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Concomitant suppression of T_H2 and T_H17 cell responses in allergic asthma by targeting retinoic acid receptor–related orphan receptor γt

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Abstract

Background—Allergic asthma is a heterogeneous chronic inflammatory disease of the airways with a massive infiltration of eosinophils or neutrophils mediated by allergen-specific T_H2 and T_H17 cells, respectively. Therefore successful treatment of allergic asthma will require suppression of both T_H2 and T_H17 cells.

Objective—We sought to investigate the role of the T_H17 cell pathway in regulating T_H2 cell responses in allergic asthma.

Methods—Allergic asthma was induced by intranasal challenge with proteinase allergens in C57BL/6, *Il17a*$^{-/-}$*Il17f*$^{-/-}$, and retinoic acid receptor–related orphan receptor γt *(RORγt)*$^{gfp/gfp}$ mice. A pharmacologic RORγt inhibitor was used to evaluate its preventive and therapeutic effects in allergic asthma. Characteristics of allergic airway inflammation were analyzed by using flow cytometry, histology, quantitative real-time PCR, and ELISA. Mixed bone marrow chimeric mice, fate mapping analysis, short hairpin RNA transduction, and *in vitro* T-cell differentiation were used for mechanistic studies.

Results—Mice deficient in IL-17A and IL-17F, as well as RORγt, exhibited a significant reduction not only in T_H17 cell responses but also in T_H2 cell responses in an animal model of

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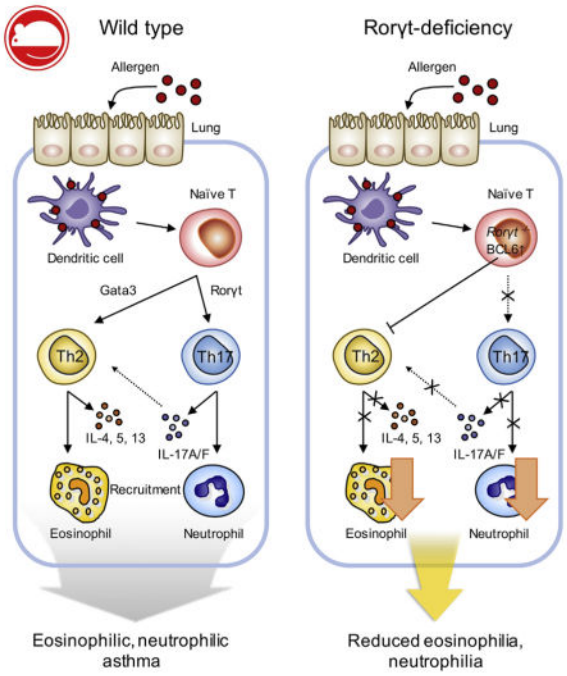
allergic asthma. Similarly, mice treated with an RORγt inhibitor had significantly diminished T_H17 and T_H2 cell responses, leading to reduced neutrophil and eosinophil numbers in the airway. RORγt-deficient T cells were intrinsically defective in differentiating into T_H2 cells and expressed increased levels of B-cell lymphoma 6 *(Bcl6). Bcl6* knockdown resulted in a remarkable restoration of T_H2 cell differentiation in RORγt- deficient T cells. Blockade of RORγt also significantly hampered the differentiation of human T_H2 and T_H17 cells from naive $CD4^+$ T cells.

Conclusion—RORγt in T cells is required for optimal T_H2 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 T_H17 and T_H2 cell responses in the airway.

Graphical Abstract



Keywords

Allergic asthma; T_H17 cell; T_H2 cell; retinoic acid receptor–related orphan receptor γt; B-cell lymphoma 6

Asthma is a heterogeneous disease of the lung and airway characterized by distinct symptoms, such as airway hyperresponsiveness, mucus production, infiltration of inflammatory granulocytes, and shortness of breath, which could be life-threatening.[1,2] The prevalence of this disease has markedly increased over the past several decades, and it has become one of the major global health problems, affecting approximately 300 million persons worldwide.[1]

Asthma can be categorized into allergic and nonallergic 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 pets, or fungi, whereas nonallergic asthma is caused by irritants, such as tobacco smoke, ozone, diesel exhaust particles, or airborne virus.[1,4]

Allergic asthma has been considered to be mediated by T_H2 cells; however, recent studies uncovered the involvement of T_H17 cells as an additional critical contributor in the pathogenesis of allergic asthma in animal models and human subjects.[5] T_H2 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 and IL-13) into the lung and airway, and induce goblet cell hyperplasia and tissue remodeling (IL-13), leading to airway hyperresponsiveness.[2,6] On the other hand, T_H17 cells have been regarded as a critical mediator of steroid-resistant neutrophilic asthma.[7–9] Increased IL-17A levels were observed in the lungs of patients with asthma, and these were positively correlated with neutrophilic inflammation, increased airway hyperresponsiveness, and a steroid-resistant type of severe asthma.[10] A mixed T_H2 and T_H17 cell response in the airways has been associated with the severity of allergic asthma.[9,11] IL-17A stimulates airway epithelial cells to secrete the chemokines CXCL1 and CXCL8, which in turn recruit neutrophils.[12] In addition, IL-17A causes airway remodeling through upregulation of α-smooth muscle actin in fibroblasts.[13] Hence T_H2 and T_H17 cells exert nonredundant pathogenic roles during the development of allergic asthma by inducing eosinophilic and neutrophilic inflammation, respectively.

Because of their critical contributions to the development of allergic asthma, a number of experimental and clinical studies have addressed whether blockade of either the T_H2 or T_H17 cell pathway ameliorates clinical symptoms of the disease. In particular, multiple clinical trials have demonstrated that antibodies against T_H2 cell cytokines (IL-4, IL-5, and 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 they have little to no effect on airway hyperresponsiveness.[14] Antibodies to IL-13 or its receptor lead to reduced airway hyperresponsiveness, with little effect in eosinophilia.[15,16] More recently, a human mAb to IL-17RA did not improve asthmatic symptoms in patients with moderate-to-severe asthma.[17]

In this regard Choy et al[18] recently reported that antibodies to T_H2 cell cytokines enhance T_H17 cell responses and neutrophilia, whereas anti–IL-17A enhances T_H2 cell responses and eosinophilia in animal models of allergic asthma. This study indicates that pulmonary T_H2 and T_H17 cell responses are mutually regulated and suggests that blockade of the T_H cell pathway alone leads to exaggeration of the other pathway. Supporting this notion, GATA-3 and IL-13 are shown to inhibit T_H17 cell differentiation.[19,20] Hence combined blockade of both T_H2 and T_H17 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 the T_H17 pathway in regulating T_H2 cell responses in allergic airway inflammation by using an animal model of proteinase-induced allergic asthma. We also aimed to investigate the clinical relevance of blocking the T_H17 pathway by analyzing T cells from patients with allergic asthma.

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 T_H17 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 nos. SNU-140602-2-7 and SNU-140217-6-8). Collection of human blood samples from healthy volunteers and subsequent experimental procedures were reviewed and approved by the Seoul National University Institutional Review Board. The protocol approval number is 1608/001-006. Collection of blood samples from patients with allergic asthma and related experimental procedures were reviewed and approved by Seoul National University Bundang Hospital Institutional Review Board (no. 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 (Seongnam, Korea). *Rorγt*$^{gfp/gfp}$, B6.SJL, and *Tcrb*$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Me). *Il17a*$^{-/-}$*Il17f*$^{-/-}$ double-deficient mice, IL-17F fate reporter mice, *Il17f*^{Cre}*R26R*^{eYFP} mice, and IL-17F reporter mice (*Il17f*^{fp}) were described previously.[21–23] All mice were maintained in a specific pathogen-free facility at Seoul National University or MD Anderson Cancer Center. Mice aged 6 to 12 weeks were used.

Reagents

Ursolic acid (UA), busulfan (Sigma-Aldrich, St Louis, Mo) and SR2211 (Calbiochem, Nottingham, United Kingdom) were dissolved in dimethyl sulfoxide (DMSO). For *in vitro* use, UA and SR2211 were further diluted with PBS (GenDEPOT, Barker, Tex). *In vitro* cell cultures of lymphoid cells were performed in RPMI 1640 supplemented with 10% FBS (Gen-DEPOT), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 55 μmol/L 2-mercaptoethanol, and 10 μg/mL gentamicin. For PLAT-E cell cultures, Dulbecco modified Eagle medium 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 (Carlsbad, Calif).

Animal models of allergic asthma

To induce allergic lung inflammation, we adopted an animal model of proteinase-induced allergic asthma induced by repeated intranasal challenge with fungal proteinase allergens.[24] In brief, mice were anesthetized with isoflurane (Terrell; Piramal, Bethlehem, Pa) and challenged intranasally with 7 μg of proteinase from *Aspergillus oryzae* (PAO; Sigma-Aldrich) plus 20 μg of ovalbumin (OVA; Grade V from Sigma-Aldrich) in 50 μL of PBS every other day for 4 times (days 0, 2, 4, and 6). For the therapeutic model, mice were challenged on days 0, 2, 4, 6, and 12. In experiments with UA, mice were injected

intraperitoneally with 150 mg/kg UA dissolved in DMSO or DMSO alone as a vehicle control. In some experiments UA was injected on days 0, 2, 4, and 6 in preventive format and days 6, 8, 10, and 12 in therapeutic format as described in Fig E1, *A* and *C*, in this article's Online Repository at www.jacionline.org. For IFN-γ neutralization, anti-mouse IFN-γ (XMG1.2; Bio X Cell, West Lebanon, NH) or rat IgG_1 (HRPN; Bio X Cell) as an isotype control were injected intraperitoneally (200 μg per mouse per injection) on days 0, 2, 4, and 6. Twenty-four hours after the last challenge, mice were killed 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 E2, *A*, in this article's Online Repository at www.jacionline.org). Alternatively, we intraperitoneally immunized mice with an OVA-alum mixture (100 μg of OVA, Imject Alum; Thermo Scientific, Waltham, Mass) on days 0 and 14 and injected them with intranasal OVA (50 μg) and intraperitoneal UA on days 25, 26, and 27 (see Fig E2, *E*). Twenty-four hours after the last challenge, mice were killed with CO_2 for further analysis.

Analysis of bronchoalveolar lavage fluid

A 20-gauge IV catheter (BD Biosciences, San Jose, Calif) was inserted into the trachea and flushed twice with 500 and 800 μL of cold PBS to collect bronchoalveolar lavage (BAL) fluid. Cytokine levels were determined by means of ELISAwith first-flushed 500 μL of BAL fluid. Ten thousand cells in BAL fluid were attached on the slide by Cytopro Centrifuge (Wescor; Logan, Utah) and stained with Diff-Quik staining kits (Sysmex, Kobe, Japan), according to the manufacturer's protocol. Absolute numbers of macrophages, eosinophils, neutrophils, and lymphocyte in BAL fluid were calculated based on total cell counts.

Analysis of lymphoid cells in the lung

The left lobe of the lung was used for histologic analysis. The rest of the lobes were placed in 3 mL of RPMI 1640 containing 0.5 mg/mL collagenase (Type IV; Gibco), 2 mg/mL Dispase (Gibco), and 25 U/ml DNase I (Bio Basic, Markham, Ontario, Canada). Lungs were first dissociated with the gentle-MACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and then digested for 30 minutes at 37°C with continuous agitation, followed by a second dissociation with the gentleMACS 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 (MP Biomedicals, Santa Ana, Calif), according to the manufacturer's instructions.

Histology

The left lobe of the lung was fixed in neutral buffered 10% formalin solution (Sigma) for at least 24 hours. Formalin-fixed samples were sent to the Pathology Center at Seoul National University College of Medicine for hematoxylin and eosin and periodic acid–Schiff staining.

Lymph node restimulation

The mediastinal lymph nodes were minced physically in cold PBS containing 1.5% FBS and filtered through a 100-μm nylon mesh. Lymphoid cells (1×10^6/mL) were cultured in the

presence of 0, 10, and 50 μg/mL OVA for 3 days. Culture supernatants were used for cytokine ELISA.

In vitro murine T_H cell differentiation

$CD4^+$ cells from the spleen and peripheral lymph nodes were positively selected with CD4 microbeads (L3T4; Miltenyi Biotec). Subsequently, naive $CD4^+$ T cells were sorted as $CD4^+CD25^-CD62L^{high}CD44^{low}$ cells with the FACSAria III cell sorter (BD Biosciences) 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 days. For T_H2 differentiation, IL-2 (10 ng/mL; eBioscience, San Diego, Calif) and IL-4 (10 ng/mL; PeproTech, Rocky Hills, NJ) were added. For IFN-γ neutralization experiment, anti–IFN-γ (5 μg/mL, XMG1.2; Bio X Cell) was added also.

Quantitative real-time PCR

Total RNA was prepared with TRIzol Reagents (Invitrogen, Carlsbad, Calif). cDNA was then synthesized with Oligo(dT) primers and reverse transcriptase included in the RevertAid cDNA synthesis kit (Thermo Fisher Scientific), and gene expression levels were examined with the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, Calif) by using iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, Calif). Data were normalized to β-actin *(Actb)* for reference. The following primers were used: *Rorγt*, 5′-CCGCTGAGAGGGCTTCAC-3′ and 5′-TGCAGGAGTAGGCCACATTACA-3′; B-cell lymphoma 6 *(Bcl6)*, 5′-CACACCCGTCCATCATTGAA-3′ and 5′-TGTCCTCACGGTGCCTTT TT-3′; interferon regulatory factor 4 *(Irf4)*, 5′-CACCAAAGCACAG AGTCACCT-3′ and 5′-TCCTCTGGATGGCTCCAGATG-3′; *Gata3*, 5′-AG AACCGGCCCCTTATGAA-3′ and 5′-AGTTCGCGCAGGATGTCC-3′; *Il4*, 5′-AGATCACGGCATTTTGAACG-3′ and 5′-TTTGGCACATCCA TCTCCG-3′; *Il5*, 5′-CGCTCACCGAGCTCTGTTG-3′ and 5′-CCAATG CATAGCTGGTGATTTTT-3′; *Il13*, 5′-GCTTATTGAGGAGCTGAGCA ACA-3′ and 5′-GGCCAGGTCCACACTCCATA-3′; and *Actb*, 5′-TGGA ATCCTGTGGCATCCATGAAAC-3′and 5′-TAAAACGCAGCTCAGTAAC AGTCCG-3′.

Mixed bone marrow chimeric mice

A day before bone marrow transfer, $Tcrb^{-/-}$ mice were injected with 35 mg/kg busulfan (Sigma-Aldrich) to ablate bone marrow cells from recipient mice. Bone marrow cells of B6.SJL ($CD45.1^{+/+}$) and $Ror\gamma t^{gfp/gfp}$ mice were obtained from femurs and tibia by flushing with cold PBS. These bone marrow cells were mixed at a 1:1 ratio and transferred into busulfan-treated $Tcrb^{-/-}$ mice through the tail vein (1.5×10^7 cells per mouse). Six to 8 weeks later, the reconstituted mice were intranasally challenged with PAO/OVA every other day for a total of 6 times. Twenty-four hours after the last challenge, mice were killed by means of CO_2 inhalation for analysis.

BCL6 knockdown in naive T cells by means of retroviral infection

Retroviral supernatants were made by transfecting PLAT-E cell lines with either negative control LMP vector or 2 kinds of LMP-shBcl6. Transfection was performed with FuGene

HD transfection reagent (Promega, Madison, Wis), according to the manufacturer's protocol. Naive T cells were sorted from splenocytes and stimulated with plate-bound αCD3 and soluble αCD28. For T_H2 differentiation, IL-2, IL-4, and αIFN-γ were added. After 24 hours, cells were infected with retroviral supernatants containing 8 μg/mL Polybrene by means of spin infection (1000*g* for 60 minutes at 30°C). After spin infection, cells were washed with PBS and again placed on the αCD3-coated plate with the same initial T_H2 differentiating condition. After another 72 hours, cells were analyzed for cytokine production by using flow cytometry.

Flow cytometry and antibodies

For intracellular cytokine analysis, cells were stimulated with phorbol 12-myristate 13-acetate (100 ng/mL; Sigma-Aldrich) and ionomycin (1 μmol/L; Sigma-Aldrich) plus Brefeldin A and monensin (both from eBioscience) before staining. IC fixation buffer and permeabilization buffer from eBioscience were used for intracellular cytokine staining, and the Foxp3 staining kit (eBioscience) was used for BCL6 staining, according to the manufacturer's instructions. Samples were analyzed with the FACSVerse flow cytometer (BD Biosciences), and acquired data were analyzed with FlowJo software (TreeStar, Ashland, Ore). The following antibodies were used for flow cytometric analysis or cell sorting: Alexa Fluor 488–conjugated antibodies to mouse CD62L (MEL-14) and IFN-γ (XMG1.2); fluorescein isothiocyanate–conjugated antibody to human CD45RA (HI100); phycoerythrin-conjugated rat IgG_1 (eBRG1; eBioscience); antibodies to mouse CD25 (PC61), IL-17A (TC11-18H10.1), and mouse/human BCL6 (IG191E/A8); peridinin-chlorophyll-protein complex/Cy5.5–conjugated antibodies to mouse CD4 (GK1.5), CD45.1 (A20), and IFN-γ; phycoerythrin/Cy7-conjugated antibodies to mouse IL-13 (eBio13A; eBioscience), mouse/human CD44 (IM7), human CD4 (OKT4), and CD45RO (UCHL1); allophycocyanin (APC)–conjugated antibodies to mouse/human IL-5 (TRFK5), human CD25 (BC96), and IL-4 (8D4-8; eBioscience); Alexa Fluor 647–conjugated antibody to mouse IL-4 (11B11); APC/Cy7-conjugated antibodies to mouse CD4 (GK1.5) and human CD3ε (OKT3); and 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 T_H2 cells was determined by staining cells with Alexa Fluor 647– and APC-conjugated antibodies 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.

ELISA

Cytokine levels in cultured supernatants or BAL fluid were measured by using ELISA kits, according to the manufacturer's protocol. For mouse IFN-γ, IL-4, and IL-5, ELISA kits were from R&D Systems (Minneapolis, Minn) or BioLegend (San Diego, Calif). For mouse IL-17A, ELISA kits were from BioLegend. For mouse IL-13 and human IL-13 and IL-17A, ELISA kits were from eBioscience.

In vitro human naive $CD4^+$ T-cell differentiation and effector memory $CD4^+$ T-cell restimulation

Human peripheral blood was obtained from healthy volunteers who are not taking any medications. PBMCs were prepared by using lymphocyte separation medium (MP

Biomedicals), according to the manufacturer's instructions. Subsequently, total $CD4^+$ T cells were isolated by means of negative selection with CD14 microbeads (Miltenyi Biotec), followed by positive selection with CD4 microbeads (Miltenyi Biotec). Naive and effector memory $CD4^+$ T cells were further sorted as $CD3^+CD4^+$ $CD45RA^+CD45RO^-CD25^-$ and $CD3^+CD4^+CD45RA^-CD45RO^+$ cells, respectively, by using the FACSAria III cell sorter (BD Biosciences). For naive T-cell differentiation, naive T cells undergoing fluorescence-activated cell sorting (FACS) 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 days. For T_H2 polarization, IL-2 (10 ng/mL), IL-4 (10 ng/mL), and anti–IFN-γ (5 μg/mL, B27; BioLegend) were added. For T_H17 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–IFN-γ (5 μg/mL), and anti–IL-4 (5 μg/mL, MP4-25D2; BioLegend) were added. TGF-β was from PeproTech, and the rest of the cytokines were from eBioscience. Effector memory $CD4^+$ T cells were stimulated with plate-coated anti-CD3 (10 μg/mL) for 48 hours in the presence of UA or DMSO.

Human PBMC isolation and *in vitro* stimulation

Human peripheral blood was obtained from allergic asthmatic patients who meet the following criteria. First, asthma was diagnosed based on a positive bronchodilator response or methacholine provocation test result. Second, positivity of the *Dermatophagoides farinae* skin response was determined with skin prick tests by using the cutoff level of both a wheal size greater than 4 mm and an allergen/histamine wheal size ratio of 1 or greater. Third, systemic corticosteroids were not administrated in the most recent month. PBMCs were prepared by using Lymphocyte Separation Medium (MP Biomedicals), according to the manufacturer's instruction. Cells (2×10^5) were stimulated with 50 μg/mL HDM extract (*D farinae*; Greer Laboratories, Lenoir, NC) in the presence of 2 μmol/L UA or DMSO as a vehicle control for 7 days.

Fate mapping study

IL-17F fate reporter mice ($Il17f^{Cre}R26R^{eYFP}$) were sensitized by means of 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 by using collagenase treatment with manual disruption. Lymphoid cells

Adoptive transfer of *in vitro*–differentiated T_H17 cells

Naive $CD4^+CD25^-CD62L^{high}CD44^{low}$ T cells were isolated from $Il17f^{rfp}$ mice crossed with OT-II mice ($Il17f^{rfp}$×OT-II mice). Naive CD4 T cells were activated with OVA peptide and irradiated antigen-presenting cells under T_H17 conditions for 4 days, and red fluorescent protein $(RFP)^+$ cells were sorted by using FACS. B6.SJL congenic ($CD45.1^+$) recipient mice were sensitized by means of 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 recipient mice on day 16. Asthma-induced B6.SJL congenic mice were assessed for IL-17A, IL-13, IL-4, and IFN-γ expression by using intracellular staining.

Statistics

Data were graphed and analyzed with Prism 6 software (GraphPad Software, La Jolla, Calif). Statistical significance between 2 groups was determined by using the 2-tailed Student *t* test. For comparison of more than 3 groups, 1-way ANOVA and Tukey tests were used. *P* values of less than .05 were considered statistically significant.

RESULTS

Il17a$^{-/-}$*Il17f*$^{-/-}$ double-knockout mice exhibit reduced T_H2 cell responses to protease allergens in the airway

To investigate the role of T_H17 cells in allergic asthma, we used *Il17a*$^{-/-}$*Il17f*$^{-/-}$(designated as double-knockout [dKO]) mice.[23] Wild-type and dKO mice were intranasally injected with mixtures of fungal PAO/OVA as a model allergen. Intranasal PAO/OVA is known to induce allergen-specific T_H2 cells, as well as T_H17 cells, in the airway, offering an ideal model to study the potential mutual regulation between the 2 T_H cell subsets.[24–26] As expected, wild-type mice expressed increased levels of IL-17A and IL-17F producers among $CD4^+$ T cells in the lungs, which were absent in dKO mice (Fig 1, *A*–*C*). Of note, frequencies and numbers of T_H2 cells were also significantly diminished in the lungs of dKO mice, and frequencies and numbers of T_H1 cells were increased compared with those in wild-type mice (Fig 1, *A*–*C*.) IL-4 and IL-5 levels in BAL fluid from dKO mice were also significantly lower than those in wild-type mice (Fig 1, *D*). A similar decrease in the frequency of T_H2 cells was also observed in $CD4^+$ T cells from BAL fluid (see Fig E3 in this article's Online Repository at www.jacionline.org). Thus combined deficiency of IL-17A and IL-17F resulted in a significant reduction of the T_H2 cell population in the airways in an animal model of allergic asthma.

A distinct T_H2-T_H17 cell population expressing both IL-17A and IL-4/IL-5 was observed in wild-type mice but not dKO mice (Fig 1, *A*). This observation raised the hypothesis that the reduced T_H2 cell responses in dKO mice might be due to the lack of these T_H2-T_H17 cells originating from T_H17 cells and that IL-$17A^-$ T_H2 cells are partially deviated from T_H17 cells because we also observed a diminished frequency of IL-$17A^-$ T_H2 cells (10.03 ± 0.56 [wild-type] vs 6.93 ± 1.02 [dKO], $P = .04$). To test this hypothesis and define the origin of the T_H2-T_H17 cell population, we first used a fate mapping system with *Il17f*^{Cre}*R26R*^{eYFP} mice,[21] which enables us to track cells that activated IL-17F expression regardless of their present expression of this cytokine. We found that few enhanced YFP^+ cells (exT_H17 cells) produced T_H2 cytokines, indicating that T_H17 cells did not become T_H2 cytokine–producing cells in the airway (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). Next, we adoptively transferred *in vitro*–generated OVA-specific T_H17 cells purified from *Il17f*^{fp}×OT-II mice[22] into congenic mice before challenging the recipient with intranasal OVA. Donor T_H17 cells appeared to be stable and did not produce T_H2 cytokines (see Fig E4, *B* and *C*). These results together indicate that T_H2-T_H17 cells found in the airways of asthmatic mice are not derived from T_H17 cells and suggest that the reduced T_H2 cell responses in dKO mice are not due to the lack of T_H17 cells.

Retinoic acid receptor–related orphan receptor γt–deficient mice exhibit reduced T_H2 cell responses to allergens in the airway

Because the lack of IL-17A and IL-17F showed reduced T_H2 cell responses in an animal model of allergic asthma, we hypothesized that T_H17 cell responses play a crucial role in generation of T_H2 cell responses. To further explore the role of T_H17 cell responses on T_H2 cells in allergic asthma, we sought to determine the role of retinoic acid receptor–related orphan receptor γt (RORγt), a signature transcriptional factor responsible for the T_H17 cell program, on T_H2 cell responses during the development of allergic asthma. We administered PAO/OVA intranasally in 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 T_H17 cells in the lung compared with wild-type mice (Fig 2, *A*–*C*). Interestingly, RORγt-deficient mice also exhibited significantly diminished T_H2 cell populations, both in frequency and number, whereas those of T_H1 cells were increased compared with values in wild-type mice (Fig 2, *AC*). Consistently, amounts of IL-4, IL-5, and IL-17A were all significantly lower in BAL fluid of RORγt-deficient mice compared with that of wild-type mice (Fig 2, *D*). Both eosinophil and neutrophil numbers in BAL fluid were also significantly reduced in the former group (Fig 2, *E*). These results demonstrate that, in addition to diminished T_H17 cell responses and neutrophilia, RORγt-deficient mice did not mount optimal T_H2 cell responses and eosinophilia in this animal model of allergic asthma.

An RORγt inhibitor suppresses both T_H2 and T_H17 cell responses in allergic asthma

Diminished pulmonary T_H2 cell responses in RORγt-deficient mice prompted us to hypothesize that targeting RORγt might be effective in suppressing both T_H2 cell– and T_H17 cell–mediated inflammation in the airway. To test this possibility, we used the RORγt inhibitor UA, which has been shown to selectively inhibit RORγt *in vitro* and *in vivo* during T_H17 cell differentiation.[28] Groups of C57BL/6 mice were intranasally administered PAO/OVA and additionally given UA or DMSO as a vehicle (see Fig E1, *A*). Consistent with the results observed in RORγt-deficient mice (Fig 2), mice treated with UA had significantly reduced frequencies and numbers of T_H2 and T_H17 cells while having increased T_H1 cell frequencies and numbers in BAL fluid (Fig 3, *A* and *B*). A similar tendency was also observed in $CD4^+$T cells in the lung (data not shown). When lymphoid cells from the mediastinal lymph nodes were restimulated with OVA, $CD4^+$ T cells from UA-treated mice produced significantly lower levels of IL-4, IL-5, and IL-17 while producing significantly higher levels of IFN-γ compared with those from vehicle-treated mice (see Fig E1, *B*). In addition, mice treated with UA exhibited significantly reduced eosinophil and neutrophil numbers in BAL fluid compared with those in vehicle-treated mice (Fig 3, *C*). Moreover, the former group had significantly reduced infiltration of inflammatory cells around the airways with reduced mucus production than seen in the latter group (Fig 3, *D*). Hence treatment with UA prevented development of T_H17 and T_H2 cell responses and blocked allergic inflammation in an animal model of allergic asthma.

We next investigated whether the RORγt inhibitor can ameliorate allergic T-cell responses in asthmatic mice. Mice were intranasally administered PAO/OVA to establish allergic airway inflammation before being injected with UA or vehicle (see Fig E1, *C*). Compared with mice treated with vehicle, mice treated with UA had a moderate but significant reduction in the

frequencies and numbers of T_H17 and T_H2 cells in BAL fluid, whereas those of T_H1 cells were increased (Fig 3, *E* and *F*). A similar tendency was also observed in OVA-restimulated lymphoid cells of the mediastinal lymph nodes (see Fig E1, *D*). Consistent with reduced T_H2 and T_H17 responses in UA-treated mice, eosinophil and neutrophil numbers in BAL fluid were all remarkably lower (Fig 3, *G*). Reduction of infiltrated inflammatory cell numbers and mucus production in the UA-treated mice were also observed (Fig 3, *H*).

To further determine whether RORγt blockade ameliorates allergic T_H2 and T_H17 cell responses in more chronic settings, we used 2 additional allergic asthma models induced by repeated intranasal PAO/OVA challenges or by systemic sensitization with OVA and alum before subsequent challenges with intranasal OVA (see Fig E2, *A* and *E*). In both models we observed a significant reduction in the numbers of both T_H17 and T_H2 cells in the BAL fluid of UA-treated mice compared with those in vehicle-treated mice (see Fig E2, *B* and *C*, and E2, *F* and *G*, respectively). Consistently, eosinophil and neutrophil numbers in BAL fluid were profoundly lower in the former than in the latter (see Fig E2, *D* and *H*). Collectively, these results demonstrate that treatment with the RORγt inhibitor UA significantly ameliorated both eosinophilic and neutrophilic inflammation in the airways in animal models of allergic asthma associated with reduced T_H2 and T_H17 cell numbers.

Diminished T_H2 cell responses by RORγt blockade are not due to increased IFN-γ levels

RORγt-deficient T cells and T cells from UA-treated mice produced higher levels of IFN-γ compared with their respective control T cells. Because IFN-γ is a strong inhibitor of T_H2 cell differentiation,[29] it is possible to surmise that the increased IFN-γ levels led to diminished T_H2 cell responses in UA-treated mice. To address this possibility, we examined whether neutralizing IFN-γ restores T_H2 cell responses in the UA-treated mice. Compared with control IgG treatment, we observed that anti–IFN-γ treatment significantly decreased the frequency of IFN-γ producers among $CD4^+$T cells in the UA-treated mice, indicating the IFN-γ–neutralizing effect of the antibody (Fig 4, *A*). By contrast, we observed comparable frequencies and numbers of T_H2 and T_H17 cells between the control IgG– and anti–IFN-γ–treated groups in the UA-treated mice (Fig 4, *A* and *B*). Consistently, differential cell counts in BAL fluid also showed that mice treated with UA exhibited reduced eosinophilia and neutrophilia regardless of anti–IFN-γ treatment (Fig 4, *C*). Thus the reduction of T_H2 cell responses by the RORγt inhibitor is independent of IFN-γ.

T cell–intrinsic function of RORγt in T_H2 cell differentiation

The observed diminished pulmonary T_H2 cell responses in RORγt-deficient mice and UA and αIFN-γ–treated mice led us to hypothesize that RORγt regulates T_H2 cell differentiation in a T cell–intrinsic manner. To determine whether RORγt plays any role in pulmonary T_H2 cell responses in a T cell–intrinsic manner *in vivo*, we generated mixed bone marrow chimeric mice by transferring a 1:1 mixture of wild-type ($CD45.1^+$) and RORγt-deficient ($CD45.2^+$) bone marrow cells into bone marrow–ablated $Tcrb^{-/-}$ mice (Fig 5, *A*). After 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 T_H2 and T_H17 cell responses in the lung and BAL fluid while

exhibiting increased T_H1 cell responses (Fig 5, *B* and *C*). Hence RORγt in T cells is required for optimal differentiation of T_H2 cells in the airway *in vivo*.

To further investigate the T cell–intrinsic role of RORγt in differentiation of T_H2 cells, we used an *in vitro* T_H2 cell differentiation system in which naive $CD4^+$ 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/IL-5–producing T_H2 cells than wild-type T cells (Fig 5, *D*). The production of IL-13 and IL-5 in the cultured supernatants was also significantly decreased in RORγt-deficient T cells (Fig 5, *E*). In addition, this inefficient differentiation of RORγt-deficient T cells into T_H2 cells was independent of IFN-γ because anti–IFN-γ treatment had little effect on the production of IL-13 and IL-5 (data not shown).

To further examine the role of RORγt in the T_H2 cell program, we analyzed mRNA transcript levels known to be associated with T_H cell differentiation. Consistent with the protein data, we observed a significant reduction in levels of *Il4*, *Il5*, and *Il13* transcripts in RORγt-deficient T cells compared with those of wild-type T cells (Fig 5, *F*). Levels of *Gata3* and *Irf4* were also slightly reduced in RORγt-deficient T cells. Interestingly, we observed that *Bcl6* expression 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 T_H2 cell signature genes but express a higher level of *Bcl6* than wild-type T cells under T_H2 cell differentiation conditions.

Because we observed an increased expression of *Bcl6* transcript in the RORγt-deficient T cells under T_H2-skewing conditions *in vitro*, we investigated whether RORγt-deficient T cells expressed increased BCL6 *in vivo*. To this end, we compared BCL6 expression in $CD44^{hi}CD62L^-CD4^+$ T cells between *Rorγt*$^{+/gfp}$ and *Rorγt*$^{gfp/gfp}$ mice. Compared with heterozygotes, RORγt-deficient mice had an increased frequency of BCL6-expressing cells among effector/memory $CD4^+$ T cell population (Fig 5, *G*). 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, whereas IFN-γ levels were comparable (Fig 5, *H*). Collectively, RORγt deficiency resulted in increased expression of BCL6 in effector memory T cells associated with diminished T_H2 and T_H17 cytokine production at steady state *in vivo*.

Downregulation of *Bcl6* restores T_H2 cell differentiation in RORγt-deficient T cells

Because BCL6 is known to suppress the differentiation of naive T cells into T_H2 cells,[30] we hypothesized that the increased *Bcl6* expression accounts for the decreased T_H2 cell differentiation in RORγt-deficient T cells. To test this hypothesis, we used 2 retro-viral short hairpin RNA constructs targeting *Bcl6* and found that they significantly diminished the expression of BCL6 (Fig 6, *A*). As depicted in Fig 6, *B*, knockdown of BCL6 in RORγt-deficient T cells resulted in a remarkable increase in the frequency of IL-13–producing T_H2 cells during T_H2 cell differentiation *in vitro*. Wild-type or BCL6-deficient naive $CD4^+$ T cells were subjected to T_H2 cell–skewing conditions to directly investigate the role of BCL6 in T_H2 cell differentiation. BCL6-deficient naive $CD4^+$ T cells exhibited an increase in the frequency of IL-4/IL-5– and IL-13–producing cells compared with that of wild-type $CD4^+$ T

cells (Fig 6, *C*). Consistently, amounts of IL-5 and IL-13 in supernatants were significantly greater in the BCL6-deficient than wild-type T cells, whereas IL-4 production was comparable (Fig 6, *D*). Thus downregulation of BCL6 restores the decreased T_H2 differentiation in RORγt-deficient T cells. These results strongly support the notion that RORγt facilitates T_H2 cell differentiation by inhibiting BCL6 expression.

Effects of RORγt inhibition in human T_H2 cell differentiation

Finally, we sought to determine the effects of RORγt inhibition on the differentiation of human T_H2 cells from naive $CD4^+$ T cells. Naive $CD4^+$ T cells from healthy donors were stimulated under T_H17 or T_H2 cell differentiation conditions in the presence or absence of UA. As expected,[28] RORγt inhibition by UA almost completely suppressed IL-17A production under T_H17-skewing conditions (Fig 7, *A*). To check the effects of RORγt inhibition on restimulation of effector/memory T_H17 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 culture (Fig 7, *A*). In addition, we also observed that addition of UA into the T_H2-skewing culture significantly inhibited the production of IL-13 compared with vehicle control (Fig 7, *B*). Consistently, the frequency of IL-4^+ cells was significantly reduced in UA-treated T cells compared with that in vehicle-treated T cells (Fig 7, *C*). However, we observed no detectable levels of IL-13 from the supernatants of anti-CD3–stimulated effector/memory $CD4^+$ T cells (Fig 7, *B*). Similarly, another RORγt inhibitor, SR2211, was found to inhibit the differentiation of human T_H2 and T_H17 cells *in vitro* (see Fig E5 in this article's Online Repository at www.jacionline.org).[31]

We next sought to explore whether inhibition of RORγt suppresses allergen-specific effector memory T_H2 and T_H17 cells in asthmatic patients. Allergic asthmatic patients were selected based on their response against the house dust mite allergen *D farinae* in skin prick tests. PBMCs from the patients were simulated with 50 μg/mL *D farinae* extract in the presence of DMSO or UA before IL-17A and IL-13 levels were measured. As shown in Fig 7, *D*, addition of UA significantly reduced production of IL-17A from *D farinae*–specific $CD4^+$ T cells. By contrast, no evident difference was observed in IL-13 levels between the DMSO- and UA-treated cells, indicating that RORγt inhibition has little role in suppressing allergen-specific effector/memory T_H2 cells. Together, these results demonstrate that RORγt inhibition resulted in suppression of both T_H2 and T_H17 cell differentiation from naive T cells while exerting little to no suppressing effector/memory T_H2 cell responses in human subjects.

DISCUSSION

Despite the nonredundant pathogenic roles of T_H2 and T_H17 cells in allergic airway inflammation, it has been unclear how the simultaneous inhibition of these 2 T_H cell subsets can be achieved. Our findings in the present study are that (1) $Il17a^{-/-}Il17f^{-/-}$ dKO mice showed reduction of T_H17 and T_H2 cell responses on proteinase allergen challenge, (2) RORγt-deficient mice exhibited significantly diminished T_H2 and T_H17 cells in the airway in response to proteinase allergen, (3) pharmacologic inhibition of RORγt also inhibited

T_H2 and T_H17 cell responses in the airway in wild-type mice in an IFN-γ–independent and T cell–intrinsic manner, (4) inhibition of T_H2 cell responses in RORγt-deficient T cells was restored by knockdown of BCL6, and (5) pharmacologic inhibition of RORγt also inhibited human T_H2 and T_H17 cell differentiation. Overall, our findings indicate that RORγt blockade can simultaneously suppress T_H2 and T_H17 cell responses during allergic asthma.

Among diverse animal models of allergic asthma, we used a PAO/OVA-induced asthma model because this model exhibits both T_H2 and T_H17 cell–mediated allergic airway inflammation, offering an ideal model to study any mutual regulation between T_H2 and T_H17 cell responses.[25] In addition, proteinase allergens used in this model contains very low amounts of endotoxin, minimizing allergen-independent inflammation, which likely interferes with interpretation of T_H2 and T_H17 cell–mediated responses.[32] RORγt-deficient mice lack lymph nodes[33]; therefore the reduced induction of T_H2 cell responses in 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 T_H2 cell differentiation. To address this issue, we examined T_H2 cell responses in RORγt-deficient T cells in the mixed chimeric *Tcrb*$^{-/-}$ mice given a 1:1 mixture of bone marrow cells from wild-type and RORγt-deficient mice, which have normal secondary lymphoid organs, including airway draining lymph nodes, and found that RORγt-deficient T cells still exhibited a profound reduction in T_H2 cell responses on intranasal allergen challenges. Therefore we concluded that RORγt in T cells is required for optimal T_H2 cell responses *in vivo*.

Because T_H2 and T_H17 cell responses play critical and nonredundant 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 human subjects.[15] However, blocking either the T_H2 or T_H17 pathway with 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 upregulation of the other pathway.[18] Similarly, a recent study by Park et al[34] demonstrated that coadministration of anti–IL-17 and anti–IL-13 reduced both T_H2 and T_H17 cell responses in the lung in an animal model of antigen-induced pulmonary arterial remodeling, whereas anti–IL-17 or anti–IL-13 alone resulted in upregulation of T_H2 or T_H17 downstream effector genes, respectively. Collectively, these reports imply that simultaneous targeting of both T_H2 and T_H17 cell responses would be a promising strategy for the treatment of allergic asthma, whereas targeting either pathway alone might be ineffective because of activation of the other pathway.

In this regard our previous study examined whether blockade of signal transducer and activator of transcription 3 (STAT3) inhibits both T_H2 and T_H17 cell responses in the airway because the activation of STAT3 is indispensable for T_H17 cell differentiation[35,36] and STAT3 has been shown recently to be required for optimal T_H2 cell differentiation.[37] Although STAT3-deficient T cells exhibited reduced T_H2 and T_H17 cell differentiation in the bronchial lymph nodes, they showed more robust T_H2 cell responses in the airway compared with STAT3-sufficient T cells, suggesting that STAT3 blockade might increase T_H2 cell responses in the airway.[26] Unlike STAT3-deficient T cells, we observed a consistent reduction of T_H2 cell responses in airways of mice with RORγt–deficient T cells, as well as in mice treated with an RORγt inhibitor. Based on these observations, we propose that

blockade of RORγt would offer a promising approach for the treatment of allergic asthma in human subjects.

What is the underlying mechanism for the diminished T_H2 cell responses caused by RORγt blockade? IFN-γ from T_H1 cell responses can suppress T_H2 and T_H17 cell responses.[38,39] However, increased T_H1 cell responses are not likely the direct the mechanism of diminished T_H2 cell responses because neutralization of IFN-γ did not restore T_H2 cell responses in RORγt-deficient T cells *in vitro*, as well as in UA-treated mice *in vivo*. Of note, we observed that the level of *Bcl6* transcript was increased in RORγt-deficient T cells stimulated under T_H2-skewing conditions and that the expression of BCL6 in $CD44^{hi}CD4^{+}$ T cells was increased in RORγt-deficient mice at steady state. The antagonism between BCL6 and RORγt or GATA-3 is well documented. BCL6 functions as a transcriptional repressor antagonizing the function of RORγt, as well as the expression of *Gata3*, leading to inhibition of T_H17 and T_H2 cell differentiation, respectively.[40,41] In addition, BCL6-deficient mice have more robust T_H2 cell–mediated inflammatory responses than wild-type mice,[42] and IL-13 is the most downregulated molecule by BCL6.[43] Importantly, we found that knockdown of BCL6 significantly restored the frequency of IL-13 producers in RORγt-deficient T cells under T_H2-skewing conditions, indicating that the increased BCL6 expression in the absence of RORγt played a critical role in the observed diminished T_H2 cell responses.

Thus we propose that RORγt blockade inhibits T_H2 cell responses, at least in 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 nonredundant and compensatory functions between IL-17A and IL-17F,[44–47] we used $Il17a^{-/-}Il17f^{-/-}$ dKO mice in our allergic asthma model and found a significantly reduced T_H2 cell response in the airways of dKO mice *in vivo*. $Il17a^{-/-}Il17f^{-/-}$ dKO mice still have intact RORγt, suggesting that the observed diminished T_H2 cell responses in dKO mice are not likely associated with reduced RORγt expression. Thus we speculate that the mechanism governing the reduced T_H2 cell responses in 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 T_H2 cell responses from nonimmune cells in the lung. Hence we propose that both RORγt and T_H17 cytokines contribute to the generation of T_H2 cell responses in the airway, presumably through distinct mechanisms. BCL6 is essential for the generation of follicular helper T cells,[49] as well as follicular regulatory T cells.[50,51] Hence one can speculate that RORγt can also play an important role in controlling germinal center reactions by modulating the expression of BCL6 in follicular helper T cells, follicular regulatory T cells, or both. Further studies will be needed to dissect the role of RORγt in germinal center reactions.

Importantly, we observed that pharmacologic inhibition of RORγt by UA also decreased significantly the differentiation of human T_H2 and T_H17 cells. UA is known to inhibit activation of STAT3 and nuclear factor κB in addition to RORγt; however, it specifically inhibits RORγt but not STAT3 or nuclear factor κB concentrations of less than 2 μmol/L, the concentration we used in the present study.[28] We also observed that another RORγt inhibitor, SR2211, also inhibited human T_H2 cell differentiation. Together with the results

obtained with RORγt-deficient T cells, these findings strongly support the notion that RORγt blockade suppresses the T_H2 cell differentiation program in mice and human subjects. In addition, the same treatment also suppressed production of allergen-induced IL-17A from the PBMCs of patients with allergic asthma. Production of T_H2 cell cytokines from the PBMCs of patients with allergic asthma was not affected by treatment with the RORγt inhibitor. Clinical longitudinal studies reported that monosensitized children with atopic asthma become polysensitized over time, indicating that new allergen-specific T_H2 or T_H17 cells continuously develops over the course of time.[52,53] Hence, although inhibition of RORγt is less efficient in controlling established memory T_H2 responses, it could eventually be beneficial for treatment of established allergic asthma by suppressing newly developing T_H2 and T_H17 cells. Supporting this notion, we observed that treatment with the RORγt inhibitor reduced all aspects of allergic asthma in our therapeutic model.

In summary, our study has unveiled a crucial role of RORγt in differentiation of T_H2 cells in allergic asthma. Based on our findings, we propose that targeting RORγt would be beneficial for the treatment of T_H2 cell–mediated eosinophilic asthma, as well as T_H17 cell–mediated neutrophilic asthma. Further immunologic and pharmacologic studies will be needed to identify effective RORγt inhibitors with few side effects before considering therapeutic use for allergic asthma in human subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

APC	Allophycocyanin
BAL	Bronchoalveolar lavage
BCL6	B-cell lymphoma 6
dKO	Double knockout
DMSO	Dimethyl sulfoxide
FACS	Fluorescence-activated cell sorting
OVA	Ovalbumin

PAO	Proteinase from *Aspergillus oryzae*
RFP	Red fluorescent protein
RORγt	Retinoic acid receptor–related orphan receptor γt
STAT3	Signal transducer and activator of transcription 3
UA	Ursolic acid
YFP	Yellow fluorescent protein

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Key messages

- RORγt in T cells is necessary for optimal T_H2 cell responses during allergic asthma.
- Inhibition of T_H2 cell responses in RORγt-deficient T cells depends on BCL6.
- Targeting RORγt might be a promising strategy for the treatment of allergic asthma through concomitant suppression of T_H2 and T_H17 cells in the airway.

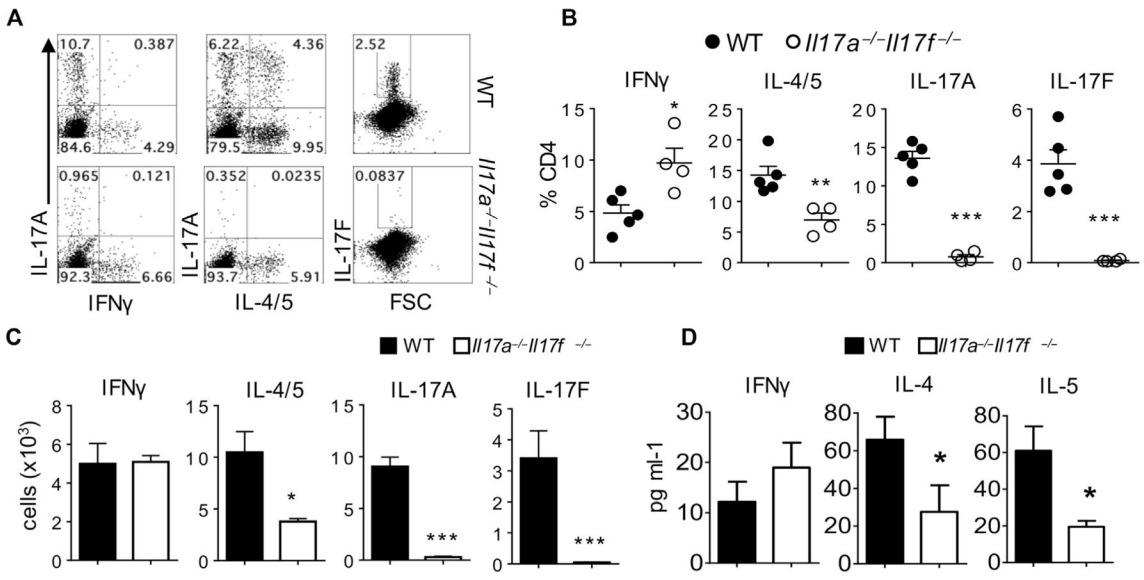

FIG. 1.

Il17a$^{-/-}$*Il17f*$^{-/-}$ dKO mice exhibit reduced T_H2 cell responses against intranasal allergens. **A,** Representative FACS plot of $CD4^+$ T cells from the lung. *FSC*, Forward scatter. **B** and **C,** Percentages (Fig 1, *B*) and absolute numbers (Fig 1, *C*) of $CD4^+$ T-cell subsets. **D,** Levels of IFN-γ, IL-4, and IL-5 in BAL fluid. The graph shows means ± SEMs. **P* < .05, ***P* < .01, and ****P* < .001. *WT*, Wild-type. were further isolated by using lymphocyte separation medium. $CD4^+$ yellow fluorescent protein $(YFP)^+$ IL-$17A^+$ cells represent stable T_H17 cells, whereas $CD4^+YFP^+$IL-$17A^-$ cells represent exT_H17 cells.

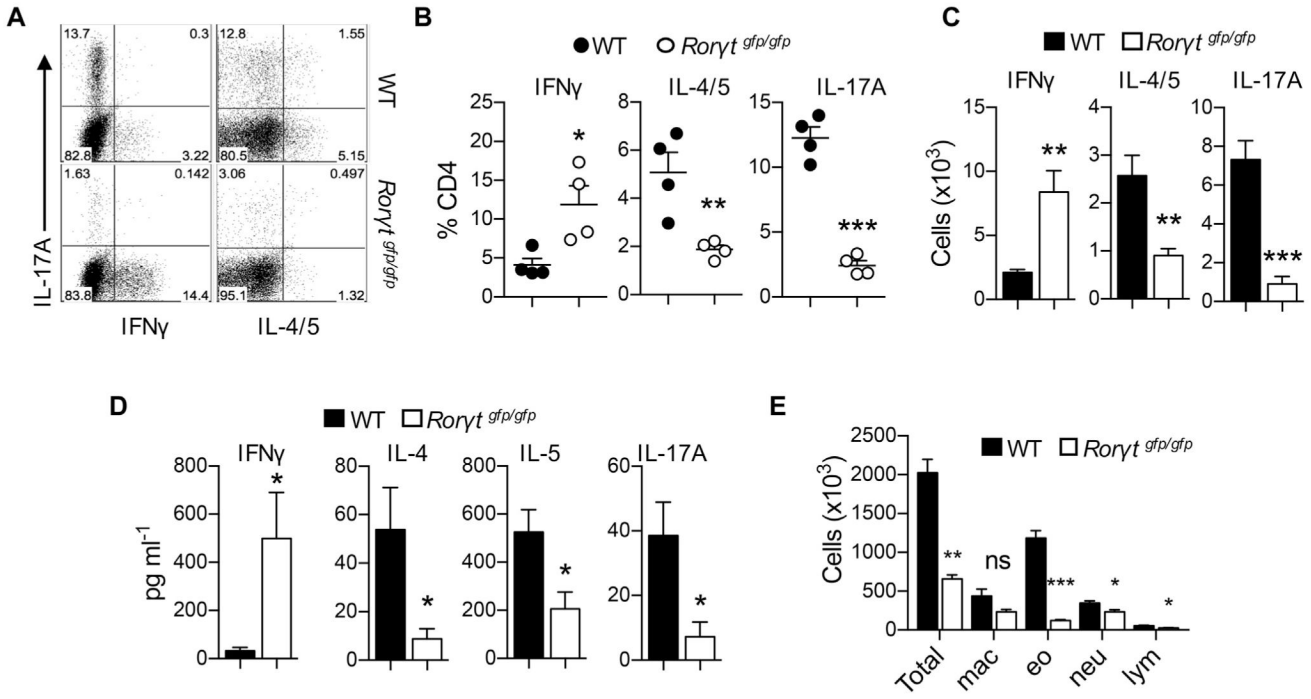

FIG. 2.

RORγt-deficient mice exhibit reduced T_H2 and T_H17 responses against intranasal allergens. **A,** Representative FACS plot of lymphocytes from the lung. **B** and **C,** Percentages (Fig 2, *B*) and absolute numbers (Fig 2, *C*) of lung $CD4^+$ T-cell subsets. **D,** Levels of IFN-γ, IL-4, IL-5, and IL-17A in BAL fluid. **E,** Absolute numbers of total cells, eosinophils *(eo)*, macrophages *(mac)*, lymphocytes *(lym)*, and neutrophils *(neu)* in BAL fluid. Data are representative of 3 independent experiments. The graph shows means ± SEMs. *$P < .05$, **$P < .01$, and ***$P < .001$. *ns*, Not significant.

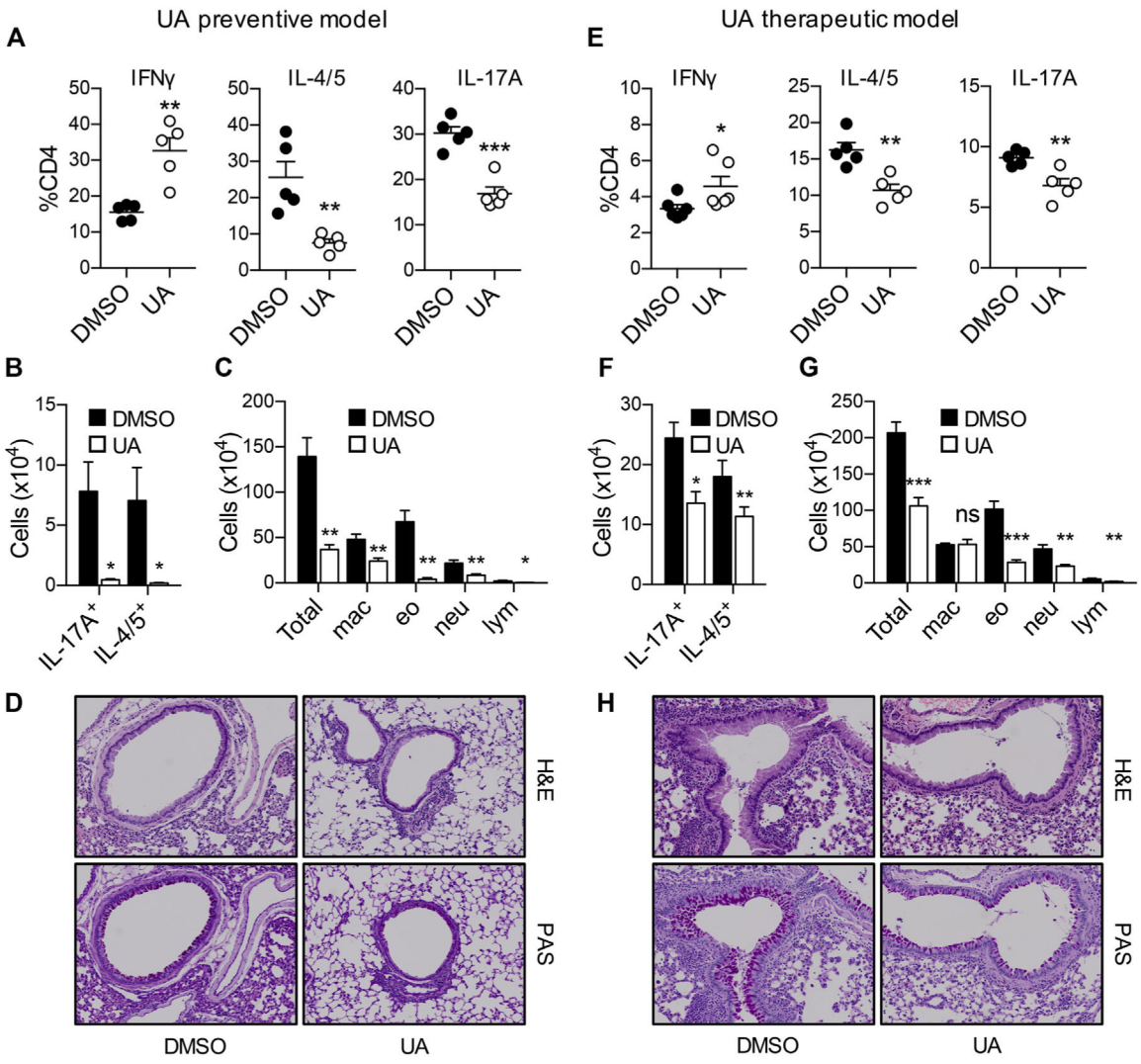

FIG. 3.

The RORγt inhibitor UA suppresses both T_H2- and T_H17-mediated allergic inflammation in the air-ways. **A–D,** Results from preventive model. **E–H,** Results from therapeutic model. Frequencies (Fig 3, *A* and *E*) and absolute numbers (Fig 3, *B* and *F*) of BAL fluid $CD4^+$ T-cell subsets. Fig 3, *C* and *G*, Absolute numbers of total cells, macrophages *(mac)*, eosinophils *(eo)*, neutrophils *(neu)*, and lymphocytes *(lym)* in BAL fluid. Fig 3, *D* and *H*, Histologic analysis of the lung. Data are representative of 3 independent experiments. *H&E*, Hematoxylin and eosin; *PAS*, periodic acid–Schiff. The graph shows means ± SEMs. $*P < .05$, $**P < .01$, and $***P < .001$. *ns*, Not significant.

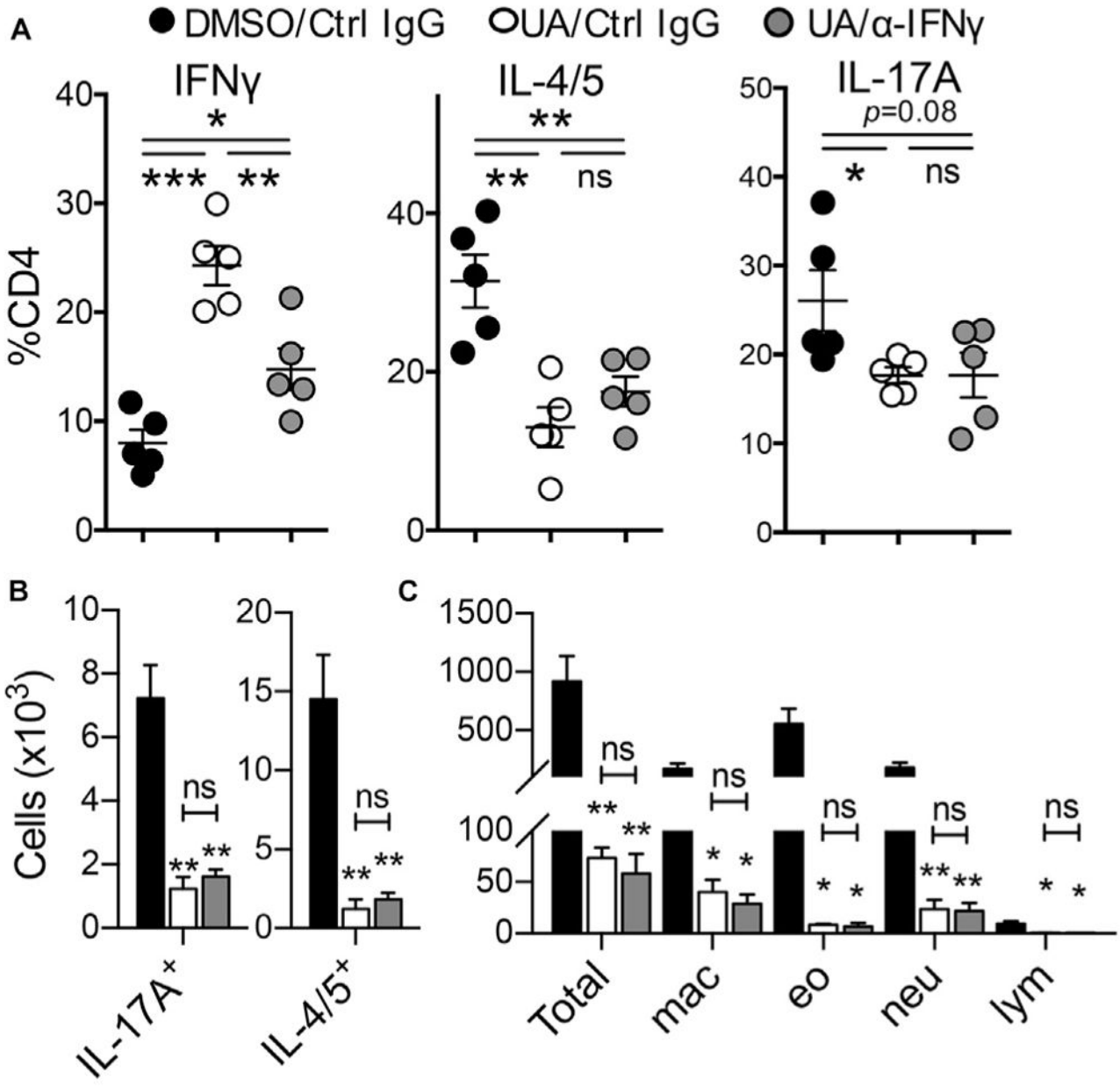

FIG. 4.

Reduction of T_H2 cell responses by UA treatment is IFN-γ independent. **A** and **B,** Frequency (Fig 4, *A*) and absolute number (Fig 4, *B*) of BAL fluid $CD4^+$ T-cell subsets. **C,** Absolute numbers of total cells, macrophages *(mac)*, eosinophils *(eo)*, neutrophils *(neu)*, and lymphocytes *(lym)* in BAL fluid. Data are representative of 3 independent experiments. The graph shows means ± SEMs. **P* < .05, ***P* < .01, and ****P* < .001. *ns*, Not significant.

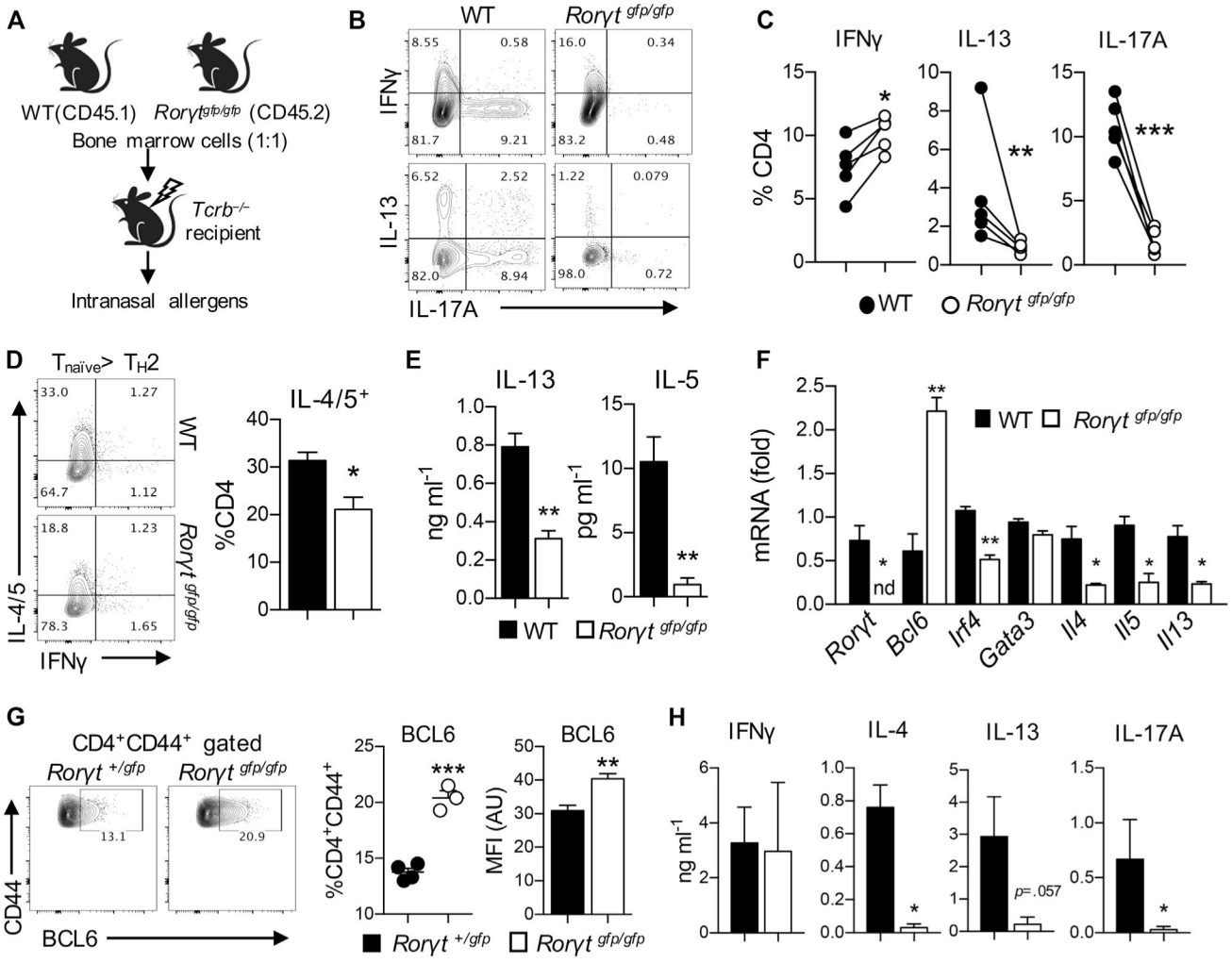

FIG. 5.

T cell–intrinsic role of RORγt in T_H2 cell differentiation. **A–C,** Results from bone marrow chimeric mice. Fig 5, *A*, Schematic outline of mixed bone marrow chimeric mouse generation. Fig 5, *B*, Representative FACS plot of lung $CD4^+$ T cells. Fig 5, *C*, Frequency of lung $CD4^+$ T-cell subsets. **D–F,** Differentiation of T_H2 cells from naive RORγt-deficient $CD4^+$ T cells. Fig 5, *D*, Expression of the indicated cytokines in $CD4^+$ T cells after differentiation. Fig 5, *E*, Levels of IL-13 and IL-5 in supernatants. Fig 5, *F*, mRNA expression of indicated genes examined by using quantitative real-time PCR. **G** and **H,** Analysis of T cells from steady-state *Rorγt*$^{+/gfp}$ or *Rorγt*$^{gfp/gfp}$ mice. Fig 5, *G*, Splenocytes were analyzed for their BCL6 expression by using flow cytometry. Fig 5, *H*, Sorted $CD44^{hi}CD62L^{-}CD4^+$ T cells were stimulated with plate-coated αCD3, and levels of the indicated cytokines were measured. Data are representative of at least 2 independent experiments. The graph shows means ± SEMs. *WT*, Wild-type. $*P < .05$, $**P < .01$, and $***P < .001$. *nd*, Not detected.

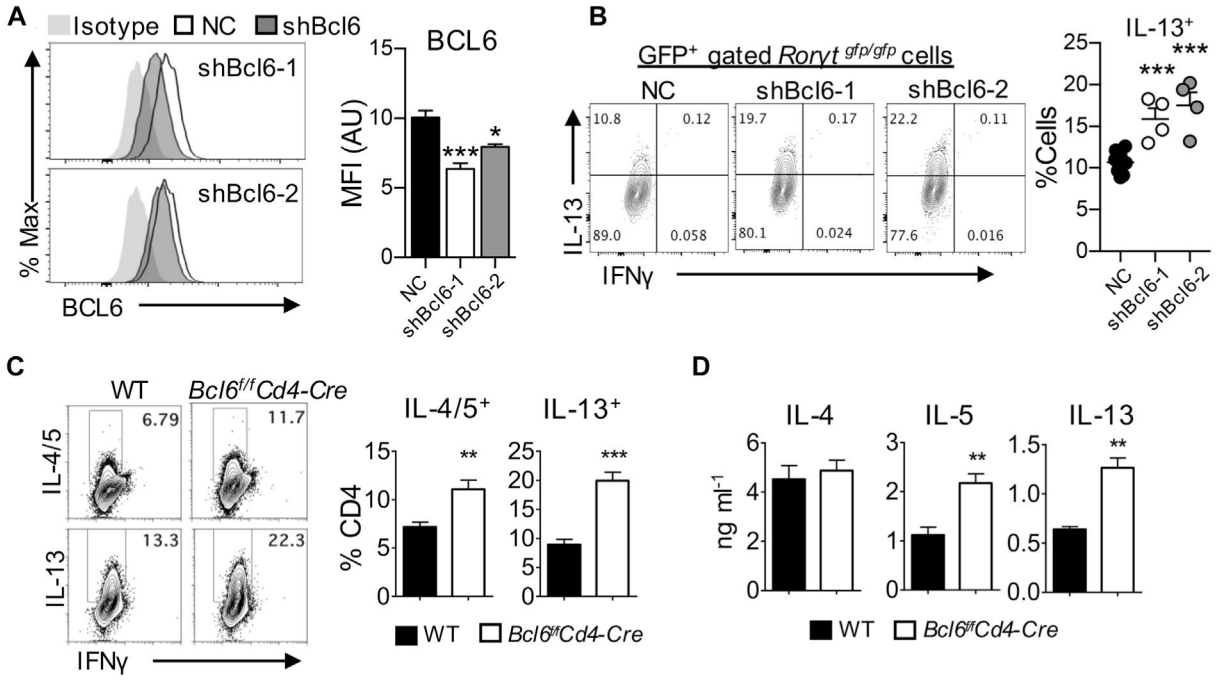

FIG. 6.

BCL6 negatively regulates T_H2 cell differentiation in RORγt-deficient T cells. **A,** Expression of BCL6 in T cells after transduction with the indicated short hairpin RNA. **B,** Frequency of IL-13–producing T cells. *GFP*, Green fluorescent protein. **C** and **D,** Naive $CD4^+$ T cells from wild-type *(WT)* or *Bcl6$^{f/f}$CD4-Cre* mice were cultured in T_H2-skweing conditions. Fig 6, *C*, Expression of the indicated cytokines were analyzed by using flow cytometry. Fig 6, *D*, Levels of IL-4, IL-5, and IL-13 in supernatants. Data are representative of at least 2 independent experiments. The graph shows means ± SEMs. **P* < .05, ***P* < .01, and ****P* < .001.

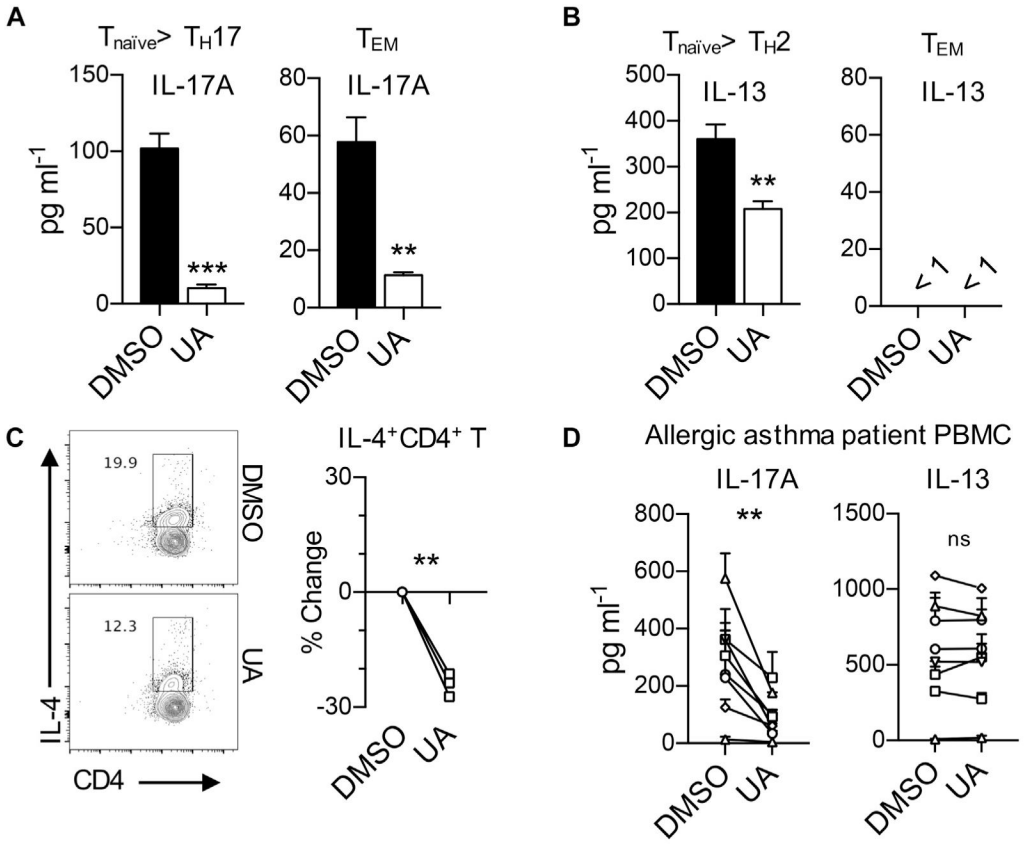

FIG. 7.

Blockade of RORγt negatively affects differentiation of human T_H2 cells. **A–C,** Human naive $CD4^+$ T cells from healthy volunteers were cultured in T_H17- or T_H2-skewing conditions, and effector memory $CD4^+$ T *(T_{EM})* cells were stimulated with plate-coated αCD3. Fig 7, *A*, Levels of IL-17A in culture media from T_H17 or T_{EM} conditions. Fig 7, *B*, Levels of IL-13 in culture media from T_H2 or T_{EM} conditions. Fig 7, *C*, Percentage changes in IL-4 producers in UA-treated $CD4^+$ T cells. Each *symbol* represents an individual donor. **D,** PBMCs from human allergic asthmatic patients were stimulated with HDM extract for 7 days. Levels of IL-17A and IL-13 are shown. Each *symbol* represents an individual patient. Data are representative of at least 3 independent experiments. The graph shows means ± SEMs. $^{**}P < .01$ and $^{***}P < .001$.