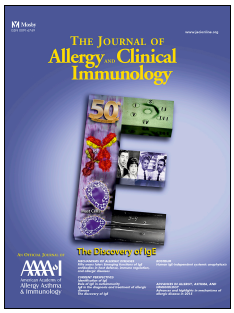
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Concomitant suppression of Th2 and Th17 cell responses in allergic asthma by targeting RORγt

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ABSTRACT

Background: Allergic asthma is a heterogeneous chronic inflammatory disease in the airway with a massive infiltration of eosinophils or neutrophils mediated by allergen-specific Th2 cells and Th17 cells, respectively. Successful treatment of allergic asthma will therefore require suppression of both Th2 and Th17 cells.

Objective: We sought to investigate the role of Th17 cell pathway in regulating Th2 cell responses in allergic asthma.

Methods: Allergic asthma was induced by intranasal challenges with proteinase allergens in C57BL/6, *Il17a*$^{-/-}$*Il17f*$^{-/-}$ and *RORγt* $^{gfp/gfp}$ mice. Pharmacological RORγt inhibitor was used to evaluate its preventive and therapeutic effects in allergic asthma. Characteristics of allergic airway inflammation were analyzed by flow cytometry, histology, qRT-PCR and ELISA. Mixed bone marrow chimeric mice, fate mapping analysis, shRNA transduction and *in vitro* T cell differentiation were employed for mechanistic studies.

Results: Mice deficient in IL-17A and IL-17F as well as in RORγt exhibited a significant reduction not only in Th17 cell responses, but also in Th2 cell responses in an animal model of allergic asthma. Similarly, mice treated with an RORγt inhibitor showed significantly diminished Th17 as well as Th2 cell responses, leading to reduced numbers of neutrophils and eosinophils in the airway. RORγt-deficient T cells were intrinsically defective in differentiating into Th2 cells and expressed increased level of *Bcl6*. Knock-down of *Bcl6* resulted in a remarkable restoration of Th2 cell differentiation in RORγt-deficient T cells. Blockade of RORγt also significantly hampered the differentiation of human Th2 cells and Th17 cells from naïve $CD4^+$ T cells.

Conclusion: RORγt in T cells is required for optimal Th2 cell differentiation by suppressing

Bcl6 expression; this finding suggests that targeting RORγt might be a promising approach for the treatment of allergic asthma by concomitantly suppressing Th17 and Th2 cell responses in the airway.

Key messages:

RORγt in T cells is necessary for optimal Th2 cell responses during allergic asthma.

Inhibition of Th2 cell responses in RORγt-deficient T cells depends on BCL6.

Targeting RORγt might be a promising strategy for the treatment of allergic asthma via concomitant suppression of Th2 and Th17 cells in the airway.

Capsule summary:

Inhibition of RORγt suppresses both Th2 and Th17 cell responses in the airway, and this can become a potential therapeutic target for the treatment of allergic asthma.

Keywords: allergic asthma, Th17 cell, Th2 cell, RORγt, BCL6

Abbreviations used:

BAL: Bronchoalveolar lavage

BCL6: B cell lymphoma 6

dKO: double knock-out

DMSO: Dimethyl sulfoxide

Gata3: GATA-binding factor 3

Irf4: Interferon regulatory factor 4

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Ova: Ovalbumin

PAO: Proteinase from *Aspergillus oryzae*

PBMC: Peripheral blood mononuclear cell

RORγt: Retinoic acid receptor–related orphan receptor γt

UA: Ursolic acid

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INTRODUCTION

Asthma is a heterogeneous disease of the lung and the airway characterized by distinct symptoms, such as airway hyper-responsiveness, mucus production, infiltration of inflammatory granulocytes and short-of-breath which could be life-threatening.[1,2] The prevalence of this disease has markedly increased over the past several decades and has now become one of the major global health problems affecting approximately 300 million people worldwide.[1] Asthma can be categorized into allergic and non-allergic asthma based on the types of triggering stimuli.[3] Allergic asthma is a common form of asthma caused by sensitization against allergens such as: pollen, house dust mites, fur dander from pet or fungi; whereas non-allergic asthma is caused by irritants such as: tobacco smoke, ozone, diesel exhaust particles or air-borne virus.[1,4]

Allergic asthma has been considered to be mediated by Th2 cells; however, recent studies uncovered the involvement of Th17 cells as an additional critical contributor in the pathogenesis of allergic asthma in animal models and in humans.[5] Th2 cells mediate eosinophilic asthma by secreting type 2 cytokines such as IL-4, IL-5, IL-9 and IL-13. These cytokines induce B cell isotype switching to IgE (IL-4), recruit eosinophils (IL-5) and mast cells (IL-9, IL-13) into the lung and the airway, induce goblet cell hyperplasia and tissue remodeling (IL-13) leading to airway hyper-responsiveness (AHR).[2,6] On the other hand, Th17 cells have been regarded as a critical mediator of steroid-resistant neutrophilic asthma.[7-9] Elevated levels of IL-17A were observed in the lungs of patients with asthma, which were positively correlated with neutrophilic inflammation, increased AHR and steroid-resistant type of severe asthma.[10] A mixed Th2 and Th17 cell response in the airways has been associated with severity of allergic asthma.[9,11] IL-17A stimulates airway epithelial cells to secrete chemokines CXCL1 and CXCL8, which in turn

recruit neutrophils.[12] In addition, IL-17A causes airway remodeling via upregulation of α-smooth muscle actin in the fibroblast.[13] Hence, Th2 cells and Th17 cells exert non-redundant pathogenic roles during the development of allergic asthma by inducing eosinophilic and neutrophilic inflammation, respectively.

Due to their critical contributions to the development of allergic asthma, a number of experimental and clinical studies have addressed whether the blockade of either Th2 cell or Th17 cell pathway ameliorates the clinical symptoms of the disease. Particularly, multiple clinical trials have demonstrated that antibodies against Th2 cell cytokines – IL-4, IL-5, IL-13 – and their receptors in patients with moderate-to-severe asthma had limited clinical benefits. For instance, antibodies to IL-5 or its receptor are generally effective in reducing eosinophilia, whereas little to no effect on airway hyper-responsiveness.[14] Antibodies to IL-13 or its receptor lead to reduced airway hyper-responsiveness with little effects in eosinophilia.[15,16] More recently, a human monoclonal antibody to IL-17RA failed to improve asthmatic symptoms in patients with moderate-to-severe asthma.[17] In this regard, Choy et al.[18] recently reported that antibodies to Th2 cell cytokines enhance Th17 cell responses and neutrophilia, while anti-IL-17A enhances Th2 cell responses and eosinophilia in animal models of allergic asthma. This study indicates that pulmonary Th2 cell and Th17 cell responses are mutually regulated, and suggests that blockade of either helper T cell pathway alone leads to the exaggeration of the other pathway. Supporting this notion, GATA-3 and IL-13 are shown to inhibit Th17 cell differentiation.[19,20] Hence, combined blockade of both Th2 cell and Th17 cell pathways might be considered to achieve therapeutic benefits in controlling asthma without adverse effects.

In the present study, we aimed to investigate the role of Th17 pathway on regulating Th2 cell responses in allergic airway inflammation by employing an animal model of proteinase-induced allergic asthma. We also aimed to investigate the clinical relevance of blocking Th17 pathway by analyzing T cells from human patients with allergic asthma.

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METHODS

Ethics statements

Animal experiments including induction of allergic lung inflammation in *Il17a*$^{-/-}$*Il17f* $^{-/-}$ double deficient mice and subsequent analysis, fate mapping and adoptive transfer of *in vitro* differentiated Th17 cells were done with protocols approved by Institutional Animal Care and Use Committees of the MD Anderson Cancer Center. All remaining animal experiments were reviewed and approved by Seoul National University Institutional Animal Care and Use Committee (IACUC#: SNU-140602-2-7 and SNU-140217-6-8). The collection of human blood samples from healthy volunteers and subsequent experimental procedures were reviewed and approved by Seoul National University Institutional Review Board. Protocol approval number is 1608/001-006. The collection of blood samples from patients with allergic asthma and related experimental procedures are reviewed and approved by Seoul National University Bundang Hospital Institutional Review Board (IRB#: B-1603/340-310). Written informed consent was obtained from all human subjects before their involvement.

Animals

C57BL/6 mice were purchased from Orient Bio. *Rorγt* $^{gfp/gfp}$, B6.SJL and *Tcrb* $^{-/-}$ mice were purchased from Jackson Laboratory. *Il17a*$^{-/-}$*Il17f* $^{-/-}$ double deficient mice, IL-17F fate reporter mice, *Il17f* ^{Cre}*R26R*^{eYFP}, and IL-17F reporter mice, *Il17f* ^{rfp}, were described previously.[21-23] All mice were maintained in specific pathogen-free facility in Seoul National University or MD Anderson Cancer Center. Mice aged 6-12 weeks were used.

Reagents

Ursolic acid (UA), bulsulfan (Sigma-Aldrich) and SR2211 (Calbiochem) were dissolved in DMSO. For *in vitro* use, UA and SR2211 were further diluted with PBS (GenDepot). *In vitro* cell cultures of lymphoid cells were performed in the RPMI1640 supplemented with 10% fetal bovine serum (FBS, GenDepot), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 55 μM 2-mercaptoethanol and 10 μg/ml gentamycin. For PLAT-E cells culture, DMEM supplemented with 10% FBS, 1 μg/ml puromycin, and 10 μg/ml blasticidin was used. All cell culture reagents except FBS were the products of Gibco.

Animal models of allergic asthma

To induce allergic lung inflammation, we adopted an animal model of proteinase-induced allergic asthma induced by repeated intranasal challenges with fungal proteinase allergens.[24] In brief, mice were anesthetized with isoflurane (Terrell from Piramal) and challenged intranasally with 7 μg of proteinase from *Aspergillus oryzae* (Sigma-Aldrich) plus 20 μg of ovalbumin (Grade V from Sigma-Aldrich) (PAO/Ova) in 50 μl of PBS every other day for four times (Days 0, 2, 4, 6). For therapeutic model, mice were challenged on days 0, 2, 4, 6 and 12. In experiments with UA, mice were injected intraperitoneally (i.p.) with 150 mg/kg of UA dissolved in DMSO or DMSO alone as a vehicle control. In some experiments, UA was injected on days 0, 2, 4, 6 in preventive format and days 6, 8, 10, 12 in therapeutic format as described in Fig. E3A and E3C in the Online Repository at www.jacionline.org. For IFNγ neutralization, anti-mouse IFNγ (XMG1.2, Bio X Cell) or rat IgG1 (HRPN, Bio X Cell) as isotype control were injected i.p. (200 μg/mouse/injection) on days 0, 2, 4, 6. 24 h or 72 h after the last challenge, mice were euthanized with CO_2 for further analysis. For chronic asthma models, we injected mice with intranasal PAO/Ova and intraperitoneal UA on days 0, 2, 4, 11, and 13 (see Fig. E4A in the Online

Repository at www.jacionline.org). Alternatively, we i.p. immunized mice with Ova-alum mixture (100 μg of Ova, Imject Alum, Thermo Scientific) on days 0 and 14 and injected them with intranasal Ova (50μg) and intraperitoneal UA on days 25, 26 and 27 (Fig. E4E). Twenty-four hours after the last challenge, the mice were euthanized with CO_2 for further analysis.

Analysis of bronchoalveolar lavage (BAL) fluid

To collect BAL fluid, 20-gauge IV catheter (BD Bioscience) was inserted into the trachea and flushed twice with 500 μl and 800 μl of cold PBS. Cytokine levels were determined by ELISA using first-flushed 500 μl of BAL fluid. Ten thousand cells in the BAL fluid were attached on the slide by cytopro centrifuge (Wescor) and stained with Diff-Quik staining kits (Sysmex Corp.) according to the manufacturer's protocol. The absolute number of macrophages, eosinophils, neutrophils, lymphocyte in BAL fluid was calculated based on total cell counts.

Analysis of lymphoid cells in the lung

Left lobe of the lung was used for histological analysis. Rest of the lobes was placed in 3 ml of RPMI1640 medium containing 0.5 mg/ml collagenase (Type IV, Gibco), 2 mg/ml dispase (Gibco) and 25 U/ml DNase I (Bio Basic Inc.). Lungs were first dissociated with gentle MACS dissociator (Miltenyi biotec) and then digested for 30 min at 37°C with continuous agitation followed by second dissociation using gentle MACS dissociator. Cells were filtered through 100 μm nylon mesh and washed with cold PBS containing 1.5% FBS. Lymphoid cells were further isolated by using lymphocyte separation medium (LSM, MP biomedicals) according to the manufacturer's instruction.

Histology

Left lobe of the lung was fixed in neutral buffered 10% formalin solution (Sigma) for at least 24 h. Formalin fixed samples were sent to Pathology Center at Seoul National University College of Medicine for Hematoxylin and eosin (H&E), Periodic acid-Schiff (PAS) staining.

Lymph node restimulation

The mediastinal lymph nodes were physically minced in cold PBS containing 1.5% FBS and filtered through 100 μm nylon mesh. The lymphoid cells (1×10^6/ml) were cultured in the presence of 0, 10, 50 μg/ml of ovalbumin for 3 d. Culture supernatants were used for cytokine ELISA.

***In vitro* murine T helper cell differentiation**

$CD4^+$ cells from spleen and peripheral lymph nodes were positively selected with CD4 microbeads (L3T4, Miltenyi biotec). Subsequently, naïve $CD4^+$ T cells were sorted as $CD4^+CD25^-CD62L^{high}CD44^{low}$ cells with FACSAria III cell sorter (BD Bioscience) and stimulated with plate-coated anti-CD3 (1 μg/ml, 145-2C11, Bio X Cell) and soluble anti-CD28 (2 μg/ml, 37.51, Bio X Cell) for 4 d. For Th2 differentiation, IL-2 (10 ng/ml, eBioscience) and IL-4 (10 ng/ml, Peprotech) were added. For IFNγ neutralization experiment, anti-IFNγ (5 μg/ml, XMG1.2, Bio X Cell) was additionally added.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was prepared using TRIzol Reagents (Invitrogen). cDNA was then synthesized using Oligo(dT) primers and reverse transcriptase included in RevertAid cDNA synthesis kit (Thermo-

fisher Scientific) and the gene expression levels were examined with Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems) using iTaq SYBR Green Supermix (Bio-Rad Laboratories). Data were normalized to *Actb* reference. The following primers were used: *Rorγt*, 5'-CCGCTGAGAGGGCTTCAC-3' and 5'-TGCAGGAGTAGGCCACATTACA-3'; *Bcl6*, 5'-CACACCCGTCCATCATTGAA-3' and 5'-TGTCCTCACGGTGCCTTTTT-3'; *Irf4*, 5'-CACCAAAGCACAGAGTCACCT-3' and 5'-TCCTCTGGATGGCTCCAGATG-3'; *Gata3*, 5'-AGAACCGGCCCCTTATGAA-3' and 5'-AGTTCGCGCAGGATGTCC-3'; *Il4*, 5'-AGATCACGGCATTTTGAACG-3' and 5'-TTTGGCACATCCATCTCCG-3'; *Il5*, 5'-CGCTCACCGAGCTCTGTTG-3' and 5'-CCAATGCATAGCTGGTGATTTTT-3'; *Il13*, 5'-GCTTATTGAGGAGCTGAGCAACA-3' and 5'-GGCCAGGTCCACACTCCATA-3'; *Actb*, 5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'.

Mixed bone-marrow chimeric mice

A day before bone-marrow transfer, *Tcrb* $^{-/-}$ mice were injected with 35 mg/kg of busulfan (Sigma-Aldrich) to ablate bone marrow cells from the recipient mice. Bone marrow cells of B6.SJL (CD45.1$^{+/+}$) and *Rorγt* $^{gfp/gfp}$ mice were obtained from femurs and tibia by flushing with cold PBS. These bone marrow cells were mixed at 1:1 ratio and transferred into busulfan-treated *Tcrb* $^{-/-}$ mice via tail vein (1.5×10^7 cells/mouse). Six to eight weeks later, the reconstituted mice were intranasally challenged with PAO/Ova every other day for six times. Twenty-four hours after the last challenge, mice were euthanized with CO_2 inhalation for analysis.

BCL6 knockdown in naïve T cells by retroviral infection.

Retroviral supernatants were made by transfecting PLAT-E cell lines with either negative control LMP vector, or two kinds of LMP-shBcl6. Transfection was performed by using FuGene HD transfection reagent (Promega) according to the manufacturer's protocol. Naïve T cells were sorted from splenocyte and stimulated with plate-bound αCD3 and soluble αCD28. For Th2 differentiation, IL-2, IL-4 and αIFN-γ were added. After 24 h, cells were infected with retroviral supernatants containing 8 μg/ml of polybrene by spin infection (1000 × g, 60 min, 30°C). After spin infection, cells were washed with PBS and again placed on the αCD3 coated plate with same initial Th2 differentiating condition. After another 72 hours, cells were analyzed for their cytokine production by flow cytometry.

Flow cytometry and antibodies

For intracellular cytokine analysis, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml, Sigma-Aldrich) and ionomycin (1 μM, Sigma-Aldrich) plus Brefeldin A and monenesin (both from eBioscience) before staining. IC fixation buffer and permeabilization buffer from eBioscience were used for intracellular cytokine staining and Foxp3 staining kit (eBioscience) was used for BCL6 staining according to the manufacturer's instruction. Samples were analyzed with FACSVerse flow cytometer (BD Bioscience) and acquired data were analyzed with FlowJo software (Treestar). Following antibodies were used for flow cytometric analysis or cell sorting: Alexa488-conjugated antibodies to mouse CD62L (MEL-14) and IFNγ (XMG1.2), FITC-conjugated antibody to human CD45RA (HI100), PE-conjugated rat IgG1 (eBRG1, eBioscience), antibodies to mouse CD25 (PC61), IL-17A (TC11-18H10.1) and mouse/human BCL6 (IG191E/A8), PerCP/Cy5.5-conjugated antibodies to mouse CD4 (GK1.5), CD45.1 (A20) and IFNγ, PE/Cy7-conjugated antibodies to mouse IL-13 (eBio13A, eBioscience),

mouse/human CD44 (IM7), human CD4 (OKT4) and CD45RO (UCHL1), APC-conjugated antibodies to mouse/human IL-5 (TRFK5), human CD25 (BC96), and IL-4 (8D4-8, eBioscience), Alexa647-conjugated antibody to mouse IL-4 (11B11), APC/Cy7-conjugated antibodies to mouse CD4 (GK1.5) and human CD3ε (OKT3), Pacific blue-conjugated antibodies to mouse CD45.2 (104) and human CD4 (RPA-T4). In some experiments, the frequency of IL-4- and/or IL-5-producing Th2 cells was determined by staining cells with Alexa647-conjugated and APC-conjugated antibody before analyzing them with a same channel in the flow cytometer. IL-4 and/or IL-5-positive cells were designated as IL-4/5$^+$ cells.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in the cultured supernatants or BALF were measured by ELISA kits according to the manufacturer's protocol. For mouse IFNγ, IL-4 and IL-5, ELISA kits are from R&D systems or BioLegend. For mouse IL-17A, ELISA kits from BioLegend were used. For mouse IL-13, human IL-13 and IL-17A, ELISA kits from eBioscience were used.

***In vitro* human naïve CD4$^+$ T cell differentiation and effector memory CD4$^+$ T cell restimulation**

Human peripheral blood was obtained from healthy volunteers who are not taking any medications. Peripheral blood mononuclear cells (PBMCs) were prepared by using lymphocyte separation medium (MP biomedicals) according to the manufacturer's instruction. Subsequently, total CD4$^+$ T cells were isolated by negative selection using CD14 microbeads (Miltenyi biotec) followed by positive selection using CD4 microbeads (Miltenyi biotec). Naïve and effector memory CD4$^+$ T cells were further sorted as CD3$^+$CD4$^+$CD45RA$^+$CD45RO$^-$CD25$^-$ and

$CD3^+CD4^+CD45RA^-CD45RO^+$ cells respectively using FACSAriaIII cell sorter (BD bioscience). For naïve T cell differentiation, FACS-sorted naïve T cells were stimulated with plate-coated anti-CD3 (10 μg/ml, OKT3, BioLegend) and soluble anti-CD28 (2 μg/ml, CD28.2, BioLegend) for 4 or 7 d. For Th2 polarization, IL-2 (10 ng/ml), IL-4 (10 ng/ml), anti-IFNγ (5 μg/ml, B27, BioLegend) were added. For Th17 polarization, IL-2 (10 ng/ml), IL-6 (20 ng/ml), TGF-β (10 ng/ml), IL-23 (20 ng/ml), IL-21 (20 ng/ml), IL-1β (20 ng/ml), anti-IFNγ (5 μg/ml), anti-IL-4 (5 μg/ml MP4-25D2, BioLegend) were added. TGF-β was from Peprotech and the rest of cytokines were from eBioscience. Effector memory $CD4^+$ T cells were stimulated with plate-coated anti-CD3 (10 μg/ml) for 48 h in the presence of UA or DMSO.

Human PBMC isolation and *in vitro* stimulation

Human peripheral blood was obtained from allergic asthma patients who meet following criteria. (1) Asthma was diagnosed with positivity in bronchodilator response or methacholine provocation test. (2) The positivity of *Dermatophagoides farinae* (*D. Farinae*) skin response was determined with skin prick test using cut-off level of both wheal size more than 4mm and allergen/histamine wheal size ratio (A/H ratio) ≥ 1. (3) Systemic corticosteroid was not administrated within recent one month. PBMCs were prepared by using lymphocyte separation medium (MP biomedicals) according to the manufacturer's instruction. 2 x 10^5 cells were stimulated with 50 μg/ml HDM extract (*Dermatophagoides farinae* from Greer laboratories) in the presence of 2 μM UA or DMSO as a vehicle control for 7 d.

Fate mapping study

IL-17F fate reporter mice, *Il17f* ^{Cre}*R26R*^{eYFP}, were sensitized by an intraperitoneal injection of Ova-alum on days 0 and 17 and challenged with intranasal Ova on days 27, 28 and 29. Lung mononuclear cells were prepared using collagenase treatment with manual disruption. Lymphoid cells were further isolated by using lymphocyte separation medium. $CD4^+YFP^+IL\text{-}17A^+$ cells represent stable Th17 cells while $CD4^+YFP^+IL\text{-}17A^-$ · cells represent exTh17 cells.

Adoptive transfer of *in vitro* differentiated Th17 cells

Naive $CD4^+CD25^-CD62L^{high}CD44^{low}$ T cells were isolated from *Il17f* ^{rfp} mice crossed with OT-II mice (*Il17f* ^{rfp} x OT-II). Naïve CD4 T cells were activated with OVA peptide and irradiated APC under Th17 conditions for 4 days, and RFP^+ cells were sorted by FACS. B6.SJL congenic ($CD45.1^+$) recipient mice were sensitized by an intraperitoneal injection of OVA-alum on days 0 and 17 and challenged with intranasal OVA on days 27, 28 and 29. RFP^+ cells (1×10^6) were transferred to the recipient mice on day 16. Asthma induced B6.SJL congenic were assessed for IL-17A, IL-13, IL-4, IFN-γ expression by intracellular staining.

Statistics

Data were graphed and analyzed by Prism 6 software (GraphPad). Statistical significance between two groups was determined by two-tailed student's t-test. For the comparison of more than three groups, one-way ANOVA test and Tukey's test were used. P values less than 0.05 was considered statistically significant.

RESULTS

Il17a$^{-/-}$*Il17f*$^{-/-}$ double knockout mice exhibit reduced Th2 cell responses to protease allergens in the airway.

To investigate the role of Th17 cells in allergic asthma, we employed *Il17a*$^{-/-}$*Il17f*$^{-/-}$ (designated as double knockout; dKO) mice.[23] Wild-type and dKO mice were intranasally injected with mixtures of fungal proteinase from *Aspergillus oryzae* and ovalbumin (PAO/Ova), as a model allergen. Intranasal PAO/Ova is known to induce allergen-specific Th2 cells as well as Th17 cells in the airway, offering an ideal model to study the potential mutual regulation between the two Th cell subsets.[24-26] As expected, wild-type mice expressed increased level of IL-17A- and IL-17F-producers among CD4$^+$ T cells in the lungs, which were absent in dKO mice (Fig. 1A-C). Of note, the frequency and number of Th2 cells were also significantly diminished in the lungs of dKO mice, while those of Th1 cells were increased, compared to wild-type mice (Fig. 1A-C). The levels of IL-4 and IL-5 in the bronchoalveolar lavage (BAL) fluid from dKO mice were also significantly lower than those from wild-type mice (Fig. 1D). Similar decrease in the frequency of Th2 cell was also observed in CD4$^+$ T cells from BAL fluid (see Fig. E1 in the Online Repository at www.jacionline.org). Thus combined deficiency of IL-17A and IL-17F resulted in a significant reduction of Th2 cell population in the airway in an animal model of allergic asthma.

A distinct Th2-Th17 cell population expressing both IL-17A and IL-4/5 was observed in the wild-type mice, but not in the dKO mice (Fig. 1A). This observation raised a hypothesis that the reduced Th2 cell responses in the dKO mice might be due to the lack of these Th2-Th17 cells originated from Th17 cells and that IL-17A^{neg}Th2 cells are partially deviated from Th17 cells, since we also observed a diminished frequency of IL-17A^{neg} Th2 cells (10.03 ± 0.56 (WT) vs

6.93 ± 1.02 (dKO), $p = 0.04$). To test this hypothesis and to define the origin of the Th2-Th17 cell population, we first utilized fate mapping system with *Il17f* ^{Cre}*R26R*^{eYFP} mice[21] that enables us to track cells that activated the expression of IL-17F regardless of their present expression of this cytokine. We found that few eYFP^{+} cells (exTh17 cells) produced Th2 cytokines, indicating that Th17 cells did not become Th2 cytokine-producing cells in the airway (see Fig. E2A in the Online Repository at www.jacionline.org). Next, we adoptively transferred *in vitro* generated ovalbumin-specific Th17 cells purified from *Il17f* ^{rfp}×OT-II mice[22] into congenic mice, before challenging the recipient with intranasal ovalbumin. The donor Th17 cells appeared to be stable and did not produce Th2 cytokines (Fig. E2B and C). These results together indicate that Th2-Th17 cells found in the airway of asthmatic mice are not derived from Th17 cells, and suggest that the reduced Th2 cell responses in the dKO mice are not due to the lack of Th17 cells.

RORγt-deficient mice exhibit reduced Th2 cell responses to allergens in the airway.

Since the lack of IL-17A and IL-17F showed reduced Th2 cell responses in an animal model of allergic asthma, we hypothesized that Th17 cell responses play a crucial role in the generation of Th2 cell responses. To further explore the role of Th17 cell responses on Th2 cells in allergic asthma, we sought to determine the role of RORγt, a signature transcriptional factor responsible for Th17 cell program, on Th2 cell responses during the development of allergic asthma. We administered PAO/Ova intranasally into wild-type and *Rorγt* $^{gfp/gfp}$ (RORγt-deficient) mice. As expected,[27] RORγt-deficient mice showed a profound reduction in the frequency and number of Th17 cells in the lung compared to wild-type mice (Fig. 2A-C). Interestingly, RORγt-deficient mice also exhibited a significantly diminished Th2 cell population, both in frequency and number, while those of Th1 cells were increased compared to wild-type mice (Fig. 2A-C). Consistently,

the amounts of IL-4, IL-5, and IL-17A were all significantly lower in the BAL fluid of RORγt-deficient mice compared to those of wild type mice (Fig. 2D). The numbers of both eosinophils and neutrophils in the BAL fluid were also significantly reduced in the former group (Fig 2E). These results demonstrate that, in addition to diminished Th17 cell responses and neutrophilia, RORγt-deficient mice failed to mount optimal Th2 cell responses and eosinophilia in this animal model of allergic asthma.

An RORγt inhibitor suppresses both Th2 and Th17 cell responses in allergic asthma.

Diminished pulmonary Th2 cell responses in RORγt-deficient mice prompted us to hypothesize that targeting RORγt might be effective in suppressing Th2 cell- as well as Th17 cell-mediated inflammation in the airway. To test this possibility, we employed an RORγt inhibitor ursolic acid (UA) that has been shown to selectively inhibit RORγt *in vitro* and *in vivo* during Th17 cell differentiation.[28] Groups of C57BL/6 mice were intranasally administered with PAO/Ova and were additionally given UA or DMSO as a vehicle (Fig. E3A). Consistent with the results observed in RORγt-deficient mice (Fig. 2), mice treated with UA showed significantly reduced frequencies and numbers of Th2 cells and Th17 cells while showing increased Th1 cells in the BAL (Fig. 3A and B). Similar tendency was also observed in $CD4^+$ T cells in the lung (data not shown). When lymphoid cells from the mediastinal lymph node were restimulated with Ova, $CD4^+$ T cells from the UA-treated mice produced significantly lower levels of IL-4, 5 and 17 while producing significantly higher level of IFN-γ, compared to those from vehicle-treated mice (Fig. E3B). In addition, mice treated with UA exhibited significantly reduced numbers of eosinophils and neutrophils in the BAL fluid, compared with vehicle-treated mice (Fig. 3C).

Moreover, the former group showed significantly reduced infiltration of inflammatory cells around the airway with reduced mucus production than the latter group (Fig. 3D). Hence, treatment with UA prevented the development of Th17 cell and Th2 cell responses and blocked allergic inflammation in an animal model of allergic asthma.

We next asked if the RORγt inhibitor can ameliorate allergic T cell responses in asthmatic mice. Mice were intranasally administered with PAO/Ova to establish allergic airway inflammation before being injected with UA or vehicle (Fig. E3C). Compared to mice treated with vehicle, mice treated with UA showed a moderate but significant reduction in the frequencies and numbers of Th17 and Th2 cells in the BAL, while those of Th1 cells were increased (Fig. 3E and F). Similar tendency was also observed in the Ova-restimulated lymphoid cells of the mediastinal lymph node (Fig. E3D). Consistent with reduced Th2 and Th17 responses in the UA-treated mice, the numbers of eosinophil and neutrophil in the BAL fluid were all remarkably lower (Fig. 3G). Reduction of the infiltrated inflammatory cells as well as mucus production in the UA-treated mice was also observed (Fig. 3H).

To further determine if blockade of RORγt ameliorates allergic Th2 and Th17 cell responses in more chronic settings, we employed two additional allergic asthma models induced by repeated intranasal PAO/Ova challenges or by systemic sensitization with ovalbumin and alum before subsequent challenges with intranasal ovalbumin (Fig. E4A and E4E). In both models, we observed a significant reduction in the numbers of both Th17 and Th2 cells in the BAL fluid of UA-treated mice compared with vehicle-treated mice (Fig. E4B/C and E4F/G). Consistently, the numbers of eosinophils and neutrophils in the BAL fluid were profoundly lower in the former

than the latter (Fig. E4D and E4H). Collectively, these results demonstrate that treatment with an RORγt inhibitor UA significantly ameliorated both eosinophilic and neutrophilic inflammation in the airway in animal models of allergic asthma, associated with reduced Th2 cell and Th17 cell.

Diminished Th2 cell responses by RORγt blockade are not due to increased IFNγ

RORγt-deficient T cells as well as T cells from UA-treated mice produced higher levels of IFNγ compared to their respective control T cells. Since IFNγ is a strong inhibitor of Th2 cell differentiation,[29] it is possible to surmise that the increased IFNγ led to the diminished Th2 cell responses in the UA-treated mice. To address this possibility, we examined if neutralizing IFNγ restores Th2 cell responses in the UA-treated mice. Compared with control IgG treatment, we observed that anti-IFNγ treatment significantly decreased the frequency of IFNγ producers among $CD4^+$ T cells in the UA-treated mice, indicating the IFNγ-neutralizing effect of the antibody (Fig. 4A). By contrast, we observed comparable frequencies and numbers of Th2 cells and Th17 cells between control IgG- and anti-IFNγ-treated group in the UA-treated mice (Fig. 4A and B). Consistently, differential cell count of BAL also showed that mice treated with UA exhibited reduced eosinophilia and neutrophilia regardless of anti-IFNγ treatment (Fig. 4C). Thus, the reduction of Th2 cell responses by the RORγt inhibitor is independent of IFNγ.

T cell intrinsic function of RORγt in Th2 cell differentiation

The observed diminished pulmonary Th2 cell responses in the RORγt-deficient mice as well as in the UA and αIFNγ-treated mice led us to hypothesize RORγt regulates Th2 cell differentiation in a T cell-intrinsic manner. To determine if RORγt play any role in pulmonary Th2 cell responses in a T cell-intrinsic manner *in vivo*, we generated mixed bone marrow chimeric mice

by transferring 1:1 mixture of wild-type ($CD45.1^+$) and RORγt-deficient ($CD45.2^+$) bone marrow cells into bone marrow-ablated *Tcrb*$^{-/-}$ mice (Fig. 5A). Following reconstitution, the chimeric mice were intranasally challenged with PAO/Ova. Compared with wild-type $CD4^+$ T cells, RORγt-deficient $CD4^+$ T cells exhibited significantly reduced Th2 cell and Th17 cell responses in the lung and in the BAL while exhibiting increased Th1 cell responses (Fig. 5B and C). Hence, RORγt in T cells is required for the optimal differentiation of Th2 cells in the airway *in vivo*.

To further investigate the T cell-intrinsic role of RORγt in the differentiation of Th2 cells, we utilized an *in vitro* Th2 cell differentiation system, in which naïve $CD4^+$ T cells from *Rorγt* $^{gfp/gfp}$ mice were stimulated with plate-coated αCD3 in the presence of soluble αCD28, IL-2 and IL-4. Of note, RORγt-deficient T cells showed a significantly lower frequency of IL-4/5-producing Th2 cells than wild-type T cells (Fig. 5D). The production of IL-13 and IL-5 in the cultured supernatants was also significantly decreased in RORγt-deficient T cells (Fig. 5E). In addition, this inefficient differentiation of RORγt-deficient T cell into Th2 cell was independent of IFNγ since anti-IFNγ treatment showed little effect in the production of IL-13 and IL-5 (data not shown). To further examine the role of RORγt in Th2 cell program, we analyzed the levels of mRNA transcripts known to be associated with helper T cell differentiation. Consistent with the protein data, we observed a significant reduction in the levels of *Il4*, *Il5* and *Il13* transcripts in RORγt-deficient T cells compared to those of wild-type T cells (Fig. 5F). The levels of *Gata3* and *Irf4* were also slightly reduced in RORγt-deficient T cells. Interestingly, we observed that the expression of *Bcl6* was significantly higher in RORγt-deficient T cells than wild-type T cells. These data demonstrate that RORγt-deficient T cells express lower levels of Th2 cell signature genes, but express higher level of *Bcl6* than wild-type T cells under Th2 cell differentiation

condition.

Since we observed an increased expression of *Bcl6* transcript in the RORγt-deficient T cells under Th2-skewing condition *in vitro*, we asked if RORγt-deficient T cells expressed increased BCL6 *in vivo*. To this end, we compared the expression of BCL6 in $CD44^{hi}CD62L^{-}CD4^{+}$ T cells between *Rorγt* $^{+/gfp}$ and *Rorγt* $^{gfp/gfp}$ mice. Compared to heterozygotes, RORγt-deficient mice had an increased frequency of BCL6-expressing cells among effector/memory $CD4^{+}$ T cell population (Fig. 5G). When the $CD44^{hi}CD62L^{-}CD4^{+}$ T cells were subjected to plate-coated anti-CD3 stimulation, RORγt-deficient T cells produced significantly less IL-4, IL-13, and IL-17A than wild-type T cells, while the level of IFNγ was comparable (Fig. 5H). Collectively, RORγt deficiency resulted in an increased expression of BCL6 in effector memory T cells associated with diminished Th2 and Th17 cytokine production in steady state *in vivo*.

Downregulation of *Bcl6* restores Th2 cell differentiation in RORγt-deficient T cells.

Since BCL6 is known to suppress the differentiation of naïve T cells into Th2 cells,[30] we hypothesized that the increased *Bcl6* accounts for the decreased Th2 cell differentiation in RORγt-deficient T cells. To test this hypothesis, we utilized two retroviral shRNA constructs targeting *Bcl6* and found that they significantly diminished the expression of BCL6 (Fig. 6A). As depicted in Figure 6B, the knock-down of BCL6 in RORγt-deficient T cells resulted in a remarkable increase in the frequency of IL-13-producing Th2 cells during Th2 cell differentiation *in vitro*. To directly investigate the role of BCL6 in Th2 cell differentiation, wild-type or BCL6-deficient naïve $CD4^{+}$ T cells were subjected to Th2 cell-skewing condition. BCL6-deficient naïve $CD4^{+}$ T cells exhibited an increase in the frequency of IL-4/5 and IL-13

producing cells compared to that of wild-type $CD4^+$ T cells (Fig. 6C). Consistently, the amounts of IL-5 and IL-13 in the supernatants were significantly higher in the BCL6-deficient T cells than wild-type T cells while the production of IL-4 was comparable (Fig. 6D). Thus, downregulation of BCL6 restores the decreased Th2 differentiation in RORγt-deficient T cells. These results strongly support the notion that RORγt facilitates Th2 cell differentiation by inhibiting the expression of BCL6.

Effects of RORγt inhibition in human Th2 cell differentiation.

We finally sought to determine the effects of RORγt inhibition on the differentiation of human Th2 cell from naïve $CD4^+$ T cells. Naïve $CD4^+$ T cells from healthy donors were stimulated under Th17 cell or Th2 cell differentiation condition in the presence or absence of UA. As expected,[28] RORγt inhibition by UA almost completely suppressed IL-17A production under Th17-skewing condition (Fig. 7A). To check the effects of RORγt inhibition on the restimulation of effector/memory Th17 cells, $CD3^+CD4^+CD45RA^-CD45RO^+$ T cells from the same healthy volunteers were stimulated with plate-bound CD3 antibody. IL-17A production was significantly suppressed by the addition of UA in the culture (Fig. 7A). In addition, we also observed that the addition of UA into the Th2-skewing culture significantly inhibited the production of IL-13 compared to vehicle control (Fig. 7B). Consistently, the frequency of $IL\text{-}4^+$ cells was significantly reduced in UA-treated T cells compared to vehicle-treated T cells (Fig. 7C). However, we observed no detectable level of IL-13 from the supernatants of anti-CD3 stimulated effector/memory $CD4^+$ T cells (Fig. 7B). Similarly, another RORγt inhibitor, SR2211 was found to inhibit the differentiation of human Th2 cell as well as Th17 cells *in vitro* (see Fig. E5A and E5B in the Online Repository at www.jacionline.org).[31]

We next sought to explore if the inhibition of RORγt suppresses allergen-specific effector-memory Th2 cells and Th17 cells in asthma patients. Allergic asthma patients were selected based on their response against house dust mite allergen, *Dermatophagoides farinae* (*D. farinae*), in skin-prick-test. Peripheral blood mononuclear cells (PBMCs) from the patients were simulated with 50 μg/ml of *D. farinae* extract in the presence of DMSO or UA before the levels of IL-17A and IL-13 were measured. As shown in Figure 7D, the addition of UA significantly reduced the production of IL-17A from *D. farinae*-specific $CD4^+$ T cells. By contrast, no evident difference was observed in the levels of IL-13 between the DMSO- and UA-treated cells, indicating that the inhibition of RORγt have little role in suppressing allergen-specific effector/memory Th2 cells. Together, these results demonstrate that RORγt inhibition resulted in the suppression of both Th2 cell and Th17 cell differentiation from naïve T cells, while exerting little to no of in suppressing effector/memory Th2 cell responses in humans.

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DISCUSSION

Despite the non-redundant pathogenic roles of Th2 cells and Th17 cells in allergic airway inflammation, it has been unclear how the simultaneous inhibition of these two helper T cell subsets can be achieved. Our findings in the present study are (i) *Il17a*$^{-/-}$*Il17f*$^{-/-}$ dKO mice showed reduction of Th17 and Th2 cell response upon proteinase allergen challenge, (ii) RORγt-deficient mice exhibited significantly diminished Th2 as well as Th17 cells in the airway in response to proteinase allergen, (iii) pharmacological inhibition of RORγt also inhibited Th2 cell as well as Th17 cell responses in the airway in wild-type mice in an IFNγ-independent and T cell-intrinsic manner, (iv) the inhibition of Th2 cell responses in RORγt-deficient T cells was restored by the knock-down of BCL6, and (v) pharmacological inhibition of RORγt also inhibited the differentiation of human Th2 cell as well as Th17 cell differentiation. Overall, our findings indicate that blockade of RORγt can simultaneously suppress Th2 cell as well as Th17 cell responses during allergic asthma.

Among diverse animal models of allergic asthma, we employed PAO/Ova-induced asthma model since this model exhibits both Th2 cell- and Th17 cell-mediated allergic airway inflammation, offering an ideal model to study any mutual regulation between Th2 and Th17 cell responses.[25] In addition, proteinase allergens used in this model contains very low amount of endotoxin, minimizing allergen-independent inflammation which likely interferes with the interpretation of Th2 cell- and Th17 cell-mediated response.[32] RORγt-deficient mice lack lymph nodes;[33] therefore the reduced induction of Th2 cell responses in the RORγt-deficient mice could be due to the lack of airway-draining lymph nodes where antigen-induced T cell differentiation occurs, rather than a role of RORγt in Th2 cell differentiation. To address this issue, we examined Th2

cell responses in RORγt-deficient T cells in the mixed chimeric *Tcrb* $^{-/-}$ mice given 1:1 mixture of bone marrow cells from wild-type and RORγt-deficient mice, which have normal secondary lymphoid organs including the airway draining lymph nodes, and found that RORγt-deficient T cells still exhibited a profound reduction in Th2 cell responses upon intranasal allergen challenges. Therefore, we concluded that RORγt in T cell is required for optimal Th2 cell responses *in vivo.*

Since Th2 and Th17 cell responses play critical and non-redundant roles in the pathogenesis of allergic asthma, there have been a number of attempts to block either pathway in animal models as well as in humans.[15] However, blocking either Th2 or Th17 pathway by anti-IL-13 or anti-IL-17 alone has been shown not to be sufficient for the treatment of allergic asthma because such treatment resulted in the upregulation of the other pathway.[18] Similarly, a recent study by Park et al.[34] demonstrated that co-administration of anti-IL-17 and anti-IL-13 reduced both Th2 cell and Th17 cell responses in the lung in an animal model of antigen-induced pulmonary arterial remodeling while anti-IL-17 or anti-IL-13 alone resulted in the upregulation of Th2 or Th17 downstream effector genes, respectively. Collectively, these reports imply that simultaneous targeting of both Th2 and Th17 cell responses would be a promising strategy for the treatment of allergic asthma, while targeting either pathway alone might be ineffective due to the activation of the other pathway. In this regard, our previous study examined if blockade of STAT3 inhibits both Th2 and Th17 cell responses in the airway since the activation of STAT3 is indispensable for Th17 cell differentiation,[35,36] and STAT3 has been recently shown to be required for optimal Th2 cell differentiation.[37] Although STAT3-deficient T cells exhibited reduced Th2 cell and Th17 cell differentiation in the bronchial lymph nodes, they showed more robust Th2 cell responses in

577 the airway compared with STAT3-sufficient T cells, suggesting that blockade of STAT3 might
578 increase Th2 cell responses in the airway.[26] Unlike STAT3-deficient T cells, we observed a
579 consistent reduction of Th2 cell responses in the airway of RORγt–deficient T cells as well as in
580 mice treated with an RORγt inhibitor. Based on these observations, we propose that blockade of
581 RORγt would offer a promising approach for the treatment of allergic asthma in humans.
582
583 What is the underlying mechanism for the diminished Th2 cell responses by the blockade of
584 RORγt? IFNγ from Th1 cell responses can suppress Th2 cell, as well as Th17 cell responses.[38,39]
585 However, increased Th1 cell responses are not likely the direct mechanism of the diminished Th2
586 cell responses since neutralization of IFNγ failed to restore Th2 cell responses in RORγt-
587 deficient T cells *in vitro* as well as UA-treated mice *in vivo*. Of note, we observed that the level
588 of *Bcl6* transcript was elevated in RORγt-deficient T cells stimulated under Th2-skewing
589 condition, and that the expression of BCL6 in $CD44^{hi}CD4^{+}$ T cells was increased in RORγt-
590 deficient mice in steady state. The antagonism between BCL6 and RORγt or GATA-3 is well
591 documented. BCL6 functions as transcriptional repressor antagonizing the function of RORγt as
592 well as the expression of *Gata3*, leading to the inhibition of Th17 and Th2 cell differentiation,
593 respectively.[40,41] In addition, BCL6-deficient mice develop more robust Th2 cell-mediated
594 inflammatory responses than wild-type mice[42] and IL-13 is the most downregulated molecule by
595 BCL6.[43] Importantly, we found that knock-down of BCL6 significantly restored the frequency of
596 IL-13-producers in RORγt-deficient T cells under Th2-skewing condition, indicating that the
597 increased BCL6 in the absence of RORγt played a critical role in the observed diminished Th2
598 cell responses. Thus we propose that blockade of RORγt inhibits Th2 cell responses, at least in
599 part, by inducing the expression of BCL6 in T cells. At this moment, it is not clear how RORγt

regulates BCL6 and further studies are needed to identify the underlying molecular mechanisms. To rule out any possible non-redundant and compensatory functions between IL-17A and IL-17F,[44-47] we employed *Il17a*$^{-/-}$*Il17f* $^{-/-}$ dKO mice in our allergic asthma model and found a significantly reduced Th2 cell response in the airway of the dKO *in vivo*. *Il17a*$^{-/-}$*Il17f* $^{-/-}$ dKO mice still have intact RORγt, suggesting that the observed diminished Th2 cell responses in the dKO mice are not likely associated with reduced RORγt expression. Thus we speculate that the mechanism governing the reduced Th2 cell responses in the dKO mice differs from that induced by RORγt-deficiency. It is possible that IL-17A and IL-17F stimulate the production of soluble factors such as complement[48] that could facilitate Th2 cell responses from non-immune cells in the lung. Hence, we propose that both of RORγt and Th17 cytokines contribute to the generation of Th2 cell responses in the airway, presumably through distinct mechanisms. BCL6 is essential for the generation of follicular helper T (Tfh) cells[49] as well as follicular regulatory T (Tfr) cells.[50, 51] Hence one can speculate that RORγt may also play an important role in controlling germinal center reactions by modulating the expression of BCL6 in Tfh and/or Tfr cells. Further studies will be needed to dissect the role of RORγt in germinal center reactions.

Importantly, we observed that the pharmacological inhibition of RORγt by UA also significantly decreased the differentiation of human Th2 cells, as well as Th17 cells. UA is known to inhibit the activation of STAT3, NF-κB in addition to RORγt; however, it specifically inhibits RORγt- but not STAT3, nor NF-κB- at concentrations lower than 2 μM, the concentration we used in the present study.[28] We also observed that another RORγt inhibitor SR2211 also inhibited the differentiation of human Th2 cell. Together with the results obtained with RORγt-deficient T

622 cells, these findings strongly support the notion that blockade of RORγt suppresses Th2 cell
623 differentiation program in mice as well as in humans. In addition, the same treatment also
624 suppressed the production of allergen-induced IL-17A from the PBMCs of human allergic
625 asthma patients. The production of Th2 cell cytokines from the PBMCs of human allergic asthma
626 patients was not affected by the treatment of RORγt inhibitor. Clinical longitudinal studies
627 reported that mono-sensitized children with atopic asthma become poly-sensitized over time
628 indicating that new allergen-specific Th2 or Th17 cells continuously develops over the course of
629 time.[52,53] Hence, though the inhibition of RORγt is less efficient in controlling established
630 memory Th2 responses, it could eventually be beneficial for the treatment of established allergic
631 asthma by suppressing newly developing Th2 cells and Th17 cells. Supporting this notion, we
632 observed that the treatment with the RORγt inhibitor reduced all aspects of allergic asthma in our
633 therapeutic model.
634
635 In summary, our study has unveiled a crucial role of RORγt in the differentiation of Th2 cells in
636 allergic asthma. Based on our findings, we propose that targeting RORγt would be beneficial for
637 the treatment of Th2 cell-mediated eosinophilic asthma as well as Th17 cell-mediated
638 neutrophilic asthma. Further immunologic and pharmacologic studies will be needed to identify
639 effective RORγt inhibitors with few side effects before considering therapeutic use for allergic
640 asthma in humans.

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FIGURE LEGENDS

FIG 1. *Il17a*$^{-/-}$*Il17f*$^{-/-}$ dKO mice exhibit reduced Th2 cell responses against intranasal allergens. A, Representative FACS plot of $CD4^+$ T cells from the lung Percentage (**B**) and absolute number (**C**) of $CD4^+$ T cell subsets. **D,** Levels of IFNγ, IL-4 and IL-5 in BAL fluid. The graph shows means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001.

FIG 2. **RORγt-deficient mice exhibit reduced Th2 as well as Th17 responses against intranasal allergens. A**, Representative FACS plot of lymphocytes from the lung. Percentage (**B**) and absolute number (**C**) of lung $CD4^+$ T cell subsets. **D**, Levels of IFNγ, IL-4, IL-5 and IL-17A in the BAL fluid. **E**, Absolute number of total cells, eosinophils, macrophages, lymphocytes and neutrophils in the BAL fluid. Data are representative of three independent experiments. The graph shows means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001 ns, Not significant. mac, macrophages. eo, eosinophils. neu, neutrophils. lym, lymphocytes.

FIG 3. **An RORγt inhibitor, UA suppresses both Th2- and Th17-mediated allergic inflammation in the airway. A-D**, Results from preventive model. **E-H**, Results from therapeutic model. Frequency (**A** and **E**) and absolute number (**B** and **F**) of BAL $CD4^+$ T cell subsets. **C** and **G**, Absolute number of total cells, macrophages, eosinophils, neutrophils and lymphocytes in BAL fluid. **D** and **H**, Histological analysis of the lung. Data are representative of three independent experiments. The graph shows means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001. ns, Not significant.

FIG 4. **Reduction of Th2 cell responses by UA treatment is IFNγ-independent.** Frequency (**A**)

and absolute number (**B**) of BAL $CD4^+$ T cell subsets. **C**, Absolute number of total cells, macrophages, eosinophils, neutrophils and lymphocytes in BAL fluid. Data are representative of three independent experiments. The graph shows means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001.

FIG 5. **T cell intrinsic role of RORγt in Th2 cell differentiation.** (**A-C**) Results from bone-marrow chimeric mice **A**, A schematic outline of mixed bone marrow chimeric mice generation. **B**, Representative FACS plot of lung $CD4^+$ T cells. **C**, Frequency of lung $CD4^+$ T cell subsets. (**D-F**) Differentiation of Th2 cells from naïve RORγt-deficient $CD4^+$ T cells. **D**, The expression of the indicated cytokines in $CD4^+$ T cells after differentiation. **E**, The levels of IL-13 and IL-5 in the supernatants. **F**, mRNA expression of indicated genes examined by quantitative real time PCR. (**G-H**) Analysis of T cells from steady state *Rorγt* $^{+/gfp}$ or *Rorγt*$^{gfp/gfp}$ mice. **G**, Splenocytes were analyzed for their BCL6 expression by flow cytometry. **H**, Sorted $CD44^{hi}CD62L^{-}CD4^+$ T cells were stimulated with plate-coated αCD3, and the levels of the indicated cytokines were measured. Data are representative of at least two independent experiments. The graph shows means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001. nd, not detected.

FIG 6. **BCL6 negatively regulates Th2 cell differentiation in RORγt-deficient T cells. A**, Expression of BCL6 in T cells after transduction with the indicated shRNA. ~~on day 4.~~ **B**, The frequency of IL-13 producing T cells. (**C** and **D**) Naïve $CD4^+$ T cells from WT or *Bcl6*$^{f/f}$*CD4-Cre* were cultured in Th2-skweing condition. **C**, The expression of the indicated cytokines were analyzed by flow cytometry. **D**, The levels of IL-4, IL-5 and IL-13 in the supernatant. Data are representative of at least two independent experiments. The graph shows means ± sem. *P < 0.05,

691 **P < 0.01, ***P < 0.001.

692

693 **FIG 7**. **Blockade of RORγt negatively affects the differentiation of human Th2 cells.** Human
694 naïve $CD4^+$ T cells from healthy volunteers were cultured in Th17- or Th2-skewing condition
695 and effector memory $CD4^+$ T (T_{EM}) cells were stimulated with plate-coated αCD3 (**A-C**). **A**,
696 Levels of IL-17A in the culture media from Th17 condition or T_{EM}. **B**, Levels of IL-13 in the
697 culture media from Th2 condition or T_{EM}. **C**, The percent changes of IL-4 producers in the UA-
698 treated $CD4^+$ T cells. Each symbol represents individual donor. **D,** PBMCs from human allergic
699 asthma patients were stimulated with HDM extract for 7 d. Levels of IL-17A and IL-13. Each
700 symbol represents individual patient. Data are representative of at least three independent
701 experiments. The graph shows means ± sem. **P < 0.01, ***P < 0.001.

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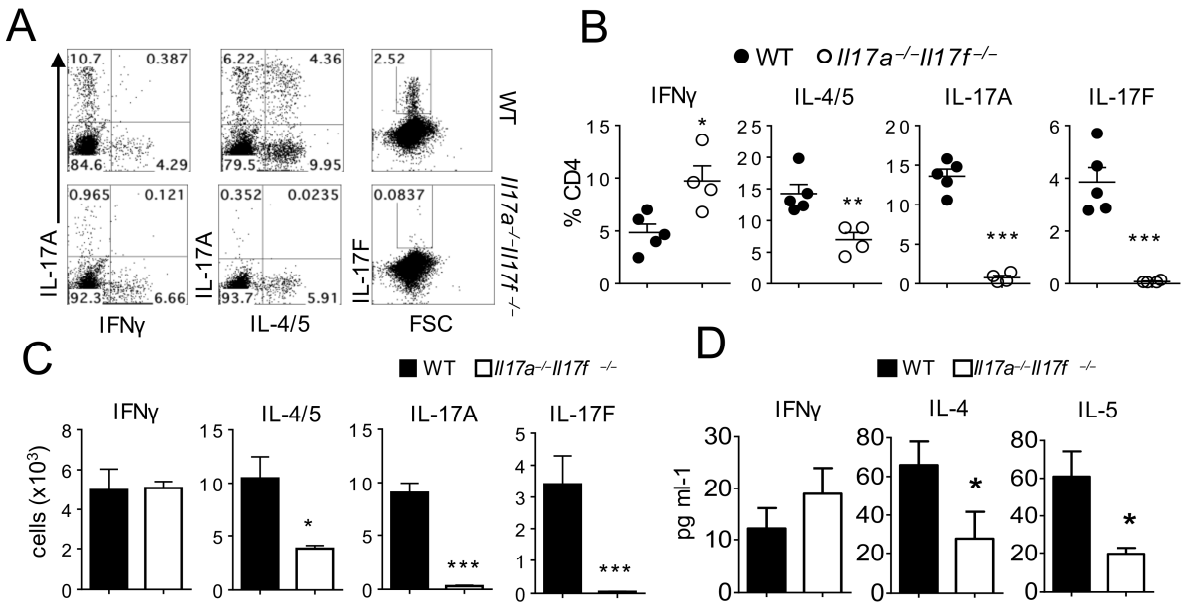
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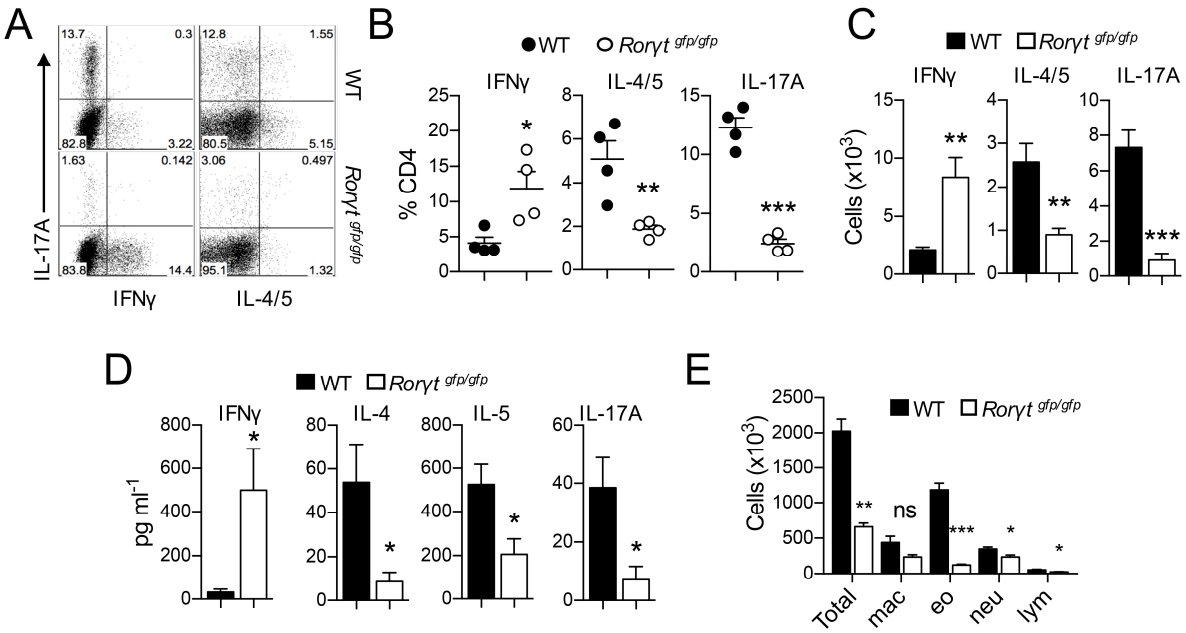
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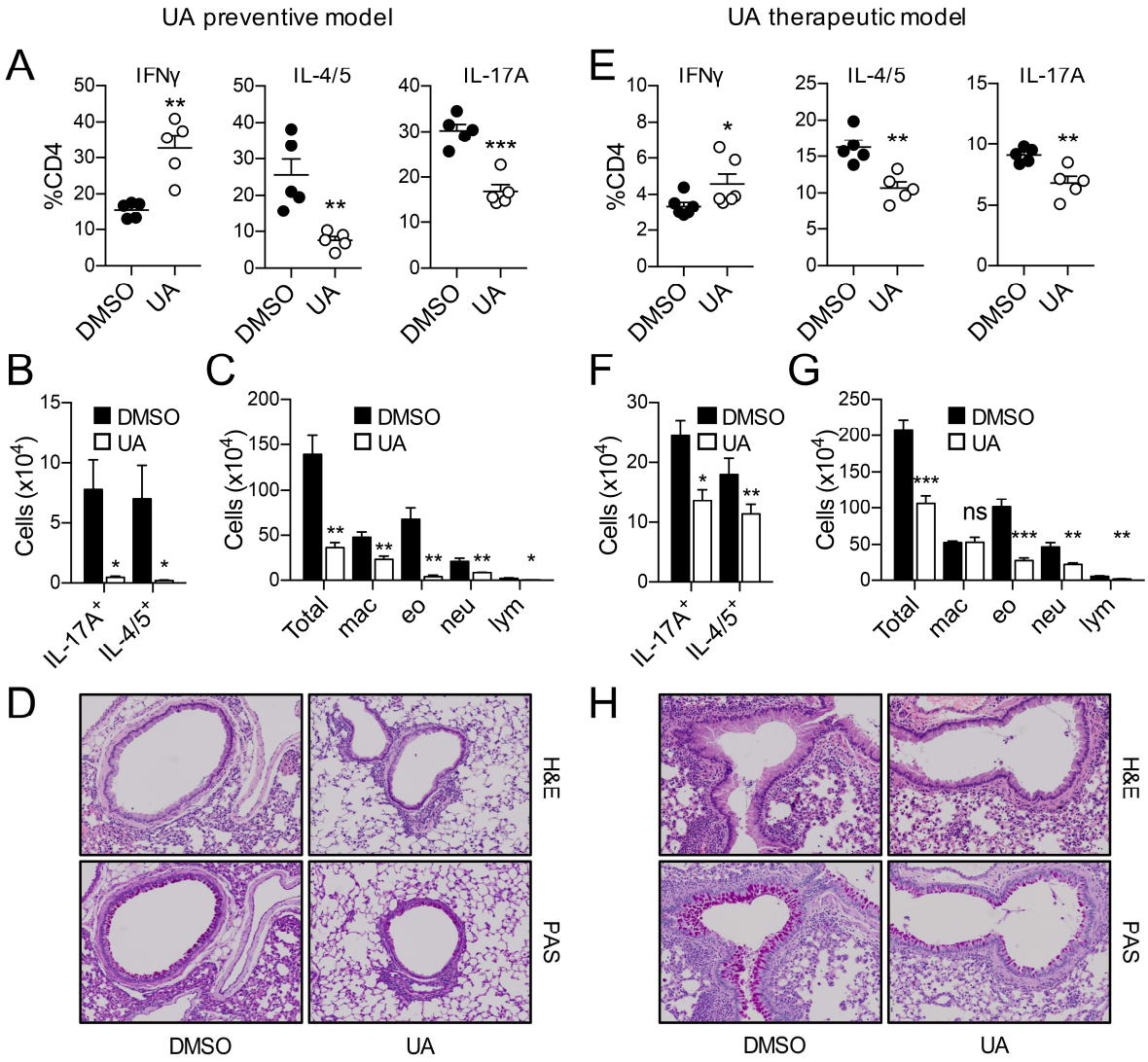


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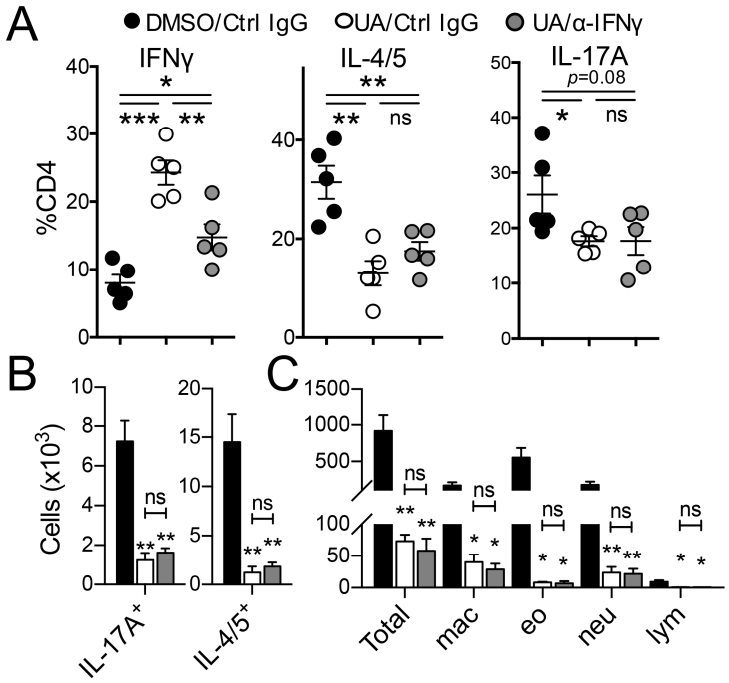
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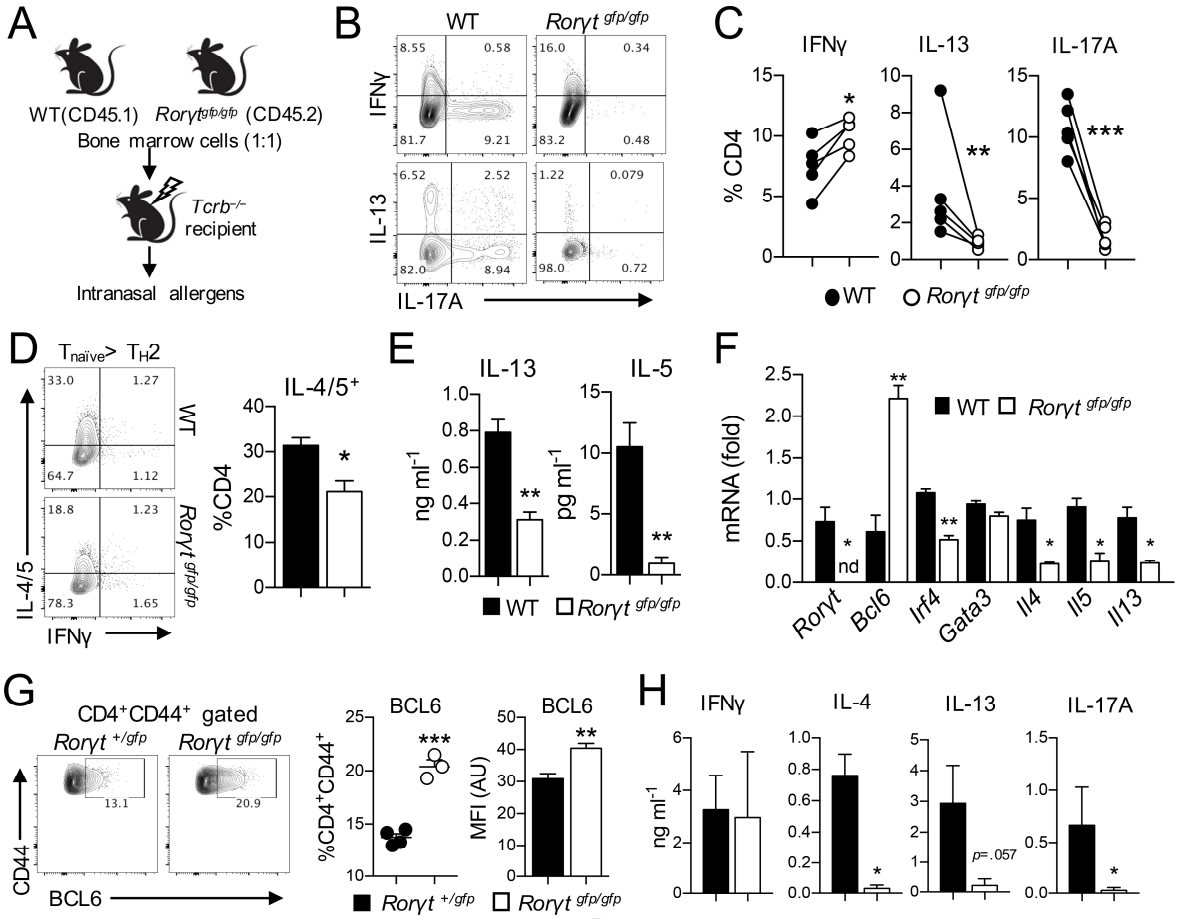
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UA preventive model
UA therapeutic model
A
IFNγ
IL-4/5
IL-17A
%CD4
DMSO
UA
E
B
C
F
G
Cells (x10^4)
IL-17A+
IL-4/5+
Total
mac
eo
neu
lym
ns
D
H
H&E
PAS

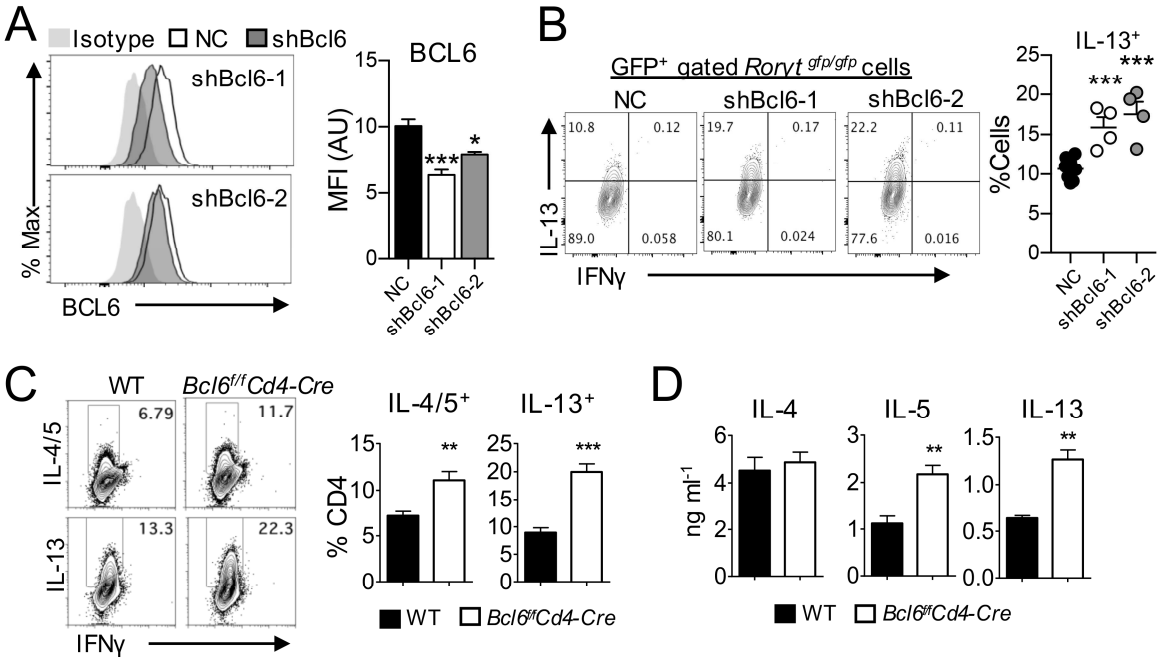
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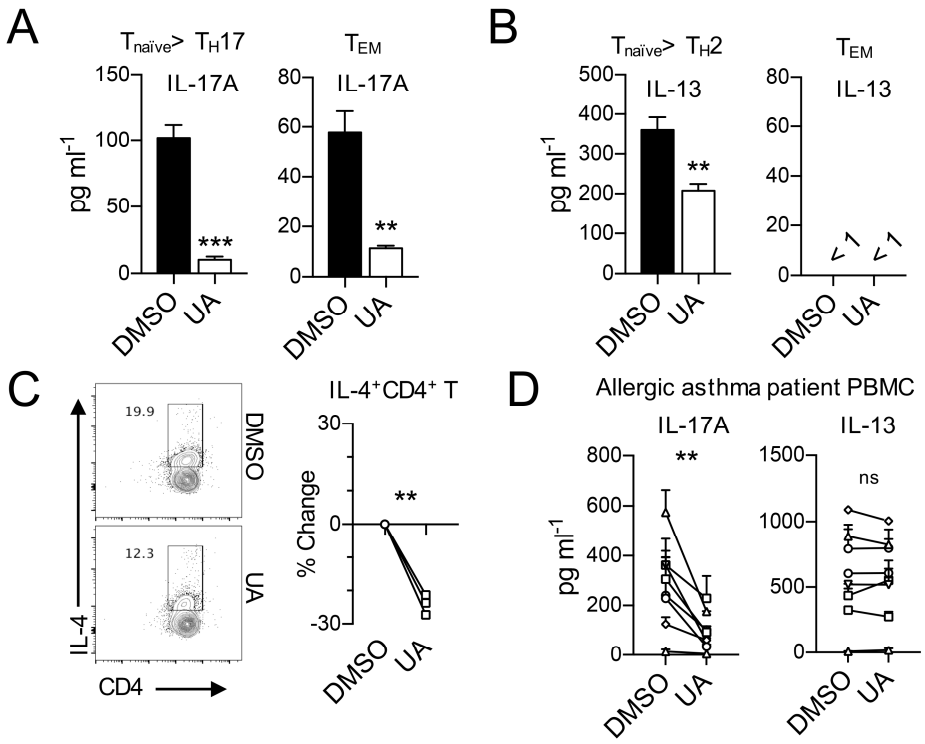
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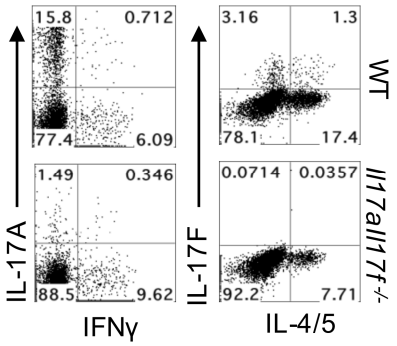
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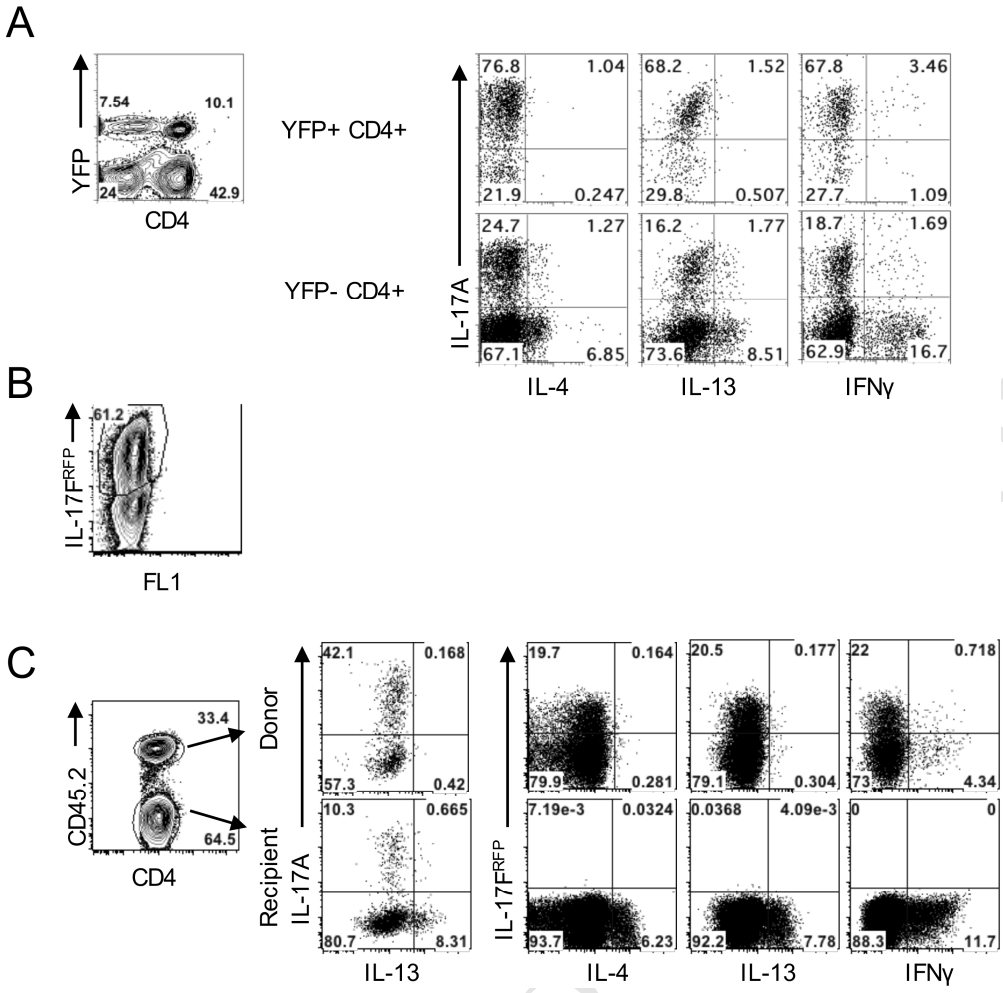
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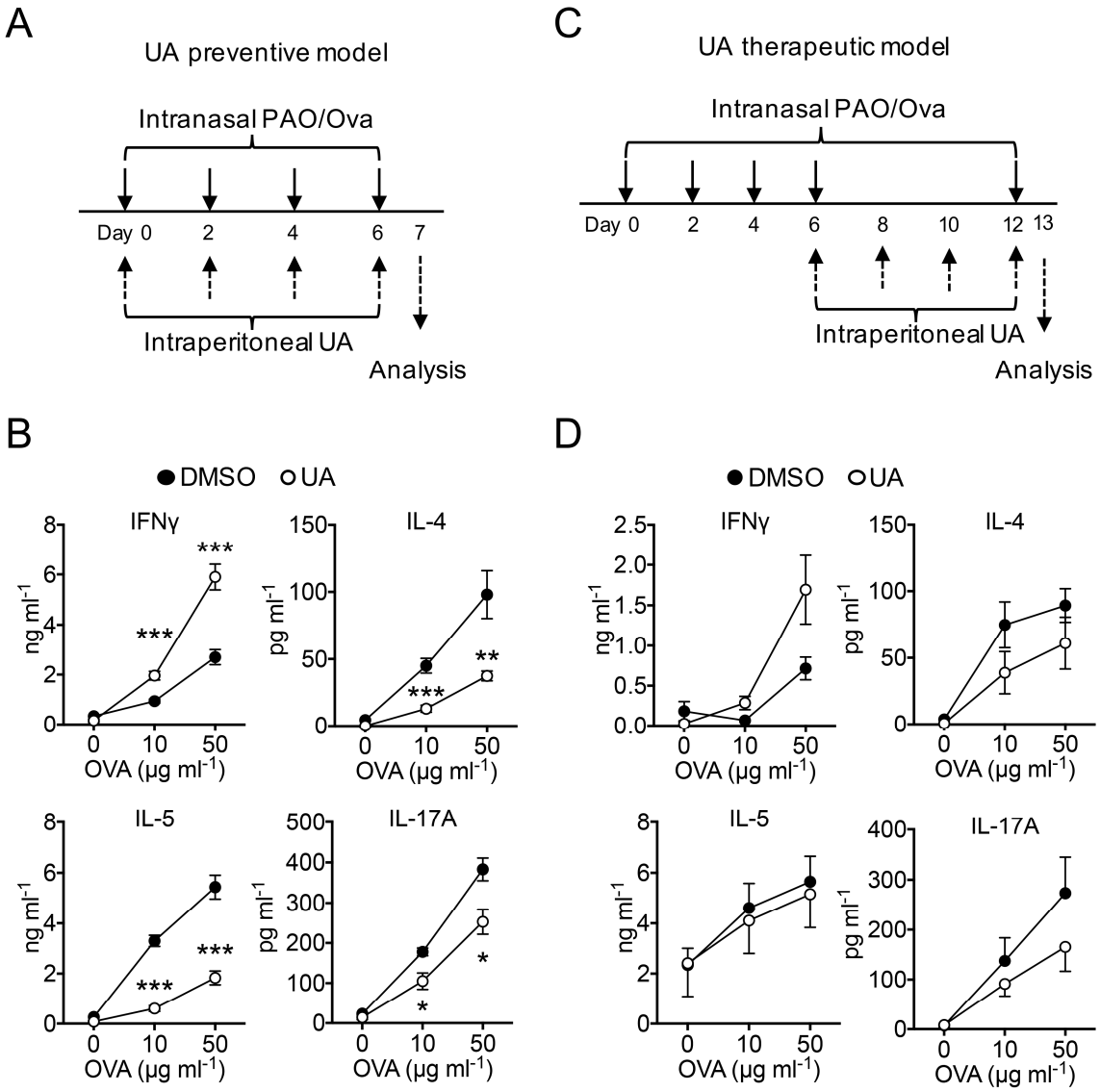
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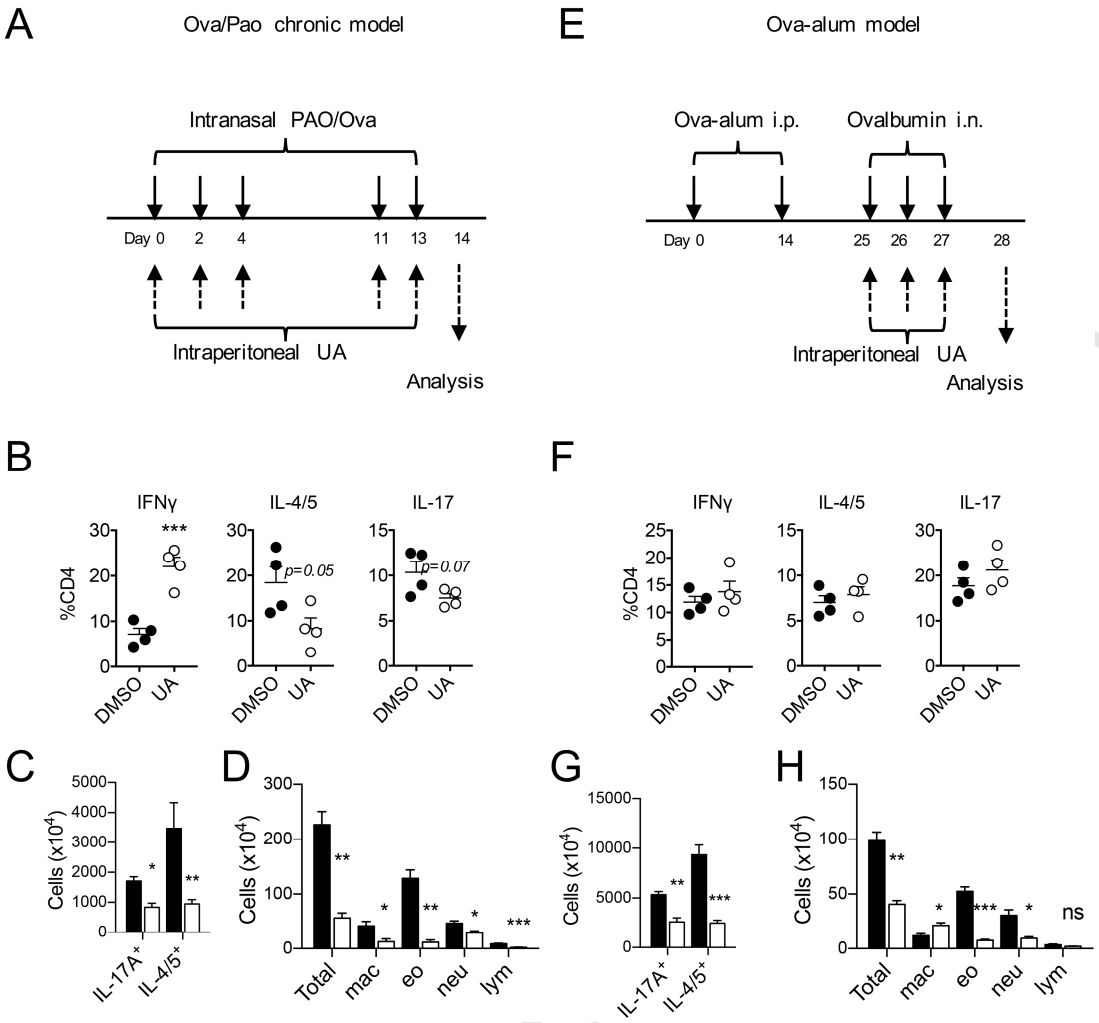
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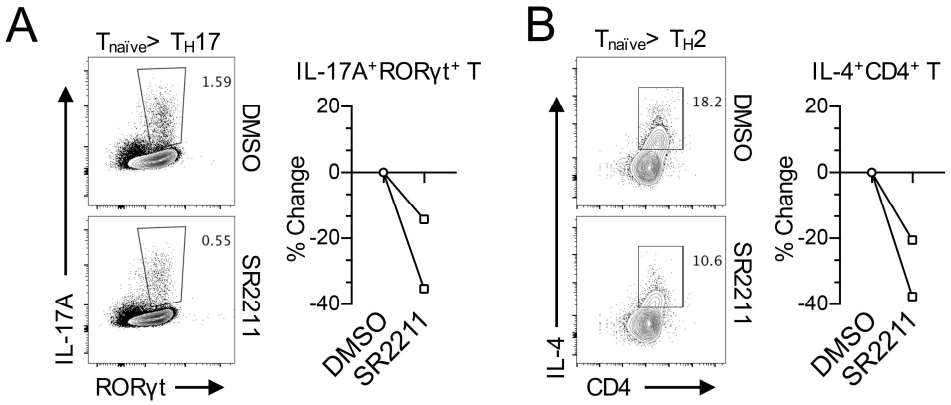

A
UA preventive model
Intranasal PAO/Ova
Day 0
2
4
6
7
Intraperitoneal UA
Analysis
C
UA therapeutic model
Intranasal PAO/Ova
Day 0
2
4
6
8
10
12
13
Intraperitoneal UA
Analysis
B
DMSO
UA
IFNγ
IL-4
IL-5
IL-17A
ng ml-1
pg ml-1
OVA (μg ml-1)
D
DMSO
UA
IFNγ
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OVA (μg ml-1)

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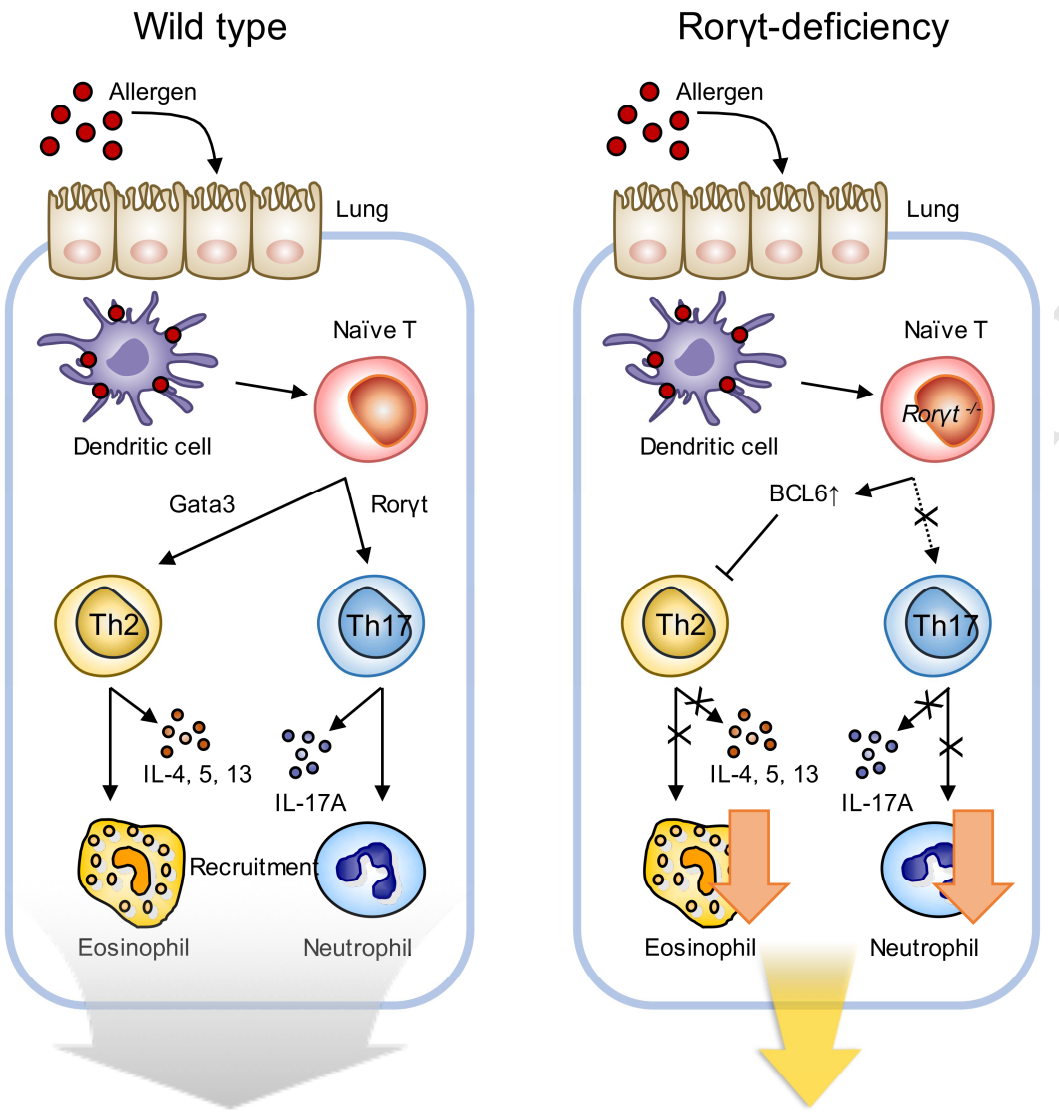


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Wild type
Allergen
Lung
Naïve T
Dendritic cell
Gata3
Rorγt
Th2
Th17
IL-4, 5, 13
IL-17A
Recruitment
Eosinophil
Neutrophil
Asthmatic symptoms
Rorγt-deficiency
Allergen
Lung
Naïve T
Dendritic cell
Rorγt -/-
BCL6↑
Th2
Th17
IL-4, 5, 13
IL-17A
Eosinophil
Neutrophil
Relief of asthma symptoms

SUPPLYMENTARY FIGURE LEGENDS

FIG E1. Representative FACS plot of BAL CD4$^+$ T cells of mice in Fig 1. BAL cells were gated on CD45.2$^+$TCRβ$^+$CD4$^+$. Data are representative of two independent experiments.

FIG E2. Plasticity and stability of Th17 cells during allergic asthma. A, Intracellular staining of IL-17A and other cytokines. **A**, The expression of indicated cytokines in the lung eYFP$^+$ or eYFP$^-$ CD4$^+$ T cells. (**B-C**) Naïve CD4$^+$CD25$^-$CD62L^{high}CD44^{low} T cells from *Il17f* ^{rfp} × OT-II mice were stimulated under Th17 conditions, and RFP$^+$ cells were FACS-sorted (**B**) and transferred into B6.SJL congenic mice (CD45.1$^+$) before challenging the recipients with allergens. The frequencies of the BAL CD4$^+$ T cell subsets were analyzed by flow cytometry (**C**). Data are representative of two independent experiments. n = 3-4 per experiment.

FIG E3. **Preventive and therapeutic effects of UA on allergic asthma.** A Schematic diagram of experimental protocol for UA preventive model used in Fig. 3 A-D (**A**) or for UA therapeutic model used in Fig. 3 E-H (**C**). (**B** and **D**), Lymphoid cells from the mediastinal lymph nodes were restimulated with ovalbumin and the production of IFNγ, IL-4, IL-5 and IL-17A in the culture media was determined. Data are representative of three independent experiments. The graph shows means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001.

FIG E4. Effects of UA on chronic asthma model. A and **E**, Schematic diagrams of experimental protocol for chronic model using PAO/Ova (**B-D**) and OVA-alum (**F-H**) respectively. The frequency (**B** and **F**) and absolute number (**C** and **G**) of BAL CD4$^+$ T cell

subsets. **D** and **H**, Absolute number of total cells, macrophages, eosinophils, neutrophils and lymphocytes in BAL fluid. The graph shows means ± sem. *P < 0.05, **P < 0.01, ***P < 0.001.

FIG E5. SR2211, another RORγt inhibitor, negatively regulates the differentiation of human Th2 cells. A, Representative FACS plot for *in vitro* Th17 differentiation and the percent changes of IL-17^{+}RORγt^{+} T cells. **B**, Representative FACS plot for *in vitro* Th2 differentiation and the percent changes of IL-4 producing T cells. Each symbol represents individual donor.

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