

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Surfactant protein D alleviates eosinophil-mediated airway inflammation and remodeling in patients with aspirin-exacerbated respiratory disease

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Abstract

Background: Surfactant protein D (SPD) is a member of the collectin family that lines the airway epithelial cells with host defense. However, the role of SPD in the pathogenesis of aspirin-exacerbated respiratory disease (AERD) is still unclear.

Methods: The serum SPD level was measured in patients with AERD ($n = 336$), those with aspirin-tolerant asthma (ATA, $n = 442$), and healthy controls (HC, $n = 104$). Polymorphisms of *SFTPD* in the study subjects were analyzed. The effect of LTE4 on SPD production through eosinophil infiltration was investigated in BALB/c mice. The protective function of SPD against eosinophils inducing inflammation and remodeling was assessed in vitro/vivo. The potential efficacy of nintedanib against airway remodeling through the production of SPD was evaluated.

Results: The serum SPD level was significantly lower ($P < .001$) in AERD compared with ATA patients, and negatively correlated with fall in FEV₁ (%) after lysine-aspirin bronchoprovocation test and/or the urinary LTE4 level. In addition, polymorphism of *SFTPD* at rs721917 was significantly different in the study subjects (odds ratio, 2.124; 95% confidence intervals, 1.310–3.446; $P = .002$). LTE4-exposed mice showed an increased eosinophil count with a decreased SPD level in bronchoalveolar lavage fluid. Eosinophils increased α -smooth muscle actin expression in airway epithelial cells, which was attenuated by SPD treatment. Furthermore, nintedanib protected the airway epithelial cells against eosinophils by enhancing the production of SPD.

Conclusion: The decreased level of SPD in AERD was associated with airway inflammation/remodeling under the eosinophilic condition, suggesting that modulation of SPD may provide a potential benefit in AERD.

KEYWORDS

aspirin-exacerbated respiratory disease, eosinophils, inflammation, remodeling, surfactant protein D

1 | INTRODUCTION

The clinical feature of aspirin-exacerbated respiratory disease (AERD) is moderate to severe asthma comorbid with chronic rhinosinusitis (CRS)/nasal polyps, which is associated with cysteinyl leukotriene (cysLT) overproduction and eosinophilic inflammation in the upper and lower airway mucosa.¹ Asthma exacerbation and severe asthma which are more prevalent in AERD induce declines in lung function (lower FEV₁ value and increased airway hyperresponsiveness), which do not fully respond to current anti-inflammatory treatment²; CRS/nasal polyps found in AERD do not respond to conventional treatment and sinus surgeries. However, underlying mechanisms and an interaction with epigenetic or environmental factors are not fully understood.

Eosinophilia, which is more prominent in patients with AERD, is associated with more severe airway inflammation and remodeling.³ Effector cells, including mast cells and eosinophils, are major sources of cysLTs that play key roles in the pathogenesis of AERD,^{4,5} resulting in persistent cysLT-mediated amplification of innate type 2 immunity. Moreover, eosinophils play an important role in orchestrating tissue remodeling.^{6,7} Increased expression of vimentin and α -smooth muscle actin (α -SMA) and loss of E-cadherin through the Smad3 signaling are involved in the airway remodeling of asthmatic airway.⁸ Furthermore, cell-cell interactions between eosinophils and epithelial cells also lead to airway remodeling.⁹

Surfactant protein D (SPD) mainly produced by airway epithelial cells is a component of innate immunity within the airways.¹⁰ SPD binds to pathogens and induces phagocytosis by interacting with phagocytic cells such as macrophages and neutrophils.^{11,12} SPD also modifies airway inflammation by suppressing mast cells and eosinophils.¹³⁻¹⁵ A recent study has suggested that SPD deficiency may be involved in severe asthma, reflecting increased epithelial damage, or permeability within the airways.¹⁶ SPD dysregulation is evident in several pulmonary diseases, but the role of SPD in AERD has not been determined.

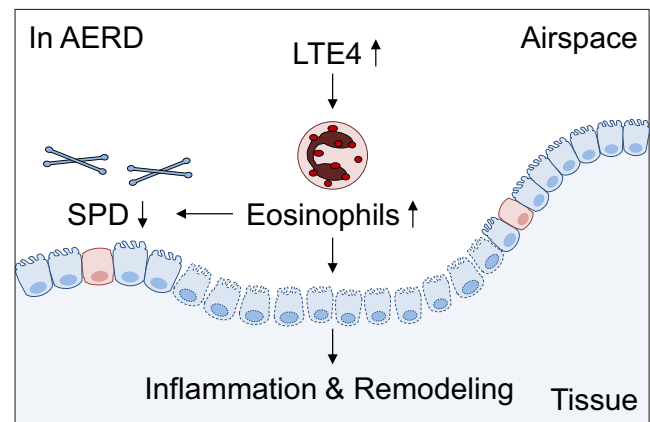
Nintedanib is an intracellular inhibitor of tyrosine kinases approved for treatment of idiopathic pulmonary fibrosis and cancer.^{17,18} Moreover, nintedanib effectively ameliorates airway inflammation and remodeling in mice.¹⁹ A recent study demonstrated a novel function of nintedanib that upregulates the expression of SPD in the lungs through c-Jun N-terminal kinase (JNK)-activator protein-1 (AP-1) pathway.²⁰ However, the effect of nintedanib on airway inflammation, especially on eosinophil and epithelial cells by enhancing production of SPD, has not been clarified.

This study was conducted to elucidate the role of SPD in the pathogenetic mechanisms of airway inflammation and remodeling in AERD and to evaluate the protective role of SPD against eosinophils.

2 | METHODS

2.1 | Study subjects

This study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108). All patients provided



GRAPHICAL ABSTRACT

High level of leukotriene E4 induces eosinophil infiltration in the lungs, leading to the reduction of surfactant protein D in patients with AERD. Decreased level of surfactant protein D and increased number of eosinophils enhance airway inflammation/remodeling. Modulation of surfactant protein D may provide a potential benefit in airway epithelium against eosinophils.

written informed consent at the time of recruitment. We enrolled patients with AERD (n = 336), those with aspirin-tolerant asthma (ATA, n = 442), and healthy controls (HC, n = 104). Asthma diagnosis was based on typical symptoms (airway obstruction with reversibility and increased airway hyperresponsiveness). Spirometry was performed to evaluate the degree of airway obstruction. Methacholine bronchial challenge test was conducted to examine the degree of airway hyperresponsiveness. Sputum eosinophil and neutrophil counts were measured as previously described.²¹ Atopy status was defined as at least 1 positive result in skin prick tests using commonly inhaled allergens (Bencard, Bradford, UK): *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, dog, cockroach, tree pollen mixture (alder, ash, beech, birch, elm, hazel, oak, plane, poplar, and willow), grass pollen mixture (velvet grass, orchard, rye grass, timothy grass, kentucky bluegrass, and *Meadow fescue*), mugwort, ragweed, Japanese hops, *Aspergillus*, and *Alternaria*. Serum total IgE was measured using the ImmunoCAP system (Thermo Fisher Scientific, Waltham, CA, USA). All sera were frozen and kept at -80°C and measured simultaneously. The presence of rhinosinusitis and nasal polyps was determined using paranasal sinus X-rays, CT scans, and/or rhinoscopy. The diagnosis of AERD was based on a positive response to lysine-aspirin bronchoprovocation test (L-ASA BPT) as previously described.²² ATA patients were asthmatic ones with a negative response to L-ASA BPT. HC had no histories of asthma or other allergic diseases. To determine the cutoff value for a diagnostic test, the application of 95% confidence intervals of the mean was used. An interval obtained by subtracting standard deviation (SD) from mean shows that the chance of a test value coming outside less than 15%. The lower limit of this interval (mean—SD) was considered the cutoff value.

2.2 | Urinary LTE4 measurement

Urine samples were collected before the L-ASA BPT and stored at -80°C in aliquots until required. The LTE4 metabolite level was analyzed as previously described.²³ Briefly, the LTE4 level was quantified using Mass Hunter Quantitative Analysis B.07.00 (Agilent Technologies, Santa Clara, CA, USA). LTE4-d5 (Cayman Chemical Company, Ann Arbor, MI, USA) was used as a deuterated internal standard. Creatinine was also measured to normalize the actual concentrations of LTE4.

2.3 | Genetic polymorphism analysis

Genomic DNA was isolated from the peripheral blood using the Puregene DNA purification kit (Gentra system, Minneapolis, MN, USA), according to the manufacturer's instructions. Single nucleotide polymorphism (SNP) genotyping was performed by the primer extension method using a SNaPshot ddNTP primer extension kit (Applied Biosystems, Foster City, CA, USA) as previously described.²⁴ The target SNPs and primer sequences for extension are indicated in Table S2.

2.4 | Peripheral blood eosinophil isolation

AERD patients' peripheral blood was collected during the disease-stable state while maintaining antiasthmatic medications, including inhaled corticosteroid. Patients who had been maintained on systemic corticosteroids were excluded. Blood was put into BD Vacutainer tubes containing acid citrate dextrose solution (BD Biosciences, Franklin Lakes, NJ, USA) and layered on Lymphoprep solution (Axis-Shield, Oslo, Norway), followed by centrifugation at $879 \times g$ for 25 minutes. The layer containing granulocytes was separated and placed in Hank's balanced salt solution (HBSS) buffer, with 2 mmol/L ethylenediaminetetraacetic acid (EDTA) and 2% dextran, for 20 minutes. Red blood cells were eliminated by hypotonic lysis. Eosinophils were separated from neutrophils by immunomagnetic cell sorting (Miltenyi Biotec Inc., Auburn, CA, USA) according to the manufacturer's recommendations.

2.5 | Cell culture

Human airway epithelial cell lines, A549 cells (American Type Culture Collection, Manassas, VA, USA), were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (50 $\mu\text{g/mL}$). A549 cells were treated with human LTE4 (Cayman Chemical Company) in serum-free medium. To investigate the effect of SPD against eosinophils, human recombinant SPD (R&D Systems, Minneapolis, MN, USA) was preincubated with eosinophils for 30 minutes. Then airway epithelial cells were cocultured with the eosinophils in the medium supplemented with 2% fetal bovine serum for 24 hours. To promote production of SPD, A549 cells were pretreated with nintedanib (Cayman Chemical Company) for 48 hours.

2.6 | ELISA

SPD, IFN- γ , IL-4, IL-5, and IL-8 (R&D Systems) were measured using ELISA kits, according to the manufacturer's instructions.

2.7 | Western blot

Cells were lysed with lysis buffer containing protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA). Following SDS-PAGE and transfer to a PVDF membrane (Bio-Rad, Hercules, CA, USA), each molecular size of nitrocellulose membrane was incubated with primary antibody as follows: anti-E-cadherin (Cell Signaling Technology), anti-vimentin (Cell Signaling Technology), anti-phospho-Smad3 (Abcam, Cambridge, MA, USA), anti-Smad3 (Abcam), anti- α -SMA (Abcam), and anti-SPD (Abcam).

2.8 | Confocal microscopy

The airway epithelial cells seeded on L-lysine-coated slides (Polysciences, Warrington, PA, USA) were cocultured with or without eosinophils. Primary antibodies including anti- α -SMA (Abcam) and anti-SPD (Abcam) were incubated for 1 hour, respectively. Then, appropriate Alexa Fluor[®] anti-IgG secondary antibodies (Invitrogen Life Technologies, Carlsbad, CA, USA) were used. Nuclei were stained with 4, 6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA). The cells were examined under a Zeiss LSM 710 confocal microscope with a $63 \times$ oil objective lens (Carl Zeiss, Oberkochen, Germany). Images were analyzed using ZEN 2009 software (Carl Zeiss).

2.9 | Mice

Six-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were used under specific pathogen-free conditions. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Ajou University (IACUC-2016-0028) in accordance with the guidelines of "Allergy." To induce airway inflammation and remodeling in mice, eosinophils collected from patients with AERD (2×10^6 in 20 μL of phosphate-buffered saline, PBS) were instilled intrabronchially.

2.10 | Airway resistance measurement

The airway resistance to inhaled methacholine (Sigma-Aldrich) was measured using the FlexiVent System (SCIREQ, Montreal, Canada) as previously described.²⁵

2.11 | Lung histology

Lungs were fixed in 4% formalin, embedded in paraffin, and cut into 5- μm sections. H&E stain was conducted to investigate immune cell infiltration. Masson's trichrome stain was performed to determine airway smooth muscle and collagen in the lungs. Tissue sections

were evaluated using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.12 | Statistical analysis

Continuous variables were analyzed by Student's *t* test or one-way ANOVA with Bonferroni's post hoc test. Pearson's chi-squared test was applied for categorical variables. Spearman correlation analysis identified the associations between continuous variables. $P < .05$ was considered statistically significant. Statistical analyses were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA, USA) was used to produce graphs.

3 | RESULTS

3.1 | Comparison of serum SPD level between AERD and ATA patients

The study subjects' clinical characteristics are shown in Table S1. The prevalence of severe CRS ($P < .001$) and nasal polyps ($P < .001$) was significantly higher, while baseline FEV₁ ($P = .027$) and PC₂₀ methacholine levels ($P = .002$) were significantly lower in

TABLE 1 Comparison between asthmatic patients with high SPD and those with low SPD

Clinical parameters	SPD		P value
	High	Low (< 891.1 pg/mL)	
Age (y)	44.8 ± 14.9/294	44.4 ± 13.6/82	.831
Female sex (%)	62.4/294	69.5/82	.245
Atopy (%)	47.5/278	49.4/77	.798
Paranasal sinusitis (%)	56.2/80	64.7/34	.533
Nasal polyp (%)	45.9/74	29.7/37	.151
Baseline FEV ₁ (%)	89.1 ± 20.2/227	86.2 ± 20.0/68	.445
Fall of FEV ₁ (%)	14.6 ± 11.1/107	19.9 ± 12.2/40	.013
PC ₂₀ (mg/mL)	5.6 ± 6.4/177	6.2 ± 7.5/50	.611
Total IgE (kU/L)	390.0 ± 708.0/276	343.9 ± 544.7/77	.596
TEC (/ μ L)	386.7 ± 372.9/238	399.3 ± 354.3/68	.804
Sputum Eos (%)	21.6 ± 33.3/169	20.7 ± 34.1/58	.862
Sputum Neu (%)	60.7 ± 34.7/151	58.9 ± 34.6/46	.756

Eos, eosinophils; FEV₁, forced expiratory volume in 1 s; IgE, immunoglobulin E; Neu, neutrophils; PC₂₀, the provocative concentration of methacholine required to cause a 20% fall in FEV₁.

Values are given as n (%) for categorical variables and as mean ± SD for continuous variables. *P* values were obtained by Pearson's chi-squared test for categorical variables and Student's *t* test for continuous variables.

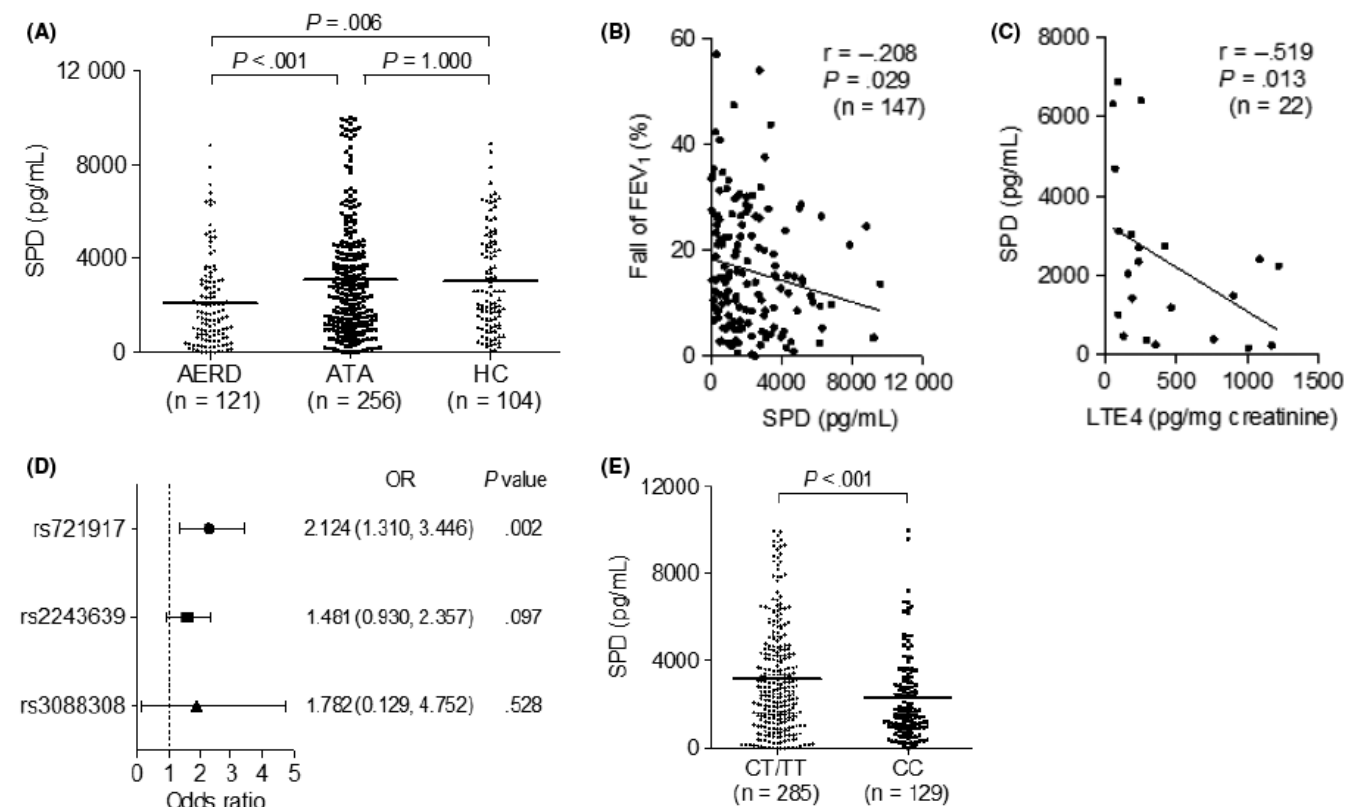


FIGURE 1 Comparison of the serum SPD level between the study subjects. (A) Low level of serum SPD in patients with AERD. *P* values were obtained by one-way ANOVA with Bonferroni's post hoc test. (B) Correlations between the serum SPD level and (B) FEV₁ fall after L-ASA BPT/(C) the urinary LTE4 level. The correlations are represented as Spearman correlation coefficient *r* (*P* value). (D) Odds ratio of each polymorphism of *SFTPD* given by a recessive analysis model. (E) SPD level according to rs721917 genotype. *P* values were obtained by Student's *t* test. CC, CC homozygous alleles; CT, CT heterozygous alleles; TT, TT homozygous alleles

AERD than in ATA groups. To investigate the involvement of SPD in AERD pathogenesis, we compared the serum SPD level between AERD and ATA patients: The serum SPD level was significantly lower in AERD than in ATA patients ($P < .001$, Figure 1A). When patients were divided into SPD high/low groups (the cutoff value, 891.1 pg/mL), SPD-low group had greater FEV₁ (%) fall after the L-ASA BPT ($P = .013$, Table 1). Moreover, a negative correlation was found between the serum SPD level and the fall of FEV₁ (%) after the L-ASA BPT ($r = -.208$, $P = .029$; Figure 1B) and/or urinary LTE4 level in patients with AERD ($r = -.519$, $P = .013$; Figure 1C), indicating that decreased SPD level may be associated with cysLT-mediated airway inflammation in AERD.

3.2 | Effect of genetic polymorphisms of *SFTPD* on the SPD level in patients with AERD

Genetic polymorphisms of *SFTPD* are as described in Table S2. Three targeted SNPs of *SFTPD* were analyzed to investigate an association between genetic variation and the phenotype of AERD. The genotype and allele frequencies of rs721917 in the AERD group were significantly different from those in the ATA group (OR = 2.124, $P = .002$; Figure 1D): The homozygous CC genotype was significantly higher in AERD than in ATA and HC groups ($P = .002$ and $P = .038$, respectively; Table S3). However, the frequency of either rs2243639 or rs3088308 showed no significant difference in study subjects (Table S3). With rs721917, asthmatic patients with the CC

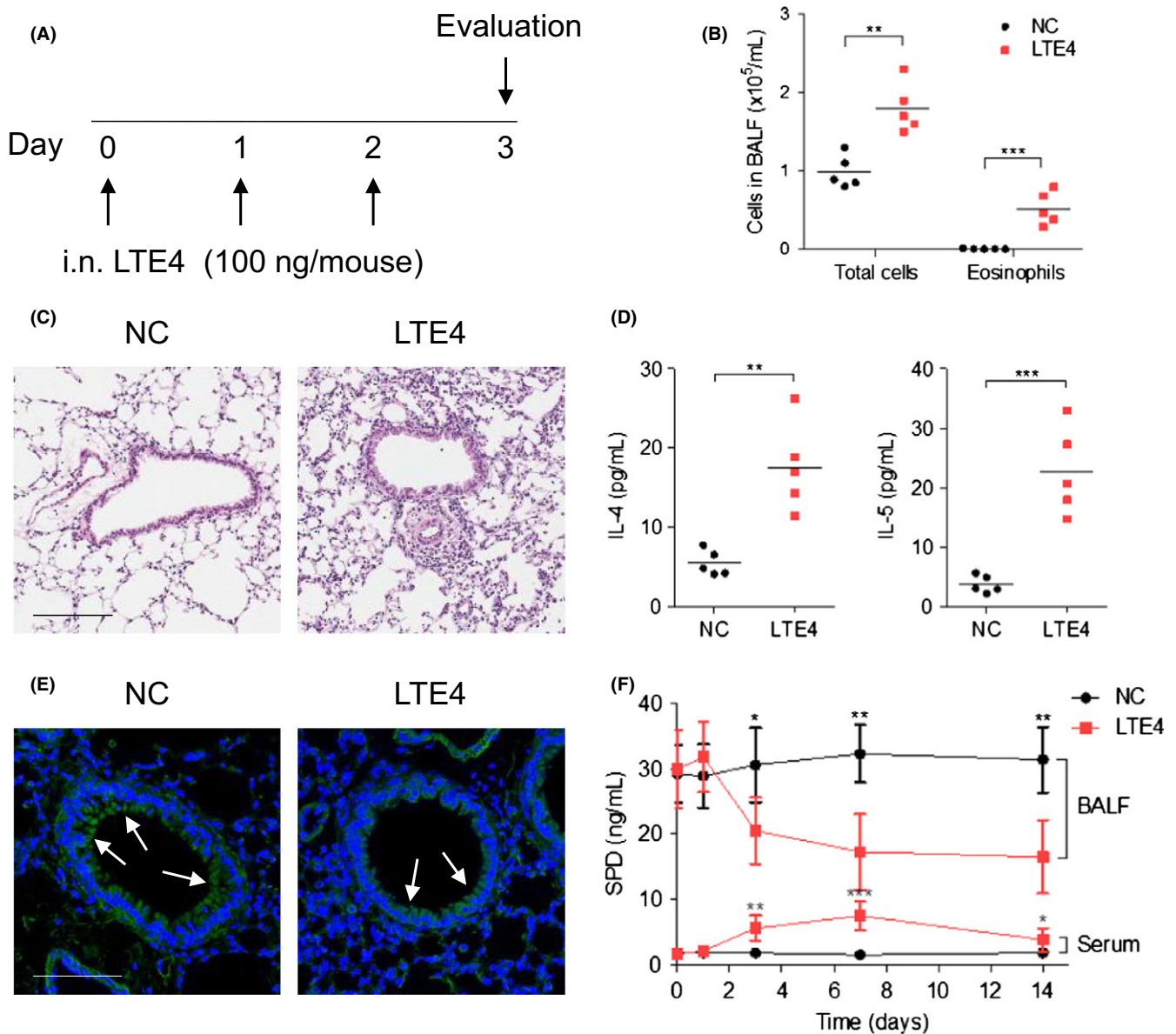


FIGURE 2 Effect of LTE4 in the lungs of BALB/c mice. A, Experimental schedule for LTE4 treatment. B, Differential cell number in BALF. C, Lung histology stained with H&E. Scale bar, 200 μm . D, Concentrations of IL-4 and IL-5 in BALF. E, Immunofluorescence staining of nuclei (blue) and SPD (green) in the lungs. Scale bar, 100 μm . F, SPD levels in BALF and serum. Data are presented as means, $n = 5$. * $P < .05$, ** $P < .01$, and *** $P < .001$ obtained by Unpaired t test

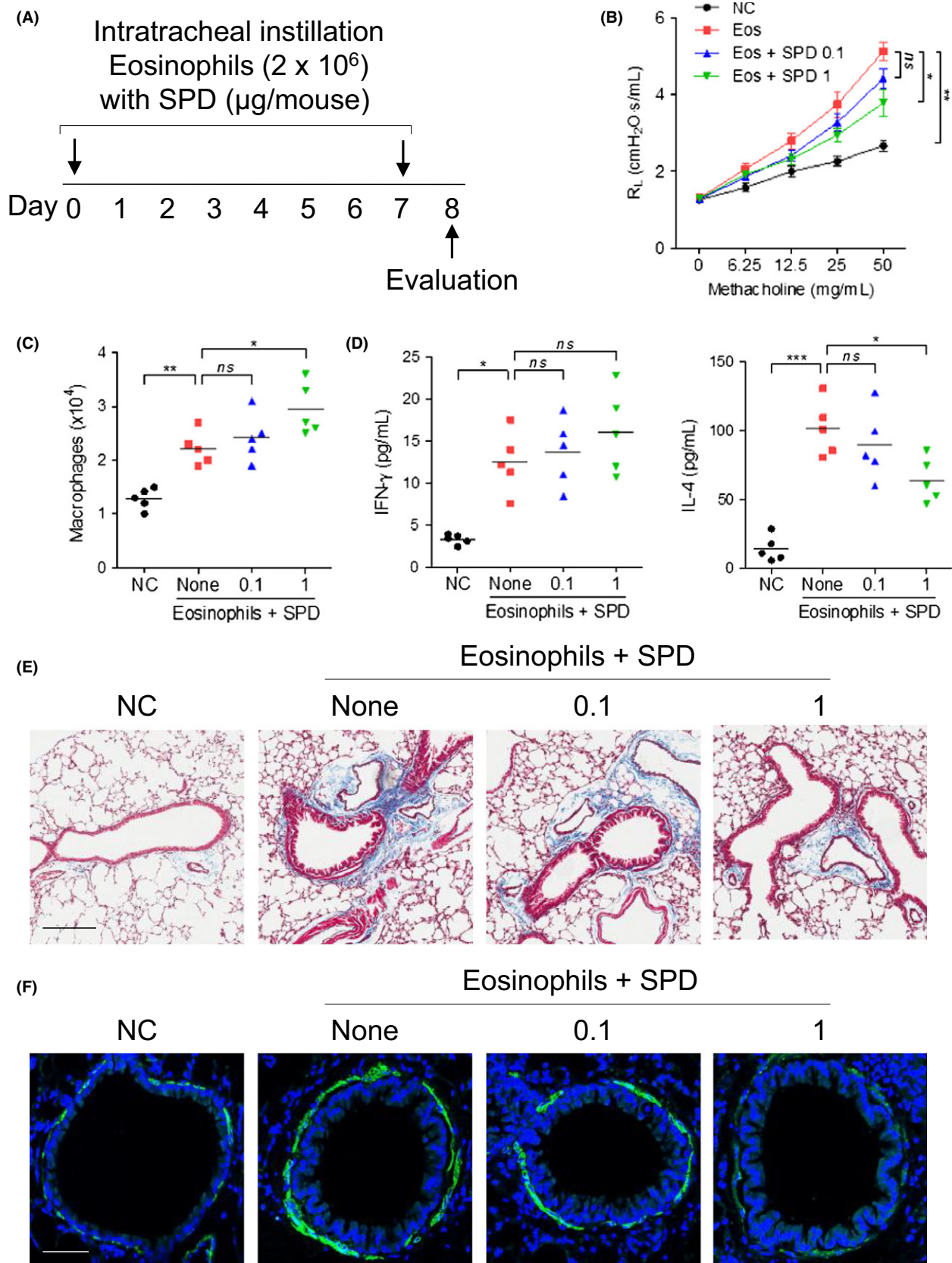


FIGURE 3 Role of SPD against eosinophils in BALB/c mice. A, Experimental schedule for eosinophil instillation. B, Airway hyperresponsiveness. C, Macrophage number. D, Concentrations of IFN- γ and IL-4. E, Lung histology stained with Masson trichrome. Scale bar, 200 μm . F, Representative immunofluorescence staining of nuclei (blue) and α -SMA (green) in the lungs. Scale bar, 50 μm . Data are presented as means, $n = 5$. * $P < .05$, ** $P < .01$, and *** $P < .001$ obtained by Bonferroni's *post hoc* test. ns, not significant

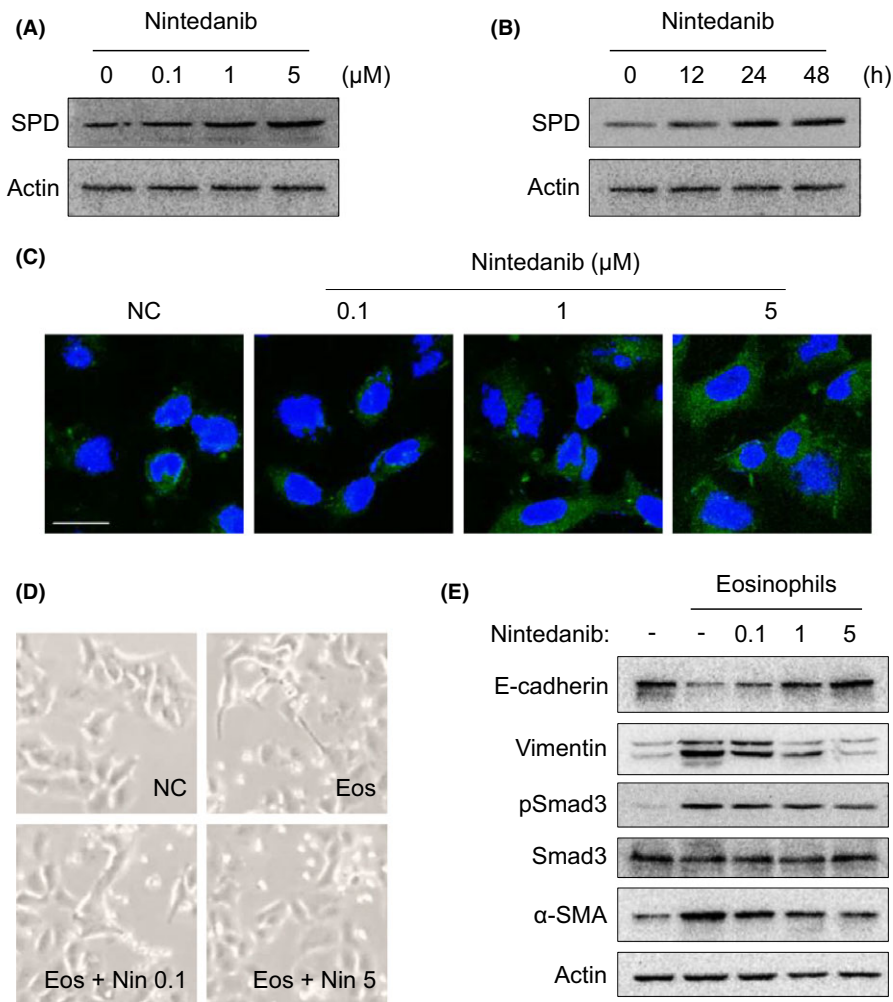


FIGURE 4 Effect of nintedanib on SPD production in A549 cells. Nintedanib upregulated SPD expression in A, a dose- and B, time-dependent manner. C, Confocal images of SPD localization on A549 cells (DAPI, blue; SPD, green). Scale bar, 20 μ m. D, Changes in A549 cell morphology by nintedanib treatment in the presence of eosinophils from patients with AERD. E, Inhibition of Smad3 signaling induced by eosinophils. Eos, eosinophils; Nin, nintedanib

genotype had a significantly lower level of serum SPD compared to those with heterozygous CT or homozygous TT genotype ($P < .001$, Figure 1E). Clinical features according to the rs721917 are summarized in Table S4.

3.3 | Effect of LTE4 on airway inflammation, remodeling, and SPD production

To evaluate the roles of LTE4 in the lungs, mice were treated with LTE4 as follows (Figure 2A). LTE4 significantly increased the number of immune cells, especially eosinophils (Figure 2B). Lung histology images also showed that LTE4 had the ability to enhance inflammatory cell infiltration (Figure 2C). Moreover, LTE4 markedly induced airway inflammation through IL-4 and IL-5 production (Figure 2D). This study further investigated the effect of LTE4 on the SPD level. Immunostaining indicated that SPD localized on epithelium was reduced by LTE4 treatment (Figure 2E). The SPD level in BALF was also decreased but increased in serum initially. However, continuous exposure to LTE4 resulted in depletion of SPD in the lungs and reduction in the SPD level in sera (Figure 2F). LTE4 affected the SPD level in the lungs by increasing eosinophil number rather than directly inhibiting SPD expression as eosinophils strongly induced

airway remodeling by upregulating expression of α -SMA (Figure S1A). In addition, eosinophils induced airway inflammation by producing pro-inflammatory cytokines in airway epithelial cells (Figure S1B), indicating that eosinophils enhanced by LTE4 play key roles in airway inflammation, remodeling, and SPD reduction.

3.4 | Role of SPD in eosinophil-mediated airway inflammation and remodeling

To investigate the effects of SPD on airway epithelial cells, A549 cells were cocultured with peripheral blood eosinophils from AERD patients with or without SPD. SPD treatment reduced α -SMA expression in airway epithelial cells in the presence of eosinophils (Figure S1C). Immunostaining revealed decreased expression of α -SMA in the cells by SPD treatment (Figure S1D). In addition to in vitro experiments, SPD function against eosinophils was tested in mice (Figure 3A). SPD-treated mice recovered from airway hyperresponsiveness compared to those instilled with eosinophils alone (Figure 3B). The number of macrophages was significantly increased in the SPD-treated groups (Figure 3C). The IFN- γ level in BALF of mice treated with SPD was higher, but not statistically significant between the SPD-treated and nontreated groups. However, the IL-4

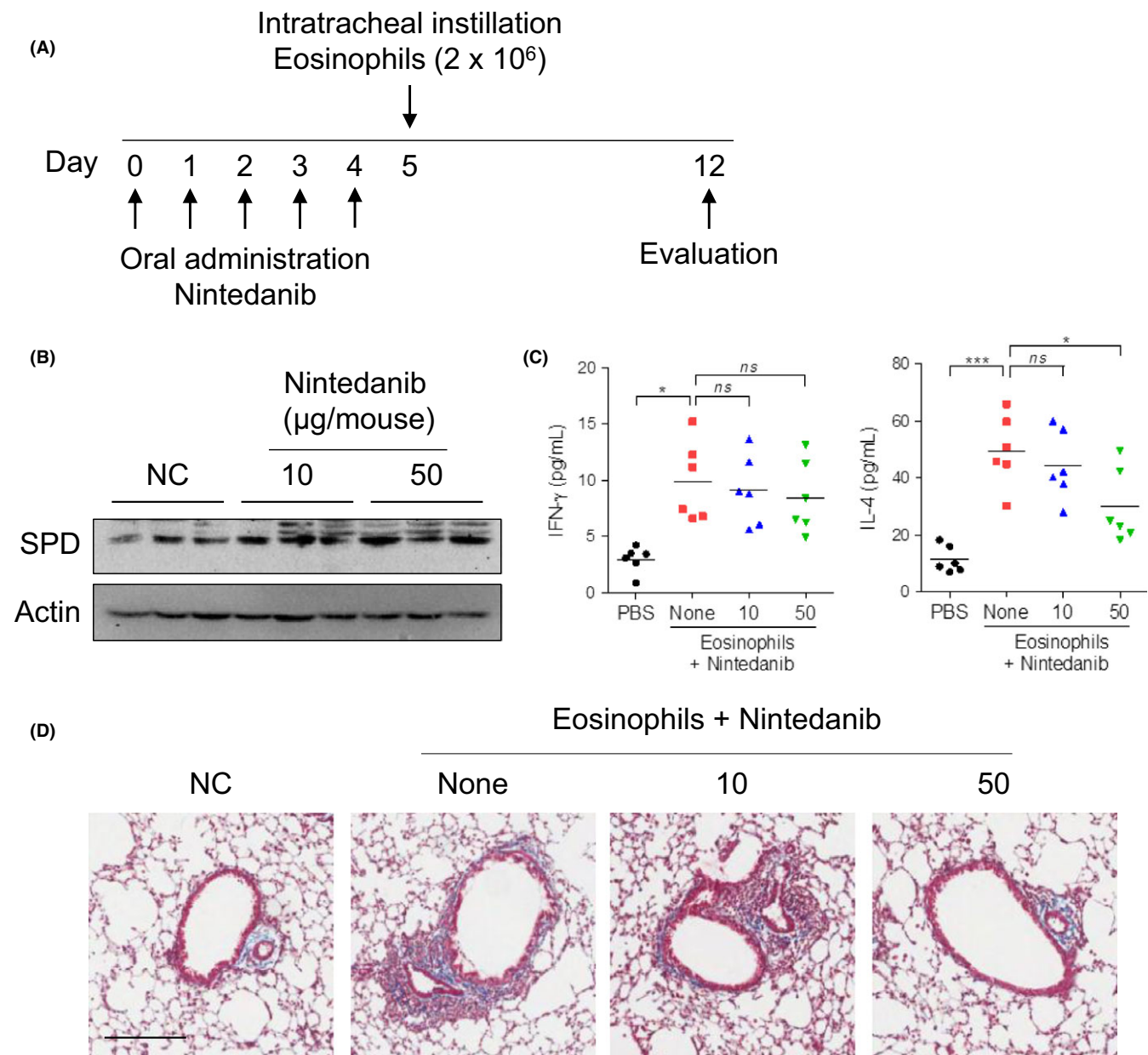


FIGURE 5 Role of SPD produced by nintedanib against eosinophils in BALB/c mice. A, Experimental schedule for nintedanib treatment. B, SPD expression in the lungs. C, Concentrations of IFN- γ and IL-4 in BALF. D, Lung histology stained with Masson trichrome. Scale bar, 200 μm . Data are presented as means, $n = 6$. * $P < .05$, and *** $P < .001$ obtained by one-way ANOVA with Bonferroni's *post hoc* test. *ns*, not significant

level decreased in the SPD-treated group compared with the non-treated group (Figure 3D). Moreover, the enhanced fibrotic area, mediated by eosinophils, was alleviated by SPD treatment (Figure 3E), indicating reduced airway remodeling. Immunostaining also revealed downregulation of α -SMA in the lung tissue (Figure 3F).

3.5 | Protective function of nintedanib through the production of SPD

Nintedanib induced SPD production from A549 cells in a dose- and time-dependent manner (Figure 4A,B). Confocal images showed

localization of SPD on cell surface, which were increased by nintedanib treatment (Figure 4C). To demonstrate a protective function of nintedanib against eosinophils, A549 cells were cocultured with eosinophils from patients with AERD. Nintedanib-pretreated cells maintained their original shapes, while untreated cells changed to the elongated shape (Figure 4D). Moreover, expressions of α -SMA, vimentin, and pSmad3 were inhibited by nintedanib treatment (Figure 4E). Airway epithelial cells cocultured with eosinophils under the trans-well system showed similar results of decreased expression of α -SMA through inhibition of Smad3 signaling (Figure S2). Furthermore, nintedanib was orally administered to confirm the efficacy in

mice (Figure 5A). Production of SPD in the lungs was enhanced during the 5-day treatment (Figure 5B). Mice instilled with eosinophils showed elevated IFN- γ and IL-4 production, but nintedanib significantly ameliorated these cytokines (Figure 5C). Additionally, eosinophil-induced airway remodeling was attenuated under the SPD-rich condition (Figure 5D), indicating that nintedanib may have potential benefits in asthmatic patients with peripheral eosinophilia and decreased serum SPD levels.

4 | DISCUSSION

This is the first study to demonstrate a lower serum SPD level in patients with AERD. LTE4 could decrease the SPD level and increase airway inflammation by mediating eosinophilic inflammation. Moreover, SPD treatment reduced airway inflammation and remodeling through inhibition of eosinophil-epithelial cell interactions. Nintedanib treatment has a protective function against eosinophils by promoting SPD production from epithelial cells. These findings suggest that SPD may be critical in the pathogenic mechanisms of airway inflammation/remodeling occurring in the upper and airway mucosa of patients with AERD through regulation of eosinophils.

AERD is associated with cysLT overproduction and more activated status of eosinophils in upper and lower airway mucosa.²⁶⁻²⁸ As for cysLTs, several medications have been suggested as the therapy to block leukotriene receptors or to inhibit leukotriene synthesis.²⁹⁻³¹ However, AERD was not completely managed by these drugs. Steroid is an effective eosinophil apoptosis-inducing agent; however, as eosinophilic inflammation in patients with AERD is not completely controlled, further treatment modalities are needed. Based on these hypotheses, this study was conducted to investigate the role of SPD mainly expressed in airway epithelial cells may play a role in cysLT/eosinophil-mediated airway inflammation. In the present study, we demonstrated a significantly lower serum SPD level in the AERD group than in the ATA group, in which genetic polymorphisms may affect SPD production in AERD group, as previous reports have shown that genetic polymorphisms of SPD contribute to the development of allergic rhinitis and lung inflammations.³¹⁻³³ In addition, there were negative correlations between the serum SPD level and FEV₁ fall after the L-ASA BPT and/or the urinary LTE4 level in patients with AERD. Moreover, LTE4 treatment in mice has shown reduced expression of localized SPD in airway epithelial cells and decreased levels of SPD in BALF/sera with BAL eosinophilia. These findings suggest that a higher cysLT level in patients with AERD could reduce SPD production, which may contribute to eosinophilic inflammation in AERD.

SPD is a lipid and protein complex that is involved in pathogen clearance and immune cell regulation.¹⁰ In SPD-deficient mice, allergic immune responses, especially Th2 response that enhances IL-13 production, are increased by allergen exposure.³² In addition, *Sftpd*^{-/-} mice have an impaired systemic Th2 response at baseline as the signaling through Toll-like receptors increases by endotoxin

exposure.³³ Considering that airway eosinophilia is closely associated with the severity of upper and lower airway inflammation and poor outcome of patients with AERD, the current study focused on the role of SPD against eosinophils in airway inflammation. In the present study, LTE4 treatment in mice could induce reduction of localized SPD expression in airway epithelial cells and decreased SPD level in BALF. However, SPD levels in sera were increased after a short-term exposure (which may be derived from an alveolar-to-vascular leakage). These findings suggest that LTE4 itself may have minimal effects on SPD or α -SMA expression in airway epithelial cells, but if exposed to eosinophils, SPD expression can be downregulated, while α -SMA expression is upregulated. When SPD was treated with eosinophils in mice, the IFN- γ level in BALF was increased, and the IL-4 level was decreased, which are comparable with that of a previous study demonstrating that SPD shifts immune responses from Th2 to Th1, leading to the modulation of allergic reactions.³⁴ Increased IFN- γ production may indicate a protective function of SPD. Furthermore, the number of macrophages in BALF was increased by SPD treatment. SPD promotes the activation of macrophages and contributes to the clearance of apoptotic cells.³⁵ In an in vitro study, SPD treatment in the presence of eosinophils could reduce expression of α -SMA, a remodeling marker, in airway epithelial cells. These findings suggest that SPD may inhibit eosinophil-induced airway inflammation/remodeling by enhancing macrophage infiltration to remove SPD-bound eosinophils. AERD patients with higher eosinophilia and lower serum SPD levels suffer from more severe clinical symptoms through enhanced eosinophil-mediated airway inflammation/remodeling as proposed in Figure S3.

Nintedanib was originally developed as a potent intracellular inhibitor of tyrosine kinases; however, it also upregulated SPD production in epithelial cells.²⁰ In this study, nintedanib significantly increased SPD production, which protected airway epithelial cells from eosinophils. This observation indicates that nintedanib has multiple functions through a new mechanism that is distinct from the tyrosine kinase inhibition. Previously, SPD treatment has already been suggested to reduce neonatal chronic lung disease, cystic fibrosis, and emphysema.³⁶ Although further studies are needed, we suggest that nintedanib may provide potential benefits via suppressing eosinophilic inflammation in patients with AERD.

The present study has some limitations: First, the SPD level in BALF of asthmatic patients could not be measured, which may have provided direct evidence to explain SPD roles in the lungs. Secondly, the mechanism through which SPD inhibits interactions between eosinophils and epithelial cells has not yet been completely established. Although SPD binds to CD32 (Fc γ II receptor) on eosinophils,¹⁴ further analyses may show whether SPD can affect other adhesion molecules on the surface of eosinophils.³⁷ Thirdly, patients with AERD need to be categorized into more specific endotypes because all patients had wide ranges of peripheral/sputum eosinophil counts, survivals, and activities, which may have been associated with the clinical outcome.³⁸ AERD endotypes are necessary for more targeted and precise treatment.³⁹

In conclusion, a decreased SPD level may contribute to the pathogenesis of AERD through elevating eosinophil-mediated airway inflammation/remodeling. Therefore, enhancing SPD production might be a new therapeutic approach to AERD patients with eosinophilia.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Youngwoo Choi designed and performed the experiments and wrote the manuscript. Dong-Hyun Lee and Hoang Kim Tu Trinh performed the experiments. Ga-Young Ban, Han-Ki Park, Yoo Seob Shin, and Seung-Hyun Kim joined in designing the experiments. Hae-Sim Park supervised all the processes.

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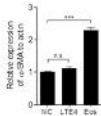
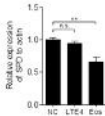
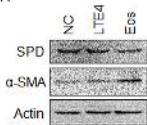
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SUPPORTING INFORMATION

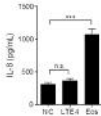
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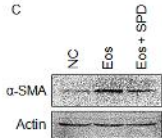
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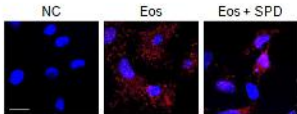
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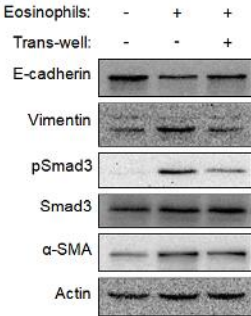


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Supporting Information

Surfactant protein D alleviates eosinophil-mediated airway inflammation and remodeling in patients with aspirin-exacerbated respiratory disease

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Supplementary Tables

Table S1 Demographic data of the study subjects

Clinical parameter	AERD	ATA	HC	P value		
				AERD vs. ATA	AERD vs. HC	ATA vs. HC
Age (y)	43.2 ± 13.9 / 336	44.7 ± 14.4 / 442	31.6 ± 10.6 / 104	0.036	<0.001	<0.001
Female sex (%)	64.9 / 336	61.8 / 442	54.5 / 104	0.372	0.012	0.062
Atopy (%)	52.1 / 330	48.9 / 412	28.0 / 99	0.384	<0.001	<0.001
Paranasal sinusitis (%)	54.5 / 253	22.0 / 159	NA	<0.001	NA	NA
Nasal polyp (%)	42.1 / 271	18.2 / 104	NA	<0.001	NA	NA
Baseline FEV ₁ (%)	84.9 ± 21.0 / 319	88.5 ± 20.7 / 338	NA	0.027	NA	NA
Fall of FEV ₁ (%)	22.1 ± 11.0 / 281	6.7 ± 4.1 / 186	NA	<0.001	NA	NA
PC ₂₀ (mg/mL)	4.0 ± 6.1 / 248	5.7 ± 6.3 / 251	NA	0.002	NA	NA
Total IgE (kU/L)	358.4 ± 550.1 / 328	379.9 ± 648.3 / 411	NA	0.632	NA	NA
TEC (/μL)	413.9 ± 412.2 / 310	387.6 ± 373.7 / 345	NA	0.392	NA	NA
Sputum Eos (%)	23.9 ± 35.2 / 217	21.4 ± 32.5 / 216	NA	0.431	NA	NA
Sputum Neu (%)	54.5 ± 34.6 / 166	59.8 ± 33.2 / 192	NA	0.526	NA	NA

AERD, aspirin-exacerbated respiratory disease; ATA, aspirin-tolerant asthma; HC, healthy controls; FEV₁, forced expiratory volume in 1 s; methacholine PC₂₀, the provocative concentration of methacholine required to cause a 20% fall in FEV₁; IgE, immunoglobulin E; TEC, total eosinophil count; Eos, eosinophils; Neu, neutrophils; NA, not applicable.

Values are given as n (%) for categorical variables and as mean ± SD for continuous variables.

P values were obtained by Pearson chi-square test for categorical variable and Student's *t* test for continuous variables.

Table S2 Primer sequence for *SFTPD* polymorphism analysis

Target SNPs		Primer sequence
2538C>T (rs721917) Exon 2	Forward	CCTAAACCATGTCCATGAAG
	Reverse	CAGTTCCAATGACCATTCC
	Genotyping	TGAAGACCTACTYCCACAGAACAA
7140G>A (rs2243639) Exon 5	Forward	AGTCTCCCCACTTCTCTCTC
	Reverse	AGATTCTCTCCATGTTCCAG
	Genotyping	ACTGCTCACCTGCTGCCCCTG
10994T>A (rs3088308) Exon 8	Forward	CACACAGGCTGGTGGACA
	Reverse	CACCCCAGTTGGCTCAGA
	Genotyping	GGCTGGTGGACAGTTGGCC

Table S3 Genetic association of *SFTPD* polymorphisms in the study subjects

Genotype	AERD, n (%)	ATA, n (%)	HC, n (%)	AERD vs. ATA		AERD vs. HC		ATA vs. HC	
				OR (95% CI) [§]	P value	OR (95% CI) [§]	P value	OR (95% CI) [§]	P value
rs721917									
CC	47 (42.3)	56 (25.7)	27 (28.4)	reference		reference		reference	
CT+TT	64 (57.7)	162 (74.3)	68 (71.6)	1.310 (2.124, 3.446)	0.002	1.850 (1.032, 3.315)	0.038	0.871 (0.508, 1.493)	0.615
rs2243639									
GG	64 (59.3)	111 (49.6)	58 (62.4)	reference		reference		reference	
AG+AA	44 (40.7)	113 (50.4)	35 (37.6)	1.481 (0.930, 2.357)	0.097	0.878 (0.497, 1.550)	0.653	0.593 (0.361, 0.972)	0.037
rs3088308									
TT	109 (98.2)	209 (98.6)	92 (96.8)	reference		reference		reference	
AT+AA	2 (1.8)	3 (1.4)	3 (3.2)	0.782 (0.129, 4.752)	0.528	1.777 (0.291, 10.866)	0.789	2.272 (0.450, 11.468)	0.308

[§]Odds ratio are given by recessive analysis model.

P values were obtained by Pearson chi-square test.

Table S4 Clinical features according to the rs721917 in asthmatic patients

Clinical parameter	CT+TT	CC	<i>P</i> value
Age (y)	44.8 ± 13.7 / 226	43.9 ± 15.8 / 103	0.626
Female sex (%)	62.4 / 226	65.0 / 103	0.643
Atopy (%)	46.9 / 211	50.0 / 102	0.609
Paranasal sinusitis (%)	59.4 / 64	60.0 / 40	0.950
Nasal polyp (%)	40.7 / 59	42.5 / 40	0.857
Baseline FEV ₁ (%)	88.5 ± 21.9 / 174	89.1 ± 18.0 / 95	0.800
Fall of FEV ₁ (%)	15.5 ± 11.8 / 83	18.8 ± 14.1 / 55	0.137
PC ₂₀ (mg/mL)	6.2 ± 6.8 / 127	5.4 ± 6.4 / 67	0.432
Total IgE (kU/L)	365.7 ± 663.9 / 209	400.7 ± 695.3 / 100	0.670
TEC (/μL)	400.8 ± 390.5 / 178	363.7 ± 292.6 / 92	0.423
Sputum Eos (%)	19.9 ± 31.7 / 136	23.5 ± 34.4 / 71	0.446
Sputum Neu (%)	60.7 ± 33.1 / 111	62.0 ± 34.6 / 65	0.816

FEV₁, forced expiratory volume in 1 s; methacholine PC₂₀, the provocative concentration of methacholine required to cause a 20% fall in FEV₁; IgE, immunoglobulin E; TEC, total eosinophil count; Eos, eosinophils; Neu, neutrophils.

Values are given as n (%) for categorical variables and as mean ± SD for continuous variables.

P values were obtained by Pearson's chi-squared test for categorical variables and Student's *t* test for continuous variables.

Eosinophils

In AERD

LTE₄↑, SPD↓

SPD

Airway epithelial cells

