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1 **Airway epithelial integrin β4 suppresses allergic inflammation by decreasing**
2 **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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41 **Introduction**

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

51 Allergic asthma is a chronic airway disorder that is characterized with
52 epithelial desquamation, airway inflammation and airway hyperresponsiveness (AHR)
53 to non-specific spasmogen (Matucci et al, 2018). Although $CD4^+$ Th2 cells (Th2 cells)
54 are widely recognized to orchestrate the development of the disease by expressing
55 Th2 cytokines (e.g. interleukin (IL)-4, IL-5 and IL-13), there is compelling evidence
56 that airway epithelium plays a vital role in the induction of aberrant immune
57 responses underlying the pathogenesis of allergic asthma due to its' frontline location
58 to directly contact aerosol allergen and other environmental insultants (Lambrecht &
59 Hammad, 2015). Indeed, AECs from asthma patients are often damaged and loss their
60 homeostasis status including detachment, fragility and abnormal repair ability etc.
61 (KleinJan, 2015; Wang et al, 2008). Upon allergen exposure, the repair process of
62 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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85 5, 17 and 22 are the main chemokines that selectively bind to CCR4 (Holgate, 2012).
86 Indeed, high levels of CCL17 and CCL22 have been found not only in the lung and
87 bronchoalveolar lavage fluid (BALF) of asthma patients but also in those of
88 ovalbumin (OVA) sensitized and challenged mice (Gonzalo et al, 1999; Hijnen et al,
89 2004). CCL17 and CCL22 are predominantly produced by AECs and pulmonary
90 macrophages, respectively (Gonzalo et al, 1999; Kawasaki et al, 2001). In particular,
91 the former chemokine is increasingly being recognized as a key epithelial secreted
92 attractant for Th2 cells recruitment to the asthmatic airways (Hijnen et al, 2004).
93 Indeed, clinical studies have demonstrated that the level of CCL17 positively
94 correlates with the degree of airway obstruction and fractional exhaled nitric oxide
95 (FeNo) of asthma patients (Ying et al, 2005). However, the mechanisms how AECs
96 contribute to the development of asthma by employing CCL17 still remain largely
97 unknown.

98 Interestingly, ITGB4 is located at the basal surface of AECs and regulates the
99 stable adhesion of these cells to the underlying basement membrane (Pan et al, 2016).
100 ITGB4 has a unique long cytoplasmic domain subunit that could recruit a range of
101 signaling molecules. ITGB4 plays a key role in the activation of several intracellular
102 signaling pathways including phosphatidylinositol 3-kinase (PI3-K) (Shaw et al,
103 1997), extracellular signal-regulated kinases (ERK) 1/2 (Chen et al, 2010) and NF-κB
104 (Nikolopoulos et al, 2004), indicating that this integrin potentially contributes to the
105 instigation of subsequent immune response and inflammation. Although there is a
106 correlation between ITGB4 and the induction of allergic asthma, much remains

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

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

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121 ## Results

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

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

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

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

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

154 **ITGB4 negatively impacts on CCL17 production**

155 Both clinic and animal studies have shown that Th2 cells orchestrate the development
156 of allergic asthma (KleinJan, 2015). To determine how ITGB4 contributes the
157 activation of Th2 cells, we examined the levels of CCL3, CCL5, CCL17 and CCL22
158 that could bind to CCR4, a Th2 chemotactic receptor (Assarsson et al, 2004; Qiu et al,
159 2018; Yoshie & Matsushima, 2015). Significant higher levels of CCL17 protein and
160 transcript were found in the BALF and lung of HDM exposed ITGB4$^{-/-}$ mice, as
161 compared to those of HDM exposed ITGB4$^{+/+}$ mice (Fig 3A and B). Interestingly,
162 there was no significant difference in the transcript levels of CCL3, CCL5 and CCL22
163 in cultured AECs of HDM exposed ITGB4$^{-/-}$ mice, as compared to those of HDM
164 exposed ITGB4$^{+/+}$ mice (Fig EV1). Sputum samples from patients with allergic

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

170 **ITGB4 inhibits the EGFR phosphorylation of AECs**

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

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

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

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

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

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

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

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

220 As a key role for CCL17 is indicated in the pathogenesis, we neutralized this
221 chemokine with anti-CCL17 mAb in HDM exposed ITGB4$^{-/-}$ mice. Treatment with
222 anti-CCL17 mAb significantly and greatly decreased the levels the chemokine in
223 lung, as compared to isotype Ab treatment (Fig 7A). Neutralization of CCL17
224 significantly suppressed exaggerated AHR, airway inflammation in HDM exposed
225 ITGB4$^{-/-}$ mice (Fig 7B and C). Of note, anti-CCL17 mAb treatment reduced the levels
226 of CD4$^{+}$IL-4$^{+}$ T cells and CD4$^{+}$IL-13$^{+}$ T cells but not CD4$^{+}$IFN-$\gamma^{+}$ T cells and
227 CD4$^{+}$IL-17$^{+}$ T cells in the lung of HDM exposed ITGB4$^{-/-}$ mice (Fig 7D). Similarly,
228 neutralizing CCL17 decreased the protein and transcript levels of IL-4, IL-5, and IL-
229 13, but not those of IFN-$\gamma$ and IL-17A in the BALF and lung of HDM exposed
230 ITGB4$^{-/-}$ mice, as compared to isotype Ab treatment (Fig 7E and F).

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231 **Discussion:**

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

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

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

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

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

280 Although the production of CCL17 by AECs is known, it is still largely
281 obscure how integrins contribute to the increased expression of CCL17 in allergen
282 induced asthma. With ITGB4$^{-/-}$ mice, we were able to identify that the
283 synchronization between ITGB4 and EGFR essentially orchestrates CCL17
284 production in AECs through the action of multiple downstream signaling pathways
285 including EGFR, ERK1/2 and NF-κB (Fig 4-5). Studies in cancer research have
286 shown that interaction between ITGB4 and EGFR phosphorylation has a significant
287 impact on the progression and metastasis of hepatocellular carcinoma cells, mammary
288 tumour cells and mammary tumour cells (Bon et al, 2007; Huafeng et al, 2018; Leng
289 et al, 2016). EGFR, a receptor tyrosine kinase, can induce phosphorylation of multiple
290 intracellular transcriptional regulators including ERK1/2, and PI3K pathway (Jiang et
291 al, 2017; Zhu et al, 2015). Among these signaling regulators, EGFR is essential for the
292 activation of ERK signaling that critically mediate the production of pro-
293 inflammatory factors (Huang et al, 2017). Our results are in line with the
294 aforementioned findings, and have identified that a direct interaction between ITGB4
295 and EGFR in airway epithelial cells drives the development of Th2 cells-associated
296 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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319 allergic asthma by the regulation of Th2 and Th17 responses.

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

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329 **Methods**

330 **Collection of sputum samples**

331 Asthmatic patients (defined by clinical diagnosis with evidence of bronchodilator
332 reversibility testing, clinical assessment and a confirmed history of atopy with raised,
333 allergen-specific IgE or positive radioallergosorbent test to at least one aero-allergen )
334 (Matucci et al, 2018) were recruited and categorized by induced sputum inflammatory
335 cell counts as allergic asthma. Participants provided written informed consent,
336 approved by the No.20180308 Central south University Research Ethics Committees.
337 The demographic and clinical characteristics are shown in Table EV1. Induced
338 sputum samples were obtained using nebulized hypertonic (4.5%) saline, treated with
339 dithiothreitol and counts and viability (trypan blue exclusion) were determined by
340 hemocytometer. The plugs were treated with a volume of dithiothreitol representing 4
341 times the mucus weight. Then, an equal volume of PBS was added to all samples
342 before filtration with a 0.45 μm nylon filter. The supernatants were collected for
343 CCL17 quantitation.

344 **Animals**

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

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

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

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

368 **Measurement of lung function**

369 Airway resistance was measured using a direct plethysmography (Biosystems XA;
370 Buxco Electronics, USA), as previously described (Liu et al, 2010b). In brief, mice
371 were anesthetized with a mixture containing xylazine (10 mg/kg) and ketamine (100
372 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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395 (ab52894, Abcam) in 1% BSA/PBS overnight at 4°C, washed with 1% BSA/PBS and
396 incubated with Cy3-conjugated or FITC-conjugated goat anti-rabbit IgG (10 mg/ml;
397 GE Healthcare) for 45 min respectively, then washed with 1% BSA/PBS and stained
398 with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 10 min at RT. Slips
399 were fixed on slides. ITGB4 and EGFR in AECs were visualized using a fluorescence
400 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with a ×100
401 objective lens. Images were captured using a digital camera (Axio-Cam ICc3, Spectra
402 Service, Ontario, NY) and analysed using AxioVision Rel. 4.7 software (Zeiss).
403 Sampling was performed on eight to ten different areas for 40-60 cells of each slide.

404 **Flow cytometry**

405 Single cell suspensions from lungs were prepared as previously described (Teijaro et
406 al, 2011) and were used for flow cytometric analysis. In brief, cell suspensions were
407 prepared by enzymatically digesting the lung tissue using dispase (Discovery
408 Labware; Corning, Bedford, MA). Following incubation with surface marker
409 antibodies anti-CD45, (clone 104，BD Biosciences, San Jose, CA), anti-CD3 (clone
410 17A2，BD Biosciences, San Jose, CA), anti-CD4 (clone GK1.5, BD Biosciences,
411 San Jose, CA) and isotype controls (Rajavelu et al, 2015), cells were fixed using 4%
412 PFA and permeabilized using Cytofix/Cytoperm solution (BD Biosciences, San Jose,
413 CA). Cells were then incubated with anti-IFN-γ (Cat. no. 12-7311), anti-IL-4 (Cat. no.
414 12-7041), anti-IL-13 (Cat. no. 12-7133) and anti-IL-17 (Cat. no. 12-7177) antibodies
415 (eBioscience, San Diego, CA). Numbers of positive cells were quantified by flow
416 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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439 (1μM, an EGFR phosphorylation inhibitor), U0126 (10μM, an ERK phosphorylation
440 inhibitor), or SC75741 (5μM, a NF-κB p65 inhibitor) respectively. Levels of ITGB4,
441 CCL17, EGFR, phosphorylated EGFR (p-EGFR), ERK, phosphorylated ERK (p-
442 ERK) and NF-κB p65 were determined respectively with anti-mouse antibodies
443 against these proteins by western blot as previously reported (Jiang et al, 2014).
444 GAPDH and Lamin B1 were used as controls as indicated.

445 **Immunoprecipitation**

446 Protein were extracted with radioimmunoprecipitation assay (RIPA) buffer: 50
447 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate,
448 0.1%SDS, 5 mmol/L EDTA, pH=7.4. Protein concentration was measured by BCA
449 protein determination kit (PC0020, Solarbio, China). Immunoprecipitations were
450 performed overnight using the appropriate dilution of a polyclonal anti-ITGB4
451 antibody (Ab197772, Abcam) added to the lysates (1 μg proteins), under rotatry
452 mixing for 1 at 4°C h. The immune complexes were allowed to bind to Protein A-
453 Sepharose beads at 4°C for 3 h, washed (15 min, 4°C) with RIPA buffer and
454 resuspended in loading buffer. Immunoprecipitates were subjected to SDS, 10%
455 PAGE and immunoblotted on a polyvinylidene difluoride membrane.
456 Immunocomplexes were stained with anti-ITGB4 (Ab197772, Abcam) as well as
457 EGFR (Ab52894, Abcam) using ECL according to the manufacturer's guidelines
458 (NCI4106, Thermo Pierce, USA).

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468 **Author contributions**

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

473 **Conflict of interest**

474 The authors declare that they have no conflict of interest.

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641 **Figure legend.**

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

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

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

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

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

655

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

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

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

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670 **Figure 3. CCL17 production and Th2 cells infiltration are further elevated by**
671 **the deficiency of ITGB4.**
672 A, B Mice were sensitized to HDM on days 0, 7, 14 or 21. Some mice were treated
673 with 1% Dox in drinking water to specifically delete ITGB4 in airway
674 epithelial cells. Control non-sensitized mice received PBS. CCL17 protein in
675 BALF (n = 8) and the levels of CCL17 transcripts in lung (n = 6) were
676 determined by ELISA and qPCR, respectively. Values represented as mean ±
677 SEM. **P < 0.01 compared with controls using an unpaired, Student's t test.
678 C CCL17 expression detected in induced sputum samples from allergic asthmatic
679 patients (n = 41) and control subjects (n = 35). Values represented as mean ±
680 SEM. **P < 0.01 using an unpaired, Student's t test.
681
682 **Figure 4. Physical interaction between ITGB4 and EGFR and the**
683 **phosphorylation of EGFR in airway epithelial cells of ITGB4$^{+/+}$ or**
684 **ITGB4$^{-/-}$ mice.**
685 A Airway epithelial cells were isolated from the lung of ITGB4$^{+/+}$ or ITGB4$^{-/-}$
686 mice. Cells were cultured and then stimulated with or without EGF (1 ng/ml)
687 for 60 min. Western blot staining of stimulated AECs for p-EGFR and total
688 EGFR, and p-EGFR/EGFR ratios normalized to vehicle control. Values
689 represented as mean ± SEM for six samples from one experiment and
690 representative of 3 independent experiments. **P<0.01 using an unpaired,
691 Student's t test.
692 B Immunoprecipitation of stimulated AECs for interaction between ITGB4 and
693 EGFR.
694 C Immunofluorescence staining of stimulated AECs for co-localization of ITGB4
695 and EGFR, bars: 10 μm.
696
697 **Figure 5. ITGB4 deficiency leads to augmented activation of EGFR, ERK1/2 and**
698 **NF-κB pathways.**

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699 A Airway epithelial cells were isolated from the lung of ITGB4$^{+/+}$ or ITGB4$^{-/-}$
700 mice. Cells were cultured and then stimulated with or without EGF (1 ng/ml)
701 for 60 min. (A) Western blot staining of stimulated AECs for phosphorylated
702 EGFR (p-EGFR), phosphorylate ERK1/2 (p-ERK1/2), total EGFR, total
703 ERK1/2, and CCL17 in the presence or absence of EGFR tyrosine
704 phosphorylation inhibitor AG1478.
705 B Western blot staining of stimulated AECs for p-ERK1/2, total ERK1/2 and
706 CCL17 in the presence or absence of ERK1/2 tyrosine phosphorylation
707 inhibitor U0126.
708 C Western blot staining of stimulated AECs for CCL17 in the presence or absence
709 of NF-κB inhibitor SC75741.
710 D Western blot staining of nuclear extracts of stimulated ITGB4$^{-/-}$ AECs for NF-
711 κB p65 in the presence or absence of AG1478, U1026 and SC75741, NF-κB
712 p65/Lamin B1 ratios normalized to vehicle control. All values represented as
713 mean ± SEM for six samples from one experiment and representative of 3
714 independent experiments. **P<0.01 using an unpaired, Student's t test.
715
716 **Figure 6. Blockade of EGFR phosphorylation inhibits both CCL17 production**
717 **and subsequent enhanced AHR, lung inflammation in HDM exposed**
718 **ITGB4$^{-/-}$ mice.**
719 A, B AECs-specific ITGB4 conditional knock out mice were constructed and
720 exposed HDM (HDM+) or PBS (HDM-) on days 0, 7, 14 or 21. AECs were
721 isolated from these mice and some cells were treated with EGFR inhibitor
722 AG1478 or control vehicle. The protein levels of EGFR, p-EGFR and CCL17
723 in AECs was detected by western blot. Values represented as mean ± SEM for
724 5 samples from one experiment and representative of 3 independent
725 experiments. **P<0.01 using an unpaired, Student's t test. Some mice
726 received injection with either AG1478 or same volume of the control vehicle.
727 C Two days after last HDM exposure, AHR was represented as airway resistance

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728 in response to methacholine. Data represent the mean ± SEM of 6 mice per
729 group. **P < 0.01 by 2-way ANOVA followed by Fisher post hoc test.
730 D, E Lung histology was assessed (n = 8) and the levels of IFN-$\gamma^+$CD4$^+$, IL-4$^+$CD4$^+$,
731 IL-13$^+$CD4$^+$ and IL-17A$^+$CD4$^+$ T cells in lung were determined (n = 10), bars:
732 50 μm. Values represented as mean ± SEM. **P < 0.01 compared with
733 controls using an unpaired, Student's t test.
734
735 **Figure 7. Neutralization of CCL17 reduces exaggerated AHR, airway**
736 **inflammation and Th2 responses in HDM sensitized ITGB4$^{-/-}$ mice.**
737 A Mice were treated with 1% Dox in drinking water to specifically delete ITGB4
738 in airway epithelial cells and were also sensitized with either HDM or PBS on
739 days 0, 7, 14 or 21. Some mice received treatment with either anti-CCL17 or
740 isotype control antibodies. Two days after the final challenge, CCL17 protein
741 in lung was determined by western blot, ratios of CCL17/GAPDH normalized
742 to vehicle control. Values represented as mean ± SEM for 6 samples from one
743 experiment and representative of 3 independent experiments. **P<0.01 using
744 an unpaired, Student's t test.
745 B Two days after last HDM exposure, AHR was represented as airway resistance
746 in response to methacholine. Data represent the mean ± SEM of 6 mice per
747 group. **P < 0.01 by 2-way ANOVA followed by Fisher post hoc test.
748 C, D Lung histology was assessed (n = 8) and the levels of IFN-$\gamma^+$CD4$^+$, IL-4$^+$CD4$^+$,
749 IL-13$^+$CD4$^+$ and IL-17A$^+$CD4$^+$ T cells in lung were determined (n = 10), bars:
750 50 μm.Values represented as mean ± SEM. **P < 0.01 compared with
751 controls using an unpaired, 2-tailed Student's t test.
752 E, F Two days after the final challenge, the levels of IFN-γ, IL-4, IL-13, and IL-17A
753 protein in BALF (n = 8) and their transcripts in lung (n = 6) were examined by
754 ELISA and qPCR, respectively. Values represented as mean ± SEM. *P< 0.05
755 compared with controls using one-way ANOVA followed
756 by Dunnett's post hoc test.

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757

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

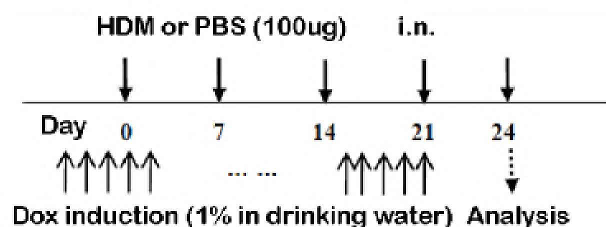
759 ITGB4$^{+/+}$ or ITGB4$^{-/-}$ mice were exposed to HDM on days 0, 7, 14 or 21. The

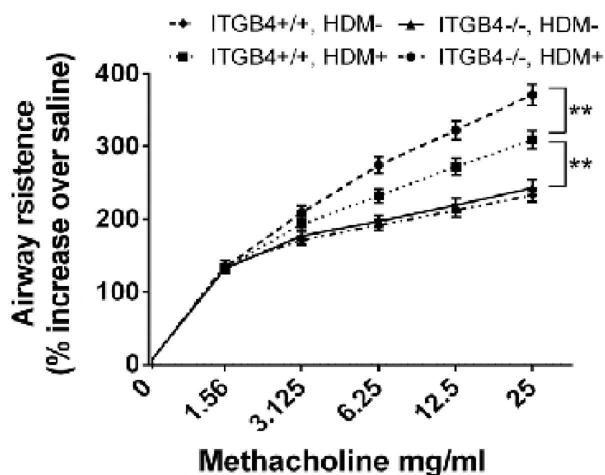
760 level of CCL22, CCL3 and CCL5 transcripts in airway epithelial cells (n = 10)

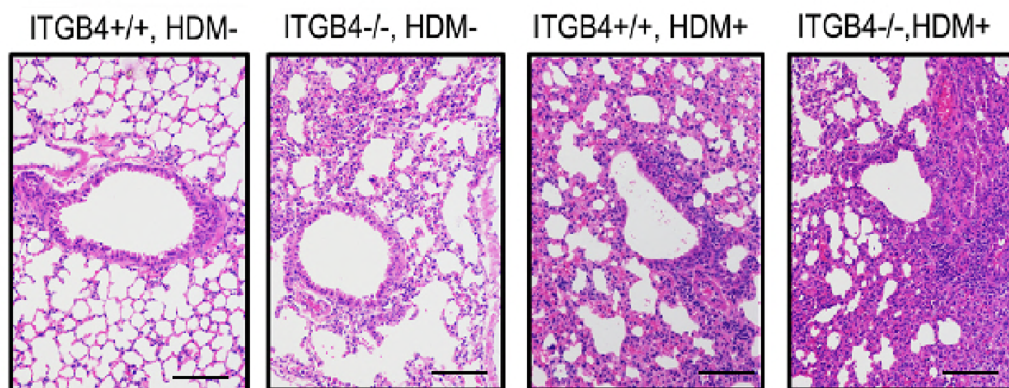
761 were detected by qPCR. Values represented as mean ± SEM. **P< 0.01

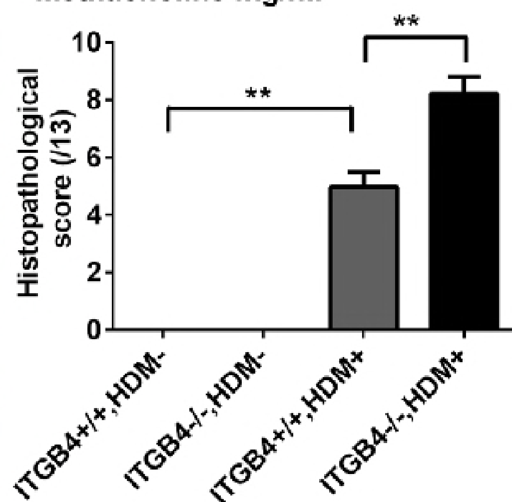
762 compared with controls using controls using an unpaired, 2-tailed Student's t

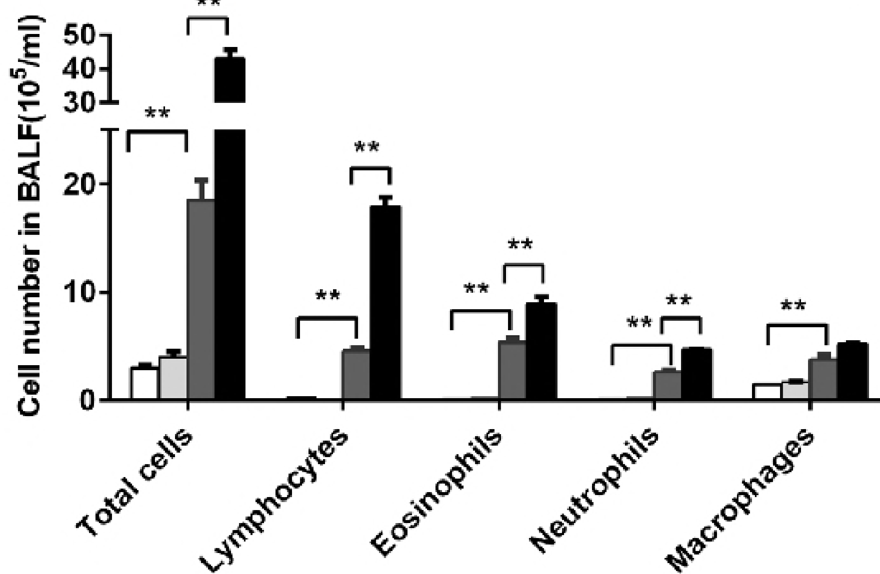
763 test.

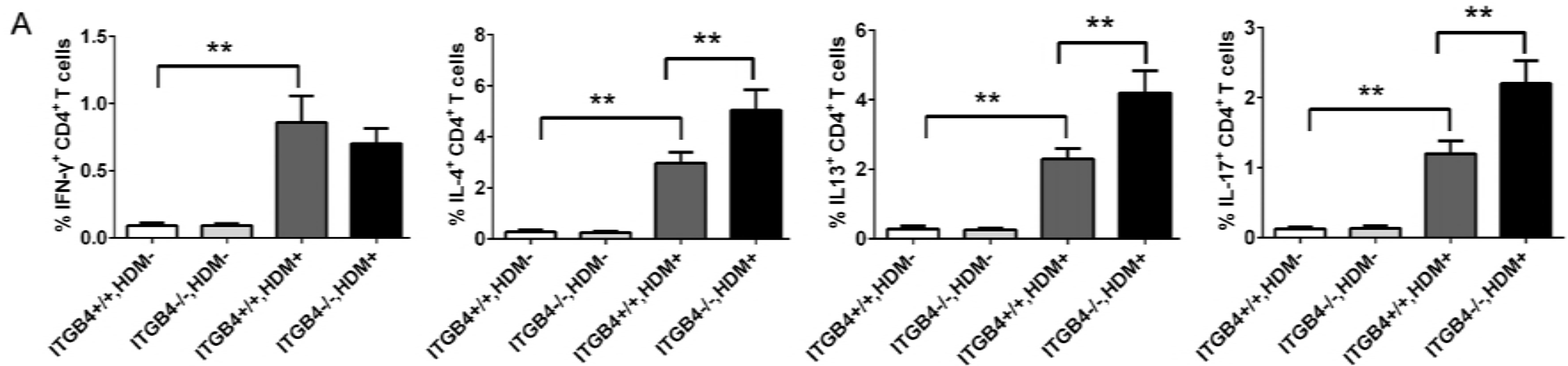
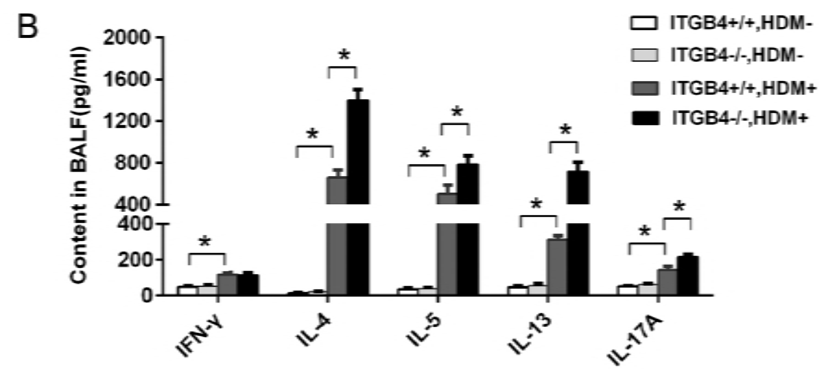
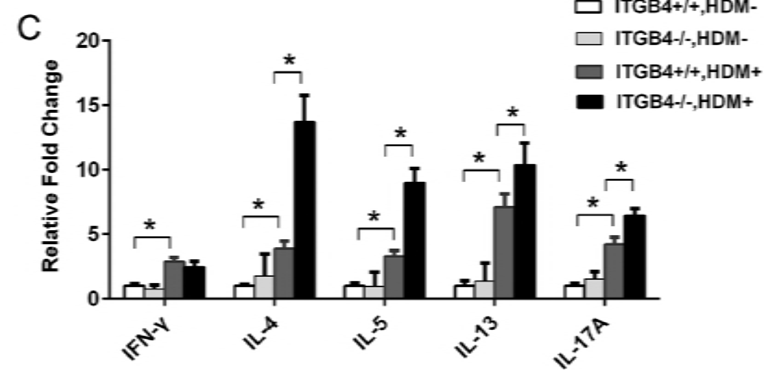

A
HDM or PBS (100ug)
i.n.
Day
0
7
14
21
24
Dox induction (1% in drinking water)
Analysis

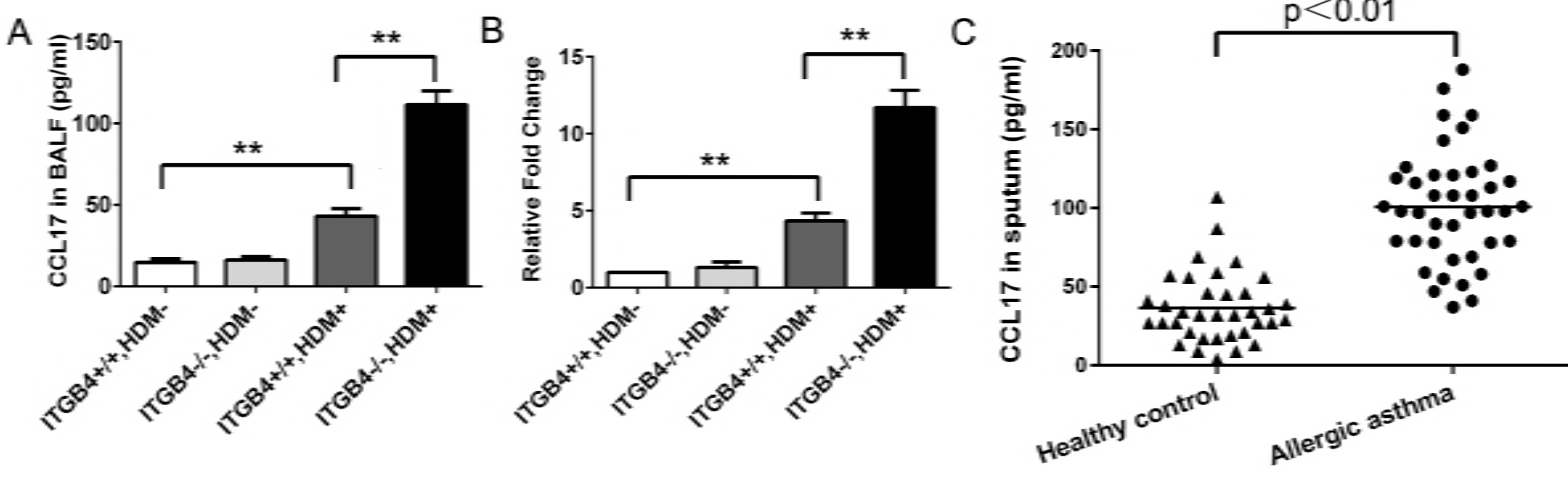

B
ITGB4+/+, HDM-
ITGB4-/-, HDM-
ITGB4+/+, HDM+
ITGB4-/-, HDM+
Airway rsistence
(% increase over saline)
Methacholine mg/ml
**
**

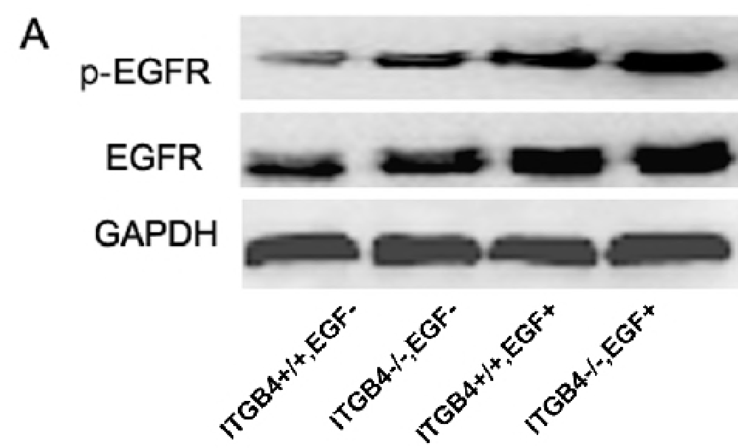

C
ITGB4+/+, HDM-
ITGB4-/-, HDM-
ITGB4+/+, HDM+
ITGB4-/-,HDM+

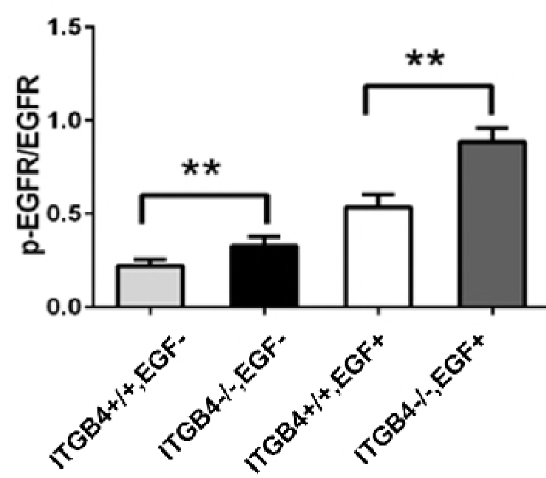

Histopathological score (/13)
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
**
**

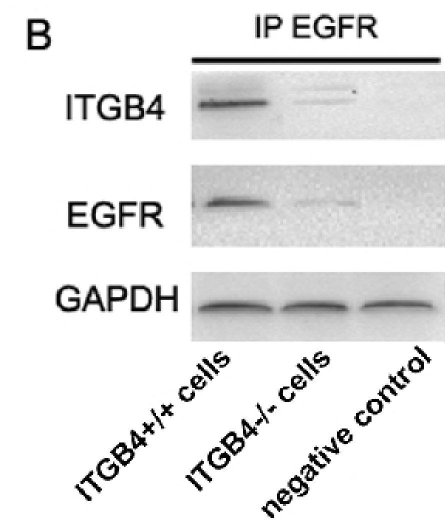

D
Cell number in BALF(10^5/ml)
Total cells
Lymphocytes
Eosinophils
Neutrophils
Macrophages
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+

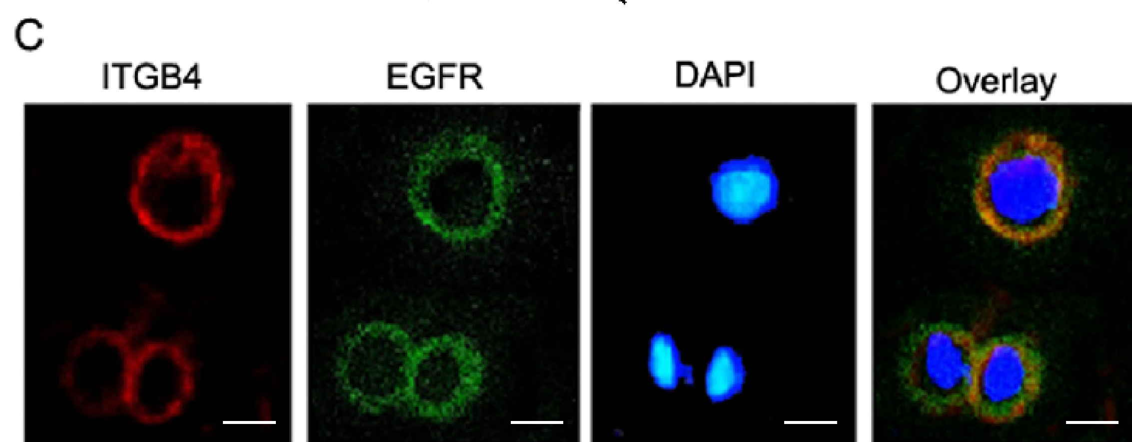

A
% IFN-γ+ CD4+ T cells
% IL-4+ CD4+ T cells
% IL13+ CD4+ T cells
% IL-17+ CD4+ T cells
**
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+

B
Content in BALF(pg/ml)
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
IFN-γ
IL-4
IL-5
IL-13
IL-17A

C
Relative Fold Change
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
IFN-γ
IL-4
IL-5
IL-13
IL-17A

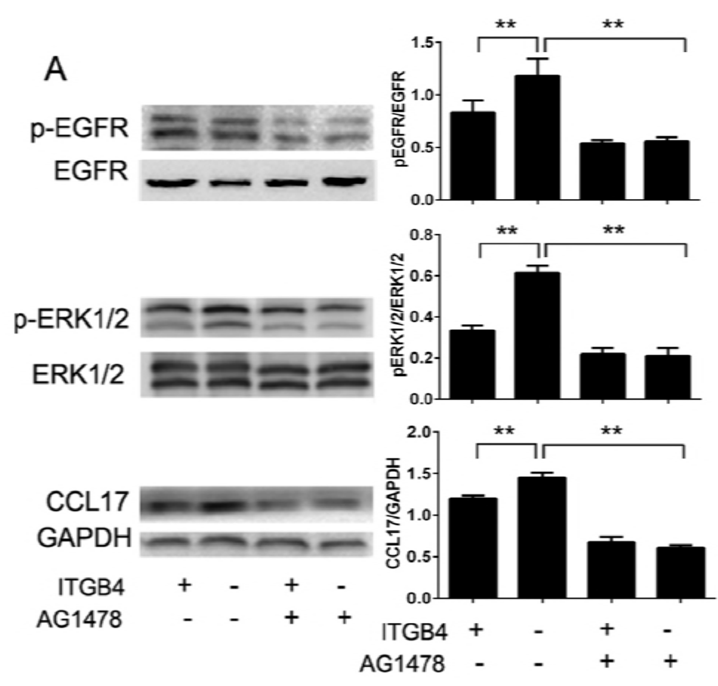

A
CCL17 in BALF (pg/ml)
150
100
50
0
**
**
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
B
Relative Fold Change
15
10
5
0
**
**
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+
C
p<0.01
CCL17 in sputum (pg/ml)
200
150
100
50
0
Healthy control
Allergic asthma

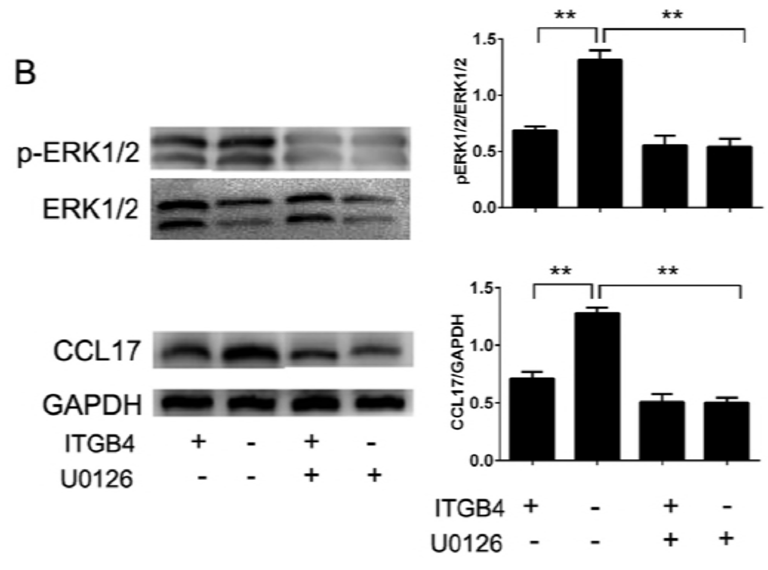

A
p-EGFR
EGFR
GAPDH
ITGB4+/+,EGF-
ITGB4-/-,EGF-
ITGB4+/+,EGF+
ITGB4-/-,EGF+

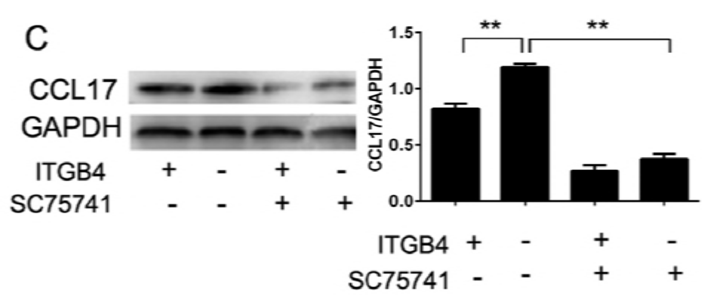

p-EGFR/EGFR
1.5
1.0
0.5
0.0
**
**
ITGB4+/+,EGF-
ITGB4-/-,EGF-
ITGB4+/+,EGF+
ITGB4-/-,EGF+

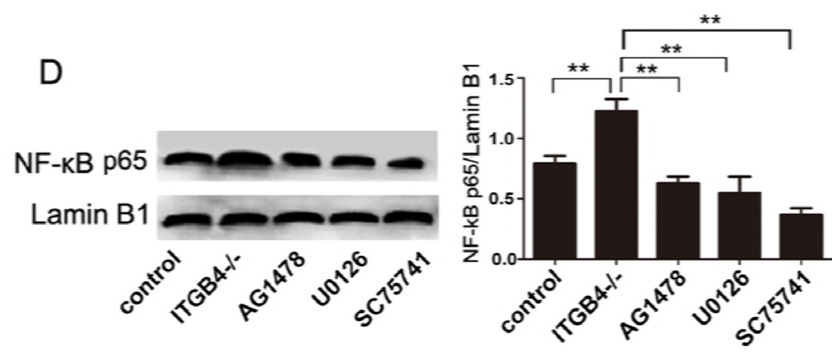

B
IP EGFR
ITGB4
EGFR
GAPDH
ITGB4+/+ cells
ITGB4-/- cells
negative control

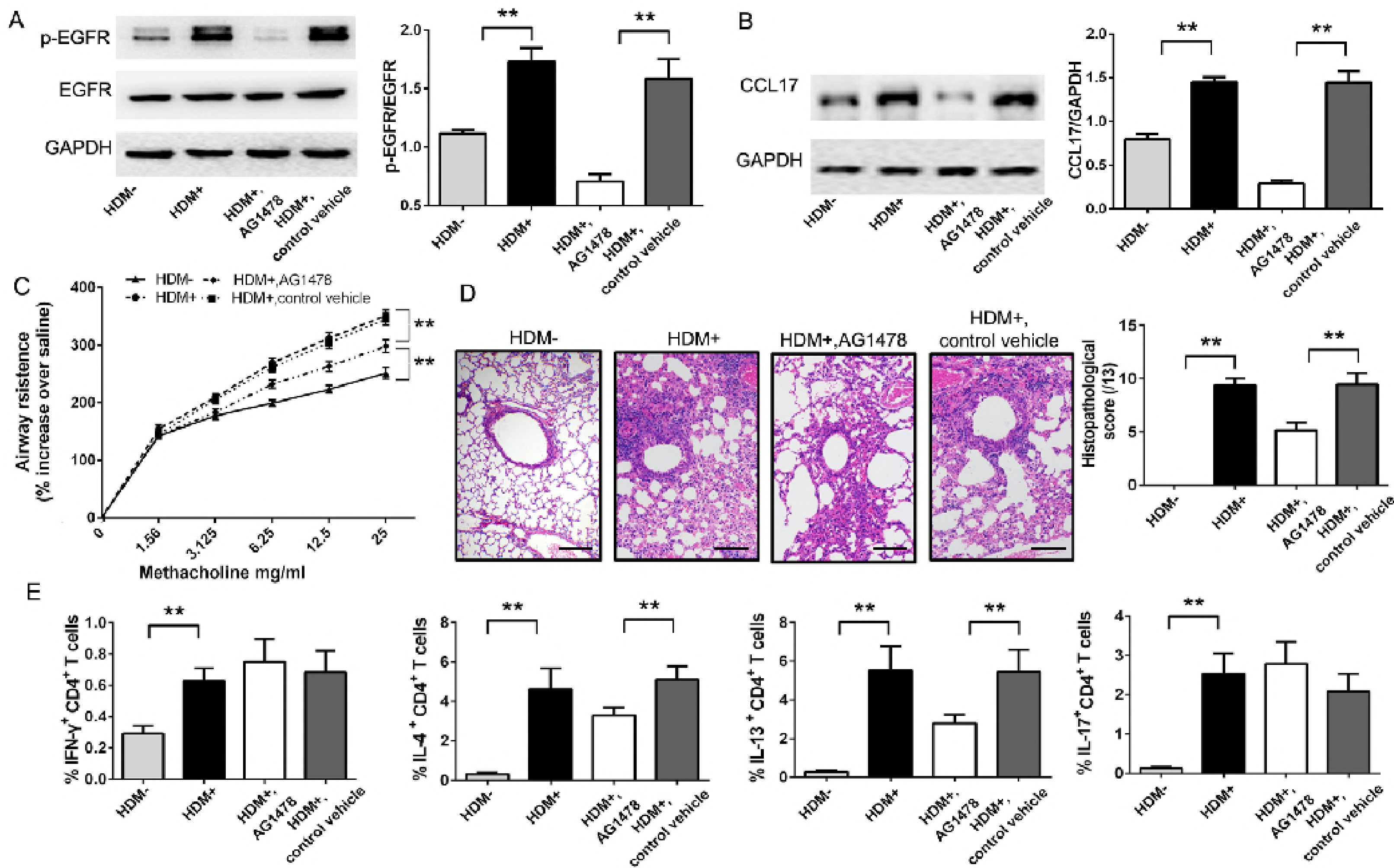

C
ITGB4
EGFR
DAPI
Overlay

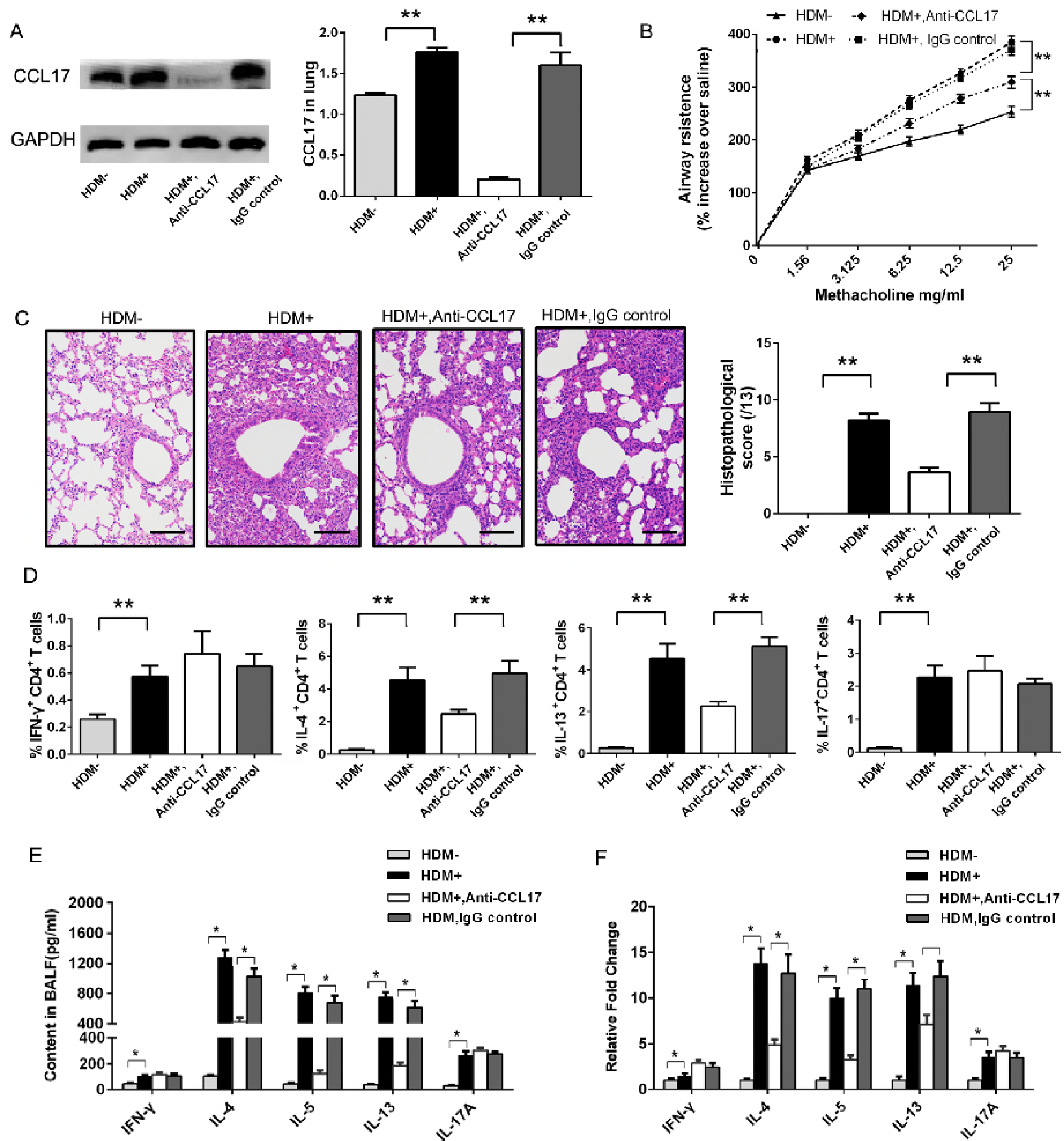

A
p-EGFR
EGFR
p-ERK1/2
ERK1/2
CCL17
GAPDH
ITGB4 + - + -
AG1478 - - + +
pEGFR/EGFR
pERK1/2/ERK1/2
CCL17/GAPDH
**
ITGB4 + - + -
AG1478 - - + +


B
p-ERK1/2
ERK1/2
CCL17
GAPDH
ITGB4 + - + -
U0126 - - + +
pERK1/2/ERK1/2
CCL17/GAPDH
**
ITGB4 + - + -
U0126 - - + +


C
CCL17
GAPDH
ITGB4 + - + -
SC75741 - - + +
CCL17/GAPDH
**
ITGB4 + - + -
SC75741 - - + +


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

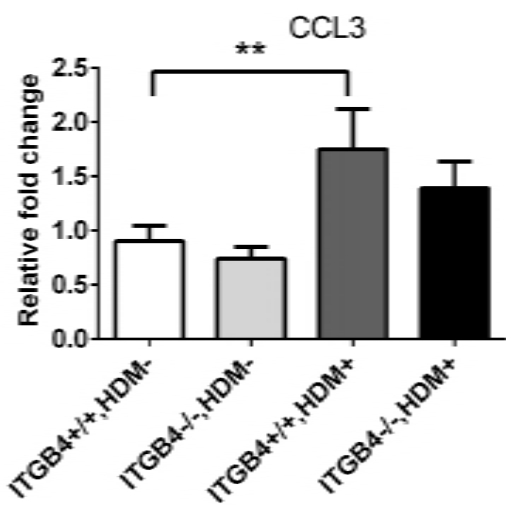
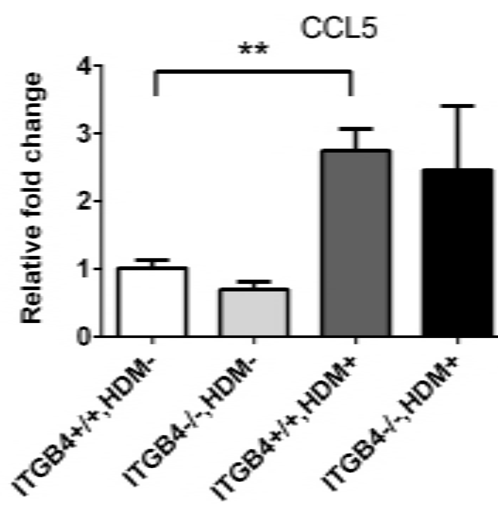
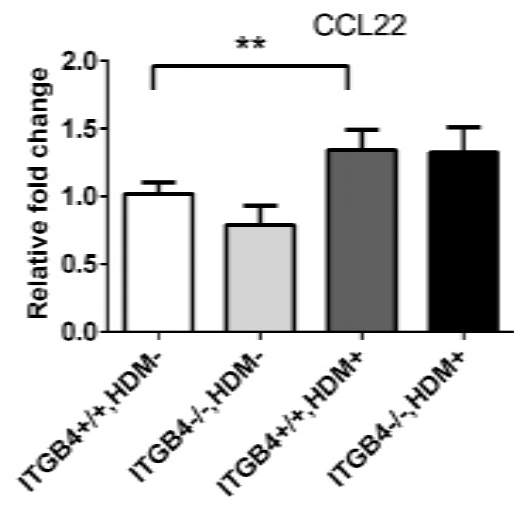

CCL3
**
Relative fold change
2.5
2.0
1.5
1.0
0.5
0.0
ITGB4+/+,HDM-
ITGB4-/-,HDM-
ITGB4+/+,HDM+
ITGB4-/-,HDM+

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' |