IL-22 induces Reg3γ and inhibits allergic inflammation in house dust mite–induced asthma models

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Previous studies have shown that IL-22, one of the Th17 cell–related cytokines, plays multiple roles in regulating allergic airway inflammation caused by antigen-specific Th2 cells; however, the underlying mechanism remains unclear. Here, we show that allergic airway inflammation and Th2 and Th17 cytokine production upon intratracheal administration of house dust mite (HDM) extract, a representative allergen, were exacerbated in IL-22–deficient mice. We also found that IL-22 induces Reg3γ production from lung epithelial cells through STAT3 activation and that neutralization of Reg3γ significantly exacerbates HDM-induced eosinophilic airway inflammation and Th2 cytokine induction. Moreover, exostatin-like 3 (EXTL3), a functional Reg3γ binding protein, is expressed in lung epithelial cells, and intratracheal administration of recombinant Reg3γ suppresses HDM-induced thymic stromal lymphopoietin and IL-33 expression and accumulation of type 2 innate lymphoid cells in the lung. Collectively, these results suggest that IL-22 induces Reg3γ production from lung epithelial cells and inhibits the development of HDM-induced allergic airway inflammation, possibly by inhibiting cytokine production from lung epithelial cells.

INTRODUCTION

Asthma is an increasing global health problem that is characterized by the infiltration of eosinophils and lymphocytes into the airways, mucus production, and airway hyper-responsiveness to a variety of stimuli (Martinez and Vercelli, 2013; Lambrecht and Hammad, 2015). A series of studies have revealed that these characteristics are caused by Th2 cells, which secrete IL-4, IL-5, IL-9, and IL-13. Adoptive transfer of in vitro–generated antigen-specific Th2 cells has demonstrated that Th2 cells are sufficient to reproduce most asthma-like features (Cohn et al., 1997). Furthermore, experiments using mice lacking Th2 cytokines have illuminated the importance of Th2 cytokines in promoting allergic airway inflammation (Lambrecht and Hammad, 2015). These studies have provided strong evidence that antigen-specific Th2 cells and their cytokines are the major players that cause asthma.

However, the view that asthma is an exclusively Th2 cell–mediated disease has been changed by recent findings that not only Th2 cytokines but also other T cell–related cytokines, such as IL-17A and IL-22, are expressed in the airway in patients with asthma (Molet et al., 2001; Rankin et al., 2016). Furthermore, in the airways of patients with asthma, Th2-biased inflammation was observed in only 50% of patients with asthma (Woodruff et al., 2009) and that clinical trials with antibodies against Th2 cytokines have shown therapeutic benefits only in a restricted subset of patients (Chung, 2015). These results suggest that although Th2 cells and their cytokines play major roles, there should be more players involved in the development of asthma.

Another helper T cell subset shown to regulate the development of asthma is Th17 cells. We have previously shown that adoptive transfer of antigen-specific Th17 cells enhances Th2 cell–dependent eosinophilic airway inflammation and airway responsiveness (Wakashin et al., 2008). We have also shown that IL-17A produced by Th17 cells provokes neutrophilic inflammation (Wakashin et al., 2008), one of the main characteristics of patients with severe asthma. Moreover, cluster analyses using clinical phenotypes and sputum cellular patterns have revealed that a considerable proportion of patients with asthma shows a neutrophil-dominated inflammation and that the severity of airway neutrophilia is correlated with frequent exacerbation and poor responses to inhaled corticosteroids (Moore et al., 2010, 2014). The relationship

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Abbreviations used: BALF, bronchoalveolar lavage fluid; CCSP, Clara cell–specific protein; DOX, doxycycline; HDM, house dust mite; ILC, innate lymphoid cell; MLN, mediastinal lymph node; qPCR, quantitative PCR; RNA-seq, RNA sequencing; TSLP, thymic stromal lymphopoietin.

The Rockefeller University Press
J. Exp. Med. 2017 Vol. 214 No. 10 3037–3050
https://doi.org/10.1084/jem.20162108

between Th17 cells and the development of severe asthma is further underscored by the fact that the levels of IL-17 in bronchoalveolar lavage fluid (BALF) positively correlate with the severity of asthma (Moore et al., 2014). These results suggest that in addition to Th2 cells, Th17 cells and their cytokines are involved in the pathophysiology of asthma.

Recently, the role of IL-22, which was considered one of the Th17 cytokines, in the development of asthma has been evaluated by several groups. Consistent with the fact that IL-22 has both pro- and anti-inflammatory properties (Rutz et al., 2014), studies focusing on IL-22 in asthma have yielded conflicting results. We and others have shown that IL-22 inhibits the development of allergic airway inflammation (Takahashi et al., 2011; Pennino et al., 2013). We have also shown that IL-22 inhibits IL-25 production from lung epithelial cells (Takahashi et al., 2011), consistent with a recent finding that IL-22 is involved in the crosstalk between immune responses and epithelial cell functions (Dudakov et al., 2015). In contrast, Besnard et al. (2011) reported that allergic airway inflammation is reduced by IL-22 neutralization during the sensitization phase, whereas IL-22 neutralization during the antigen challenge phase enhances allergic airway inflammation, with increased Th2 cytokine production in OVA-induced asthma models. These data suggest the multiple roles of IL-22 in the pathogenesis of asthma; however, the underlying mechanisms remain unclear.

In this study, we examined the role of IL-22 in the development of allergic airway inflammation induced by intratracheal administration of a common natural allergen, house dust mites (HDMs). Our results suggest that IL-22 induces the expression of Reg3γ, an antimicrobial protein, from lung epithelial cells in a STAT3-dependent manner and thereby inhibits the development of HDM-induced allergic airway inflammation possibly through the inhibition of epithelial cytokine expression.

RESULTS

HDM-induced allergic airway inflammation is exacerbated in *IL22*$^{-/-}$ mice

Recent evidence has suggested that IL-22 regulates the development of allergic airway inflammation. To gain further insight into the roles of IL-22 in asthma under relatively physiological conditions, we evaluated the involvement of IL-22 in the development of allergic airway inflammation induced by intratracheal administration of a common natural allergen, HDMs, but not in usual OVA-induced asthma models. WT mice and IL-22-deficient (*IL22*$^{-/-}$) mice were intratracheally sensitized with HDMs on day 0 and day 7 and then challenged with daily HDM administration from day 15 to day 18. 48 h after the last HDM challenge, the numbers of eosinophils, neutrophils, and CD4$^+$ T cells in the BALF were measured (Fig. S1 A). Consistent with our previous results (Takahashi et al., 2011), *IL22*$^{-/-}$ mice exhibited enhanced eosinophil, neutrophil, and CD4$^+$ T cell recruitment into the airways compared with WT mice (Fig. 1 A). The proportion of eosinophils in the BALF was also significantly increased in *IL22*$^{-/-}$ mice (Fig. 1 B). Histological analyses revealed a significant increase in inflammatory cell infiltration around the bronchus (Fig. 1 C) and in goblet cell numbers (Fig. 1 D) in *IL22*$^{-/-}$ mice, indicating that the lack of IL-22 signaling exacerbates allergen-induced airway inflammation. Serum levels of HDM-specific IgG1 and those of total IgE were also significantly increased in *IL22*$^{-/-}$ mice (Fig. 1, E and F), while HDM-specific IgE was under the detection limit in both WT mice and *IL22*$^{-/-}$ mice. Airway responsiveness to methacholine was enhanced in HDM-sensitized and HDM-challenged *IL22*$^{-/-}$ mice compared with HDM-sensitized and HDM-challenged WT mice (Fig. 1 G). We also evaluated HDM-induced cytokine production of cells isolated from draining lymph nodes and lung in HDM-sensitized and HDM-challenged mice to examine recall responses to HDM in vitro (Fig. S1 B). As shown in Fig. 1 H, HDM-induced production of IL-5, IL-13, and IL-17A by mediastinal lymph node (MLN) cells was significantly increased in *IL22*$^{-/-}$ mice compared with WT mice. Similarly, HDM-induced production of IL-5, IL-13, and IL-17A by lung-infiltrating cells was significantly increased in *IL22*$^{-/-}$ mice (Fig. 1 I). Collectively, these results indicate that IL-22 inhibits the development of HDM-induced Th2 and Th17 responses and thus suppresses allergic airway inflammation.

IL-22 is produced mainly by CD4$^+$ T cells that do not produce IFN-γ, IL-5, IL-13, or IL-17A

Earlier studies showed that NK-like cells (Kumar et al., 2013), αβ TCR$^+$ cells (Scriba et al., 2008; Hamada et al., 2009; Takahashi et al., 2011), γδ T cells (Yao et al., 2010), NKT cells (Paget et al., 2012), and innate lymphoid cells (ILCs; Carrega et al., 2015) in the lung are capable of producing IL-22. To clarify the cell populations that produce IL-22 in HDM-induced asthma models, we next searched for IL-22-producing cells in the lung of HDM-sensitized and HDM-challenged mice by intracellular cytokine staining (Fig. S1 A). As shown in Fig. 2 A, most IL-22$^+$ cells in the lung were CD3ε$^+$ cells. Further analysis of the IL-22$^+$ CD3ε$^+$ cells revealed that IL-22 was produced predominantly by αβ TCR$^+$ CD4$^+$ T cells and that small numbers of γδ T cells and CD8$^+$ T cells also produced IL-22 (Fig. 2 A). Although previous studies have shown that ILCs and NCR$^+$ cells produce IL-22 under certain pathological conditions (Kumar et al., 2013; Carrega et al., 2015), our analysis showed that neither ILCs (Lin$^-$ Thy1.2$^+$) nor NCR$^+$ cells produce IL-22 (Fig. 2 B and not depicted) in HDM-induced asthma models. Consequently, the frequency (Fig. 2 C) and absolute number (Fig. 2 D) of IL-22-producing CD4$^+$ T cells (IL-22$^+$ CD4$^+$ T cells) were significantly higher than those of IL-22-producing CD8$^+$ T cells, γδ T cells, NK-like cells, or ILCs.

To further examine the characteristics of IL-22$^+$ CD4$^+$ T cells in the lung, we examined the expression of IFN-γ, IL-5, IL-13, and IL-17A in IL-22$^+$ CD3ε$^+$ CD4$^+$ T cells. As shown in Fig. 2 E, most of the IL-22$^+$ CD4$^+$ T cells did

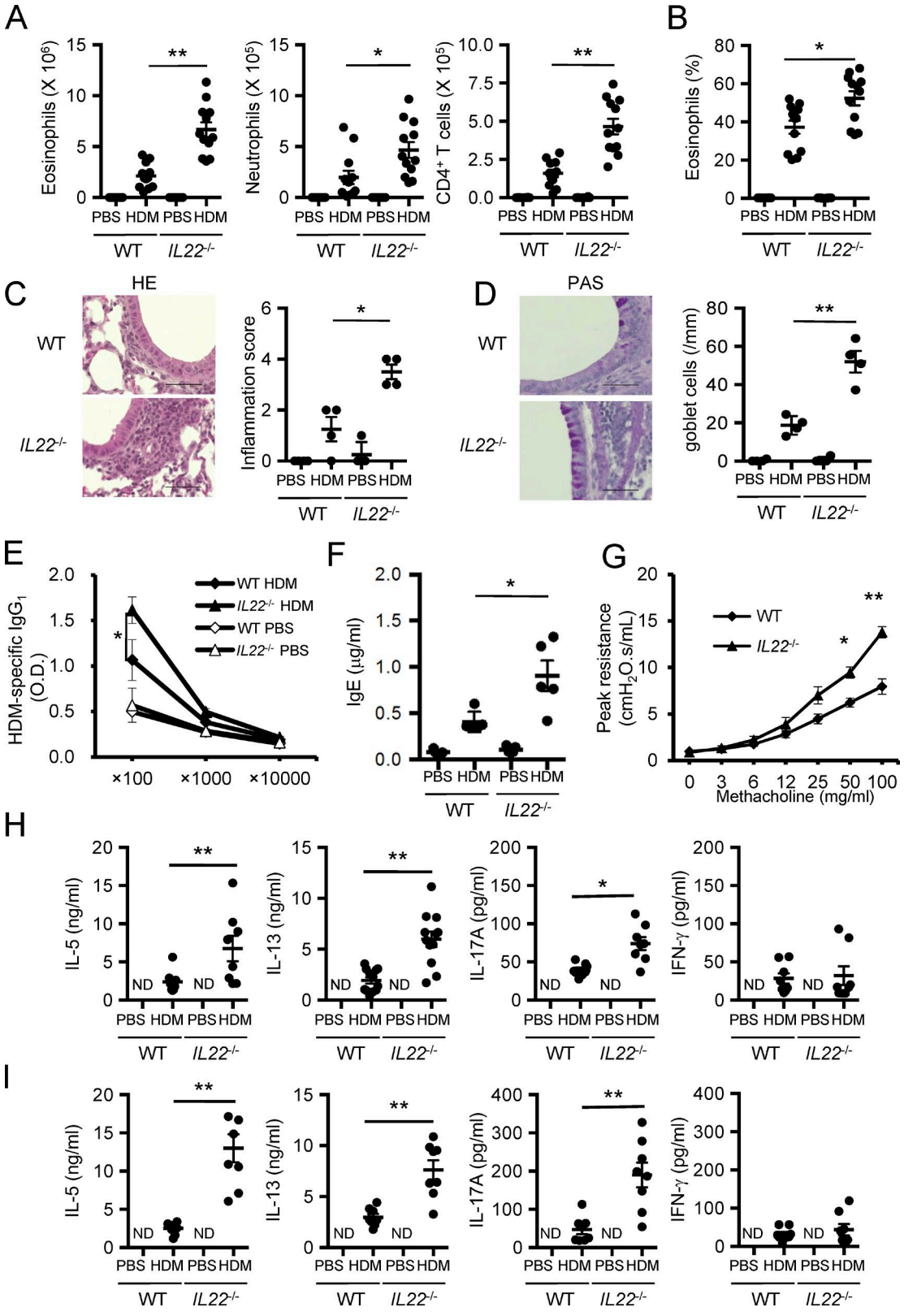

Figure 1. **HDM-induced allergic airway inflammation is exacerbated in *IL22*$^{-/-}$ mice.** WT mice and *IL22*$^{-/-}$ mice were sensitized and challenged with HDM extract or PBS (as a control), as described in Fig. S1 A. (A and B) The absolute numbers of eosinophils, neutrophils, and CD4$^+$ T cells (A) and the frequency of eosinophils (B) in the BALF were evaluated 48 h after the last HDM challenge. Data are mean ± SEM for 12 mice in each group from four in-

not produce IFN-γ, IL-5, IL-13, or IL-17A. The frequency of IL-22$^+$ CD4$^+$ T cells was significantly higher than that of IFN-γ$^+$ CD4$^+$ T cells or IL-17$^+$ CD4$^+$ T cells but tended to be lower than that of IL-13$^+$ CD4$^+$ T cells or IL-5$^+$ CD4$^+$ T cells (Fig. 2 F). Moreover, IL-22$^+$ CD4$^+$ T cells did not express master transcription factors, such as T-bet, GATA3, or RORγt (Fig. 2 G). These results suggest that IL-22$^+$ CD4$^+$ T cells in the lung of HDM-induced asthma models are distinct from Th1, Th2, and Th17 cells.

IL-22 induces Reg3γ expression in lung epithelial cells in a STAT3-dependent manner

To address the molecular mechanisms by which IL-22 inhibits the development of HDM-induced allergic airway inflammation, we examined the expression of IL-22 receptor in the lung. Quantitative PCR (qPCR) analysis revealed that IL-22 receptor 1 (IL-22R1) was expressed in EpCAM$^+$ CD45$^-$ lung pan-epithelial cells (Messier et al., 2012) but not immune cells (Fig. 3 A). Immunostaining confirmed that IL-22R1 was expressed mainly in lung epithelial cells (Fig. 3 B), suggesting that lung epithelial cells are likely to be a target of IL-22.

We next performed an unbiased comprehensive screening of genes induced by IL-22 administration in the lung by RNA sequencing (RNA-seq) analysis. We identified 18 differentially expressed genes (Fig. 3, C and D), and among them, we focused on Reg3γ, because Reg3 family members have recently been shown to possess not only antimicrobial activity but also immune-modulatory function (Lai et al., 2012; Lörchner et al., 2015). Consistently, we found that Reg3γ was one of genes whose expression was reduced in the lung in HDM-sensitized and HDM-challenged *IL22*$^{-/-}$ mice as compared with that in HDM-sensitized and HDM-challenged WT mice (Fig. 3, E and F).

To confirm that Reg3γ is expressed in lung epithelial cells, we evaluated the expression levels of Reg3γ mRNA in isolated EpCAM$^+$ CD45$^-$ lung epithelial cells by qPCR analysis. Reg3γ mRNA was highly expressed in EpCAM$^+$ CD45$^-$ cells, and the expression levels were further enhanced in EpCAM$^+$ CD45$^-$ cells isolated from HDM-sensitized and HDM-challenged mice (Fig. 3 G). Importantly, among Reg3 family members, Reg3γ was the most highly expressed in the lung (Fig. 3 G). Immunostaining for Reg3γ confirmed that Reg3γ expression was enhanced in the epithelial cells in HDM-sensitized and HDM-challenged mice and that the induction was less significant in *IL22*$^{-/-}$ mice (Fig. 3 H). Consistently, the levels of Reg3γ in the BALF were significantly increased in HDM-sensitized and HDM-challenged WT mice, and the increase was less obvious in *IL22*$^{-/-}$ mice (Fig. 3 I). The kinetic analysis revealed that the frequency of IL-22$^+$ CD4$^+$ T cells in the lung and the levels of Reg3γ in the BALF increased during the challenge phase but not in the sensitization phase in HDM-induced asthma models with similar kinetics (Fig. 3, J and K), suggesting the roles of IL-22 and Reg3γ in the challenge phase in this model.

Because STAT3 activation mediates IL-22-dependent epithelial cell responses (Pickert et al., 2009), we next examined the requirement of STAT3 for Reg3γ induction. Reduction of STAT3 expression in lung epithelial cells was achieved by the administration of doxycycline (DOX) to Clara cell–specific protein (CCSP)–rtTA/tetO-Cre/*STAT3*$^{fl/fl}$ mice (Fig. 3 L). By using DOX-treated CCSP-rtTA/tetO-Cre/*STAT3*$^{fl/fl}$ mice (Fig. S1 C), we examined whether epithelial STAT3 expression is required for Reg3γ expression and found that HDM-induced Reg3γ expression in lung epithelial cells was significantly attenuated in DOX-treated CCSP-Cre/*STAT3*$^{fl/fl}$ mice compared with WT mice (Fig. 3 M). Collectively, these results suggest that IL-22 induced by HDM stimulation enhances Reg3γ production in lung epithelial cells through STAT3 activation.

Reg3γ inhibits the development of HDM-induced allergic airway inflammation

We next examined whether Reg3γ functions as a downstream effector molecule of IL-22 for the suppression of HDM-induced allergic airway inflammation. Because Reg3γ-deficient mice develop colitis spontaneously (Loonen et al., 2014), which could alter the systemic inflammatory responses, we examined the effect of Reg3γ neutralization by intratracheal administration of neutralizing antibody (Choi et al., 2013). In this experiment, HDM-sensitized WT mice were administered once with anti-Reg3γ antibody or control antibody 12 h before the first HDM challenge (Fig. S1 D). Importantly, anti-Reg3γ antibody significantly increased the numbers of eosinophils and CD4$^+$ T cells but not of neutrophils in the BALF in HDM-induced asthma models (Fig. 4 A). The effect of Reg3γ neutralization was further underscored by an increase in eosinophilic peribronchial and perivascular inflammation (Fig. 4 B) and in goblet cell hyperplasia (Fig. 4 C). HDM-specific IgG1 production (Fig. 4 D) and airway hyper-responsiveness (Fig. 4 E) were also elevated

dependent experiments. *, P < 0.05; **, P < 0.01 by unpaired Student's *t* test. (C and D) Representative photomicrographs of lung sections with hematoxylin and eosin (HE) staining and histological inflammatory scores (C) and those with periodic acid–Schiff (PAS) staining and the numbers of goblet cells (D) at 48 h after the last HDM challenge. Data are mean ± SEM for 4 mice in each group from two independent experiments. *, P < 0.05; **, P < 0.01 by unpaired Student's *t* test. Bars, 50 µm. (E and F) Serum levels of HDM-specific IgG1 (E) and of total IgE (F). Data are mean ± SEM for 5 mice in each group from three independent experiments. *, P < 0.05 by unpaired Student's *t* test. (G) Airway resistance to methacholine was measured 48 h after the last HDM challenge. Data are mean ± SEM for 5 mice in each group from two independent experiments. *, P < 0.05; **, P < 0.01 by unpaired Student's *t* test. (H and I) MLN cells (H) and single-cell suspensions of the lung (I) were isolated from HDM-sensitized and HDM-challenged mice and stimulated with HDM or PBS for 5 d (Fig. S1 B). The levels of IL-5, IL-13, IL-17A, and IFN-γ in the culture supernatants were measured using ELISA. Data are mean ± SEM for 12 mice in each group from four independent experiments. *, P < 0.05; **, P < 0.01 by unpaired Student's *t* test.

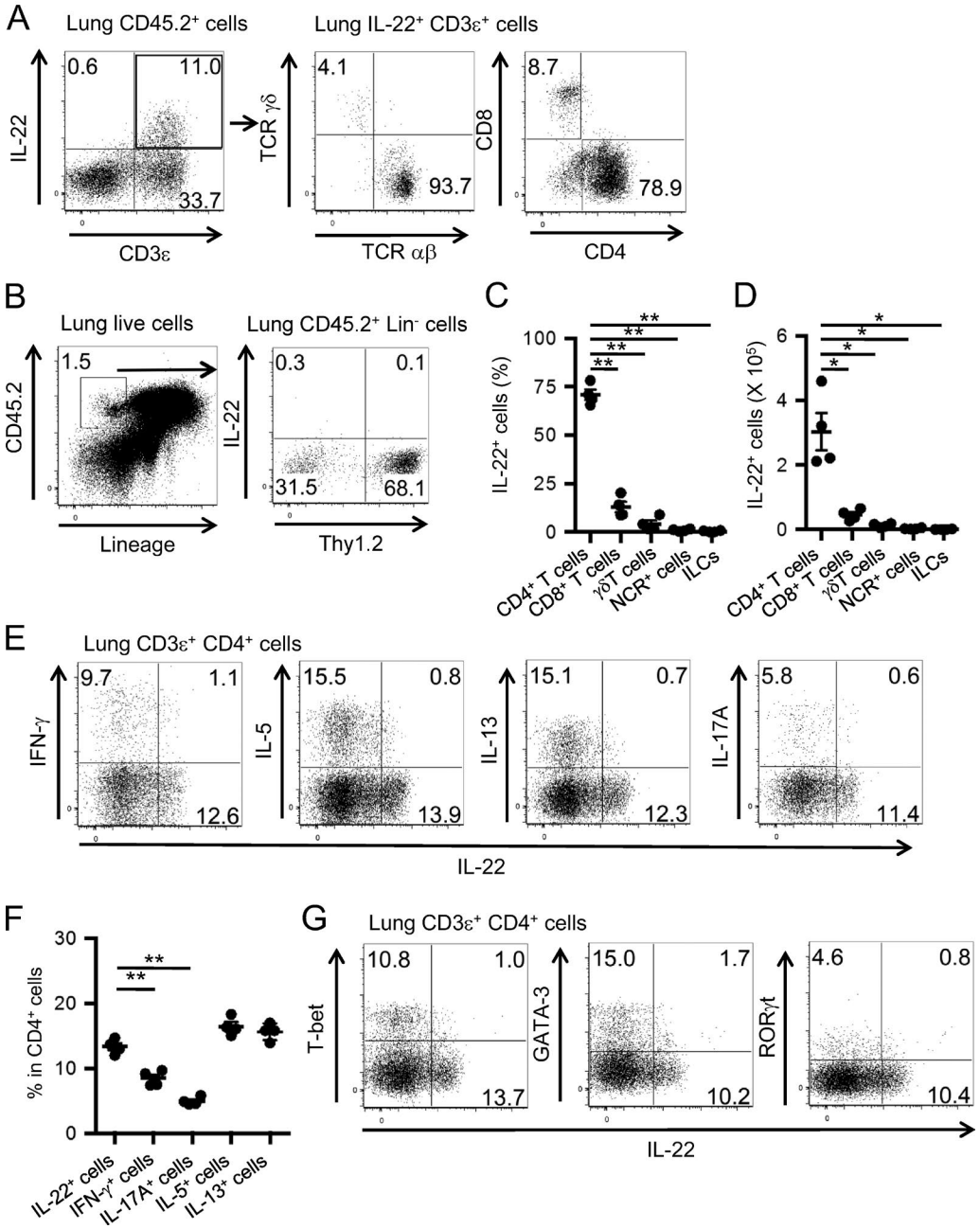

Figure 2. **CD4⁺ T cells are major IL-22–producing cells in the lungs in HDM-induced asthma models.** (A and B) Single-cell suspensions of lung-infiltrating cells of HDM-sensitized and HDM-challenged mice (Fig. S1 A) were stimulated with PMA + ionomycin for 4 h and then analyzed for the expression of intracellular IL-22 together with surface expression of indicated markers. Shown are representative FACS plots with the percentage of cells from three independent experiments. (C and D) Percentages (C) and absolute numbers (D) of IL-22–producing CD4⁺ CD3ε⁺ cells, CD8⁺ CD3ε⁺ cells, γδTCR⁺ CD3ε⁺ cells, NCR1⁺ cells, and Lin⁻ Thy1.2⁺ ILCs in the lung are shown. Data are mean ± SEM for four mice in each group from two independent experiments. *, $P < 0.05$; **, $P < 0.01$ by one-way ANOVA and Tukey's test. (E and F) Shown are representative FACS profiles of intracellular staining for IL-22 versus IFN-γ, IL-5, IL-13, or IL-17A of CD4⁺ CD3ε⁺ cells (E) and percentages of IL-22–, IFN-γ–, IL-17A–, IL-5–, or IL-13–producing CD4⁺ CD3ε⁺ cells (F) in the lung. Data are mean ± SEM for four mice in each group from two independent experiments. **, $P < 0.01$ by one-way ANOVA and Tukey's test. (G) Representative FACS profiles of intracellular staining for IL-22 versus T-bet, GATA3, or RORγt of CD4⁺ CD3ε⁺ cells in the lung from three independent experiments.

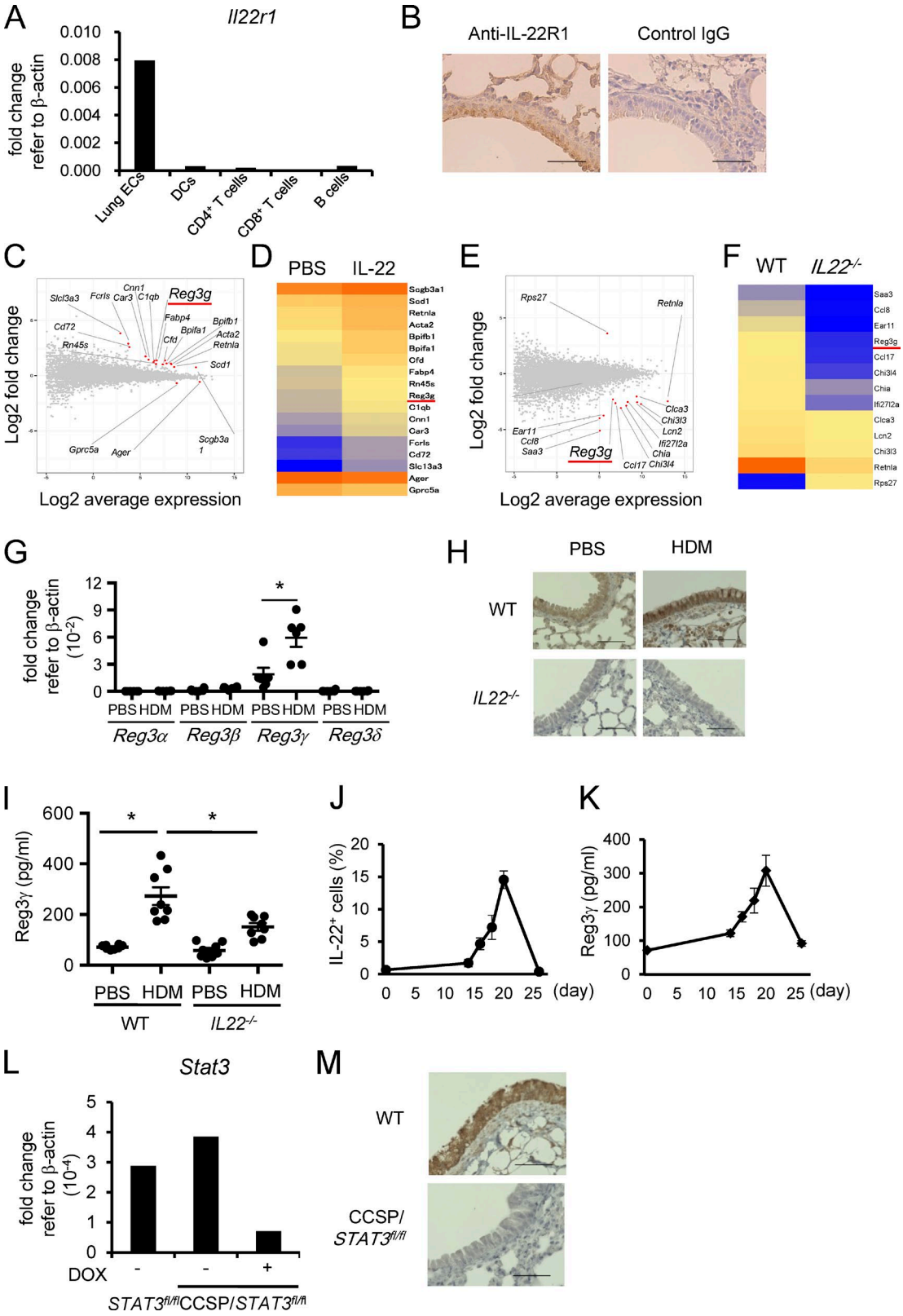

Figure 3. **IL-22 induces Reg3γ expression in lung epithelial cells in a STAT3-dependent manner.** (A) Expression levels of *IL22R1* mRNA in EpCAM$^+$ CD45$^-$ lung epithelial cells (EC), DCs, CD4$^+$ T cells, CD8$^+$ T cells, and B cells were evaluated using qPCR. (B) Shown are representative microphotographs of anti-IL-22R1 or control IgG staining of lung section. Shown are representative data from two independent experiments. Bars, 50 µm. (C and D) IL-22 or

by the intratracheal administration of anti-Reg3γ antibody. Furthermore, the production of IL-5 and IL-13 from MLN cells upon in vitro stimulation with HDM was enhanced when mice were treated with anti-Reg3γ antibody (Fig. 4 F). Meanwhile, the frequency of IL-22$^+$ CD4$^+$ T cells in the lung (Fig. 4 G) as well as the levels of IL-22 production (Fig. 4 H) was comparable between mice treated with anti-Reg3γ antibody and control antibody. On the other hand, intratracheal administration of recombinant Reg3γ significantly decreased the number of eosinophils in the BALF not only in WT mice (Fig. 4 I) but also in *IL22*$^{-/-}$ mice (Fig. 4 J) in the HDM-induced asthma model (Fig. S1 D). Collectively, these data suggest that Reg3γ acts as one of effector molecules downstream of IL-22 for the suppression of HDM-induced allergic airway inflammation in mice.

Reg3γ inhibits HDM-induced expression of thymic stromal lymphopoietin (TSLP) and IL-33 in the lung

We next explored the mechanisms by which Reg3γ inhibits the development of allergic responses to HDM. Recently, EXTL3 has been shown to be a binding partner of REG3A, a human orthologue of Reg3γ, and to act as its functional receptor (Lai et al., 2012). We therefore examined the expression of EXTL3 mRNA in EpCAM$^+$ CD45$^-$ lung epithelial cells as well as DCs, CD4$^+$ T cells, CD8$^+$ T cells, and B cells isolated from spleen, because recent studies have clearly shown that not only immune cells but also lung epithelial cells are involved in the regulation of asthmatic responses (Hammad and Lambrecht, 2015). Importantly, expression levels of EXTL3 mRNA were higher in EpCAM$^+$ CD45$^-$ lung epithelial cells than those in DCs, CD4$^+$ T cells, CD8$^+$ T cells, or B cells (Fig. 5 A). EXTL3 mRNA was further increased in EpCAM$^+$ CD45$^-$ cells isolated from HDM-sensitized and HDM-challenged mice compared with those from PBS-treated control mice (Fig. 5 B). Because lung epithelial cells produce cytokines, such as TSLP, IL-25, and IL-33 and accelerate the development of Th2-type immune responses in the lung (Hammad and Lambrecht, 2015), we examined whether Reg3γ inhibits epithelial cytokine expression in lung upon HDM stimulation (Fig. S1 E). Importantly, intratracheal administration of recombinant Reg3γ significantly inhibited HDM-induced TSLP and IL-33 expression in the lung at mRNA levels (Fig. 5 C) and protein levels (Fig. 5 D). Consistently, recruitment of type 2 innate lymphoid cells (ILC2s) into the lung was significantly attenuated in the mice treated with Reg3γ compared with that in control mice (Fig. 5 E). Collectively, these results suggest that Reg3γ functions on the lung epithelial cells and inhibits TSLP and IL-33 production, leading to the suppression of ILC2 accumulation and eosinophilic airway inflammation.

DISCUSSION

In this study, we show a novel mechanism underlying IL-22-mediated suppression of HDM-induced asthmatic responses. We found that HDM-induced Th2 and Th17 cytokine production and eosinophilic airway inflammation were exacerbated in *IL22*$^{-/-}$ mice (Fig. 1). We also found that IL-22 was produced mainly by CD4$^+$ T cells, which are negative for IL-17A or RORγt, suggesting that IL-22-producing CD4$^+$ T cells in the lung are distinct from Th17 cells (Fig. 2). Transcriptome analysis of IL-22-stimulated lung revealed that Reg3γ was induced in lung epithelial cells upon IL-22 stimulation in a STAT3-dependent manner (Fig. 3). In addition, the neutralization of Reg3γ significantly exacerbated HDM-induced eosinophilic airway inflammation, Th2 cytokine production, and airway hyper-responsiveness (Fig. 4). Furthermore, EXTL3, a functional receptor for Reg3γ, was expressed on lung epithelial cells, and recombinant Reg3γ inhibited HDM-induced TSLP and IL-33 expression in the lung (Fig. 5). Collectively, these results suggest that the induction of Reg3γ expression in lung epithelial cells could be involved in IL-22-mediated suppression of allergic airway inflammation.

Consistent with the fact that IL-22 exerts both proinflammatory and anti-inflammatory properties in many inflammatory contexts (Rutz et al., 2014), previous studies examining the roles of IL-22 in allergic airway inflam-

PBS was intratracheally administered to WT mice. 24 h after administration, total RNAs were prepared from the lung and subjected to RNA-seq analysis. (C) Shown is an M-A plot based on log ratios (IL-22 − PBS [$\log_2$]) and means ([IL-22 + PBS]/2[$\log_2$]) of all transcripts. Red dots indicate differentially expressed genes (DEGs) with q value (false discovery rate [FDR]) < 0.01. (D) Shown is a heat map of DEGs. (E and F) WT and *IL22*$^{-/-}$ mice were sensitized and challenged with HDM as described in Fig. S1 A. 48 h after the last HDM administration, total RNAs were prepared from the lung and subjected to RNA-seq analysis. (E) Shown is an M-A plot based on log ratios (WT − *IL22*$^{-/-}$ [$\log_2$]) and means ([WT + *IL22*$^{-/-}$]/2[$\log_2$]) of all transcripts. Red dots indicate DEGs with q value (FDR) < 0.01. (F) Shown is a heat map of DEGs. (G) WT mice were sensitized and challenged with HDM or PBS as described in Fig. S1 A. EpCAM$^+$CD45$^-$ epithelial cells were isolated from the lung by flow cytometry, and expression levels of mRNAs for Reg3 family members were evaluated using qPCR. Data are mean ± SEM for six mice in each group from three independent experiments. *, P < 0.05 by unpaired Student's *t* test. (H and I) WT mice and *IL22*$^{-/-}$ mice were sensitized and challenged with HDM or PBS as described in Fig. S1 A. Shown are representative microphotographs of anti-Reg3γ staining of lung section (H) and levels of Reg3γ in BALF (I). Data are mean ± SEM for eight mice in each group from two independent experiments. *, P < 0.05 by one-way ANOVA and Tukey's test. Bars, 50 µm. (J and K) WT mice were sensitized and challenged with HDM as described in Fig. S1 A. Shown are kinetics of IL-22$^+$ CD4$^+$ T cells in the lung (J) and the levels of Reg3γ in BALF (K). Data are mean ± SEM for eight mice in each group from two independent experiments. (L) EpCAM$^+$ CD45$^-$ lung epithelial cells were isolated from control *STAT3*$^{fl/fl}$ mice or CCSP-rtTA/tetO-Cre/*STAT3*$^{fl/fl}$ mice (CCSP/*STAT3*$^{fl/fl}$ mice) that were administered with or without DOX for 7 d, and levels of *STAT3* mRNA were evaluated by qPCR. (M) HDM-sensitized WT mice and CCSP/*STAT3*$^{fl/fl}$ mice were given DOX and then challenged with HDM for four consecutive days as described in Fig. S1 C. 48 h later, the lung sections were subjected to immunostaining with anti-Reg3γ antibody. Shown are representative microphotographs from two independent experiments. Bars, 50 µm.

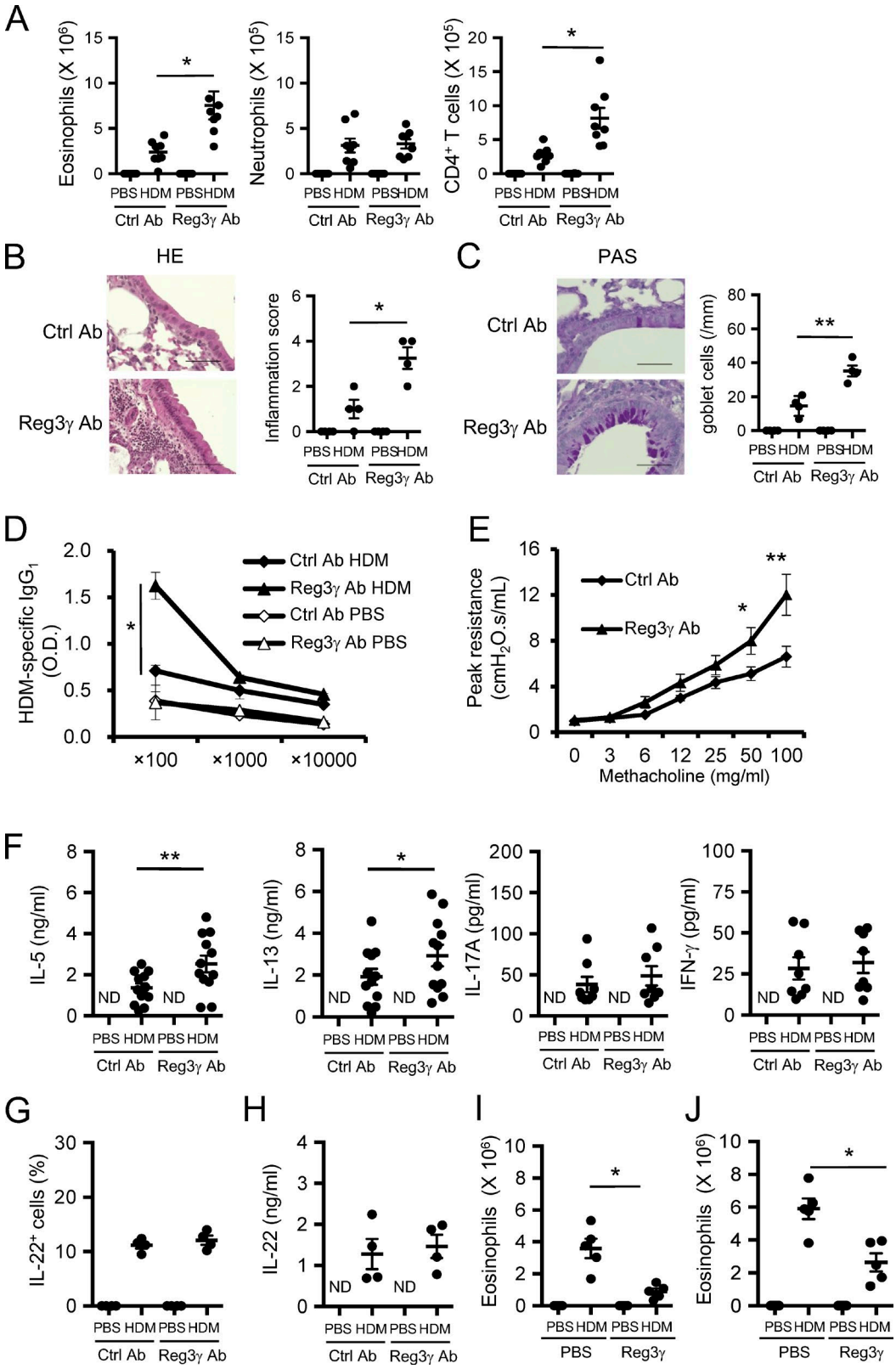

Figure 4. **HDM-induced allergic airway inflammation is exacerbated by the administration of anti-Reg3γ antibody.** (A–H) HDM-sensitized WT mice were administered intratracheally with anti-Reg3γ antibody or control antibody and then challenged with HDM as described in Fig. S1 D. (A) 48 h after the last HDM challenge, the numbers of eosinophils, neutrophils, and CD4+ T cells in the BALF were evaluated. Data are mean ± SEM for 8 mice in

mation have yielded conflicting results. In this study, we show that IL-22 is expressed predominantly in $CD4^+$ T cells at the challenge phase but not the sensitization phase in HDM-induced asthma models (Figs. 2 and 3) and plays an inhibitory role against eosinophilic airway inflammation (Fig. 1). IL-22 has recently been shown to be an essential cytokine to maintain homeostasis and barrier function against pathogens at epithelial surfaces (Sonnenberg et al., 2011). HDM extracts contain various allergenic proteins, including Der p1, a cysteine protease that disrupts epithelial barrier by several distinct mechanisms (Wills-Karp et al., 2010). Because asthma is considered to be caused at least in part by the defects in epithelial barrier function in the airways (Hammad and Lambrecht, 2015), our finding that IL-22 prevents asthmatic responses at the epithelium supports the consideration. Further studies on the localization and the interacting cells of IL-22^+ $CD4^+$ T cells in the lung may lead to a better understanding of the mechanism of IL-22-mediated suppression of allergic airway inflammation.

We found that the major IL-22 producers in HDM-induced asthmatic responses are $CD4^+$ T cells, which are distinct from Th17 cells (Fig. 2). We have previously shown that $CD4^+$ T cells are major sources of IL-22 during OVA-induced asthma models (Takahashi et al., 2011). In contrast, Taube et al. (2011) showed that IL-22 is produced predominantly by ILCs in the lung in mice challenged with OVA in unsensitized OVA-specific T cell receptor transgenic mice. We found here that $CD4^+$ T cells that solely produce IL-22 were the dominant producer of IL-22 in HDM-sensitized and HDM-challenged mice, whereas ILCs did not produce IL-22 in this experimental setting (Fig. 2). We speculate that the difference in IL-22 producer could be derived from the sensitization status. Namely, ILCs produce IL-22 mainly in the early phase of immune responses, when well-differentiated T cells are limited, whereas $CD4^+$ T cells produce IL-22 mainly in late phase of immune responses. Studies with IL-22 reporter mice are needed for further understanding of IL-22-producing cells in vivo.

We identified Reg3γ as one of IL-22-target genes in lung epithelial cells by an unbiased comprehensive screening of genes regulated by IL-22 (Fig. 3). We also found that intratracheal administration of HDM induces the expression of Reg3γ but not other Reg3 family members in $EpCAM^+$ $CD45^-$ lung epithelial cells (Fig. 3 G). In addition, we found that HDM-induced Reg3γ expression in lung epithelial cells was reduced in IL-$22^{-/-}$ mice (Fig. 3, H and I) and that Reg3γ induction was not obvious in mice lacking STAT3 expression in lung epithelial cells (Fig. 3 M), indicating that HDM induces Reg3γ expression specifically in lung epithelial cells through IL-22-STAT3 pathways. Our findings are consistent with those of a previous study by Zheng et al. (2008) showing that IL-22 treatment of ex vivo colonic tissues results in the up-regulation of genes encoding many antimicrobial proteins, including S100A8, S100A9, Reg3β, and Reg3γ, and that IL-22 is required for the induction of the Reg family proteins, including Reg3α and Reg3γ, in colonic epithelial cells during bacterial infection.

Although Reg3γ expression was reduced in *IL22*$^{-/-}$ mice, a considerable amount of Reg3γ was still expressed in the BALF in *IL22*$^{-/-}$ mice in HDM-induced asthma models (Fig. 3 I), suggesting the presence of other factor(s) in the induction of Reg3γ expression. Because STAT3 is a downstream signal transducer of IL-22 to express Reg3γ, one of the candidates would be the molecule that activates STAT3 in epithelial cells. This speculation is further supported by the fact that innate-like intraepithelial lymphocytes induce herpes virus entry mediator–mediated STAT3 activation in epithelial cells and lead to Reg3γ expression, which is required for immune responses against bacteria (Shui et al., 2012).

We show that Reg3γ is involved in the suppression of HDM-induced asthmatic responses. We found that HDM-induced eosinophilic airway inflammation (Fig. 4, A and B), mucus production (Fig. 4 C), and airway hyper-reactivity (Fig. 4 E) were significantly enhanced by the administration of neutralizing anti-Reg3γ antibody. We also found that HDM-induced Th2 cytokine production of MLN cells in HDM-sensitized mice was enhanced when mice were treated with anti-Reg3γ antibody (Fig. 4 F), suggesting that Reg3γ is involved in T cell differentiation in HDM-induced asthma models. On the other hand, we found that intratracheal administration of Reg3γ inhibited HDM-induced eosinophilic airway inflammation in both WT mice and *IL22*$^{-/-}$ mice (Fig. 4, I and J). Collectively, these results suggest that Reg3γ functions as an effector molecule underlying IL-22-mediated suppression of HDM-induced allergic airway inflammation.

each group from three independent experiments. *, $P < 0.05$ by unpaired Student's *t* test. (B and C) Representative photomicrographs of lung sections with hematoxylin and eosin (HE) staining and histological inflammatory scores (B) and those with periodic acid–Schiff (PAS) staining and the numbers of goblet cells (C). Data are expressed as mean ± SEM for 4 mice in each group from two independent experiments. *, $P < 0.05$; **, $P < 0.01$ by unpaired Student's *t* test. Bars, 50 µm. (D) Serum levels of HDM-specific IgG1. Data are mean ± SEM for 8 mice in each group from three independent experiments. *, $P < 0.05$ by unpaired Student's *t* test. (E) Airway resistance to methacholine at 48 h after the last HDM challenge. Data are mean ± SEM for 10 mice in each group from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ by unpaired Student's *t* test. (F) MLN cells were stimulated with HDM for 5 d. Data are mean ± SEM for 12 mice in each group from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ by unpaired Student's *t* test. (G and H) The frequency of IL-22^+ $CD4^+$ T cells in the lung (G) and the levels of IL-22 in the supernatants of single-cell suspensions of the lung that were stimulated with HDM (H). Data are mean ± SEM for 4 mice in each group from two independent experiments. (I and J) HDM-sensitized WT mice (I) and *IL22*$^{-/-}$ mice (J) were intratracheally injected with recombinant Reg3γ protein or PBS and then challenged with HDM as described in Fig. S1 D. 48 h after the last HDM challenge, the number of eosinophils in BALF was evaluated. Data are mean ± SEM for 5 mice in each group from two independent experiments. *, $P < 0.05$ by unpaired Student's *t* test.

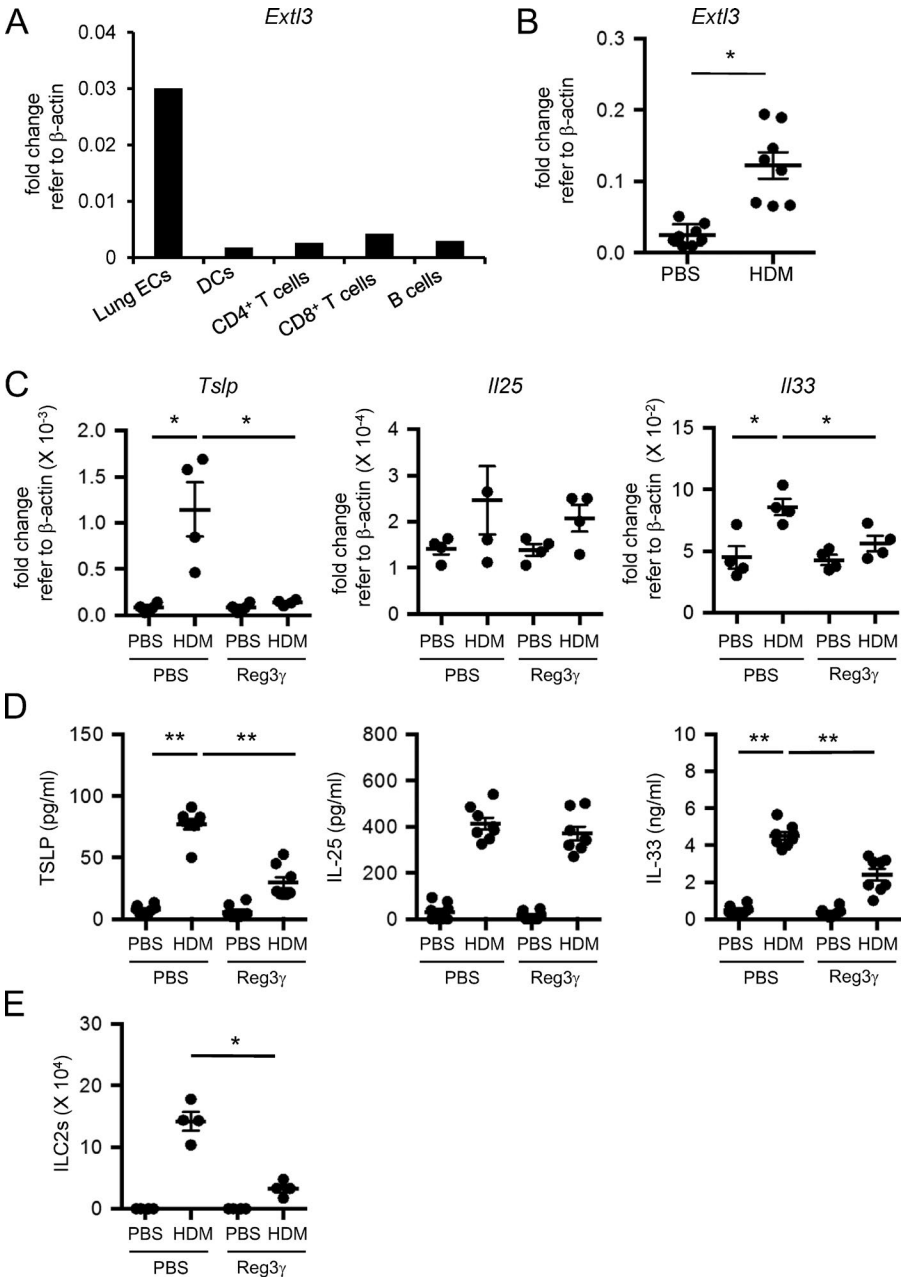

Figure 5. **Reg3γ inhibits the expression of TSLP and IL-33 in the lung.** (A) Expression levels of *EXTL3* mRNA in EpCAM$^+$ CD45$^-$ lung epithelial cells, DCs, CD4$^+$ T cells, CD8$^+$ T cells, and B cells were evaluated by qPCR. Shown are representative data from two independent experiments. (B) EpCAM$^+$ CD45$^-$ lung epithelial cells were isolated from HDM-sensitized and HDM-challenged mice or PBS-treated mice 48 h after the last challenge. The expression levels of *EXTL3* mRNA were evaluated by qPCR. Data are mean ± SEM for eight mice in each group from three independent experiments. *, $P < 0.05$ by unpaired Student's *t* test. (C and D) WT mice were administered intratracheally with HDM (50 µg) together with recombinant Reg3γ (10 µg) or PBS (Fig. S1 E). (C) 4 h later, the expression levels of mRNA for TSLP, IL-25, and IL-33 in whole lung were evaluated using qPCR. Data are mean ± SEM for four mice in each group from two independent experiments. *, $P < 0.05$ by one-way ANOVA and Tukey's test. (D) 16 h later, protein levels of TSLP, IL-25, and IL-33 in lung homogenates were determined using ELISA. Data are mean ± SEM for eight mice in each group from three independent experiments. **, $P < 0.01$ by one-way ANOVA and Tukey's test. (E) HDM-sensitized WT mice were administered intratracheally with recombinant Reg3γ or vehicle and then challenged with HDM as described in Fig. S1 D. 48 h after the last HDM challenge, the number of ILC2s (Lin$^-$ Thy1.2$^+$ ST2$^+$ cells) in the lung was evaluated using flow cytometry. Data are mean ± SEM for four mice in each group from two independent experiments. *, $P < 0.05$ by unpaired Student's *t* test.

Regarding the mechanism underlying Reg3γ-mediated suppression of allergic airway inflammation, we found that EXTL3, a functional receptor of Reg3γ (Lai et al., 2012), is expressed in lung epithelial cells (Fig. 5 A) and that the intratracheal administration of Reg3γ inhibits HDM-induced expression of IL-33 and TSLP (Fig. 5, C and D). These data suggest that Reg3γ directly functions on lung epithelial cells and suppresses cytokine production that enhances allergic airway inflammation. However, because some cell types besides epithelial cells are capable of producing IL-33 and TSLP, we cannot exclude the possibility that Reg3γ suppresses the cytokine production from other cellular sources. Further studies on the producers of these cytokines are required to understand detailed mechanisms underlying the Reg3γ-mediated suppression.

It has recently been shown that antimicrobial proteins, including Reg3 family members regulate cellular responses beyond antimicrobial activity. Lai et al. (2012) showed that Reg3γ and its human orthologue REG3A are induced by IL-22 and enhance proliferation and differentiation of keratinocytes during skin injury. In addition, Reg3β, another member of Reg3 family proteins, has been reported to enhance myocardial healing by accumulating M2 macrophages (Lörchner et al., 2015). We showed here that Reg3γ plays a role in the regulation of allergic airway inflammation presumably by acting on epithelial cells. However, we cannot

exclude the possibility that Reg3γ changes the microbiota in the lung and indirectly affects the epithelial cell function and subsequent immune responses because microbiota in the lung has recently been implicated in the pathogenesis of asthma (Huang et al., 2015; Huang and Boushey, 2015). Because fungal sensitization also causes allergic airway inflammation, the alteration of fungal microbiota in the lung may also be involved in Reg3γ-mediated suppression of asthmatic responses. Further studies to assess the microbiota in the lung are needed to fully clarify how Reg3γ suppresses the development of HDM-induced allergic airway inflammation.

In conclusion, our data suggest that IL-22 inhibits the development of HDM-induced allergic airway inflammation by inhibiting cytokine production possibly through the induction of Reg3γ in lung epithelial cells. Although further studies are needed, our results indicate that Reg3γ is a crucial regulator of epithelial immune responses during allergic inflammation and suggest that Reg3γ and its receptor have therapeutic potential for asthma.

MATERIALS AND METHODS

Mice

C57BL/6 mice (Charles River Laboratories) and *IL22*$^{-/-}$ mice on a C57BL/6 background (Kreymborg et al., 2007) were housed in microisolator cages under specific pathogen-free conditions. Mice carrying floxed alleles for STAT3 (*STAT3*$^{fl/fl}$ mice; Takeda et al., 1999) were crossed with CCSP-rtTA/tetO-Cre mice in which Cre recombinase is expressed by DOX treatment in lung epithelial cells (Perl et al., 2002). STAT3 deletion was achieved by the administration of DOX (2 mg/ml) dissolved in a sucrose MediGel (ClearH2O; Fig. S1 C). The Chiba University Animal Care and Use Committee approved animal procedures used in this study.

HDM-induced allergic airway inflammation

Mice were sensitized and challenged with intratracheal administration of HDM extracts (Greer Laboratories) as described previously with minor modifications (Norimoto et al., 2014). In brief, mice were sensitized intratracheally with 50 μg HDM in 25 μl PBS twice at a 7-d interval. 7 d after the last sensitization, mice were challenged with 5 μg HDM for four consecutive days (Fig. S1 A). In some experiments, mice were administered intratracheally with anti-Reg3γ antibody (100 μg; Abgent) or recombinant Reg3γ (10 μg; R&D Systems) as described previously (Choi et al., 2013) and challenged with HDM (Fig. S1 D). 48 h after the last HDM challenge, the numbers of eosinophils, neutrophils, and lymphocytes recovered in BALF were evaluated as described elsewhere (Plantinga et al., 2013). Airway resistance was measured by FlexiVent FX (SCIREQ) as described previously (Wakashin et al., 2008).

HDM-induced in vitro cytokine production

Cytokine production from draining lymph node cells was evaluated as described previously (Hammad et al., 2009). In brief, mice were sensitized and challenged with HDM as described above. Cells isolated from MLNs (10^6 cells) or single-cell suspensions prepared from the lung were stimulated with HDM (30 μg/ml) for 5 d in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μM β-mercaptoethanol, and 2 mM L-glutamine (complete RPMI medium) in a round-bottom 96-well plate (Fig. S1 B). The levels of cytokines in the culture supernatants were measured using ELISA kits according to the manufacturer's instructions.

HDM-induced cytokine expression in lung

Mice were administered intratracheally with HDM (50 μg) or PBS together with 10 μg recombinant Reg3γ or vehicle (Fig. S1 E). 4 h after administration, mRNA expression for TSLP, IL-25, and IL-33 in whole lung was analyzed using qPCR. The protein levels of TSLP, IL-25, and IL-33 in lung homogenates were evaluated uising ELISA at 16 h after the HDM administration.

IL-22–producing cells in the lung

Single-cell suspensions were prepared from the lungs of HDM-sensitized and HDM-challenged mice and stimulated with PMA (20 ng/ml; Calbiochem) and ionomycin (1 μg/ml; Calbiochem) for 4 h in the presence of brefeldin A (10 μM; BD Bioscience) and monensin (2 μM; Sigma-Aldrich). Cells were stained with indicated antibodies against surface molecules together with intracellular cytokines as described previously (Suto et al., 2008). Where indicated, cells were stained with T-bet, GATA3, or RORγt together with IL-22 using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience).

Histological analysis of the lung

Lung sections (3 μm thick) were stained with hematoxylin and eosin or periodic acid–Schiff according to standard protocols. Histological score and the number of goblet cells were evaluated as described elsewhere (Takahashi et al., 2011; Zaiss et al., 2015).

RNA-seq analysis

Total RNA was isolated from the lung of C57BL/6 mice administered intratracheally with IL-22 (100 ng) or PBS and the lung of WT mice or *IL22*$^{-/-}$ mice sensitized and challenged with HDM by using the RNeasy Mini kit (QIAGEN). RNA-seq was performed on an Illumina HiSeq 1500 using a TruSeq Rapid SBS kit (Illumina) in a 50-base single-end mode. mRNA profiles were calculated using Cufflinks software and expressed as fragments per kilobase of exon model per million mapped fragments. An M-A plot was obtained on the basis log ratios and the mean of all transcripts in lung of mice treated with IL-22 and PBS or lung of HDM-sensitized and HDM-challenged WT mice and *IL22*$^{-/-}$ mice. Differentially expressed genes were determined by a weighted mean difference method using the TCC package in R software (Kadota et al., 2008). The RNA-seq data are available at Gene Expression Omnibus database under accession no. GSE100858.

qPCR analysis

qPCR was performed using a standard protocol on an ABI PRISM 7300 instrument (Applied Biosystems) using a SYBR green reagent (Power SYBR Green PCR Master Mix; Applied Biosystems). The sequences of PCR primers are as follows: IL-22R1, forward primer: 5′-GCTCGCTGC AGCACACTACCAT-3′; reverse primer, 5′-TGAGTGTGG GGTGGACCAGCAT-3′; Reg3α, forward primer: 5′-GGC ACCGAGCCCAATG-3′; reverse primer: 5′-GGATTT CTCTCCCATGCAAAGT-3′; Reg3β, forward primer: 5′-ACTCCCTGAAGAATATACCCTCC-3′; reverse primer: 5′-CGCTATTGAGCACAGATACGAG-3′; Reg3γ, forward primer: 5′-TTCCTGTCCTCCATGATCAAAA-3′; reverse primer: 5′-CATCCACCTCTGTTGGGTTCA-3′; Reg3δ, forward primer: 5′-ACCACAGACCTGGGCTAATG-3′; reverse primer: 5′-AGTCCAATCCAGATGTATGGGAA-3′; STAT3, forward primer: 5′-GGAGGAGTTGCAGCA AAAAG-3′; reverse primer: 5′-TGTGTTTGTGCCCAG AATGT-3′; EXTL3, forward primer: 5′-GGCTATACC ATGTTGCGGAAT-3′; reverse primer: 5′-AGTGAGCAA TGAGGGGGAAGA-3′; TSLP, forward primer: 5′-ACG GATGGGGCTAACTTACAA-3′; reverse primer: 5′-AGT CCTCGATTTGCTCGAACT-3′; IL-25, forward primer: 5′-ACAGGGACTTGAATCGGGTC-3′; reverse primer: 5′-TGGTAAAGTGGGACGGAGTTG-3′; IL-33, forward primer: 5′-TCCAACTCCAAGATTTCCCCG-3′; reverse primer: 5′-CATGCAGTAGACATGGCAGAA-3′; and β-actin, forward primer: 5′-GCTCTGGCTCCTAGCACCAT-3′; reverse primer: 5′-GCCACCGATCCACACAGAGT-3′.

Lung epithelial cell isolation

Lung epithelial cells were isolated by a two-step sorting method as described previously (Yokota et al., 2017). In brief, lung single-cell suspensions were prepared using Dispase (1,000 PU/ml; EIDIA), and then CD45.2$^+$ hematopoietic cells were depleted by MACS sorting. Isolated CD45.2$^-$ cells were subjected to further purification of EpCAM$^+$ CD45.2$^-$ cells by a SH800 cell sorter (Sony Biotechnology). The resultant cells were >95% pure EpCAM$^+$ CD45.2$^-$ cells, which represent pan-epithelial cells (Messier et al., 2012).

Immunohistochemistry

Lung was fixed with 10% neutral buffered formalin for 24 h before paraffin embedding. Sections were stained with anti-IL-22R1 antibody (Abcam), anti-phospho-STAT3 antibody (Cell Signaling Technology), or anti-Reg3γ antibody (Abcam) according to standard protocols.

ELISA

The amounts of IL-5, IL-13, IFN-γ, IL-22, TSLP, IL-25, and IL-33 were determined using ELISA kits from R&D Systems. The levels of IL-17A were determined using an ELISA kit from BioLegend. The levels of total IgE in sera and the levels of Reg3γ in BALF were determined using ELISA kits from Roche and LifeSpan BioSciences, respectively. The levels of HDM-specific IgG1 were evaluated as described previously (Cates et al., 2004).

Data analysis

Data are summarized as means ± SEM, unless otherwise indicated. The statistical analysis of the results was performed using the unpaired *t* test or ANOVA as appropriate. P-values <0.05 were considered significant.

Online supplemental material

Fig. S1 shows schematic diagrams of experimental protocols.

ACKNOWLEDGMENTS

We thank Dr. J.A. Whitsett for CCSP-rtTA/tetO-Cre mice. We also thank Drs. K. Suzuki, A. Suto, and K. Ikeda for valuable discussion and Ms. J. Iwata for animal care.

This work was supported in part by Institute for Global Prominent Research, Chiba University, and Grants-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

The authors declare no competing financial interests.

Author contributions: T. Ito and K. Hirose designed the study, conducted the experiments, analyzed data, and wrote the manuscript. A. Saku, K. Kono, H. Takatori, and T. Tamachi contributed experiments. Y. Goto, J.-C. Renauld, and H. Kiyono provided valuable materials and advice. H. Nakajima designed the study and wrote the manuscript.

Submitted: 13 December 2016

Revised: 29 May 2017

Accepted: 10 July 2017

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