

Amelioration of allergic airway inflammation in mice by regulatory IL-35 through dampening inflammatory dendritic cells

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Keywords

allergic asthma; animal models; dendritic cells; interleukin-35; mediastinal lymph node.

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Abstract

Background: IL-35, a new member of the IL-12 family, is an inhibitory cytokine produced by regulatory T and B lymphocytes that play a suppressive role in the inflammatory diseases.

This study focuses on the cellular mechanism regulating the anti-inflammatory activity of IL-35 in asthmatic mice.

Methods: Ovalbumin-induced asthmatic and humanized asthmatic mice were adopted to evaluate the *in vivo* anti-inflammatory activities of IL-35. For monitoring the airway, Penh value (% baseline) was measured using a whole-body plethysmograph.

Results: In this study using ovalbumin-induced asthmatic mice, we observed that intraperitoneal injection of IL-35 during the allergen sensitization stage was more efficient than administration in the challenge stage for the amelioration of airway hyper-responsiveness. This was reflected by the significantly reduced concentration of asthma-related Th2 cytokines IL-5 and IL-13, as well as eosinophil counts in bronchoalveolar lavage fluid (all $P < 0.05$). IL-35 also significantly attenuated the accumulation of migratory CD11b⁺CD103[−] dendritic cells (DC) in the mediastinal lymph node (mLN) and lung of mice (all $P < 0.05$). IL-35 markedly inhibited the ovalbumin-induced conversion of recruited monocytes into inflammatory DC, which were then substantially reduced in mLN to cause less T-cell proliferation (all $P < 0.05$). Further study using the humanized asthmatic murine model also indicated human IL-35 exhibited a regulatory impact on allergic asthma.

Conclusion: Our findings suggest that IL-35 can act as a crucial regulatory cytokine to inhibit the development of allergic airway inflammation via suppressing the formation of inflammatory DC at the inflammatory site and their accumulation in the draining lymph nodes.

Abbreviations

AHR, airway hyper-responsiveness; BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived DC; DC, dendritic cells; HDM, house dust mite; mLN, mediastinal lymph node; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells.

Allergic asthma, one of the most common forms of bronchial inflammation of respiratory diseases, is clinically characterized by reversible bronchial constriction and airway hyper-responsiveness (AHR). The elevated serum IgE concentration, and differentiated CD4⁺ T helper type 2 (Th2) lymphocytes in bronchial tissues and draining lymph nodes (LNs) that can secrete allergy-related proinflammatory cytokines

such as IL-4, IL-5, and IL-13, contribute essentially to the pathogenesis and exacerbation of allergic asthma (1–3).

Dendritic cells (DC) are functional antigen-presenting cells that play a pivotal role in the initiation and amplification of Th2 responses by presenting special type of peptide epitopes from pathogens or allergen-derived antigens to naive CD4⁺ T cells. In mice, transient depletion of lung CD11c⁺DC either before ovalbumin (OVA) aerosol challenge or after the induction of AHR abrogates the Th2-predominant inflammation in the airway (4). Several DC subsets can be classified according to their distinctive cell surface marker expression and lineage, including CD103⁺–CD11b⁺CD11c^{hi} conventional DC, CD103⁺CD11b⁺–CD11c^{hi} conventional DC, and CD11c^{low}B220⁺PDCA-1⁺ plasmacytoid DC (pDC) (5). Generally, the CD103⁺ DC or plasmacytoid DC are efficient to regulate the CD4⁺ Th cell activation and induce the airway or oral tolerance (6, 7). On the contrary, the conventional CD11b⁺ DC serve as an indispensable initiator for developing allergic response in the airway (8). The accumulation of CD11b⁺ DC in the lung and mediastinal lymph nodes (mLNs) is commonly observed in mice before the manifestation of characteristic clinical features of asthma. Different research groups have addressed the identity of DC precursors originating from the bone marrow and suggested that they are the major source of lymphoid organ-resident DC and migratory tissue DC in steady state (9, 10). However, during inflammation or infection, the Ly6C^{hi} inflammatory monocytes can be recruited to the inflammatory sites and differentiated into inflammatory DC, expressing cell surface CD11c, major histocompatibility complex (MHC) II, and costimulatory molecules (11, 12).

IL-35, the newest member of the IL-12 family, is a heterodimer composed of Ebi3 and p35, signaling through the receptor comprising GP130 and IL-12Rβ2, or IL-27Rα and IL-12Rβ2 (13, 14). Within the CD4⁺ Th cell population, IL-35 is produced by resting and activated regulatory T cells (Tregs) after converting conventional T cells into IL-35-dependent-induced Tregs (iT_{reg}35) (15). IL-35 also directly promotes human B lymphocytes into IL-10- or IL-35-producing regulatory B cells, attenuating autoimmune and other inflammatory diseases. Additional evidence has demonstrated that IL-35 secreted by inducible costimulator-positive regulatory T cells can efficiently suppress the Th17 activity and reduce the progression of OVA-/lipopolysaccharide (LPS)-induced

neutrophilia in AHR (16). Administration of plasmid to enhance IL-35 expression could effectively attenuate the house dust mite (HDM) allergen-specific Th2 cell line-induced airway inflammation and IgE production in mice (17).

In this study, we investigated the *in vivo* regulatory role of IL-35 in the OVA-induced eosinophilia and allergic inflammation in airway and elucidated the novel cellular mechanism for the suppression of AHR in asthmatic mice upon IL-35 treatment.

Methods

Methods are described in detail in Data S1.

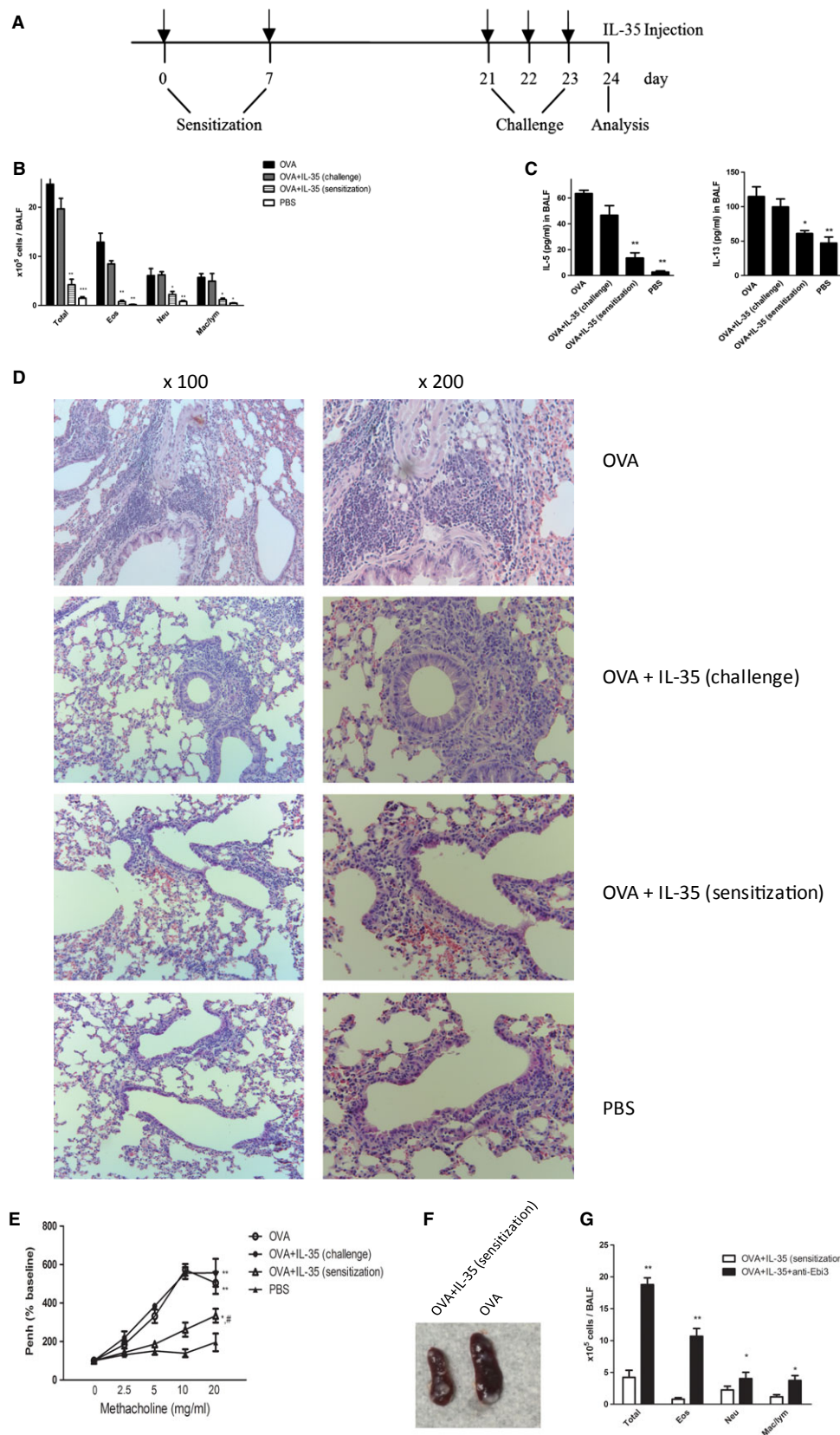
Results

Intraperitoneal injection of IL-35 during allergen sensitization relieved OVA-induced allergic airway inflammation and AHR

To investigate whether exogenous IL-35 was sufficient to exert beneficial impacts on allergic asthma, we established the OVA-induced airway inflammation in mice using the protocol shown in Fig. 1A. The inflammatory response in lung tissue was significantly suppressed when IL-35 was injected i.p. during the allergen sensitization stage (days 1–7), which was indicated by the markedly decreased number of infiltrated eosinophils, neutrophils, and mononuclear cells, as well as the reduced Th2- and allergy-related cytokines (IL-5 and IL-13) release in the bronchoalveolar lavage fluid (BALF) (Fig. 1B, C). On day 24, reduced peribronchial inflammatory infiltrates were also observed in hematoxylin and eosin (H&E)-stained lung sections from the group of mice treated with IL-35 in the sensitization stage (Fig. 1D). Interestingly, we did not find a dramatically therapeutic effect in asthmatic mice with IL-35 injection during allergenic challenge (days 21–23). For *in vivo* IL-35 treatment at the sensitization stage, mice displayed a decrease in AHR, which was indicated by the dampened Penh growth (Fig. 1E). Although the initial administration of IL-35 indeed mitigated the allergic inflammation, it could not completely eliminate the asthmatic signs such as shortness in breath of mice. Interestingly, a smaller spleen was found in mice received IL-35 during sensitization (Fig. 1F). To validate the exclusive role of IL-35 in

Figure 1 Effects of intraperitoneal injection of IL-35 during the allergen sensitization stage on airway inflammation and AHR in the OVA-induced murine model. (A) Timeline protocol of OVA-induced allergic asthma and IL-35 administration. (B) The total cell number and differential cell counts in bronchoalveolar lavage fluid (BALF) were determined using Hemacolor staining. Total, total cells; Eos, eosinophils; Neu, neutrophils; Mac/lym, macrophages/lymphocytes. (C) The concentration of IL-5 and IL-13 in BALF was determined. **P* < 0.05, ***P* < 0.01 ****P* < 0.001, compared with the group of mice induced by OVA alone. (D) Representative lung sections stained with H&E and examined at ×100 or ×200 magnification (*n* = 5). (E) Airway obstruction was mea-

sured as Penh values, Penh (% baseline) was calculated by dividing methacholine-induced Penh by PBS baseline (*n* = 3). **P* < 0.05, ***P* < 0.01, compared with control. #*P* < 0.05, compared with the group of mice induced by OVA alone. (F) Representative whole-mount spleen from mice induced by OVA with or without IL-35 treatment (*n* = 4 per group). (G) BALF cells were differentially counted after mice were treated with OVA and IL-35 in the presence or absence of anti-Ebi3 antibody. **P* < 0.05, ***P* < 0.01. The analysis was conducted 24 h after the last OVA exposure (day 24). Bar charts were presented with mean ± SD (*n* = 4 per group). AHR, airway hyper-responsiveness; OVA, ovalbumin.



modulating the immune response, we applied a neutralizing antibody to Ebi3, which displays an ability to specifically interrupt the activity of IL-35 (13). As a result, the regulatory role of IL-35 on infiltrating cells in BALF was reversed by the neutralizing antibody (Fig. 1G). Collectively, the above-mentioned data suggest that the IL-35 is capable of inhibiting the Th2-predominant allergic immune response and AHR in asthmatic mice sensitized to OVA.

Accumulation of CD11b+CD103[−]DC in mLN and lung is impaired in OVA-induced asthmatic mice upon IL-35 treatment

As the pulmonary inflammation induced by OVA could be significantly suppressed by IL-35 treatment, we next address the mechanism by which IL-35 functions as a regulatory cytokine. We then evaluate whether IL-35 has any effect on the DC accumulation in mLN or lung. Indeed, mice administered with murine IL-35 on days 1 and 7 during OVA immunization displayed a significant decrease in the percentage and absolute number of the CD11b+CD103[−]DC subset in mLN (Fig. 2A,B). Consistently, we observed a significant reduction in the percentage and absolute number of CD11b+CD103[−]DC that accumulated in the lung of IL-35-treated mice compared with OVA-alone-treated mice when examined 24 h after the last OVA aerosol challenge (Fig. 2C, D). No significant difference in the percentage of pDC in the lung could be detected in both groups of mice with or without IL-35 treatment (Fig. 2E). To address whether the decreased proportion of CD11b+CD103[−]DC in the mLN from IL-35-treated mice was due to inhibited proliferation in mLN, we assessed the proliferation of CD11b+CD103[−]DC in mLN by determining its 5-bromodeoxyuridine (BrdU) incorporation on day 8 (19). Equivalently low levels of BrdU⁺ cells were observed in these two groups of mice with or without IL-35 treatment (Fig. 2F). These data therefore indicated the decreased proportion of CD11b+DC in mLN upon IL-35 treatment was likely due to reduced migration but not IL-35-suppressed *in situ* proliferation, when compared with OVA-alone-treated mice. Taken together, the above data indicated that the administration of IL-35 caused an impaired DC trafficking to the lung and draining mLN.

CD11c⁺ cells in mLN of IL-35-treated mice were less efficient to induce Th2-predominant allergic inflammation

We then explored whether IL-35-mediated decrease in DC from mLN was sufficient to account for the attenuated Th2-

mediated allergic inflammation. First, we evaluated the ability of mLN CD11c+DC to induce T-cell proliferation. As shown in Fig. 3A, DC from IL-35-treated mice displayed the attenuated ability in promoting CD4⁺Th cell proliferation, compared with that from OVA-alone-treated mice. Moreover, we observed a markedly reduced mRNA transcription of Th2-related cytokines IL-4, IL-5, and IL-13 in mLN cells from IL-35-treated mice upon *in vitro* OVA restimulation (Fig. 3B). Meanwhile, a consistent decrease in protein level of asthma-related IL-5 and IL-13 was also observed in the supernatant of OVA-restimulated mLN cells from mice treated with IL-35 (Fig. 3C).

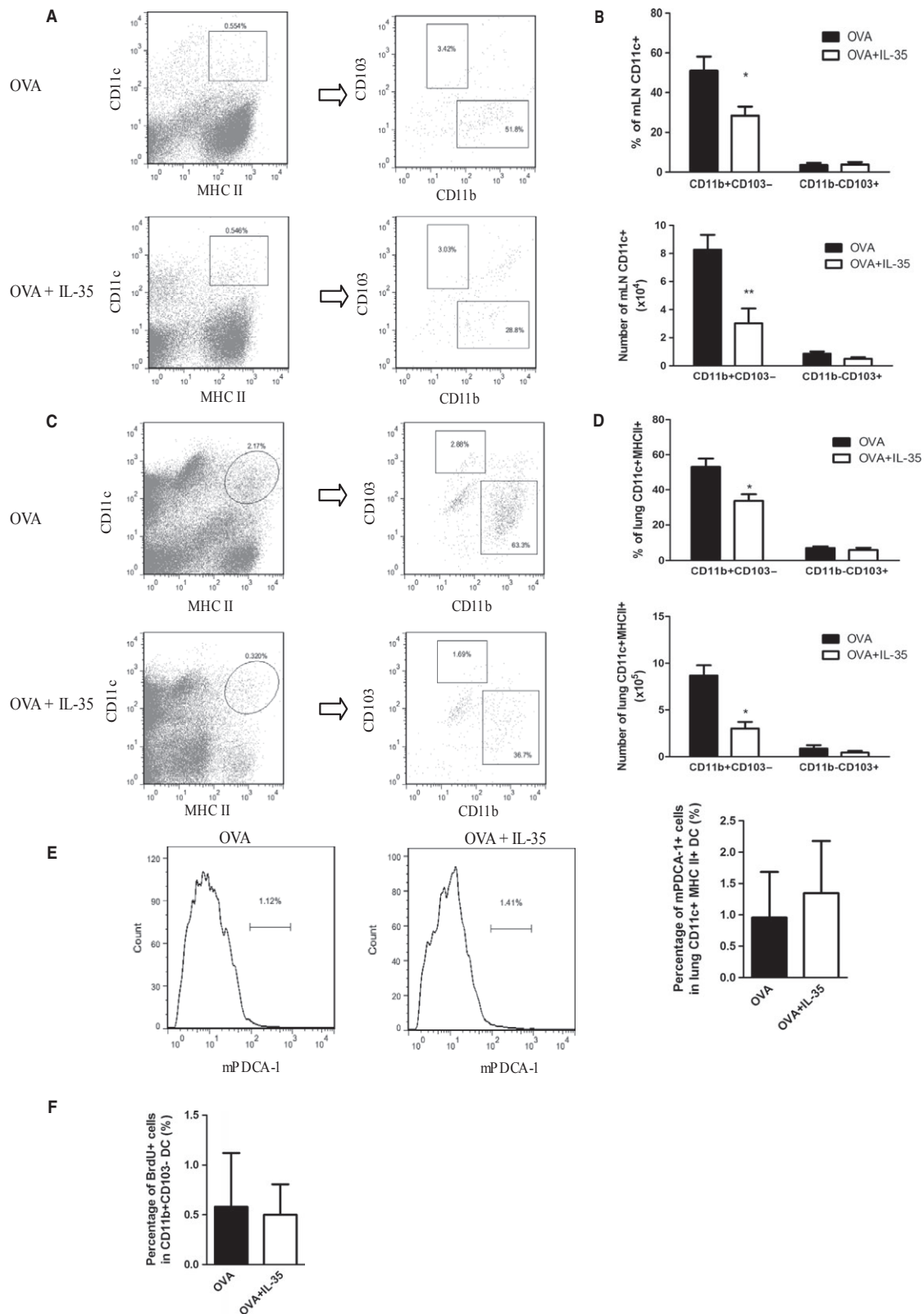
We then explored whether DC in mLN of OVA-treated mice with or without IL-35 treatment had any divergent properties to induce Th2-mediated pulmonary inflammation. CD11c+DC from mLN of mice treated with OVA/alum alone or combined OVA/alum and IL-35 were enriched using magnetic cell sorting. Such enriched CD11c+DC were then adaptively transferred intratracheally (i.t.) into recipient mice. After challenge with OVA, mice instilled with CD11c+DC isolated from mLN in IL-35-treated mice displayed a moderately dampened AHR provocation when compared with DC from OVA-treated mice without IL-35 injection (Fig. 4A). Furthermore, mice instilled with CD11c+DC from IL-35-plus OVA-treated mice developed less peribronchiolar infiltration and mucus production in the airway, with a lower percentage of IL-4-producing CD4⁺ Th2 cells compared to OVA-alone-treated mice *in vivo* (Fig. 4B,C). Overall, these results suggested that CD11c+DC in mLN from IL-35-treated mice are less effective to induce asthmatic feature than those from mLN of mice induced by OVA alone, thereby indicating the reduced accumulation of CD11b+DC in mLN should be an explanation for the IL-35-mediated suppression against OVA-induced airway inflammation.

IL-35 inhibited the production of inflammatory DC as well as its migration to mLN for promoting T-cell proliferation

Inflammatory monocytes could be recruited into the peritoneal cavity after mice were injected with OVA and alum adjuvant (20). This kind of monocyte can take up antigens, transform into inflammatory DC, migrate into the mLN, and function as DC. To elucidate whether IL-35 has any effect on the inflammatory DC, we generated the Ly6C⁺ inflammatory monocytes from bone marrow and determined the gene expression of potential receptor for IL-35 in murine monocytes, including *gp130*, *il12rb2*, and *il27ra*. Similar levels of the three gene expressions were found in the untreated,

Figure 2 Effects of IL-35 on the OVA-induced CD11b+CD103[−]DC accumulation in mLN and lung of mice. (A) Representative dot plots of CD11b+CD103[−] and CD11b[−]CD103+DC in mLN on day 8 and (B) percentages and absolute numbers of mLN DC subsets were analyzed. (C) Representative dot plots of CD11b+CD103[−] and CD11b[−]CD103+DC in lung on day 24 and (D) percentages and absolute numbers of lung DC subsets were analyzed. (E) Representative histogram of murine plasmacytoid dendritic cell antigen

(mPDCA)-1+ plasmacytoid DC in CD11c+MHC II+DC from lung on day 24 (left panel) and percentage of mPDCA-1+ plasmacytoid DC (right panel) were analyzed. (F) BrdU was i.p. injected 48 h before killing, the BrdU⁺ cells in the CD11b+ CD11c+DC in the mLN on day 8 were then analyzed using flow cytometry. *n* = 5 per group. **P* < 0.05, ***P* < 0.01, compared with the group of mice induced by OVA alone. Bar charts were presented with mean ± SD (*n* = 4 per group). mLN, mediastinal lymph node; OVA, ovalbumin.



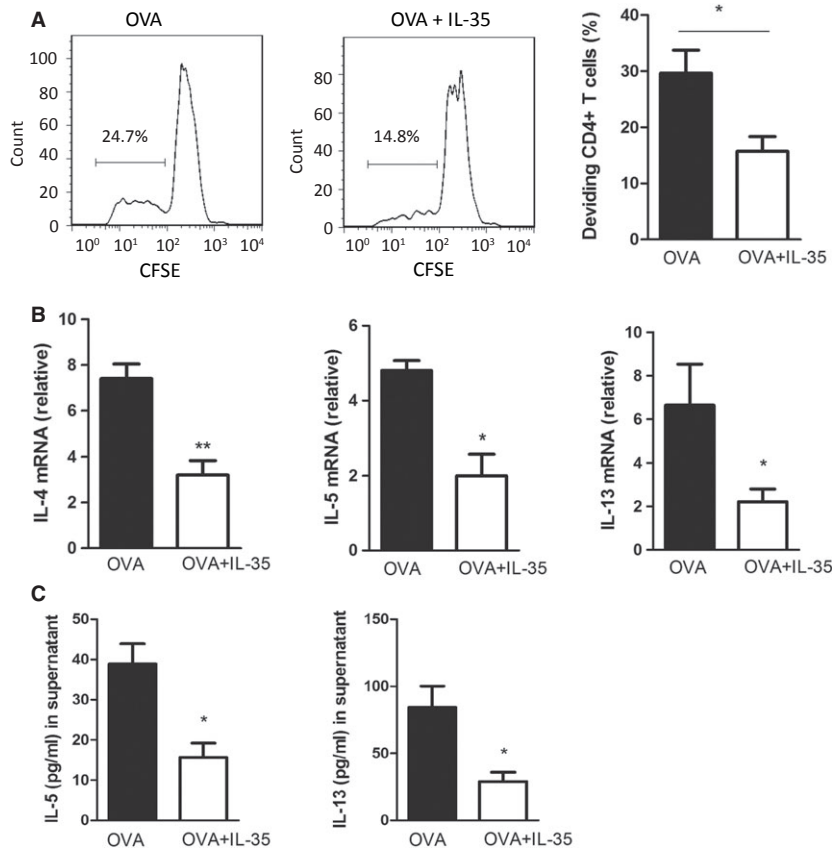


Figure 3 *In vitro* effects of CD11c⁺ cells isolated from mLN of IL-35-treated mice on the activation of T cells. (A) CD11c⁺ cells were enriched from mLN of mice, treated either with combined IL-35 and OVA or OVA alone on day 8, and then cocultured with CFSE-labeled CD4⁺ T cells for 4 days. CFSE dilution was evaluated by flow cytometry. Representative histograms (left panel) and bar chart (right panel) of T-cell proliferation were assessed by flow cytometry. Representative histograms were shown from three

independent experiments with similar results. mLN cells were cultured and restimulated with OVA (100 µg/ml) for 72 h, (B) the mRNA level of IL-4, IL-5, and IL-13 was determined by Q-PCR, and (C) the concentration of IL-5 and IL-13 in supernatant was measured by Milliplex MAP kit. Bar charts were presented with mean ± SD (*n* = 4 per group). **P* < 0.05, ***P* < 0.01, compared with control. mLN, mediastinal lymph node; OVA, ovalbumin.

OVA/alum or combined OVA/alum and IL-35-stimulated monocytes *in vitro* (Fig. 5A). Then, recipient mice were injected i.p. with OVA/alum or OVA/alum together with IL-35, followed by the intraperitoneal transportation with monocytes. Twenty-four hours later, the significantly decreased CD11c expression and slightly reduced MHC II and CD86 expression were found on the CD11b⁺Ly6C⁺F4/80^{int} monocytes in mice received IL-35 (Fig. 5B). Moreover, the number of CD11c⁺Ly6C⁺ inflammatory DC in mLN strongly decreased in the IL-35-treated mice 36 h after the monocyte injection (Fig. 5C). Notably, mice with IL-35 treatment also displayed decreased expression of CD86 on CD11c⁺Ly6C⁺ DC in mLN with a reduced proportion of MHC II-positive DC (Fig. 5D). To analyze the capacity of inflammatory DC in inducing T-cell division, the CD4⁺ Th cells were isolated from OVA-sensitized mice and labeled with carboxyfluorescein succinimidyl ester (CFSE) and then injected i.v. into the recipient mice on day 0. Then, mono-

cytes stimulated with OVA/alum with or without IL-35 for 24 h were injected i.p. into recipient mice on day 1. On day 3, the T-cell proliferation in the mLN of mice received IL-35 and OVA/alum-treated monocytes was attenuated compared with that in mice injected with OVA-/alum-treated monocytes (Fig. 5E).

To address whether there is any functional effect of IL-35 on the conventional DC, we generated the bone marrow-derived dendritic cells (BMDC). Similar to monocytes, comparable gene expression of *gp130*, *il12rb2*, and *il27ra* could be observed in BMDC (data not shown). We also found comparable proportions of BMDC that took up OVA in the presence or absence of IL-35 *in vitro* (Fig. S1A). Similar to IL-10, IL-35 treatment did not lead to any difference in the CD86 and MHC II expression on OVA-pulsed BMDC (Fig. S1B). The expression of transcription factor interferon regulatory factor4 (IRF4) in DC is critical to polarize Th2 response, and we found that mRNA transcription of IRF4

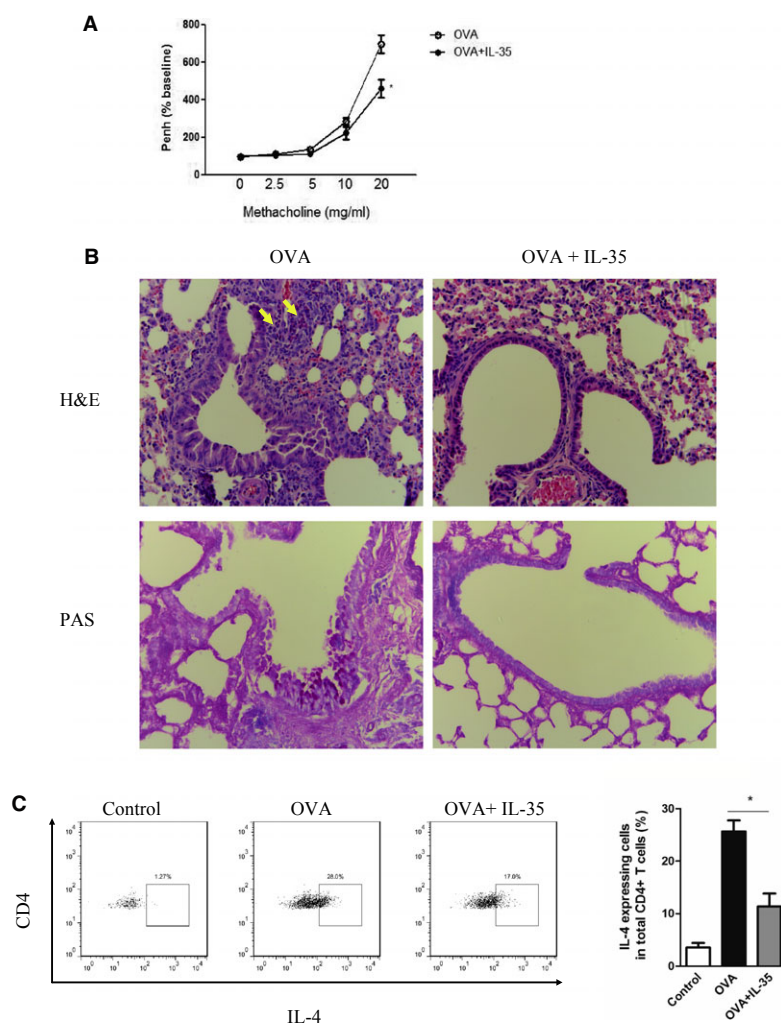


Figure 4 *In vivo* effects of CD11c⁺ cells isolated from mLN of IL-35-treated mice on the induction of Th2-mediated airway inflammation. CD11c⁺ cells were enriched from mLN of mice treated either with combined IL-35 and OVA/alum or OVA/alum alone on day 8. The enriched CD11c⁺ cells were then adoptively transferred to untreated recipient mice. Mice were killed and analyzed after the last challenge with OVA on day 20. (A) AHR of mice was analyzed by the measurement of lung Penh in response to increasing concentration of methacholine, Penh (% baseline) was calculated by dividing methacholine-induced Penh by PBS baseline ($n = 3$).

* $P < 0.05$. (B) Representative photomicrographs of lung sections stained with H&E (upper panel) and periodic acid–Schiff (PAS, lower panel), and examined at 200 $\times$ magnification ($n = 3$). Yellow arrows indicated the infiltrated eosinophils around bronchus. (C) The representative dot plots (left panel, $n = 3$) and bar chart (right panel) of the percentages of IL-4-positive Th2 lymphocytes in CD4⁺ Th cells were analyzed using flow cytometry. Bar charts were presented with mean $\pm$ SD ($n = 4$ per group). mLN, mediastinal lymph node; OVA, ovalbumin; AHR, airway hyper-responsiveness.

was not strikingly disturbed in OVA-stimulated BMDC with or without IL-35 (Fig. S1C). As expected, the chemokine transcriptions, including CCL2, CCL5, CCL17, CCL20, and CCL22, in OVA-stimulated BMDC showed no remarkable difference with or without IL-35 treatment (Fig. S1C). Taken together, these data revealed that IL-35 interrupted the conversion of the recruited monocytes to inflammatory DC and caused an impaired accumulation of inflammatory DC in the mLN and subsequent T-cell proliferation. However, IL-35

could not cause any influence to OVA-induced activation of BMDC *in vitro*.

Recombinant human IL-35 moderates AHR in humanized murine model of allergic asthma

To identify whether IL-35 exhibits similar effects in humans as well as in mice, we employed the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice reconstituted

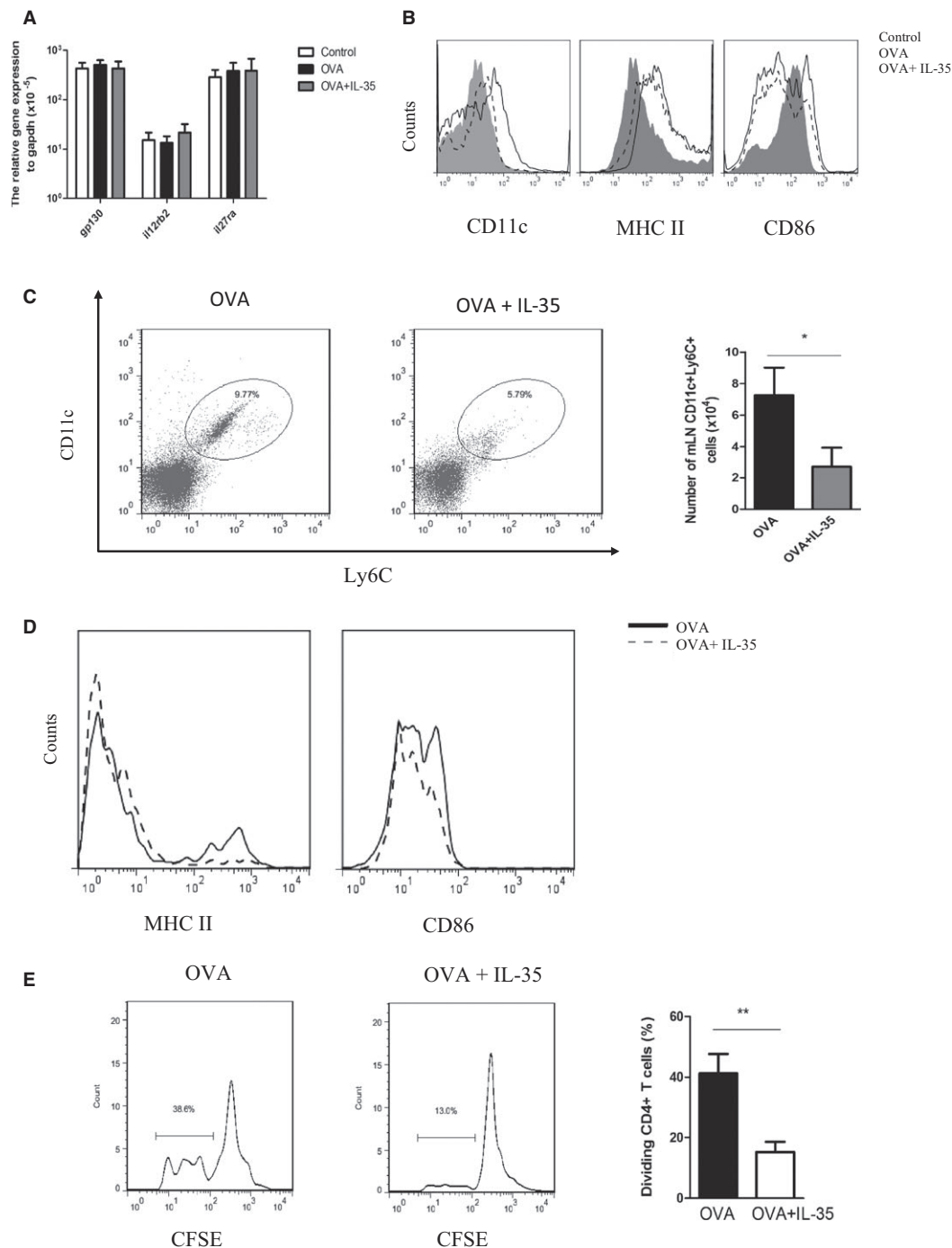


Figure 5 Effects of IL-35 on the OVA-mediated monocytes conversion into inflammatory DC. (A) The mRNA levels of *gp130*, *il12b2*, and *il27ra* in monocytes stimulated with OVA/alum (20 μ g/ml) with or without IL-35 (100 ng/ml) were determined after 24-h incubation. Bar charts are presented with mean $\pm$ SD. (B) The expression of CD11c, MHC II, and CD86 on CD11c+Ly6C+F4/80^{int} peritoneal monocytes was analyzed using flow cytometry. (C) Representative dot plots (left panel) and absolute number (right

panel) of CD11c+Ly6C+ cells in mLN after 36-h incubation are shown (four mice per group). (D) The expression of MHC II and CD86 on CD11c+Ly6C+ cells in mLN was evaluated using flow cytometer. (E) Representative histograms (left panel) and bar chart (right panel) of T-cell proliferation were assessed 48 h after the i.p. injection with monocytes. * $P < 0.05$, ** $P < 0.01$, compared with control. mLN, mediastinal lymph node; OVA, ovalbumin.

with human peripheral blood mononuclear cells (PBMC) from allergic patients with asthmatic sensitized to HDM. In line with a previous study (21), airway inflammation was established 14 days after the last HDM aerosol, and we detected a stably existed human CD45+ cells in the blood of SCID mice 21 days after human PBMC injection (Fig. S2). *In vivo* i.p. injection of human IL-35 reduced AHR in the humanized SCID model, as reflected by the relatively lower rate of Penh growth (Fig. 6A), although it could not restore AHR back to the healthy control level. The cellular infiltration around the bronchus was substantially mitigated when IL-35 was injected together with the human PBMC, as demonstrated in the H&E-stained lung sections (Fig. 6B). Therefore, our results demonstrated that human IL-35 administration could downregulate the HDM-induced airway allergic inflammation in humanized SCID mice. However, we did not observe any significant change in the expression of

CD11c, MHC II, or CD86 on the primary human monocytes upon *in vitro* treatment with IL-35 (50 or 100 ng/ml, data not shown).

Discussion

It had been found that *Ebi3*^{−/−} mice are more susceptible to leishmaniasis (22), and *IL-12α*^{−/−} mice are more susceptible to *Leishmania major* infection, experimental autoimmune encephalomyelitis, and collagen-induced arthritis (23–25). These observations indicate that IL-35 plays inhibitory role in the immunologic responses against the exogenous or endogenous antigens in host. Regarding the allergic airway diseases, Huang et al. found that IL-35 effectively suppressed Blo t 5-specific Th2 cell-mediated airway inflammation. However, the detailed cellular mechanisms of the inhibitory activity of IL-35 remain unclear (17). In fact, Whitehead et al.

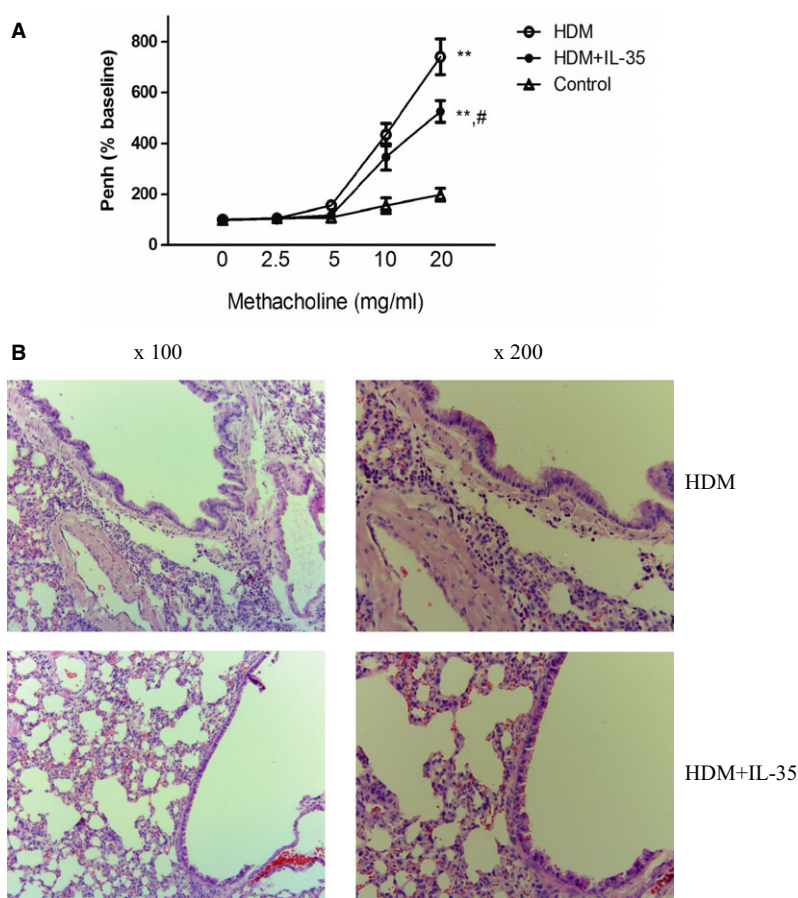


Figure 6 *In vivo* effects of human recombinant IL-35 on the development of asthmatic feature in humanized murine model. SCID mice were reconstituted with 2×10^7 human PBMC and then challenged with HDM on days 1, 3, and 7. (A) On day 21, AHR was evaluated by the measurement of lung Penh in response to increasing concentration of methacholine. Penh (% baseline) was calculated by dividing methacholine-induced Penh by PBS baseline. $**P < 0.01$, compared with control. $\#P < 0.05$, compared with the

group of HDM. (B) Lung tissue from humanized SCID mice (PBMC from allergic patient with asthmatic patient) treated with HDM or combined treatment of HDM and IL-35 was stained with H&E. Representative photomicrographs are from three independent experiments ($n = 4$ for each group). Original magnification was $\times 100$ and $\times 200$. AHR, airway hyper-responsiveness; SCID, severe combined immunodeficiency; HDM, house dust mite; PBMC, peripheral blood mononuclear cells.

(16) had identified that the deficiency of IL-35 in mice gave rise to the IL-17A-producing Th17 cells, which was required to maintain the AHR after multiple allergen challenges. Although we employed the OVA-/alum-induced eosinophilia asthma model, the consistent phenomenon could be observed, which is reflected by the elevated concentration of serum OVA-specific IgE in mice treated by the anti-Ebi3 antibody compared with the OVA-activated mice with or without IL-35 treatment (Fig. S3). In the current study, we reported a novel mechanism that IL-35 modulated the suppression of inflammatory DC at the inflammatory site and impaired their accumulation in the draining LNs in allergic asthmatic mice.

CCR2 + Ly6C⁺ monocytes have been regarded as the precursors of inflammatory DC. They are recruited in the inflammatory or infectious sites, where they took up antigen, expressed the DC-like cell surface molecules, and acquired the elevated potential to promote the effector T cells (20, 26, 27). We found that the monocyte transformation into DC was inhibited in the presence of IL-35, as indicated by the significantly decreased expression of DC marker CD11c and slightly reduced expression of MHC II and CD86. Moreover, the IL-35 caused a decreased accumulation of CD11b+CD103⁺DC, leading to the attenuated T-cell proliferation and Th2 cell-mediated allergic immune response. These results revealed, for the first time, in addition to T or B cells, IL-35 may directly suppress the production of inflammatory DC, thereby causing an impaired adaptive immune response. In this regard, IL-35 may be a potential inhibitory cytokine for the antigen recognition, which is indispensable for the antigen-specific immune response in host. Interestingly, several studies have reported that the IL-35 could favor the tumor cell growth through either suppressing the antigen-specific CD8⁺Treg cells or enhancing myeloid cell accumulation and angiogenesis (28, 29). The tumor-specific antigen could be recognized by DC to provoke a subsequent immune reaction to help for the tumor cell clearance (30, 31). Based on our results, it would be worth to further evaluate whether IL-35-mediated suppression on inflammatory monocyte transformation toward inflammatory DC has any contribution to the IL-35-promoted tumor cells growth.

In addition, IL-35 did not induce any direct effect on the *in vitro* OVA uptake, costimulatory molecule expression, or chemokine production in OVA-stimulated BMDC. However, we could not preclude the possibility that IL-35 may have some influence on the functions of conventional DC in OVA-induced airway inflammation *in vivo*. No difference was found in either the absolute number or the percentage of CD11b⁺CD103⁺DC in mLN or lung of asthmatic mice after IL-35 injection. We also did not observe any obvious change in the proportion of peritoneal CX3CR1⁺CD11c⁺F4/80^{hi} macrophages, which can activate CD103⁺DC to promote tolerance, in mice sensitized to OVA and alum after IL-35 administration (data not shown). Therefore, IL-35 is not likely to suppress the Th2-predominant airway inflammation by regulating the accumulation of CD103⁺DC in mLN.

Furthermore, we have adopted the novel chimeric allergic asthmatic mice with peripheral humanized blood cells to

show that IL-35 could exhibit the regulatory effect by down-regulating HDM-induced airway allergic inflammation and AHR in allergic asthma. These results suggested that the human IL-35 exerts a similar suppressive role in the development of allergic inflammation by modulating the inflamed human PBMC, although the mechanism remains unraveling. This humanized murine model mimicking human allergic asthma therefore provided relevant biochemical basis for the clinical implication of IL-35 for the treatment of allergic asthma in human. However, further study is required to elucidate the direct effect of human IL-35 on human PBMC *in vitro*.

Together, our current study should have demonstrated for the first time that the regulatory cytokine IL-35 substantially suppresses the recruited monocytes conversion to inflammatory DC and causes impaired accumulation of CD11b+CD103⁺DC in mLN and lung, thereby leading to the decreased airway allergic inflammation. It had been reported that people with genetic predisposition suffer from high risk of developing specific IgE-mediated response to common aeroallergens (34). Dendritic cells with IgE binding can present inhaled allergens and induce an allergic inflammation through the activation of the T or B lymphocytes in the secondary lymphatic systems (35). Therefore, IL-35 may be used as a preventive treatment for individuals having genetic risk of allergic diseases. Together with the results using humanized asthmatic mice, IL-35 may also be a promising therapeutic agent for patients with allergic diseases.

Author contributions

JD and CKW wrote the manuscript and contributed to the study hypothesis, literature search, and data interpretation. JD, ZC, DJ, and MC performed the experiments. CKW and CWKL obtained funding, supervised the study, and contributed to critical revision.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.**Figure S1.** Effects of IL-35 on OVA-stimulated BMDC.**Figure S2.** Human PBMC in SCID mice.**Figure S3.** Serum OVA-specific IgE level in asthmatic mice with different treatment.**Table S1.** Primer sequences used in Q-PCR.**References**

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Data S1: Methods

Methods

Mice

Inbred female BALB/c mice (8 weeks old) and non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (5–6 week old) were bred under specific pathogen-free conditions and maintained at our Laboratory Animal Services Center, The Chinese University of Hong Kong. Experiments were conducted in accordance with the principles outlined in the Animal Experimentation Ethics Committee Guide for the Care and Use of Laboratory Animals, as approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Patients

Patients diagnosed with allergic asthma aged 20 - 30 years with house dust mites (HDM) sensitization (n = 10) and sex- and age-matched non-atopic healthy control subjects (n = 5) were recruited from the Allergy Clinic of The Prince of Wales Hospital, Hong Kong. EDTA blood (50 ml) obtained from each subject was diluted with phosphate buffered saline (PBS) and PBMC were then harvested using Ficoll gradient centrifugation (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Purified PBMC (2×10^7) were used for intraperitoneal injection (i.p.) into SCID mice for the construction of humanized mice. All the recruited subjects were ethnic Chinese. The above protocol were approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong-New Territories East Cluster Hospitals, and informed written consent was obtained from all subjects or their parents in accordance with the 1964 Declaration of Helsinki and its later amendments.

OVA-induced allergic asthmatic mice model

Allergic airway inflammation was induced as previously described (18). Briefly, mice were sensitized with i.p. OVA (20 µg, Sigma-Aldrich Corp., St Louis, MO, USA) adsorbed to 1 mg Imject Alum (Thermo Fisher Scientific Inc, Rockford, IL, USA) or PBS on days 1 and 7, challenged twice with intranasal 1 % OVA or PBS aerosol on days 21, 22 and 23. Murine IL-35 (400 ng/mouse, Adipogen International, Inc. San Diego, USA) was injected i.p. just before the sensitization or challenge. In some experiments, mice were pre-treated i.p. with anti-Ebi3 antibody (500 ng/mouse; Shenandoah Biotechnology Inc., Warwick, PA, USA) 1 hour before the IL-35 administration. Mice were examined 24 hours after the last challenge. For monitoring AHR, Penh was measured by exposing conscious mice to aerosolized PBS for setting a baseline value, followed by increasing concentrations of aerosolized methacholine using a whole body plethysmograph (Buxco-Force Pulmonary Maneuvers, Buxco Research Systems, Wilmington, NC, USA). Lung tissues were obtained from sacrifice mice and embedded in paraffin, and 5 µm sections were prepared for H&E and periodic acid-Schiff (PAS) staining before examination by light microscopy (Leica DM6000B, Leica Microsystems Inc., Buffalo Grove, IL, USA).

Generation of murine bone marrow-derived DC (BMDC) and bone marrow-derived monocytes

BMDC were generated as previously described (32). Bone marrow cell suspensions were obtained from femurs and tibias of female Balb/c mice, and cultured in complete medium [RPMI 1640 (Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FCS, Life Technologies), 50 µM 2-mercaptoethanol (Sigma-Aldrich Corp., St. Louis, MO, USA), 2 mM glutamine (Life Technologies),

and 0.1 mM nonessential amino acids (Life Technologies)] supplemented with 20 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml murine IL-4 (PepProTech Inc., Rocky Hill, NJ, USA). Non-adherent cells were removed on day 2, and the medium was refreshed every 3 days. BMDC were harvested on day 8.

We obtained the bone marrow derived monocytes according to the protocol in previous publication (33). Bone marrow cell suspensions were cultured in the medium (M199 + 10% fetal calf serum (FCS), + 1% Penicillin/Streptomycin) supplemented with 20 ng/ml recombinant murine M-CSF (PepProTech). Monocytes were then harvested on day 5.

Purification of CD11c⁺ cells and the co-culture of CD11c⁺ cells and CD4⁺ T cells

Single cell suspension was prepared from mLN (five female Balb/c mice in a pool), all the CD11c⁺ cells were isolated using excessive anti-CD11c magnetic microbeads (Miltenyi Biotec) according to the manufacturer's instructions. To obtain OVA sensitized CD4⁺ T cells, mice were injected i.p. with OVA-alum on days 1 and 7. Ten days later, CD4⁺ T cells were isolated from the spleen of primed mice using anti-CD4 magnetic microbeads (Miltenyi Biotec). CD11c⁺ cells and carboxyfluorescein succinimidyl ester (CFSE) labeled CD4⁺ T cells (1×10^5) were co-cultured in 0.5 ml RPMI 1640 medium supplemented with 10% FCS in 24-well plate. The proliferation of T cells was determined by measuring the CFSE dilution after 4 days.

Allergic features of mice induced by the adoptive transfer of DC from mLN

On days 1 and day 7, mice were instilled with enriched CD11c⁺ cells (1×10^6 /mouse)

either from OVA plus IL-35-treated mice or OVA alone treated mice, followed by the intratracheal (i.t.) injection of 50 µg OVA on days 13, 15, 17 and 19. The analysis was performed 24 hours after the last challenge of OVA.

Assay of cytokines in BALF and culture supernatant

The concentrations of Th2-related cytokines IL-5 and IL-13 were measured using Mouse Cytokine Milliplex MAP assay kit (Merck Millipore, Billerica, MA, USA) with the Bio-Plex 200 suspension array system (Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometry

To identify the DC subsets, mLN and lung tissue were treated with collagenase (2 mg/ml, Life Technologies) and DNAase I (40 U/ml, Life Technologies). Single cell suspension was prepared by filter the digested tissue with a 70 µm mesh cell strainer. After the red blood cells lysis, cells were stained with the following fluorescence dye conjugated antibodies: anti-CD103, anti-CD11b, anti-CD11c, anti-I-A/I-E, anti-F4/80, anti-Ly6C, anti-CD86, anti-BrdU and corresponding iso-typical control antibodies (Biolegend Corp., San Diego, CA, USA). For the intracellular staining, cells were stained with anti-CD4-FITC followed by fixation with 4% paraformaldehyde and permeabilization with perm buffer (BD Pharmingen Corp., San Diego, CA, USA). Then the cells were analyzed by flow cytometry (FACSCalibur flow cytometer, BD Biosciences Corp., San Jose, CA, USA) after stained with anti-IL-4-APC (BioLegend).

Q-PCR

Total RNA was extracted using TRIzol reagent (Life Technologies) and reverse transcribed into first-strand complementary DNA using PrimeScript™ RT Master Mix

(Takara Bio Inc., Shiga, Japan). The quantitative expression of target genes was measured using FastStart Universal SYBR Green Master (ROX) (Roche Applied Science, Basel, Switzerland) with corresponding primers (Table S1). The GAPDH house keeping gene was used as the internal reference and the relative gene expression was calculated using $2^{-\Delta Ct}$ ($Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$).

OVA-mediated the conversion of monocytes to inflammatory DC

The mice were injected i.p. with OVA-alum (20 $\mu\text{g}/\text{mouse}$) with or without murine IL-35 (400 ng/mouse), followed by the adoptive transfer with monocytes ($2 \times 10^6/\text{mouse}$). Then peritoneal and mLN cells were harvested 24 hours or 36 hours later for flow cytometric analysis. For the analysis of T cell proliferation in mLN, the OVA sensitized CD4⁺ T cells ($1 \times 10^7/\text{mouse}$) were labeled with CFSE and injected intravenously (i.v.) on day 0. Then monocytes ($2 \times 10^6/\text{mouse}$) stimulated *in vitro* with OVA-alum (20 $\mu\text{g}/\text{ml}$) with or without IL-35 (100 ng/ml) for 24 hours were injected i.p. on day 1. T cell proliferation was assessed with flow cytometry on day 3.

Humanized SCID murine model with allergic asthma

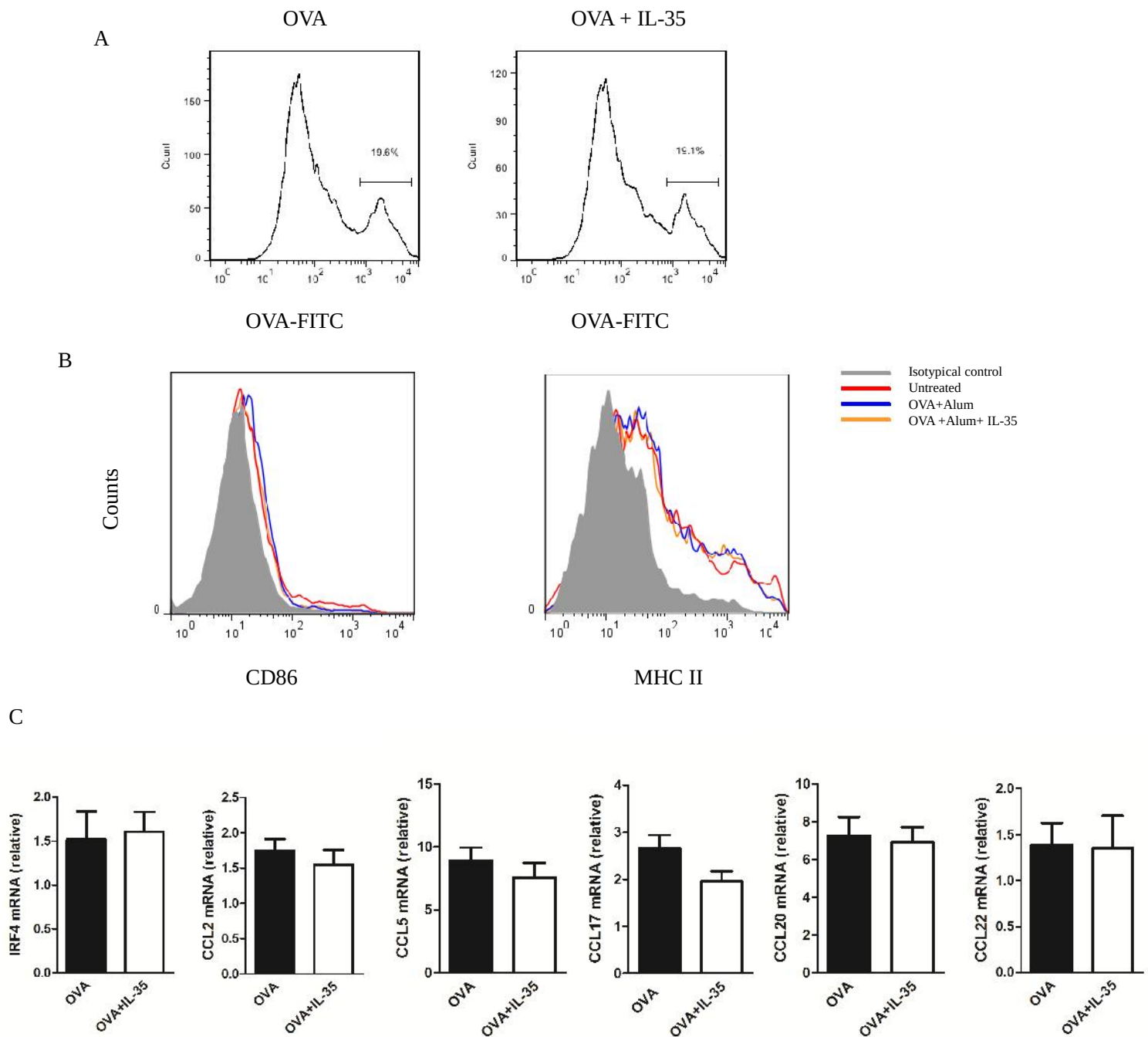
Humanized asthmatic murine model using SCID mice were established as previously reported (21). 2×10^7 PBMC from asthmatic patients with HDM sensitization or sex-age-matched healthy control subjects were i.p. injected into SCID mice on day 0, with or without i.p. injection with human recombinant IL-35 (500 ng/mouse , Enzo Life Sciences, Inc., Farmingdale, NY, USA) on days 0, 1 and 3. HDM (Greer laboratories, Lenoir, NC, USA) (50 mg) was then i.t. instilled on days 1, 3 and 7. The humanized SCID mice were scarified for analysis on day 21.

Statistical analysis

All data were expressed as mean $\pm$ SD. Differences between groups were assessed by one-way ANOVA analysis or student t test. $P < 0.05$ was considered significant. All

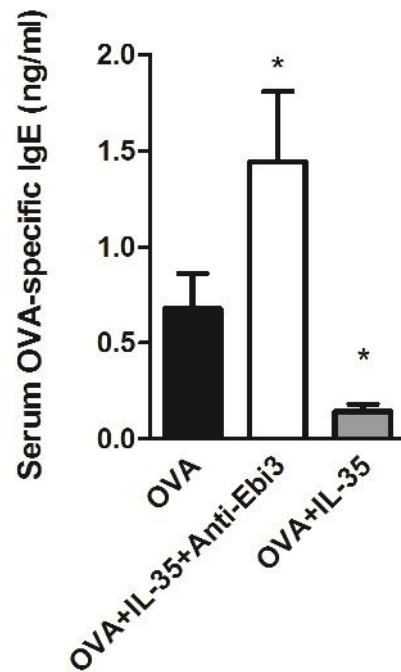
analyses were performed using the SPSS statistical software for Windows, version 20
(SPSS Inc., Chicago, IL, USA).

Supplementary Figure S1



Supplementary Figure S1 Effects of IL-35 on OVA-stimulated BMDC. (A) BMDC were treated by FITC-OVA-alum (20 μ g/ml) with or without IL-35 (100 ng/ml) *in vitro* for 24 hours, and phagocytosed FITC-OVA were detected using flow cytometry. (B) BMDC were stimulated *in vitro* with OVA-alum (20 μ g/ml) with or without IL-35 (100 ng/ml) for 24 hours, the surface expression of CD86 and MHC II expression were detected using flow cytometry. (C) BMDC were *in vitro* stimulated with OVA-alum (20 μ g/ml) with or without IL-35 (100 ng/ml) for 24 hours, then the mRNA expression of interferon regulatory factor 4 (IRF4), CCL2, CCL5, CCL17, CCL20 and CCL22 were detected. Bar charts are presented by comparing the relative gene expression between BMDC with or without IL-35 treatment (n = 4). *P < 0.05.

Supplementary Figure S3



Supplementary Figure S3 Mice with asthma were induced using OVA. For the anti-Ebi3 treatment, the OVA treated mice were i.p injected with or without anti-Ebi3 antibody (500 ng/mouse) and murine IL-35 (400 ng/mouse) on day 0 and 7. Mice were scarified on day 24 and the serum concentrations of OVA-specific IgE were measured by ELISA (Biolegend). OVA, mice were treated by OVA alone; OVA+IL-35+Anti-Ebi3, mice were treated by OVA, together with murine IL-35 and anti-Ebi3 antibody; OVA+IL-35, mice were treated by OVA and IL-35. * $p < 0.05$, when compared with the group of mice received OVA only. Columns and error bars represent mean $\pm$ SEM (n = 4 per group).

Supplementary Table S1 Primer sequences used in Q-PCR

Genes		Primer sequences (5'-3')
Mouse- <i>gapdh</i>	Forward	GCCTGGAGAAACCTGCCAAGTA
	Reverse	TGAAGTCGCAGGAGACAACCTG
Mouse- <i>gp130</i>	Forward	AATCGGACCCACTTGA
	Reverse	GAGCGGCTTGTTTGAG
Mouse- <i>il12rb2</i>	Forward	GTGGACCAAACAATCTG
	Reverse	CGTATGCGGAAGTGAAG
Mouse- <i>il27ra</i>	Forward	TCCAGACGCCATTCTTAGATC
	Reverse	CCCAGTCCACCACATACTCC
Mouse- <i>ccl2</i>	Forward	TCTGGGCCTGCTGTTTACA
	Reverse	CTGTCACACTGGTCACTCCTA
Mouse- <i>ccl5</i>	Forward	GGGTACCATGAAGATCTCTGCA
	Reverse	TTGGCGGTTTCCTT CGAGTGA
Mouse- <i>ccl17</i>	Forward	GCCATCGTGTTTCTGACTGT
	Reverse	TGAGGGAGGAAGGCTTTATT
Mouse- <i>ccl20</i>	Forward	AATGGCCTGCGGTGGCAA
	Reverse	CATCGGCCATCTGTCTTGTGA
Mouse- <i>ccl22</i>	Forward	GATAACCGTCAAGAACCGAGAT
	Reverse	GGAGTAGGCATTGGGTAAGG
Mouse- <i>irf4</i>	Forward	GACCAGTCACACCCAGAAATCCC
	Reverse	GTTCTGTGTCACCTGGCAACC
Mouse- <i>il4</i>	Forward	AGATCATCGGCATTTTGAACG
	Reverse	TTTGGCACATCCATCTCCG
Mouse- <i>il5</i>	Forward	CGCTCACCGAGCTCTGTTG
	Reverse	CCAATGCATAGCTGGTGATTTT
Mouse- <i>il13</i>	Forward	GCTTATTGAGGAGCTGAGCAACA
	Reverse	GGCCAGGTCCACACTCCATA