Intelectin contributes to allergen-induced IL-25, IL-33, and TSLP expression and type 2 response in asthma and atopic dermatitis

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The epithelial and epidermal innate cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) have pivotal roles in the initiation of allergic inflammation in asthma and atopic dermatitis (AD). However, the mechanism by which the expression of these innate cytokines is regulated remains unclear. Intelectin (ITLN) is expressed in airway epithelial cells and promotes allergic airway inflammation. We hypothesized that ITLN is required for allergen-induced IL-25, IL-33, and TSLP expression. In two asthma models, *Itln* knockdown reduced allergen-induced increases in Il-25, Il-33, and Tslp and development of type 2 response, eosinophilic inflammation, mucus overproduction, and airway hyperresponsiveness. *Itln* knockdown also inhibited house dust mite (HDM)-induced early upregulation of Il-25, Il-33, and Tslp in a model solely inducing airway sensitization. Using human airway epithelial cells, we demonstrated that HDM-induced increases in ITLN led to phosphorylation of epidermal growth factor receptor and extracellular-signal regulated kinase, which were required for induction of IL-25, IL-33, and TSLP expression. In two AD models, *Itln* knockdown suppressed expression of Il-33, Tslp, and Th2 cytokines and eosinophilic inflammation. In humans, ITLN1 expression was significantly increased in asthmatic airways and in lesional skin of AD. We conclude that ITLN contributes to allergen-induced Il-25, Il-33, and Tslp expression in asthma and AD.

INTRODUCTION

Asthma is an increasingly common disease, which is characterized by allergic airway inflammation, airway hyperresponsiveness, mucus overproduction, and peribronchial fibrosis.[1] Atopic dermatitis (AD) is a chronic inflammatory skin disease with a worldwide prevalence of 1–20%,[2] and is frequently associated with asthma.[3] The type 2 immune response has pivotal roles in the pathogenesis of both asthma and the acute phase of AD. Type 2 cytokines such as IL-4, IL-5, and IL-13 are expressed in asthmatic airways and in acute eczematous lesions.[4–6]

The epithelial- and epidermal-derived cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) are upregulated during innate immune responses to allergen in asthma and AD and prime type 2 immune responses by acting on innate and adaptive immune cells.[7,8] TSLP activates dendritic cells[9] and also directly promotes naive $CD4^+$ T cells to differentiate into type 2 helper T cells.[10] IL-25, IL-33, and TSLP can stimulate group 2 innate lymphoid cells to release type 2 cytokines.[11–14] IL-25,[15–18] IL-33,[19] and TSLP[10] have each been reported to be both necessary and sufficient for type 2 cytokine production, eosinophilic airway inflammation, mucous metaplasia, and airway hyperresponsiveness in mouse asthma models. Overexpression of TSLP in skin keratinocytes is sufficient to induce a type 2 response and AD-like phenotype in mice.[20] In subjects with asthma, bronchial epithelial expression of TSLP,[21] IL-25,[22,23], and IL-33[24] have been reported to be upregulated. In the skin of subjects with AD, the expression of

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Received 29 April 2016; accepted 18 January 2017; published online 22 February 2017. doi:10.1038/mi.2017.10

IL-33 and TSLP is increased in epidermal keratinocytes.[20,25] However, the mechanism by which the expression of IL-25, IL-33, and TSLP is regulated in bronchial epithelial cell and epidermal keratinocytes remains unknown. Identifying the epithelial- and epidermal-derived mediator that regulates the expression of these cytokines is essential for understanding the molecular mechanism of initiation of asthma and AD.

Lectins are a group of molecules that have important roles in innate immune response to pathogens.[26–28] Human intelectin-1 (ITLN1) is a soluble lectin that recognizes galactofuranose in carbohydrate chains of the bacterial cell wall.[29] ITLN1 has been shown to bind *Mycobacterium bovis* bacillus Calmette–Guérin,[30] *Streptococcus pneumonia*,[31] *Vibrio cholerae* and other enteric bacterial pathogen.[32] ITLN expression is increased rapidly after gastrointestinal nematode parasite infection in mice and sheep.[33–35] These data suggest that ITLN may have a role in innate immune response in pathogen defense. ITLN1 is upregulated in bronchial epithelial brushings and induced sputum of subjects with asthma.[36–38] It has been reported that a single-nucleotide polymorphism in ITLN1 is associated with increased asthma risk.[37] Mouse Itln expression is also increased in ovalbumin (OVA) allergic mice and IL-13-overexpressing mice.[36,39] We have previously reported that Itln contributes to airway eosinophilic inflammation in OVA allergic mice.[40] Because ITLN is implicated in the innate immune response, we hypothesize that ITLN is upregulated at early stage after allergen sensitization of the airway and is required for the expression of IL-25, IL-33, and TSLP.

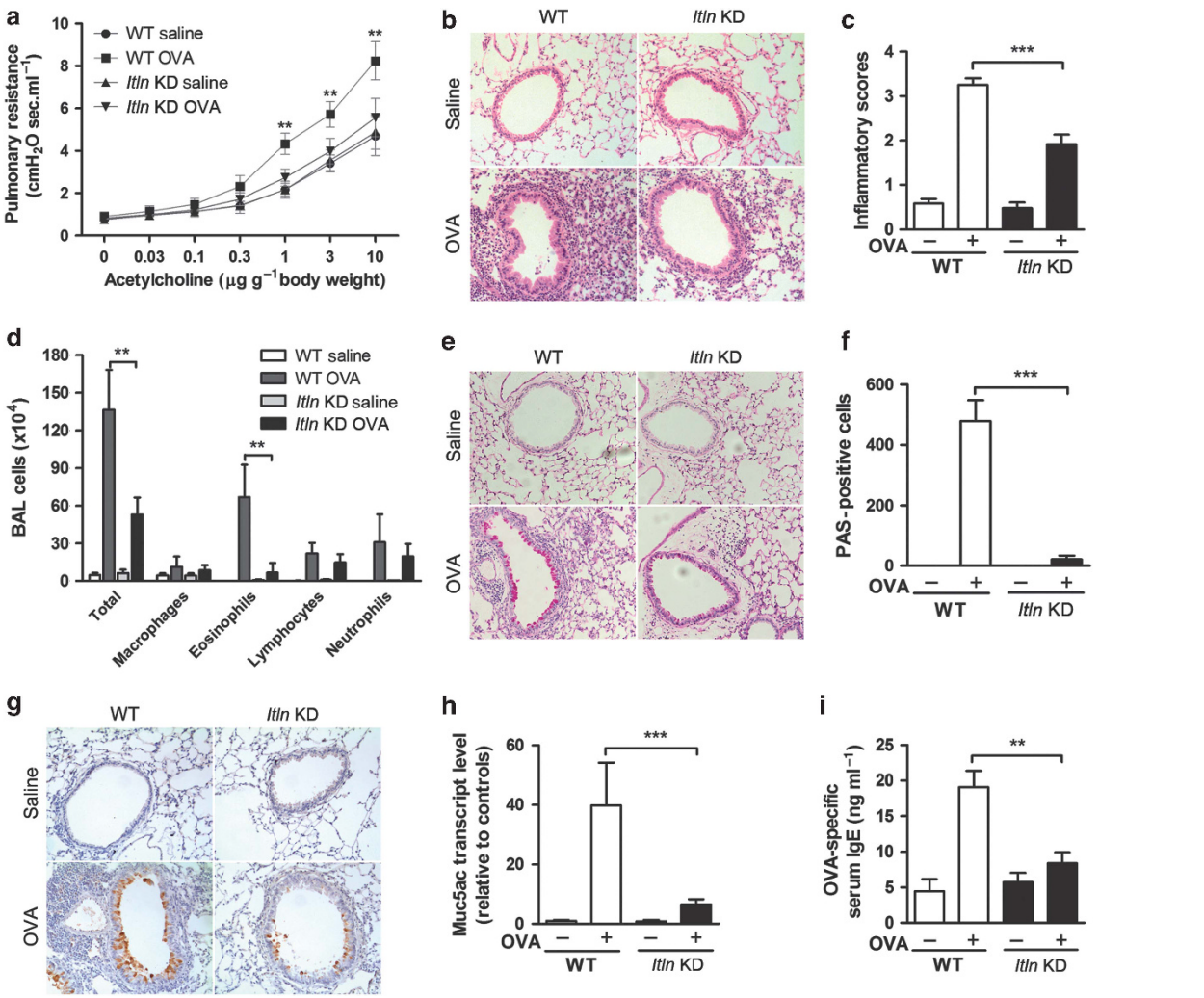

Figure 1 *Itln* KD mice are protected from AHR, airway inflammation, mucus production, and allergic response in the OVA asthma model. (**a**) Pulmonary resistance in response to different concentration of intravenous acetycholine in WT and *Itln* KD mice after sensitization and challenge with OVA or saline. $n = 6$–8 mice per group. (**b**) H&E staining of representative lung sections. Original magnification, $\times 200$. (**c**) Inflammatory scores of lung sections were calculated as described in Methods. (**d**) Counts for macrophages, eosinophils, lymphocytes and neutrophils in BAL fluids. $n = 6$–8 mice per group. (**e**) Periodic acid-Schiff (PAS) staining for mucus in representative lung sections. Original magnification, $\times 200$. (**f**) The numbers of PAS-staining-positive cells were counted in four random fields for each lung section at $\times 200$ magnification. (**g**) Immunohistochemistry for Muc5ac in representative lung sections at $\times 200$ magnification. (**h**) *Muc5ac* transcripts levels were determined by quantitative PCR. (**i**) Serum OVA-specific IgE in peripheral blood was determined by ELISA. $n = 6$–8 mice per group. Data are mean ± s.e.m. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. *Itln* KD mice challenged with OVA. Results are representative of three individual experiments.

To test our hypothesis, we developed a transgenic mouse in which Itln expression was conditionally suppressed by short hairpin RNA (shRNA) against *Itln*. We investigated the role of Itln in the pathogenesis of asthma models induced by OVA or house dust mite (HDM), and in AD models induced by vitamin D3 analog calcipotriol (MC903) or HDM. We further examined the role of Itln in the HDM-induced Il-25, Il-33, and Tslp expression at the sensitization phase of asthma. Moreover, we examined the expression of ITLN1 in subjects with asthma and AD.

RESULTS

Itln knockdown mice are protected from allergen-induced airway hyperresponsiveness, eosinophilic inflammation, and mucus metaplasia in two allergic asthma models

Itln knockdown (KD) mice and control mice on the same background (C57BL/6) were sensitized and challenged by intranasal administration of OVA or saline. We measured the mRNA levels of *Itln1* (the only *Itln* gene in C57BL/6 mice[34]) by quantitative PCR. *Itln1* expression was significantly increased in the lung of OVA-challenged WT mice compared with saline-challenged WT mice. However, the increase of *Itln1* expression was markedly suppressed, reduced by 58.8% in protein level, in the lung tissue in OVA-challenged ITLN KD mice compared with WT mice (**Supplementary Figure S1 online**).

We investigated the role of Itln in AHR, airway inflammation, and mucus overproduction. Airway response to acetylcholine was the same in saline-challenged WT and *Itln* KD mice. However, OVA-challenged *Itln* KD mice were significantly protected from AHR when compared with WT mice (**Figure 1a**). The numbers of total cells and eosinophils in bronchoalveolar lavage fluid and the numbers of inflammatory cells around the conducting airways assessed by Hematoxylin and Eosin staining were significantly lower ($>50\%$ reduction) in OVA-challenged *Itln* KD mice when compared with WT mice (**Figures 1b–d**). The numbers of periodic acid-schiff (PAS)-stain-positive, MUC5AC-stain-positive cells and the levels of *Muc5ac* transcripts were markedly reduced in OVA-challenged *Itln* KD mice compared with WT mice (**Figures 1e–h**). Serum levels of OVA-specific immunoglobulin E (IgE) were significantly decreased in OVA-challenged *Itln* KD mice when compared with WT mice (**Figure 1i**). Our data indicated that Itln has a key role in the development of AHR, airway inflammation, mucus overproduction, and allergic response in a murine asthma model induced by OVA.

We also investigated the role of Itln in the pathogenesis of asthma using the HDM asthma model, as this allergen is more relevant to human asthma. *Itln1* transcript level increased in the lung of HDM-challenged WT mice but was significantly suppressed in HDM-challenged *Itln* KD mice (**Supplementary Figure S2a**). Similar to our findings in OVA asthma model, HDM-challenged *Itln* KD mice were protected from AHR when compared with WT mice (**Supplementary Figure S2b**). The numbers of total cells and eosinophils in bronchoalveolar lavage fluid, inflammatory cells around conducting airways, and mucus-containing cells were significantly less in HDM-challenged *Itln* KD mice when compared with WT mice (**Supplementary Figure S2c–e**). Total serum IgE levels were decreased in HDM-challenged *Itln* KD mice when compared with WT mice (**Supplementary Figure S2f**). These data indicate that Itln has a key role in the development of AHR, airway inflammation, mucus overproduction, and allergic response in the HDM asthma model.

Itln is involved in the development of Th2 immune responses in allergic asthma models

We next investigated whether Itln is required for the development of Th2 immune response in mouse OVA asthma model. We analyzed the expression of the Th2 cytokines Il-4, Il-5, and Il-13 in mouse lung by quantitative PCR and enzyme-linked immunosorbent assay (ELISA). OVA challenge induced the expression of Il-4, Il-5, and Il-13 in lung tissue and bronchoalveolar lavage fluid of WT mice. However, the increase of these cytokines was significantly suppressed in OVA-challenged *Itln* KD mice (**Figures 2a–f**). We also evaluated the immune response by intracellular cytokine staining of the T-cell subsets isolated from lung tissue and mediastinal lymph node. We found that percentages of type 2 helper T cells ($IL\text{-}4^+CD4^+$) were increased in lung tissue and draining lymph node of OVA-challenged WT mice, but significantly decreased in OVA-challenged *Itln* KD mice (**Figures 2g** and **h**). Our data indicate that Itln contributes to the development of Th2 immune response in asthma.

We also determined the expression of Il-4, Il-5, and Il-13 in HDM-sensitized and challenged mouse lung by quantitative PCR. We found that the expression of these cytokines was increased in lung tissue of HDM-challenged WT mice, but significantly suppressed in HDM-challenged *Itln* KD mice (**Supplementary Figure S3a-c**).

Itln contributes to the upregulation of the airway epithelial cytokines IL-25, IL-33, and TSLP after exposure to allergen

Il-25, Il-33, and Tslp are key players in the innate immune response of epithelial cells to allergens and promote Th2 immune responses in asthma.[7] OVA (**Figure 2i–k**) and HDM (**Supplementary Figure S3d–i**) challenge each increased lung Il-25, Il-33, and Tslp protein levels in WT mice, but these increases were significantly suppressed in *Itln* KD mice. Our data indicate that Itln is required, at least in part, for upregulation of Il-25, Il-33, and Tslp in the airway epithelium after allergen sensitization and challenge. These cytokines were still induced in OVA and HDM-challenged *Itln* KD mice compared with control *Itln* KD mice, which could be attributed to the incomplete knockdown of Itln expression.

To test our hypothesis that Itln may participate in airway innate immune responses to allergen by regulating the expression of Il-25, Il-33, and Tslp, we used a model inducing sensitization without obvious airway inflammation to examine the kinetics of Itln1, Il-25, Il-33, and Tslp expression at the allergen sensitization phase.[41] In this model, there were only three consecutive daily intranasal challenges with HDM. We found that HDM induced the expression of Itln1, Il-25, Il-33,

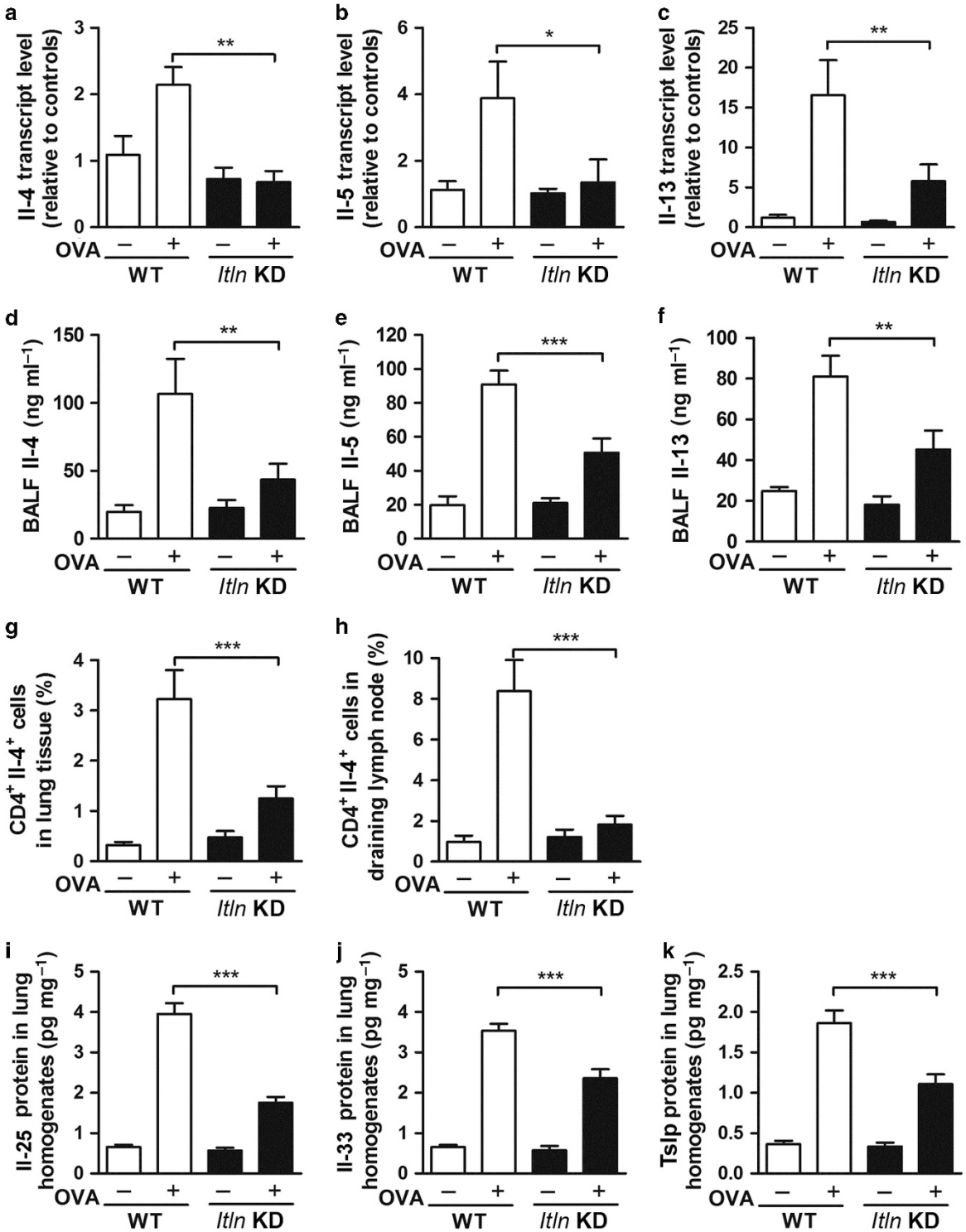

Figure 2 Itln contributes to the expression of Th2 cytokines and Il-25, Il-33, Tslp in the OVA asthma model. (**a**–**c**) The mRNA levels of Il-4, Il-5, and Il-13 in mouse lung were determined by quantitative PCR. (**d**–**f**) The protein levels of Il-4, Il-5, and Il-13 in mouse lungs were determined by ELISA. (**g**, **h**) Percentages of CD4 + Il4 + cells in the lungs and mediastinal lymph nodes were determined by flow cytometry after intracellular staining. (**i**–**k**) The protein levels of Il-25, Il-33, and Tslp in mouse lung homogenates were determined by ELISA. $n = 6$–8 mice per group. Data are mean ± s.e.m. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. *Itln* KD mice challenged with OVA. Results are representative of three individual experiments.

and Tslp at 2 and 6 h after the last HDM exposure in the lung of WT mice (**Figures 3a–d**). However, HDM-induced upregulations of Itln1, Il-25, Il-33, and Tslp were markedly suppressed in ITLN KD mice (**Figures 3e-h**). Our data indicate that Itln is involved in the upregulation of Il-25, Il-33, and Tslp in the sensitization phase of asthma.

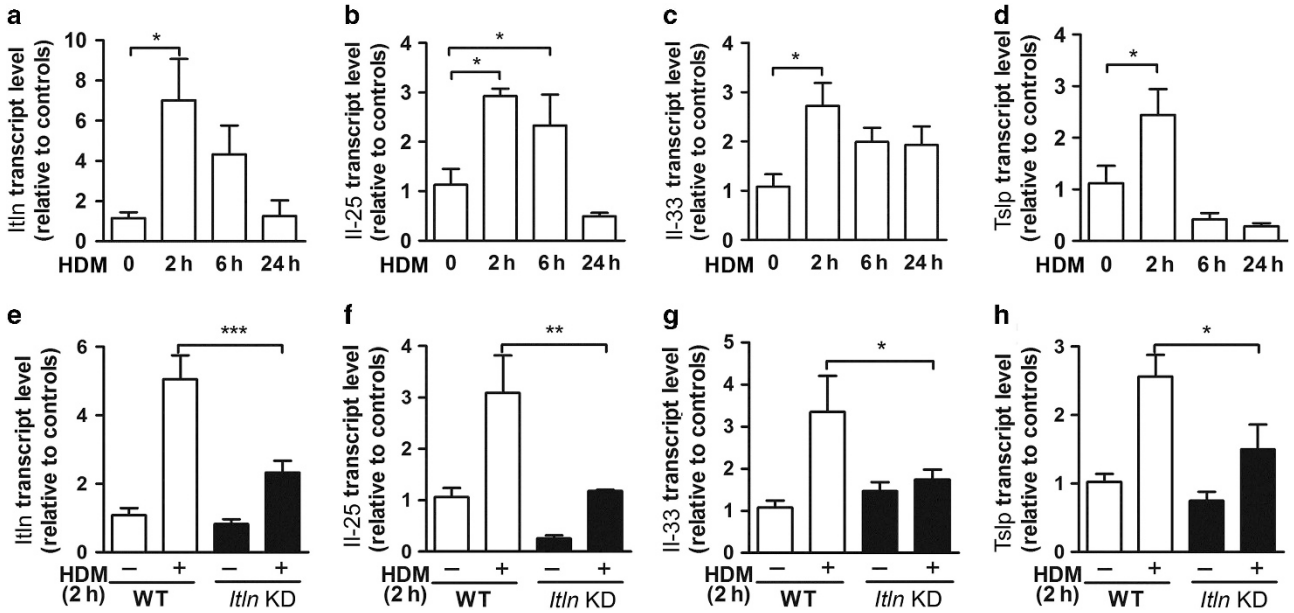

Figure 3 Itln contributes to HDM-induced Il-25, Il-33, and Tslp expression in mouse lungs. (**a**–**d**) The kinetics of Itln1, Il-25, Il-33, and Tslp expression in the lungs of WT mice after airway exposure to HDM were determined by quantitative PCR. $n = 6$ mice per group. Data are mean ± s.e.m. $^{*}P < 0.05$ vs. WT treated with vehicle. (**e**–**h**) The expression of Itln1, Il-25, Il-33, and Tslp in *Itln* KD mice and WT mice after airway exposure to HDM for 2 h were determined by quantitative PCR. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. WT mice exposed to HDM for 2 h. Results are representative of three independent experiments.

We next sought to establish an *in vitro* system to confirm and extend our *in vivo* finding that airway ITLN1 has a key role in IL-25, IL-33, and TSLP expression after HDM exposure. We examined the expression of these cytokines in BEAS-2B human bronchial epithelial cells after exposure to HDM for 0, 2, 6, 24 h. HDM induced ITLN1, IL-25, IL-33, and TSLP expression in BEAS-2B cells at 2 and 6 h. However, HDM-induced increases in these cytokines were suppressed when ITLN1 expression was silenced by RNA interference (**Figures 4a–h** and **Supplementary Figure S4a–c**). Pretreatment of BEAS-2B cells with galactose, which binds to ITLN1 and can be used as an inhibitor of ITLN1,[29] also inhibited HDM-induced IL-25, IL-33, and TSLP mRNA expression (**Supplementary Figure S4d–f**). Our data indicate that ITLN1 is necessary for allergen-induced IL-25, IL-33, and TSLP expression in human airway epithelial cells.

As the activation of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinases (ERK) has been implicated in the expression of IL-33 and TSLP expression in keratinocytes,[42,43] we examined whether the ERK cascade is involved in HDM-induced IL-25, IL-33 and TSLP expression in BEAS-2B cells. We found that the EGFR inhibitors AG1478 and PD153035 and the ERK inhibitor U0126 each blocked HDM-induced IL-25, IL-33, and TSLP expression in BEAS-2B cells (**Supplementary Figure S5a–i**). Furthermore, HDM stimulation increased EGFR and ERK phosphorylation at 2 and 4 h, respectively, and *ITLN1* shRNA transfection inhibited HDM-induced EGFR and ERK phosphorylation (**Figures 4i–k**). These data indicate that the activation of EGFR and ERK mediates HDM-induced IL-25, IL-33, and TSLP expression, and that ITLN1 expression contributes to HDM-induced EGFR and ERK activation in human bronchial epithelial cells.

Itln contributes to IL-33 and TSLP expression and Th2 inflammation in two AD models

AD is another allergic disease characterized by Th2 inflammation. We investigated the role of Itln in a murine model of AD induced by topical application of the vitamin D3 analog calcipotriol (MC903) to the ear.[44,45] *Itln1* transcript levels were increased in the lesional skin exposed to MC903 for 9 days in WT mice, but markedly reduced in *Itln* KD mice (**Figure 5d**). Immunohistochemistry showed many Itln-positive keratinocytes in the skin exposed to MC903 in WT mice (**Figure 5e**). MC903-treated WT mice had red, scaly skin with markedly increased ear thickness and proliferation of keratinocytes, but these pathological changes were significantly attenuated in MC903-treated *Itln* KD mice (**Figures 5a** and **e**). Dermal infiltration of eosinophils was abundant in MC903-treated WT mice but reduced in *Itln* KD mice (**Figure 5b**). Moreover, serum IgE level was increased in MC903-exposed WT mice but significantly reduced in *Itln* KD mice (**Figure 5c**). Our data indicate that Itln contributes to MC903-induced allergic skin inflammation.

Furthermore, we examined the expression of Th2 cytokines in ear tissue by quantitative PCR. MC903 induced the expression of Il-4, Il-5, and Il-13 in ear tissues of WT mice. However, increases of these cytokines were suppressed in MC903-exposed *Itln* KD mice (**Supplementary Figure S6a–c**). Tslp has been reported to be expressed in keratinocytes and have a pivotal role in MC903-induced AD.[45] We found the level of Tslp and Il-33 transcripts but not Il-25 transcript was markedly increased in the lesional ear of MC903-exposed WT mice. However, the upregulation of Tslp and Il-33 were suppressed in *Itln* KD mice (**Supplementary Figure S6d–e**).

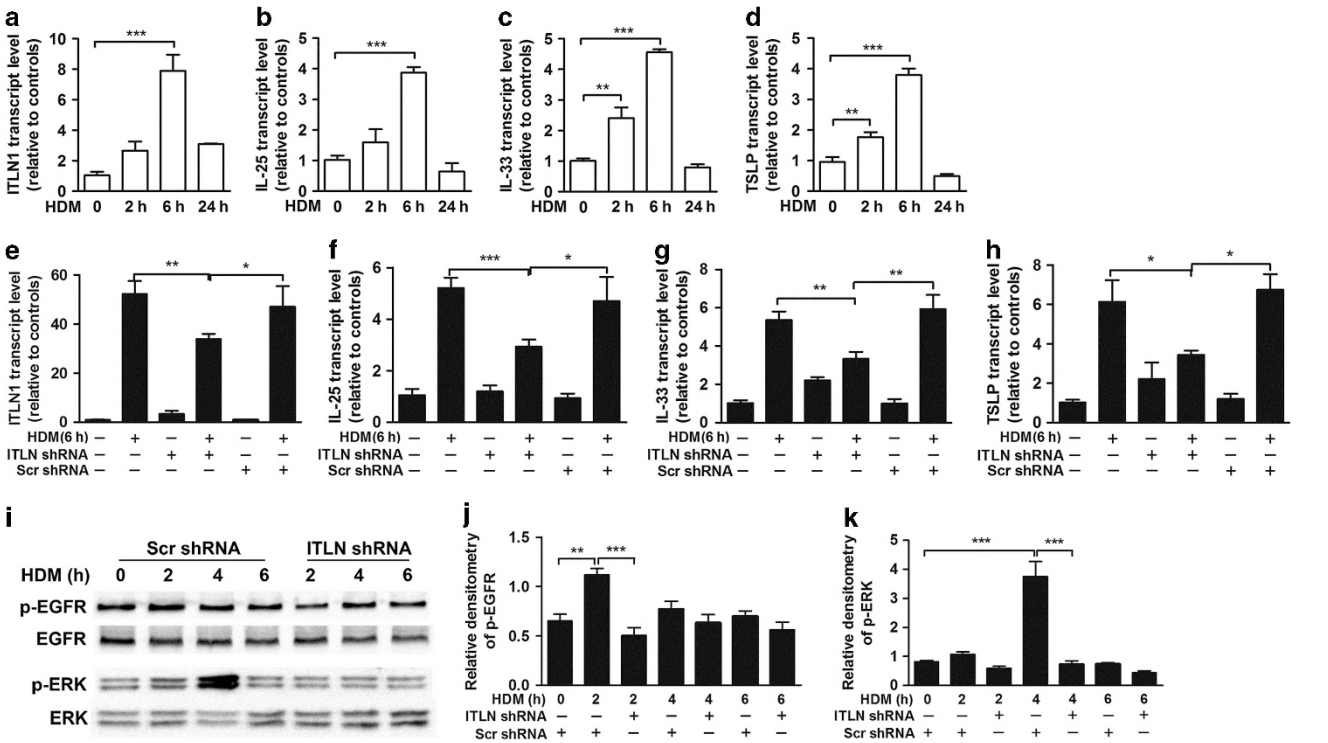

Figure 4 ITLN1 contributes to HDM-induced IL-25, IL-33, and TSLP expression and the activation of EGFR and ERK pathway in human bronchial epithelial cells. (**a–d**) The kinetics of ITLN1, IL-25, IL-33, and TSLP expression in BEAS-2B cells after exposure to HDM were determined by quantitative PCR. $n = 4$ wells per group. Data are mean ± s.e.m. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. BEAS-2B cells treated with PBS. (**e–h**) The expression of ITLN1, IL-25, IL-33, and TSLP in BEAS-2B cells transfected with shRNA against ITLN1 or scrambled shRNA and exposed to HDM for 6 h. $n = 4$ wells per group. Data are mean ± s.e.m. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. BEAS-2B cells treated with *ITLN1* shRNA. (**i**) HDM stimulation increased EGFR and ERK phosphorylation, but *ITLN1* shRNA transfection inhibited HDM-induced EGFR and ERK phosphorylation. Densitometry of phospho-EGFR relative to total EGFR (**j**), and phosphor-ERK relative to total ERK (**k**). Data are mean ± s.e.m from triplicates. $^{***}P < 0.001$. The data are representative of three independent experiments.

Our data indicate that Itln is required, at least in part, for the expression of Tslp, Il-33, and Th2 cytokines in MC903-induced AD. Tslp and Il-33 were still induced in MC903-exposed *Itln* KD mice compared with control *Itln* KD mice, which could be attributed to the incomplete knockdown of Itln expression.

We also used another AD model induced by HDM to investigate the role of Itln in allergic skin inflammation.[46] Itln expression was increased in the ear skin of WT mice exposed to HDM for 5 weeks. However, the upregulation of Itln1 was suppressed in *Itln* KD mice. Ear thickness, eosinophil infiltration, and serum IgE level were increased in HDM-exposed WT mice but reduced in HDM-exposed *Itln* KD mice (**Supplementary Figure S7a–d**). Moreover, Tslp, Il-33, and Th2 cytokine expression was significantly increased in the ear of HDM-exposed WT mice but markedly suppressed in *Itln* KD mice (**Supplementary Figure S7e–j**).

ITLN1 expression is increased in the airway of subjects with asthma and in the lesional skin of AD

We recruited 23 healthy controls and 48 subjects with asthma. Subject characteristics are summarized in **Supplementary Table S1**. Subjects with asthma had lower forced expiratory volume (FEV_1) and methacholine PD_{20} and higher serum IgE levels and blood eosinophil numbers than healthy control subjects.

We examined *ITLN1* transcript levels in bronchial brushings from healthy controls and subjects with asthma by quantitative PCR. *ITLN1* transcript levels were markedly increased in subjects with asthma when compared with healthy controls (**Figure 6a**). ITLN1 immunostaining in bronchial biopsy samples revealed that ITLN1-positive cells were mainly airway mucous cells and submucosal gland cells (**Figure 6b**).

We investigated whether ITLN1 expression is associated with IL-25 expression in epithelial brushings. We previously reported that the transcript levels of IL-25, but not IL-33 or TSLP, were increased in bronchial brushings from subjects with asthma compared to healthy controls.[47] We found epithelial *ITLN1* transcript levels were correlated with *IL-25* levels when all subjects were included ($\rho = 0.378$, $P = 0.001$) (**Figure 6c**). However, this correlation was weak when only subjects with asthma were included ($\rho = 0.13$, $P = 0.379$).

Furthermore, epithelial *ITLN1* transcript levels were significantly correlated with the levels of eosinophils in induced sputum when all subjects were included ($\rho = 0.696$, $P < 0.0001$), and this correlation remained significant when only subjects with asthma were included ($\rho = 0.622$, $P < 0.0001$) (**Figure 6d**). The correlations between epithelial *ITLN1* transcript levels and FEV_1, methacholine PD_{20}, and eosinophils in bronchial biopsies were also significant when all

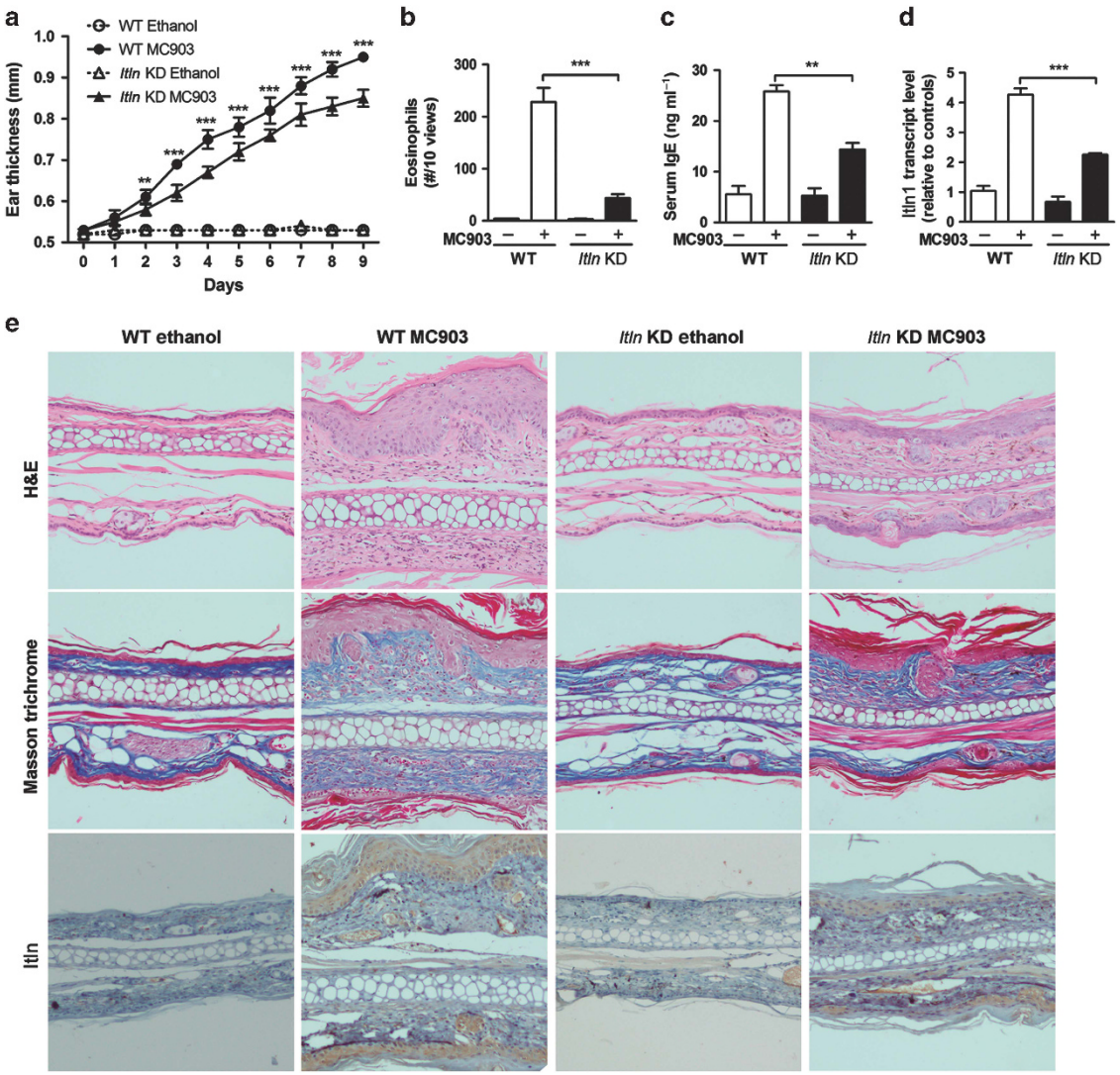

Figure 5 *Itln* KD mice are protected from allergic skin inflammation in MC903-induced AD model. (**a**) Ear thickness was measured daily before the daily application of MC903 or ethanol to the right ear. $n = 5$–8 mice per group. (**b**) The number of eosinophils in the ear was counted in 10 randomly selected views of one representative ear section from each mouse. (**c**) Serum total IgE level in peripheral blood was determined by ELISA. $n = 5$–8 mice per group. (**d**) *Itln* transcript levels in ear tissue were determined by quantitative PCR. $n = 5$–8 mice per group. (**e**) H&E staining, Masson trichrome staining, and Itln immunohistochemistry of representative ear section. Original magnification, × 200. Data are mean ± s.e.m. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. *Itln* KD mice after MC903 exposure. Results are representative of three independent experiments.

subjects were included, but these correlations were weak when only subjects with asthma were included (**Supplementary Figure S8**).

As ITLN1 is secreted protein, we measured ITLN1 protein levels in induced sputum and plasma. We found that plasma and sputum ITLN1 protein levels were significantly increased in subjects with asthma when compared with healthy controls (**Figures 6e** and **g**). Sputum ITLN1 protein levels were significantly correlated with the levels of eosinophils in induced sputum when all subjects were included ($\rho = 0.85$, $P < 0.0001$). Importantly, this correlation remained significant when only subjects with asthma were included ($\rho = 0.73$, $P < 0.0001$) (**Figure 6f**). This indicates that sputum ITLN1 may be a useful non-invasive biomarker for airway eosinophilia. Sputum and plasma ITLN1 protein levels were also significantly correlated with FEV_1, methacholine PD_{20}, and eosinophils in bronchial biopsies, and plasma ITLN1 protein levels were correlated with eosinophils in induced sputum when all subjects were included (**Supplementary Figures S9** and **S10**), but these correlations were weak when only subjects with asthma were included.

We also examined the expression of ITLN1 protein in skin biopsies of subjects with AD and control subjects by immunohistochemistry. ITLN1 was expressed in the keratinocytes of lesional skin of subjects with chronic AD. However, there were few if any ITLN1-positive cells in control subjects (**Figure 7**).

DISCUSSION

In the present study, we demonstrated that Itln, a molecule expressed in airway epithelial cells and skin keratinocytes, has a key role in the epithelial and epidermal expression of Il-25,

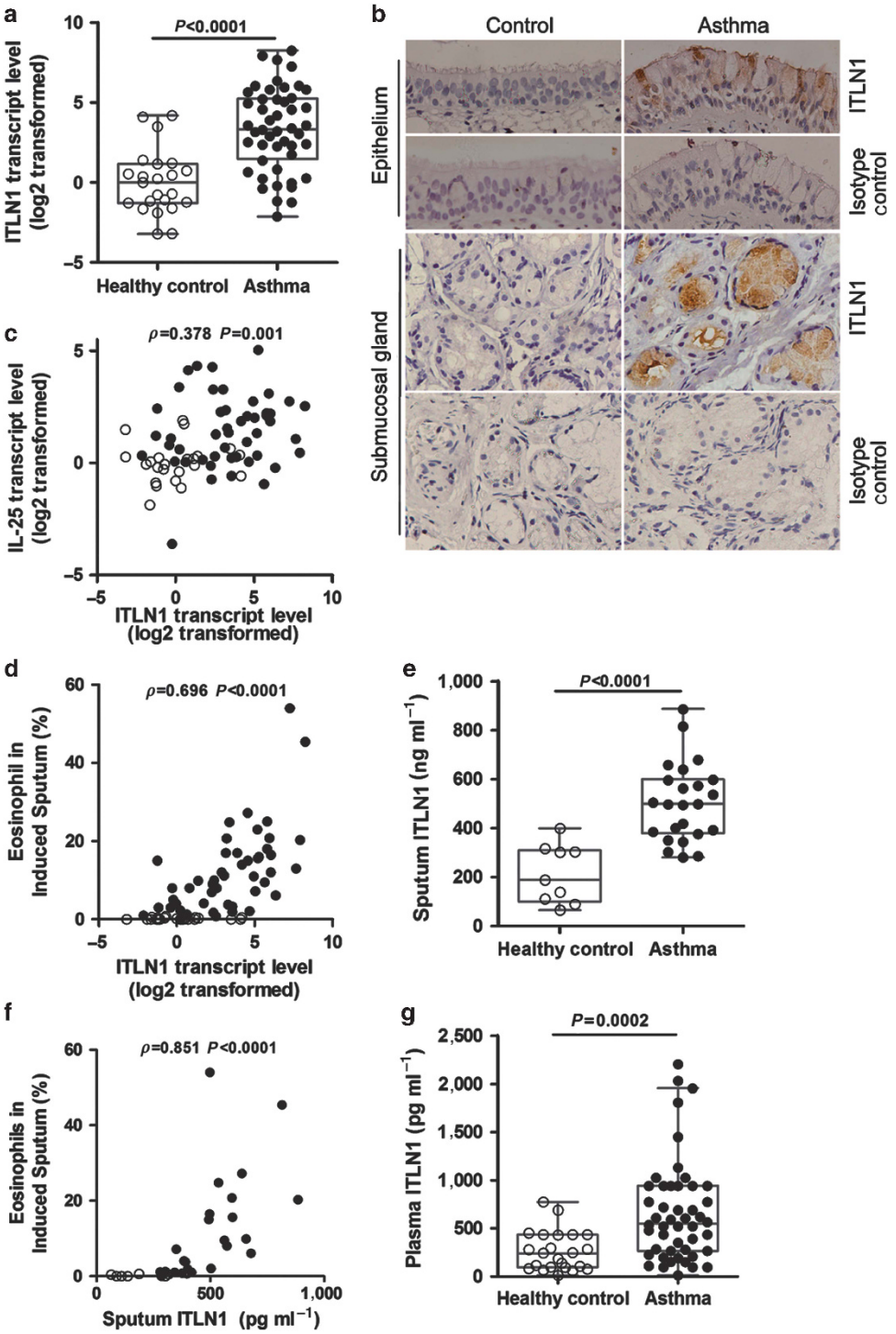

Figure 6 The expression of ITLN1 is increased in the airway and plasma of subjects with asthma, and is correlated with induced sputum eosinophils. (**a**) The transcript levels of *ITLN1* in bronchial brushings of subjects with asthma ($n = 48$) and healthy controls ($n = 32$) were determined by quantitative PCR. Values were relative to median value of healthy controls and were log2 transformed. (**b**) Immunohistochemistry of ITLN1 in epithelium and submucosal gland in representative sections of bronchial biopsies from subjects with asthma and healthy controls. Original magnification, × 400. Correlation between *ITLN1* transcript levels and (**c**) *IL-25* transcript levels in bronchial brushings, and (**d**) the percentage of eosinophils in induced sputum of subjects with asthma ($n = 48$) and healthy control ($n = 23$). (**e**) Sputum ITLN1 protein levels of subjects with asthma ($n = 24$) and healthy controls ($n = 9$) were determined by ELISA. (**f**) Correlation between sputum ITLN1 protein levels and the percentage of eosinophils in induced sputum of subjects with asthma ($n = 24$) and healthy control ($n = 9$). (**g**) Plasma ITLN1 protein levels of subjects with asthma ($n = 48$) and healthy controls ($n = 32$) were determined by ELISA.

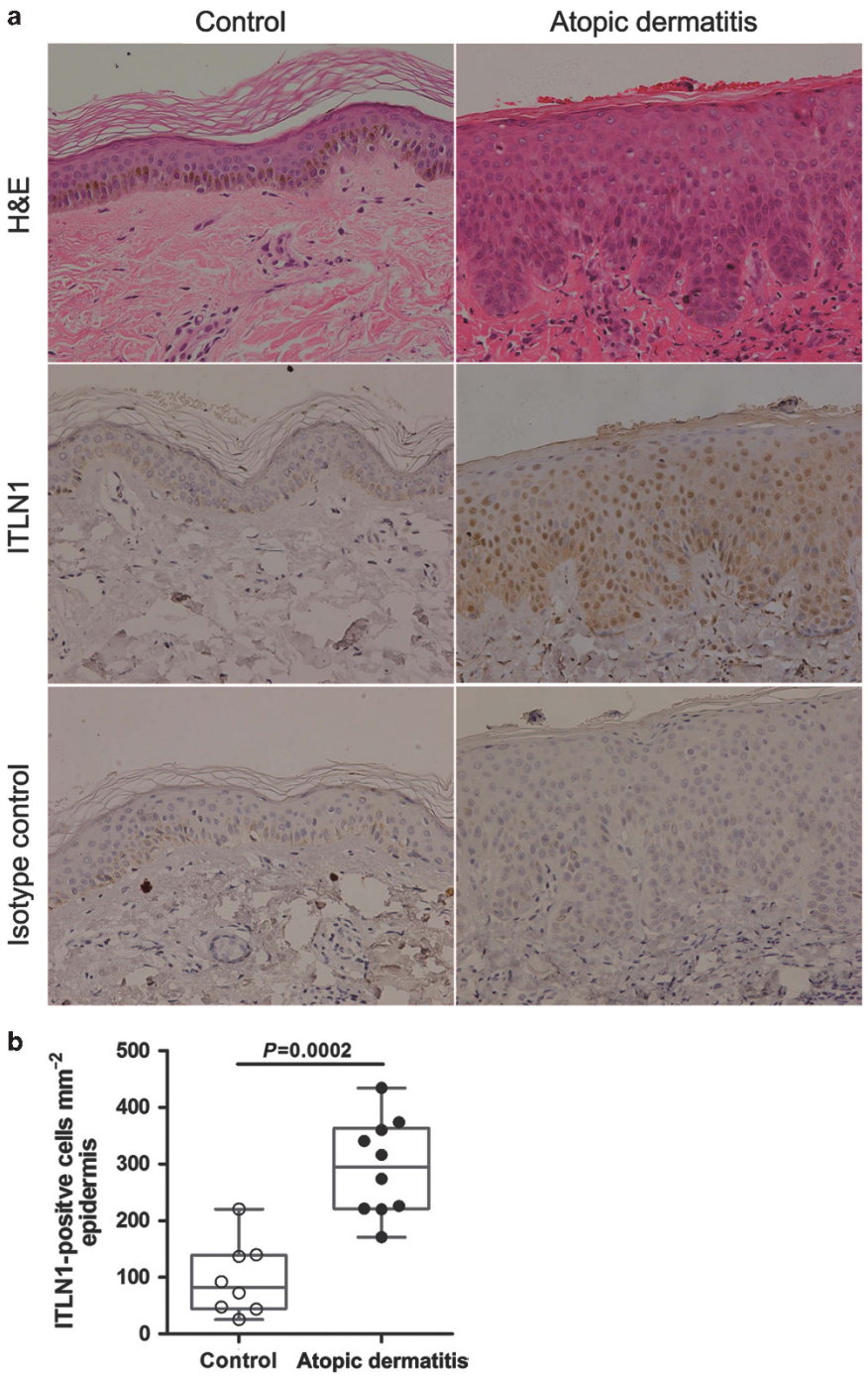

Figure 7 ITLN1 expression is increased in the epidermis of lesional skin of subjects with atopic dermatitis. (**a**) H&E staining and immunohistochemistry of ITLN1 in representative sections of normal skin of control subjects and in the lesional skin of subjects with atopic dermatitis. Original magnification, ×400. (**b**) Quantification of ITLN1 staining positive cells in the skin of control subjects ($n=8$) and subjects with atopic dermatitis ($n=10$) by counting ITLN1 staining positive cells in the epidermis in 6–8 randomly selected views of each section.

Il-33, Tslp, and the type 2 immune response in murine models of allergic asthma and AD, respectively. We found that Itln1 is upregulated rapidly in the airway after allergen exposure and contributes to allergen-induced Il-25, Il-33, and Tslp expression at the sensitization phase of asthma. We also demonstrated that ITLN1 expression is increased in the airway of subjects with asthma and in lesional skin of subjects with AD.

Human ITLN1 is a soluble lectin that recognizes galactofuranose in carbohydrate chains of the bacterial cell wall.[29] ITLN expression is increased rapidly after gastrointestinal nematode parasite infection in mice and sheep.[33–35] Human ITLN1 has been shown to bind *Mycobacterium bovis* bacillus Calmette–Guérin[30] and *S. pneumonia*.[31] In a recent study, Hatzios *et al.*[32] found that ITLN bound to *V. cholerae* and other

enteric bacterial pathogen. ITLN was degraded by *V. cholerae* protease, which may contribute to the infection of *V. cholera*. These reports suggest that ITLN has a role in the innate immune response of pathogen defense.

We have previously reported that airway eosinophilic inflammation and mucus production were inhibited after airway transfection with *Itln* shRNA in an OVA asthma model.[40] In the present study, we generated transgenic *Itln* KD mice and found that AHR, airway eosinophilic inflammation, mucus overproduction, and type 2 immune response were significantly suppressed in *Itln* KD mice not only in the OVA asthma model but also in an HDM asthma model. With *Itln* KD mice, we were able to further investigate the underlying mechanism by which Itln contributes to the pathogenesis of asthma. We found that Itln contributes to the expression of epithelial cytokines Il-25, Il-33, Tslp after OVA or HDM sensitization, and challenge. Because Il-25, Il-33, and Tslp are key players in the innate immune response of epithelial cells exposed to allergens,[41] our findings suggest Itln may have a role in the innate immune response. Pemberton *et al.*[34] reported that Itln expression is increased early in enteric epithelial cells after nematode parasite infection and its expression peaks at time of worm expulsion in resistant mouse strain. Voehringer and colleagues[48] reported that there was substantial increase of Itln expression in Rag-deficient mice after parasite infection, suggesting the upregulation of Itln after parasite infection is independent of adaptive immunity. These findings prompted us to further investigate the role of Itln in the allergen-induced expression of epithelial proinflammatory cytokines.

To further investigate the role of Itln in the expression of IL-25, IL-33, and TSLP at the sensitization phase of asthma, we used a protocol in which mice are exposed to HDM intranasally for 3 consecutive days. This protocol induces sensitization without obvious airway inflammation.[41] We found the expression of Il-25, Il-33, and Tslp were induced at 2–6 h after the last exposure to HDM. Itln1 expression was also induced early (at 2 h) after the last exposure to HDM. However, HDM-induced Il-25, Il-33, and Tslp expression was suppressed in *Itln* KD mice. In *in vitro* experiments, HDM induced the expression of ITLN1, IL-25, IL-33, and TSLP in human bronchial epithelial cells at 2–6 h. However, HDM-induced IL-25, IL-33, and TSLP expression was suppressed when ITLN1 expression was inhibited by RNA interference or when ITLN1 function was blocked by addition of galactose. Our findings provide evidence that ITLN may have a role in the innate immune response to airway allergen exposure by contributing to the expression of IL-25, IL-33, and TSLP. Our study has several limitations. First, we used negative littermates as wild-type controls in mouse experiments. Mice overexpressing a scrambled shRNA would be a better control. Second, Itln knockdown did not completely block the expression of innate cytokines in mouse and *in vitro* experiments. This could be attributed to the incomplete suppression of Itln expression by RNA interference.

As the EGFR and ERK pathway have been previously implicated in regulating IL-33 and TSLP expression in keratinocytes,[42,43] we examined the possibility that ITLN might participate in HDM-induced IL-25, IL-33, and TSLP expression via effects on EGFR and ERK activation. Consistent with this possibility, we found that EGFR and ERK phosphorylation was inhibited by *ITLN1* RNA interference and that EGFR and ERK activity were required for HDM-induced IL-25, IL-33, and TSLP expression in BEAS-2B cells. Therefore, ITLN1 may participate in HDM-induced IL-25, IL-33, and TSLP expression, at least in part, by contributing to the activation of EGFR and ERK activation. The mechanism by which ITLN contributes to EGFR and ERK pathway activation is not yet known.

For the first time, we demonstrated that ITLN is highly expressed in the epidermis of lesional skin of subjects with atoptic dermatitis. Itln expression is also increased in epidermal keratinocytes of MC903 and HDM-induced AD models. The phenotypes of AD including ear swelling, reddening, epidermal hyperplasia, and dermal infiltration of eosinophils were significantly reduced in *Itln* KD mice. Consistent with previous studies, we found that MC903 and HDM induced the expression of Il-4, Il-5, Il-13, Il-33, and Tslp in lesional skin.[25,45] We demonstrated that Itln contributed to the expression of these cytokines in these two different models of AD. These findings provide further evidence that Itln plays a critical role in innate immune response to allergen exposure by regulating the expression of innate cytokines.

Consistent with our findings in mouse experiments, we demonstrated that ITLN1 expression is increased in airway epithelium of subjects with asthma and in lesional skin of subjects with AD. Epithelial *ITLN1* transcript levels were correlated with the levels of eosinophils in induced sputum in subjects with asthma. Sputum and plasma ITLN1 protein levels were also markedly increased in subjects with asthma, and sputum ITLN1 protein levels were significantly correlated with sputum eosinophilia in subjects with asthma. This is consistent with a recent study that reported that ITLN1 protein levels were increased in induced sputum of subjects with asthma during acute exacerbations and that ITLN1 levels were associated with sputum eosinophila.[38]

Taken together with previous reports, our data suggest a dual role for Itln in type 2 immune responses. As we show here, Itln contributes to initiating type 2 responses by regulating expression of Il-25, Il-33, and Tslp. Although knockdown of Itln reduced allergen-induced expression of these cytokines, previous studies showed that transgenic overexpression of Itln in type 2 pneumocytes did not induce type 2 responses,[48] suggesting that the activity of ITLN depends upon other factors that are induced during innate immune responses. In addition to its role in initiation of type 2 responses, Itln also acts as an effector molecule that is induced as a consequence of type 2 immune responses. Itln/ITLN expression is increased rapidly in mouse lung and human bronchial epithelial cells after stimulation with IL-13, a type 2 cytokine,[49] and helminth-induced Itln expression is dependent on the Il-13 and Il-4 signaling molecule Stat6.[48] As Itln contributes to establishment of type 2 responses and is also produced as a consequence of

these responses, Itln may contribute to the amplification of type 2 responses in asthma and AD.

METHODS

Subjects. We recruited 23 healthy control subjects and 48 subjects with asthma; most of these subjects (21 controls and 43 with asthma) were also included in a previous study.[47] All subjects were Chinese and were recruited from Tongji Hospital. Subjects with asthma were diagnosed by a physician; had symptoms of episodic cough, wheeze and/or breathlessness; and had accumulated dosage of methacholine provoking a 20% fall of forced expiratory volume in the first second (FEV_1 PD_{20}) <2.5 mg and/or $\geq 12\%$ increase in FEV_1 following inhalation of 200 μg salbutamol. Healthy control subjects had no respiratory symptoms, normal spirometric values, and methacholine PD_{20} >2.5 mg. None of the subjects had ever smoked or received inhaled or oral corticosteroid or leukotriene antagonist. For each subject, we recorded demographic information, collected blood and induced sputum samples, and performed spirometry and allergen skin prick testing with a panel of 14 aeroallergens. We performed bronchoscopy with brushing and endobronchial biopsies. We brushed 10 sites within the subsegmental bronchi of right middle and lower lobes (10 gentle upward and downward strokes per site). We used forceps to biopsy left lower lobe carinae and fixed samples in polyoxymethylene.

We collected skin biopsies from the lesional skin of 10 subjects with chronic AD and from the nonlesional skin of 8 control subjects who underwent hemangioma or adipoma surgery. AD patients were diagnosed according to the criteria described previously.[50] Patients had not received topical medication for at least 1 week or systemic medication for at least 4 weeks before skin biopsies.

Generation of inducible transgenic shRNA (*Itln* knockdown) mice. We used C57BL/6 mice (Vital River, Beijing, China) to generate inducible transgenic shRNA mice because it has been reported that there is a natural deletion of *Itln2* gene in the genomic DNA of C57BL/6 mice.[34] The shRNA that was proven to suppress the expression of *Itln1* in our previous study[40] was cloned into the pINV-7 vector (TaconicArtemis GmbH, Cologne, Germany) to generate the inducible *Itln* shRNA transgenic construct (**Supplementary Figure 1A**). The sequence of *Itln1* shRNA (sense strand) was: 5′-AA GGAAAGTGTTGGACTGACATTCAAGACGTGTCAGTCCAACA CTTTCCTT-3′. pINV-7 vector is a bimodal DNA construct harboring an shRNA cassette under the control of a H1 promoter with a tetracycline operator site and a cassette driving the expression of a codon-optimized tetracycline repressor (TetR) from the CAGGS promoter.[51] The TetR binds to tetracycline operator and prohibits the expression of shRNA in absence of tetracycline or doxycycline, whereas the shRNA is expressed in presence of tetracycline or doxycycline. The *Itln* shRNA transgenic construct was used for pronuclear microinjection to generate five transgenic mouse lines. We checked for the functionality of the tetracycline-inducible shRNA expression system by treating mice with 2 mg ml^{-1} doxycycline in the drinking water containing 50 mg ml^{-1} sucrose. We chose one transgenic line in which Itln1 expression was mostly suppressed for all experiments. Eight- to 12-week-old mice were used in all experiments, and negative littermates were used as wild-type controls. Both *Itln* KD mice and control mice were given doxycycline starting from 5 days before allergen sensitization.

Murine models of asthma. (1) OVA-alum model. As described previously,[52] 8– 12-week-old female C57BL/6 mice (Vital River, Beijing, China) were sensitized by intraperitoneal administration of OVA (100 μg in a total volume of 200 μl; Sigma-Aldrich, St Louis, MO, USA, Grade V) mixed with 0.5 mg aluminum hydroxide three times at weekly intervals (day 0, 7, 14). Control mice received adjuvant alone. Beginning 1 week after the last injection, mice were challenged five times by intranasal administration of OVA (1,000 μg in 50 μl saline) at daily intervals (day 21, 22, 23, 24, 25). Control mice were challenged with saline alone. (2) HDM model. Mice were anesthetized with ether before intranasal aspiration of 40 μl of the solution of lyophilized HDM extract (2.5 mg ml^{-1}; Greer Laboratories, San Diego, CA, USA) or saline on days 0, 1, 21, 22, and 23.[53] Twenty-four hours after the last challenge of OVA or HDM, mice were anesthetized with tribromoethanol (400 mg kg^{-1} of body weight). We measured pulmonary resistance in response to a range of concentrations of intravenous acetylcholine using the forced oscillation technique with the FlexiVent system (SCIREQ, Montreal, QC, Canada) as previously described.[54]

Murine model of allergen airway sensitization. Mice were anesthetized with ether before intranasal aspiration of 40 μl of the solution of lyophilized HDM extract (2.5 mg ml^{-1}; Greer Laboratories) or saline for 3 consecutive days. Mice were killed on 2, 6, and 24 h after the last exposure.

Murine models of AD. (1) MC903 model. We resuspended calcipotriol (MC903; Sigma-Aldrich) in ethanol at 50 μM and applied 1 nM (20 μl) daily to the outer and inner surfaces of the right ear for 9 days as described previously.[45] We used ethanol as a vehicle control (20 μl per ear). Ear thickness was measured daily using vernier calipers. (2) HDM model. As described previously,[55] we applied 25 μl of the solution of lyophilized HDM extract (10 mg ml^{-1}; Greer Laboratories) to both surfaces of the right ear weekly for 5 weeks after the ear was stripped with tape. Ear thickness was measured before each MC903 or HDM application. Ear samples and serum were collected 24 h after the last application of MC903 or HDM. Ear samples were fixed with formalin, embedded in paraffin, and stained with hematoxylin and eosin. Two investigators counted eosinophils blindly in 10 fields randomly defined in one representative ear section of each mouse.

Cell culture and treatment. BEAS-2B cells (CRL-9609, American Type Culture Collection) were cultured in Dulbecco's Modified Eagle's medium/high glucose (HyClone, Logan, UT, USA) in six-well plates. When cells were 90% confluent, 4 μg hITLN1 shRNA or scrambled shRNA plasmid in 250 μl of Opti-MEMI medium was mixed with 10 μl lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) diluted in 240 μl of medium and added to each well. Six hours later, the media were substituted with Dulbecco's Modified Eagle's medium with or without HDM (25 μg ml^{-1}; Greer Laboratories). At the indicated time points, cells were harvested for quantitative RT-PCR and western blotting, and the media were collected for ELISA. The sequence of *ITLN1* shRNA (sense strand) was: 5′-GCATCTTATTACTCACCC TATCTCGAGATAGGGTGAGTAATAAGATGC-3′. The sequence of scrambled shRNA (sense strand) was: 5′-GCCCATTCTTATCAT ACTCATCTCGAGATGAGTATGATAAGAATGGGC-3′.

For inhibition of ITLN with galactose, galactose (Sigma-Aldrich) was added to the medium (final concentration 30 mM) 1 h before HDM stimulation. EGFR inhibitor PD153035 (Selleck, Houston, TX, USA; final concentration 5 μM) and AG1478 (Selleck, final concentration 10 μM), and MEK inhibitor U0126 (Selleck, final concentration 10 μM) were added to the medium 1 h before HDM stimulation.

Quantitative RT-PCR. We isolated total RNA from bronchial epithelial brushings, mouse lungs and BEAS-2B cells using TRIzol (Invitrogen) and synthesized first-strand cDNA using the PrimeScript RT reagent kit (Takara, Japan). The transcript levels of human *ITLN1, IL-25, IL-33, TSLP* in BEAS-2B cells and bronchial brushings, and mouse *Itln1, Muc5ac, Il-4, Il-5, Il-13, Il-17a, Il-25, Il-33, Tslp* in mouse lungs were determined by using the Takara Perfect Real Time PCR kit, Takara SYBR Premix ExTaq polymerase, and an ABI Prism 7500 PCR System. The primers used are listed in **Supplementary Table 2**. The cycle threshold (Ct) of each gene transcript was normalized to the mean Ct of *β-actin* and *GAPDH*. Fold differences were determined by the $2^{-\Delta\Delta Ct}$ method.[56]

Western blotting. Mouse lungs and BEAS-2B cells were harvested at the indicated time points, washed twice with cold PBS, and lysed in

RIPA lysis buffer (Goodbio technology, Beijing, China) containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After 30 min on ice, lysates were centrifuged at 15,000 × g for 5 min to remove insoluble material. 50 μg protein samples were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred and immuno-probed with rabbit-anti-mouse Itln antibody (1:1,000; Proteintech Group, Rosemont, IL, USA). Antibody was detected using horseradish peroxidase-conjugated anti-rabbit IgG (1:3,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) followed by ECL Western blot detection reagent (Beyotime Biotech, Shanghai, China). Blots were stripped and then re-probed for β-actin (1:3,000; Abcam, Cambridge, UK). Densitometry was performed using ImageJ (National Institutes of Health, USA) and the protein level of ITLN was indexed to beta-actin. For phosphorylated EGFR, total EGFR, phosphorylated ERK and total ERK western blotting, rabbit monoclonal phospho-EGFR (Tyr1068) (1:1,000), EGFR (1:1,000), phospho-p44/42 ERK1/2 (Thr202/Tyr204) (1:2,000) and ERK1/2 antibody (1:1,000) from Cell Signaling Technology (Danvers, MA, USA) were used.

Histology, immunohistochemistry, PAS staining. We stained 2 μm sections with hematoxylin and eosin or rabbit-anti-human ITLN (94–145) antibody (1:400; Phoenix Pharmaceuticals, Burlingame, CA, USA) or an isotype matched control rabbit IgG (1:2,000; Abmart, Shanghai, China) for endobronchial and skin biopsies. For endobronchial biopsies, observers who were blinded to the clinical status of the subjects counted numbers of eosinophils per mm^2 submucosa, and calculated basement membrane thickness. We used the mean of 50 measurements taken over a distance of at least 1 mm to calculate basement membrane thickness as previously described.[57]

Mouse lungs were fixed for 5 min by instillation of 4% paraformaldehyde-PBS (Sigma) through a tracheal catheter at a transpulmonary pressure of 15 cmH_2O and lungs were fixed in 4% paraformaldehyde-PBS overnight. Five-μm-thick sections were used for immunohistochemistry with rabbit-anti-human ITLN (94–145) antibody (1:400; Phoenix Pharmaceuticals) or an isotype matched control rabbit IgG (1:2,000; Abmart). Antibodies were detected using the DAB kit (Zhongshan Goldenbridge Biotechnology, Beijing, China) as directed by the manufacturer.

Lung sections were also stained with PAS (Goodbio technology, Beijing, China) staining for detection of mucus. The number of PAS-staining-positive cells was counted in four random fields for each lung section at × 200 magnification.[58]

Assessment of airway inflammation. Cell counts for macrophages, eosinophils, lymphocytes, and neutrophils in bronchoalveolar lavage fluid were performed as previously described.[36] Paraffin-embedded 5-μm lung sections were stained with hematoxylin and eosin. The severity of peribronchial inflammation was scored by a blinded observer using the following features: 0, normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of > 4 cells deep.[59]

Assessment of serum IgE levels. Blood was collected by cardiac puncture from antigen or vehicle-treated mice after airway responsiveness measurements in mouse models of allergic airway inflammation or 24 h after the last challenge in mouse models of allergic skin inflammation. Serum IgE levels were measured by ELISA with OVA-specific IgE (Cayman Chemical Company, Ann Arbor, MI, USA) and total IgE (RayBiotech, Norcross, GA, USA).

ELISA. Mouse Il-4, Il-5, Il-13 in bronchoalveolar lavage fluid and Il-25, Il-33, Tslp in mouse lung homogenate were determined using commercially available ELISA sets (R&D Systems, USA). ELISA was performed according to the manufacturer's instructions. All samples and standards were measured in duplicate.

Flow cytometry. Mouse lung and mediastinal lymph nodes were isolated, minced, dispersed through nylon filters and washed. Cells were incubated for 4 h at 37 °C in Rosewell Park Memorial Institute medium containing phorbol myristate acetate, ionomycin, and brefeldin A (Sigma-Aldrich). Cells were then resuspended in phosphate-buffered saline/2% fetal calf serum and stained with Cd4, Il-4, and Il-17A antibodies (Biolegend, San Diego, CA, USA). Samples were analyzed on a FACSort flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Statistics. We analyzed data using Prism version 5 (GraphPad Software, La Jolla, CA, USA) and SPSS version 19 (SPSS, New York City, NY, USA). For normally distributed data we calculated means ± s.d. and used parametric tests (one-way analysis of variance with Tukey correction or unpaired *t*-test) to compare across groups. For non-normally distributed data we calculated medians (with interquartile ranges) and used non-parametric tests (Kruskal–Wallis test with Dunn intergroup comparison or Mann–Whitney test). We analyzed correlation using Spearman's rank order correlation.

Study approval. All experiments were approved by the ethics committee of Tongji Hospital, Huazhong University of Science and Technology.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (grants 81670019 and 81170022), Ministry of Science and Technology of China (grant 2016YFC1304400), and NIH grant U19 AI 077439. We thank the patients who volunteered for this study; Xiaoling Rao and Zhengyun Wang for bronchoscopy support; Wang Ni, Shixin Chen and Kun Zhang for spirometry measurement; Xiaozhu Huang and Xin Ren from University of California, San Francisco for suggestions in measurement of mouse pulmonary resistance.

DISCLOSURE

The authors declared no conflict of interest.

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