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## sphingosine -1 Phosphate Receptor-1 signaling maintains endothelial cell barrier function and protects against immune complex-induced vascular injury

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### Abstract

**Objective:** Immune complex (IC) deposition activates neutrophils (PMN), increases vascular permeability and leads to organ damage in SLE and RA. The bioactive lipid sphingosine-1-phosphate (S1P), acting via S1P receptor 1 (S1P<sub>1</sub>), is a key regulator of endothelial cell (EC) barrier function. We hypothesized that augmenting EC integrity via S1P<sub>1</sub> signaling would attenuate inflammatory injury mediated by ICs.

**Methods:** In vitro barrier function was assessed in human umbilical vein endothelial cells (HUVECs) by Electric Cell-substrate Impedance Sensing (ECIS). Phosphorylation of myosin light chain2 (p-MLC2) and VE-Cadherin staining in HUVECs was assessed by immunofluorescence. Reverse Arthus reaction (RAR) in skin and lung was performed in mice with S1P<sub>1</sub> deleted from ECs (ECKO) and mice treated with S1P<sub>1</sub> agonists and antagonists.

**Results:** S1P<sub>1</sub> agonists prevented loss of barrier function in HUVEC treated with IC-activated PMN. S1P<sub>1</sub> ECKO and WT mice treated with S1P<sub>1</sub> antagonists had amplified RAR, whereas specific S1P<sub>1</sub> agonists attenuated skin and lung RAR in WT mice. ApoM-Fc, a novel S1P chaperone, mitigated EC cell barrier dysfunction induced by activated PMN in vitro and attenuated lung RAR. S1P<sub>1</sub> agonists and ApoM-Fc markedly reduced p-MLC2 and disruption VE-Cadherin, manifestations of cell contraction and destabilization of adherence junctions, respectively, induced by activated PMN.

**Conclusion:** S1P<sub>1</sub> signaling in ECs modulates vascular responses to IC deposition. S1P<sub>1</sub> agonists and ApoM-Fc enhance the EC barrier, limit leukocyte escape from capillaries, and

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provide protection from inflammatory injury. The S1P/S1P<sub>1</sub> axis is a new target to attenuate tissue responses to IC deposition and mitigate end organ damage.

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## INTRODUCTION

Systemic lupus erythematosus and rheumatoid arthritis, though complex and heterogeneous, share the basic pathophysiologic mechanisms of immune complex (IC) deposition in tissues and neutrophil activation that cause end organ damage. Circulating ICs induce neutrophil activation by both Fc and complement receptors which trigger release of pro-inflammatory chemokines and cytokines that lead to endothelial cell (EC) barrier dysfunction and increase vascular permeability [1] [2]. Loss of EC barrier integrity has been implicated in inflammatory injury in mouse models of rheumatoid arthritis (RA) [3] and systemic lupus erythematosus (SLE) [4]. When the EC integrity is compromised, plasma proteins extravasate and neutrophils transmigrate via paracellular (inter-endothelial) routes orchestrated by activated adhesion molecules [5]. Paracellular transmigration is mediated in part by phosphorylation of VE-cadherin and transient separation of adherens junctions which creates intercellular gaps [6, 7]. Enhancing barrier integrity with pharmacological or genetic approaches has been shown to impede both vascular leak and neutrophil transmigration in animals treated with LPS or histamine, respectively, to induce vascular permeability [8, 9]. We hypothesized that augmenting EC barrier function would attenuate inflammatory injury mediated by ICs. This approach has the potential to limit IC deposition and leukocyte transmigration into tissues, yet EC integrity has not been pharmacologically targeted in either SLE or RA.

Sphingosine-1-phosphate (S1P), a bioactive lipid secreted mainly by RBCs, ECs, and activated platelets, is a key regulator of EC barrier function and lymphocyte egress out of lymph nodes and thymus [10, 11]. S1P interacts with five broadly expressed GPCRs, S1P<sub>1-5</sub>. When S1P bind EC S1P<sub>1</sub>, it stabilizes adherens junctions by increasing the translocation of GTPase Rac and VE-cadherin to the cell periphery, and resultant homotypic VE-cadherin interactions cause cell spreading and closure of intercellular gaps [12–14]. Blocking S1P degradation or administration of S1P<sub>1</sub> agonists have been shown to diminish lung inflammation in response to LPS and acute lung injury with bleomycin [15, 16]. We asked whether S1P<sub>1</sub> agonists could decrease inflammation in an animal model of IC-mediated vascular injury, the reverse Arthus reaction (RAR). In this model, ICs activate neutrophils via Fc receptors to release reactive oxygen species, proteolytic enzymes, cytokines and chemokines. Complement receptors are required in some animal models, depending upon the strain and organ in which the RAR is performed [17, 18]. Although vascular injury is a critical pathophysiologic component of the RAR, pharmacologic targeting of the EC barrier has not been studied. In this study, we show that S1P<sub>1</sub> agonists, including a novel engineered S1P chaperone - ApoM-Fc [19], prevent barrier dysfunction in response to activated neutrophil-induced injury in vitro and diminish neutrophil transmigration and red blood cell (RBC) extravasation in vivo. These data support a novel approach to limiting inflammation by enhancing EC barrier integrity and thus, have implications for the treatment of patients with IC-mediated injury.

## MATERIALS AND METHODS

### Reagents

Specific S1P<sub>1</sub> agonists, CYM-5442 (Santa Cruz Biotechnology) and SEW-2871 and a specific S1P<sub>1</sub> antagonist, W146 (Cayman Chemical), were dissolved in DMSO. A novel engineered S1P chaperone, ApoM-Fc loaded with S1P- [19], was used in vitro (10 µg/ml) and in vivo (4 mg/kg IP). Human recombinant C5a (R&D Systems; 100 ng/ml) was used to activate PMNs. The following antibodies were used for western blot and/or immunofluorescence: ApoM (ab91656, Abcam), anti-VE-Cadherin (C19, Santa Cruz Biotechnology), and anti-p-MLC2 (T18/S19, Cell Signaling).

### In vitro formation of Immune complexes

Goat anti-ovalbumin IgG (500 µg in 300 µl PBS, MP Biomedicals) was incubated with 50 µg ovalbumin for 60 min at 37°C. ICs were kept at 4°C overnight, and then centrifuged for 5 min at 10,000 g. After washing in cold PBS, the pellet was resuspended in 300 µl PBS. Previous work by other laboratories has shown that goat IgG binds human and mouse Fc receptors [20, 21].

### Human umbilical vein endothelial cell (HUVEC) culture and Electrical Cell Substrate Impedance (ECIS) assays

HUVECs (passages 2 to 8, 1.5X10<sup>5</sup>), were cultured on fibronectin-coated 8 well ECIS plates (8W10E, Applied BioPhysics) in M199 supplemented with HEPES, 10% heat-inactivated FBS, 10 mM Hepes, penicillin and streptomycin (50 mg/ml), and EC growth factors (Sigma). Cells were plated 18 hrs before use and were serum starved for 2 hrs. In experiments using PMNs, ICs (1:20 dilution) and C5a (100ng/ml) were added to HUVECs 15 min prior to the addition of PMNs. HUVECs treated with unactivated neutrophils were used as a control to validate each experimental run. A AC current (4 kHz) was applied (ECIS Theta, Applied Biophysics), and resistance measurements were obtained every 5 min.

### Neutrophil isolation and activation

10 ml of human blood from healthy donors was collected in EDTA. Neutrophils (PMN) were isolated using Polymorphoprep™ (Axis-Shield), washed in HBSS without cations, resuspended at 1–5X10<sup>5</sup>, and used within 1 hr of isolation. Protocols for human subjects were approved by Institutional IRB at HSS and written informed consent was obtained.

### Immunofluorescence staining

HUVECs (3X10<sup>5</sup>) were cultured on fibronectin-coated culture slides and used when confluent. HUVECs were serum starved for 2 hrs before addition of S1P<sub>1</sub> agonists or antagonists or ICs (1:20 dilution) and C5a (100 ng/ml). PMNs (1X10<sup>5</sup>) were added 10 min after the addition of IC and C5a. After a 30 min incubation at 37°C, supernatants were discarded and wells were washed in PBS. Cells were fixed in PFA (4%) at RT and permeabilized with Triton-X100 (0.1%). The cells were blocked with 1% FBS for 1hr at RT. To detect phosphorylated myosin light chain2 (p-MLC2) and VE-cadherin, samples were incubated with anti-p-MLC2 (5 µg/ml) and anti-VE-cadherin (5µg/ml) antibodies overnight.

After 3 washes, samples were incubated with fluorescent secondary antibodies (Invitrogen, Alexa 488 and Alexa 568) for 1 hr at RT. Samples were imaged using an Olympus FluoView FV10i confocal microscope.

## Mice

Animal experiments were performed under the guidelines set by the Institutional Animal Care and Use Committee at Weill Cornell Medicine, New York. Male C57BL/6J mice (8–10 weeks) were obtained from Jackson Laboratories. EC specific gene deletion of S1PR1: *S1pr1<sup>fl/fl</sup>* mice [22] were crossed with *Cdh5-CreERT2* mice [23] to establish Cre positive *S1pr1<sup>fl/fl</sup>* (S1P<sub>1</sub> ECKO) mice and Cre negative controls. To induce EC knockdown of S1P<sub>1</sub>, 8–10-week-old Cre+ *S1pr1<sup>fl/fl</sup>* mice and control Cre- *S1pr1<sup>fl/fl</sup>* mice were treated with tamoxifen (10mg/ml, 200  $\mu$ l IP) for 5 days. Deletion of S1P<sub>1</sub> exon 2, containing the entire coding region, was confirmed by PCR (supplemental Figure 1). Mice were used 2–4 weeks after tamoxifen administration. *Apom<sup>-/-</sup>* mice were a gift from L. B. Nielsen and C. Christoffersen, Copenhagen, Denmark [24]. Absence of apolipoprotein M was confirmed on western blot of mouse plasma (supplemental Figure 1).

## Reverse Arthus Reaction in skin

Anesthetized mice were shaved and injected with intradermal goat anti-ovalbumin IgG (60  $\mu$ g/30  $\mu$ l) or PBS alone. Immediately thereafter, ovalbumin (400  $\mu$ g; Sigma) and Evans Blue (EB; 0.5% in 150  $\mu$ l PBS) were injected via tail vein. Some mice received intradermal W146 (10 $\mu$ g) or vehicle concomitantly with anti-ovalbumin IgG. After 4 hrs, mice were sacrificed and the diameter of EB leakage was measured. 6 mm punch skin samples were weighed and treated with formamide (500  $\mu$ l at 60°C overnight) to extract EB and supernatants were measured at 610 nm. In preliminary experiments, we found that nonspecific goat IgG injected intradermally did not elicit a reaction after IV injection with ovalbumin and EB and that injection of goat anti-ovalbumin IgG intradermally did not elicit a reaction after IV injection of EB without ovalbumin.

## Reverse Arthus Reaction in lung

Mice were anesthetized and goat anti-ovalbumin IgG (250  $\mu$ g) was administered intranasally; immediately thereafter, mice were injected with IV 0.5% EB and ovalbumin (750  $\mu$ g in 150  $\mu$ l PBS). CYM-5442 (0.5mg/kg) or SEW-2874 (10 mg/kg) was administered IP (in 25% tween: 75% water) starting 2 hrs prior to RAR and then every 5 hrs. In some experiments, W146 (10  $\mu$ g) or vehicle was administered intranasally concomitantly with anti-ovalbumin IgG. 24 hrs after RAR, mice were anesthetized and broncho-alveolar lavage (BAL) was performed. 500  $\mu$ l of PBS with 1mM EDTA was administered and collected 3 times. Samples (300  $\mu$ l) were stained with Wright-Giemsa after cytopsin. Cell counts were obtained with a hemocytometer (ADVIA) and the number of PMN was calculated by multiplying the total number of WBCs by the percentage of PMN assessed on cytopsin.

## Western Blotting

Plasma samples (1  $\mu$ l) from ApoM<sup>-/-</sup> and WT mice were denatured at 95°C for 5 min after addition of 10%  $\beta$ -mercaptoethanol. Proteins were electrophoresed and transferred onto

nitrocellulose. Transferred proteins were blocked for 1hr and then probed with anti-ApoM antibody (10 µg/ml).

### Statistics

All data were presented as the mean ± standard error of mean (S.E.M). Comparisons between different groups were made using the unpaired T-test. Statistical significance was set at P<0.05.

## RESULTS

### Mice deficient in EC specific S1P<sub>1</sub> show amplified inflammation and hemorrhage in response to lung RAR

The RAR is a well-established model of acute IC-mediated vascular injury and inflammation characterized by neutrophil activation and vascular leak [25, 26]. Because IC deposition in microvessels correlates with vascular permeability [27], we posited that decreased EC barrier function and the consequent increase in IC deposition would enhance inflammation. To test this hypothesis, we performed lung RAR in mice with ECs lacking S1P<sub>1</sub> (EC knock out, ECKO). At baseline, S1P<sub>1</sub> ECKO mice injected with IV EB alone showed increased pulmonary extravasation of EB compared to controls, as previously reported [28]; notably, BAL fluid from S1P<sub>1</sub> ECKO mice injected with EB did not show increased numbers of RBCs or WBCs (data not shown). In contrast, after RAR, S1P<sub>1</sub> ECKO mice demonstrated marked increase in EB extravasation and lung weight (Figures 1A and B) indicative of vascular leak. BAL from S1P<sub>1</sub> ECKO mice exposed to RAR also showed a greater number of WBCs and RBCs compared to Cre-negative littermate controls (Figures 1C and D). WBCs in BAL fluid were comprised mostly of neutrophils (range: 50–80%) and alveolar macrophages (Figures 1E and F). Taken together, these findings demonstrate impaired barrier function and increased inflammation in response to RAR in S1P<sub>1</sub> ECKO mice.

### W146, a specific S1P<sub>1</sub> antagonist, increases lung and skin RAR

ApoM, a constituent of high density lipoprotein (HDL), carries approximately 65% of circulating S1P while albumin carries 30%. Mice lacking ApoM have a 50% reduction in circulating S1P compared to controls [29], and ApoM delivery of S1P is more protective of barrier function and more anti-inflammatory than albumin delivery [30] [31]. Therefore, we hypothesized that *ApoM*<sup>-/-</sup> mice would have an increased inflammatory response to RAR. Surprisingly, *ApoM*<sup>-/-</sup> mice and WT mice had similar numbers of PMN and RBCs in BAL fluid after RAR (Figure 2A and B) and similar EB extravasation in their lungs. ApoM deficiency was confirmed by western blot of plasma samples (supplemental Figure 1B). We considered the possibility that local levels of S1P were elevated by platelets and/or EC release during RAR and obscured the effects of ApoM deficiency. At higher levels of S1P (~500nM), albumin and ApoM have been shown to be equally effective at reducing TNF-α induced VCAM-1 in ECs in vitro [32].

We hypothesized that partial blockade of S1P<sub>1</sub> with a S1P<sub>1</sub> antagonist would render *ApoM*<sup>-/-</sup> mice more vulnerable to IC-mediated injury than WT mice, in spite of high local levels of albumin S1P. In preliminary studies, systemic administration of W146 IV (10 mg/kg or a

total of 250 µg) induced significant pulmonary leakage, as has been published ([33], supplemental Figure 2C) and we verified that W146 decreased baseline HUVEC resistance in ECIS (supplemental Figure 2a). We reasoned that administration lower doses of intranasal W146 (10 µg) would not induce vascular leakage at baseline, but would limit S1P<sub>1</sub> availability and unmask the protective effect of ApoM-mediated delivery of S1P during RAR. Indeed, low dose W146 administered intranasally to *ApoM*<sup>-/-</sup> concomitant with anti-ovalbumin IgG markedly increased extravasation of RBCs and PMNs from blood vessels into the alveolar space (Figures 2B and C), while treatment of WT mice with W146 at the same dose did not alter RAR. These data demonstrate that decreased signaling of S1P<sub>1</sub> (induced by partial blockade of S1P<sub>1</sub> with W146) increases vulnerability to endothelial barrier dysfunction during IC-mediated inflammation which can be overcome by delivery of S1P by ApoM.

Because IC-mediated injury in skin is common to patients with SLE, we sought to confirm that modulation of S1P<sub>1</sub> signaling also affects skin RAR. Similar to lung RAR, there was no evidence that ApoM deficiency increased skin RAR, and intradermal injection of low doses of W146 alone did not increase EB leak (supplemental figure 3). However, in contrast to our findings in the lung RAR in WT mice, low dose W146 (10 µg) increased the skin RAR when administered concomitantly with anti-ovalbumin IgG, compared to reaction sites (Figures 2C–E). The finding that low dose W146 augmented skin RAR, but not lung RAR in WT mice may be explained by a lower density of S1P<sub>1</sub> in skin compared to lung [34]. These data show that low doses of locally administered W146 does not induce skin or lung leakage at baseline but augments vascular leak in IC-mediated injury in skin of WT mice and in lung of *ApoM*<sup>-/-</sup> mice. These data further support the concept that blockade of S1P<sub>1</sub> receptors increases inflammation in response IC deposition.

### **S1P<sub>1</sub> agonist CYM-5442 increases barrier function, and mitigates vascular injury induced by activated PMN injury in vitro and in vivo.**

Given that blockade of S1P<sub>1</sub> signaling increases IC-driven injury, we hypothesized that augmentation of EC barrier function would limit inflammation in response to ICs. First, we first tested the ability capacity of CYM-5442, a specific S1P<sub>1</sub> agonist, to increase EC barrier function in vitro with ECIS. CYM-5442 induced a rapid increase in EC resistance at doses of 50–200 nM that returned to baseline after 1.5–2 hrs (supplemental figure 2B; n=3). Then we modeled the neutrophil-mediated EC injury characteristic of the RAR in vitro by exposing HUVEC to PMNs activated by IC and C5a and assessed barrier function in the presence or absence of S1P<sub>1</sub> agonists. No detectable changes were detected when HUVECs were treated with unstimulated PMN (Figure 3A). C5a or ICs alone did not induce a decrease in HUVEC resistance in ECIS after 1–2 hrs (data not shown) but C5a- and IC-activated PMNs caused a marked decrease in resistance within 30 min that was sustained for 8 hrs (Figure 3A). CYM-5442 (200nM) rescued the PMN-induced drop in resistance (Figure 3A), supporting the possibility that CYM-5442 diminishes inflammatory injury in the RAR by protecting EC barrier function.

Because CYM-5442 is a S1P<sub>1</sub> agonist that can act as a functional antagonist, inducing internalization and degradation of S1P<sub>1</sub> in a dose dependent manner, we first determined the

dose of CYM-5442 that induced EB leakage. At 10 mg/kg, a dose that has been shown to induce maximal lymphopenia and yield plasma concentrations of approximately 1 $\mu$ M [35], there was significant EB leakage (supplemental figure 2C, 3<sup>rd</sup> panel). Because our ECIS experiments showed that CYM-5442 was protective of EC barrier at concentrations of 50–200 nM, we hypothesized that administration of CYM-5442 at 0.5 mg/kg to mice would yield plasma concentration such that it would function as an S1P<sub>1</sub> agonist (50–200 nM) and increase EC barrier function. In mice treated with CYM-5442 (0.5mg/kg), the lung RAR was blunted. BAL fluid contained fewer PMNs and RBCs in (Figure 3B and C), but EB extravasation or lung weights were not decreased. Treatment with a much weaker S1P<sub>1</sub> agonist, SEW-2871 (10 mg/kg; [35]) resulted in a similar attenuation of WBCs and RBCs in BAL after lung RAR, but no significant change in EB extravasation or lung weights, (supplemental Figure 4). We confirmed the protective effect of CYM-5442 for IC-driven inflammation in skin RAR. Treatment with intradermal CYM-5442 reduced vascular leak as demonstrated by a decrease in EB extravasation and tissue edema as assessed by formamide extraction of EB and skin biopsy weights, respectively (Figures 3E and F). The finding that CYM-5442 was effective at blocking EB extravasation in the skin, but not the lung, during RAR is consistent with our observation that W146 exacerbated skin, but not lung RAR, in WT mice.

#### **ApoM-Fc, a novel S1P chaperone, protects ECs from loss of barrier integrity induced by activated PMN and attenuates lung RAR.**

A novel biologic chaperone of S1P, ApoM-Fc was recently shown to increase circulating S1P levels by approximately 60% and to attenuate myocardial injury after ischemia-reperfusion injury and reduce brain injury in a mouse model of stroke [19]. In addition, ApoM-Fc increased barrier function to a greater extent than albumin-S1P and did not induce lymphopenia, suggesting specificity to activation of EC S1P receptors, particularly S1P<sub>1</sub> [19]. We examined the ability of ApoM-Fc to attenuate IC-mediated injury initiated by activated PMN in vitro and in vivo. ApoM-Fc rescued EC barrier function in HUVECs exposed to IC- and C5a- activated PMN in a sustained manner (Figure 4A). In comparison, albumin-S1P (200 nM) increased resistance, but the effect was transient (Figure 4A).

To determine whether ApoM-Fc is an effective means to deliver S1P to ECs and mitigate IC-mediated injury in vivo, we treated mice with ApoM-Fc and initiated lung RAR. ApoM-Fc attenuated the inflammatory response in lung RAR indicated by a decrease in PMN and RBCs in BAL fluid (Figure 4B and C), but like CYM-5442 and SEW-2871, there was no effect on EB leakage or lung weights (Figure 4D or E).

#### **S1P<sub>1</sub> agonists CYM-5442 and ApoM-Fc limit endothelial cell phosphorylation of MLC and VE-Cadherin junction disassembly in response to IC and C5a activated neutrophils**

Activated neutrophils have been shown to induce the Rho GTPase pathway in EC which contributes to barrier breach, vascular leak [36] and neutrophil migration [37]. Phosphorylation of myosin light chain (p-MLC) of myosin II induces RhoA-mediated assembly of stress fibers and focal adhesions, mediating cell contraction. We hypothesized that S1P protected ECs from injury induced by activated neutrophils and prevented loss of barrier integrity by inhibiting the Rho pathway. To test this experimentally, we compared p-

MLC staining in HUVECs exposed to PMN activated with IC and C5a to that of HUVECs exposed to unstimulated PMN (control HUVECs, Figure 5, top panel). Treatment with activated PMNs markedly increased HUVEC p-MLC staining, which appeared to localize to stress fibers (Figure 5, middle panel). p-MLC staining was attenuated in HUVECs pre-incubated with ApoM-Fc and then treated with IC-activated PMN, (Figure 5, bottom panel). Because Rho pathway-mediated endothelial hyper-permeability is associated with disruption of VE-cadherin at adherens junctions [38], we determined whether ApoM-Fc protected VE-cadherin localization in HUVECs treated with IC- and C5a-activated PMNs. HUVECs treated with activated PMN showed marked loss of VE-Cadherin staining, whereas HUVECs pretreated with ApoM-Fc showed mostly intact staining (Figure 5, bottom panel). Similarly, HUVEC pretreated with CYM-5442 were protected from PMN induced injury, demonstrating preserved VE-cadherin and attenuated p-MLC (supplemental Figure 5). Collectively, these data suggest that S1P agonists protect ECs from neutrophil-mediated induction of the Rho pathway.

## DISCUSSION

We present genetic and pharmacological evidence that S1P<sub>1</sub> signaling in ECs modulates vascular responses to IC deposition. Deletion of S1P<sub>1</sub> in ECs augmented vascular leakage and inflammation induced by the RAR suggesting protective role for S1P<sub>1</sub>. This was supported by evidence that W146, a specific S1P<sub>1</sub> antagonist, decreased EC barrier function *in vitro* and increased skin and lung RAR. Finally, two S1P<sub>1</sub> agonists, CYM-5442, SEW-2871, and a novel biologic chaperone of S1P, ApoM-Fc, protected EC barrier integrity from IC activated PMN induced injury *in vitro* and *in vivo*. Collectively, our results demonstrate that S1P<sub>1</sub> signaling enhances the EC barrier, limits leukocyte escape from capillaries, and provides protection from inflammatory injury. In addition, we show that S1P<sub>1</sub> agonists prevent VE-cadherin disruption and phosphorylation of MLC, a potential mechanism by which they maintain vascular barrier integrity despite pathogenic mediators released by activated PMN.

While the S1P<sub>1</sub> agonists were effective in decreasing inflammation, they did not significantly reduce EB extravasation or plasma escape from the microvasculature. Enhanced S1P<sub>1</sub> signaling during IC-mediated inflammation may not be sufficient to block transudation of small molecules, such as albumin, but it appears sufficient to prevent transmigration of PMN and leakage of RBC at sites of barrier breaks. It is also possible that S1P<sub>1</sub> agonists decrease migration of inflammatory cells at sites of inflammation, at least in part, by influencing the phosphorylation status of VE-cadherin; phosphorylation of T685 on the cytoplasmic domain of VE-Cadherin supports permeability while de-phosphorylation of T731 supports leukocyte transmigration [39]. In contrast to our findings in the lung, CYM-5442 decreased EB extravasation and skin weights in the skin RAR. This may be due to the lower density of S1P<sub>1</sub> receptors in the skin compared to the lung. Alternatively, there may be differential expression of S1P family receptors or other receptors on ECs that determine vascular barrier function in different vascular beds. The ability of low dose W146 to augment the skin, but not lung RAR, in WT mice also supports differences in S1P<sub>1</sub> density and/or other receptors influencing permeability in the skin and lung.



Increasing barrier function has been shown to limit inflammation in models of ischemia-reperfusion injury and several other models of acute injury [40] [41]. Although the RAR is also an acute model of inflammation, increasing barrier function to limit the number of activated neutrophils transmigrating into tissues may have long term consequences on organ function. PMN influx has been identified as key mediator of renal damage in nephrotoxic serum transfer, a model of IC-initiated glomerulonephritis [42]. Reducing exposure to neutrophil-derived chemokines, cytokines, reactive oxidants and proteolytic enzymes limits acute damage and allows resolution of damage. The critical role of microvascular integrity in limiting joint damage and PMN infiltration is also supported in the K/BxN serum-transfer model of rheumatoid arthritis [3]. Indeed, blood vessels in joints appear to be more susceptible to IC-mediated injury than vasculature in other tissues [43].

Despite the body of evidence demonstrating the key role of microvascular in regulation of tissue injury induced by inflammation, pharmacological targeting of ECs to increase barrier function in RA and SLE has not been pursued. Drug discovery and development for SLE and RA targeting the S1P/S1P<sub>1</sub> axis has focused solely on restricting egress of pathogenic lymphocytes from lymph nodes and induction of circulating lymphopenia [44]. Because of their ability to induce lymphopenia, S1P<sub>1</sub> antagonists have been studied in mouse models of inflammatory arthritis [45], and while blocking autoreactive T cells from entering target tissues is beneficial for reducing inflammation and organ damage, the effects on ECs may be deleterious. Indeed, prolonged treatment of mice with the S1P<sub>1</sub> antagonist NIBR-0213 to treat adjuvant-induced arthritis decreased joint inflammation, but long term administration resulted in pulmonary edema, perivascular fibrosis, and functional lung impairment [46]. Because patients with SLE and RA frequently have lung involvement, they may be more vulnerable to pulmonary vascular leak and inflammation.

The clinical efficacy of FTY-720, a nonspecific S1P receptor agonist/functional antagonist that also induces lymphopenia, for the treatment of multiple sclerosis has encouraged development of several S1P<sub>1</sub> specific agonists/antagonists for the treatment of other autoimmune diseases [44]. In animal models of lupus nephritis, both FTY-720 and a specific S1P<sub>1</sub> receptor KRP-203 induced lymphopenia, reduced renal injury, and increased survival [47, 48]. A problem with this approach is that depending on the dose and frequency of its delivery, FTY-720 has been shown to induce and/or exacerbate vascular leak in the lung and brain [49, 50]; the mechanism by which FTY-720 can impair vascular integrity involves S1P<sub>1</sub> phosphorylation, internalization, and proteasomal degradation [33]. CYM-5442, while efficacious at low doses in our studies, had a limited therapeutic window because it, too, increased vascular leakage at high doses consistent with its ability to induce S1P<sub>1</sub> internalization and proteasome degradation [35]. ApoM-Fc has the potential to effectively enhance delivery S1P without leading to excessive S1P<sub>1</sub> internalization. An additional advantage of ApoM-Fc is that its half-life is 4 days compared to 4–5 hrs for CYM-5442 or SEW-2871. Finally, ApoM-Fc does not induce lymphopenia, but formal studies on the effects of ApoM-Fc on leukocyte function are ongoing to determine whether it is immunosuppressive.

Novel approaches to target the S1P/S1P<sub>1</sub> axis, particularly in systemic diseases that put patients at risk for pulmonary vascular leak, are needed to augment barrier function without

causing degradation of S1P. Such interventions could be used as adjuncts to immunosuppressive therapy, allowing simultaneous targeting to decrease production of autoantibodies and to attenuate tissue responses to IC deposition. Our data support further studies in models of chronic inflammatory injury to determine whether S1P<sub>1</sub> augmented EC barrier function will mitigate end organ damage.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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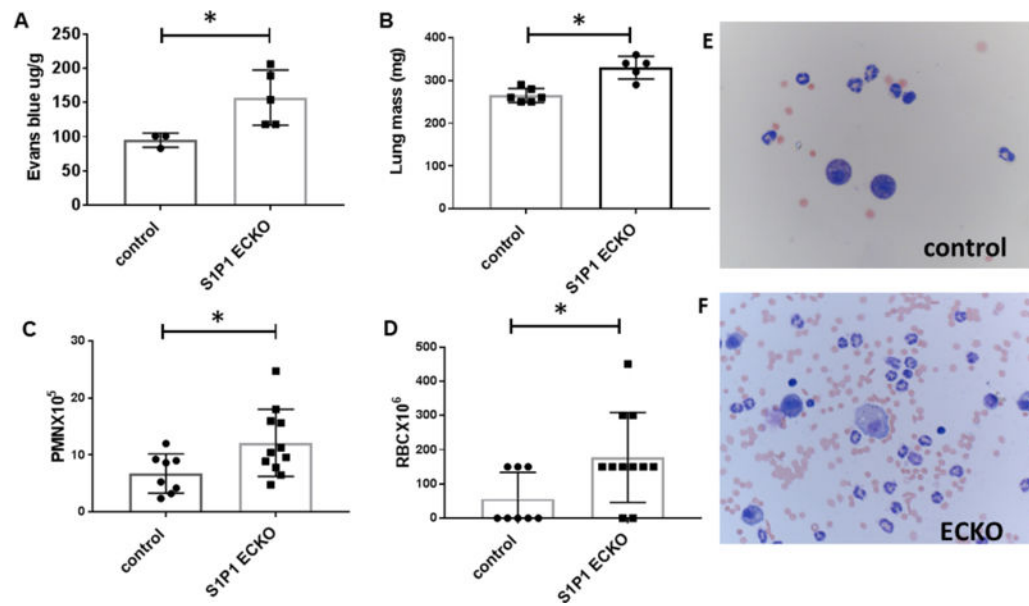
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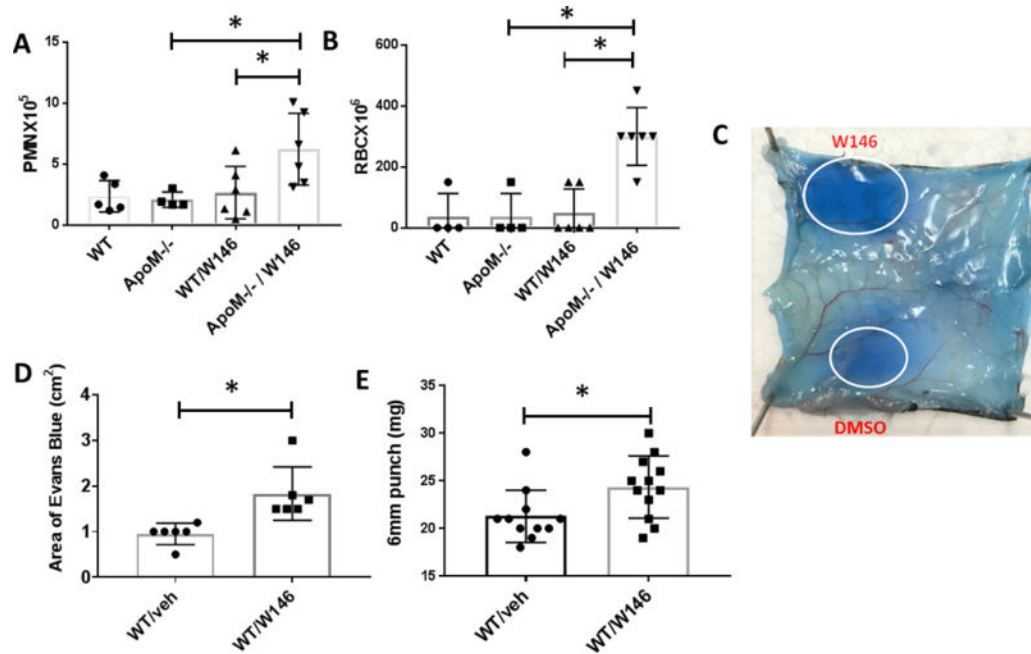
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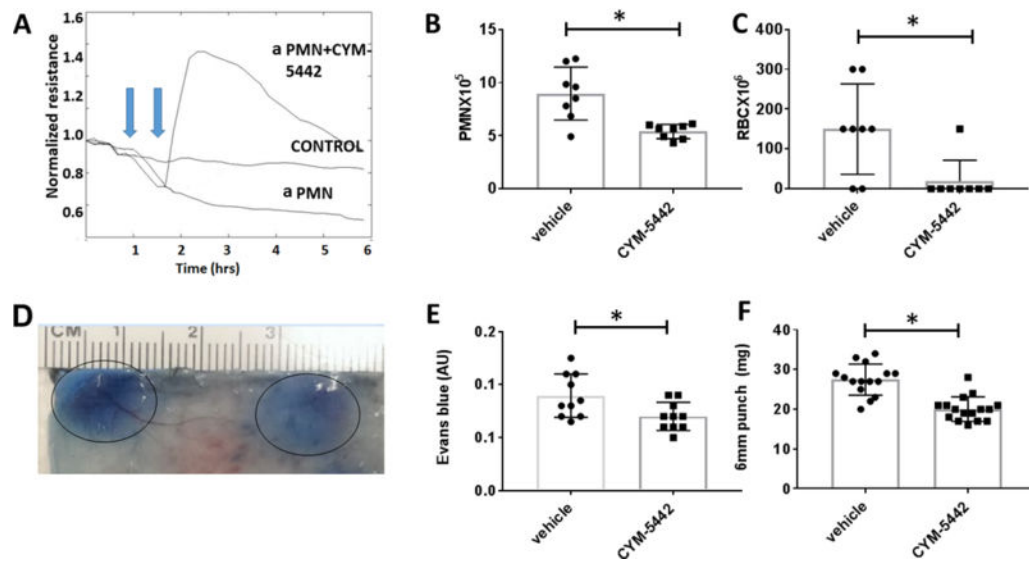
**Figure 1. S1P<sub>1</sub> ECKO mice show accentuated lung RAR compared to Cre-negative littermate controls similarly treated with tamoxifen.**

(A) After RAR, Evans blue leakage into lung was quantified by formamide extraction (n=3–5 mice/group, \*p=0.04). (B) Lung mass, a surrogate marker of edema, was measured after RAR (n=5–6 mice/group, \*p=0.0007). BAL fluid was assessed for (C) total numbers of PMNs (n=8–11 mice/group; \*p=0.03) and (D) RBCs (n=8–11 mice/group; \*p=0.03). Representative Wright-Giemsa stain of BAL fluid after lung RAR in control (upper panel, E) and ECKO mice (lower panel, F).



**Figure 2. SIP<sub>1</sub> antagonist W146 increased lung RAR in ApoM<sup>-/-</sup> mice and skin RAR in WT mice.**

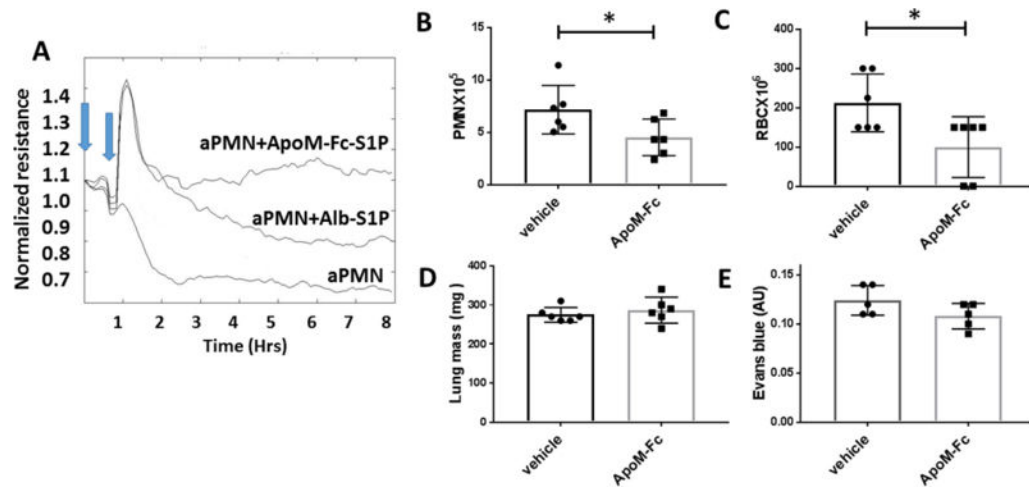
For the lung RAR, mice were treated with intranasal anti-ovalbumin IgG followed by ovalbumin with 0.5% Evans blue IV, and after 24 hrs subjected to bronchoalveolar lavage. Mice received W146 (10  $\mu$ g) or vehicle intranasally concomitant with anti-ovalbumin IgG. (A) PMN counts (n=6 mice/group \*p=0.04) and (B) RBC counts (n=6 mice/group \*p=0.005) were performed. (C) For skin RAR, mice were injected intradermally with goat anti-ovalbumin IgG or PBS, followed by ovalbumin and Evan's blue IV, and mice killed after 4hrs. Some mice received intradermal W146 (10  $\mu$ g) with anti-ovalbumin IgG. A representative skin sample after RAR with intradermal W146 or vehicle is shown. (D) Skin RAR was assessed by measuring the diameter of the major and minor axis and determining area of EB leakage at the injection site (n=6; \*p=0.006), and (E) the weight of 6 mm punch biopsies (n=11–12; \*p=0.02).



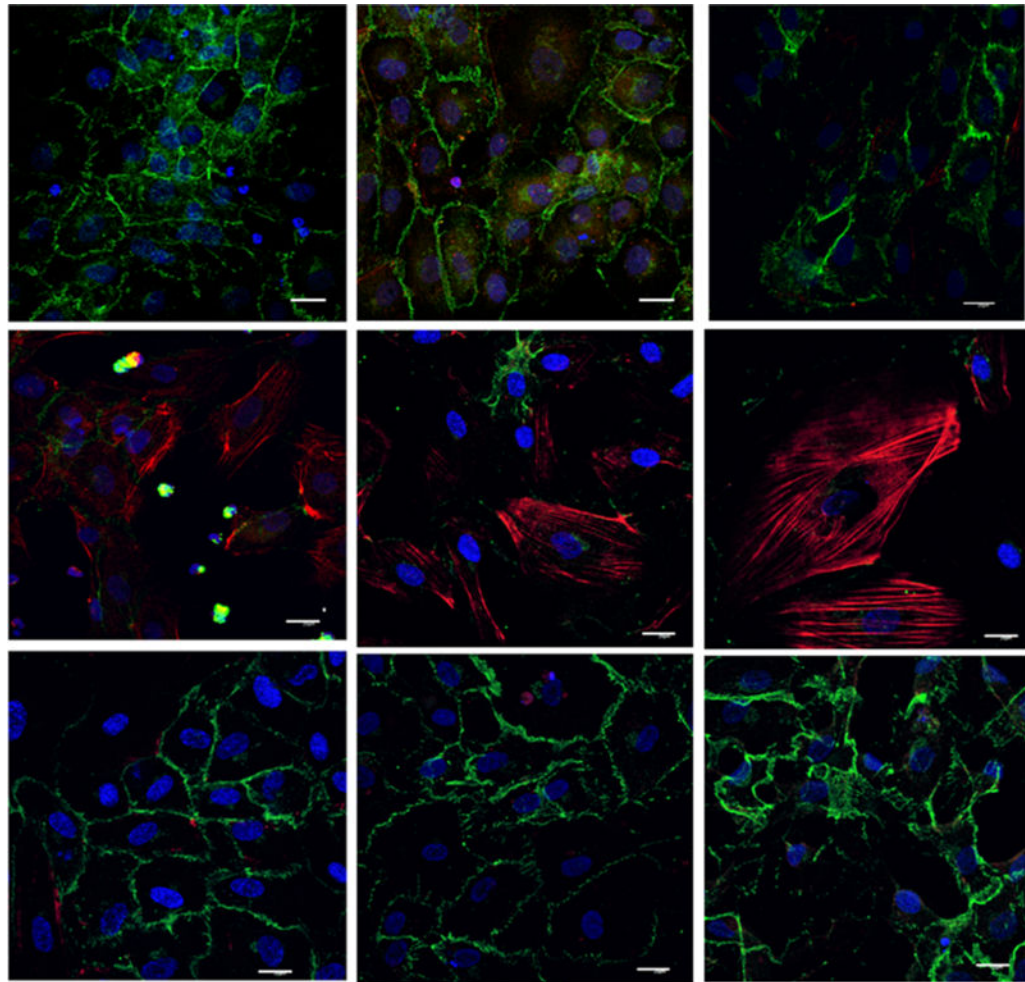
**Figure 3. SIP1 agonist CYM-5442 protected ECs against IC activated PMN induced injury in vitro and in vivo.**

(A) CYM-5442 rescued PMN-induced EC barrier dysfunction. IC and C5a activated PMN (aPMN) ( $1 \times 10^5$ ) were added to HUVECs (1<sup>st</sup> blue arrow) and resistance was measured at 5 min intervals. CYM-5442 was added 30 min (2<sup>nd</sup> blue arrow) after the addition of activated PMN, and resistance was measured at 5 min intervals. The control tracing (middle) represents ECs treated with unstimulated PMN. ECIS tracing is representative of experiments with 3 different healthy PMN donors. (B, C) Lung RAR was performed in WT C57Bl/B6 mice. CYM-5442 (0.5mg/kg IV) attenuated lung RAR as assessed by BAL PMNs (n=8 mice/group, \*p=0.002) and RBCs (n=8 mice/group, \*p=0.01). (D-F) Skin RAR was performed in WT mice with CYM-5442 or vehicle control. (D) A representative sample skin injected with CYM-5442 (10  $\mu$ g) vs. DMSO control is shown. Skin RAR was quantified by measuring (E) area of EB extravasation (n=10 mice, \*p=0.02), and (F) weights of 6 mm punch biopsies (n=15 mice; \*p<0.0001).





**Figure 4. ApoM-Fc rescued IC activated PMN induced EC injury in vitro and in vivo.** (A) IC and C5a activated PMN (aPMN) ( $1 \times 10^5$ ) were added to HUVECs (1<sup>st</sup> blue arrow) and 30 min later (2<sup>nd</sup> blue arrow) ApoM-Fc ( $10 \mu\text{g/ml}$ ) loaded with S1P was added or albumin-S1P (Alb-S1P,  $200 \text{ nM}$ ). EC resistance was measured in ECIS at 5 min intervals. ECIS tracing is representative of experiments with 3 different healthy PMN donors. (B-E) Mice were treated with ApoM-Fc or PBS at the initiation of lung RAR. At 24 hrs inflammation and injury was assessed by (B) PMNs in BAL ( $n=6$  mice/group,  $* p < 0.05$ ), (C) RBCs in BAL ( $n=6$  mice/group;  $p=0.03$ ), (D) lung mass (E), and EB extravasation.



**Figure 5. ApoM-Fc decreases myosin light chain phosphorylation (p-MLC) and attenuates disruption of VE-cadherin in HUVECs treated with activated PMNs.**

HUVECs were treated for 30 min with IC and C5a activated PMNs ( $1 \times 10^5$ ) and stained for VE-Cadherin (green) and p-MLC (red). Nuclei were stained with DAPI. Top panel: unstimulated PMN, middle panel: IC and C5a activated PMN; bottom panel: HUVECs pretreated with ApoM-Fc prior to incubation with IC and C5a activated PMN. Images representative of 3 experiments with 3 different healthy PMN donors. (scale bar = 20 microns)