

Research Article

iNKT cells with high PLZF expression are recruited into the lung via CCL21-CCR7 signaling to facilitate the development of asthma tolerance in mice

Xintong Feng^{1,2} , Caiqi Zhao³, Ling Li³, Jingjing Feng^{1,2}, Wei He^{1,2}, Tianyun Shi^{1,2}, Na Li^{1,2}, Zhijun Jie^{1,2} and Xiao Su³¹ Department of Pulmonary and Critical Care Medicine, Shanghai Fifth People's Hospital, Fudan University, Shanghai, China² Center of Community-Based Health Research, Fudan University, Shanghai, China³ Unit of Respiratory Infection and Immunity, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China

Establishment of immune tolerance is crucial to protect humans against asthma. Promyelocytic leukemia zinc finger (PLZF) is an emerging suppressor of inflammatory responses. CCL21-CCR7 signaling mediates tolerance development. However, whether PLZF and CCL21-CCR7 are required for the development of asthma tolerance is unknown. Here, we found that *Zbtb16* (coding PLZF) and *Ccl21* were upregulated in OVA-induced asthma tolerance (OT) lungs by RNA-seq. PLZF physically interacted with GATA3 and its expression was higher in GATA3⁺ Th2 cells and ILC2s in OT lungs. *Zbtb16*-knockdown in lymphocytes promoted the differentiation of CD3e⁺CD4⁺ T cells, particularly those producing IL-4 and IL-5. Moreover, iNKT cells with high expression of PLZF were recruited into the lungs via draining lymph nodes during tolerance. Blockade of CCL21-CCR7 signaling in OT mice decreased the PLZF⁺ cell population, abolished CCR7-induced PLZF⁺ iNKT recruitment to the lungs, enhanced Th2 responses and exacerbated lung pathology. In OT mice, respiratory syncytial virus (RSV) infection impeded PLZF⁺ cell and CCR7⁺PLZF⁺ iNKT cell recruitment to the lungs and increased airway resistance. Collectively, these results indicate that PLZF could interact with GATA3 and restrain differentiation of IL-4- and IL-5-producing T cells, iNKT cells with high PLZF expression are recruited to the lungs via CCL21-CCR7 signaling to facilitate the development of asthma tolerance.

Keywords: Asthma tolerance · CCL21-CCR7 · Invariant natural killer T cells · PLZF · Respiratory syncytial virus



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

Introduction

Asthma is a major worldwide health problem causing episodes of wheezing, coughing, and breathlessness in susceptible indi-

viduals, affecting approximately 300 million individuals worldwide including people from childhood to old age [1]. Early-life exposure to allergens might promote the establishment of immune tolerance by inducing the anergy or deletion of specific T cells and

Correspondence: Xiao Su and Zhijun Jie
e-mail: xsu@ips.ac.cn; jiezhjxh@163.com

[Correction added on 11 September 2020 after first online publication: second corresponding author Zhijun Jie added]

the generation of T-regulatory cells [2,3]. Oral tolerance (OT) is a state of local and systemic immune unresponsiveness induced by oral administration of an innocuous antigen [4]. Oral treatment with specific antigens to induce tolerance is also an excellent strategy for allergy immunotherapy (AIT) and is widely used clinically to prevent allergic and autoimmune diseases [5]. Our group and other groups have established mouse models of asthma tolerance by breastfeeding or oral feeding with ovalbumin (OVA) (OT mice) [6–8]. However, to date, the mechanisms by which asthma tolerance is established and disrupted have remained elusive. Elucidation of the regulatory mechanisms underlying natural and acquired tolerance is highly important for both asthma prevention and AIT.

Recently, the highly conserved transcription factor (TF) promyelocytic leukemia zinc finger (PLZF, encoded by the *Zbtb16* gene) has emerged as an important regulator of immune processes. PLZF plays a crucial role in cell growth, attenuating the proliferation of mature and reactive T cells, and induces apoptosis [9,10]. PLZF is expressed in innate lymphoid cells (ILCs), CD8⁺ T cells, CD4⁺ T cells [11], specifically high expressed in invariant natural killer T (iNKT) cells as a signature transcriptional regulator, essential for the differentiation, development, and acquisition of function of iNKT cells [12,13].

As a transcriptional repressor, PLZF can extensively limit the expression of inflammatory genes and suppress inflammatory signaling programs [14]. Moreover, in a model of transplantation immune tolerance, transgenic expression of PLZF in T cells confers effector properties resembling those of iNKT cells and renders the cells more susceptible to apoptosis, leading to impaired expansion following alloactivation [15]. Allogeneic transplantation of PLZF-transgenic T cells in vivo can initiate “suicidal” regulation of effector cells, increase Tregs, and reduce the incidence of graft-versus-host response, thus, inducing some degree of graft immune tolerance [15].

Accumulating evidence strongly suggests that PLZF-expressing iNKT cells may play a role in immune tolerance. A lack or reduction of iNKT cells is closely related to increased susceptibility to autoimmune diseases and even lead to the aggravation of these diseases; however, adoptive transfer of iNKT cells or specific overexpression of their T-cell receptors (TCRs) can alleviate the symptoms of autoimmune disease [16–18]. In addition, clinical studies have verified defects in the quantity and function of iNKT cells in patients with various autoimmune diseases [19–21]. The latest studies have suggested that iNKT cells also contribute to tolerance to cow's milk in children [22, 23]. Treatment with alpha-galactosylceramide (α -GalCer) combined with CD1d, a specific marker of iNKT cells, before allergen sensitization can prevent T-helper 2 (Th2) cell responses, promoting iNKT cell-mediated induction of Tregs and inducing peripheral tolerance in asthma [24–26]. Thus, PLZF-expressing iNKT cells might be a novel target for studying asthma tolerance establishment and disruption.

In addition, CCR7 and its ligands (CCL19 and CCL21) are well-known mediators inducing tolerance to inhaled antigens. These

mediators induce T-cell anergy, promote apoptosis, and regulate the homing of various populations of immune cells [27]. CCL21 isoforms, particularly CCL21Ser (encoded by the *Ccl21a* gene, predominantly detectable in the immune system), are necessary and sufficient for the migration of thymocytes and the establishment of self-tolerance in T cells in the absence of CCL19 [28, 29], and CCR7-mediated cellular responses to CCL21 may last longer than those to CCL19 [27]. CCR7 plays an indispensable role in the differentiation of thymic iNKT effector subsets, and CCR7⁺ iNKT cells are the predominant emigrating iNKT cell subset [30].

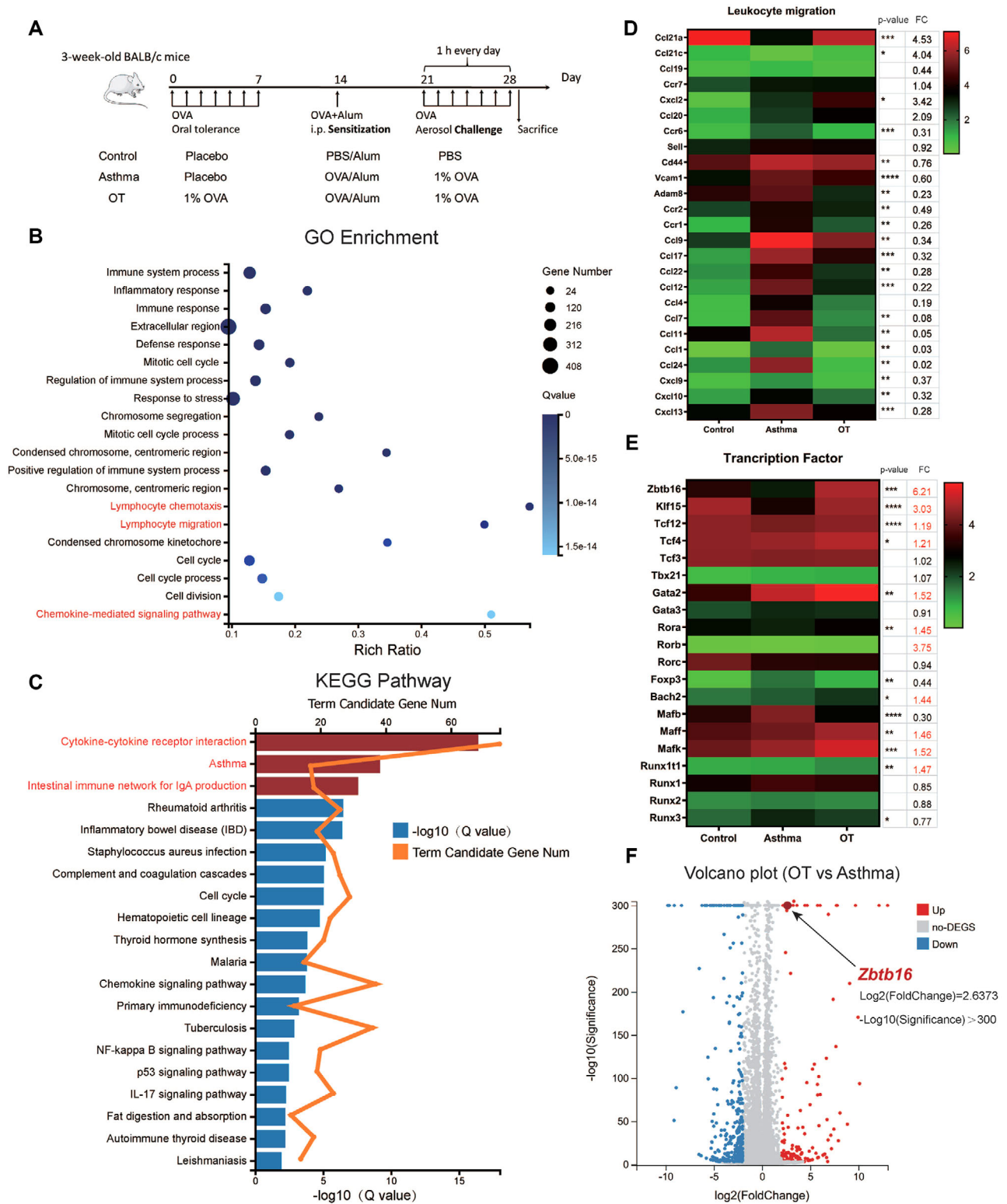
Early-life infection with viruses, particularly respiratory syncytial virus (RSV), is a factor that can disrupt asthma tolerance and aggravate asthma from childhood to adulthood [31]. In addition, early infection with RSV impairs maternally transferred tolerance and increases susceptibility to allergic asthma [7]. Our previous work revealed that RSV infection impairs OT to asthma by recruiting IL-17A-producing cells to the lungs [8]. However, whether the ability of RSV infection to disrupt asthma tolerance is attributed to PLZF-expressing iNKT cells via CCL21-CCR7 signaling needs further investigation.

Here, we hypothesized that PLZF-expressing iNKT cells and CCL21-CCR7 signaling might be involved in the establishment of asthma tolerance. Blockade of CCL21-CCR7 signaling or infection with RSV disrupts asthma tolerance by reducing PLZF expression and iNKT cell recruitment. The findings of this study will deepen our understanding of asthma tolerance establishment and RSV impairing tolerance mechanisms. Manipulation of the TF PLZF and PLZF-expressing iNKT cells might be novel therapeutic approaches for asthma.

Results

Zbtb16 and *Ccl21a* genes are upregulated in asthma-tolerant lungs

To study gene profiles and signaling pathways involved in asthma tolerance, we performed RNA-seq analysis on lung tissue harvested from control, asthma, and OT mouse models at d28 (Fig. 1A). The quality control and statistical analysis of RNA-seq data were based on the sequencing analysis platform of BGI Genomics and described in detailed in the Materials and Methods section. Via gene ontology (GO) enrichment analysis, we found that the most highly enriched genes were related to the terms: lymphocyte chemotaxis, chemokine-mediated signaling pathway, and lymphocyte migration process (Fig. 1B). Through Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, we determined that the significantly differentially expressed genes were involved in the cytokine-cytokine receptor interaction, asthma, and intestinal immune network for IgA production pathways (Fig. 1C). Lymphocyte chemotaxis- and migration-related genes differed between asthmatic and OT lungs. Among these genes, compared to that in the control group *Ccl21a* expression in the lungs was downregulated in the asthma group and



upregulated in the OT group (fold change = 4.53) (Fig. 1D). We also analyzed 3144 TF genes and identified 20 genes that were differentially expressed among control, asthmatic, and OT lungs. *Zbtb16* (encoding PLZF) was significantly upregulated (fold change = 6.21) in OT lungs compared to asthmatic lungs (Fig. 1E). In addition, the volcano plot of the differential gene expression analysis results revealed that *Zbtb16* was one of the significantly upregulated differential gene in OT lungs compared to asthmatic lungs (Fig. 1F). In the volcano plot, we set a threshold 300 in y-axis to better exhibit those genes with extremely high values of $-\log_{10}(\text{Significant})$. These findings suggested that PLZF, CCL21, and chemotaxis might contribute to asthma tolerance.

PLZF and CCL21-CCR7 signaling are upregulated in asthma-tolerant lungs

To examine whether PLZF and CCL21-CCR7 signaling are upregulated in asthma-tolerant lungs, we performed RT-qPCR assays and first confirmed that the *Zbtb16*, *Ccr7*, and *Ccl21a* expression levels were increased in asthma-tolerant lungs compared to asthmatic lungs (Fig. 2A). By flow cytometric analysis, we found that the number of PLZF⁺ cells was reduced in asthmatic lungs compared to control lungs; however, the number of lung PLZF⁺ cells was increased in OT compared to asthma (Fig. 2B and C). Similarly, the number of lung CCR7⁺ cells in OT was also increased compared with that in asthma (Fig. 2D and E). Furthermore, approximately a quarter of lung CCR7⁺ cells expressed PLZF, and the percentage of CCR7⁺PLZF⁺ cells was significantly increased in OT lungs compared to both asthmatic and control lungs (Fig. 2F and G). These findings indicate that PLZF⁺ cell counts and CCL21-CCR7 signaling are increased and might be involved in the development of OVA feeding-induced asthma tolerance.

PLZF is increased in Lin[−] and Lin⁺ cells and physically interacts with GATA3 in asthma tolerance

To identify which PLZF-expressing cell population is increased and associated with asthma tolerance, we analyzed the percentage of PLZF⁺ cells among both Lin[−] and Lin⁺ cell populations in control, asthmatic, and asthma-tolerant lungs. Most PLZF-expressing cells lacked lineage markers, a characteristic of progenitor cells,

haemopoietic stem cells, and innate immune cells. Lin[−]PLZF⁺ cells in lung were significantly reduced in asthma compared to control but increased in the asthma tolerance (Fig. 3A and B). More importantly, Lin⁺PLZF⁺ cells were also increased in OT lungs compared to asthmatic lungs and were particularly increased compared to control lungs (Fig. 3C). This population, although small, might be specifically induced by OT and involved in tolerance formation.

Considering that GATA3 is a key TF in Th2 cells and ILC2s in asthma, regulating the secretion of type 2 cytokines, we sought to examine whether PLZF is coexpressed with GATA3 during the development of asthma tolerance. Via flow cytometric analysis, we found that the number of lung Lin[−]PLZF⁺GATA3⁺ cells was significantly reduced in asthma compared to control but increased in OT compared with asthma (Fig.). Moreover, the number of Lin⁺PLZF⁺GATA3⁺ cells was increased in OT lungs compared to both asthma and control lungs (Fig. 3D and F). Although PLZF expression was upregulated in OT lungs (Fig. 2C), GATA3 expression was downregulated on the contrary (Fig. 3G). To further explore whether GATA3 and PLZF physically interact, we transfected 293T cells with plasmids encoding Flag-tagged PLZF and HA-tagged GATA3 or with empty vector. Cells were collected, and total protein was extracted for coimmunoprecipitation. In the extracts, the anti-Flag antibody-precipitated product bound with the anti-GATA3 antibody-precipitated product; vice versa, the anti-HA antibody-precipitated product bound with the anti-PLZF antibody-precipitated product (Fig. 3H). These findings suggest that PLZF is coexpressed with GATA3 and physically interacts with GATA3, which controls the function of GATA3 during OT possibly by reducing its transcription.

PLZF expression in Th2 cells and ILC2s is reduced in asthmatic lungs but increased in OT lungs

GATA3 is a signature transcriptional regulator in Th2 cells and ILC2s and controls the transcription of Th2 cytokines [32, 33]. We examined PLZF expression in the populations of Th2 cells and ILC2s among the isolated lung cells from control, asthmatic, and asthma-tolerant mice by flow cytometry. The percentages of PLZF⁺ Th2 cells and PLZF⁺ ILC2s were reduced in asthmatic lungs compared to control lungs but were increased in asthma-tolerant lungs compared to asthmatic lungs (Fig. 4A–D). These findings suggest

Figure 1. *Zbtb16* and *Ccl21a* genes are upregulated in the asthma-tolerant lungs. (A) Experimental procedures for establishing asthmatic and oral tolerized mouse models. (B) GO pathway enrichment and analysis of differential genes. The x-axis is the enrichment ratio [Rich Ratio = Term Candidate Gene Num/Term Gene Num]. The y-axis is the size of the GO Pathway, bubble indicating the number of genes commented on GO Pathway, the color represents the enriched Q value, and the darker the color represents the smaller the Q value. (C) KEGG pathway enrichment and analysis of differential genes. The length of the lower x-axis column represents the size of Q value ($-\log_{10}(\text{Q value})$), line on the upper x is the number of differentially expressed genes on the KEGG Pathway. (D–E) Heatmap showing expression value ($\log_2(\text{FPKM}+1)$) of key transcription factor genes and chemokines and surface molecules genes related to lymphocyte migration in the whole lung. The transverse axis represents the mean value of $\log_2(\text{FPKM}+1)$ of the sample in each group, and the longitudinal axis represents the gene name. The color of patch redder represents the higher level of expression, and bluer represents the lower expression. p-values were compared between the asthma and OT groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (unpaired two-tailed Student's t test). FC represents gene expression fold change of OT/Asthma. (F) Volcano plot of up- or downregulated gene expression between asthma and OT. Cut off $\log_2(\text{fold change (FC)})$ value = 2. x-axis represents the difference value after the $\log_2$ conversion, and the y-axis represents the significance value after the $\log_{10}$ conversion. Red dots represent the upregulation of differentially expressed genes (DEGs), blue dots represent the downgrade of DEG, gray dots represent non-DEG. Results of RNA-sequence represented one experiment including 11 samples totally (n = 3–4 in each group).

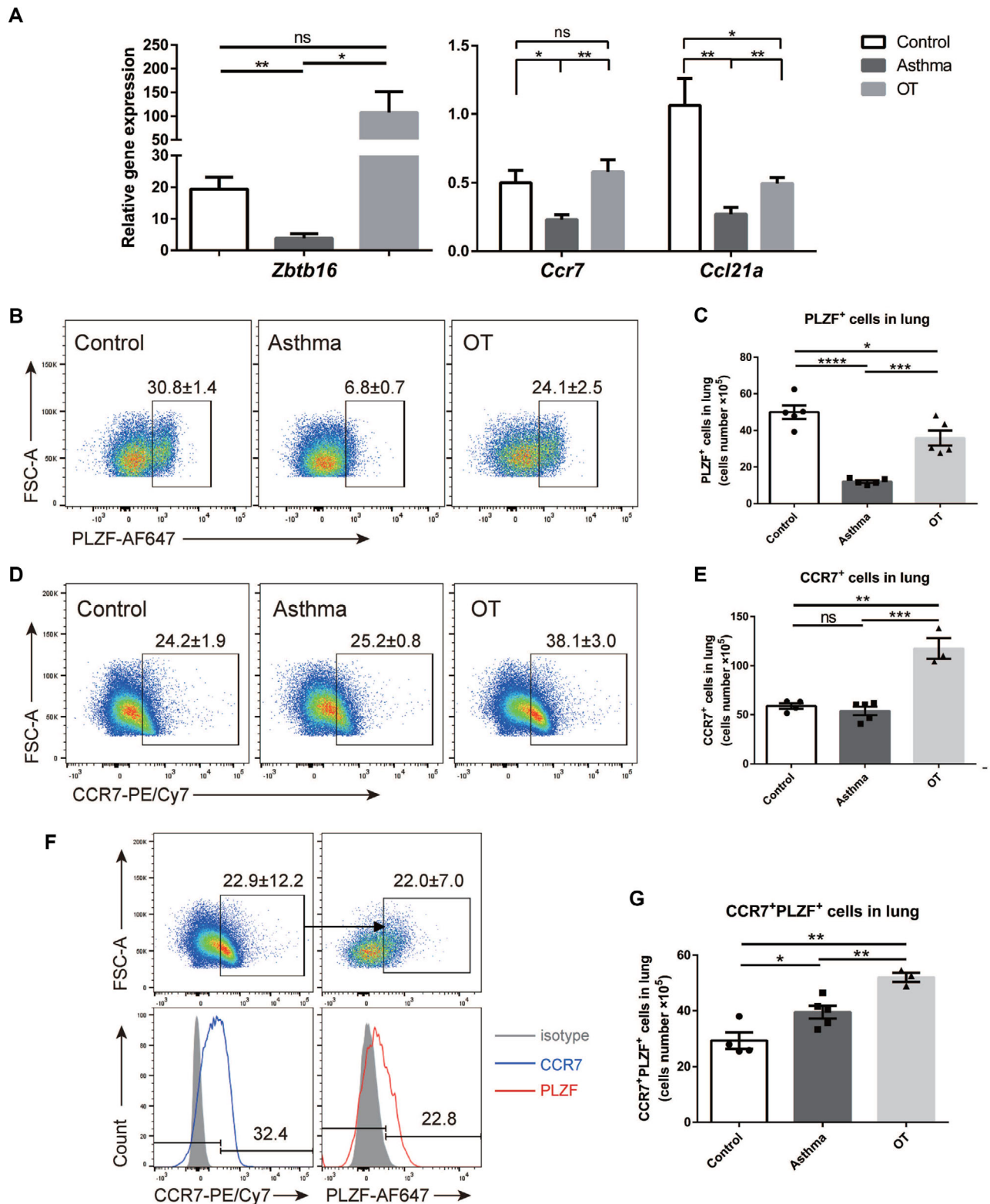


Figure 2. PLZF and CCL21-CCR7 signaling are upregulated in the asthma-tolerant lungs. The mice were subjected to treatment as described in Fig. 1 and subsequently analyzed the parameters in control, asthma and OT mice. (A) Gene expression of *Zbtb16* and *Ccr7*, *Ccl21a* in the whole lung as determined by RT-qPCR analysis. (B–G) Flow cytometry analysis of cell population in the lung. The numbers represent gating percentages of total live cells (FVS⁻) in each group. B–C, PLZF⁺ cells; D–E, CCR7⁺ cells; F–G, PLZF⁺CCR7⁺ cells, histograms in F show the CCR7 (blue) and PLZF (red) expression separately in sample and isotype control (gray). Data are means ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns, not significant (unpaired two-tailed Student's *t* test). Results are one representative experiment of at least two independent experiments (*n* = 3–5 in each group).

that PLZF might interact with GATA3 to suppress its function in Th2 cell and ILC2 populations [34], thereby mediating asthma tolerance.

***Zbtb16* knockdown promotes CD3e⁺CD4⁺ lymphocytes differentiation and cytokines secretion**

The above results confirmed that PLZF can physically interact with GATA3 and that these two TFs showed inverse expression changes in lung Th2 cells and ILC2s, indicating the possible inhibitory role of PLZF. However, whether PLZF regulates the function of lymphocytes is less certain. Here, we isolated splenic lymphocytes from WT mice and cultured them *in vitro* (Fig. 5A), knocked down *Zbtb16* in these lymphocytes by transfection of *Zbtb16*-siRNA, and examined the transfection and gene knockdown efficiency 24 h later by immunofluorescence and RT-qPCR (Fig. 5B). Then, to simulate asthma or RSV infection in the pulmonary environment *in vivo*, we used the supernatant of OVA-treated (36 h) BEAS-2B cells, RSV-treated (24 h) MLE cells, untreated BEAS-2B or untreated MLE cells to stimulate *Zbtb16*-knockdown lymphocytes for 12 h. We then used flow cytometry to analyze the total count of live cells; counts of CCR7 and CD3e⁺CD4⁺ T cells; and levels of the type 2 cytokines IL-4 and IL-5 (Fig. 5C–J). *Zbtb16*-siRNA transfection in lymphocytes knocked down *Zbtb16* expression by approximately 20–50% (Fig. 5B); the knockdown efficiency from low to high followed the order of *Zbtb16*-siRNA1, *Zbtb16*-siRNA2, and *Zbtb16*-siRNA3. The results showed that after *Zbtb16* knockdown, CD3e⁺CD4⁺ T cells were significantly increased, and CD3e[−]CD4[−] T cells were sharply decreased (Fig. 5C). After OVA or RSV treatment, CD3e⁺CD4⁺ T cells were markedly increased in the negative control (NC) groups, and *Zbtb16* knockdown led to a further increase of CD3e⁺CD4⁺ T cells. However, as the efficiency of *Zbtb16* knockdown increased, the increase in CD3e⁺CD4⁺ T cells was slightly reduced but still nearly exceeded that in the NC groups (Fig. 5D). This result indicated that *Zbtb16* can suppress CD3e⁺CD4⁺ T lymphocyte differentiation. Given that PLZF is a key TF for cell growth, excessive deficiency of PLZF might compromise cell survival and differentiation. We also analyzed the secretion of IL-4 and IL-5 from CD3e⁺CD4⁺ T cells and found that both cytokines were increased after *Zbtb16* knockdown in the vehicle control, OVA-stimulated, and RSV-stimulated groups (Fig. 5E–H). However, the increased reduction in *Zbtb16* expression was followed by reductions in the cytokine levels. These findings suggest that cell viability is decreased in the setting of excessive PLZF deficiency. Finally, we assessed CCR7 expression in lymphocytes. In the vehicle control group, CCR7⁺ cells were increased after *Zbtb16* knockdown, owing to the increased differentiation of T cells (Fig. 5J). However, after treatment with OVA or RSV, CCR7 expression was substantially decreased (compared with the untreated group), and *Zbtb16* deficiency further significantly reduced CCR7 expression (Fig. 5I and J). Thus, these findings indicate that *Zbtb16* expression in lymphocytes can suppress CD3e⁺CD4⁺ T-cell differentiation and IL-4/IL-5 secretion but that during OVA or RSV challenge, *Zbtb16* could maintain CCR7 expression in lymphocytes.

PLZF and CCR7 are coexpressed in asthma-tolerant lungs and mainly distributed in the airway submucosa

To examine the amount and distribution of PLZF⁺ cells in the lung airway and the relation of PLZF with CCR7, we labelled the TF PLZF and the membrane protein CCR7 in lung tissue of OT mice by immunofluorescence and screened the cells by confocal microscopy (Fig. 6). The image in Fig. 6A shows numerous PLZF-expressing cells in the lungs. Most of these cells were distributed in the airway submucosa, and few were distributed in the airway epithelium, indicating that PLZF-expressing cells are immune cells or lung parenchymal cells. Moreover, PLZF and CCR7 were coexpressed in many lung cells, and a small amount of PLZF protein was expressed in the cytoplasm (Fig. 6B and C).

PLZF-expressing CCR7⁺ iNKT cells are increased in the lungs, HLNs, and MLNs in asthma tolerance

CCR7-expressing iNKT cells have been reported to be the predominant emigrating cell subset that contributes to the development of tolerance [30]. To identify the CCR7⁺PLZF⁺ cell population that functions in the development of asthma tolerance, we performed flow cytometric analysis of PLZF⁺ iNKT and CCR7⁺PLZF⁺ iNKT cells from control, asthmatic, and asthma-tolerant lungs. We defined CD3e⁺TCRβ⁺α-GalCer/CD1d tetramer⁺ cells as iNKT cells in the lung cell population, there are quite a number of iNKT cells in PLZF⁺ cells (Fig. 7A) and about 80% of iNKT cells expressed PLZF in OT lungs (Fig. 7C). Most iNKT cells in the lungs and LNs expressed CCR7, and the number of PLZF⁺ iNKT and CCR7⁺PLZF⁺ iNKT cells was increased in asthma-tolerant lungs compared with asthmatic lungs (Fig. 7B).

We also analyzed the changes in CD3e⁺TCRβ⁺α-GalCer/CD1d tetramer⁺ iNKT (Fig. 7D) and CCR7⁺ iNKT cells in HLNs and mesenteric lymph node (MLNs) from control, asthma, and asthma-tolerant lungs. The percentages of total iNKT and CCR7⁺ iNKT cells were increased in asthma-tolerant HLNs and MLNs compared to their asthmatic counterparts (Fig. 7E and F). These findings indicate that HLNs and MLNs might be involved in the trafficking of CCR7⁺ iNKT cells and recruitment of iNKT cells to the lung during the establishment of asthma tolerance.

CCL21-CCR7 signaling blockade reduces PLZF⁺ and CCR7⁺ iNKT cells in the lungs and LNs in OT

To further examine whether CCL21-CCR7 signaling regulates the PLZF⁺, CCR7⁺PLZF⁺, and CCR7⁺ iNKT cell populations in asthma-tolerant lungs, we applied an anti-CCL21 neutralizing antibody to block CCL21-CCR7 signaling in asthma-tolerant lungs (Fig. 8A). By flow cytometric analysis, we found that the percentage of CCR7⁺ cells was reduced in anti-CCL21 antibody-treated OT lungs compared to isotype antibody-treated OT lungs (Fig. 8B). After blockade of CCL21, the percentages and numbers of PLZF⁺ and CCR7⁺PLZF⁺ cells were markedly reduced in

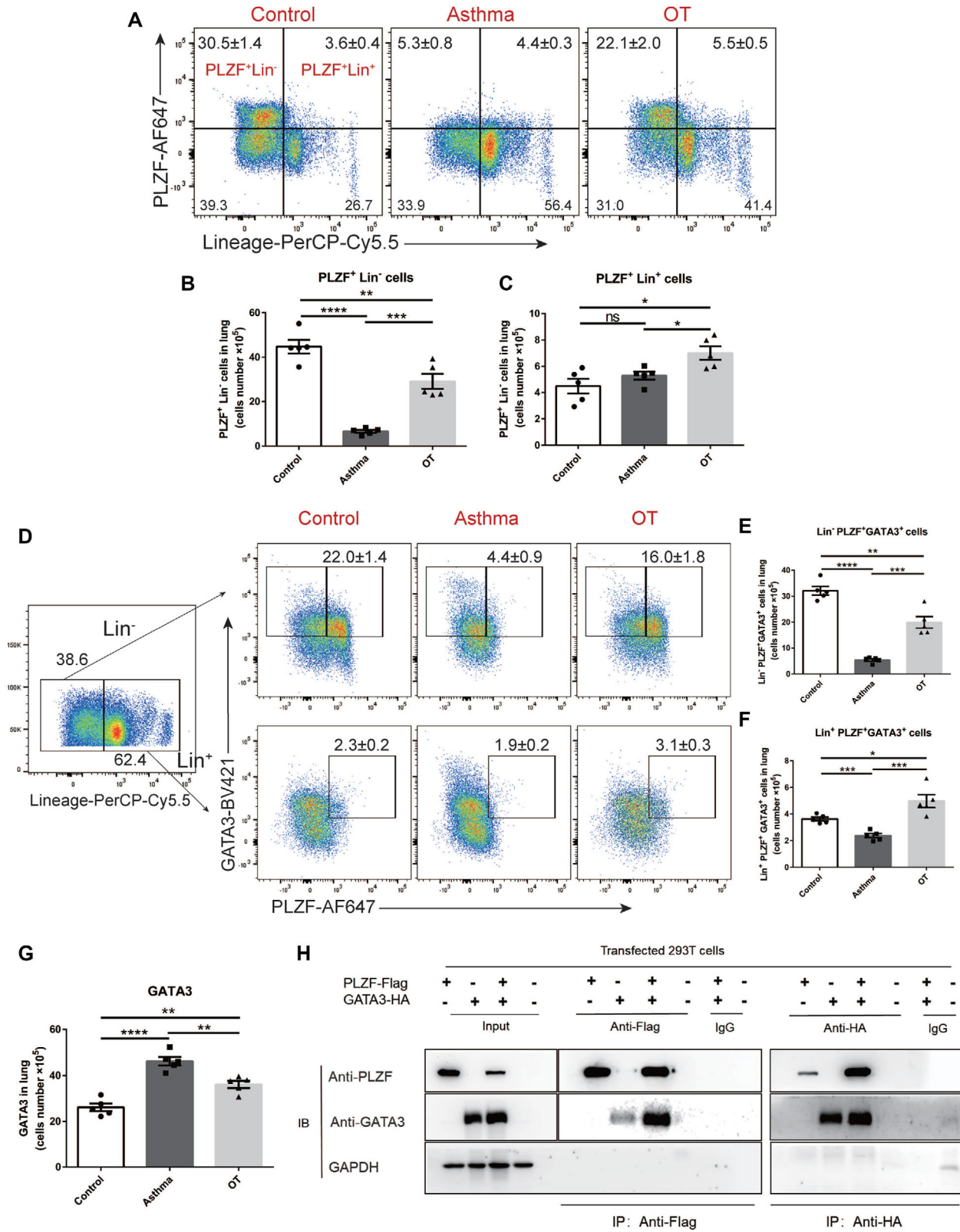


Figure 3. PLZF is increased in Lin⁺ and Lin⁺ cells and physically interacts with GATA3 in asthma tolerance. (A–C) Flow cytometry analysis for comparison of Lineage and PLZF expression in the lung of asthma and OT model (Lineage antibodies are including CD3e, CD11b, CD45R/B220, Ly-76, Ly-6G, and Ly-6C). (D–G) Flow cytometry analysis for comparison of GATA3 and PLZF expression in Lin⁺ (D–E) and Lin⁺ (D–F) subset separately among the three groups. The numbers represent gating percentages of parent gates in each group. (H) Coimmunoprecipitation of PLZF and GATA3. Nonimmunoprecipitated material (10% input) was analyzed as a control for appropriate expression of proteins programmed by transfected plasmids. Data are means $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, ns, not significant (unpaired two-tailed Student's *t* test). Results are from one representative experiment of at least two independent experiments (n = 5 in each group).

anti-CCL21 antibody-treated OT lungs compared to isotype antibody-treated OT lungs (Fig. 8C–E). More importantly, the percentages and numbers of total iNKT and CCR7⁺ iNKT cells were also significantly reduced in CCL21 antibody-treated OT lungs compared with isotype antibody-treated OT lungs (Fig. 8F–H). Similarly, we further labelled iNKT and CCR7⁺ iNKT cells in HLNs and MLNs and found that these cells were significantly

decreased in the anti-CCL21 antibody-treated LNs compared to the isotype antibody-treated LNs in the OT mice (Fig. 8I–N). These blocking experiments confirmed that the recruitment of PLZF⁺ iNKT cells to the lungs is dependent on CCL21–CCR7 signaling in the setting of OT and that these iNKT cells might partially be trafficked from the intestinal immune system through MLNs and HLNs.

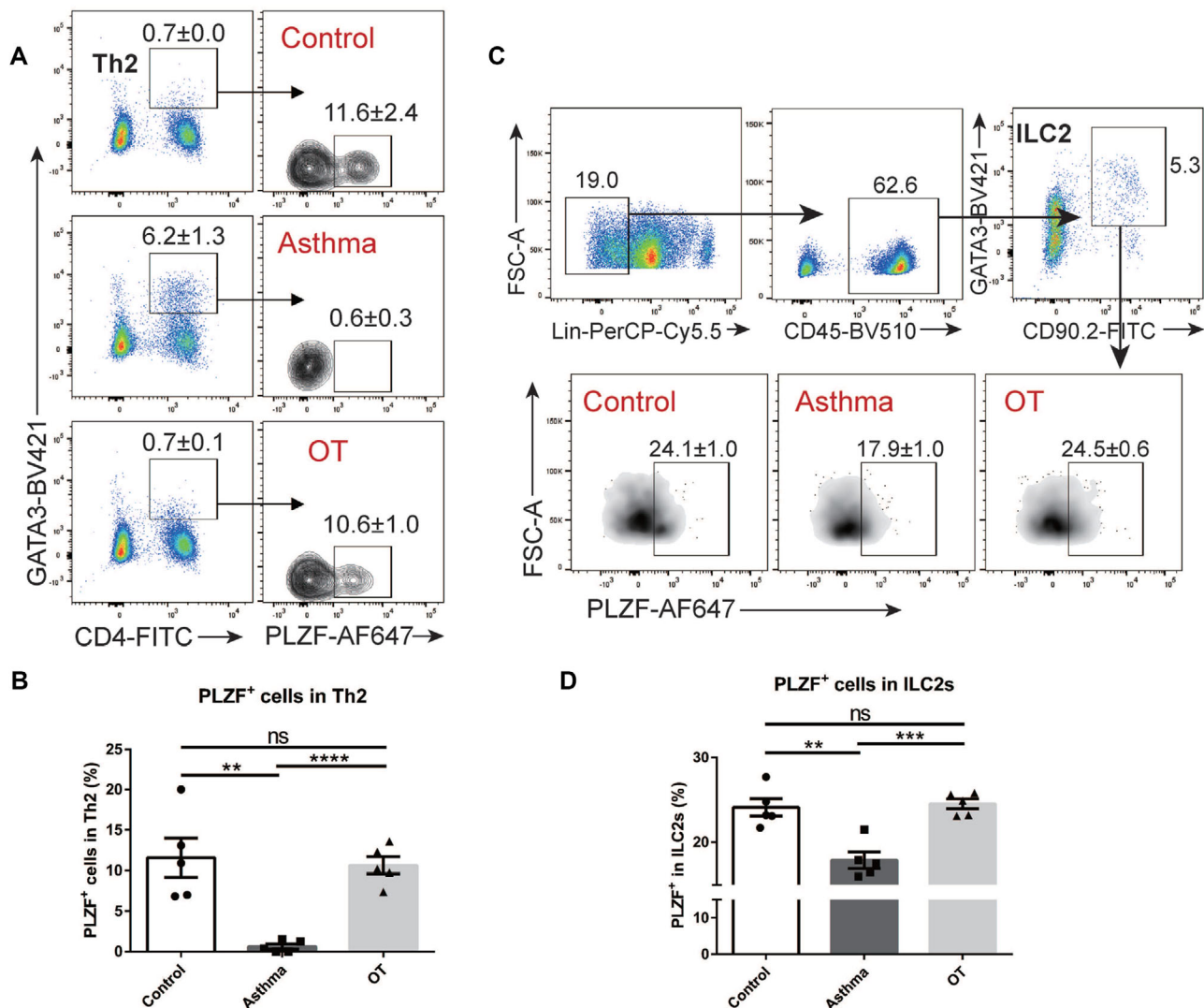


Figure 4. PLZF in Th2 cells and ILC2s is reduced in asthmatic lungs but increased in OT lungs. (A–D) PLZF expression frequencies (%) of Th2 and ILC2s were separately shown in lung by flow cytometry analysis. A–B, Th2 are gating as CD4⁺ GATA3⁺. C–D, ILC2s are defined as Lineage⁺ CD45⁺ CD90.2⁺ GATA3⁺. The numbers represent gating percentages of parent gates in each group. Data are means $\pm$ SEM. ** p < 0.01, *** p < 0.001, **** p < 0.0001, ns, not significant (unpaired two-tailed student's *t* test). Results are from one representative experiment of at least two independent experiments (n = 5 in each group).

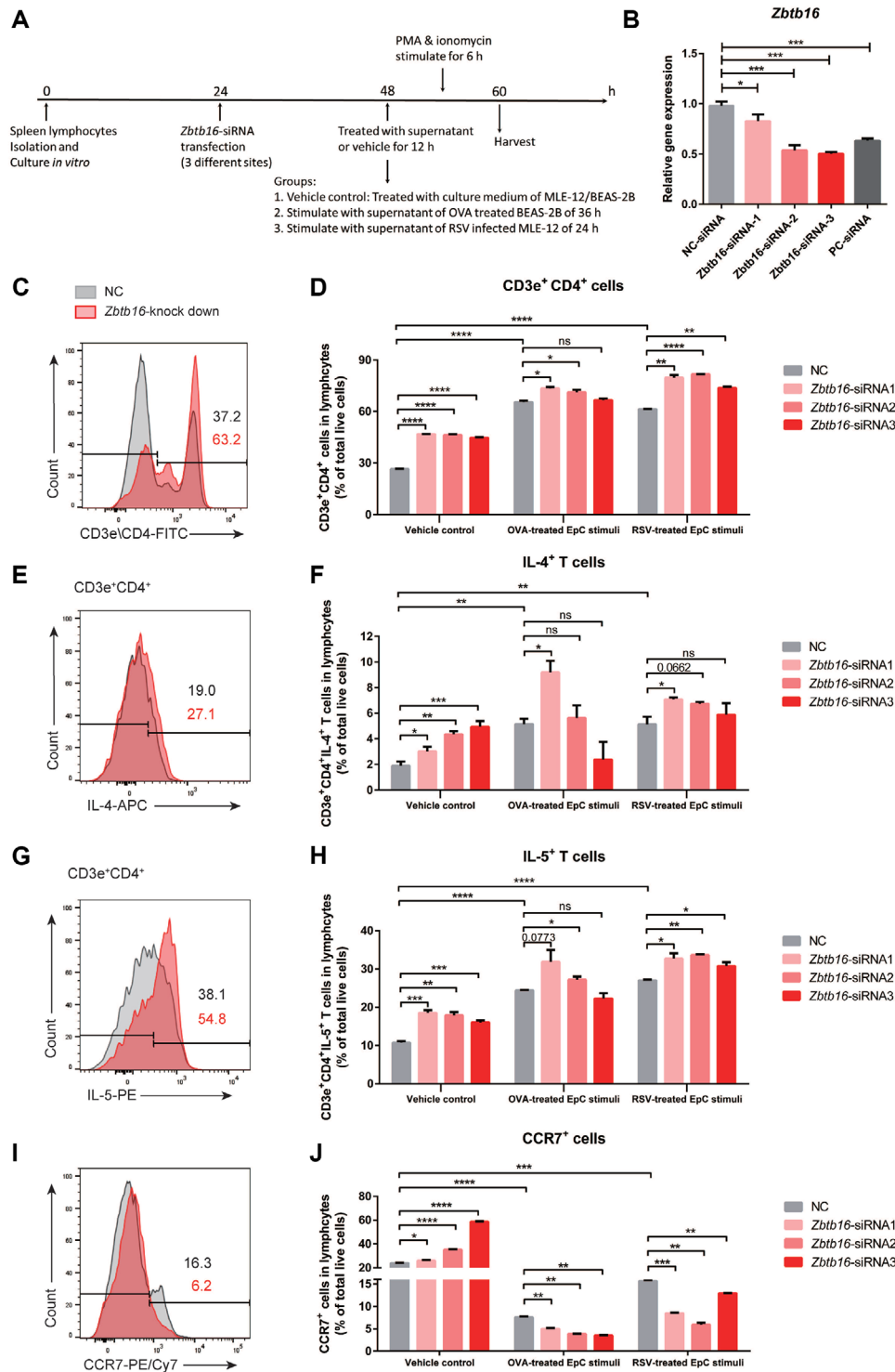


Figure 5. Zbtb16 knockdown promotes CD3e⁺CD4⁺ lymphocytes differentiation and cytokines secretion. (A) Zbtb16 knockdown procedures in mice spleen lymphocyte *in vitro*. (B) Gene expression of Zbtb16 measured by RT-qPCR in mice spleen lymphocytes after transfection of Zbtb16-siRNA. (C–J) Flow cytometry analysis of CD3e⁺CD4⁺ T cells, CD3e⁺CD4⁺IL-4⁺, CD3e⁺CD4⁺IL-5⁺ T cells, and CCR7⁺ cells in total live lymphocytes among Vehicle Control, OVA- or RSV-treated lung epithelial cells supernatant stimuli groups. Histograms show comparison between the Negative Control (NC, grey) and Zbtb16-knockdown (red) group, the numbers represent the positive gating percentages of total live cells (FVS⁺). Statistical analysis graphs show frequencies (%) of total live lymphocytes (FVS⁺) in flow cytometry. Data are means ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns, not significant (unpaired two-tailed student's *t* test). Results are from one representative experiment of at least two independent experiments (*n* = 2–4 in each group).

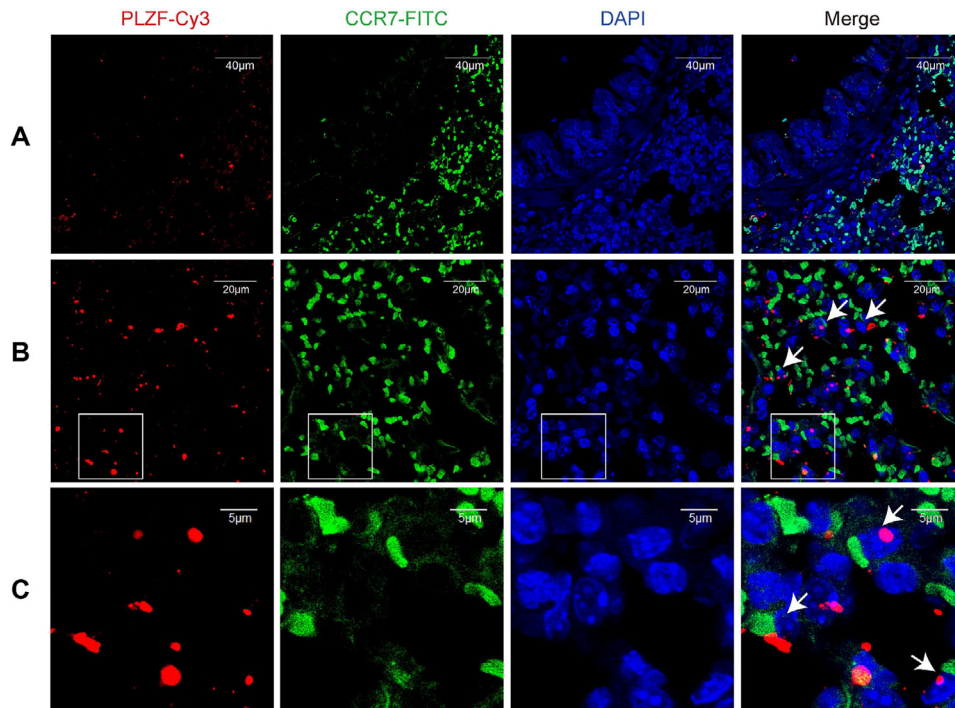


Figure 6. PLZF and CCR7 are coexpressed in OT lungs and mainly distributed in the airway submucosa. (A–C) Representative confocal images of lung sections labeled with PLZF-Cy3 (red), CCR7-FITC (green) and DAPI (blue) from oral tolerance mice in objective magnification $\times 60$. A shows the airway and the adjacent submucosa. B shows submucosal lung tissue. C shows the magnified section in B. The white arrows indicate PLZF and CCR7 coexpressing cells in lung tissue. Scale bars, 5 μm . Results are representative sections from the lungs of 4–5 OT mice in one experiment of at least two independent experiments.

CCL21-CCR7 signaling blockade exacerbates airway lesions and increases type 2 responses to impair OT

After anti-CCL21 antibody blockade in asthma-tolerant groups, the pathological lesions in anti-CCL21 antibody-treated OT lungs were exacerbated compared with those in isotype antibody-treated OT lungs, as manifested by bronchial mucosal hyperplasia, goblet cell proliferation, inflammatory cell recruitment, and the presence of mucoid exudate in the airways (Fig. 9A).

Although the plasma IgE level did not differ between the anti-CCL21 antibody-treated OT and isotype antibody-treated OT groups (Fig. 9B), but flow cytometric analysis showed that the numbers of Th2 cells, ILC2s and IL-4⁺ or IL-5⁺ ILC2s in anti-CCL21 antibody-treated OT lungs were increased compared to those in isotype antibody-treated OT lungs (Fig. 9C–I). By qPCR analysis of gene expression in lung samples, we confirmed that *Ccr7*, *Il33*, *Il4*, and *Il13* expression levels were reduced in anti-CCL21 antibody-treated OT lungs compared to isotype antibody-treated OT lungs (Fig. 9J). These findings indicate that CCL21-CCR7 signaling is required for the development of asthma tolerance.

RSV infection compromises asthma tolerance by inhibiting recruitment of iNKT cells to the lung

We infected one group of tolerized mice with RSV (strain A2, 10⁶ PFU/mouse) via three intranasal doses before sensitization and

challenge, at the same time another group of OT mice received three equal doses of UV-irradiated RSV (UV-RSV) (Fig. 10A). On day 28, the airway resistance was measured as an indicator of airway hyperresponsiveness, it was significantly increased in RSV-infected OT mice compared to UV-RSV-challenged OT mice (Fig. 10B), indicating that RSV infection can disrupt asthma tolerance, as shown previously [8]. The flow cytometric analysis in lung showed that the numbers of PLZF⁺, CCR7⁺, PLZF⁺ iNKT, and CCR7⁺ PLZF⁺ iNKT cells were also reduced in RSV-infected OT mice compared to UV-RSV-challenged OT mice (Fig. 10C–G). This result implies that RSV may disrupt OT via the same mechanism as OVA by suppressing PLZF- and CCL21-CCR7-dependent iNKT recruitment. The flow cytometry analysis of HLN and MLN also showed iNKT and CCR7⁺ iNKT cells were reduced in RSV-infected OT mice compared to UV-RSV-challenged OT mice (Fig. 10I–K). These results indicate that RSV infection might disrupt asthma tolerance by impairing CCL21-CCR7-mediated trafficking of iNKT cells through HLN and MLN, thus, reducing iNKT cell recruitment to the lungs.

Discussion

Defect in natural tolerance is a leading cause of allergic diseases. Severe RSV infection in childhood is associated with an increased prevalence and persistence of allergic asthma [35]. A better understanding of the mechanisms by which tolerance is induced and

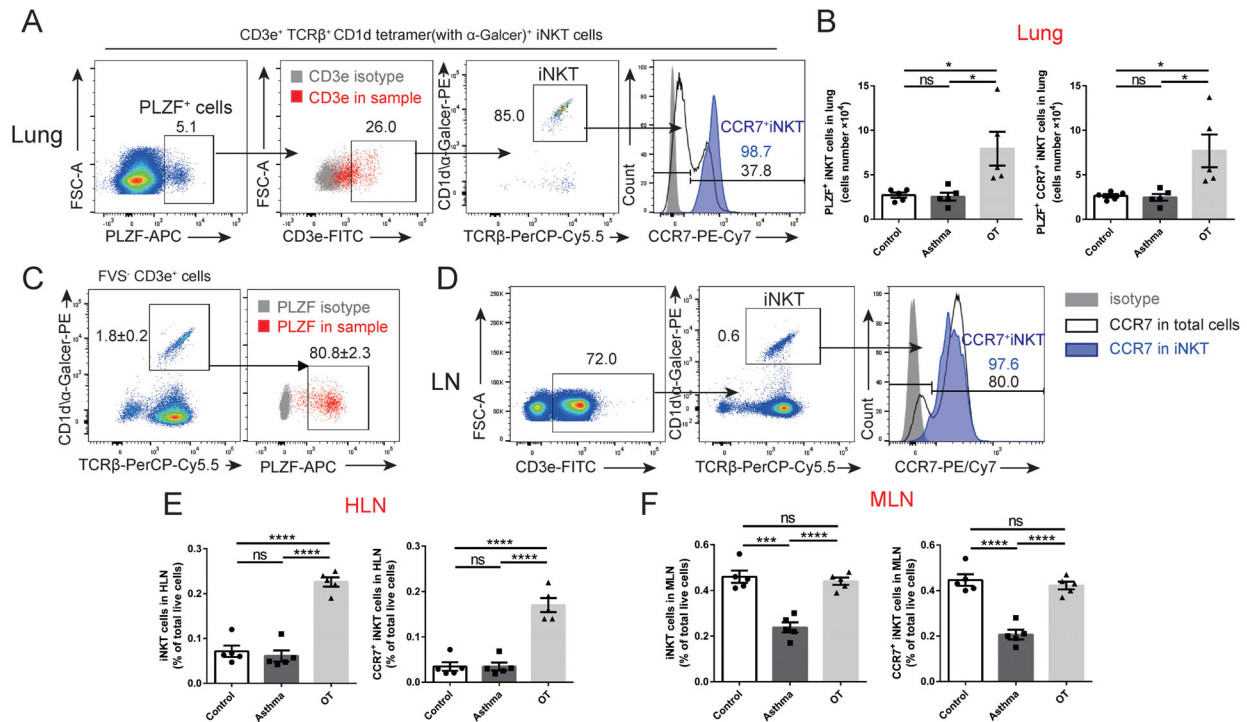


Figure 7. PLZF-expressing CCR7⁺ iNKT cells are increased in the lungs, HLNs and MLNs in asthma tolerance. (A) Flow cytometry gating strategy of PLZF⁺ CD3e⁺ TCRβ⁺ α-Galcer/CD1d tetramer⁺ CCR7⁺ iNKT populations in the lungs. The numbers represent gating percentage of parent gates in each graph. (B) Numbers of PLZF⁺ iNKT and CCR7⁺ PLZF⁺ iNKT cells in the lung by flow cytometry analysis. (C) PLZF⁺ frequencies of total iNKT cells in the OT lungs. (D) Gating strategy of CD3e⁺ TCRβ⁺ α-Galcer/CD1d tetramer⁺ iNKT cells in the LNs. The numbers represent gating percentages of parent gates in each group. (E–F) Frequencies of iNKT and CCR7⁺ iNKT cells in the HLN and MLN separately by flow cytometry analysis. Data are means ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns, not significant (unpaired two-tailed student's *t* test). Results are from one representative experiment of at least two independent experiments (*n* = 5 in each group). HLN, hilar lymph nodes; MLN, mediastinal lymph nodes.

subverted could guide the development of new therapeutic targets for asthma. In this study, we provided the first evidence that PLZF is a transcriptional regulator of asthma tolerance. Interestingly, both PLZF and CCL21-CCR7 signaling contribute to the development of asthma tolerance (Fig. 1,2,7–9). In particular, CCR7⁺ PLZF⁺ and CCR7⁺ PLZF⁺ iNKT cells in the lungs and draining lymph nodes are essential for the development of asthma tolerance, because CCL21-CCR7 blockade reduces CCR7⁺ PLZF⁺ iNKT cells in the lungs, HLNs, and MLNs (Fig. 8) and recapitulates the asthma-like phenotype in OT mice (Fig. 9).

PLZF is expressed by a common precursor of ILCs [36]. Since PLZF plays a vital role in the early development of ILCs, PLZF-deficient mice have an ILC2 defect and a decreased type 2 response in asthma [37]. In our study, PLZF expression in OT lungs was greatly induced at the mRNA level by oral feeding of OVA and at the protein level by CCL21 signaling-driven recruitment of CCR7⁺ PLZF⁺ immune cells. Regarding the role of PLZF during the development of OT, we speculate that PLZF is a negative regulator of GATA3 that controls the transcription of genes encoding Th2 cytokines (*IL4*, *IL5*, and *IL13*). Therefore, another important finding of this study is that PLZF physically binds to GATA3 (Fig. 3H). PLZF and GATA3 are coexpressed in both the Lin[−] and Lin⁺ cell populations (Fig. 3D–F), strongly suggesting that PLZF is a limiting factor for GATA3 transcription. The results of siRNA-mediated *Zbtb16* knockdown in splenic lymphocytes in vitro support this

notion, since *Zbtb16* deficiency to a certain extent promoted the differentiation of CD3e⁺ CD4⁺ T cells, particularly those producing IL-4 and IL-5 (Fig. 5). However, excessive knockdown might limit the survival and development of lymphocytes, indicating that complete deletion of *Zbtb16* in vivo might impair the growth and self-renewal of immune cells. However, the *Zbtb16*-transgenic mouse model might be more suitable for evaluating the effect of PLZF on asthma tolerance in vivo for further research.

PLZF is a signature TF in iNKT cells that directs their innate-like effector differentiation [38]. Thus far, we have not determined the exact mechanism by which oral feeding of OVA can increase iNKT cells in asthma-tolerant lungs. Microbial exposure at mucosal surfaces, particularly during early postnatal development, has been reported to regulate NKT cell trafficking and function in the intestine and lungs and to determine susceptibility to NKT cell-mediated inflammatory disorders [39]. Viral infections in early life may also alter the iNKT cell compartment; indeed, neonatal infection with influenza virus led to iNKT cell-dependent protection against adult airway hyperreactivity in a mouse model of asthma [39,40]. However, unlike influenza infection, RSV infection early in life may promote later development of asthma in humans [41]. Our data demonstrated that PLZF-expressing iNKT cells are increased in HLNs and MLNs in asthma tolerance (Fig. 7 and 8); this increase is regulated by CCL21-CCR7 signaling, indicating that iNKT cells might be driven by a high

concentration of CCL21 in tolerant lungs to traffic through LNs to the lungs. Blockade of CCL21 reduced PLZF⁺ iNKT cells in tolerant lungs and HLNs (Fig. 8), suggesting that recruitment of CCR7⁺PLZF⁺ iNKT cells might increase this cell population in asthma-tolerant lungs. However, RSV infection subverted this tolerance by decreasing PLZF and CCL21-CCR7 signaling and, thus, reduced iNKT cell recruitment to the lungs (Fig. 10). These findings may help to explain the RSV-induced recurrent asthma symptoms of patients from a novel perspective. iNKT cell-specific deletion of *Zbtb16* is thus warranted to elucidate the role of PLZF⁺ iNKT cells in the establishment of asthma tolerance.

Recently, a few studies have suggested the potential role of iNKT cells in suppressing asthma inflammation. For instance, iNKT cells could mediate induction of Tregs, this evidence was supported by findings that intraperitoneal administration of α -GalCer before allergen sensitization could promote the expansion and suppressive activity of lung CD4⁺FoxP3⁺ Treg cells [24–26]. These effects could suppress regulated Th2-cell responses and decrease maturation of lung DCs [24]. However, the relationship between CCL21-CCR7 and iNKT recruited in tolerant lung and if RSV infection could impair tolerance by inhibiting iNKT recruitment are not well-defined yet. Our studies not only answer the questions above, firstly elucidate the role of iNKT cells recruitment via CCL21-CCR7 signaling in asthma tolerance, but through analyzing our RNA-seq database, we also found that some glycolipid (for example, α -GalCer) synthesis related genes were upregulated in the tolerant lungs (data not shown). Considering that mucosal immune system is an integrated global organ and there is a gut-lung cross talk in mediating mucosal inflammatory diseases [42], feeding OVA might change gut and lung microbiome and their metabolites which might be essential for expansion of iNKT cells and induction of OT [3,43]. Therefore, the relationship among oral feeding of OVA, lung and gut microbiome and metabolism, and development of iNKT is worthy of investigation in the future.

In conclusion, we revealed that PLZF and CCL21-CCR7 signaling are increased in immune cells, especially iNKT cells, during the establishment of oral OVA feeding-induced asthma tolerance. RSV infection (via suppression of CCR7 signaling) and CCL21 blockade reduced PLZF⁺ cells and PLZF⁺ iNKT cells in tolerant lungs, impeded the establishment of asthma tolerance, and recapitulated an asthma-like phenotype. Our findings are the first to suggest the role of PLZF in asthma tolerance and its relation with the CCL21-CCR7 axis a relationship that provides novel insights into the mechanisms of asthma tolerance and the impact of RSV infection on the development of asthma tolerance. Moreover, our findings will be useful for developing new treatments for asthma patients in the setting of RSV infection.

Materials and methods

Animals

Male BALB/c mice (3–4 wk old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China).

Under specific-pathogen-free conditions, the mice were housed in groups with 12 h dark/light cycles and with free access to food and water. Anesthesia was induced with an intraperitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg) before mice sacrificed. All the animal studies were approved by the Committees on Animal Research of the Institute Pasteur of Shanghai, Chinese Academy of Sciences (Animal Ethics Review Number: A2018042).

Experimental procedures for establishing the asthmatic and tolerized mouse models

As described previously [8], BALB/c mice (3–4 wk old) were divided into three groups (Fig. 2A): (i) Control: mice were fed placebo for 1 week, rested for 1 week, intraperitoneally administered one dose of alum (50 μ L of alum gel in 70 μ L of PBS) during a 1-week period, and aerosolized with PBS (1 h/d) for 1 week; (ii) Asthma: mice were fed placebo for 1 week, rested for 1 week, intraperitoneally administered one dose of OVA/alum (50 μ g of OVA and 50 μ L of alum gel in 70 μ L of PBS) during a 1-week period, and aerosolized with OVA (10 mg/mL in PBS, 1 h/d) for 1 week; (iii) OT: mice were fed OVA (1% dissolved in sterile water) for 1 week, rested for 1 week, intraperitoneally administered one dose of OVA/alum during a 1-week period, and aerosolized with OVA (10 mg/mg in PBS, 1 h/d) for 1 week. (iv) OT+anti-CCL21: mice were treated as described for the OT group, except that an anti-CCL21-neutralizing antibody was injected at a dose of 10 μ g/mouse via the tail vein 12 h before intraperitoneal OVA sensitization and at a dose of 1 μ g/mouse 1 h before aerosol OVA challenge each day. Specifically, control OT mice were administered an intraperitoneal injection of an equal concentration of isotype antibody and received the corresponding treatments. Mouse CCL21/6Ckine and isotype antibodies were purchased from R&D Bio-Techne, and neutralization of CCL21 in mice was evaluated according to the recommended assays.

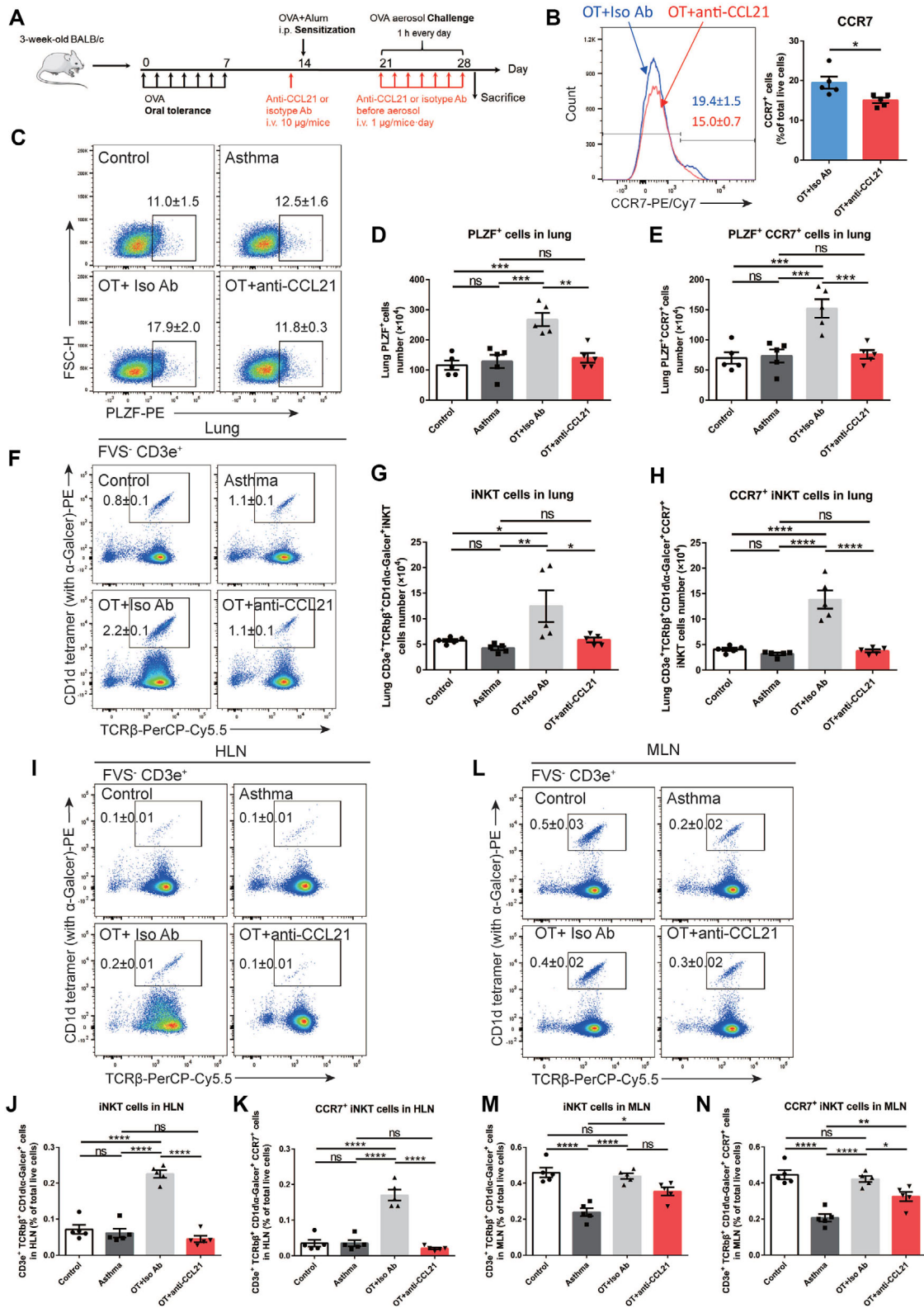
RSV titration and infection

HEp-2 cells (ATCC, Rockville, MD, USA) were maintained in DMEM/F12 supplemented with 10% FBS. The A2 strain of RSV was friendly provided by School of Basic Medical Sciences (Fudan University, Shanghai, China) and propagated in a HEp-2 cell. Cytopathic effect appeared 3–5 days later. At maximal cytopathic effect, the cells were harvested and disrupted by sonication. Viral titers were determined by standard plaque assay on HEp-2 cells.

In the UV-RSV-infected OT and RSV-infected OT group, the mice after OT were intranasally instilled with UV-RSV or RSV 80 μ L separately (strain A2, 10⁶ PFU/mouse) at days 7, 10, and 13, and then were treated by OVA sensitization and challenge the same as the asthma group from the days 15 to 28.

Airway resistance index

Airway reactivity was assessed by methacholine (MCh, Sigma, St. Louis, MO) provocation testing, whereas respiratory



mechanics were recorded using invasive lung function assessment (AniRes2005 V3.5, Animal Pulmonary Function Analysis System, Beijing Bestlab Technology Co., Ltd, China). Briefly, the mice were intraperitoneally anesthetized and ventilated. The cannula of the trachea was connected to a pressure transducer to measure transpulmonary pressure. Airflow and tidal volume were determined using a flow transducer. MCh (0.025, 0.05, 0.1, and 0.2 mg/kg) were injected to the mice by external jugular vein and pulmonary resistance (RL) was measured for 5 min in each dose. RL was calculated by dividing transpulmonary pressure by airflow. The percentage of RL change was presented in the results.

Quantitative PCR analysis

Total RNA was extracted from whole cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. RNA concentration was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using a reverse transcriptase kit (FastQuant RT Kit, Tiangen Biotech), followed by quantitative RT-PCR analysis (SYBR Green, TaKaRa). Reaction was detected on ABI 7900HT Fast Real-Time PCR System. The sequences of the used primers were as follows: *Ccl21a*, 5'-AAGGCAGTGATGGAGGGGT-3' (forward) and 5'-CTTAGAGTGCTCCGGGGT-3' (reverse); *Ccr7*, 5'-TGACGAGTCGGTGTGCTTC-3' (forward) and 5'-GGTAGGTA TCCGTCATGGTCTTG-3' (reverse); *Zbtb16*, 5'-CTGGGACTTTGTG CGATGTG-3' (forward) and 5'-CGGTGGAAGAGGATCTCAACA-3' (reverse); *Il4*, 5'-AAGAACACCACAGAGAGTGAGCTC-3' (forward) and 5'-TTTCAGTGATGTGGACTTGGA CTC-3' (reverse); *Il13*, 5'-AAAGCAACTGTTTCGCCACG-3' (forward) and 5'-CCTCT CCCAGCAAAGTCTG-3' (reverse); *Il33*, 5'-TCCTTGCTTGGC AGTATCCA-3' (forward) and 5'-TGCTCAATGTGTCAACAGACG-3' (reverse). The relative expression levels of corresponding genes were determined by the $2^{-\Delta\Delta CT}$ method [44], normalized by GAPDH.

Isolation of cells from the lung and lymph nodes

After anesthetized, mice were performed tracheal intubation, then blood was taken from the left ventricle for plasma extraction and IgE measurement, and mice were died from acute loss of blood. The chest was opened and the left atrial appendage was removed, we perfused the lung through tracheal using 1.5 mL of enzyme mix according to the Lung Dissociation Kit (Miltenyi Biotec), inflated

each lung lobe, and ligate after lung infusion. And then the whole lung was carefully stripped from thymus, heart, efferent and afferent vessels, trachea and connective tissue, transferred into the gentleMACS C Tube containing 2 mL of enzyme mix. Single cell suspension of the lung tissue was prepared using the gentleMACS™ Octo Dissociator with Heaters according to the manufacturer's instructions. Cells were collected by centrifugation at $335 \times g$ for 10 min at 4°C. Residual erythrocytes were lysed with ACK lysis buffer, and the cells were rinsed twice with cold PBS/0.5% BSA and at last filter with a 70 μ m cell strainer. The single cells were collected and resuspend in staining buffer for flow cytometry. Total cells were diluted and counted by a hemocytometer.

Mesenteric lymph nodes were collected and digested in 1.5 mL HBSS medium (containing 5% FBS, 100 U/mL penicillin/streptomycin) with collagenase IA (1 mg/mL final concentration) and DNase IV (20 μ g/mL final concentration) at 37°C for 30 min. The digested lymph nodes were gently crushed in a 70 μ m cell strainer using a 5 mL syringe plunger, and then the cells were rinsed through the strainer with cold PBS/0.5% BSA. After lysing erythrocytes, the single cells were collected and resuspended in staining buffer for flow cytometry.

Isolation and culture of lymphocytes from the spleen, transfection siRNA, and treatment procedures

As described in Fig. 5A, 6–8-week-old WT BALB/c mice were euthanatized, the whole spleen of each mouse was removed and ground gently on nylon strainer by syringe piston into a centrifuge tube. Mouse Lymphocyte Separation Medium (Dakewe™) was used for density gradient separation of spleen lymphocytes. The lymphocytes were counted by a hemocytometer and cultured in RPMI 1640 containing 10% v/v FBS and 1% v/v penicillin/streptomycin in vitro. About 24 h later, the lymphocytes were transfected with 100 nM *Zbtb16*-siRNA (designed on three different sites), NC-siRNA or Positive Control-siRNA separately through Polyplus-transfection® reagent for 24–72 h. At 48 h, immunofluorescence and RT-qPCR were performed to test the transfection and *Zbtb16*-knock down efficiency. Prepared supernatant of OVA treated BEAS-2B (for 36 h, BEAS-2B was normal bronchial epithelial cells that suitable for OVA stimulation in vitro), RSV treated MLE (MOI = 5, for 24 h, MLE-12 was commonly used pulmonary epithelial cells in RSV infection research in vitro) and untreated supernatant of BEAS-2B or MLE as vehicle control were used to stimulate *Zbtb16*-knock down lymphocytes and NC-controls for 12 h. Six hours before harvest, each group was incubated with 50 ng/mL PMA,

Figure 8. Flow cytometry analysis of CCR7 and (or) PLZF expressing cells, iNKT and CCR7⁺ iNKT cells in lung, HLN and MLN. (A) CCL21-blockade asthma tolerance model described in Methods section. (B) Comparison of CCR7⁺ cells in lung between isotype antibody-treated OT (blue) and anti-CCL21 antibody-treated OT (red) by flow cytometry. (C–E) Analysis of total PLZF⁺, CCR7⁺ PLZF⁺ cell numbers in total live cells of the lung. (F–H) Analysis of CCR7⁺iNKT, CCR7⁺PLZF⁺iNKT cell numbers in total live cells of the lung. (I–N) Analysis of CCR7⁺iNKT, CCR7⁺PLZF⁺iNKT cell frequencies in total live cells of HLN and MLN. The number in each flow cytometry graph represent gating percentage of total live cells (FVS⁺) in each group. Data are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant (unpaired two-tailed student's t test, corrected by one-way ANOVA with Turkey post-hoc test). Results are from one representative experiment out of at least two independent experiments ($n = 5$ in each group). HLN, hilar lymph nodes; MLN, mediastinal lymph nodes.

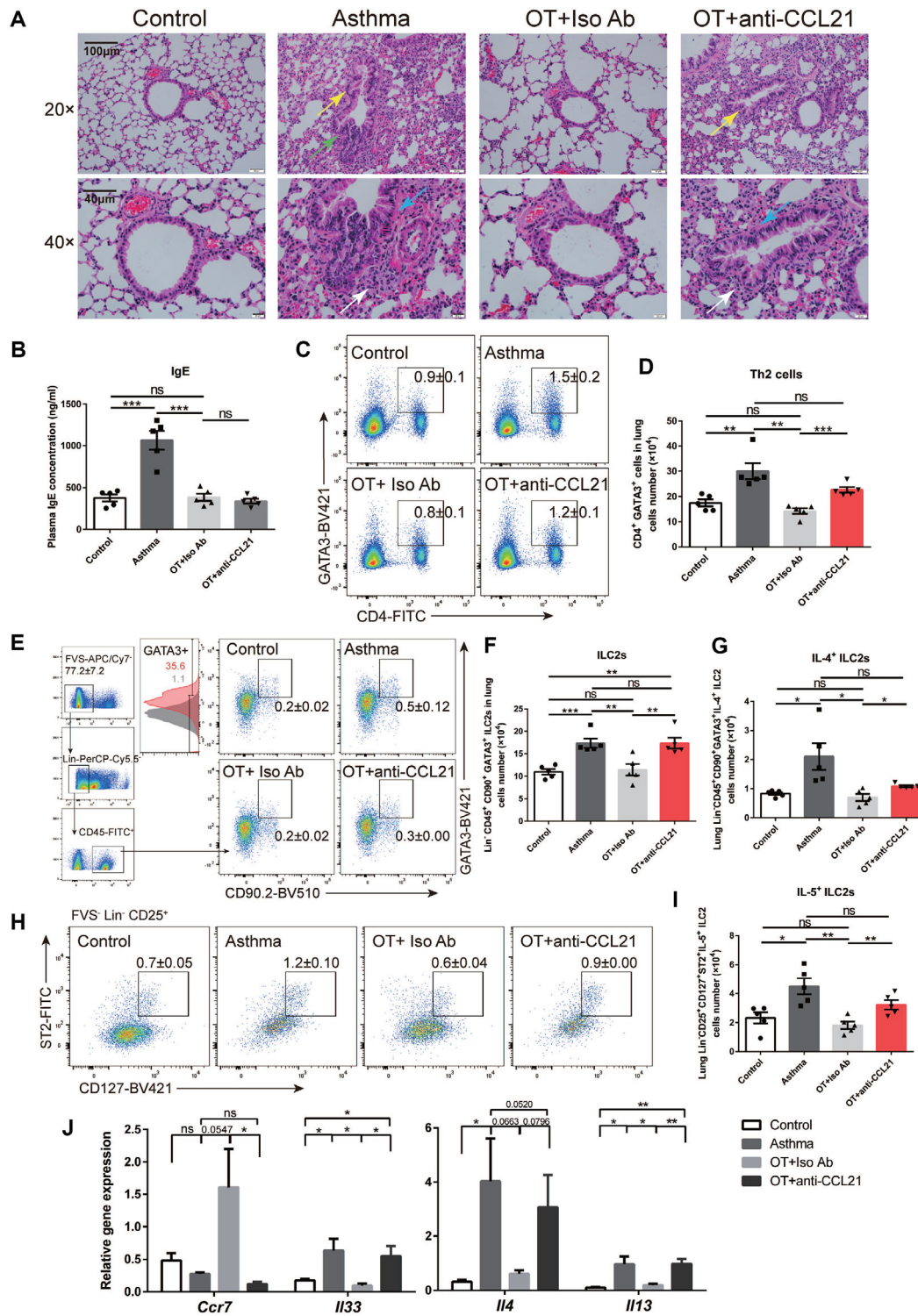


Figure 9. Lung inflammation in CCL21-blockade asthma tolerance model measured by pathology, flow cytometry (Th2 and ILC2s) and RT-qPCR (type 2 cytokines). (A) Representative microphotographs of lung histological sections from control, asthma, tolerized, and anti-CCL21 tolerized mice. Yellow arrow indicates thickness of bronchial mucous hyperplasia and goblet cells hyperplasia; white arrow points to peribronchial inflammatory cell infiltration; blue arrow denotes thickening epithelial base membrane; and green arrows indicate bronchial constriction and mucoid exudate. Objective magnification $\times 20$; inserts were from the same section with a magnification $\times 40$. (B) Plasma IgE levels (ng/ml) were measured by ELISA. (C–I) Flow cytometric analysis of frequencies and numbers of CD4⁺GATA3⁺Th2 (C–D), Lin[−]CD45⁺CD90.2⁺GATA3⁺IL-4⁺ILC2s (E–G), Lin[−]CD25⁺CD127⁺ST2⁺IL-5⁺ ILC2s (H–I) in the lungs. The number in each flow cytometry graph represent gating percentage of total live cells (FVS[−]) in each group. (J) Gene expression of *Ccr7*, *Il33*, *Il4*, and *Il13* in the whole lung as determined by qPCR analysis. Data are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant (unpaired two-tailed student's *t* test, corrected by one-way ANOVA with Turkey post-hoc test). Results are from one representative experiment of at least two independent experiments.

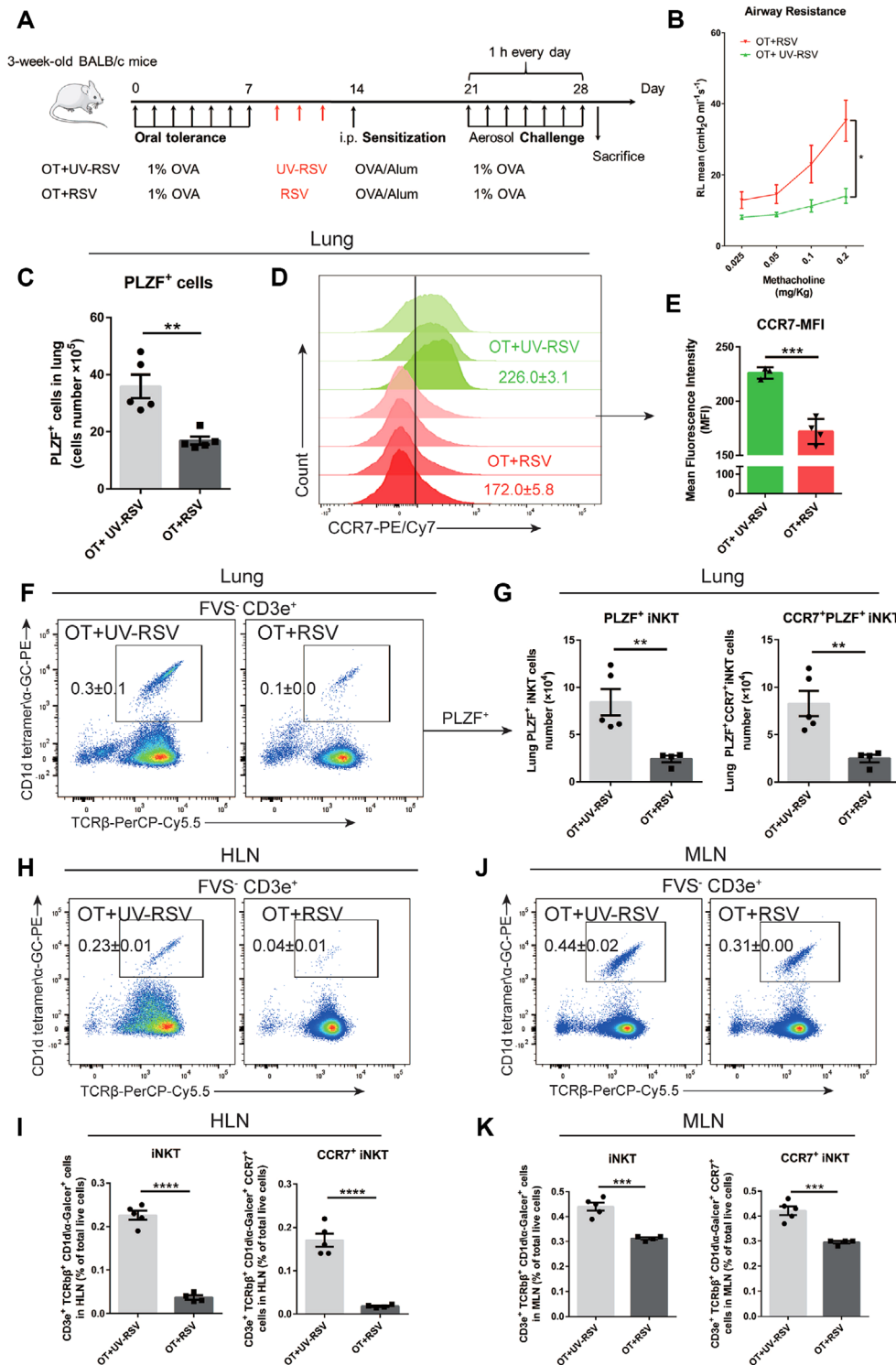


Figure 10. RSV infection compromises asthma tolerance by inhibiting recruitment of iNKT cells to the lung. (A) The procedures inducing asthma tolerance, and tolerance with early RSV infection in mice. (B) Airway resistance index at d28 in mouse model (means ± SEM, $n = 3$, $^*p < 0.05$, unpaired two-tailed student's t test). (C) Numbers of PLZF⁺ cells in lungs by flow cytometric analysis. (D-E) The MFI of CCR7 expression in lung between OT + UV-RSV (green) and OT + RSV (red) by flow cytometry. (F-K) Frequencies and numbers of iNKT (including CCR7⁺ iNKT and PLZF⁺CCR7⁺iNKT) cells in the lung, HLN, and MLN by flow cytometric analysis. The number in each flow cytometry graph represent gating percentage of total live cells (FVS⁺) in each group. Data are means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ (unpaired two-tailed student's t test). Results are from one representative experiment of at least two independent experiments ($n = 4-5$ in each group).

1 $\mu\text{g/mL}$ ionomycin, and 1 $\mu\text{g/mL}$ GolgiPlugTM protein transport inhibitor for 6 h to stimulate cytokine secretion. At 60 h, the lymphocytes were harvested and stained by Fixable Viability Stain to distinguish live cells, and then stained by other antibodies for flow cytometry.

Flow cytometry

The methods of flow cytometry adhere to the guidelines [45]. Cell suspensions were incubated with purified anti-CD16/32 (clone 2.4G2) for 10 min on ice to block Fc receptors. Fixable Viability Stain 780 (FVS 780, BD, Bioscience) was stained for 15 min in room temperature to all live samples to exclude dead cells. Fluorochrome or biotin-labeled mAbs (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 & Ly-6C (RB6-8C5); CD4 (RM4-5 or GK1.5), CD25 (PC61), Thy1.2/CD90.2 (53-2.1 or 30-H12), CD127 (SB/199), KLRG1 (2F1), TCR β (H57-597), GATA3 (L50-823), PLZF (R17-809), IL-5 (TRFK5), IFN- γ (XMG1.2), IL-17A (TC11-18H10), and IgG1 (A85-1) were purchased from BD Biosciences. CCR7 (4B12), IL-33R α /ST2 (DIH9), GATA3 (16E10A23), and PLZF (9E-12) were purchased from BioLegend. CD45 (30-F11), ROR γ T (B2D), PLZF (9E12), IL-4 (11B11) were purchased from eBioscience. T1/ST2 (DJ8) was purchased from MD Biosciences. Mouse CD1d tetramer (with α -GalCer loaded) and CD1d Tetramer (without α -GalCer loaded, NC) were purchased from MBL (Medical & biological laboratories Co., LTD.). Data were collected with LSRFortessaTM flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore). Collected events were gated on FVS⁺ leukocytes, and doublets were excluded. As an isotype control, IgG antibody was preincubated with the cells at a 25-fold excess to allow setting for positive and negative gates.

The fixation and intracellular staining with the TF Buffer Set (BD PharmingenTM) was used for intracellular TF staining. For intracellular cytokines staining, cell suspensions were incubated with 50 ng/mL PMA, 1 $\mu\text{g/mL}$ ionomycin, and 1 $\mu\text{g/mL}$ GolgiPlugTM protein transport inhibitor for 6 h to stimulate cytokine secretion, then Cytofix/Cytoperm/GolgiPlug Kit (BD PharmingenTM) was used to intracellular cytokines staining.

Lung immunofluorescence and microscopy

Primary antibody anti-PLZF mouse mAb (2A9, Active MotifTM) and Anti-CCR7 Rabbit pAb (ServicebioTM) or Rabbit mAb IgG Isotype Control (DA1E, CSTTM) were used at the indicated final concentrations for immunofluorescence. Cy3-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, and DAPI (all from ServicebioTM) were used as fluorescent secondary antibodies. Images were acquired by Olympus FV1200 Laser Scanning Microscopes.

Coimmunoprecipitation

Protein extracts from 293T cells transfected with expression plasmids encoding PLZF-Flag or GATA3-HA were immunoprecipitated (IP) by Protein A Agarose Beads and Protein G Agarose Beads (CSTTM) with anti-Flag (DYKDDDDK Tag Mouse mAb (9A3), CSTTM) or anti-HA antibody (Santa cruzTM) or IgG antibody (Rabbit mAb IgG Isotype Control (DA1E), CSTTM), and then were analyzed by western blotting with anti-PLZF mAb (2A9, Active MotifTM) or anti-GATA3 mAb (D13C9, Cell Signaling TechnologyTM).

RNA-seq analysis

This project was based on the sequencing analysis platform of BGI Genomics Co Ltd. Bowtie2 was used to align clean reads to the genome sequence, and then used RSEM (a software package for RNA-seq reads to calculate gene and transcript subtype expression levels) to calculate the gene expression level of each sample. The GO and KEGG analysis were performed separately to classify the differential genes according to the GO or KEGG annotation results and official classification, and then use the phyper function in R software for enrichment analysis. Then the FDR correction is performed on the *P*-value, and the function of *Q*-value ≤ 0.05 is generally regarded as significant enrichment. Differentially expressed gene (DEG) detection in heatmap analysis was performed according to the method described before [46]. Then calculate the *p*-value according to the normal distribution through Benjamini Y., Hochberg, Y., and Storey, J. and Tibshirani, R. two strategies to correct *p*-values to *Q*-values. In order to improve the accuracy of DEGs, we defined genes with a difference multiple of more than two times and *Q*-value ≤ 0.001 , and screened them for significantly differentially expressed genes.

Statistical analysis

Prism 7 (GraphPad Software) was used to perform unpaired two-tailed Student's *t* test (independent two-sample) and one-way ANOVA with Tukey's multiple comparisons test (independent samples more than three) on datasets for which statistical significance is indicated (except the RNA-sequencing data). All figures of functional data show mean $\pm$ SEM.

Acknowledgements: This study was supported by Minhang Leading Talent Development Plan (201807) and Key Research of Shanghai Fifth People's Hospital (2019WYZD03); Youth Program of Shanghai Municipal Commission of Health and Family Planning (20164Y0264); National Natural Science Foundation of China (NSFC) programs 81730001, 91942305, and 81970075; the

Strategic Leading Project (B) of CAS XDPB0303; the International Collaboration project of CAS 153831KYSB20170043; and Innovative Research Team of High-level Local Universities in Shanghai. The authors greatly appreciate Prof. Bin Wang research group (Basic Medical Sciences, Fudan University) for friendly providing RSV A2 strain; Prof. Dapeng Zhou (Tongji University) for his kind help and suggestion; and Lianping Cheng for breeding animals.

Author contributions: X.S. and Z.J.J. conceived and designed the experiments, analyzed data, provided funds, and wrote the manuscript. X.T.F. performed experiments, collected, analyzed data, and wrote the draft. C.Q.Z. and L.L. assisted with plasmid construction, transfection, co-IP, and animal experiments. J.J.F., W.H., N.L., and T.Y.S. assisted with animal experiments, helped to design, and interpret the experiments.

Conflict of interest: The authors declare no commercial or financial conflict of interest.

Peer review: The peer review history for this article is available at <https://publons.com/publon/10.1002/eji.202048798>

References

- Fahy, J. V., Type 2 inflammation in asthma: present in most, absent in many (vol 15, pg 57, 2015). *Nat. Rev. Immunol.* 2015. 15: 130.
- Tournoy, K. G., Provoost, S., Van Hove, C. and Joos, G., The role of immune tolerance in asthma pathogenesis. *Curr. Allergy Asthma Rep.* 2006. 6: 437–443.
- Levan, S. R., Stamnes, K. A., Lin, D. L., Panzer, A. R., Fukui, E., McCauley, K., Johnson, C. C. et al., Elevated faecal 12,13-diHOME concentration in neonates at high risk for asthma is produced by gut bacteria and impedes immune tolerance. *Nat. Microbiol.* 2019. 4: 1851–1861.
- Tordesillas, L. and Berin, M. G., Mechanisms of oral tolerance. *Clin. Rev. Allergy Immunol.* 2018. 55: 107–117.
- Faria, A. M. and Weiner, H. L., Oral tolerance: therapeutic implications for autoimmune diseases. *Clin. Dev. Immunol.* 2006. 13: 143–157.
- Verhasselt, V., Milcent, V., Cazareth, J., Kanda, A., Fleury, S., Dombrowicz, D., Glaichenhaus, N. et al., Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nat. Med.* 2008. 14: 170–175.
- Krishnamoorthy, N., Khare, A., Oriss, T. B., Raundhal, M., Morse, C., Yarlagaadda, M., Jr., Ray, A. et al., Early infection with respiratory syncytial virus impairs regulatory T cell function and increases susceptibility to allergic asthma. *Nat. Med.* 2012. 18: 1525–1530.
- Shi, T., He, Y., Sun, W., Wu, Y., Li, L., Jie, Z. and Su, X., Respiratory Syncytial virus infection compromises asthma tolerance by recruiting interleukin-17A-producing cells via CCR6-CCL20 signaling. *Mol. Immunol.* 2017. 88: 45–57.
- McConnell, M. J., Chevallier, N., Berkofsky-Fessler, W., Giltane, J. M., Malani, R. B., Staudt, L. M. and Licht, J. D., Growth suppression by acute promyelocytic leukemia-associated protein PLZF is mediated by repression of c-myc expression. *Mol. Cell. Biol.* 2003. 23: 9375–9388.
- Bernardo, M. V., Yelo, E., Gimeno, L., Campillo, J. A. and Parrado, A., Identification of apoptosis-related PLZF target genes. *Biochem. Biophys. Res. Commun.* 2007. 359: 317–322.
- Eidson, M., Wahlstrom, J., Beaulieu, A. M., Zaidi, B., Carsons, S. E., Crow, P. K., Yuan, J. et al., Altered development of NKT cells, gammadelta T cells, CD8 T cells and NK cells in a PLZF deficient patient. *PLoS One* 2011. 6: e24441.
- Kovalovsky, D., Uche, O. U., Eladad, S., Hobbs, R. M., Yi, W., Alonzo, E., Chua, K., Pandolfi, P. P. et al., The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat. Immunol.* 2008. 9: 1055–1064.
- Savage, A. K., Constantinides, M. G., Han, J., Picard, D., Martin, E., Li, B., Lantz, O. et al., The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 2008. 29: 391–403.
- Sadler, A. J., Rossello, F. J., Yu, L., Deane, J. A., Yuan, X., Wang, D., Irving, A. T. et al., BTB-ZF transcriptional regulator PLZF modifies chromatin to restrain inflammatory signaling programs. *Proc. Natl. Acad. Sci. USA* 2015. 112: 1535–1540.
- Ghosh, A., Holland, A. M., Dogan, Y., Yim, N. L., Rao, U. K., Young, L. F., Sant'angelo, D. et al., PLZF confers effector functions to donor T cells that preserve graft-versus-tumor effects while attenuating GVHD. *Cancer Res.* 2013. 73: 4687–4696.
- Kronenberg, M. and Gapin, L., The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol.* 2002. 2: 557–568.
- Hammond, K. J. and Godfrey, D. I., NKT cells: potential targets for autoimmune disease therapy? *Tissue Antigens* 2002. 59: 353–363.
- Wilson, S. B. and Delovitch, T. L., Janus-like role of regulatory iNKT cells in autoimmune disease and tumour immunity. *Nat. Rev. Immunol.* 2003. 3: 211–222.
- Van Kaer, L., alpha-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat. Rev. Immunol.* 2005. 5: 31–42.
- Liu, R., La Cava, A., Bai, X. F., Jee, Y., Price, M., Campagnolo, D. I., Christadoss, P. et al., Cooperation of invariant NKT cells and CD4+CD25+ T regulatory cells in the prevention of autoimmune myasthenia. *J. Immunol.* 2005. 175: 7898–7904.
- Ly, D., Mi, Q. S., Hussain, S. and Delovitch, T. L., Protection from type 1 diabetes by invariant NK T cells requires the activity of CD4+CD25+ regulatory T cells. *J. Immunol.* 2006. 177: 3695–3704.
- Cianferoni, A., Saltzman, R., Saretta, F., Barni, S., Dudek, E., Kelleher, M. and Spergel, J. M., Invariant natural killer cells change after an oral allergy desensitization protocol for cow's milk. *Clin. Exp. Allergy* 2017. 47: 1390–1397.
- Sozanska, B., Raw cow's milk and its protective effect on allergies and asthma. *Nutrients* 2019. 11: 469.
- Chen, Q., Guo, X., Deng, N., Liu, L., Chen, S., Wang, A., Li, R. et al., alpha-Galactosylceramide treatment before allergen sensitization promotes iNKT cell-mediated induction of Treg cells, preventing Th2 cell responses in murine asthma. *J. Biol. Chem.* 2019. 294: 5438–5455.
- Chen, Q., Guo, X., Deng, N., Liu, L., Chen, S., Wang, A., Li, R. et al., alpha-galactosylceramide generates lung regulatory T cells through the activated natural killer T cells in mice. *J. Cell. Mol. Med.* 2019. 23: 1072–1085.
- Jiang, S., Game, D. S., Davies, D., Lombardi, G. and Lechler, R. I., Activated CD1d-restricted natural killer T cells secrete IL-2: innate help for CD4+CD25+ regulatory T cells? *Eur. J. Immunol.* 2005. 35: 1193–1200.
- Forster, R., Davalos-Misslitz, A. C. and Rot, A., CCR7 and its ligands: balancing immunity and tolerance. *Nat. Rev. Immunol.* 2008. 8: 362–371.

- 28 Britschgi, M. R., Favre, S. and Luther, S. A., CCL21 is sufficient to mediate DC migration, maturation and function in the absence of CCL19. *Eur. J. Immunol.* 2010. 40: 1266–1271.
 - 29 Kozai, M., Kubo, Y., Katakai, T., Kondo, H., Kiyonari, H., Schaeuble, K., Luther, S. A. et al., Essential role of CCL21 in establishment of central self-tolerance in T cells. *J. Exp. Med.* 2017. 214: 1925–1935.
 - 30 Wang, H. and Hogquist, K. A., CCR7 defines a precursor for murine iNKT cells in thymus and periphery. *eLife* 2018. 7.
 - 31 Holt, P. G. and Sly, P. D., Viral infections and atopy in asthma pathogenesis: new rationales for asthma prevention and treatment. *Nat. Med.* 2012. 18: 726–735.
 - 32 Landry, D. B., Engel, J. D. and Sen, R., Functional Gata-3 binding-sites within murine Cd8-alpha upstream regulatory sequences. *J. Exp. Med.* 1993. 178: 941–949.
 - 33 Leiden, J. M., Transcriptional regulation of T-cell receptor genes. *Annu. Rev. Immunol.* 1993. 11: 539–570.
 - 34 Miaw, S. C., Choi, A., Yu, E., Kishikawa, H. and Ho, I. C., ROG, repressor of GATA, regulates the expression of cytokine genes. *Immunity* 2000. 12: 323–333.
 - 35 Sigurs, N., Aljassim, F., Kjellman, B., Robinson, P. D., Sigurbergsson, F., Bjarnason, R. and Gustafsson, P. M., Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. *J. Thorax.* 2010. 65: 1045–1052.
 - 36 Constantinides, M. G., McDonald, B. D., Verhoef, P. A. and Bendelac, A., A committed precursor to innate lymphoid cells. *Nature* 2014. 508: 397–+.
 - 37 Verhoef, P. A., Constantinides, M. G., McDonald, B. D., Urban, J. F., Sperling, A. I. and Bendelac, A., Intrinsic functional defects of type 2 innate lymphoid cells impair innate allergic inflammation in promyelocytic leukemia zinc finger (PLZF)-deficient mice. *J. Allergy Clin. Immunol.* 2016. 137: 591.
 - 38 Mao, A. P., Constantinides, M. G., Mathew, R., Zuo, Z., Chen, X., Weirauch, M. T. and Bendelac, A., Multiple layers of transcriptional regulation by PLZF in NKT-cell development. *Proc. Natl. Acad. Sci. USA* 2016. 113: 7602–7607.
 - 39 Brennan, P. J., Brigl, M. and Brenner, M. B., Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat. Rev. Immunol.* 2013. 13: 101–117.
 - 40 Chang, Y. J., Kim, H. Y., Albacker, L. A., Lee, H. H., Baumgarth, N., Akira, S., Savage, P. B. et al., Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity. *J. Clin. Invest.* 2011. 121: 57–69.
 - 41 Ascough, S., Paterson, S. and Chiu, C., Induction and subversion of human protective immunity: contrasting influenza and Respiratory Syncytial virus. *Front. Immunol.* 2018. 9: 323.
 - 42 Tulic, M. K., Piche, T. and Verhasselt, V., Lung-gut cross-talk: evidence, mechanisms and implications for the mucosal inflammatory diseases. *Clin. Exp. Allergy* 2016. 46: 519–528.
 - 43 Satitsuksanoa, P., Jansen, K., Globinska, A., van de Veen, W. and Akdis, M., Regulatory immune mechanisms in tolerance to food allergy. *Front. Immunol.* 2018. 9: 2939.
 - 44 Livak, K. J. and Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 2001. 25: 402–408.
 - 45 Cossarizza, A., Chang, H. D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W. W. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur. J. Immunol.* 2019. 49: 1457–1973.
 - 46 Wang, L., Feng, Z., Wang, X., Wang, X. and Zhang, X., DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics* 2010. 26: 136–138.
- Abbreviations:** AHR: airway hyperresponsiveness · CCL21: C-C motif chemokine ligand 21 · CCR7: C-C motif chemokine receptor 7 · GATA3: GATA Binding Protein 3 · HLN: hilar lymph node · ILC2: group 2 innate lymphoid cell · MLN: mesenteric lymph node · OT: oral tolerance · OVA: ovalbumin · PLZF: promyelocytic leukemia zinc finger · RSV: respiratory syncytial virus
- Full correspondence:** Prof. Xiao Su, Unit of Respiratory Infection and Immunity, Institute Pasteur of Shanghai, Chinese Academy of Sciences. 320 Yueyang Road, Life Science Research BLD B114, Shanghai 200031, China
e-mail: xsu@ips.ac.cn
Prof. Zhijun Jie, Department of Pulmonary and Critical Care Medicine, Shanghai Fifth People's Hospital, Fudan University, Shanghai 200240, China
e-mail: jiejzlxh@163.com
- Received: 8/6/2020
Revised: 29/6/2020
Accepted: 23/7/2020
Accepted article online: 26/7/2020