

Platelet and Erythrocyte Sources of S1P Are Redundant for Vascular Development and Homeostasis, but Both Rendered Essential After Plasma S1P Depletion in Anaphylactic Shock

Salomé L. Gazit,* Boubacar Mariko,* Patrice Thérond,* Benoit Decouture, Yuquan Xiong, Ludovic Couty, Philippe Bonnin, Véronique Baudrie, Sylvain M. Le Gall, Blandine Dizier, Nesrine Zoghdani, Jessica Ransinan, Justin R. Hamilton, Pascale Gaussem, Pierre-Louis Tharaux, Jerold Chun, Shaun R. Coughlin, Christilla Bachelot-Loza, Timothy Hla, Benoit Ho-Tin-Noé, Eric Camerer

Rationale: Sphingosine-1-phosphate (S1P) signaling is essential for vascular development and postnatal vascular homeostasis. The relative importance of S1P sources sustaining these processes remains unclear.

Objective: To address the level of redundancy in bioactive S1P provision to the developing and mature vasculature.

Methods and Results: S1P production was selectively impaired in mouse platelets, erythrocytes, endothelium, or smooth muscle cells by targeted deletion of genes encoding sphingosine kinases -1 and -2. S1P deficiency impaired aggregation and spreading of washed platelets and profoundly reduced their capacity to promote endothelial barrier function *ex vivo*. However, and in contrast to recent reports, neither platelets nor any other source of S1P was essential for vascular development, vascular integrity, or hemostasis/thrombosis. Yet rapid and profound depletion of plasma S1P during systemic anaphylaxis rendered both platelet- and erythrocyte-derived S1P essential for survival, with a contribution from blood endothelium observed only in the absence of circulating sources. Recovery was sensitive to aspirin in mice with but not without platelet S1P, suggesting that platelet activation and stimulus–response coupling is needed. S1P deficiency aggravated vasoplegia in this model, arguing a vital role for S1P in maintaining vascular resistance during recovery from circulatory shock. Accordingly, the S1P₂ receptor mediated most of the survival benefit of S1P, whereas the endothelial S1P₁ receptor was dispensable for survival despite its importance for maintaining vascular integrity.

Conclusions: Although source redundancy normally secures essential S1P signaling in developing and mature blood vessels, profound depletion of plasma S1P renders both erythrocyte and platelet S1P pools necessary for recovery and high basal plasma S1P levels protective during anaphylactic shock. (*Circ Res.* 2016;119:e110–e126. DOI: 10.1161/CIRCRESAHA.116.308929.)

Key Words: anaphylaxis ■ endothelium ■ shock ■ sphingosine-1-phosphate ■ vascular endothelial function ■ vascular permeability ■ vascular tone regulation

Sphingosine-1-phosphate (S1P) is a bioactive metabolite of the sphingomyelin pathway that plays key roles in vascular development and homeostasis.¹ Most vascular roles of S1P have been attributed to activation of G protein–coupled S1P

receptor signaling, although S1P may also have receptor-independent functions.^{1–3} In the developing vasculature, S1P₁ signaling prevents angiogenic hypersprouting and contributes to vascular stabilization.^{4–6} In the mature vasculature, endothelial

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From the INSERM U970, Paris Cardiovascular Research Centre, 75015 Paris, France (S.L.G., B.M., L.C., V.B., S.M.L.G., N.Z., J.R., P.-L.T., E.C.); Université Sorbonne Paris Cité, Paris, France (S.L.G., B.M., B. Decouture, L.C., P.B., V.B., S.M.L.G., B. Dizier, N.Z., J.R., P.G., P.-L.T., C.B.-L., B.H.-T.-N., E.C.); AP-HP, Hôpital Bicêtre, Service de Biochimie, 94275 Le Kremlin Bicêtre, France (P.T.); Lip(Sys)2-Biochimie appliquée, Université Paris-Sud, Université Paris-Saclay, 92290 Châtenay-Malabry, France (P.T.); INSERM U1140, Faculté de Pharmacie, 75006 Paris, France (B. Decouture, B. Dizier, P.G., C.B.-L.); Center for Vascular Biology, Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, Cornell University, New York (Y.X., T.H.); AP-HP, Hôpital Lariboisière, Physiologie Clinique–Explorations-Fonctionnelles, INSERM U965, 75010, Paris, France (P.B.); Australian Centre for Blood Diseases & Department of Clinical Haematology, Monash University, Melbourne, Australia (J.R.H.); AP-HP, Hôpital Européen Georges Pompidou, Service d'Hématologie Biologique, Paris, France (P.G.); Department of Molecular and Cellular Neuroscience, Dorris Neuroscience Center, The Scripps Research Institute, La Jolla, CA (J.C.); Cardiovascular Research Institute, University of California, San Francisco (S.R.C.); and INSERM U698, 75018 Paris, France (B.H.-T.-N.).

*These authors contributed equally to this article.

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

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Nonstandard Abbreviations and Acronyms

BM	bone marrow
CLEC-2	C-type lectin-like receptor 2
PAF	platelet-activating factor
S1P	sphingosine-1-phosphate
S1Pless	S1P deficient
Sphk	sphingosine kinase
TEER	transendothelial electric resistance
WT	wild-type

S1P₁ signaling stabilizes endothelial adherens junctions and promotes blood flow, whereas smooth muscle cell S1P₂ and S1P₃ promote vascular contraction and, thereby, regulate vascular tone.^{5,7–10} Circulating S1P plays a key role in the activation of vascular receptors because lack of blood-borne S1P impairs vascular development, perturbs homeostatic maintenance of vascular integrity, and dramatically increases anaphylaxis mortality.^{11,12} Plasma S1P, presumed to be saturating for endothelial luminal S1P receptors, is provided by several types of cells and carried on different chaperones.^{1,13} Although this may argue that redundancy between vascular sources of S1P secures essential functions of S1P signaling, several recent studies suggest nonredundant roles for both erythrocyte- and platelet-derived S1P, as well as bias in S1P delivery to plasma chaperones and in chaperone-dependent S1P signaling.^{11,14–17} Considering the critical roles of S1P signaling in vascular development and homeostasis, it is important to define the relative and potentially unique contribution of the main sources of circulating S1P to the activation of different vascular receptors and the potential effects of S1P source exhaustion or depletion on vascular function. Notably, sources of circulating S1P can be selectively depleted in disease states; high-density lipoprotein and platelet pools have been shown to be exhausted in severe sepsis and myocardial infarction, respectively.^{18–20} If these sources are indeed nonredundant, such exhaustion could have profound effects on vascular function. Depending on the level of redundancy, anemia, thrombocytopenia, or inhibition of tissue-specific S1P metabolism, transporters or release mechanisms could also result in S1P deficiency-associated vascular dysfunction.

Plasma S1P is provided by erythrocytes and endothelial cells (which also provide lymph S1P) and is carried by high-density lipoprotein— and low-density lipoprotein-associated apolipoprotein M, as well as by albumin (Figure 1A).^{1,21–23} S1P is actively removed from interstitial fluids, generating a gradient that is required for lymphocyte egress to blood and lymph.²⁵ Two mammalian sphingosine kinase (Sphk) isoforms (1 and 2) are partly redundant for S1P generation.²⁶ Platelets and erythrocytes do not efficiently degrade S1P and, therefore, have a high S1P synthetic capacity^{23,27–29} (Figure 1A). Embryonic bleeding and death associated with erythrocyte S1P deficiency argues that erythrocyte-derived S1P may play a unique role in endothelial S1P₁ activation.¹¹ This and the observation that platelets do not contribute to plasma S1P^{21,23} question the functional importance of platelet-derived S1P in homeostasis. Yet a doubling of S1P

levels in serum relative to plasma suggests that platelet activation has the potential to substantially augment local S1P concentrations,²⁷ potentially engaging receptors that are not saturated by plasma S1P, for example, on vascular smooth muscle cells, or changing the bias or balance of receptor activation.^{30,31} Intriguingly, in this regard, a recent study argued a key role for platelet S1P release and paracrine activation of endothelial S1P₁ after activation of platelet immunoreceptor tyrosine-based activation motif–coupled receptors.¹⁴ In a model of immunization-induced bleeding in draining lymph nodes, individual platelets were shown to gain subendothelial access at high endothelial venules. In the absence of the platelet immunoreceptor tyrosine-based activation motif receptor C-type lectin-like receptor 2 (CLEC-2), reticular epithelial podoplanin, or platelet S1P, lymph node bleeding was observed. Platelet immunoreceptor tyrosine-based activation motif–coupled receptors maintain vascular integrity during vascular development and inflammation and mediate blood/lymph separation^{32–34}; the role for platelet-derived S1P as a downstream mediator in these settings remains to be explored. Another recent study demonstrated an important cell-autonomous role for S1P in platelet activation and thrombosis, arguing that platelet S1P is relevant for platelet S1P₁ activation even in the presence of erythrocyte-derived plasma S1P.¹⁵ Importantly, however, selective ablation of S1P production in megakaryocytes was not used to confirm a role of platelet S1P in either study. S1P release by platelets appears to be largely dependent on thromboxane formation and sensitive to nonselective cyclooxygenase inhibitors, such as aspirin (acetylsalicylic acid) and ibuprofen.³⁵ Platelet S1P release may, thus, be inhibited by over-the-counter nonsteroidal anti-inflammatory drugs, highlighting the importance of revealing its functions. While the role for S1P production in endothelium has not been directly tested, Sphk1 is dynamically regulated by both G protein–coupled receptor and tyrosine kinase receptors and has been proposed to be important for barrier protective signaling in endothelium by S1P₁ transactivation.^{36,37}

To explore roles for platelet-derived S1P and redundancy between circulating and resident S1P sources in vascular development and homeostasis, we generated mice with isolated or combined tissue-specific deficiency of Sphks 1 and 2 in megakaryocytes/platelets, erythrocytes, endothelial cells, and smooth muscle cells, and compared effects of S1P source deficiency to effects of thrombocytopenia or deficiency in vascular S1P receptors. Using washed platelets from platelet S1P-deficient mice, we observed autocrine roles for S1P in platelet activation and spreading and a key paracrine role for S1P in modulation of endothelial barrier function by platelets. Yet experiments comparing the impact of platelet S1P deficiency to that of other S1P source deficiencies or thrombocytopenia during developmental-, physical-, inflammatory-, and irradiation-induced vascular challenge all argued that roles observed for platelet S1P *ex vivo* were compensated for by other mechanisms and S1P sources *in vivo*. Accordingly, experiments designed to test essential roles proposed for platelet-derived S1P in thrombosis and lymph node vascular integrity and erythrocyte-derived S1P in

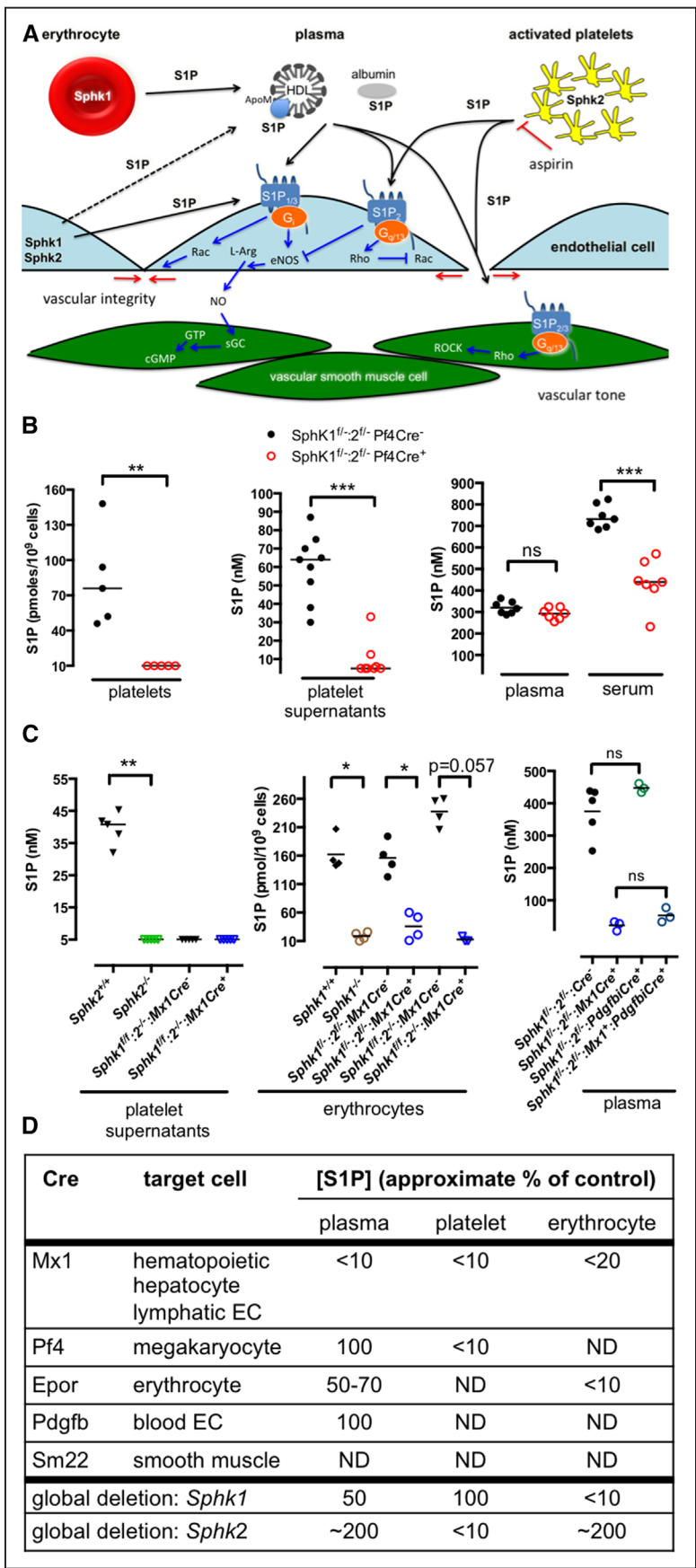


Figure 1. Generation of sphingosine-1-phosphate (S1P)-deficient mice. A, Schematic overview of S1P signaling in the vasculature. S1P receptors on endothelial cells and possibly smooth muscle cells play important roles in regulating vascular development, endothelial barrier function, and vascular tone. Multiple cell types provide S1P to plasma, where it binds to high-density lipoprotein (HDL), albumin, and low-density lipoprotein (LDL). B–C, S1P concentrations as determined by high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry in lysed platelets, supernatants of washed and activated platelets, erythrocytes, plasma, and serum from mice with single global deficiency or compound tissue-specific deficiency in Sphk1 and Sphk2 in megakaryocytes (*Sphk1^{fl/fl};*2^{fl/fl};*Pf4Cre⁺*), blood endothelium (*Sphk1^{fl/fl};*2^{fl/fl};*PdgfbCre⁺*), hematopoietic cells, hepatocytes, and lymphatic endothelium (*Sphk1^{fl/fl};*2^{fl/fl};*Mx1Cre⁺*), or the latter combined (*Sphk1^{fl/fl};*2^{fl/fl};*Mx1Cre⁺*;*PdgfbCre⁺*). Compound gene deletion was performed either by tissue-specific recombination of one loxP-flanked copy of each kinase gene in a context where the other copy was deleted in all cells (*f/f*;*–/–*) or by tissue-specific recombination of 2 loxP flanked copies of one kinase gene in a context where both copies of the other kinase gene were deleted in all cells (*f/f*;*–/–*). D, Overview of Cre alleles, their target cells, and effect of Sphk deletion on S1P levels in platelets, erythrocytes, and plasma under homeostasis as determined above or published elsewhere.^{11,23,24} Note that erythrocytes and platelets make near exclusive use of Sphk1 and Sphk2, respectively, for S1P production and that while relevant target cells of Mx1Cre (erythrocytes and lymphatic endothelial cells) continuously provide S1P to plasma, platelets only release their S1P content on activation and, thus, contribute to serum but not plasma S1P. Blood endothelium does not contribute measurably to plasma S1P, but may still constitute a relevant source for activation of vascular receptors. ApoM indicates apolipoprotein M; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; Epore, erythropoietin receptor; L-Arg, L-arginine; Mx1, myxovirus resistance-1; ND, not determined; NO, nitric oxide; Pdgfb, platelet-derived growth factor beta; Pf4, platelet factor 4; Rac, Rac GTPase; ROCK, Rho-associated protein kinase; SM22, smooth muscle protein 22; and Sphk2, sphingosine kinase 2.

vascular development with targeted genetic approaches failed to confirm the need for any single source when competing sources were left intact. On the other hand, we observed that plasma S1P is depleted during anaphylactic shock, rendering S1P delivery by both platelets and erythrocytes essential for recovery.

Methods

Mice

Genetically mutant mice used in this study were of mixed C57BL/6J:129SVJ background and compared with age- and sex-matched littermate controls. Wild-type (WT) C57BL/6J mice were purchased from Charles River or Janvier laboratories. Each point in dot-plots represents a sample from one mouse with the exception of plasma S1P measurements for which plasma from several mice of the same genotype were in some cases pooled. Genes encoding mouse Sphk1 and Sphk2 are designated *Sphk1* and *Sphk2*, respectively. Generation of global (−) and conditional (f) knockout alleles for these genes and their postnatal deletion in hematopoietic cells by polyI:C induction of the Mx1Cre transgene³⁸ to generate plasma S1P deficient (S1Pless) mice (*Sphk1*^{fl/fl};2^{−/−};Mx1Cre⁺ and *Sphk1*^{fl/fl};2^{fl/fl};Mx1Cre⁺) has been described.²³ Mice deficient in S1P production in megakaryocytes and platelets (*Sphk1*^{fl/fl};2^{fl/fl};Pf4Cre⁺ and *Sphk1*^{fl/fl};2^{−/−};Pf4Cre⁺) were generated using a Pf4Cre allele.³⁹ *S1pr1* floxed, *S1pr2*, and *S1pr3* knockout and *Pdgfb*CreERT alleles have been described.^{40–43} Endothelial-specific deletion of *S1pr1* was achieved by oral administration of 20 µg tamoxifen to *S1pr1*^{fl/fl};PdgfbCreERT neonates thrice between p1 and p5.

Endothelial Barrier Function

Transendothelial electric resistance (TEER) was assessed using electric cell–substrate impedance sensing (Applied Biophysics) at 4000 Hz.⁴⁴ Human umbilical vein endothelial cells (PromoCell) were plated at 10⁵ cells/fibronectin coated 8W10E(+) well. The day after, cells were equilibrated (2 hours) in serum-free endothelial cell basal medium (PromoCell) before establishing baseline resistance (1 hour). 10⁷ washed platelets were then added, followed by a protease-activated receptor-4–activating peptide (500 µM). Where indicated, endothelial cells were pretreated with the S1P₁ antagonist W146 (1 µM) or vehicle control before the addition of platelets.

Inflammation-Associated Bleeding

Models of inflammation-associated bleeding have been described elsewhere.^{14,34,45} Briefly, reverse passive Arthus reaction was elicited subcutaneously with anti-BSA IgG (60 µg) immediately followed by BSA (50 µg/g) intravenously. Mice were euthanized 4 hours later, and biopsies of control and inflamed skin were photographed for blinded scoring of petechiae, harvested, weighed, homogenized, and sonicated in cold phosphate-buffered saline for measurement of hemoglobin content and myeloperoxidase activity. Lung inflammation was induced by intranasal inoculation with 25 µg *Pseudomonas aeruginosa* lipopolysaccharide. Six hours later, bronchoalveolar lavage was performed and hemoglobin and blood cell content quantified. Immunization was induced by injection of 20 µL of an emulsion of complete Freund's adjuvant/Ovalbumin phosphate-buffered saline in the right hock.¹⁴ One week later, mice were anesthetized and transcardially perfused to remove intravascular blood, and lymph node bleeding was assessed.

Study Approval

Experimental procedures were approved by the animal ethics committee of the Paris Descartes University and the French Ministry of Research and Higher Education (03686.02; 02822.02; 03651.02).

Statistical Analyses

Statistical significance of differences in survival outcome was assessed with the Gehan–Breslow–Wilcoxon test; differences in sphingolipid levels, calcium signaling, platelet aggregation and adhesion, Evans

Blue leak, myeloperoxidase, leukocyte counts, bleeding, thrombosis, hemoglobin, edema, hematocrits with the Mann–Whitney test; and P-selectin exposure, TEER, paw swelling, cardiac and blood flow parameters by 2-way analysis of variance. Cross-comparisons between experimental groups in Figure 6E were performed by Kruskal–Wallis test followed by Dunn's multiple comparison test. **P*<0.05; ***P*<0.01; ****P*<0.0001; *****P*<0.00001; ns, not significant; n, number of animals included.

Additional details concerning the mouse lines and materials, as well as additional methods, online figures, and figure legends can be found in the [Online Data Supplement](#).

Results

Effects of Global and Tissue-Restricted Sphk Deficiencies on Circulating Pools of S1P

We generated mice with compound deficiency of Sphk1 and Sphk2 in megakaryocytes/platelets (platelet S1Pless), erythrocytes, endothelial cells, smooth muscle cells, or all cells providing S1P to plasma (plasma S1Pless) by crossing *Sphk1* and *Sphk2* conditional (floxed, f) and global (−) knockout alleles²³ to Cre recombinase genes driven by platelet factor 4 (*Pf4Cre*),³⁹ erythropoietin receptor (*Epor-Cre*),¹¹ platelet-derived growth factor beta (*Pdgfb*CreERT2),⁴⁰ smooth muscle protein 22-alpha (*SM22-Cre*), and Myxovirus resistance-1 (*Mx1Cre*)²³ promoters, respectively. S1P source mutants were first generated with 1 floxed allele and 1 knockout allele for each kinase, with controls differing from knockouts only by the presence of the Cre allele (*Sphk1*^{fl/fl};2^{fl/fl};Cre^{+/−}), although variants where the gene for one kinase was deleted in a tissue-specific manner in a context where the gene for the other kinase was absent in all cells (eg, *Sphk1*^{fl/fl};2^{−/−};Cre^{+/−}) were also generated for comparison with published reports. Plasma S1Pless mice and the erythrocyte S1Pless mice have already been described, and they have <10% of normal plasma and <2% of normal erythrocyte S1P, respectively.^{11,23} S1P levels in lysed platelets and activation-induced S1P release from washed platelets from platelet S1Pless mice (*Sphk1*^{fl/fl};2^{fl/fl};Pf4Cre⁺) were both below detection limits for most donors, with a matching reduction in serum, but not plasma (Figure 1B). No significant change was observed in sphingosine levels in plasma, serum, or platelet supernatants (Online Figure I). Intriguingly, Sphk1 and Sphk2 were almost exclusively required for erythrocyte and platelet S1P production, respectively (Figure 1C). As a consequence, both classical plasma S1Pless mice,^{12,14,23} which are rendered profoundly plasma S1P deficient by postnatal deletion of *Sphk1* in hematopoietic cells and lymphatic endothelial cells in a *Sphk2*^{−/−} background (*Sphk1*^{fl/fl};2^{−/−};Mx1Cre⁺) and their Cre[−] controls, were devoid of platelet S1P (Figure 1C). Deletion of *Sphk1* and *Sphk2* in blood endothelium had no impact on plasma S1P levels even in the context of compound deletion in hematopoietic cells and lymphatic endothelium (Figure 1C). Collectively, these observations confirm that erythrocytes and lymphatic endothelial cells are the main sources of plasma S1P under homeostasis (without contribution from platelets or blood endothelium),²³ suggest cell-selective use of Sphks that has important consequences for the interpretation of reports of nonredundant roles of S1P sources, and argue that megakaryocyte deletion of Sphks yields platelets that remain S1Pless and do not scavenge S1P from plasma (Figure 1D).

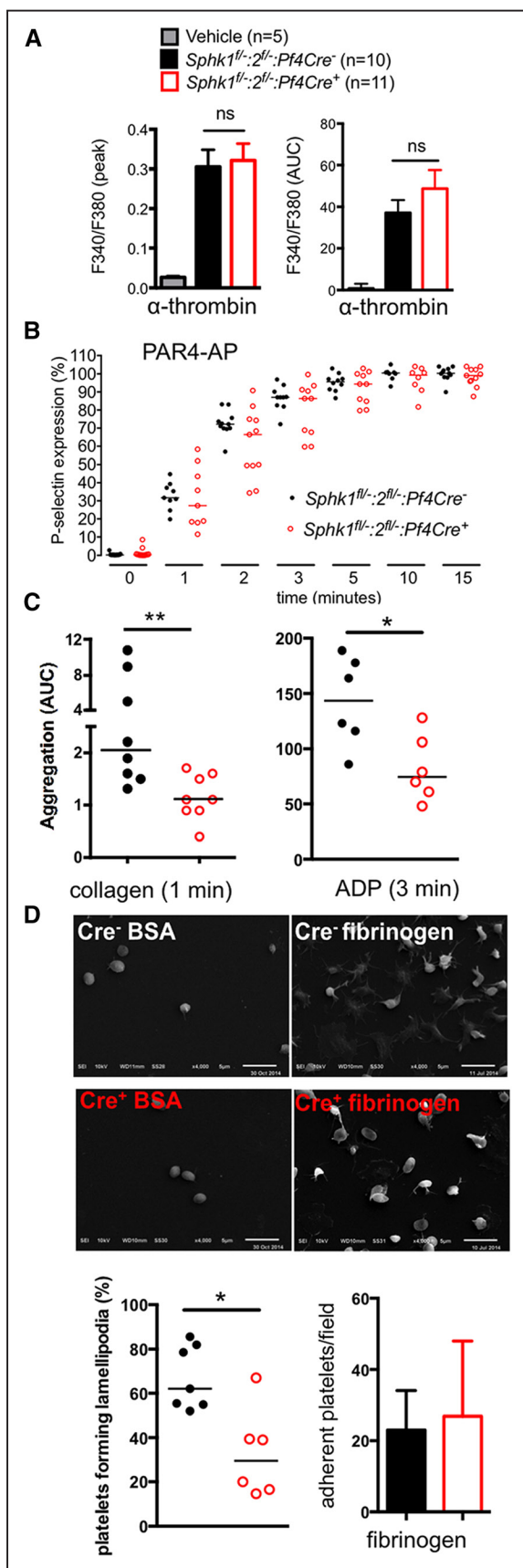


Figure 2. Platelet sphingosine-1-phosphate (S1P) deficiency impairs functional responses in washed platelets. The function of sphingosine kinase (Sphk) 1- and 2-deficient (*Continued*)

Sphk Deficiency Impairs Aggregation and Spreading of Washed Platelets

Because S1P has been reported to induce platelet shape change,²⁷ we evaluated the function of S1Pless platelets. Maximal and cumulative increases in cytosolic-free calcium in response to α -thrombin were indistinguishable between washed S1Pless and littermate control platelets (Figure 2A). P-selectin exposure in response to a peptide agonist for the thrombin receptor protease-activated receptor-4 was found to be grossly normal, although delayed in platelets from some platelet S1Pless donors (Figure 2B). A delayed response was seen in aggregation to collagen, while ADP-induced aggregation was also impaired at later times (Figure 2C). Platelet spreading (lamellipodia formation) on fibrinogen was reduced with platelet S1P deficiency (Figure 2D). These observations suggest an autocrine role for platelet S1P release in platelet activation and, thus, a potential role for S1P in hemostasis and thrombosis and are consistent with recent observations by Urtz et al.¹⁵

Platelets Promote Endothelial Barrier Function Ex Vivo by Release of S1P

We next addressed the importance of S1P release for the capacity of platelets to promote TEER, which reflects on endothelial barrier function. We observed a robust increase in TEER when washed platelets from WT mice were added to human umbilical vein endothelial cell grown to confluence on fibronectin-coated microelectrodes (Figure 3A). When the platelets were activated with protease-activated receptor-4-agonist peptide, we observed a rapid further increase in TEER that was succeeded by a decrease and subsequent recovery (Figure 3A). The initial increase in barrier function observed without deliberate activation of the platelets was blunted with the S1P₁ antagonist W146 and may reflect constitutive release of S1P from the plasma membrane.⁴⁶ W146 also reduced basal resistance, consistent with tonic barrier-promoting S1P signaling in endothelium.^{9,12} S1Pless platelets were strongly impaired in their ability to promote barrier function (Figure 3B), as were platelets from *Sphk2*^{-/-} donors, confirming a dominant role for Sphk2 in S1P production (Figure 3C and 3D). Unlike mass spectrometry (Figure 1B), this assay uncovered a minor but significant contribution of Sphk1 to platelet S1P release (*Sphk1*^{fl/fl};*2*^{-/-};*Mx1Cre*⁺ versus *Sphk1*^{fl/fl};*2*^{-/-};*Mx1Cre*⁻; Figure 3D, left panel). These results confirm that S1P is a key endothelial barrier-promoting factor released by platelets.⁴⁴

Figure 2 Continued. platelets was addressed ex vivo using washed platelets by assessing (A) α -thrombin (10 nmol/L)-induced changes in cytosolic $[Ca^{2+}]$ by fura-2 ratiometric analysis, (B) protease-activated receptor-4-agonist peptide (PAR4-AP; 200 μ M)-induced P-selectin exposure by flow cytometry, (C) collagen- (0.75 μ g/mL) and ADP (1.5 μ M)-induced aggregation by densitometry, and (D) spreading on fibrinogen by scanning electron microscopy. Sphk 1- and 2-deficient platelets show normal calcium signaling, slightly delayed P-Selectin exposure, delayed aggregation to collagen (difference observed at 1 but not 3 minutes), and reduced aggregation to ADP and spreading on fibrinogen (red) relative to platelets from littermate control mice (black). Platelet adhesion to fibrinogen and spreading on BSA were not different between the groups. AUC indicates area under the curve.

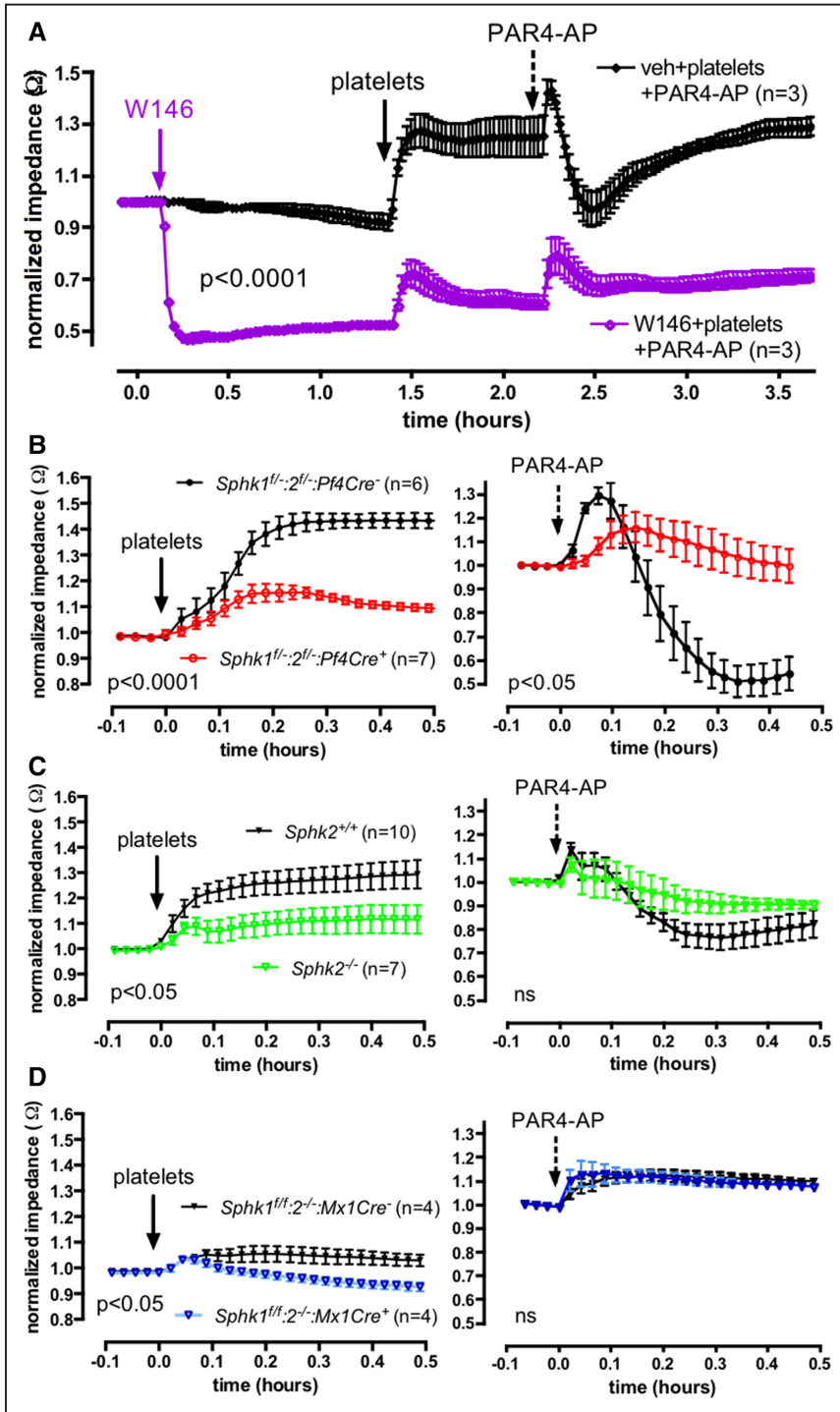


Figure 3. Sphingosine-1-phosphate (S1P) is critical for the capacity of platelets to promote endothelial barrier function ex vivo. Human umbilical vein endothelial cell (HUVEC) were cultured on gold electrodes and electric impedance, reflecting transendothelial electric resistance and barrier function, and measured. After an initial stabilization period, 10^7 washed platelets from wild-type (A) or indicated sphingosine kinase (Sphk)-deficient (B–D, left) mice or littermate controls were added to HUVEC monolayers (B–D, left) and then activated with protease-activated receptor-4-agonist peptide (PAR4-AP; 500 μ M; B–D, right). Endothelial cells in A were treated with the S1P₁ antagonist W146 (1 μ M, purple) or vehicle control (black) for 1 hour before the addition of platelets. In A, impedance was normalized before addition of W146 or vehicle control. In B–D, impedance was normalized before platelet addition in left panels and before PAR4 addition in right panels. The PAR4-AP did not significantly alter barrier function in HUVECs in the absence of platelets, and platelets alone had negligible effects on impedance of fibronectin- or collagen-coated electrodes.

Platelet- and Erythrocyte-Derived S1P Are Redundant for Vascular Development and Blood/Lymph Separation

Because our ex vivo observations were consistent with a proposed paracrine role of S1P downstream of platelet CLEC-2,¹⁴ we next addressed whether platelet S1P deficiency was associated with developmental defects reminiscent of deficiency in CLEC-2 signaling.³² Blood/lymph mixing at E14.5 after PF4Cre-mediated deletion of a LoxP-flanked CLEC-2 allele suggests that PF4Cre mediates efficient excision by this

time.³³ However, although thrombocytopenic *Nf-e2-/-* embryos⁴⁷ isolated in parallel exhibited both brain bleeds and blood in lymphatic structures as reported for CLEC-2-deficient embryos, neither phenotype was observed with platelet S1P deficiency at E12.5 or E14.5 (Figure 4A).⁴⁸

Erythrocyte-derived S1P was recently reported to be essential for vascular development¹¹ and is likely to compensate for developmental platelet S1P deficiency. Considering that platelets have strong S1P-mediated paracrine actions on cultured endothelium (Figure 3) and appear in circulation

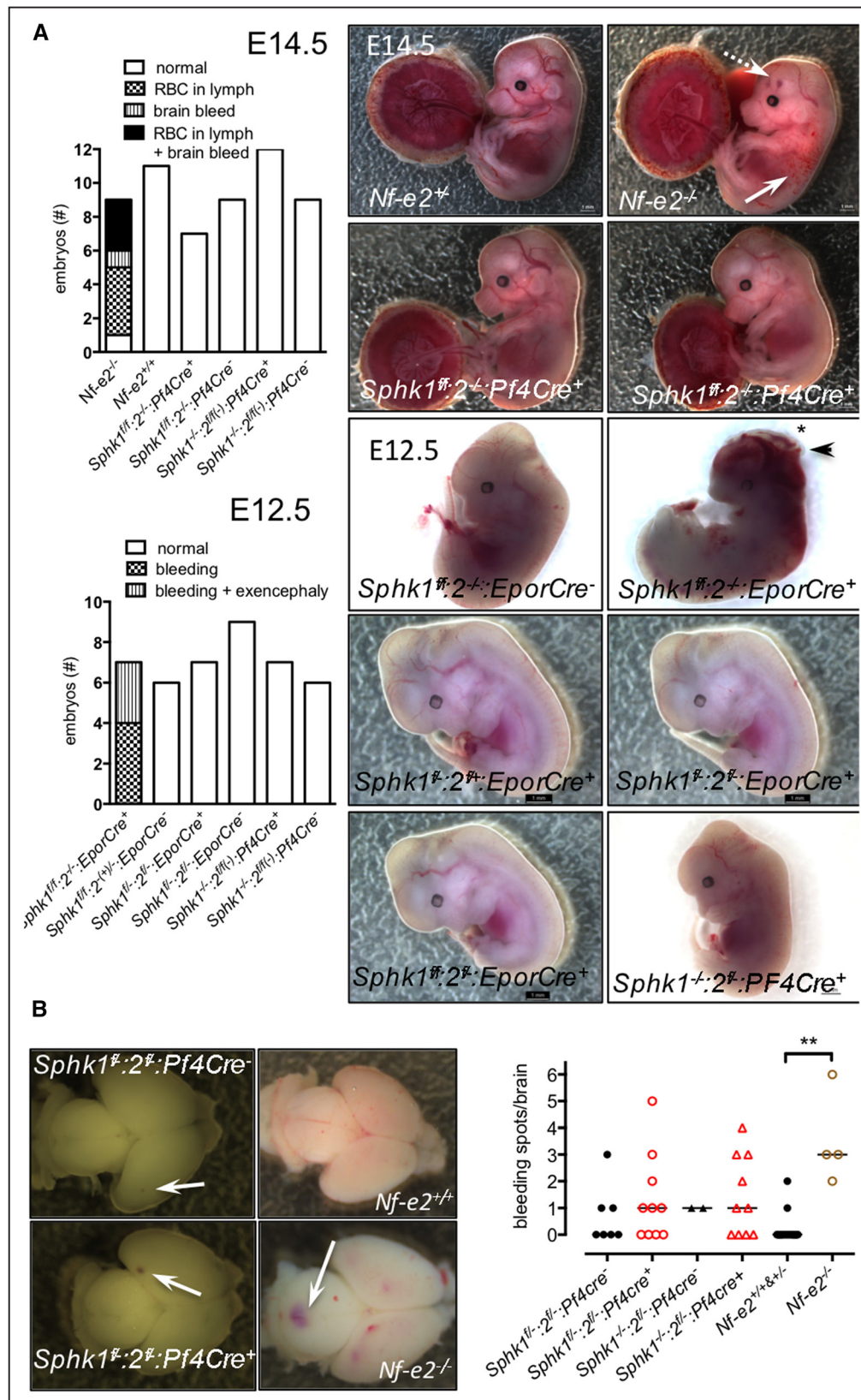


Figure 4. Platelet- and erythrocyte-derived sphingosine-1-phosphate (S1P) are dispensable for developmental blood-lymph separation and vascular integrity. **A**, Embryos with defective thrombopoiesis (*Nf-e2^{-/-}*) or rendered deficient in megakaryocyte sphingosine kinase (*Sphk1*) in a *Sphk2^{-/-}* background (*Sphk1^{fl/fl};2^{-/-};Pf4Cre⁺*) were recovered at E14.5 and inspected for the presence of blood in lymphatic vessels (white arrow) and intracranial hemorrhage (hatched white arrow). Note the presence of both phenotypes in *Nf-e2^{-/-}* embryos but not littermate controls or platelet S1P-deficient embryos. Although embryos rendered deficient in erythrocyte *Sphk1* in a *Sphk2^{-/-}* background (*Sphk1^{fl/fl};2^{-/-};EporCre⁺*) showed widespread bleeding (black arrowhead) and, in some cases, (Continued)

Figure 4 Continued. exencephaly (asterisk) at E12.5, this was not observed when *Sphk1* was deleted in erythrocytes in a *Sphk2*^{-/-} background (*Sphk1*^{fl/fl}:*2*^{fl/-}:*EpoR*Cre), nor when *Sphk1* and 2 were both deleted specifically in erythrocytes (*Sphk1*^{fl/-}:*2*^{fl/-}:*EpoR*Cre⁺) or when both erythrocyte and platelet sources were impaired (*Sphk1*^{fl/-}:*2*^{fl/fl}:*Pf4*Cre⁺). The left panel summarizes observations in collected embryos. **B**, *Sphk*-deficient and *Nf-e2*^{-/-} neonates were euthanized on postnatal day 0 to 2 and brains dissected after transcardial phosphate-buffered saline (PBS) perfusion to look for evidence of hemorrhage. **Left**, Representative brains. **Right**, Summary. No significant difference in bleeding prevalence was observed with platelet- or platelet- and erythrocyte-S1P deficiency, whereas bleeding was significantly more frequent in *Nf-e2*^{-/-} than in corresponding littermate controls. Images were acquired in PBS immersion with a Leica M165FC stereo microscope.

around E10.5,⁴⁹ it is surprising that platelet S1P cannot also compensate for a lack of erythrocyte-derived S1P. Notably, bleeding and death reported for erythrocyte S1P deficiency were only observed when *Sphk1* was deleted in erythrocytes in embryos that were also *Sphk2*^{-/-} (*Sphk1*^{fl/fl}:*Sphk2*^{-/-}:*EpoR*Cre⁺), not in embryos with one intact allele of *Sphk2* (*Sphk1*^{fl/fl}:*Sphk2*^{+/-}:*EpoR*Cre⁺), even if adults with the latter genotype showed near complete absence of erythrocyte S1P.¹¹ To address if this reflected a redundant role for *Sphk1* and *Sphk2* in erythrocytes, we performed erythrocyte-restricted deletion of both kinases. Although we uniformly observed severe bleeding in *Sphk1*^{fl/fl}:*Sphk2*^{-/-}:*EpoR*Cre⁺ embryos dissected at E12.5 as reported,¹¹ no embryonic bleeding and normal postnatal survival were observed when deficiency of both kinases was restricted to erythrocytes (*Sphk1*^{fl/-}:*Sphk2*^{fl/-}:*EpoR*Cre⁺; Figure 4A), arguing against a nonredundant role for erythrocytes in S1P provision. Embryonic bleeding was also not observed in *Sphk1*^{fl/-}:*Sphk2*^{fl/-}:*Pf4*Cre⁺ embryos (Figure 4A), predicted to lack both erythrocyte and platelet S1P (Figure 1), suggesting that endothelium-derived S1P may also be sufficient to support development.

Birth trauma also induced cerebral bleeding at a frequency that was enhanced with *Nf-e2*^{-/-} deficiency (Figure 4B). Although neonatal brain bleeding was observed also in *Sphk*-deficient mice, it did not increase with platelet or platelet- and erythrocyte-S1P deficiency (Figure 4B).

Collectively, these observations argue that although erythrocytes may be sufficient,¹¹ no single cellular source is essential for providing bioactive S1P to support S1P₁ signaling during embryonic development.

Redundancy of S1P Sources in Vascular Integrity, Hemostasis, and Thrombosis in Adult Mice

As previously reported, lack of *Sphk1* and *Sphk2* in all cells that provide S1P to plasma was associated with constitutive vascular leak to lung in adult mice (Figure 5A).¹² This was phenocopied by postnatal deletion of *S1pr1* in endothelial cells (*S1pr1*^{fl/fl}:*Pdgfrb*Cre⁺) and, thus, reflected lack of endothelial S1P₁ signaling (Figure 5A). By contrast, we did not observe significant vascular leak with isolated deficiency in S1P production in platelets, erythrocytes, or endothelial cells (Figure 5A), arguing source redundancy also in adult mice, under homeostasis. As platelet S1P is likely to be engaged only during vascular inflammation or injury, we explored the role for this source further. Release of platelet S1P after CLEC-2 activation might suggest a role for platelet S1P in supporting vascular integrity during inflammation³⁴; yet, platelet S1P deficiency did not enhance histamine-induced paw edema (Figure 5B) as was observed for plasma S1P deficiency,¹² and neither platelet- (*Sphk1*^{fl/-}:*2*^{fl/-}:*Pf4*Cre⁺)

nor plasma- (*Sphk1*^{fl/-}:*2*^{fl/-}:*Mx1*Cre⁺ or *Sphk1*^{fl/fl}:*2*^{-/-}:*Mx1*Cre⁺) S1P deficiency triggered bleeding in models of lung (Figure 5C) or skin (Figure 5D) inflammation even if platelet depletion did. Platelet S1P deficiency also did not influence neutrophil recruitment in these models (Figure 5C and 5D).⁵⁰ Besides paracrine action on endothelium, our ex vivo observations supported a possible cell autonomous role for S1P in platelet activation, consistent with Urtz et al.¹⁵ We, therefore, next addressed if platelet S1P deficiency impaired hemostasis in adult mice and if protection from thrombosis reported in *Sphk2* global knockouts resulted from S1P deficiency in platelets.¹⁵ However, we found no effect of platelet S1P deficiency on tail-bleeding times nor on time to ferric chloride-induced occlusion of the carotid artery (Figure 5E and 5F). To address whether platelet-derived S1P might prevent subtle vascular injury, we then transplanted irradiated WT mice with *Nf-e2*^{-/-} or platelet S1Pless hematopoietic progenitor cells. Mice transplanted with *Nf-e2*^{-/-} fetal liver cells uniformly displayed cerebral hemorrhage 10 to 13 days after irradiation, which was not observed in controls (Figure 5G). However, bleeding was not observed after transplantation of bone marrow (BM)-derived cells from even profoundly platelet S1Pless *Sphk1*^{fl/fl}:*Sphk2*^{-/-}:*Pf4*Cre⁺ donors (Figure 5G). These results suggest that despite autocrine and paracrine actions observed ex vivo (Figures 2 and 3), other S1P sources and other mechanisms will in most cases render platelet-derived S1P redundant for platelet activation and vascular integrity in vivo.

Platelets and Erythrocytes Provide Redundant Sources of Lymph Node–Protective S1P After Immunization

Surprised that the essential role reported for platelet S1P downstream of CLEC-2 signaling after immunization¹⁴ did not extend to other settings where CLEC-2 signaling has been implicated, we addressed whether platelet S1P deficiency indeed sensitizes to lymph node bleeding. Supporting protective roles for both platelets and S1P, we observed blood in draining lymph nodes of both platelet-depleted and plasma S1Pless mice (*Sphk1*^{fl/fl}:*2*^{-/-}:*Mx1*Cre⁺) 1 week after immunization in the right hock (Figures 6A–6C).⁵¹ Greatly reduced bleeding in chimeric *Sphk1*^{fl/fl}:*2*^{-/-}:*Mx1*Cre⁺ mice transplanted with WT BM also supported a role for a circulating S1P source (Figure 6C). However, platelet S1P was not essential because removal of even the minor *Sphk1*-derived S1P in *Sphk2*^{-/-} platelets did not enhance bleeding (*Sphk1*^{fl/fl}:*2*^{-/-}:*Pf4*Cre⁺; Figure 6C). Accordingly, erythrocyte-derived S1P was sufficient to prevent bleeding because transfusion of washed, packed erythrocytes on days 4 and 6 after immunization prevented bleeding in *Sphk1*^{fl/fl}:*2*^{-/-}:*Mx1*Cre⁺ mice (Figure 6C). Erythrocytes from

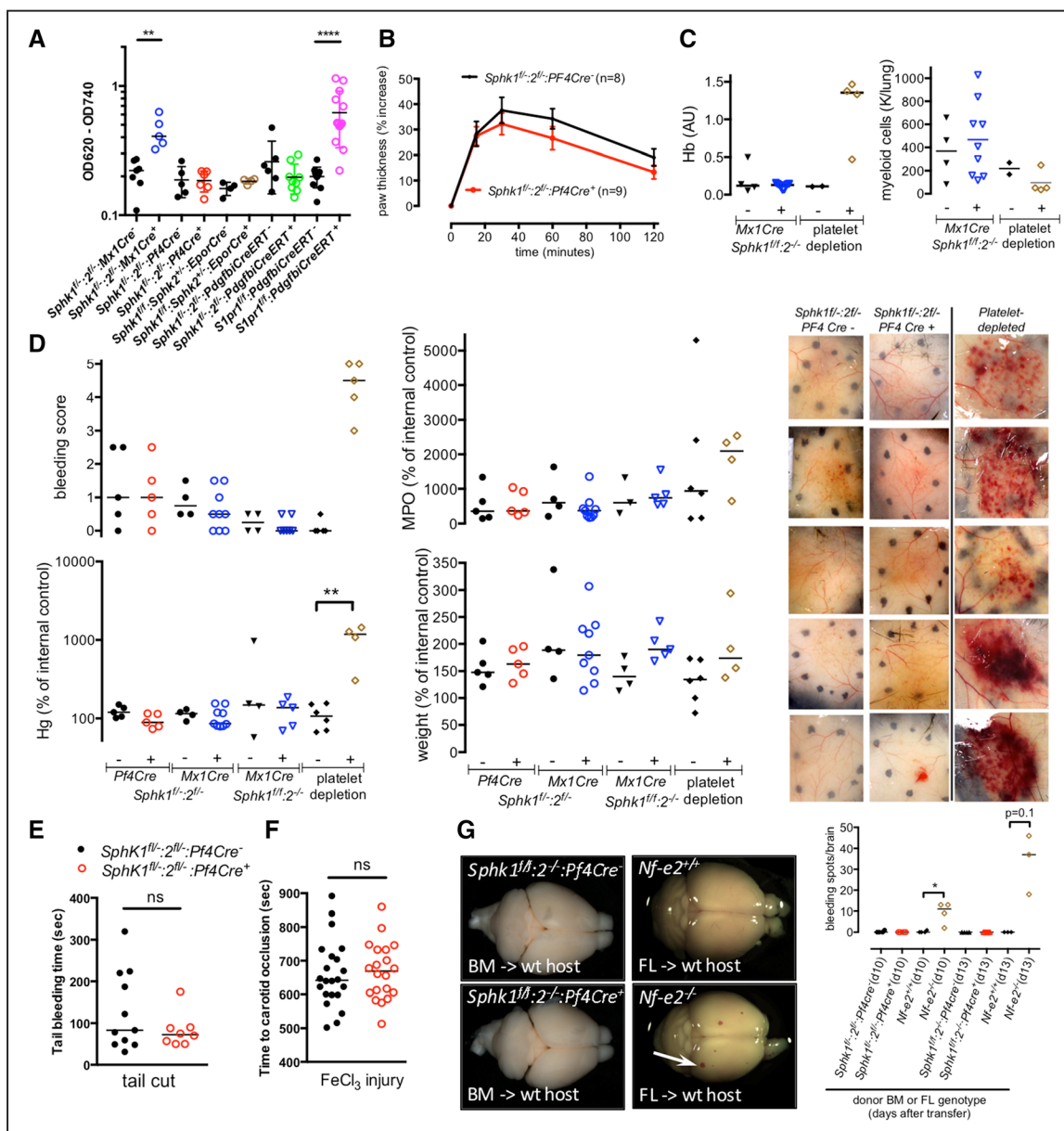


Figure 5. Effects of sphingosine-1-phosphate (S1P) deficiencies on vascular integrity, hemostasis, thrombosis, and leukocyte recruitment in adult mice. **A**, Constitutive Evans Blue leak to lung in mice deficient in S1P sources or the endothelial S1P₁ receptor. **B**, Histamine (60 µg)-induced paw swelling normalized for increase in paw diameter after vehicle injection in the contralateral paw. **C**, Hemoglobin and myeloid cells in bronchoalveolar lavage 6 hours after intranasal inoculation of lipopolysaccharide (LPS; 25 µg). Platelet depletion triggered bleeding and reduced leukocyte accumulation in lung, whereas S1P deficiency did not affect either parameter. **D**, Hemoglobin, bleeding score, myeloperoxidase (MPO) activity, and weight of skin biopsies 4 hours after semisimultaneous subcutaneous injection of anti-BSA and intravenous injection of BSA (left). Bleeding, MPO activity, and weight were all increased in reverse passive Arthus reaction (rpA) biopsies relative to control independent of genotype. Representative images (right) show occasional mild petechiae at sites injected with anti-BSA antibody in *Sphk1^{fl/-};2^{fl/-}* mice independent of *Pf4Cre* status. **E** and **F**, Bleeding times after removal of the distal 3 mm of the tail (**E**) and time to arterial occlusion after 4 minutes exposure of the carotid artery to a FeCl₃ (15%) swab (**F**). Platelet S1P deficiency did not influence either end point, nor hemoglobin content in collected blood in **E** or in embolization in **F**. **G**, C57BL/6 wild-type mice were transplanted with platelet S1P-deficient bone marrow (BM) or *Nf-e2^{-/-}* fetal liver (FL) cells the day after Cesium 137 irradiation. Ten or 13 days (d) later, brains were removed after transcardial perfusion. Left, Representative brains 13 days after transplantation; right, summary. Platelet- but not platelet S1P deficiency resulted in brain bleeding after irradiation.

Sphk2^{-/-} donors were used to exclude possible contribution of S1P by residual platelets in the erythrocyte preparation. Furthermore, we did not observe increased bleeding in *Sphk2^{-/-}* relative to WT littermates nor in platelet S1Pless mice generated with a strategy allowing comparison with littermates proficient in platelet S1P (*Sphk1^{fl/-};2^{fl/-};Pf4Cre⁺*),

while the bleeding tendency persisted in the corresponding pan-hematopoietic S1Pless mice (*Sphk1^{fl/-};2^{fl/-};Mx1Cre⁺*) (Figures 6D and 6E). Although these observations support a role for circulating S1P in preserving lymph node integrity after immunization, they argue against a nonredundant role for the platelet source.

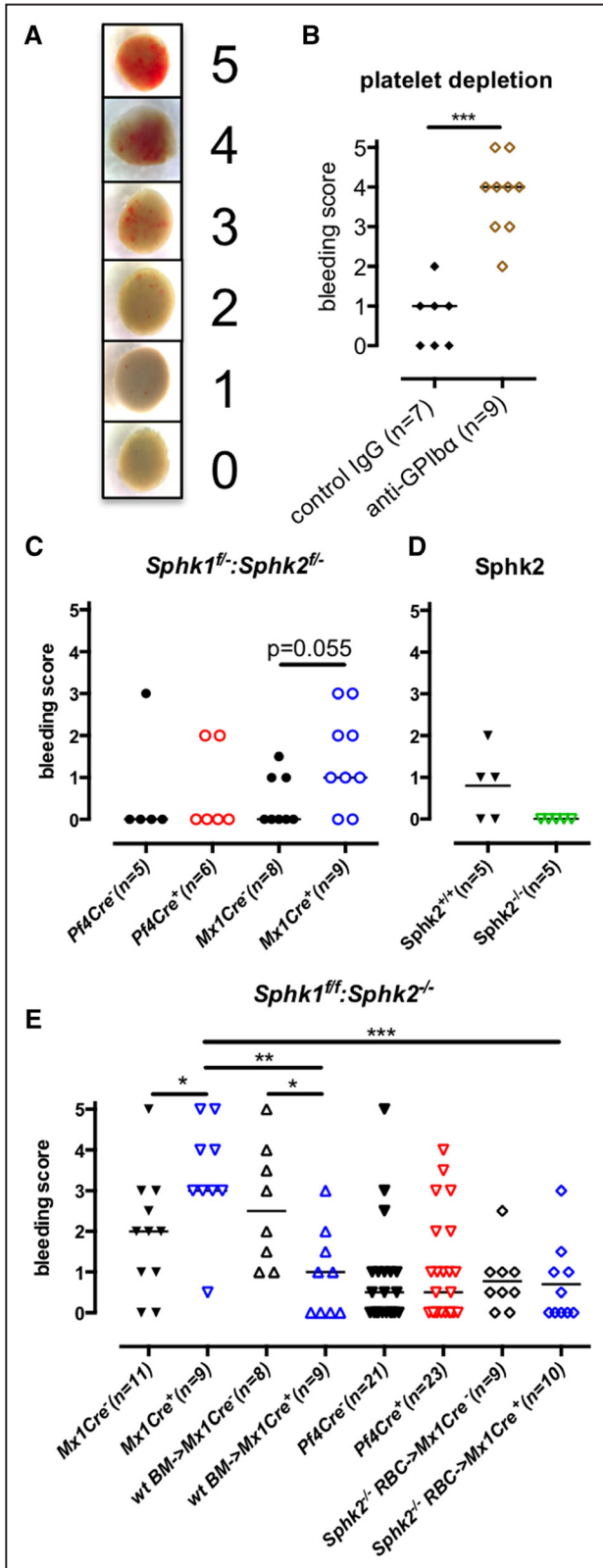


Figure 6. Platelets and sphingosine-1-phosphate (S1P), but not platelet-derived S1P, are essential for lymph node hemostasis after immunization. A–E, Mice were immunized in the right hock, and draining and control lymph nodes isolated 1 week later for gross assessment of bleeding. Bleeding on the efferent surface of the right popliteal lymph node was scored in a blinded manner according to the scale shown in A. In B, anti-GPIIb/IIIa antibodies were administered to C57BL/6 males the day (Continued)

Platelet and Erythrocyte Sources of S1P Are Both Required for Recovery From Anaphylactic Shock

Compound erythrocyte, platelet, and lymphatic endothelial S1P deficiency in plasma S1Pless mice leads to constitutive vascular leak and sensitizes mice to vascular leak and death from passive systemic anaphylaxis induced with immune complexes or with platelet-activating factor (PAF), a key mediator of anaphylactic shock in mice.^{12,52} Even if PAF does not directly activate mouse platelets,⁵³ intravital microscopy revealed an enhancement of transient and stable interactions between platelets and the endothelium of mesenteric vessels after PAF challenge (Figure 7A and Online Videos I and II). A halving in platelet S1P content 40 minutes after challenge suggested that they also release S1P in this context (Figure 7B). Strikingly, however, despite engagement of the platelet pool of S1P and its continued production by erythrocytes, plasma levels were reduced by >80% on average (85 ± 47 versus 451 ± 52 nmol/L; $P > 0.0001$; Figure 7B). Depletion of plasma S1P to levels at or below the threshold needed to saturate S1P receptors hinted at greater dependence on circulating S1P sources in this context. Accordingly, chimeric WT mice with *Sphk1^{fl/fl}:2^{-/-}:Mx1Cre⁺* BM were more sensitive to PAF challenge than controls with *Sphk1^{fl/fl}:2^{-/-}:Mx1Cre⁻* BM (Figure 7C) even if nonhematopoietic sources provide sufficient S1P to sustain lymphocyte trafficking and vascular integrity in these mice under homeostatic conditions (Online Figure IIA).²³ Arguing a necessary role for both circulating cellular sources of S1P, sensitization to PAF challenge was also observed in platelet S1P-deficient mice without exacerbation of vascular leak, conferred by transplantation of BM-derived cells from these mice, and observed in mice with isolated lack of erythrocyte-derived S1P (Figure 7C; Online Figure IIB). By contrast, impaired S1P production in blood endothelium (*Sphk1^{fl/fl}:2^{-/-}:PdgfrbCre⁺*) or smooth muscle cells (*Sphk1^{fl/fl}:2^{-/-}:SM22Cre⁺*) in isolation had no effect on survival, although a significant contribution of endothelium-derived S1P could be observed in the context of combined erythrocyte, platelet, and plasma S1P deficiency (*Sphk1^{fl/fl}:2^{-/-}:Mx1Cre⁺:PdgfrbCre⁻* versus *Sphk1^{fl/fl}:2^{-/-}:Mx1Cre⁺:PdgfrbCre⁺*; Figure 7D). Addressing if inhibition of S1P release from platelets with nonsteroidal anti-inflammatory drugs³⁵ would influence recovery from anaphylaxis, we observed that pretreatment with aspirin or ibuprofen mirrored platelet S1P deficiency in exacerbation of PAF-induced mortality (Figure 7E; Online Figure III). In stark contrast, aspirin treatment was protective in mice that lack platelet S1P (Figure

Figure 6 Continued. before harvest and thrombocytopenia confirmed immediately before transcardial perfusion. Thrombocytopenia induced bleeding in draining lymph nodes (B), while deficiency in platelet-derived S1P (*Sphk1^{fl/fl}:2^{-/-}:Pf4Cre⁺* or *Sphk2^{-/-}*) did not (C and D). Lack of all circulating sources of S1P (*Sphk1^{fl/fl}:2^{-/-}:Mx1Cre⁺*), was associated with a bleeding tendency (C). In a background globally deficient in sphingosine kinase (*Sphk2*) (E), deletion of *Sphk1* in hematopoietic cells and lymphatic endothelial cells (*Sphk1^{fl/fl}:2^{-/-}:Mx1Cre⁺*), but not in megakaryocytes (*Sphk1^{fl/fl}:2^{-/-}:Pf4Cre⁺*), was associated with lymph node bleeding. Sensitization to bleeding associated with plasma S1P deficiency could be rescued by transplantation of wild-type bone marrow (WT BM) cells or by transfusions of erythrocytes from *Sphk2^{-/-}* donors (E). Images were acquired in phosphate-buffered saline (PBS) immersion with a Leica M165FC stereo microscope.

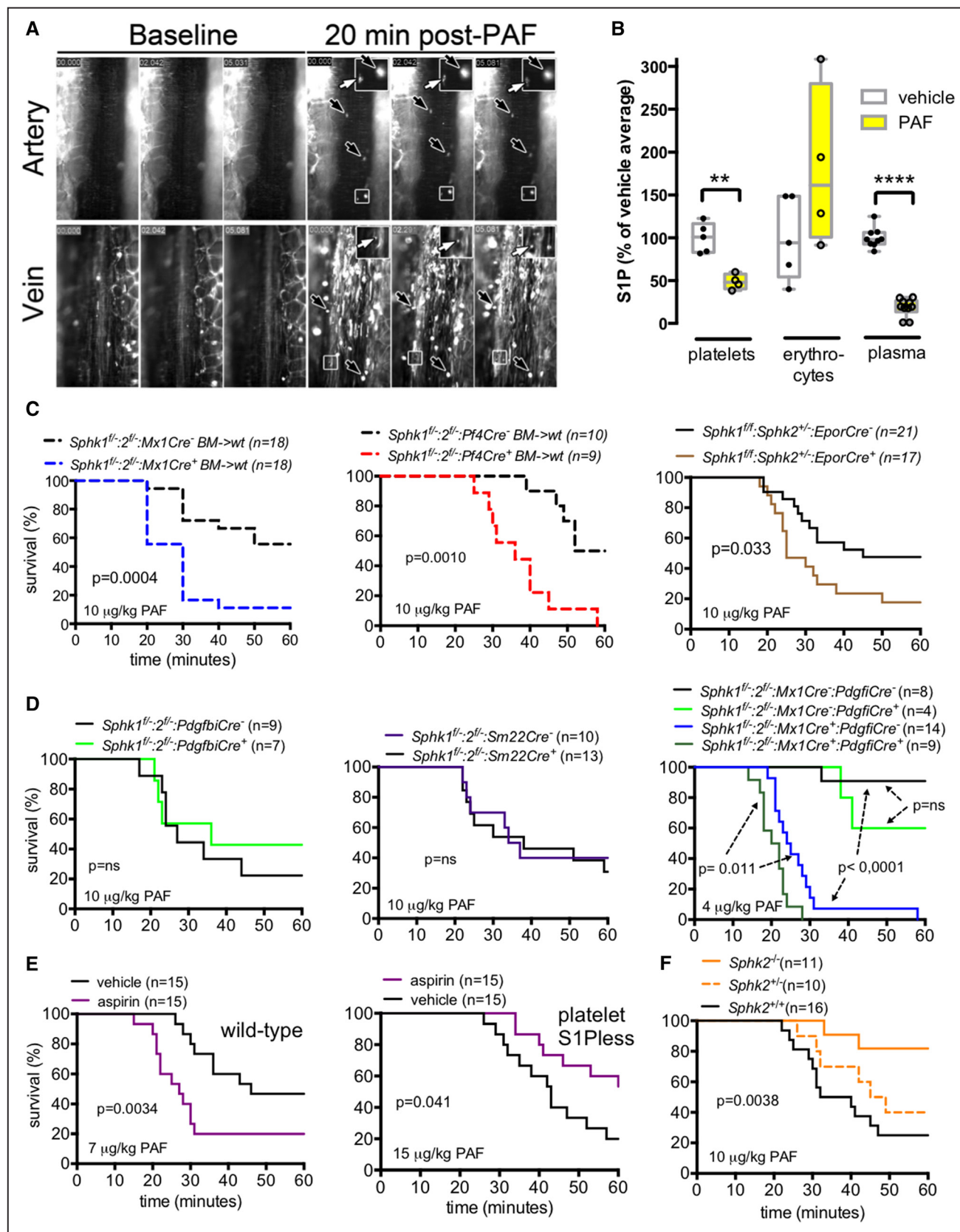


Figure 7 Continued. respectively. Insets show higher-magnification views of squared regions. Images are representative of observations made in 6 mice. **B**, S1P concentrations in platelets, erythrocytes, and plasma of C57BL/6J wild-type mice 40 minutes after PAF (4 μ g/kg) or vehicle challenge. Note S1P depletion from platelets and plasma, but not erythrocytes. **C–F**, Mice were injected with PAF at indicated concentrations and survival monitored for 60 minutes. Note that S1P deficiency in circulating cells; in chimeric wild-type (WT) mice with sphingosine kinase (Sphk) deficiency in all bone marrow (BM) cells (*Sphk1^{fl/-};2^{fl/-};Mx1Cre⁺*) or selectively in megakaryocytes (*Sphk1^{fl/-};2^{fl/-};Pf4Cre⁺*) or mice lacking S1P production in erythrocytes (*Sphk1^{fl/-};2^{fl/-};EporCre⁺*) all showed a significant increase in mortality relative to controls (**C**). By contrast, S1P deficiency in the vessel wall, either in blood endothelium (*Sphk1^{fl/-};2^{fl/-};PdgfrbCre⁺*) or in smooth muscle cells (*Sphk1^{fl/-};2^{fl/-};Sm22Cre⁺*) did not increase mortality in isolation, although deficiency in blood endothelium significantly increased mortality in the context of pan-hematopoietic deficiency (*Sphk1^{fl/-};2^{fl/-};PdgfrbCre⁺;Mx1Cre⁺*) (**D**). Aspirin significantly increased mortality in wild-type mice, but not in chimeric wild-type mice with *Sphk1^{fl/-};2^{fl/-};Pf4Cre⁺* BM (**E**). A higher PAF dose was used in the latter because of protective effects of Sphk2 deficiency (**F**), which may be conferred by high plasma S1P levels in these mice.

7E). Suggesting a dependence on S1P dose rather than unique roles for S1P sources, the requirement for platelet S1P was overcome in mice globally deficient in Sphk2, which paradoxically have high plasma S1P levels and were protected from mortality (Figure 7F).⁵⁴ Unlike platelet deficiency and consistent with aspirin-treated platelets maintaining the capacity to prevent inflammation-associated bleeding,³⁴ no overt bleeding was observed in aspirin-treated animals after PAF challenge (0/10 animals analyzed). Thus, anaphylactic shock is associated with rapid depletion of plasma S1P, which renders S1P contribution by both platelet and erythrocyte sources necessary for recovery and supraphysiological plasma S1P levels as observed in *Sphk2^{-/-}* mice protective.

Circulating S1P Promotes Anaphylaxis Survival by Maintaining Vascular Tone

S1P has been suggested to provide protection in mouse models of anaphylaxis by promoting vascular integrity, vascular tone, and glomerular filtration.^{12,54–57} Although we originally focused on the role of S1P₁ in protecting vascular integrity during systemic anaphylaxis,¹² Olivera et al later argued the importance of S1P₂ signaling and vascular tone regulation.⁵⁵ In support of Olivera et al, endothelial-selective S1P₁ deficiency had no effect on PAF survival (Figure 8A), despite inducing constitutive vascular leak (Figure 5A). To address if increased lethality associated with S1P deficiency was instead of hemodynamic origin and S1P₂-dependent, we monitored blood pressure and heart rate after PAF injection. PAF induced a rapid drop in blood pressure independent of genotype, which was followed by continued decline and death in plasma S1Pless and *S1pr2^{-/-}* mice, contrasting gradual recovery in most controls (Figure 8B). In a likely last attempt to maintain blood pressure, death was preceded by transient tachycardia in most S1P signaling-deficient animals (Figure 8B). Although this supports a role for S1P in maintaining vascular tone, genotype-dependent differences were unremarkable relative to differences in survival and did not reveal effects of isolated platelet or erythrocyte S1P deficiency (Online Figure IV). Profound anesthesia, temperature control, and compensatory pathways engaged to maintain pressure may have masked the contribution of S1P signaling to vascular tone in these experiments. We, therefore, next assessed renal artery and basilar trunk blood flow velocities, left ventricular systolic function, and cardiac output before and 30 minutes after PAF administration under mild isoflurane anesthesia by noninvasive echocardiography and ultrasound pulsed Doppler analysis (Figure 8C; Online Figure V). With these techniques, we observed a striking decrease in systolic and diastolic left ventricular diameters, cardiac

output, and organ perfusion in plasma S1Pless mice, suggesting exaggeration of circulatory shock with distributive and hypovolemic components, leading to a drop in venous return to the heart and, thus, in cardiac output (Figure 8C).^{58–60} A similar phenotype was observed with platelet S1P deficiency and in S1P₂-deficient mice (Figure 8C and Online Figure V).⁴¹ Although S1P₂ deficiency was associated with significantly increased mortality in these experiments (Figure 8D), sensitization was not as strong as for plasma S1P deficiency. Accordingly, protective effects of Sphk2 deficiency (Figure 7F), presumed to reflect high plasma S1P concentrations, persisted in an *S1pr2^{-/-}* background (Figure 8E). *S1pr2^{-/-}* sensitization was not exacerbated by compound deficiency of S1P₃,^{41,42} the second smooth muscle contractile S1P receptor (Figure 8F). This could argue that lack of S1P₁ signaling and exacerbated vascular leak nevertheless contributes to poor venous return and more severe circulatory shock in plasma S1Pless mice (Figure 8C; Online Figure V).¹² Collectively, and consistent with Olivera et al,⁵⁵ these observations suggest that although S1P promotes both vascular integrity through S1P₁ and vascular tone through S1P₂, the latter dominates but does not fully account for the survival benefit of S1P signaling in anaphylaxis.

Discussion

We have explored functional roles for platelet-derived S1P in vitro and compared effects of platelet S1P deficiency to thrombocytopenia and to erythrocyte, endothelial, smooth muscle, and plasma S1P deficiency on vascular development and homeostasis in vivo. Deletion of *Sphk1* and *Sphk2* in mouse megakaryocytes abrogated S1P release from platelets without affecting plasma S1P levels, supporting the notion that platelets do not contribute substantially to plasma S1P under homeostasis.^{21,23} S1P deficiency partially impaired platelet aggregation and spreading and nearly eliminated the ability of platelets to promote endothelial barrier function in vitro. However, it did not result in bleeding or vascular fragility or protect from arterial thrombosis in vivo. Despite its importance in isolated systems, platelet S1P, thus, seems to be redundant for hemostasis, thrombosis, and vascular integrity when other S1P sources or other protective mechanisms are intact. Although these observations contrast essential roles recently reported for platelet- as well as erythrocyte-derived S1P for S1P₁ activation during vascular development,¹¹ immunization,¹⁴ and thrombosis,¹⁵ such requirements were not observed in this study when S1P deficiency was induced specifically in the relevant cells in a genetic context where major competing sources were left intact. Contrasting the source redundancy

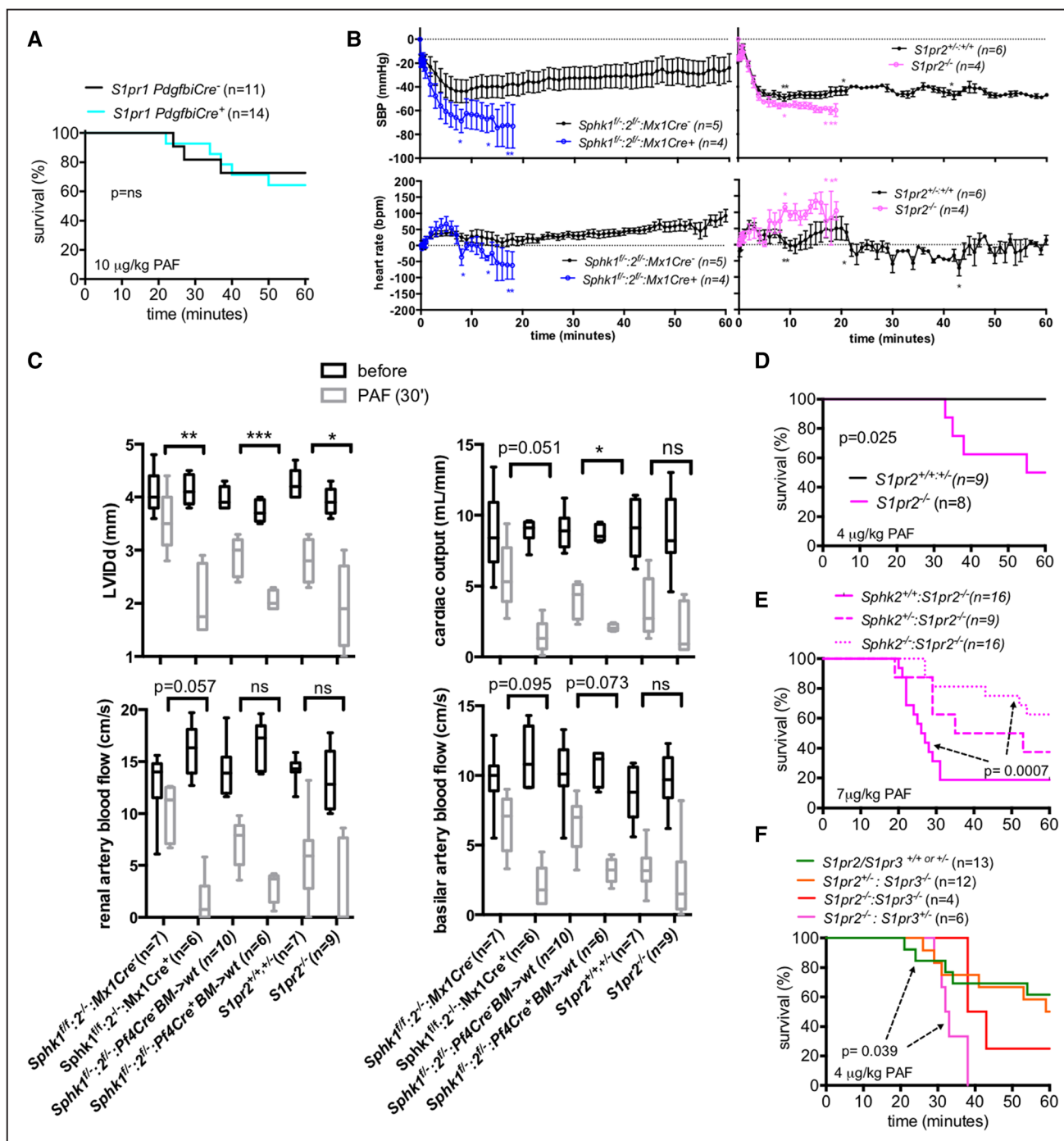


Figure 8. Sphingosine-1-phosphate (S1P) promotes recovery from anaphylactic shock by S1P₂-mediated enhancement of vascular tone. **A**, Mice selectively lacking S1P₁ in blood endothelium (*S1pr1*^{fl/fl};PdgfbiCre⁺) were challenged with platelet-activating factor (PAF) and survival monitored for 60 minutes. **B**, Plasma S1Pless (*Sphk1*^{fl/fl};2^{fl/fl};Mx1Cre⁺), S1P₂ deficient mice (*S1pr2*^{-/-}), and littermate controls were challenged with PAF (2 µg/kg) under pentobarbital anesthesia, and blood pressure and heart rate were monitored for 60 minutes and normalized to prestimulation values. Asterisk indicates death of an animal. **C**, Plasma S1Pless (*Sphk1*^{fl/fl};2^{fl/fl};Mx1Cre⁺), chimeric wild-type (WT) mice with megakaryocyte sphingosine kinase (Sphk)1- and 2-deficient bone marrow (BM; *Sphk1*^{fl/fl};2^{fl/fl};P4Cre⁺), S1P₂-deficient mice (*S1pr2*^{-/-}), and respective controls were challenged with PAF (4 µg/kg) and left ventricular systolic function and blood flow velocities in the right renal artery and basilar trunk determined 30 minutes later by echocardiography and ultrasound pulsed Doppler analysis under light (0.5%) isoflurane anesthesia. Left ventricular internal diameter in diastole (LVIDd), cardiac output, right renal artery, and basilar trunk mean blood flow velocities before and after PAF challenge. **D–F**, Mice globally deficient in S1P₂ either alone (*S1pr2*^{-/-}; **D**) or in conjunction with Sphk2 (*S1pr2*^{-/-};Sphk2^{-/-}; **E**) or S1P₃ (*S1pr2*^{-/-};S1pr3^{-/-}; **F**) and littermate controls were challenged with PAF at indicated concentrations and survival monitored for 60 minutes. Note that only S1P₂ deficiency sensitizes to PAF, independent of S1P₃, although residual protection by Sphk2 deficiency in a *S1pr2*^{-/-} background suggests the involvement of second receptor.

observed in vascular development, homeostasis, and local inflammation, rapid and profound depletion of the plasma pool rendered S1P delivery by both platelets and erythrocytes essential for recovery in a model of systemic anaphylaxis where S1P₂ signaling maintains vascular tone. A contribution of bioactive S1P from the vessel wall was revealed only in the absence of both circulating sources. Aspirin, which impairs S1P release from platelets,³⁵ sensitized to anaphylaxis mortality in an S1P-dependent manner.

Because cells of nonhematopoietic origin contribute enough S1P to sustain plasma S1P levels well above the K_d for S1P₁ binding,^{21,23,61} it is surprising that the absence of erythrocyte and, especially, platelet S1P would lead to a deficit in activation of S1P₁ on cells in contact with plasma as has recently been reported.^{14,15,62} Our observations suggest that the importance of single S1P sources was exaggerated by the genetic context in which these studies were performed. Although we confirm S1P dependence in the same models, we fail to confirm necessary roles for platelet- or erythrocyte-derived S1P when using more specific genetic tools. When S1P release from platelets was proposed to provide a critical barrier-protective signal to S1P₁ on high endothelial venules after platelet activation by CLEC-2/podoplanin, this was tested using mice that lack *Sphk1* in cells that provide S1P to plasma and *Sphk2* in all cells.^{14,23} Our results argue that also the *Sphk2*^{-/-} littermate controls used were devoid of platelet S1P and that the Cre-dependent differences observed, therefore, could not have reflected on a role for the platelet S1P source. Moreover, although we also observed bleeding with plasma S1P deficiency that could be rescued by transplantation of WT BM-derived cells, this was not replicated by megakaryocyte-specific deletion and could be reversed by transfusion of erythrocytes. Thus, although platelet S1P may be sufficient to prevent lymph node bleeding in this context, it is not necessary. The critical role of erythrocyte-derived S1P in engaging S1P₁ to coordinate vascular developmental was also demonstrated in an *Sphk2*^{-/-} background¹¹ and, thus, did not address the potential contribution of *Sphk2*-derived S1P from platelets and possibly endothelial cells. When we tested this directly by generating embryos lacking S1P in erythrocytes without impairing competing sources, development proceeded normally. Finally, when platelet-derived S1P was shown to be important for autocrine activation of platelet S1P₁ in thrombosis, this was performed with mice deficient in *Sphk2* in all cells,¹⁵ which also display thrombocytopenia and supraphysiological plasma S1P levels.^{54,63} Although we also observed functional defects in S1P-deficient platelets *ex vivo*, we did not confirm protection against ferric chloride-induced carotid thrombosis when *Sphk2* was selectively deleted in megakaryocytes/platelets. Thus, although S1P coordinates vascular development and integrity and possibly platelet activation and spreading, no single cellular source of S1P seems to be essential for these processes. This is not surprising, as depletion of any single source should not reduce plasma S1P to levels below S1P receptor saturation.^{21,23,61} Because we also did not observe spontaneous vascular leak with compound *Sphk1* and *Sphk2* deficiency in endothelium, these data underscore the importance of plasma S1P,

which is maintained by several redundant sources, to maintain vascular integrity during embryogenesis and postnatal homeostasis.

Considering that we did not find support for a barrier-protective role for S1P downstream of platelet CLEC-2 in lymph nodes, it is perhaps not surprising that we also did not observe blood/lymph mixing phenotypes or sensitization to local inflammation-induced hemorrhage in platelet S1Pless mice. These experiments also did not reveal a role for platelet-derived S1P in modulating neutrophil recruitment or neutrophil-mediated damage.⁵⁰ If S1P accounts for most endothelial barrier-protective capacity of platelets *in vivo* as we observe *ex vivo*, this may favor models in which platelets protect against inflammation-induced hemorrhage by S1P-independent suppression of leukocyte-mediated injury or by physical prevention of erythrocyte escape⁶⁴ rather than by paracrine actions on the endothelium.¹⁴ Our results do not support a key role for S1P as a platelet-derived mediator in any of these models and suggest that only profound S1P deficiency is associated with hemorrhage and only in unique situations, such as during vascular development and tissue remodeling.

The surprising observation that plasma S1P was profoundly depleted in a model of anaphylactic shock argued that the amount of S1P available for receptor activation can drop below saturating levels during systemic inflammation, rendering the contribution of all residual cellular sources of S1P essential. Although the mechanisms of S1P depletion remain to be addressed, nonredundant roles for both platelet- and erythrocyte-derived S1P identified in this model contrasted models of local inflammation and suggested a contribution from both circulating sources, independent of any qualitative differences. Protection in mice with supraphysiological homeostatic plasma S1P levels argued that these sources were, nevertheless, not adequate to sustain S1P receptor activation. This is consistent with reports that S1P infusion can promote recovery from histamine-induced shock.⁵⁵ Exacerbated hypotension, loss of vascular tone, and poor blood return to the heart observed in mice lacking S1P argued that S1P could contribute to both distributive and hypovolemic components of shock in the PAF model.⁵⁸ Suggesting a dominant role in maintaining vascular tone,⁵⁵ sensitization with platelet S1P deficiency was not associated with enhanced vascular leak or bleeding, and endothelial deficiency of S1P₁ did not affect survival, despite inducing constitutive vascular leak to lung. By contrast, deficiency of S1P₂, which promotes smooth muscle contractility and myogenic tone, was associated with severe vasoplegia and increased lethality. A persistent survival benefit afforded by *Sphk2* deficiency in an *S1pr2*^{-/-} background may, nevertheless, suggest a contribution of S1P₁ or another receptor to S1P protection.⁸

A protective role for platelet-derived S1P in anaphylaxis begs the question of whether antithrombotic or anti-inflammatory therapies impair platelet S1P release and, thus, may influence recovery from circulatory shock. Thromboxane synthesis plays an important role in platelet S1P release, evidenced in part by the inhibitory effect of nonsteroidal anti-inflammatory drugs.³⁵ This predicted that nonsteroidal anti-inflammatory drugs might impair recovery from PAF-induced shock similar to platelet S1P deficiency, which is indeed what we observed

with both aspirin and ibuprofen. Remarkably, the effect of aspirin was inverted in mice lacking platelet S1P. It remains to be determined if the protective role of platelet S1P extends to other inflammatory conditions known to be aggravated by thrombocytopenia.^{65,66}

In conclusion, our observations with tissue-specific ablation of S1P production in mice suggest that provision of S1P to plasma from multiple cellular sources normally secures sufficient S1P receptor activation to sustain vascular development, vascular integrity and tone, and possibly hemostasis through platelet activation and spreading. However, systemic inflammation may lead to rapid depletion of plasma S1P, rendering the contribution of each S1P source critical. In this context, S1P source preservation becomes key. Thus, inhibition of platelet S1P release with aspirin impaired recovery from anaphylactic shock in mice. S1P depletion has not been addressed in human anaphylaxis, but was recently reported in septic shock.¹⁹ To appreciate if monitoring and preservation of plasma S1P may provide diagnostic or therapeutic value in circulatory shock, it will be important to address if S1P is also depleted in other severe inflammatory conditions and explore mechanisms of S1P depletion in future studies. The suboptimal engagement of S1P₂ signaling during recovery from systemic anaphylaxis also argues potential therapeutic utility of S1P₂ agonists for maintenance of vascular tone.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- The bioactive lipid sphingosine-1-phosphate (S1P) critically supports vascular development and vascular integrity in mice primarily via the S1P₁ receptor.
- Platelets have been suggested to support vascular integrity after activation of C-type lectin-like receptor 2 by paracrine actions of platelet-derived S1P on endothelial S1P₁.
- S1P deficiency dramatically impairs recovery in mouse models of anaphylactic shock by aggravating loss of vascular integrity and tone.

What New Information Does This Article Contribute?

- Without S1P production, platelet aggregation and spreading is impaired, and platelets lose their capacity to promote endothelial barrier function in vitro.
- Blocking S1P production in platelets, erythrocytes, endothelial cells, or smooth muscle cells does not impair vascular development or integrity, including in conditions where platelet C-type lectin-like receptor 2 is essential, suggesting that redundant S1P sources normally secure these functions in vivo.
- In a mouse model of systemic anaphylaxis, rapid and profound depletion of plasma S1P renders S1P delivery by both platelets and erythrocytes necessary for survival.

S1P is critical for vascular development and function, but the relative importance of different cell types for provision of bioactive S1P is unknown. Dynamic endothelial S1P production may relay barrier protection by other mediators; erythrocytes provide the bulk of plasma S1P and may provide essential S1P during vascular development; and platelet S1P release may protect vascular integrity during inflammation and after immunization. However, when S1P production was selectively impaired in each of these cell types, we found them to be largely redundant for vascular development and function. Accordingly, impairing any single cellular source does not bring plasma S1P below levels predicted as receptor-saturating under homeostasis. In a model of systemic anaphylaxis, we observed rapid depletion of plasma S1P to <20% of basal levels. This rendered circulating sources of S1P essential for recovery, and blocking S1P release with aspirin impaired survival. Although S1P supports vascular integrity and vascular tone in this model via S1P₁ and S1P₂, respectively, only the latter was essential for the survival benefit of S1P in anaphylaxis. Thus, rapid depletion of plasma S1P may contribute to cardiovascular shock during systemic inflammation, suggesting potential diagnostic value in monitoring S1P levels in human disease and therapeutic benefits in preserving S1P signaling.

Platelet and erythrocyte sources of S1P are redundant for vascular development and homeostasis, but both rendered essential after plasma S1P depletion in anaphylactic shock.

Salome L. Gazit, Boubacar Mariko, Patrice Therond, Benoit Decouture, Yuquan Xiong, Ludovic Couty, Philippe Bonnin, Veronique Baudrie, Sylvain M. Le Gall, Blandine Dizier, Nesrine Zoghiani, Jessica Ransinan, Justin R. Hamilton, Pascale Gaussem, Pierre-Louis Tharaux, Jerold Chun, Shaun R. Coughlin, Christilla Bachelot-Loza, Timothy Hla, Benoit Ho-Tin-Noé and Eric Camerer

Supplemental Material

Supplemental Methods, Figure and Video Legends, References and Figures

Supplemental Methods

Characterization of mutant mice. The same induction protocol was used in alternative strategies to generate *Sphk1^{fl/-}:2^{fl/-}:Mx1Cre⁺* and *Sphk1^{fl/-}:2^{fl/-}:Mx1Cre⁻* littermate controls. Controls were also polyI:C-treated, and successful Mx1-Cre mediated excision was always confirmed by complete blood cell (CBC) counts with a HemaVet (Drew Scientific)¹. PolyI:C-induced *Sphk1^{fl/-}:2^{fl/-}:Mx1Cre⁺* and *Sphk1^{fl/-}:2^{-/-}:Mx1Cre⁺* mice were only used for experiments if plasma S1P deficiency was reflected in peripheral lymphocyte counts below 2.5 K/ μ l and 60% of total white blood cells. Lymphopenia was as pronounced in *Sphk1^{fl/-}:2^{fl/-}:Mx1Cre⁺* as has already been described in *Sphk1^{fl/-}:2^{-/-}:Mx1Cre⁺* mice¹. The efficiency and specificity of deletion with the *Pdgfb-iCreERT* allele with our tamoxifen induction protocol was confirmed by analysis vasculature of different organs (retina, brain, heart, lung) in adult mice crossed with reporter alleles activated by Cre excision².

Bone marrow transplantation. Recipient mice were exposed to 6.5 Gy Cesium 137 radiation twice with a 3-4 hour interval. The day after, each mouse received 10⁷ bone marrow cells as indicated i.v. under isoflurane anesthesia. Chimeric mice were used >4 weeks after transplantation. Transplantation efficiency was determined to be >95 % in parallel experiments.

Collection of blood, plasma, serum, erythrocytes, platelets and platelet supernatants. Blood was collected from the retro-orbital venous plexus under isoflurane anesthesia with EDTA-coated capillaries for CBC analysis, platelet rich plasma (PRP) or platelet poor plasma (PPP) collection or in uncoated capillaries for serum preparation. Serum was prepared by centrifugation of coagulated whole blood for 10 minutes at 500 g. PPP was prepared for S1P measurements by centrifugation of anticoagulated blood for 10 minutes at 500 g twice. PRP was prepared for platelet isolation by centrifugation of anticoagulated blood for 10 minutes at 80 g after addition of a 1:1 volume of PIPES buffer (20 mM Pipes, 150 mM NaCl, pH 6.5). PRP was then collected into tubes containing 1/1 volume of wash buffer (140 mM NaCl, 10 mM NaHCO₃, 2.5 mM KCl, 0.5 mM Na₂HPO₄, 1.0 mM MgCl₂, 6.46 mM trisodium citrate, 0.1 % w/v dextrose, 0.35% Bovine Serum Albumin (BSA; Fraction V), pH 6.5) supplemented with prostaglandin I₂ (PGI₂, 1 μ M, Sigma P6188) and apyrase (100 mU/ml, Sigma A6535) and spun 10 minutes at 500G for platelet isolation. Platelets were then resuspended and washed twice in the same buffer. For S1P release, washed platelets were resuspended to 400 k/ μ l in 200 μ l HBSS with 0.35 % lipid free BSA and stimulated with thrombin (30 nM) and convulxin (250 ng/ml) for 30 minutes at 37 °C. Platelets were then spun down for 10 minutes at 500 g and the supernatant analysed by mass spectrometry. For erythrocyte collection, blood was drawn into 25% ACD and spun for 10 minutes at 80g. Preparation of washed, packed erythrocytes for S1P quantification and transfusions was performed as previously described¹.

LC-MS/MS analysis of sphingoid bases in plasma, serum and platelet supernatants: 200 μ L of plasma, serum, or 200 μ L of platelet supernatant, 100 μ L of an internal standard solution containing 0.1 μ g/mL C17-SPH and 0.1 μ g/mL C17-S1P and 1 mL of methanol were mixed, vortexed and centrifuged at 12,000 g for 5 min. Supernatants were transferred into glass vials prior to injection (4 μ L) into the UPLC-MS/MS system for analysis, with the autosampler set at 4°C. The UPLC-MS/MS consisted of a Waters Acquity and a Triple Quadripole (TQD) tandem mass spectrometer. Chromatographic separation was obtained using an Acquity UPLC HSS T3 column (100mm Lx2.1mm i.d., 1.7 μ m particle size, Waters, Milford, MA, USA) with a guard column of the same material maintained at 65°C. The mobile phase consisted of water containing 30 mM ammonium acetate and formic acid 0.1% (eluent A) and methanol (eluent B) at a flow rate of 0.5 mL/min in a gradient mode. The following linear elution gradient was used: 0-0.2 min, 100% A; 0.2-0.7 min, 100% to 10% A; 0.7-1.3 min, 10%A/90%B; 1.3-1.6 min, 10% to

100% A. Between runs the system was re-equilibrated with 100% eluent A for 3.4 min. The mass spectrometer was operated in the positive ion mode with an electrospray voltage of 3600V. Sphingoid bases were quantified using multiple reaction monitoring (MRM) and peak areas were integrated with Waters QuanLynx software.

HPLC quantification of S1P concentrations in platelets and erythrocytes: Methanol (1 ml) containing 2.5 μ l concentrated HCl was added to washed platelets or erythrocytes in 50 μ L PBS. C17-S1P (15 pmol) was added as internal standard and lipids were extracted by addition of 1 ml chloroform, 200 μ l NaCl (4 M) and 100 μ l NaOH (3 M). The alkaline aqueous S1P-containing phase devoid of other sphingoid bases and of the majority of hydrophobic PL was transferred to a clean tube and the organic phase re-extracted with 0.5 ml methanol, 0.5 ml of 1M NaCl and 3N NaOH (50 μ l). The alkaline aqueous phases were combined, acidified with 100 μ l concentrated HCl and extracted twice with 1.5 ml chloroform. The organic phases were evaporated and the dried lipids were dissolved in a mixture (50 μ l) of methanol and 0.07 M K₂HPO₄ (9:1 v/v). A derivatization mixture of 10 mg *o*-phthalaldehyde, 200 μ l ethanol, 10 μ l 2-mercaptoethanol and 10 ml boric acid (3% v/w) was prepared and adjusted to pH 10.5 with KOH 10N. Five μ l of the derivatization mixture was added to the lipids and the solution incubated for 15 minutes at 37°C. The derivatives were analyzed with a Hewlett Packard HPLC system using an RP 18 Kromasil column (2.1 mm i.d. x 150 mm) maintained at 45°C. Separation was performed with the isocratic eluent containing methanol: K₂HPO₄ (0.07 M) (78:22 v/v) at a flow rate of 0.25 mL/min. The derivatives were detected selectively using a Hewlett Packard spectrofluorometer with an excitation wavelength of 334 nm and an emission wavelength of 440 nm. S1P was quantified by comparison of its fluorescent signal with that of the derivative of the internal standard (coefficients of variation, <5%).

Calcium signaling. Washed platelets were loaded with fura-2 AM (4 μ M) in HBSS supplemented with PGI₂ (1 μ M), Apyrase (100 mU/ml) and EDTA (1 mM) for 40 minutes at 37 °C, spun down and re-suspended to 100 k/uL in calcium-free HBSS supplemented with 10 mM HEPES and 100 μ M EGTA. 100 μ L aliquots of loaded platelets were assayed per well in 96-well black-walled clear-bottomed plates using a FlexStation (Molecular Devices) plate reader warmed at 37°C. Fluorescence emission at 510 nm after alternating 340 nm and 380 nm excitation wavelengths was monitored every 3.5 seconds for 5 minutes. Human α -thrombin (10 nM) or vehicle control was added with a robotic injector after establishment of baseline emission ratio. Results were analysed using SoftMax Pro software. Fluorescence ratio (340/380 nm) was normalized to basal fluorescence ratio for each well, and maximal responses and area under the curve (AUC) determined for each sample. Each sample was from an independent mouse.

Platelet preparation for aggregation and secretion. Blood was collected into ACD-C solution (13 mM citric acid, 12.6 mM sodium citrate, 11mM D-glucose) by cardiac puncture. To optimize the yield of platelet recovery, blood was diluted 2:1 with wash buffer (36mM citric acid, 5 mM D-glucose, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 103 mM NaCl, pH = 6.5 in presence of 0.1 U/mL apyrase and 10 μ M PGE1). Diluted platelet-rich plasma (PRP) was obtained by centrifugation at 170 g for 7 min, diluted in wash buffer (1:1) with inhibitors (as above) and centrifuged 10 min at 750 g. The platelet pellet was resuspended in wash buffer and centrifuged as above. Finally, the platelets were resuspended to 3.5x10⁸/mL in HEPES buffer (10 mM HEPES, 140 mM NaCl, 3 mM KCl, 5 mM NaHCO₃, 0.5 mM MgCl₂, 10 mM D-glucose, pH = 7.35), counted using a veterinary counter MS9 (MS Laboratoires) added 2 mM calcium chloride depending of the experiment to be performed.

Platelet secretion. Platelet secretion was measured with a Becton Dickinson FACScalibur flow cytometer (BD Biosciences, Le Pont de Claix, France). Washed platelets (1.5x10⁸/mL) were stimulated with PAR4-AP (200 μ M, Bachem, Weil am Rhein, Germany). At different time-points a 5 μ L platelet aliquot was diluted with Hepes buffer and incubated for 10 min with anti-CD62P-PE monoclonal antibody or with IgG1-PE isotype control under saturating concentration, as recommended by the

manufacturer (Emfret Analytics, Eibelstadt, Germany). Before analysis, a further dilution step (1:10) was performed, and a total of 10 000 platelet events was recorded. The increase in CD62P-positive cells after agonist exposure is expressed as the percentage of positive cells relative to isotype control.

Platelet aggregation. Platelet aggregation was measured using a Biodata aggregometer (Biodata, PAP-8E). Washed platelets ($3.5 \cdot 10^8$ /mL) were pre-incubated for 2 minutes at 37°C. Platelet aggregation was then induced by addition of collagen (0.75 µg/ml) or ADP (1.5 µM). For ADP-induced aggregation, fibrinogen (5 µg/mL) was added just prior to pre-incubation. Aggregation was registered as change in light transmission and was calculated as the maximum of aggregation curve (% aggregation) and the area under the aggregation curve (AUC).

Platelet spreading assessed by scanning electron microscopy (SEM). Washed platelets ($3 \cdot 10^8$ /mL) were dropped on glass slides coated with either 2% BSA (resting conditions) or fibrinogen (0.2 mg/mL). After 30 minutes of adhesion, a drop of fixation solution (0.1 M sodium cacodylate, 2 mM CaCl_2 , 2% PFA, 4% glutaraldehyde) was added, and the slides incubated overnight in a bath of 0.1 M sodium cacodylate/2 mM CaCl_2 . The day after, samples were fixed with 1% osmium tetroxide, washed and dehydrated with an ethanol series (25 %, 50 %, 75%, 95%, 100%) and dried by HMDS. Samples were then gold coated with a JEOL JFC-1300 Auto fine coater (JEOL Europe SAS, Croissy Sur Seine, France) and examined with a JEOL JSM-6510 LV.

Tail bleeding times. A 3 mm transection was performed of the tail of mice anesthetized with ketamine 50 mg/kg (Clorketam 1000®, Vétoquinol S.A, Magny-Vernois, France) and xylazine 6 mg/kg (Rompun®, Bayer Santé, Puteaux, France). The tail was immediately immersed in 10 ml of 0.9% isotonic saline at 37°C, and the time to permanent cessation of bleeding recorded. Tail bleeding was monitored for at least 90 seconds beyond this time point, to ensure that bleeding did not re-start.

Arterial thrombosis. Mice were anesthetized with with ketamine 50mg/kg (Clorketam 1000®, Vétoquinol S.A, Magny-Vernois, France) and xylazine 6mg/kg (Rompun®, Bayer Santé, Puteaux, France), and the left carotid artery exposed and dissected away from the vagus nerve and surrounding tissues. Carotid artery blood flow was monitored with a Doppler flow meter equipped with a transonic flow probe (Model MA0.5PSB, Transonic System Inc, Ithaca, NY). Thrombosis was induced by depositing a 3 mm² filter paper (Whatman No. 2) soaked in FeCl_3 (15%, Sigma) on the carotid artery 5 mm upstream of the flowmeter probe and leaving it for 4 minutes. Blood flow was monitored 5 minutes beyond cessation.

Developmental and perinatal bleeding. Spontaneous developmental and perinatal hemorrhage was addressed in Sphk deficient and, for comparison, platelet deficient ($Nfe2^{-/-}$) embryos and neonates. Embryos were harvested 12.5 or 14.5 days after conception (E), grossly inspected for cerebral hemorrhage, evidence for blood in lymphatic structures and other abnormalities in a blinded manner, and genotyped. Neonates (p1-p3) were anesthetized with ketamine/xylazine (100 and 10 mg/kg, respectively) and transcardially perfused with saline before harvesting the brain and scoring for hemorrhage in a blinded manner.

Irradiation- induced bleeding. 5-8 week old C57BL/6 males were exposed to 6.5 Gy Cesium 137 radiation twice with a 4 hour interval. The day after, each mouse received $5 \cdot 10^6$ E14.5 fetal liver cells ($Nfe2^{-/-}$ or $Nfe2^{+/+}$) or 10^7 bone marrow cells (Sphk deficient or littermate control donors) i.v. under isoflurane anesthesia. 10 or 13 days (as indicated) after irradiation, mice were anesthetized with ketamine/xylazine (80-100 and 5-10 mg/kg, respectively), and transcardially perfused with 1 % PFA after collection of blood for determination of platelet numbers. Brain were then harvested and photographed, and the number of bleeding spots quantified by an examiner blinded to genotype.

Spontaneous and agonist- induced vascular leak. Paw diameter was measured with a caliper (Mitutoyo) before, 15, 30, 60 and 120 minutes after injection of histamine (60 μ g in 20 μ L PBS/BSA) in the right posterior footpad and vehicle control in the contralateral footpad. Swelling was normalized to pre-injection values and corrected for responses to vehicle control.

Reverse passive Arthus reaction (detailed description). Shaved mice were anesthetized with ketamine/xylazine (100 and 10 mg/kg, respectively) and the reverse passive Arthus reaction (rpA) reaction elicited by s.c. injection of anti-BSA IgG (60 μ g in 20 μ L saline) immediately followed by i.v. injection of BSA (50 μ g/g) in saline. Mice were sacrificed 4 hours later and biopsies of control and inflamed skin harvested with 5 mm diameter biopsy punches. Skin biopsies were then weighed, homogenized and sonicated in cold PBS. Tissue lysates were centrifuged and a 30 μ L aliquot of each supernatant was collected for measurement of hemoglobin content by addition of formic acid and absorbance reading at 405 nm. Pelleted tissue lysates were then resuspended and diluted 1:1 (vol:vol) with potassium phosphate buffer (50 mM final) supplemented with hexadecyltrimethylammonium bromide (HTAB, 0.9 % final, Sigma, St. Louis, MO) to extract myeloperoxidase (MPO). After 5 minute centrifugation at 10000 g, MPO activity was measured in supernatants by adding 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma). The reaction was stopped with sulfuric acid and the absorbance at 450 nm was recorded using a Multiskan plate reader (Thermo Labsystems Nepean Ontario Canada).

Pneumonia (detailed description). Mice were anesthetized with ketamine/xylazine (100 mg/kg, 10 mg/kg, respectively) and inoculated intranasally with 25 μ g *Pseudomonas aeruginosa* LPS prepared in a final volume of 20 μ L PBS (10 μ L/nostril). 6 hours later, the trachea was cannulated with an 18-gauge angiocath, and bronchoalveolar lavage (BAL) performed 3 times with 0.6 mL sterile PBS. Hemoglobin content was determined by absorbance reading at 405 nm and blood cells in BAL quantified using a HemaVet cell analyzer (Drew Scientific).

Immunization and analysis of HEV bleeding (detailed description). Mice received an injection of 20 μ L of an emulsion of complete Freund's adjuvant/Ovalbumin PBS in the right hock and 20 μ L PBS in the contralateral hock under isoflurane anesthesia as described by Herzog et al.³. Where indicated, mice were given 350 μ L washed, packed erythrocytes from Sphk1^{ff}:2^{-/-} donors on day 4 and 6 after immunization or depleted of platelets by injection of 2 μ g/g of anti-GP1b α or rat IgG control (Emphret Analytics, Eibelstadt, Germany) on day 6 after immunization by retro-orbital injection. Blood was drawn in EDTA immediately prior to transcardial perfusion to confirm antibody-induced thrombocytopenia. On day 7 after immunization, mice were anesthetized and transcardially perfused with 1% PFA to remove intravascular blood. Right and left popliteal and inguinal LNs were dissected and photographed. Bleeding on the efferent side was scored in a blinded fashion on a scale from 0-5, where 0 represents no bleeding and 5 represents strong bleeding. When bleeding was observed in the popliteal lymph nodes, mesenteric, para-aortic and axial LNs were also inspected for signs of bleeding. Strong bleeding in the ipsilateral popliteal lymph node, observed with platelet depletion and in plasma S1Pless mice, was sometimes associated with bleeding also in ipsilateral inguinal, contralateral popliteal and para-aortic lymph nodes, but not in axial or contralateral inguinal lymph nodes.

Anaphylactic shock modeled with PAF injection. Passive systemic anaphylaxis was mimicked by i.v. injection of platelet activating factor (PAF) at indicated concentrations^{4,5}. Mice were anesthetized with isoflurane (2%) and PAF administered via the retro-orbital venous plexus. Where indicated, 3-4 drops of blood were collected into EDTA coated capillaries from the contralateral retro-orbital plexus 10 minutes after injection for CBC analysis (HemaVet, Drew Scientific). Behavior and survival were followed for 60 minutes, after which all surviving animals were euthanized. Mice displaying spasms and loss of righting reflex were euthanized before this endpoint and counted as non-survivors. Aspirin (100 mg/kg), ibuprofen (40 mg/kg) or vehicle was administered i.p. 1 hour prior to PAF injection where indicated.

Intravital microscopy. Wild-type C57/BL6J mice were anesthetized with ketamine/xylazine (100 mg/kg, 10 mg/kg, respectively), and the mesentery was carefully exposed and spread on a Petri dish via a midline laparotomy incision. For staining of circulating platelets and leukocytes, mice were injected with rhodamine-6G (3 mg/kg mouse) through the retro-orbital venous sinus, and placed under an upright fluorescence microscope (MacroFluo, Leica Microsystems, Nanterre, France) equipped with a thermostated heating plate and a 5X objective, and connected to a sCMOS camera (Orca-Flash-4.0, Hamamatsu Photonics, Hamamatsu, Japan) for image acquisition using Metamorph software (Molecular Devices, Downingtown, PA). After 10 min during which baseline cell interactions in arteries and veins were recorded, PAF (4 μ g/kg mouse) was injected through the retro-orbital venous sinus and cell interactions were followed for up to 30 min.

Echocardiography and ultrasound pulsed Doppler blood flow recordings. Before and 30 minutes after i.v. injection of PAF (4 μ g/kg), mice were anesthetized with isoflurane (0.5%), placed on a heating mat and subjected to ultrasound measurements using an echocardiograph (Acuson S 3000, Siemens, Erlangen, Germany) equipped with a 14-MHz linear transducer (14L5 SP). Heart rate and left ventricle and pulmonary artery internal diameters were measured as well as peak systolic, end-diastolic, and time-averaged mean blood flow velocities in the pulmonary artery for cardiac output calculation, and in the right renal artery and basilar trunk for renal and brain perfusion as previously described⁶⁻⁸.

Blood pressure and heart rate monitoring. Acute pressor responses were measured in mice anesthetized with sodium pentobarbital (60 mg/kg i.p.). Flexible plastic catheters were introduced into the left carotid artery and jugular vein. The arterial catheter was connected to a pressure transducer (SS13L) and a Biopac System MP35. The MP35 unit converts incoming signals into digital signals that were processed using an algorithm based on feature extraction to detect and measure the characteristic of BP cycle, BSL 4.0 (Biopac Systems, Inc.). A syringe was introduced into the venous catheter and used for bolus injection of PAF (2 μ g/kg) after an initial stabilization period of >30 minutes. Blood pressure and heart rate were then recorded for 60 minutes.

Supplemental Figure Legends

Online Figure I. Levels of sphinganine and sphingosine in platelet S1P deficient mice. Dihydro-S1P (dhS1P) concentrations in serum (A) and sphingosine concentrations in plasma, serum and platelet supernatants (B) from mice deficient in megakaryocyte *Sphk1&2* and littermate controls as determined by LC/MS/MS.

Online Figure II. Vascular leak and anaphylaxis survival in mice lacking S1P in circulating cells. A. Chimeric wild-type (wt) mice with pan-hematopoietic *Sphk1&2*-deficient or control bone marrow (BM) were assessed for Evans Blue leak to brain and lung (left panel) or bled for quantification of plasma S1P concentrations by LC/MS/MS (right panel). **B.** Megakaryocyte/platelet *Sphk1&2* deficient mice and littermate controls were challenged with PAF (10 µg/kg) and survival monitored for 60 minutes. Increase in hematocrits 10 minutes after PAF challenge relative to pre-challenge values are shown in the right panel.

Online Figure III. Ibuprofen sensitizes to PAF-induced shock. Wt C57BL/6 males were treated with ibuprofen, challenged with PAF at 7 (A) or 12 (B) µg/kg PAF 1 hour later, and survival monitored for 60 minutes.

Online Figure IV. Effect of S1P signaling deficiencies on blood pressure and heart rate after PAF challenge. Mice lacking S1P production in all cells that provide S1P to plasma (*Sphk1^{ff}:2^{-/-}:Mx1Cre⁺*; A), S1P₂ in all cells (*S1pr2^{-/-}*; B), S1P production selectively in megakaryocytes/platelets (*Sphk1^{ff}:2^{-/-}:Pf4Cre⁺*; C) or S1P production selectively in erythrocytes (*Sphk1^{ff}:2^{-/-}:EporCre⁺*; D), and their respective littermate controls were challenged with PAF (2 µg/kg) under pentobarbital anesthesia and blood pressure and heart rate monitored for 60 minutes and normalized to pre-stimulation values. Asterisk indicates the death of an animal. SBP, systolic blood pressure; DBP, diastolic blood pressure. SBP and HR for A and B are shown in Figure 8B. Mean +/- SEM is shown. Two-way ANOVA did not reveal statistically significant differences between genotypes for any group.

Online Figure V. Echocardiography and ultrasound pulsed Doppler blood flow analysis before and after PAF treatment. Example of parameters assessed before and after i.v. injection of PAF (4 µg/kg) in “Plasma S1Pless” (*Sphk1^{ff}:2^{-/-}:Mx1Cre⁺*) mouse and one littermate control (*Sphk1^{ff}:2^{-/-}:Mx1Cre⁻*). Echocardiography and pulsed Doppler blood flow recordings were carried out under light (0.5%) isoflurane anesthesia and with thermoregulation starting 30 minutes after PAF injection and took ~7-8 minutes to complete. For some severely affected mice (*Sphk1^{ff}:2^{-/-}:Mx1Cre⁺* and *Sphk2^{-/-}*) we were not able to record peripheral blood flow velocities. 3-5 recordings were made of each parameter in each mouse. Mice that died during or before the recordings (exclusively *Sphk1^{ff}:2^{-/-}:Mx1Cre⁺* and *Sphk2^{-/-}*) were excluded. Note that mice with severe circulatory shock at 30 minutes that survived showed almost complete functional recovery at 2 hours. Measurements were performed on a Siemens Acuson S 3000 (Erlangen, Germany) equipped with a 14-MHz linear transducer (14L5 SP).

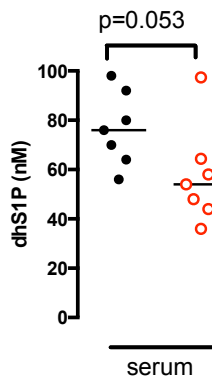
Online Videos

PAF-induced anaphylactic shock causes interactions between platelets, leukocytes, and the vessel wall in both arteries and veins. Intravital microscopy imaging of interactions between platelets, leukocytes, and the vessel wall before and 20 minutes after PAF-induced anaphylaxis. Five second time-lapse microscopy videos taken in arteries (**Online Video I**) and veins (**Online Video II**) before (left panels) and 20 minutes after (right panels) PAF (4 $\mu\text{g/kg}$) injection, as indicated. PAF injection led to transient interactions between platelets, leukocytes and the vessel wall in both arteries and veins, as well as to firm adhesion (≥ 5 sec). White and black arrows indicate firmly adherent platelets and leukocytes, respectively. Erythrocytes, which do not bind rhodamine-6G, can also be observed as black spheres adhering firmly to the venular wall 20 minutes after PAF injection. Videos are representative of observations made in 6 wild-type mice.

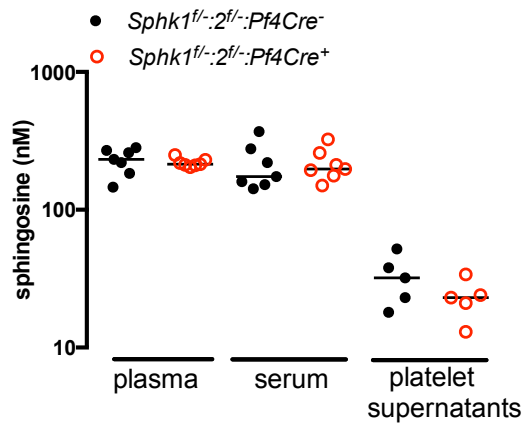
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A

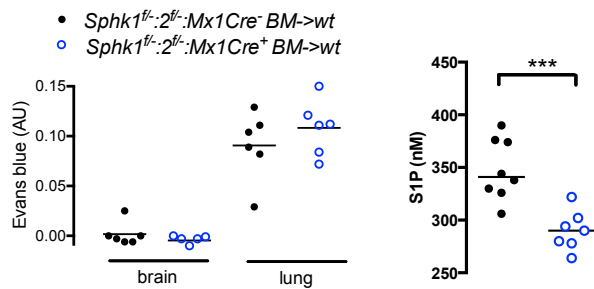


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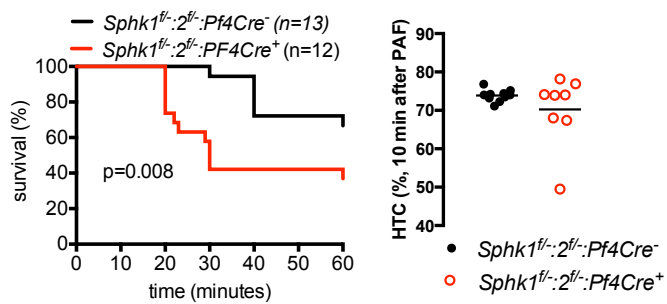


Online Figure I

A

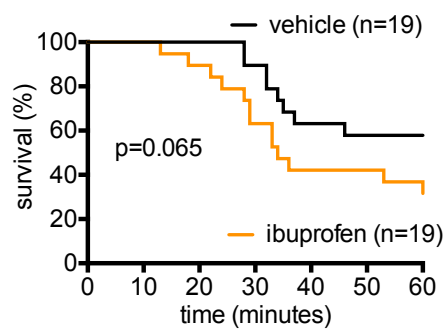


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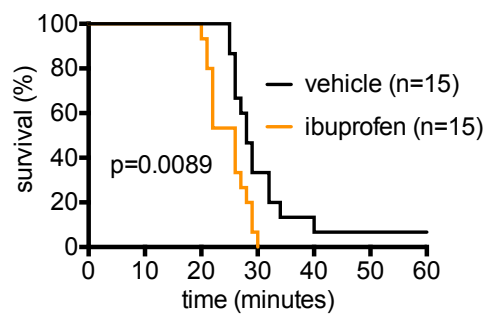


Online Figure II.

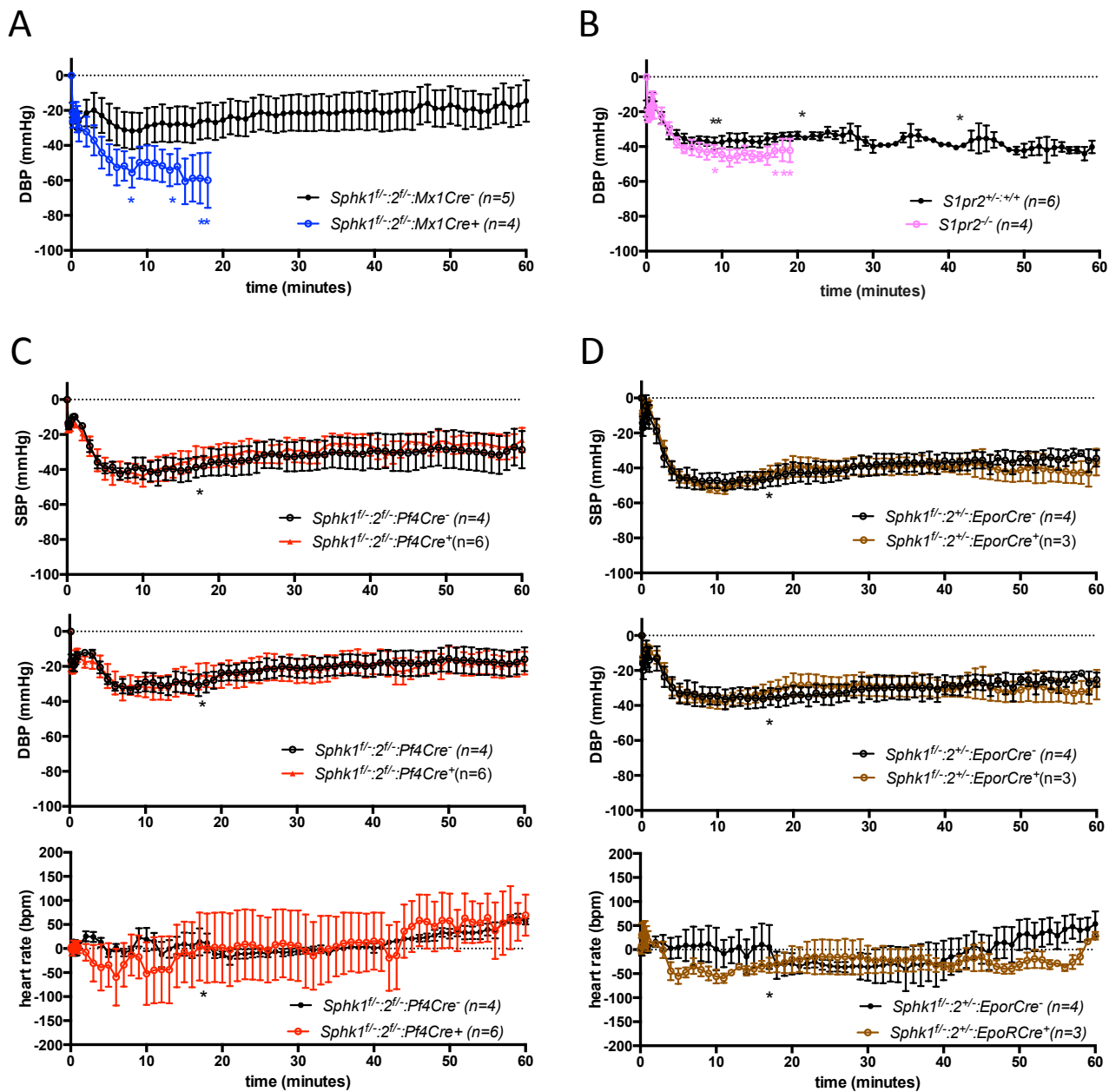
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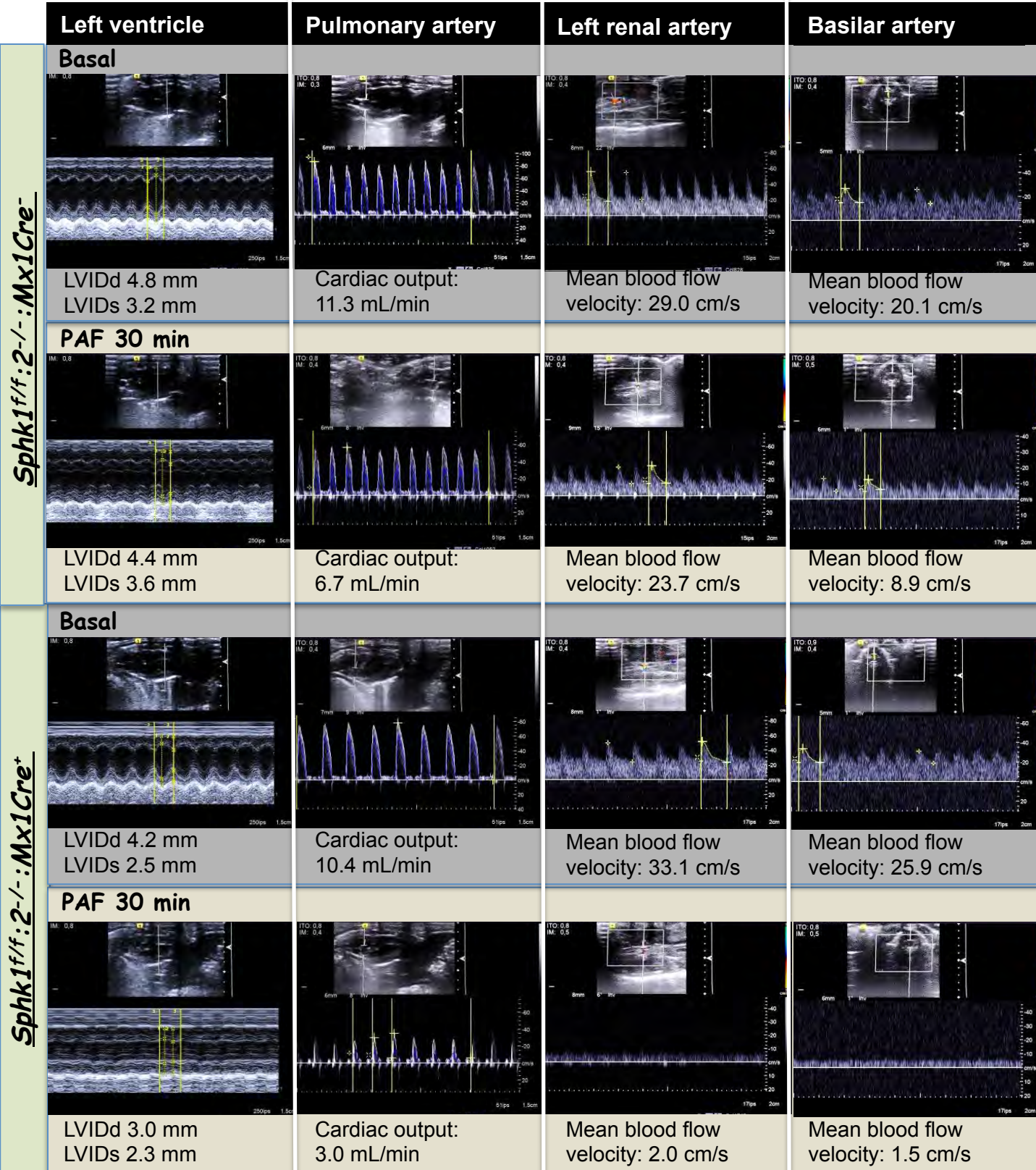
B



Online Figure III



Online Figure IV.



Online Figure V