Sphingosine-1-phosphate receptor 2 protects against anaphylactic shock through suppression of endothelial nitric oxide synthase in mice

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Original Article

Sphingosine-1-phosphate receptor-2 protects against anaphylactic shock through suppression of eNOS in mice[1]

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ABSTRACT

Background: Sphingosine-1-phosphate receptor-2, $S1P_2$, is expressed in vascular endothelial cells (EC). However, the role of $S1P_2$ in vascular barrier integrity and anaphylaxis is not well understood. Endothelial nitric oxide (NO) synthase (eNOS) generates NO to mediate vascular leakage, compromising survival in anaphylaxis. We recently observed that endothelial $S1P_2$ inhibits Akt, an activating kinase of eNOS.

Objective: We tested the hypothesis that endothelial $S1P_2$ might suppress eNOS, exerting a protective effect against endothelial barrier-disruption and anaphylaxis.

Methods: Mice deficient in $S1P_2$ and eNOS underwent antigen challenge or platelet-activating factor (PAF) injection. Analyses were performed to examine vascular permeability and the underlying mechanisms.

Results: *S1pr2* deletion augmented vascular leakage and lethality after either antigen challenge or PAF injection. PAF injection induced activation of Akt and eNOS in the aorta and lung of *S1pr2*-null mice, which were augmented compared with wild-type mice. Consistently, PAF-induced increase in cyclic GMP level in the aorta was enhanced in *S1pr*-null mice. Genetic *Nos3* deletion or pharmacological eNOS blockade protected *S1pr2*-null mice from aggravation of barrier-disruption after antigen challenge and PAF injection. EC isolated from *S1pr2*-null mice exhibited greater stimulation of Akt and eNOS with enhanced NO production in response to S1P or PAF, compared with wild-type EC. Moreover, *S1pr2*-deficient EC showed more severe disassembly of adherens junctions with augmented *S*-nitrosylation of β-catenin in response to PAF, which was restored by pharmacological eNOS blockade.

Conclusion: $S1P_2$ diminishes harmful robust eNOS stimulation and thereby attenuates vascular barrier-disruption, suggesting potential usefulness of $S1P_2$ agonists as novel

therapeutic agents for anaphylaxis.

Key Messages

●Endothelial $S1P_2$ plays a protective role against acute barrier disruption during anaphylaxis.

●The barrier-protective effect of $S1P_2$ is mediated through suppression of eNOS.

Capsule Summary

Endothelial $S1P_2$ protects against vascular leakage in anaphylaxis through suppressing production of nitric oxide, which disrupts the endothelial barrier, and could be a promising novel target for treating anaphylaxis.

Key words: sphingosine-1-phosphate, $S1P_2$, vascular permeability, anaphylaxis, endothelial nitric oxide synthase, nitric oxide, nitrosylation, β-catenin, VE-cadherin, adherens junction

Abbreviations used:

AJ: adherens junctions

BSA: bovine serum albumin

cGMP: cyclic GMP

EC: endothelial cell

eNOS: endothelial NO synthase

HCT: hematocrit

L-NAME: Nω-L-nitro-argininemethyl ester

MLEC: mouse lung endothelial cell

NO: nitric oxide

OVA: ovalbumin

PAF: platelet-activating factor

PI3K: phosphatidylinositol 3-kinase

PTEN: phosphatase and tensin homolog deleted from chromosome 10

S1P: sphingosine-1-phosphate

VEGF: vascular endothelial growth factor-A

WT: wild-type

INTRODUCTION

Sphingosine-1-phosphate (S1P) has been identified as an important regulator of vascular and immune functions.[1, 2] S1P is synthesized by sphingosine kinases-1 and -2 in a wide variety of cells.[3] Plasma S1P, which is present at the concentration of ~10^{-6} M, is derived from erythrocytes, vascular endothelial cells (EC) and other cells.[4-6] Most of the biological activities of S1P are mediated by the S1P-specific G protein-coupled receptor family $S1P_1$~ $S1P_5$.[7] Among these, $S1P_1$, $S1P_2$ and $S1P_3$ are widely expressed major receptor subtypes. EC in culture express readily detectable levels of $S1P_1$ and $S1P_3$ whereas $S1P_2$ expression is much lower or undetectable.[8, 9] However, we recently observed by analyzing LacZ-knockin mice at the *S1pr2* locus that EC in a variety of organs express $S1P_2$ in vivo.[10] $S1P_1$ and $S1P_2$ exert distinct effects on cell responses, including migration and proliferation, through the preferential coupling to G_i and $G_{12/13}$, respectively.[11]

Platelet-activating factor (PAF), histamine and leukotrienes are edematogenic mediators that disrupt vascular barrier integrity, which is sustained mainly by the endothelial adherens junction (AJ) comprising VE-cadherin and its associated proteins.[12] In anaphylaxis, these edematogenic mediators are released mainly from mast cells through antigen engagement of mast cell-associated IgE and act on vascular endothelium and smooth muscle to cause severe vascular leakage and hypotension.[13] To establish novel therapies for anaphylaxis, it is important to better understand the pathophysiological mechanisms underlying vascular barrier disruption. Both in vitro and in vivo studies have documented that S1P maintains vascular endothelial barrier function via endothelial $S1P_1$.[14,15] A subsequent study showed that plasma S1P is required to prevent vascular leakage in anaphylaxis after antigen challenge or injections of PAF and histamine.[16] Thus, the plasma S1P–endothelial $S1P_1$ axis plays

a central role in maintaining vascular barrier integrity.[17] In contrast to $S1P_1$, pharmacological blockade of $S1P_2$ was reported to reduce histamine-induced vascular leakage.[18] More recently, it has been shown that genetic deletion of *S1pr2* attenuated vascular leakage and hypothermia in anaphylaxis after antigen challenge.[19] *S1pr2* deletion was accompanied by diminished elevation of plasma histamine and cytokines after antigen challenge, suggesting that stimulation of mast cell activation by $S1P_2$ is a determinant of vascular hyperpermeability and anaphylaxis. Unlike this report, however, another group showed that $S1P_2$ was rather vital for recovery from histamine-induced hypothermia through regulating vascular tone and histamine clearance, which suggests that $S1P_2$ exerts a protective effect against anaphylaxis at a site distal to mast cell activation.[20, 21] Thus, the in vivo role of $S1P_2$ in anaphylaxis is not yet fully understood.

Previous studies demonstrated that endothelial nitric oxide (NO) synthase (eNOS or NOS3)-derived NO is involved in an increase of vascular permeability caused by PAF and vascular endothelial growth factor-A (VEGF).[22-24] The activity of the calmodulin-dependent enzyme eNOS is stimulated by an increase in intracellular free Ca^{2+}, interaction with regulatory proteins, and phosphorylation mediated largely by Ca^{2+}-independent protein kinases including Akt and protein kinase A.[25] Phosphorylation of eNOS sensitizes eNOS to Ca^{2+}, resulting in augmented activation of eNOS. Most edematogenic mediators, including PAF and histamine, act through G protein-coupled receptors. Importantly, anaphylactic shock has been shown to depend on eNOS activation mediated by endothelial $G_{q/11}$ and phosphatidylinositol 3-kinase (PI3K)-Akt.[26,27] We recently found that endogenous $S1P_2$ mediates inhibition of Akt in EC,[10] unlike $S1P_1$ which stimulates eNOS via Akt.[28] These observations raise the possibility that $S1P_2$ might suppress eNOS through inhibition of Akt, exerting a

rescue effect on vascular hyperpermeability.

In the present study, we tested this hypothesis by determining how genetic *Nos3* deletion and pharmacological eNOS blockade affected $S1P_2$-mediated effects on vascular hyperpermeability in anaphylaxis in mice. Our results indicate that $S1P_2$ protects mice from vascular barrier disruption elicited by either antigen challenge or PAF injection and that this protective effect of $S1P_2$ is mediated through suppression of anaphylaxis-associated eNOS stimulation. These observations provide the basis for targeting $S1P_2$ as a novel treatment of anaphylaxis.

METHODS

Supplemental information can be found in the Methods section in this article's Online Repository.

Mice

All animal experiments were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and were approved by the Committee on Animal Experimentation of Kanazawa University. *S1pr2*-null (*S1pr2*$^{-/-}$) mice were previously described.[10] *S1pr2*$^{-/-}$ mice (129Ola;C57BL/6J mixed background) were back-crossed with C57BL/6J mice (#000664, Charles River) once. In some experiments, we employed another *S1pr2*–deleted (*S1pr2*$^{LacZ/LacZ}$) mouse in which LacZ is knocked in at the *S1pr2* locus.[10] *Nos3*$^{-/-}$ mice (#002684, C57BL/6J background) were obtained from the Jackson Laboratory. *S1pr2*$^{-/-}$; *Nos3*$^{-/-}$ mice and wild-type (WT) littermates were generated by heterozygous matings.

Statistics

All values are expressed as mean ± SEM. Data were analyzed using unpaired, two-tailed Student's *t*-test, and two-way ANOVA followed by Bonferroni post test to determine statistical significance using GraphPad Prism software. Values of $P < 0.05$ were considered statistically significant.

RESULTS

$S1P_2$ protects mice from vascular hyperpermeability in anaphylaxis

We studied how $S1P_2$ affected vascular hyperpermeability and mortality using two models of active systemic anaphylaxis, which predominantly depends on PAF.[29] Mice had been sensitized with bovine serum albumin (BSA) or ovalbumin (OVA) and challenged with these antigens. After BSA challenge, *S1pr2*$^{-/-}$ mice displayed greater extravasation of i.v. injected Evans blue in the lung compared with WT mice (Fig 1, A and B). Consistent with this, *S1pr2*$^{-/-}$ mice showed higher hematocrit (HCT) due to augmented plasma leakage compared with WT mice (Fig 1, C). Evans blue leakage under saline administration was similar in *S1pr2*$^{-/-}$ and WT mice, suggesting that basal vascular permeability was not altered in *S1pr2*$^{-/-}$ mice. *S1pr2*$^{-/-}$ mice began to die within 10 min after the antigen challenge and all *S1pr2*$^{-/-}$ mice died by 30 min, whereas WT mice started to die as late as 25 min and 45% of WT mice survived the antigen challenge at 60 min (Fig 1, D). Similarly, in OVA-sensitized *S1pr2*$^{-/-}$ mice, challenge with OVA augmented Evans blue leakage and HCT elevation compared with those in WT mice (Fig E1).

We next studied the vascular permeability responses to i.v. injection of the anaphylactic mediator PAF. *S1pr2*$^{-/-}$ mice showed greater PAF-induced extravasation of Evans blue dye in the lung than did WT mice (Fig E2, A and B). Histological analysis of the lungs showed more pronounced accumulation of perivascular fluid in *S1pr2*$^{-/-}$mice after PAF injection, compared with WT mice (Fig E2, C). *S1pr2*$^{-/-}$ mice exhibited higher HCT compared with that of WT mice at 10 min after PAF injection (Fig E2, D). Consequently, survival after PAF injection was severely impaired in *S1pr2*$^{-/-}$ mice: 90% of WT mice survived PAF challenge whereas less than 10% of *S1pr2*$^{-/-}$ mice survived (Fig E2, E). We also analyzed the effect of PAF on dermal

vascular permeability in *S1pr2*$^{-/-}$ and WT mice using a Miles assay. Basal vascular permeability was similar in *S1pr2*$^{-/-}$ and WT mice (Fig E2, F). Intradermal injection of PAF induced a dose-dependent increase in leakage of Evans blue dye in both genotypes, with greater leakage of Evans blue in *S1pr2*$^{-/-}$ mice compared with WT mice. *S1pr2*$^{-/-}$ mice also displayed enhanced dermal leakage of Evans blue dye after local histamine injection compared with WT mice (data not shown). To delineate how the genetic background of mice affected the anaphylactic responses, we analyzed *S1pr2*$^{LacZ/LacZ}$ mice[10] that had been back-crossed with C57BL/6 mice 4 times and WT littermates. *S1pr2*$^{LacZ/LacZ}$ mice showed exaggerated responses of Evans blue leakage and HCT elevation after PAF challenge compared with WT mice (Fig E3), which were similar to those in *S1pr2*$^{-/-}$ mice. In these experiments, we anesthetized mice for a very short time (~1 min) with ether. In the non-anesthetized condition, we observed essentially the same exaggerated responses in HCT and death after PAF injection in *S1pr2*$^{-/-}$ mice compared with WT mice (Fig E4). These data indicate that *S1pr2*$^{-/-}$ mice show enhanced vascular permeability with increased mortality in anaphylaxis.

Akt/eNOS activation is augmented in aorta and lung of *S1pr2*$^{-/-}$ mice after PAF challenge

S1pr2$^{-/-}$ and WT mice, which received saline injection, showed similar levels of basal phosphorylation of Akt (Fig 2, A and C) and eNOS (Fig 2, B and D) in both aorta and lung. PAF injection stimulated Akt phosphorylation in aorta from both *S1pr2*$^{-/-}$and WT mice but with a greater increase in *S1pr2*$^{-/-}$ mice (Fig 2, A). In contrast, PAF-induced activation of ERK or p38MAPK was not different between *S1pr2*$^{-/-}$and WT mice (data not shown). Notably, PAF injection increased eNOS phosphorylation in aorta from both mouse groups with a greater degree of stimulation in *S1pr2*$^{-/-}$ mice (Fig 2, B).

Similarly to aorta, PAF increased phosphorylation in Akt and eNOS in lung from both *S1pr2*$^{-/-}$ and WT mice with greater degrees of stimulation in *S1pr2*$^{-/-}$ mice (Fig 2, C and D). NO synthesized by eNOS stimulates the formation of cyclic GMP (cGMP) in vascular wall. The basal level of cGMP in aorta was similar between *S1pr2*$^{-/-}$ and WT mice (Fig 2, E). PAF injection induced a larger increase in aortic cGMP level in *S1pr2*$^{-/-}$ mice than in WT mice. These observations raise the possibility that the exaggerated increase in vascular permeability in *S1pr2*$^{-/-}$ mice might be due to augmented activation of the Akt/eNOS signaling pathway and generation of NO in the vasculature.

Pharmacological inhibition of NO synthase or genetic deletion of *Nos3* protect *S1pr2*$^{-/-}$ mice from vascular hyperpermeability in anaphylaxis

Administration of Nω-L-nitro-argininemethyl ester (L-NAME) 3 h before PAF injection prevented the exaggerated HCT elevation after PAF challenge in *S1pr2*$^{-/-}$ mice (Fig E5, A). L-NAME also protected *S1pr2*$^{-/-}$ mice against PAF-induced lethality (Fig E5, B). These observations suggest that NO is involved in PAF-induced vascular leakage and lethality in *S1pr2*$^{-/-}$mice. To elucidate the role of eNOS in exaggerated PAF-induced vascular leakage in *S1pr2*$^{-/-}$mice, we crossed *S1pr2*$^{-/-}$ mice with *Nos3*$^{-/-}$ mice to generate *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ compound mutant mice. The HCT after PAF injection in *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice was lower than that in *S1pr2*$^{-/-}$ mice and was not different from that in WT mice or *Nos3*$^{-/-}$ mice (Fig 3, A). Thus, augmentation of the PAF-induced HCT elevation in *S1pr2*$^{-/-}$mice was abolished by *Nos3* deficiency. Consistently, *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice were almost completely rescued from PAF-induced lethality (Fig 3, B).

We also studied the effects of *Nos3* deficiency on vascular responses in

anaphylaxis after BSA challenge. The magnitude of the increase in plasma histamine level after antigen challenge was similar in WT, *S1pr2*$^{-/-}$, *Nos3*$^{-/-}$, and *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice (Fig E6, A). The HCT at 10 min after antigen challenge in *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice was lower than that in *S1pr2*$^{-/-}$ mice and similar to that in WT mice and *Nos3*$^{-/-}$ mice (Fig 3, C). These observations suggest that eNOS in the vascular endothelium is responsible for *S1pr2* deficiency-induced aggravation of vascular hyperpermeability in anaphylaxis. We studied the hypotensive response to an antigen challenge in these mutant mice. A previous study[30] documented that in *S1pr2*$^{-/-}$ mice, the in vivo pressor response and the in vitro vascular contractile response to phenylephrine were diminished, the latter of which was endothelium-dependent. *S1pr2*$^{-/-}$ mice showed significantly lower steady state systolic and diastolic blood pressure than WT mice while both *Nos3*$^{-/-}$ mice and *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice displayed higher systolic blood pressure than WT mice (Table E1). Antigen challenge induced a rapid drop in blood pressure to a level of ~40 mmHg in anesthetized WT mice**,** and hypotension persisted for more than 40 min (Fig 3, D). In *S1pr2*$^{-/-}$ mice, blood pressure declined more rapidly and became unmeasurably low, leading to death within 25 min. Under the same condition, *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice and *Nos3*$^{-/-}$ mice showed only a mild**,** transient reduction in blood pressure and recovered by 50 min. These observations indicate that *S1pr2*$^{-/-}$ mice exhibit severe fatal circulatory shock and that deletion of *Nos3* gene protects both *S1pr2*$^{-/-}$ and WT mice from anaphylactic shock. Antigen challenge in WT mice induced a decline in core body temperature (mean change of -1.6°C at 20 min) (Fig E6, B). *S1pr2*$^{-/-}$ mice displayed more severe hypothermia compared with WT mice. *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice and *Nos3*$^{-/-}$ mice exhibited only a mild, transient decrease in body temperature, which was less marked compared with that in WT mice. Consistent with all these data, *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice were protected from antigen

challenge-induced lethality: *S1pr2*$^{-/-}$ single mutant mice started to die at 8 min after antigen challenge with 85% mortality at 60 min, whereas *S1pr2*$^{-/-}$;*Nos3*$^{-/-}$ mice began to die at 45 min and mortality at 60 min was 38% (Fig 3, E). *Nos3*$^{-/-}$ single mutant mice were also protected from antigen challenge-induced lethality as reported previously.[26]

S1P$_2$ negatively regulates Akt–eNOS pathway in vascular EC

In lung EC (MLEC) from WT mice, PAF stimulated phosphorylation of both Akt and eNOS (Fig 4, A and B). S1P alone did not affect phosphorylation of Akt or eNOS but rather reduced PAF-induced phosphorylation of eNOS in WT MLEC. In contrast to WT MLEC, S1P by itself stimulated phosphorylation of Akt and eNOS in *S1pr2*$^{-/-}$ MLEC. This is most likely because S1P$_1$, which is a major S1P receptor in *S1pr2*$^{-/-}$ MLEC, mediated S1P-induced phosphorylation of Akt and eNOS whereas S1P$_2$ counteracted S1P$_1$-mediated phosphorylation of Akt and eNOS in WT MLEC. Furthermore, PAF stimulated phosphorylation of Akt and eNOS to a greater degree in *S1pr2*$^{-/-}$ MLEC compared with WT MLEC. Unlike WT MLEC, S1P did not reduce PAF-induced phosphorylation of Akt and eNOS in *S1pr2*$^{-/-}$ MLEC, so that phosphorylation of Akt and eNOS in *S1pr2*$^{-/-}$ MLEC stimulated with S1P plus PAF was substantially greater than in WT MLEC. We examined how S1P$_2$ affected the production of NO in MLEC. In MLEC isolated from WT mice, either PAF or VEGF but not S1P stimulated the generation of NO (Fig 4, C). In *S1pr2*$^{-/-}$ MLEC, PAF and VEGF stimulated NO production to a greater degree compared with WT EC. Moreover, S1P by itself modestly stimulated NO production in *S1pr2*$^{-/-}$ MLEC, which was consistent with the results of eNOS phosphorylation (Fig 4, B). In addition, S1P slightly enhanced PAF-induced NO production in *S1pr2*$^{-/-}$ MLEC. These observations

suggest that $S1P_2$ exerts inhibitory effects on NO production in EC through reducing phosphorylation of Akt and eNOS.

$S1P_2$ contributes to maintaining AJ through inhibiting PAF-induced *S*-nitrosylation of β-catenin

NO mediates disruption of endothelial AJ, which comprise VE-cadherin and intracellular associated proteins including β-catenin and p120 catenin,[12] and increases vascular permeability.[22, 23, 25] Recent studies[31, 34] demonstrated that VEGF stimulation of eNOS induced *S*-nitrosylation of β-catenin, leading to disassembly of AJ complexes. We examined how $S1P_2$ influenced PAF-induced disassembly of AJ and *S*-nitrosylation of β-catenin in a monolayer of MLEC. Immunofluorescent staining with anti-VE-cadherin and anti-β-catenin antibodies showed that VE-cadherin and β-catenin were uniformly distributed at cell-cell contacts in both WT and *S1pr2*$^{-/-}$ MLEC in a non-treated condition, indicating that AJ were formed (Fig 5, A). PAF treatment resulted in a discontinuous, zigzag pattern of staining of both VE-cadherin and β-catenin in both WT and *S1pr2*$^{-/-}$ MLEC, indicating disruption of AJ. PAF-induced disruption of AJ was more severe in *S1pr2*$^{-/-}$ MLEC than in WT MLEC. Pretreatment with L-NAME almost totally prevented disruption of AJ in both WT and *S1pr2*$^{-/-}$ MLEC. We analyzed *S*-nitrosylation of β-catenin in MLEC treated with PAF and S1P in the presence and absence of L-NAME. In WT EC, PAF plus S1P tended to increase *S*-nitrosylation of β-catenin but not significantly (Fig 5, B). In contrast, PAF plus S1P substantially increased *S*-nitrosylation of β-catenin in *S1pr2*$^{-/-}$ MLEC. L-NAME abolished *S*-nitrosylation of β-catenin induced by PAF plus S1P in both WT and *S1pr2*$^{-/-}$ MLEC. These results suggest that $S1P_2$ inhibits PAF-induced disassembly of AJ through mechanisms involving inhibition of NO production and thereby

prevention of *S*-nitrosylation of β-catenin.

DISCUSSION

The present study provides evidence supporting that $S1P_2$ is protective against acute vascular barrier disruption, based on the composite results obtained in two anaphylaxis models of antigen challenge and PAF injection. Our study also revealed the mechanisms underlying the protective effect of $S1P_2$: $S1P_2$ suppressed harmful robust activation of eNOS, which plays a key role in vascular leakage in anaphylaxis. Thus, our study identified the role of a novel endothelial signaling pathway comprising $S1P_2$–Akt inhibition–eNOS inhibition, which plays a protective role against vascular barrier-disruption and may represent a promising new target for treating vascular hyperpermeability.

We studied the effects of *S1pr2* deficiency on acute vascular barrier disruption in active anaphylaxis and PAF injection. In the active anaphylaxis model, our data demonstrated that *S1pr2* deficiency exacerbated vascular leakage, hypotension, hypothermia and lethality without causing an increase in plasma histamine level (Figs 1 and 3, and Fig E6). Moreover, *S1pr2* exacerbated vascular leakage and lethality provoked by injection of the anaphylactic mediator PAF (Fig E2). These results together imply that $S1P_2$ alleviates acute vascular permeability largely by acting at a site distal to the release of anaphylactic mediators from mast cells; i.e., very likely at the vasculature itself. Our observations are consistent with the studies by Olivera et al.,[20, 21] which showed that histamine injection-induced hypothermia and hypotension were aggravated in *S1pr2*$^{-/-}$ mice, but are not consistent with that by Oskeritzian et al.,[19] which showed that *S1pr2* deficiency alleviated vascular leakage and hypothermia after antigen challenge, contrasting to our study. The findings by Oskeritzian et al.[19] also differ from our observations, in that PAF or histamine injection-induced vascular leakage and hypothermia were not different between

S1pr2$^{-/-}$ and WT mice. The reasons for the discrepancies between their study and the present study are not clear. However, it is possible that a difference in the experimental models, i.e., passive anaphylaxis[19] vs. active anaphylaxis (our study), might have led to the different results. The genetic backgrounds of mice were not exactly identical but very similar (mixed background of C57BL/6 x 129/Sv[19] vs. back-crossing once or twice of C57BL/6 x 129/Ola with C57BL/6 (our study)).

The present study demonstrated that endothelial $S1P_2$ inhibits eNOS (Fig 4). *S1pr2* deficiency de-inhibits eNOS in the vasculature in vivo (Fig 2). NO generated by eNOS is involved in vascular hyperpermeability in response to pro-inflammatory mediators such as PAF and VEGF.[22-24] Moreover, acute hypotension and lethality after antigen challenge and PAF injection entirely depend on the PI3K/Akt/eNOS pathway.[26] Our results also showed that inhibition of eNOS action by genetic or pharmacological manipulation in *S1pr2*$^{-/-}$ mice markedly improved vascular leakage, hypotension and survival rate after antigen challenge, thus abrogating the effects of *S1pr2* deficiency (Fig 3 and Fig E5). All these observations together indicate that $S1P_2$ provides a protective effect against vascular barrier disruption largely by suppressing eNOS.

NO regulates many biological processes including vasorelaxation, through stimulating soluble guanylate cyclase and cGMP production.[32] However, the previous study[26] showed that a specific inhibitor of soluble guanylate cyclase or genetic deletion of soluble guanylate cyclase-α1, a major isoform in the vasculature, did not protect mice from PAF-induced lethality, suggesting that NO might mediate shock in a manner independent of cGMP. In addition to guanylate cyclase–cGMP, NO may affect biological processes through causing *S*-nitrosylation of proteins.[33] AJ control endothelial barrier function.[12] Recent studies showed that VEGF induced

S-nitrosylation of β-catenin, resulting in the dissociation of β-catenin from VE-cadherin and disassembly of AJ complexes.[31, 34] In pulmonary EC, PAF and S1P induced *S*-nitrosylation of β-catenin with disassembly of VE-cadherin and β-catenin at cell-cell contacts, which was augmented by *S1pr2* deficiency (Fig 5). Of note, pharmacological blockade of eNOS restored the aggravation of *S*-nitrosylation of β-catenin and AJ disassembly by *S1pr2* deficiency. Therefore, our data together with these recent observations indicate that $S1P_2$ exerts a protective effect on barrier functions through mechanisms involving inhibition of *S*-nitrosylation of β-catenin and consequent disassembly of the AJ.

Akt is a major Ca^{2+}-independent eNOS-activating protein kinase.[35] *S1pr2* deficiency augmented activation of Akt as well as eNOS in the vasculature (Fig 2). Therefore, it is likely that increased Akt activity at least in part mediates enhanced eNOS phosphorylation in *S1pr2*$^{-/-}$ mice. Endogenously expressed $S1P_2$ is nearly exclusively coupled to $G_{12/13}$ and downstream Rho.[36] $S1P_2$ was previously shown to stimulate 3'-specific phosphatase of phosphoinositides, "phosphatase and tensin homolog deleted from chromosome 10 (PTEN)", via Rho kinase.[37] Phosphatidylinositol-3,4,5-P_3 generated by PI3K mediates Akt stimulation. $S1P_2$-mediated stimulation of PTEN decreases the cellular level of phosphatidylinositol-3,4,5-P_3, thereby resulting in inhibition of Akt activation.[37] The previous gene knockout study by Korhonen et al.[27] showed that endothelial $G_{q/11}$-mediated signaling is crucially involved in NO formation and vascular barrier disruption induced by PAF and other mediators. $G_{q/11}$ mediates activation of the calmodulin-dependent enzyme eNOS through both mechanisms of Ca^{2+} mobilization and Akt-mediated eNOS phosphorylation.[35] Hence, the study by Korhonen et al.[27] and our study together suggest that the PAF receptor, which is a $G_{q/11}$–coupled receptor,

mediates an increase in vascular permeability through eNOS stimulation whereas $S1P_2$ counteracts this $G_{q/11}$-mediated action through $G_{12/13}$-mediated suppression of the Akt–eNOS pathway.

Among the S1P receptors, $S1P_1$ has been shown to potentiate endothelial barrier integrity in vivo and in vitro.[8, 14, 38] A recent study using sphingosine kinases-null mice indicated that plasma S1P is required for vascular integrity.[16] Furthermore, administration of synthetic $S1P_1$ agonists, which powerfully induce downregulation of $S1P_1$, but also that of $S1P_1$ antagonists induced pulmonary edema.[15, 17, 39] Therefore, $S1P_1$ seems to maintain vascular integrity in a tonic manner.[16] $S1P_1$ and $S1P_2$ activate distinct signaling pathways: $S1P_1$ mediates G_i-dependent Rac activation, which is implicated in the vascular barrier integrity-maintaining action of $S1P_1$,[8, 14] whereas $S1P_2$ mediates inhibition of Rac via $G_{12/13}$.[10, 36] Moreover, $S1P_1$ stimulates eNOS phosphorylation through G_i–PI3K–Akt, unlike $S1P_2$.[40] However, the stimulatory effect of $S1P_1$ on phosphorylation of Akt and eNOS seems to be counteracted by $S1P_2$ in WT EC, as suggested by our observation that S1P increased phosphorylation of Akt and eNOS in *S1pr2*-deficient EC but not WT EC (Fig 4). These distinct signaling properties likely underlie the differential roles of $S1P_1$ and $S1P_2$ in the regulation of barrier integrity: *S1pr2* deficiency does not affect baseline vascular permeability but prevents enhanced vascular leakage after antigen challenge or injections of PAF and histamine (Figs 1, and Figs E1 and E2).[20] Consistent with this is the previous observation that baseline permeability was not affected by the presence or absence of eNOS.[22, 23] Different from $S1P_2$, $S1P_1$ maintains not only constitutive barrier integrity but protects against barrier disruption caused by anaphylaxis and inflammation.[15, 16, 39] It is likely that $S1P_1$ protects the integrity of AJ through a mechanism different from eNOS–NO regulation. Thus, these observations together with the previous

observations[27] suggest that receptor signaling mediated by $G_{q/11}$, G_i and $G_{12/13}$ play distinct critical roles in triggering, preventing and limiting vascular leakage.

We have demonstrated that $S1P_2$ suppresses stimulation of eNOS and NO generation in EC through inhibition of Akt during anaphylaxis. As a result, $S1P_2$ protects against the disassembly of endothelial AJ by inhibiting *S*-nitrosylation of β-catenin, and thereby vascular leakage. An $S1P_2$-selective agonist may have a distinct clinical benefit over an $S1P_1$-selective agonist as a therapeutic for anaphylaxis.

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Footnote

[1]Disclosure of potential conflict of interest: None

[2]On leave from Center of Medical Functional Experiment, Yanbian University College of Medicine, China

Figure Legends

Figure 1. *S1pr2* deficiency exacerbates vascular leakage in active anaphylaxis. Mice that had been sensitized with BSA were challenged with i.v. injection of BSA. (A and B) Evans blue leakage in lungs of mice 10 min after BSA challenge or saline infusion. (A) Representative lung photographs. (B) Quantification of Evans blue leakage in lung. (C) HCT 10 min after BSA challenge. (D) Survival of BSA-challenged WT and *S1pr2*$^{-/-}$ mice. Numbers in parentheses denote numbers of mice or samples analyzed throughout the Figures. The following symbols are used throughout the Figures. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. If not marked, differences were not statistically significant.

Figure 2. *S1pr2* deficiency augments PAF injection-induced phosphorylation of Akt and eNOS in aorta and lung and production of cGMP in aorta. (A-D) PAF-stimulated phosphorylation of Akt at Ser^{473} and eNOS at Ser^{1177} in aorta (A and B) and lung (C and D) of WT and *S1pr2*$^{-/-}$ mice that were i.v. injected with saline or PAF. (E) PAF injection-stimulated increase in cGMP level in aorta. Cyclic GMP was extracted and determined using an ELISA kit.

Figure 3. Genetic eNOS deficiency restores aggravation of vascular leakage after PAF injection and antigen challenge in *S1pr2* deficient mice. (A) HCT 10 min after PAF injection in WT and *S1pr2*$^{-/-}$ mice with or without *Nos3* deficiency. (B) Survival after PAF injection of WT and *S1pr2*$^{-/-}$ mice with or without *Nos3* deficiency. (C) HCT 10 min after BSA challenge in *S1pr2*$^{+/+}$ and *S1pr2*$^{-/-}$ mice with or without *Nos3* deficiency. (D) Changes in mean arterial blood pressure after BSA challenge in *S1pr2*$^{+/+}$ and *S1pr2*$^{-/-}$ mice with or without *Nos3* deficiency. (E) Survival after BSA

challenge of *S1pr2*$^{+/+}$ and *S1pr2*$^{-/-}$ mice with or without *Nos3* deficiency.

Figure 4. PAF- and S1P-stimulated phosphorylation of Akt and eNOS and production of NO are exaggerated in *S1pr2* deficient EC. (A and B) PAF (100 nM)- and S1P (100 nM)-stimulated phosphorylation of Akt at Ser^{473} (A) and eNOS at Ser^{1177} (B) in lung endothelial cells (MLEC) isolated from WT and *S1pr2*$^{-/-}$ mice. Serum-starved MLEC were stimulated as indicated for 20 min. Top: representative blots. Bottom: quantified data. (C) PAF (50 nM)-, VEGF-A_{165} (10 ng/ml)- and S1P (100 nM)-stimulated increase in NO production in MLEC. NO released into media for 3 h was determined as NO_2^- and NO_3^-.

Figure 5. PAF-induced disruption of AJ is aggravated by *S1pr2* deficiency and rescued by a NOS inhibitor in EC. (A) Immunofluorescent staining using anti-VE-cadherin (green) or anti-β-catenin antibody (red) in MLEC. MLEC were stimulated or not with PAF (100 nM) for 30 min with or without L-NAME (1 mM) in the presence of 1% fetal bovine serum. Bars, 20 μm. (B) PAF- and S1P-induced *S*-nitrosylation of β-catenin in MLEC. Serum-starved MLEC were pretreated or not with S1P (30 nM) plus L-NAME (1 mM) or S1P alone for 10 min and then stimulated with PAF (100 nM) for 30 min, followed by analysis using the biotin-switch method and western blotting with anti-β-catenin antibody. Left: representative blots. Right: quantified data of *S*-nitroso-β-catenin.

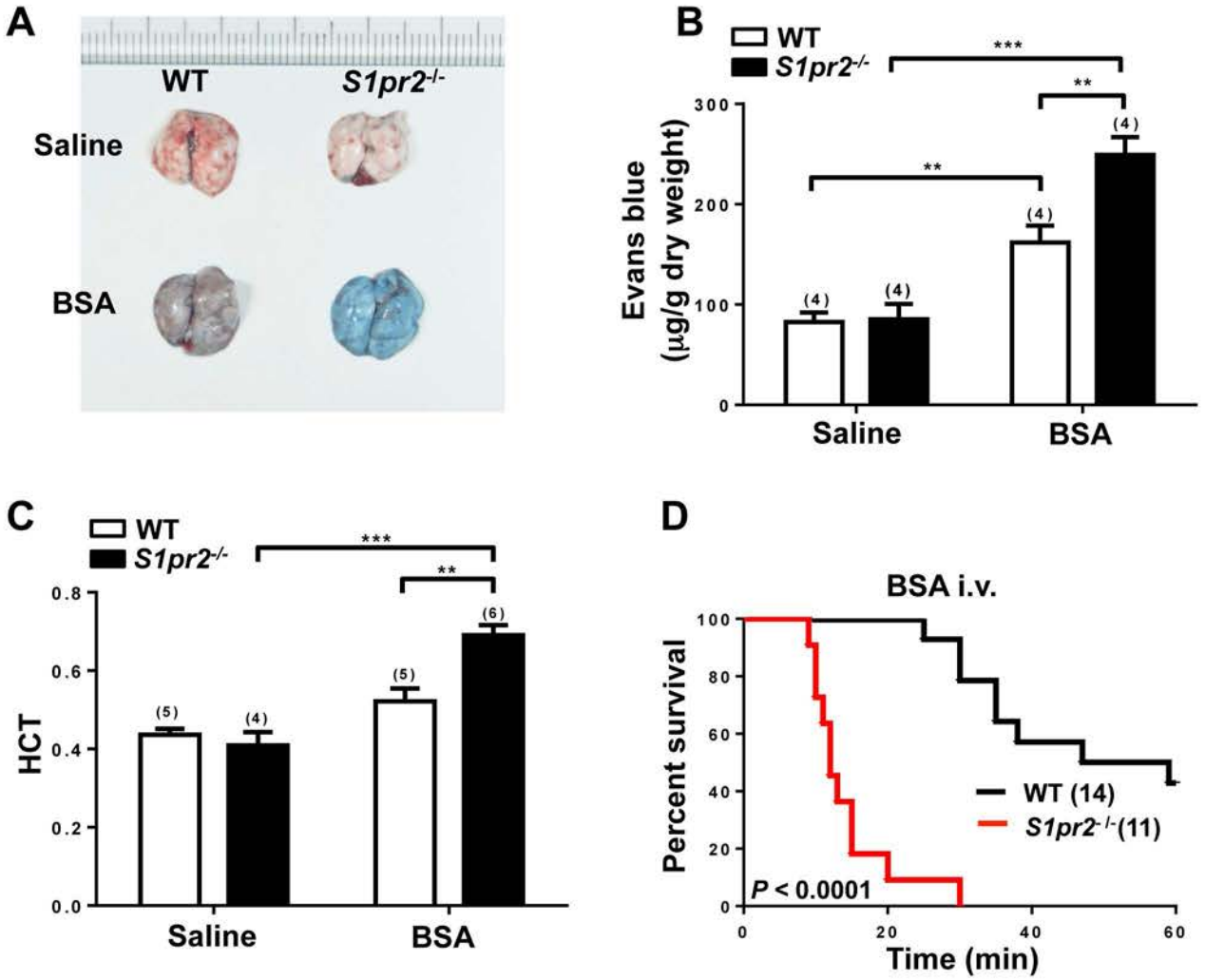
A
WT
S1pr2-/-
Saline
BSA
B
WT
S1pr2-/-
***
**
**
Evans blue
(μg/g dry weight)
300
200
100
0
(4)
(4)
(4)
(4)
Saline
BSA
C
WT
S1pr2-/-
***
**
HCT
0.8
0.6
0.4
0.2
0.0
(5)
(4)
(5)
(6)
Saline
BSA
D
BSA i.v.
Percent survival
100
80
60
40
20
0
WT (14)
S1pr2-/-(11)
P < 0.0001
0
20
40
60
Time (min)

Figure 1

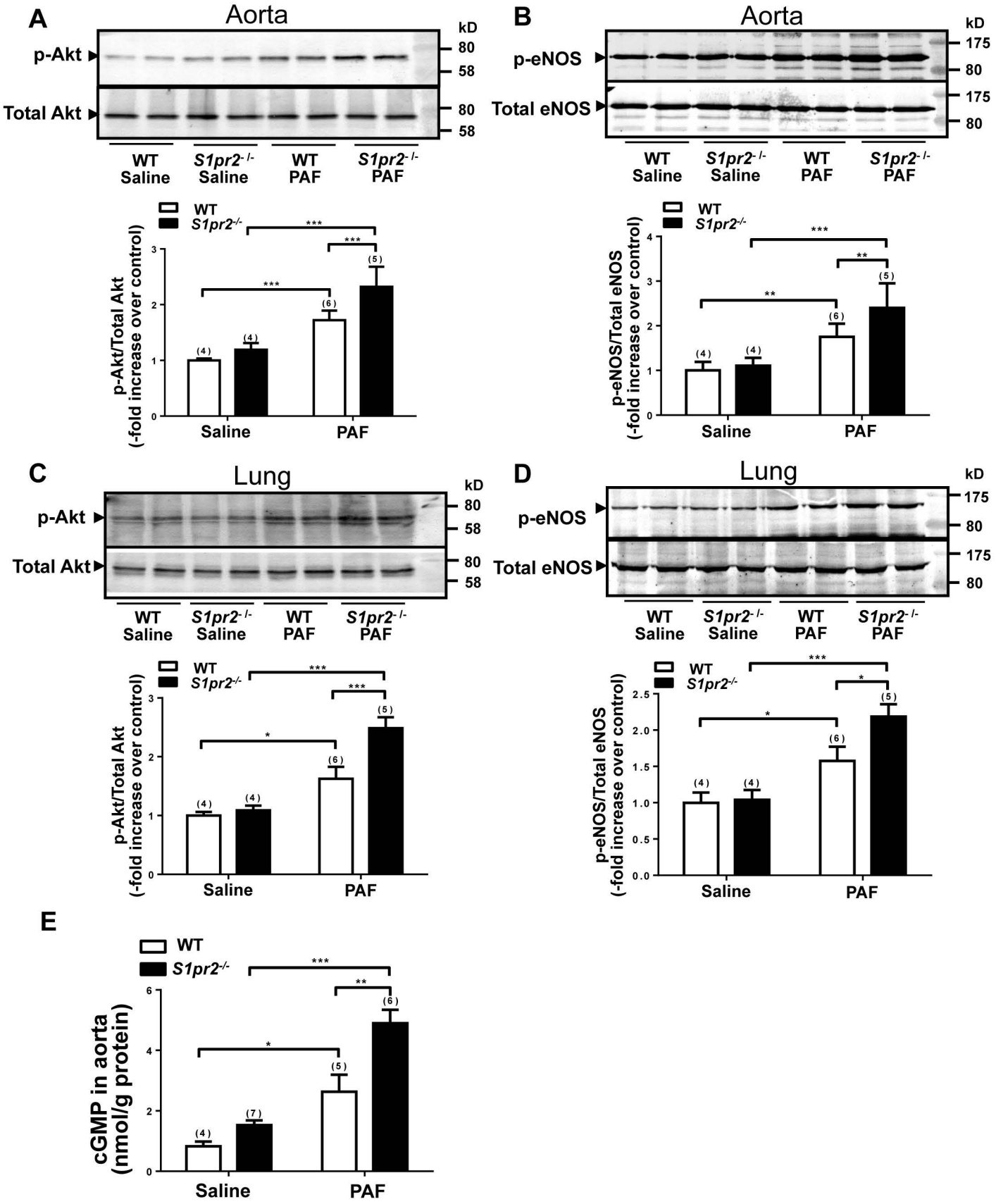
A
Aorta
p-Akt
Total Akt
kD
80
58
80
58
WT Saline
S1pr2-/- Saline
WT PAF
S1pr2-/- PAF
WT
S1pr2-/-
p-Akt/Total Akt (-fold increase over control)
Saline
PAF
B
Aorta
p-eNOS
Total eNOS
kD
175
80
175
80
p-eNOS/Total eNOS (-fold increase over control)
C
Lung
D
Lung
E
cGMP in aorta (nmol/g protein)

Figure 2

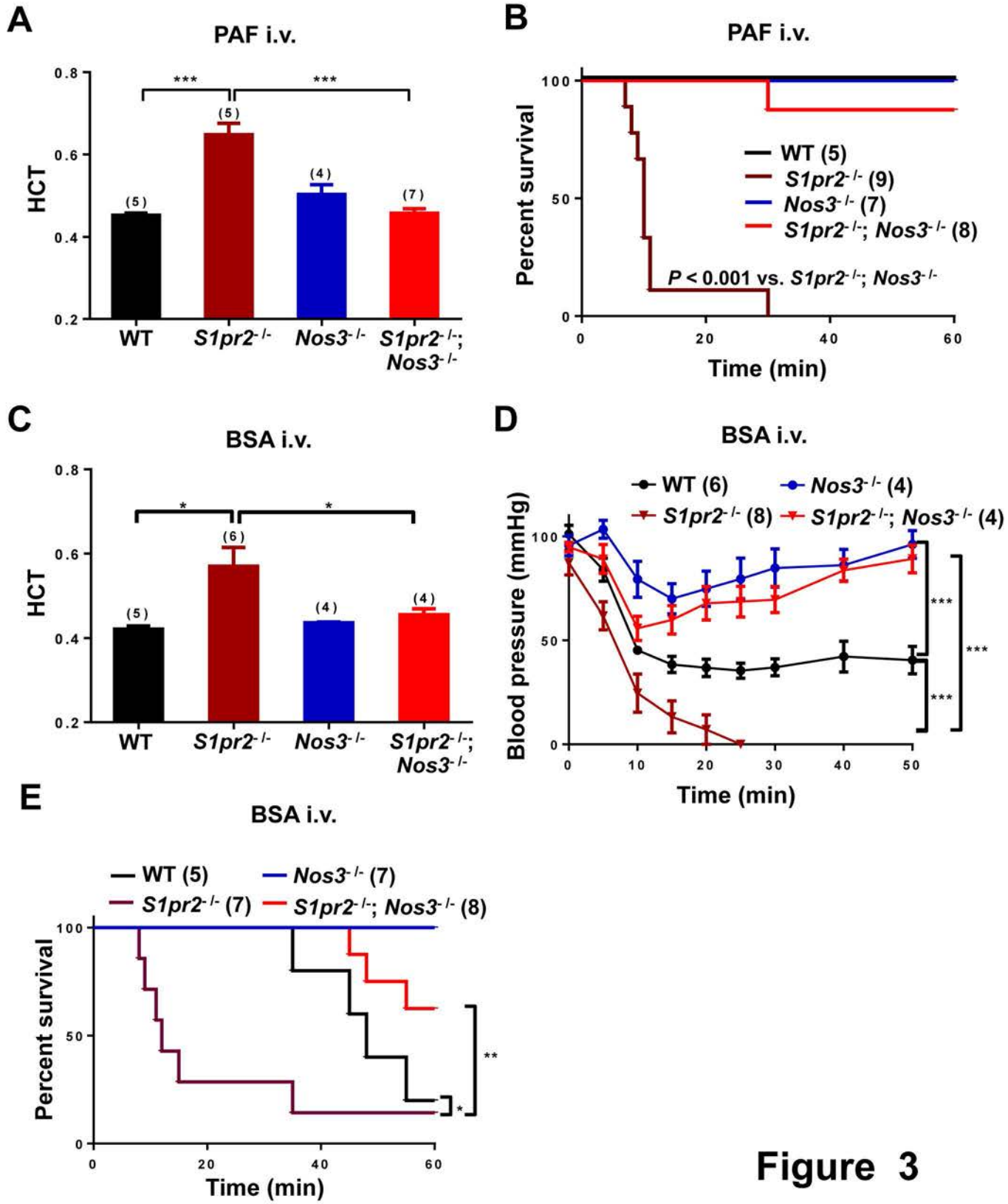
A
PAF i.v.
HCT
0.8
0.6
0.4
0.2
***
***
(5)
(5)
(4)
(7)
WT
S1pr2-/-
Nos3-/-
S1pr2-/-; Nos3-/-
B
PAF i.v.
Percent survival
100
50
0
WT (5)
S1pr2-/- (9)
Nos3-/- (7)
S1pr2-/-; Nos3-/- (8)
P < 0.001 vs. S1pr2-/-; Nos3-/-
0
20
40
60
Time (min)
C
BSA i.v.
HCT
0.8
0.6
0.4
0.2
*
*
(6)
(5)
(4)
(4)
WT
S1pr2-/-
Nos3-/-
S1pr2-/-; Nos3-/-
D
BSA i.v.
WT (6)
Nos3-/- (4)
S1pr2-/- (8)
S1pr2-/-; Nos3-/- (4)
Blood pressure (mmHg)
100
50
0
***
***
***
0
10
20
30
40
50
Time (min)
E
BSA i.v.
WT (5)
Nos3-/- (7)
S1pr2-/- (7)
S1pr2-/-; Nos3-/- (8)
Percent survival
100
50
0
**
*
0
20
40
60
Time (min)

Figure 3

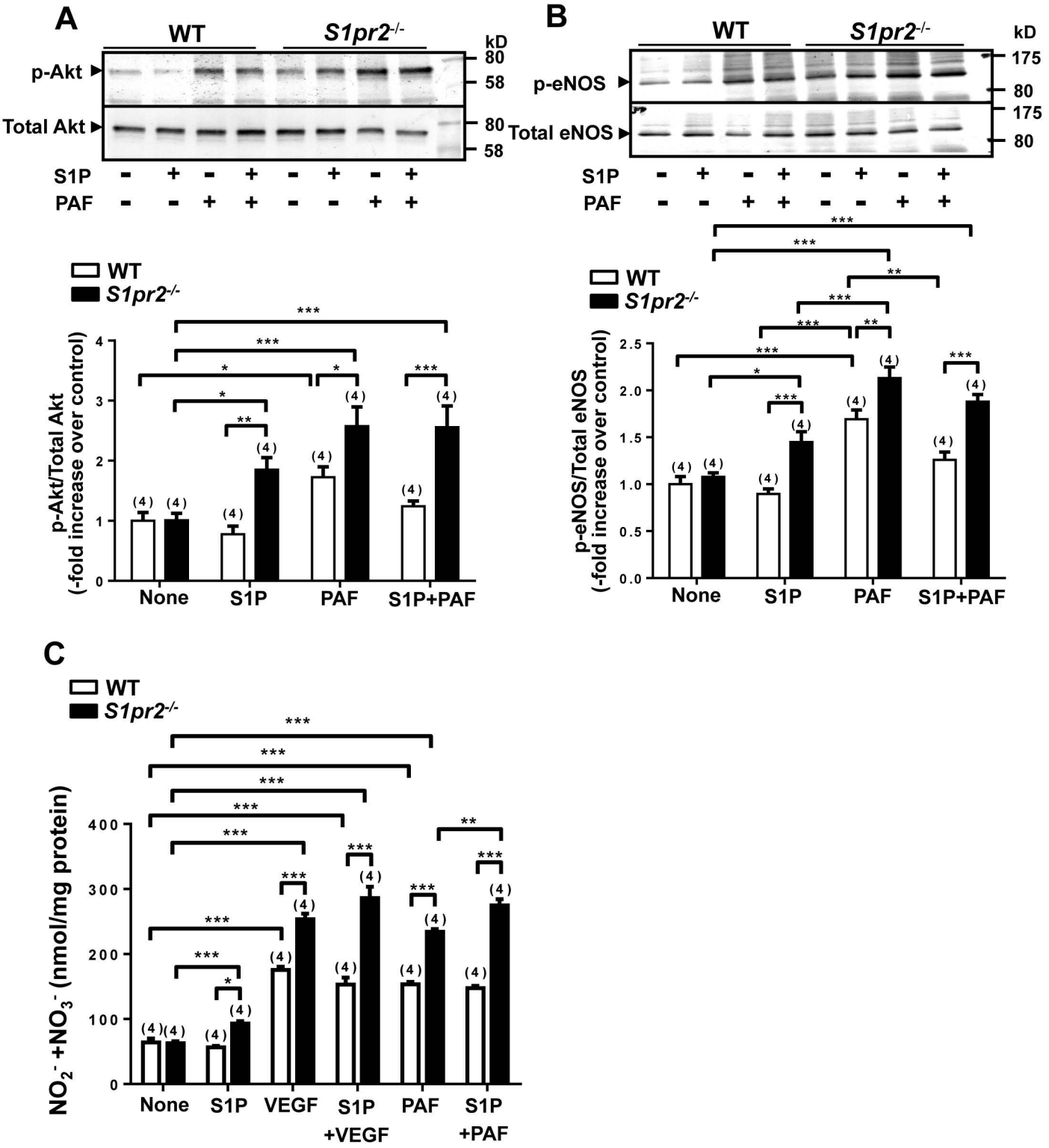
A
WT
S1pr2-/-
kD
p-Akt
Total Akt
80
58
80
58
S1P
PAF
WT
S1pr2-/-
p-Akt/Total Akt
(-fold increase over control)
None
S1P
PAF
S1P+PAF
B
WT
S1pr2-/-
kD
p-eNOS
Total eNOS
175
80
175
80
S1P
PAF
WT
S1pr2-/-
p-eNOS/Total eNOS
(-fold increase over control)
None
S1P
PAF
S1P+PAF
C
WT
S1pr2-/-
NO2- +NO3- (nmol/mg protein)
None
S1P
VEGF
S1P +VEGF
PAF
S1P +PAF

Figure 4

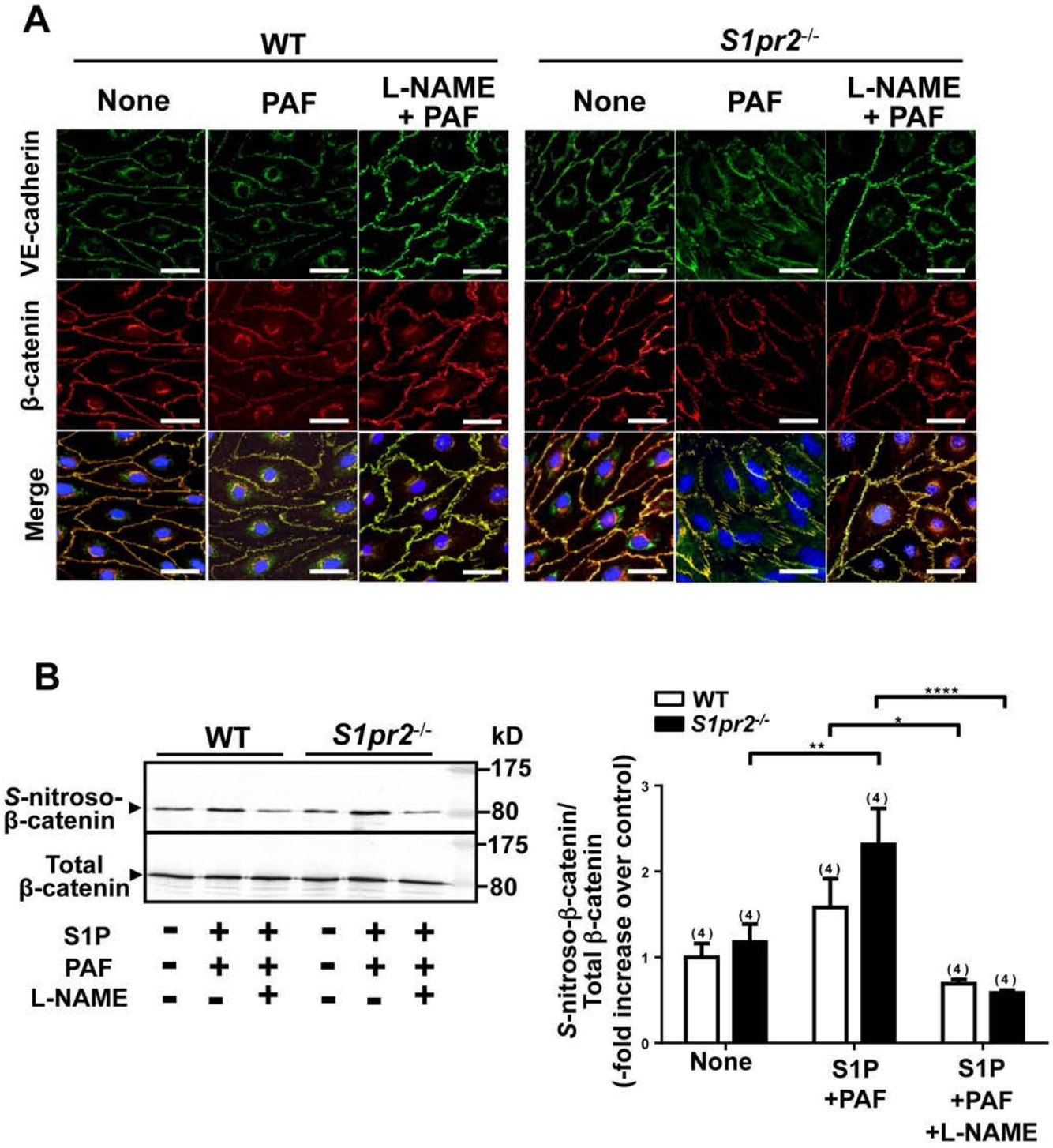
A
WT
S1pr2-/-
None
PAF
L-NAME + PAF
None
PAF
L-NAME + PAF
VE-cadherin
β-catenin
Merge
B
WT
S1pr2-/-
kD
S-nitroso-β-catenin
Total β-catenin
175
80
175
80
S1P
PAF
L-NAME
WT
S1pr2-/-
S-nitroso-β-catenin/ Total β-catenin (-fold increase over control)
None
S1P +PAF
S1P +PAF +L-NAME
**
*
****

Figure 5

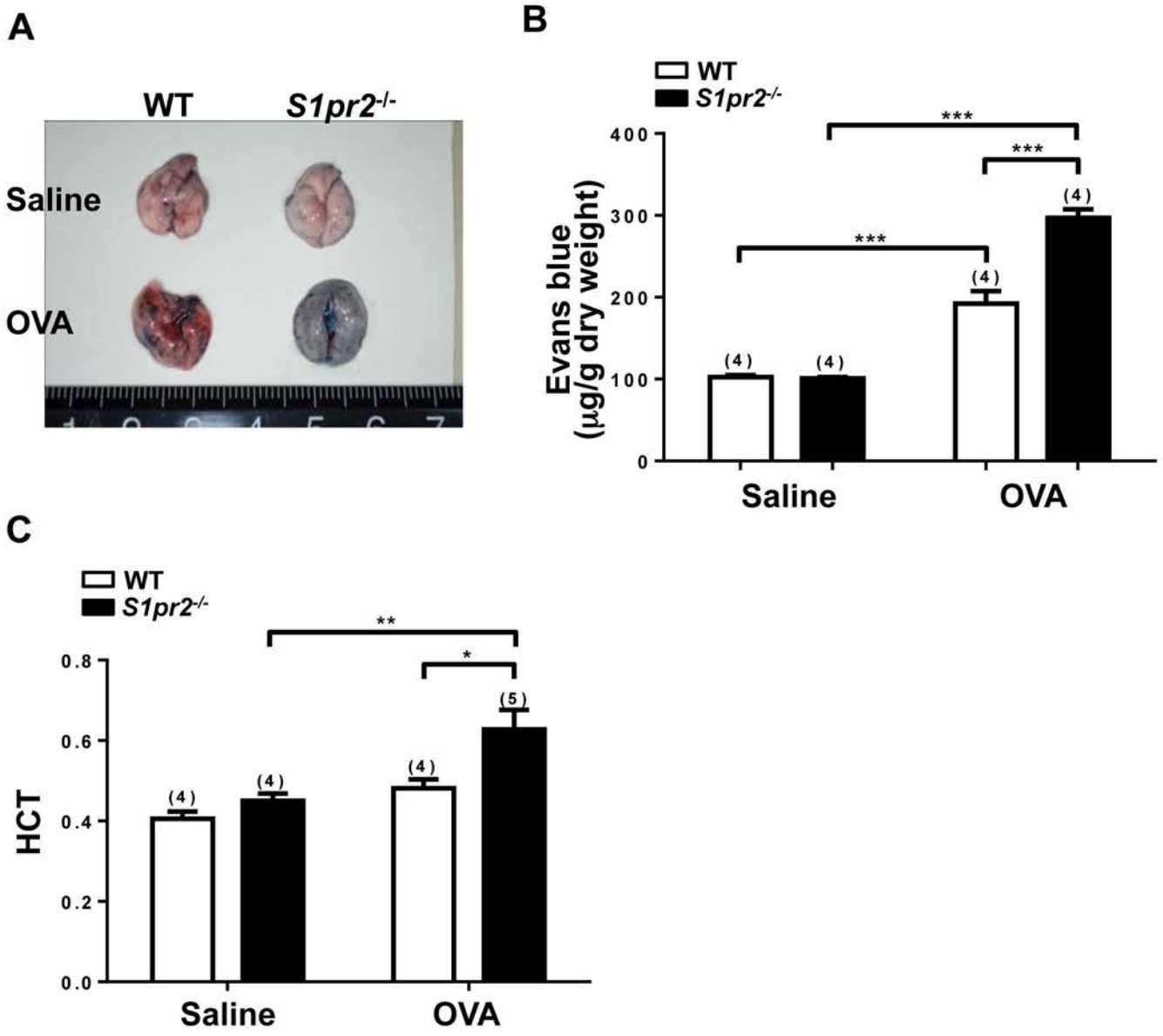
A
WT
S1pr2-/-
Saline
OVA
B
WT
S1pr2-/-
Evans blue
(μg/g dry weight)
400
300
200
100
0
***
***
***
(4)
(4)
(4)
(4)
Saline
OVA
C
WT
S1pr2-/-
HCT
0.8
0.6
0.4
0.2
0.0
**
*
(4)
(4)
(4)
(5)
Saline
OVA

Figure E1

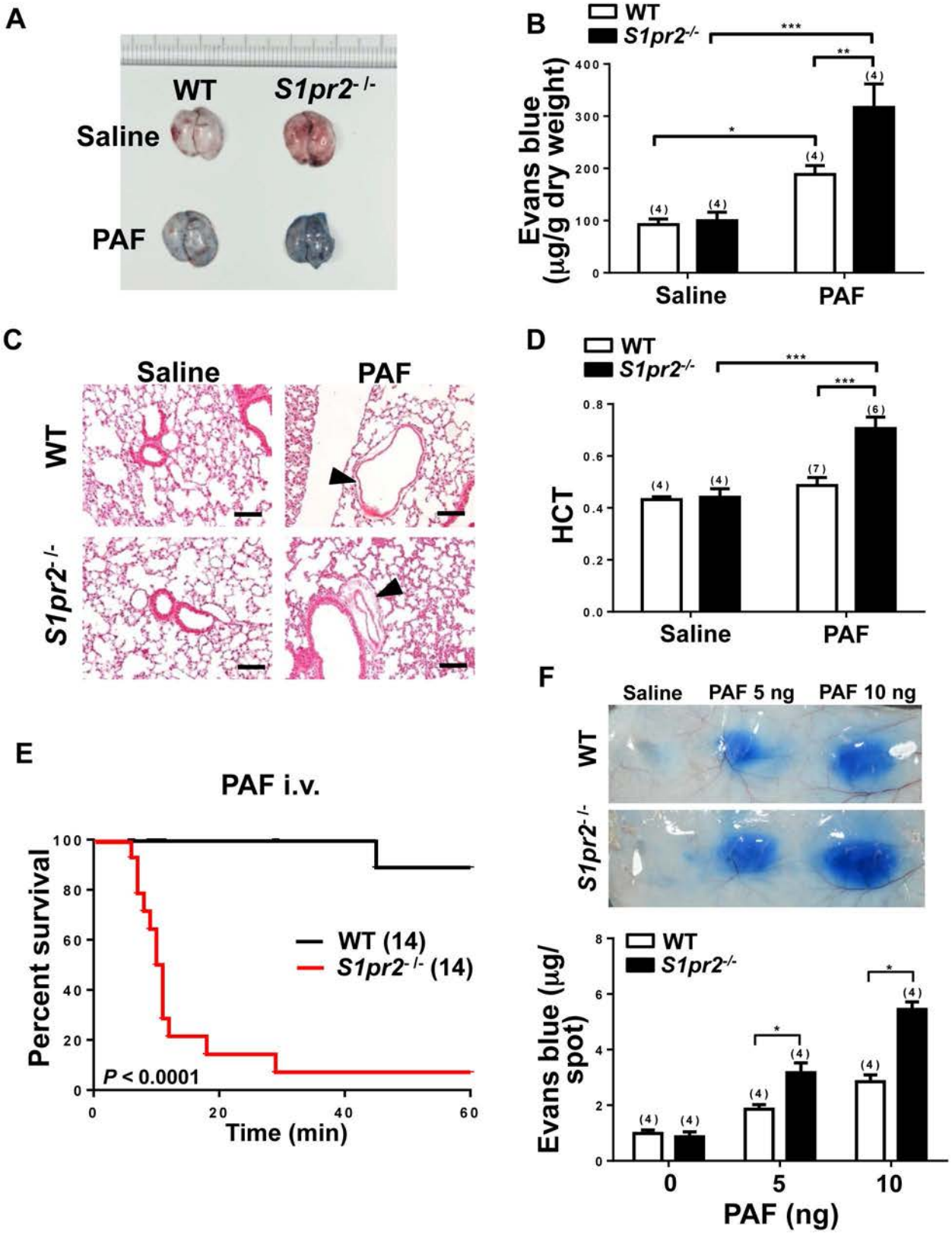
A
WT
S1pr2-/-
Saline
PAF
B
WT
S1pr2-/-
Evans blue (µg/g dry weight)
Saline
PAF
C
Saline
PAF
WT
S1pr2-/-
D
WT
S1pr2-/-
HCT
Saline
PAF
E
PAF i.v.
Percent survival
Time (min)
WT (14)
S1pr2-/- (14)
P < 0.0001
F
Saline
PAF 5 ng
PAF 10 ng
WT
S1pr2-/-
Evans blue (µg/ spot)
PAF (ng)

Figure E2

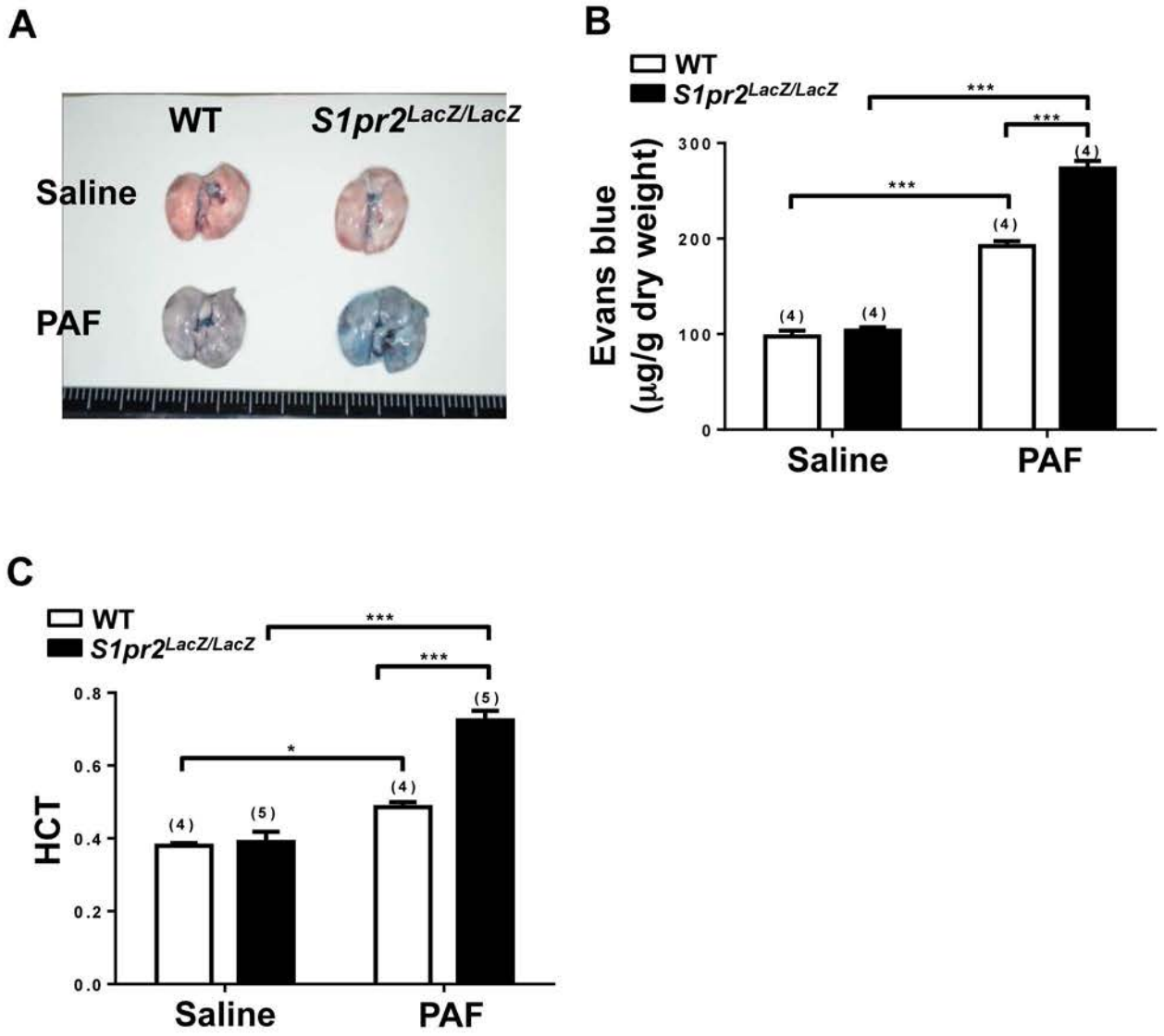
A
WT
S1pr2LacZ/LacZ
Saline
PAF
B
WT
S1pr2LacZ/LacZ
Evans blue
(μg/g dry weight)
300
200
100
0
***
***
***
(4)
(4)
(4)
(4)
Saline
PAF
C
WT
S1pr2LacZ/LacZ
HCT
0.8
0.6
0.4
0.2
0.0
***
***
*
(4)
(5)
(4)
(5)
Saline
PAF

Figure E3

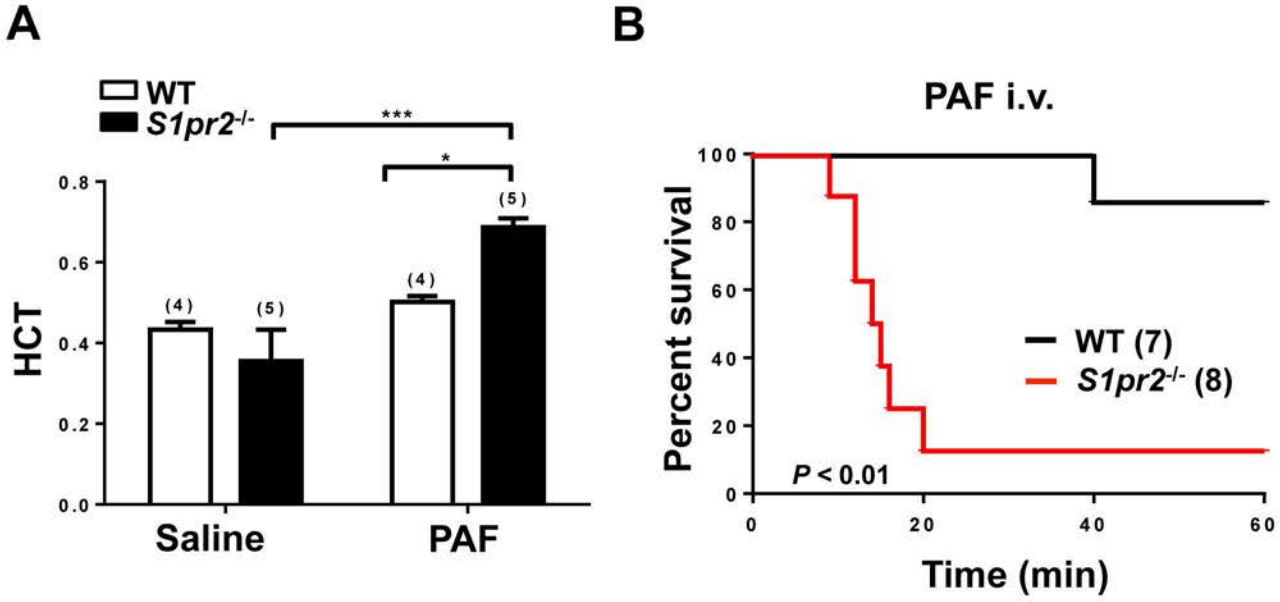
A
WT
S1pr2-/-
***
*
HCT
0.8
0.6
0.4
0.2
0.0
(4)
(5)
(4)
(5)
Saline
PAF
B
PAF i.v.
Percent survival
100
80
60
40
20
0
WT (7)
S1pr2-/- (8)
P < 0.01
0
20
40
60
Time (min)

Figure E4

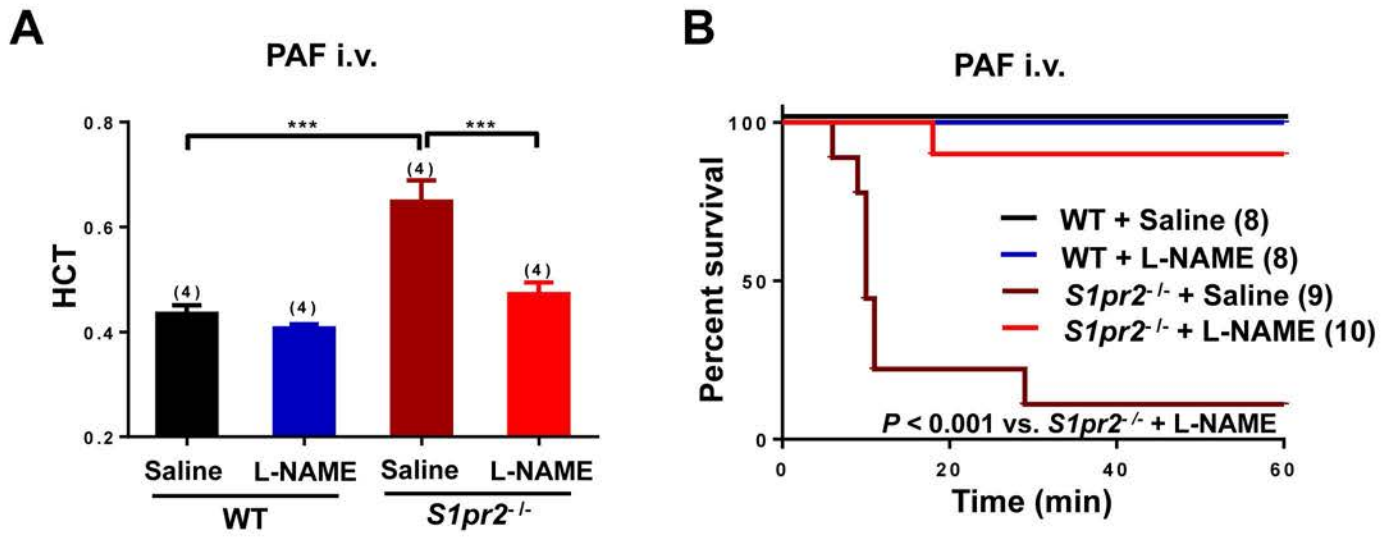
A
PAF i.v.
HCT
0.8
0.6
0.4
0.2
***
***
(4)
(4)
(4)
(4)
Saline
L-NAME
Saline
L-NAME
WT
S1pr2-/-
B
PAF i.v.
Percent survival
100
50
0
WT + Saline (8)
WT + L-NAME (8)
S1pr2-/- + Saline (9)
S1pr2-/- + L-NAME (10)
P < 0.001 vs. S1pr2-/- + L-NAME
0
20
40
60
Time (min)

Figure E5

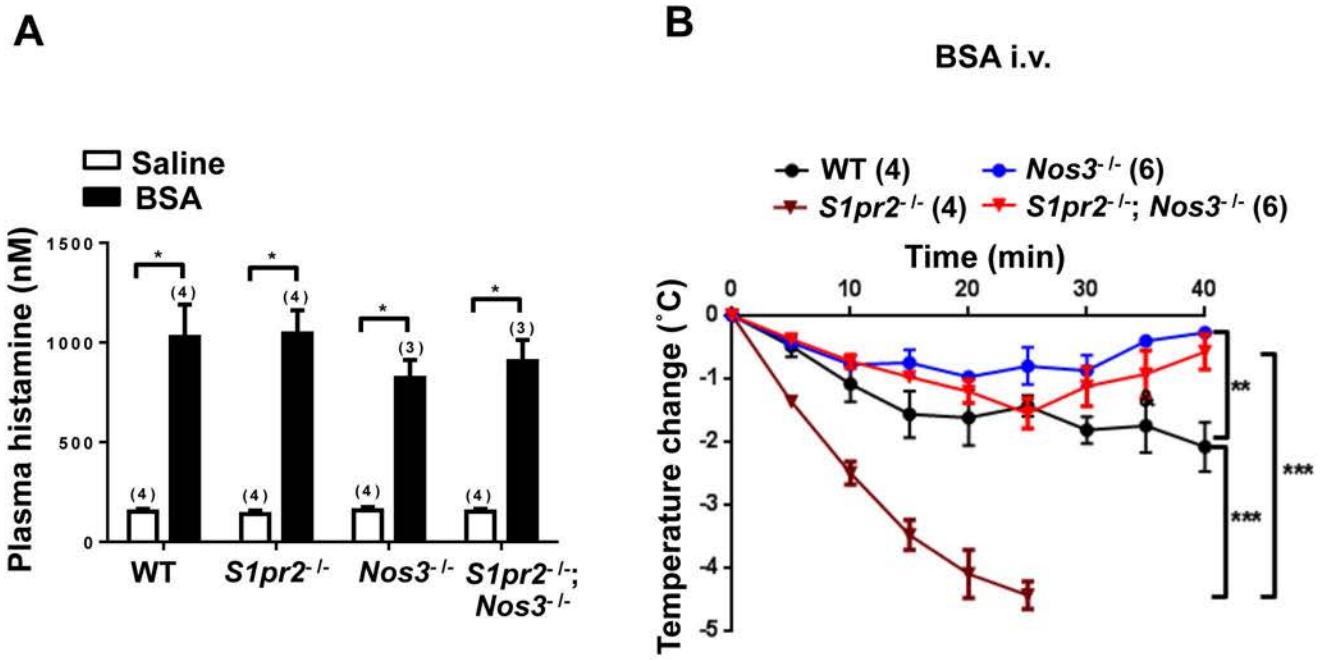
A
Saline
BSA
Plasma histamine (nM)
1500
1000
500
0
(4)
(4)
(4)
(4)
(4)
(4)
(3)
(3)
*
*
*
*
WT
S1pr2-/-
Nos3-/-
S1pr2-/-; Nos3-/-
B
BSA i.v.
WT (4)
Nos3-/- (6)
S1pr2-/- (4)
S1pr2-/-; Nos3-/- (6)
Time (min)
0
10
20
30
40
Temperature change (˚C)
0
-1
-2
-3
-4
-5
**
***
***

Figure E6