

ORIGINAL ARTICLE

Vagal- α 7nAChR signaling attenuates allergic asthma responses and facilitates asthma tolerance by regulating inflammatory group 2 innate lymphoid cellsXintong Feng¹, Ling Li², Jingjing Feng¹, Wei He¹, Na Li¹, Tianyun Shi¹, Zhijun Jie¹ & Xiao Su²¹ Department of Pulmonary and Critical Care Medicine, Shanghai Fifth People's Hospital, Fudan University, Shanghai, China² Unit of Respiratory Infection and Immunity, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China**Keywords**Asthma tolerance, Inflammatory group 2 innate lymphoid cells, Sphingosine-1-phosphate, Vagal circuits, α 7 nicotinic acetylcholine receptor**Correspondence**

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Immunology & Cell Biology 2021; **99**: 206–222**Abstract**

Disorders of immune tolerance may lead to allergic asthma. Group 2 innate lymphoid cells (ILC2s) and inflammatory ILC2s (iILC2s) are key players in asthma. The vagus nerve innervating the airways releases acetylcholine or neuropeptides (i.e. calcitonin gene-related peptide) via pulmonary C-fibers (PCFs), which could regulate ILC2 activity upon binding the α 7 nicotinic acetylcholine receptor (α 7nAChR, coded by *Chrna7*) or neuropeptide receptors. Whether and how α 7nAChR and PCFs regulate asthma and the formation of asthma tolerance via ILC2s or iILC2s are poorly understood. We used vagotomized, PCF degeneration and *Chrna7* knockout mice to investigate ovalbumin (OVA)-induced asthma and oral OVA feeding-induced asthma tolerance. Our results revealed that vagotomy could generally suppress lung ILC2s and iILC2s, which mitigated allergic asthma responses but disrupted asthmatic tolerance. Removal of neuropeptides by PCF degeneration also reduced lung ILC2s and iILC2s, attenuating asthma responses, but did not affect asthma tolerance. In comparison, deletion of *Chrna7* increased resident ILC2s and trafficking iILC2s in the lung, worsened allergic inflammation and disrupted oral tolerance. Mechanistically, deletion of *Chrna7* in asthma-tolerant conditions upregulated T helper 2 cytokine- (*Il4*, *Il13* and *Il25*) and sphingosine-1-phosphate (S1P)-related genes (*S1pr1* and *Sphk1*). Blockade of S1P reduced iILC2 recruitment into asthmatic lungs. Our work is the first to demonstrate that vagal- α 7nAChR signaling engaging with iILC2s and S1P not only alleviates asthma but also facilitates asthma tolerance. These findings may provide a novel therapeutic target for attenuating asthma by enhancing asthmatic tolerance.

INTRODUCTION

Asthma is one of the most common chronic airway inflammatory diseases in humans. Type 2 inflammation is a principal mechanism of asthma, mainly mediated by T helper 2 (Th2) cells, group 2 innate lymphoid cells (ILC2s) and the type 2 cytokines they secrete, such as interleukin (IL)-4 and 13.¹ Among them, ILC2s have emerged as pivotal players in asthma in recent years, bridging innate and adaptive immune responses, as well as the nervous and immune systems.

Tracing back the etiology of asthma, deficiency in natural immune tolerance early in life is an essential factor

determining asthma prevalence. Moreover, the establishment of allergen-specific tolerance could protect patients from an excessive response to allergens.² Thus, allergen-specific immunotherapy has now been widely used to induce long-term clinical tolerance against allergens, causing a reduction in symptoms and a decrease in the need for pharmacotherapy, thereby improving quality of life for patients.^{3,4} Investigating the mechanisms underlying asthma tolerance is beneficial to exploit new targets to induce stable tolerance and prevent asthma and allergic disease. More than 15 epidemiological studies so far have shown that raw cow milk can reduce the risk of allergic diseases, confirming the tolerance-inducing effect of intestinal exposure to

antigens.^{5–8} Based on the classic model of breast milk-transferred asthma tolerance,^{9,10} our previous studies successfully established a mouse model of asthma tolerance by directly feeding ovalbumin (OVA) in early life.¹¹ The mechanisms of allergy-protective effects of such oral tolerance (OT) include T-cell anergy, deletion or homing.¹² However, the tolerance mechanism is systematic and far more complex. Here, for the first time, we investigate whether and how neuroimmune mechanisms regulate asthma and OT via ILC2s.

The airway epithelial cell-derived alarmins including IL-25, IL-33 and thymic stromal lymphopoietin can promote expansion of ILC2s.¹³ Studies have recently revealed that there are also various types of neurotransmitter receptors expressed on ILC2s, such as $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR),¹⁴ $\beta 2$ -adrenergic receptor,¹⁵ neuromedin U receptor 1 (NMUR1)¹⁶ and calcitonin gene-related peptide (CGRP) receptor,¹⁷ that could convey a neural signal to regulate the behavior of ILC2s in the immune response, particularly allergic asthma.¹⁸ Following activation, ILC2s produce robust amounts of type 2 cytokines IL-4, IL-5 and IL-13 to promote eosinophilic inflammation and airway hyperreactivity; thus, they play an essential role in the pathogenesis of asthma.¹⁹

Among ILC2s, recent studies have demonstrated that there is an inflammatory ILC2 (iILC2) population responsive to IL-25 that complements IL-33-responsive natural ILC2s (nILC2s). In response to the challenge of allergens or pathogens, iILC2s, as transient progenitors of ILCs, may develop into nILC2-like cells.²⁰ Notably, iILC2s are trafficking cells that arise from the resting ILC2s residing in the intestinal lamina propria and are recruited to diverse tissues under a sphingosine-1-phosphate (S1P) chemotactic gradient that promotes iILC2 entry and accumulation in peripheral sites, including the lung, via the lymphatics and blood. Once iILC2s access peripheral sites they are involved in defense against pathogens and tissue repair.²¹ However, whether these iILC2s are involved in asthma development and oral OVA feeding-induced asthma tolerance remains elusive.

Since the introduction of the “inflammatory reflex” theory,^{22–24} the crosstalk between the vagus nerve and the immune system has been well documented over the years. The latest insights have inspired people to consider the lung as a sensory and neuroimmune organ innervated with a large number of nerves; the afferent arc of the reflex senses injury and antigen and then activates efferent neural circuits, including the cholinergic anti-inflammatory pathway, modulating immune responses and the progression of inflammatory diseases.²⁵ Recent research has newly identified “pulmonary neuroendocrine cells,” secreting neuropeptide γ -aminobutyric acid or CGRP to

activate ILC2s and sensing exogenous airway signals, such as allergens, to amplify allergic asthma responses.¹⁷

The vagal nerve innervates the airways, containing cholinergic neurons and peptidergic neurons. Approximately 75% of the afferents in the vagal branches innervating the entire respiratory tract are capsaicin-sensitive C-fibers belonging to peptidergic neurons.^{26–28} Pulmonary C-fibers (PCFs) are located in the mucosa, lung parenchyma and space between epithelial cells in the airways^{27,29} and express neuropeptides, including CGRP and substance P.³⁰ Studies have shown that ILC2s are localized adjacent to C-fiber sensory neurons and express neuropeptide receptors.^{17,31} Recent studies have also revealed that selective ablation of vagal C-fiber neurons (e.g. capsaicin injection) in mice prevented allergen-induced airway hyperreactivity, thus indicating that PCFs are required for airway inflammation in asthma.^{32,33}

Distinct from PCFs, the $\alpha 7$ nAChR is activated by the neurotransmitter acetylcholine (ACh) to mediate rapid excitatory synaptic transmission.³⁴ $\alpha 7$ nAChR signaling plays a key role in activating the cholinergic anti-inflammatory pathway. Namely, $\alpha 7$ nAChR and $\alpha 7$ nAChR-expressing immune cells, together with the afferent and efferent neuronal arcs that form the information integrating center of the central nervous system, coordinate an inflammatory reflex to regulate neuroimmune responses.²⁵ Recent studies suggest that $\alpha 7$ nAChR-specific agonists can attenuate ILC2-dependent airway hyperreactivity,¹⁴ indicating that $\alpha 7$ nAChR could restrain inflammation in asthma. Thus, we hypothesize that $\alpha 7$ nAChR plays a pivotal role in asthma tolerance by regulating ILC2s, as well as the newly discovered iILC2s.

In this study, we explored whether and how the vagus nerve and its acting components, PCF and $\alpha 7$ nAChR, regulate asthma and the development of asthma tolerance. In particular, we targeted the role of S1P-dependent iILC2 trafficking and vagal $\alpha 7$ nAChR in regulating asthma and the formation of asthma tolerance. According to the evidence, we conclude that vagal $\alpha 7$ nAChR signaling can attenuate allergic asthma responses and facilitate asthma tolerance by regulating ILC2s.

RESULTS

Disruption of vagal circuits reduces ILC2s and attenuates OVA-induced allergic inflammation, but promotes inflammatory ILC2s to impair asthma tolerance

Previous studies have shown that vagotomy can alleviate allergen-induced airway hyperreactivity, reducing allergic inflammation in animal models of asthma,^{35,36} but whether vagal circuits affect ILC2 populations in asthma and asthma tolerance is not very clear. Based on the latest

studies, classic $CD45^+Lin^-CD90.2^+GATA3^+$ ILC2s (or nILC2s defined by $CD45^+Lin^-CD127^+CD90.2^{hi}KLRG1^{lo}$) are naturally resting cells in the lung and cannot traffic between organs.^{20,21} By contrast, intestinally-derived iILC2s (defined as $CD45^+Lin^-ST2^+CD90.2^+KLRG1^{hi}$ or $CD45^+Lin^-CD127^+CD90.2^+KLRG1^{hi}$ populations)^{20,21} could migrate to the lung and contribute to inflammation in an S1P-dependent manner.^{21,37} To test whether the disruption of vagal signals could impair asthma tolerance and impact iILC2 activities in the lung, or reduce iILC2 recruitment to the lung in our asthma model, we unilaterally excised the right cervical vagus nerve of mice (vagotomy), and 10 days later, we established an OVA-induced asthma and OVA feeding-induced asthma tolerance model (Figure 1a). At the end point of the model, we harvested the blood and lung tissue for further measurement. Plasma immunoglobulin E (IgE) showed that vagotomy did not change the IgE level in the OVA-induced OT group but significantly reduced the IgE level in the asthma group (Figure 1b). Analysis of ILC2s in the lung by flow cytometry revealed that the classic population ($Lin^-CD45^+CD90.2^+GATA3^+$ ILC2s) was reduced after vagotomy in asthma but not changed with OT between the vagotomy and sham groups (Figure 1c, d). We then examined the proportion of $CD45^+Lin^-ST2^+CD90.2^+KLRG1^{hi}$ iILC2s in the lung and found that the disruption of vagal circuits can reduce iILC2s in asthma but conversely increase iILC2s in OT (Figure 1e, f). These findings indicate that during the development of asthma tolerance, disruption of vagal circuits might influence the iILC2 populations recruited to the lung rather than nILC2s residing in the airway mucosa.

To further investigate the influence of vagal circuit disruption in asthma and OT, we measured the gene expression of a select set of asthma-associated cytokines, the neuropeptide CGRP and S1P-related receptors and synthase in the whole lung by real-time quantitative PCR. The results showed that after vagotomy, type 2 inflammation represented by *Il4* and *Il13* and the ILC2 alarmin *Il25* were decreased in asthma, and slightly elevated in OT (Figure 2a), corresponding to the reduced level of iILC2s in the lung. Thus, we predict that vagal circuits play an important role in asthma tolerance by regulating iILC2s. The relative gene expression of the neuropeptide CGRP (encoded by *Calca*) implied that vagotomy directly induced a reduction in CGRP expression in asthma, but CGRP expression remained relatively unchanged upon induction of tolerance, compared with vagotomy alone (Figure 2b). Whether this increase is a result of the deficiency in the main inhibitory component in vagal circuits during tolerance, increasing neuroendocrine cell secretion of CGRP or enhancing PCF compensatory activation after vagotomy, still needs further research. In addition, S1P receptor 1

(*S1pr1*), which regulates the migration of immune cells,^{21,38} and the key S1P synthase (sphingosine kinase 1, *Sphk1*)³⁹ were also decreased in asthma, and both were enhanced in OT after vagal circuit disruption (Figure 2c, d). We next investigated Th2 responses after vagotomy by flow cytometry analysis in the lung (Figure 2e, f) and found that vagotomy could also reduce Th2 responses in the asthma model but did not have a significant effect on Th2 cells in the OT condition. Thus, iILC2s instead of Th2 cells might be involved in the disruption of asthma tolerance after vagotomy. The results indicate that vagal circuits regulate lung iILC2s, as well as the S1P-related gene expression, in both asthma and tolerance. However, the vagal regulation may be complicated, because it integrates the effects of different components in the vagus, including cholinergic neurons and peptidergic neurons. Thus, studies regarding the function of these two components in regulating asthma tolerance appear to be warranted.

Inhibition of S1P signaling reduces iILC2s in the lung and attenuates allergic inflammation

While previous work has revealed that iILC2s can migrate to the lung in an S1P-dependent manner during pathogen infection,²¹ this pathway has not been confirmed in OVA-induced asthma. Therefore, we used FTY720 (also called fingolimod), which antagonizes the S1P signaling pathway,²¹ to investigate whether we could block iILC2 recruitment to the lung during asthma in an S1P-dependent manner. Before asthma sensitization, mice were treated with FTY720 or vehicle intraperitoneally (Figure 3a), and the control or OT group received equivalent vehicle at the same time. After treatment with FTY720, the plasma IgE level in the asthma group was significantly decreased compared with that in the vehicle group (Figure 3b). Flow cytometric analysis of lung $CD45^+Lin^-ST2^+CD90.2^+KLRG1^{hi}$ iILC2s in the FTY720-treated asthma group also showed decreases in iILC2 numbers to levels seen in the OT and control groups (Figure 3c, d). The results indicate that S1P can mediate iILC2 egress to the lung in the asthma model. Therefore, vagal circuits may play a role in both asthma and OT by regulating iILC2 egress through S1P signaling.

Degeneration of pulmonary C-fibers in vagal circuits alleviates allergic inflammatory responses by regulating lung ILC2s

PCFs are capsaicin-sensitive nerves, and recent studies have revealed that degeneration of PCFs by capsaicin pretreatment but not downstream receptor TRPV1

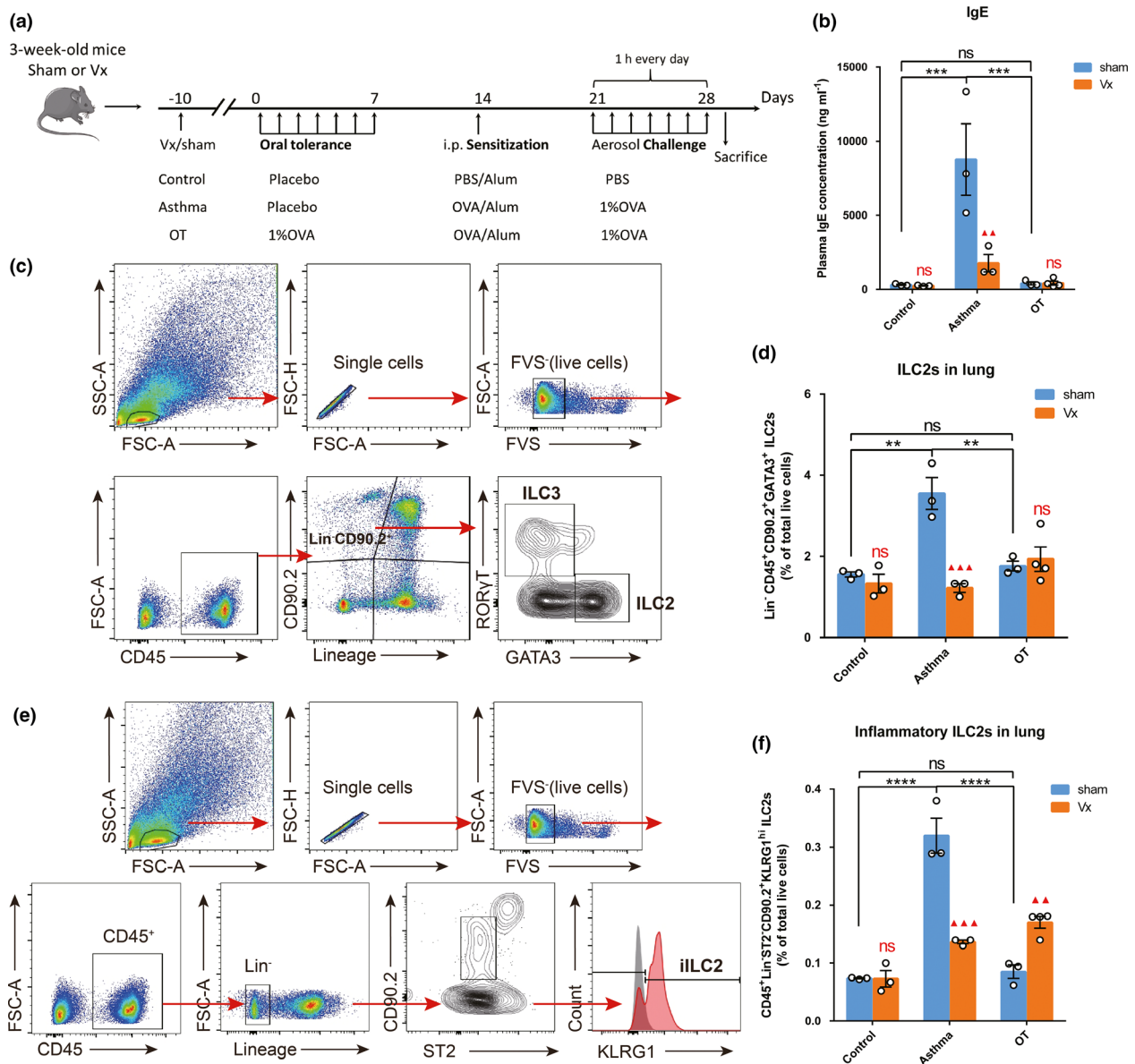


Figure 1. Disruption of vagal circuits reduces group 2 innate lymphoid cells (ILC2s) in the asthma model but promotes inflammatory ILC2s (iILC2s) in the asthma tolerance model. **(a)** Experimental procedures for establishing asthma and oral tolerance (OT) mouse models. Mice were divided into control, asthma and OT, and 10 days before the beginning of the model, each group was pretreated by right or sham cervical vagotomy and subdivided into vagotomy (Vx) and sham. The detailed procedures had been described in the "Methods". Mice were killed at day 28 to obtain samples for further measurement. **(b)** Plasma immunoglobulin E (IgE) levels measured by ELISA. **(c–f)** Flow cytometry analysis of Lin⁺CD45⁺CD90.2⁺GATA3⁺ ILC2s and CD45⁺Lin⁺ST2⁺CD90.2⁺KLRG1^{hi} iILC2s in the whole lung was performed separately among the six groups. The gating strategies are shown in **c** and **e**, and the figures below use the similar strategy for the first three gates. All the analyses are based on FVS⁺ (live cells). Data are mean $\pm$ s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; ns, not significant between the compared groups. $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$ and $\blacktriangle\blacktriangle\blacktriangle P < 0.001$ represent comparison of adjacent bar of Vx and sham in each group by the unpaired two-tailed Student's *t*-test. Multiple comparisons of groups were corrected by one-way ANOVA with Tukey's test; $n = 3$ or 4 in each group, and results are representative of two independent experiments. FSC-A, forward scatter area; FSC-H, forward scatter height; FVS, fixable viability stain; i.p., intraperitoneally; OVA, ovalbumin; PBS, phosphate-buffered saline; SSC-A, side scatter area.

(transient potential receptor vanilloid 1) blockade can decrease allergic airway inflammatory responses, including eosinophils, Th2 cells and inflammatory

cytokines, as well as reduce asthmatic hyperreactivity and airway remodeling in an asthma model.^{30,32,33,40,41} However, whether PCFs have effects on ILC2s,

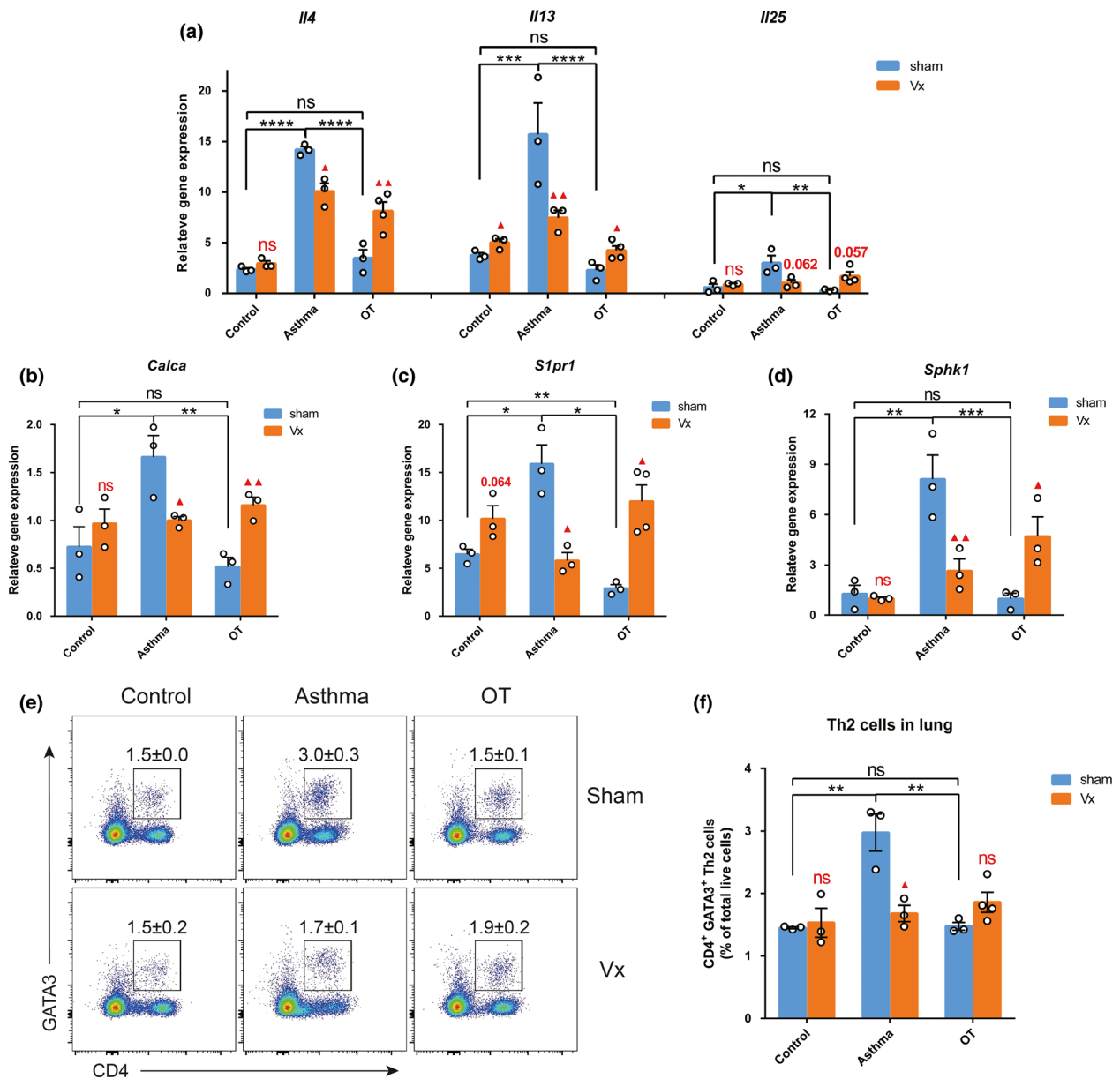


Figure 2. Disruption of vagal circuits differentially regulates gene expression and T helper 2 (Th2) cells of the lung in the asthma and oral tolerance models. The experiments were performed in the asthma and oral tolerance models after vagotomy as described in Figure 1a. **(a–d)** Relative gene expression of cytokines *Il4*, *Il13*, *Il25*, neuropeptide calcitonin gene-related peptide (CGRP; encoded by *Calca*), *S1pr1* and *Sphk1* in the whole lung among the six groups as determined by real-time quantitative PCR (RT-qPCR) analysis. **(e, f)** Flow cytometric analysis of CD4⁺GATA3⁺ Th2 cells in the whole lung among the six groups. The frequencies represent the gating percentages of total live cells in each group. All the analyses were based on FVS⁺ (live cells). Data are mean ± s.e.m. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, not significant between the compared groups. ▲*P* < 0.05 and ▲▲*P* < 0.01 represent comparison of adjacent bars of vagotomy (Vx) and sham in each group by the unpaired two-tailed Student's *t*-test, corrected by one-way ANOVA with Tukey's multiple comparisons test; *n* = 3 or 4 in each group, and results are representative of two independent experiments.

especially iILC2s, in asthma and tolerance is not very clear. Therefore, we performed degeneration of the PCF (named KPCF) via subcutaneous capsaicin injection in neonatal mice, and 3 weeks later, the mice that passed

the PCF degeneration test (described in the “Methods”) were challenged with our asthma and tolerance models (Figure 4a). KPCF-pretreated mice showed a significant reduction in plasma IgE during asthma, but no change

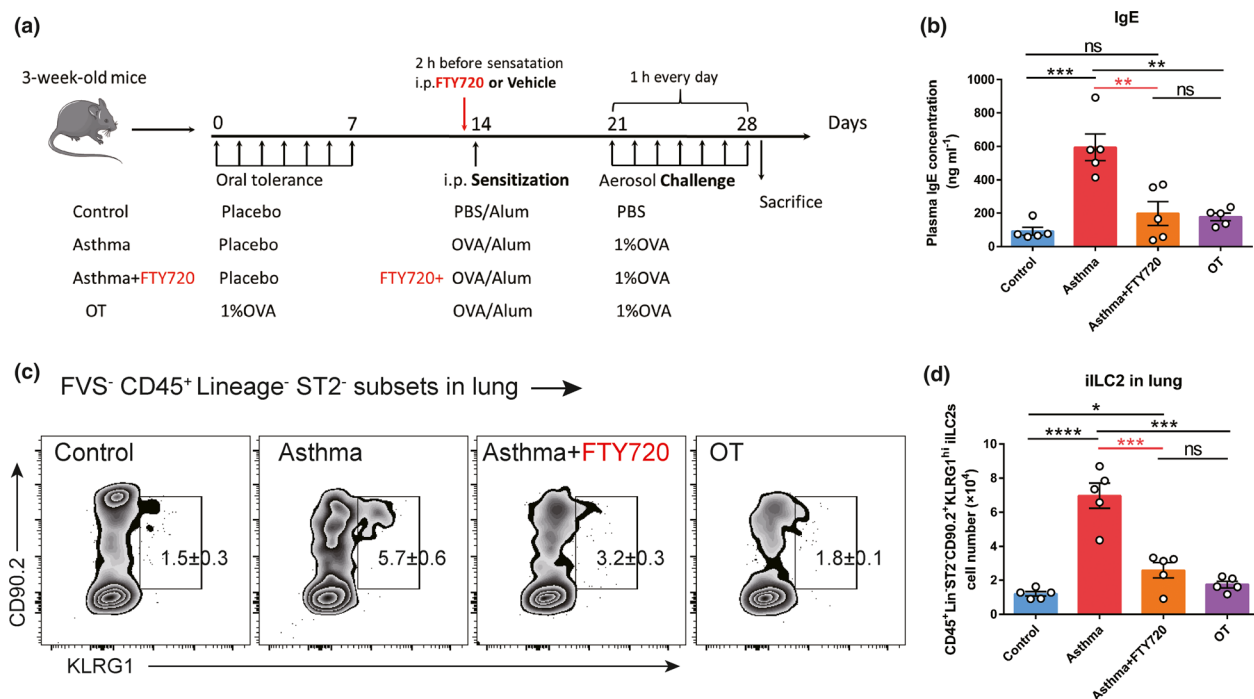


Figure 3. Inhibition of sphingosine-1-phosphate (S1P) signaling reduces group 2 innate lymphoid cells (ILC2s) in the lung and attenuates allergic inflammation. **(a)** Experimental procedures for S1P-signaling inhibition in the asthma model: at day 14 mice in the asthma group were injected intraperitoneally with 1 mg kg⁻¹ FTY720 or equal volume of vehicle 2 h before sensitization. Mice in the control or oral tolerance (OT) groups were also injected with vehicle. The remaining treatment in models was given as before. **(b)** Plasma immunoglobulin E (IgE) levels measured by ELISA. **(c, d)** Flow cytometry analysis of CD45⁺Lin⁻ST2⁻CD90.2⁺KLRG1^{hi} inflammatory ILC2s (iILC2s) in the whole lung among the four groups. The representative gates and frequencies (mean ± s.e.m.) of iILC2s in total live cells in each group are exhibited in **c**, with bar graphs and analysis in **d**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, not significant between the compared groups, with an unpaired two-tailed Student's *t*-test; *n* = 5 in each group, and results are representative of two independent experiments. i.p., intraperitoneally; PBS, phosphate-buffered saline.

in IgE was observed in the OT group between the intact and KPCF groups (Figure 4b). Similarly, in asthma, KPCF-treated mice exhibited substantially decreased frequencies of ILC2s, as well as iILC2s, in the lung, whereas comparable frequencies of ILC2s and iILC2s were observed between the OT KPCF-treated and PCF intact groups (Figure 4c–f). Moreover, KPCF mice in the asthma group exhibited lower levels of type 2 cytokine (*Il4*, *Il13* and *Il25*) and *S1pr1* messenger RNA expression compared with those in the intact PCF mice, with no significant change in OT (Figure 4g–j). Results suggest that the gene expression of this S1P receptor, and potentially downstream signaling, may not be altered by PCF during OT. Consistent with these results, PCF appears to be a more effective factor in vagal circuits to promote inflammation in the lung during asthma than in OT. The subsequent change in *S1pr1* expression otherwise indicates that S1P signaling is regulated by PCF in asthma and might be an important mechanism of neuroimmune regulation in vagal circuits.

Deficiency of $\alpha 7$ nAChR increases ILC2s and aggravates allergic inflammation in asthma model

The findings above imply that PCF might be the proinflammatory factor in vagal circuits regulating S1P-induced iILC2 egress to the lung during asthma, but the effector component in OT in vagus tissue is not PCF. Based on existing work suggesting that $\alpha 7$ nAChR activated on ILC2s can inhibit ILC2 effector function and repress ILC2-dependent airway hyperreactivity,¹⁴ we postulated that a pulmonary parasympathetic inflammatory reflex,^{23,24} in which $\alpha 7$ nAChR-expressing ILC2s cells are efferent arms of this reflex, is triggered in our OVA-induced OT model to restrain ILC2-mediated inflammation.

First, we used $\alpha 7$ nAChR knockout ($\alpha 7$ nAChR^{-/-}) or wild-type (WT) mice in our asthma model (Figure 5a). After the last OVA challenge, $\alpha 7$ nAChR^{-/-} mice, compared with WT animals, exhibited higher levels of plasma IgE (Figure 5b), as well as increased frequencies and numbers of classic ILC2s and

CD45⁺Lin⁻CD127⁺CD90.2⁺KLRG1^{hi} iILC2s in the lung, whereas numbers of CD45⁺Lin⁻CD127⁺CD90.2^{hi}KLRG1^{lo} nILC2s²¹ were more comparable (Figure 5c–g). This result complements a previous study that showed ameliorated asthma upon treatment with an agonist to activate $\alpha 7$ nAChR on ILC2s.¹⁴ Hence, engagement of $\alpha 7$ nAChR could ameliorate allergic inflammation by reducing the numbers of ILC2s in the lung or restraining the recruitment of iILC2s.

Deficiency of $\alpha 7$ nAChR impairs asthma tolerance by increasing ILC2s

To further address the role of $\alpha 7$ nAChR in suppressing iILC2s in asthma and understand whether it subsequently induces OVA-induced asthma tolerance, we established our asthma tolerance model in $\alpha 7$ nAChR^{-/-} mice, in parallel with our asthma model (Figure 6a). Strikingly, during asthma tolerance, $\alpha 7$ nAChR^{-/-} mice exhibited increased frequencies and numbers of ILC2s, as well as type 2 cytokine expression in the lung (Figure 6c–e), but there was no change in plasma IgE levels between WT and $\alpha 7$ nAChR^{-/-} mice (Figure 6b). Remarkably, the increased numbers of ILC2s in OT-challenged $\alpha 7$ nAChR-deficient mice approximated the levels during asthma in WT mice. However, despite this finding, the associated type 2 cytokine messenger RNA response was less enhanced in tolerized $\alpha 7$ nAChR-deficient mice compared with WT asthmatic mice (Figure 6e). This discrepancy may be attributed to Th2 and other non- $\alpha 7$ nAChR-regulated effector cells maintaining tolerance. Furthermore, $\alpha 7$ nAChR deficiency was associated with significantly higher *S1pr1* and *Sphk1* expression in both the asthma and OT groups (Figure 6f, g), indicating that $\alpha 7$ nAChR can inhibit *S1pr1* and *Sphk1* gene expression. Importantly, $\alpha 7$ nAChR deficiency in OT mice resulted in aggravated inflammation in pulmonary alveoli, bronchi and mesenchyme, characterized by increased bronchial mucosal hyperplasia, goblet cell proliferation, inflammatory cell infiltration and the presence of mucoid exudate in the airways, compared with WT OT mice (Figure 6h). Moreover, lung pathology in OT $\alpha 7$ nAChR-deficient mice was similar to that in WT asthmatic lungs.

$\alpha 7$ nAChR is required for attenuating ILC2-dependent inflammation by reducing iILC2 recruitment to the lung

iILC2s are transient progenitors of ILCs that can arise from intestinal ILC2s and egress via the gut-draining mesenteric lymph node (MLN) to tissues.^{20,21} To examine whether $\alpha 7$ nAChR-induced inhibition of ILC2s in asthma tolerance is associated with reduced iILC2 accumulation in

the lung, we analyzed the ILC2 subsets in both the lung and the MLN. We observed that OT $\alpha 7$ nAChR^{-/-} mice exhibited significantly more iILC2s in the lung (Figure 7a–e) and fewer iILC2s in the MLN compared with WT mice, mirroring results in the asthma model (Figure 7f, g). $\alpha 7$ nAChR deficiency also resulted in significantly decreased classic ILC2s in the MLN of both asthma and OT mice (Figure 7h, i), which corresponds to the increase in iILC2s and nILC2s in the lung (Figure 6a–e). These results suggest that lung iILC2 and nILC2 numbers are closely associated with total MLN ILC2s and that they might be partly transformed from trafficking iILC2s.

Collectively, these results suggest a previously unrecognized regulatory role for $\alpha 7$ nAChR that operates between the parasympathetic nervous system and the innate immune system to control iILC2 egress to the lung in both asthma and tolerance. Specifically, $\alpha 7$ nAChR signaling could limit the recruitment of iILC2s and thus represent a potential therapeutic target for the treatment of ILC2-mediated asthma.

DISCUSSION

Despite increasing evidence that the lung is a sensory organ,^{14,16,17,32} most neuroimmune studies have focused on inflammation in the asthma model itself, and neuroimmune regulation of asthma tolerance has remained largely a neglected field. In this study, we evaluated the effect of different vagal circuit components on iILC2s in both asthma and OT models.

Our results showed that the integrated vagal circuits exhibit proinflammatory effects during asthma by facilitating ILC2 expansion, type 2 inflammatory cytokine expression and IgE production in the lung. These disease-promoting effects appeared to be majorly attributed to one of the afferent arc components, PCFs. However, the other component, $\alpha 7$ nAChR, that engages the cholinergic anti-inflammatory pathway also played an essential role in attenuating the allergic inflammatory response above and promoting OVA-induced OT to alleviate asthma. Compared with WT mice, mice lacking $\alpha 7$ nAChR exhibited significantly increased resident ILC2s and trafficking iILC2s in the lung during asthma. Even more strikingly, tolerized $\alpha 7$ nAChR-deficient mice displayed remarkably more ILC2s and iILC2s in the lung than WT tolerized mice. Selective accumulation of ILC2s and iILC2s in the lung correlated nicely with increased expression of type 2 pathogenic cytokines in asthma, such as IL-4 and IL-13 and aggravated lung lesions. Impressively, these $\alpha 7$ nAChR-dependent effects on asthma tolerance complement previous work showing that $\alpha 7$ nAChR on ILC2s reduces ILC2-effector function and represses ILC2-dependent airway hyperreactivity in

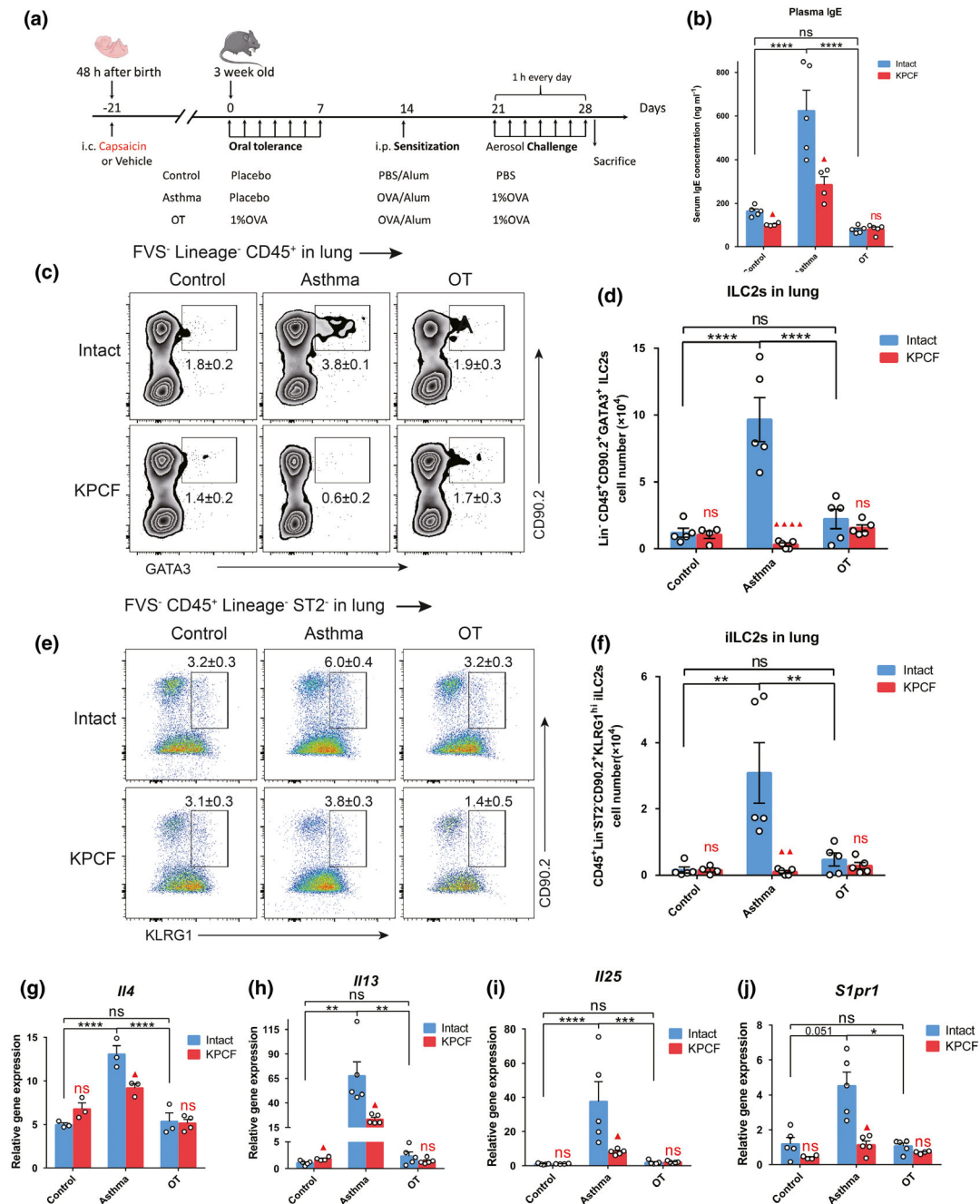


Figure 4. Degeneration of pulmonary C-fiber (PCF) in vagal circuits attenuates allergic inflammatory responses via suppressing lung group 2 innate lymphoid cells (ILC2s). **(a)** Experimental procedures for PCF degeneration: neonatal B6 mice, within 48 h of birth, were injected subcutaneously (s.c.) with a single dose of capsaicin solution (50 mg kg⁻¹) or its vehicle in an equal volume. At 3 weeks of age, mice assessed as having a complete degeneration of the C-fibers and intact controls were separately divided into the control, asthma and oral tolerance (OT) groups. **(b)** Plasma immunoglobulin E (IgE) levels measured by ELISA. **(c–f)** Flow cytometry analysis of Lin⁻CD45⁺CD90.2⁺GATA3⁺ ILC2s **(c, d)** and CD45⁺Lin⁻ST2⁺CD90.2⁺KLRG1^{hi} inflammatory ILC2s (iILC2s) **(e, f)** in the whole lung performed separately among the six groups. The representative gates and frequencies (mean $\pm$ s.e.m.) of ILC2s and iILC2s in the parent gate in each group were exhibited in **c** and **e**, with bar graphs and analysis in **d** and **f**. **(g–j)** Relative gene expression of cytokines *Il4*, *Il13*, *Il25* and *S1pr1* in the whole lung as determined by real-time quantitative PCR analysis. Data are mean $\pm$ s.e.m.; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$; ns, not significant between the compared groups; $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$ and $\blacktriangle\blacktriangle\blacktriangle P < 0.0001$ represent comparison of adjacent bars between the KPCF and intact groups by the unpaired two-tailed Student's *t*-test, corrected by one-way ANOVA with Tukey's multiple comparisons test; $n = 4$ or 5 in each group, and results are representative of two independent experiments. OVA, ovalbumin; PBS, phosphate-buffered saline.

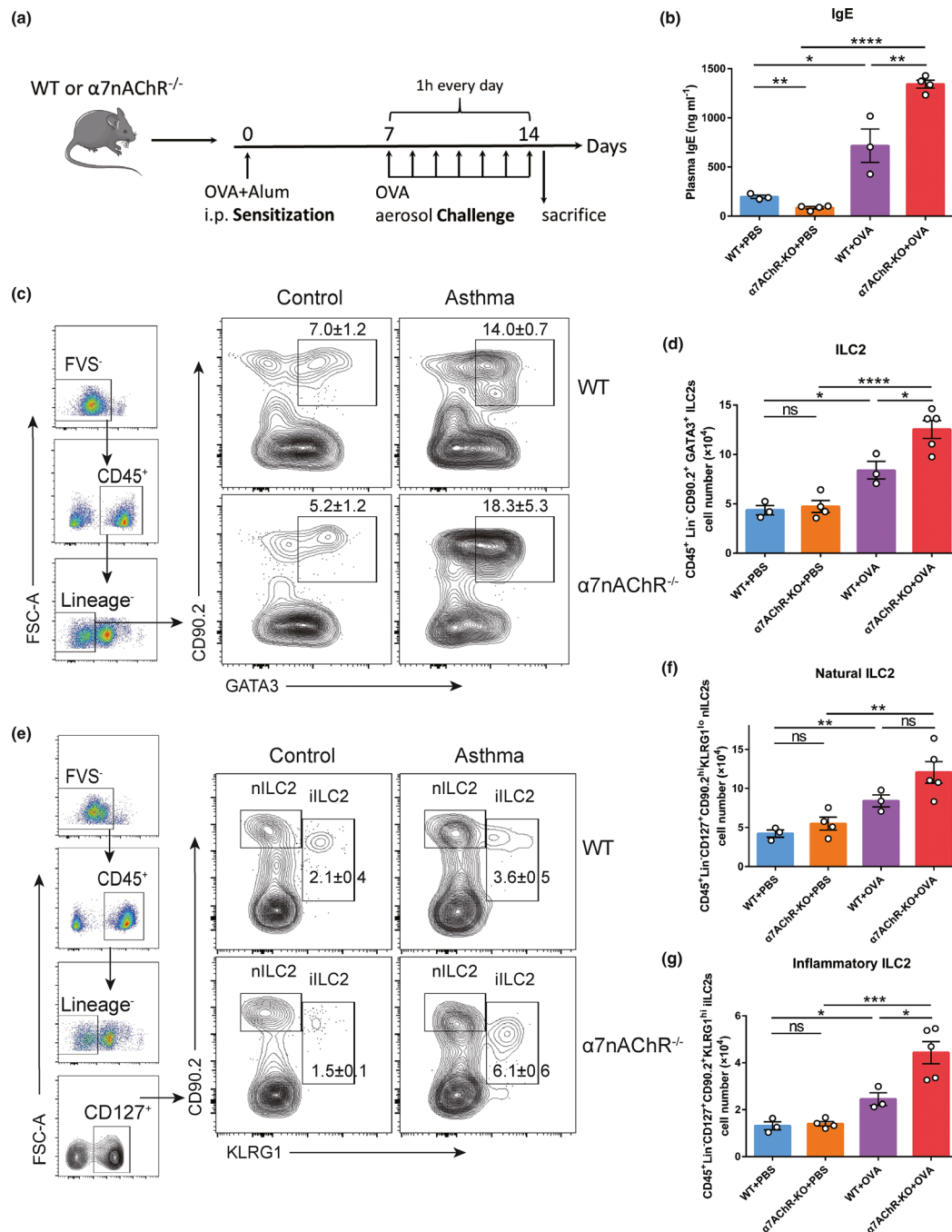


Figure 5. Deficiency of α7 nicotinic acetylcholine receptor (α7nAChR) increases group 2 innate lymphoid cells (ILC2s) and aggravates allergic inflammation in the asthma model. **(a)** Experimental procedures for the asthma model in α7nAChR-deficiency mice: 6–8-week-old wild-type or α7nAChR^{-/-} mice were sensitized and challenged with ovalbumin (OVA) to establish asthma, and at the end of the experimental period (day 14) samples were collected for the following experiments. **(b)** Plasma immunoglobulin E (IgE) levels measured by ELISA. **(c–g)** Flow cytometry analysis of CD45⁺Lin⁻CD90.2⁺GATA3⁺ ILC2s, CD45⁺Lin⁻CD127⁺CD90.2^{hi}KLRG1^{lo} natural ILC2s (nILC2s) and CD45⁺Lin⁻CD127⁺CD90.2^{hi}KLRG1^{hi} inflammatory ILC2s (iILC2s) in the whole lung performed separately among the four groups. nILC2s and iILC2s were gated in one panel. The percentages of ILC2s, nILC2s and iILC2s in the parent gate are exhibited in **c** and **e**, and cell numbers were analyzed in **d**, **f** and **g**. Data are means ± s.e.m. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, not significant between the compared groups, with the unpaired two-tailed Student's *t*-test; *n* = 3 or 5 in each group, and results are representative of three independent experiments. PBS, phosphate-buffered saline; FSC-A, forward scatter area; i.p., intraperitoneally; KO, knock out; WT, wild type.

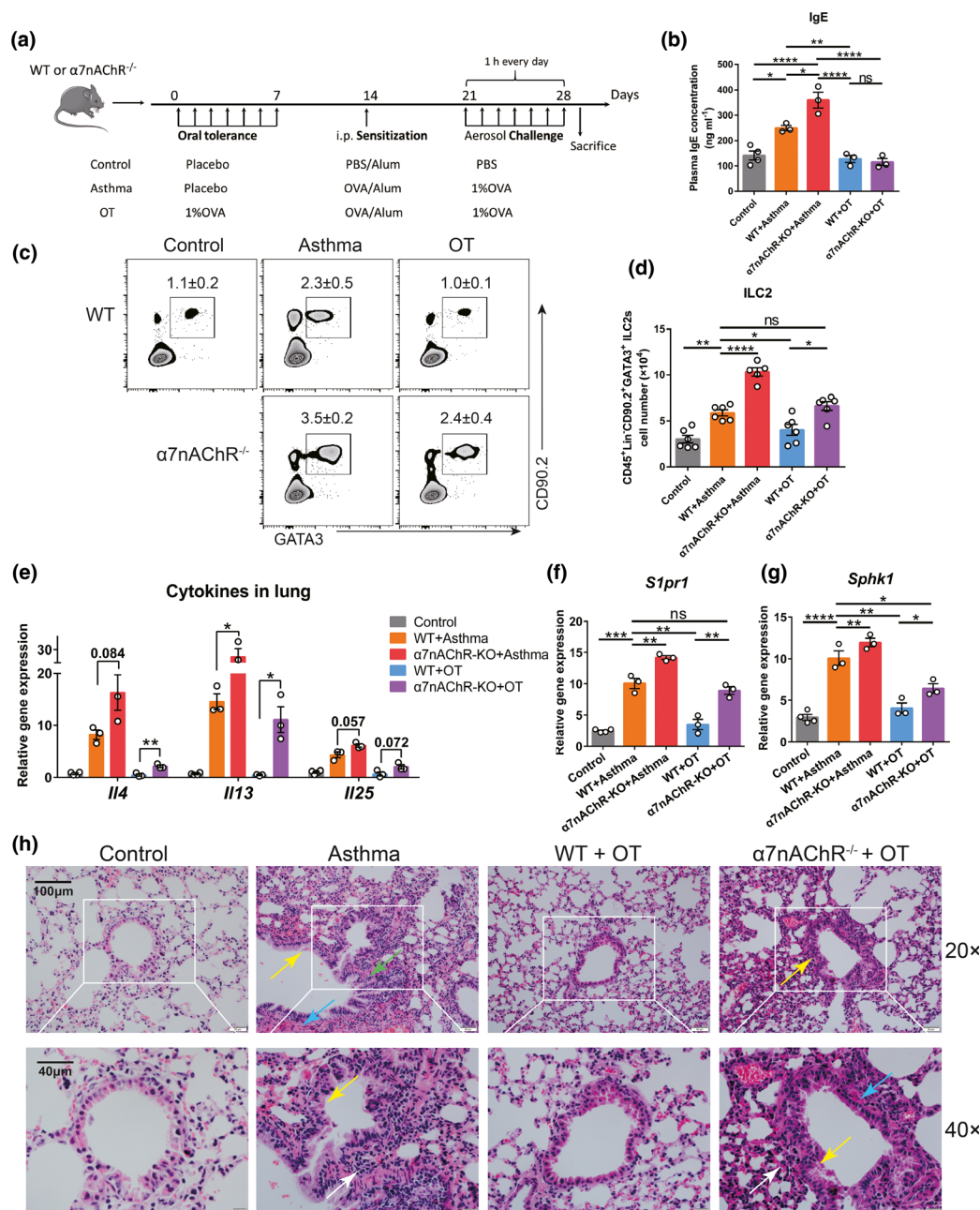


Figure 6. Deficiency of $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) can impair asthma tolerance by increasing group 2 innate lymphoid cells (ILC2s). **(a)** Experimental procedures for the asthma and oral tolerance model in $\alpha 7$ nAChR-deficiency mice. **(b)** Plasma immunoglobulin E (IgE) levels measured by ELISA. **(c, d)** Flow cytometry analysis of CD45⁺Lin⁻CD90.2⁺GATA3⁺ ILC2s in the whole lung among the five groups. The representative gates and frequencies (mean $\pm$ s.e.m.) of ILC2s in total live cells in each group are exhibited in **c**, and numbers of ILC2s were analyzed and shown in **d**. **(e–g)** Relative gene expression of cytokines *Il4*, *Il13*, *Il25* and *S1pr1* and *Sphk1* in the whole lung as determined by real-time quantitative PCR analysis. **(h)** Representative microphotographs of lung histological sections stained with hematoxylin and eosin from mice in the control and asthma groups, oral tolerance (OT) in wild-type (WT) mice and OT in $\alpha 7$ nAChR^{-/-} mice. Yellow arrows indicate thickness of bronchial mucous hyperplasia and goblet cells hyperplasia; white arrows point to peribronchial inflammatory cell infiltration; blue arrows denote thickening epithelial base membrane and green arrow indicates bronchial constriction and mucoid exudate. Objective magnification 20 $\times$; inserts were from the same section with a magnification 40 $\times$. Data are means $\pm$ s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant between the compared groups, with an unpaired two-tailed Student's *t*-test corrected by one-way ANOVA with Tukey's multiple comparisons test; n = 5 or 6 in each group, and results are representative of two independent experiments. KO, knock out; OVA, ovalbumin.

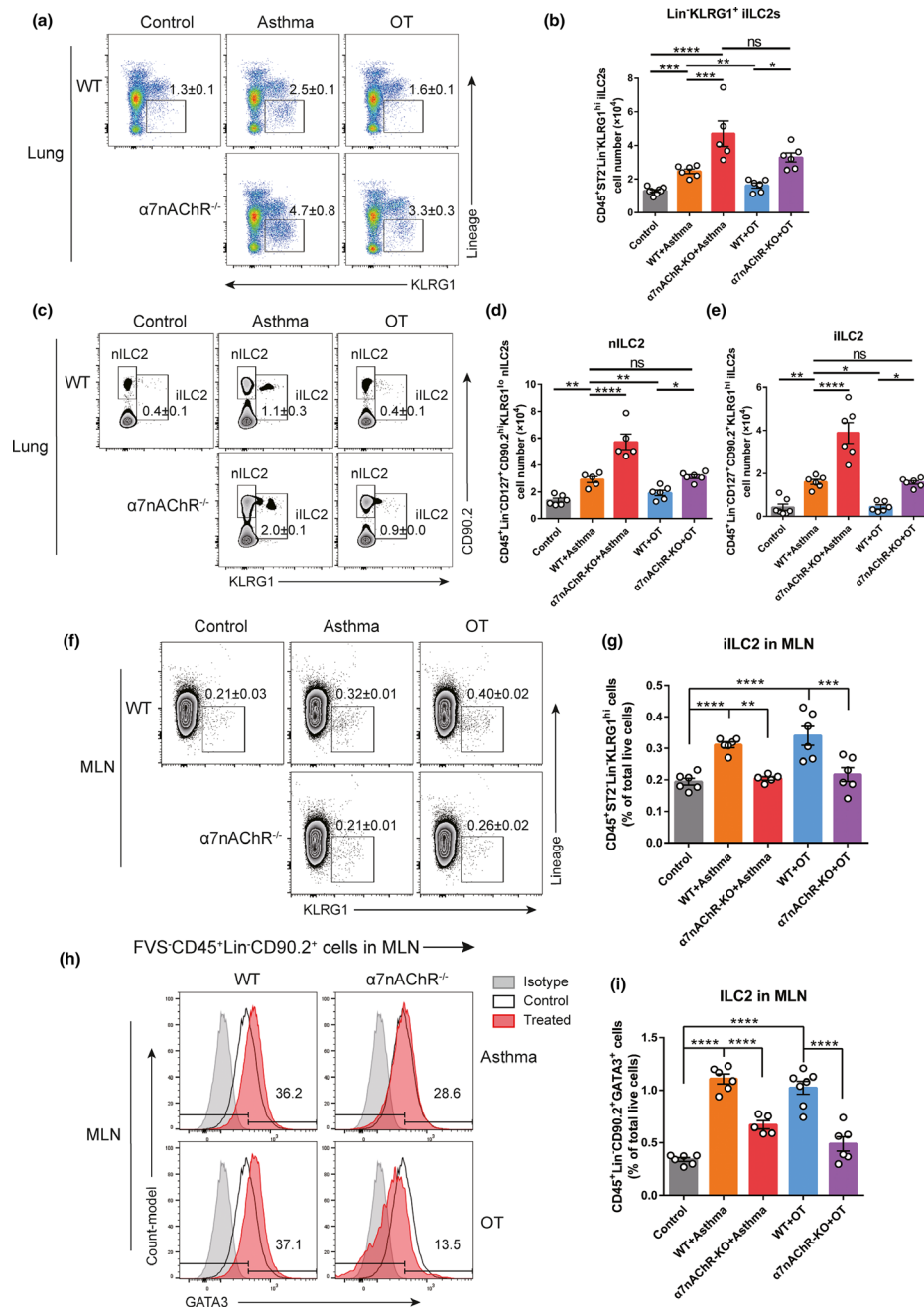


Figure 7. α7 nicotinic acetylcholine receptor (α7nAChR) is required for attenuating group 2 innate lymphoid cell (ILC2)-dependent inflammation by reducing inflammatory ILC2 (iILC2) recruitment to the lung. The experiments were performed in asthma and oral tolerance models in α7nAChR-deficient mice as described in Figure 6a. **(a–e)** Flow cytometry analysis of CD45⁺ST2⁺Lin⁺KLRG1^{hi} iILC2s **(a, b)**, CD45⁺Lin⁺CD127⁺CD90.2^{hi}KLRG1^{lo} natural ILC2s **(c, d)** and CD45⁺Lin⁺CD127⁺CD90.2⁺KLRG1^{hi} iILC2s **(c, e)** in the whole lung performed separately among the five groups. The representative gates and frequencies of total live cells in each group are exhibited in **a** and **c**, with cell numbers analyzed and shown in **b, d** and **e**. **(f–i)** Flow cytometry analysis of CD45⁺ST2⁺Lin⁺KLRG1^{hi} iILC2s and CD45⁺Lin⁺CD90.2⁺GATA3⁺ iILC2s in gut-draining mesenteric lymph node (MLN) performed separately among the five groups. **f** shows the representative gates and frequencies of total live cells in each group. **h** shows the percentage of GATA3⁺ ILC2s of the parent gate among the treated groups (red) compared with the control group (black) by histograms. Data are means ± s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; ns, not significant between the compared groups, with an unpaired two-tailed Student's *t*-test corrected by one-way ANOVA with Tukey's multiple comparisons test; *n* = 5 or 6 in each group, and results are representative of two independent experiments. KO, knock out; OT, oral tolerance; WT, wild type.

OVA-induced asthma,¹⁴ and we now propose a model where $\alpha 7$ nAChR plays a pivotal role in asthma tolerance by regulating resident ILC2s in the lung, as well as iILC2 recruitment to the lung to dampen nILC2 expansion. Therefore, we can explain the anti-inflammatory effect of $\alpha 7$ nAChR in asthma from the new perspective of maintaining immune tolerance.

The peptidergic C-fibers and the cholinergic anti-inflammatory pathway in vagal circuits exhibited opposing effects on asthma or asthma tolerance. The vagus showed a combined effect determined by alterations in the predominant role of these two pathways, and the integrated effect was also different between asthma and tolerance. In the asthma model, the disruption of vagal signals by vagotomy in mice induced dramatic amelioration of asthma, including decreased plasma IgE levels, Th2 cells, ILC2 and iILC2 populations, as well as type 2 cytokines and neuropeptide expression in the lung, compared with sham controls. Similarly, degeneration of PCFs in asthma also significantly alleviated allergic inflammation, whereas $\alpha 7$ nAChR deficiency aggravated inflammation. Thus, we suppose that $\alpha 7$ nAChR could restrain asthma inflammation to some extent, but the PCF dominates in vagal circuits during the pathological state. Nevertheless, in asthma tolerance when mice acquire the capacity to maintain normal physiological status to OVA challenge, the $\alpha 7$ nAChR anti-inflammatory pathway plays the predominant role in constraining the immune response to allergens, as $\alpha 7$ nAChR knockout impaired asthma tolerance in the lung, while PCF degeneration did not have overt effects on tolerance. These results imply that vagal circuits exert complicated regulation of the immune system in diverse conditions and that enhancing $\alpha 7$ nAChR¹⁴ and depressing PCF can both attenuate asthma inflammation in the lung. In particular, $\alpha 7$ nAChR signaling also plays an essential role in promoting asthma tolerance, and this may become a new therapeutic target to induce asthma tolerance. In addition, there appears to be inter-regulation between $\alpha 7$ nAChR and PCF responses, but this requires further investigation.

Further insights into the mechanisms by which $\alpha 7$ nAChR achieves asthma tolerance have been garnered from close examination of ILC2s, and the newly characterized iILC2s, in asthma inflammatory responses. Almost all iILC2s are reportedly positive for the proliferation marker Ki67, which indicates they exhibit a high proliferative potential.²⁰ More recent data also suggest that S1P-dependent inter-organ trafficking of iILC2s from the gut to the lung contributes to type 2 inflammation in antihelminth defense; however, the S1P inhibitor FTY720⁴² did not affect IL-25-induced intestinal ILC2 proliferation and only blocked iILC2 tissue accumulation.²¹ Our study attempts to close the gap in

knowledge surrounding the role of iILC2s in asthma and their neuroimmune regulatory mechanisms. In our study, we found that although iILC2s constitute a small population, as transient progenitors of ILCs,²⁰ they are significantly increased in asthmatic lungs compared with control lungs, and decrease upon achieving OT, which is analogous to Th2 cell and ILC2 changes. This indicates that iILC2s can also be activated in asthma and depressed in tolerance, contributing to allergic inflammation and joining nILC2s to amplify responses.

In this study, we focused on the function of vagal $\alpha 7$ nAChR signaling in regulating iILC2 recruitment in the lung. We performed FTY720 treatment to confirm that the iILC2s were indeed recruited via S1P signaling. Results showed that S1P blockade by FTY720 could reduce iILC2s in asthmatic lungs to levels comparable to control and OT mouse lung levels. Hence, we have demonstrated that blockade of S1P can attenuate inflammation in asthma by reducing ILC2s, in addition to blocking S1P-dependent egress of Th2 lymphocytes.³⁸ Based on these findings, we further examined whether $\alpha 7$ nAChR could regulate iILC2s and related S1P signaling in asthma. Results indicated that $\alpha 7$ nAChR^{-/-} mice exhibit an increased predisposition for the accumulation of iILC2s and enhancement of S1P signaling in the lung, with ensuing reduction in iILC2s in the MLN, regardless of asthma or OT. In other words, $\alpha 7$ nAChR is a key negative regulatory factor of iILC2s and related S1P-dependent egress in asthma; in this way, $\alpha 7$ nAChR can contribute to restraining allergic inflammation and facilitating OT. However, we cannot rule out the possibility that apart from the inhibition of resident ILC2s and trafficking of iILC2s, there are other mechanisms underlying $\alpha 7$ nAChR regulation in asthma. Still, the novel role of $\alpha 7$ nAChR that we have exploited is of great significance and deserves in-depth research in the future.

Previous studies have shown that vagotomy, PCF degeneration or $\alpha 7$ nAChR activation can alleviate allergen-induced airway hyperreactivity and reduce allergic airway inflammation in asthma models, including eosinophils and Th2 cells infiltration and inflammatory cytokines production.^{14,30,32,33,35,36,40,41} Consequently, our research has a minor flaw in that we mainly focused on changes in ILC2 numbers. As ILC2s are not the only drivers of allergic airway inflammation, in the future we will explore the specific mechanism of the vagus nerve in regulating all type 2 immune cells step-by-step, as well as related chemokines and cytokines that play a role in asthma tolerance.

In conclusion, our study is consistent with the concept that neuroimmune regulation is indispensable in asthma. Moreover, targeting neuroreceptors, such as $\alpha 7$ nAChR, can not only inhibit the allergic response but also promote the establishment of asthma tolerance. Thus, we propose a

model in Figure 8. The vagus nerve is widely distributed in the airway with two components: peptidergic nerves represented by the PCF and cholinergic nerves represented by $\alpha 7$ nAChR signaling, which play different roles in the pathogenesis of allergic asthma. Neuropeptides, such as CGRP, substance P and NMU, released from PCF endings function like ILC2 alarmins (IL-25, IL-33, thymic stromal lymphopoietin) and increase the number of ILC2s and iILC2s and the production of type 2 cytokines in the lung. By contrast, $\alpha 7$ nAChR signaling, which releases ACh, could attenuate allergic inflammation and promote asthma tolerance by inhibiting ILC2 responses and S1P-dependent recruitment of iILC2s in the lung during allergic inflammation. Future studies will probe deeply into the role of $\alpha 7$ nAChR in regulating iILC2s and S1P-dependent gut–lung circulation in OT. FTY720, which antagonizes the S1P signaling pathway,^{21,38,39,42–44} will also be useful in future neuroimmune research to investigate the role of various vagal components (such as PCF, NMU and $\alpha 7$ nAChR) in regulating S1P-dependent trafficking of different immune cells. The coordination between different components in vagal circuits is also important, and the interaction between different neural pathways is worth exploring as well. Our work has uncovered the role of $\alpha 7$ nAChR in asthma tolerance and examined the possible underlying mechanisms by which $\alpha 7$ nAChR regulates ILC2s and iILC2s. A better understanding of the neuroimmune regulation axis in tolerance disruption and asthma development is essential as we attempt to exploit new immunotherapies, such as targeting $\alpha 7$ nAChR, to promote tolerance establishment.

METHODS

Animals

$\alpha 7$ nAChR-deficient mice (C57BL/6 background, B6.129S7-Chrna7^{tm1Bay}, number 003232) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The WT and $\alpha 7$ nAChR-deficient mice used in this study were littermates and cohoused under the same conditions at 22°C on a 12-h light/dark cycle with free access to food and water. Anesthesia was induced with pentobarbital sodium (50 mg kg⁻¹) injected intraperitoneally. The animal studies were approved by the Committee on Animal Research of the Institut Pasteur of Shanghai, Chinese Academy of Sciences.

Mouse models of allergen-induced asthma and oral tolerance

OVA-induced asthma and OT animal models were established as previously described.¹¹ The mice used in the experiments were 3–4 weeks of age. The asthma group mice were fed with placebo from day 0 for 1 week, sensitized intraperitoneally

with OVA/alum [50 µg of OVA (Sigma-Aldrich, Saint Louis, Missouri, USA) and 50 µL of alum gel (Thermo Scientific, Rockford, IL, USA) in 70 µL of phosphate-buffered saline (PBS)] once on day 14 and challenged via aerosol with OVA (10 mg mL⁻¹ in PBS), which lasted for 1 h per day from day 21 for 1 week; the OT group mice were fed OVA (1% dissolved in sterile water) from day 0 for 1 week, and the rest of the procedures were the same as those for the asthma group; the control group mice were given equivalent placebo, alum or PBS at the same time as the other groups. On day 28, all mice in the model were killed for experiments.

Unilateral vagotomy

At 1–2 weeks before the establishment of the asthma and tolerance mice models, right or sham cervical vagotomy was performed with mice under anesthesia and aseptic operation as described previously.⁴⁵ It has been reported that the right nerve controls cardiac function, thus this side was not chosen for vagus nerve stimulation,⁴⁶ and no mice died of heart failure after the right-side vagotomy.

FTY720 treatment

Fingolimod (FTY720) HCl was purchased from Selleck Chemicals (Houston, TX, USA). On day 14 in the animal model described above, 2 h before OVA sensitization, mice were injected intraperitoneally with 2.0 µg g⁻¹ bodyweight^{38,47} FTY720 or with vehicle (0.9% saline) only as a control.

Neonatal capsaicin treatment

As previously described, neonatal mice were treated with capsaicin (50 mg kg⁻¹, subcutaneously; Alomone Labs, Jerusalem, Israel) or vehicle alone (1:1:8 vol/vol/vol ethanol–Tween 80–saline) at 48 h postnatal.⁴⁸ Then, mice (3–4 weeks after capsaicin pretreatment) were OVA fed, sensitized and challenged to establish asthma tolerance as described above. One day before OVA feeding (approximately 3 weeks after capsaicin administration), eye-wiping tests were first performed to assess whether complete degeneration of the C-fibers had occurred after neonatal treatment with capsaicin.⁴⁹ For this test, we administered 20 µL of a 0.01% capsaicin solution to the eyes of each mouse and counted the number of wiping movements that occurred for 60 s. The maximal final concentration of ethanol in this solution was 0.001%, and it had no effect *per se*.³³ The mice that wiped their eyes less than five times were defined to have been desensitized by neonatal capsaicin treatment, and the others that did not meet the standard were not used in further experiments.

ELISA

The whole blood of each mouse was collected from the left ventricle on day 28. The blood was centrifuged at 1000g for 15 min to collect plasma. The plasma IgE was measured using

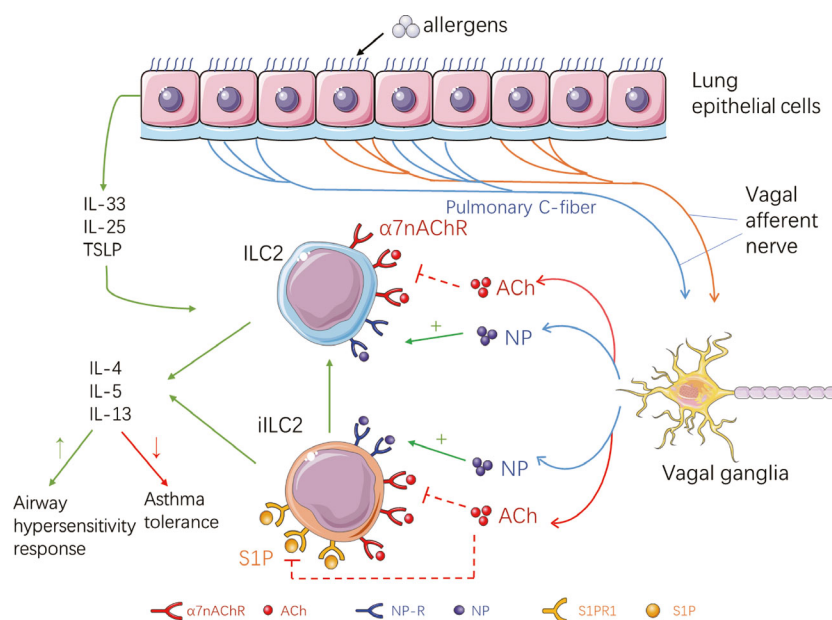


Figure 8. In this study, we found that neuropeptides released from pulmonary C-fiber (PCF) endings could enhance airway hypersensitivity in asthma by enhancing group 2 innate lymphoid cells (ILC2s) and inflammatory ILC2s (iILC2s) and type 2 cytokine [interleukin (IL)-4, 5 and 13] production. However, $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) signaling activated by acetylcholine can suppress allergic inflammation or facilitate oral ovalbumin (OVA) feeding-induced asthmatic tolerance by reducing ILC2 or iILC2 responses and sphingosine-1-phosphate (S1P)-dependent iILC2 recruitment into the lung. ACh, acetylcholine; NP, neuropeptide; S1PR1, sphingosine-1-phosphate receptor 1; TSLP, thymic stromal lymphopoietin.

a commercial ELISA kit (BD Biosciences, San Diego, CA, USA) according to the manual provided by the manufacturer.

Real-time quantitative PCR analysis

Following the manufacturer's instructions, total RNA was extracted from the whole lung using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Complementary DNA was synthesized using a reverse transcriptase kit (FastQuant RT Kit, TIANGEN Biotech™, Beijing, China) and then subjected to real-time quantitative PCR analysis (SYBR Green master mix, Yeasen, Shanghai, China). The reaction was detected on an ABI 7900HT fast real-time PCR system (Foster City, CA, USA). The sequences of the primers used for the target genes are listed in Supplementary table 1. The relative expression levels of detected genes were determined by the $2^{-\Delta\Delta CT}$ method⁵⁰ and normalized by *Gapdh* levels.

Isolation of cells from the lungs and lymph nodes

Mice were killed and perfused with cold PBS. Where indicated, lungs were removed and washed twice in Roswell Park Memorial Institute-1640 medium (HyClone, Logan, Utah, USA) supplemented with 25 mM HEPES, L-glutamine

and 1% penicillin/streptomycin (Sigma-Aldrich, USA). The excised lungs were minced, ground and transferred into a conical tube containing 5 mL of digestive solution [collagenase type 1A (1 mg mL^{-1} , Sigma-Aldrich™, USA) plus type IV bovine pancreatic DNase I grade II, from bovine pancreas ($20 \text{ } \mu\text{g mL}^{-1}$, Sigma-Roche, USA) in Hanks' balanced salt solution (HyClone, Logan, Utah, USA) containing 5% fetal bovine serum (Gibco, USA), 100 U mL^{-1} penicillin and $100 \text{ } \mu\text{g mL}^{-1}$ streptomycin]. The postcaval lobe was fixed in buffered formalin for histological analysis. Single-cell suspensions of the remaining lung parenchymal tissue were prepared with a gentleMACS lung dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The cells were collected by centrifugation for 10 min at $335g$ at 4°C . Erythrocytes were lysed by the ACK lysing buffer (Beyotime, Shanghai, China). The cell pellets were washed with cold PBS/0.5% bovine serum albumin and filtered by a $70\text{-}\mu\text{m}$ cell strainer. The cells were diluted and counted with a hemocytometer.

MLNs were collected. The lymph nodes were digested by collagenase in 1.5-mL centrifuge tubes at 37°C for 30 min. A plunger of a 5-mL syringe was used to gently crush the digested lymph nodes in a $70\text{-}\mu\text{m}$ cell strainer. Cold Roswell Park Memorial Institute-1640 medium was used to flush cells through the strainer. The cell pellets were collected by centrifugation after lysing the erythrocytes. The cells were diluted and counted with a hemocytometer.

Flow cytometry

Cell suspensions were incubated with purified anti-CD16/32 (clone 2.4G2, BD Horizon, USA) for 10 min at 2–8°C to block Fc receptors. All live samples were stained with Fixable Viability Stain 780 (FVS 780; BD Horizon, NJ, USA) for 15 min away from light at room temperature to exclude dead cells. All analyses were based on FVS[−] (live cells). Fluorochrome or biotin-labeled monoclonal antibodies (clones denoted in parentheses) against Lineage Antibody Cocktail with Isotype Control, including CD3e (145-2C11), CD11b (M1/70), CD45R/B220 (RA3-6B2), Ly-76 (TER-119), Ly-6G and Ly-6C (RB6-8C5), CD45 (30-F11), Thy1.2/CD90.2 (53-2.1 or 30-H12), CD127 (SB/199), KLRG1 (2F1) and GATA3 (L50-823), were purchased from BD Biosciences. Anti-ST2 (DIH9) and anti-ROR γ T (B2D) antibodies were purchased from BioLegend (CA, USA) and eBioscience (Thermo Fisher Scientific, USA), respectively. Anti-T1/ST2 (DJ8) antibodies were purchased from MD Biosciences (Minnesota, USA). Cells were first stained for surface markers for 30 min on ice protected from light and then fixed, permeabilized and stained with intracellular markers by a Transcription Factor Buffer Set (BD Pharmingen, CA, USA) according to the instructions. As an isotype control, fluorescein-conjugated IgG antibodies were preincubated with cells to set the positive and negative gates. Data were collected with an LSRFortessa flow cytometer (BD Biosciences, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). All collected cells were gated on FVS[−] leukocytes, and doublets were excluded.

Lung histology

The lungs of the model mice were removed and fixed in fresh formalin for 48 h. After paraffin embedding, each sample was cut into sections and stained with hematoxylin and eosin. A pathologist blinded to the experimental design visualized each lung section and gave a report.

Statistical analysis

Data are expressed as means $\pm$ s.e.m. Statistical analyses were performed using the two-tailed Student's *t*-test (between two groups) and one-way ANOVA with Bonferroni's or Tukey's *post hoc* test (multiple comparisons among three or more groups) with Prism software (GraphPad Software, La Jolla, CA, USA). *P* values < 0.05 were considered to be statistically significant: **P* < 0.05; ***P* < 0.01; ****P* < 0.001, *****P* < 0.0001.

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AUTHOR CONTRIBUTION

Xintong Feng: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Visualization; Writing-original draft. **Ling Li:** Data curation; Formal analysis; Methodology. **Jingjing Feng:** Data curation; Formal analysis; Methodology. **Wei He:** Formal analysis; Software. **Na Li:** Investigation; Methodology. **Tianyun Shi:** Methodology. **Zhijun Jie:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision. **Xiao Su:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Writing-review & editing.

CONFLICT OF INTEREST

The authors confirm that all funding sources supporting the work and all institutional or corporate affiliations are disclosed in the manuscript, and no commercial or financial conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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