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IL-37 inhibits IL-4/IL-13-induced CCL11 production and lung eosinophilia in murine allergic asthma

Running title: IL-37 inhibits CCL11 and eosinophilia in murine lung

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Abstract

Background: IL-37 is emerging as an anti-inflammatory cytokine, particularly in innate inflammation. However, the role of IL-37 in Th2-mediated allergic lung inflammation remains uncertain. We sought to determine the role and the underlying mechanisms of IL-37 in the development of house dust mites (HDM)-induced murine asthma model.

Methods: We examined the effect of IL-37 administration during the sensitization or challenge phase on Th2-mediated allergic asthma induced by inhaled HDM. Cellular source of CCL11 and distribution of IL-37 receptors, IL-18R α and IL-1R8, were determined in HDM-exposed lungs. Finally, we examined the effect of IL-37 on CCL11 production and STAT6 activation in different primary lung structural cell types upon IL-4/IL-13 stimulation.

Results: IL-37 had no effect on HDM sensitization, but when administrated during the challenge phase, significantly attenuated pulmonary eosinophilia, CCL11 production and airway hyper-reactivity (AHR). Interestingly, IL-37 treatment had no significant effects on lung infiltrating T cells and Th2 cytokine production. Intranasal co-administration of CCL11 reversed the inhibiting effect of IL-37 on HDM-induced pulmonary eosinophilia and AHR. Furthermore, we demonstrated that CCL11 was primarily expressed by fibroblasts and airway smooth muscle cells (AMSC), while IL-37 receptors by tracheobronchial epithelial cells (TEC). In vitro study showed that IL-37 inhibited IL-4/IL-13-induced STAT6 activation and CCL11 production by fibroblasts and AMSC, which was dependent on its direct action on TEC. Moreover, cell-contact was required for the inhibitory effect of IL-37-treated TEC.

Conclusions: IL-37 attenuates HDM-induced asthma, possibly by inhibiting IL-4/IL-13-induced CCL11 production by fibroblasts and AMSC via its direct act on TEC.

Key words: Allergic asthma, CCL11, IL-37, lung structural cell, Th2-mediated eosinophilia

Introduction

Asthma is a complex disease characterized by airway inflammation and airway hyper-reactivity (AHR), affecting 300 million people worldwide (1). Allergic asthma is the most common form, and widely believed to be mediated by dysregulated type 2 inflammatory response to common inhaled environmental allergens (2, 3). Type 2 cytokines, IL-4, IL-5, and IL-13, primarily produced by antigen-specific Th2 cells, play critical roles in allergic asthma by acting on immune cells and lung structural cells, leading to IgE isotype switch, pulmonary eosinophilia, mucus secretion and bronchoconstriction (4, 5).

Pulmonary eosinophilia is a cardinal feature of allergic asthma (2, 6). The cooperation of several cytokines and chemokines is required for asthmatic eosinophilia (7-9). IL-5 is a well-known cytokine and critical for most aspects of eosinophils including development, mobilization, survival and activation(10). However, IL-5 has a minor role in eosinophil chemotaxis, instead, CCL11 is believed to be critical for attracting eosinophil into the lungs (11, 12). IL-4/IL-13 signaling contributes to pulmonary eosinophilia by inducing CCL11 production, as interruption of IL-4, IL-13 or downstream STAT6 markedly decreased CCL11 production and pulmonary eosinophilia in several asthma models (13-15). In vitro studies

demonstrated that IL-4/IL-13 could induce CCL11 secretion by several lung structural cells in a STAT6-dependent manner (16-18). However, it remains unclear about the major source of CCL11 and the possible negative regulators of CCL11 expression during allergic lung inflammation.

IL-37 is a unique member of IL-1 family with broad anti-inflammatory functions (19). The early study showed that endogenous IL-37 exerts its anti-inflammatory effect via interaction with nucleus Smad3 (20). More attention has been paid to the extracellular function of IL-37, which recently has been reported to be mediated by IL-18R α and IL-1R8 (21). Most studies have focused on the role of IL-37 in limiting innate inflammation primarily by inhibiting pro-inflammatory cytokine production by macrophages or epithelial cells (20, 21). Although so far there is no IL-37 homologue found in the mouse, murine cells are able to respond to human IL-37, as transgenic mice expressing human IL-37 gene or human IL-37 treatment exhibited a remarkable protection in several mouse models of acute inflammation or injury (22-25). However, little is known about how IL-37 modulated adaptive immune response, despite a previous study demonstrating that IL-37 suppressed hapten-specific T cell differentiation by targeting dendritic cells (DC) maturation (26). Emerging evidence suggests that IL-37 may be involved in allergic asthma, as decreased IL-37 expression was reported in allergic asthmatic children (27, 28) and IL-37 administration during the challenge phase was shown to reduce asthma phenotypes in a conventional ovalbumin (OVA)-induced asthma model (28). However, it remains unknown whether IL-37 inhibited the development of allergic Th2 response via airway sensitization, and the mechanism by which IL-37 attenuated allergic lung inflammation.

In this study, we took advantage of a mouse model of allergic asthma induced by house dust mites (HDM), a clinically relevant allergen, via mucosal sensitization route, to study the role of IL-37 in the development of allergic lung inflammation by intranasally (i.n.) administering recombinant human IL-37 during the sensitization phase or challenge phase.

Materials and methods

Mice

Female BALB/c mice purchased from Chinese Academy of Science (Shanghai, China) were housed in the animal facility of Fudan University under specific pathogen-free barrier conditions. Experimental procedures were in accordance with the Animal Care and Use Committee at Fudan University.

Reagents

HDM was purchased from Greer laboratory (Lenoir, NC, USA). Fluorochrome labeled antibodies for flow cytometry: mouse anti-CD4 and CD8 (both from Biolegend, San Diego, CA, USA); recombinant cytokines used: human IL-37b (E. coli-derived Val46-Asp218, endotoxin <0.10 EU/ μ g), mouse CCL11 (both from R&D, Minneapolis, MN, USA); mouse IL-4 and IL-13 (both from Peprotech, Rocky hill, NJ, USA). For cell culture, media D/F12K, Ham's F12, DMEM, RPMI-1640 and FBS were purchased from Gibco (Waltham, MA, USA).

Allergic asthma mouse model and pharmacologic interventions

HDM-induced allergic asthma model was established as previously described (29). Bronchoalveolar lavage fluid (BALF) total cells were counted, and differential leukocytes were determined from stained cytopspins by Weight-Giemsa. AHR was measured as previously described using Buxco's modular and invasive system (BUXCO Electronics Inc., NY, USA) (30). For intervention experiments, IL-37 (200 ng/mouse) was i.n. administrated 30 min before HDM sensitization or 30 min before HDM challenge. In some experiments, CCL11 (200 ng/mouse) was i.n. administrated 30 min after HDM challenge.

ELISA

The concentrations of IL-4, IL-5, IL-3, IL-17A, IFN- γ , and CCL11 were determined by mouse ELISA kits (R&D).

Histopathological analysis of lung

Lung sections were stained with hematoxylin and eosin (H&E) or Periodic Acid Schiff (PAS). Eosinophils were counted blinded in 10 random high-power fields of H&E stained sections at a magnification of 200 X.

Primary cell Isolation and stimulation

The isolation of tracheobronchial epithelial cells (TEC) and airway smooth muscle cells (AMSC) were performed as previously described with modification (31, 32). For isolation of alveolar epithelial cells (AEC), lung tissues without trachea and bronchi tissue were cultured in D/F12K medium until AEC migrated from the lung explants. For isolation of lung fibroblasts, lung tissues without trachea and bronchi tissue were digested with collagenase I followed by 40 min incubation for precluding macrophages. The purity of lung fibroblasts, ASMC, AEC and TEC were determined by flow cytometry or immunofluorescence staining of specific markers (Fig. S1).

Lung fibroblasts, ASMC, or AEC were starved with low serum medium (1% FBS), and TEC with 5% FBS for 24 h followed by IL-4 and IL-13 stimulation (each 10 ng/ml) for 30 min or 24 h. In some experiments, lung fibroblasts and ASMC were pre-treated with IL-37 at different concentrations for 30 min. For direct co-culture system, TEC were seeded in a 24-well plate with lung fibroblasts and ASMC (1×10^5 each). For indirect co-culture system, TEC and lung fibroblasts or ASMC (each for 1×10^5) were seeded in 24-well transwell plates (Corning, 0.4 μ M). The co-cultured cells were pretreated with IL-37 (50 ng/ml) for 30 min, then followed by IL-4 and IL-13 stimulation.

Western blot

Cell lysates were incubated with the following primary antibodies against phosphor-STAT6 (Tyr641) (1:1000), total-STAT6 (1:1000), GAPDH (1:1000) followed by incubation with HRP-conjugated secondary anti-rabbit antibodies (1:3000) (all from Cell Signaling Technology, Beverly, MA, USA). Blots were developed with the ECL detection system (Pierce/Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative PCR analysis

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was generated with High-capacity cDNA Reverse Transcription kit (TaKaRa Bio, Otsu shiga, Japan). Quantitative real-time PCR was performed with SYBR green Gene Expression Assay (TaKaRa Bio). The relative expression of target genes was calculated using the comparative method for relative quantitation by normalization to the internal control β -actin. The primer sequences of all genes for PCR were in Table 1.

Statistical analysis

Differences between groups were analyzed by unpaired Student's t-tests or ANOVA (Prism 5; GraphPad Software, San Diego, CA).

Results

IL-37 treatment during the challenge phase, but not the sensitization phase, attenuates pulmonary eosinophilia and AHR in HDM-induced allergic asthma

We first investigated the role of IL-37 in HDM sensitization by i.n. administering IL-37 to BALB/c mice during the sensitization phase (Fig. S2A). IL-37 did not affect inflammatory infiltrates in BALF and lung tissues, as well as AHR following HDM challenge (Fig. S2B-D). Consistently, we found that IL-37 failed to inhibit HDM-induced expression of IL-1 α and IL-33 (Fig. S3), both of which were reported to be required for HDM sensitization (33). These data suggest that IL-37 did not affect HDM sensitization. In contrast, IL-37 administration during the challenge phase (Fig. 1A) significantly reduced the numbers of total cells and eosinophils, with no effect on macrophages, lymphocytes and neutrophils, in BALF (Fig. 1B). Consistently, IL-37 greatly decreased the magnitudes of peribronchial inflammatory infiltrates mainly consisting of eosinophils, which was further confirmed by eosinophil counts in H&E stained lung sections (Fig. 1C, D). However, there was no difference in the extent of PAS-positive staining and gene expression of Muc5ac, a marker of mucus cell hyperplasia (Fig. 1C and data not shown) (34), suggesting that IL-37 did not affect mucus secretion. Importantly, IL-37 significantly reduced AHR responding to methacholine (Fig. 1E). Collectively, our data demonstrate that IL-37 administration during the challenge phase attenuates pulmonary eosinophilia and AHR in HDM-induced allergic asthma.

IL-37 treatment has no significant effect on T cell infiltration and cytokine production

Accumulating evidence demonstrates that Th2 cells mediate pulmonary eosinophilia by producing IL-4, IL-5 and IL-13 in allergic asthma. However, flow cytometric analysis showed that IL-37 treatment had no significant inhibition on the numbers of CD4⁺ and CD8⁺ T cells in BALF of asthmatic mice (Fig. 2 A, B), which is consistent with unaltered BALF lymphocytes (Fig. 1B). Furthermore, IL-37 did not significantly affect the concentrations of IL-4, IL-5 and IL-13 in lung tissue homogenates (Fig. 2C). These data suggest that IL-37 did not affect airway infiltration and the activation of Th2 cells in HDM-induced allergic asthma. Th17 cells were reported to be involved in HDM-induced allergic asthma (35), and Th1 cells to counteract allergic Th2 responses (36). IL-37 also had no effect on the production of IL-17A and IFN- γ in lungs (Fig. 2C), further supporting that IL-37 did not inhibit the activation of

effector T cells in our model. Similar results were obtained when IL-37 was administered during the challenge phase to OVA-induced asthma model (Fig. S4A-G).

The inhibitory effect of IL-37 on HDM-induced allergic asthma is mediated by reduced lung CCL11 production

Pulmonary eosinophilia is dependent on CCL11 in allergic asthma (12). Consistent with reduced pulmonary eosinophilia, IL-37 treatment significantly decreased CCL11 production in lungs of asthmatic mice (Fig. 3A and Fig. S4H). We next investigated whether the supplement of exogenous CCL11 could abolish the inhibitory effects of IL-37 on HDM-induced asthma by i.n. administering CCL11 into mice together with IL-37 or alone during the challenge phase. CCL11 alone did not affect HDM-induced allergic asthma (Fig. S5). Importantly, CCL11 co-administration completely restored pulmonary eosinophilia and AHR, with no effect on the infiltration of other inflammatory cells in BALF and mucus secretion as determined by PAS staining in IL-37-treated mice (Fig. 3B-E). Notably, CCL11 co-administration did not affect Th2 cytokine production in lungs of IL-37-treated asthmatic mice (Fig. 3F). Our findings that either reduced AHR caused by IL-37 treatment or restored AHR by exogenous CCL11 was not associated with altered expression of IL-13, suggesting an IL-13-independent mechanism by which IL-37 inhibited AHR. Cysteinyl leukotrienes (cys-LTs) is another potent bronchoconstrictor agonist, and the antagonist of the receptor of cys-LTs was clinically used for treating asthma (37). IL-37 treatment significantly reduced lung expression of leukotriene C4 synthase (LTC₄S), the key enzyme for cys-LTs synthesis, while co-administration with CCL11 completely restored lung expression of LTC₄S in IL-37-treated mice (Fig. S6). Collectively, our results demonstrate that the inhibitory effect of IL-37 on HDM-induced allergic asthma is due to the reduction in CCL11 production.

Lung fibroblasts and ASMC are the major cell sources for CCL11 in HDM-induced asthma

We next explored the underlying mechanism by which IL-37 inhibited CCL11 production in lungs of asthmatic mice. Considering that many lung cell types are reported to express CCL11 (16-18), we first attempted to identify the major cell source of CCL11 in our model. To this end, CD45⁺ leukocytes and CD45⁻ cells were sorted from enzymatically digested lungs by FACS and respective CCL11 gene expression was evaluated. CD45⁻ cells sorted from lungs of naïve mice expressed baseline level of CCL11, which greatly increased (7.31±0.83-fold) in those from HDM-induced asthmatic mice (Fig. 4A). Expectedly, CD45⁻ cells from IL-37-treated mice had greatly decreased CCL11 gene expression (Fig. 4A). In contrast, very little, if any, CCL11 gene expression was detected in CD45⁺ leukocytes from naïve or HDM-induced asthmatic mice (Fig. 4A). To further determine which CD45⁻ cell type primarily expressed CCL11, lung structural cells including fibroblasts, ASMC, AEC, and TEC were isolated from naïve mice. Relatively high baseline gene levels of CCL11 were detected in fibroblasts and ASMC, but not AEC and TEC (Fig. 4B). Markedly up-regulated CCL11 gene expression was detected in lung fibroblasts, and ASMC, but not AEC and TEC, isolated from HDM-induced asthmatic mice (Fig. 4B), indicating CCL11 is primarily produced by lung fibroblasts and ASMC in HDM-induced allergic asthma.

IL-37 treatment fails to directly inhibit IL-4/IL-13-induced CCL11 secretion by lung fibroblasts and ASMC in vitro

IL-4 and IL-13 have been reported to be critical for inducing CCL11 expression in allergic asthma (4, 15, 38). Given our above findings that IL-37 inhibited CCL11 with no significant effect on Th2 cytokine production, we hypothesized that IL-37 could inhibit IL-4/IL-13-induced CCL11 production, thereby leading to reduced pulmonary eosinophilia. In vitro study revealed that stimulation with IL-4/IL-13 greatly induced CCL11 expression at mRNA and protein levels by cultured fibroblasts and ASMC, but not AEC and TEC, isolated from naïve mice (Fig. 4C, D), further confirming lung fibroblasts and ASMC as major cell sources of CCL11 in type 2 inflammation-dominated lung microenvironment. However, in contrast to significantly decreased CCL11 expression in lung fibroblasts and ASMC from IL-37-treated mice (Fig. 4B), IL-37 pretreatment at different concentrations had no effect on CCL11 secretion in the culture of lung fibroblasts and ASMC upon IL-4/IL-13 stimulation (Fig. 4E, F). These data clearly suggest that the suppressive effect of IL-37 on CCL11 production is not caused by its direct act on lung fibroblasts and ASMC, instead, IL-37 may target other cell types to indirectly suppress IL-4/IL-13-induced CCL11 production by lung fibroblasts and ASMC.

IL-37 receptors, IL-18R α and IL-1R8, are highly expressed in TEC

To identify the target cells of IL-37 in our model, we first examined the cellular distribution of two identified IL-37 receptors, IL-18R α and IL-1R8. Much higher baseline gene levels of both receptors were detected in CD45⁻ cells than those in CD45⁺ leukocytes (Fig. 5A). Gene expression of both receptors was not altered in either CD45⁻ cells or CD45⁺ leukocytes, sorted from lungs of HDM-induced asthmatic mice (Fig. 5A). Furthermore, both receptors were expressed in TEC at high baseline levels, while in lung fibroblasts and ASMC at very low levels (Fig. 5B). This is consistent with the unresponsiveness of lung fibroblasts or ASMC to IL-37 inhibition. Relatively high baseline level of IL-1R8 was detected in AEC (Fig. 5B, right), which is consistent with previous findings that IL-1R8 is preferentially expressed by epithelial cells (39). Gene expression of both receptors was unaltered in all four types of lung structural cells isolated from HDM-induced asthmatic mice (Fig. 5B). Consistently, IL-4/IL-13 stimulation had no effect on IL-18R α and IL-1R8 expression in cultured lung fibroblasts, ASMC, AEC, or TEC (Fig. 5C). Collectively, these results suggest that TEC, which express two receptors of IL-37 at highest levels, is likely to be the major target cell of IL-37 in lungs.

IL-37 targets on TEC to indirectly suppress IL-4/IL-13-induced CCL11 production by lung fibroblasts and ASMC in a cell-contact manner

We next investigated whether the direct act of IL-37 on TEC is needed to inhibit IL-4/IL-13-induced CCL11 production by lung fibroblasts and ASMC. To this end, we first performed direct co-culture of TEC with lung fibroblasts or ASMC, which were pretreated with IL-37 for 30 min followed by IL-4/IL-13 stimulation. We found that IL-37 pretreatment significantly reduced CCL11 concentrations in supernatants from co-culture of TEC and lung fibroblasts or ASMC (Fig. 6A). It is known that STAT6 is required for IL-4/IL-13-induced CCL11 expression (14, 18). We demonstrated that IL-4/IL-13 stimulation rapidly induced strong STAT6 phosphorylation in cells from co-culture system, which was greatly reduced by IL-37 pretreatment (Fig. 6B). Moreover, IL-37 pretreatment had no effect on IL-4/IL-13-induced STAT6 activation in TEC (Fig. 6B), suggesting decreased p-STAT6 levels could be attributed to the reductions in STAT6 activation in co-cultured lung fibroblasts or

ASMC. To find whether this reduction required cell-cell contact, TEC and fibroblasts or ASMC were cultured in transwell plates, which precluded direct cell-cell contact but allowed communication via soluble factors. In contrast to direct co-culture system, IL-37 pretreatment of TEC failed to inhibit IL-4/IL-13-induced CCL11 production by lung fibroblasts or ASMC in the indirect co-culture system (Fig. 6C). Similarly, supernatants from IL-37-pretreated TEC also failed to inhibit IL-4/IL-13-induced CCL11 by lung fibroblasts or ASMC (Fig. S7). Collectively, our results demonstrate that IL-37 suppresses IL-4/IL-13-induced CCL11 production by lung fibroblasts or ASMC via its direct act on TEC, which is dependent on direct cell-cell contact.

Discussion

We here revealed a previously unidentified role of IL-37 in suppressing CCL11 production by lung fibroblasts and ASMC via its direct action on TEC, leading to attenuated pulmonary eosinophilia and AHR in a HDM-induced asthma model.

In contrast to OVA-induced asthma model that relies on non-mucosal intraperitoneal sensitization to OVA with alum as adjuvant, asthma model induced by airway sensitization with HDM is more relevant to human asthma. Given previous studies showing IL-37 as an inhibitor of innate immunity and the importance of pro-inflammatory cytokines in HDM sensitization (21, 33), we speculated that IL-37 might interfere with airway sensitization with HDM. However, IL-37 administration during the sensitization phase failed to affect the phenotypes of allergic asthma following airway challenge with HDM. In sharp contrast, IL-37 administration during the challenge phase significantly inhibited pulmonary eosinophilia and AHR. However, IL-37 treatment appeared not to affect Th2 cell differentiation, recruitment and reactivation in response to HDM, as evidenced by comparable airway infiltration of CD4⁺ T cells and T cell-associated cytokine production in both lungs and mediastinal lymph nodes (Fig. S2E, F). The previous study using OVA model showed that IL-37 treatment inhibited the Th2 cytokine levels in BALF, despite of unaltered lymphocyte infiltration (28). However, it is notable that the concentrations of IL-4, IL-5 and IL-13 they measured were very low (IL-5 at highest level was about 15 pg/ml and IL-4 and IL-13 both only about 3 pg/ml). Furthermore, we confirmed the inability of IL-37 to inhibit Th2 cytokine levels by using the same OVA model. Consistent with unchanged IL-13 levels, there was no change in mucus secretion following IL-37 treatment in both models. Furthermore, we also found that the administration of IL-37 had no effect on serum levels of total IgE, emphasizing a local anti-inflammatory effect of IL-37 (Fig. S8).

In spite of unchanged Th2 cytokine production, we found that IL-37 treatment greatly suppressed the production of CCL11, a critical eosinophil chemokine, in HDM-induced asthmatic mice. Importantly, co-administration of exogenous CCL11 completely restored pulmonary eosinophilia and AHR with no effect on Th2 cell response, suggesting that reduced CCL11 production mediates the inhibitory effect of IL-37 on HDM-induced allergic asthma. It is also worth noting that reduced AHR in IL-37-treated asthmatic mice is accompanied by decreased expression of LTC₄S, indicative of less cys-LTs synthesis, but unchanged production of IL-13, both of which are known to be responsible for the development of AHR (40, 41). Since eosinophils are known to be a primary source of cys-LTs

during allergic inflammation, it is likely that IL-37 inhibits pulmonary eosinophilia, which release less cys-LTs, leading to reduced AHR independently of IL-13.

Furthermore, we revealed that IL-37 suppressed CCL11 production by impairing IL-4/IL-13 signaling. We first identified lung fibroblasts and ASMC as the major cell sources of CCL11 in HDM-induced allergic asthma. Interestingly, in contrast to the efficient inhibition in vivo, addition of IL-37 failed to inhibit IL-4/IL-13-induced CCL11 production by these cells in vitro, indicating of indirect inhibitory effect. Although IL-37 has not been identified in mouse, its functional receptors IL-18R α and IL-1R8 are actually present in mouse, both of which have been reported to be involved in allergic asthma (42, 43). We found that lung fibroblasts and ASMC expressed these two receptors at very low levels, which is consistent with their unresponsiveness to IL-37 inhibition in our study. Macrophages are reported to respond to IL-37 upon stimulation with Toll-like receptor (TLR) agonists (20, 21). However, lung CD45⁺ cells isolated from naïve or asthmatic mice expressed very little IL-37 receptors, suggesting they are unlikely to be the target cells of IL-37 in our model. Notably TEC, but not AEC, constitutively expressed IL-37 receptors at very high levels, which is consistent with airway epithelial cells at different anatomical location serves distinct function in responses to different stimuli (44). Importantly, direct cell-cell contact with IL-37-pretreated TEC was required for the effective inhibition of IL-4/IL-13-induced STAT6 activation and CCL11 production by lung fibroblasts and ASMC. Moreover, IL-37 did not affect IL-4/IL-13-induced STAT6 activation in TEC, emphasizing the inability of IL-37 to directly inhibit IL-4/IL-13 signaling. It would be worth determining the critical molecule expressed by IL-37-treated TEC that mediates the inhibition of IL-4/IL-13 signaling and consequent CCL11 expression in the future study.

According to the literature, human IL-37 exerts similar biological function in both human and mouse cells under different inflammatory context such as suppression of cytokine production stimulated by different stimuli (20, 21, 45). Moreover, mouse expresses both functional receptors of IL-37, IL-18R α and IL-1R8, with a similar distribution pattern to that observed in human (21, 39), further suggesting a similar response to IL-37. Actually, transgenic expression of human IL-37 or administration of human IL-37 have been extensively used in the study of IL-37 in mouse models of inflammatory diseases, which also demonstrated a correlation with some corresponding human diseases (22-24, 26). Thus, IL-37 is very likely to play a similar anti-inflammatory role in human allergic asthma.

In summary, our study demonstrated that IL-37 had no effect on HDM sensitization, but markedly inhibited pulmonary eosinophilia and AHR when administered during the challenge phase. Furthermore, we demonstrated that IL-37 had no significant effect on Th2 cytokine production, instead, impaired IL-4/IL-13 signaling in lung fibroblasts and ASMC indirectly by acting on TEC, leading to reduced CCL11 production. Thus, our study reveals the underlying mechanism of the suppressive effect of IL-37 on HDM-induced allergic asthma, and which may have implications for the development of new therapeutic approach for allergic asthma.

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Figure legends

Figure 1. Administration of IL-37 in the challenge phase attenuates house dust mites (HDM)-induced eosinophilic airway inflammation and airway hyper-reactivity (AHR).

(A) Protocol for HDM-induced allergic airway inflammation and the time points of IL-37 intervention during the sensitization phase. (B) The differential cell counts in BALF. (TC: total cells; Mac: macrophages; Eos: eosinophils; Lym: lymphocytes; Neu: neutrophils) (C) Representative photomicrographs of lung sections stained with H&E and PAS. Original magnification, 200 X. (D) Numbers of eosinophils were counted in ten random 200 X high-power fields (HPF). (E) Airway resistance to methacholine was measured at 72 hours after the final HDM challenge by using Buxco's modular and invasive system.

Columns and error bars represent mean $\pm$ SEM. n=5 per group. *p< 0.05, **p< 0.01, ***p< 0.001. NS=no significance. Similar results were obtained from two or four independent experiments.

Figure 2. Administration of IL-37 during challenge phase has no significant effect on T cell response in lungs upon house dust mites (HDM) challenge.

(A) Representative flow cytometric analysis of the percentages of CD4⁺ and CD8⁺ cells. (B) The absolute cell numbers of CD4⁺ and CD8⁺ cells calculated based on the numbers of total cells and respective percentage in BALF. (C) ELISA analysis of Th cell cytokine concentrations in the lung tissue homogenates.

Columns and error bars represent mean $\pm$ SEM. n=15 per group. **p< 0.01, ***p< 0.001. NS=no significance.

Figure 3. IL-37 inhibits lung production of CCL11, and exogenous CCL11 administration abolishes the inhibitory effect of IL-37 on HDM-induced allergic asthma.

(A) The concentration of CCL11 in the lung tissue homogenates of HDM-induced asthmatic mouse with or without IL-37 treatment. (B-F) Following co-administration of CCL11 and IL-37, the differential cell counts in BALF (B), representative photomicrographs of lung sections stained with H&E and PAS. Original magnification, 200 X (C), numbers of eosinophils in peribronchial tissue of lung sections (D), and airway resistance to methacholine (E), ELISA analysis of concentrations of IL-4, IL-5, and IL-13 in the lung tissue homogenates (F).

Columns and error bars represent mean $\pm$ SEM. n=5 per group. *p< 0.05, **p< 0.01, ***p< 0.001. NS=no significance. Similar results were obtained from two independent experiments.

Figure 4. IL-37 fails to directly inhibit CCL11 production, which is primarily produced by lung fibroblasts and airway smooth muscle cells (ASMC).

(A and B) Gene expression of CCL11 in isolated CD45⁻ and CD45⁺ cells (A), as well as fibroblasts, ASMC, alveolar epithelial cells (AEC), and tracheobronchial epithelial cells (TEC) (B) isolated from digested lung of HDM-induced asthmatic mice with or without IL-37 treatment. Cells from lung of PBS treated mice were used as control. (C and D) CCL11 expression at mRNA (C) and protein levels (D) in fibroblasts, ASMC, AEC, or TEC isolated from lungs of naïve mice with or without IL-4/IL-13 stimulation. (E and F) CCL11 concentrations in the culture of IL-4/13-stimulated lung fibroblasts (E) and ASMC (F) which was pretreated with IL-37 at different concentration for 30 min.

Columns and error bars represent mean $\pm$ SEM. n=3-5 per group. *p< 0.05, **p< 0.01, ***p< 0.001. NS=no significance. Similar results were obtained from three or four independent experiments. In E and F, columns and error bars are mean $\pm$ SEM of three replicate wells from a representative of three experiments.

Figure 5. IL-37 receptors, IL-18R α and IL-1R8, is primarily expressed on tracheobronchial epithelial cells (TEC). (A and B) Gene expression of IL-18R α and IL-1R8 in isolated CD45⁻ cells and CD45⁺ cells (A), as well as lung fibroblasts, airway smooth muscle cells (ASMC), alveolar epithelial cells (AEC), and TEC (B) from digested lung of house dust mites (HDM)-induced asthmatic mice or PBS controls. (C) Gene expression of IL-18R α and IL-1R8 in isolated lung fibroblasts, AEC, TEC, and ASMC from lungs of naive mice stimulated with or without IL-4/IL-13.

Columns and error bars represent mean $\pm$ SEM. n=3 per group. NS=no significance. Similar results were obtained from two or three independent experiments.

Figure 6. IL-37 targets on TEC to indirectly suppress IL-4/IL-13-induced CCL11 production and STAT6 activation by/in lung fibroblasts and ASMC in a cell-contact manner. For direct co-culture system, lung fibroblasts or ASMC were directly co-cultured with TEC at a ratio of 1:1. For indirect co-culture system, lung fibroblasts or ASMC were co-cultured with TEC in the transwell plates at a 1:1 ratio. Cells in both co-culture systems were pretreated with IL-37 for 30 min followed by IL-4/IL-13 stimulation. (A) ELISA analysis of CCL11 concentrations in supernatant of direct co-culture system. (B) Representative western blots showing the levels of phosphorylated STAT6 (p-STAT6) and total STAT6 (t-STAT6) in direct co-cultured cells or cultured TEC alone 30 min after IL-4/IL-13 stimulation. (C) ELISA analysis of CCL11 concentrations in supernatant of indirect co-culture system.

Columns and error bars are mean $\pm$ SEM of three replicate wells from a representative of three experiments. *p< 0.05, **p< 0.01, NS=no significance.

Table 1. Primer sequences for quantitative PCR.

Gene	Forward primer	Reverse primer
Mu β -actin	5'-CCAGCCTTCCTTCTTGGGTATG-3'	5'-TGTGTTGGCATAGAGGTCTTTACG-3'
Mu IL-1R8	5'-CTCCGTGACTCCTTCCTCTG-3'	5'-TGGGATCTTTGTCATCCTGA-3'
Mu IL-18R α	5'-ACTTTTGCTGTGGAGACGTTAC-3'	5'-CCGGCTTTTCTCTATCAGTGAAT-3'
Mu CCL11	5'-GAATCACCAACAACAGATGCAC-3'	5'-ATCCTGGACCCACTTCTTCTT-3'
Mu IL-1 α	5'-CCGTGTTGCTGAAGGAGT-3'	5'-GACTTTGTTCTTTGGTGGC-3'
Mu IL-33	5'-ACT ATG AGT CTC CCT GTC CTG-3'	5'-ACG TCA CCC CTT TGA AGC-3'
Mu LTC ₄ S	5'-CCTGTGCGGACTGTTCTACC-3'	5'-CTCGCGTATAGGGGAGTCAG-3'

Figure 1

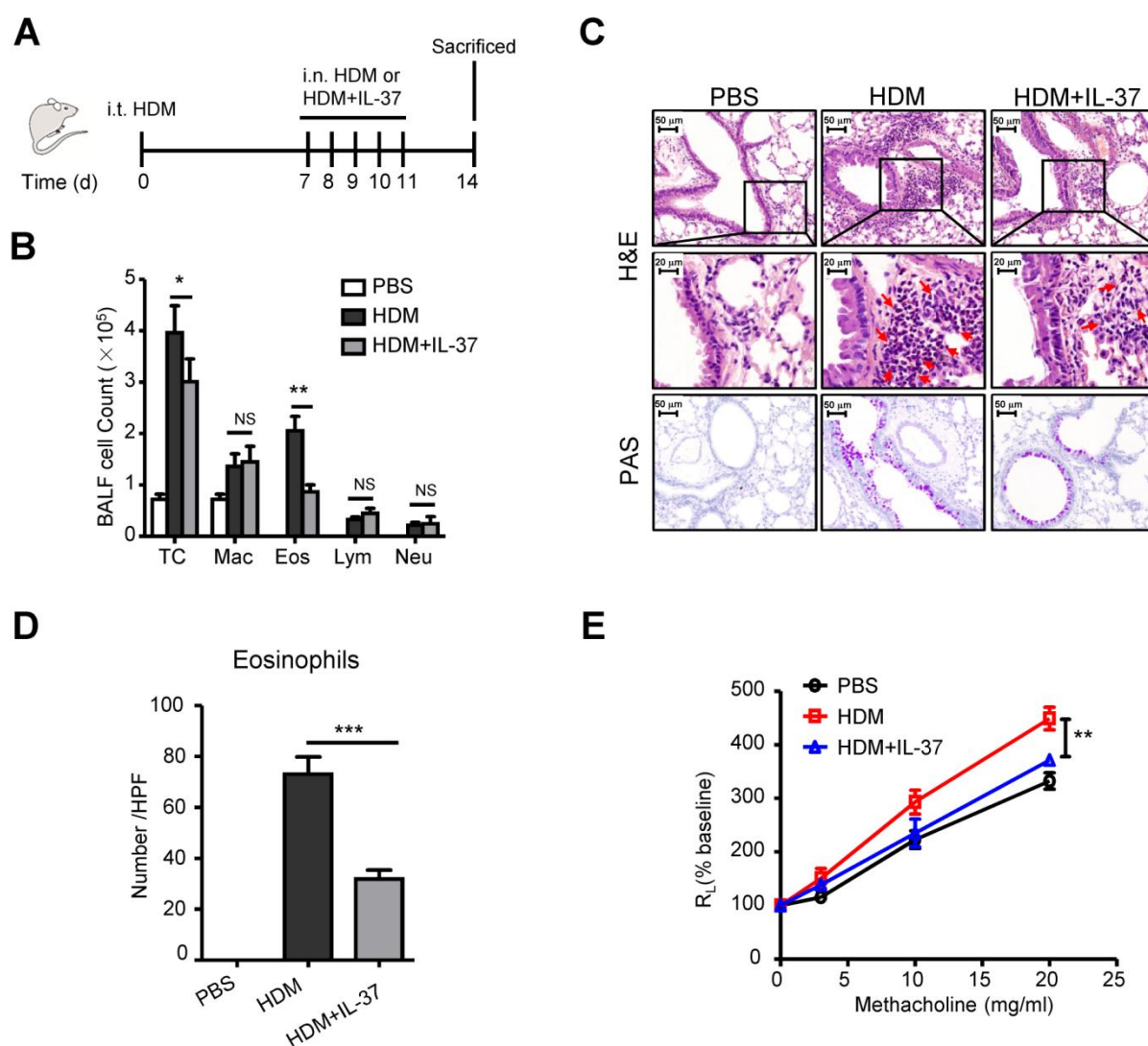


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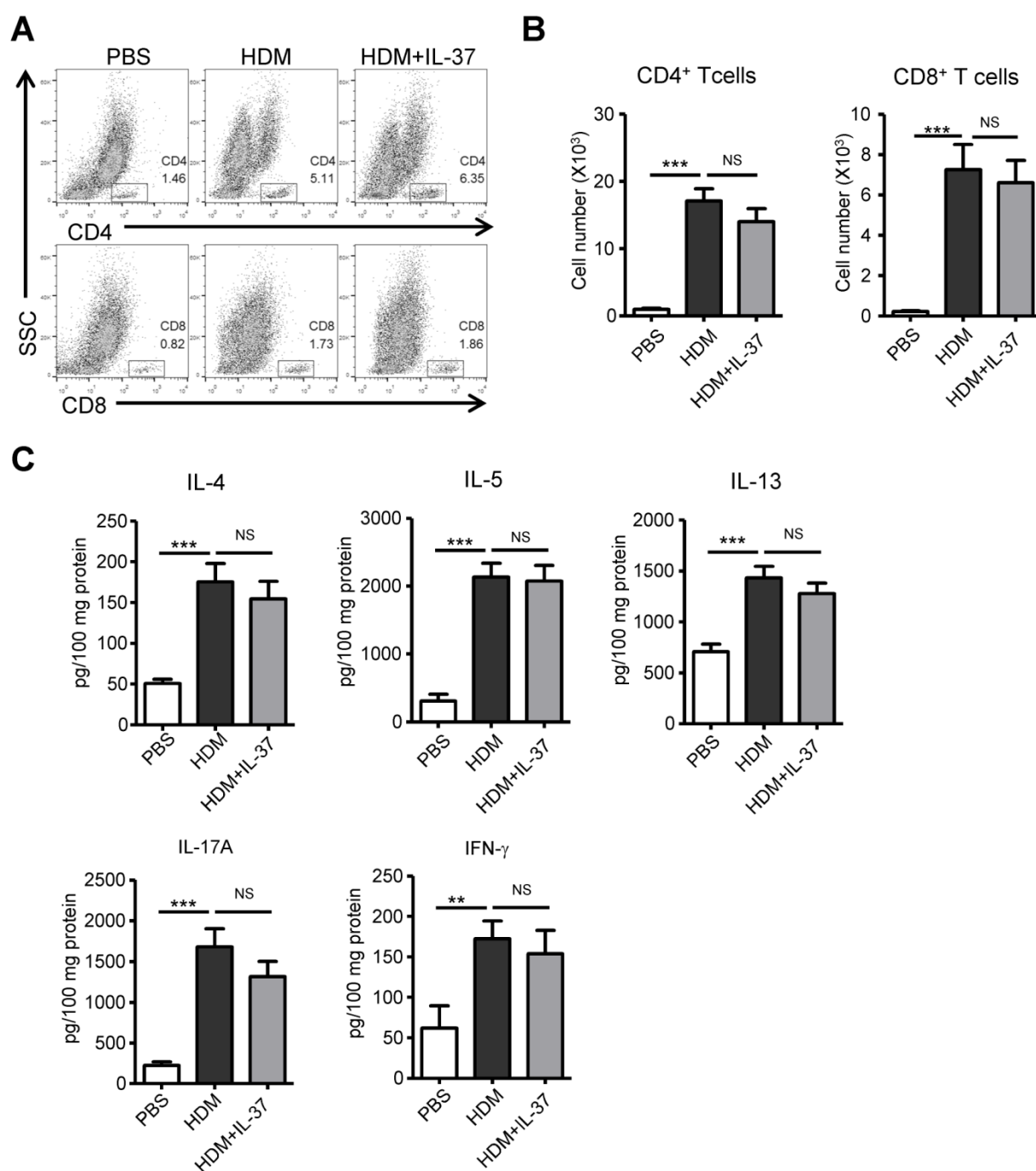


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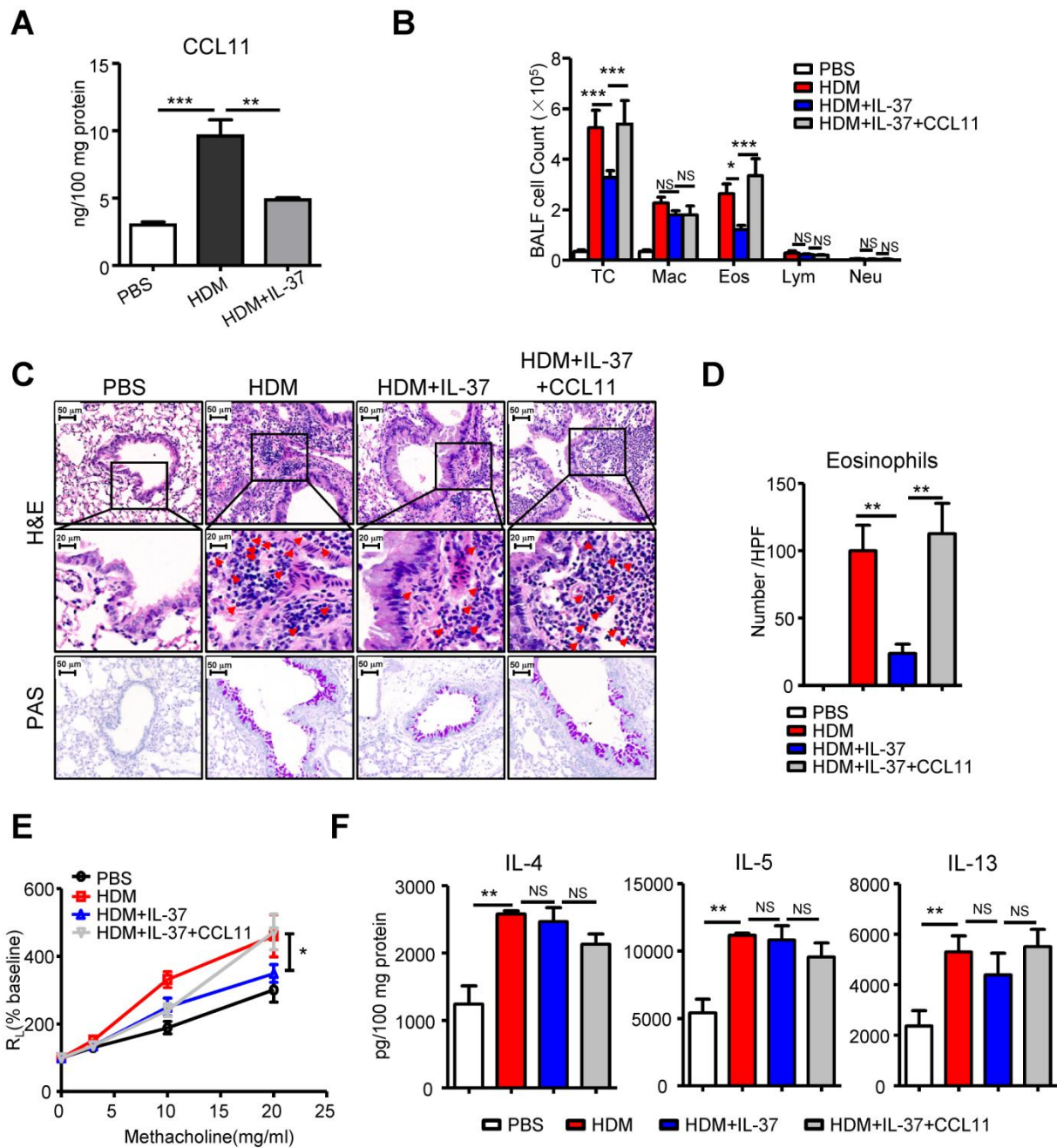


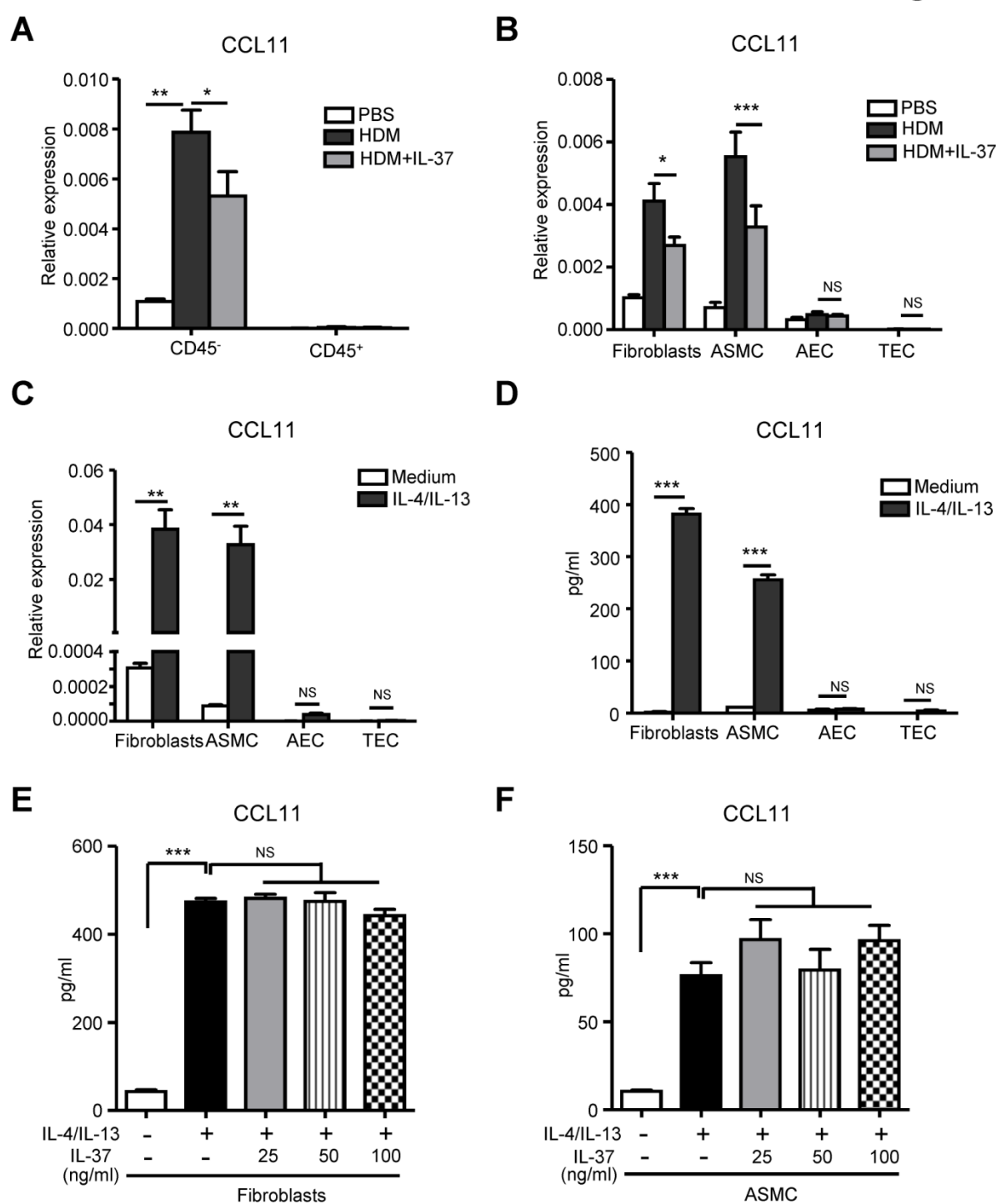
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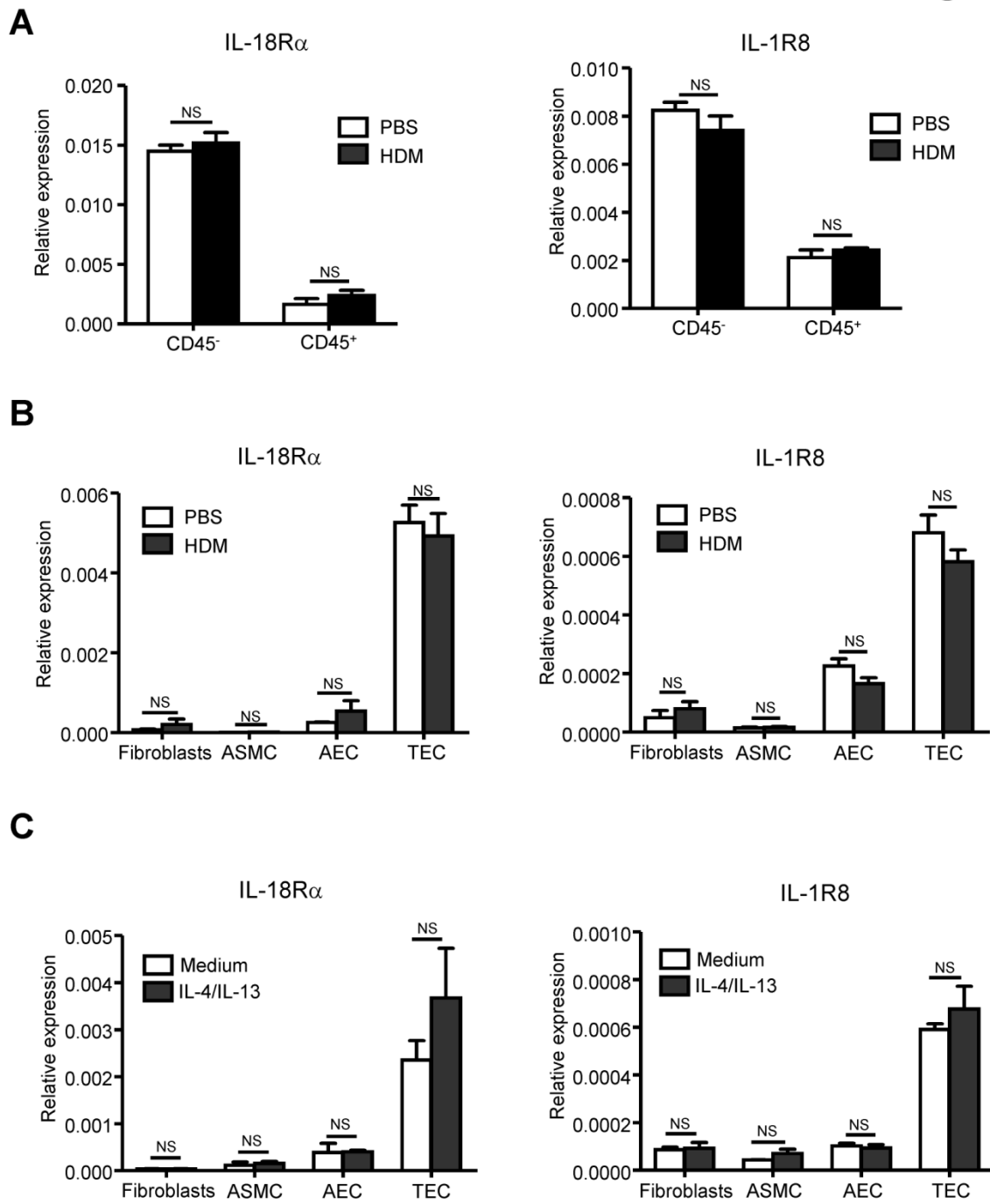
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Figure 6

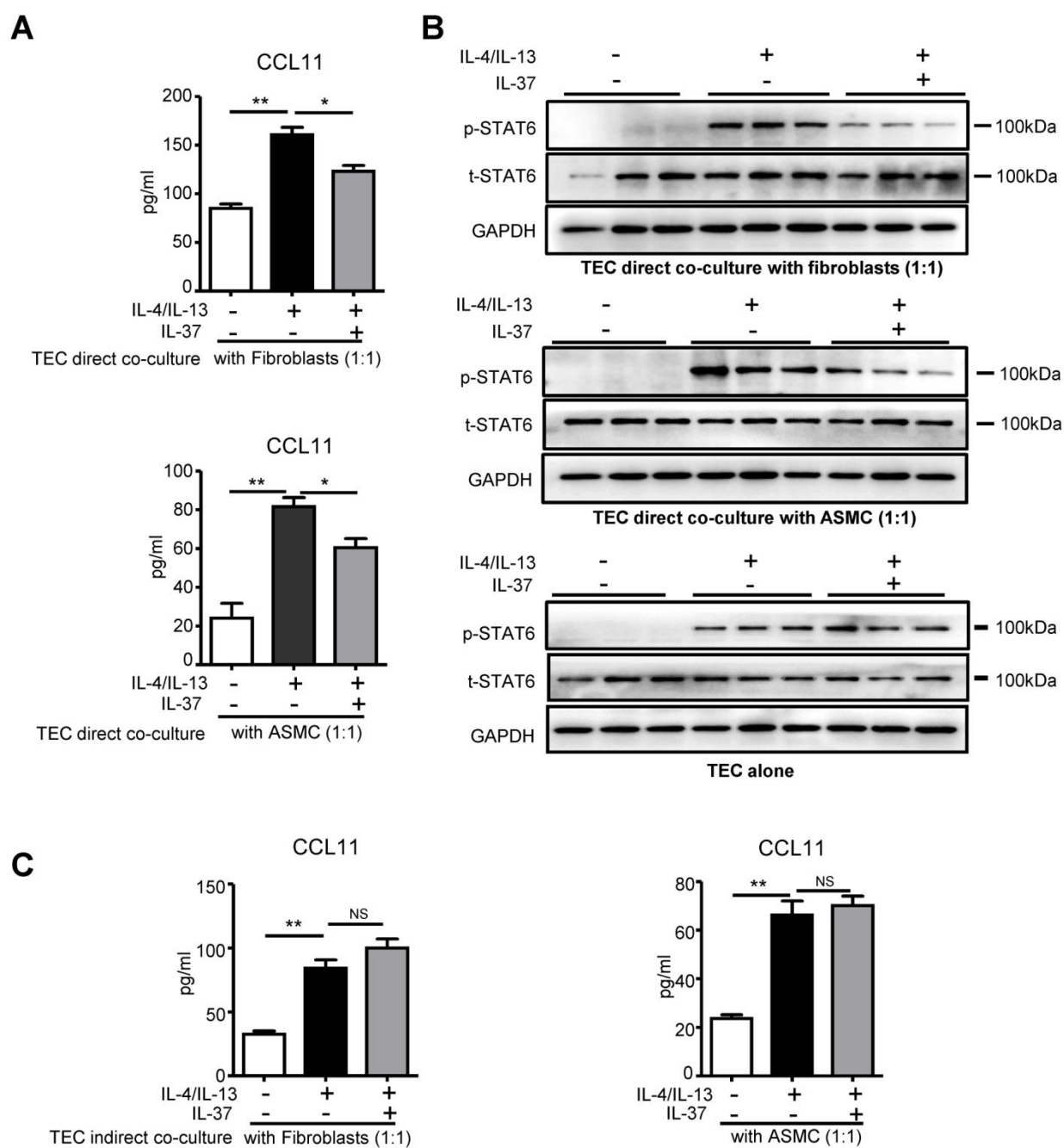
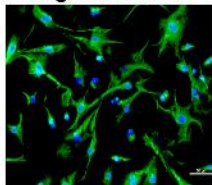


Figure S1

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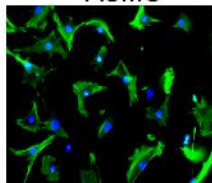
Lung fibroblasts



DAPI Vimentin

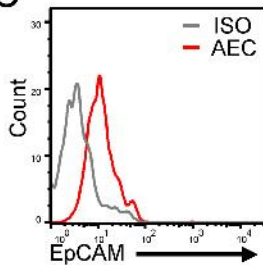
B

ASMC



DAPI α -SMA

C



D

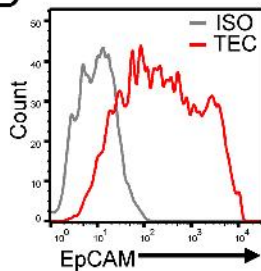


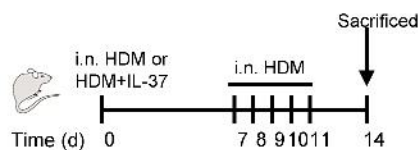
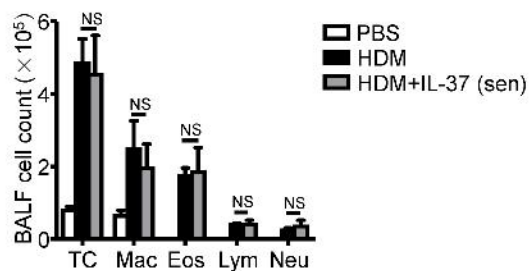
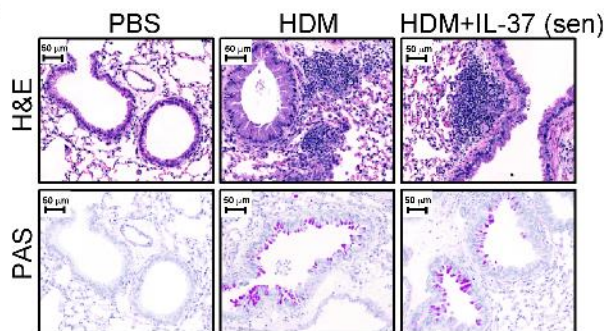
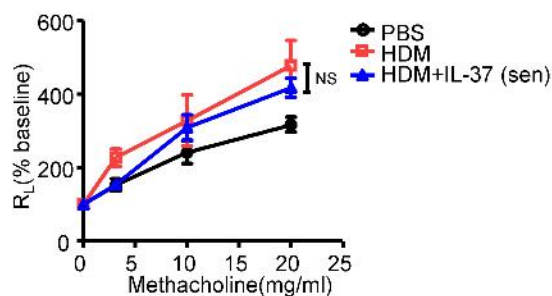
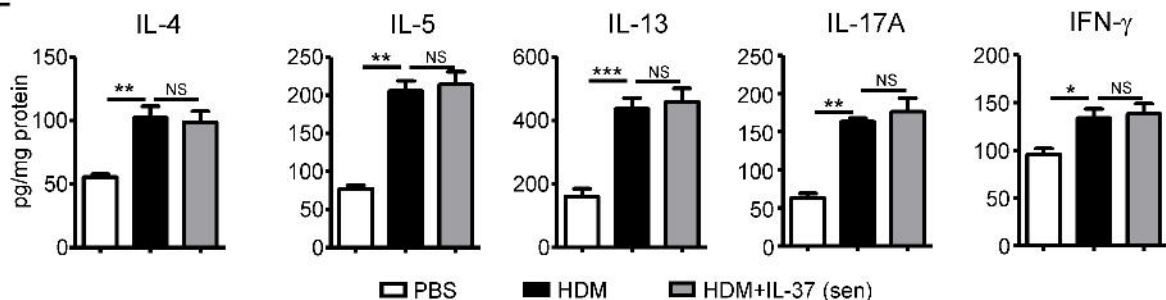
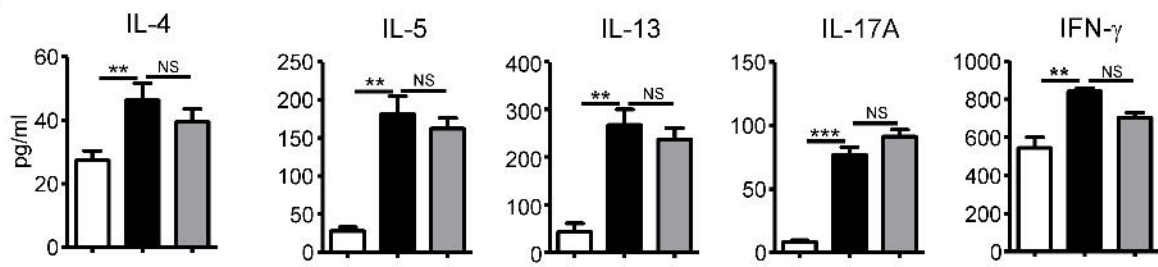
Figure S2**A****B****C****D****E****F**

Figure S3

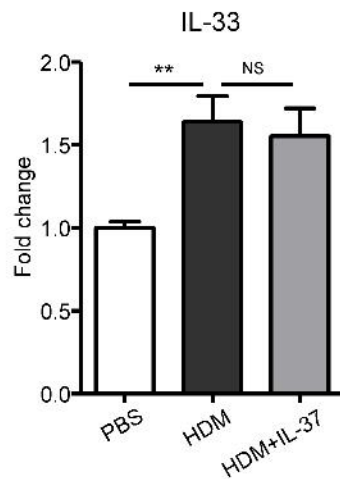
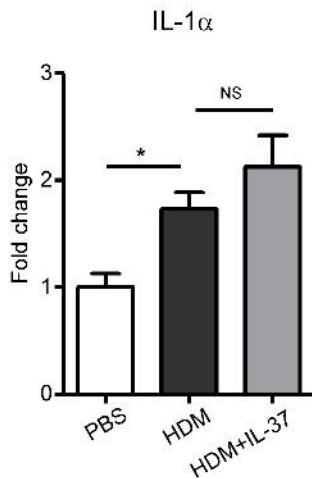


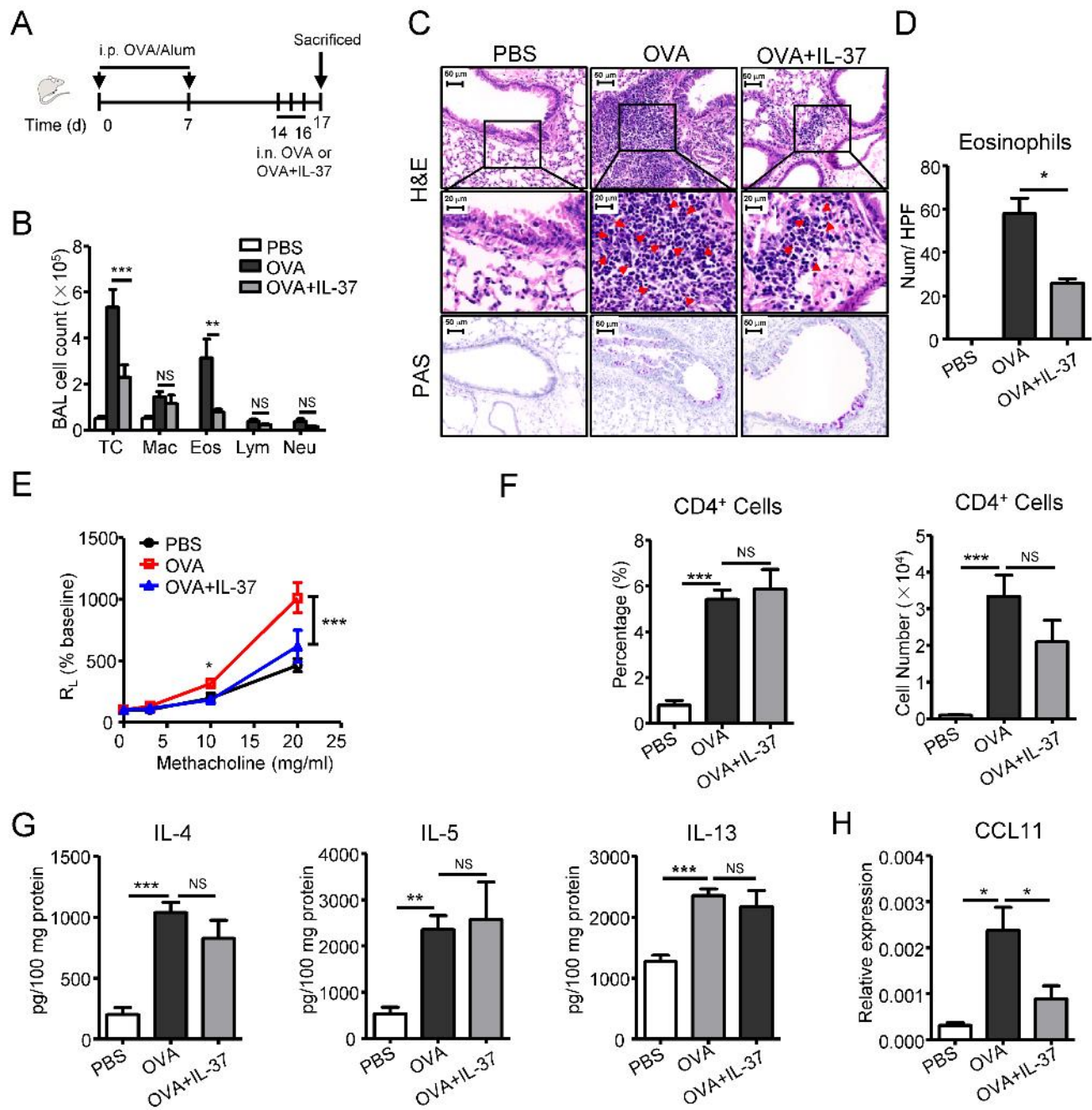
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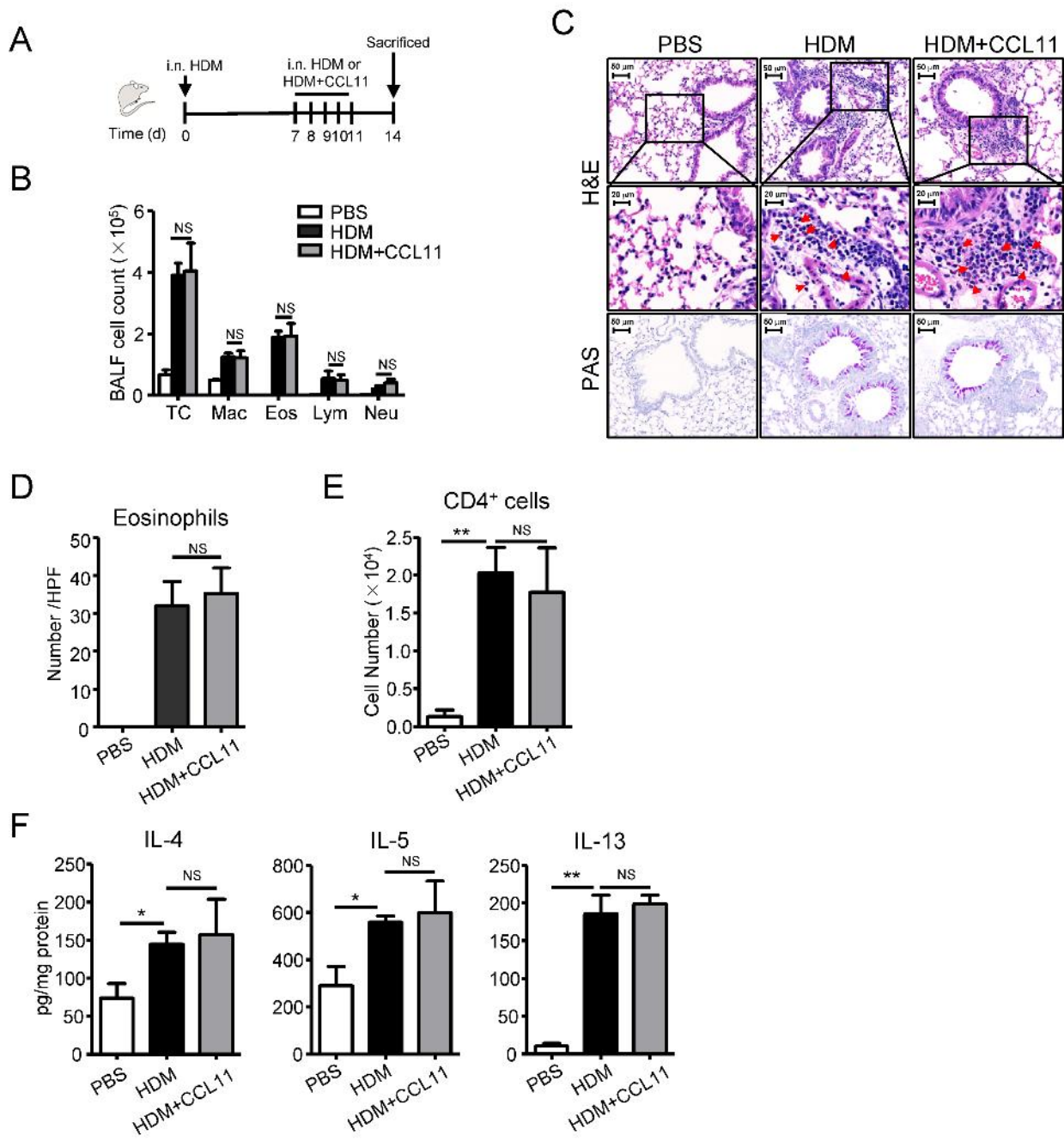
Figure S5

Figure S6

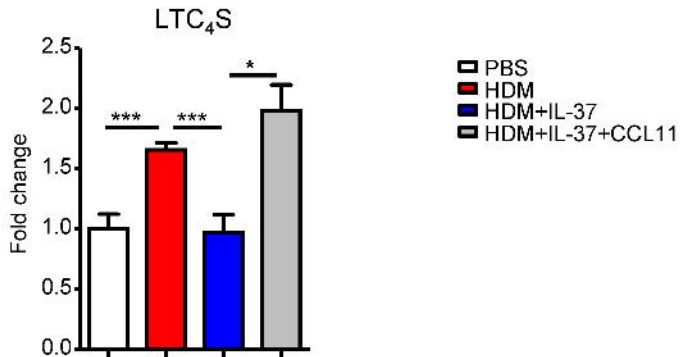


Figure S7

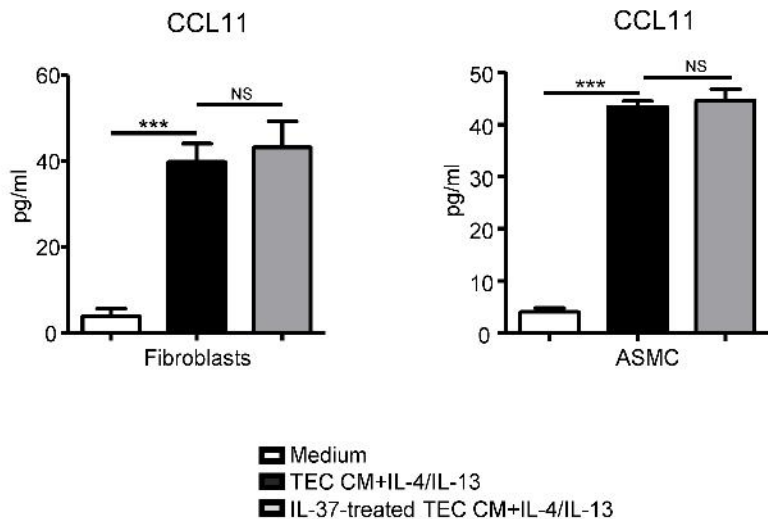
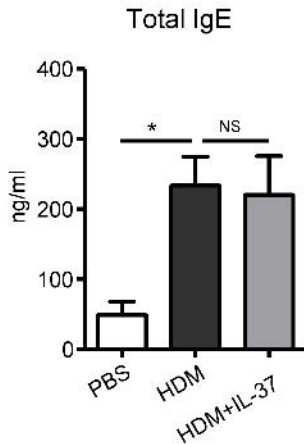
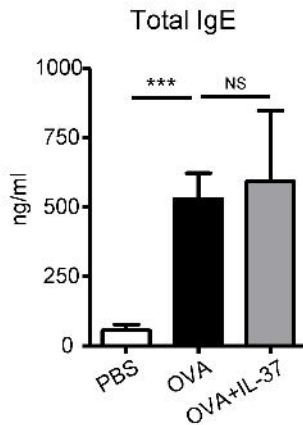


Figure S8

A



B



IL-37 inhibits IL-4/IL-13-induced CCL11 production and lung eosinophilia in murine allergic asthma

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Figure S1. The purity of isolated lung structural cells. (A and B) Immunofluorescence staining of cultured lung fibroblasts with vimentin (green) (A) and airway smooth muscle cells (ASMC) with α -SMA antibodies (B) and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (blue). (C and D) Representative flow cytometric analysis of the expression of EpCAM, the epithelial cell marker, in isolated alveolar epithelial cells (AEC) (C) and tracheobronchial epithelial cells (TEC) (D).

Figure S2. Administration of IL-37 during the sensitization phase fails to impair house dust mites (HDM)-induced pulmonary eosinophilia and airway hyper-reactivity (AHR).

(A) Protocol for HDM-induced allergic airway inflammation and the time points of IL-37 intervention during the sensitization phase. (B) The differential cell counts in bronchoalveolar lavage fluid (BALF). (TC: total cells; Mac: macrophages; Eos: eosinophils; Lym: lymphocytes; Neu: neutrophils) (C) Representative photomicrographs of lung sections. Original magnification, 200 X. (D) Airway resistance to methacholine. (E and F) The concentration of Th cytokines in lung homogenates (E) and the culture of mediastinal lymph nodes (MLN) (F).

Columns and error bars represent mean $\pm$ SEM. n=5 per group. *p< 0.05, **p< 0.01, ***p< 0.001. NS=no significance. Similar results were obtained from two independent experiments.

Figure S3. Administration of IL-37 has no effect on the expression of IL-1 α and IL-33 in lungs following HDM exposure. Gene expression of IL-1 α and IL-33 in lungs 2 h after single HDM (10 μ g/mouse) challenge.

Columns and error bars represent mean $\pm$ SEM. n=4-5 per group. *p< 0.05, **p< 0.01. NS=no significance. Similar results were obtained from two independent experiments.

Figure S4. Administration of IL-37 attenuates ovalbumin (OVA)-induced pulmonary

eosinophilia and airway hyper-reactivity (AHR). (A) Protocol for OVA-induced allergic airway inflammation and the time points of IL-37 intervention. (B) The differential cell counts in bronchoalveolar lavage fluid (BALF). (TC: total cells; Mac: macrophages; Eos: eosinophils; Lym: lymphocytes; Neu: neutrophils) (C) Representative photomicrographs of lung sections stained with H&E and PAS. Original magnification, 200 X. (D) Numbers of eosinophils were counted in ten random 200 X high-power fields (HPF). (E) Airway resistance to methacholine. (F) The percentage and number of Infiltrating CD4⁺ T cells in BALF. (G) ELISA analysis of protein levels of Th2 cytokines in lung tissues homogenates. (H) Gene expression of CCL11 in lungs.

Columns and error bars represent mean $\pm$ SEM. n=4 per group. *p< 0.05, **p< 0.01, ***p< 0.001. NS=no significance. Similar results were obtained from two independent experiments.

Figure S5. Exogenous CCL11 has no effect on HDM-induced pulmonary eosinophilia and Th2 cell response. (A) Protocol for HDM-induced allergic airway inflammation and the time points for administering CCL11. (B) The differential cell counts in bronchoalveolar lavage fluid (BALF). (TC: total cells; Mac: macrophages; Eos: eosinophils; Lym: lymphocytes; Neu: neutrophils) (C) Representative photomicrographs of lung sections stained with H&E and PAS. Original magnification, 200 X. (D) Numbers of eosinophils were counted in ten random 200 X high-power fields (HPF). (E) The number of infiltrating CD4⁺ T cells in BALF. (F) ELISA analysis of concentrations of IL-4, IL-5, and IL-13 in lung tissue homogenates.

Columns and error bars represent mean $\pm$ SEM. n=4-6 per group. *p< 0.05, **p< 0.01, ***p< 0.001. NS=no significance. Similar results were obtained from two independent experiments.

Figure S6. IL-37 reduces leukotriene C4 synthase (LTC₄S) expression in lungs of HDM-induced asthmatic mice, which is restored by co-administration of exogenous CCL11. Gene expression of LTC₄S in lungs determined by quantitative real-time PCR. Columns and error bars represent mean $\pm$ SEM. n=5 per group. *p< 0.05, ***p< 0.001. Similar results were obtained from two independent experiments.

Figure S7. The conditioned medium (CM) of IL-37-pretreated tracheobronchial epithelial cells (TEC) fails to inhibit IL-4/IL-13-induced CCL11 production by lung fibroblasts and airway smooth muscle cells (ASMC). ELISA analysis of protein levels of IL-4/IL-13 induced CCL11 in the supernatant of lung fibroblasts and ASMC with or without the addition of CM from IL-37-pretreated TEC.

Columns and error bars are mean $\pm$ SEM of three replicate wells from a representative of three experiments. ***p< 0.001. NS=no significance. Similar results were obtained from three independent experiments.

Figure S8. Administration of IL-37 has no effect on the concentration of total IgE in serum both of HDM-induced and OVA-induced asthma model. (A and B) ELISA analysis of the concentration of total IgE in serum of HDM- (A) and OVA-induced asthmatic mice (B) with or without IL-37 administration.

Columns and error bars represent mean $\pm$ SEM. n=4-5 per group. *p< 0.05, ***p< 0.001. NS=no significance. Similar results were obtained from two or three independent experiments.