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ORIGINAL ARTICLE

DEL-1, as an anti-neutrophil transepithelial migration molecule, inhibits airway neutrophilic inflammation in asthma

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Abstract

Background: Neutrophil migration into the airways is a key process in neutrophilic asthma. Developmental endothelial locus-1 (DEL-1), an extracellular matrix protein, is a neutrophil adhesion inhibitor that attenuates neutrophilic inflammation.

Methods: Levels of DEL-1 were measured in exhaled breath condensate (EBC) and serum in asthma patients by ELISA. DEL-1 modulation of neutrophil adhesion and transepithelial migration was examined in a co-culture model in vitro. The effects of DEL-1-adenoviral vector-mediated overexpression on ovalbumin/lipopolysaccharide (OVA/LPS)-induced neutrophilic asthma were studied in mice in vivo.

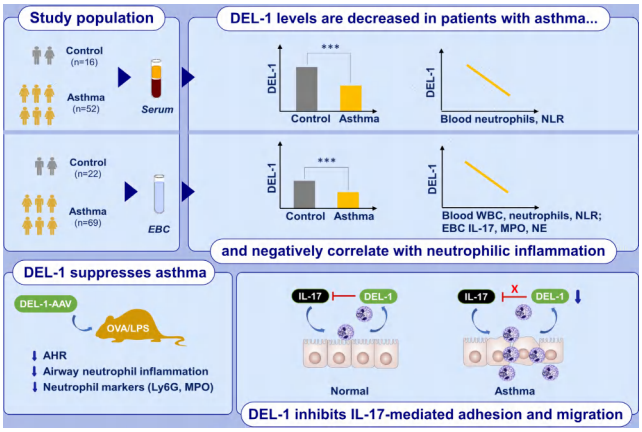
Results: DEL-1 was primarily expressed in human bronchial epithelial cells and was decreased in asthma patients. Serum DEL-1 concentrations were reduced in patients with severe asthma compared with normal subjects (567.1 ± 75.3 vs. 276.8 ± 29.36 pg/mL, $p < .001$) and were negatively correlated to blood neutrophils ($r = -0.2881$, $p = .0384$) and neutrophil-to-lymphocyte ratio (NLR) ($r = -0.5469$, $p < .0001$). DEL-1 concentrations in the EBC of severe asthmatic patients (113.2 ± 8.09 pg/mL) were also lower than normal subjects (193.0 ± 7.61 pg/mL, $p < .001$) and were positively correlated with the asthma control test (ACT) score ($r = 0.3678$, $p = .0035$) and negatively related to EBC IL-17 ($r = -0.3756$, $p = .0131$), myeloperoxidase (MPO) ($r = -0.5967$, $p = .0055$), and neutrophil elastase (NE) ($r = -0.5488$, $p = .0009$) expression in asthma patients. Neutrophil adhesion and transepithelial migration in asthma patients were associated with LFA-1 binding to ICAM-1 and inhibited by DEL-1. DEL-1 mRNA and protein expression in human bronchial epithelial cells were regulated by IL-17. Exogenous DEL-1 inhibited IL-17-enhanced neutrophil adhesion and migration. DEL-1 expression was decreased while neutrophil infiltration was increased in the airway of a murine model of neutrophilic asthma. This was prevented by DEL-1 overexpression.

Abbreviations: 16HBE, human bronchial epithelial cell line; AAV, adeno-associated virus; AHR, airway hyperresponsiveness; DEL-1, developmental endothelial locus-1; EBC, exhaled breath condensate; IL-17, interleukin 17; LPS, lipopolysaccharide; Ly6G, lymphocyte antigen 6 family member G; MPO, myeloperoxidase; NE, neutrophil elastase; NLR, neutrophil-to-leukocyte ratio; OVA, ovalbumin; WBC, white blood cells.

Man Jia and Heng Fu contributed equally to this work.

Conclusions: DEL-1 down-regulation leads to increased neutrophil migration across bronchial epithelial cells and is associated with neutrophilic airway inflammation in asthma.

KEYWORDS
asthma, bronchial epithelial cells, DEL-1, neutrophil transepithelial migration



GRAPHICAL ABSTRACT
EBC and serum DEL-1 levels are decreased in asthma and are correlated with neutrophil related markers. IL-17 induces downregulation of DEL-1 in 16HBE, whereas exogenous DEL-1 significantly suppresses IL-17-induced neutrophil adhesion and transepithelial migration in asthma. DEL-1 overexpression attenuates airway neutrophil infiltration in neutrophilic asthmatic mice.

1 | INTRODUCTION

Bronchial asthma is a complicated, heterogeneous chronic airway disease involving diverse pathobiological mechanisms (endotypes) with different clinical presentations (phenotypes).[1] Airway inflammatory phenotypes in asthma are categorized as eosinophilic, neutrophilic, mixed granulocytic, and paucigranulocytic based on sputum eosinophil and neutrophil proportions.[2] Although airway eosinophilia is a common feature of asthma, it is not a universal finding, whilst eosinophils may not be the key effector cells in all patients.[3] A large number of studies have shown the presence of airway neutrophils which may act as a driver of airways inflammation and damage, particularly in the more severe forms of asthma.[4–6] Excessive airway neutrophilia is reported in 20%–30% of asthmatic subjects.[7] These patients tend to have reduced responsiveness to steroid therapy, poorer asthma control and higher risks of exacerbation.[5] Increasing evidence suggest that neutrophil-dominated airway inflammation in asthma is closely related to severe asthma and sudden fatal asthma.[5,6]

Neutrophil trafficking from the bloodstream to the lung epithelium is a prerequisite for airway neutrophilia. Vascular neutrophils undergo rolling, activation, and adhesion to endothelial cells and finally traffic from blood vessels to the lung.[8] Lung interstitial neutrophils undergo further transepithelial migration as a final process that facilitates the rapid trafficking of neutrophils to the airway lumen.[9] At present, the molecular mechanism of neutrophil transendothelial migration and activation are understood to a certain extent, although the regulation of neutrophil lung trafficking, a key step in neutrophilic asthma, remains unclear.

Neutrophil surface receptors such as β2 integrin lymphocyte function related antigen-1 (LFA-1, also called CD11b/CD18) and macrophage differentiation antigen-1 (MAC-1, also called CD11a/CD18) bind to their ligand intercellular adhesion molecule-1 (ICAM-1, mainly expressed in endothelial and epithelial cells) to promote neutrophil-endothelial cell interactions.[10,11] LFA-1 and MAC-1 play different roles in the transmigration process of neutrophils through endothelial cells with LFA-1 mainly mediating the firm adhesion of neutrophils to vascular endothelium while MAC-1 mainly facilitates neutrophil crawling into the endothelial cavity.[12–14] Neutrophils adhere to epithelial cells in a β2 integrin-dependent manner.[15,16] The percentage of LFA-1-positive neutrophils is decreased in peripheral blood whilst being increased in induced sputum a short time after allergen challenge in asthmatic patients.[16] In addition, ICAM-1 expression is increased in airway epithelial cells of asthmatic subjects with low expression in other mucosal cell types.[17,18] LFA-1 and ICAM-1 inhibitors greatly reduced neutrophil numbers in bronchoalveolar lavage fluid (BALF) and the inflammatory response in mice exposed to rhinovirus and lipopolysaccharide (LPS) to induce neutrophilic inflammation.[19,20] These data indicate a key role for LFA-1/ICAM-1-dependent cell adhesion pathway in neutrophilic airway inflammation in asthma.

Developmental endothelial locus-1 (DEL-1), is an extracellular matrix protein secreted by endothelial cells, and contains an Arg-Gly-Asp (RGD) sequence that can bind to integrin and participates in integrin-mediated cell adhesion.[21,22] DEL-1 acts as an endogenous anti-adhesive factor by competitively binding to LFA-1, thereby preventing neutrophils from adhering to vascular endothelial cells.[23]

We hypothesized that DEL-1 plays an important regulatory role in the migration of neutrophils from tissues into the airways, which regulates airway neutrophilic inflammation in asthma. We aimed to examine the expression and regulation of DEL-1 in human asthma, the functional effect of DEL-1 on human airway epithelial cell neutrophil transmigration and in a murine model of neutrophilic asthma.

2 | METHODS

2.1 | Patients' exhaled breath condensate (EBC), serum, and lung tissue collection

Human EBC and serum samples were collected from outpatients with asthma at the First Affiliated Hospital of Nanjing Medical University and from community-based healthy volunteers. The EBC was collected by using an EcoScreen condenser (Jaeger).[24] Lung tissues were obtained from participants after surgical resection for lung cancer. The clinical and demographic characteristics of the patients are shown in Tables S1–S3.

2.2 | Animal experiments

Four-week-old female BALB/c mice were divided into five groups: control, ovalbumin (OVA)/Lipopolysaccharide (LPS), OVA/LPS + anti-IgG, OVA/LPS + anti-IL-17, and OVA/LPS + DEL-1 overexpressing group. The DEL-1 overexpressing group of mice received 1×10^{11} viral genomes (vg) of AAV6 adeno-associated virus (Shandong Weizhen Biotechnology Co., Ltd.) expressing the DEL-1 gene by nasal aspiration on Day 0.[25] Sensitization with OVA and LPS was performed on Day 21, Day 22, Day 23, and Day 28, followed by OVA challenges on Day 35, Day 36, Day 42, and Day 43. Before each challenge, the anti-IgG and anti-IL-17 groups were treated with neutralizing antibodies.[26] The experimental protocol details can be found in Figure S1.

2.3 | Immunofluorescence

Neutrophils and human bronchial epithelial (16HBE) cells were cultured individually on cell slides or together on transwell inserts. Cells were fixed, permeabilized, and blocked. Primary and secondary antibodies were applied, followed by washing and mounting. Visualization was performed using a Zeiss fluorescence microscope for neutrophils and 16HBE cells, and a Leica Stellaris confocal microscope for the co-culture experiments.

2.4 | Neutrophil-bronchial epithelial cell adhesion and migration

16HBE cells were cultured on collagen-coated Transwell chambers. ICAM-1 blocking antibody, DEL-1 recombinant protein, and IL-17 were added to the cell monolayer. Human neutrophils from peripheral blood were labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and added to the upper chamber of the Transwell. Neutrophils in the LFA-1 and MAC-1 groups were pretreated with specific blocking antibodies. Fluorescence intensity was measured to calculate the relative adhesion rate of neutrophils. The migration ability of neutrophils across the bronchial epithelial cells was assessed by counting the number of neutrophils that migrated to the lower chamber and characteristics of the patients can be found in Supporting Information and Table S4.

2.5 | Immunohistochemistry

Human and mouse lung tissues were fixed with 4% paraformaldehyde and subsequently embedded in paraffin, then deparaffinized and dehydrated. For immunohistochemistry staining using a rabbit monoclonal anti-DEL-1 antibody and an anti-MPO antibody (both Cell Signaling Technology Inc.) (1:100) and a rabbit polyclonal anti-Ly6G antibody (Proteintech Group Inc) (1:100) were used.

2.6 | Enzyme-linked immunosorbent assay (ELISA)

The levels of human EBC DEL-1, IL-4, IL-5, IL-13, IL-17, neutrophil elastase (R&D) and MPO (SEA601Hu, USCN Life Science) as well as DEL-1 (CSB-EL007914MO, Cusabio) in mouse BALF were measured by ELISA according to the manufacturer's instructions.

2.7 | Statistical analysis

All data were represented as mean ± standard error mean (SEM) and $p < .05$ was considered significant. The statistical analyses were performed using GraphPad Prism software v9.0 (GraphPad Software, Inc.). Experiments with multiple comparisons were evaluated by one-way ANOVA followed by Bonferroni's post-test or Brown–Forsythe and Welch ANOVA tests (for normally distributed parameters) and Kruskal–Wallis test (non-normal distributed parameters) for multiple data sets. Comparisons between two groups were performed with an unpaired Student's *t*-test for normally distributed parameters and with Mann–Whitney test for non-normal distributed parameters. Correlations were determined using Pearson's Correlation analysis

(normally distributed parameters) and Spearman's Correlation analysis. (non-normal distributed parameters).

3 | RESULTS

3.1 | DEL-1 expression is decreased in bronchial epithelial cells of lung tissue and in serum of asthmatic patients and is negatively related to the blood neutrophils

To explore the expression of DEL-1 in human lung tissue, we collected lung tissues from eight healthy controls and seven patients with asthma for immunohistochemical staining. Both healthy controls and patients with asthma underwent surgical lung resection due to the presence of lung cancer. DEL-1 protein was predominantly expressed in bronchial epithelial cells but levels were significantly lower in the bronchial epithelium in patients with asthma than in healthy control participants. However, there was no significant change in expression in vascular endothelial cells (Figure 1A,B).

We further examined the level of DEL-1 in the serum of asthmatic patients and found that serum DEL-1 levels in patients with severe asthma (276.8 ± 29.36 pg/mL) were significantly decreased compared to non-severe asthma (428.0 ± 30.11 pg/mL, $p < .05$) and normal subjects (567.1 ± 75.3 pg/mL, $p < .001$) (Figure 1C). Serum DEL-1 levels were significantly negatively correlated with blood neutrophils ($r = -0.2881$, $p = .0384$) and neutrophil-to-lymphocyte ratio (NLR) ($r = -0.5469$, $p < .0001$), but not with the total white blood cells (WBC) ($r = -0.0528$, $p = .7099$) or eosinophils ($r = 0.0744$, $p = .6002$) (Figure 1D). Serum DEL-1 was not obviously related to the lung function in asthmatic patients (Figure S2A).

We divided asthma patients into those with allergic, high blood eosinophil or high blood neutrophil groups (see the Supporting Information for details). There was no significant difference in serum DEL-1 levels between the asthmatic patients in the allergic group (379.2 ± 31.54 pg/mL), the high blood eosinophil group (402.1 ± 42.24 pg/mL) or the high blood neutrophil group (339.6 ± 26.13 pg/mL) compared to the healthy control group (567.1 ± 75.3 pg/mL) (Figure 1E).

In order to explore the expression of DEL-1 in airway inflammation phenotypes of asthma, we examined its concentration in supernatants of induced sputum in patients with asthma. However, DEL-1 concentrations in induced sputum of asthmatic patients were below the lower limit of detection (data not shown). Therefore, we analyzed the GSE147880 dataset in the Gene Expression Omnibus (GEO), which contains transcriptome data on induced sputum from asthmatic patients. Through the utilization of single sample gene set enrichment analysis (ssGSEA), we identified CLCA1, POSTN, and SERPINB2 as characteristic genes associated with Type 2 (T2) asthma. Based on the median T2 score by ssGSEA, we categorized the patients into two groups: T2 high type and T2 low type asthma. Subsequently, we found that the expression level of induced sputum DEL-1 in patients with T2-low asthma was significantly lower compared to patients with T2-high asthma ($p < .01$) (Figure 1F).

3.2 | DEL-1 expression is decreased in EBC of asthmatic patients and is related to the level of asthma control and neutrophil related markers

DEL-1 expression was decreased in EBC samples of non-severe asthmatic patients (141.0 ± 5.3 pg/mL) and severe asthmatic patients (113.2 ± 8.09 pg/mL) compared with healthy controls (193.0 ± 7.61 pg/mL, both $p < .001$) (Figure 2A). DEL-1 concentrations decreased according to asthma control: well-controlled group (143.5 ± 6.97 pg/mL), not-well-controlled group (131.9 ± 7.49 pg/mL), and very poorly controlled group (109.2 ± 9.83 pg/mL) (Figure 2B). The DEL-1 levels (117.4 ± 8.94 pg/mL) in the EBC of the patients with an ACT score less than 20 points (asthma not controlled) were decreased compared with the DEL-1 levels (158.0 ± 7.74 pg/mL) in the EBC of patients with an ACT = 25 points (asthma completely controlled) (Figure 2B). Moreover, the expression level of DEL-1 was positively correlated with the ACT score of asthma patients ($r = 0.3678$, $p = .0035$) (Figure 2C). There was no significant correlation between EBC DEL-1 level and lung function in patients with asthma (Figure S2B).

EBC DEL-1 levels in patients with high blood neutrophils were lower than that in those with allergic asthma or with high blood eosinophils (90.57 ± 6.49 vs. 122.1 ± 7.64 vs. 128.0 ± 7.28 pg/mL, $p < .05$) (Figure 2D). There was a negative correlation between the concentration of EBC DEL-1 and white blood cell count ($r = -0.5014$, $p = .0001$), neutrophils ($r = -0.6551$, $p < .0001$) and NLR ($r = -0.5109$, $p < .0001$) in the blood while there was no correlation with blood eosinophils ($r = -0.0287$, $p = .8368$) (Figure 2E). The concentration of T2 cytokines, such as IL-4 (24.75 ± 1.67 vs. 47.18 ± 3.99 vs. 51.43 ± 10.29 pg/mL, $p < .001$), IL-5 (3.57 ± 0.38 vs. 5.48 ± 0.50 vs. 6.82 ± 0.61 pg/mL, $p < .01$), IL-13 (12.26 ± 1.12 vs. 21.19 ± 2.41 vs. 28.80 ± 3.73 pg/mL, $p < .01$) as well as biomarkers of neutrophilic airway inflammation, such as IL-17 (21.71 ± 1.48 vs. 34.03 ± 0.80 vs. 35.93 ± 1.81 pg/mL, $p < .001$), MPO (7.39 ± 0.28 vs. 13.68 ± 0.26 vs. 17.14 ± 2.13 ng/mL, $p < .001$) and neutrophil elastase (NE) (26.55 ± 2.77 vs. 44.46 ± 3.38 vs. 58.19 ± 3.93 pg/mL, $p < .001$) were increased in EBC of non-severe asthma patients and severe asthma patients compared with those in the control group (Figure 2F). EBC DEL-1 levels in asthma patients were significantly negatively correlated with IL-17 ($r = -0.3756$, $p = .0131$), MPO ($r = -0.5967$, $p = .0055$) and NE ($r = -0.5488$, $p = .0009$). In contrast, there was no correlation between DEL-1 and IL-4 ($r = -0.2326$, $p = .2854$), IL-5 ($r = -0.1720$, $p = .3467$) or IL-13 ($r = 0.0514$, $p = .7875$) in EBC of asthma patients (Figure 2G).

3.3 | DEL-1 production in 16HBECs is inhibited by IL-17, IL-4 and LPS

The expression of DEL-1 mRNA levels in human bronchial epithelial cells (16HBECs) was significantly decreased by IL-17 stimulation for 6 and 48 h, IL-4 stimulation for 24 h and LPS stimulation for 48 h compared with the control group (all $p < .05$) (Figure 3A). The Th1 cytokines

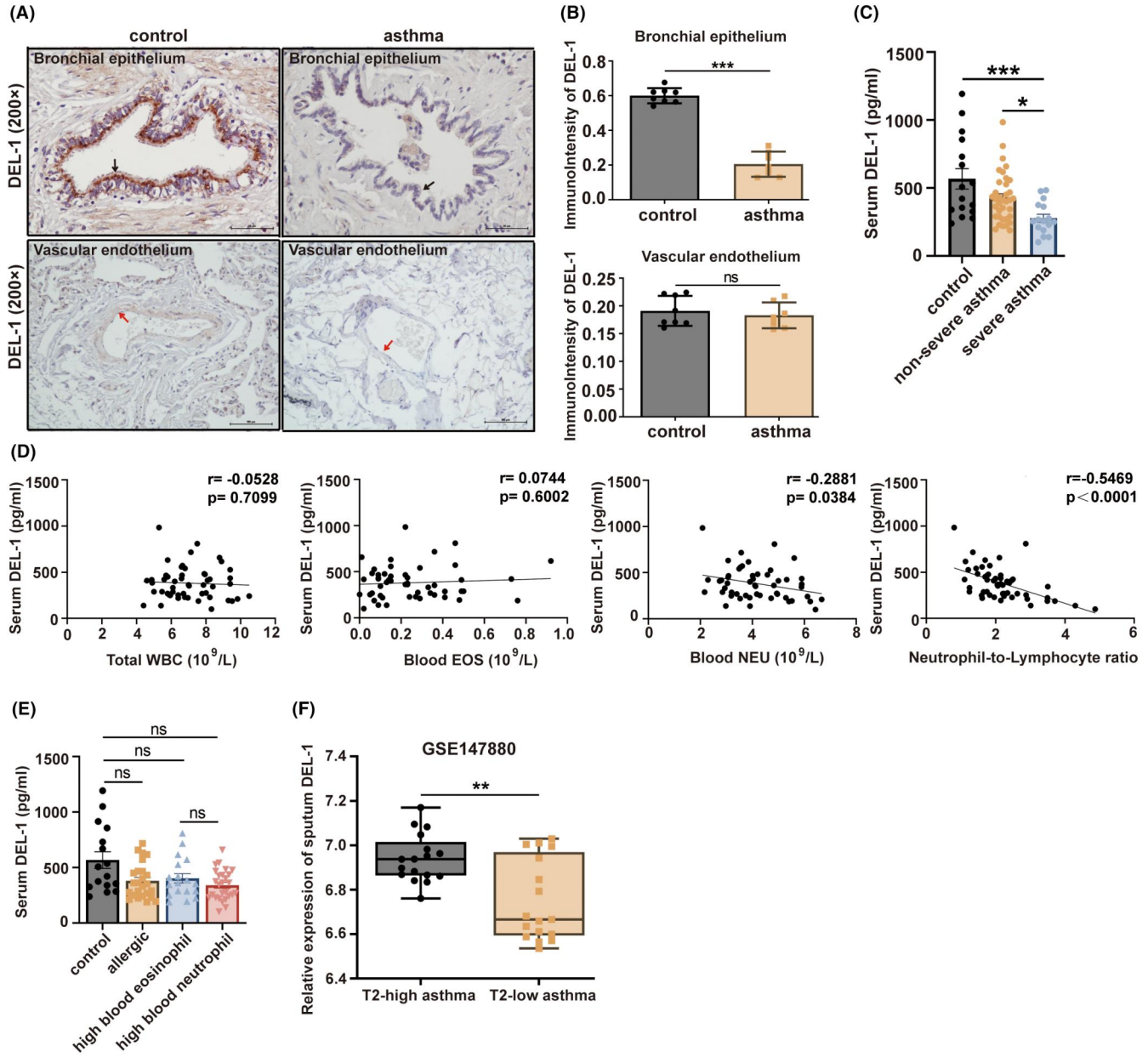


FIGURE 1 DEL-1 expression is decreased in bronchial epithelial cells of lung tissue and in serum of asthmatic patients and is negatively related to the blood neutrophils. (A) Representative images showing induced DEL-1 protein immunostaining in patients with asthma ($n=7$) compared with that seen in healthy subjects ($n=8$). The black arrow represents bronchial epithelial cells; Red arrow represents vascular endothelial cells. (B) DEL-1 protein relative expression was analyzed by Image-pro plus 6.0 and presented graphically. (C) DEL-1 levels of serum in healthy controls ($n=16$), non-severe asthma ($n=36$) and severe asthma ($n=16$). (D) The relationship between serum DEL-1 in patients with asthma ($n=52$) and the counts of white blood cells (WBC), eosinophils (EOS), neutrophils (NEU), and neutrophil to lymphocyte ratio (NLR) in peripheral blood using Pearson's analysis. (E) The concentration of DEL-1 in the serum of patients with allergic asthma ($n=25$), high blood eosinophil ($n=18$) and high blood neutrophil ($n=27$) compared to the healthy control ($n=16$). (F) The expression of induced sputum DEL-1 in patients with T2-low asthma ($n=17$) and with T2-high asthma ($n=17$). Data are presented as mean ± SEM using unpaired Student's t test in (B) and Mann–Whitney test in (F), one-way ANOVA followed by Bonferroni's post-test in (C) and Brown-Forsythe and Welch ANOVA tests in (E). Ns (not significant), $^{*}p<.05$, $^{**}p<.01$ and $^{***}p<.001$ compared with respective control groups.

IFN-γ and TNF-α and another Th2 cytokine IL-13 had no significant effect on the levels of DEL-1 mRNA in 16HBE cells (Figure 3A). IL-17 (20 ng/mL), IL-4 (20 ng/mL) and LPS (20 μg/mL) also down-regulated DEL-1 protein expression in 16HBE cells (all $p<.05$) (Figure 3B). In addition, IL-17 (20 ng/mL) and IL-4 (20 ng/mL) down-regulated DEL-1 secretion by 16HBECs in a time-dependent manner ($p<.01$) (Figure 3C).

3.4 | Transbronchial epithelial cell migration of neutrophils in asthma is associated with LFA-1 binding to ICAM-1 and inhibited by DEL-1

Approximately 24 h post-seeding, the 16HBE cells were observed to form confluent monolayers by evaluating the TEER (Figure 4A).

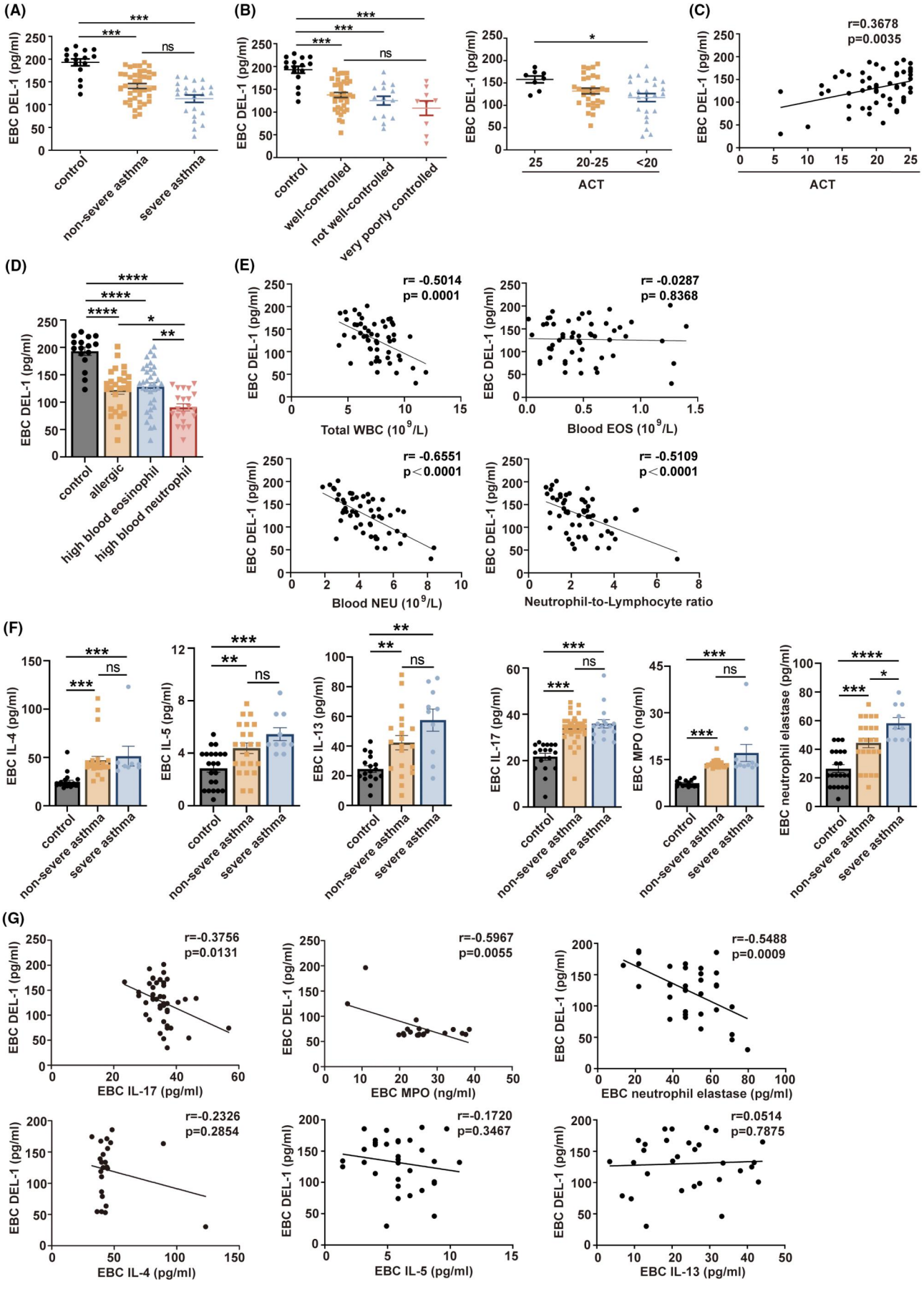


FIGURE 2 DEL-1 expression is decreased in EBC of asthmatic patients and is related to the level of asthma control and neutrophil related markers. (A) DEL-1 levels of EBC in healthy controls ($n=16$), non-severe asthma ($n=40$) and severe asthma ($n=23$). (B) Decreased levels of DEL-1 were associated with the degree of asthma symptom control according to the asthma control test (ACT) scores (left panel). The levels of DEL-1 in EBC of asthmatic patients with ACT score equal to 25 points ($n=8$), 20–24 points ($n=30$), and less than 25 points ($n=24$) (right panel). (C) Correlation between DEL-1 level in EBC and ACT score in asthma patients ($n=61$) using Pearson's Correlation. (D) Levels of DEL-1 in the EBC of patients with allergic asthma ($n=27$), high blood eosinophil ($n=34$) and high blood neutrophil ($n=21$) compared to the healthy control ($n=16$). (E) The correlation between DEL-1 expression in EBC of asthma patients ($n=54$) and the counts of white blood cells (WBC), eosinophils (EOS), neutrophils (NEU) and neutrophil-to-lymphocyte ratio (NLR) in patient's blood using Pearson's Correlation analysis. (F) IL-4 (control, $n=22$; non-severe asthma, $n=26$; severe asthma, $n=8$), IL-5 (control, $n=22$; non-severe asthma, $n=22$; severe asthma, $n=10$), IL-13 (control, $n=27$; non-severe asthma, $n=20$; severe asthma, $n=10$), IL-17 (control, $n=18$; non-severe asthma, $n=46$; severe asthma, $n=16$), MPO (control, $n=14$; non-severe asthma, $n=24$; severe asthma, $n=10$) and neutrophil elastase (control, $n=21$; non-severe asthma, $n=23$; severe asthma, $n=10$) expressions in EBC samples from asthmatic patients and healthy control volunteers were detected by ELISA. (G) The relationship between the EBC DEL-1 levels and the EBC neutrophil-related markers IL-17 ($n=43$), MPO ($n=20$), neutrophil elastase (n = 33) levels and the EBC levels of the Th2-cytokines IL-4 ($n=23$), IL-5 ($n=32$) as well as IL-13 ($n=30$) in patients with asthma using Pearson's Correlation. Data are presented as mean ± SEM using one-way ANOVA followed by Bonferroni's post-test in (A) and Brown-Forsythe and Welch ANOVA tests in (B), (D) and (F). Ns (not significant), $^{*}p<.05$, $^{**}p<.01$, $^{***}p<.001$ and $^{****}p<.0001$ compared with respective control groups.

To determine the potential molecular mechanisms of neutrophil adherence to bronchial epithelial cells in asthma, the co-localization of ICAM-1 on 16HBECs and of LFA-1 on neutrophils extracted from the blood of asthma patients was detected by immunofluorescence. LFA-1 and its ligand, ICAM-1 were co-localised in this co-culture model (Figure 4B) suggesting a possible role for ICAM-1/LFA-1 interaction in neutrophil-bronchial epithelial adhesion and migration in asthma.

Then BCECF-AM stained neutrophils extracted from peripheral blood of healthy subjects and asthma patients were seeded on the 16HBE cells and were used for the study of neutrophil-bronchial epithelial cell adhesion and migration (Figure 4C–F). The adhesion and migration ability of neutrophils derived from asthma patients was slightly increased compared to healthy controls but this did not reach significance (black control bars, Figure 4C–F). Blocking neutrophil LFA-1 in asthma patients and healthy subjects significantly inhibited neutrophil adhesion (Figure 4C,D) and migration (Figure 4E,F). Exogenous DEL-1 significantly suppressed both baseline and IL-17-induced neutrophil adhesion (Figure 4C,D) and transepithelial migration (Figure 4E,F), suggesting that neutrophil-bronchial epithelial cell migration in asthma is driven by IL-17 and can be inhibited by DEL-1.

3.5 | DEL-1 expression is decreased while neutrophil infiltration is increased in airway in a murine model of neutrophilic asthma

To clarify the expression and distribution of DEL-1 in asthma and its correlation with airway neutrophils, we established an OVA-induced asthma mouse model (conventional sensitization) and an OVA/LPS-induced neutrophilic asthma mouse model (see Figure S1 for details). As in human asthma, DEL-1 expression was mainly localized to the bronchial epithelial cells and was significantly reduced in OVA-induced asthma mice and OVA/LPS-induced neutrophilic asthma mice (Figures 5A,B). The number of white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), and eosinophils (EOS) in the BALF of asthmatic mice in the two groups were significantly increased compared with those of control mice (Figure 5C). DEL-1 levels were also significantly suppressed in the BALF of OVA-induced asthma mice (336.6 ± 25.04 vs. 680.8 ± 90.11 pg/mL, $p<.05$) and OVA/LPS-induced neutrophilic asthma mice (156.2 ± 11.29 vs. 744.8 ± 72.23 pg/mL, $p<.001$) compared with the respective controls (Figure 5D). In contrast, OVA and OVA/LPS models both resulted in significantly enhanced levels of BALF IL-17 and IL-4 (Figures 5D).

BALF DEL-1 levels were negatively correlated with BALF IL-17 ($r=-0.7712$, $p=.0250$; $r=-0.994$, $p<.001$) and the number of neutrophils in BALF ($r=-0.7754$, $p=.0700$; $r=-0.9741$, $p=.0002$) of the OVA and OVA/LPS models of asthma respectively (Figure 5E). In contrast, BALF levels of DEL-1 did not significantly correlate with BALF IL-4 expression in either the OVA-induced asthma mice ($r=-0.6777$, $p=.0648$) or the OVA/LPS-induced neutrophilic asthma mice ($r=0.3111$, $p=.4618$) (Figure 5F).

3.6 | DEL-1 overexpression attenuates airway neutrophil infiltration in neutrophilic asthmatic mice

To further confirm the involvement of DEL-1 in neutrophilic inflammation in asthma, we overexpressed DEL-1 and used an anti-IL-17 antibody in the neutrophilic asthma model (Figure S1). The trachea wall was significantly thickened with epithelial cell–cell adherence being clearly damaged in the neutrophilic asthma model (Figure 6A). The inflammatory score, a marker of inflammatory cell infiltration, was also significantly increased by OVA/LPS challenge (Figure 6A,B). In addition, the expression of the neutrophil markers Ly6G and MPO were both increased in the lungs of mice with neutrophilic asthma (Figure 6A,C). These OVA/LPS-induced inflammatory and remodelling changes were alleviated in the anti-IL-17 antibody-treated animals and in mice overexpressing DEL-1 (Figure 6A–C).

The airway resistance of neutrophilic asthma mice was significantly higher than that in the control group ($p<.05$, Figure 6D), in contrast,

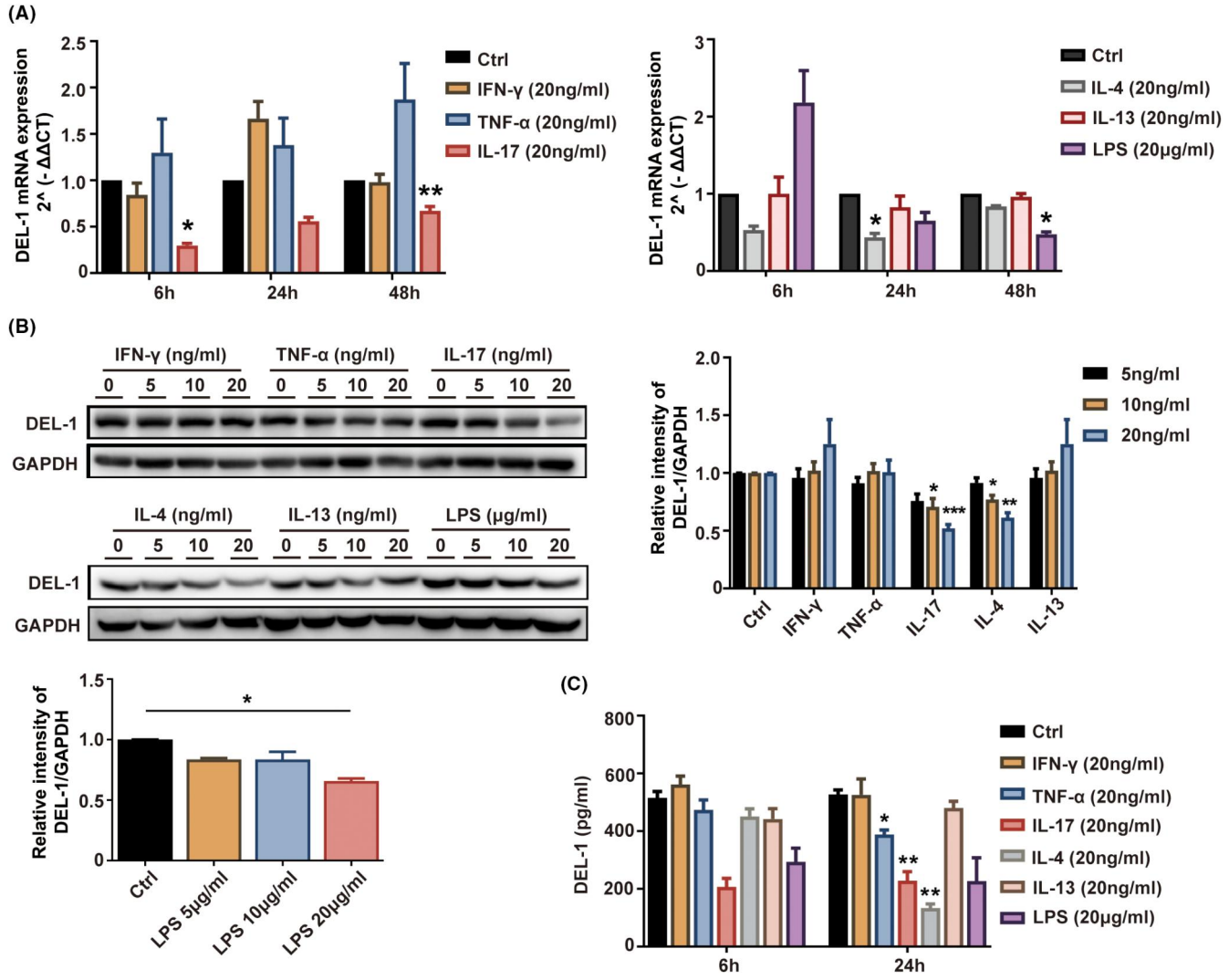
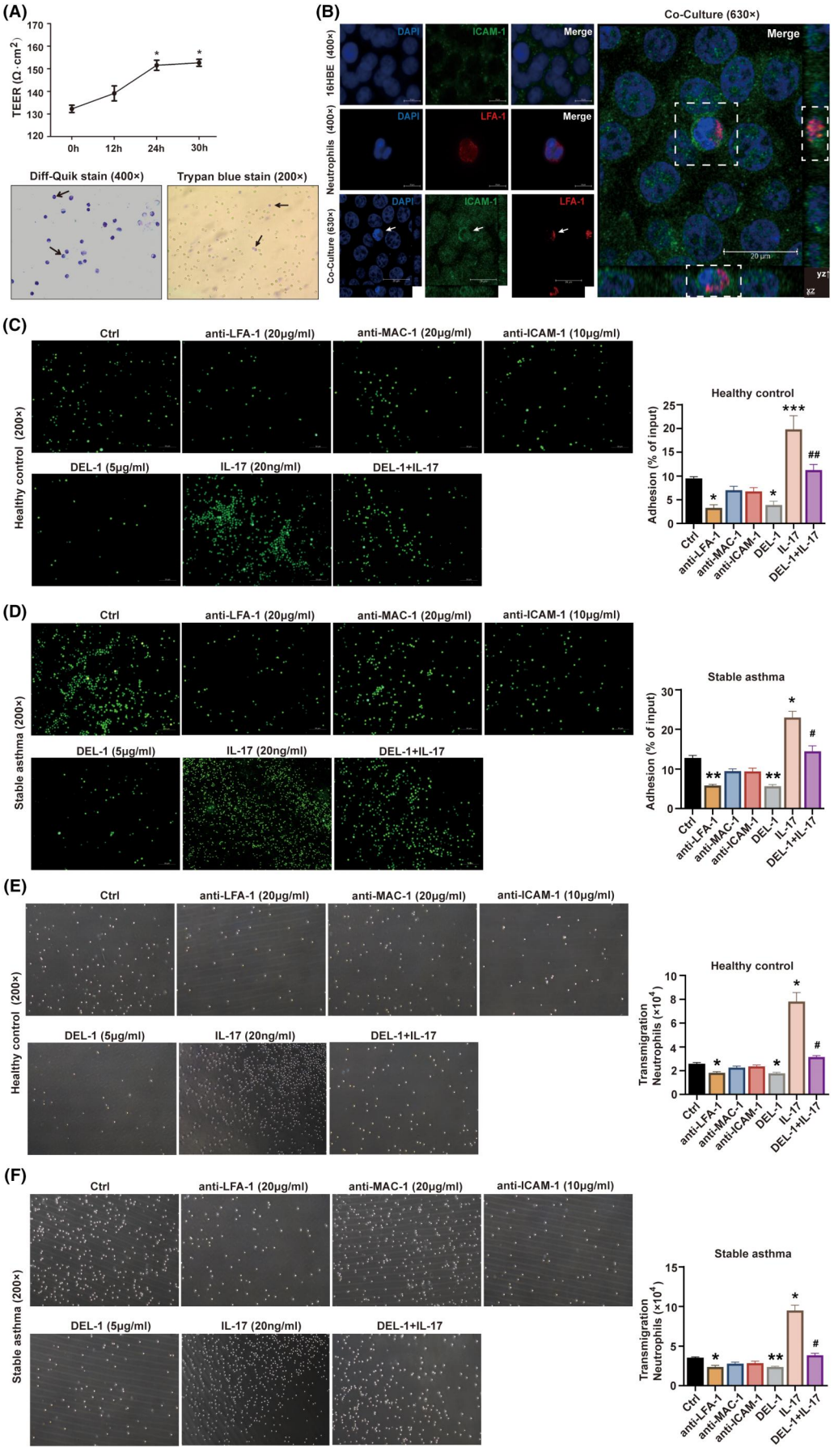
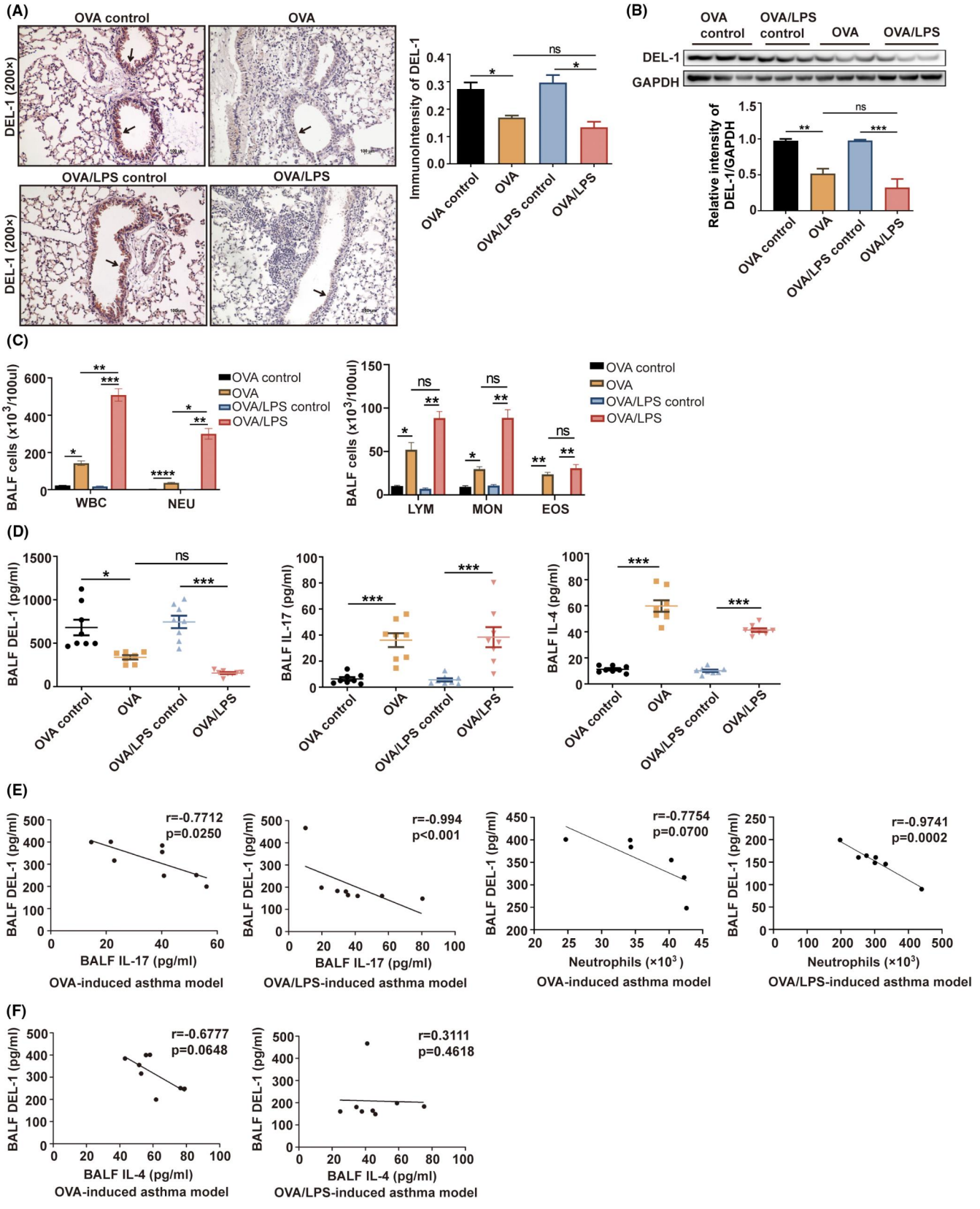


FIGURE 3 IL-4, IL-17, and LPS down-regulate DEL-1 expression in bronchial epithelial cells. (A) 16HBE cells were treated with IFN-γ (20 ng/mL), TNF-α (20 ng/mL), IL-17 (20 ng/mL) as well as IL-4 (20 ng/mL), IL-13 (20 ng/mL) and LPS (20 μg/mL) for 6, 24 and 48 h, and DEL-1 mRNA expression was measured by RT-qPCR. (B) 16HBE cells were exposed to different concentrations of IFN-γ, TNF-α, IL-17, IL-4, IL-13, and LPS for 24, 48, 72 h, and DEL-1 protein production was determined by Western blotting (WB). (C) Each stimulating factor interfered with 16HBE cells for 6 and 24 h, respectively. The secretion level of DEL-1 in the cell supernatant was detected by ELISA. Data are presented as mean ± SEM of three independent experiments using one-way ANOVA followed by Brown-Forsythe and Welch ANOVA tests in (A–C). Ns (not significant), $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$, compared with control (Ctrl).

FIGURE 4 DEL-1 inhibits IL-17-mediated neutrophil-bronchial epithelial cell adhesion and migration in asthma. (A) Transepithelial electrical resistance (TEER) changes of 16HBE cells growing on the upper layer of Transwell chamber at 0, 12, 24, and 30 h; Diff-Quik staining to identify the purity of neutrophils (400×, left panel); Trypan blue staining to identify the viability of neutrophils (200×, right panel). (B) Immunofluorescence staining of ICAM-1 in 16HBE cells (green) and LFA-1 in neutrophils (red). Counterstaining of the nuclei was performed using DAPI (blue). The results showed that LFA-1 is co-localized with ICAM-1 (scale bar 20 μm). (C) Fluorescence microscope observation of BCECF-AM labeled neutrophils (green) from healthy subjects ($n = 4$) adhered to 16HBECs (Left). The specific groups are as follows: negative control group (Ctrl), neutrophil LFA-1 blocking group (anti-LFA-1), neutrophil MAC-1 blocking group (anti-MAC-1), bronchial epithelial cell ICAM-1 blocking group (anti-ICAM-1), human recombinant DEL-1 protein (5 μg/mL) intervention group (DEL-1), IL-17 (20 ng/mL) stimulation group (IL-17), DEL-1 (5 μg/mL) intervention IL-17 (20 ng/mL) treated bronchial epithelial cell group (DEL-1 + IL-17). The relative adhesion rate of neutrophils in each group (Right). (D) Fluorescence microscope observation of BCECF-AM labeled neutrophils (green) from asthmatic patients ($n = 5$) adhered to 16HBECs (Left). The relative adhesion rate of neutrophils in each group (Right). (E) Light microscope observation of neutrophils from healthy subjects ($n = 4$) migrating to the lower chamber of Transwell (Left). The number of neutrophils in the lower chamber was counted by the hemocytometer (Right). (F) Light microscope observation of neutrophils from asthmatic patients ($n = 5$) migrating to the lower chamber of Transwell (Left). The number of neutrophils in the lower chamber was counted by the hemocytometer (Right). Data are presented as mean ± SEM of three independent experiments using one-way ANOVA followed by Brown-Forsythe and Welch ANOVA tests in (A) and (C–F). Compared with the negative control group (Ctrl), $^{*}p < .05$, $^{**}p < .01$; compared with the IL-17 stimulation group (IL-17), $^{\#}p < .05$, $^{\#\#}p < .01$.





the airway resistance of mice either overexpressing DEL-1 or in anti-IL-17 antibody-treated animals were decreased ($p < .05$, Figure 6D). These effects were not associated with any significant changes in peripheral blood cell counts (Figure 6E). In contrast, the BALF total cell number as well as BALF lymphocytes (LYM) and neutrophils (NEU) were significantly increased in OVA/LPS-treated "neutrophilic asthma" mice, whilst these were attenuated in mice overexpressing DEL-1 or receiving the anti-IL-17 antibody-treated animals (Figure 6F).

FIGURE 5 The expression of DEL-1 is decreased in lung tissue and BALF of asthmatic mice, which is negatively correlated with the level of IL-17 and neutrophils in BALF. (A) Immunohistochemical analysis of DEL-1 expression in PBS-exposed control mice (control), OVA-treated asthma mice (OVA), OVA/LPS induced-neutrophilic asthma mice (original magnification×200, scale bar 100 μm, black arrow) (Left) and scored (Right). (B) The WB detection image of DEL-1 protein in the lung tissue homogenate of each group of mice (above graph) and the relative expression of DEL-1 protein (below graph). (C) The total number of white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MON), and eosinophils (EOS) in the BALF for each group of mice. (D) The concentrations of DEL-1, IL-17 and IL-4 in the BALF for each group were measured by ELISA. (E) Correlation between DEL-1 in the BALF of mice in the OVA-induced asthma group ($n = 6–8$) and the neutrophilic asthma group ($n = 7–8$) with BALF levels of IL-17 and BALF neutrophils using Pearson's Correlation. (F) The relationship between levels of BALF DEL-1 and BALF IL-4 expression in OVA-induced asthma mice ($n = 8$) and OVA/LPS-induced neutrophilic asthma mice ($n = 8$) using analysis of Pearson's Correlation. The data were analyzed using Kruskal–Wallis test in (A) and (B), one-way ANOVA followed by Bonferroni's post hoc analysis in (C) and (D). Ns (not significant), $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ compared to respective controls in (A–D).

4 | DISCUSSION

We demonstrated that DEL-1 concentrations in serum and EBC were reduced in subjects with asthma compared with healthy control subjects, and were related to the level of asthma control, blood neutrophil count, and neutrophil-related biomarkers. In addition, we found that DEL-1 was significantly expressed on human lung bronchial epithelial cells, and was decreased in an OVA/LPS-induced neutrophilic asthma mouse model. OVA combined with LPS resulted in increased neutrophil infiltration in lung tissue as well as BALF and increased airway resistance, all of which were prevented by DEL-1 adenoviral-mediated overexpression. In vitro experiments demonstrated that IL-17, IL-4, and LPS down-regulated DEL-1 expression in bronchial epithelial (16HBE) cells and that exogenous addition of DEL-1 inhibited the ability of neutrophils obtained from patients with asthma to adhere and migrate through bronchial epithelial cells. These results indicated that decreased DEL-1 may be involved in airway neutrophilic inflammation in severe asthma.

DEL-1 is an extracellular matrix protein secreted by endothelial cells during embryonic development but its expression becomes static after birth and it may not be expressed in the endothelial cells in adulthood.[21] We found that DEL-1 was mainly localized to bronchial epithelial cells in human lung tissue, and its expression level was much higher than in vascular endothelial cells, which have been the major focus of previous research.[23,27] We also found that the expression of DEL-1 was significantly decreased in the serum and EBC of asthma patients. The expression of DEL-1 in the lung tissue of asthmatic mice was also decreased, especially in neutrophilic asthmatic mice.

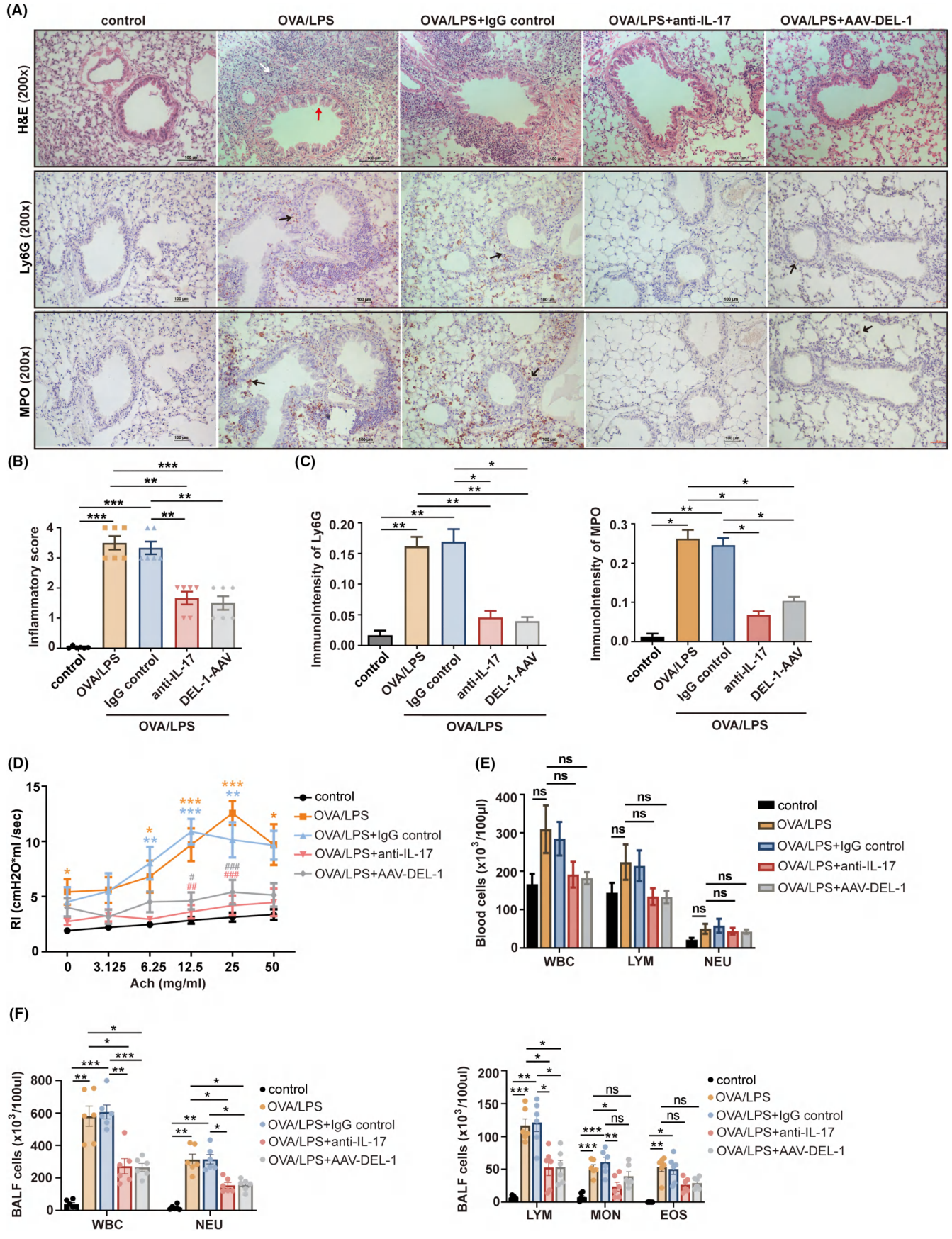
As a primary defense against external challenge, neutrophils are the first white blood cells recruited to the damaged airway.[28] The migration of intravascular neutrophils mainly depends on the β2 integrin on its surface which binds to cell adhesion molecules (such as ICAM-1) on vascular endothelial cells.[8,9] Previous studies have shown that LFA-1 has a stronger pro-adhesion effect and an affinity for ICAM-1 than MAC-1 and promotes neutrophil adhesion to bronchial epithelial cells.[13,14] Our study found that blocking LFA-1 significantly inhibited the transbronchial epithelial migration of neutrophils obtained from patients with asthma, while blocking MAC-1 alone had no obvious inhibitory effect, suggesting that the migration of neutrophils to the airway may be dependent on LFA-1. Therefore, inhibiting the expression of cell surface adhesion molecules or interfering with their ligand binding may be a potential mechanism for alleviating airway inflammation in asthma.

Integrins recognize extracellular matrix proteins by binding to cell surface arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD) sequences and thereby mediate recognition and adhesion between neutrophils and the extracellular matrix.[29] DEL-1 is also called EGF-like repeat and discoidin I-like domain-containing protein 3 (EDIL3) because it contains three repeated epidermal growth factor (EGF)-like domains (E1, E2, E3) and two discoidin-I (discoidin-I)-like domains (C1, C2). The second EGF domain (E2) of DEL-1 contains an important sequence that can bind integrin: the RGD sequence.

Previous studies have reported that IL-17 participates in the activation and migration of neutrophils by directly or indirectly inducing epithelial cells, endothelial cells and other mesenchymal cells to secrete inflammatory factors that promote the chemotaxis and survival of neutrophils.[30] Clinical studies have confirmed that the number of induced sputum and blood neutrophils in asthma patients is directly proportional to the expression of Th17-related factors.[31] Our study demonstrated that the expression of DEL-1 was decreased in bronchial epithelial cells following exposure to injurious factors such as IL-17, which causes a large number of neutrophils to adhere to and migrate out of bronchial epithelial cells. Exogenous DEL-1 inhibited IL-17-induced neutrophil adhesion and migration of neutrophils obtained from patients with asthma to airway epithelial cells suggesting that DEL-1 is involved in IL-17-mediated migration of neutrophils across the bronchial epithelium in asthma.

An anti-inflammatory effect of DEL-1 is reported in other inflammatory diseases. Local supplementation of DEL-1 can reverse periodontal inflammation and LFA-1-dependent neutrophil infiltration and IL-17 production in aged mice.[27] Similarly, DEL-1 is also involved in inflammation of the central nervous system,[32] blood-mediated inflammation immediately after pancreatic islet transplantation,[33] adrenal dysfunction associated with systemic inflammation,[34] and salivary gland inflammation.[35] In the lungs where DEL-1 is abundantly expressed, DEL-1 also acts as a "gatekeeper". Overexpression of DEL-1 through mesenchymal stem cells can inhibit LPS-induced lung injury and reduce the number of BALF neutrophils.[36] DEL-1 treatment of BALF cells from patients with asthma inhibits the expression of IL-17 and eosinophil-related cytokines.[37]

In order to explore the role of DEL-1 in the formation of airway inflammation in bronchial asthma and the possibility of targeted therapy, we used AAV6 to construct a mouse model of neutrophil



asthma with DEL-1 overexpression which generated similar effects as blocking IL-17. Airway hyperresponsiveness, neutrophil activation markers, and inflammatory cell infiltration in the trachea and surrounding blood vessels in the lung tissue of asthmatic mice overexpressing DEL-1 were all reduced compared with asthmatic mice. In addition to suppressing lung inflammation, DEL-1 overexpression

FIGURE 6 DEL-1 inhibits inflammatory cell infiltration in lung tissue of neutrophilic asthma mice. (A) H&E staining to observe the infiltration of inflammatory cells in the trachea and surrounding blood vessels in the lung tissues of PBS control group (control), OVA/LPS-induced neutrophilic asthma group (OVA/LPS), isotype antibody control group (OVA/LPS + IgG control), IL-17 neutralizing antibody intervention group (OVA/LPS + anti-IL-17), DEL-1 overexpression intervention group (OVA/LPS + AAV-DEL-1). IHC staining to observe the expression of Ly6G and MPO in the lung tissues of each group of mice (original magnification×200, black arrow). (B) Inflammation scores of each group mice. (C) The relative expression of Ly6G and MPO in the lung tissues of mice in each group ($n = 6$/group) and were analyzed by Image-pro plus 6.0. (D) The airway responsiveness of mice in different concentrations of Ach (0–50 mg/mL). RI represents airway resistance. (E) The total number of peripheral blood white blood cells (WBC), lymphocytes (LYM), and neutrophils (NEU) in each group of mice. (F) WBC, NEU, LYM, monocyte (MON), and eosinophil (EOS) counts in the BALF for each group of mice. The data were analyzed using Kruskal–Wallis test in (B) and (C), one-way ANOVA followed by Brown–Forsythe and Welch ANOVA tests in (D) and Bonferroni's post hoc analysis in (E) and (F). Ns (not significant), $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ compared to respective controls in (A–F). $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ compared to the OVA/LPS group in (D).

reduced the total number of white blood cells, lymphocytes, and neutrophils in BALF. These results suggest that DEL-1 may be an effective anti-inflammatory factor to inhibit airway neutrophilic inflammation in asthma.

This study has some limitations. One of the major limitations is that the correlation between sputum DEL-1 and neutrophilia could not be determined in patients as the levels of DEL-1 in induced sputum were below the lower limit of detection, which might be attributed to the induced sputum preparation process, including sample acquisition, handling, processing, and the use of the reducing agent dithiothreitol (DTT).[38,39] Furthermore, we were unable to accurately assess sputum granulocyte levels due to technical problems with immunocytochemistry which prevented us from defining the sputum neutrophil status of patients from whom we collected EBC and serum. Another limitation is that experiments in vitro were carried out with 16HBE cells in submerged culture rather than primary HBECs grown at ALI. Future studies will investigate the interaction mechanism of adhesion molecules in the process of neutrophils across bronchial epithelium in neutrophilic asthma. Furthermore, although previous studies using AAV6 have shown no effect of the adenoviral vector alone on lung inflammation,[40] we did not have a separate AAV6 vector control arm in our study.

In summary, we use a combination of data from human clinical samples and in vitro and in vivo models of neutrophilic asthma and neutrophil migration to show a potential role for reduced DEL-1 in mediating severe neutrophilic asthma. Our study extends previous work showing decreased DEL-1 in BALF of allergic asthmatics[37] to show a greater reduction in patients with severe neutrophilic asthma. Down-regulation of DEL-1 and the subsequent increase in neutrophil migration into the airways of patients with severe asthma suggests that this pathway may be useful in targeting patients with this form of severe asthma.

AUTHOR CONTRIBUTIONS

Man Jia, Xinyu Jiang, Xin Yao, and Ian M Adcock designed this study. Man Jia, Heng Fu, Lina Wang, and Jiayan Xu performed the animal experiments. Man Jia and Heng Fu performed the ex vivo experiments. Man Jia, Heng Fu, Xinyu Jiang, Jiayan Xu, Shujuan He and Fan Zhang recruited the patients. Man Jia, Xinyu Jiang, Shujuan He, and Fan Zhang collected samples. Man Jia, Xinyu Jiang, Lei Yao, and Peng Sun performed the analysis of data and interpretation. Man Jia wrote the manuscript. Yi Liu assisted in collecting lung tissue specimens. Xin Yao, Peter J. Barnes, and Ian M Adcock critically revised the manuscript. All the authors read and approved the final paper.

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CONFLICT OF INTEREST STATEMENT

All the authors declare no commercial or financial conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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