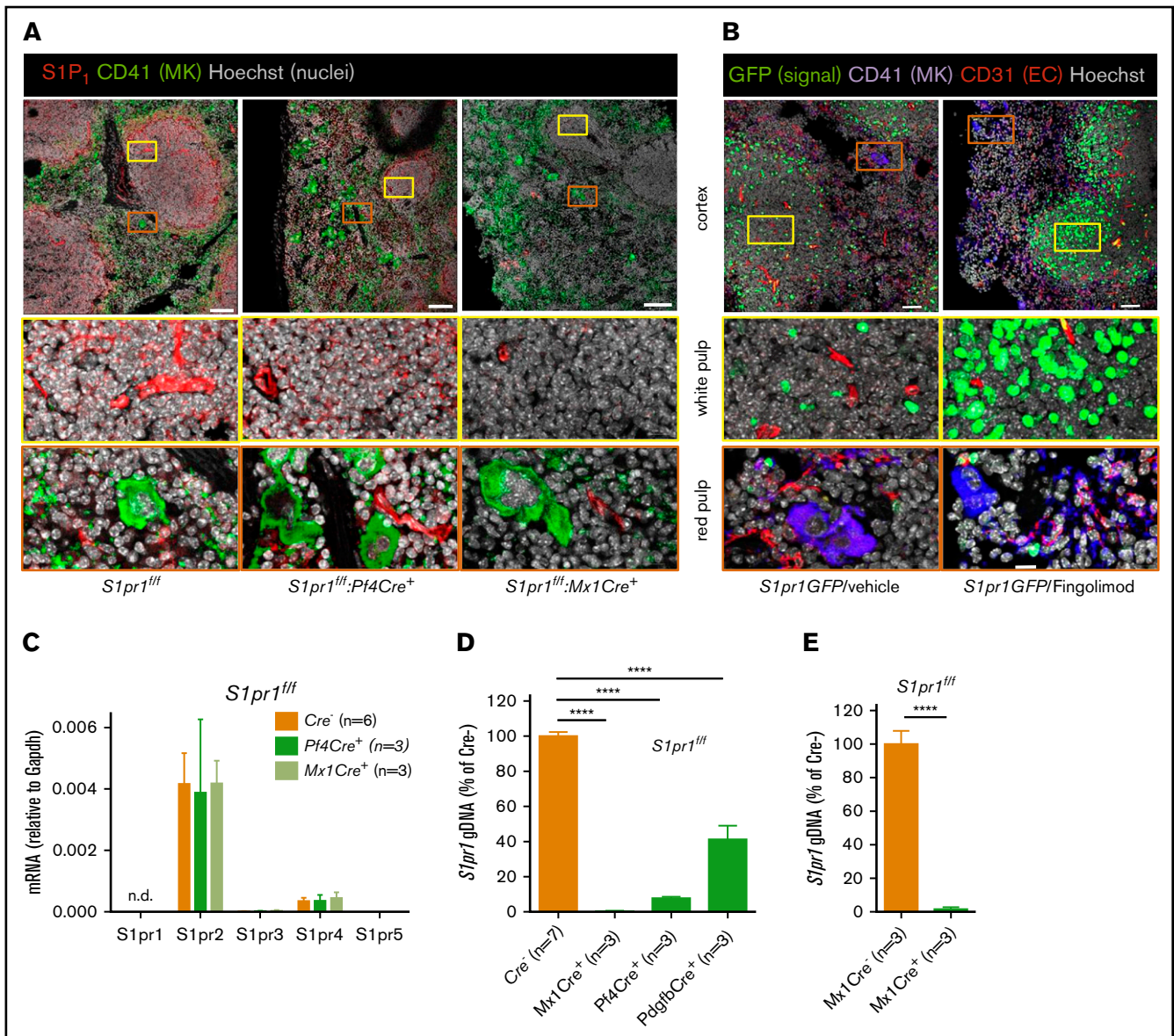


Figure 2. S1P₁ is not expressed in murine MKs. (A) Expression of S1P₁ (red) and the MK marker CD41 (green) in spleen of mice with or without MK-selective (*S1pr1^{fl/fl};Pf4Cre⁺*) or pan-hematopoietic (*S1pr1^{fl/fl};Mx1Cre⁺*) S1P₁ deletion. Note S1P₁ expression in blood vessels and white pulp, but not in MKs, irrespective of gene deletion. Scale bars represent 50 μ m. (B) Constitutive (left) and fingolimod-induced (1 mg/kg, 24 hours; right) S1P₁ signaling in spleens of S1P₁ signaling reporter mice.³⁴ Note constitutive and fingolimod-enhanced S1P₁ signaling (reflected by nuclear GFP accumulation in green) in blood vessels (in red) and cells within the white pulp, but not MKs (in blue). Scale bars represent 50 μ m. (C) Abundance of S1PR transcripts in BM-derived MKs from *S1pr1*-deficient mouse lines relative to *Gapdh*. Note lack of *S1pr1* expression (mRNA) or compensatory upregulation of other receptors after 3 days of culture. (D) Abundance of nonexcised *S1pr1* in genomic DNA (gDNA) from BM-derived MKs from *S1pr1*-deficient mouse lines after 5 days of culture relative to pooled *S1pr1^{fl/fl}* littermate controls. (E) Relative abundance of nonexcised *S1pr1* in genomic DNA from freshly isolated BM cells from *S1pr1*-deficient mice passed through 70- μ m filters. Statistical analysis by Mann-Whitney *U* test. n.d., not detectable.

shown). Transcriptional analysis also did not reveal compensatory upregulation of other S1P receptors in the absence of S1P₁ (Figure 2C). Analysis of genomic DNA from BM-derived MKs from the same lines showed more than 98%, more than 90%, and more than 50% excision of *S1pr1* with *Mx1Cre*, *Pf4Cre*, and *PdgfbCre*, respectively (Figure 2D). As ~70% of fluorescence-activated cell sorter sortable cells (including progenitors and excluding very mature MKs) from these cultures expressed CD41,

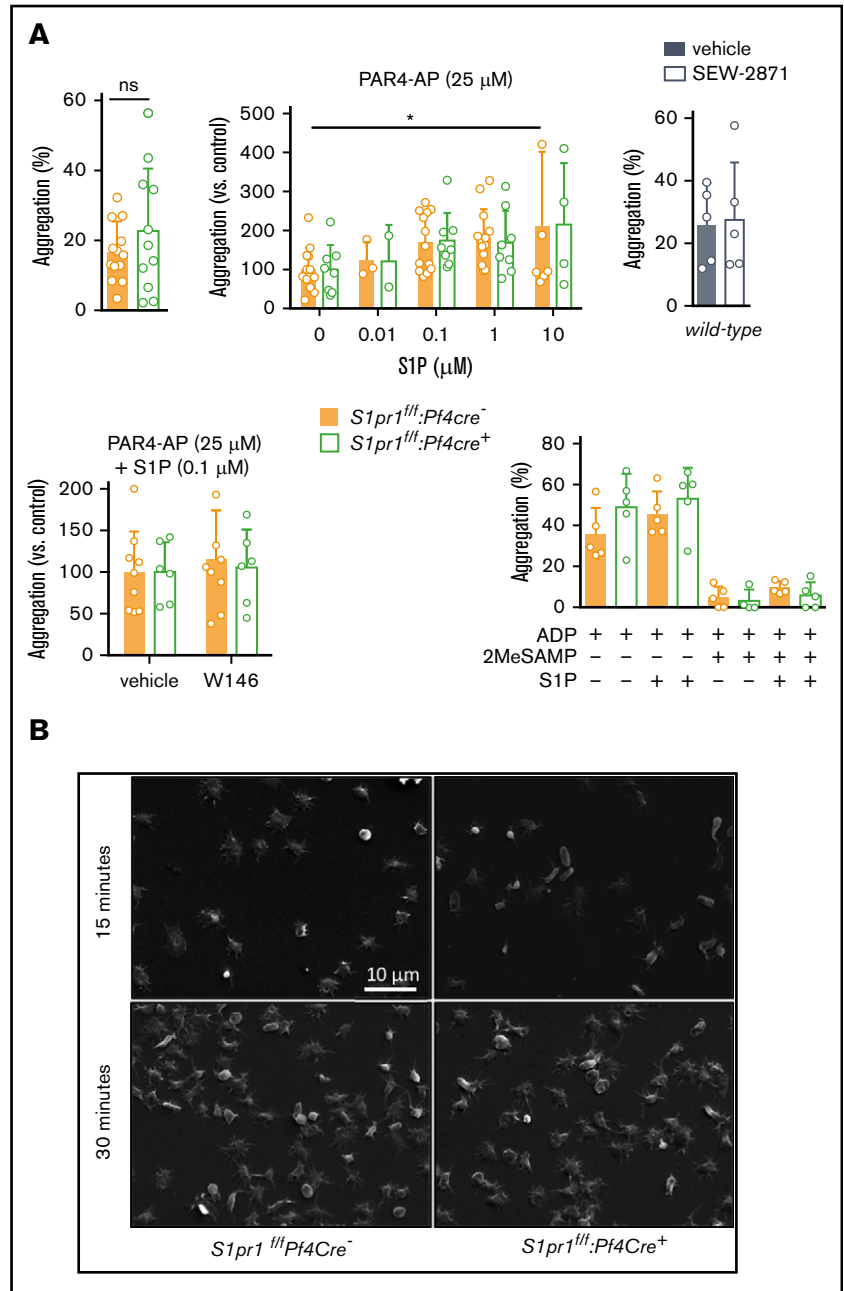
we conclude that *Mx1Cre* and *Pf4Cre* both yielded near complete genomic excision in MKs, whereas excision with *PdgfbCre* was either partial or more restricted to mature MKs. Accordingly, *PdgfbCre* showed partial activation of an eYFP reporter in splenic MKs (supplemental Figure 6). This argues that S1P₁ is not expressed on murine MKs, militating against even a nonessential role for MK S1P₁ in thrombopoiesis, and further arguing that increased platelet counts observed with pan-hematopoietic S1P₁ deficiency reflect actions in MK progenitors.

Figure 3. Platelet S1P₁ is dispensable for platelet aggregation and spreading in mice.

Platelets from mice in which *S1pr1* was deleted in MKs (*S1pr1^{fl/fl}:Pf4Cre⁺*; green), littermate controls (orange), or wild-type mice (gray) were isolated, washed, and tested for their capacity to aggregate (A) and spread (B).

(A, upper) Platelet aggregation in response to submaximal concentrations (25 μM) of PAR4-AP (thrombin receptor agonist) in the absence (left) or presence of exogenous S1P (0.1-10 μM; middle) or of S1P₁ agonist SEW-2871 (0.5 μM; right). (A, lower) Platelet aggregation in response to submaximal PAR4-AP in the presence of S1P (0.1 μM) in the presence or absence of S1P₁ antagonist W146 (10 μM; left) or in response to the weak platelet agonist ADP (2 μM; right), with and without exogenous S1P (10 μM) or P2Y12 antagonism (2MeSAMP, 40 μM) to address potential redundancy with P2Y12, which, similar to S1P₁, is Gα_i coupled.

(B) Representative scanning electron microscopy images (upper) showing the extent of platelet spreading 15 and 30 minutes after plating on fibrinogen in the presence of S1P (0.5 μM; quantification in supplemental Figure 7). Note that although S1P did not trigger aggregation (not shown), it slightly increased PAR4-AP induced aggregation. However, neither aggregation nor spreading was influenced by selective S1P₁ modulation or *S1pr1* deficiency. S1P also could not compensate for the absence of functional P2Y12 by alternative engagement of Gα_i. Statistical analyses by 2-way analysis of variance or the Mann-Whitney *U* test, as appropriate. Mean ± standard deviation is shown, symbols represent the number of mice.



S1P₁ is dispensable for activation and spreading of mouse platelets

These results also question a reported role for S1P₁ in platelet aggregation and thrombosis in mice.⁷ S1P alone did not trigger aggregation of washed murine platelets (not shown), although it slightly enhanced PAR4 activating peptide (PAR4-AP)-induced aggregation (Figure 3A). This effect of S1P persisted in the absence of *S1pr1*, and pharmacologic S1P₁ modulators did not affect PAR4-AP-induced platelet aggregation (Figure 3A). S1P₁ deficiency also did not affect ADP-induced platelet aggregation in the presence or absence of exogenous S1P at a concentration suggested to modulate platelet activation,⁷ even when sensitizing the system by inhibiting P2Y12-mediated Gα_i activation with

2MeSAMP. Platelet adhesion and spreading on fibrinogen were also unaffected by *S1pr1* deletion, S1P addition, or S1P₁ antagonism (Figure 3B; supplemental Figure 7). Thus, aggregation, spreading, and thrombosis phenotypes reported in *Sphk*-deficient platelets^{6,7} and mice⁷ likely reflect on activation of a different receptor, receptor-independent effects, or thrombocytopenia,²² rather than on a role for S1P₁ in platelet activation.⁷

Deregulated S1P production by Mx1Cre-sensitive hematopoietic cells suppresses thrombopoiesis in *Sphk2*^{-/-} mice

Increased platelet counts in *Sphk1^{fl/fl}:2^{fl/fl}:Mx1Cre⁺* mice (Figure 1B) not only argue against a necessary role for the S1P gradient²⁰ but also

against a necessary intracellular, receptor-independent role for S1P in platelet production.²² The latter was deduced from the observation that mice globally deficient in Sphk2 display relative thrombocytopenia and defective PP fragmentation.²² We confirmed a 25% reduction in circulating platelets and a slight increase in mean platelet volume (MPV) in *Sphk2*^{-/-} mice; an intermediate phenotype in *Sphk2*^{+/-} littermates suggested a dose-dependent effect of Sphk2 deficiency (Figure 4A). As reported, the phenotype was present, although less profound, when the line was inbred to C57BL/6J background, and Sphk1 deficiency did not influence platelet counts despite being associated with a ~50% reduction in plasma S1P levels (supplemental Figure 8A).²² Bone density, BM progenitors, platelet life span, and spleen size were unaltered, and genotype-dependent differences in platelet counts persisted after splenectomy (supplemental Figure 8B-F). Despite thrombocytopenia, MK numbers were higher in spleen and BM, consistent with a defect in thrombopoiesis (supplemental Figure 8G-H).²² Although PPs appeared larger, as reported by Zhang et al,²² we did not observe a decrease in the capacity of fetal liver-derived MKs to produce PPs and shed platelets (supplemental Figure 8I). Concordantly, isolated deletion of *Sphk2* in MKs had no effect on platelet counts (Figure 4B). Consistent with a paracrine effect of Sphk2 deficiency on MK function, *Sphk2* deletion with Lyve1Cre, active in lymphatic endothelial cells, some blood endothelial cells, macrophages, and other CD45+ cells,⁴⁸ did reduce platelet counts (Figure 4B). This presented the possibility that the apparent increase in platelet counts in plasma S1Pless mice (*Sphk1*^{fl/fl}:*Sphk2*^{fl/fl}:*Mx1Cre*⁺; Figure 1B) represented a rescue of *Sphk2*^{+/-}-induced thrombocytopenia (Figure 4A) with pan-hematopoietic Sphk1 deficiency. This would imply that the *Sphk2*^{-/-} phenotype results from a redistribution of sphingosine toward Sphk1 rather than from loss of Sphk2-derived S1P (Figure 4C).¹⁶ Consistent with this notion, sphingosine did not build up in BM cells, as observed in plasma and RBCs of *Sphk2*^{-/-} mice; Sphk1 expression remained unaltered (Figure 4D).⁹ To test this possibility more directly, we deleted *Sphk1* in Mx1Cre-sensitive cells in a background globally deficient in Sphk2 (*Sphk1*^{fl/fl}:*Sphk2*^{-/-}:*Mx1Cre*⁺). Consistent with our hypothesis, this returned platelet counts to wild-type levels (Figure 4E). The rescue was conferred by bone marrow transplantation (Figure 4F), but was not reproduced with selective Sphk1 deletion in MKs in a *Sphk2*^{-/-} background (Figure 4G). Compound deletion of the 2 kinases did not significantly affect MKP numbers or platelet life span, and therefore did not appear to provide rescue by an independent mechanism (Figure 4H). Thus, instead of reflecting on a critical intracellular signaling role for Sphk2-derived S1P in MKs, thrombocytopenia in Sphk2-deficient mice appears to arise from paracrine effects of S1P generated on redistribution of hematopoietic cell sphingosine to Sphk1.

Deregulated S1P production suppresses thrombopoiesis via S1P₂

The above results suggest that Sphk2 deficiency induces thrombocytopenia by a receptor-dependent mechanism, and we further show that S1P₁ can suppress megakaryopoiesis. However, S1P₁ antagonism did not ameliorate thrombocytopenia in *Sphk2*^{-/-} mice, whereas antagonism of S1P₂, which is expressed on murine MKs (Figure 2C),^{20,23} did (Figure 5A). S1P₂ deficiency did not by itself affect platelet production (Figure 5B), but when *S1pr2*^{+/-} intercrosses were performed in an *Sphk2*^{-/-} background, S1P₂ deficiency rescued *Sphk2*^{-/-}-induced thrombocytopenia (Figure 5C).

Conversely, when *Sphk2*^{+/-} intercrosses were performed in an *S1pr2*^{-/-} background, Sphk2 deficiency no longer induced thrombocytopenia (Figure 5D vs Figure 4A). MPVs were also normalized by S1P₂ deficiency (Figure 5C-D). Transmission electron microscopy revealed a high density of MKs in *Sphk2*^{-/-} BM (Figure 5E). Among these, we observed peri-sinusoidal MKs with scarce demarcation membrane systems (DMS), poorly resolved DMS regions sometimes without granules, and low-contrast MK “ghosts” that appeared to be undergoing necrosis. This contrasted with *Sphk2*^{+/+}:*S1pr2*^{+/+} and *Sphk2*^{-/-}:*S1pr2*^{-/-} BM, in which most mature MKs were large with well-defined DMS. This suggests that aberrant S1P₂ activation impairs MK maturation. S1P₂ is known to repel B cells when they encounter high S1P concentrations at the germinal center perimeter, a process that depends on Rho kinase, which also negatively regulates platelet formation by suppressing the actions of Rac1 and Cdc42 on cytoskeletal reorganization and microtubule assembly.^{17,49-51} Consistent with a role for the Rho pathway, the Rho kinase inhibitor Y-27632 significantly increased platelet counts in Sphk2-deficient mice with no effect on littermate controls (Figure 5F). Collectively, these observations suggest that Sphk1-derived S1P suppresses MK maturation in Sphk2-deficient mice by aberrant activation of S1P₂ and Rho kinase downstream.

Although compound deficiencies of Sphk1 and S1P₂ reversed Sphk2 deficiency-induced thrombocytopenia, it is noteworthy that neither fully normalized the MK phenotype in *Sphk2*^{-/-} mice. Neither compound deficiency eliminated the higher density of MKs in *Sphk2*^{-/-} spleens (supplemental Figure 9), compound Sphk1 deficiency did not normalize MPV in *Sphk2*^{-/-} mice (Figure 4E), *Sphk2*^{-/-} PP extensions appear thicker also ex vivo (supplemental Figure 8I²²), and Sphk-deficient platelets display defective activation and spreading ex vivo.^{6,22} These persistent phenotypes may reflect on imbalanced membrane lipids within *Sphk2*^{-/-} MKs and platelets, although not a simple buildup of upstream metabolites, as we observed a paradoxical decrease in sphingosine levels in Sphk-deficient platelets (Figure 5G).^{6,22}

Discussion

We here address roles for S1P in platelet production and function, using genetic and pharmacologic approaches in mice. Contrasting recent literature, our observations support neither a necessary role for the S1P gradient or MK/platelet S1P₁ in platelet production or aggregation nor a necessary intracellular signaling role for S1P in platelet production. They instead reveal that S1P₁ signaling continuously restrains megakaryopoiesis and that S1P₂ signaling can further suppress platelet production when sphingosine metabolism is disturbed in the hematopoietic niche.

Three recent reports have positioned S1P as a critical facilitator of platelet biogenesis and signaling by S1P₁-dependent attraction of PPs into BM sinusoids and subsequent shedding during platelet budding from MKs,²⁰ receptor-independent regulation of platelet shedding by Sphk2-derived S1P,²² and amplification of platelet aggregation by an autocrine S1P₁ activation loop acting downstream of conventional platelet agonists.⁷ Our observations do not fully support either model, and argue that S1P plays a limited role in platelet biology but major disturbances in S1P metabolism or signaling.

First, we do not observe a necessary role for S1P₁-mediated blood sensing in platelet formation. Genetic impairment of S1P provision to plasma or S1P breakdown in tissue, both with profound effects

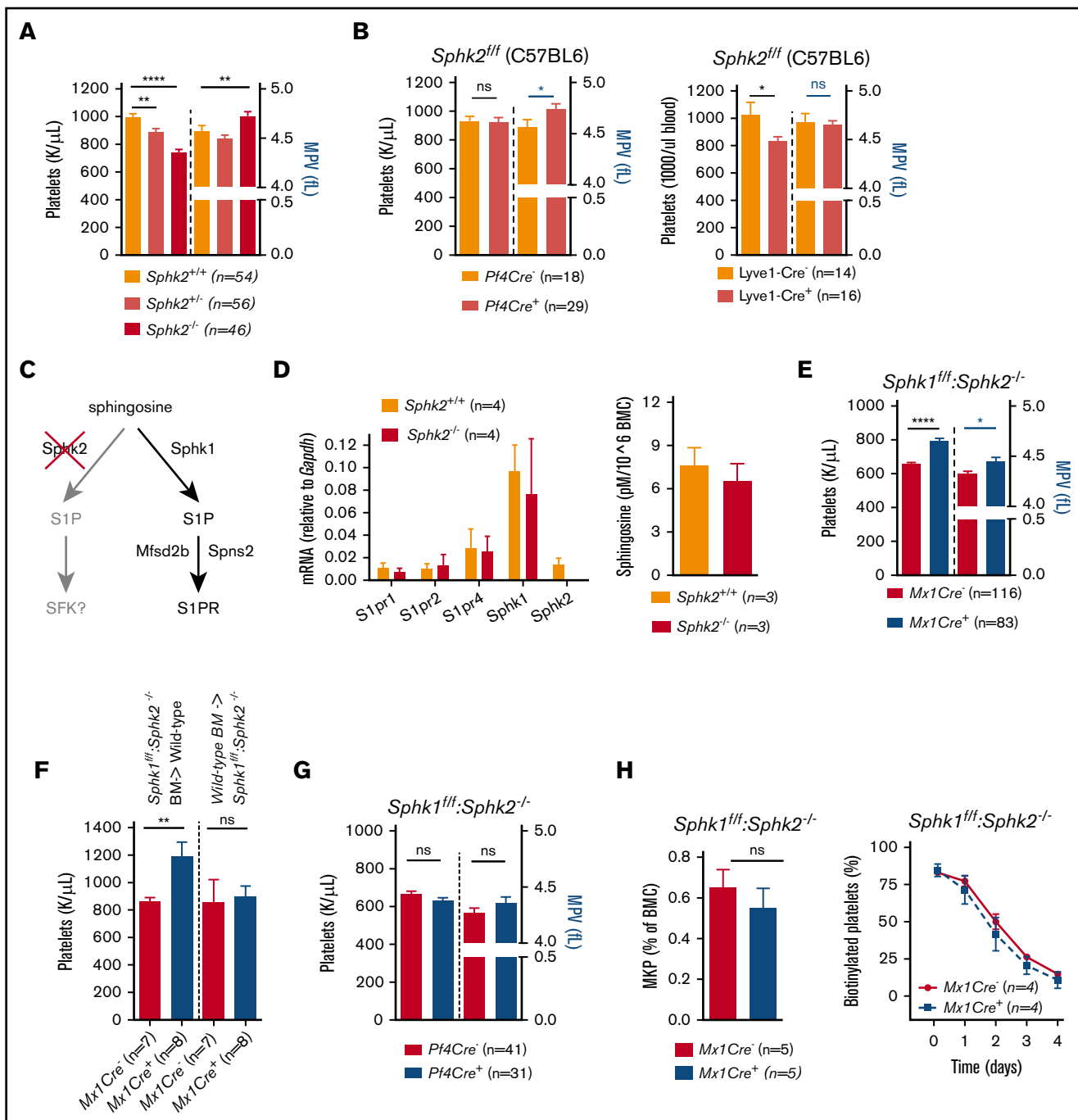


Figure 4. Sphk2 deficiency induces thrombocytopenia by redirection of sphingosine to Sphk1. (A-B) Platelet counts and MPV from *Sphk2* heterozygous intercrosses in C57BL/6J:129SVJ mixed background. (B) Effect of MK (Pf4Cre)- and lymphatic endothelium/CD45+ (Lyve1Cre)-selective *Sphk2* deletion on platelet counts. (C) Thrombocytopenia in *Sphk2*^{-/-} mice could be explained by redistribution of sphingosine to Sphk1 rather than by loss of Sphk2-derived S1P. This, in turn, could impair MK maturation by a receptor-dependent mechanism after S1P export by Spns2 or Mfsd2b, depending on cell type. (D) Impact of *Sphk2* deficiency on the expression of Sphks and S1PRs and levels of sphingosine in total bone marrow cells (S1P was below the detection threshold). (E) Effect of deletion of *Sphk1* in Mx1Cre-sensitive cells on *Sphk2* deficiency-induced thrombocytopenia and MPV. (F) Effect of transplantation of BM cells from mice lacking Sphk1&2 in Mx1Cre-sensitive cells to lethally irradiated wild-type recipients and vice versa on platelet counts in the host. Note that the rescue conferred by Sphk1 deficiency is BM cell-derived, as the *Sphk2*^{-/-} phenotype itself.²² (G) Effect of deletion of *Sphk1* in MKs on *Sphk2* deficiency-induced thrombocytopenia and MPV. (H) Effect of compound Sphk1 deficiency on MKP and platelet life span in *Sphk2*^{-/-} mice. Statistical analyses by Mann-Whitney *U* test or 2-way analysis of variance.

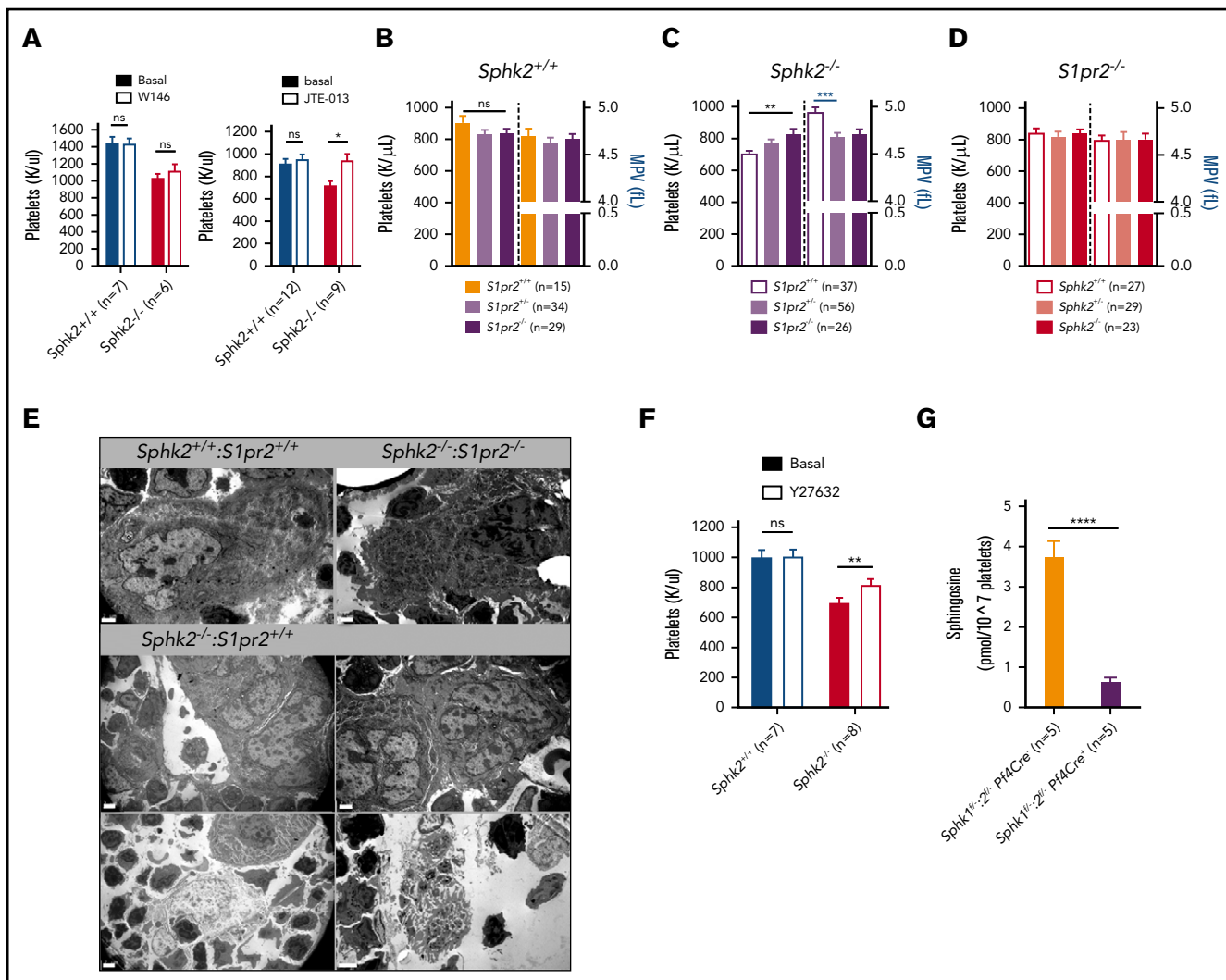


Figure 5. Sphk2 deficiency induces thrombocytopenia by aberrant S1P₂ activation. (A) Effects of S1P₁ (W146, 10 mg/kg, left) or S1P₂ (JTE-013, 1.2 mg/kg) antagonism on Sphk2 deficiency-induced thrombocytopenia (24-hour platelet counts). (B-D) Platelet counts and MPV in litters from independent intercrosses of *S1pr2*^{+/-} in a wild-type background (B), *S1pr2*^{+/-} in a Sphk2-deficient background (C), and of Sphk2^{+/-} in a S1P₂-deficient background (D). Note that although S1P₂ deficiency does not itself affect platelet counts, it rescues Sphk2 deficiency-induced thrombocytopenia. (E) Transmission electron micrographs of bone marrow from *Sphk2*^{+/+}, *Sphk2*^{-/-}, and *Sphk2*^{-/-}:*S1pr2*^{-/-} mice. Although the majority of MKs from *Sphk2*^{+/+} and *Sphk2*^{-/-}:*S1pr2*^{-/-} mice were singular and large, with a mature appearance and well-defined demarcation membrane systems (upper), MKs in *Sphk2*^{-/-} were highly heterogeneous, with clusters of immature MKs or mature MKs with limited DMS next to blood sinusoids (middle), low-contrast MK “ghosts” that appeared to be undergoing necrosis (bottom left, next to a normal MK) and platelet release within the bone marrow (bottom right). Representative images from *n* = 4 mice per genotype are shown. Scale bars, 2 μ m. (F) Effect of a bolus injection of the Rho kinase inhibitor Y27632 (10mg/kg) on platelet counts in *Sphk2*^{-/-} and *Sphk2*^{+/+} controls. Note a significant increase in platelet counts only in the knockout. (G) Sphingosine content of Sphk deficient platelets. Statistical analyses by 2-way analysis of variance (A,F) or the Mann-Whitney *U* test.

on lymphocyte trafficking, did not reduce platelet counts, nor did complete or partial deletion of MK *S1pr1* by 4 different transgenic approaches. Also at variance with Zhang et al,²⁰ we did not observe effects of S1P₁-selective pharmacological modulation on platelet counts when controlling for effects of vehicle and prior bleeding, nor did we confirm S1P₁ expression on murine MKs ex vivo or in situ. Our experiments were carried out in different strain backgrounds, littermate controlled, and sufficiently powered to reveal an important role for S1P gradient sensing. These observations are also in line with clinical experience, which has not revealed thrombocytopenia as an important adverse effect of S1P₁-targeting drugs.^{52,53}

In direct contrast, we observed elevated platelet counts with widespread neonatal deletion of either *Sphk1&2* or *S1pr1*. Although modest, this effect was highly significant and replicated with constitutive *S1pr1* deletion in hematopoietic cells, but not in MKs. Thrombocytosis was not conferred by transplantation of *S1pr1*-deficient bone marrow or induced with adult deletion or acute pharmacological S1P₁ modulation, suggesting developmental or delayed effects. A slight increase in MKs and a concomitant decrease in RBC counts suggested that S1P₁ may drive the differentiation of a common progenitor toward the erythroid lineage. Whether this reflects a direct role for S1P₁ signaling in cell fate decisions or indirect effects on the hematopoietic niche or stem cell trafficking^{42,45,46} remains to be determined.

Second, our results do not support a critical intracellular role for Sphk2-derived S1P in platelet production. As reported in a second Zhang et al study,²² we observed mild thrombocytopenia in *Sphk2*^{-/-} mice. Yet whereas Zhang et al deduced an intracellular role for Sphk2-derived S1P from observations in mice globally deficient in Sphk2, we did not reproduce thrombocytopenia with Pf4Cre-mediated selective deletion in MKs, even if this results in near complete loss of platelet S1P.⁶ Reversal of Sphk2 deficiency-induced thrombocytopenia with *Sphk1* deletion in hematopoietic cells further suggested that the phenotype reflected on an increase rather than a decrease in S1P production, a hypothesis substantiated by similar reversal with S1P₂ deficiency. Although our interpretation of how Sphk2 deficiency induces thrombocytopenia differs, detailed characterization of the phenotype by Zhang et al remains consistent with our data. Elegant 2-photon microscopy showed abnormal extension of proplatelets into BM sinusoids of *Sphk2*^{-/-} mice, followed by retraction without efficient platelet shedding.²² Our results suggest that the activation of S1P₂ and Rho kinase downstream could contribute to impaired platelet shedding, and that disturbed membrane lipid composition could also contribute to the gross aspects of the phenotype. S1P₂ has been reported to mediate blood repulsion in osteoclast precursors¹⁹ and to confine B cells to germinal centers by preventing their exit to high S1P environment.¹⁷ One could thus imagine high plasma S1P as observed in Sphk2-deficient animals to constitute a repulsive cue during platelet formation. Yet transfer of thrombocytopenia,²² but not high S1P levels,⁹ with transfer of *Sphk2*^{-/-} bone marrow cells does not support this model, and *Sphk2*^{-/-} PPs extended far into the bone marrow sinusoids before retracting.²² Impaired MK maturation was reminiscent of compound deficiency in *Cdc42* and *Rac1*, and our observations would be consistent with G $\alpha_{12/13}$ -coupled S1P₂ suppressing *Cdc42* and *Rac1* through RhoA, thus inhibiting terminal MK maturation or platelet shedding.^{20,49}

Third, our results argue against an important role for S1P₁ in amplifying platelet aggregation. Urtz et al reported protection from arterial thrombosis with global Sphk2 deficiency and reduced platelet aggregation in response to thrombin and other agonists in platelets derived from these mice.⁷ This phenotype was attributed to a lack of activation of platelet S1P₁ by platelet-derived S1P, based mainly on studies performed in human platelets or whole blood.⁷ Although we previously confirmed defective aggregation and spreading in Sphk-deficient platelets in the absence of exogenous S1P, we did not observe protection from thrombosis in mice with MK-selective Sphk deficiency.⁶ Our current study shows no effect of *S1pr1* deletion in assays in which we observed clear effects of Sphk deficiency,⁶ arguing against an autocrine platelet signaling loop involving S1P₁. S1P₁ modulation was shown to affect human platelet activation in whole blood,⁷ and it is possible that another S1P receptor takes on this function in mice, and that S1P₁ plays a more important role in humans. Yet as plasma S1P should already saturate S1P₁,^{54,55} it is unclear how platelets would sense further elevation of S1P levels after platelet activation by an S1P₁-dependent mechanism.

Whereas the current dogma would predict substantial effect of S1P₁ modulation on platelet counts and thrombosis, our observations argue that these effects are likely to be minimal. Even if developmental deficiency of S1P₁ in Mx1Cre-sensitive cells resulted in a modest increase in platelet counts, this was not

observed with adult deletion, and neither chronic treatment with oral fingolimod nor acute dosing with S1P₁ modulators had a measurable effect on platelet counts. In contrast, even partial deficiency in Sphk2 reduced platelet counts by redistribution of sphingosine to Sphk1, a kinase that is frequently upregulated in cancer, inflammation, and other disease conditions. Increased Sphk1 activity in cells within the hematopoietic niche capable of S1P export could thus be predicted to suppress platelet production. This could be relevant to cancer, Gaucher disease, and other conditions in which there is evidence of both S1P pathway deregulation and thrombocytopenia.⁵⁶

In conclusion, our observations argue that the S1P gradient and MK S1P₁ are both dispensable for platelet formation, and that S1P₁ is not critically involved in platelet activation or spreading in mice. Although it remains possible that S1P₁ is expressed and functional on human MKs and platelets, the current model, which predicts that S1P₁ modulation could be associated with a risk of bleeding and thrombosis, is based extensively on experiments performed in mice. The model therefore warrants revision, especially in light of clinical experience and with recent expansion of S1P₁-directed therapies to pathologies associated with considerable bleeding risk.

Acknowledgments

The authors thank the Cellular and Molecular Imaging Platform, CRP2-UMS 3612 CNRS-US25 Inserm-IRD-Université Paris Descartes, Blandine Dizier, and Paris Cardiovascular Research Center (PARCC) platform staff for support, Camille Brunaud (PARCC) and Philippe Rameau (Gustave Roussy, Integrated Biology Core Facility, Villejuif, France) for cell sorting and flow cytometry analysis, and William Vainchenker and Alain Schmitt for helpful advice.

This work was funded by The Leducq Foundation (SphingoNet) (R.L.P. and E.C.), the French National Research Agency (ANR-10-MIDI-0003) (E.C.), Fondation de France (E.C.), the Intramural Research Program of the National Institutes of Health, the National Institute of Diabetes and Digestive and Kidney Diseases (R.L.P.), Higher Education Commission, Pakistan (H.N. and R.I.), the Marie Curie Prestige program (A.N.), the French Society of Arterial Hypertension (B.M.), and Promex Stiftung für die Forschung (B.D.).

H.N. is a graduate student at Paris Descartes University. This work is submitted in partial fulfillment of the requirement of the PhD.

Authorship

Contribution: H.N., N.Z., L.C., A.L., A.N., M.L.A., B.M., R.I., Y.A., P.H.B., S.L.G., S.P.-C., B.D., V.B., E.D.C., M.K., A.B., P.T., and E.C. designed and performed experiments and analyzed data; E.C., H.N., and A.N. wrote the manuscript; and M.L.A., M.K., P.G., P.-L.T., J.C., S.P., N.D., R.L.P., and C.B.-L. provided reagents and conceptual advice and critically reviewed the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: A.N., 0000-0003-0567-6790; E.D.C., 0000-0003-0942-2819; M.K., 0000-0003-2447-4350; P.G., 0000-0002-9139-2147; P.-L.T., 0000-0002-6062-5905; J.C., 0000-0003-3964-0921; R.L.P., 0000-0003-0456-1270; E.C., 0000-0002-6271-7125.

Correspondence: Eric Camerer, INSERM U970, Paris Cardiovascular Research Center, 56 Rue Leblanc, 75015 Paris, France; e-mail: eric.camerer@inserm.fr.

References

1. Proia RL, Hla T. Emerging biology of sphingosine-1-phosphate: its role in pathogenesis and therapy. *J Clin Invest*. 2015;125(4):1379-1387.
2. Obinata H, Hla T. Sphingosine 1-phosphate in coagulation and inflammation. *Semin Immunopathol*. 2012;34(1):73-91.
3. Mendelson K, Evans T, Hla T. Sphingosine 1-phosphate signalling. *Development*. 2014;141(1):5-9.
4. Olivera A, Mizugishi K, Tikhonova A, et al. The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis. *Immunity*. 2007;26(3):287-297.
5. Xiong Y, Yang P, Proia RL, Hla T. Erythrocyte-derived sphingosine 1-phosphate is essential for vascular development. *J Clin Invest*. 2014;124(11):4823-4828.
6. Gazit SL, Mariko B, Thérond P, et al. Platelet and erythrocyte sources of S1P are redundant for vascular development and homeostasis, but both rendered essential after plasma S1P depletion in anaphylactic shock. *Circ Res*. 2016;119(8):e110-e126.
7. Urtz N, Gaertner F, von Bruehl ML, et al. Sphingosine 1-phosphate produced by sphingosine kinase 2 intrinsically controls platelet aggregation in vitro and in vivo. *Circ Res*. 2015;117(4):376-387.
8. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol*. 2005;25(24):11113-11121.
9. Sensken SC, Bode C, Nagarajan M, Peest U, Pabst O, Gräler MH. Redistribution of sphingosine 1-phosphate by sphingosine kinase 2 contributes to lymphopenia. *J Immunol*. 2010;184(8):4133-4142.
10. Cyster JG, Schwab SR. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu Rev Immunol*. 2012;30:69-94.
11. Pappu R, Schwab SR, Cornelissen I, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science*. 2007;316(5822):295-298.
12. Venkataraman K, Lee YM, Michaud J, et al. Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. *Circ Res*. 2008;102(6):669-676.
13. Hänel P, Andréani P, Gräler MH. Erythrocytes store and release sphingosine 1-phosphate in blood. *FASEB J*. 2007;21(4):1202-1209.
14. Vu TM, Ishizu AN, Foo JC, et al. Mfsd2b is essential for the sphingosine-1-phosphate export in erythrocytes and platelets. *Nature*. 2017;550(7677):524-528.
15. Mendoza A, Bréart B, Ramos-Perez WD, et al. The transporter Spns2 is required for secretion of lymph but not plasma sphingosine-1-phosphate. *Cell Reports*. 2012;2(5):1104-1110.
16. Yanagida K, Hla T. Vascular and immunobiology of the circulatory sphingosine 1-phosphate gradient. *Annu Rev Physiol*. 2017;79(1):67-91.
17. Green JA, Suzuki K, Cho B, et al. The sphingosine 1-phosphate receptor S1P₂ maintains the homeostasis of germinal center B cells and promotes niche confinement. *Nat Immunol*. 2011;12(7):672-680.
18. Skoura A, Michaud J, Im DS, et al. Sphingosine-1-phosphate receptor-2 function in myeloid cells regulates vascular inflammation and atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2011;31(1):81-85.
19. Ishii M, Kikuta J, Shimazu Y, Meier-Schellersheim M, Germain RN. Chemorepulsion by blood S1P regulates osteoclast precursor mobilization and bone remodeling in vivo. *J Exp Med*. 2010;207(13):2793-2798.
20. Zhang L, Orban M, Lorenz M, et al. A novel role of sphingosine 1-phosphate receptor S1pr1 in mouse thrombopoiesis. *J Exp Med*. 2012;209(12):2165-2181.
21. Hla T, Galvani S, Rafii S, Nachman R. S1P and the birth of platelets. *J Exp Med*. 2012;209(12):2137-2140.
22. Zhang L, Urtz N, Gaertner F, et al. Sphingosine kinase 2 (Sphk2) regulates platelet biogenesis by providing intracellular sphingosine 1-phosphate (S1P). *Blood*. 2013;122(5):791-802.
23. Golfier S, Kondo S, Schulze T, et al. Shaping of terminal megakaryocyte differentiation and proplatelet development by sphingosine-1-phosphate receptor S1P4. *FASEB J*. 2010;24(12):4701-4710.
24. Bigaud M, Guerini D, Billich A, Bassilana F, Brinkmann V. Second generation S1P pathway modulators: research strategies and clinical developments. *Biochim Biophys Acta*. 2014;1841(5):745-758.
25. Pyne S, Adams DR, Pyne NJ. Sphingosine kinases as druggable targets [published online ahead of print 20 February 2018]. *Handb Exp Pharmacol*. doi: 10.1007/164_2018_96.
26. Weske S, Vaidya M, Reese A, et al. Targeting sphingosine-1-phosphate lyase as an anabolic therapy for bone loss. *Nat Med*. 2018;24(5):667-678.
27. Zhu Z, Fu Y, Tian D, et al. Combination of the immune modulator fingolimod with alteplase in acute ischemic stroke: a pilot trial. *Circulation*. 2015;132(12):1104-1112.
28. Fu Y, Hao J, Zhang N, et al. Fingolimod for the treatment of intracerebral hemorrhage: a 2-arm proof-of-concept study. *JAMA Neurol*. 2014;71(9):1092-1101.
29. Allende ML, Tuymetova G, Lee BG, Bonifacino E, Wu YP, Proia RL. S1P1 receptor directs the release of immature B cells from bone marrow into blood. *J Exp Med*. 2010;207(5):1113-1124.
30. Ramos-Perez WD, Fang V, Escalante-Alcalde D, Cammer M, Schwab SR. A map of the distribution of sphingosine 1-phosphate in the spleen. *Nat Immunol*. 2015;16(12):1245-1252.

31. Choi JW, Gardell SE, Herr DR, et al. FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. *Proc Natl Acad Sci USA*. 2011;108(2):751-756.
32. Allende ML, Bektas M, Lee BG, et al. Sphingosine-1-phosphate lyase deficiency produces a pro-inflammatory response while impairing neutrophil trafficking. *J Biol Chem*. 2011;286(9):7348-7358.
33. Camerer E, Regard JB, Cornelissen I, et al. Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. *J Clin Invest*. 2009;119(7):1871-1879.
34. Kono M, Tucker AE, Tran J, Bergner JB, Turner EM, Proia RL. Sphingosine-1-phosphate receptor 1 reporter mice reveal receptor activation sites in vivo. *J Clin Invest*. 2014;124(5):2076-2086.
35. Tiedt R, Schomber T, Hao-Shen H, Skoda RC. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood*. 2007;109(4):1503-1506.
36. Schmitt A, Drouin A, Massé JM, Guichard J, Shagraoui H, Cramer EM. Polymorphonuclear neutrophil and megakaryocyte mutual involvement in myelofibrosis pathogenesis. *Leuk Lymphoma*. 2002;43(4):719-724.
37. Bektas M, Allende ML, Lee BG, et al. Sphingosine 1-phosphate lyase deficiency disrupts lipid homeostasis in liver. *J Biol Chem*. 2010;285(14):10880-10889.
38. Claxton S, Kostourou V, Jadeja S, Chambon P, Hodivala-Dilke K, Fruttiger M. Efficient, inducible Cre-recombinase activation in vascular endothelium. *Genesis*. 2008;46(2):74-80.
39. Swendeman SL, Xiong Y, Cantalupo A, et al. An engineered S1P chaperone attenuates hypertension and ischemic injury. *Sci Signal*. 2017;10(492):eaal2722.
40. Sanna MG, Wang SK, Gonzalez-Cabrera PJ, et al. Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P1 antagonist in vivo. *Nat Chem Biol*. 2006;2(8):434-441.
41. Sanna MG, Liao J, Jo E, et al. Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem*. 2004;279(14):13839-13848.
42. Massberg S, Schaerli P, Knezevic-Maramica I, et al. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell*. 2007;131(5):994-1008.
43. Joseph C, Quach JM, Walkley CR, Lane SW, Lo Celso C, Purton LE. Deciphering hematopoietic stem cells in their niches: a critical appraisal of genetic models, lineage tracing, and imaging strategies. *Cell Stem Cell*. 2013;13(5):520-533.
44. Sartawi Z, Schipani E, Ryan KB, Waeber C. Sphingosine 1-phosphate (S1P) signalling: Role in bone biology and potential therapeutic target for bone repair. *Pharmacol Res*. 2017;125(Pt B):232-245.
45. Golan K, Vagima Y, Ludin A, et al. S1P promotes murine progenitor cell egress and mobilization via S1P1-mediated ROS signaling and SDF-1 release. *Blood*. 2012;119(11):2478-2488.
46. Ishii M, Egen JG, Klauschen F, et al. Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis [published correction appears in *Nature*. 2010;465(7300):966]. *Nature*. 2009;458(7237):524-528.
47. Rankin EB, Wu C, Khatri R, et al. The HIF signaling pathway in osteoblasts directly modulates erythropoiesis through the production of EPO. *Cell*. 2012;149(1):63-74.
48. Pham TH, Baluk P, Xu Y, et al. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med*. 2010;207(1):17-27.
49. Pleines I, Dütting S, Cherpokova D, et al. Defective tubulin organization and proplatelet formation in murine megakaryocytes lacking Rac1 and Cdc42. *Blood*. 2013;122(18):3178-3187.
50. Chang Y, Auradé F, Larbret F, et al. Proplatelet formation is regulated by the Rho/ROCK pathway. *Blood*. 2007;109(10):4229-4236.
51. Chen Z, Naveiras O, Balduini A, et al. The May-Hegglin anomaly gene MYH9 is a negative regulator of platelet biogenesis modulated by the Rho-ROCK pathway. *Blood*. 2007;110(1):171-179.
52. Kappos L, Radue EW, O'Connor P, et al; FREEDOMS Study Group. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *N Engl J Med*. 2010;362(5):387-401.
53. Cohen JA, Barkhof F, Comi G, et al; TRANSFORMS Study Group. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. *N Engl J Med*. 2010;362(5):402-415.
54. Lee MJ, Van Brocklyn JR, Thangada S, et al. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science*. 1998;279(5356):1552-1555.
55. Rosen H, Sanna MG, Cahalan SM, Gonzalez-Cabrera PJ. Tipping the gatekeeper: S1P regulation of endothelial barrier function. *Trends Immunol*. 2007;28(3):102-107.
56. Mistry PK, Liu J, Sun L, et al. Glucocerebrosidase 2 gene deletion rescues type 1 Gaucher disease. *Proc Natl Acad Sci USA*. 2014;111(13):4934-4939.