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Airway epithelial integrin β4 suppresses allergic inflammation by decreasing CCL17 production

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Abstract

Airway epithelial cells (AECs) play a key role in asthma susceptibility and severity. Integrin β4 (ITGB4) is a structural adhesion molecule that is downregulated in the airway epithelium of asthma patients. Specific ITGB4 deficiency in AECs induces exaggerated Th2 responses, severe allergen-induced airway inflammation and airway hyperresponsiveness (AHR) in mouse model of allergic asthma. However, the underlying mechanisms remain unexplored. In this study, we determine the role of ITGB4 of AECs in the regulation of Th2 response and in the induction of asthma and identify the underpinning molecular mechanisms. We found that ITGB4 deficiency led to exaggerated Th2 cells infiltration, inflammation and AHR and higher production of CCL17 in HDM treated mice. ITGB4-regulated CCL17 production in AECs was regulated by EGFR, ERK and NF-κB pathways. EFGR-antagonist treatment or the neutralization of CCL17 by antibody inhibited exaggerated pathological marks in HDM-challenged ITGB4-deficient mice. Together, these results demonstrated that ITGB4 of AECs negatively regulates the development of Th2 responses of allergic asthma by down-regulation of EGFR and CCL17 pathway.

Keywords: ***ITGB4, airway epithelial cells, EGFR, CCL17, asthma, Th2 cells***

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Introduction

Airway epithelial cells (AECs) form a barrier to environment hazardous stimuli by tight intercellular junctions and adhesion on basal membrane (Roche et al, 1993). Integrins play important roles in the adhesion, tissue repair and homeostasis of these cells (Pan et al, 2017). Among these molecules, integrin β4 (ITGB4) has been shown to regulate the adhesion of AECs on basal membrane through hemidesmosomal structure that is a specialized adhesion micro-structure attached to the extracellular matrix (Dowling et al, 1996). Previously, ITGB4 is implicated in the pathogenesis of allergic asthma, and its' level is significantly decreased in AECs of asthma patients (Liu et al, 2010a; Xiang et al, 2014).

Allergic asthma is a chronic airway disorder that is characterized with epithelial desquamation, airway inflammation and airway hyperresponsiveness (AHR) to non-specific spasmogen (Matucci et al, 2018). Although $CD4^+$ Th2 cells (Th2 cells) are widely recognized to orchestrate the development of the disease by expressing Th2 cytokines (e.g. interleukin (IL)-4, IL-5 and IL-13), there is compelling evidence that airway epithelium plays a vital role in the induction of aberrant immune responses underlying the pathogenesis of allergic asthma due to its' frontline location to directly contact aerosol allergen and other environmental insultants (Lambrecht & Hammad, 2015). Indeed, AECs from asthma patients are often damaged and loss their homeostasis status including detachment, fragility and abnormal repair ability etc. (KleinJan, 2015; Wang et al, 2008). Upon allergen exposure, the repair process of epithelial layer of asthma patients is disrupted and is unable to restore the integrity of

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63 airway epithelial barrier (Georas & Rezaee, 2014; Heijink et al, 2014). As a result,
64 lesion of airway epithelial barrier promotes the exposure of inhaled allergens to
65 submucosal region whereby antigen presentation process is greatly accelerated.
66 Furthermore, clinical studies have shown that high levels of epidermal growth factor
67 receptor (EGFR) and transcription growth factor β (TGF-β) are produced by AECs of
68 asthma patients (Boxall et al, 2006; Puddicombe et al, 2000), indicating the stressed
69 repair process of airway epithelium. Mouse studies have also added to our
70 understanding of how AECs can contribute to the pathogenesis of the disease. One
71 example is that increased permeability of the airway epithelium to HDM exposure is
72 associated with the heightened activity of nuclear factor of kappa B (NF-κB) and pro-
73 inflammatory responses (Stacey et al, 1997). Indeed, a number of studies have shown
74 that AECs direct subsequent immune responses through releasing various
75 proinflammatory mediators to recruit and activate immune cells (Hallstrand et al,
76 2014; Holgate et al, 2009).

77 Infiltration of T lymphocytes and other immune cells is exquisitely modulated
78 by pro-inflammatory mediators including chemokines (Castan et al, 2017). Distinctive
79 patterns of chemokine receptors are differentially expressed on the surface of T helper
80 cell subsets, among which several chemokine receptors drive the selective recruitment
81 of Th2 cells into the asthmatic airways (Kim et al, 2001; Lukacs, 2001). For example,
82 C-C chemokine receptor (CCR) 4 is preferentially expressed on Th2 cells, whose
83 activation critically regulates the infiltration of these cells into the asthmatic airway
84 upon allergen inhalation (Panina-Bordignon et al, 2001). Chemokine ligand (CCL) 3,

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5, 17 and 22 are the main chemokines that selectively bind to CCR4 (Holgate, 2012). Indeed, high levels of CCL17 and CCL22 have been found not only in the lung and bronchoalveolar lavage fluid (BALF) of asthma patients but also in those of ovalbumin (OVA) sensitized and challenged mice (Gonzalo et al, 1999; Hijnen et al, 2004). CCL17 and CCL22 are predominantly produced by AECs and pulmonary macrophages, respectively (Gonzalo et al, 1999; Kawasaki et al, 2001). In particular, the former chemokine is increasingly being recognized as a key epithelial secreted attractant for Th2 cells recruitment to the asthmatic airways (Hijnen et al, 2004). Indeed, clinical studies have demonstrated that the level of CCL17 positively correlates with the degree of airway obstruction and fractional exhaled nitric oxide (FeNo) of asthma patients (Ying et al, 2005). However, the mechanisms how AECs contribute to the development of asthma by employing CCL17 still remain largely unknown.

Interestingly, ITGB4 is located at the basal surface of AECs and regulates the stable adhesion of these cells to the underlying basement membrane (Pan et al, 2016). ITGB4 has a unique long cytoplasmic domain subunit that could recruit a range of signaling molecules. ITGB4 plays a key role in the activation of several intracellular signaling pathways including phosphatidylinositol 3-kinase (PI3-K) (Shaw et al, 1997), extracellular signal-regulated kinases (ERK) 1/2 (Chen et al, 2010) and NF-κB (Nikolopoulos et al, 2004), indicating that this integrin potentially contributes to the instigation of subsequent immune response and inflammation. Although there is a correlation between ITGB4 and the induction of allergic asthma, much remains

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unknown about the underlying mechanisms. In particular, little is known about the contribution of ITGB4 pathway to Th2 regulated immune responses. Therefore, we sought to further unravel the role of such pathway in this study.

Using our well-established conditional knockout *in vivo* system, we were able to show that ITGB4 in AECs negatively regulates the development of Th2 responses and allergic asthma with a mouse model of house dust mite (HDM) induced asthma. We have also demonstrated a critical link between ITGB4 and CCL17 that underpins the activation of Th2 cells. We further explore this inflammatory paradigm through the administration of AG1478, a compound that has been demonstrated to bind antagonistically to EGFR (Wang et al, 2010), as a novel intervention strategy to disrupt the activation of ERK pathway and thus the Th2 responses. These findings highlight the critical role of ITGB4, EGFR and CCL17 in the regulation of Th2-inflammatory features and indicate a novel pathway that regulates key pathophysiological features of allergic asthma.

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Results

Deficiency of ITGB4 in AECs aggravates the development of HDM induced allergic asthma

AECs specific ITGB4 conditionally knock out mice were treated with doxycycline to deplete this integrin as previously described (Fig 1A) (Liu et al, 2018). Mice were exposed HDM to induce allergic asthma. HDM exposure resulted in significantly increased airway reactivity and elevated levels of inflammatory cells in BALF and lung of $ITGB4^{+/+}$ mice, as compared to PBS exposed control. Following HDM exposure, $ITGB4^{-/-}$ mice exhibited significantly higher AHR than that of $ITGB4^{+/+}$ mice (Fig 1B). HDM exposed $ITGB4^{-/-}$ mice displayed significantly elevated histopathological scores, as compared to either PBS exposed $ITGB4^{-/-}$ mice or HDM exposed $ITGB4^{+/+}$ mice (Fig 1C). In line with aforementioned findings, the levels of BALF inflammatory infiltrates of HDM exposed $ITGB4^{-/-}$ mice also augmented significantly when compared to those of HDM exposed $ITGB4^{+/+}$ mice (Fig 1D). These observations reveal a negative role of ITGB4 in the regulation of the development of allergic asthma.

AECs-specific ITGB4 deficiency increases the numbers of Th2 and Th17 cells and the levels of their cytokines

To further characterize the impact of ITGB4 defect on the activation of T cells, we examined the infiltration of Th1, Th2 and Th17 cells by flow cytometry and the levels of their cytokines in lung by ELISA and qPCR, respectively. The levels of $CD4^{+}IL\text{-}4^{+}$ T cells, $CD4^{+}IL\text{-}13^{+}$ T cells and $CD4^{+}IL\text{-}17^{+}$ T cells increased significantly in the lung

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of both ITGB4$^{+/+}$ and ITGB4$^{-/-}$ groups following HDM exposure as compared to respective PBS control groups, and the levels of CD4^{+}IFN-γ^{+} T cells did not display significant changes (Fig 2A). Of note, the infiltration of Th2 and Th17 cells were significantly greater in HDM exposed ITGB4$^{-/-}$ animals than that of ITGB4$^{+/+}$ animals. Likewise, the protein and transcript levels of IL-4, IL-5, IL-13 and IL-17A were significantly elevated as demonstrated in the BALF of HDM exposed ITGB4$^{-/-}$ group, when compare to those of HDM exposed ITGB4$^{+/+}$ group (Fig 2B and C). HDM exposure resulted in significantly increased levels of these cytokines in BALF and lung of ITGB4$^{+/+}$ mice, as compared to those PBS exposed control. These data provide evidence that ITGB4 negatively regulates the activity of Th2 and Th17 cells but not Th1 cells.

ITGB4 negatively impacts on CCL17 production

Both clinic and animal studies have shown that Th2 cells orchestrate the development of allergic asthma (KleinJan, 2015). To determine how ITGB4 contributes the activation of Th2 cells, we examined the levels of CCL3, CCL5, CCL17 and CCL22 that could bind to CCR4, a Th2 chemotactic receptor (Assarsson et al, 2004; Qiu et al, 2018; Yoshie & Matsushima, 2015). Significant higher levels of CCL17 protein and transcript were found in the BALF and lung of HDM exposed ITGB4$^{-/-}$ mice, as compared to those of HDM exposed ITGB4$^{+/+}$ mice (Fig 3A and B). Interestingly, there was no significant difference in the transcript levels of CCL3, CCL5 and CCL22 in cultured AECs of HDM exposed ITGB4$^{-/-}$ mice, as compared to those of HDM exposed ITGB4$^{+/+}$ mice (Fig EV1). Sputum samples from patients with allergic

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asthma and healthy subjects were also collected to determine the secretion of CCL17. By using ELISA, levels of CCL17 were found significantly higher in the sputum of patients with allergic asthma, as compared to that of healthy controls (Fig 3C). Taken together, these findings indicate that ITGB4 in AECs critically regulates the expression of CCL17 and is associated with the pathogenesis of allergic asthma.

ITGB4 inhibits the EGFR phosphorylation of AECs

ITGB4 has been shown to regulate EGFR phosphorylation in liver cancer cells, breast cancer cells and gastric cancer cells, which is an important signalling mechanism for ERK activation (Bon et al, 2007; Huafeng et al, 2018; Leng et al, 2016). To determine whether ITGB4 regulates EGFR phosphorylation in our model, we treated isolated AECs from $ITGB4^{+/+}$ and $ITGB4^{-/-}$ mice with recombinant EGF to investigate the levels of EGFR phosphorylation. Basal level of EGFR phosphorylation in $ITGB4^{-/-}$ AECs was higher than that in $ITGB4^{+/+}$ AECs in the absence of EGF (Fig 4A). However, EGF significantly enhanced EGFR phosphorylation in $ITGB4^{-/-}$ AECs, as compared to that in $ITGB4^{+/+}$ AECs. Furthermore, assays with immunoprecipitation (Fig 4B) and immunofluorescence (Fig 4C) revealed a direct binding between ITGB4 and EGFR in cultured AECs. These results suggest that ITGB4 negatively regulates EGFR phosphorylation through physical association with the receptor.

ITGB4 regulates the production of CCL17 through the activation of EGFR, ERK and NF-κB pathways

To determine the role of EGFR mediated signaling pathways in ITGB4-regulated CCL17 production, we examined the expression of CCL17 in isolated AECs using

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multiple inhibitors, including AG1478 for EGFR phosphorylation, U0126 for ERK1/2 phosphorylation and SC75741 for NF-κB p65 activation. Western blot analysis revealed that treatments with AG1478, U0126 or SC75741 significantly inhibited the levels of p-EGFR, p-ERK1/2 or nucleus NF-κB p65 in ITGB4$^{-/-}$ AECs than those in ITGB4$^{+/+}$ AECs, respectively (Fig 5A, B and C). Importantly, these inhibitor treatments blocked heightened CCL17 production in ITGB4$^{-/-}$ AECs. ERK activated pathways has been described to induce phosphorylation and translocation of the transcription factor NF-κB to the nucleus (37). Meanwhile, NF-κB inhibitors have been reported to block CCL17 expression in the alveolar epithelial cell line (Berin et al, 2001). To verify the possible involvement of NF-κB in the upregulation of CCL17, we studied p65 subunit of the active NF-κB complex upon stimulation with AG1478, U0126 or SC75741. As anticipated, treatments with AG1478, U0126 and SC75741 reduced the levels of nucleus NF-κB p65 whose binding sites are present in the CCL17 promoter in ITGB4$^{-/-}$ AECs (Fig 5D). Together, these findings indicate that blocking EGFR, ERK and NF-κB pathways suppresses augmented CCL17 production in ITGB4$^{-/-}$ AECs following HDM exposure.

Blockade of EGFR phosphorylation inhibits CCL17 production and exaggerated AHR, airway inflammation and Th2 cells infiltration

To investigate the role of EGFR signaling pathway in ITGB4-regulated CCL17 production, we blocked EGFR phosphorylation with AG1478 both *in vitro* and *in vivo* by using ITGB4$^{-/-}$ AECs and mice. Administration of AG1478 inhibited the levels of p-EGFR in AECs, as compared to vehicle treatment (Fig 6A). Furthermore, inhibition

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of EGFR phosphorylation by AG1478 led to significantly decreased levels of CCL17 in cultured ITGB4$^{-/-}$ AECs (Fig 6B). Exaggerated AHR and histopathological scores were also significantly reduced by EGFR blockade in HDM exposed ITGB4$^{-/-}$ mice, as compared to those of vehicle treatment (Fig 6C and D). Flow cytometry revealed that AG1478 administration significantly reduced the levels of IL-4^{+} CD4^{+} T cells and IL-13^{+} CD4^{+} T cells but not IFN-γ^{+}CD4^{+} T cells and IL-17^{+} CD4^{+} T cells in the lung of HDM treated ITGB4$^{-/-}$ mice (Fig 6E). These data suggest that EGFR signaling pathway underpins ITGB4-regulated CCL17 production in AECs and thus contributes to the pathogenesis of Th2 inflammation.

Neutralization of CCL17 diminishes exaggerated AHR, airway inflammation and Th2 responses in HDM exposed ITGB4$^{-/-}$ mice

As a key role for CCL17 is indicated in the pathogenesis, we neutralized this chemokine with anti-CCL17 mAb in HDM exposed ITGB4$^{-/-}$ mice. Treatment with anti-CCL17 mAb significantly and greatly decreased the levels the chemokine in lung, as compared to isotype Ab treatment (Fig 7A). Neutralization of CCL17 significantly suppressed exaggerated AHR, airway inflammation in HDM exposed ITGB4$^{-/-}$ mice (Fig 7B and C). Of note, anti-CCL17 mAb treatment reduced the levels of CD4^{+}IL-4^{+} T cells and CD4^{+}IL-13^{+} T cells but not CD4^{+}IFN-γ^{+} T cells and CD4^{+}IL-17^{+} T cells in the lung of HDM exposed ITGB4$^{-/-}$ mice (Fig 7D). Similarly, neutralizing CCL17 decreased the protein and transcript levels of IL-4, IL-5, and IL-13, but not those of IFN-γ and IL-17A in the BALF and lung of HDM exposed ITGB4$^{-/-}$ mice, as compared to isotype Ab treatment (Fig 7E and F).

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Discussion:

Integrins are critical molecules for airway epithelial integrity and their expression is dramatically altered during chronic airway diseases such as allergic asthma (Huang et al, 1996; Qiu et al, 2017; Sundaram et al, 2017). However, the functional roles of these molecules in the regulation of aberrant immune responses and in the pathogenesis of disease are only beginning to be understood. We have previously reported that ITGB4 is downregulated in AECs of asthma patients and shown that it negatively regulates thymic stromal lymphopoietin (TSLP) production and antigen presenting process by DCs in an AECs-specific conditional knockout mouse system (Liu et al, 2018). In this study, we observed a negative regulatory role of ITGB4 in modulating the activity of Th2 cells through promoting CCL17 production in ITGB4$^{-/-}$ mice, indicating an unidentified role of this integrin in the mechanisms underpinning the development of allergic asthma. Furthermore, our findings establish a new link between ITGB4, EGFR and CCL17 in AECs, and highlight the potential of targeting this pathway for the treatment of allergic asthma.

AECs are known as the first cell barrier to inhaled pollutants and allergen, and play a vital role in driving immune responses in respiratory disorders. Not only as the structural component that maintains epithelial architecture, integrins also play a key role in asthma pathogenesis. A recent study has shown that α5β1 integrin deficiency protects mice from cytokine-enhanced bronchoconstriction in a mouse model of asthma (Sundaram et al, 2017). However, it is difficult to specifically identify the mechanism for this protection, since integrin has been reported to regulate a wide

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range of biological processes by targeting intracellular pathways including FAK，Src，Ras, RhoA, EGFR, ERK, p53, etc. (Leng et al, 2016; Mitra & Schlaepfer, 2006; Stewart & O'Connor, 2015). In this context, our observation that the specific disruption of ITGB4 in AECs protects animal from allergic asthma makes this problem more tractable and suggests a central role of AECs in the induction of allergic asthma. In our study, exposure to HDM in the airways of ITGB4$^{-/-}$ mice led to exaggerated airway inflammation and heightened AHR (Fig 1), which is in line with our previous findings (Liu et al, 2010b). We have also shown that the effects of ITGB4 deficiency in AECs are not just limited on pathophysiological changes in airway but also on the activation of T helper cells as AECs-specific ITGB4 negatively regulates the infiltration of Th2 and Th17 cells and the production of their cytokines (Fig 2). This observation suggests that ITGB4 contributes to the disease progress through the regulation of Th2 and Th17 activation.

The infiltration of T helper cells is exquisitely regulated by a wide range of chemokines (Castan et al, 2017; Guerreiro et al, 2011). While changes in levels of other CCR4-binding chemokines (e.g. CCL3, CCL5 and CCL22) did not show significant differences, the level of CCL17 was significantly elevated in the BALF and the lung of HDM exposed ITGB4$^{-/-}$ mice (Fig 3). Notably, a significantly increased level of CCL17 was detected in the sputum of allergic asthma patients. Indeed, CCL17 plays a crucial role in the recruitment of Th2 cells into lung, as demonstrated by a mouse model of RSV infection in the lung (Monick et al, 2007). Several lines of evidence have shown that CCL17 can be produced by alveolar

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macrophages and dendritic cells as well (Ait Yahia et al, 2014; Staples et al, 2012). However, it is difficult to determine the exact roles of these immune cells in the regulation of CCL17. In this regard, our study is important in understanding the pathogenesis of allergic asthma, by demonstrating that the AEC-derived CCL17 is critical for the activation of Th2 cells.

Although the production of CCL17 by AECs is known, it is still largely obscure how integrins contribute to the increased expression of CCL17 in allergen induced asthma. With ITGB4$^{-/-}$ mice, we were able to identify that the synchronization between ITGB4 and EGFR essentially orchestrates CCL17 production in AECs through the action of multiple downstream signaling pathways including EGFR, ERK1/2 and NF-κB (Fig 4-5). Studies in cancer research have shown that interaction between ITGB4 and EGFR phosphorylation has a significant impact on the progression and metastasis of hepatocellular carcinoma cells, mammary tumour cells and mammary tumour cells (Bon et al, 2007; Huafeng et al, 2018; Leng et al, 2016). EGFR, a receptor tyrosine kinase, can induce phosphorylation of multiple intracellular transcriptional regulators including ERK1/2, and PI3K pathway (Jiang et al, 2017; Zhu et al, 2015). Among these signaling regulators, EGFR is essential for the activation of ERK signaling that critically mediate the production of pro-inflammatory factors (Huang et al, 2017). Our results are in line with the aforementioned findings, and have identified that a direct interaction between ITGB4 and EGFR in airway epithelial cells drives the development of Th2 cells-associated allergic responses. Interestingly, AG1478 treatment completely blocked the

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297 phosphorylation of EGFR and reduced CCL17 production in the AECs of HDM
298 exposed ITGB4$^{-/-}$ mice to basal level. It also significantly inhibited the development
299 of AHR, airway inflammation and the infiltration of Th2 cells (Fig 6). Furthermore,
300 neutralizing CCL17 dramatically suppressed the manifestation of the disease in our
301 model. Therefore, our data have shown that EGFR and CCL17 critically contribute to
302 the disease, as demonstrated by its' impact on Th2 cells infiltration, exaggerated AHR,
303 airway inflammation in HDM exposed ITGB4$^{-/-}$ mice.

304 Interestingly, we have also found that ITGB4 defect leads to heightened
305 infiltration of Th17 cells and higher production of IL-17A (Fig 2). The importance of
306 Th17 cells and their cytokines is implied not only in autoimmune diseases but also in
307 allergic asthma (Chakir et al, 2003; Molet et al, 2001). Although EGFR antagonist or
308 CCL17 mAb treatments significantly suppressed the migration of Th2 cells in HDM
309 exposed ITGB4$^{-/-}$ mice, they had no impact on Th17 cells infiltration. Furthermore,
310 CCL17 mAb treatment did not affect IL-17A production. This may be related the
311 increased TSLP production in AECs (Liu et al, 2018). Indeed, unaffected Th17
312 function is likely the reason why there exists residual AHR and airway inflammation
313 after EGFR antagonist or anti-CCL17 mAb treatments in HDM exposed ITGB4$^{-/-}$
314 mice. These observations indicate the differential and profound roles of ITGB4 in the
315 induction of allergic asthma and highlight the complex and heterogeneous nature of
316 allergic asthma. Furthermore, the infiltration of Th1 cells and the production of IFN-γ
317 were not affected after EGFR-inhibitor or anti-CCL17 mAb treatments in HDM
318 exposed ITGB4$^{-/-}$ mice, suggesting a specific role of ITGB4 in the pathophysiology of

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allergic asthma by the regulation of Th2 and Th17 responses.

In conclusion, our data provide an insight that ITGB4 defect in AECs leads to elevated Th2 responses and exaggerated AHR and airway inflammation. By focusing on the role of ITGB4, we have developed models that allow dissection of the mechanisms predisposing to the development of Th2 responses, allergic inflammation and AHR. Here we demonstrate the importance of integrated signaling events between ITGB4, EGFR, ERK1/2 and NF-κB pathways specifically in AECs for the induction of allergic disease which may be clinically relevant. Understanding the contribution of this molecular network within AECs to the pathogenesis of allergic asthma may provide new therapeutic approaches for the treatment of the disease.

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Methods

Collection of sputum samples

Asthmatic patients (defined by clinical diagnosis with evidence of bronchodilator reversibility testing, clinical assessment and a confirmed history of atopy with raised, allergen-specific IgE or positive radioallergosorbent test to at least one aero-allergen) (Matucci et al, 2018) were recruited and categorized by induced sputum inflammatory cell counts as allergic asthma. Participants provided written informed consent, approved by the No.20180308 Central south University Research Ethics Committees.

The demographic and clinical characteristics are shown in Table EV1. Induced sputum samples were obtained using nebulized hypertonic (4.5%) saline, treated with dithiothreitol and counts and viability (trypan blue exclusion) were determined by hemocytometer. The plugs were treated with a volume of dithiothreitol representing 4 times the mucus weight. Then, an equal volume of PBS was added to all samples before filtration with a 0.45 μm nylon filter. The supernatants were collected for CCL17 quantitation.

Animals

Control wild type (WT) and AECs-specific ITGB4 conditionally knocked out ($ITGB4^{-/-}$) mice were housed under barrier conditions in air-filtered, temperature-controlled units under a 12-hour light-dark cycle and with free access to food and water. The generation of AECs-specific ITGB4 conditionally knocked out mice was described previously (Liu et al, 2018). Briefly, to produce $ITGB4^{-/-}$ mice, doxycycline (Dox; 1% in drinking water) was administered to 8-week-old CCSP–rtTA $^{tg/-}$/TetO-

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Cre $^{tg/-}$/ITGB4 $^{fl/fl}$ mice. All animal studies were approved by the No.201803079 Central South University at XiangYa Animal Care and Use Committee. All the methods were carried out in accordance with the relevant guidelines and regulations.

Induction of allergic asthma and administration of anti-CCL17 neutralizing antibody and EGFR antagonist

For the induction of allergic asthma, mice were intranasally (i.n.) exposed to 100 μg HDM on days 0, 7, 14 and 21 as previous described (Draijer et al, 2013). Nonsensitized mice were i.n. treated with phosphate-buffered saline (PBS). On day 24, AHR was assessed, and then inflammatory infiltrates and histological changes in the lung were quantified as described previously (Nguyen et al, 2018). Some of ITGB4$^{-/-}$ mice were intraperitoneally (i.p.) treated with 150 μg anti-mouse CCL17 monoclonal antibody (mAb, clone 110904, R&D Systems) or isotype control mAb (MAB006; R&D Systems, USA) in 200 μl PBS, twice a week for 3 weeks as previously described (Lee et al, 2018). Some of ITGB4$^{-/-}$ mice were given intraperitoneal (i.p.) injections of EGFR inhibitor AG1478 (20mg/kg, Calbiochem, San Diego, CA) which was dissolved in CMC-Na (0.5%) or control vehicle three times weekly over the course of HDM treatment period (Wang et al, 2010).

Measurement of lung function

Airway resistance was measured using a direct plethysmography (Biosystems XA; Buxco Electronics, USA), as previously described (Liu et al, 2010b). In brief, mice were anesthetized with a mixture containing xylazine (10 mg/kg) and ketamine (100 mg/kg) by i.p. injection (ratio 1:4, respectively; 150 μl per mouse). A cannula was

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373 then inserted into the trachea, and mice were ventilated with a tidal volume of 8 ml/kg
374 at a rate of 145 breaths/min. Increasing doses of aerosolized methacholine (1.56,
375 3.125, 6.25, 12.5, 25mg/ml, Sigma-Aldrich) were delivered intratracheally (i.t.).
376 Airway resistance was presented as percentage increase over baseline (saline
377 challenge).

378 **BALF collection and cell counting**

379 BALF was collected and processed as previously described (Nguyen et al, 2016). In
380 brief, the lung was lavaged with 0.5 ml ice-cold PBS with 0.1 mM EDTA twice. Red
381 blood cells were removed by using hypotonic red blood cell lysis buffer and BALF
382 was then centrifuged to collect cellular infiltrate. Total cell numbers were quantified
383 using a haemocytometer and cells were cytospun onto glass slides (ThermoFisher
384 Scientific, Scoresby, Victoria, Australia). Differential leukocyte counts were
385 determined based on morphological criteria by light microscopy (×100) on May-
386 Grunwald and Giemsa-stained slides.

387 **Lung histology and immunohistochemical staining**

388 Lungs lobes from mice were removed, fixed in 4% paraformaldehyde (5 ml), and
389 embedded in paraffin blocks. Then, sections were stained with hematoxylin and eosin
390 (H&E) (Sigma, St. Louis, MO) (Nguyen et al, 2018; Wang et al, 2013).
391 Histopathological changes (inflammatory infiltrates) were scored blindly at
392 morphological criteria, according to previous publications (Starkey et al, 2014).

393 Immunofluorescent staining was also performed on primary mouse AECs
394 using the following antibodies: anti-ITGB4 (Ab182120, Abcam), anti-EGFR

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(ab52894, Abcam) in 1% BSA/PBS overnight at 4°C, washed with 1% BSA/PBS and incubated with Cy3-conjugated or FITC-conjugated goat anti-rabbit IgG (10 mg/ml; GE Healthcare) for 45 min respectively, then washed with 1% BSA/PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 10 min at RT. Slips were fixed on slides. ITGB4 and EGFR in AECs were visualized using a fluorescence microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with a ×100 objective lens. Images were captured using a digital camera (Axio-Cam ICc3, Spectra Service, Ontario, NY) and analysed using AxioVision Rel. 4.7 software (Zeiss). Sampling was performed on eight to ten different areas for 40-60 cells of each slide.

Flow cytometry

Single cell suspensions from lungs were prepared as previously described (Teijaro et al, 2011) and were used for flow cytometric analysis. In brief, cell suspensions were prepared by enzymatically digesting the lung tissue using dispase (Discovery Labware; Corning, Bedford, MA). Following incubation with surface marker antibodies anti-CD45, (clone 104，BD Biosciences, San Jose, CA), anti-CD3 (clone 17A2，BD Biosciences, San Jose, CA), anti-CD4 (clone GK1.5，BD Biosciences, San Jose, CA) and isotype controls (Rajavelu et al, 2015), cells were fixed using 4% PFA and permeabilized using Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA). Cells were then incubated with anti-IFN-γ (Cat. no. 12-7311), anti-IL-4 (Cat. no. 12-7041), anti-IL-13 (Cat. no. 12-7133) and anti-IL-17 (Cat. no. 12-7177) antibodies (eBioscience, San Diego, CA). Numbers of positive cells were quantified by flow cytometry (FACSCanto flow cytometer, BD Biosciences, San Jose, CA). Data were

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417 collected on a FACSCanto flow cytometer and analysed with FlowJo software
418 (version 7.6, Tree Star, Inc).

419 **ELISA assay**

420 Levels of IFN-γ, IL-4，IL-5, IL-13, IL-13, IL-17 and CCL17 were determined with
421 ELISA assays according to the manufacturer's protocols (Sigma, St. Louis, MO,
422 USA).

423 **RNA extraction, RT-PCR and quantitative RT-PCR**

424 Total RNA was prepared using TRIzol reagent (Invitrogen) and phenolchloroform
425 extraction from whole-lung tissues of mice and quantified on a SmartSpecTM Plus
426 spectrophotometer (Bio-rad, USA) (Liu et al, 2012). cDNA was synthesized by RT-
427 PCR using oligo d(T) primer (Invitrogen) on a T100 thermal cycler (Bio-Rad).
428 Quantitative PCR (qPCR) was performed on a CFX96 Touch™ Deep Well Real-Time
429 PCR Detection System (Bio-rad，USA) using TaqMan Gene Expression Master Mix
430 (Applied Biosystems) with thermal cycling conditions. Primer sequences were
431 described in Table EV2. Resulting mRNA levels were normalized to GAPDH and
432 expressed as a fold-change relative to control samples.

433 **Western blot**

434 AECs were isolated from mouse lung as previously described (Brockman-Schneider
435 et al, 2008). CCSP (sc-365992, Santa Cruze) was used to sort $CCSP^{+}$ AECs from
436 $ITGB4^{-/-}$ mice by flow cytometry. $CCSP^{+}$ AECs were cultured and used as $ITGB4^{-/-}$
437 AECs. Fifty μg of cell protein was separated by 10% SDS-PAGE and transferred to a
438 polyvinylidene difluoride membrane. Some AECs were pretreated with AG1478

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(1μM, an EGFR phosphorylation inhibitor), U0126 (10μM, an ERK phosphorylation inhibitor), or SC75741 (5μM, a NF-κB p65 inhibitor) respectively. Levels of ITGB4, CCL17, EGFR, phosphorylated EGFR (p-EGFR), ERK, phosphorylated ERK (p-ERK) and NF-κB p65 were determined respectively with anti-mouse antibodies against these proteins by western blot as previously reported (Jiang et al, 2014). GAPDH and Lamin B1 were used as controls as indicated.

Immunoprecipitation

Protein were extracted with radioimmunoprecipitation assay (RIPA) buffer: 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1%SDS, 5 mmol/L EDTA, pH=7.4. Protein concentration was measured by BCA protein determination kit (PC0020, Solarbio, China). Immunoprecipitations were performed overnight using the appropriate dilution of a polyclonal anti-ITGB4 antibody (Ab197772, Abcam) added to the lysates (1 μg proteins), under rotatry mixing for 1 at 4°C h. The immune complexes were allowed to bind to Protein A-Sepharose beads at 4°C for 3 h, washed (15 min, 4°C) with RIPA buffer and resuspended in loading buffer. Immunoprecipitates were subjected to SDS, 10% PAGE and immunoblotted on a polyvinylidene difluoride membrane. Immunocomplexes were stained with anti-ITGB4 (Ab197772, Abcam) as well as EGFR (Ab52894, Abcam) using ECL according to the manufacturer's guidelines (NCI4106, Thermo Pierce, USA).

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Author contributions

CL, MY and XQQ conceived and designed this study. CL, LY, XZ, YX, XZD and YZZ carried out the experiments. YX, XPQ, HJL, LQ and QWQ contributed to the interpretation of the results. CL and MY wrote the paper. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Ait Yahia S, Azzaoui I, Everaere L, Vorng H, Chenivesse C, Marquillies P, Duez C, Delacre M, Grandjean T, Balsamelli J, Fanton d'Andon M, Fan Y, Ple C, Werts C, Boneca IG, Wallaert B, Chamaillard M, Tsicopoulos A (2014) CCL17 production by dendritic cells is required for NOD1-mediated exacerbation of allergic asthma. *Am J Respir Crit Care Med* **189:** 899-908

Assarsson E, Kambayashi T, Schatzle JD, Cramer SO, von Bonin A, Jensen PE, Ljunggren HG, Chambers BJ (2004) NK cells stimulate proliferation of T and NK cells through 2B4/CD48 interactions. *J Immunol* **173:** 174-180

Berin MC, Eckmann L, Broide DH, Kagnoff MF (2001) Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. *Am J Respir Cell Mol Biol* **24:** 382-389

Bon G, Folgiero V, Di Carlo S, Sacchi A, Falcioni R (2007) Involvement of alpha6beta4 integrin in the mechanisms that regulate breast cancer progression. *Breast cancer research : BCR* **9:** 203

Boxall C, Holgate ST, Davies DE (2006) The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur Respir J* **27:** 208-229

Brockman-Schneider RA, Amineva SP, Bulat MV, Gern JE (2008) Serial culture of murine primary airway epithelial cells and ex vivo replication of human rhinoviruses. *Journal of immunological methods* **339:** 264-269

Castan L, Magnan A, Bouchaud G (2017) Chemokine receptors in allergic diseases. *Allergy* **72:** 682-690

Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, Boulet LP, Hamid Q (2003) Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* **111:** 1293-1298

Chen W, Garcia JG, Jacobson JR (2010) Integrin beta4 attenuates SHP-2 and MAPK signaling and reduces human lung endothelial inflammatory responses. *Journal of cellular biochemistry* **110:** 718-724

Dowling J, Yu QC, Fuchs E (1996) Beta4 integrin is required for hemidesmosome formation, cell adhesion and cell survival. *The Journal of cell biology* **134:** 559-572

Draijer C, Robbe P, Boorsma CE, Hylkema MN, Melgert BN (2013) Characterization of macrophage phenotypes in three murine models of house-dust-mite-induced asthma. *Mediators of inflammation* **2013:** 632049

Georas SN, Rezaee F (2014) Epithelial barrier function: at the front line of asthma immunology and allergic airway inflammation. *J Allergy Clin Immunol* **134:** 509-520

Gonzalo JA, Pan Y, Lloyd CM, Jia GQ, Yu G, Dussault B, Powers CA, Proudfoot AE, Coyle AJ, Gearing D, Gutierrez-Ramos JC (1999) Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J Immunol* **163:** 403-411

Guerreiro R, Santos-Costa Q, Azevedo-Pereira JM (2011) The chemokines and their receptors: characteristics and physiological functions. *Acta medica portuguesa* **24 Suppl 4:** 967-976

Hallstrand TS, Hackett TL, Altemeier WA, Matute-Bello G, Hansbro PM, Knight DA (2014) Airway epithelial regulation of pulmonary immune homeostasis and inflammation. *Clinical immunology* **151:** 1-15

Heijink IH, Nawijn MC, Hackett TL (2014) Airway epithelial barrier function regulates the pathogenesis of allergic asthma. *Clin Exp Allergy* **44:** 620-630

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Hijnen D, De Bruin-Weller M, Oosting B, Lebre C, De Jong E, Bruijnzeel-Koomen C, Knol E (2004) Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell- attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis. *J Allergy Clin Immunol* **113:** 334-340

Holgate ST (2012) Innate and adaptive immune responses in asthma. *Nat Med* **18:** 673-683

Holgate ST, Roberts G, Arshad HS, Howarth PH, Davies DE (2009) The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis. *Proc Am Thorac Soc* **6:** 655-659

Huafeng J, Deqing Z, Yong D, Yulian Z, Ailing H (2018) A cross-talk between integrin beta4 and epidermal growth factor receptor induces gefitinib chemoresistance to gastric cancer. *Cancer cell international* **18:** 50

Huang L, Pu J, He F, Liao B, Hao B, Hong W, Ye X, Chen J, Zhao J, Liu S, Xu J, Li B, Ran P (2017) Positive feedback of the amphiregulin-EGFR-ERK pathway mediates PM2.5 from wood smoke-induced MUC5AC expression in epithelial cells. *Sci Rep* **7:** 11084

Huang XZ, Wu JF, Cass D, Erle DJ, Corry D, Young SG, Farese RV, Jr., Sheppard D (1996) Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin. *The Journal of cell biology* **133:** 921-928

Jiang C, Zhu Y, Zhou Z, Gumin J, Bengtsson L, Wu W, Songyang Z, Lang FF, Lin X (2017) TMEM43/LUMA is a key signaling component mediating EGFR-induced NF-kappaB activation and tumor progression. *Oncogene* **36:** 2813-2823

Jiang R, Cai J, Zhu Z, Chen D, Wang J, Wang Q, Teng Y, Huang Y, Tao M, Xia A, Xue M, Zhou S, Chen AF (2014) Hypoxic trophoblast HMGB1 induces endothelial cell hyperpermeability via the TRL-4/caveolin-1 pathway. *J Immunol* **193:** 5000-5012

Kawasaki S, Takizawa H, Yoneyama H, Nakayama T, Fujisawa R, Izumizaki M, Imai T, Yoshie O, Homma I, Yamamoto K, Matsushima K (2001) Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J Immunol* **166:** 2055-2062

Kim CH, Rott L, Kunkel EJ, Genovese MC, Andrew DP, Wu L, Butcher EC (2001) Rules of chemokine receptor association with T cell polarization in vivo. *J Clin Invest* **108:** 1331-1339

KleinJan A (2015) Airway inflammation in asthma: key players beyond the Th2 pathway. *Curr Opin Pulm Med* **22:** 46-52

Lambrecht BN, Hammad H (2015) The immunology of asthma. *Nat Immunol* **16:** 45-56

Lee MC, Saleh R, Achuthan A, Fleetwood AJ, Forster I, Hamilton JA, Cook AD (2018) CCL17 blockade as a therapy for osteoarthritis pain and disease. *Arthritis research & therapy* **20:** 62

Leng C, Zhang ZG, Chen WX, Luo HP, Song J, Dong W, Zhu XR, Chen XP, Liang HF, Zhang BX (2016) An integrin beta4-EGFR unit promotes hepatocellular carcinoma lung metastases by enhancing anchorage independence through activation of FAK-AKT pathway. *Cancer letters* **376:** 188-196

Liu C, Qin X, Liu H, Xiang Y (2012) Downregulation of integrin beta4 decreases the ability of airway epithelial cells to present antigens. *PLoS One* **7:** e32060

Liu C, Xiang Y, Liu H, Li Y, Tan Y, Zhu X, Zeng D, Li M, Zhang L, Qin X (2010a) Integrin beta4 was downregulated on the airway epithelia of asthma patients. *Acta Biochim Biophys Sin* **42:** 538-547

Liu C, Xiang Y, Liu HJ, Gao G, Howard ST, Zhu XL, Qin XQ (2010b) Involvement of integrin beta4 in ozone stress-induced airway hyperresponsiveness. *Biochem Biophys Res Commun* **397:** 290-295

Liu C, Yuan L, Zou Y, Yang M, Chen Y, Qu X, Liu H, Jiang J, Xiang Y, Qin X (2018) ITGB4 is essential for containing HDM-induced airway inflammation and airway hyperresponsiveness. *Journal*

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of leukocyte biology **103:** 897-908

Lukacs NW (2001) Role of chemokines in the pathogenesis of asthma. *Nat Rev Immunol* **1:** 108-116

Matucci A, Vultaggio A, Maggi E, Kasujee I (2018) Is IgE or eosinophils the key player in allergic asthma pathogenesis? Are we asking the right question? *Respir Res* **19:** 113

Mitra SK, Schlaepfer DD (2006) Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol* **18:** 516-523

Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, Olivenstein R, Elias J, Chakir J (2001) IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* **108:** 430-438

Monick MM, Powers LS, Hassan I, Groskreutz D, Yarovinsky TO, Barrett CW, Castilow EM, Tifrea D, Varga SM, Hunninghake GW (2007) Respiratory syncytial virus synergizes with Th2 cytokines to induce optimal levels of TARC/CCL17. *J Immunol* **179:** 1648-1658

Nguyen TH, Maltby S, Simpson JL, Eyers F, Baines KJ, Gibson PG, Foster PS, Yang M (2016) TNF-alpha and Macrophages Are Critical for Respiratory Syncytial Virus-Induced Exacerbations in a Mouse Model of Allergic Airways Disease. *J Immunol* **196:** 3547-3558

Nguyen TH, Maltby S, Tay HL, Eyers F, Foster PS, Yang M (2018) Identification of IFN-gamma and IL-27 as Critical Regulators of Respiratory Syncytial Virus-Induced Exacerbation of Allergic Airways Disease in a Mouse Model. *J Immunol* **200:** 237-247

Nikolopoulos SN, Blaikie P, Yoshioka T, Guo W, Giancotti FG (2004) Integrin beta4 signaling promotes tumor angiogenesis. *Cancer Cell* **6:** 471-483

Pan L, Zhao Y, Yuan Z, Farouk MH, Zhang S, Bao N, Qin G (2017) The Integrins Involved in Soybean Agglutinin-Induced Cell Cycle Alterations in IPEC-J2. *Molecules and cells* **40:** 109-116

Pan L, Zhao Y, Yuan Z, Qin G (2016) Research advances on structure and biological functions of integrins. *SpringerPlus* **5:** 1094

Panina-Bordignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C, Buonsanti C, Miotto D, Mapp C, Villa A, Arrigoni G, Fabbri LM, Sinigaglia F (2001) The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* **107:** 1357-1364

Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE (2000) Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **14:** 1362-1374

Qiu C, Liu W, Shi F, Fen M, Ren L, Qi H (2017) Silencing of beta1 integrin regulates airway remodeling by regulating the transcription of SOCEassociated genes in asthmatic mice. *Mol Med Rep* **16:** 2645-2651

Qiu SL, Kuang LJ, Tang QY, Duan MC, Bai J, He ZY, Zhang JQ, Li MH, Deng JM, Liu GN, Zhong XN (2018) Enhanced activation of circulating plasmacytoid dendritic cells in patients with Chronic Obstructive Pulmonary Disease and experimental smoking-induced emphysema. *Clinical immunology* **195:** 107-118

Rajavelu P, Chen G, Xu Y, Kitzmiller JA, Korfhagen TR, Whitsett JA (2015) Airway epithelial SPDEF integrates goblet cell differentiation and pulmonary Th2 inflammation. *J Clin Invest* **125:** 2021-2031

Roche WR, Montefort S, Baker J, Holgate ST (1993) Cell adhesion molecules and the bronchial epithelium. *The American review of respiratory disease* **148:** S79-82

Shaw LM, Rabinovitz I, Wang HH, Toker A, Mercurio AM (1997) Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. *Cell* **91:** 949-960

Stacey MA, Sun G, Vassalli G, Marini M, Bellini A, Mattoli S (1997) The allergen Der p1 induces NF-

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606 kappaB activation through interference with IkappaB alpha function in asthmatic bronchial epithelial
607 cells. *Biochem Biophys Res Commun* **236:** 522-526
608 Staples KJ, Hinks TS, Ward JA, Gunn V, Smith C, Djukanovic R (2012) Phenotypic characterization of
609 lung macrophages in asthmatic patients: overexpression of CCL17. *J Allergy Clin Immunol* **130:** 1404-
610 1412 e1407
611 Starkey MR, Nguyen DH, Essilfie AT, Kim RY, Hatchwell LM, Collison AM, Yagita H, Foster PS,
612 Horvat JC, Mattes J, Hansbro PM (2014) Tumor necrosis factor-related apoptosis-inducing ligand
613 translates neonatal respiratory infection into chronic lung disease. *Mucosal Immunol* **7:** 478-488
614 Stewart RL, O'Connor KL (2015) Clinical significance of the integrin alpha6beta4 in human
615 malignancies. *Lab Invest* **95:** 976-986
616 Sundaram A, Chen C, Khalifeh-Soltani A, Atakilit A, Ren X, Qiu W, Jo H, DeGrado W, Huang X,
617 Sheppard D (2017) Targeting integrin alpha5beta1 ameliorates severe airway hyperresponsiveness in
618 experimental asthma. *J Clin Invest* **127:** 365-374
619 Teijaro JR, Walsh KB, Cahalan S, Fremgen DM, Roberts E, Scott F, Martinborough E, Peach R,
620 Oldstone MB, Rosen H (2011) Endothelial cells are central orchestrators of cytokine amplification
621 during influenza virus infection. *Cell* **146:** 980-991
622 Wang Q, Li H, Yao Y, Xia D, Zhou J (2010) The overexpression of heparin-binding epidermal growth
623 factor is responsible for Th17-induced airway remodeling in an experimental asthma model. *J Immunol*
624 **185:** 834-841
625 Wang W, Li X, Zheng D, Zhang D, Huang S, Zhang X, Ai F, Wang X, Ma J, Xiong W, Zhou Y, Li G,
626 Shen S (2013) Dynamic changes of peritoneal macrophages and subpopulations during ulcerative
627 colitis to metastasis of colorectal carcinoma in a mouse model. *Inflamm Res* **62:** 669-680
628 Wang Y, Bai C, Li K, Adler KB, Wang X (2008) Role of airway epithelial cells in development of
629 asthma and allergic rhinitis. *Respir Med* **102:** 949-955
630 Xiang Y, Zhou XY, Tan YR, Tan ML, Liu HJ, Liu C, Qu XP, Qin XQ (2014) Analysis on the relevance
631 of asthma susceptibility with the alteration of integrin beta 4 expression. *PLoS One* **9:** e95533
632 Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, Robinson D, Zhang G, Zhao J, Lee TH,
633 Corrigan C (2005) Thymic stromal lymphopoietin expression is increased in asthmatic airways and
634 correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* **174:** 8183-
635 8190
636 Yoshie O, Matsushima K (2015) CCR4 and its ligands: from bench to bedside. *International*
637 *Immunology* **27:** 11-20
638 Zhu G, Fan Z, Ding M, Zhang H, Mu L, Ding Y, Zhang Y, Jia B, Chen L, Chang Z, Wu W (2015) An
639 EGFR/PI3K/AKT axis promotes accumulation of the Rac1-GEF Tiam1 that is critical in EGFR-driven
640 tumorigenesis. *Oncogene* **34:** 5971-5982

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Figure legend.

Figure 1. AHR and allergic disease of the lung is markedly exaggerated in the absence of airway epithelial ITGB4.

A Mice were sensitized to HDM on days 0, 7, 14 or 21. Some mice were treated with 1% Dox in drinking water to specifically delete ITGB4 in airway epithelial cells. Control non-sensitized mice received PBS.

B AHR was represented as airway resistance in response to methacholine. Data represent the mean ± SEM of six mice per group. **P < 0.01 by 2-way ANOVA followed by Fisher post hoc test.

C Lung histology was assessed (n = 8), bars: 50 μm. Values represented as mean ± SEM. **P < 0.01 compared with controls using an unpaired, Student's t test.

D BALF inflammatory cell was counted (n = 10). Values represented as mean ± SEM. **P< 0.01 compared with controls using one-way ANOVA followed by Dunnett's post hoc test.

Figure 2. **The infiltration T helper cells and the levels of BALF cytokine of ITGB4$^{+/+}$ or ITGB4$^{-/-}$ mice**.

A Mice were sensitized to HDM on days 0, 7, 14 or 21. Some mice were treated with 1% Dox in drinking water to specifically delete ITGB4 in airway epithelial cells. Control non-sensitized mice received PBS. Two days after the final challenge, the infiltration of IFN-γ^+, IL-4^+, IL-13^+ and IL-$17A^+$ T lymphocyte in lung of ITGB4$^{+/+}$ and ITGB4$^{-/-}$ mice was assessed by flow analysis (n = 12). Values represented as mean ± SEM. **P < 0.01 compared with controls using an unpaired, Student's t test.

B, C Two days after the final challenge, the levels of IFN-γ, IL-4, IL-13, and IL-17A protein in BALF (n = 8) and their transcripts in lung (n = 6) were examined by ELISA and qPCR, respectively. Values represented as mean ± SEM. *P< 0.05 compared with controls using one-way ANOVA followed by Dunnett's post hoc test.

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Figure 3. CCL17 production and Th2 cells infiltration are further elevated by the deficiency of ITGB4.

A, B Mice were sensitized to HDM on days 0, 7, 14 or 21. Some mice were treated with 1% Dox in drinking water to specifically delete ITGB4 in airway epithelial cells. Control non-sensitized mice received PBS. CCL17 protein in BALF (n = 8) and the levels of CCL17 transcripts in lung (n = 6) were determined by ELISA and qPCR, respectively. Values represented as mean ± SEM. **P < 0.01 compared with controls using an unpaired, Student's t test.

C CCL17 expression detected in induced sputum samples from allergic asthmatic patients (n = 41) and control subjects (n = 35). Values represented as mean ± SEM. **P < 0.01 using an unpaired, Student's t test.

Figure 4. Physical interaction between ITGB4 and EGFR and the phosphorylation of EGFR in airway epithelial cells of ITGB4$^{+/+}$ or ITGB4$^{-/-}$ mice.

A Airway epithelial cells were isolated from the lung of ITGB4$^{+/+}$ or ITGB4$^{-/-}$ mice. Cells were cultured and then stimulated with or without EGF (1 ng/ml) for 60 min. Western blot staining of stimulated AECs for p-EGFR and total EGFR, and p-EGFR/EGFR ratios normalized to vehicle control. Values represented as mean ± SEM for six samples from one experiment and representative of 3 independent experiments. **P<0.01 using an unpaired, Student's t test.

B Immunoprecipitation of stimulated AECs for interaction between ITGB4 and EGFR.

C Immunofluorescence staining of stimulated AECs for co-localization of ITGB4 and EGFR, bars: 10 μm.

Figure 5. ITGB4 deficiency leads to augmented activation of EGFR, ERK1/2 and NF-κB pathways.

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A Airway epithelial cells were isolated from the lung of ITGB4$^{+/+}$ or ITGB4$^{-/-}$ mice. Cells were cultured and then stimulated with or without EGF (1 ng/ml) for 60 min. (A) Western blot staining of stimulated AECs for phosphorylated EGFR (p-EGFR), phosphorylate ERK1/2 (p-ERK1/2), total EGFR, total ERK1/2, and CCL17 in the presence or absence of EGFR tyrosine phosphorylation inhibitor AG1478.

B Western blot staining of stimulated AECs for p-ERK1/2, total ERK1/2 and CCL17 in the presence or absence of ERK1/2 tyrosine phosphorylation inhibitor U0126.

C Western blot staining of stimulated AECs for CCL17 in the presence or absence of NF-κB inhibitor SC75741.

D Western blot staining of nuclear extracts of stimulated ITGB4$^{-/-}$ AECs for NF-κB p65 in the presence or absence of AG1478, U1026 and SC75741, NF-κB p65/Lamin B1 ratios normalized to vehicle control. All values represented as mean ± SEM for six samples from one experiment and representative of 3 independent experiments. **P<0.01 using an unpaired, Student's t test.

Figure 6. Blockade of EGFR phosphorylation inhibits both CCL17 production and subsequent enhanced AHR, lung inflammation in HDM exposed ITGB4$^{-/-}$ mice.

A, B AECs-specific ITGB4 conditional knock out mice were constructed and exposed HDM (HDM+) or PBS (HDM-) on days 0, 7, 14 or 21. AECs were isolated from these mice and some cells were treated with EGFR inhibitor AG1478 or control vehicle. The protein levels of EGFR, p-EGFR and CCL17 in AECs was detected by western blot. Values represented as mean ± SEM for 5 samples from one experiment and representative of 3 independent experiments. **P<0.01 using an unpaired, Student's t test. Some mice received injection with either AG1478 or same volume of the control vehicle.

C Two days after last HDM exposure, AHR was represented as airway resistance

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in response to methacholine. Data represent the mean ± SEM of 6 mice per group. **P < 0.01 by 2-way ANOVA followed by Fisher post hoc test.

D, E Lung histology was assessed (n = 8) and the levels of IFN-γ^+CD4$^+$, IL-4$^+$CD4$^+$, IL-13$^+$CD4$^+$ and IL-17A$^+$CD4$^+$ T cells in lung were determined (n = 10), bars: 50 μm. Values represented as mean ± SEM. **P < 0.01 compared with controls using an unpaired, Student's t test.

Figure 7. Neutralization of CCL17 reduces exaggerated AHR, airway inflammation and Th2 responses in HDM sensitized ITGB4$^{-/-}$ mice.

A Mice were treated with 1% Dox in drinking water to specifically delete ITGB4 in airway epithelial cells and were also sensitized with either HDM or PBS on days 0, 7, 14 or 21. Some mice received treatment with either anti-CCL17 or isotype control antibodies. Two days after the final challenge, CCL17 protein in lung was determined by western blot, ratios of CCL17/GAPDH normalized to vehicle control. Values represented as mean ± SEM for 6 samples from one experiment and representative of 3 independent experiments. **P<0.01 using an unpaired, Student's t test.

B Two days after last HDM exposure, AHR was represented as airway resistance in response to methacholine. Data represent the mean ± SEM of 6 mice per group. **P < 0.01 by 2-way ANOVA followed by Fisher post hoc test.

C, D Lung histology was assessed (n = 8) and the levels of IFN-γ^+CD4$^+$, IL-4$^+$CD4$^+$, IL-13$^+$CD4$^+$ and IL-17A$^+$CD4$^+$ T cells in lung were determined (n = 10), bars: 50 μm.Values represented as mean ± SEM. **P < 0.01 compared with controls using an unpaired, 2-tailed Student's t test.

E, F Two days after the final challenge, the levels of IFN-γ, IL-4, IL-13, and IL-17A protein in BALF (n = 8) and their transcripts in lung (n = 6) were examined by ELISA and qPCR, respectively. Values represented as mean ± SEM. *P< 0.05 compared with controls using one-way ANOVA followed by Dunnett's post hoc test.

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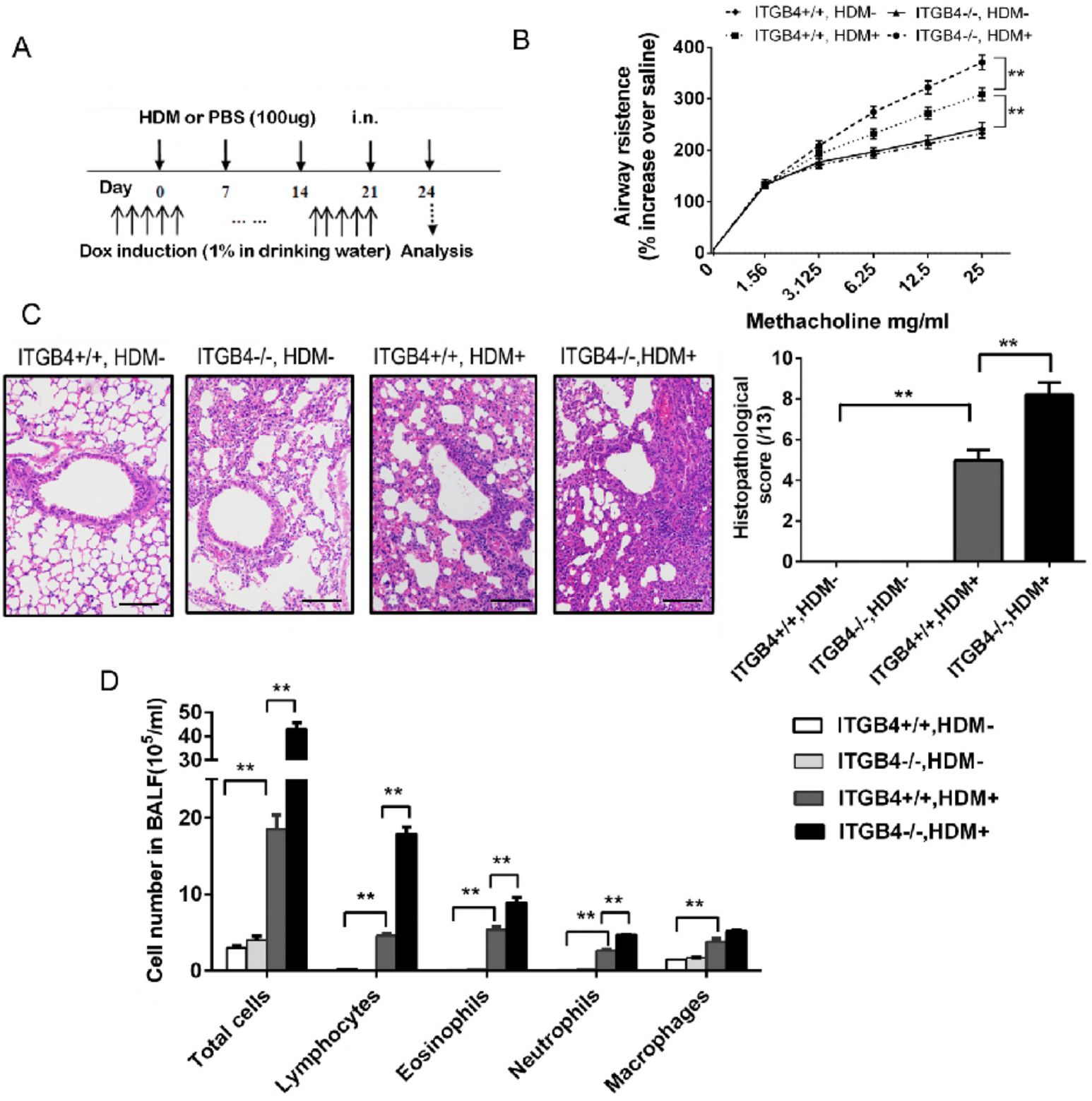
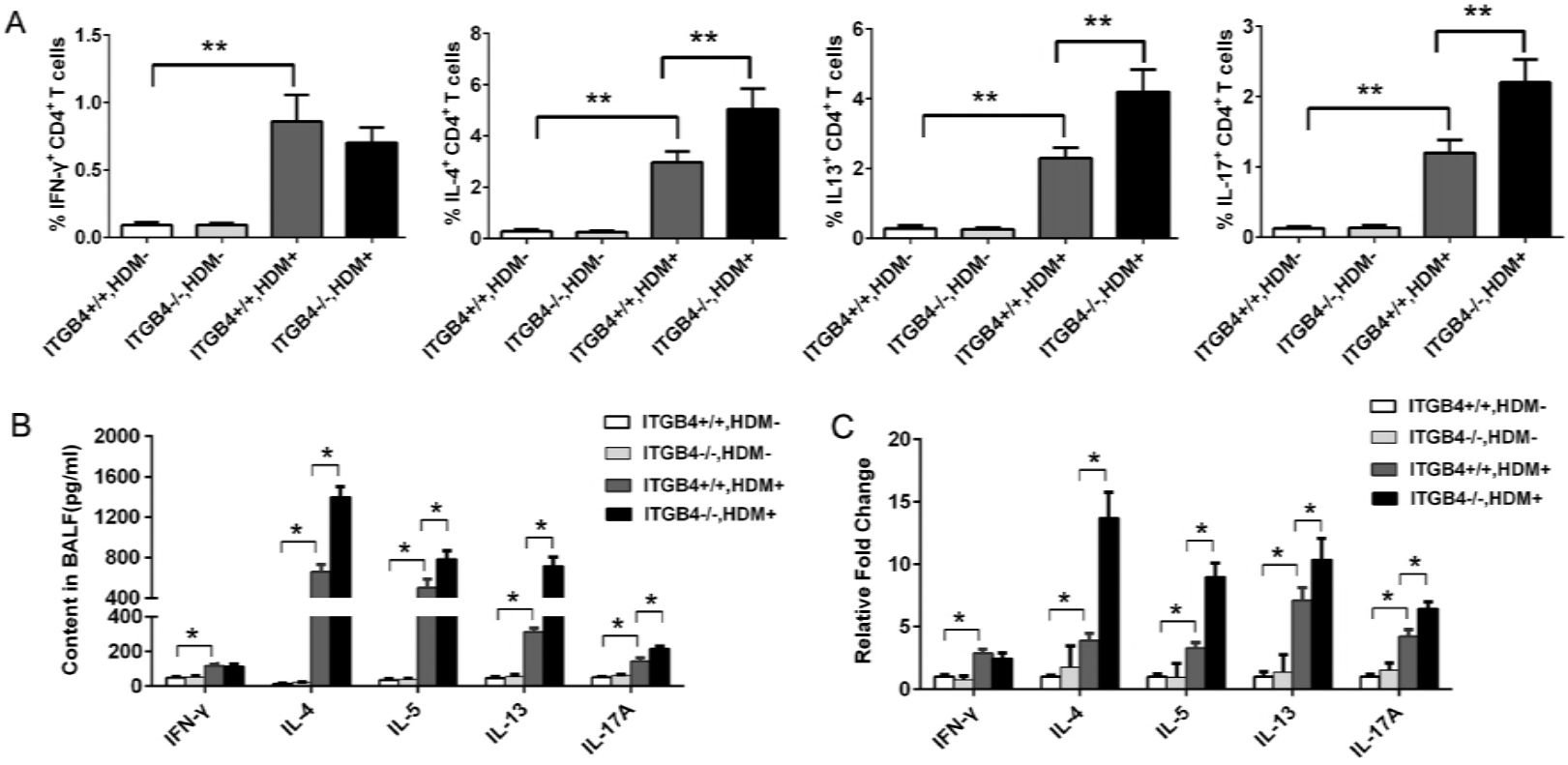
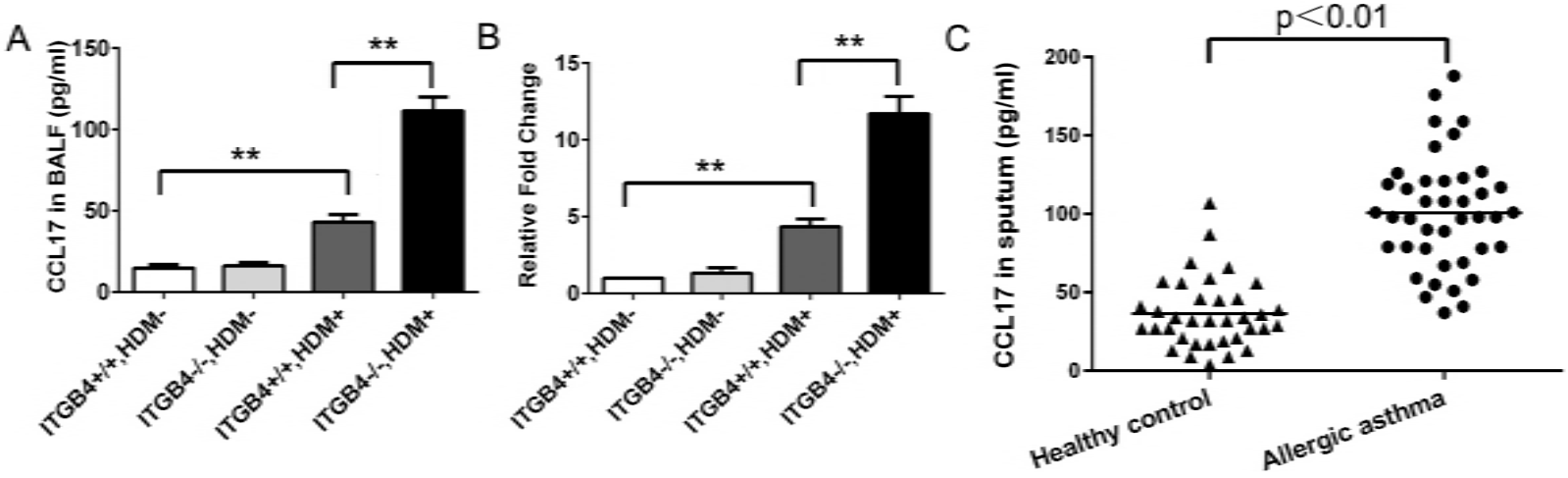
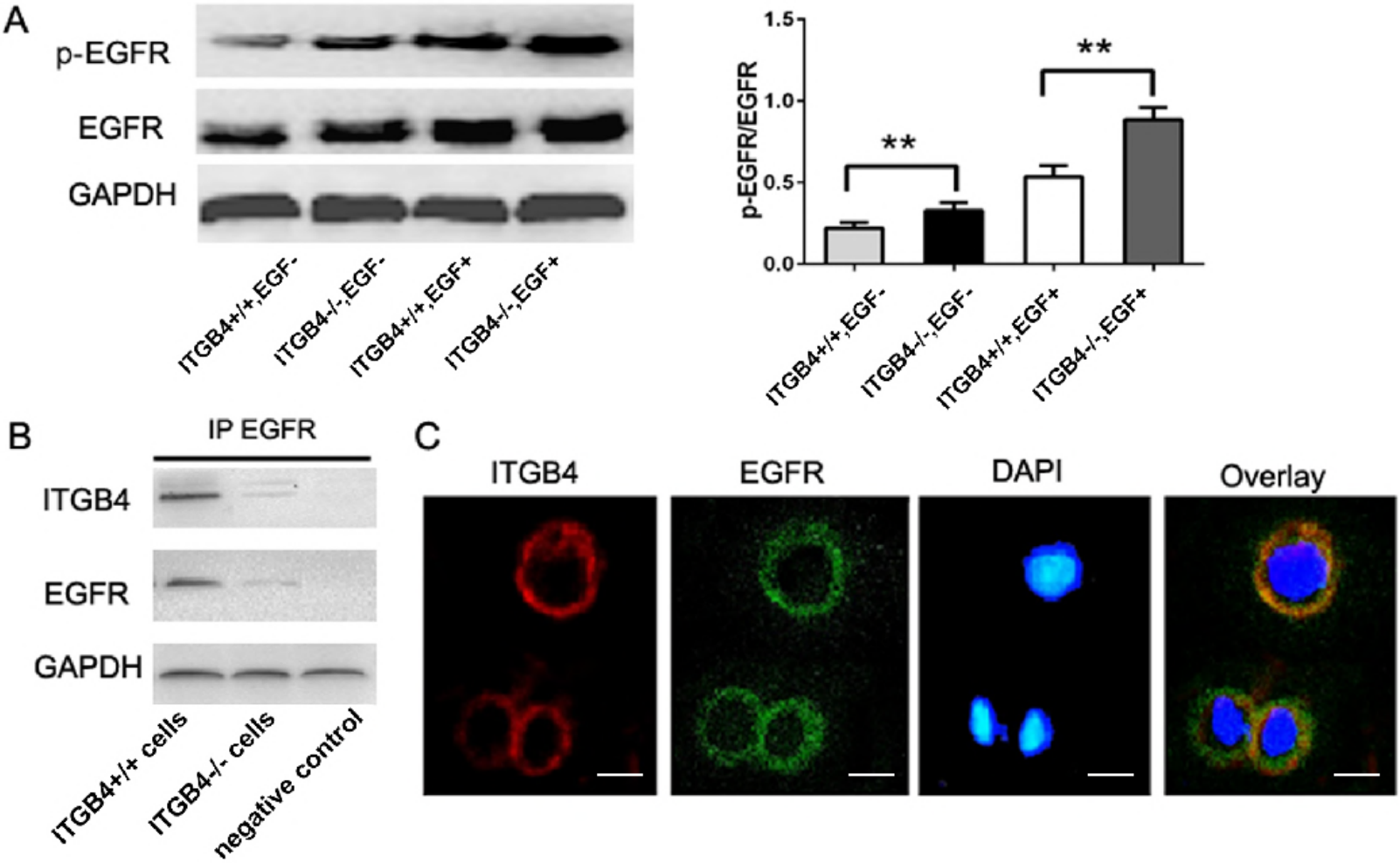
Figure EV1. ITGB4 has no impact on the expression of CCL3, CCL5 and CCL22.

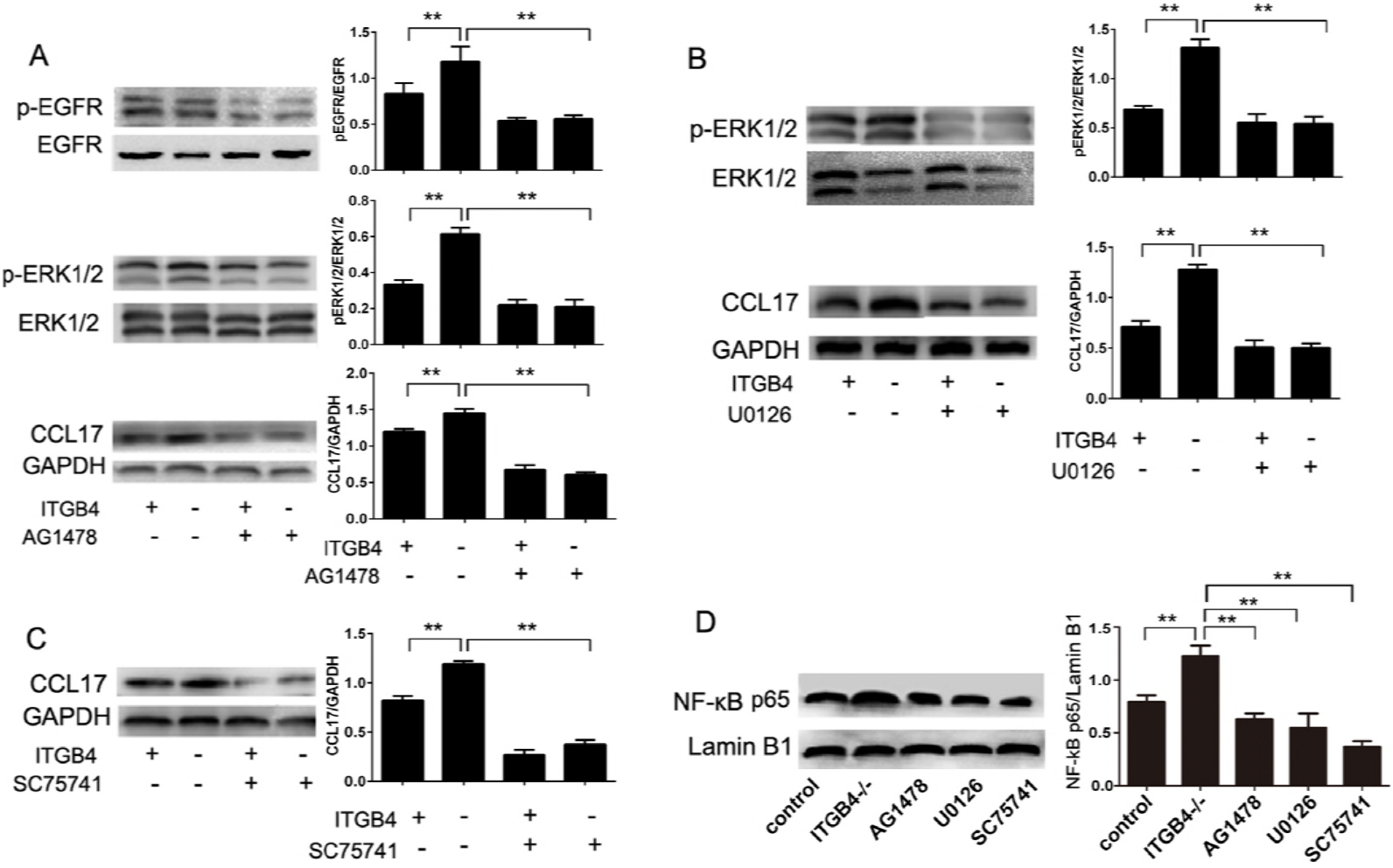
ITGB4$^{+/+}$ or ITGB4$^{-/-}$ mice were exposed to HDM on days 0, 7, 14 or 21. The level of CCL22, CCL3 and CCL5 transcripts in airway epithelial cells (n = 10) were detected by qPCR. Values represented as mean ± SEM. **P< 0.01 compared with controls using controls using an unpaired, 2-tailed Student's t test.

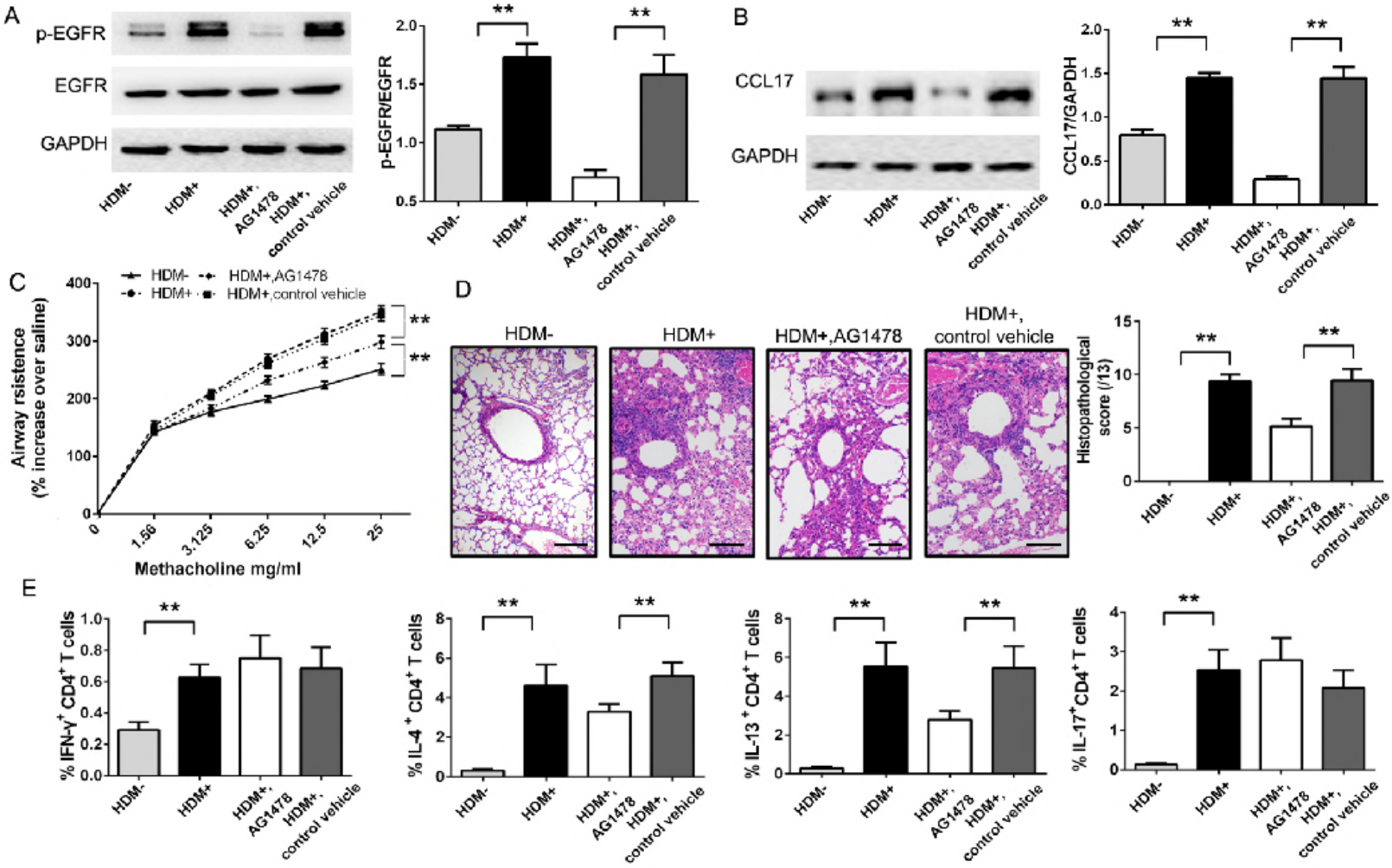

A
HDM or PBS (100ug)
i.n.
Day
0
7
14
21
24
Dox induction (1% in drinking water)
Analysis
B
ITGB4+/+, HDM-
ITGB4-/-, HDM-
ITGB4+/+, HDM+
ITGB4-/-, HDM+
Airway rsistence (% increase over saline)
400
300
200
100
0
**
**
0
1.56
3.125
6.25
12.5
25
Methacholine mg/ml
C
ITGB4+/+, HDM-
ITGB4-/-, HDM-
ITGB4+/+, HDM+
ITGB4-/-,HDM+
Histopathological score (/13)
10
8
6
4
2
0
**
**
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
D
Cell number in BALF(10^5/ml)
50
40
30
20
10
0
**
**
**
**
**
**
**
**
**
**
Total cells
Lymphocytes
Eosinophils
Neutrophils
Macrophages
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+

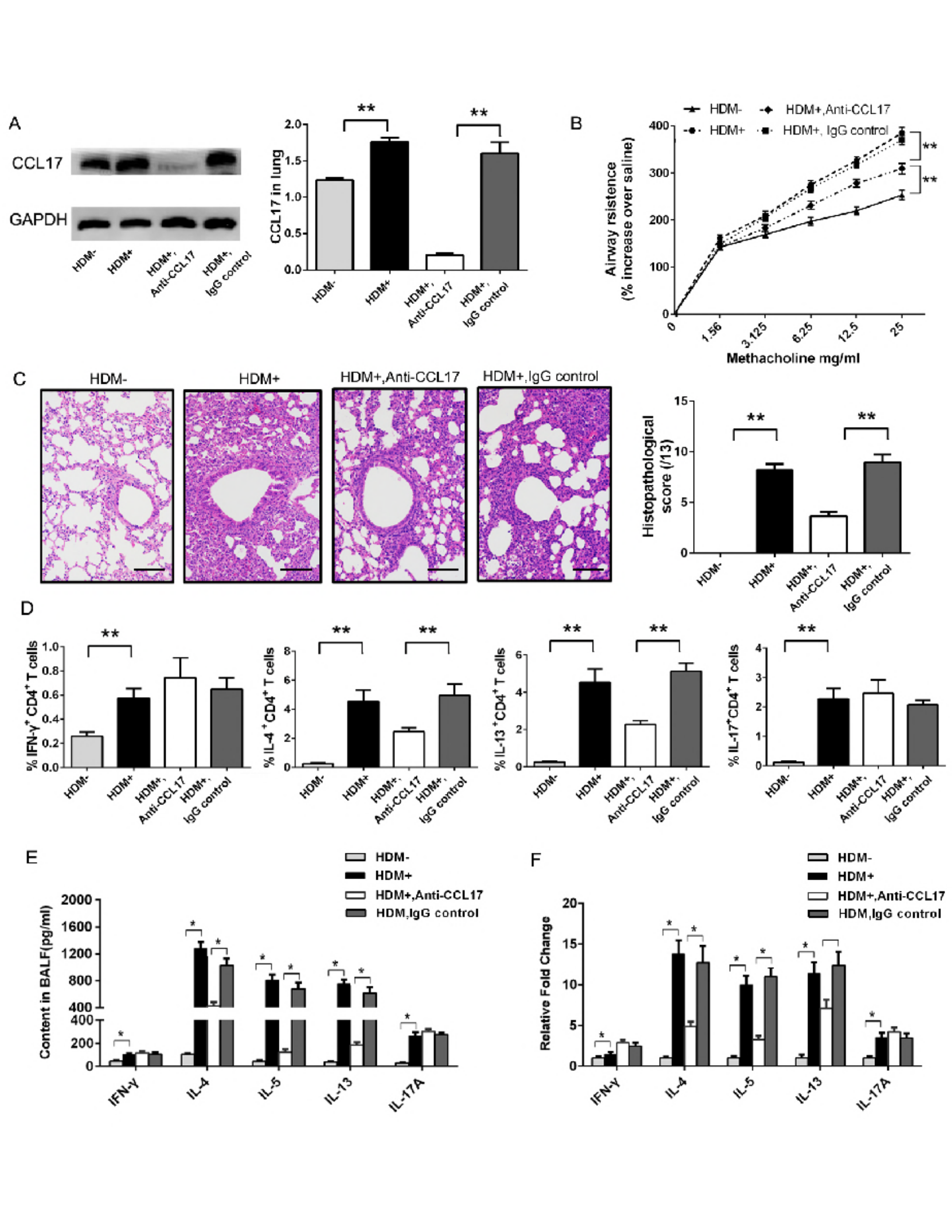

A
% IFN-γ+ CD4+ T cells
% IL-4+ CD4+ T cells
% IL13+ CD4+ T cells
% IL-17+ CD4+ T cells
**
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
B
Content in BALF(pg/ml)
IFN-γ
IL-4
IL-5
IL-13
IL-17A
*
C
Relative Fold Change


A
CCL17 in BALF (pg/ml)
150
100
50
0
**
**
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
B
Relative Fold Change
15
10
5
0
**
**
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
C
p<0.01
CCL17 in sputum (pg/ml)
200
150
100
50
0
Healthy control
Allergic asthma


A
p-EGFR
EGFR
GAPDH
ITGB4+/+,EGF-
ITGB4-/-,EGF-
ITGB4+/+,EGF+
ITGB4-/-,EGF+
p-EGFR/EGFR
1.5
1.0
0.5
0.0
**
**
B
IP EGFR
ITGB4
EGFR
GAPDH
ITGB4+/+ cells
ITGB4-/- cells
negative control
C
ITGB4
EGFR
DAPI
Overlay


A
p-EGFR
EGFR
p-ERK1/2
ERK1/2
CCL17
GAPDH
ITGB4 + - + -
AG1478 - - + +
pEGFR/EGFR
pERK1/2/ERK1/2
CCL17/GAPDH
B
p-ERK1/2
ERK1/2
CCL17
GAPDH
ITGB4 + - + -
U0126 - - + +
pERK1/2/ERK1/2
CCL17/GAPDH
C
CCL17
GAPDH
ITGB4 + - + -
SC75741 - - + +
CCL17/GAPDH
D
NF-κB p65
Lamin B1
control
ITGB4-/-
AG1478
U0126
SC75741
NF-κB p65/Lamin B1


A
p-EGFR
EGFR
GAPDH
HDM-
HDM+
HDM+, AG1478
HDM+, control vehicle
p-EGFR/EGFR
B
CCL17
GAPDH
CCL17/GAPDH
C
HDM-
HDM+
HDM+,AG1478
HDM+,control vehicle
Airway rsistence (% increase over saline)
Methacholine mg/ml
D
HDM+,AG1478
HDM+, control vehicle
Histopathological score (/13)
E
% IFN-γ+ CD4+ T cells
% IL-4+ CD4+ T cells
% IL-13+ CD4+ T cells
% IL-17+CD4+ T cells


A
CCL17
GAPDH
HDM-
HDM+
HDM+, Anti-CCL17
HDM+, IgG control
CCL17 in lung
B
HDM-
HDM+
HDM+,Anti-CCL17
HDM+, IgG control
Airway rsistence (% increase over saline)
Methacholine mg/ml
C
HDM-
HDM+
HDM+,Anti-CCL17
HDM+,IgG control
Histopathological score (/13)
D
% IFN-γ+ CD4+ T cells
% IL-4 +CD4+ T cells
% IL-13 +CD4+ T cells
% IL-17+CD4+ T cells
E
HDM-
HDM+
HDM+,Anti-CCL17
HDM,IgG control
Content in BALF(pg/ml)
IFN-γ
IL-4
IL-5
IL-13
IL-17A
F
Relative Fold Change

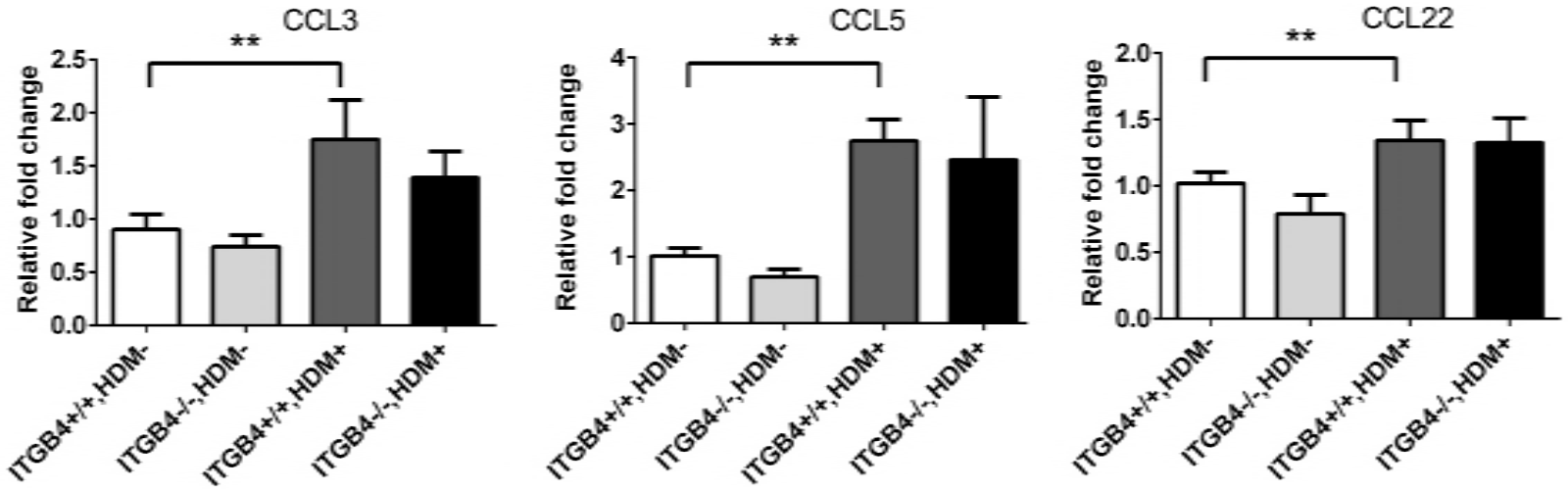

CCL3
**
Relative fold change
2.5
2.0
1.5
1.0
0.5
0.0
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
CCL5
**
Relative fold change
4
3
2
1
0
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
CCL22
**
Relative fold change
2.0
1.5
1.0
0.5
0.0
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+

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Table EV1: Characteristics of patients with allergic asthma and controls

	Control	Asthmatics
Number	n = 35	n = 41
Gender (f/m)	17/18	19/22
Age	27 (18-39)	29 (16-45)
Atopy, n (%) **	7 (20)	46 (71)
Total IgE (IU/ML)**	65 (7-182)	284 (29-1257)
FEV1(%)*	105 (92–134)	92 (45–114)
FEV1:FVC*	91 (78–99)	79 (51–86)

FEV1, forced expiratory volume in 1s; FVC, forced vital capacity.
Data are presented as mean±SD or median (range).

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Table EV2: TaqMan primers for qRT-PCR.

Mouse gene in Taqman expression assay	Primer sequence
IFN-γ	Forward: 5'-AGCAAGGCGAAAAAGGATGC-3'
	Reverse: 5'-TCATTGAATGCTTGGCGCTG-3'
IL-4	Forward: 5'-CCAAACGTCCTCACAGCAAC-3'
	Reverse: 5'-AGGCATCGAAAAGCCCGAA-3'
IL-5	Forward: 5'-AAGCAATGAGACGATGAGGCT-3'
	Reverse: 5'-CCCCACGGACAGTTTGATTCT-3'
IL-13	Forward: 5'-CTTGCTTGCCTTGGTGGTCT-3'
	Reverse: 5'-TCCATACCATGCTGCCGTTG-3'
IL-17A	Forward: 5'-TACCTCAACCGTTCCACGTC-3'
	Forward: 5'-TACCTCAACCGTTCCACGTC-3'
	Reverse: 5'-TTTCCCTCCGCATTGACACA-3'
CCL3	Forward: 5'-ACT GCC CTT GCT GTT CTT CT-3'
	Reverse: 5'-CTG CCG GTT TCT CTT AGT CA-3'
CCL5	Forward: 5'-GTGCTCCAATCTTGCAGTCG-3'
	Reverse: 5'-AGAGCAAGCAATGACAGGGAA-3'
CCL17	Forward: 5'-TACCATGAGGTCACTTCAGATGC-3'
	Reverse: 5'-GCACTCTCGGCCTACATTGG-3'
CCL22	Forward: 5'- CTTGCTGTGGCAATTCAGACC-3'
	Reverse: 5'-ACTAAACGTGATGGCAGAGGG-3'