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SUPPORTING INFORMATION

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Immunomodulatory function of surfactant protein D in eosinophilic asthma

To the Editor,

Asthma is a chronic inflammatory disease in the lungs with several phenotypes.¹ Traditionally, asthma has long been considered a Th2 response mediated by multiple factors.² In particular, periostin (a secreted matricellular protein) contributes to eosinophilic inflammation.³ In addition, it has been suggested that periostin induces transforming growth factor (TGF)- β 1 production, leading to structural changes in the airways.⁴ Recently, we have demonstrated the function of surfactant protein D (SPD) in patients with aspirin-exacerbated respiratory disease (AERD), a representing phenotype of eosinophilic asthma.⁵ All these molecules are released from airway epithelium and are involved in airway inflammation; however, little is known about the relation of SPD with type 2 cytokines in the process of eosinophilic asthma.

This study enrolled 884 adult patients and 230 healthy control subjects (HCs). The patients with asthma showed significantly higher serum levels of periostin and TGF- β 1 than HCs ($P < 0.001$ and $P < 0.001$, respectively). When patients with asthma were classified according to peripheral total eosinophil count (TEC), patients with eosinophilic asthma ($\text{TEC} \geq 300$ cells/ μL) had a higher

prevalence of severe asthma, a higher total IgE, and a lower PC₂₀ methacholine than those with noneosinophilic asthma ($P = 0.005$, $P < 0.001$, and $P = 0.026$, respectively; Table S1). In addition, the serum levels of periostin and TGF- β 1 were significantly higher in patients with eosinophilic asthma ($P < 0.001$ and $P = 0.006$, respectively; Table S1) than those with noneosinophilic asthma ($\text{TEC} < 300$ cells/ μL).

Furthermore, when patients with asthma were classified by the cutoff value (116.7 ng/mL) of serum periostin (high/low periostin group), an interval (mean+2SD) indicating the chance of a test value coming outside less than 5%, TEC as well as serum levels of TGF- β 1 and SPD was significantly higher in the high periostin group than in the low periostin group ($P = 0.011$, $P = 0.001$, and $P = 0.040$, respectively; Table S2). Considering that periostin is a systemic biomarker for eosinophilic airway inflammation and associated with TGF- β 1 activation, significantly positive correlations were found between serum periostin and TEC ($r = 0.207$, $P < 0.001$; Figure S1A) or TGF- β 1 levels ($r = 0.221$, $P < 0.001$; Figure S1B). These observations were weaker than expected due to the complexity that several cells including immune cells and structural cells are the potential

sources of periostin and TGF- β 1. In addition, significantly negative correlations were found between serum periostin and FEV₁ (%) value ($r = -0.104$, $P = 0.032$; Figure S2A) or between TGF- β 1 and PC₂₀ methacholine value ($r = -0.129$, $P = 0.028$; Figure S2B). These findings suggest that periostin/TGF- β 1 may contribute to develop airway obstruction and severe asthma in adult asthmatics.

Many previous reports have suggested that SPD is involved in several lung diseases including asthma, but the role of SPD in eosinophilic asthma is still unclear.⁶ When the relationship between SPD and periostin or TGF- β 1 was analyzed, positive correlations were found between serum SPD and periostin ($r = 0.126$, $P = 0.014$; Figure S2C) or TGF- β 1 levels ($r = 0.109$, $P = 0.039$; Figure S2D). These findings suggest that epithelial damage under chronic inflammatory conditions in asthmatic airway may result in the efflux of SPD from epithelial cells into vessels,⁷ although an inverse association between SPD and markers for type 2 inflammation may be easier to explain protective function of SPD in asthma. In this study, to confirm the role of SPD in asthma using *in vivo* models, we recruited 2 different mouse models of asthma

(sensitized by ovalbumin with alum or LPS) as shown in Figure 1A. Both models showed significantly increased airway hyperresponsiveness (Figure 1B), while cell proportions (especially eosinophilia) in bronchoalveolar lavage fluid (BALF) were significantly different between the 2 groups ($P < 0.05$ for all, Figure 1C). Moreover, compared to ovalbumin (with LPS)-sensitized models, significantly higher levels of Th2 cytokines as well as higher levels of periostin/TGF- β 1/SPD were noted in the BALF of ovalbumin (with alum)-sensitized mice (Figure 1D), which is considered a model of eosinophilic asthma. Several biologics have been developed to manage eosinophilic asthma using the antagonists of Th2 cytokines,⁸ but still some patients are not completely controlled. Therefore, this study investigated the effect of SPD as a novel therapeutic agent in the regulation of eosinophilic asthma. We selected the optimal concentration of SPD for the treatment as described in Figure S3. Ovalbumin-sensitized mice were treated with SPD intranasally 1 h before every challenge, which were compared with the effect of dexamethasone and montelukast, 2 major anti-asthmatic medications. All treatments significantly reduced airway

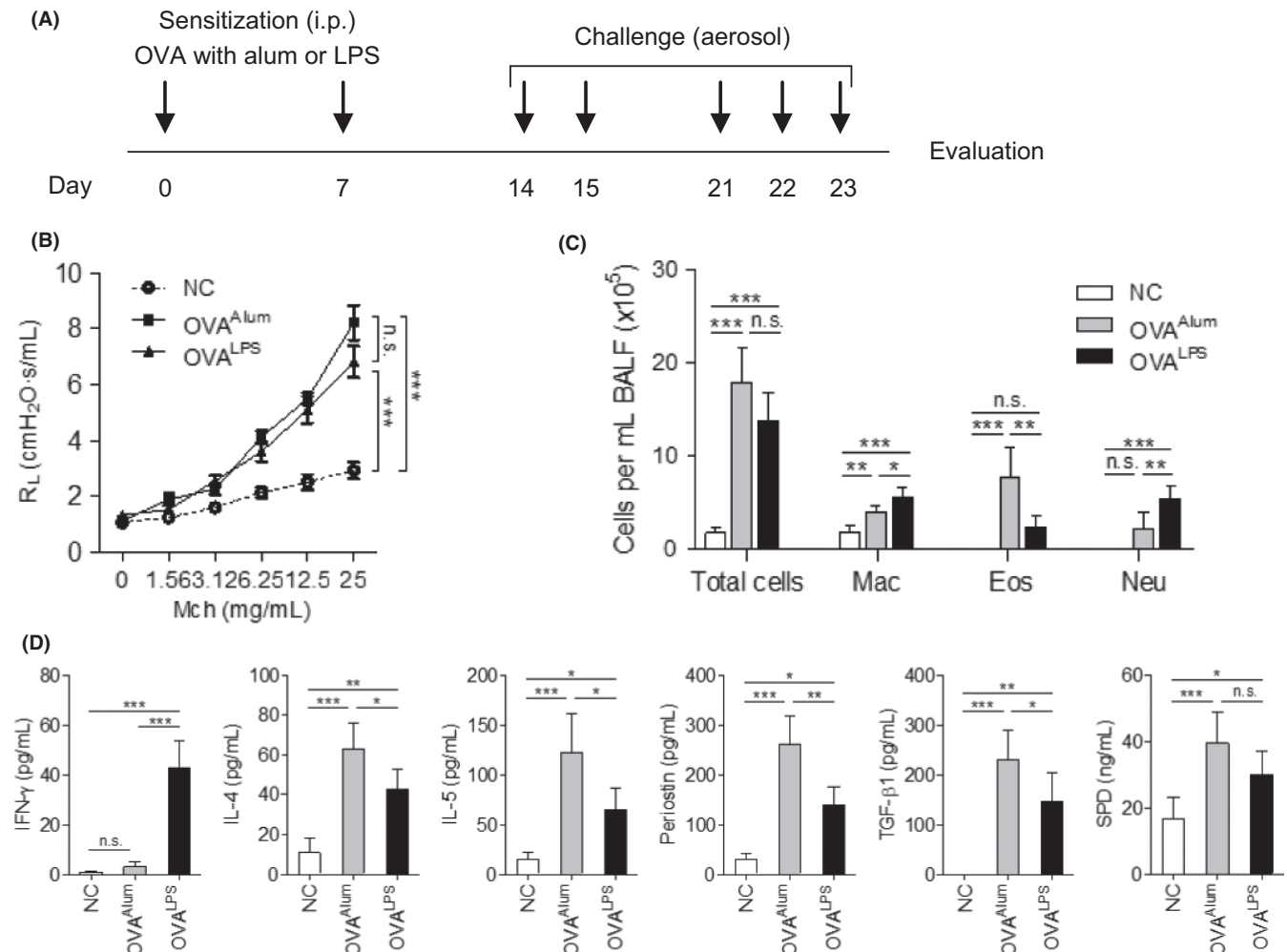


FIGURE 1 Comparisons of immune responses in 2 different asthma mouse models. A, Experimental schedule for inducing asthma. B, Airway hyperresponsiveness. MCh, methacholine. C, Differential cell count. D, Concentration of cytokines in BALF. Data are represented as means $\pm$ SD, $n = 5$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ obtained by one-way ANOVA with Bonferroni's *post hoc* test. n.s., not significant. Mac, macrophages; Eos, eosinophils; Neu, neutrophils

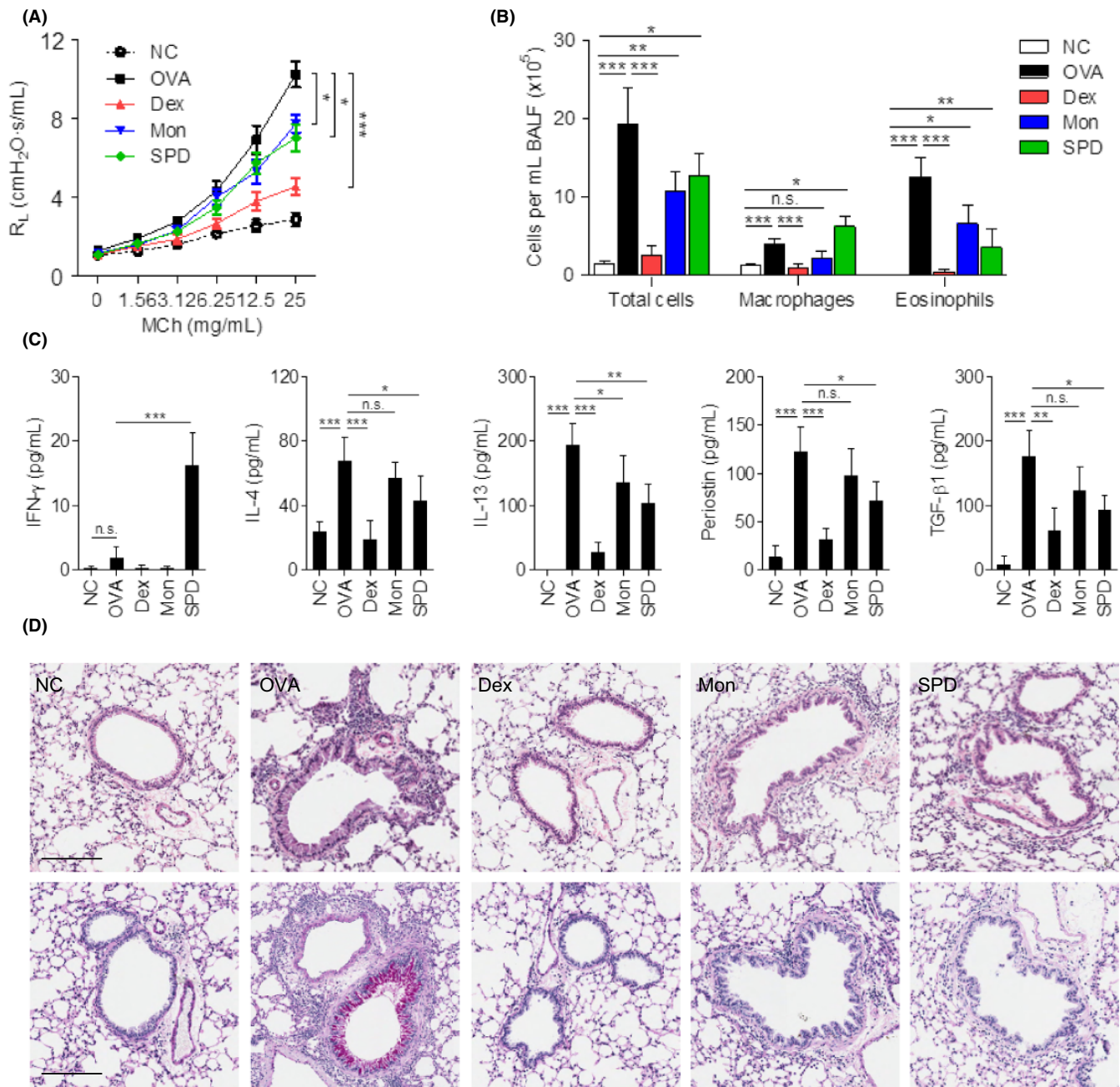


FIGURE 2 Immunomodulatory function of SPD in ovalbumin (with alum)-sensitized mice. A, Airway hyperresponsiveness. B, Differential cell count. C, Concentration of cytokines in BALF. D, Lung histology stained with hematoxylin and eosin (upper) or PAS (lower). Scale bar, 200 μ m. Data are represented as means $\pm$ SD, $n = 5$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ obtained by one-way ANOVA with Bonferroni's *post hoc* test. n.s., not significant

hyperresponsiveness (Figure 2A) and the number of total immune cells in BALF (Figure 2B); however, SPD decreased the number of eosinophils, but increased macrophages in BALF. Dexamethasone and montelukast attenuated the levels of periostin and TGF- β 1 as well as Th2 cytokines (IL-4/IL-13); however, SPD enhanced IFN- γ production (Figure 2C). In addition, T cells isolated from the lungs of SPD-treated mice showed increased Th1, but decreased Th2 cytokine production when stimulated with anti-CD3/28 antibodies (Figure S4). Although a specific mechanism of SPD for the

induction of Th1 response is not completely elucidated, a previous study has revealed that SPD increases Th1 cytokines such as IFN- γ and IL-12 by modulating the function of alveolar macrophages.⁹ Nevertheless, lung histology stained with hematoxylin and eosin or PAS showed a protective function of SPD in the airways (Figure 2D). Therefore, we suggest that SPD may be a novel molecule with the potential benefits to manage patients with eosinophilic asthma who do not respond well to conventional anti-inflammatory agents.

In conclusion, increased levels of epithelium-derived molecules (periostin and TGF- β 1) contribute to develop eosinophilic asthma, in which SPD may provide a new therapeutic option as an immunomodulator through shifting immune responses from Th2 to Th1.

FUNDING INFORMATION

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

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Tolerability of iobitridol in patients with non-immediate hypersensitivity reactions to iodinated contrast media

To the Editor,

Non-immediate reactions (NIRs) with iodinated contrast media (ICMs) are increasing, particularly with iodixanol.^{1,2} Iobitridol has low cross-reactivity with other ICMs and produces less NIRs. We evaluated iobitridol as an alternative ICM in patients with NIRs with ICMs.

This is a retrospective study of 142 patients that consulted our Allergy Service between July 2011 and July 2017 due to suspected NIRs to ICMs. All patients signed a written informed consent.

Skin tests [prick, intradermal (ID) and patch tests (PT)] were performed with 4 ICMs (iohexol, iodixanol, ioversol and iobitridol), according to ENDA/EAACI recommendations³ (Appendix S1). If STs

with iobitridol were negative, a single-blinded placebo-controlled drug provocation test (CDPT) with iobitridol was performed. The first day, 5 mL and 20 mL of iobitridol were administered intravenously at hourly intervals. If tolerated, two 50 mL doses were administered with an interval of 1 hour the following week. Before entering CDPTs, all possible risks were carefully reviewed.

The characteristics of the sample are shown in Table S1. Iodixanol was the most frequently involved ICM (52.8%) followed by iohexol (16.2%), iobitridol (2.1%) and ioversol (1.4%). The ICM was unknown in 39 cases (27.5%). Maculopapular exanthemas (MPE) were the most frequent clinical manifestation (66.9%), although more

Supporting Information

Immunomodulatory function of surfactant protein D in eosinophilic asthma

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Methods

Study Subjects

This study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108). All patients provided written informed consent at the time of recruitment. Adult patients with eosinophilic asthma (n=422), those with non-eosinophilic asthma (n=462) and healthy control subjects (HC, n=230) were enrolled. The diagnosis of asthma was based on typical symptoms, spirometry results (airway obstruction with reversibility) and increased airway hyperresponsiveness to methacholine. In addition, the severe asthma group was classified following the ATS/ERS guidelines.¹ 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 blue grass and *Meadow fescue*), mugwort, ragweed, Japanese hops, *Aspergillus* and *Alternaria*. Eosinophilic asthma was defined when study subjects had a large number of peripheral eosinophils (more than 300 eosinophils/ μ L).² To determine the cutoff value for high or low periostin, 95% confidence intervals of mean were used. An interval obtained by adding standard deviation (SD) from mean shows the chance of a test value coming outside less than 5%. The higher limit of this interval (mean + 2SD) was considered the cutoff value.

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). To induce eosinophilic asthma, mice were injected intraperitoneally with 75 μ g

ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO, USA) with 2 mg alum (Thermo FisherScientific, Waltham, CA USA) or 10 µg LPS (Sigma-Aldrich) for sensitization. Then, mice were challenged with 1% ovalbumin in saline using a nebulizer. To investigate the effect of human recombinant SPD (R&D Systems, Minneapolis, MN, USA) in ovalbumin-induced asthmatic mice, 10 µg of SPD was injected intranasally 1 h before each challenge. 1 mg/kg dexamethasone (Sigma-Aldrich) or 10 mg/kg montelukast (Sigma-Aldrich) were administered orally for comparison with SPD.

Differential cell count

The total number of inflammatory cells was counted. After Diff-quick staining (Dade Behring, Dudingon, Switzerland) of bronchoalveolar lavage fluid (BALF) pellets in a cytopsin preparation, the types of inflammatory cells were determined by counting 300 cells, which were classified as macrophages, lymphocytes, neutrophils, or eosinophils.

Measurement of airway resistance

Airway resistance to inhaled methacholine (Sigma-Aldrich) was measured using the flexiVent System (SCIREQ, Montreal, Canada). Each mouse was injected with aerosol methacholine at increasing concentrations (0, 1.56, 3.12, 6.25, 12.5 and 25 mg/mL), and the peak airway responses to the inhaled methacholine were recorded.

T-cell stimulation

T cells were isolated from the lungs using immunomagnetic cell sorting (Miltenyi Biotec Inc., Auburn, CA, USA) according to the manufacturer's recommendations. These cells (5×10^5) were seeded on a 24-well plate (TPP) and stimulated with anti-CD3/CD28 antibodies (1 µg/mL each; eBioscience, San Diego, CA, USA) for 15 h.

Lung histology

Lungs were fixed in 4% formalin, embedded in paraffin, and cut into 5- μ m sections. Tissue sections were stained with H&E or PAS, and observed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

ELISA

SPD, IFN- γ , IL-4, IL-5, IL-13, TGF- β 1 (R&D Systems), eosinophil-derived neurotoxin (MBL, Sakae, Japan) and periostin (human: Shino-Test Corporation, Tokyo, Japan; mouse: R&D Systems) were measured by using ELISA kits, according to the manufacturer's recommendations.

Statistical analysis

Continuous variables were analyzed using Student's *t* test or one-way ANOVA with Bonferroni's *post hoc* test. Pearson's Chi-squared test was applied for categorical variables. Statistical analyses were performed using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA). A *P* value of < 0.05 was considered to indicate statistical significance. GraphPad Prism 5.0 software (GraphPad Inc., San Diego, CA, USA) was used to produce graphs.

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Table S1. Clinical characteristics of eosinophilic (TEC $\geq$ 300)/non-eosinophilic (TEC $<$ 300) adult asthmatic patients and healthy control subjects

Variables	Asthma		HCs (n=230)	<i>P</i> value	
	Eosinophilic (n=422)	Non-eosinophilic (n=462)		Asthma vs. HC	Eosinophilic vs. Non-eosinophilic
Age (y)	43.2 $\pm$ 13.7	45.7 $\pm$ 15.8	32.0 $\pm$ 11.1/230	<0.001	0.015
Female sex (%)	57.1/387	64.8/439	56.0/230	0.117	0.026
Atopy (%)	57.6/415	53.2/452	21.8/165	<0.001	0.218
Severe asthma (%)	19.2/422	12.3/462	ND	ND	0.005
Baseline FEV ₁ (%)	86.2 $\pm$ 20.8/369	87.7 $\pm$ 21.7/378	ND	ND	0.321
PC ₂₀ (mg/mL)	8.3 $\pm$ 21.2/315	11.9 $\pm$ 19.3/321	ND	ND	0.026
Total IgE (kU/L)	472.4 $\pm$ 829.6/417	309.9 $\pm$ 500.1/456	79.7 $\pm$ 126.9/65	<0.001	<0.001
Sputum Eos (%)	30.2 $\pm$ 36.6/265	15.6 $\pm$ 29.1/261	ND	ND	<0.001
Sputum Neu (%)	52.8 $\pm$ 64.9/220	64.9 $\pm$ 31.6/215	ND	ND	<0.001
Periostin (ng/mL)	88.06 $\pm$ 42.3/225	71.5 $\pm$ 33.7/223	57.9 $\pm$ 29.5/141	<0.001	<0.001
TGF- β 1 (ng/mL)	31.2 $\pm$ 16.6/197	26.9 $\pm$ 14.3/205	25.5 $\pm$ 11.3/175	<0.001	0.006
SPD (ng/mL)	3.2 $\pm$ 2.8/155	3.1 $\pm$ 2.9/226	2.8 $\pm$ 2.3/36	0.359	0.708

Values are given as n (%) for categorical variables and as mean $\pm$ 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.

TEC, total eosinophil count ; 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; Eos, eosinophils; Neu, neutrophils; SPD, surfactant protein D; ND, no data.

Table S2. Comparison of clinical characteristic between asthmatic patients with high (≥ 116.7 ng/mL) and low (< 116.7 ng/mL) periostin levels

Variables	High (n=89)	Low (n=578)	<i>P</i> value
Age (y)	43.5 $\pm$ 14.2	43.0 $\pm$ 14.2	0.710
Female sex (%)	61.2	65.0	0.537
Atopy (%)	44.5	40.3	0.336
Severe asthma (%)	37.6	18.8	< 0.001
Baseline FEV ₁ (%)	85.4 $\pm$ 22.2	88.4 $\pm$ 20.3	0.264
PC ₂₀ (mg/mL)	6.5 $\pm$ 10.0	10.5 $\pm$ 13.8	0.015
TEC (cells/ μ L)	666.7 $\pm$ 785.3	405.9 $\pm$ 587.8	0.011
Total IgE (kU/L)	530.9 $\pm$ 1146.5	327.2 $\pm$ 570.9	0.189
Sputum Eos (%)	25.3 $\pm$ 36.6	18.9 $\pm$ 31.9	0.187
Sputum Neu (%)	54.4 $\pm$ 37.2	60.4 $\pm$ 33.1	0.317
TGF- β 1 (ng/mL)	36.0 $\pm$ 15.6	30.5 $\pm$ 21.5	0.001
SPD (ng/mL)	3.7 $\pm$ 3.6	2.9 $\pm$ 2.7	0.040

Values are given as n (%) for categorical variables and as mean $\pm$ 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.

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

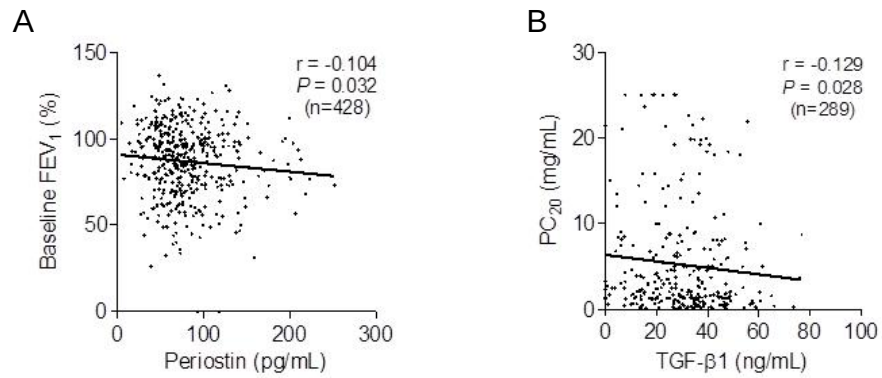


Figure S1. Correlation between periostin/TGF-β1 and lung function decline. Correlations between (A) periostin/baseline FEV₁ and (B) TGF-β1/PC₂₀ metahocline. The data are presented as Spearman correlation coefficient r (P value). TEC, total eosinophil count.

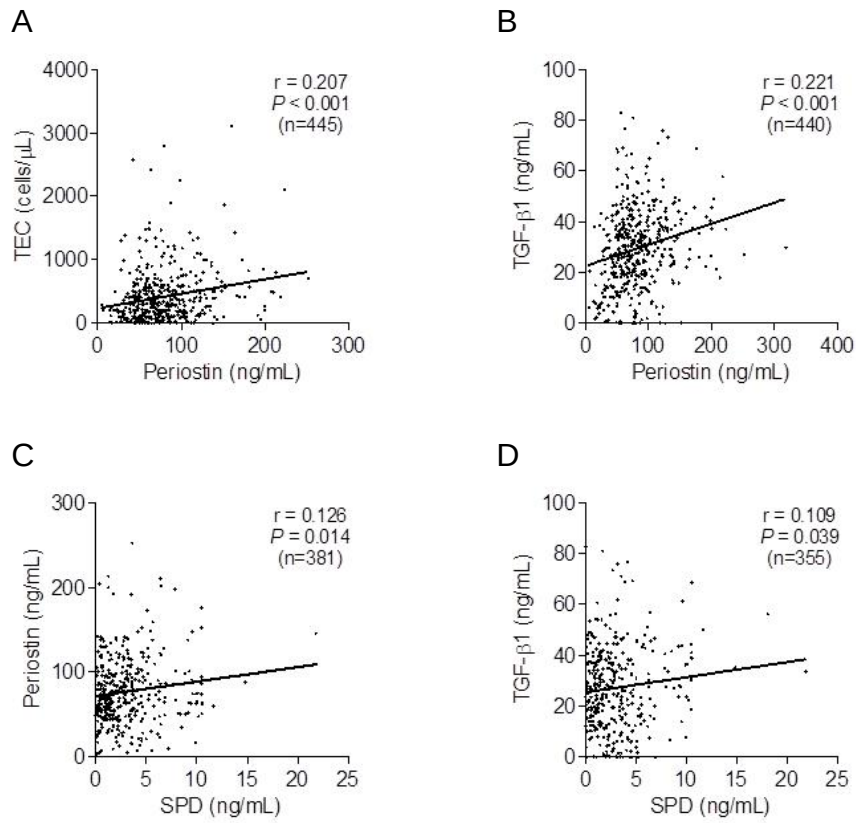
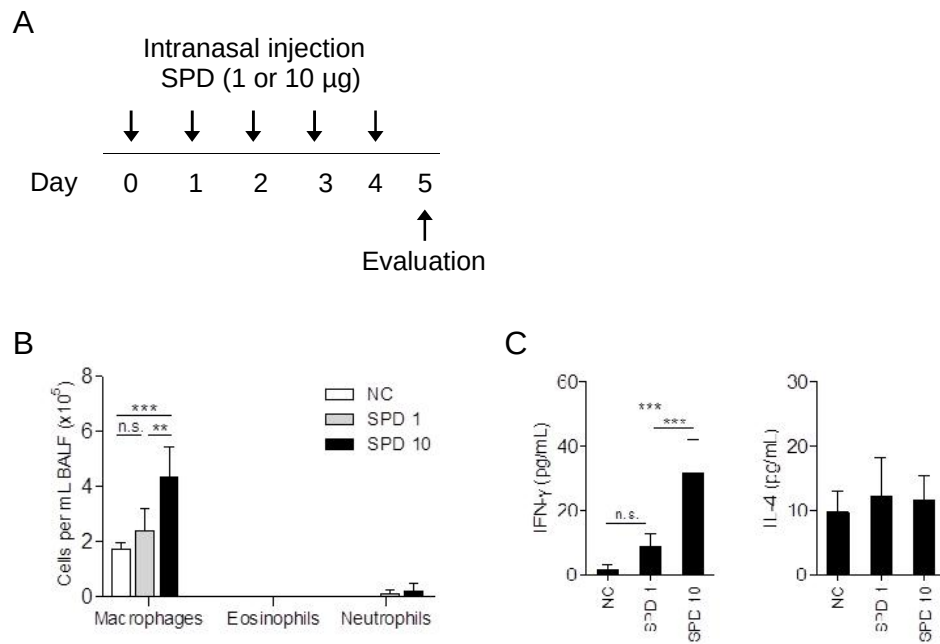
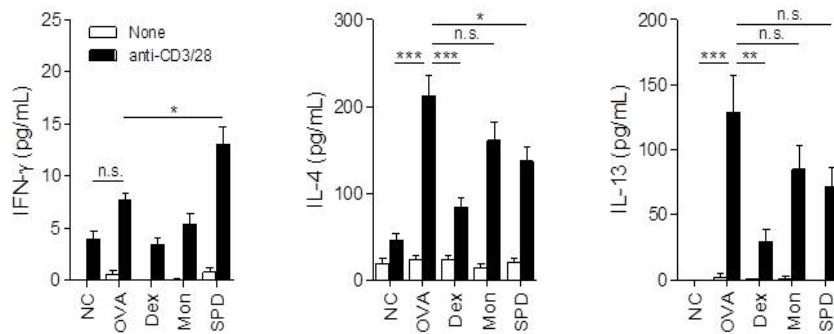


Figure S2. Correlation between serum periostin and TEC or TGF- β 1 levels (S2A, S2B) and between serum SPD and periostin or TGF- β 1 levels (S2C, S2D). The data are presented as Pearson correlation coefficient r (P value). TEC: total eosinophil count.



28

29 **Figure S3.** Effect of SPD on immune responses in normal mice. (A) Experimental schedule
30 for SPD treatment. (B) Differential cell count. (C) Concentration of cytokines in BALF. Data
31 are represented as means $\pm$ SD, $n = 5$. $**P < 0.01$ and $***P < 0.001$ obtained by one-way
32 ANOVA with Bonferroni's *post hoc* test. n.s., not significant.



33

34 **Figure S4.** Cytokines released from T cells in the lungs of allergic mice. Data are represented
35 as means $\pm$ SD, $n = 5$. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, obtained by one-way
36 ANOVA with Bonferroni's *post hoc* test. n.s., not significant.