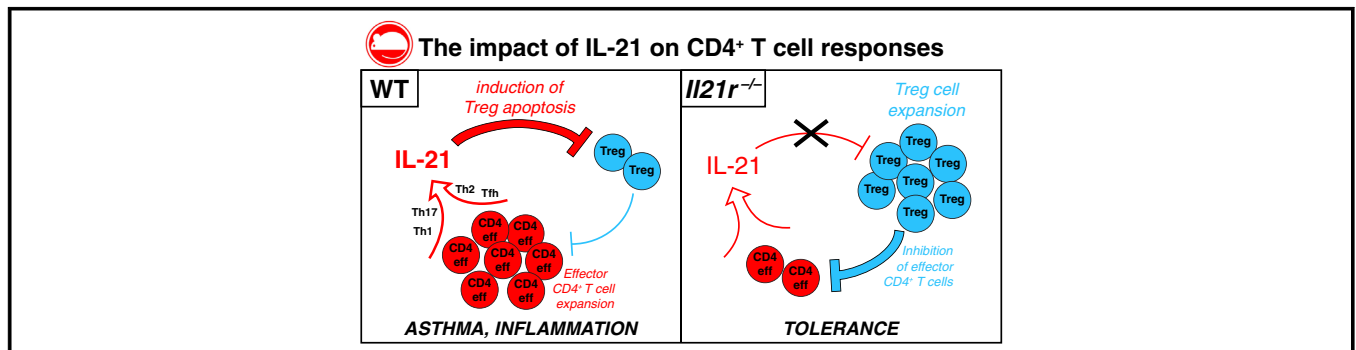


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GRAPHICAL ABSTRACT



Background: IL-21 is a key player of adaptive immunity, with well-established roles in B-cell and cytotoxic T-cell responses. IL-21 has been implicated in promotion of effector CD4⁺ T cells and inhibition of forkhead box P3–positive regulatory T (Treg) cells, but the mechanism and functional relevance of these findings remain controversial.

Objective: We sought to understand the mechanisms by which IL-21 controls effector CD4⁺ cell responses and Treg cell homeostasis. **Methods:** We used IL-21 receptor-deficient mice to study the effect of IL-21 on T-cell responses in models of asthma and colitis. We used mixed bone marrow chimeras and adoptive transfer of naive CD4⁺ T cells and Treg cells into lymphopenic mice to assess the cell-intrinsic effects of IL-21. Using various *in vitro* T-cell assays, we characterized the mechanism of IL-21-mediated inhibition of Treg cells.

Results: We show that IL-21 production by T_H2 and follicular helper T/ex-follicular helper T cells promotes asthma by inhibiting Treg cells. *Il21r*^{-/-} mice displayed reduced

generation of T_H2 cells and increased generation of Treg cells. In mixed chimeras we demonstrate that IL-21 promotes T_H2 responses indirectly through inhibition of Treg cells. Depleting Treg cells in *Il21r*^{-/-} mice restored T_H2 generation and eosinophilia. Furthermore, IL-21 inhibited Treg cell generation in mice with colitis. Using competitive transfer of *Il21r*^{+/+} and *Il21r*^{-/-} CD4⁺ cells, we show that IL-21 directly inhibited expansion of differentiated Treg cells but was dispensable for T_H1/T_H17 effectors. We show that IL-21 sensitizes Treg cells to apoptosis by interfering with the expression of Bcl-2 family genes.

Conclusion: IL-21 directly promotes apoptosis of Treg cells and therefore indirectly sustains generation of inflammatory T_H cells and related effector responses. (J Allergy Clin Immunol 2019;143:2178-89.)

Key words: *IL-21, asthma, colitis, regulatory T cells, mucosal immunity*

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IL-21 is a member of the IL-2 cytokine family with pleiotropic functions that profoundly affect adaptive immune responses. IL-21 is mainly produced by activated CD4⁺ and natural killer T cells and acts on a variety of immune cells by signaling through a heterodimeric receptor composed of IL-21 receptor (IL-21R) and IL-2 receptor γ . Several early reports highlighted the important function of this cytokine in B-cell biology. IL-21 was found to critically promote immunoglobulin class-switching and antibody production in follicular B cells.¹⁻³ Interestingly, IL-21 can promote both survival and expansion or death of B cells dependent on the presence or absence of additional costimulatory factors.⁴⁻⁶ IL-21 can also act on myeloid cells by promoting differentiation to alternatively activated macrophages⁷ and by controlling survival and activation of conventional dendritic cells.^{8,9}

Abbreviations used

BAL:	Bronchoalveolar lavage
FACS:	Fluorescence-activated cell sorting
FoxP3:	Forkhead box P3
HDM:	House dust mite
ICOS:	Inducible T-cell costimulator
ILC2:	Type 2 innate lymphoid cell
IL-21R:	IL-21 receptor
LN:	Lymph node
OVA:	Ovalbumin
PD-1:	Programmed cell death protein 1
Rag1:	Recombination-activating gene 1
ROR:	Retinoic acid–related orphan receptor
STAT:	Signal transducer and activator of transcription
T _{HH} :	Follicular helper T
Treg:	Regulatory T
WT:	Wild-type

The role of IL-21 in T-cell responses has received a lot of attention over the last years. Work from our group and others showed the importance of IL-21 for sustained CD8⁺ T-cell responses during chronic infection.^{10–12} IL-21 also plays an important role for CD4⁺ T-cell polarization and effector function. Loss of IL-21R results in impaired type 2 CD4⁺ T-cell responses in animal models of allergic airway inflammation and helminth infection.^{7,13,14} Furthermore, a number of *in vitro* studies suggested a key role of IL-21 on CD4⁺ T-cell polarization: early reports indicated that IL-21 could inhibit TGF- β –dependent forkhead box P3 (FoxP3)⁺ regulatory T (Treg) cell induction while promoting T_H17 differentiation.^{15,16} Although the effect of IL-21 on T_H17 differentiation was put into perspective by reports showing normal T_H17 responses in *Il21r*^{−/−} animals in mouse models of autoimmune encephalomyelitis and myocarditis,^{17,18} an effect of IL-21 on Treg cell function was further corroborated by *in vitro* and *in vivo* studies. In particular, IL-21 was shown to interfere with Treg cell–mediated suppression of conventional T cells^{19–21} and to inhibit Treg cell differentiation/expansion *in vivo* during chronic viral infection²² and in graft-versus-host responses.²³ This inhibitory effect of IL-21 was also confirmed for human Treg cells.^{20,24,25}

The profound effect of IL-21 on immune activation and adaptive immunity has ignited the interest of the medical community for the clinical application of IL-21 and IL-21–blocking agents in cancer and autoimmune pathologies, respectively.²⁶ However, given the pleiotropic activities of IL-21 on different immune cells, determining the relative importance of individual activities in immune responses is crucial to fully understand the possible implications of the therapeutic modulation of IL-21 signaling. In particular, the precise mechanism by which IL-21 affects effector CD4⁺ T-cell differentiation and function, as well as Treg cell differentiation and/or homeostasis, *in vivo* is still unclear. The ultimate effect and significance of potential inhibitory effects of IL-21 on Treg cells during immune responses *in vivo* still needs to be clarified.

In this work we have unveiled IL-21 as a critical negative regulator of Treg cells in the context of allergic airway inflammation. By using different adoptive cotransfers of Treg cells and conventional T cells from wild-type (WT) and *Il21r*^{−/−} mice into lymphopenic hosts, we investigate the nature of IL-21–dependent Treg cell inhibition *in vivo* and define an important

direct effect of this cytokine on the homeostasis of fully differentiated Treg cells.

METHODS

Mice

C57BL/6, recombination-activating gene (*Rag1*)^{−/−}, *Il21r*^{−/−} (C57BL/6 N7), and DEREK mice were bred and maintained in individually ventilated cages under specific pathogen-free conditions at BioSupport AG (Schlieren, Switzerland) or at the ETH Phenomics Facility (Zurich, Switzerland). *Il21*-mCherry/*Il2*-emGFP dual-reporter transgenic mice²⁷ were kindly provided by Warren Leonard (National Institutes of Health, Bethesda, Md). For experiments, age-matched mice 6 to 10 weeks of age were used. Swiss federal and local animal ethics committees approved the described animal experiments.

Cell preparation

Single-cell suspensions from spleens, peripheral (inguinal), and mesenteric lymph nodes (LNs) and Peyer patches were prepared by pressing the organs through 40- μ m pore-size strainers (BD Biosciences, San Jose, Calif) in PBS supplemented with 2% FBS. For isolation of lamina propria lymphocytes, the colon was cut into small pieces and incubated for 30 minutes under agitation at 37°C in HBS–2% FCS (Ca/Mg free) containing 5 mmol/L EDTA and 2 mmol/L dithiothreitol. After thorough vortexing, the supernatant, which contained intraepithelial lymphocytes, was discarded. The procedure was repeated 2 more times without dithiothreitol to remove further epithelial cells. The remaining tissue was then digested for 80 minutes under agitation at 37°C in HBS supplemented with 1 mg/mL collagenase IV (Sigma, St Louis, Mo). The mixture was vortexed, and the LPL-containing supernatant was filtered through 40- μ m pore-size strainers, washed, and resuspended in PBS–2% FCS.

Flow cytometry

For surface staining, cells were resuspended in PBS–2% FCS, briefly incubated with Fc receptor blocking mAb (clone 2.4G2), and subsequently incubated at 4°C with the relevant fluorescently labeled surface antibodies for 15 minutes. Cells were incubated 4 hours at 37°C with phorbol 12-myristate 13-acetate (10^{−7} mol/L; Sigma), ionomycin (1 μ g/mL; Sigma), and monensin (2 μ g/mL; Sigma) to detect cytokine expression. For intracellular cytokine staining, restimulated cells were surface stained, fixed with 4% formalin for 10 minutes, and permeabilized with PBS–2% FCS supplemented with 0.5% saponin. Cells were then incubated with fluorescently labeled cytokine-specific antibodies for 30 minutes at room temperature. For staining of nuclear proteins, the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, Calif) was used. After staining, cells were washed and resuspended in PBS–2% FCS for flow cytometric analysis by using a FACSCalibur or a LSRFortessa (BD Biosciences). Fluorescence-activated cell sorting (FACS) data were then analyzed with FlowJo software (TreeStar, Ashland, Ore). Intracellular staining of IL-21 using an IL-21R–human Fc fusion protein was performed, as previously described.¹¹ Type 2 innate lymphoid cells (ILC2s) were gated as CD45⁺ lineage (Ter119, Siglec-F, CD11b, CD11c, Gr-1, CD3, T-cell receptor β , and CD19)–negative CD90⁺ ST2⁺ cells.

The following antibodies were used: anti-CD3 ϵ (clone 145-2C11), anti-CD4 (GK1.5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD19 (eBio1D3), anti-CD25 (PC61), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD45RB (C363-16A), anti-CD90.2 (30-H12), anti-CXCR5 (SPRCL5), anti-FoxP3 (FJK-16s), anti-inducible T-cell costimulator (ICOS; C398.4), anti-IFN- γ (XMG1.2), anti-IL-4 (11B11), anti-IL-5 (TRFK5), anti-IL-13 (eBio13A), anti-IL-17A (eBio17B7), anti-IL-9 (RM9A4), anti-Gr-1 (RB6-8C5), anti-programmed cell death protein 1 (PD-1; J43), anti-retinoic acid–related orphan receptor (ROR) γ t (Q31-378), anti-Siglec-F (E50-2440), anti-ST2 (DIH9), anti-T-cell receptor β (H57-597), anti-Ter-119 (Ter1-19), and anti-interferon regulatory factor 1 (D5E4; from CST, Danvers, Mass). For nonviable cell exclusion, Fixable Viability Dye eFluor 780 (Thermo Fisher, Waltham, Mass) was used. For

analysis of cell proliferation, Cell Proliferation Dye eFluor 670 was used. Annexin V staining was performed according to the manufacturer's protocol. The listed reagents were purchased from BioLegend (San Diego, Calif), Thermo Fisher, and BD Biosciences.

Ovalbumin- and house dust mite-induced airway allergic inflammation and bronchoalveolar lavage analysis

Groups of mice were immunized through intraperitoneal administration of 100 μ g of ovalbumin (OVA; Sigma) adsorbed to 200 μ L of alum adjuvant. On days 7 and 8, mice were challenged by means of intratracheal inoculation with 20 μ g of OVA in 50 μ L of PBS. On day 11, mice were euthanized, and after cannulating the trachea, bronchoalveolar lavage (BAL) was performed by flushing the airways 3 times with 0.4 mL of PBS. For house dust mite (HDM)-induced asthma, mice were immunized by means of intratracheal instillation of 10 μ g of HDM (Greer Allergy Immunotherapy; Greer Laboratories, Lenoir, NC) in 50 μ L of PBS. Mice were challenged intratracheally 5 times from day 7 until day 11 with 10 μ g of HDM in 50 μ L of PBS and analyzed on day 14.

Histology

For histologic analysis of the lungs, the mice were euthanized, the trachea was cannulated, and the lungs were inflated with 0.5 mL of 10% formalin. Lungs were subsequently processed and stained with Alcian blue (pH 2.5) and periodic acid-Schiff.

Treg cell depletion

A protocol of OVA-induced airway allergic inflammation was used. In addition, mice were given 1 μ g of diphtheria toxin (VWR Merck Bioscience, Radnor, Pa) in 200 μ L of PBS by means of intraperitoneal injection on days 1, 3, 5, 7, and 9.

Mixed bone marrow chimeras

WT CD45.1/CD45.2 mice were lethally irradiated with 2 sequential exposures to a radiation source (2×600 rad, with an interval of 4 hours). Bone marrow cells were freshly harvested from the femurs and tibiae of donor WT (CD45.1⁺), WT (CD45.2⁺), and *Il21r*^{-/-} (CD45.2⁺) animals. Irradiated mice were then reconstituted by means of intravenous injection of equal numbers of CD45.1⁺ and CD45.2⁺ bone marrow cells. Recipient mice were subsequently treated for 5 weeks with antibiotics, and experiments were performed after 8 weeks.

Adoptive transfer of CD4 T cells into *Rag1*-deficient mice

Naive CD25⁻CD45RB^{hi}CD4⁺ cells were purified with a FACS Aria cell sorter (BD Biosciences) and injected intravenously into *Rag1*^{-/-} mice (4×10^5 cells per mouse). For the cotransfer of naive CD4⁺ T cells and Treg cells, CD25⁺CD45RB^{lo}CD4⁺ Treg cells (5×10^4 WT and 5×10^4 *Il21r*^{-/-} cells) were also FACS sorted and injected with naive CD4⁺ T cells. Assessment of colitis severity was performed based on weight loss and thickening/shortening of the colon.

In vitro assays

For *in vitro* Treg cell differentiation, CD45RB^{hi}CD25⁻FoxP3-EGFP⁻CD4⁺ T cells purified by FACS sorting from DREG mice were stimulated with plate-bound anti-CD3 ϵ (145-2C11, 2 μ g/mL) and soluble anti-CD28 (37.51, 2 μ g/mL) in the presence of human TGF- β (3 ng/mL; Sigma) and, when required, recombinant murine IL-21 (100 ng/mL; eBioscience). Stimulation of sorted primary Treg cells was performed with plate-bound anti-CD3 ϵ (145-2C11, 2 μ g/mL) and anti-CD28 (37.51, 2 μ g/mL). Fresh IL-2 (5 ng/mL; eBioscience) was added every day, and IL-21 (100 ng/mL)

was added every second day. For *in vitro* suppression assays, WT Treg cells were FACS sorted from DREG mice carrying congenic marker CD45.1 and cultured *in vitro* together with equal numbers of IL-21R-deficient eFluor670-labeled naive CD45RB^{hi}CD25⁻FoxP3-EGFP⁻CD4⁺ T cells and IL-21R-deficient CD11c⁺ DCs at a ratio of 1:5. Cells were stimulated with soluble anti-CD3 ϵ (145-2C11, 250 ng/mL) in the presence or absence of IL-21 (100 ng/mL). After 3 days, cells were stained with a viability dye and analyzed by using flow cytometry. For experiments using cell death inhibitors, Q-VD-OPH (10 μ mol/L; Sigma), Necrostatin-1 (100 μ mol/L; Merck, Whitehouse Station, NJ) and Ferrostatin-1 (2 μ mol/L; ChemBridge, San Diego, Calif) were added daily. To assess the impact of IL-21 on mRNA expression in Treg cells, cells cultured for 3 days in the suppression assay with or without IL-21 were stained for CD4, CD11c, and CD45.1 and with a viability dye; Treg cells were FACS sorted and suspended in TRI Reagent (Molecular Research Center, Cincinnati, Ohio) for quantitative RT-PCR analysis.

Quantitative RT-PCR

Total RNA was isolated from sorted cells by using TRI Reagent and reverse transcribed with GoScript (Promega, Madison, Wis). Quantitative RT-PCR was performed with the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, Mass) on an iCycler (Bio-Rad Laboratories, Hercules, Calif). Expression was normalized to β -tubulin. The following primers were used: Bcl-2 forward, AAC ATC GCC CTG TGG ATG AC; Bcl-2 reverse, AAA CAG AGG TCG CAT GCT GG; Bcl-XL forward, CGG ATT GCA AGT TGG ATG GC; Bcl-XL reverse, TGC TGC ATT GTT CCC GTA GA; Bax forward, GCT AGC AAA CTG GTG CTC AA; Bax reverse, CTT GGA TCC AGA CAA GCA GC; Bak forward, CCT TCG GGG TCT TCG TCT TT; Bak reverse, GGA GAC CAC CGT CAC TTG TC; Bim forward, CCC GGA GAT ACG GAT TGC AC; and Bim reverse, GTC TTC AGC CTC GCG GTA AT.

Statistical analysis

Two-tailed paired and unpaired *t* tests were performed with Prism 4.0 software (GraphPad Software, La Jolla, Calif).

RESULTS

Lack of IL-21R leads to reduced type 2 allergic airway inflammation and expansion of the Treg cell population

We and others previously reported a key role of IL-21 in the development of type 2 allergic airway inflammation.^{13,14} Consistently, OVA/alum sensitization followed by repeated airway challenge resulted in impaired eosinophilia (Fig 1, A), reduced mucus production (Fig 1, B), and fewer CD4⁺ T cells infiltrating the lungs and airways (Fig 1, C) in the absence of IL-21R. T_H2 polarization was also significantly diminished among CD4⁺ T cells in BAL fluid but not in the lungs (Fig 1, D). Consequently, this resulted in strongly reduced numbers of T_H2 cytokine (ie, IL-4, IL-5, and IL-13)-producing CD4⁺ T cells in the BAL fluid and lungs of *Il21r*^{-/-} mice compared with WT control mice (Fig 1, E). IL-21R deficiency did not affect development of ILC2s (Fig 1, F, and see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). Importantly, similar results were obtained in the HDM model of asthma (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org).

To identify the source of IL-21 in this response, we induced asthma in *Il21*-mCherry/*Il21*-emGFP dual reporter transgenic mice.²⁷ We found a considerable upregulation of IL-21 expression in the lungs of immunized mice compared with naive control animals (Fig 1, G). The vast majority of IL-21-expressing (mCherry⁺) cells in the lungs consisted of CD4⁺ T cells (Fig 1, G), most of which coexpressed ICOS and PD-1 on their surfaces

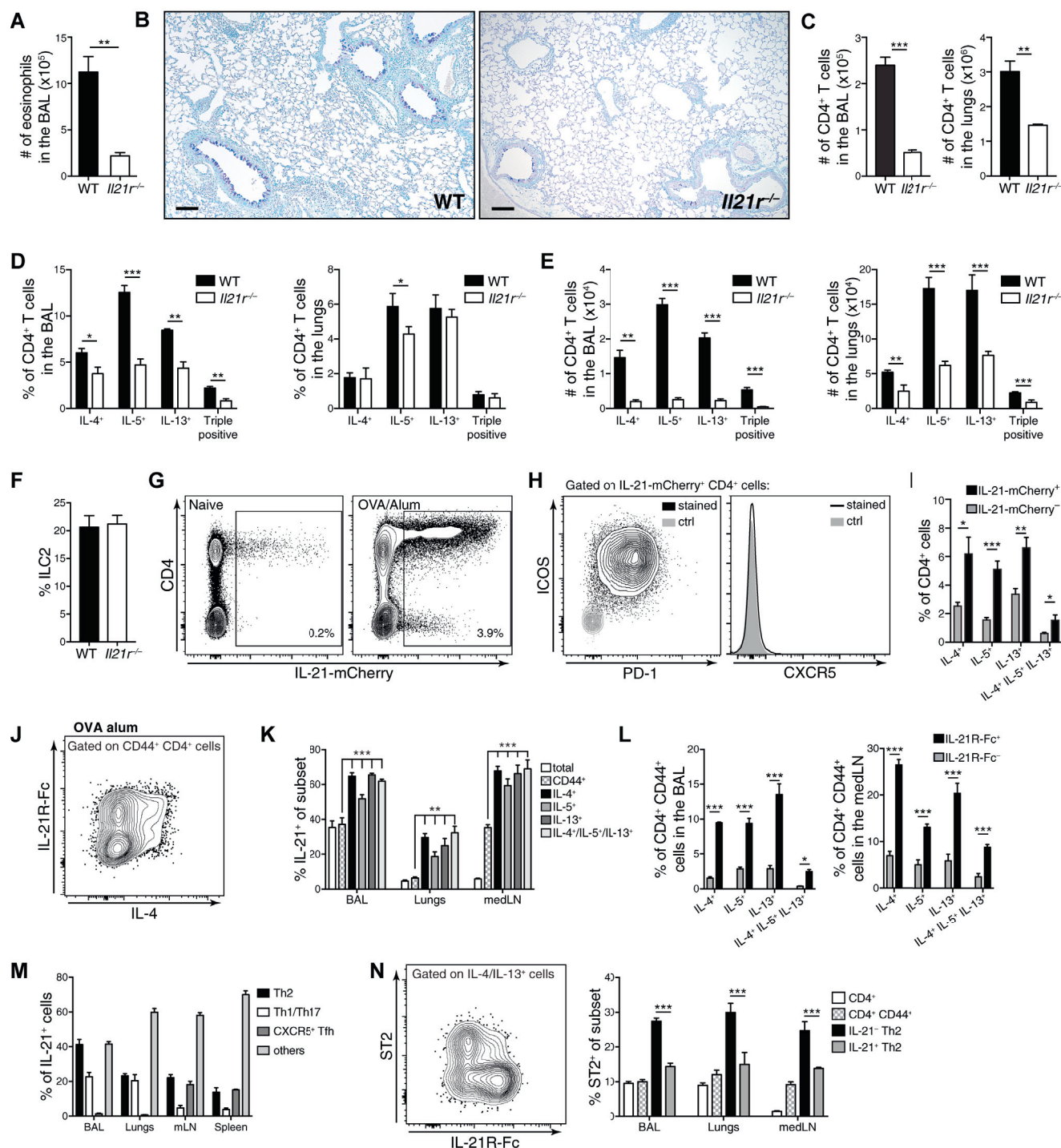


FIG 1. IL-21 production by extrafollicular and type 2 T_H cells controls the severity of allergic airway inflammation in a mouse model of asthma. **A–E**, OVA-induced asthma in WT and *Il21*^{-/-} mice. Fig 1, A, Eosinophil numbers in the airways. Fig 1, B, Alcian blue/periodic acid-Schiff staining of lungs. Scale bar = 0.1 mm. Fig 1, C, Numbers of CD4⁺ T cells. Fig 1, D and E, Percentages (Fig 1, D) and numbers (Fig 1, E) of cytokine-producing CD4⁺ cells. **F**, Percentage of ILC2s among CD90⁺Lin⁻ cells in lungs (4–5 mice per group >3 times with comparable results). **G–I**, OVA-induced asthma in *Il21*-mCherry/*Il2*-emGFP reporter mice. Fig 1, G, Expression of IL-21 in lungs of naive/immunized animals. Fig 1, H, Expression of indicated markers by IL-21⁺ T cells (lungs). Fig 1, I, Expression of T_H2 cytokines in FACS-sorted IL-21⁺/IL-21⁻ lung cells (n = 7). **J–N**, C57BL/6 mice (n = 4) were immunized as above. Expression of IL-4/IL-5/IL-13 and IL-21 (using an IL-21R-Fc fusion) was assessed. Fig 1, J, Expression of IL-4 and IL-21 by CD4⁺ cells in BAL fluid. Fig 1, K, Percentage of IL-21⁺ cells among total CD4⁺ T cells and in T_H2 cytokine⁺ effector CD4⁺ T cells. Fig 1, L, Expression of T_H2 cytokines among IL-21⁺ and IL-21⁻ CD44⁺CD4⁺ cells. Fig 1, M, Coexpression of T_H2 (IL-4 and IL-13), T_H1/T_H17 (IFN-γ/IL-17A), and T_{FF} (CXCR5/PD-1/ICOS) markers among IL-21⁺ cells. Fig 1, N, Expression of ST2 and IL-21 in IL-4/IL-13⁺ T_H2 cells and expression of ST2 by total and IL-21^{+/+} effectors (3 times with similar results). *P < .05, **P < .01, and ***P < .001.

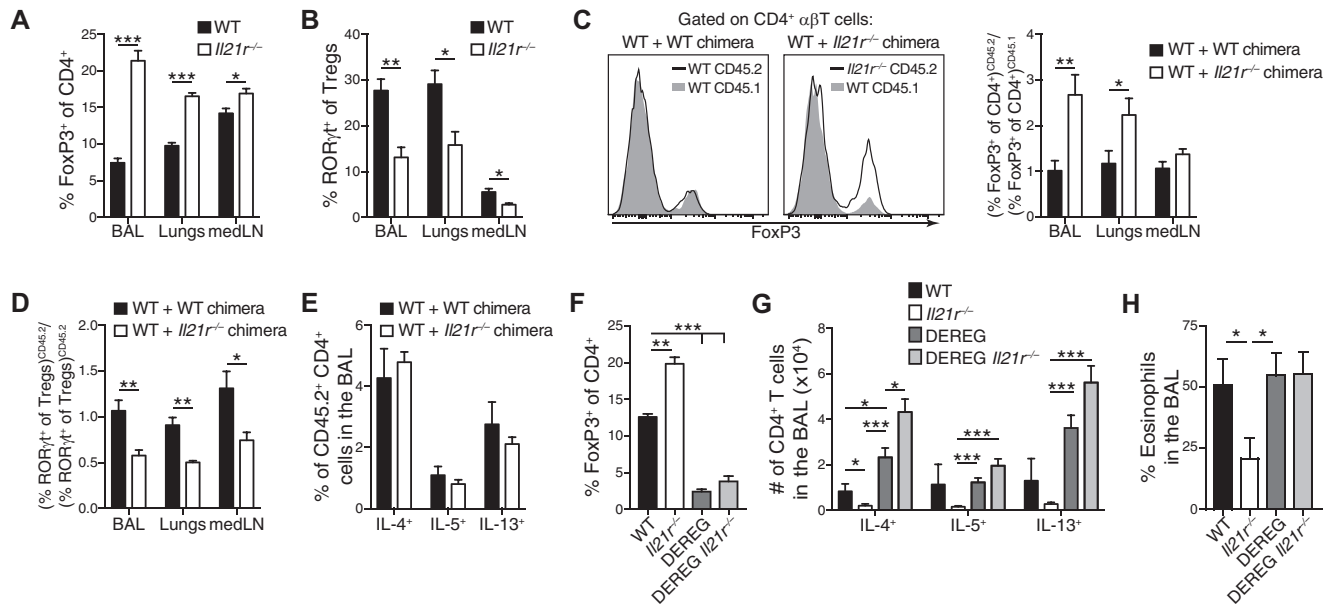


FIG 2. IL-21 deficiency causes expansion of airway-infiltrating Treg cells and reduction of inflammation in a mouse model of asthma. **A** and **B**, Analysis of WT and *Il21*^{-/-} mice after OVA-induced asthma: percentage of FoxP3⁺ cells among CD4⁺ T cells (Fig 2, A) and expression of RORγt among Treg cells (Fig 2, B). Results are representative of more than 3 independent experiments with 4 to 5 mice per group. **C-E**, FACS analysis of mixed (WT plus *Il21*^{-/-}) and control (WT plus WT) bone marrow chimeras after OVA-induced asthma (5 mice per group). Fig 2, C, FoxP3 expression among BAL fluid CD4⁺ T cells in the CD45.1⁺ versus CD45.2⁺ compartments and ratio of FoxP3 expression between CD45.2⁺ and CD45.1⁺ CD4⁺ T cells. Fig 2, D, Ratio of RORγt expression between CD45.2⁺ and CD45.1⁺ Treg cells. Fig 2, E, Expression of IL-4, IL-5, and IL-13 among CD45.2⁺ CD4⁺ T cells. *medLN*, Mediastinal lymph node. **F-H**, Allergic airway inflammation was induced in WT, *Il21*^{-/-}, DEREG, and DEREG *Il21*^{-/-} mice treated with diphtheria toxin to deplete FoxP3⁺ Treg cells. Shown are percentages of Treg cells (Fig 2, F), numbers of IL-4/IL-5/IL-13-producing cells (Fig 2, G), and percentages of eosinophils (Fig 2, H) in the airways. Results are representative of more than 3 experiments using 4 to 6 mice per group with comparable results. **P* < .05, ***P* < .01, and ****P* < .001.

but lacked expression of the follicular helper T (T_{FH}) cell marker CXCR5 (Fig 1, H). IL-33 receptor (ST2), a T_H2 lineage marker, was comparably expressed in IL-21-producing and non-IL-21-producing CD4⁺ cells in the lungs (see Fig E1, C). Notably, comparing non-IL-21-producing (mCherry⁻) and IL-21-producing (mCherry⁺) CD4⁺ T cells sorted from the lungs of asthmatic mice, it was the latter that predominantly produced also IL-4, IL-5, and/or IL-13 (Fig 1, J).

To confirm this finding using an alternative approach, we performed intracellular IL-21 staining in CD4⁺ T cells isolated from the lungs of asthmatic mice using an IL-21R-human Fc fusion protein. We found that T_H2 cytokine-producing cells showed increased expression of IL-21 compared with the remaining CD44⁺ effector CD4⁺ T cells in the airways, lungs, and mediastinal LNs (Fig 1, J and K). Consistently, IL-21-producing CD4⁺ T cells showed enriched coexpression of IL-4, IL-5, and/or IL-13 compared with IL-21-negative effectors (Fig 1, L), confirming the findings obtained with *Il21*-mCherry/*Il2*-emGFP reporter mice.

We then aimed to better characterize the source of IL-21 in asthmatic mice and assessed the coexpression of T_H subset markers among IL-21-producing CD4⁺ T cells. In the airways and lungs, a large fraction of IL-21 production was derived from IL-4/IL-13-expressing T_H2 cells (Fig 1, M). Expectedly, the T_{FH} marker CXCR5 was not found on IL-21-producing cells in lungs and airways but was found on a sizable fraction of IL-21⁺ cells in mediastinal LNs and spleens. Nonetheless, the majority of

IL-21 producers across all organs consisted of CD4⁺ T cells that lacked expression of signature cytokines from T_H1, T_H2, or T_H17 and was also CXCR5⁻ (Fig 1, M). Notably, although *in vitro*-polarized T_H9 cells can also produce IL-21 (see Fig E1, D),²⁸ we found neglectable frequencies of IL-9 producers in the lungs of asthmatic mice, suggesting that IL-21 producers did not originate from IRF1⁺/IL-9⁺ cells (see Fig E1, E). These IL-21 single producers might include “ex-T_{FH}” cells that migrated out of the follicle losing CXCR5 without acquiring any additional effector functionality.

We then compared expression of ST2 and IL-21 by T_H2 cells. Expectedly ST2⁺ cells showed enriched expression of T_H2 signature cytokines (see Fig E1, F). Intriguingly, IL-21-producing T_H2 cells showed strongly reduced expression of ST2 compared with IL-21-negative T_H2 cells (Fig 1, N). Again, we obtained similar results in the HDM model of asthma, with IL-21 originating from both T_H2 cells and T_{FH}/ex-T_{FH} cells (see Fig E2, C-E) and differential expression of IL-21 and IL-33 receptor in subpopulations of T_H2 cells (see Fig E2, F).

Interestingly, further characterization of the lymphocyte compartment revealed a significantly increased population of FoxP3⁺ Treg cells in the airways of IL-21R-deficient mice (Fig 2, A, and see Fig E3, A, in this article's Online Repository at www.jacionline.org), whereas Treg cell numbers were comparable in thymi, LNs, and spleens of naive *Il21*^{-/-} and WT mice (data not shown).²² Interestingly, a considerable fraction of Treg cells infiltrating the airways of asthmatic WT mice coexpressed the

transcription factor ROR γ t, and IL-21R deficiency inhibited ROR γ t expression in Treg cells (Fig 2, B).

Taken together, these data demonstrate that IL-21 can be produced by classical T_H2 cells (in coproduction with IL-4, IL-13, and/or IL-5) and classical T_{FF} (CXCR5⁺PD-1⁺) cells, as well as a population of CD4⁺ T cells lacking CXCR5 and all subset signature cytokines, which are probably derived from T_{FF} cells (ex-T_{FF}). Moreover, absence of IL-21 increases Treg cell numbers in lungs and airways of asthmatic mice.

Cell-autonomous IL-21R signaling directly mediates Treg cell inhibition but is not required for generation of T_H2 effectors

Because the absence of IL-21R resulted in an impairment in the generation of T_H2 cells and reduced asthmatic features, as well as an increased percentage of Treg cells, we next asked which of these effects was directly mediated by IL-21 in a cell-intrinsic manner. To address this question, we generated mixed bone marrow chimera by reconstituting lethally irradiated CD45.1⁺CD45.2⁺ WT hosts with a 1:1 mixture of bone marrow cells derived from WT (CD45.1⁺) and *Il21r*^{-/-} (CD45.2⁺) mice. Recipients containing a mixture of congenic CD45.1⁺ and CD45.2⁺ WT bone marrow served as controls. Chimeras were sensitized with OVA/alum and subjected to intratracheal challenges with OVA before cellular analysis. Importantly, IL-21R-deficient Treg cells outcompeted WT Treg cells and accumulated preferentially in the airways and lungs of asthmatic mice (Fig 2, C, and see Fig E3, D), demonstrating a cell-autonomous inhibitory effect of IL-21 on the recruitment/expansion of Treg cells during allergic airway inflammation. Moreover, we again observed reduced ROR γ t expression in *Il21r*^{-/-} compared with *Il21r*^{+/+} Treg cells (Fig 2, D). Notably, T_H2 cytokine production by CD4⁺ effector T cells was unaffected in the absence of cell-autonomous IL-21R signaling, both in BAL fluid (Fig 2, E, and see Fig E3, B) and lung tissue (see Fig E3, C). This shows that IL-21R signaling is not intrinsically required to generate T_H2 cells during allergic airway inflammation, implying that impaired accumulation of T_H2 cells observed in lung and airways of allergen-treated *Il21r*^{-/-} full knockout mice (Fig 1, E) was an indirect effect, possibly partially mediated by enhanced numbers of Treg cells. Analogous to T_H2 cells, we could exclude a cell-intrinsic effect of IL-21R signaling on accumulation of eosinophils in the lungs and airways (see Fig E3, E).

Interestingly, when assessing total infiltration of Treg cells in the airways of mixed WT plus *Il21r*^{-/-} chimeras, we observed that the increased fraction of CD45.2⁺ *Il21r*^{-/-} Treg cells led to a significant increase in total Treg cell numbers compared with control chimeras (see Fig E3, F). However, this increase was not as pronounced as that of full *Il21r* knockout mice (Fig 2, A). Interestingly, we also observed reduced infiltration in the airways and lungs of mixed WT plus *Il21r*^{-/-} chimeras (see Fig E3, G and H). Consequently, total numbers of airway- and lung-infiltrating eosinophils and T cells were reduced in mixed WT plus *Il21r*^{-/-} chimeras, as were numbers of T_H2 cytokine-secreting CD4⁺ T cells (see Fig E3, I and J).

Notably, the population of Treg cells was also inflated in the airways of *Il21r*^{-/-} HDM-immunized mice, although to a lesser extent compared with the OVA/alum model (see Fig E2, G). Analysis of mixed bone marrow chimeras revealed that this effect was also directly mediated by a cell-intrinsic effect of IL-21 (see Fig

E2, H). Moreover, similar to what we observed in the OVA/alum model, differentiation of T_H2 cells was independent of cell-intrinsic IL-21R signaling also in the HDM model (see Fig E2, I).

To investigate whether the inflated population of Treg cells might be responsible for the diminished T_H2 response in *Il21r*^{-/-} mice, we crossed *Il21r*^{-/-} mice to FoxP3-DTR transgenic (DEREG) mice.²⁹ Treatment of these mice with diphtheria toxin effectively removed the majority of FoxP3⁺ cells (Fig 2, F). Concomitantly, recruitment of CD4⁺ T cells (see Fig E3, K) and development of T_H2 cytokine-producing cells was strongly boosted both in the presence and absence of IL-21R (Fig 2, G, and see Fig E3, L and N). Moreover, eosinophilia in Treg cell-depleted *Il21r*^{-/-} DEREG mice was restored to levels observed in Treg cell-sufficient WT mice (Fig 2, H, and see Fig E3, M and N). The massive T_H2 cytokine response mounted by *Il21r*^{-/-} mice in the absence of Treg cells reinforces the finding from chimeric mice showing that IL-21 signaling is not intrinsically required for the development of T_H2 cells. Restoration of T_H2 cytokine production and eosinophil infiltration in *Il21r*^{-/-} mice by depletion of Treg cells indicates that increased numbers of Treg cells in *Il21r*^{-/-} mice at least partially account for reduced allergic airway inflammation in *Il21r*^{-/-} mice. However, the contribution of additional effects of IL-21 on other immune cells cannot be excluded.

Collectively, our results from mixed chimeric and Treg cell-depleted mice highlight a redundant role of IL-21 in the generation of T_H2 cells during asthma and define a strong cell-intrinsic effect of IL-21 in the inhibition of Treg cells.

IL-21 inhibits peripheral generation of Treg cells during T cell-driven colitis

Next, we investigated whether IL-21R signaling inhibits Treg cells autonomously in other proinflammatory models. Therefore we analyzed the role of IL-21 in mediating CD4⁺ T-cell polarization and expansion in T-cell transfer colitis, an inflammatory bowel disease model, which is promoted by T_H1/T_H17 cells and inhibited by Treg cells.³⁰⁻³³ Use of this model offers the possibility of cotransferring a variety of combinations of WT and IL-21R-deficient CD4⁺ T cells, allowing us to study the cell-autonomous effects of IL-21 and to track the fate of the transferred cells.

We first sorted naive CD25⁻CD45RB^{hi}CD4⁺ T cells from WT and *Il21r*^{-/-} mice and transferred them separately into *Rag1*^{-/-} hosts (Fig 3, A). Seven weeks later, homeostatic expansion in a lymphopenic environment resulted in a large fraction of WT CD4⁺ T cells in spleens, mesenteric LNs, and lamina propria that produced IL-21 (Fig 3, B, and see Fig E4, A, in this article's Online Repository at www.jacionline.org). Comparable frequencies of IL-17 and IFN- γ producers were found on transfer of *Il21r*^{-/-} CD4⁺ T cells (Fig 3, C), indicating that IL-21R is dispensable for T_H1 and T_H17 differentiation. In contrast, transferred naive donor Foxp3⁻CD4⁺ T cells lacking IL-21R showed a significantly enhanced conversion to FoxP3⁺ Treg cells (Fig 3, D, and see Fig E4, B). Notably, the severity of colitis was comparable between groups (data not shown), indicating that the magnitude of *de novo* Treg cell differentiation was not sufficient to prevent the development of colitis.

To assess the effect of IL-21 on CD4 T-cell expansion, as well as its effect on the differentiation of naive CD4⁺ T cells into effector cells and induced Treg cells in a competitive setting,

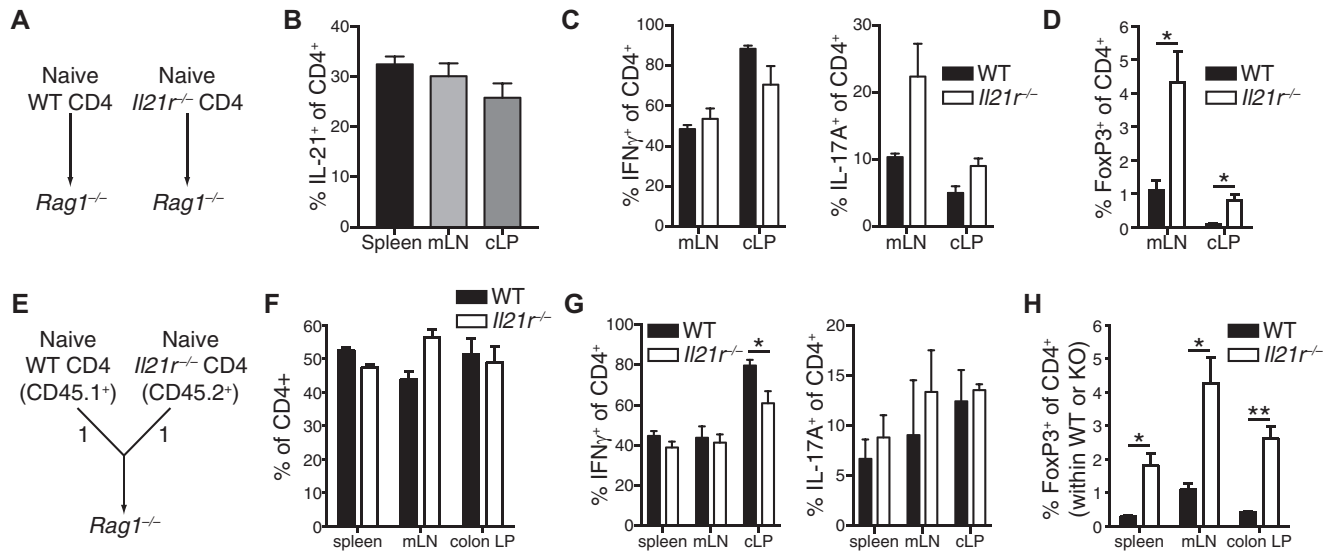


FIG 3. IL-21 inhibits peripheral generation of FoxP3⁺ Treg cells during systemic CD4⁺ T cell-driven inflammation. **A–D**, Naive CD4⁺ T cells isolated from *Il21*-mCherry/*Il2*-emGFP reporter mice or from WT or *Il21*^{-/-} mice were transferred separately into lymphopenic *Rag1*^{-/-} hosts (Fig 3, A). After 7 weeks, animals were euthanized and analyzed. Fig 3, B, Expression of IL-21 by transferred CD4⁺ T cells obtained from *Il21*-*Il2* reporter mice. Fig 3, C, Cells derived from mesenteric LNs (mLN) and colonic lamina propria (cLP) were stimulated *in vitro*, and production of IFN-γ and IL-17A was assessed by using intracellular cytokine staining. **D**, Percentage of FoxP3⁺ Treg cells among CD4⁺ T cells from mLN and cLP. Data are representative of 3 experiments using 3 to 4 mice per group with similar results. **E–H**, Naive CD4⁺ T cells purified from WT (CD45.1⁺) or *Il21*^{-/-} (CD45.2⁺) mice were cotransferred into lymphopenic *Rag1*^{-/-} hosts (Fig 3, E). After 7 weeks, CD4⁺ T cells from animals were isolated from different organs and characterized by using flow cytometry. Fig 3, F, Percentage of WT (CD45.1⁺) and *Il21*^{-/-} (CD45.2⁺) cells among total CD4⁺ T cells. Fig 3, G, Intracellular expression of IFN-γ and IL-17A in WT or *Il21*^{-/-} CD4⁺ T cells. Fig 3, H, Percentage of FoxP3⁺ cells among CD45.1⁺ and CD45.2⁺ CD4⁺ T cells. **P* < .05 and ***P* < .01.

we cotransferred FACS-sorted naive CD25⁻CD45RB^{hi} CD4⁺ T cells from WT (CD45.1) and *Il21*^{-/-} (CD45.2) mice into *Rag1*^{-/-} hosts (Fig 3, E). WT and *Il21*^{-/-} CD4⁺ T cells expanded (Fig 3, F) and differentiated to cytokine-producing effectors comparably, except for a slight reduction in IFN-γ producers among the *Il21*^{-/-} cells residing in the colonic lamina propria (Fig 3, G). However, IL-21R-deficient CD4⁺ T cells again showed a significantly enhanced proportion of *de novo*-induced Treg cells (Fig 3, H). Importantly, the purity of the transferred WT and *Il21*^{-/-} cell populations after sorting was comparable (see Fig E4, C and D).

These data prove a cell-autonomous direct inhibitory effect of IL-21 in the generation/expansion of Treg cells during inflammation. Also, similar to what we saw during T_H2-mediated allergic airway inflammation (Fig 2, E and G, and see Fig E2, B and C), even in this T_H1/T_H17-mediated colitis model, we observed no direct effect of the absence of IL-21R on the generation and expansion of proinflammatory effector CD4 T cells.

IL-21 affects the expansion of FoxP3⁺ Treg cells during intestinal inflammation

The results shown thus far prove a direct inhibitory effect of IL-21 on Treg cells. However, whether IL-21 affects differentiation, expansion, or survival of Treg cells is still unclear. Therefore, to discern the nature of the effect of IL-21 on Treg cells, we performed a different type of transfer experiment using the colitis model. In this case we isolated CD25⁺CD45RB^{lo}CD4⁺ Treg cells (>95% FoxP3⁺) from WT (CD45.1⁺CD45.2⁺) and

Il21^{-/-} (CD45.2⁺) mice and cotransferred these together with naive CD25⁻CD45RB^{high}CD4⁺ T cells from WT (CD45.1) mice (Fig 4, A, left panel). This enabled us to observe the effects of IL-21R signaling on the expansion/maintenance of already differentiated Treg cells in an inflammatory context. A prominent enrichment of IL-21R-deficient Treg cells could be observed (Fig 4, A, right panel; Fig 4, B; and see Fig E4, E), demonstrating a potent inhibitory effect of IL-21 on the expansion or maintenance of natural Treg cells during inflammation. However, these data do not exclude an additional role for IL-21 in *de novo* Treg cell differentiation.

IL-21 induces FoxP3 downregulation and promotes apoptosis of induced and natural Treg cells

To determine the underlying mechanism of Treg cell inhibition by IL-21, we performed a time-course analysis of induction of FoxP3 in naive CD4 cells cultured in Treg cell-polarizing conditions in the presence or absence of IL-21. At the same time, we tracked cell division by labeling cells with the dye eFluor670. Addition of IL-21 from the start allowed us to discern whether IL-21 (1) inhibits FoxP3 induction and Treg cell conversion, (2) hampers proliferation of already differentiated Treg cells, or (3) promotes expansion of FoxP3⁻ cells. The results showed that IL-21 had no effect on induction of FoxP3 expression in the first 2 days, whereas inhibition became visible after 3 days of culture (Fig 5, A). In addition, proliferation of FoxP3⁺ and FoxP3⁻ cells remained unaffected by addition of IL-21 (Fig 5, B). These data suggest that IL-21 acts mainly on already

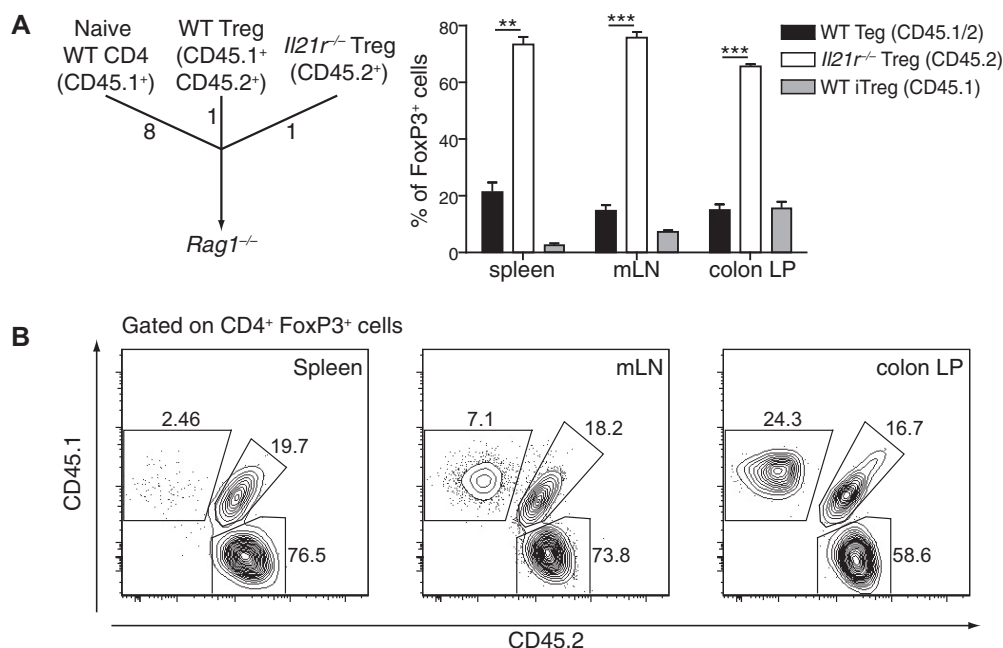


FIG 4. IL-21 inhibits expansion of differentiated FoxP3⁺ Treg cells. **A**, *Rag1*-deficient mice were injected intravenously with a mixture of 4×10^5 naive CD45.1⁺ WT CD4⁺ T cells, 5×10^4 CD45.1⁺ CD45.2⁺ WT Treg cells, and 5×10^4 CD45.2⁺ *Il21*^{-/-} Treg cells. After 7 weeks, mice were euthanized, and CD4⁺ T cells were analyzed. *Right*, Percentage of FoxP3⁺ Treg cells derived from the 3 different transferred CD4⁺ T-cell populations after *in vivo* expansion. LP, Lamina propria; mLN, mesenteric lymph node. ***P* < .01 and ****P* < .001. **B**, Representative FACS analysis of the data shown in Fig 4, A. Data are representative of 3 experiments (*n* = 3–4 mice per group) with comparable results.

differentiated Treg cells rather than inhibiting the induction of FoxP3 expression.

To address this possibility, we next polarized naive CD4⁺ T cells (>99% purity) from DEREG (FoxP3-EGFP) mice in the presence of TGF-β to Treg cells for 2 days. Subsequently, we sorted the *de novo*-generated Treg (FoxP3-EGFP⁺) cells, labeled them with the dye eFluor 670, and continued to culture them in Treg cell-polarizing conditions (ie, IL-2 and TGF-β) in the presence or absence of IL-21 to assess the effect of IL-21 on already differentiated Treg cells. Interestingly, IL-21 promoted loss of FoxP3 expression and generation of “ex-Treg” cells (Fig 5, C and D). Treatment with the cytokine had no effect on net proliferation of the differentiated Treg cells (Fig 5, E). These results suggest that IL-21 strongly affects the stability of *in vitro*-differentiated Treg cells. However, *in vitro*-generated Treg cells are known to be unstably committed to their lineage because of incomplete demethylation of the FoxP3 locus.^{34–36}

To determine whether IL-21 affected FoxP3 expression also in stable natural Treg cells, we sorted FoxP3-EGFP⁺ Treg cells (purity >99.5%) from DEREG mice (Fig 6, A) and stimulated them with anti-CD3/anti-CD28 and IL-2 in the presence or absence of IL-21. As shown in Fig 6, B, IL-21 induced a gradual loss of FoxP3 expression without affecting their proliferative capacity. IL-21 not only increased the fraction of FoxP3⁺ cells, it also reduced FoxP3 expression levels in the remaining proliferating Treg cells (Fig 6, C). Notably, this effect became visible only at day 3 of culture, when the Treg cells started to robustly proliferate (Fig 6, C).

Taken together, these data demonstrate that IL-21 negatively regulates FoxP3 expression and the maintenance or stability of proliferating Treg cells both *in vivo* and *in vitro*. Of note, although

supplementation of IL-21 during *in vitro* Treg cell differentiation led to reduced generation of Treg cells, it also led to upregulation of RORγt among the remaining Treg cells, similar to IL-6 (see Fig E5, A, in this article's Online Repository at www.jacionline.org), suggesting that concurrent activation of signal transducer and activator of transcription (STAT) 3 during TGF-β-driven differentiation of Treg cells might favor generation of RORγt⁺ Treg cells.

To investigate whether IL-21 can promote Treg cell death and thereby explain our *in vivo* observations, we performed a classical *in vitro* suppression assay. We cocultured Treg cells purified from *Il21*^{+/-} DEREG and *Il21*^{-/-} DEREG mice together with naive CD4⁺ T cells and dendritic cells (both derived from *Il21*^{-/-} mice to exclude potential confounding effects of IL-21 on these cells) with soluble anti-CD3 in the presence or absence of IL-21. After 3 days, cells were analyzed by using flow cytometry (see Fig E5, B). IL-21 administration did not impair the suppressive capacity of Treg cells during the brief course of the experiment (see Fig E5, C) but resulted in increased percentages of dead Treg cells and therefore a reduction in total numbers of viable WT Treg cells (Fig 6, D). Accordingly, the percentage of Annexin V⁺ apoptotic Treg cells was also increased in the presence of IL-21 (Fig 6, E). Importantly, IL-21 had no effect on *Il21*R-deficient Treg cells (Fig 6, D). Quantitative RT-PCR analysis of Bcl-2 family genes in the Treg cells purified from these cultures revealed that treatment with IL-21 led to reduced expression of prosurvival family members *Bcl2* and *Bcl2l1*-XL isoform (which encode Bcl-2 and Bcl-XL, respectively) and increased expression of the proapoptotic member *Bcl2l1* (which encodes Bim; Fig 6, F), suggesting that IL-21 might affect Treg cell survival by sensitizing them to apoptotic cell death. Indeed, the

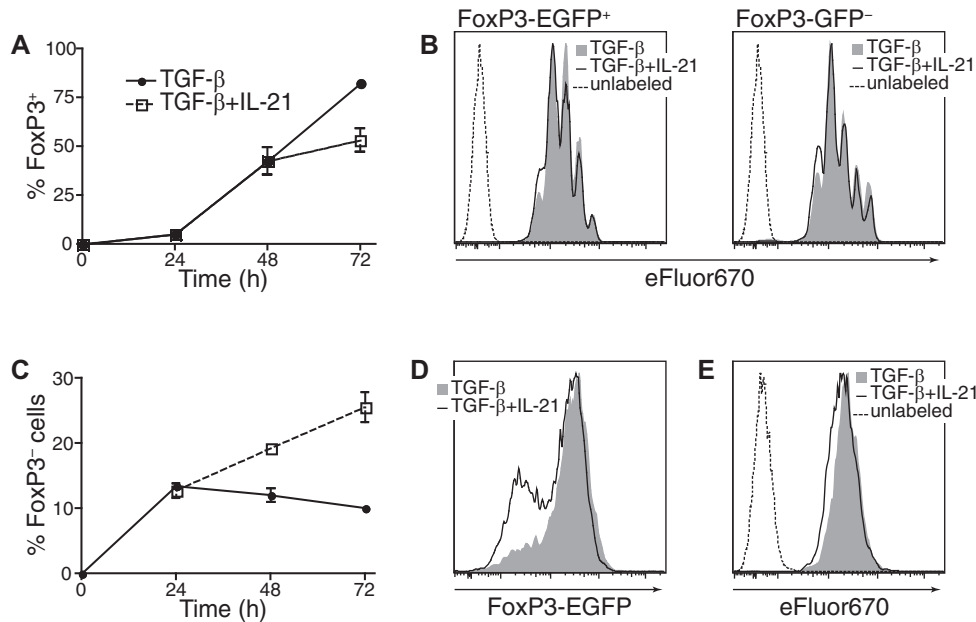


FIG 5. IL-21 induces loss of FoxP3 in *de novo*-generated Treg cells. **A**, Naive FoxP3-EGFP⁺CD25⁻CD45RB^{hi}CD4⁺ T cells were FACS-sorted from spleens and skin-draining LNs of DEREG mice. Cells were labeled with the cell proliferation dye eFluor670 and cultured in Treg cell-polarizing conditions with or without IL-21. The percentage of induced FoxP3-EGFP⁺ cells at the indicated time points is shown. The graph shows averages $\pm$ SEMs of triplicate observations. **B**, eFluor670-labeled naive FoxP3-EGFP⁺CD25⁻CD45RB^{hi}CD4⁺ T cells were cultured as in Fig 5, A. Representative proliferation profiles of FoxP3-EGFP⁺ and FoxP3-EGFP⁻ cells after 3 days of culture, as seen by dilution of eFluor670, are shown. **C**, Naive FoxP3-EGFP⁻CD25⁻CD45RB^{hi}CD4⁺ T cells were FACS sorted from DEREG mice and cultured for 2 days in Treg cell-inducing conditions. FoxP3-GFP⁺ cells from this culture were then sorted (purity >99%), labeled with eFluor670, and put back in culture with the unlabeled FoxP3⁻ fraction in the presence of TGF- β or IL-2 and with or without IL-21. The percentage of FoxP3⁻ cells among eFluor670⁺ cells at the indicated time points was determined by using flow cytometry. Shown are average percentages $\pm$ SEMs of triplicate observations. Representative histograms of the data shown in Fig 5, C, depicting FoxP3 expression (**D**) and dilution of the proliferation dye (**E**) after 72 hours of culture in the indicated conditions.

induction of Treg cell death by IL-21 could be largely averted by supplementation of the pancaspase inhibitor Q-VD-OPH but not by administration of RIPK1 and the necroptosis inhibitor Necrostatin-1 or the ferroptosis inhibitor Ferrostatin-1 (Fig 6, G). Importantly, IL-21 was responsible for apoptosis of Treg cells also *in vivo*: Treg cells in the airways of asthmatic WT mice were more apoptotic compared with Treg cells isolated from the airways of *Il21r*^{-/-} mice (Fig 6, H). These data show that IL-21 can promote the death of activated Treg cells by means of caspase-mediated apoptosis.

DISCUSSION

IL-21 is a proinflammatory cytokine that has been previously shown to support CD8⁺ T-cell responses during chronic viral infections,¹⁰⁻¹² as well as T_H2 effector responses in models of asthma and nematode infection.^{7,13,14} *In vitro* studies indicated that IL-21 promotes T_H17 cell differentiation at the expense of TGF- β -induced Treg cell induction.^{15,16} Although the *in vivo* relevance of these findings has been challenged by subsequent reports,^{17,18} further studies implicated an inhibitory role of IL-21 Treg cell development, function, or both in both mice and human subjects.^{19,20,22-24,37-39}

In this work we focused on unraveling the function of IL-21 in Treg cells and effector CD4⁺ T-cell biology. We previously

reported impaired type 2 allergic airway inflammation in *Il21r*^{-/-} mice. In the present study we show that this is associated with increased accumulation of Treg cells in the airways of *Il21r*^{-/-} mice. Depletion of Treg cells in *Il21r*^{-/-} mice led to re-appearance of eosinophils and T_H2 cytokine-producing cells in the lungs, suggesting that the inflated Treg cell population is at least partially responsible for impaired allergic airway responses in the absence of IL-21R. Indeed, when Treg cells were depleted, T_H2 cytokine production was comparable in the presence and absence of IL-21R, implicating that IL-21 is not intrinsically required for T_H2 development. Analysis of mixed bone marrow chimeras corroborated this finding: IL-21R signaling was superfluous for T_H2 cell differentiation in a competitive setting.

Although it has been suggested that IL-21 suppresses Treg cells indirectly by decreasing IL-2 production in conventional CD4⁺ T cells,²¹ the mixed bone marrow chimera data unambiguously demonstrate a direct cell-intrinsic inhibitory effect of IL-21 on Treg cells. A recent report highlighted the importance of Treg cells in the inhibition of allergic responses and how pathogenic T_H2 cells escape Treg cell-mediated suppression.⁴⁰ In light of the pleiotropic effects mediated by IL-21 on a variety of immune cells, we cannot exclude that IL-21 contributes to the pathogenesis of asthma through a mechanism beyond Treg cell suppression.⁴¹ Notably, in our experiments we did not observe impaired development of ILC2s in asthmatic *Il21r*^{-/-} mice in

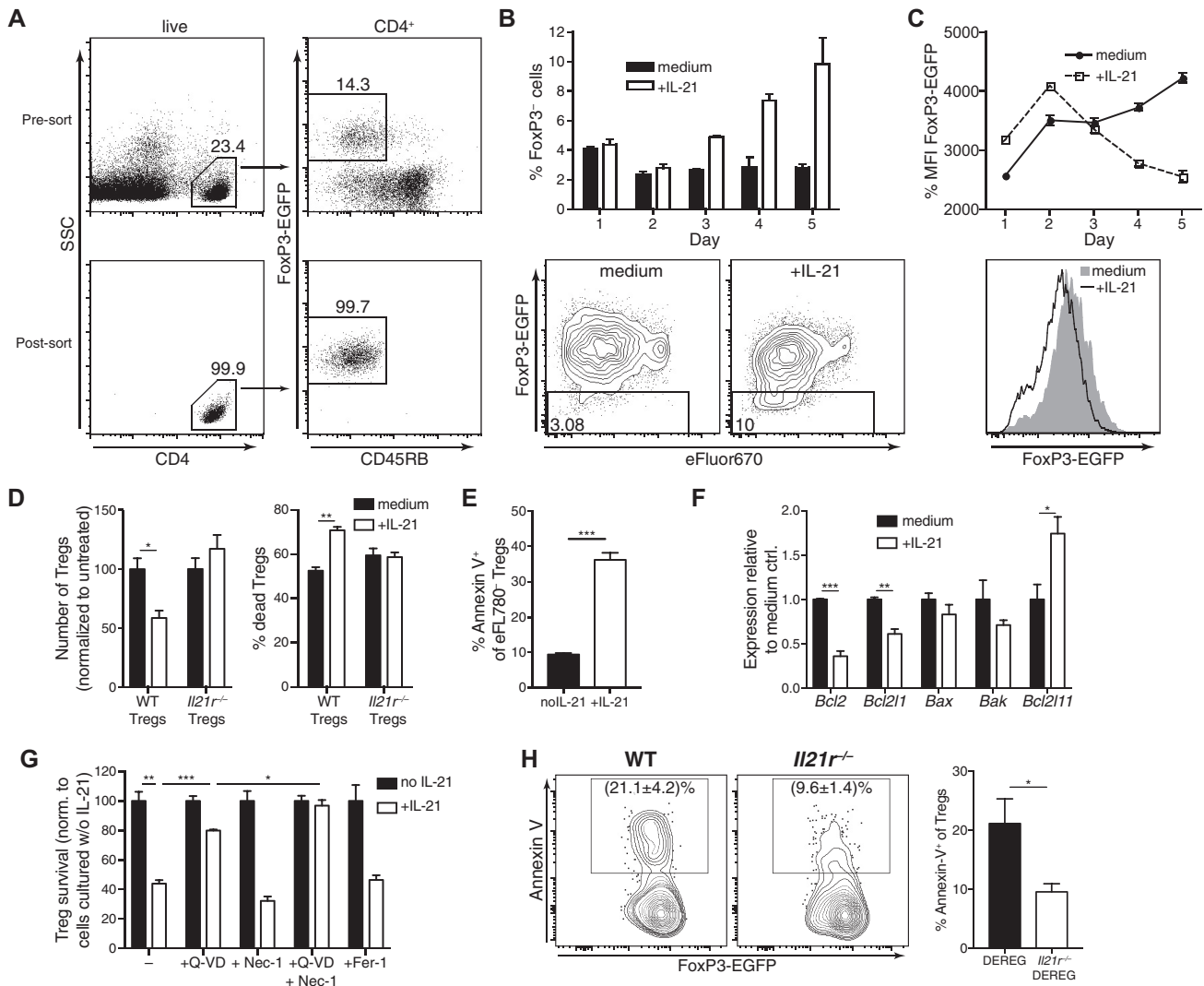


FIG 6. IL-21 causes loss of FoxP3 and apoptotic death in activated Treg cells. **A**, FoxP3-EGFP⁺ CD45RB^{lo} CD4⁺ Treg cells were isolated from DEREG mice (purity >99.5%). SSC, Side scatter. **B**, Cells were cultured with anti-CD3/anti-CD28 or IL-2 and with or without IL-21. Percentage of FoxP3⁺ cells at indicated time points and representative FACS plots at day 5. **C**, Mean fluorescence intensity of FoxP3-EGFP in Treg cells at indicated time points and expression of FoxP3 at day 5. Results are representative of 3 experiments. **D–F**, Treg cells were purified from DEREG CD45.1 mice and cultured for 3 days alongside IL-21R-deficient eFluor670-labeled naive CD4⁺ T cells and CD11c⁺ DCs with anti-CD3 and with or without IL-21. **D**, Numbers of live Treg cells and percentages of dead Treg cells. IL21^{-/-} Treg cells served as controls for the purity/specificity of recombinant IL-21. **E**, Annexin V staining in Treg cells. **F**, Expression of Bcl-2 family genes in Treg cells purified after 3 days of culture, as described in Fig 6, **D**. **G**, Treg cells were cultured as in Fig 6, **D**, and the cell death inhibitors Q-VD, Necrostatin-1 (*Nec-1*), and Ferrostatin-1 (*Fer-1*) were supplemented daily. Plot shows survival of Treg cells after 3 days. Results are representative of 3 experiments with similar results. **H**, DEREG and DEREG IL21^{-/-} mice (*n* = 4 per group) were immunized with OVA/alum, as described in Fig 1. After intratracheal challenge with OVA, BAL fluid cells were isolated, and Annexin V staining was performed and analyzed by using flow cytometry. **P* < .05, ***P* < .01, and ****P* < .001.

contrast to a previous report.¹⁴ Although we cannot explain the reasons for this difference, it should be noted that mixed bone marrow experiments performed by the other laboratory did not reveal a defect in IL-21R-deficient ILC2s, indicating that IL-21 is not directly required for expansion of ILC2s during allergic airway inflammation.¹⁴

Our study also revealed that several CD4⁺ T-cell subsets produce IL-21, including classical TH2 cells (in coproduction with IL-4, IL-13, and/or IL-5), classical CXCR5⁺PD-1⁺ TH17 cells, and a population lacking CXCR5 and all subset signature

cytokines, which are probably ex-TH17 cells. IL-21-producing TH17 and ex-TH17 cells were typically present in draining LNs and spleens, whereas IL-21-producing TH2 cells predominated in the asthmatic airways in the OVA/alum and HDM models. Intriguingly, we found that classical TH2 cells can be separated by ST2 staining into 2 populations that differentially express IL-21 and ST2 (IL-21⁺ST2⁻ TH2 cells and IL-21⁻ST2⁺ TH2 cells), which might have distinct developmental pathways. It is tempting to speculate that these IL-21⁺ST2⁻ TH2 cells might be ex-TH17 cells. A recent study showed that TH17 cells can migrate

out of the follicle and convert into T_H2 cytokine producers in asthmatic mice.⁴² Whether IL-21⁺ST2⁺ T_H2 cells in LNs and lungs are actually ex- T_{FH} cells remains to be addressed in a fate-mapping approach.

IL-21-mediated restraining of Treg cells was not only observed in the asthma model. Previously, we reported inflation of IL-21R-deficient Treg cells in the type 1 response elicited during viral infections.²² Similarly, cotransfer of naive CD4⁺ T cells from WT and *Il21r*^{-/-} mice into *Rag1*^{-/-} hosts resulted in selective expansion of Treg cells lacking IL-21R. Although IL-21 was suggested to promote the expansion of FoxP3⁺CD4⁺ T cells *in vitro*,³⁹ we found that expression of IL-21R on non-Treg cells did not affect their capacity to proliferate and differentiate into T_H1 and T_H17 effectors in lymphopenic hosts, indicating that effector CD4⁺ T cells are not intrinsically dependent on IL-21 to achieve their full functionality. Together with previous reports,^{6,17,18} our data show that IL-21 is in principle not required for T_H2 , T_H1 , and T_H17 development. Instead, IL-21 can promote T_H polarization and effector responses indirectly by restraining Treg cells intrinsically. Plausible mechanisms include a modulation of Treg cell induction or an effect on the homeostasis of differentiated Treg cells. Cotransfer of purified Treg cells from WT and *Il21r*^{-/-} mice into *Rag1*^{-/-} recipients showed an expansion advantage of IL-21R-deficient Treg cells compared with their WT counterpart, proving that IL-21 directly affects the expansion/maintenance of already differentiated natural Treg cells.

In vitro we show that IL-21 does not inhibit the induction of FoxP3⁺ expression but rather affects the maintenance and the stability of its expression in *de novo*-differentiated Treg cells. Because previous studies highlighted that *in vitro*-generated Treg cells only unstably commit to their lineage,³⁴⁻³⁶ we also assessed the effect of IL-21 on natural Treg cells and confirmed its negative effect on FoxP3 expression. IL-6 was also shown to promote downregulation of FoxP3 in proliferating Treg cells. Mechanistically, IL-6-driven activated STAT3 can compete with STAT5 for binding to the conserved non-coding DNA sequence element 2 region of the FoxP3 locus, causing recruitment of DNA methyltransferases and thereby inhibiting FoxP3 expression.⁴³ Given that IL-21R also predominantly signals through STAT3, it is plausible that IL-21-mediated inhibition of FoxP3 expression can occur through a similar mechanism. Others have shown that activation of Treg cells can lead to loss of FoxP3 expression, which might sensitize them to undergo programmed cell death.^{44,45} Our results indicate that IL-21 accelerates loss of FoxP3 and simultaneously sensitizes Treg cells to apoptosis.

Activated Treg cells treated with IL-21 showed downregulation of the prosurvival factors Bcl-2 and Bcl-XL and upregulation of the proapoptotic Bcl-2 family member Bim. Intriguingly, induction of Bim through IL-21 was also observed in B cells⁵ and dendritic cells.⁹ This imbalance in the expression of proapoptotic and prosurvival members of the Bcl-2 family coincided with an increased sensitivity of IL-21-treated Treg cells to apoptosis, an effect that could be largely rescued by administration of the pancaspase inhibitor Q-VD-OPh. Interestingly, although the necroptosis inhibitor Necrostatin-1 alone did not prevent IL-21-driven death of Treg cells, in conjunction with Q-VD it completely restored Treg cell survival. This suggests that, in a condition in which apoptosis is inhibited, IL-21 might drive at least part of the Treg cells to undergo necroptotic cell death. Therefore, our results show that IL-21 sensitizes activated Treg cells to apoptotic

cell death and that this sensitization is associated with deregulated expression of Bcl-2 family genes. Future efforts will have to focus on defining the precise mechanisms mediating the actual induction of cell death in Treg cells sensitized by IL-21.

In the asthma model we found that a substantial fraction of the airway-infiltrating Treg cells coexpressed the transcription factor ROR γ t. This subpopulation of Treg cells was previously described to play an important role in suppression of immune pathology in the intestine.⁴⁶⁻⁴⁸ Although IL-21R-deficient animals challenged with OVA/alum showed a greatly increased population of Treg cells, the relative fraction of ROR γ t⁺ cells among *Il21r*^{-/-} Treg cells was reduced. Analysis of mixed bone marrow chimeras confirmed reduced expression of ROR γ t⁺ cells among IL-21R-deficient Treg cells; however, the fraction of ROR γ t⁺ Treg cells among total CD4⁺ T cells was comparable between the WT and *Il21r*^{-/-} compartment. Also, although IL-21, similar to IL-6, inhibited TGF- β -mediated *in vitro* generation of Treg cells, it also led to upregulation of ROR γ t in the remaining Treg cells, probably by activating STAT3 signaling.^{16,49} Upregulation of ROR γ t in Treg cells by IL-21 might merely be an epiphenomenon with no strong effect on Treg cell functionality, but our results do not exclude the possibility that ROR γ t⁺ Treg cells might be resistant to IL-21-driven apoptosis.

Altogether, our results further confirm the role of IL-21 as a key regulator of T cell-mediated immunity. In addition to the known function in supporting CD8⁺ T cells, here we show that IL-21 promotes effector T-cell responses by directly inhibiting homeostasis of FoxP3⁺ Treg cells.

In light of the results presented here, the effect of IL-21 on development of effector CD4⁺ T-cell responses should be reconsidered. Initially described to be a T_H17 -specific cytokine, there is accumulating evidence that IL-21 production is not confined to a particular subset and, depending on the model, can be found in T_H1 , T_H17 , T_{FH} , and, as shown here, T_H2 cells. Although a number of *in vitro* studies implicated IL-21 as a key polarizing factor for T_H17 and an inhibitor of T_H1 differentiation,⁵⁰ our *in vivo* experiments prove that cell-intrinsic IL-21R signaling has no effect on differentiation/expansion of effector T_H1 , T_H2 , or T_H17 subsets. On the basis of our results, we propose that IL-21 influences effector CD4⁺ T-cell responses indirectly through inhibition of Treg cell homeostasis. These findings suggest new possible therapeutic strategies to promote or inhibit Treg cell survival in the treatment of immune pathologies, chronic infections, and cancer.

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Key messages

- IL-21 directly inhibits Treg cell homeostasis and is not intrinsically required for generation of T_H2/T_H17 cells in mouse models of asthma and colitis.
- In the absence of IL-21, the inflated population of Treg cells is responsible for reduced expansion of proinflammatory effector CD4⁺ T cells.
- IL-21 sensitizes Treg cells to apoptosis by interfering with the expression of Bcl-2 family genes.

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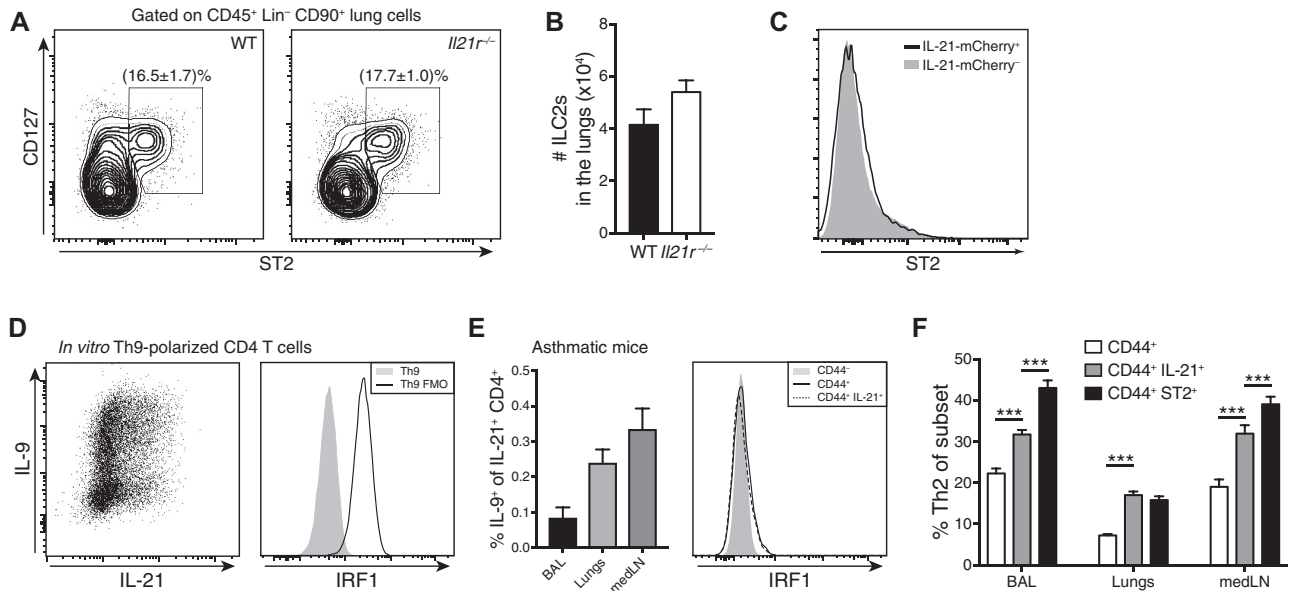


FIG E1. Effect of IL-21 on ILC2s and characterization of the sources of IL-21 and T_H2 cytokines in the OVA/alum model of asthma. **A**, Representative plot depicting the gating strategy to analyze ILC2s in the lungs. Numbers depict percentages ± SEMs of ILC2s among Lin⁻CD90⁺ cells in OVA/alum-immunized WT and *Il21*^{-/-} mice. **B**, Total numbers of ILC2s isolated from the lungs of WT and *Il21*^{-/-} mice (4 mice per group were used). **C**, Histogram overlay depicting expression of the T_H2 marker ST2 in *Il21*-mCherry⁺ and *Il21*-mCherry⁻ CD4 T cells isolated from the lungs of immunized *Il21*-*Il2* reporter mice. **D**, Coexpression of IL-21 and IL-9 (left panel) and expression of IRF1 (right panel) by *in vitro*-polarized T_H9 cells. **E**, Expression of IL-9 by IL-21-producing CD4⁺ T cells in OVA/alum-immunized mice (n = 4 mice). **F**, Bar graph shows percentages of cells positive for IL-4/IL-13 among total effector CD4⁺ T cells and among IL-21⁺, or ST2⁺ cells (n = 4 mice). ***P < .001.

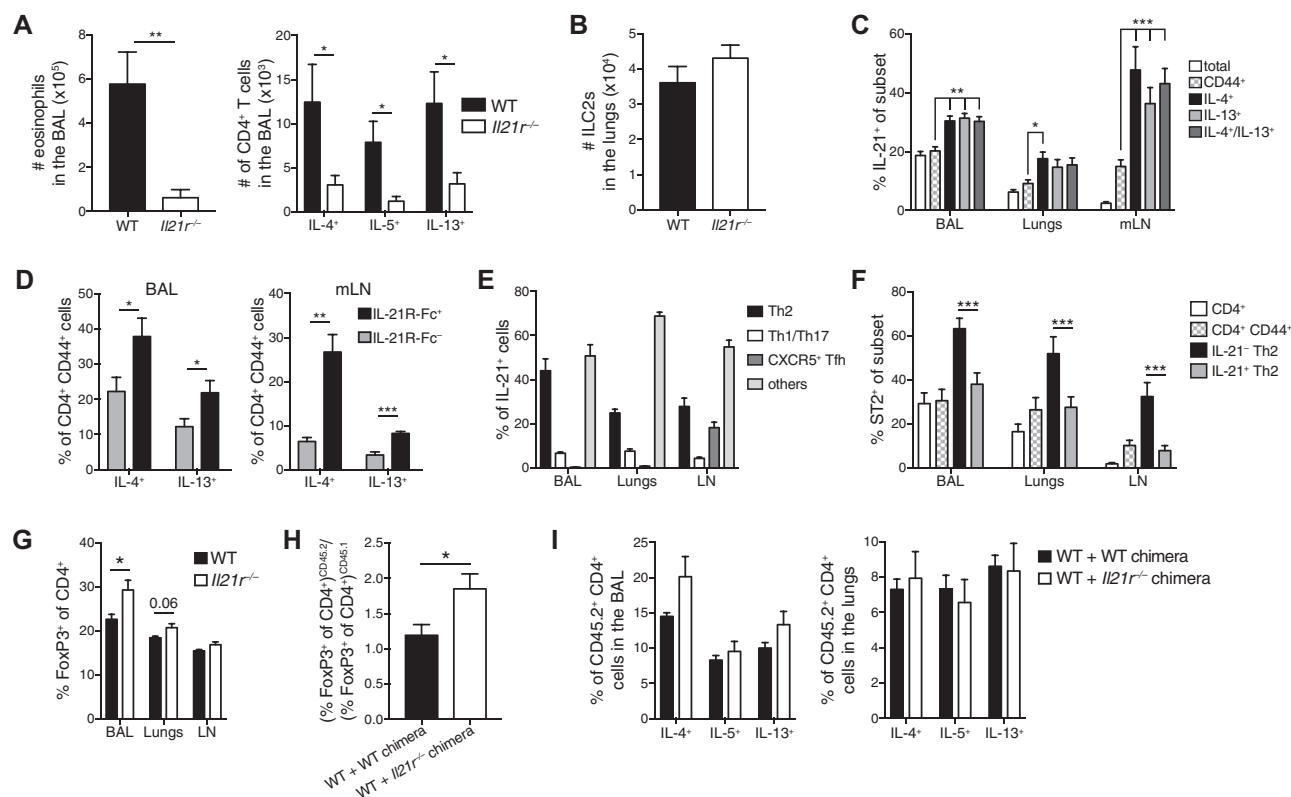


FIG E2. Characterization of the role of IL-21 in the HDM model of asthma. **A** and **B**, HDM-induced asthma in WT and *Il21*^{-/-} mice (5 and 6 mice per group). Fig E2, **A**, Number of eosinophils and cytokine-producing CD4⁺ T cells in BAL fluid. Fig E2, **B**, Number of ILC2s in lungs. **C-F**, WT mice (n = 4) were immunized with HDM. Fig E2, **C**, Percentage of expression of IL-21 in the indicated subsets in the various organs. *mLN*, Mediastinal lymph node. Fig E2, **D**, Expression of IL-4 and IL-13 among IL-21⁺ and IL-21⁻ effector CD4⁺ T cells. Fig E2, **E**, Coexpression of IL-4 and IL-13, T_H1/Th17 (IFN- γ and IL-17A), and T_{FH} (CXCR5, PD-1, and ICOS) markers by IL-21⁺ cells. Fig E2, **F**, Percentage of expression of ST2 by total CD4⁺ T cells and by IL-21⁺ and IL-21⁻ T_H2 (IL-4/IL-13⁺) cells. **G**, Percentage of Treg cells of WT and *Il21*^{-/-} mice after HDM-induced asthma (5-6 mice per group). **H** and **I**, Mixed bone marrow chimeras were immunized and challenged intratracheally with HDM (5-6 mice per group). Fig E2, **H**, The bar graph shows the ratio of FoxP3 expression between CD45.2⁺ and CD45.1⁺ CD4⁺ T cells in BAL fluid. Fig E2, **I**, The bar graph depicts expression of IL-4, IL-5, and IL-13 among CD45.2⁺ CD4⁺ T cells in BAL fluid and lungs of HDM-immunized mixed bone marrow chimeras. **P* < .05, ***P* < .01, and ****P* < .001.

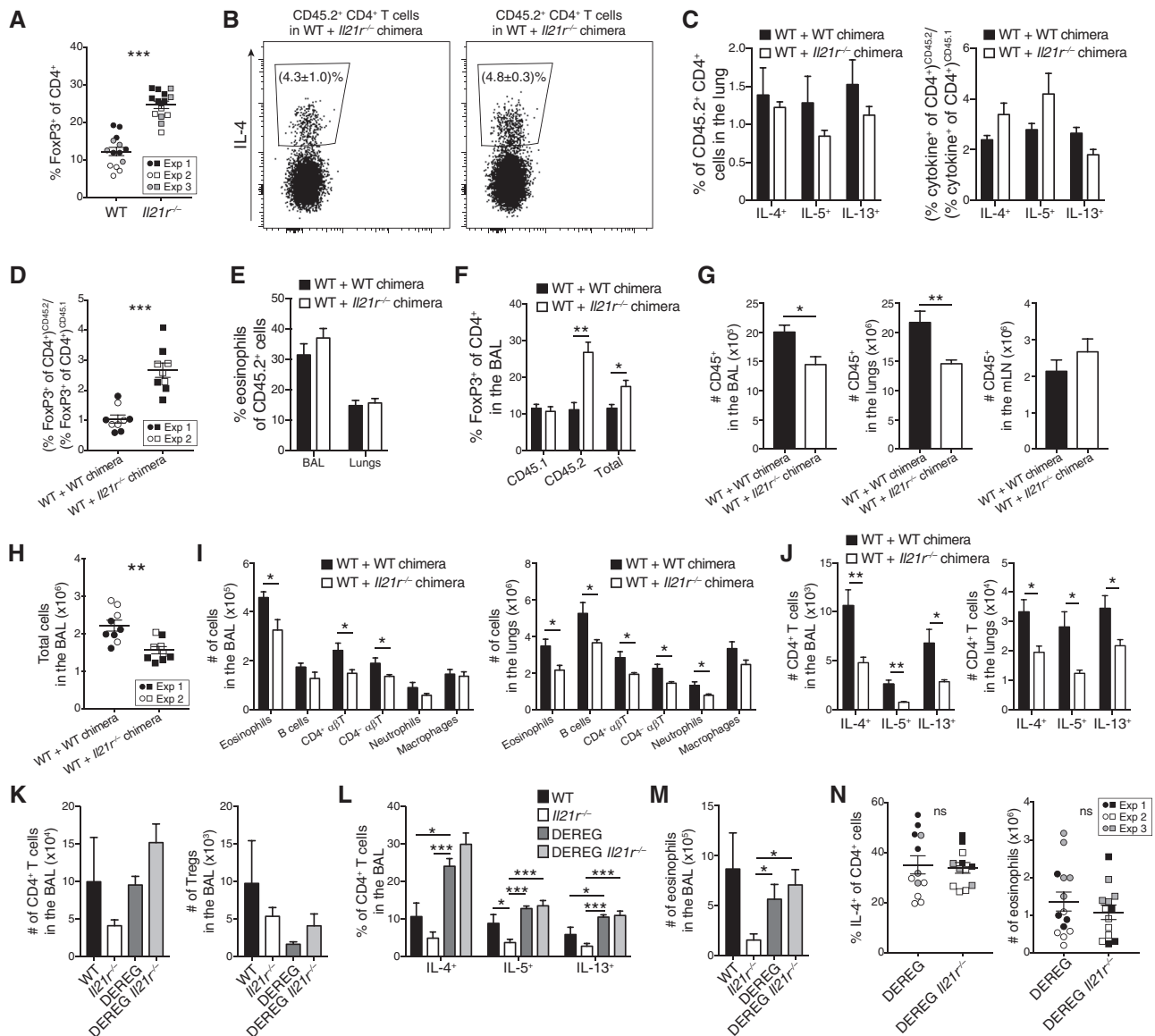


FIG E3. Cell-autonomous IL-21R signaling directly inhibits Treg cells but is not required for TH2 polarization and eosinophil infiltration in OVA/alum-induced asthma. **A**, Compiled statistic from 3 separate experiments showing Treg cell percentages in BAL fluid after OVA-induced asthma. Each dot represents a mouse (data from the same experiment depicted in the same color). **B–J**, FACS analysis of cells from OVA-immunized mixed bone marrow chimeras (5 mice per group). Fig E3, **B**, Expression of IL-4 by CD45.2⁺CD4⁺ T cells (BAL). Fig E3, **C**, Cytokine expression by CD45.2⁺CD4⁺ cells and ratio of cytokine-expressing CD45.2⁺CD45.1⁺CD4⁺ T cells (lungs). Fig E3, **D**, Compiled data from 2 independent experiments showing percentage of BAL fluid Treg cells in OVA-immunized chimeras. Fig E3, **E**, Percentage of eosinophils among CD45.2⁺ cells. Fig E3, **F**, Percentage of FoxP3⁺ cells among CD4⁺ T cells in each compartment. Fig E3, **G**, Cell numbers in indicated organs. Fig E3, **H**, Compiled statistics from 2 separate experiments depicting numbers of BAL fluid cells in OVA-immunized chimeras. Fig E3, **I**, Numbers of immune cells in chimeras. Fig E3, **J**, Numbers of TH2 cytokine-positive cells. **K–N**, OVA-induced asthma in WT, *Il21r*^{-/-}, DEREG, and DEREG *Il21r*^{-/-} mice treated with diphtheria toxin (4–6 mice per group). Fig E3, **K**, Numbers of CD4⁺ T cells and Treg cells (BAL). Fig E3, **L**, Expression of IL-4/IL-5/IL-13 by BAL fluid CD4⁺ T cells. Fig E3, **M**, Eosinophil numbers (BAL). Fig E3, **N**, Compiled data from 3 experiments showing IL-4 production and eosinophil infiltration in the airways of DEREG and DEREG *Il21r*^{-/-} mice. * $P < .05$, ** $P < .01$, and *** $P < .001$. ns, Not significant.

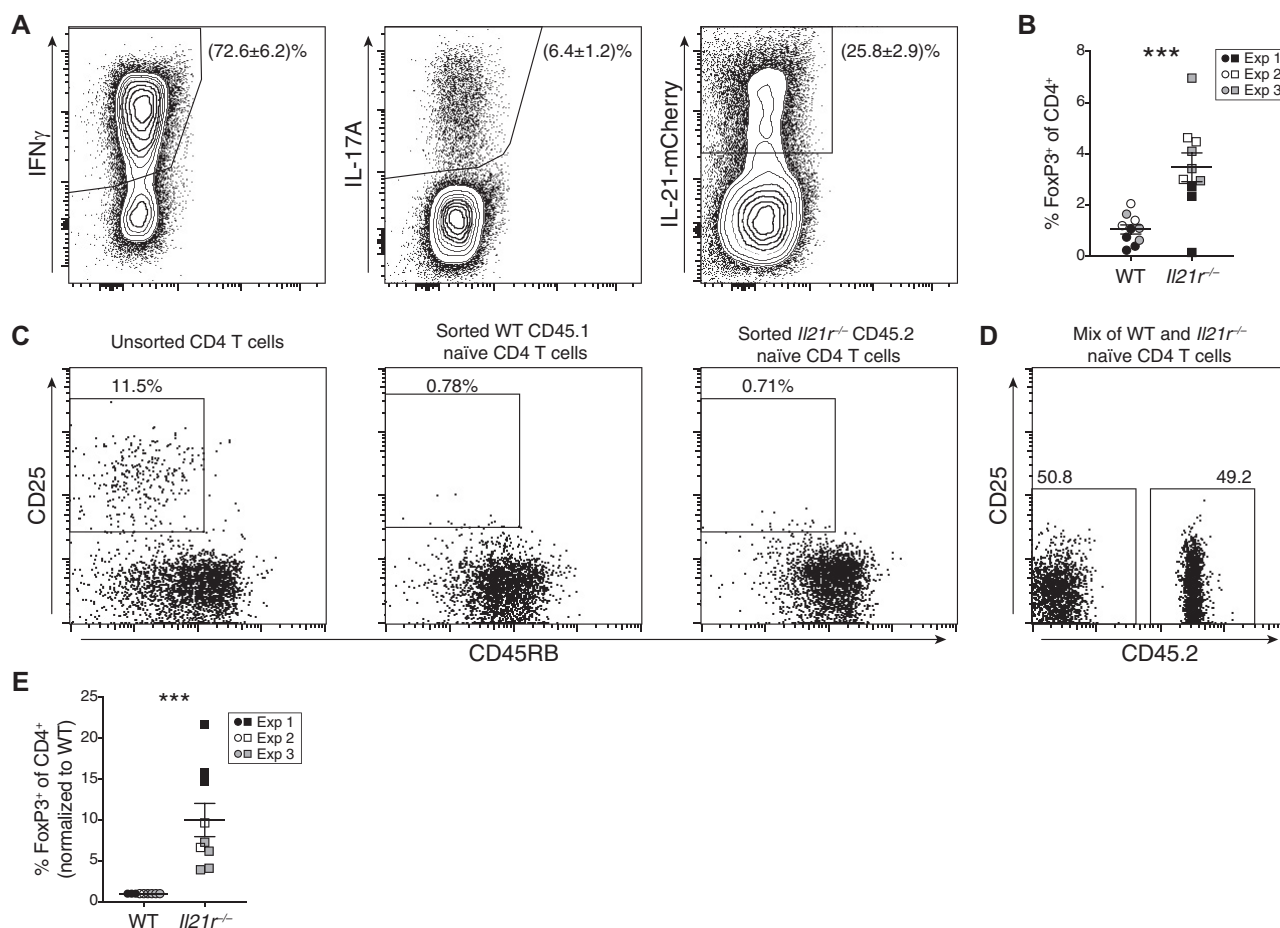


FIG E4. Cytokine expression by CD4⁺ T cells in mice with colitis and purity of CD4⁺ T cells for adoptive transfer. **A**, Naïve CD25⁻D45RB^{hi}CD4⁺ T cells were isolated from *Il21*-mCherry/*Il2*-emGFP reporter mice and transferred into *Rag1*^{-/-} hosts (n = 4). Expression of IFN-γ, IL-17A, and IL-21 among transferred CD4⁺ T cells obtained from colonic lamina propria at week 7. **B**, Naïve CD4⁺ T cells purified from WT and *Il21r*^{-/-} mice were injected separately into *Rag1*^{-/-} hosts. Plot depicts compiled data from 3 distinct experiments showing percentages of Treg cells among transferred CD4⁺ T cells. Each dot represents a single mouse (data from the same experiment shown in the same color). **C** and **D**, Naïve CD25⁻CD45RB^{hi}CD4⁺ T cells were purified from CD45.1 WT and CD45.2 *Il21r*^{-/-} mice. Fig E4, C, Expression of CD25 and CD45RB before and after sorting. Fig E4, D, Purified WT and *Il21r*^{-/-} naïve CD4⁺ T cells were mixed at a 1:1 ratio before injection into lymphopenic mice. The dot plot depicts the composition of the injected mix. **E**, *Rag1*^{-/-} mice were injected with a mixture of naïve CD45.1⁺ WT CD4⁺ T cells, CD45.1⁺CD45.2⁺ WT Treg cells, and CD45.2⁺*Il21r*^{-/-} Treg cells. After 7 weeks, CD4⁺ T cells were analyzed. The plot depicts the knockout/WT ratio among Treg cells (compiled data from 3 experiments). ***P < .001.

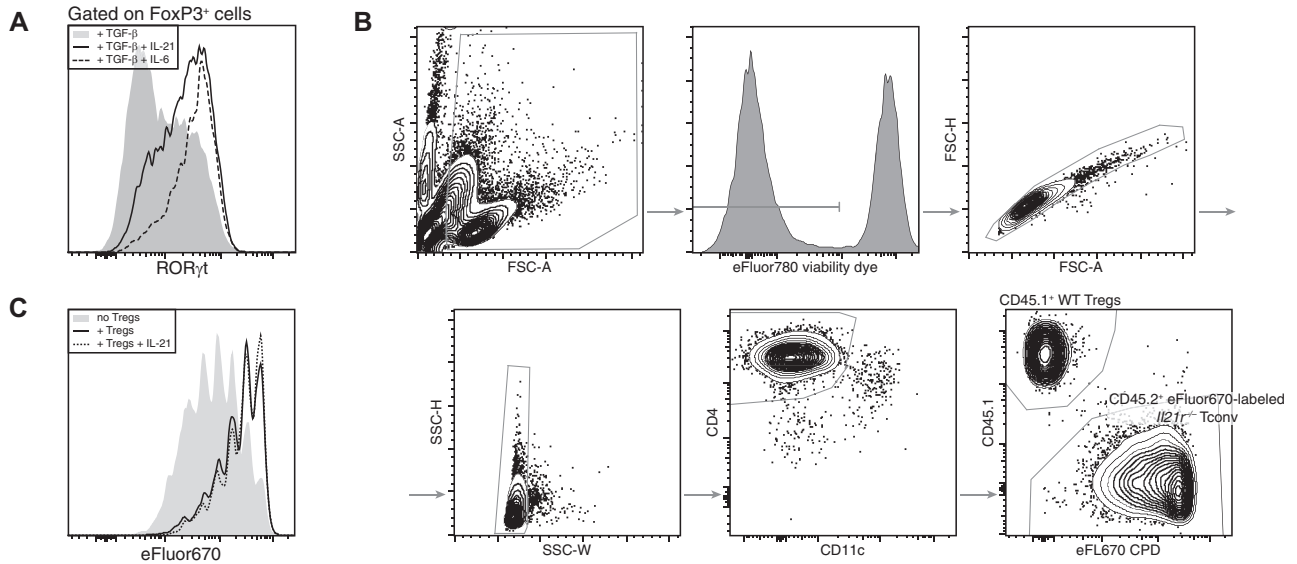


FIG E5. Effect of IL-21 on *in vitro*-cultured Treg cells. **A**, Naive FoxP3-EGFP⁻CD25⁻CD45RB^{hi} CD4⁺ T cells were FACS-sorted from spleens and skin-draining LNs of DERE mice. Cells were cultured in Treg cell-polarizing conditions in the presence or absence of IL-21 or IL-6. After 3 days, expression of RORγt and FoxP3 was assessed by means of intracellular staining. The histogram overlay shows expression of RORγt among FoxP3⁺ cells cultured in the indicated conditions. **B** and **C**, Treg cells were purified from spleens and skin-draining LNs of DERE mice expressing congenic marker CD45.1 and stimulated *in vitro* together with IL-21R-deficient eFluor670-labeled naive CD4⁺ T cells and CD11c⁺ DCs, with soluble anti-CD3 in the presence or absence of IL-21. After 3 days, cells were stained with a viability dye and analyzed by using flow cytometry. Fig E5, **B**, Gating strategy for the experiment shown in Fig 6, **D-G**. Fig E5, **C**, Histogram overlay depicts the dilution of eFluor670 among responding conventional T cells in the presence or absence of Treg cells and IL-21. *FSC*, Forward scatter; *SSC*, side scatter.