Title

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Journal

Circulation Research, 119(8)

ISSN

0009-7330

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Publication Date

2016-09-30

DOI

10.1161/circresaha.116.308929

Peer reviewed

Published in final edited form as:

Circ Res. 2016 September 30; 119(8): e110–e126. doi:10.1161/CIRCRESAHA.116.308929.

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

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Abstract

Rationale—Sphingosine-1-phosphate (S1P) signaling is essential for vascular development and postnatal vascular homeostasis. The level of redundancy in S1P sources sustaining these processes remains unclear.

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Disclosures: None.

Objective—To address S1P source redundancy in the regulation of vascular development, integrity and tone.

Methods and Results—S1P production was selectively impaired in mouse platelets, erythrocytes, endothelium, and/or smooth muscle cells by targeted deletion of genes encoding sphingosine kinases (Sphks) 1&2. S1P deficiency blunted aggregation and spreading of washed platelets and profoundly impaired 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. $S1P_2$ mediated most of the survival benefit of S1P, while endothelial $S1P_1$ was dispensable for survival despite its importance for maintaining vascular integrity. Accordingly, S1P deficiency aggravated vasoplegia, arguing a vital role for S1P in maintaining vascular resistance during recovery from circulatory shock.

Conclusions—While source redundancy secures essential roles of S1P in vascular development and integrity, profound depletion of plasma S1P during anaphylactic shock renders both erythrocyte and platelet S1P pools necessary for recovery and high basal plasma S1P levels protective.

Subject Terms

Vascular Biology; Platelets; Hemodynamics; Vascular Disease; Developmental Biology; Blood Pressure; Cell Signaling/ Signal Transduction; Endothelium/Vascular Type/Nitric Oxide

Keywords

sphingosine-1-phosphate; endothelium; vascular endothelial function; vascular permeability; vascular tone regulation; shock; anaphylaxis

Introduction

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 ($S1P_{1-5}$) signaling, although S1P may also have receptor-independent functions.[1-3] In the developing vasculature, $S1P_1$ signaling prevents angiogenic hyper-sprouting and contributes to vascular stabilization.[4-6] In the mature vasculature, endothelial $S1P_1$ signaling stabilizes endothelial adherens junctions and promotes blood flow while smooth muscle cell $S1P_2$ and $S1P_3$ promote vascular contraction and thereby regulate vascular tone.[5, 7-10] Circulating S1P plays a key role in activation of vascular receptors, as 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 lumenal S1P receptors, is provided by several types of cells, and carried on different chaperones.[1, 13]

While this may argue that redundancy between vascular sources of S1P secures essential functions of S1P signaling, several recent studies suggest non-redundant 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 sensitivity of vascular homeostasis to S1P source exhaustion or deficiency. Notably, sources of circulating S1P can be selectively depleted in disease states; HDL and platelet pools have been shown to be exhausted in severe sepsis and myocardial infarction, respectively.[18-20] If these sources are indeed non-redundant, such exhaustion could have profound effects on vascular function. Depending on the level of redundancy and the threshold requirement for receptor activation, anemia, thrombocytopenia, or inhibition of tissue-specific S1P metabolism, transporters or release mechanisms could also result in S1P-associated vascular dysfunction.

Plasma S1P is provided by erythrocytes and endothelial cells (which also provide lymph S1P), and is carried by HDL- and LDL- associated apolipoprotein M as well as by albumin (Figure1A).[1, 21-23] S1P is actively removed from interstitial fluids, generating a gradient that is required for lymphocyte trafficking to blood and lymph.[24] Two mammalian sphingosine kinase (Sphk) isoforms (1&2) are partly redundant for S1P generation.[25] Platelets and erythrocytes do not efficiently degrade S1P and therefore have a high S1P synthetic capacity (Figure 1A;[23, 26-28]). Embryonic bleeding and death associated with erythrocyte S1P deficiency argues that erythrocyte-derived S1P may play a unique role in endothelial $S1P_1$ 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,[26] potentially engaging receptors that are not saturated by plasma S1P, e.g. on vascular smooth muscle cells, or changing the bias or balance of receptor activation.[29, 30] Intriguingly in this regard, a recent study argued a key role for platelet S1P release and paracrine activation of endothelial $S1P_1$ after activation of platelet ITAM-coupled receptors.[14] In a model of immunization-induced bleeding in draining lymph nodes, individual platelets were shown to gain sub-endothelial access at high endothelial venules. In the absence of the platelet immunoreceptor tyrosine-based activation motif (ITAM) receptor C-type lectin-like receptor 2 (CLEC-2), reticular epithelial podoplanin or plasma S1P, lymph node bleeding was observed. Platelet ITAM-coupled receptors maintain vascular integrity during vascular development and inflammation and mediate blood/lymph separation;[31-33] 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 $S1P_1$ 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 non-selective cyclooxygenase inhibitors such as aspirin (acetylsalisylic acid) and ibuprofen.[34] Platelet S1P release may thus be inhibited by over-the-counter non-steroidal anti-

inflammatory drugs (NSAIDs), 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 GPCR and tyrosine kinase receptors and has been proposed to be important for $S1P_1$ transactivation for barrier protective signaling in endothelium.[35, 36]

To explore roles for platelet-derived S1P and redundancy between circulating S1P sources in vascular development and homeostasis, we generated mice with isolated or combined tissue-specific deficiency of Sphks 1 & 2 in megakaryocytes/platelets, erythrocytes and endothelial 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 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 to age- and sex-matched littermate controls. Wild-type 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 Sphk 1&2 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 Mx1-Cre transgene[63] to generate plasma S1Pless mice (*Sphk1*$^{f/f}$*:2*$^{-/-}$*:Mx1Cre*$^+$ and *Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1Cre*$^+$) has been described.[23] Mice deficient in S1P production in megakaryocytes and platelets (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Pf4Cre*$^+$ and *Sphk1*$^{f/f}$*:2*$^{-/-}$*:Pf4Cre*$^+$) were generated using a *Pf4-Cre* allele.[37] *S1pr1* floxed, *S1pr2* and *S1pr3* knockout and *PdgfbiCreERT* alleles have been described.[38, 55, 56, 64] Endothelial specific deletion of *S1pr1* was achieved by oral administration of 20 μg tamoxifen to *S1pr1*$^{f/f}$*:PdgfbiCreERT* neonates thrice between p1 and p5.

Endothelial barrier function

Trans-endothelial electrical resistance (TEER) was assessed using electrical cell-substrate impedance sensing (ECIS, Applied Biophysics) at 4,000 Hz [40]. Human umbilical vein endothelial cells (HUVEC; PromoCell) were plated at 10^5 cells/fibronectin coated

8W10E(+) well. The day after, cells were equilibrated (2h) in serum-free endothelial cell basal medium (PromoCell) before establishing baseline resistance (1h). 10^7 washed platelets were then added, followed by a PAR4 activating peptide (PAR4-AP; 500 μM). Where indicated, endothelial cells were pre-treated with the $S1P_1$ 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, 33, 65]. Briefly, rpA was elicited s.c. with anti-BSA IgG (60 μg) immediately followed by BSA (50 μg/g) i.v. Mice were sacrificed 4 hours later and biopsies of control and inflamed skin photographed for blinded scoring of petechiae, harvested, weighed, homogenized and sonicated in cold PBS for measurement of hemoglobin content and myeloperoxidase (MPO) activity. Pneumonia was induced by intranasal inoculation with 25 μg Pseudomonas aeruginosa LPS. 6 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 Freunds adjuvant/Ovalbumin PBS in the right hock.[14] One week later, mice were anesthetized and transcardially perfused to remove intravascular blood and lymph node bleeding 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, MPO, 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 two-way ANOVA. 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.

Supplemental Material (SM)

Additional details concerning the mouse lines and materials, as well as additional methods, online figures and figure legends can be found in the SM online.

Results

Effects of global and tissue-restricted Sphk deficiencies on circulating pools of S1P

We generated mice with compound deficiency of Sphk1&2 in megakaryocytes/platelets (platelet S1Pless), erythrocytes, endothelial cells, smooth muscle cells, or all cells providing S1P to plasma (plasma S1Pless) by crossing *Sphk1&2* conditional (floxed, f) and global (-)

knockout alleles[23] to Cre recombinase genes driven by platelet factor 4 (*Pf4-Cre*),[37] erythropoietin receptor (*Epor-Cre*)[11], platelet-derived growth factor beta (*Pdgfb-iCreERT2*),[38] smooth muscle protein 22-alpha (*SM22-Cre*), and Myxovirus resistance-1 (*Mx1-Cre*)[23] promoters, respectively. S1P source mutants were first generated with one floxed allele and one knockout allele for each kinase, with controls differing from knockouts only by the presence of the Cre allele ($Sphk1^{f/-}:2^{f/-}:Cre^{+/-}$), 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 (e.g. $Sphk1^{f/f}:2^{-/-}:Cre^{+/-}$) were also generated for comparison with published reports. Plasma S1Pless mice and the erythrocyte S1Pless mice have already been described, and 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 ($Sphk1^{f/-}:2^{f/-}:Pf4Cre^{+}$) 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 ($Sphk1^{f/f}:2^{-/-}:Mx1Cre^{+}$) and their Cre^{-} controls were devoid of platelet S1P (Figure 1C). Deletion of *Sphk1&2* 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 non-redundant roles of S1P sources, and argues that megakaryocyte deletion of Sphks yields platelets that remain S1P deficient and do not scavenge S1P from plasma (Figure 1D).

Sphk deficiency impairs aggregation and spreading of washed platelets

As S1P has been reported to induce platelet shape change,[26] 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 PAR4 (PAR4-AP) was 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 impaired also at later times (Figure 2C). Platelet spreading (lamellipodia formation) on fibrinogen was reduced with platelet S1P deficiency (Figure 2D). These observations may suggest an autocrine role for platelet S1P release in platelet activation and thus a 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 trans-endothelial electrical resistance (TEER), which reflects upon endothelial barrier

function. We observed a robust increase in TEER when washed platelets from wild-type mice were added to HUVEC grown to confluence on fibronectin-coated microelectrodes (Figure 3A). When the platelets were activated with PAR4-AP, 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 $S1P_1$ antagonist W146 and may reflect constitutive release of S1P from the plasma membrane.[39] 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 D). Unlike mass spectrometry (Figure 1B), this assay uncovered a minor but significant contribution of Sphk1 to platelet S1P release (*Sphk1*$^{f/f}$*:2*$^{-/-}$*:Mx1Cre*$^{+}$ vs. *Sphk1*$^{f/f}$*:2*$^{-/-}$*:Mx1Cre*$^{-}$, Figure 3D, left panel). These results argue that S1P is a key endothelial barrier-promoting factor released by platelets.[40]

Platelet- and erythrocyte-derived S1P are redundant for vascular development and blood/lymph separation

As our ex vivo observations were consistent with a proposed paracrine role of S1P downstream of platelet CLEC-2,[14] we next addressed if platelet S1P deficiency was associated with developmental defects reminiscent of deficiency in CLEC-2 signaling.[31] Blood/lymph mixing at E14.5 after PF4-Cre-mediated deletion of a LoxP-flanked CLEC-2 allele suggests that PF4-Cre mediates efficient excision by this time.[32] However, while thrombocytopenic *Nf-e2*$^{-/-}$ embryos[41] 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;[42]).

Erythrocyte-derived S1P was recently reported to be essential for vascular development,[11] 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 around E10.5,[43] 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 was only observed when *Sphk1* was deleted in erythrocytes in embryos that were also *Sphk2*$^{-/-}$ (*Sphk1*$^{f/f}$*:Sphk2*$^{-/-}$*:EpoR-Cre*$^{+}$), not in embryos with one intact allele of *Sphk2* (*Sphk1*$^{f/f}$ *: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&2 in erythrocytes, we performed erythrocyte-restricted deletion of both kinases. While we uniformly observed severe bleeding in *Sphk1*$^{f/f}$*:Sphk2*$^{-/-}$*:EpoR-Cre*$^{+}$ embryos dissected at E12.5 as reported,[11] no embryonic bleeding and normal postnatal survival was observed when deficiency of both kinases was restricted to erythrocytes (*Sphk1*$^{f/-}$*:Sphk2*$^{f/-}$*EpoR-Cre*$^{+}$)(Figure 4A). Embryonic bleeding was also not observed in *Sphk1*$^{-/-}$*:Sphk2*$^{f/-}$ *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.

Birthing 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 are sufficient, no single cellular source is essential for providing bioactive S1P to support $S1P_1$ signaling during embryonic development.

Redundancy of S1P sources in vascular integrity, hemostasis and thrombosis in adult mice

As previously reported, lack of Sphk1&2 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*$^{f/f}$*:PdgfbiCre*$^{+}$), and thus reflected lack of endothelial $S1P_1$ 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 under homeostatic conditions in adult mice. As we predicted platelet S1P 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 inflammation,[33] 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*$^{f/-}$*:2*$^{f/-}$*:Pf4Cre*$^{+}$) nor plasma- (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1Cre*$^{+}$ *or Sphk1*$^{f/f}$*:2*$^{-/-}$*:Mx1Cre*$^{+}$) 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 D).[44] 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 was due to 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 F). To address if platelet-derived S1P might prevent subtle vascular injury, we then transplanted irradiated wild-type mice with *Nf-e2*$^{-/-}$ or platelet S1Pless hematopoietic progenitor cells. Mice transplanted with *Nf-e2*$^{-/-}$ fetal liver cells uniformly displayed cerebral hemorrhage 10-13 days after irradiation that was not observed in controls (Figure 5G). However, bleeding was not observed after transplantation of bone marrow (BM)-derived cells from even profoundly platelet S1P deficient *Sphk1*$^{f/f}$*:Sphk2*$^{-/-}$*:Pf4Cre*$^{+}$ 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 immunization[14] 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*$^{f/f}$*:2*$^{-/-}$*:Mx1Cre*$^{+}$) one week after immunization in the right hock (Figures 6A-C).[45] Greatly reduced bleeding in chimeric *Sphk1*$^{f/f}$*:2*$^{-/-}$*:Mx1Cre*$^{+}$ mice transplanted with wild-type BM also supported a role for a circulating S1P source (Figure 6C). However, platelet S1P was not essential as removal of even the minor Sphk1-derived S1P in *Sphk2*$^{-/-}$ platelets did not enhance bleeding (*Sphk1*$^{f/f}$*:2*$^{-/-}$*:Pf4Cre*$^{+}$;Figure 6C). Accordingly, erythrocyte-derived S1P was sufficient to prevent bleeding as transfusion of washed, packed, erythrocytes on days 4 and 6 after immunization prevented bleeding in *Sphk1*$^{f/f}$*:2*$^{-/-}$*:Mx1Cre*$^{+}$ mice (Figure 6C). Erythrocytes from *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 wild-type littermates nor in platelet S1Pless mice generated with a strategy allowing comparison with littermates proficient in platelet S1P (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Pf4Cre*$^{+}$), while the bleeding tendency persisted in the corresponding pan-hematopoietic S1P deficient mice (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1Cre*$^{+}$) (Figures 6D and E). While these observations support a role for circulating S1P in preserving lymph node integrity after immunization, they argue against a non-redundant role for the platelet source.

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 PAF, a key mediator of anaphylactic shock in mice.[12, 46] Even if PAF does not directly activate mouse platelets,[47] 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 vs. 451±52 nM, $p>0.0001$; Figure 7B). Depletion of plasma S1P to levels at or below the threshold needed to sustain S1P receptor activation hinted at greater dependence on circulating S1P sources in this context. Depletion of plasma S1P to levels at or below the threshold needed to sustain S1P receptor activation hinted at greater dependence on circulating S1P sources in this context. Accordingly, chimeric wild-type mice with *Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1-Cre*$^{+}$ BM were more sensitive to PAF challenge than controls with *Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1-Cre*$^{-}$ BM (Figure 7C), even if non-hematopoietic sources provide sufficient S1P to sustain lymphocyte trafficking and vascular integrity in these mice under homeostatic conditions (Online Figure IIA; [23]). Arguing an essential 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 and

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, loss of S1P production in blood endothelium (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:PdgfbiCre*$^{+}$) or smooth muscle cells (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:SM22Cre*$^{+}$) 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*$^{f/-}$*:2*$^{f/-}$*:Mx1Cre*$^{+}$*:PdgfbiCre*$^{-}$ vs. *Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1Cre*$^{+}$*:PdgfbiCre*$^{+}$; Figure 7D). Addressing if inhibition of S1P release from platelets with NSAIDs [34] would influence recovery from anaphylaxis, we observed that pre-treatment with aspirin or ibuprofen mirrored platelet S1P deficiency in exacerbation of PAF-induced mortality (Figure 7E and Online Figure III). In stark contrast, aspirin treatment was protective in mice that lack platelet S1P (Figure 7E). Suggesting a dependence on S1P dose rather than selective effects of S1P sources, the requirement for platelet S1P was overcome in mice globally deficient in Sphk2, which paradoxically have very high plasma S1P levels and were protected from mortality (Figure 7F).[48] Unlike platelet deficiency and consistent with aspirin-treated platelets maintaining the capacity to prevent inflammation-associated bleeding,[33] 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 supra-physiological 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, 48-51] While we originally focused on the role of $S1P_1$ in protecting vascular integrity during systemic anaphylaxis,[12] Olivera et al later argued the importance of $S1P_2$ signaling and vascular tone regulation.[49] In support of Olivera et al, endothelial selective $S1P_1$ 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 $S1P_2$ dependent, we first 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 non-invasive echocardiography and ultrasound pulsed Doppler analysis (Figure 8C and 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)[52-54]. A similar phenotype was observed with platelet S1P deficiency and in $S1P_2$ deficient mice (Figure 8C and Online Figure 5).[55] While $S1P_2$ 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 $S1P_3$,[55, 56] the second smooth muscle contractile S1P receptor (Figure 8F). This could argue that 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,[49] these observations suggest that while S1P promotes both vascular integrity through $S1P_1$ and vascular tone through $S1P_2$, 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/or plasma S1P deficiency on vascular development and homeostasis in vivo. Deletion of *Sphk1&2* 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.[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 appears to be redundant for hemostasis, thrombosis and vascular integrity when other S1P sources and/or other protective mechanisms are intact. While these observations contrast essential roles recently reported for platelet- as well as erythrocyte-derived S1P for $S1P_1$ 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 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 $S1P_2$ 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,[34] sensitized to anaphylaxis mortality in an S1P-dependent manner.

As cells of non-hematopoietic origin contribute enough S1P to sustain plasma S1P levels well above the Kd for $S1P_1$ binding,[21, 23, 57] it is surprising that the absence of erythrocyte and especially platelet S1P would lead to a deficit in activation of $S1P_1$ on cells in contact with plasma as has recently been reported.[14, 15, 58] 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 $S1P_1$ 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 upon a role for the platelet S1P source. Moreover, although we also observed bleeding with plasma S1P deficiency that could be rescued by transplantation of wild-type 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 $S1P_1$ to coordinate vascular developmental was also demonstrated in an *Sphk2*$^{-/-}$ background,[11] 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 $S1P_1$ in thrombosis, this was performed with mice deficient in Sphk2 in all cells,[15] which also display thrombocytopenia and supra-physiological plasma S1P levels.[48, 59] While 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 appears 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, 57] As we also did not observe spontaneous vascular leak with compound Sphk1&2 deficiency in endothelium, these data underscore the importance of plasma S1P, which is maintained by multiple redundant sources, to maintain vascular integrity during embryogenesis and post-natal 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.[44] 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 escape[60] 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 model, 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 S1P available for receptor activation can drop below saturating levels during systemic inflammation, rendering the contribution of all residual cellular sources of S1P essential. While the mechanisms of S1P depletion remain to be addressed, non-redundant 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 supra-physiological 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.[49] 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.[52] Suggesting a dominant role in maintaining vascular tone,[49] sensitization with platelet S1P deficiency was not associated with enhanced vascular leak or bleeding, and endothelial deficiency of $S1P_1$ did not affect survival despite inducing constitutive vascular leak to lung. By contrast, deficiency of $S1P_2$, 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 $S1P_1$ or another receptor to S1P protection.[8]

A protective role for platelet-derived S1P in anaphylaxis begs the question of whether anti-thrombotic or -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 NSAIDs.[34] This predicted that NSAIDs 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 inversed 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.[61, 62]

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 sub-optimal engagement of $S1P_2$ signaling during recovery from systemic anaphylaxis also argues potential therapeutic utility of $S1P_2$ agonists for maintenance of vascular tone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Anatol Kontush for helpful advice, and the administrative and technical staff at INSERM U960 for support. We also thank Alexina Orsoni and René Lai-Kuen for technical assistance and the Cellular and Molecular Imaging Platform, CRP2 - UMS 3612 CNRS - US25 Inserm-IRD - Université Paris Descartes.

Sources of Funding: This work was supported by INSERM, Marie Curie Actions (IRG-249193), the French National Research Agency (ANR-10-MIDI-0003), Fondation Leducq (SphingoNet), Fondation de France, the Ile-de-France Region (CORDDIM, E.C.), the French Society of Arterial Hypertension (B.M.), Canceropole (S.G.), and the National Insitutes of Health (HL65590; S.R.C),

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Nonstandard Abbreviations and Acronyms

BM	bone marrow
BT	basilar trunk
CLEC-2	C-type lectin-like receptor 2
ECKO	endothelial cell knockout
GPCR	G protein-coupled receptor

HDL	high density lipoprotein
HUVEC	human umbilical vein endothelial cell
ITAM	immunoreceptor tyrosine-based activation motif
LDL	low density lipoprotein
LPS	lipopolysaccharide
MPO	myeloperoxidase
NSAID	non-steroidal anti-inflammatory drug
PAF	platelet-activating factor
PBS	phosphate buffered saline
PAR4-AP	protease-activated receptor-4 agonist peptide
rpA	reverse passive Arthus reaction
S1P	sphingosine-1-phosphate
$S1P_n$	sphingosine-1-phosphate receptor-n
S1Pless	S1P deficient
SD	standard deviation
SEM	standard error of the mean
Sphk	sphingosine kinase
TEER	trans-endothelial electrical resistance
WT	wild-type

Novelty and Significance

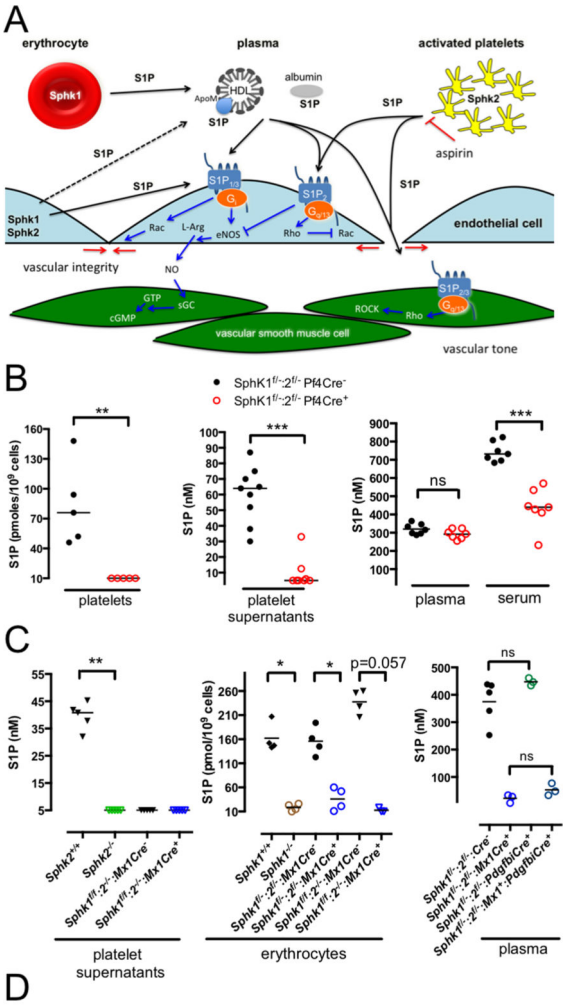
What Is Known?

- The bioactive lipid sphingosine-1-phosphate (S1P) critically supports vascular development and vascular integrity in mice primarily via the $S1P_1$ receptor.
- Platelets have been suggested to support vascular integrity after activation of C-type lectin-like receptor 2 (CLEC-2) by paracrine actions of platelet-derived S1P on endothelial $S1P_1$.
- 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 CLEC-2 is essential, suggesting that redundant S1P sources normally secure these functions.
- 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 basal plasma S1P below levels predicted as receptor-saturating. In a model of systemic anaphylaxis, we observed rapid depletion of plasma S1P to <20 % of basal levels. This rendered both circulating sources of S1P essential for recovery, and blocking S1P release with aspirin impaired survival. While S1P supports vascular integrity and vascular tone in this model via $S1P_1$ and $S1P_2$, respectively, only the latter was essential for the survival benefit of S1P. 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.



Cre	target cell	[S1P] (approximate % of control)		
		plasma	platelet	erythrocyte
Mx1	hematopoietic hepatocyte lymphatic EC	<10	<10	<20
Pf4	megakaryocyte	100	<10	ND
Epor	erythrocyte	50-70	ND	<10
Pdgfb	blood EC	100	ND	ND
Sm22	smooth muscle	ND	ND	ND
global deletion: *Sphk1*		50	100	<10
global deletion: *Sphk2*		~200	<10	~200

Figure 1. Generation of 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 HDL, albumin, and LDL. **B-C**. S1P concentrations as determined by HPLC and LC-MS/MS in lysed platelets, supernatants of washed and activated platelets, erythrocytes, plasma and/or serum from mice with single global deficiency or compound tissue-specific deficiency in Sphk1&2 in megakaryocytes (*Sphk1$^{f/-}$:*

2$^{f/-}$*:Pf4Cre*$^{+}$), blood endothelium (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:PdgfbiCre*$^{+}$), hematopoietic cells, hepatocytes and lymphatic endothelium (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1Cre*$^{+}$) or the latter combined (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1Cre*$^{+}$*:PdgfbiCre*$^{+}$). 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 two 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, 66] ND, not determined. Note that erythrocytes and platelets make near exclusive use of Sphk1 and Sphk2, respectively, for S1P production, and that while relevant target cells of Mx1-Cre (erythrocytes and lymphatic endothelial cells) continuously provide S1P to plasma, platelets only release their S1P content upon activation and thus contribute to serum but not plasma S1P. Blood endothelium does not contribute measurably to plasma S1P, but mays still constitute a relevant source for activation of vascular receptors.

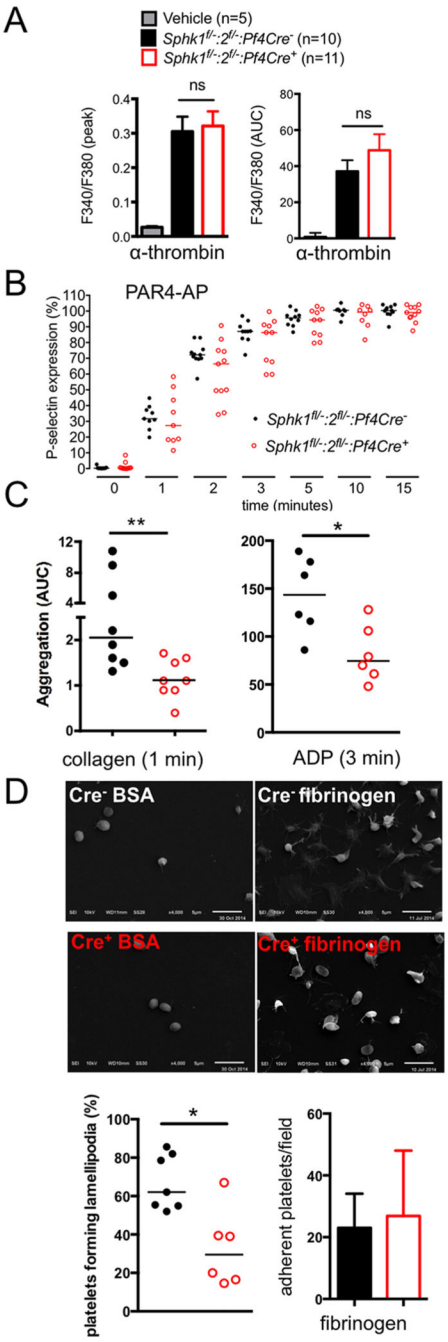


Figure 2. Platelet S1P deficiency impairs functional responses in washed platelets

The function of Sphk 1&2 deficient platelets was addressed *ex vivo* using washed platelets by assessing (**A**) α-thrombin (10 nM)- induced changes in cytosolic [Ca^{2+}] by fura-2 ratiometric analysis **(B)** PAR4-AP (200 μM) - induced P-selectin exposure by flow cytometry, **(C)** collagen (0.75 μg/mL) and ADP (1.5 μM) - induced aggregation by densitometry (AUC, area under the curve), and **(D)** spreading on fibrinogen. Sphk 1&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.

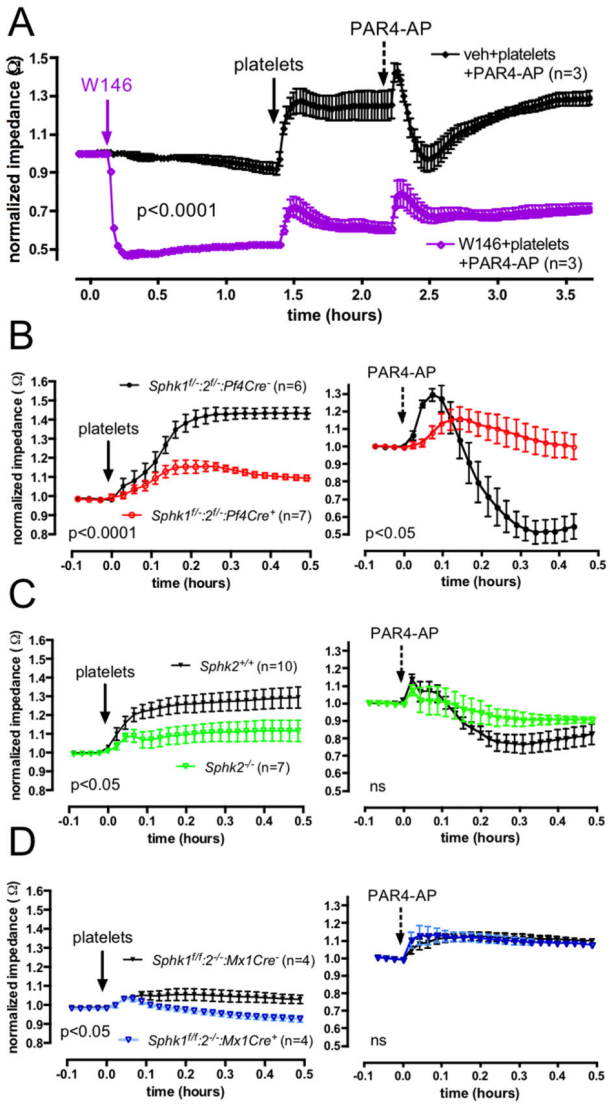


Figure 3. S1P is critical for the capacity of platelets to promote endothelial barrier function *ex vivo*

HUVEC were cultured on gold electrodes and electrical impedance, reflecting transendothelial electrical resistance and barrier function, measured. After an initial stabilization period, 10^7 washed platelets from wild-type (**A**) or indicated Sphk deficient (**B-D**) mice or littermate controls were added to HUVEC monolayers (B-D, left panel), and then activated with PAR4-AP (500 μM) (B-D, right panel). Endothelial cells in (A) were treated with the $S1P_1$ antagonist W146 (1 μM, purple) or vehicle control (black) for 1 hour prior to the addition of platelets. In A, impedance was normalized before addition of W146 or vehicle control. In B-E, 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.

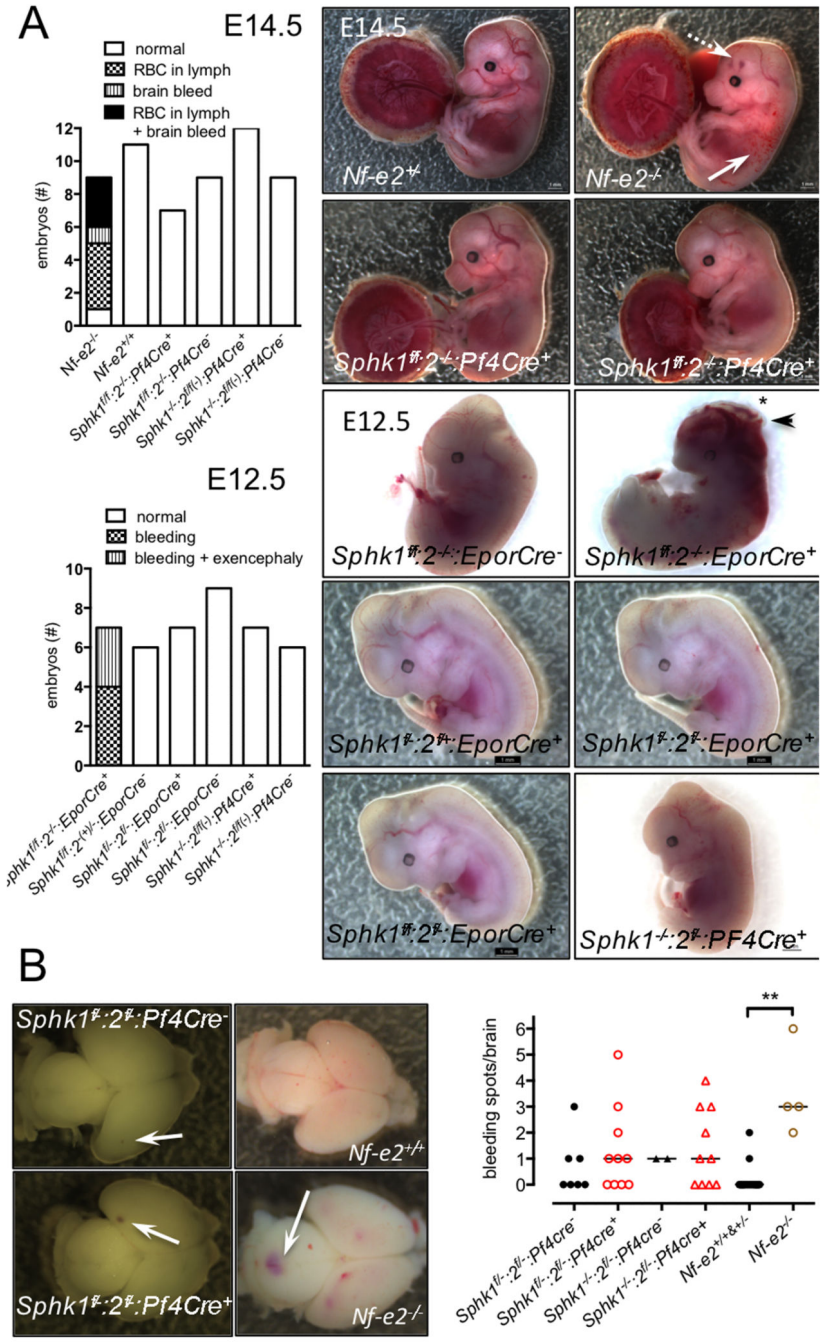


Figure 4. Platelet- and erythrocyte-derived S1P are dispensable for developmental blood-lymph separation and vascular integrity

A. Embryos with defective thrombopoiesis (*Nf-e2*$^{-/-}$) or rendered deficient in megakaryocyte Sphk1 in a *Sphk2*$^{-/-}$ background (*Sphk1*$^{f/f}$*: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. While embryos rendered deficient in erythrocyte Sphk1 in a *Sphk2*$^{-/-}$ background (*Sphk1*$^{f/f}$*:2*$^{-/-}$*:EporCre*$^{+}$) showed widespread bleeding (black arrowhead) and in some cases exencephaly (asterisk) at

E12.5, this was not observed when *Sphk1* was deleted in erythrocytes in a $Sphk2^{+/-}$ background ($Sphk1^{f/f}:2^{f/-}:EporCre$), nor when *Sphk1&2* were both deleted specifically in erythrocytes ($Sphk1^{f/-}:2^{f/-}:EpoRCre^{+}$), or when both erythrocyte and platelet sources were impaired ($Sphk1^{-/-}:2^{f/(f)-}:Pf4Cre^{+}$). The left panel summarizes observations in collected embryos. **B.** Sphk deficient and $Nf\text{-}e2^{-/-}$ neonates were sacrificed on postnatal day 0-2 and brains dissected after transcardial PBS perfusion to look for evidence of hemorrhage. Left panel: representative brains. Right panel: summary. No significant difference in bleeding prevalence was observed with platelet- or platelet- and erythrocyte-S1P deficiency, whereas bleeding was significantly more frequent in $Nf\text{-}e2^{+/-}$ than in corresponding littermate controls. Images were acquired in PBS immersion with a Leica M165FC stereo microscope.

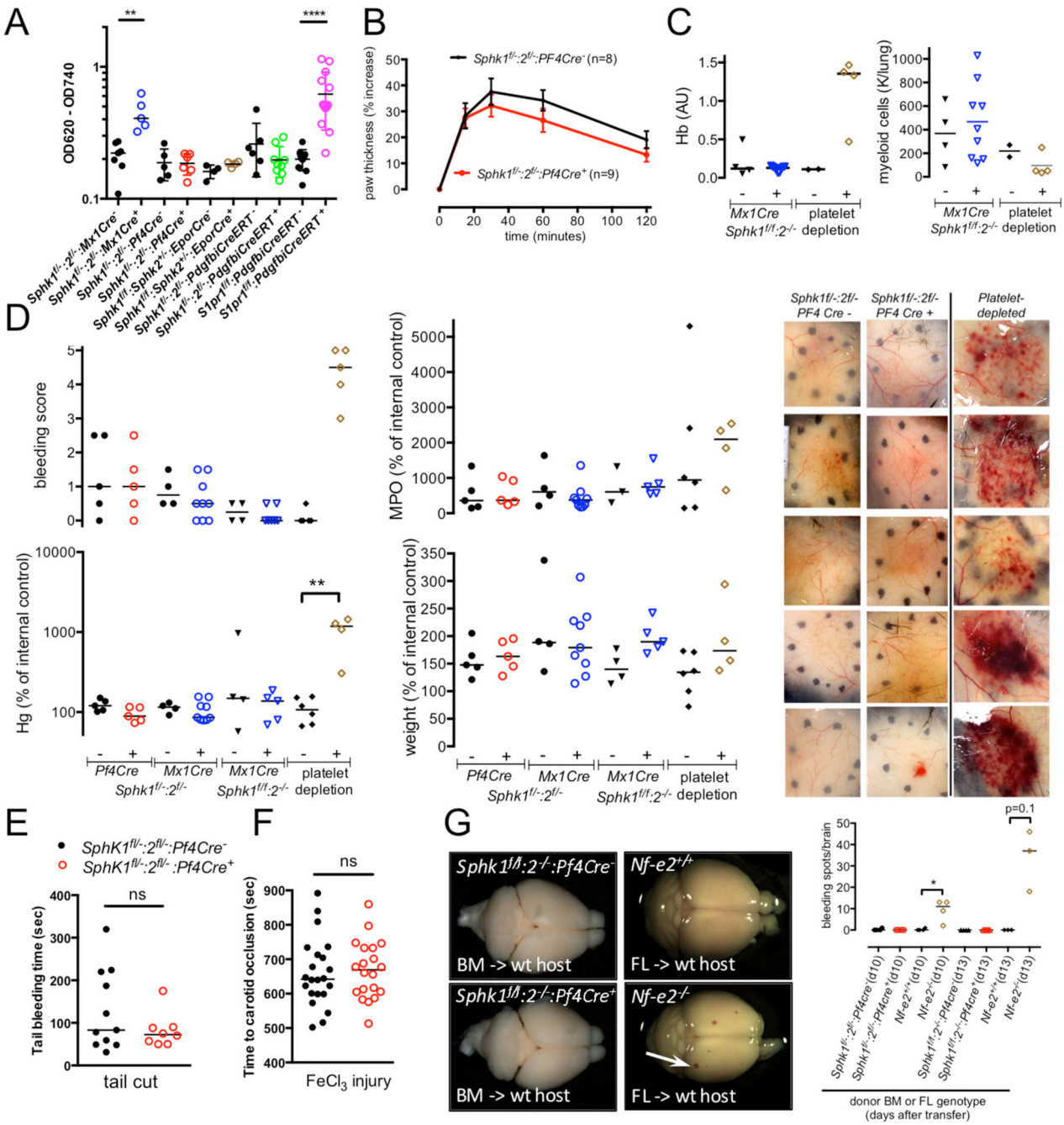


Figure 5. Effects of S1P deficiencies on vascular integrity, hemostasis, thrombosis, and leukocyte recruitment in adult mice

A. Constitutive Evans Blue leak to lung in mice deficient in S1P sources or the endothelial $S1P_1$ receptor. **B**. Histamine (60 mg) –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 LPS (25 mg). 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 semi-simultaneous s.c. injection of anti-BSA and i.v. injection of BSA (left panel). Bleeding, MPO activity, and weight were all increased in rpA biopsies relative to control independent of genotype. Representative images (right panel) show occasional mild petechiae at sites injected with anti-BSA antibody in *Sphk1*$^{f/-}$*:2*$^{f/-}$ mice independent of *Pf4-Cre* status. **E-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 $FeCl_3$ (15%) swab (**F**). Platelet S1P deficiency did not influence either endpoint, 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. 10 or 13 days (d) later, brains were removed after transcardial perfusion. Left panel, representative brains 13 days after transplantation; right panel, summary. Platelet- but not platelet S1P-deficiency resulted in brain bleeding after irradiation.

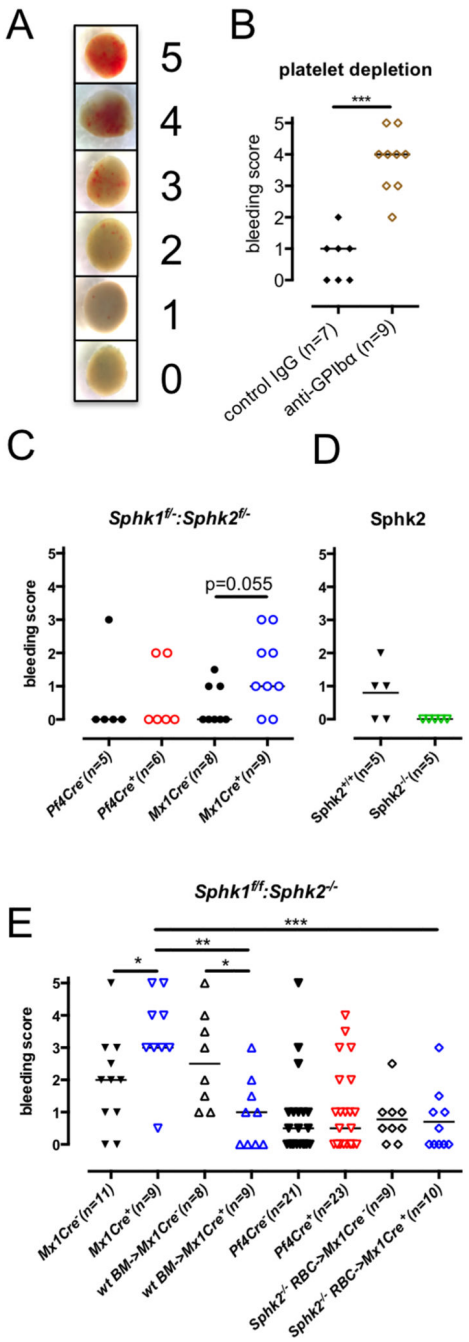


Figure 6. Platelets and S1P, but not platelet-derived S1P, are essential for lymph node hemostasis after immunization

A-E. Mice were immunized in the right hock and draining and control LNs isolated one 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-GPIbα antibodies were administered to C57BL/6 males the day before harvest, and thrombocytopenia confirmed immediately before transcardial perfusion. Thrombocytopenia induced bleeding in draining lymph nodes (**B**) while deficiency in platelet-derived S1P

(*Sphk1*$^{f/-}$:*2*$^{f/-}$:*Pf4Cre*$^{+}$ or *Sphk2*$^{-/-}$) did not. **C-D.** Lack of all circulating sources of S1P (*Sphk1*$^{f/-}$:*2*$^{f/-}$:*Mx1Cre*$^{+}$) was associated with a bleeding tendency (**C**). In a background globally deficient in Sphk2 (**E**), deletion of *Sphk1* in hematopoietic cells and lymphatic endothelial cells (*Sphk1*$^{f/f}$:*2*$^{-/-}$:*Mx1Cre*$^{+}$), but not in megakaryocytes (*Sphk1*$^{f/f}$:*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 PBS immersion with a Leica M165FC stereo microscope.

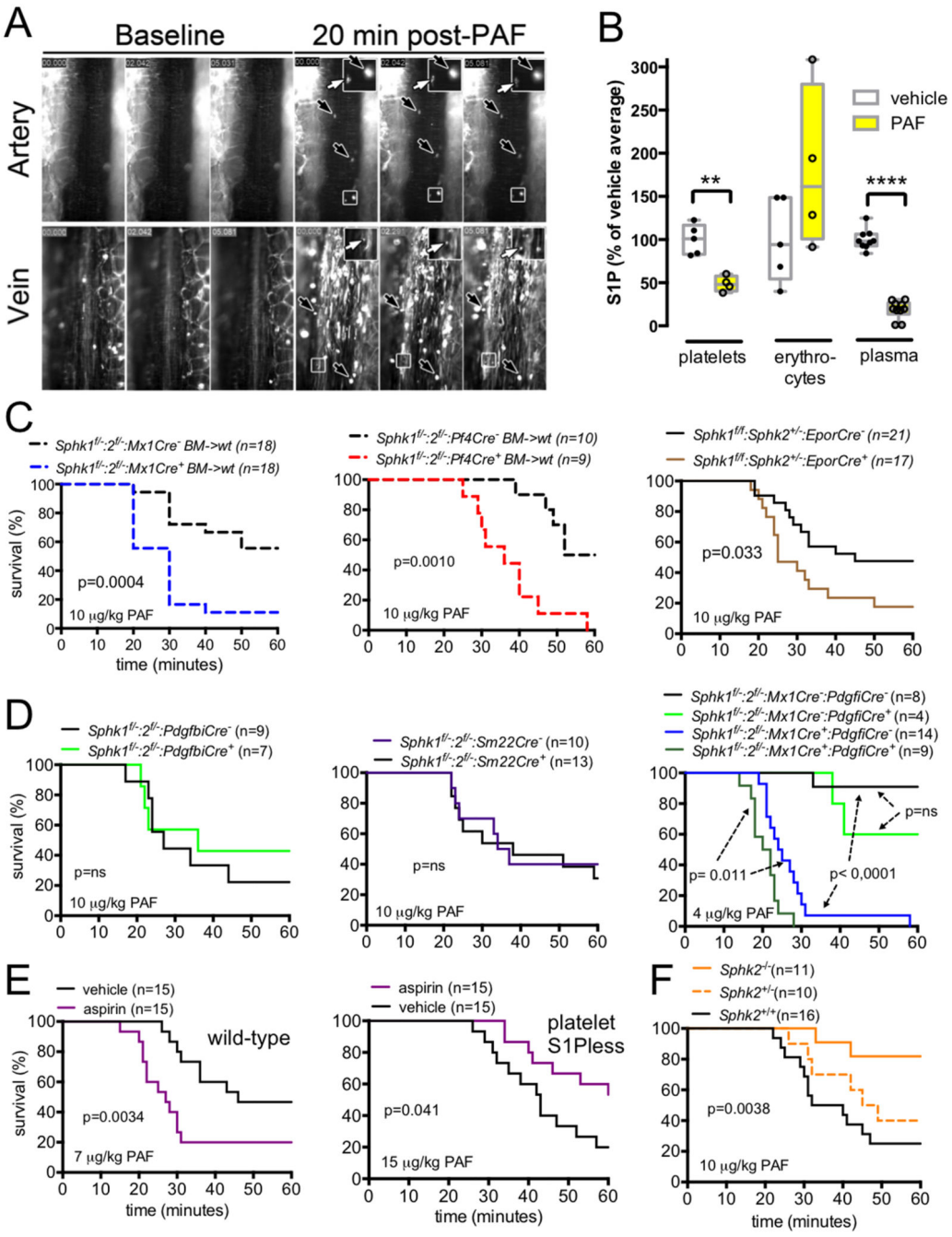


Figure 7. Plasma S1P depletion renders otherwise redundant S1P sources essential for recovery from anaphylactic shock

A. Intravital imaging of interactions between platelets, leukocytes, and the vessel wall before and 20 minutes after PAF (4 μg/kg)-induced anaphylaxis. Images extracted from time-lapse microscopy videos in the Online Supplement. 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. 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 μg/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 Sphk deficiency in all bone marrow (BM) cells (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Mx1Cre*$^{+}$), or selectively in megakaryocytes (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Pf4Cre*$^{+}$) or mice lacking S1P production in erythrocytes (*Sphk1*$^{f/f,+/-}$*: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*$^{f/-}$*:2*$^{f/-}$*:PdgfbiCre*$^{+}$) or in smooth muscle cells (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Sm22Cre*$^{+}$) did not increase mortality in isolation, although deficiency in blood endothelium significantly increased mortality in the context of pan-hematopoietic deficiency (*Sphk1*$^{f/-}$*:2*$^{f/-}$*:PdgfbiCre*$^{+}$*:Mx1Cre*$^{+}$) (**D**). Aspirin significantly increased mortality in wild-type mice, but not in chimeric wild-type mice with *Sphk1*$^{f/f}$*:2*$^{-/-}$*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.

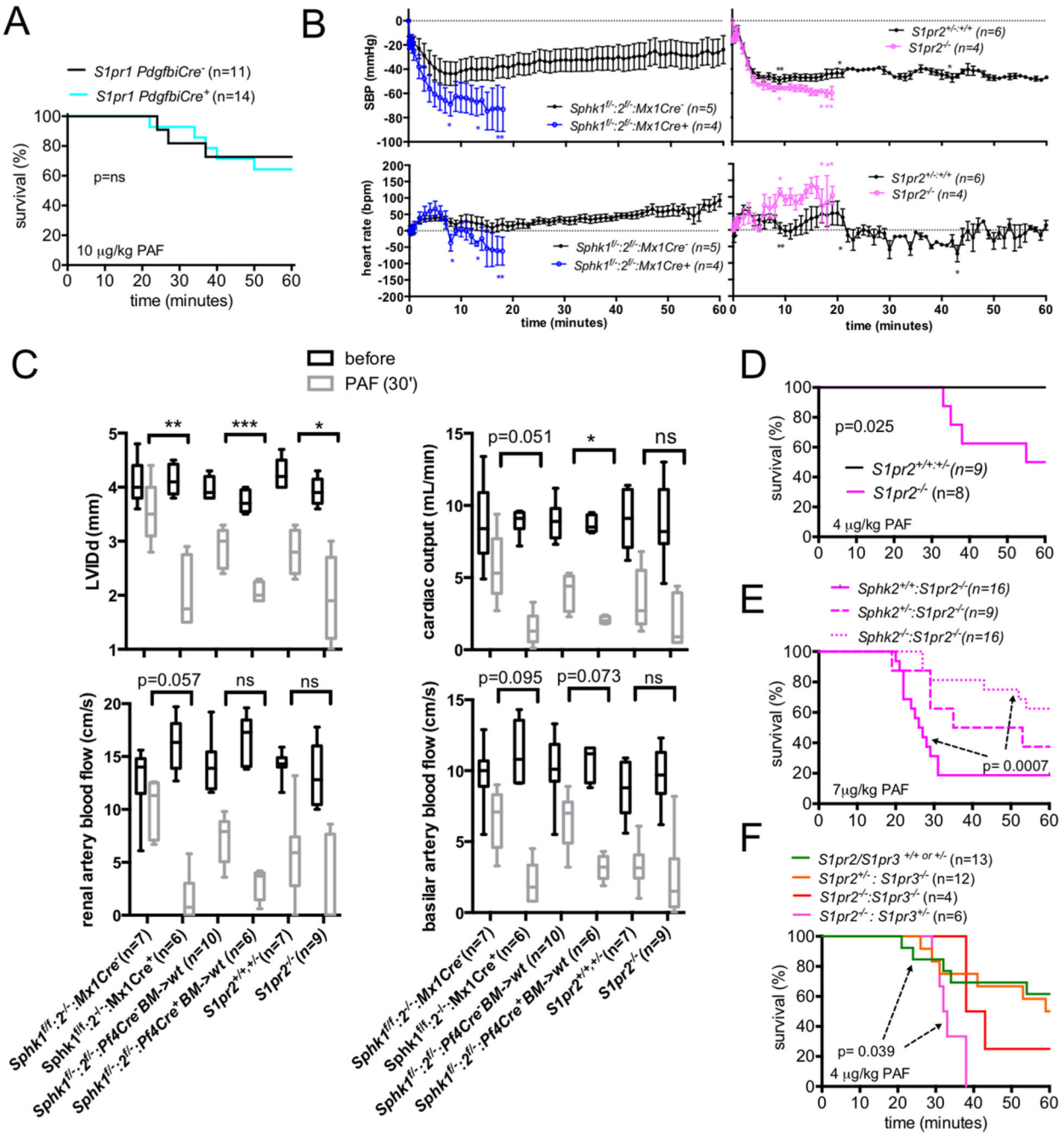


Figure 8. S1P promotes recovery from anaphylactic shock by $S1P_2$-mediated enhancement of vascular tone

A. Mice selectively lacking $S1P_1$ in blood endothelium (*S1pr1$^{f/f}$:PdgfbiCre^{+}*) were challenged with PAF and survival monitored for 60 minutes. **B**. Plasma S1Pless (*Sphk1$^{f/-}$:2$^{f/-}$:Mx1Cre^{+}*), $S1P_2$ deficient mice (*S1pr2$^{-/-}$*) and 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 death of an animal. **C.** Plasma S1Pless (*Sphk1$^{f/f}$:2$^{-/-}$:Mx1Cre^{+}*), chimeric wild-type (wt) mice with

megakaryocyte Sphk1&2 deficient bone marrow (BM)(*Sphk1*$^{f/-}$*:2*$^{f/-}$*:Pf4Cre*$^{+}$), $S1P_2$ 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. Upper panel: 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 $S1P_2$ either alone (*S1pr2*$^{-/-}$; **D**) or in conjunction with Sphk2 (*S1pr2*$^{-/-}$*:Sphk2*$^{-/-}$; **E)** or $S1P_3$ (*S1pr2*$^{-/-}$*:S1pr3*$^{-/-}$; **F**) and littermate controls were challenged with PAF at indicated concentrations and survival monitored for 60 minutes. Note that only $S1P_2$ deficiency sensitizes to PAF, independent of $S1P_3$, although residual protection by Sphk2 deficiency in a *S1pr2*$^{-/-}$ background suggests the involvement of second receptor.