

Contribution of dipeptidyl peptidase 10 to airway dysfunction in patients with NSAID-exacerbated respiratory disease

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Abstract

Background: Genetic variants of dipeptidyl peptidase 10 (DPP10) have been suggested to contribute to the development of NSAID-exacerbated respiratory disease (NERD). However, the mechanisms of how DPP10 contributes to NERD phenotypes remain unclear.

Objective: To demonstrate the exact role of DPP10 in the pathogenesis of NERD.

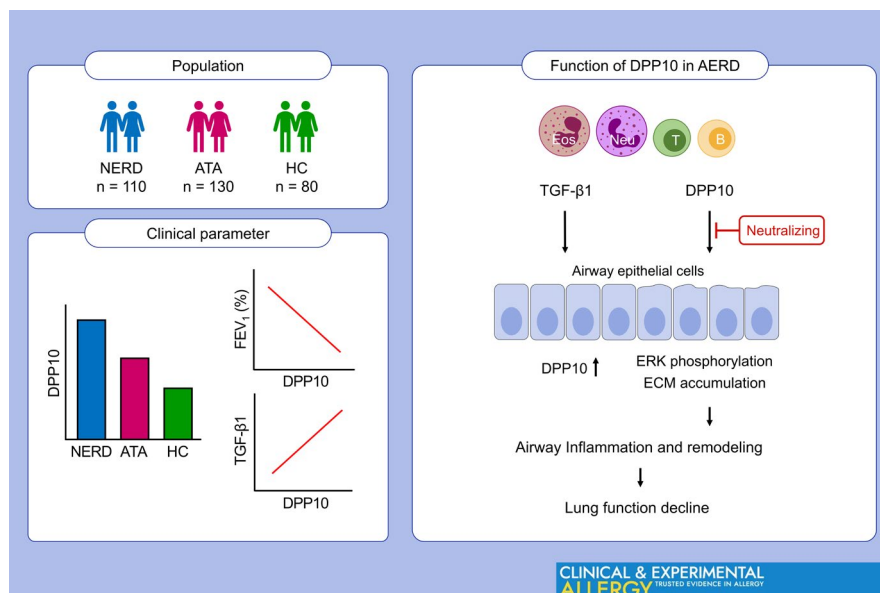
Methods: Patients with NERD ($n = 110$), those with aspirin-tolerant asthma (ATA, $n = 130$) and healthy control subjects (HCs, $n = 80$) were enrolled. Clinical characteristics were analysed according to the serum DPP10 levels in both NERD and ATA groups. The function of DPP10 in airway inflammation and remodelling was investigated with *in vitro*, *ex vivo* and *in vivo* experiments.

Results: NERD patients had higher levels of serum DPP10 and TGF- β 1 with lower FEV₁ than ATA patients or HCs ($p < .05$ for each). NERD patients with higher DPP10 levels had higher TGF- β 1, but lower FEV₁ ($p < .05$ for all), whilst no differences were noted in ATA patients. Moreover, the serum DPP10 levels had a positive correlation with TGF- β 1 ($r = 0.384$, $p < .001$), but a negative correlation with FEV₁ ($r = -0.230$, $p = .016$) in NERD patients. In *in vitro* studies, expression of DPP10 in airway epithelial cells was enhanced by TGF- β 1 treatments. Furthermore, DPP10 was found to be produced from immune cells and this molecule induced the ERK phosphorylation in airway epithelial cells, which was suppressed by anti-DPP10 treatment. In asthmatic mouse models, increased levels of DPP10 in the serum and TGF- β 1 in the bronchoalveolar lavage fluid were noted, which were suppressed by anti-DPP10 treatment. Moreover, anti-DPP10 treatment inhibited the ERK phosphorylation and extracellular matrix deposition in the lungs.

Conclusions and Clinical Relevance: These findings suggest that increased production of DPP10 may contribute to TGF- β 1-mediated airway dysfunction in NERD patients, where blockade of DPP10 may have potential benefits.

KEYWORDS

asthma, DPP10, eosinophil, inflammation, lung function, NERD, remodelling



GRAPHICAL ABSTRACT

The study found higher levels of serum DPP10 and TGF-β1 with lower FEV₁ in NERD patients than ATA or HCs. TGF-β1 contributes to increase DPP10 expressed in airway epithelial cells, whilst DPP10 derived from immune cells induced airway inflammation and remodelling by the ERK phosphorylation and ECM accumulation. Therefore, DPP10 neutralisation could be a novel therapeutic option for NERD patients.

1 | INTRODUCTION

NSAID-exacerbated respiratory disease (NERD) has common characteristics such as eosinophilic rhinosinusitis with nasal polyposis and moderate to severe asthma accompanying lower lung function with decreased FEV₁ (%). With the overproduction of cysteinyl leukotrienes, eosinophils have been recognised as major effector cells in NERD, driving persistent airway inflammation. Therefore, patients with NERD had poor clinical outcomes with lung dysfunction compared to those with aspirin-tolerant asthma (ATA), which are not perfectly controlled by current anti-inflammatory agents and leukotriene modifiers.¹⁻³ In addition, several immune cells (mast cells, basophils, neutrophils, monocytes and innate lymphoid cells) have been involved in the severity of NERD,⁴ implicating that other factors could be responsible for persistent airway inflammation and clinical phenotypes.

In addition to immune cells, airway epithelium (the first-line barrier) has been suggested to be a key player in the modulation of immune responses in airway inflammation.⁵ Airway epithelium responds to various stimuli by releasing alarmin cytokines such as IL-33, IL-25 and thymic stromal lymphopoietin, resulting in the activation of immune cells.⁶ These cytokines were elevated in response to more activated eosinophils, leading to persistent airway inflammation in severe asthma.⁷ Moreover, a novel molecule called dipeptidyl peptidase 10 (DPP10) has also been shown to be expressed in airway epithelium.⁸ To date, DPP10 was identified to be associated with asthma and lung function decline with positional cloning.⁹⁻¹¹ In

Key messages

- Higher serum DPP10 and TGF-β1 levels with lower lung function are noted in NERD patients.
- TGF-β1 and immune cells contribute to increase DPP10 in airway epithelial cells.
- DPP10 induce airway inflammation via the ERK pathway and fibrotic remodelling with ECM deposition.

addition, our previous study reported DPP10 as a genetic biomarker for NERD because one SNP with high frequency of TT genotypes has a strong association with higher levels of serum DPP10 in NERD patients and it had a positive correlation with eosinophilic inflammation markers including 15-hydroxyeicosatetraenoic acid and YKL-40.¹² However, another study has demonstrated a protective effect of DPP10 in asthma by using DPP10 mutant mice.¹³ Although the role of DPP10 in the development of asthma is still controversial, this molecule may provide a novel pathophysiologic mechanism and therapeutic target for NERD.

To clarify the role of DPP10 in the pathogenesis of NERD, this study determined (1) the clinical significance of DPP10 in NERD patients, (2) the involvement of DPP10 in airway inflammation and remodelling with *in vitro*, *ex vivo* and *in vivo* experiments and (3) the effect of anti-DPP10 on airway inflammation in asthmatic mouse models.

2 | MATERIALS AND METHODS

2.1 | Patient recruitment and clinical characteristics

The study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108). Patients with NERD ($n = 110$), those with aspirin-tolerant asthma (ATA, $n = 130$) and healthy control subjects (HCs, $n = 80$) were recruited into the present study. All the study subjects submitted informed consent, and sera were collected at the initial visit. Asthma was diagnosed following the GINA guideline.¹⁴ Especially, NERD was defined as a typical clinical history (recurrent exacerbations of upper or lower respiratory reactions after ingestion of aspirin/NSAIDs) and/or a positive response to the lysine-aspirin bronchial provocation test (Lys-ASA BPT). The Lys-ASA BPT was conducted with increasing doses of Lys-ASA solution up to 300 mg/ml as previously reported.^{15,16} The asthmatics were in a stable condition, and the levels of FEV₁ (%) were higher than 70% of the predicted value in the Lys-ASA BPT. All anti-asthmatic drugs, including leukotriene modifiers, were stopped for at least 3 days before the Lys-ASA BPT. The result of the Lys-ASA BPT was considered positive if FEV₁ (%) decreased by more than 20% after the challenge. Subjects who showed negative results to the Lys-ASA BPT or denied any changes in upper or lower respiratory tract symptoms after ingestion of aspirin/NSAIDs were defined as ATA. In addition, the degree of airway obstruction was evaluated using Spirometry. Atopic 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, *Aspergillus* and *Alternaria*. To evaluate the presence of chronic rhinosinusitis and nasal polyps, paranasal sinus X-rays, CT scans and/or rhinoscopy were used. Total IgE was measured using the ImmunoCAP system (ThermoFisher Scientific, Waltham, CA, USA). Concentrations of DPP10 (MyBioSource, San Diego, CA, USA) and TGF- β 1 (R&D Systems, Minneapolis, MN, USA) were measured using enzyme-linked immunosorbent assay kits according to the manufacturer's recommendations respectively. To determine the cut-off value for high- or low-DPP10 groups, 95% confidence intervals of mean were used. An interval obtained by adding standard deviation (SD) from mean shows the chance of a test value falling outside less than 5%. The higher limit of this interval (mean +2 SD, ≥ 10.7 ng/ml) was considered the cut-off value.

2.2 | Stimulation of airway epithelial cells

A549 cells (American Type Culture Collection, Manassas, VA, USA) were purchased and cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% foetal bovine serum (ThermoFisher Scientific). They were seeded on a 24-well plate (5×10^5) and treated

with human recombinant TGF- β 1 (R&D Systems) in 2 different doses (10 or 100 ng/ml) for 2 h or in a time-dependent manner at the fixed dose (100 ng/ml). In addition, A549 cells were treated with human DPP10 recombinant (Abcam, Cambridge, UK) with a dose-dependent manner (1 or 10 ng/ml) for 30 min. After stimulation, the cells were lysed in RIPA buffer (Abcam) with protease/phosphatase inhibitors (Cell Signaling Technology, Danvers, MA, USA) for Western blot.

2.3 | Human peripheral blood cell isolation

Peripheral blood from asthmatic patients was collected in a BD Vacutainer tube containing acid citrate dextrose solution (BD biosciences, Franklin Lakes, NJ, USA) and layered on a Lymphoprep solution (Axis-Shield, Oslo, Norway), followed by centrifugation at 879 g at 20°C for 25 min. The layer containing granulocytes was separated and placed in Hank's balanced salt solution buffer with 2 mmol/L ethylenediaminetetraacetic acid and 2% dextran for 20 min. After removing red blood cells by hypotonic lysis, eosinophils and neutrophils were isolated by magnetic activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). At the same time, the cloudy layers containing peripheral mononuclear cells were collected. After removing red blood cells, T and B cells were isolated using respective isolation kits (Miltenyi Biotec). Each cell type was immediately lysed (2×10^6) to perform Western blotting or cultured (1×10^5) on a 24-well plate in RPMI-1640 medium (Sigma-Aldrich) with 2% foetal bovine serum (ThermoFisher Scientific). After 24 h, the supernatant of each cell was collected for Western blotting. In addition, each cell type (1×10^5) was cocultured with A549 cells (American Type Culture Collection; 5×10^5) seeded on a 24-well plate in RPMI-1640 medium with 2% foetal bovine serum for 24 h, with or without anti-DPP10 antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 10 ng/ml).

2.4 | In vivo studies

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Ajou University (IACUC-2016-0022). For sensitisation, (1) 6-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were intraperitoneally treated with 75 μ g ovalbumin (OVA; Sigma-Aldrich) and 2 mg aluminium hydroxide (alum; ThermoFisher Scientific); (2) 6-week-old male C57BL/6J mice (Jackson Laboratory) were intranasally treated with 75 μ g OVA and 10 μ g lipopolysaccharide (LPS; Sigma-Aldrich). For challenges, 50 μ g OVA was intranasally introduced for 5 days in each group. To compare effects between dexamethasone (Sigma-Aldrich; 1 mg/kg) and anti-DPP10 antibody (Santa Cruz Biotechnology; 1 or 10 ng/mouse) on airway inflammation, mice were intranasally treated with the agents during challenges. To investigate the immunogenicity or toxicity of anti-DPP10 antibody, it was intranasally treated (10 ng/ml) for 5 days without OVA sensitisation. The total cell number in bronchoalveolar lavage fluid (BALF) was calculated by Diff-quick staining (Dade Behring, Dudingen, Switzerland). In addition, concentrations of DPP10 (LifeSpan BioSciences, Seattle, WA,

USA) as well as TGF- β 1, CXCL-1 and MMP-9 (R&D Systems) in the serum were measured using enzyme-linked immunosorbent assay kits. For lung histology, the left lung tissues stained with H&E or Masson's trichrome were evaluated using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.5 | Enzyme-linked immunosorbent assay

Kits for measuring several mediators were as follows: (1) DPP10 (MyBioSource) and TGF- β 1 (R&D Systems) for human samples; (2) DPP10 (LifeSpan BioSciences), IFN- γ (R&D Systems), IL-6 (R&D Systems), CXCL1 (R&D Systems), TGF- β 1 (R&D Systems) and matrix metalloproteinase-9 (MMP-9; R&D Systems) for mouse samples.

2.6 | Western blot analysis

The antibodies used were as follows: DPP10 (Santa Cruz Biotechnology), extracellular signal-regulated kinase (ERK; Cell Signaling Technology, MN, USA), phospho-ERK (Cell Signaling Technology), fibronectin (Santa Cruz Biotechnology), T-bet (ThermoFisher Scientific) and actin (Santa Cruz Biotechnology). The relative fold changes of band intensity were analysed using ImageJ (National Institutes of Health).

2.7 | Confocal microscopy

After the isolation of neutrophils, eosinophils and PBMC from peripheral blood of asthmatic patients, each cell type was diluted in

100 μ l of cold PBS with the same count, respectively (1×10^5), followed by centrifugation at 750 revolutions per minute for 3 min for the attachment onto the slides using Cytospin 4 (ThermoFisher Scientific). The cells were immediately fixed in 4% paraformaldehyde for 30 min and incubated with 0.2% Triton X-100 (Sigma-Aldrich) for 10 min. After blocking with 5% normal donkey serum (Abcam) for 1 h, the slides were incubated with diluted anti-DPP10 (Santa Cruz Biotechnology; 1:200) at 4°C overnight and Alexa Fluor 594 donkey anti-mouse (ThermoFisher Scientific; 1:200) for 1 h, followed by the incubation with 4, 6-diamidino-2-phenylindole (Sigma-Aldrich; 1 μ g/ml) for 10 min. Fluorescent images were examined under confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany).

2.8 | Statistical analysis

All statistical analyses were performed using IBM SPSS software, version 25.0 (IBM Corp., Armonk, NY, USA). GraphPad Prism 8.0 software (GraphPad Inc., San Diego, CA, USA) was used to create graphs.

3 | RESULTS

3.1 | Higher levels of serum DPP10 and lower FEV₁ in patients with NERD

Clinical characteristics of the study subjects are described in Table 1. Typical features of NERD, including the presence of CRS, nasal polyps and falls in FEV₁ (%) after lysine-aspirin challenge, were significantly higher in NERD patients than in ATA patients ($p < .05$ for

TABLE 1 Demographic data from the study subjects

Variables	NERD (n = 110)	ATA (n = 130)	HC (n = 80)	p value		
				NERD vs. ATA	NERD vs. HC	ATA vs. HC
Age (years)	44.6 $\pm$ 12.4/110	42.3 $\pm$ 13.0/130	30.1 $\pm$ 7.9/77	.158	.001	.001
Female sex (%)	71.8/110	60.8/130	57.1/77	.072	.037	.608
Atopy (%)	46.4/110	54.6/130	14.5/62	.203	.001	.001
CRS (%)	54.2/96	37.3/67	NA	.034	NA	NA
Nasal polyp (%)	42.9/105	10.5/76	NA	.001	NA	NA
Baseline FEV ₁ (%)	84.1 $\pm$ 21.3/110	89.5 $\pm$ 18.3/116	NA	.043	NA	NA
Fall of FEV ₁ (%)	16.7 $\pm$ 5.8/60	7.2 $\pm$ 5.2/43	NA	.001	NA	NA
Total IgE (kU/l)	243.3 $\pm$ 287.6/107	284.1 $\pm$ 290.3/122	NA	.288	NA	NA
TEC (/ μ l)	427.9 $\pm$ 391.8/110	388.1 $\pm$ 423.2/107	NA	.473	NA	NA
TGF- β 1 (ng/ml)	30.1 $\pm$ 11.4/83	25.7 $\pm$ 12.1/47	19.8 $\pm$ 13.6/39	.042	.037	.001
DPP10 (ng/ml)	13.4 $\pm$ 8.8/110	11.2 $\pm$ 6.2/130	6.2 $\pm$ 2.2/80	.026	.001	.001

Notes: 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 by Student's t test for continuous variables.

Abbreviations: ATA, aspirin-tolerant asthma; CRS, chronic rhinosinusitis; FEV₁, forced expiratory volume in 1 s; NA, not applicable; NERD, NSAID-exacerbated respiratory disease; TEC, total eosinophil count; TGF- β 1, transforming growth factor beta 1.

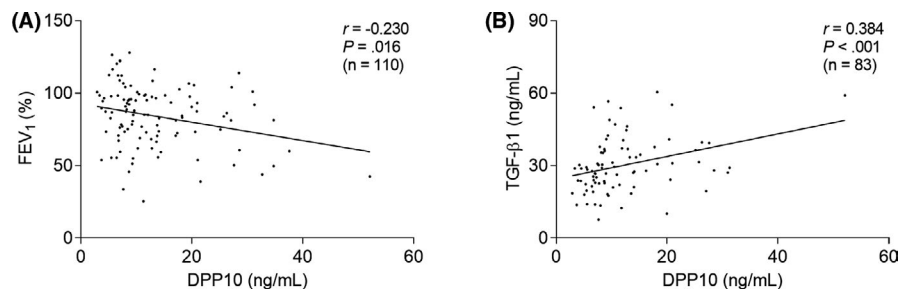


FIGURE 1 Association between DPP10 and FEV₁ (%)/TGF-β1 levels in patients with NERD. (A) A significant negative correlation between serum DPP10 and FEV₁ (%) and (B) a significant positive correlation between serum DPP10 and TGF-β1 levels in patients with NERD. Data are presented as Spearman's rank correlation coefficient *r* (*p* value). FEV₁, forced expiratory volume in 1 s; TGF-β1, transforming growth factor beta 1

TABLE 2 Characteristics of asthmatic patients with high (≥ 10.7 ng/ml) and low (< 10.7 ng/ml) DPP10 levels

Variables	NERD		<i>p</i> value	ATA		<i>p</i> value
	High (<i>n</i> = 53)	Low (<i>n</i> = 57)		High (<i>n</i> = 56)	Low (<i>n</i> = 74)	
Age (y)	45.0 ± 13.3/53	44.4 ± 11.6/57	.803	42.9 ± 12.2/56	42.0 ± 13.7/74	.694
Female sex (%)	64.2/53	78.9/57	.085	62.5/56	59.5/74	.725
Atopy (%)	39.6/53	52.6/57	.172	58.9/56	51.4/74	.390
Nasal polyps (%)	39.2/51	46.3/54	.464	12.5/32	9.1/44	.633
Baseline FEV ₁ (%)	79.8 ± 20.5/53	88.1 ± 21.4/57	.041	88.5 ± 20.1/46	90.1 ± 17.0/70	.661
Fall of FEV ₁ (%)	16.2 ± 6.4/34	17.3 ± 4.9/26	.446	6.7 ± 4.1/21	7.6 ± 6.2/22	.598
Total IgE (kU/L)	216.4 ± 181.9/53	269.7 ± 362.5/54	.340	283.6 ± 280.7/55	284.5 ± 300.1/67	.986
TEC (cells/μL)	452.1 ± 452.8/53	405.4 ± 327.6/57	.535	461.7 ± 315.4/42	405.1 ± 481.8/65	.607
TGF-β1 (ng/ml)	33.6 ± 12.1/34	27.7 ± 10.3/49	.020	31.8 ± 15.8/13	23.4 ± 9.8/34	.093

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

Abbreviations: ATA, aspirin-tolerant asthma; FEV₁, forced expiratory volume in 1 s; NERD, NSAID-exacerbated respiratory disease; TEC, total eosinophil count; TGF-β1, transforming growth factor beta 1.

all), whilst baseline FEV₁ (%) levels were significantly lower in NERD patients ($p = .043$ for all). Other clinical parameters, such as total IgE and total eosinophil count (TEC), were not significantly different between the 2 groups. When measured in all study subjects, serum DPP10 levels were higher in ATA patients than in HCs ($p = .001$). Moreover, significantly higher levels of serum DPP10 were noted in NERD patients than in ATA patients and in HCs ($p = .026$ and $p = .001$ respectively). In addition, serum DPP10 levels had a negative correlation with baseline FEV₁ (%) in NERD patients ($r = -0.230$, $p = .016$; Figure 1A), but not in ATA patients. These findings indicate that higher levels of serum DPP10 may be involved in FEV₁ (%) decline in NERD patients.

3.2 | Association between DPP10 and TGF-β1 in patients with NERD

To investigate the pathophysiological implication of DPP10 production in association with airway inflammation or remodelling, TGF-β1 levels were measured in the study subjects. As a result, higher levels of serum TGF-β1 were noted in NERD patients than in ATA

patients and in HCs ($p = .042$ and $p = .037$ respectively; Table 1) as well as in ATA patients than in HCs ($p = .001$). When NERD or ATA patients were divided into the high-DPP10 and low-DPP10 groups according to the cut-off value (10.7 ng/ml), the levels of serum TGF-β1 were significantly higher in the high-DPP10 group than in the low-DPP10 group within NERD patients ($p = .020$; Table 2), but not within ATA patients. Furthermore, a positive correlation between the levels of serum TGF-β1 and DPP10 was noted in NERD patients ($r = 0.384$, $p < .001$; Figure 1B). These results suggest that the contribution of DPP10 to airway inflammation and remodelling could be related to TGF-β1 in NERD patients.

3.3 | Induction of DPP10 expression in airway epithelial cells by TGF-β1

The present study investigated the correlation between TGF-β1 and DPP10 in AECs which are known as major source cells of DPP10. With the activation of the ERK pathway, TGF-β1 derived the increase of DPP10 expression in AECs, being highest at 2 h after the treatment (Figure 2). These findings indicated that the increased

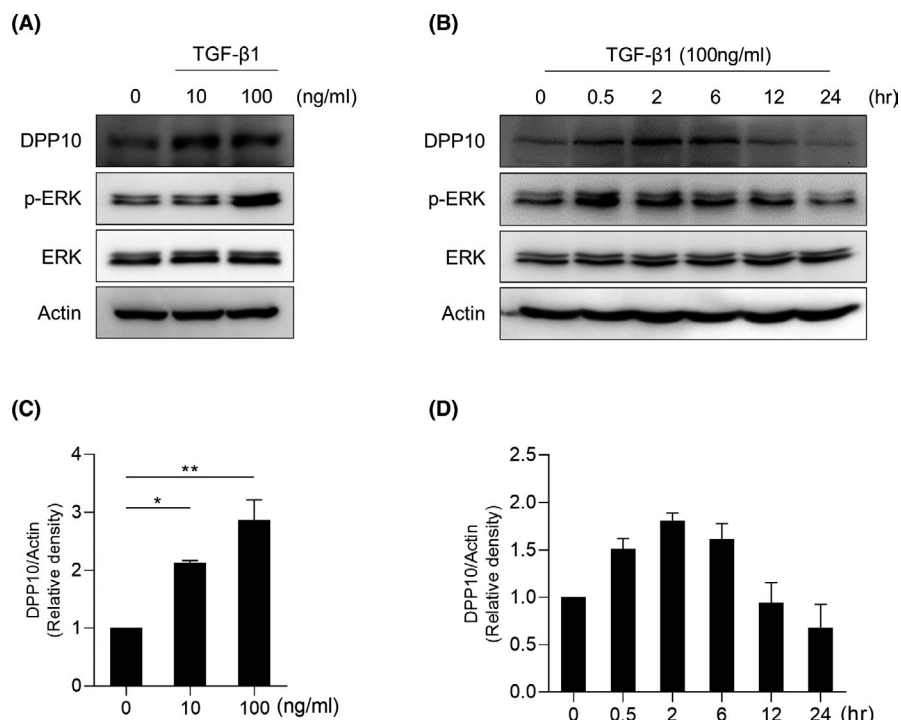


FIGURE 2 Induction of DPP10 expression by TGF-β1 in airway epithelial cells. (A) Induction of DPP10 and ERK in A549 cells by TGF-β1 in (A) dose- and (B) time-dependent manners. (C, D) Quantitative data of DPP10 expression in A549 cells treated with TGF-β1. Data are represented as means ± SEM, $n = 3$. * $p < .05$ and ** $p < .01$ were obtained by one-way ANOVA with Tukey's HSD test. TGF-β1, transforming growth factor beta 1

levels of serum DPP10 could be induced in response to higher TGF-β1 levels in NERD patients than ATA patients or HCs.

3.4 | Involvement of DPP10 in airway inflammation via the ERK pathway

As DPP10 predominantly affects airway epithelium, the further study observed a specific signalling pathway related to airway inflammation. As a result, DPP10 was found to be expressed in various immune cells, including eosinophils, neutrophils and PBMC (T cells and B cells) from asthmatic patients by immunofluorescence and Western blot analysis (Figures 3A and S1). Moreover, DPP10 directly induced the phosphorylation of ERK in AECs (Figure 3B and 3C). These DPP10-producing immune cells also strongly enhanced the phosphorylation of ERK in AECs, which was suppressed by anti-DPP10 treatment (Figure 3D and 3E). These data indicate that DPP10 produced from multiple immune cells may contribute to inflammatory responses in the airways via the ERK pathway.

3.5 | Effects of DPP10 neutralisation on airway inflammation and remodelling

To evaluate whether DPP10 production is elevated in the development of asthma, mice were sensitised in 2 models using (1) OVA and alum and (2) OVA and LPS. After sequential challenges, both models showed increased counts of immune cells, such as macrophages, neutrophils and eosinophils, compared to controls (Figure 4A). Between them, OVA plus LPS-treated mice showed higher DPP10 expression in the lung tissues and DPP10 levels in the serum than OVA plus

alum-treated mice as controls (Figure 4B and 4C), whilst TGF-β1 production in BALF was increased in both models (Figure 4D). Based on these results, anti-DPP10 antibody was treated in OVA plus LPS-treated mice as depicted in Figure 5A. Finally, significant reductions in the counts of immune cells, especially in neutrophils, were found in BALF in a dose-dependent manner (Figure 5B). In addition, anti-DPP10 treatment reduced the levels of TGF-β1, CXCL-1 and MMP-9 in BALF, whereas dexamethasone did not (Figure 5C). In the lung tissues, the ERK pathway was inhibited by anti-DPP10 antibody and dexamethasone. However, the expression of fibronectin was reduced by anti-DPP10 antibody, not by dexamethasone (Figure 5D). Immunohistochemistry analysis showed that anti-DPP10 antibody significantly reduced immune cell infiltration, epithelium thickness, mucus production and collagen deposition (Figure 5E). When the lung T cells were observed, both anti-DPP10 antibody and dexamethasone could attenuate IFN-γ and IL-6 production in BALF as well as T-bet expression in the lungs (Figure 6). To investigate the immunogenicity or toxicity of anti-DPP10 antibody in the normal state, the agent was treated in C56BL/6J without any sensitisation. As a result, there were no significant differences in the differential cell counts (Figure S2A) and the levels of inflammatory mediators (Figure S2B and S2C) when compared to controls. These data suggest that DPP10 derived immune cells may induce airway inflammation and fibrotic remodelling with extracellular matrix (ECM) deposition.

4 | DISCUSSION

This is the first study to demonstrate the contribution of DPP10 to the phenotype of NERD with lower FEV₁ (%) values. NERD

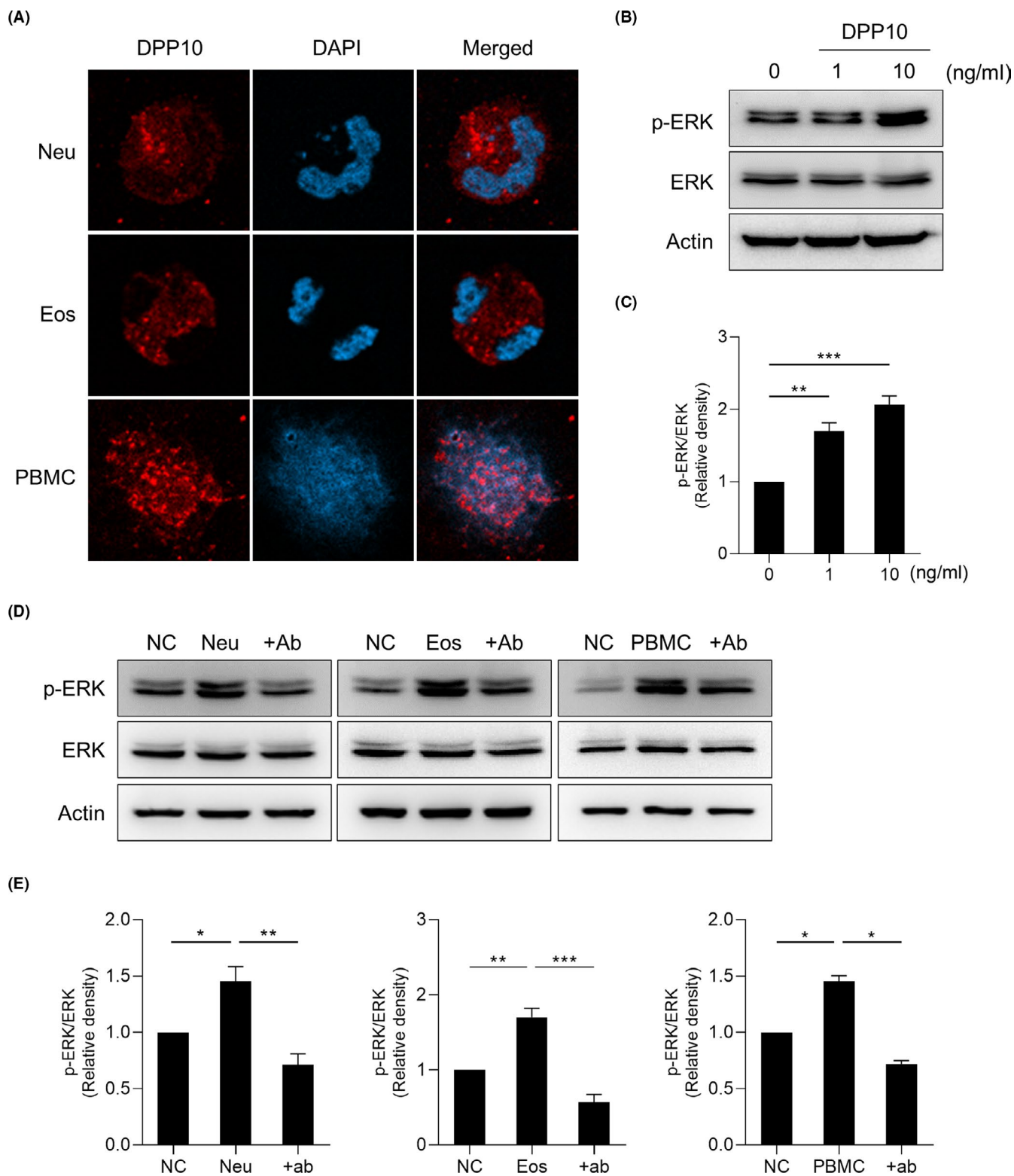


FIGURE 3 Investigation on the role of DPP10 into inflammatory response in airway epithelial cells with immune cells from asthmatic patients. (A) Immunofluorescence analysis of DPP10 expression in neutrophils, eosinophils and PBMCs from asthmatic patients. (B) Western blot analysis of the ERK phosphorylation and (C) quantitative data of p-ERK in A549 cells treated with DPP10. (D) Western blot analysis of ERK phosphorylation and (E, F and G) quantitative data of p-ERK in A549 cells cocultured with multiple immune cells with or without anti-DPP10 antibody treatment. Data are represented as means $\pm$ SEM, $n = 3$. * $p < .05$, ** $p < .01$ and *** $p < .001$ were obtained by one-way ANOVA with Tukey's HSD test. Neu, neutrophils; Eos, eosinophils; PBMC, peripheral blood mononuclear cell; Ab, anti-DPP10 antibody

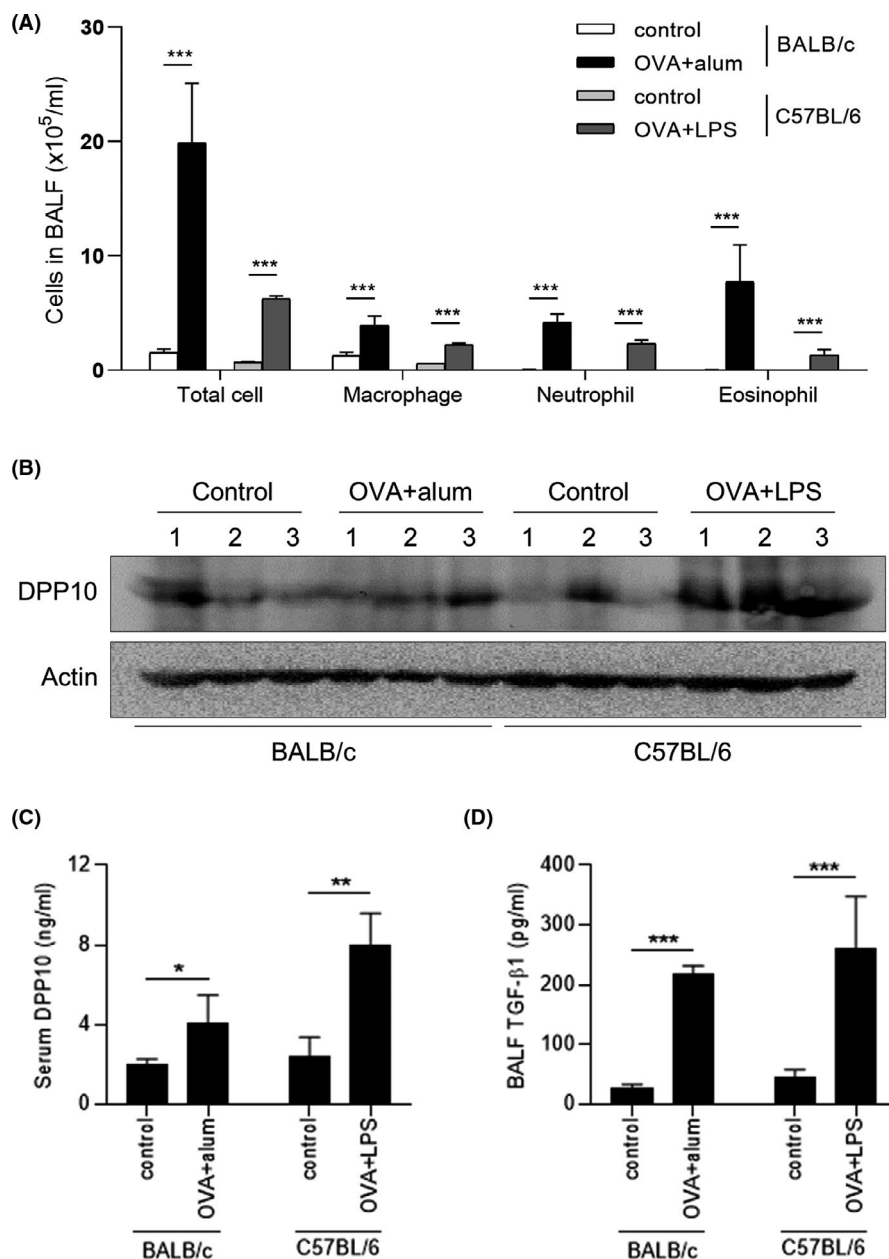


FIGURE 4 Increased levels of DPP10 in 2 types of asthmatic mouse models. (A) Differential cell count. (B) Comparison of DPP10 expression in the lung tissues. Levels of (C) DPP10 in the serum and (D) TGF- β 1 in BALF. Data are represented as means $\pm$ SEM, $n = 5$. * $p < .05$, ** $p < .01$ and *** $p < .001$ were obtained by unpaired t test. Abbreviations: BALF, bronchoalveolar lavage fluid; LPS, Lipopolysaccharide; n.s., not significant; OVA, ovalbumin; alum, aluminium hydroxide; TGF- β 1, transforming growth factor beta 1

patients showed higher levels of serum DPP10 than ATA patients. In addition, significantly positive correlations between DPP10 and TGF- β 1 levels, but a negative correlation between DPP10 and FEV₁ (%), were noted in patients with NERD (not in those with ATA). Through the *in vitro* experiments, the consistent results for the relationship between DPP10 and TGF- β 1 were confirmed in AECs. Furthermore, the role of DPP10-producing immune cells (neutrophils, eosinophils, T cells and B cells) in driving airway inflammation via the ERK pathway with increased levels of TGF- β 1 and MMP-9 was demonstrated. These findings provide a new insight into the pathogenic mechanisms of NERD that DPP10 induces inflammation and remodelling in the airway with close interactions between immune cells and epithelial cells, resulting in FEV₁ (%) decline and airway dysfunction. Therefore, regulation of DPP10 may be a novel therapeutic approach for the management of NERD patients.

Although DPP10 is the only gene identified in both genome-wide association studies and positional cloning to be associated with asthma in limited populations,¹¹ very little is known about its biological function in the lungs.¹⁷ To date, DPP10 has been thought to be primarily expressed in the brain, adrenal gland and pancreas.^{18,19} In addition to this current knowledge, the present study suggested eosinophils, neutrophils, T cells and B cells as novel sources of DPP10. Moreover, TGF- β 1 was found to increase the expression of DPP10 in AECs. Previous studies have shown that TGF- β 1 is not only produced from eosinophils,²⁰ but also expressed in neutrophils,²¹ macrophages²² and lymphocytes.²³ Nevertheless, eosinophils are still known to be a main source of TGF- β 1 in asthmatic airways.²⁴ Therefore, the predominance of eosinophils and the involvement of various immune cells releasing TGF- β 1 could contribute to the increased levels of serum DPP10 in AECs of NERD patients. Moreover, the study demonstrated that DPP10 released

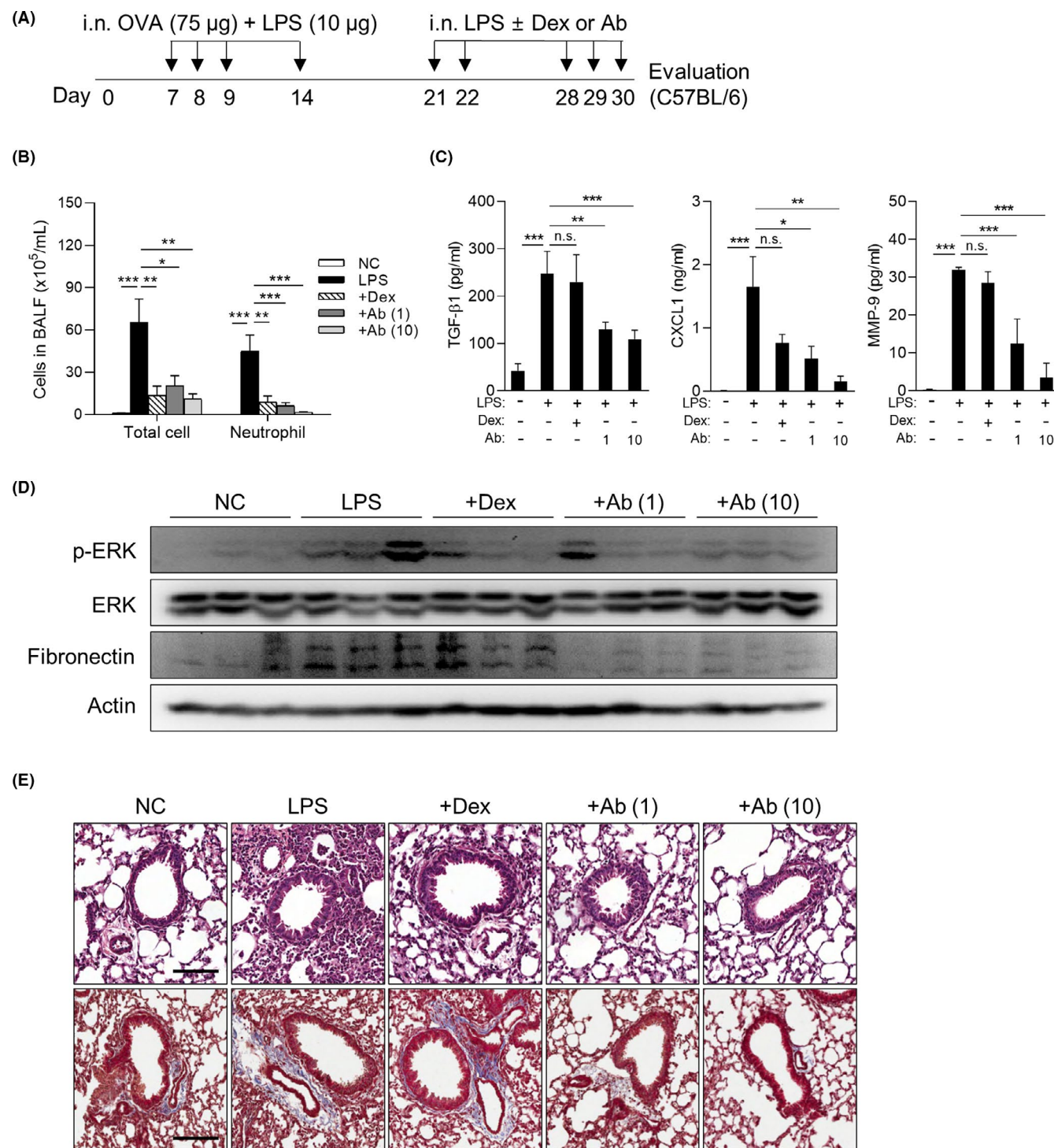


FIGURE 5 Regulation of airway inflammation and fibrotic remodelling in asthmatic mice by DPP10 neutralisation. (A) Experimental schedule. (B) Differential cell count. (C) Levels of several mediators including TGF- β 1, CXCL1 and MMP-9 in BALF. Data are represented as means $\pm$ SEM, $n = 5$. * $p < .05$, ** $p < .01$ and *** $p < .001$ were obtained by one-way ANOVA with Tukey's HSD test. n.s., not significant. (D) The phosphorylation of ERK and expression of fibronectin in the lung. (E) Histological analysis of lung tissues stained with H&E (upper panel) or trichrome (lower panel). Scale bar, 100 nm. Abbreviations: Ab, anti-DPP10 antibody; BALF, bronchoalveolar lavage fluid; CXCL-1, (C-X-C motif) ligand 1; Dex, dexamethasone; LPS, lipopolysaccharide; MMP-9, matrix metalloproteinase-9; OVA, ovalbumin; alum, aluminium hydroxide

from various immune cells could drive airway inflammation via the ERK pathway and fibrotic remodelling with ECM deposition. It is well known that NERD patients had lower lung functions than ATA

patients,²⁵ which was replicated in the present study. In addition, this study found a negative correlation between DPP10 and FEV₁ (%) levels in NERD patients, but not in ATA patients. Taken together,

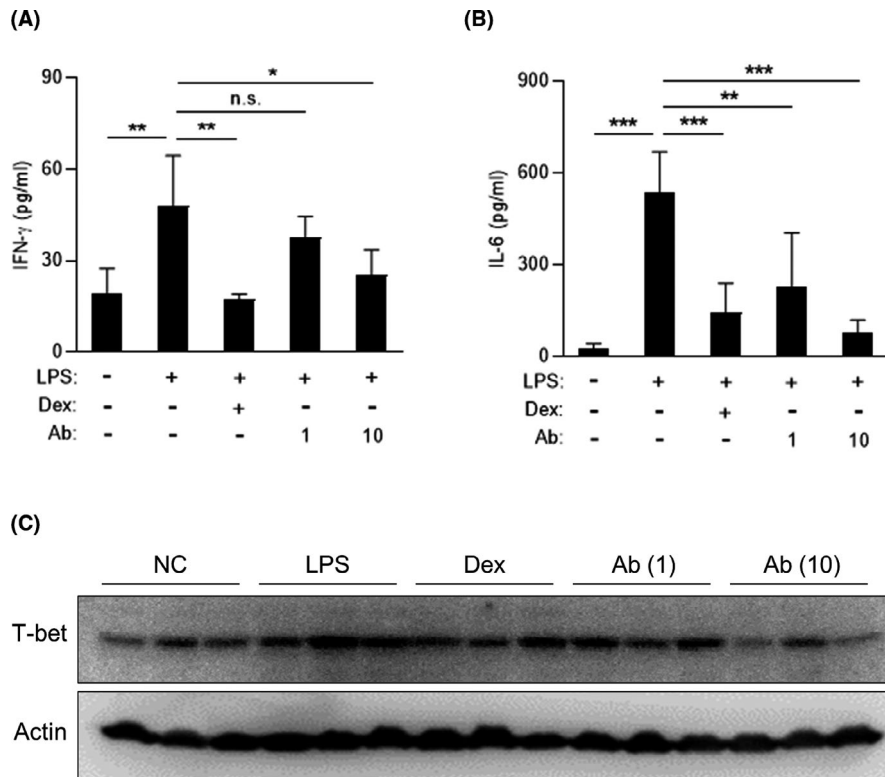


FIGURE 6 Production of cytokines released from lung CD4+ T cells. Levels of (A) IFN- γ and (B) IL-6 in the BALF. Data are represented as means $\pm$ SEM, $n = 5$. * $p < .05$, ** $p < .01$ and *** $p < .001$ were obtained by one-way ANOVA with Tukey's HSD test. (C) Expression of T-bet in CD4+ T cells isolated from the lungs. Abbreviations: Ab, anti-DPP10 antibody; Dex, dexamethasone; IFN- γ , interferon gamma; LPS, lipopolysaccharide

higher levels of DPP10 in NERD patients could worsen airway inflammation and remodelling, leading to more severe symptoms and lower lung function than ATA patients.

To investigate how DPP10 contributes to airway inflammation or remodelling, DPP10 neutralisation was performed in *in vivo* studies. Although the levels of DPP10 in serum and TGF- β 1 in BALF were increased in both asthmatic mouse models, the distribution of DPP10 in the lung tissues was quite different. Based on this result, anti-DPP10 antibody was treated in C57BL/6J mice treated with OVA plus LPS. As a result, reduced counts of immune cells and levels of TGF- β 1 and other inflammatory mediators (CXCL-1, MMP-9, IFN- γ and IL-6) were found in BALF. Furthermore, the accumulation of ECM proteins was decreased in the lungs. It is well established that TGF- β 1 and MMP-9 develop fibrotic remodelling^{26,27} and accumulate fibronectin and collagen in asthmatic airways and lungs.²⁸⁻³⁰ These lead to the progressive structural and functional changes with smooth muscle contraction in the airways.³¹ Collectively, our study further demonstrated that blocking of DPP10 could attenuate airway remodelling by regulating TGF- β 1 and MMP-9, whilst dexamethasone could not reduce the levels of inflammatory mediators or ECM deposition. Several studies reported the limitation of corticosteroids to preventing TGF- β 1-mediated airway remodelling³² or altering ECM composition.^{33,34} Taken together, these findings imply that the blockade of DPP10 could be applied to control airway inflammation and remodelling in NERD patients with steroid resistance.

Many studies have demonstrated that the mitogen-activated protein kinase (MAPK) families are not only responsible for the induction of inflammatory mediators and activation of various immune cells,³⁵ but also affect normal airway function in various aspects

and contribute to inflammation and remodelling in asthmatic airways.^{36,37} In the present study, DPP10 was found to directly activate the ERK pathway. Moreover, DPP10 expressed in inflammatory cells (eosinophils, neutrophils, T cells and B cells) enhanced the phosphorylation of the ERK pathway in AECs. This activated pathway was remarkably suppressed by DPP10 neutralisation *in vivo* and *ex vivo*, indicating that DPP10 released from immune cells could be involved in the ERK phosphorylation in asthmatic airways. Previously, various clinical trials with kinase inhibitors have been performed in asthmatic patients with steroid resistance,³⁸ because long-term use of corticosteroids is known to be associated with various systemic side-effects.³⁹ The present study suggested that DPP10 may not be involved in cysLT-related airway inflammation (data not shown); however, it first verified a close link between DPP10 and the ERK pathway of airway epithelium. Therefore, the suppression of the MAPK signalling pathway by DPP10 neutralisation could be a potential therapeutic strategy for NERD patients.

There are 2 limitations to the present study. One is that the mechanism of how TGF- β 1 induces DPP10 production in AECs has not been confirmed. However, we highlighted the positive correlation between serum DPP10 and TGF- β 1 levels in NERD patients and a negative correlation between DPP10 and FEV₁ (%). In addition, increased DPP10 levels noted in NERD patients may be derived from increased production of DPP10 by TGF- β 1 in AECs or multiple immune cells, driving airway inflammation and tissue remodelling; the blockade of DPP10 could reduce TGF- β 1 production in *ex vivo* experiments. The other is that the effect of DPP10 on airway smooth muscle contraction has not been evaluated. However, in the present study, DPP10 was shown to be related to ECM composition, such as

fibronectin and collagen. Therefore, alterations in ECM components by DPP10 could affect the contractility of airway smooth muscles. Moreover, DPP10 could possibly affect nervous systems as this molecule (a potassium channel-associated protein) modulates neuronal Kv4 channels.

In conclusion, the results of this study suggest that DPP10 can be related to FEV₁ (%) decline and lung dysfunction via TGF- β 1-mediated airway inflammation and fibrotic tissue remodelling in NERD patients. Therefore, regulation of DPP10 production or function could be suggested as a novel target treatment option for restoring airway dysfunction in NERD patients.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

S. Sim and Y. Choi designed the experiments, analysed the data and wrote the paper. D.H. Lee and H.R. Lee performed the experiments. Y.S. Shin helped design the experiments. H.S. Park provided overall supervision for the entire study.

ETHICAL APPROVAL

The human studies were approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108), and all the study subjects submitted informed consents. The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Ajou University (IACUC-2016-0022).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplementary Information

Contribution of dipeptidyl peptidase 10 to airway dysfunction in patients with NSAID-exacerbated respiratory disease

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Running title: The pathogenic role of DPP10 in NERD

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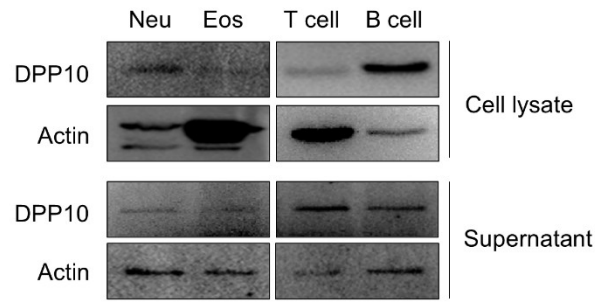


Figure S12. Western blot analysis of DPP10 expression in multiple immune cells from asthmatic patients. Comparison of DPP10 in lysate or culture supernatant in the same counts (2×10^6) of neutrophils, eosinophils, T cells, and B cells. Neu, neutrophil; Eos, eosinophils.

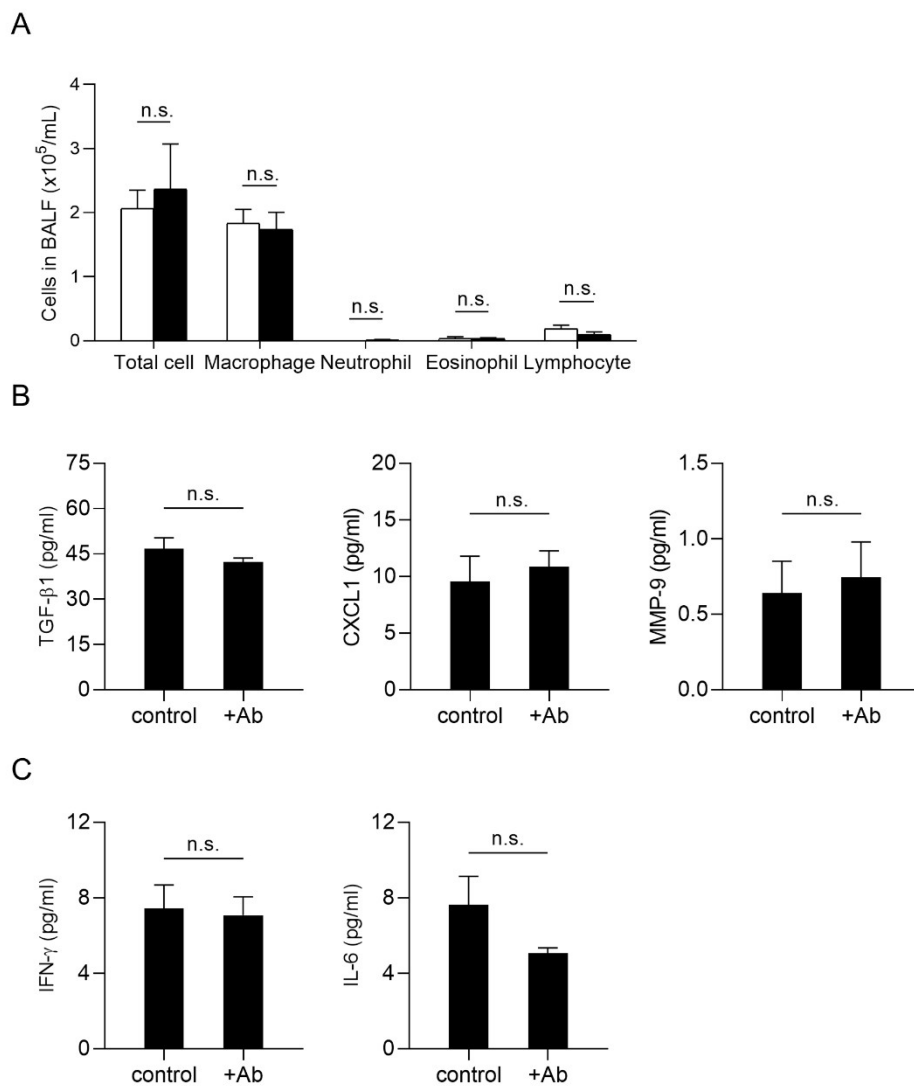


Figure S2. Comparison of airway inflammation in controls and anti-DPP10 antibody-treated mouse model without OVA sensitization. (A) Differential cell count. Concentrations of (B) TGF- β 1, CXCL-1, and MMP-9 and (C) cytokines released from lung CD4⁺ T cells including IFN- γ and IL-6 in BALF. Data are represented as means $\pm$ SEM, n = 3. *P* values were obtained by Unpaired *t* test. n.s., not significant. BALF, bronchoalveolar lavage fluid; Ab, anti-DPP10 antibody; TGF- β 1, transforming growth factor beta 1; CXCL-1, (C-X-C motif) ligand 1; MMP-9, matrix metalloproteinase-9, IFN- γ , interferon gamma.