

Role of sphingosine kinase 1 in allergen-induced pulmonary vascular remodeling and hyperresponsiveness

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Background: Immunologic processes might contribute to the pathogenesis of pulmonary arterial hypertension (PAH), a fatal condition characterized by progressive pulmonary arterial remodeling, increased pulmonary vascular resistance, and right ventricular failure. Experimental allergen-driven lung inflammation evoked morphologic and functional vascular changes that resembled those observed in patients with PAH. Sphingosine kinase 1 (SphK1) is the main pulmonary contributor to sphingosine-1-phosphate (S1P) synthesis, a modulator of immune and vascular functions. **Objective:** We sought to investigate the role of SphK1 in allergen-induced lung inflammation.

Methods: SphK1-deficient mice and C57Bl/6 littermates (wild-type [WT] animals) were subjected to acute or chronic allergen exposure. **Results:** After 4 weeks of systemic ovalbumin sensitization and local airway challenge, airway responsiveness increased less in SphK1^{-/-} compared with WT mice, whereas pulmonary vascular responsiveness was greatly increased and did not differ between strains. Acute lung inflammation led to an increase in eosinophils and mRNA expression for S1P phosphatase 2 and S1P lyase in lungs of WT but not SphK1^{-/-} mice. After repetitive allergen exposure for 8 weeks, airway responsiveness was not augmented in SphK1^{-/-} or WT mice, but pulmonary vascular responsiveness was increased in both strains, with significantly higher vascular responsiveness in SphK1^{-/-} mice compared with that seen in WT mice. Increased vascular responsiveness was accompanied by remodeling of the small and intra-acinar arteries.

Conclusion: The data support a role for SphK1 and S1P in allergen-induced airway inflammation. However, SphK1 deficiency increased pulmonary vascular hyperresponsiveness, which is a component of PAH pathobiology. Moreover, we show for the first time the dissociation between inflammation-induced remodeling of the airways and pulmonary vasculature. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Pulmonary arterial hypertension, allergen-induced lung inflammation, vascular remodeling, perfused mouse lung, sphingosine kinase, sphingosine 1-phosphate

Pulmonary arterial hypertension (PAH) is a progressive and fatal condition characterized by pulmonary arterial remodeling, leading to increased vascular resistance and right ventricular failure.^{1,2} In the lungs of patients with PAH, different types of structural change have been observed,^{2,3} including remodeling of the pulmonary arterial wall, endothelial dysfunction, and thrombosis.² Pulmonary arterial smooth muscle cell hyperplasia and hypertrophy are frequent.³ Beyond remodeling, pulmonary vasoconstriction is assumed to be an important early component of the hypertensive process.⁴ Together, these changes contribute to progressive PAH and right ventricular dysfunction, thereby reducing both quality of life and life expectancy for patients with PAH.¹

Idiopathic PAH is rare, but PAH can be associated with a variety of clinical conditions.⁵ There is strong circumstantial evidence for an immunologic pathogenesis of PAH, which is associated with diverse inflammatory diseases, including infections, autoimmune disorders, hypothyroidism, and hypersensitivity pneumonitis.^{2,5} Thus increasing attention is being focused on the proinflammatory state of the vessel wall in the progression of PAH.⁶ Notably, in different models of allergen-driven pulmonary inflammation, we and others recently observed morphologic and functional changes that resemble those observed in patients with PAH.⁷⁻¹⁰ Perivascular immune responses^{7,8} and important features of PAH-associated vascular remodeling were noted in murine pulmonary vessels after systemic and pulmonary exposure to ovalbumin (OVA) or *Aspergillus fumigatus* antigen,^{9,10} including increased proliferation of endothelial and smooth muscle cells, enlarged diameter and length of microvessels, and increased smooth muscle mass in large pulmonary vessels with augmented expression of procollagen I and III.⁹ The alterations in the lung protein expression profile during allergic inflammation partly correlated with those observed in hypoxia,¹¹ which is known to induce PAH and increased vascular responses to vasoconstrictors.² Moreover, T_H2-driven lung inflammation evoked dramatic pulmonary vascular hyperresponsiveness.⁷

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Abbreviations used

BAL:	Bronchoalveolar lavage
CT:	Threshold cycle
G-CSF:	Granulocyte colony-stimulating factor
HPRT:	Hypoxanthine guanine phosphoribosyl transferase
MCh:	Methacholine
OVA:	Ovalbumin
PAH:	Pulmonary arterial hypertension
Ppa:	Pulmonary arterial pressure
S1P:	Sphingosine-1-phosphate
S1P1:	Sphingosine-1-phosphate receptor subtype 1
S1Ply:	Sphingosine-1-phosphate lyase
S1PP:	Sphingosine-1-phosphate phosphatase
SphK1:	Sphingosine kinase 1
WT:	Wild-type

The bioactive sphingolipid mediator sphingosine-1-phosphate (S1P) is involved in multiple signaling pathways that are coupled to both inflammation and the control of air and blood flow in the lungs.^{12,13} Activation of various plasma membrane receptors leads to a rapid increase in intracellular S1P levels through sphingosine kinase (SphK) stimulation.¹³ SphK has 2 isoforms that are differentially expressed.¹³ The highest expression of SphK1 mRNA occurs in the lung, kidney, blood, and spleen, whereas the SphK2 isoform is predominantly expressed in the brain, liver, and heart.^{12,13} S1P stimulates leukocytes, mast cells, and epithelial cells.¹³ In the lung SphK1/S1P signaling is involved in the modulation of airway tone,^{14,15} the preservation of microvascular barrier function,¹⁶ and the maintenance of vascular tone.¹²

In patients with allergic asthma, increased levels of S1P were recovered in bronchoalveolar lavage (BAL) fluid after ragweed antigen challenge. Moreover, the SphK1/S1P system was coupled to T_H2 lymphocyte trafficking,¹⁷ mast cell degranulation and chemotaxis,¹⁵ the phenotype of lung fibroblasts,¹⁸ and the development of asthma-induced airway hyperreactivity.¹⁴ In murine models of allergic asthma, SphK1 protein expression was increased.¹⁴ Specific SphK1 knockdown by small interfering RNA reduced pulmonary eosinophilic inflammation, as well as IL-4, IL-5, and eotaxin levels in BAL fluid, airway mucus production, and airway hyperresponsiveness,¹⁹ thereby suggesting a therapeutic potential of SphK modulation in allergic airway disease. On the other hand, dendritic cells, which play a crucial role in the pathogenesis of allergic asthma, express S1P receptor subtype 1 (S1P1). Stimulation of S1P1 abrogated experimental asthma by altering dendritic cell function.²⁰ Thus the exact role of the SphK1/S1P system in allergic lung inflammation, especially in T_H2-evoked pulmonary arterial remodeling, is currently unknown.

In this study SphK1-deficient mice and their littermates were investigated in both an acute and a chronic model of allergen-evoked lung inflammation. Analysis of the mRNA expression profile of enzymes involved in S1P metabolism and S1P receptors, as well as functional and morphologic analysis of airways and lung vasculature, were performed.

METHODS**Animals, sensitization, and allergen exposure**

Animal procedures were approved by local authorities.

Acute lung inflammation. Mice (C57BL/6 [wild-type [WT] and SphK1^{-/-} mice) were systemically sensitized to OVA on days 0, 14, and 21.

Nonsensitized mice (PBS/OVA) and OVA/OVA mice were exposed to aerosolized OVA on days 28, 29, and 30 and killed at day 32.

Chronic lung inflammation.²² Mice were systemically sensitized to OVA on days 0 and 14 (OVA/OVA). All mice were repetitively exposed to aerosolized OVA starting at day 21, and killed at day 51. For further details, see the [Methods](#) section of this article's Online Repository at www.jacionline.org.

Airway and vascular responsiveness, lung edema, and cytokine levels

Murine lungs were isolated, perfused, and ventilated.^{7,23} Methacholine (MCh) or U46619 was infused for 0.5 or 3 minutes, respectively, and changes in airway resistance and pulmonary arterial pressure were determined. Lung edema was quantified by assessing the wet/dry weight ratio. Cytokines were measured from BAL fluid supernatants (Bio-Rad, Hercules, Calif). For further details, see the [Methods](#) section of this article's Online Repository.

Collection of lung tissue

After flushing, the left lungs were ligated and quick frozen for RNA analysis and proteomics. The right lungs were inflated with 4% paraformaldehyde for immunohistochemical and ultrastructural analysis. For quantitative morphologic analysis, whole lungs were processed.

Real-time quantitative RT-PCR

RNA was isolated by using RNeasy (Qiagen, Doncaster, Australia). Contaminating DNA was removed on a column with DNase (Qiagen). Equal amounts of RNA were reverse transcribed (iScript, Bio-Rad), and PCR reactions were prepared in triplicate (iQ-SYBRgreen, Bio-Rad). Primers specific for hypoxanthine guanine phosphoribosyl transferase (HPRT) were used for standardization (see [Table E1](#) in this article's Online Repository at www.jacionline.org). The relative expression was calculated by using the $-\Delta\Delta CT$ method.²⁴ For further details, see the [Methods](#) section of this article's Online Repository at www.jacionline.org.

Immunocytochemistry

Serially sectioned lung specimens of 12 μ m in thickness were blocked and incubated with primary antibodies and secondary reagents (see [Table E2](#) in this article's Online Repository at www.jacionline.org) and evaluated by means of sequential scanning with a confocal microscope (TCS SP5; Leica, Bensheim, Germany).

Electron microscopy

The cardiac lung lobe was postfixed with 1% osmium tetroxide, stained *en bloc* with 2% uranyl acetate, dehydrated, cleared in propylene oxide, and embedded in Durcupan resin (Sigma, St Louis, Mo). Gold to silver sections were cut with a diamond knife at 100 nm. Sections were collected on coated slotted copper grids, stained with lead citrate, and viewed at an accelerating voltage of 80 kV (JEOL JEM 1200-EX).

Quantitative morphology

Paraffin-embedded sections stained with hematoxylin and eosin were used for stereologic quantifications.²⁵ Volume densities were determined by superimposing a grid over the image with Adobe Photoshop (Adobe Systems, Inc, Mountain View, Calif) and counting grid points intersecting arterial walls and lumina or the airway epithelium, wall, and lumen.

Statistics

Data were expressed as means $\pm$ SEMs. Differences were considered statistically significant at a *P* value of less than .05. For further details, see the [Methods](#) section of this article's Online Repository.

RESULTS

Airway and pulmonary vascular physiology and inflammation

Both naive and PBS/OVA-treated SphK1^{-/-} mice had lower airway resistance than seen in naive or PBS/OVA-treated WT mice, respectively (Fig 1, A, and data not shown). PBS/OVA-treated SphK1^{-/-} mice had lower airway responsiveness compared with that seen in PBS/OVA-treated WT mice (Fig 1, B). Pulmonary vascular resistance and responsiveness did not differ between naive groups and was not altered by age in WT mice, whereas pulmonary vascular responsiveness was decreased in 14- and 18-week-old SphK1^{-/-} mice compared with that seen in 10-week-old SphK1^{-/-} mice (See Fig E1 in this article's Online Repository at www.jacionline.org).

Acute OVA sensitization and challenge increased baseline airway resistance in both WT and SphK1^{-/-} mice (Fig 1, A) and increased airway responsiveness in OVA/OVA-treated SphK1^{-/-} and OVA/OVA-treated WT mice compared with that seen in corresponding PBS/OVA-treated mice (Fig 1, B). OVA/OVA-treated WT mice showed higher airway responsiveness compared with OVA/OVA-treated SphK1^{-/-} mice, especially at higher doses of MCh (Fig 1, B).

Pulmonary arterial pressure in isolated perfused lungs was not altered by acute allergen-induced inflammation (Fig 1, C). Pulmonary vascular responsiveness was greatly increased in both OVA/OVA-treated groups and did not differ between SphK1^{-/-} and WT mice (Fig 1, D). Immune cells were increased after allergen exposure in both WT and SphK1^{-/-} mice. The numbers of eosinophils were higher in the lungs of WT compared with SphK1^{-/-} mice (Fig 1, E).

After chronic repetitive allergen exposure for 8 weeks, neither airway resistance (Fig 2, A) nor airway responsiveness (Fig 2, B) was increased in OVA/OVA-treated mice compared with that seen in the PBS/OVA-treated groups. The numbers of eosinophils in OVA/OVA-treated mice were increased compared with those seen in PBS/OVA-treated mice (Fig 2, E) but were only 5% to 10% of those seen in acute inflammation. Surprisingly, pulmonary vascular responsiveness was further increased significantly in OVA/OVA-treated WT and SphK1^{-/-} mice after chronic allergen exposure compared with that seen in PBS/OVA WT and SphK1^{-/-} mice (Fig 2, D). Furthermore, the vascular responsiveness was significantly greater in OVA/OVA-treated SphK1^{-/-} mice compared with that seen in OVA/OVA-treated WT mice (Fig 2, D) and increased compared with that after acute exposure.

Lung cytokine profile

In acute allergen-induced inflammation, levels of cytokines, including IL-4, IL-5, IL-13, IL-12p40, RANTES, and granulocyte colony-stimulating factor (G-CSF), were increased in BAL fluid of WT and SphK1^{-/-} mice compared with levels seen in corresponding PBS/OVA-treated mice (Fig 3). BAL levels of IL-2, RANTES, KC, and G-CSF were higher in OVA/OVA-treated SphK1^{-/-} mice than in OVA/OVA-treated WT mice.

After chronic allergen exposure, IL-4 and IL-13 levels were reduced almost to baseline levels, whereas IL-5 and IL-12p40 levels were still increased. BAL fluid cytokine levels did not differ between SphK1^{-/-} and WT mice after 8 weeks of OVA/OVA treatment.

In BAL fluid of PBS/OVA-treated SphK1^{-/-} mice, KC and G-CSF levels were increased compared with those seen in PBS/

OVA-treated WT mice. Levels of eotaxin, IL-10, IFN- γ , and TNF- α were low and did not differ significantly among the examined groups (data not shown).

Lung mRNA expression profile

SphK1 mRNA was detected in lungs from WT but not SphK1^{-/-} mice. In WT PBS/OVA-treated mice rank orders of expression patterns for enzymes involved in S1P synthesis were SphK2 > SphK1 > neutral ceramidase; for S1P receptors, they were S1P1 > S1P3 > S1P2; and for S1P-degrading enzymes, they were sphingosine-1-phosphate phosphatase (S1PP) 1 > sphingosine-1-phosphate lyase (S1Ply) > S1PP2.

After chronic allergen exposure, mRNA expression of S1P-synthesizing and S1P-degrading enzymes did not change significantly between SphK1^{-/-} mice and WT mice, whereas acute allergen challenge led to upregulation of S1PP2 (see Fig E2, A, and Tables E3 and E4 in this article's Online Repository at www.jacionline.org) and S1Ply (see Fig E2, B and Tables E3 and E4) mRNA in WT but not SphK1^{-/-} mice. Furthermore, acute inflammation induced downregulation of SphK2 mRNA in lungs from SphK1^{-/-} mice (see Fig E2, C and Tables E3 and E4).

Expression levels of S1P receptor subtypes were not affected by acute allergen challenge, whereas chronic allergen exposure significantly downregulated S1P1 mRNA in lungs from SphK1^{-/-} mice (see Fig E2, D and Tables E3 and E4).

Lung vascular structure

The increased pulmonary vascular responsiveness in SphK1^{-/-} and WT mice was accompanied by means of vascular remodeling (Fig 4, A-F). Staining with the proliferation marker Ki-67 showed positive nuclei in smooth muscle cells of the intra-acinar and peribronchial arteries of lungs from SphK1^{-/-} and WT mice after acute allergen challenge (Fig 4, G and H). Furthermore, endothelial cells, epithelial cells, and CD68⁺ monocytes/macrophages were also Ki-67 positive.

Prominent remodeling of the vascular wall was present in the arteries of smaller airways, with a significant increase in the volume density of smooth muscle per total vessel area after OVA/OVA treatment (Fig 5, A). Individual small pulmonary and intra-acinar arteries of lungs from SphK1^{-/-} mice showed the most increase in wall thickness but were on average not significantly different from those of lungs from WT mice (Fig 5, A). Remodeling was not present in the main pulmonary arteries in the region of the lung hilum, and morphometric analysis of pulmonary veins showed no difference between SphK1^{-/-} and WT mice after either acute or chronic allergen challenge.

Regression analysis of vascular responsiveness and wall thickness (volume density of vascular smooth muscle) of the small pulmonary arteries showed a significant linear regression between wall thickness and vascular reactivity in mice after chronic allergen challenge (WT mice, $P = .002$; SphK1^{-/-} mice, $P = .018$; Fig 5, B).

At the ultrastructural level, remodeled arteries showed a characteristic arrangement of longitudinally oriented smooth muscle cells within the intimal layer luminal to the internal elastic lamina (Fig 6). The internal elastic lamina was thickened in remodeled arteries of SphK1^{-/-} and WT mice (Fig 6). No additional elastic lamina or basement membrane was seen between intimal smooth muscle cells and endothelial cells (Fig 6).

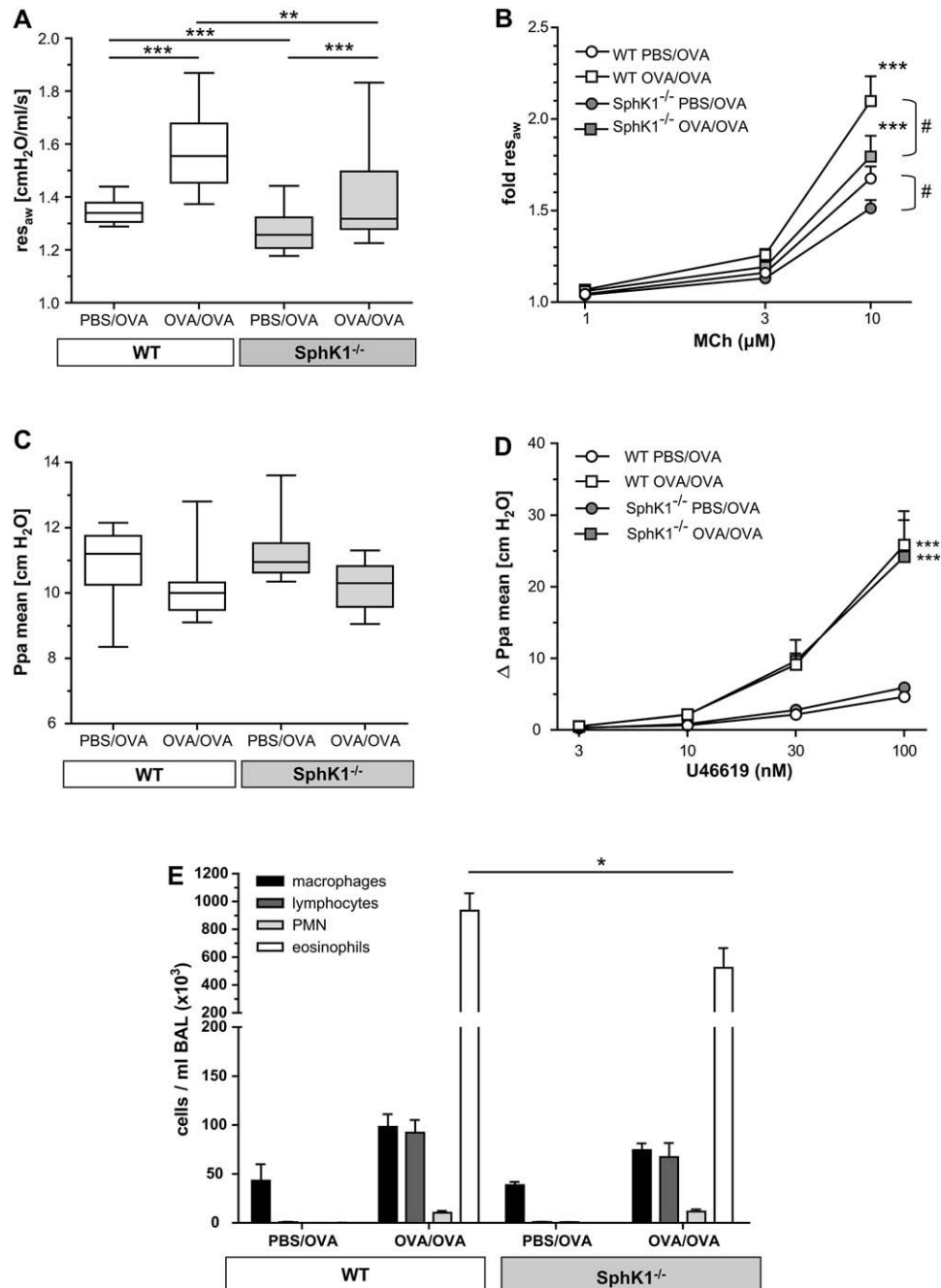


FIG 1. Acute allergen-induced inflammation. Airway resistance (res_{aw} ; **A**), airway responsiveness to MCh infusion (**B**), Ppa (**C**), and pulmonary vascular responsiveness to infused U46619 (**D**) were monitored in isolated perfused and ventilated murine lungs. BAL fluid leukocytes were counted and analyzed by means of microscopic analysis (**E**). Values are shown as means $\pm$ SEMs (WT mice: $n = 10$ -16 per group; SphK1^{-/-} mice: $n = 8$ -9 per group). * $P < .05$, ** $P < .01$, and *** $P < .001$ versus corresponding PBS/OVA or as indicated. # $P < .05$.

Lung edema was quantified in SphK1^{-/-} and WT mice after allergen sensitization and challenge to analyze a potential effect of SphK1 on endothelial barrier function in allergen-induced inflammation. Notably, neither SphK1 deficiency nor allergic lung inflammation altered the lung wet/dry weight ratio (data not shown). However, at the ultrastructural level, OVA/OVA-treated lungs compared with PBS/OVA-treated lungs showed luminal enlarged spaces between capillary endothelial cells, whereas interendothelial spaces did not differ between chronically

challenged WT and SphK1^{-/-} mice (see Fig E3 in this article's Online Repository at www.jacionline.org).

DISCUSSION

The current study strengthens evidence for a decreased asthmatic phenotype in allergen-evoked acute lung inflammation in SphK1^{-/-} mice. Notably, in contrast to the reduction of airway hyperresponsiveness, vascular hyperresponsiveness was unaltered

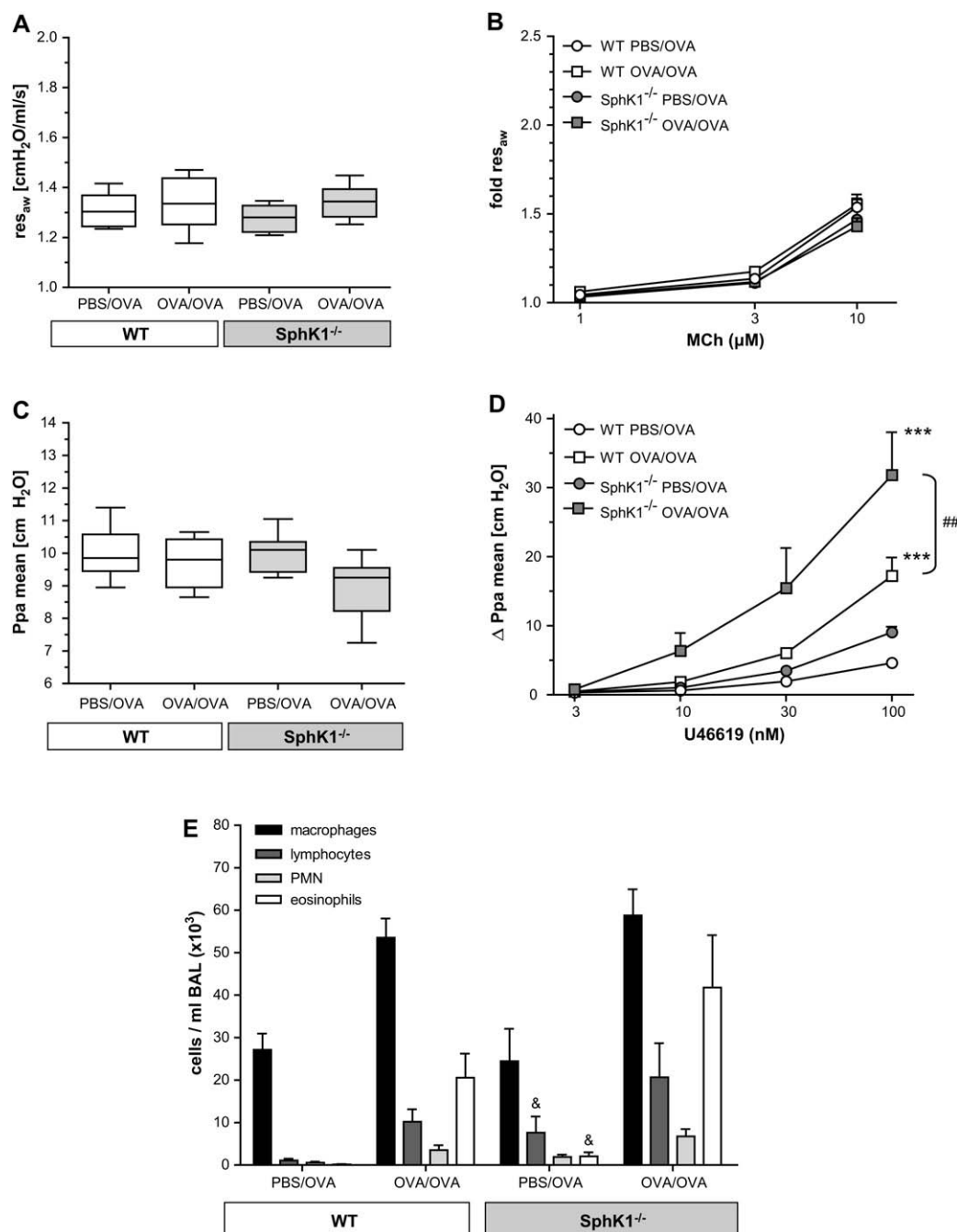
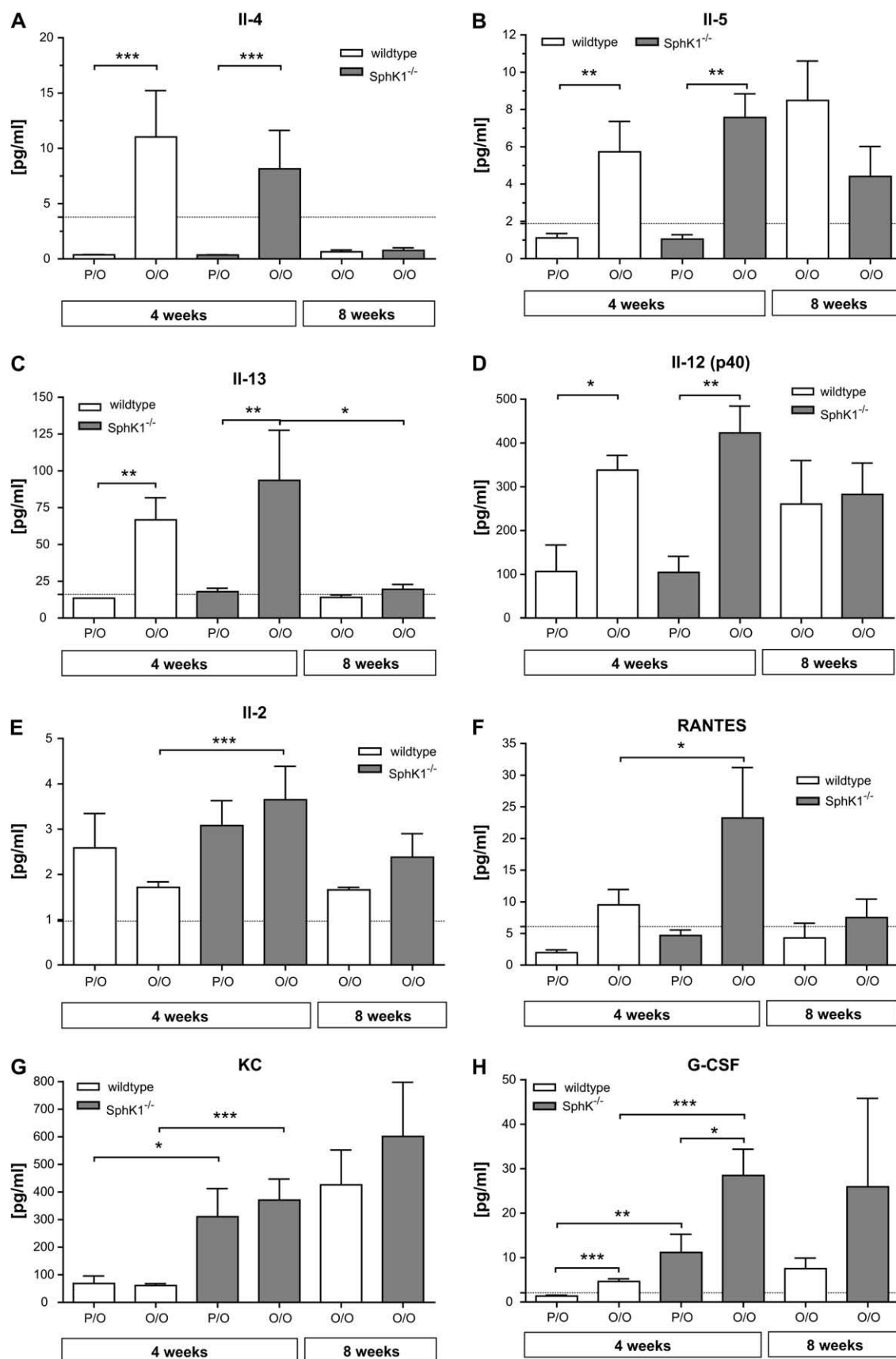


FIG 2. Chronic allergen-induced inflammation. Airway resistance (res_{aw} ; **A**), airway responsiveness to MCh infusion (**B**), Ppa (**C**), and pulmonary vascular responsiveness to infused U46619 (**D**) were monitored in isolated perfused and ventilated murine lungs. BAL fluid leukocytes were counted and analyzed by means of microscopic analysis (**E**). Values are shown as means $\pm$ SEMs (WT mice: $n = 10$ per group; SphK1^{-/-} mice: $n = 7-9$ per group). ## $P < .01$ and *** $P < .001$ versus corresponding PBS/OVA-treated group. & $P < .05$ versus the WT PBS/OVA-treated group).

in SphK1^{-/-} mice compared with that seen in WT mice during acute lung inflammation. Moreover, after long-term allergen exposure, pulmonary arterial hyperresponsiveness was aggravated compared with that seen in WT mice associated with maintained pulmonary arterial remodeling. This is the first clear demonstration of dissociation between T_H2-induced airway pathology and T_H2-induced alterations in pulmonary vascular morphology and function. Furthermore, the current data suggest a possible

involvement of SphK1 in the development of PAH accompanying inflammation.

SphK1 is the predominant contributor to pulmonary S1P synthesis and is upregulated in areas of inflammation after OVA inhalation, evoking increased S1P levels in BAL fluid.^{14,26} Reduction of SphK1 expression by means of RNA interference, as well as SphK1 inhibition, decreased allergen-induced airway inflammation after 4 weeks of OVA sensitization and challenge.^{19,26}



Consistent with these findings, we now have observed reduced airway resistance and reduced eosinophilia in lungs of SphK1^{-/-} mice compared with that seen in lungs of WT mice after 4 weeks of OVA sensitization and challenge. However, in contrast to SphK1 small interfering RNA-treated BALB/c mice,¹⁹ T_H2 cytokine levels were not reduced in SphK1^{-/-} C57Bl/6 mice. This divergence might be attributed to significantly lower T_H2 cytokine responses to allergen sensitization in C57Bl/6 WT mice compared with those seen in BALB/c mice.²⁷ Locally synthesized S1P is a bronchoconstrictor, which might sufficiently explain the reduced airway responsiveness in naive and PBS/OVA-treated SphK1^{-/-} mice compared with that seen in WT mice.^{28,29}

As observed previously in BALB/c mice,⁷ 4 weeks of exposure to OVA induced pulmonary vascular hyperresponsiveness in C57Bl/6 WT mice. SphK1^{-/-} mice showed comparable vascular hyperresponsiveness after 4 weeks of OVA exposure. Similarly, perivascular inflammation occurred in the lungs of both WT and SphK1^{-/-} mice, even though IL-2, KC, RANTES, and G-CSF levels were significantly increased in SphK1^{-/-} mice compared with those seen in WT mice. The increase in vascular smooth muscle cells expressing the proliferation marker Ki-67 after antigen exposure suggests a link between ongoing vascular remodeling and perivascular inflammation. Indeed, after prolonged allergen challenge, muscularization of small pulmonary and intra-acinar arteries was increased. Moreover, hyperplastic smooth muscle cells appeared in the intimal layer and showed a characteristic longitudinal orientation, as observed in pulmonary arteries of patients with chronic obstructive pulmonary disease with pulmonary hypertension.³⁰

Most importantly, long-term allergen challenge increased vascular responsiveness to the thromboxane receptor agonist U46619 in SphK1^{-/-} mice more than in WT mice. Pharmacologic interaction between thromboxane and sphingosine under inflammatory conditions in rat coronary arteries has been suggested,³¹ and sphingosine values might be increased in SphK1^{-/-} mice. However, in the acute allergen-induced inflammation model, neither allergen-sensitized nor PBS/OVA-treated SphK1^{-/-} mice had altered vascular responsiveness compared with that seen in WT mice. Furthermore, regression analysis demonstrated a strong relation between pulmonary vascular wall thickness and responsiveness. Therefore pharmacologic interaction between thromboxane and sphingosine might be of minor importance in the model currently investigated, whereas the structural changes might have significant consequences on vascular function in the lung.

We further investigated expression levels of enzymes generating, dephosphorylating, or degrading S1P in the lungs. Under noninflammatory conditions, the absence of SphK1 did not have a detectable effect on relative mRNA expression levels of SphK2, S1PP1, S1PP2, S1Ply, and S1P1-3 receptors. However, lung inflammation after 4 weeks of systemic allergen sensitization and local airway challenge evoked differences in the expression levels of SphK2, S1PP2, S1Ply, and the S1P1 receptor subtype. As expected from previous work,³² acute antigen-induced inflammation caused upregulation of S1PP2 mRNA levels in WT mice.

FIG 3. Pulmonary cytokine production was quantified in BAL supernatants by using the cytokine multiplex assay. Values are shown as means ± SEMs (n = 5-8 per group). P/O, PBS/OVA; O/O, OVA/OVA. *P < .05, **P < .01, and ***P < .001. Dotted lines indicate the detection limit of the cytokine assay and are missing if all values were within the working range.

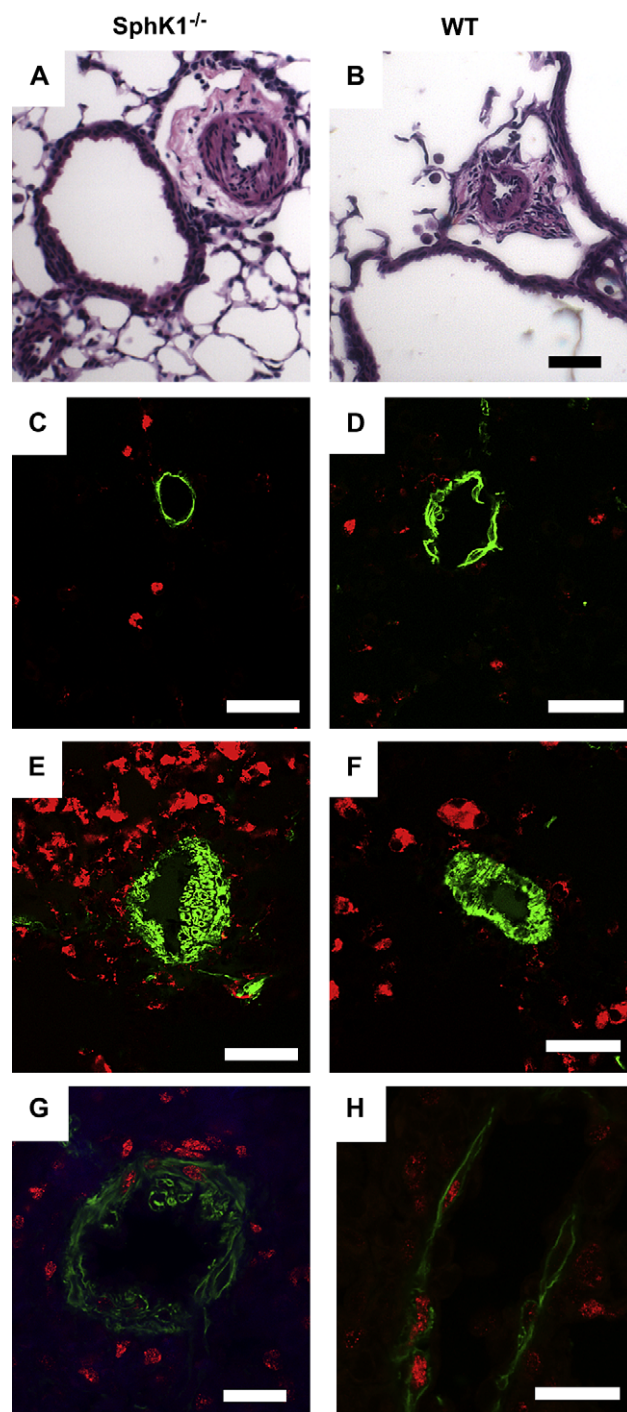


FIG 4. Chronic allergen-induced lung inflammation (**A-F**). Histochemistry of small pulmonary arteries (hematoxylin and eosin staining [Fig 4, **A** and **B**]) and double-labeling immunohistochemistry (α -smooth muscle actin [α -SMA] green/CD68 red [Fig 4, **C-F**]; α -SMA green/Ki67 red [Fig 4, **G** and **H**]) of intra-acinar arteries in lungs from SphK1^{-/-} mice (Fig 4, **A** and **E**) and WT mice (Fig 4, **B** and **F**) after OVA/OVA treatment are shown. Fig 4, **C** and **D**, show intra-acinar arteries of the SphK1^{-/-} and WT PBS/OVA-treated groups. Acute allergen-induced lung inflammation is shown in Fig 4, **G** and **H**. Ki67-immunoreactive nuclei were found in smooth muscle cells of the SphK1^{-/-} (Fig 4, **G**) and WT (Fig 4, **H**) OVA/OVA-treated groups. These photomicrographs are representative of 3 to 5 mice examined per group. Bars = 50 μ m (Fig 4, **A-F**); 20 μ m (Fig 4, **G** and **H**).

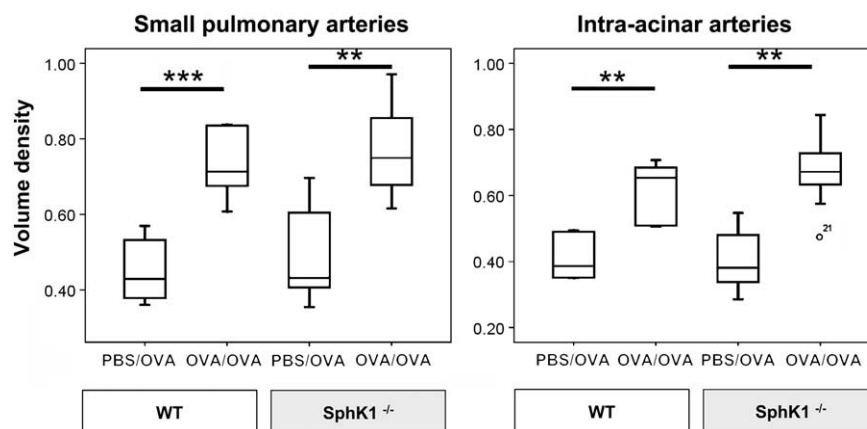


FIG 5. Chronic allergen-induced lung inflammation ($n = 6-9$ per group.). **A**, Quantitative morphometric analysis: volume density of vascular walls in small pulmonary arteries and intra-acinar arteries in SphK1^{-/-} and WT mice after PBS/OVA or OVA/OVA treatment. **B**, Regression analysis of the vascular responsiveness to U46619 and the volume density of small pulmonary arterial walls in SphK1^{-/-} and WT mice.

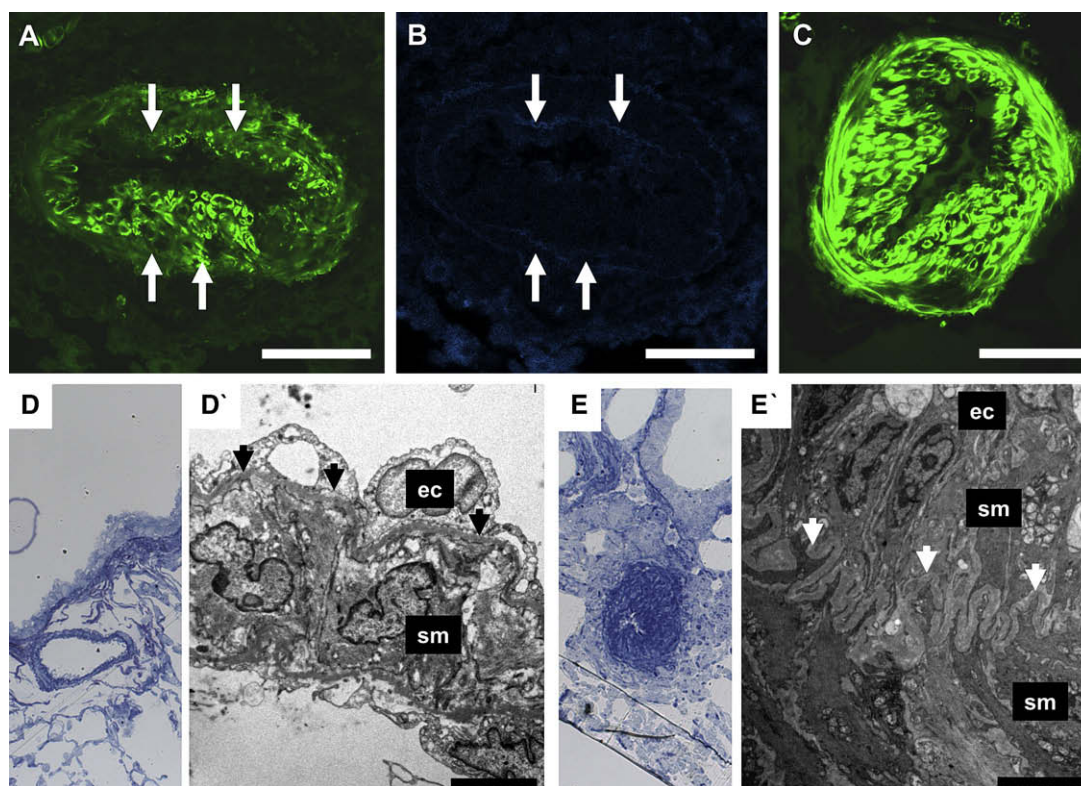


FIG 6. Immunohistochemistry (A-C), toluidine blue staining (D and E), and ultrastructural analysis (D' and E') of arteries from SphK1^{-/-} mice (Fig 6, C and D) showed the presence of α-smooth muscle actin-immunoreactive smooth muscle cells luminal to the internal elastic lamina (arrows in Fig 6, A and B). Fig 6, C, shows a different orientation of smooth muscle cells in the media and intimal layer. OVA/OVA (Fig 6, E and E') but not PBS/OVA (Fig 6, D and D') arteries of SphK1^{-/-} mice contained smooth muscle (sm) luminal to the internal elastic lamina (arrows). Note the increased thickness of the internal elastic lamina in chronically challenged arteries (Fig 6, E'). ec, Endothelial cells. These photomicrographs are representative for 3 to 5 mice examined per group. Bars: 50 μm (Fig 6, A-C); 5 μm (Fig 6, D' and E').

Surprisingly, a comparable upregulation of S1PP2 mRNA was absent in SphK1^{-/-} mice. Similarly, we also detected an inflammation-dependent increase in mRNA for the S1P-degrading enzyme S1Ply in the lungs of WT mice but not SphK1^{-/-} mice. The

absence of increased mRNA expression for S1P-degrading enzymes might be a consequence of the reduced S1P synthesis in response to inflammation in SphK1^{-/-} C57Bl/6 mice. This further underscores the important role for SphK1, the S1P it

generates, and subsequent regulation of SphK2, S1PP2, and S1Ply synthesis not only as components of a proinflammatory signaling pathway in the airways but also for anti-inflammatory pathways in pulmonary arteries. In line with these findings, S1P receptor activation in the alveolar region decreases epithelial barrier function but enhances endothelial barrier function in capillaries.^{12,33}

The SphK/S1P pathway has been proposed as a target to design novel therapies for asthma.¹⁴ However, our observations on SphK1^{-/-} mice provide evidence that inhibiting SphK1 activity actually might be detrimental in allergic inflammation of the lung. Reduction of SphK1 activity could contribute to the development of PAH associated with inflammation. Further work will be required to understand the complex links between SphK/S1P signaling, inflammation, and the pathogenesis of airway and pulmonary vascular disease.

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Key messages

- In acute allergen-induced inflammation, airway hyperresponsiveness was reduced in the absence of SphK1, but vascular hyperresponsiveness persisted.
- In chronic inflammation vascular hyperresponsiveness increased with prominent remodeling of the small and intra-acinar arteries.
- In chronic inflammation pulmonary vascular hyperresponsiveness was increased in SphK1^{-/-} mice compared with WT mice.

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METHODS

Animals, sensitization, and allergen exposure

All animal procedures were approved by local governmental and institutional authorities. The "Principles of laboratory animal care" were followed. Generation of SphK1^{-/-} mice has been described previously.^{E1} All mice were maintained under specific pathogen free conditions. Littermates were used in all experiments to control for background effects.

For acute allergen-induced lung inflammation, mice (C57BL/6 [WT] and SphK1^{-/-} mice,^{E1} age matched, 8–12 weeks of age) were systemically sensitized by means of intraperitoneal injection of 30 µg of OVA (Sigma, Deisenhofen, Germany) and 1.8 µg of Al(OH)₃ (Pierce, Rockford, Ill) in 100 µL of PBS on days 0, 14, and 21 (OVA/OVA-treated group). Nonsensitized mice received 1.8 µg of Al(OH)₃ in 100 µL of PBS (PBS/OVA-treated group). On days 28, 29, and 30, all mice were exposed to aerosolized OVA (30 mg/mL) in 0.9% saline for 30 min/d and killed at day 32.

Chronic allergen-induced lung inflammation was induced as described previously.^{E2} Mice (C57BL/6 [WT] and SphK1^{-/-} mice,^{E1} 8–12 weeks of age) were sensitized by means of intraperitoneal injection of 50 µg of OVA and 0.4 µg of Al(OH)₃ in 200 µL of PBS on days 0 and 14 (OVA/OVA-treated group). Nonsensitized mice received 0.4 µg of Al(OH)₃ in 200 µL of PBS (PBS/OVA-treated group). On day 21, all mice were exposed to aerosolized OVA (10 mg/mL) in 0.9% saline for 30 minutes 3 times per day (at 1-hour intervals) and then every second day thereafter for 8 days, followed by 4 weeks with 2 challenges per week. The mice were killed at day 51. The aerosol was generated by using a nebulizer connected to a closed chamber of 800 cm³ (FMI, Seeheim, Germany).

Isolated perfused murine lung

Murine lungs were prepared as described previously.^{E3,E4} Lungs were perfused with 37°C sterile Krebs-Henseleit hydroxyethyl amylopectin buffer (1 mL min⁻¹; Serag-Wiesner, Naila, Germany) in a nonrecirculating fashion, and left atrial pressure was adjusted at +2.2 cm H₂O. Pulmonary arterial pressure (Ppa) was continuously monitored. After isolation, lungs were ventilated with constant negative pressure (−4.5/−9 cm H₂O, 90 breaths/min) in a closed chamber. Hyperinflation (−24 cm H₂O) was performed at 4-minute intervals. The chamber pressure was continuously measured with a differential pressure transducer, and airflow velocity was monitored by means of a pneumotachograph connected to a second differential pressure transducer. Signals were amplified and registered with Pulmodyn software, and the data for airway resistance were analyzed as described previously.^{E5} All hardware and software were purchased from HSE Harvard Apparatus (March-Hugstetten, Germany).

Airway and vascular responsiveness in isolated perfused murine lungs

After a steady-state period of 30 minutes, MCh or the thromboxane receptor agonist U46619 (Calbiochem, Darmstadt, Germany) were administered to the perfusate for 0.5 or 3 minutes, respectively, and concentrations were increased at 12-minute intervals.^{E4} Airway resistance and Ppa were determined 30 seconds before and at the respective maximum response after MCh or U46619 administration. The change in airway resistance was expressed as factor of baseline airway resistance, and the difference in Ppa (ΔPpa) was expressed in centimeters of H₂O.

Wet/dry weight ratio

Lungs were weighed and subsequently dried in a 60°C oven for 48 hours. The ratio of wet weight to dry weight represented tissue edema.^{E6}

Real-time quantitative RT-PCR

RNA was isolated from quick-frozen lung tissue (RNeasy, Qiagen). Contaminating DNA was removed on a column by using DNase (Qiagen). Equal amounts of RNA were reverse transcribed (iScript; Bio-Rad, Regents Park, Australia), and PCR reactions were prepared in triplicate (iQ SYBR green, Bio-Rad). Primers specific for HPRT were used for standardization (Table E1). The data were normalized by subtracting the threshold cycle (CT) levels between the genes of interest and HPRT, and the ΔCT values were subtracted from 50. The relative expression was calculated by using the −ΔΔCT method.

Immunocytochemistry

Specimens for immune labeling were obtained from 5 SphK1^{-/-} and WT mice of either sex. The upper, medial, and lower lobes of the right lung were inflated through the trachea with 4% paraformaldehyde for at least 4 hours, cryoprotected, snap-frozen in isopentane cooled with liquid nitrogen, and stored at −20°C. They were serially sectioned at a thickness of 12 µm and subsequently preincubated for 1 hour with PBS containing 10% normal donkey serum, 0.1% BSA, and 0.5% Tween 20. Indirect immunofluorescence was performed by means of overnight incubation with mixtures of primary antibodies (Table E2), followed by subsequent incubation with appropriate combinations of secondary reagents (Table E2). Before incubation with the Ki-67 antiserum, sections were boiled twice for 7 minutes in 0.1 mol/L citrate buffer, pH 6, followed by overnight incubation with Ki-67 antibody in combination with α-smooth muscle actin antibody and incubation with secondary reagents (Table 2). The slides were evaluated by means of sequential scanning with a confocal laser scanning microscope (TCS SP5, Leica).

Statistics

Data from perfused murine lung studies were expressed as means ± SEMs. Differences were analyzed by using multiway repeated-measures ANOVA after logarithmic transformation to homogenize variances. Initial linear regression analysis showed that variation in baseline values predicted variations in response to MCh. Therefore responses to MCh were analyzed with a repeated-measures ANOVA by using pretest baselines and MCh doses as within-subject factors and strain (WT or SphK1^{-/-}) and sensitization status (PBS or OVA) as between-subject factors. Subsets of data were analyzed with preplanned linear contrasts or *post hoc* comparisons after Bonferroni adjustment of 95% confidence limits of the estimated means. We used the Dixon test for outliers to identify outliers in the morphologic analysis data set. One data point was identified as a genuine outlier and winsorized (ie, the data point was replaced by the next lower data point).^{E7}

For real-time quantitative PCR data analysis, a Mann-Whitney *U* test for comparison between 2 groups was conducted only when statistically significant differences were reached by using the global Kruskal-Wallis test that was performed first. Differences were considered as statistically significant at a *P* value of less than .05.

RESULTS

Airway and pulmonary vascular physiology and inflammation

Naive SphK1^{-/-} mice had lower airway resistance than naive WT mice. This difference was also seen in lungs of PBS/OVA-treated mice at baseline airway resistance levels (Fig 1, A), and PBS/OVA-treated WT mice had higher airway responsiveness compared with those seen in PBS/OVA-treated SphK1^{-/-} mice (Fig 1, B). Pulmonary vascular resistance and responsiveness did not differ between naive groups.

Both WT and SphK1^{-/-} mice showed increased baseline airway resistance in response to acute OVA sensitization and challenge (effect of OVA: $F[1,32] = 23.5$, $P < 0.001$; baseline × sensitization interaction: $F[1,32] = 9.6$, $P = 0.004$; Fig 1, A). After acute allergen exposure, OVA/OVA-treated SphK1^{-/-} and OVA/OVA-treated WT mice showed a significantly increased airway responsiveness compared with corresponding PBS/OVA-treated mice ($F[1,32] = 16.2$, $P = .001$; Fig 1, B). OVA/OVA-treated WT mice showed higher airway responsiveness compared with that seen in OVA/OVA-treated SphK1^{-/-} mice, especially at higher doses of MCh (baseline × MCh dose × challenge interaction ($F[1,32] = 6.5$, $P = .014$; Fig 1, B).

Pulmonary arterial pressure was not altered by acute allergen-induced inflammation (Fig 1, C). Pulmonary vascular responsiveness was greatly increased in both OVA/OVA-treated groups and did not differ between SphK1^{-/-} and WT mice (Fig 1, D).

Immune cells were increased after allergen exposure in both WT and SphK1^{-/-} mice. The numbers of eosinophils were higher in WT mice compared with those seen in SphK1^{-/-} mice (Fig 1, E).

After chronic repetitive allergen exposure for 8 weeks, neither airway resistance (Fig 2, A) nor airway responsiveness (Fig 2, B) was increased in OVA/OVA-treated mice compared with that seen in the PBS/OVA-treated groups. The numbers of eosinophils in OVA/OVA-treated mice were increased compared with those seen in PBS/OVA-treated mice (Fig 2, E). However, the total numbers of eosinophils in OVA/OVA-treated mice were 5% to 10% of the corresponding numbers seen during acute inflammation. Pulmonary arterial pressure was not altered by chronic allergen sensitization and challenge in WT or SphK1^{-/-} mice (Fig 2, C). Surprisingly, pulmonary vascular responsiveness was further significantly increased in OVA/OVA-treated WT and SphK1^{-/-} mice after chronic allergen exposure compared with that seen in PBS/OVA WT and SphK1^{-/-} mice ($F[1,28] = 39.7$, $P = .001$; Fig 2, D). Furthermore, the vascular responsiveness was significantly greater in OVA/OVA-treated SphK1^{-/-} mice compared with that seen in OVA/OVA-treated WT mice (baseline $\times$ U46619

dose $\times$ challenge interaction: $F[1,28] = 12.3$, $P = .002$; Fig 2, D) and increased compared with that seen after acute exposure.

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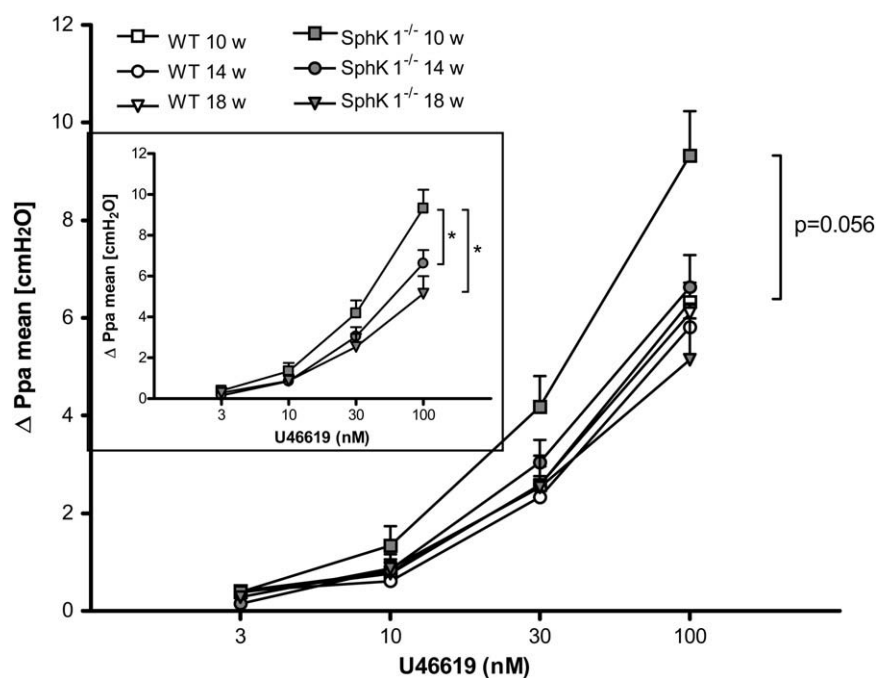


FIG E1. Effect of age on pulmonary vascular responsiveness to infused U46619 was analyzed in isolated perfused and ventilated lungs of 10-week-old (10 w), 14-week-old (14 w), and 18-week-old (18 w) SphK1^{-/-} and WT mice. Values are shown as means $\pm$ SEMs ($n = 5-7$ per group).

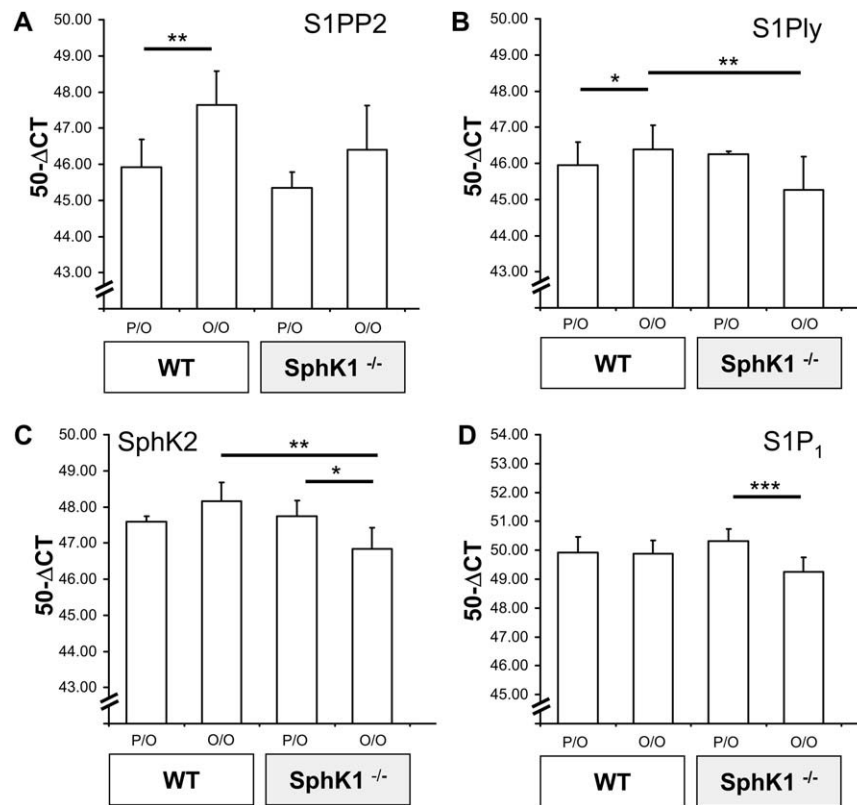


FIG E2. Quantitative RT-PCR analysis: **A-C**, acute allergen-induced lung inflammation; **D**, chronic allergen-induced lung inflammation. Acute allergen challenge led to upregulation of S1PP2 (Fig E2, **A**) and S1Ply (Fig E2, **B**) mRNA in WT mice but not in SphK1^{-/-} mice. Acute inflammation induced downregulation of SphK2 and S1P1 mRNA in lungs from SphK1^{-/-} mice (Fig E2, **C**), whereas chronic inflammation reduced S1P1 mRNA in lungs from SphK1^{-/-} mice (Fig E2, **D**). The data represent 5 to 7 lungs per group. * $P < .05$, ** $P < .01$, and *** $P < .001$.

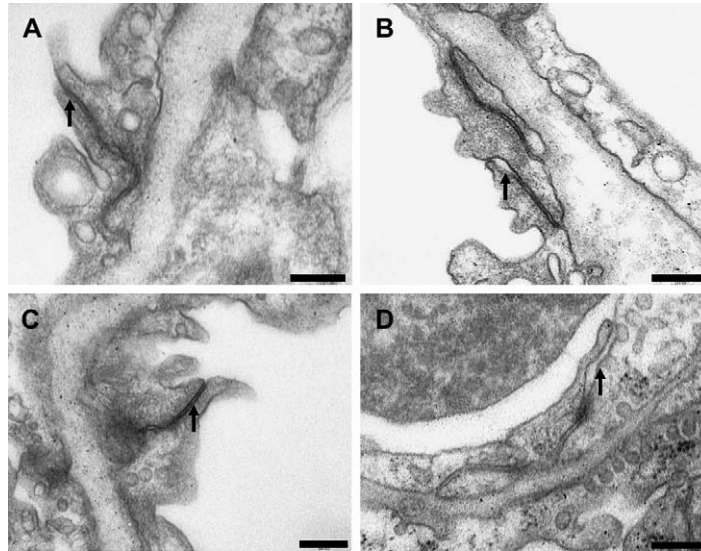


FIG E3. Ultrastructural analysis of WT (**A** and **B**) and SphK1^{-/-} (**C** and **D**) capillary endothelium showed the presence of enlarged luminal spaces between cells in OVA/OVA-treated (*arrows* in Fig E3, **B** and **D**) but not PBS/OVA-treated (Fig E3, **A** and **C**) arteries. These photomicrographs are representative of 3 to 5 mice examined per group. Bars = 200 nm.

TABLE E1. Used primer pairs

Name	GI no.	Forward primer; reverse primer	Product length (bp)
HPRT	53237103	GCCCCAAAATGGTTAAGGTT; TTGCGCTCA TCTTAGGCTTT	208
Sphingosine kinase 1 (SK1)	22094104	TGTCACCCATGAACCTGCTGTCCCTGCA CA; AGAAGGCACTGGCTCCTCCAGA GGAACAAG	?
Sphingosine kinase 2 (SK2)	42543999	GGCATTGTCACTGTGTCTGG; GCAGAGA AGAAGCGAGCAGT	210
Sphingosine phosphate lyase 1 (S1PLy)	31543693	CTGATGACCGTACCATGTGC; GAATCCC TGAGAAGGGGAAG	169
Sphingosine-1-phosphate phosphatase 1 (S1PP1)	31543695	TGGGAGCCATTTCTAGTTG; AGCAAA CTTCCCCCAAACT	156
Sphingosine-1-phosphate phosphatase 2 (S1PP2)	NM_001004173.1	TTTCTCCCTTTACCCACTG; GGCATT CCGTACTCTGCAAT	176
N-acylsphingosine amidohydrolase 2 (neutral ceramidase, nCer)	74141614	CCCTTCATTTCGGAACTTCA; CTGGATC CGCCAAGATAAAA	164
Endothelial differentiation sphingolipids G-protein- coupled receptor 1 (Edg1 = S1P1)	XM_986946.1	CTCTGCTCCTGCTTTCCATC; GCAGGC AATGAAGACACTCA	173
Endothelial differentiation, sphingolipid G protein- coupled receptor 3 (Edg3 = S1P3)	NM_010101.2	GTGTGTTTCATTGCCTGTTGG; TTGACTAGACAGCCGCACAC	208
Endothelial differentiation sphingolipids G protein- coupled receptor 5 (Edg5 = S1P2)	NM_010333.2	TCTCAGGGCATGTCACTCTG; CAGCT TTTGTCCTGCGTA	163

TABLE E2. Primary and secondary antibodies

Antigen	Host	Dilution	Source
CD68	Rat (clone FA-11)	1:500	AbD Serotec, Düsseldorf, Germany
CD31	Rat (MEC13.3)	1:500	BD PharMingen, San Jose, Calif
Biotin anti-mouse CD45.1	Mouse (clone A20)	1:200	eBioscience, San Diego, Calif
FITC anti-mouse α -SMA	Mouse (clone 1A4)	1:500	Sigma
Ki-67	Rat	1:100	DAKO, Glostrup, Denmark
Secondary antibody			
Cy5 anti-rat	Donkey	1:25	Jackson Laboratories, Bar Harbor, Me
Cy3 anti-rabbit	Donkey	1:100	Jackson Laboratories

α -SMA, α -Smooth muscle actin.

TABLE E3. Relative expression 50-ΔCT

	Acute allergen-induced inflammation				Chronic allergen induced-inflammation			
	WT		SphK1 ^{-/-}		WT		SphK1 ^{-/-}	
	P/O	O/O	P/O	O/O	P/O	O/O	P/O	O/O
S1PP2	45.91	47.64	45.35	46.41	45.53	46.16	44.70	45.28
SD	0.78	0.93	0.43	1.21	0.66	0.77	0.92	0.29
S1Ply	45.94	46.39	46.25	45.26	47.31	47.83	46.95	46.99
SD	0.65	0.67	0.08	0.93	1.61	1.36	1.60	0.81
SphK2	47.59	48.16	47.74	46.83	46.76	47.17	47.60	46.93
SD	0.15	0.52	0.44	0.59	1.17	1.07	0.77	0.89
S1P1	49.92	49.89	50.31	49.25	51.36	51.38	50.93	50.29
SD	0.54	0.45	0.42	0.50	0.57	1.03	0.41	1.06

P/O, PBS/OVA; O/O, OVA/OVA.

TABLE E4. Relative expression levels, fold increase/decrease

	Acute allergen-induced inflammation				Chronic allergen-induced inflammation			
	WT		SphK1 ^{-/-}		WT		SphK1 ^{-/-}	
	P/O	O/O	P/O	O/O	P/O	O/O	P/O	O/O
S1PP2	1	3.32 (1.74-6.32)	NS	NS	NS	NS	NS	NS
S1Ply	1	1.82 (1.13-2.93)	NS	NS	NS	NS	NS	NS
SphK2	NS	NS	1	0.53 (0.35-0.8)	NS	NS	NS	NS
S1P1	NS	NS	NS	NS	NS	NS	1	0.48 (0.34-0.68)

P/O, PBS/OVA; *O/O*, OVA/OVA; *NS*, not significant.