



ORIGINAL ARTICLE

Developmental endothelial locus-1 (Del-1) antagonizes Interleukin-17-mediated allergic asthma

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Abstract

Interleukin (IL)-17 is a major contributor to the pathogenesis of allergic asthma. Developmental endothelial locus-1 (Del-1) is an endothelial cell-secreted protein known to inhibit IL-17 expression. However, little is known about the association between Del-1 and IL-17 in the pathogenesis of allergic asthma. Using bronchoalveolar lavage fluid (BALF) and peripheral blood samples collected from allergic asthmatic patients and controls, we explored the role of Del-1 in relation to IL-17 in allergic asthma. We found that the negative correlation between Del-1 and IL-17 was significant in BALF of allergic asthmatics. Del-1 treatment inhibited the expression of IL-17, the differentiation of IL-17-secreting leukocytes and associated cytokines. Contrarily, IL-17 levels were increased after treatment with anti-Del-1 mAb. Consistent with this, Del-1 treatment led to downregulation of IL-5, CCL5 and IL-4, thus reducing secretion of eosinophil cationic protein. Furthermore, Del-1 significantly downregulated the expression of ICAM-1 and may have the potential to reduce leukocyte transendothelial migration. Our data demonstrate that Del-1 can negatively regulate IL-17 and its proinflammatory function, thereby limiting airway inflammation in allergic asthmatics, and suggest Del-1 as a potential candidate for prevention and treatment of allergic asthma.

INTRODUCTION

Allergic asthma is a complex, chronic inflammatory airway disease involving many inflammation molecules and cell types, including cytokines, chemokines, lymphocytes and eosinophils. The Th2 lymphocytes cytokines, such as interleukin (IL)-4 and -5, play an important role in the pathogenesis of allergic asthma.^{1,2} Recently, there has been increasing evidence of vital roles for IL-17 in allergic asthma.^{3,4}

IL-17 has been recognized as a proinflammatory cytokine in the pathogenesis of various diseases by regulating many inflammatory molecules, including chemokines, cytokines and adhesion molecules.^{5–7} Increased levels of IL-17 in bronchoalveolar lavage fluid (BALF), blood and sputum of patients with allergic asthma have been reported.⁸ Sequencing studies revealed that IL-17 activates stromal cells to release chemokines and cytokines and increases the expression of extracellular

matrix proteins in allergic asthma.^{9,10} In addition, IL-17 enhances eosinophilic recruitment during airway inflammation in allergic asthma.^{11,12} Hence, there might be clinical benefit by inhibiting IL-17 immunity.

Developmental endothelial locus-1 (Del-1, also known as EDIL3) is an endogenous protein which is secreted by endothelial cell and acts to suppress inflammation in various tissues and organs.^{13,14} Previous studies have shown that Del-1 interacts with leukocyte function-associated antigen 1 (LFA-1) to interfere with LFA-1/ICAM-1 leukocyte adhesion pathway.^{15–17} Del-1 was recently identified as an inhibitor of IL-17-mediated inflammation.^{18–20} Genetic deficiency of Del-1 induces IL-17-triggered inflammatory damage in mouse periodontitis models. Furthermore, our previous studies had shown the negative correlation between the mRNA expression of IL-17 and Del-1 in atopic allergic children.²¹ However, while this may indicate Del-1 as a key regulator in the IL-17 inflammation, little is

definitively known about the role of Del-1 in allergic asthma.

Herein, we sought to investigate the role of Del-1 in allergic asthma and its relationship with IL-17 inflammation. BALF from controls and asthmatics with allergic asthma was assessed for expression of Del-1 and IL-17. IL-17, relative cytokines and ECP were detected after soluble Del-1 or anti-Del-1 mAb treatment. IL-17-secreting cells and non-IL-17-secreting cells were also isolated from blood samples to determine the expression of STAT3, RORc (the human homolog of ROR γ t) and cytokines, such as IL-17, TGF- β , IL-6 after soluble Del-1 treatment. Our data demonstrate a potential role for Del-1 in the inflammatory response in allergic asthma and reveal a possible approach to treat allergic asthma.

RESULTS

Decreased Del-1 secretion is associated with increased IL-17 expression in allergic asthma

Using ELISA, Del-1 was markedly lower in BALF of asthmatic patients compared with healthy controls ($P < 0.001$, Figure 1a). Conversely, IL-17 expression was greater in BALF from allergic asthma compared with control subjects ($P < 0.001$, Figure 1b). Furthermore, linear-regression analysis of Del-1 expression *versus* IL-17 expression in BALF from allergic asthma identified a significant inverse association between Del-1 and IL-17 ($r = -0.63$; $P < 0.001$; Figure 1c). Therefore, the inverse

association between expression of Del-1 and IL-17 characterizes human allergic asthma.

Del-1 is critically involved in IL-17 levels in allergic asthma

To confirm whether Del-1 has a role in IL-17 levels in allergic asthma, BALF cells collected from allergic asthma patients were treated by Del-1. As shown in Figure 2a, IL-17 levels in the culture supernatants of BALF cells were significantly decreased following Del-1 administration compared with PBS-treated controls ($P < 0.001$). The percentage of IL-17 $^{+}$ cells was lower in BALF cells following Del-1 treatment compared with PBS-treated controls. (Figure 2b). In addition, we confirmed by qPCR the lower expression of IL-17 in BALF cells following Del-1 administration ($P < 0.01$, Figure 2c). On the contrary, after Del-1 neutralization with anti-Del-1mAb, IL-17 levels in BALF cells collected from allergic asthma patients were significantly increased compared with isotype controls ($P < 0.005$, Figure 2d). These data suggest that Del-1 is essentially involved in IL-17 levels in allergic asthma.

Del-1 has an essential role in IL-17-secreting cell function in allergic asthma

To determine whether treatment with Del-1 could directly downregulate IL-17 expression, we collected blood from allergic asthma patients and then IL-17-secreting cells and

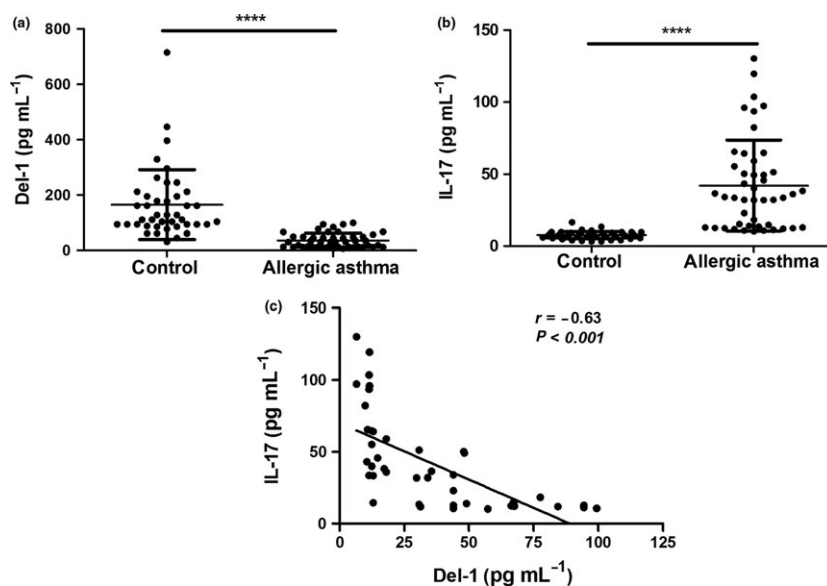


Figure 1. Lower expression of Del-1 in allergic asthma correlates with IL-17 expression. **(a)** ELISA analysis of Del-1 in BALF collected from allergic asthma patients ($n = 46$) and controls ($n = 42$). Small horizontal lines indicate the mean. **(b)** IL-17 protein in BALF collected from allergic asthma patients ($n = 46$) and controls ($n = 42$). Small horizontal lines indicate the mean. **(c)** Linear-regression analysis of Del-1 expression *versus* IL-17 expression in allergic asthma ($n = 46$). Each symbol represents an individual sample. **** $P < 0.001$. BALF, bronchoalveolar lavage fluid.

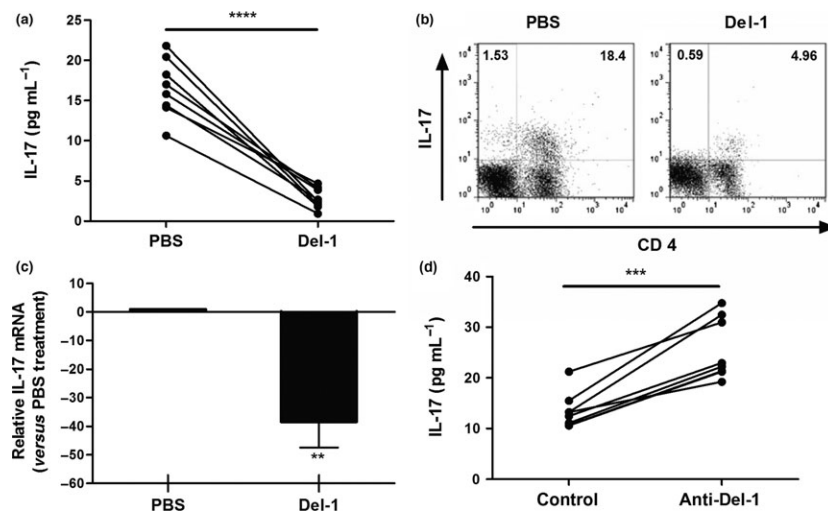


Figure 2. Effect of Del-1 or anti-Del-1 on IL-17 expression in allergic asthma patients. The cells of BALF collected from allergic asthma patients ($n = 8$) were treated with Del-1 or PBS. **(a)** After 48 h, supernatants were collected. Secretion of IL-17 was measured by ELISA. Each symbol represents an individual patient; paired data for each individual are connected by a line. **(b)** After 48 h, BALF cells were collected and stained with anti-IL-17, anti-CD4 or control IgG. IL-17 expression was analyzed by Flow cytometry. The gate was set on lymphocytes. **(c)** After 48 h, BALF cells were collected. qPCR analysis of IL-17 mRNA expression in the cells. Results were normalized to GAPDH mRNA expression and are presented relative to PBS treatment. **(d)** The cells of BALF collected from allergic asthma patients ($n = 8$) were treated with anti-Del-1 or IgG2A isotype controls. After 48 h, supernatants were collected. Secretion of IL-17 was measured by ELISA. Each symbol represents an individual patient; paired data for each individual are connected by a line. ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$. BALF, bronchoalveolar lavage fluid.

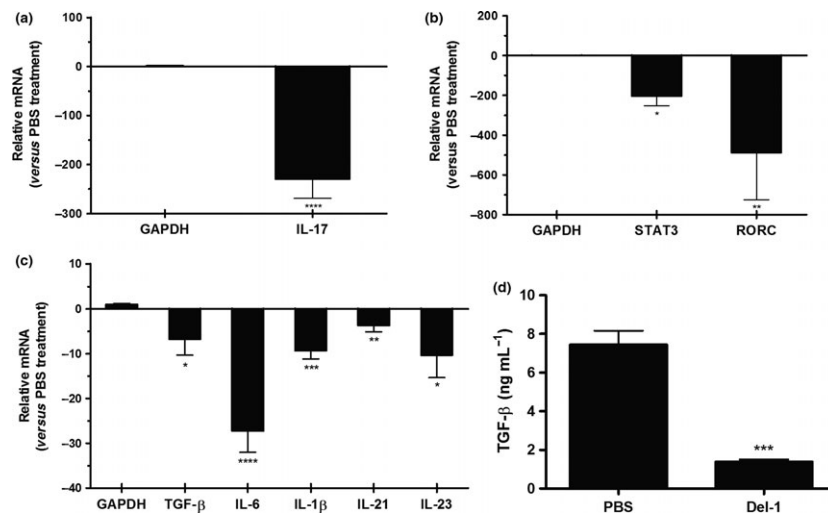


Figure 3. Del-1 inhibits IL-17-associated molecules in allergic asthma. IL-17-secreting cells and non-L-17-secreting cells were isolated by MACS from blood of allergic asthma patients ($n = 12$). IL-17-secreting cells were treated with Del-1 or PBS. After 48 h, IL-17 **(a)**, STAT3 and RORC **(b)** mRNA was determined by qPCR in IL-17-secreting cells. qPCR results were normalized to GAPDH mRNA expression and presented relative to PBS treatment. **(c)** Non-IL-17-secreting cells were treated with Del-1 or PBS. After 48 h, mRNA levels of TGF-β, IL-6, IL-1β, IL-21, and IL-23 were determined by qPCR. qPCR results were normalized to GAPDH mRNA expression and presented relative to PBS treatment. **(d)** Non-IL-17-secreting cells were treated with Del-1 or PBS. After 48 h, supernatants were collected. Secretion of TGF-β was measured by ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

non-IL-17-secreting cells were separated by MACS. Administration of Del-1 resulted in lower expression of IL-17 in IL-17-secreting cells than did similar treatment

with PBS ($P < 0.001$, Figure 3a). We next investigated Del-1 inhibited the expression of development factors of IL-17-secreting cells. We used qPCR to determine the expression

of STAT3 and RORc in IL-17-secreting cells after administration of Del-1. Samples treated with Del-1 had lower expression of STAT3 and RORc mRNA than did PBS-treated controls ($P < 0.05$, Figure 3b). Moreover, lower expression of TGF- β , IL-6, IL-1 β , IL-21 and IL-23, which are essential for development and maintenance of IL-17-secreting cells, was found by qPCR in non-IL-17-secreting cells after administration of Del-1 ($P < 0.05$, Figure 3c). In addition, TGF- β levels in the culture supernatants of non-IL-17-secreting cells were significantly decreased following Del-1 administration compared with PBS-treated controls ($P < 0.001$).

Del-1 reduces the secretion of IL-17 associated cytokines in allergic asthma

We investigated the expression of IL-17 associated cytokines in BALF cells from allergic asthmatics after treatment with Del-1. Significant changes were observed in the transcript abundance and protein levels of inflammatory molecules associated with IL-17 inflammatory in allergic asthma (Figure 4). IL-6 was downregulated following Del-1 administration ($P < 0.01$), as was expression of G-CSF, IL-4, IL-5, CCL5, CCL11 and IL-1 β ($P < 0.05$). Conversely, IL-10, an inflammatory inhibitor, was increased in samples following Del-1 treatment ($P < 0.05$). However, Del-1 administration had little effect on the expression levels of TNF- α , IL-13, CXCL2 and CXCL5 ($P > 0.05$).

Del-1 is involved in eosinophil inflammatory in allergic asthma

After administration of Del-1, ECP levels in the culture supernatants of BALF cells from allergic asthmatics were

significantly lower compared with PBS-treated samples ($P < 0.001$, Figure 5a). We next assessed whether treatment with Del-1 inhibits eosinophil-associated cytokines in non-IL-17-secreting cells separated from the blood of allergic asthma patients by MACS. We observed significant changes in the transcript abundance of G-CSF, TNF- α , IL-4, CCL5 and CXCL5 in the non-IL-17-secreting cells after administration of Del-1 ($P < 0.05$, Figure 5b).

Del-1 reduces the expression of ICAM-1 in allergic asthma

Del-1 is known to interfere with LFA-1-ICAM-1 interactions to reduce the trafficking of leukocytes into tissues.¹⁵ Figure 6 shows that addition of Del-1 to BALF cells from allergic asthmatics directly suppressed ICAM-1 mRNA expression ($P < 0.005$). Meanwhile, treatment with Del-1 significantly downregulated ICAM-1 induced by IL-17 ($P < 0.01$). Consistent with these findings, combined anti-Del-1 and IL-17 treatment resulted in greater upregulation of ICAM-1 mRNA expression than IL-17 treatment control ($P < 0.001$). These data collectively suggest the inhibitory effect of Del-1 on ICAM-1 expression in allergic asthma in the presence and absence of IL-17.

DISCUSSION

In this study, we determined the role of Del-1 in allergic asthma and its relationship with IL-17 inflammation. Our results show that Del-1 appears to play an inhibitory role in allergic asthma. Specifically, Del-1 negative regulates IL17 expression and that of other cytokines vital to

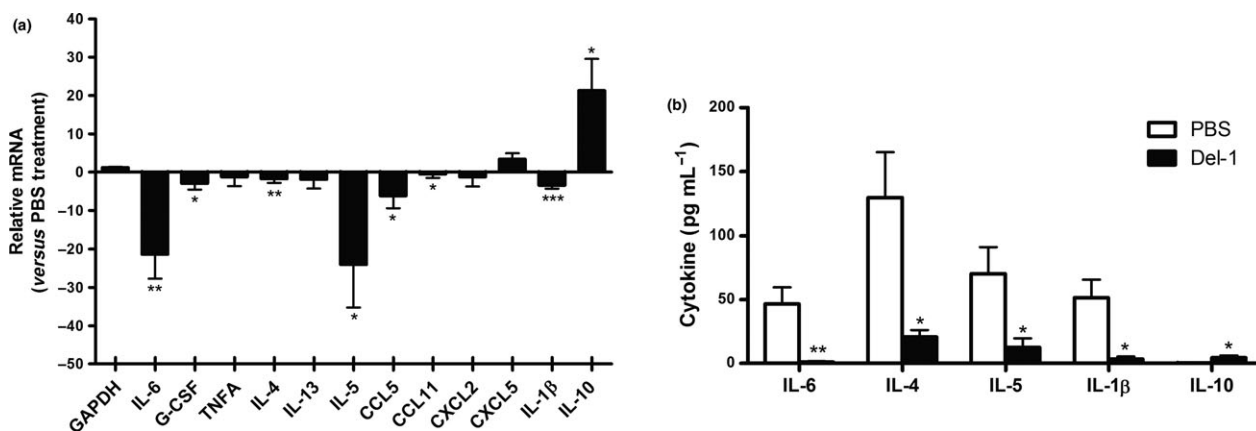


Figure 4. The expression of inflammatory molecules after Del-1 treatment in allergic asthma. The cells of BALF collected from allergic asthma patients ($n = 8$) were treated with Del-1 or PBS. After 48 h, the cells were collected and the levels of inflammatory molecules were determined by qPCR (a) and fluorescence (b). qPCR results were normalized to GAPDH mRNA expression and are presented relative to PBS treatment. * $P < 0.05$, ** $P < 0.01$. BALF, bronchoalveolar lavage fluid.

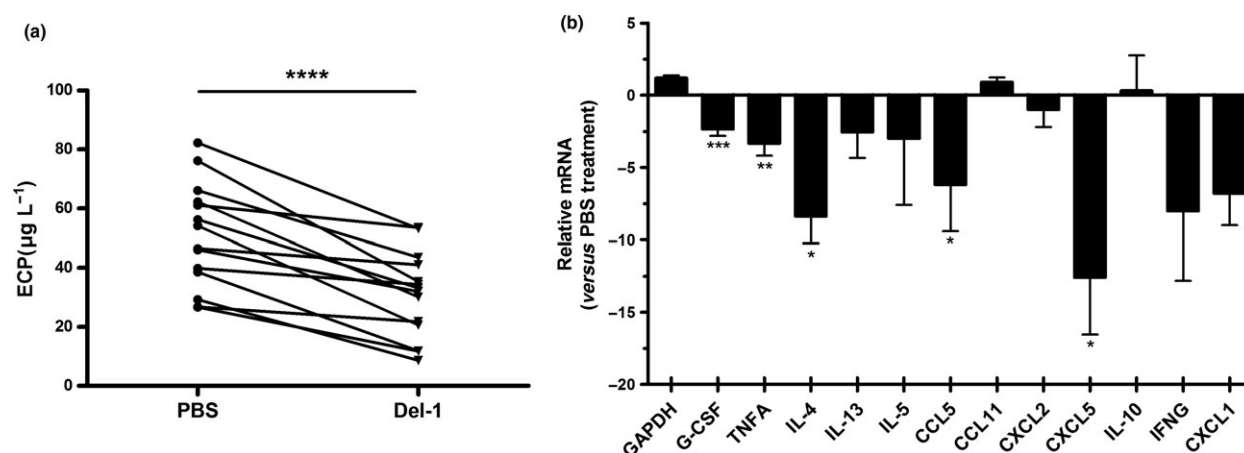


Figure 5. Administration of Del-1 reduces secretion of eosinophil-associated inflammatory molecules. **(a)** The cells of BALF collected from allergic asthma ($n = 14$) patients were treated with Del-1 or PBS. After 48 h, supernatants were collected. Eosinophil cationic protein were determined by fluoroenzymeimmunoassay. Each symbol represents an individual sample; paired data points for each individual are connected by a line. **(b)** Non-IL-17-secreting cells were isolated by MACS from blood of allergic asthma patients ($n = 12$) and treated with Del-1 or PBS. After 48 h, mRNA levels of eosinophil-associated inflammatory molecules were determined by qPCR. qPCR results were normalized to GAPDH mRNA expression and presented relative to PBS treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

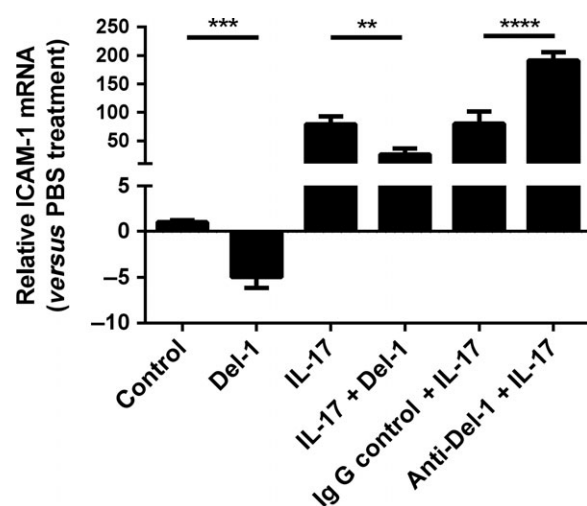


Figure 6. Del-1 treatment reduces ICAM-1 mRNA expression in allergic asthma. The cells of BALF collected from allergic asthma patients were treated with Del-1 ($n = 10$), IL-17 ($n = 10$) or anti-Del-1 ($n = 10$). After 48 h, cells were collected. ICAM-1 mRNA expression was measured by qPCR; results were normalized to GAPDH mRNA expression and are presented relative to PBS treatment. ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$. BALF, bronchoalveolar lavage fluid.

development of eosinophilia and associated inflammation-mediated pathology in allergic asthma.

Del-1 has been identified as a negative regulator of inflammation and is highly expressed in the lung.^{15,22} We found that Del-1 levels were lower in BALF collected from subjects with allergic asthma than controls (Figure 1a). Moreover, soluble Del-1 downregulated levels of ECP, which is used as a diagnostic marker for asthma

severity and corresponds with symptom onset (Figure 5a). Soluble Del-1 also decreased levels of inflammatory cytokines IL-6, G-CSF, IL-4, IL-5, CCL5, CCL11 and IL-1 β and increased expression of the anti-inflammatory cytokine IL-10 in BALF cells from allergic asthmatics (Figure 4), thus indicating that Del-1 plays a role of inhibition in the pathology of allergic asthma.

In recent years, IL-17 has been put forward as a potentially important mediator of allergic asthma. We found the increase in IL-17 to be associated with decreased Del-1 expression in allergic asthma (Figure 1c). Maekawa *et al.* reported that IL-17 inhibits the expression of Del-1 through a GSK3 β -dependent pathway.²³ In addition, Chavakis *et al.* found that Del-1 inhibited IL-17 expression in a model of periodontitis.¹⁸ These data indicated that Del-1 and IL-17 may inhibit each other in inflammation. Then our results show that soluble Del-1 also inhibited IL-17 in allergic asthma (Figures 2a–c and Supplementary figure 1). We next determined whether anti-Del-1 mAb could influence IL-17 expression in allergic asthma. After Del-1 neutralization, IL-17 levels were significantly increased (Figure 2d). These data suggest that Del-1 is critically involved in IL-17 inflammation in allergic asthma.

Moreover, we investigated the inhibitory mechanism of Del-1 by isolating IL-17-secreting cells from the blood of patients with allergic asthma and assessing IL-17 inflammation following Del-1 treatment. We found that Del-1 directly inhibited the expression of IL-17 (Figure 3a). The orphan retinoid receptor, ROR γ , which is the master transcription factor in IL-17-secreting cells, appears to be required for IL-17 production.^{24,25}

Transduction of naive T cells with ROR γ c induces the development of IL-17-secreting cells.^{26,27} In addition, activation of STAT3 is necessary to induce ROR γ c and induce expression of IL-17 by increasing the expression of ROR γ c.^{28,29} Therefore, STAT3 and ROR γ c seem to cooperate, and competent production of IL-17 depends on the presence of both transcription factors. We found that soluble Del-1 downregulated the expression of ROR γ c and STAT3 (Figure 3b). Meanwhile, cytokines such as TGF- β , IL-6, IL-1 β , IL-21 and IL-23 are known to be required for lineage commitment and the production of IL-17. TGF- β and IL-6 polarize naive T cells toward a Th17 phenotype, whereas TGF- β alone induces Treg cells.³⁰ These data suggest that IL-6 plays a pivotal role in dictating whether an immune response is dominated by Treg or Th17 cells. In this study, we found that IL-6 and TGF- β were decreased in non-IL-17-secreting cells by Del-1 treatment compared with PBS treatment (Figures 3c, d). Specifically, IL-6 was downregulated more than 30-fold. These data indicate that Del-1 could inhibit development transcription factors of IL-17-secreting cells by decreasing TGF- β and IL-6 levels. Furthermore, IL-23, IL-21 and IL-1 β are essential for the differentiation, growth and stabilization of IL-17-secreting cells.^{31,32} IL-23 serves to expand and stabilize IL-17-secreting cells, while IL-21 induces development of IL-17-secreting cells, amplifying their frequency.³³ Combinations of IL-1 β plus IL-6 or IL-1 β plus IL-23 were proposed to be the differentiation factors for human IL-17-secreting cells.^{34,35} In this study, we detected a decrease in IL-23, IL-21 and IL-1 β after treatment with Del-1. These results further suggest that Del-1 may inhibit IL-17 function in the development of allergic asthma.

A "classic" hallmark pathological change in asthma is eosinophilic inflammation in the airways.^{36,37} Eosinophilia is usually driven by large amounts of cytokines (including IL-4, IL-5 and IL-13).^{38,39} Among them, a critical cytokine for eosinophilia is IL-5, which contributes to increased eosinophil differentiation, activation and survival.⁴⁰ Together with eosinophil attracting chemokines (CCL5), IL-5 regulates the recruitment of eosinophils in tissues.⁴¹ We have demonstrated that Del-1 inhibited the expression of IL-5 and CCL5 in BALF cells of allergic asthmatics (Figure 4). In addition, various lines of evidence demonstrate the importance of IL-4 in allergic asthma. IL-4 can lead to changes in functions of allergic asthma including eosinophil infiltration, airway hyper-responsiveness and mucus overproduction.^{42,43} Our results showed that expression of IL-4 decreased following Del-1 administration (Figure 4). It has been recognized that CCL11(eotaxin), a small cytokine belonging to the CC chemokine family, selectively recruits eosinophils by inducing their chemotaxis in allergic asthma.⁴⁴ In this

study, CCL11 was downregulated following Del-1 administration (Figure 4). The decrease in these cytokines and chemokines indicate a correlation between Del-1 and eosinophils in allergic asthma. We therefore wished to determine whether Del-1 treatment could directly affect eosinophils in allergic asthma in the absence of IL-17. To do this, we collected blood cells, isolating non-IL-17-secreting cells and treated them with soluble Del-1. Our data suggest that in non-IL-17-secreting cells, Del-1 administration led to decreased expression of IL-4 and CCL5 (Figure 5b). These results indicate that Del-1 plays an important role by inhibiting expression of eosinophil associated cytokines in allergic asthma.

ICAM-1 is an important adhesion molecule which plays a vital role in migration of leukocytes to inflammation sites. Migration is dependent on interactions between LFA-1 and ICAM-1.^{45,46} Del-1, as an antagonist of LFA-1, interferes with LFA-1–ICAM-1 interactions and inhibits the expression of ICAM-1.¹⁵ In addition, IL-17 can improve ICAM-1 expression to enhance inflammation.^{47,48} Therefore, we measured the expression of ICAM-1 by qPCR in BALF cells treated with IL-17 and Del-1, either alone or in combination. Our data suggest that Del-1 downregulated ICAM-1 expression. IL-17 led to upregulation of ICAM-1, but the presence of Del-1 inhibited this upregulation. Combined anti-Del-1 and IL-17 treatment upregulated ICAM-1 mRNA expression compared with IL-17 treatment control (Figure 6). Thus, Del-1 is likely to suppress migration of leukocytes in the lungs through suppression of ICAM-1 expression in allergic asthma.

In summary, we have shown that Del-1 reduced the production of IL-17 inflammation and may inhibit eosinophilia associated inflammation factors. A lack of Del-1 correlated with the pathophysiology of allergic asthma may provide new clues to the development of allergic asthmatic inhibitors as a novel strategy in the treatment of allergic diseases. In addition, since Del-1 is an endogenous anti-inflammatory factor, Del-1 might be a safe and promising approach for the treatment of allergic asthma.

METHODS

Subjects

Forty-six atopic asthmatic subjects (22 boys and 24 girls, mean age 8.2 ± 3.6 years) were enrolled in the study. Asthma diagnosis was performed by a pneumologist based on respiratory symptoms and lung function according to Global Strategy for Asthma Management and Prevention 2014. Atopic status was evaluated by the ImmunoCAP test (Phadia, Uppsala, Sweden), detecting specific IgE antibodies against house dust mite (*Dermatophagoides pteronyssinus*), Japan escedar (*Cryptomeria japonica*), orchard grass (*Dactylis glomerata*),

Table 1. Characteristics of the subjects.

	Healthy controls	Allergic asthmatics
Total, <i>n</i>	42	46
Age, years	8.6 ± 3.4	8.2 ± 3.6
Sex, male/female	23/19	22/24
FEV1, % predicted	97.2 ± 3.3	73.2 ± 7.1*
Specific IgE, KU L ⁻¹	<0.35	72.2 ± 37.5*

Values are presented as means ± s.e.s.

FEV1, % predicted = Predicted percentage of forced expiratory volume in the first second.

**P* < 0.05 versus healthy control.

common ragweed (*Ambrosia elatior*) and cat dander (*Felis domesticus*). Patients showing more than class 4 for at least one of these allergens were enrolled. All patients stopped oral and topical application of corticosteroids and did not take any other medication for at least 4 weeks before collecting samples. Forty-two normal control subjects (23 boys and 19 girls, mean age 8.6 ± 3.4 years) were also recruited. All normal control subjects were children with normal lung function, no history or symptoms of allergy, and were not taking any medications for the preceding 4 weeks. All subject characteristics are given in Table 1. The study was approved by the local ethical committee of the Shanghai Children's Hospital.

Collection of BALF and treatment

Bronchoscopy was performed with a flexible fiberoptic bronchoscope; bronchoalveolar lavage was performed by instilling sterile saline solution in one pulmonary segment of the middle lobe or lingula. The collected BALF was then filtered. After the procedure, BALF was centrifuged at 1500g for 3 min at 4°C. The supernatants were collected and preserved at -20°C for ELISA. The cells were resuspended in PBS. Viability was routinely assessed by trypan blue exclusion and ranged between 88 and 95%. Cells (2 × 10⁶ mL⁻¹) were cultured in 1 mL of complete medium (RPMI plus 10% fetal bovine serum) and treated with 5 µg mL⁻¹ of soluble Del-1 (R&D Systems), 5 ng mL⁻¹ of IL-17 (R&D Systems), 10 µg mL⁻¹ of anti-Del-1 (clone 670421, R&D Systems), 10 µg mL⁻¹ of monoclonal mouse IgG2A (clone 20102, R&D Systems) or PBS in a 24-well plate (Corning Costar, Cambridge, MA, USA) in the presence of 5 µg mL⁻¹ anti-CD3 antibody, 2 µg mL⁻¹ anti-CD28 antibody (eBiosciences, San Diego, CA, USA), 5 ng mL⁻¹ TGF-β1, 20 ng mL⁻¹ IL-6 and 10 ng mL⁻¹ IL-23 (all from R&D Systems). After 48 h activation, cells were harvested for intracellular staining and real-time quantitative PCR (qPCR) (Supplementary figure 2). The culture supernatants were collected and preserved at -20°C for ELISA and fluoroenzymeimmunoassay.

Atopic measurements

Serums were collected by vein puncture with BD Vacutainers (BD, Plymouth, UK). Measurement of specific IgE levels in human serum and ECP levels in culture supernatants was

performed by fluoroenzymeimmunoassay using commercially available Immuno-CAP tests (Phadia). All measurements were carried out with a Phadia 250 assay instrument. IgE values of 17.5 KU L⁻¹ or greater were regarded as more than class 4.

Isolation of IL-17-secreting cells and non-IL-17-secreting cells

Heparinated blood samples were collected from asthmatic patients by vein puncture with BD Vacutainers (BD, Plymouth, UK). Fresh peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation. PBMCs were washed twice in PBS, counted, and immediately used for MACS using the IL-17 Secretion Assay – Cell Enrichment and Detection Kit (PE) (Miltenyi Biotec, Bergisch Gladbach, Germany). The assays were performed as recommended by the manufacturer. IL-17-secreting cells and non-IL-17-secreting cells were isolated. Purity of IL-17-secreting cells is 95.3%, as measured by flow cytometry (Supplementary figure 3). The viability of freshly isolated cells was >99%, as evaluated by trypan blue dye exclusion. Isolated cells (2 × 10⁶ mL⁻¹) were cultured in 1 mL of complete medium with 5 µg mL⁻¹ of soluble Del-1 (R&D Systems) in a 24-well plate in the presence of 5 µg mL⁻¹ anti-CD3 antibody, 2 µg mL⁻¹ anti-CD28 antibody (eBiosciences), 5 ng mL⁻¹ TGF-β1, 20 ng mL⁻¹ IL-6 and 10 ng mL⁻¹ IL-23 (all from R&D Systems). After 48 h activation, cells were harvested for qPCR analysis.

ELISA

The levels of IL-17 Del-1 and TGF-β in BALF and culture supernatants were measured by ELISA (Del-1 ELISA kit, R&D Systems; IL-17 ELISA kit and TGF-β kit, eBioscience) following the manufacturer's instructions. Absorbance was read at 450/570 nm on a Tecan Sunrise microplate absorbance reader (Tecan Group Ltd, Switzerland). All samples were measured in duplicate.

Flow cytometry

Cytokine-producing cells were sorted by intracellular cytokine staining using flow cytometry. Cells were resuspended in RPMI-1640 medium to a concentration of 1 × 10⁶ cells mL⁻¹ and activated with Leukocyte Activation Cocktail (BD Biosciences, San Jose, CA, USA) in the presence of brefeldin A protein transport inhibitor (BD Biosciences) for 5 h at 37°C. Cells were then washed and incubated with APC-labeled anti-human CD4 antibody (clone RPA-T4, BD Biosciences). After washing, cells were fixed with Fixation Solution (BD Biosciences) and incubated for 20 min at room temperature in the dark. After permeabilization, cells were incubated with PE-labeled anti-human IL-17 antibody (clone N49-653, BD Biosciences) for 20 min at room temperature in the dark. PE-conjugated mouse IgG1 (BD Biosciences) was used as isotype control. Cells were resuspended in PBS and analyzed on FACSCalibur (BD Biosciences) using Cell-Quest software (BD

Biosciences). The data acquired were analyzed with FlowJo software (Tree Star, Stanford, CA).

RNA isolation, cDNA synthesis and qPCR

RNA was extracted using an RNAiso Plus Kit (Takara, Tokyo, Japan). A Nanodrop instrument (Thermo Scientific, Vantaa, Finland) was used to determine concentrations of each RNA isolate. cDNA then was generated from each RNA sample using a Reverse Transcription kit (Takara, Tokyo, Japan) according to the protocol provided. qPCR was performed in the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). All reactions were performed using SYBR Premix Ex Taq (Takara, Tokyo, Japan). At the end of each reaction, cycle threshold was manually set at the level that reflected the best kinetic PCR parameters; melting curves were then acquired and analyzed. The house-keeping gene GAPDH was used as an internal control. The relative RNA expression levels were inferred from the threshold cycle (CT) values. Data were expressed as fold change using the $2^{-\Delta\Delta C_t}$ method.⁴⁹ Gene-specific primers are listed in Supplementary table 1.

Fluorescence

The levels of IL-17, IL-6, TNF- α , IL-4, IL-5, IL-1 β and IL-10 in culture supernatants of BALF cells with Del-1 treatment were measured by Fluorescence (High Sensitivity 9-Plex Human ProcartaPlex™ Panel, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The concentration of the samples was calculated by Luminex 200 (Luminex Corporation, Austin, TX, USA).

Statistical analyses

Quantitative data from multiple experiments were pooled prior to analysis and the complete data set is presented. All values were reported as standard error of the mean. Differences between samples and control were evaluated using ANOVA or Student's *t*-tests. Logistic regression was used in a model with IL-17 and Del-1 levels as dependent and independent variables, respectively. $P < 0.05$ was regarded as significant. All analyses were performed using SPSS software (version 15; SPSS, Chicago, IL, USA) and GraphPad Prism software (version 3.0; GraphPad Software, San Diego, CA, USA).

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CONFLICT OF INTEREST

The authors declare that they have no relevant conflict of interests.

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SUPPORTING INFORMATION

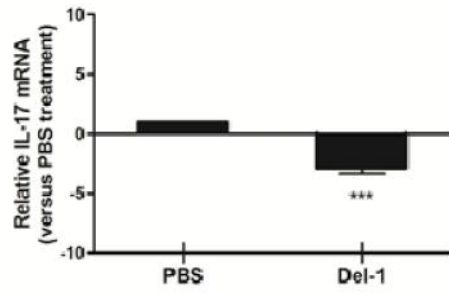
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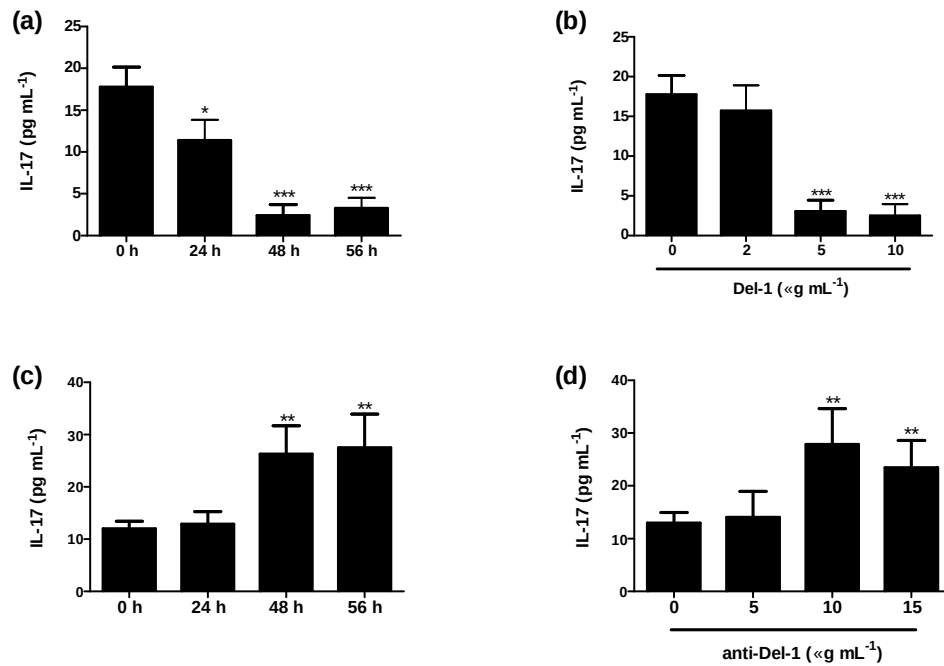
Supplementary table 1. PCR primer sequences

PCR primer	DNA sequences (5' → 3')
GAPDH	forward GAAGGTGAAGGTCGGAGTCA reverse GAAATCCCATCACCATCTTC
Del-1	forward AGGAAGCTGTGATTTGCTGGTT reverse CAGGCGAATTTATGGGAAGAAA
IL-17A	forward CAGATTACTACAACCGATCC reverse CATGTGGTAGTCCACGTTCC
CXCL1	forward AACAGCCACCAGTGAGCTTC reverse GAAAGCTTGCCTCAATCCTG
CXCL-2	forward GCTTCCTCCTTCCTTCTGGT reverse GGGCAGAAAGCTTGTCTCAA
CXCL-5	forward AAACTTTTCCATGCGTGCTC reverse TTGTCTTGATCCAGAAGCCC
ICAM-1	forward AGGGTAAGGTTCTTGCCAC reverse TGATGGGCAGTCAACAGCTA
CCL11	forward CAGGTAAAGCAGCAGGTGG reverse AGGTCTCCGCAGCACTTCT
CCL5	forward TGTAATCCCGAACCCATTTC reverse TACACCAGTGGCAAGTGCTC
IL-13	forward GATTCCAGGGCTGCACAGTA reverse GGTCAACATCACCCAGAACC
IL-5	forward TCTCCAGTGTGCCTATTCCC reverse CGAACTCTGCTGATAGCCAA
IL-4 V2	forward AGCGAGTGTCTTCTCATGG reverse TGAACAGCCTCACAGAGCAG
G-CSF	forward AGAGAGTGTCCGAGCAGCAC reverse CAAGTGAGGAAGATCCAGGG
IL-10	forward CTCATGGCTTTGTAGATGCCT

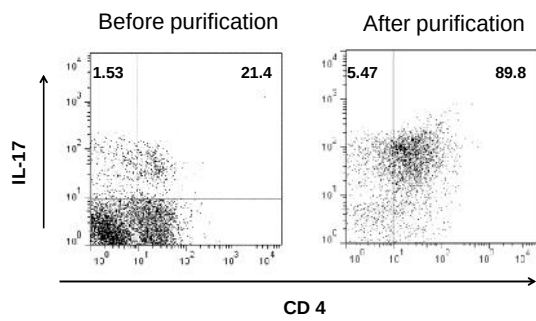
	reverse	GCTGTCATCGATTTCTTCCC
IL-1 β	forward	ACAGTGGCAATGAGGATG
	reverse	TGTAGTGGTGGTCGGAGA
IL-6	forward	GTCAGGGGTGGTTATTGCAT
	reverse	AGTGAGGAACAAGCCAGAGC
IFNG	forward	TCAGCCATCACTTGGATGAG
	reverse	CGAGATGACTTCGAAAAGCTG
TNFA	forward	AGATGATCTGACTGCCTGGG
	forward	CTGCTGCACTTTGGAGTGAT
RORC	forward	GGTGATAACCCCGTAGTGGA
	reverse	CTGCAAAGAAGACCCACACC
TGFB1	forward	CTTCCAGCCGAGGTCCTT
	reverse	CCCTGGACACCAACTATTGC
IL-21	forward	TGCTGACTTTAGTTGGGCCT
	reverse	TGTGAATGACTTGGTCCCTG
IL-23A	forward	CCCACTGGATATGGGGAAC
	reverse	AGAAGCTCTGCACACTGGC
STAT3	forward	CTGCTCCAGGTACCGTGTGT
	reverse	CCTCTGCCGGAGAAACAG



Supplementary figure 1. Effect of Del-1 on IL-17 expression on T cells obtained from the peripheral blood of healthy controls. T cells were isolated by MACS from blood of healthy controls ($n = 4$) were treated with Del-1 or PBS. After 48 h, T cells were collected. qPCR analysis of IL-17 mRNA expression in the cells. Results were normalized to GAPDH mRNA expression and are presented relative to PBS treatment. **** $P < 0.001$.



Supplementary figure 2. Time-course and dose-response studies of IL-17 expression with Del-1 and the anti-Del-1 antibody treatment. The cells of BALF collected from allergic asthma patients ($n = 6$) were treated with Del-1. **(a)** The expression of IL-17 mRNA at 0 h, 24 h 48 h or 56 h post Del-1 administration (5 µg mL⁻¹) was analyzed by RT-PCR. **(b)** IL-17 mRNA expression in BALF cells treated for 48 h with increasing doses of Del-1. The cells of BALF collected from allergic asthma patients were treated with the anti-Del-1. **(c)** The expression of IL-17 mRNA at 0 h, 24 h 48 h or 56 h post the anti-Del-1 administration (10 µg mL⁻¹) was analyzed by RT-PCR. **(d)** IL-17 mRNA expression in BALF cells treated for 48 h with increasing doses of the anti-Del-1. Results were normalized to GAPDH mRNA expression and are presented relative to 0 h or 0 µg mL⁻¹. ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.



Supplementary figure 3. IL-17-secreting cells after purification. IL-17-secreting cells were isolated by MACS from blood of allergic asthma patients and stained with anti-IL-17 and anti-CD4. The percent of IL-17-secreting T cells was analyzed by flow cytometry. The gate was set on lymphocytes.