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IL-13 Regulates Th17 Secretion of IL-17A in an IL-10–Dependent Manner

Dawn C. Newcomb,* Madison G. Boswell,* Matthew M. Huckabee,* Kasia Goleniewska,* Daniel E. Dulek,[†] Sara Reiss,* Nicholas W. Lukacs,[‡] Jay K. Kolls,[§] and R. Stokes Peebles, Jr.*

IL-13 is a central mediator of airway hyperresponsiveness and mucus expression, both hallmarks of asthma. IL-13 is found in the sputum of patients with asthma; therefore, IL-13 is an attractive drug target for treating asthma. We have shown previously that IL-13 inhibits Th17 cell production of IL-17A and IL-21 *in vitro*. Th17 cells are associated with autoimmune diseases, host immune responses, and severe asthma. In this study, we extend our *in vitro* findings and determine that IL-13 increases IL-10 production from Th17-polarized cells and that IL-13–induced IL-10 production negatively regulates the secretion of IL-17A and IL-21. To determine if IL-13 negatively regulates lung IL-17A expression via an IL-10–dependent mechanism *in vivo*, we used a model of respiratory syncytial virus (RSV) strain A2 infection in STAT1 knockout (KO) mice that increases lung IL-17A and IL-13 expression, cytokines not produced during RSV infection in wild-type mice. To test the hypothesis that IL-13 negatively regulates lung IL-17A expression, we created STAT1/IL-13 double KO (DKO) mice. We found that RSV-infected STAT1/IL-13 DKO mice had significantly greater lung IL-17A expression compared with that of STAT1 KO mice and that increased IL-17A expression was abrogated by anti-IL-10 Ab treatment. RSV-infected STAT1/IL-13 DKO mice also had increased neutrophil infiltration compared with that of RSV-infected STAT1 KO mice. Neutralizing IL-10 increased the infiltration of inflammatory cells into the lungs of STAT1 KO mice but not STAT1/IL-13 DKO mice. These findings are vital to understanding the potential side effects of therapeutics targeting IL-13. Inhibiting IL-13 may decrease IL-10 production and increase IL-17A production, thus potentiating IL-17A–associated diseases. *The Journal of Immunology*, 2012, 188: 1027–1035.

Asthma is a chronic disease that affects 300 million people worldwide (1). CD4⁺ Th2 cells are a primary driving force of allergic lung inflammation in asthma, and the Th2 cytokine IL-13 is a central mediator of airway hyperresponsiveness and airway mucus expression, both important hallmarks of asthma (2, 3). IL-13 is found in abundance in the sputum of patients with asthma (4), and therefore IL-13 is an attractive drug target for treating asthma (3, 5). Currently, there are multiple ongoing clinical trials using IL-13 antagonists for asthma therapy (6–12).

IL-13 binds to IL-13R (also known as the type II IL-4 receptor), which is composed of two subunits, IL-4R α and IL-13R α 1. We have reported recently that Th17 cells, but not Th0, Th1, or Th2 cells, in both mice and humans express the IL-13R α 1 subunit of IL-13R (13, 14). IL-13R α 1 expression on mouse T cells has been

confirmed by Wilson et al. (15). IL-13 binds IL-13R, resulting in the phosphorylation and activation of the downstream transcription factor STAT6. Once phosphorylated, STAT6 homodimerizes and translocates to the nucleus for the transcription of IL-13–dependent genes. We determined in mouse and human Th17 cells that the addition of IL-13 resulted in STAT6 phosphorylation and attenuated IL-17A and IL-21 production as well as reduced the expression of the Th17 transcription factor ROR- γ T (RORC2 in humans) (13, 14). The focus of this project is to further define the mechanism by which IL-13 negatively regulates IL-17A production in the lung.

Th17 cells are a subset of CD4⁺ T cells with distinct properties from Th1, Th2, and regulatory T cells (16, 17). Th17 cells are found in the lungs of patients with severe asthma (18, 19), and the Th17 cytokine IL-17A is present in the bronchoalveolar lavage (BAL) fluid and bronchial biopsies of patients with moderate to severe asthma (20, 21). Th17 cells also are associated with autoimmune and inflammatory disorders, such as the experimental autoimmune encephalitis (EAE) mouse model of multiple sclerosis and rheumatoid arthritis (22), and are critical for the clearance of some extracellular pathogens (16, 23–26). In mice, Th17 cell differentiation occurs when T cells are activated in the presence of TGF- β and IL-6 (26–28), and maintenance and sustainability of Th17 cells require IL-23 (16, 29, 30). Th17 cells produce a variety of cytokines, including IL-17A, IL-17F, IL-21, and IL-10 (25). IL-17A and IL-17F have similar structural homology, and both increase the production of inflammatory cytokines and mucus (31); however, IL-17A is more biologically active than IL-17F (32). IL-21 acts in an autocrine manner to promote IL-17A production and further inhibits IFN- γ production from Th1 cells (28, 33). IL-10 is an anti-inflammatory cytokine that inhibits IL-17A production from Th17 cells (34).

*Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232; [†]Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN 37232; [‡]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109; and [§]Department of Pediatrics, University of Pittsburgh, Children's Hospital of Pittsburgh, Pittsburgh, PA 15213

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Address correspondence and reprint requests to Dr. Dawn C. Newcomb, Department of Medicine, Vanderbilt University Medical Center, 1161 21st Avenue, T-1221 Medical Center North, Nashville, TN 37232. E-mail address: dawn.newcomb@vanderbilt.edu

Abbreviations used in this article: BAL, bronchoalveolar lavage; DKO, double knockout; EAE, experimental autoimmune encephalomyelitis; KO, knockout; RSV, respiratory syncytial virus; WT, wild-type.

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To determine if IL-13 regulates IL-17A production *in vivo*, we used a mouse model of respiratory syncytial virus (RSV) strain A2 infection in STAT1 knockout (KO) mice. We reported previously that RSV infection increased lung IL-13 and IL-17A expression in STAT1 KO mice but not wild-type (WT) BALB/c mice (35). Based on our *in vitro* findings that IL-13 negatively regulated IL-17A production from Th17 cells, we hypothesized that IL-13 negatively regulates IL-17A production *in vivo*. To test our hypothesis, we generated STAT1/IL-13 double KO (DKO), STAT1/IL-4 DKO, and STAT1/STAT6 DKO mice. STAT6 is a downstream transcription factor for IL-13 and IL-4 signaling. The use of STAT1/IL-13 DKO, STAT1/IL-4 DKO, and STAT1/STAT6 DKO mice will determine the independent contribution of IL-13 and IL-4, relative to total STAT6 signaling, in attenuating lung IL-17A protein expression. These studies further define the mechanisms by which IL-13 downregulates IL-17A production and are important for understanding the potential side effects in therapeutics that target IL-13 or IL-13 signaling in lung disease.

Materials and Methods

Mice

Pathogen-free 8- to 10-wk-old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) (36). STAT1 KO and IL-13 KO mice on a BALB/c background were generated as described previously (36, 37). IL-4 KO and STAT6 KO mice on a BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME). STAT1/IL-13 DKO, STAT1/IL-4 DKO, and STAT1/STAT6 DKO mice were generated by mating and subsequent genotyping. In caring for the animals, investigators adhered to the revised 1996 *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

RSV infections

The A2 strain of RSV was provided by R. Chanock (National Institutes of Health, Bethesda, MD). Viral stocks of RSV A2 were generated by the infection of HEP-2 cells and titered by plaque assay as described previously (38). Mice were anesthetized with ketamine/xylazine and infected intranasally with 1×10^6 PFU of RSV A2. Lungs were harvested 6 d postinfection. In select experiments, mice were injected *i.p.* with 100 μ g anti-IL-10 or isotype control Ab (generated from ascites fluid at the University of Michigan) 24 h before RSV infection and 3 d postinfection (39).

T cell isolation and Th17 cell polarization and activation

CD4⁺ T cells were purified from the spleens of mice as described previously (11). T cells were activated using anti-CD3 (5 μ g/ml) and anti-CD28 (1 μ g/ml) (BD Biosciences) Abs in 96-well plates for 4 d. T cells were differentiated into Th17 cells by adding mouse rIL-23 (10 ng/ml), human rTGF- β (1 ng/ml), mouse rIL-6 (20 ng/ml), anti-IFN- γ (10 μ g/ml), and anti-IL-4 (10 μ g/ml). In select cultures, anti-IL-10 (10 μ g/ml), mouse rIL-13 (10 ng/ml), or mouse rIL-21 (0.5–5 ng/ml) also was added. All of the Abs and mouse rIL-13, mouse rIL-23, and mouse rIL-21 were purchased from R&D Systems. Mouse rIL-6 and human rTGF- β were purchased from PeproTech (Rocky Hill, NJ).

Cytokine measurements

Cytokine levels were measured from cell culture supernatants or whole lung homogenates with available DuoSet or Quantikine ELISA kits (R&D Systems) following the manufacturer's instructions.

Flow cytometry

Lungs were harvested, minced, and digested for 40 min at 37°C in RPMI 1640 containing 10% FBS, collagenase type I (0.1% w/v), and DNase (500 μ g/ml). The digestion was stopped with 10 μ l of 0.5 M EDTA, and minced lung was passed through a 70- μ m strainer. For intracellular cytokines to be stained, cells were stimulated in RPMI 1640, 10% FBS, 1 μ M ionomycin (Sigma-Aldrich), 50 ng/ml PMA (Sigma-Aldrich), and 0.07% GolgiStop (BD Pharmingen) for 6 h at 37°C and 5% CO₂. Cells then were counted using a hemocytometer, and the total number of cells from the lungs of each mouse was recorded. One million cells from the lungs of each mouse

were stained for cell surface molecules and intracellular cytokines as described previously (40). We used the following Abs from BD Pharmingen: anti-CD3e, anti-CD8a, anti-CD4, anti-Ly6G (Gr-1), anti-DX5, anti- γ δ TCR, and anti-IL-17A. Anti-CD16/32 Ab (BD Pharmingen) was used to prevent nonspecific staining. Cell samples were analyzed using an LSR II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

Quantitative PCR

A two-step real-time PCR assay using SYBR green mix (Bio-Rad, Hercules, CA) was used to detect SOCS3 as described previously (41), with GAPDH being used as the housekeeping gene. Primer sequences were as follows: SOCS3, forward, 5'-GGGTGGCAAAGAAAAGGAG-3', reverse, 5'-GTTGAGCGTCAAGACCCAGT-3'; GAPDH, forward, 5'-GGCCCCCTCTGGAAAGCTGTGG-3', reverse, 5'-CCDGGCATCGAAGGTGGAAGA-3'.

Analysis of inflammatory cell infiltration into BAL fluid

BAL was performed by instilling 800 μ l saline through a tracheostomy tube and then withdrawing the fluid with gentle suction via syringe. WBCs were counted on a hemocytometer. Cytologic examination was performed on cytopsin preparations (Thermo Shandon). Cytopsin slides were fixed and stained using Three-Step Stain (Richard-Allan Scientific). Differential counts were based on counts of 200 cells using standard morphologic criteria to classify the cells as neutrophils, eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes).

Statistical analyses

Data are presented as mean \pm SEM. For Figs. 1 and 2, data shown represent a combined analysis of cell culture wells from three independent experiments. For Figs. 3–6, data shown represent a combined analysis of individual mice. Data were analyzed with ANOVA followed by a Tukey posthoc test or an unpaired two-tailed *t* test using Prism (version 4; GraphPad) with values being considered significant when *p* < 0.05.

Results

IL-13 inhibits IL-21 production, resulting in decreased IL-17A production in mouse CD4⁺ T cells

CD4⁺ T cells isolated from the spleens of WT BALB/c mice were polarized to become Th17 cells with TGF- β , IL-6, IL-23, anti-IL-4, and anti-IFN- γ in the presence of IL-13 (0–10 ng/ml). Cell culture supernatants were collected 1–4 d after activation and polarization and were examined for IL-21 and IL-17A cytokine production. IL-21 production was decreased in the cells treated with IL-13, starting 2 d after polarization and activation, and IL-13 continued to attenuate IL-21 production at days 3 and 4 of T cell polarization (Fig. 1A). IL-17A production was decreased 3 d after polarization, with maximal IL-17A production occurring 4 d after polarization (Fig. 1B). To determine if decreased IL-21 production results in inhibited IL-17A production, naive CD4⁺ T cells were activated and polarized in the presence of rIL-21 (0–5 ng/ml) as well as IL-13 (0–10 ng/ml). IL-17A production was measured 4 d after polarization, and rIL-21 restored IL-17A production in the presence of IL-13 (Fig. 1C). Taken together, these data suggest that IL-13 attenuates IL-17A production by decreasing IL-21 production.

IL-13 attenuates IL-17A and IL-21 production in an IL-10-dependent manner in mouse CD4⁺ T cells

IL-10 downregulates IL-17A production from Th17 cells (42), and IL-10 produced by Th17 cells attenuates EAE severity in mice (34). Therefore, we hypothesized that IL-13 increases IL-10 production in Th17 cells and that inhibiting IL-10 restores IL-17A production in the presence of IL-13. As hypothesized, IL-10 levels were increased in mouse Th17 cells polarized in the presence of IL-13 (Fig. 2A). Also as hypothesized, IL-10 neutralization in the presence of IL-13 restored IL-17A and IL-21 production to the levels seen in Th17 cells not exposed to IL-13 (Fig. 2B, 2C).

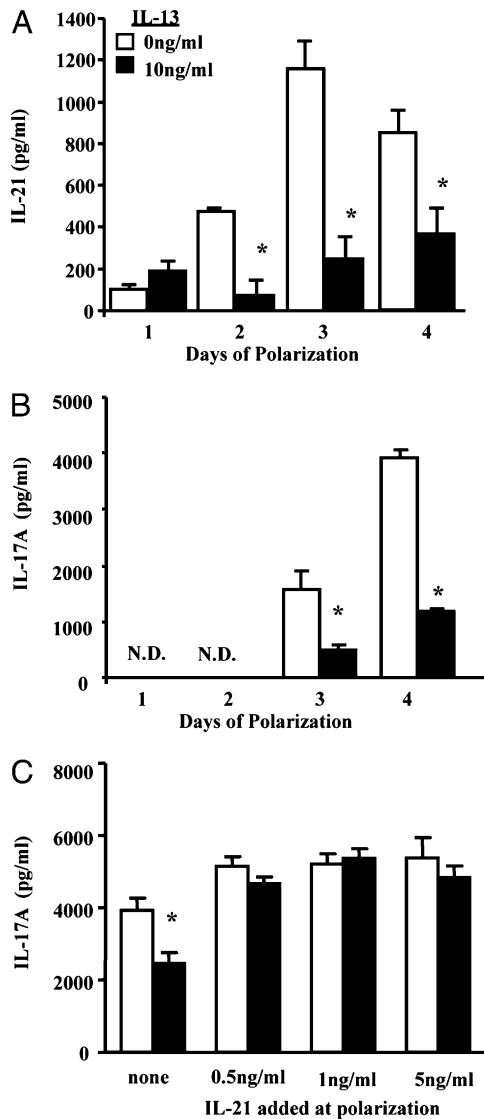


FIGURE 1. IL-13 decreases IL-17A production in an IL-21-dependent manner. *A* and *B*, CD4⁺ T cells were activated with anti-CD3 and anti-CD28 Abs with Th17-polarizing conditions in the presence of mouse rIL-13 (0 or 10 ng/ml). Cell supernatants were collected 1–4 d after polarization, and IL-21 and IL-17A were examined by ELISA. **p* < 0.05 compared with 0 ng/ml IL-13 for each day. *n* = 4–8 cell culture wells from three independent experiments. *C*, CD4⁺ T cells were activated and polarized to become Th17 cells in the presence of mouse rIL-13 (0 or 10 ng/ml) and/or mouse rIL-21 (none, 0.5, 1, or 5 ng/ml). Cell supernatants were collected 4 d after polarization, and IL-17A was examined by ELISA. *n* = 4–8 cell culture wells from three independent experiments. **p* < 0.05 compared with 0 ng/ml IL-13 when no mouse rIL-21 was added to the culture conditions.

These data suggest that IL-13 negatively regulates IL-17A and IL-21 production in an IL-10-dependent mechanism.

IL-13 negatively regulates RSV-induced IL-17A production in the lung

We have shown previously that IL-13 negatively regulates IL-17A production from Th17 cells in both mice and humans (13, 14); however, whether IL-13 regulates Th17 cytokine production in the lung is unknown. We hypothesized that IL-13 negatively regulates lung Th17 cytokine production, and we capitalized on a model of RSV A2 infection in STAT1 KO mice. As we have shown previously, RSV A2 infection in STAT1 KO mice resulted in significant

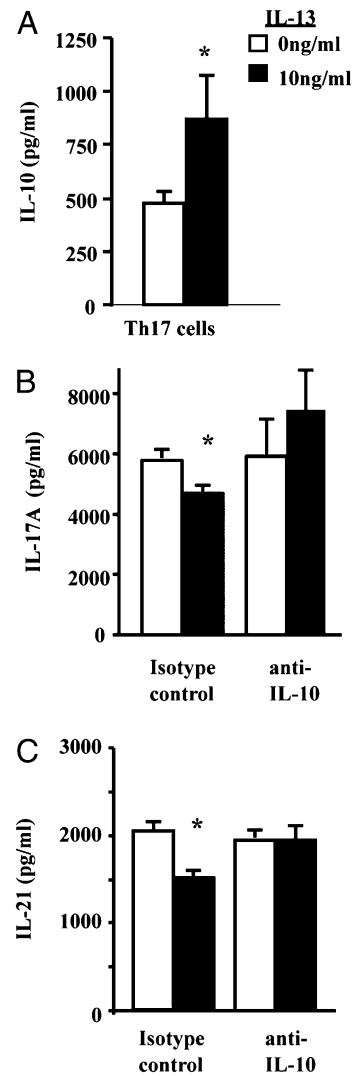


FIGURE 2. IL-13 attenuates IL-17A and IL-21 production in an IL-10-dependent manner in mouse CD4⁺ T cells. *A*, CD4⁺ T cells were activated and polarized to Th17 cells in the presence of IL-13 (0 or 10 ng/ml). Cell culture supernatants were collected 4 d after polarization and analyzed for IL-10 production. **p* < 0.05 compared with 0 ng/ml IL-13. *n* = 8 cell culture wells from three independent experiments. *B* and *C*, CD4⁺ T cells were activated and polarized to Th17 cells for 4 d with anti-IL10 (10 μg/ml) or isotype control Ab in the presence of IL-13. Cell culture supernatants were analyzed for IL-21 and IL-17A production by ELISA 4 d after polarization. *n* = 4–5 cell culture wells from two independent experiments. **p* < 0.05 compared with 0 ng/ml IL-13.

increases of lung IL-17A and IL-13 expression, whereas IL-17A and IL-13 were not detected in RSV-infected WT mice (Fig. 3A, 3D) (35). To determine the specific role of IL-13 and IL-13 signaling in RSV-induced IL-17A production in STAT1 KO mice, we generated STAT1/IL-13 DKO, STAT1/IL-4 DKO, and STAT1/STAT6 DKO mice. Both IL-13 and IL-4 activate STAT6 and negatively regulate Th17 cytokine secretion in vitro (13, 14, 17). Six days after RSV A2 infection, the known peak of lung IL-17A in protein expression in STAT1 KO mice (35), lungs were harvested and analyzed for cytokine production (Fig. 3). RSV-infected WT mice did not have detectable levels of IL-17A or IL-17F. RSV-infected STAT1 KO mice had significant increases in IL-17A, IL-17F, IL-10, and IL-13 expression compared with those of RSV-infected WT mice. RSV-infected STAT1/IL-13 DKO and STAT1/IL-4 DKO mice had significant increases in IL-17A and

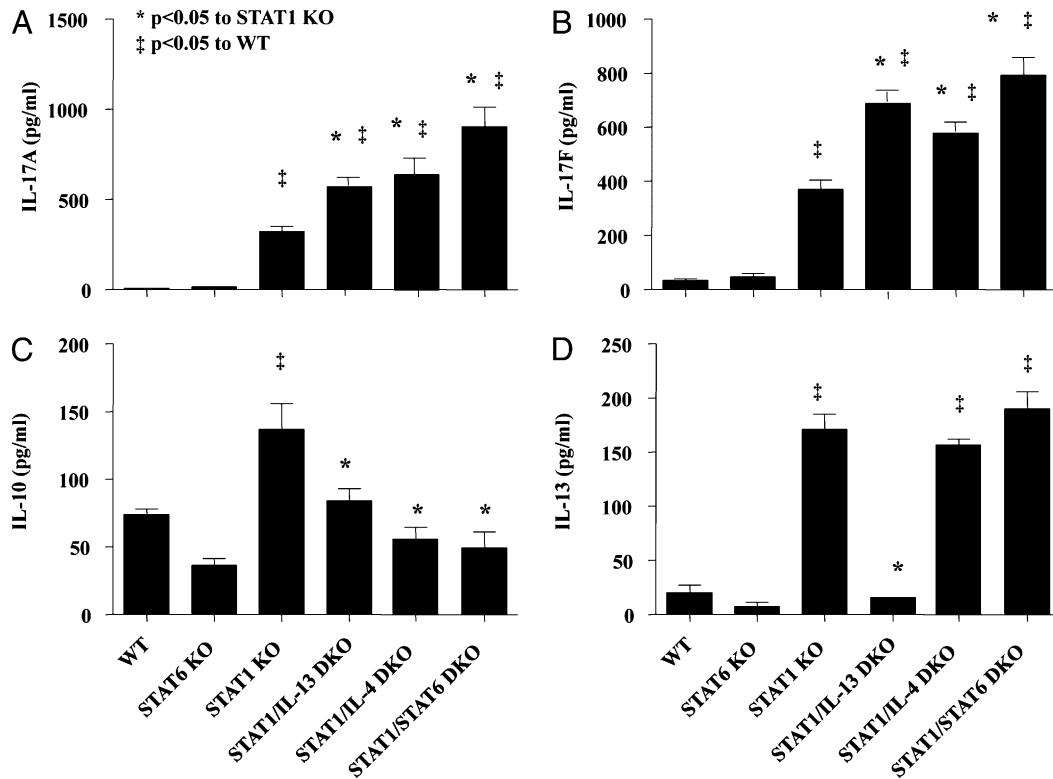


FIGURE 3. IL-13 and IL-4 independently regulate RSV-induced cytokine production in STAT1 KO mice. *A–D*, Mice were infected with RSV A2, and lung homogenates were analyzed 6 d postinfection for cytokine production by ELISA. $n = 7–10$ mice for each group from three independent experiments. * $p < 0.05$ compared with STAT1 KO mice, † $p < 0.05$ compared with WT mice.

IL-17F expression and a significant decrease in IL-10 expression compared with those of RSV-infected STAT1 KO mice. IL-17A and IL-17F production from RSV-infected STAT1/STAT6 DKO mice was ~40% greater compared with those of RSV-infected STAT1/IL-13 DKO or STAT1/IL-4 DKO mice, but this increase was not statistically significant. IL-13 was not detected in RSV-infected STAT1/IL-13 DKO mice, but IL-13 levels were not different among RSV-infected STAT1 KO, STAT1/IL-4 DKO, and STAT1/STAT6 DKO mice (Fig. 3*D*). We did not find a difference in IL-21 production in any group examined (data not shown). The peak of IL-21 production likely occurs at an earlier time point, as supported by our *in vitro* findings (Fig. 1*A*). Taken together, these data show that both IL-13 and IL-4 negatively regulate IL-17A and IL-17F production in whole lung homogenates of RSV-infected STAT1 KO mice. Based on our previous studies with IL-13 regulating IL-17A production *in vitro*, we decided to focus on the role of IL-13 in RSV-induced IL-17A production for the remainder of the study.

RSV-induced IL-17A is expressed by CD3⁺CD4⁺CD8⁻ T cells and CD3⁺CD4⁻CD8⁻ cells

IL-17A is produced from numerous cell types. Therefore, to determine the cellular source of IL-17A and investigate the role of IL-13, cells were isolated from the lungs of STAT1 KO, STAT1/IL-13 DKO, and STAT1/STAT6 DKO mice 6 d after RSV infection and were restimulated with PMA and ionomycin in the presence of GolgiStop for an analysis by flow cytometry. Cells were stained for Abs to identify CD4⁺ T cells, CD8⁺ T cells, $\gamma\delta$ T cells, Gr-1⁺ cells, and DX5⁺ cells (Fig. 4*A* and data not shown). Lungs from RSV-infected STAT1 KO mice had IL-17A-expressing CD3⁺CD4⁺CD8⁻ cells as well as CD3⁺CD4⁻CD8⁻ cells (Fig. 4*A*), but RSV-infected STAT1/IL-13 DKO mice had significant increases in IL-17A-expressing CD3⁺CD4⁺CD8⁻ cells compared with those

of RSV-infected STAT1 KO mice (Fig. 4*B*). RSV-infected STAT1/STAT6 DKO mice also significantly increased IL-17A expression by CD3⁺CD4⁺CD8⁻ cells as well as CD3⁺CD4⁻CD8⁻ cells compared with those of RSV-infected STAT1 KO mice (Fig. 4*B*). RSV-infected CD3⁺CD4⁻CD8⁻ cells were not DX5⁺ cells, $\gamma\delta$ T cells, or Gr-1⁺ cells, because no IL-17A expression was detected in the cells expressing these markers (Fig. 4*A* and data not shown). These data suggest that IL-13 negatively regulates IL-17A expression in CD3⁺CD4⁺CD8⁻ Th17 cells.

IL-10 neutralization decreases RSV-induced IL-17A production in STAT1 KO mice

Based on our *in vitro* results showing that IL-13 negatively regulated IL-17A production in an IL-10-dependent manner (Fig. 2), we hypothesized that neutralizing IL-10 *in vivo* would increase RSV-induced IL-17A production in STAT1 KO mice but have no effect on IL-17A production in RSV-infected STAT1/IL-13 DKO mice. To test these hypotheses, we *i.p.* injected mice with anti-IL-10 or isotype control Ab 1 d before RSV infection and 3 d postinfection. Mice were RSV-infected, and lungs were harvested 6 d postinfection and examined for cytokine production. IL-10 neutralization decreased the detection of secreted IL-10 in lung homogenates of STAT1 KO and STAT1/IL-13DKO mice (Fig. 5*A*). IL-10 neutralization significantly increased IL-17A and IL-17F lung production in RSV-infected STAT1 KO mice but had no effect on lung IL-17A or IL-17F production in RSV-infected STAT1/IL-13 DKO mice (Fig. 5*B, C*). IL-10 neutralization had no effect on IL-13 production (Fig. 5*D*).

IL-10 binds to IL-10R and has been shown previously to decrease IL-17A production from Th17 cells by increasing SOCS3 expression (43). Based on our findings showing that neutralizing IL-10 increases IL-17A in RSV-infected STAT1 KO mice but not RSV-infected STAT1/IL-13 DKO mice, we examined the relative

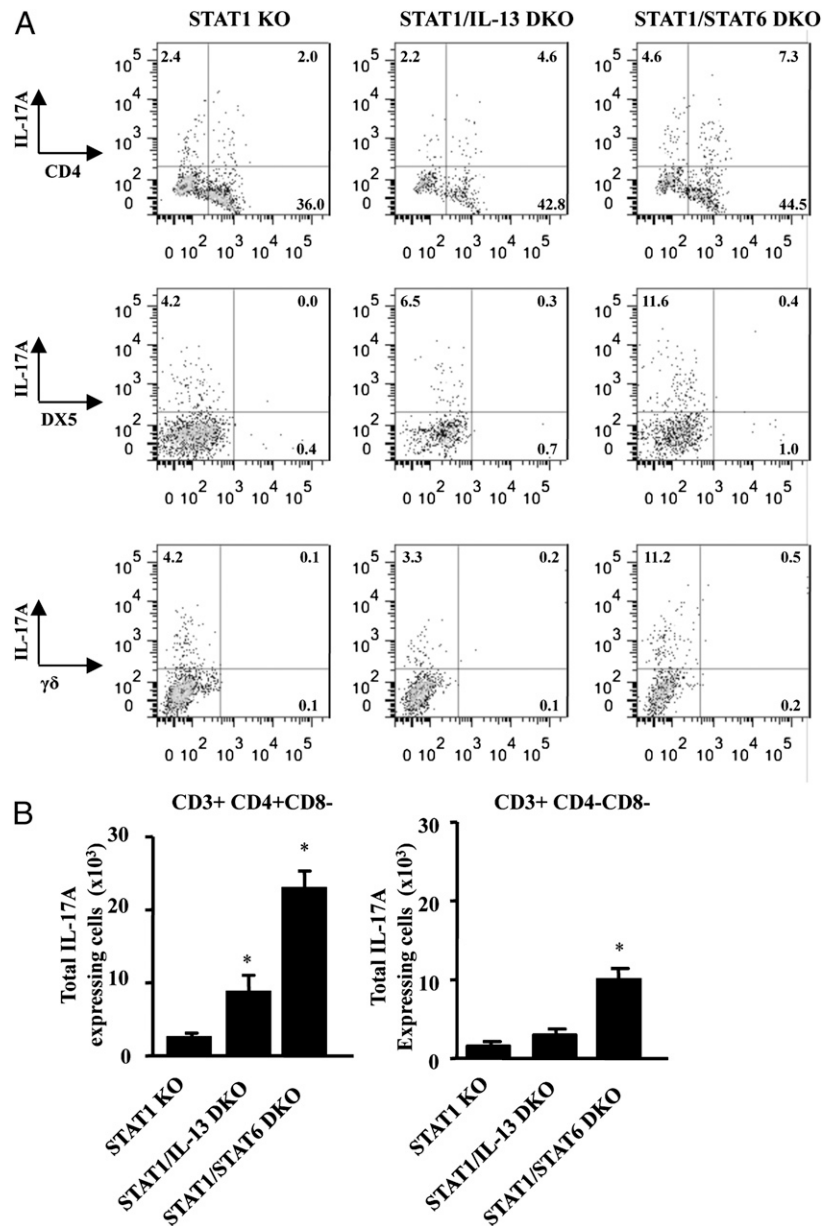


FIGURE 4. IL-17A is expressed by CD3⁺CD4⁺CD8⁻ and CD3⁺CD4⁻CD8⁻ cells in STAT1 KO, STAT1/IL-13 DKO, and STAT1/STAT6 DKO mice after RSV infection. Mice were infected with RSV A2, and lungs were harvested 6 d postinfection. A single-cell suspension from the lungs was restimulated with PMA, ionomycin, and GolgiPlug. Surface staining was performed with Abs against CD3, CD4, CD8, and $\gamma\delta$ T cells. Intracellular cytokine staining was performed with Abs against IL-17A. **A**, Representative dot plots that are CD3-gated. **B**, Total number of cells that are CD3⁺CD4⁺CD8⁻ or CD3⁺CD4⁻CD8⁻. Total cell numbers were calculated by multiplying the percentage of IL-17A⁺CD3⁺CD4⁺ or IL-17A⁺CD3⁺CD4⁻ cells by the total cell number determined by counting the cells using a hemocytometer. $n = 7$ –10 mice for each group from three independent experiments. * $p < 0.05$ compared with STAT1 KO mice.

expression levels of SOCS3 by real-time PCR from the lungs of mice 6 d after RSV infection. RSV-infected STAT1/IL-13 DKO mice treated with isotype control Ab had significantly decreased SOCS3 expression compared with that of RSV-infected STAT1 KO mice treated with isotype control Ab (Fig. 5E). IL-10 neutralization significantly decreased SOCS3 relative expression in RSV-infected STAT1 KO mice but had no effect on SOCS3 relative expression in RSV-infected STAT1/IL-13 DKO mice. Taken together, these results reveal that IL-13 negatively regulates IL-17A and IL-17F production, increasing SOCS3 expression in an IL-10-dependent manner.

IL-13 deficiency increases IL-10-mediated airway neutrophilia in RSV-infected STAT1 KO mice

RSV infection increases the infiltration of inflammatory cells into the lungs of STAT1 KO mice (35). Increased IL-17A expression increases neutrophil infiltration into target tissue (44). Therefore, we hypothesized that IL-13 deficiency would increase neutrophil infiltration into the lungs of RSV-infected STAT1 KO mice. BAL fluid was collected 6 d after RSV infection and examined for the

infiltration of inflammatory cells. In mice treated with isotype Ab, RSV-infected STAT1/IL-13 DKO mice had a statistically significant 2.5-fold increase in the infiltration of neutrophils compared with that of RSV-infected STAT1 KO mice (Fig. 6B). Neutralization of IL-10 increased the total number of inflammatory cells in BAL fluid of RSV-infected STAT1 KO mice as well as the number of neutrophils, eosinophils, and macrophages (Fig. 6). Neutralization of IL-10 did not alter the number of inflammatory cells in RSV-infected STAT1/IL-13 DKO mice. Taken together, these data show that RSV-infected STAT1/IL-13 DKO mice have increased the infiltration of neutrophils and that neutralizing IL-10 increases the infiltration of inflammatory cells in RSV-infected STAT1 KO mice.

Discussion

Our previous in vitro studies revealed that mouse CD4⁺ Th17 cells expressed functional IL-13R α 1 (13, 14). IL-13 signaling through this receptor inhibited Th17 cytokine production (13, 14), but the mechanism of action remained unknown. In this study, we de-

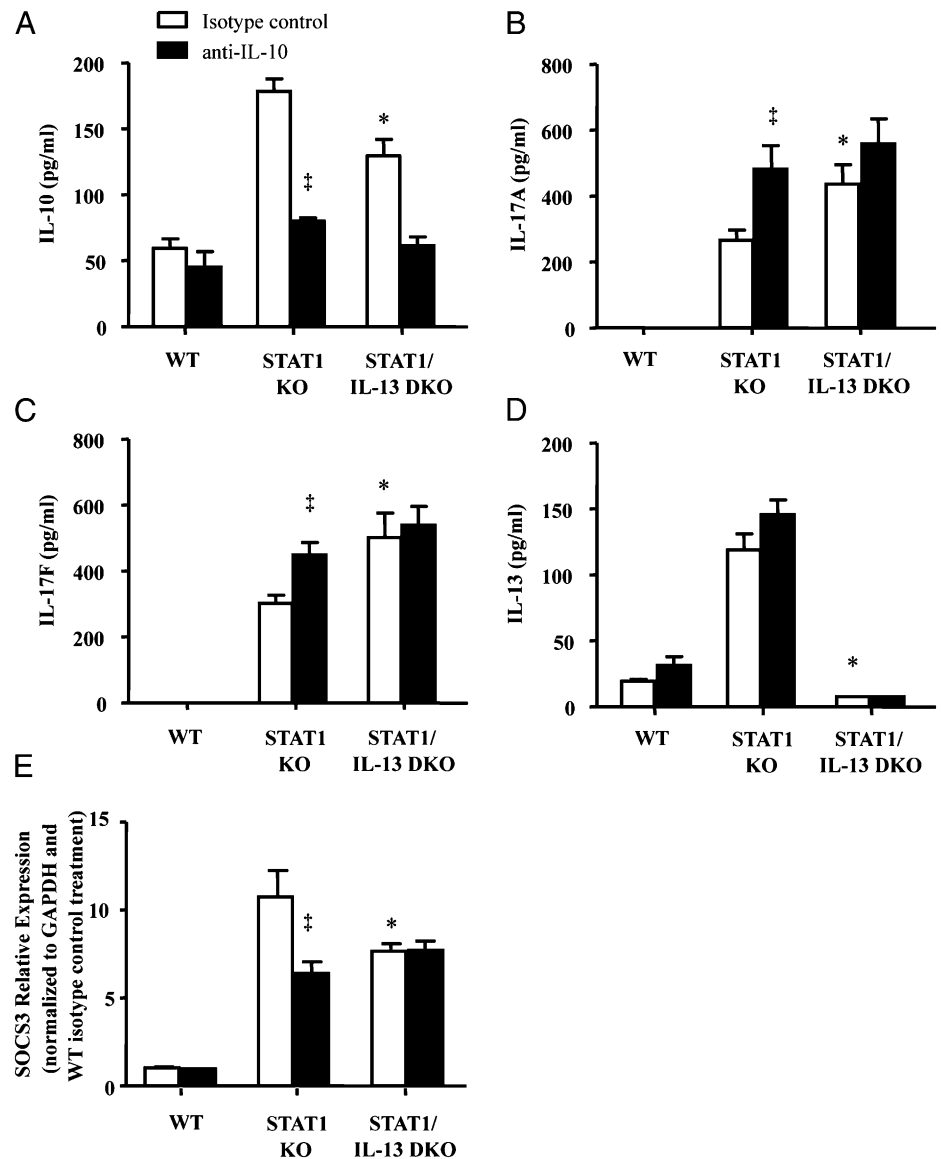


FIGURE 5. IL-10 neutralization increases RSV-induced IL-17A production in STAT1 KO mice. Mice were injected i.p. with anti-IL-10 or IgG isotype control Ab 1 d before RSV A2 infection and 3 d postinfection. *A–D*, Lungs were harvested 6 d postinfection and analyzed for cytokine production by ELISA. *E*, SOCS3 relative expression normalized to GAPDH and WT isotype control treatment. $n = 5$ mice for each group. $*p < 0.05$ compared with STAT1 KO mice treated with isotype control Ab, $*p < 0.05$ compared with isotype control Ab within strain of mice.

terminated that IL-13 increases IL-10 production, which attenuates IL-17A and IL-21 expression in vitro. Adding rIL-21 at the time of Th17 polarization restored IL-17A production in the presence of IL-13. IL-10 neutralization prevented IL-13 from attenuating IL-17A and IL-21 production in Th17 cells in vitro. These data show that IL-13 inhibits IL-17A production by increasing IL-10 expression, which in turn decreases IL-21 expression. We provide data for a mechanism by which IL-13 negatively regulates Th17 cytokine production is mediated by IL-10.

Based on these in vitro results, we hypothesized that IL-13 attenuates IL-17A production in vivo, also by an IL-10-dependent mechanism. To test this hypothesis, we used the model of RSV A2 infection in STAT1 KO mice, which induces lung IL-13 and IL-17A protein expression, whereas neither of these cytokines is produced in WT mice with RSV A2 infection (35). In the in vivo experiments reported in this study, we found that there was a significant 2-fold increase in IL-17A and IL-17F expression detected in STAT1/IL-13 DKO mice compared with that in STAT1 KO mice. IL-4, which activates STAT6, also negatively regulated IL-17A and IL-17F expression (13, 14, 17) in vivo. We found that IL-13 and IL-4 have similar inhibitory effects on IL-17A expression in RSV-infected STAT1 KO mice, and when viewed in the context of RSV-infected STAT1/STAT6 DKO mice, there

appears to be an additive effect of these cytokines in negatively regulating lung IL-17A protein expression. Our results suggest that both IL-13 and IL-4 are critical mediators of IL-17A inhibition by the STAT6 signaling pathway.

IL-17A expression was detected in CD3⁺CD4⁺CD8⁻ cells as well as CD3⁺CD4⁻CD8⁻ cells. In this model, cells expressing DX5, GR-1, or $\gamma\delta$ T cells did not produce IL-17A. DX5 is a marker that stains NK cells and a small subset of T lymphocytes, which are likely NKT cells (45). Gr-1 is expressed on granulocytes, monocytes during differentiation, and weakly on plasmacytoid dendritic cells. CD3⁺ double-negative cells (CD4⁻ and CD8⁻) have been shown previously to produce both IL-17A and IFN- γ after *Francisella tularensis* and *Listeria monocytogenes* infections in mice (24, 46). These double-negative cells were required for the clearance of *L. monocytogenes* (24), and IL-17A also was vital for the clearance of *F. tularensis* and *L. monocytogenes* infections in mice (24, 46). IL-17A expression by CD3⁺ double-negative cells was increased significantly in STAT1/STAT6 DKO mice compared with that in STAT1 KO and STAT1/IL-13 DKO mice. These data suggest that IL-17A production from CD3⁺ double-negative cells is negatively regulated by IL-4 but not IL-13.

In vitro, IL-10 production was increased significantly in Th17 cells polarized in the presence of IL-13, and IL-10 was required for

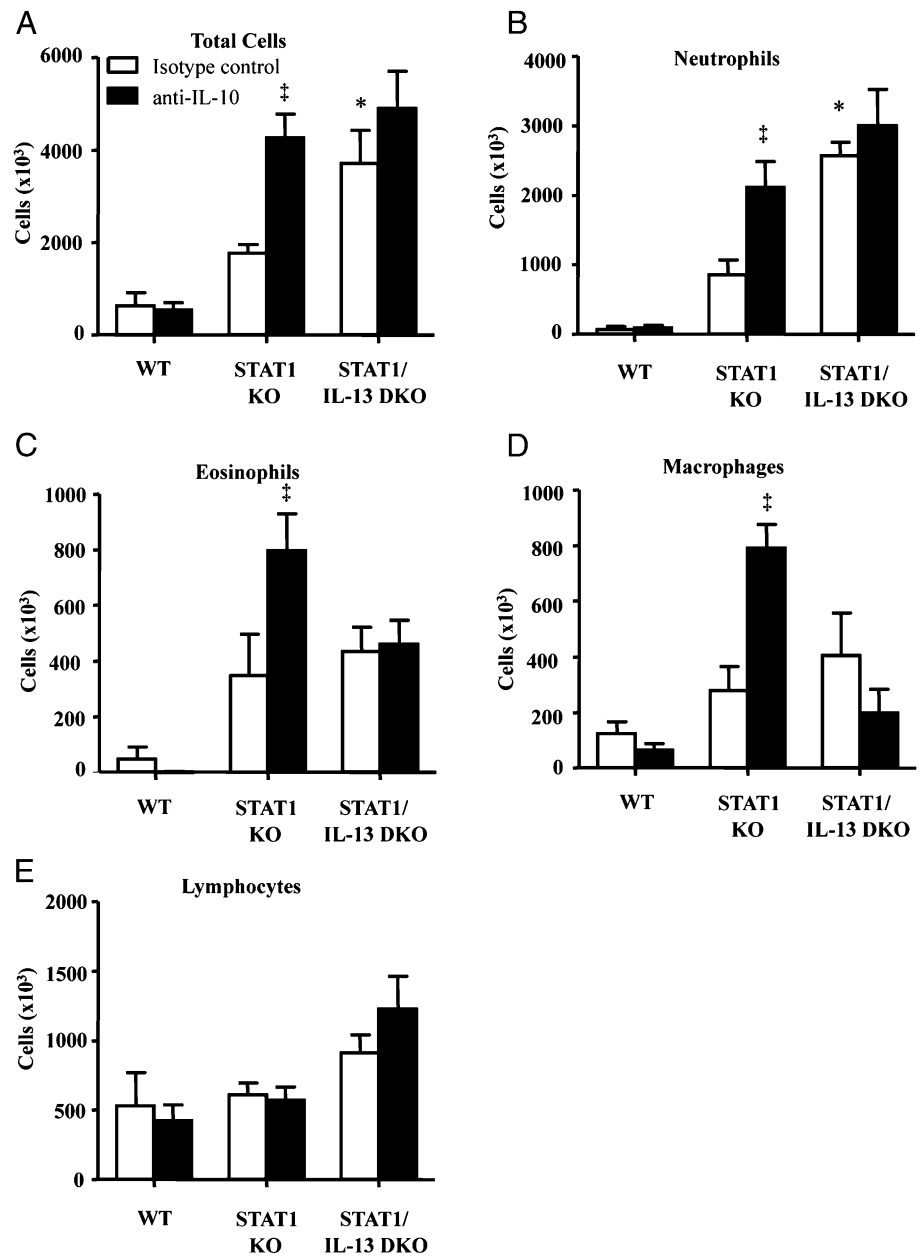


FIGURE 6. IL-13 deficiency increases IL-10-mediated airway inflammation in STAT1 KO mice. Mice were injected i.p. with anti-IL-10 or IgG isotype control Ab 1 d before RSV A2 infection and 3 d post-infection. BAL fluid was collected 6 d postinfection, and infiltration of inflammatory cells was examined. *A*, Total number of inflammatory cells in BAL fluid. *B–E*, Neutrophils, eosinophils, macrophages, and lymphocytes in BAL fluid. $n = 4–5$ mice for each group. $*p < 0.05$ compared with STAT1 KO mice treated with isotype control Ab, $^{\ddagger}p < 0.05$ compared with isotype control Ab within strain of mice.

IL-13 inhibition of IL-17 and IL-21 production. In vivo, we showed that IL-10 production was increased significantly in STAT1 KO mice compared with that in WT mice, but in the absence of IL-13 (in STAT1/IL-13 DKO mice), IL-10 production was decreased significantly. Further, when IL-10 was neutralized before RSV infection, IL-17A and IL-17F production was increased significantly in STAT1 KO mice but not in STAT1/IL-13 DKO mice. Combined, our in vitro and in vivo studies show that IL-13 increases IL-10 production and that IL-10 in turn decreases IL-17A and IL-17F production.

IL-10 has anti-inflammatory properties, and IL-10R α was expressed on IL-17A-producing CD4⁺ cells in the small intestines (47). IL-10 inhibited IL-17A production from Th1/Th17 cells, which produce IFN- γ and IL-17A, as well as Th17 cells, which only produce IL-17A, verifying that IL-10 can directly affect Th17 cells (47). These data show that IL-10R is expressed on Th17 cells and that IL-10 attenuates IL-17A production. McGeachy et al. (34) showed that IL-10 is produced by Th17 cells and that TGF- β and IL-6 were required for IL-10 production.

Further, IL-10 neutralization in the EAE model increased disease severity in mice receiving adoptive transfer of CD4⁺ T cells stimulated with TGF- β and IL-6 (34). We previously determined in human T cells that IL-13R α 1 expression on T cells required the Th17 differentiating cytokines TGF- β , IL-6, and IL-23 (14). Therefore, TGF- β and IL-6 are required for IL-13R α 1 expression, allowing for IL-13 signaling, as well as IL-10 production by Th17 cells.

IL-10 binds to IL-10R and induces STAT3 and SOCS3 expression (43). Although STAT3 is a required transcription factor for Th17 differentiation and cytokine production, IL-10 negatively regulates Th17 cytokine production by increasing SOCS3 expression, which downregulates IL-6/gp130 signaling (43). In this study, we showed that SOCS3 expression was decreased when IL-10 was neutralized in RSV-infected STAT1 KO mice but not STAT1/IL-13 DKO mice. Taken together, these studies define how IL-13 regulates Th17 cell cytokine secretion by upregulating IL-10 production and SOCS3 expression, resulting in the attenuation of IL-17A and IL-17F production.

IL-13 is a central mediator of airway hyperresponsiveness and mucus production, and IL-13 is increased in the sputum of patients with asthma (4). Therefore, therapeutics that target IL-13 and IL-13 signaling are attractive in treating allergic airway diseases including asthma, and several therapeutics are currently undergoing clinical trials (6–11). Therapeutics targeting IL-13 and IL-13 signaling pathways have a strong safety profile. However, most studies do not recruit patients with moderate to severe asthma (6, 11, 48), and none of the studies measured IL-17A levels (6–11). Patients with moderate to severe asthma have increased IL-17A⁺ cells compared with those of mild asthmatics and healthy controls (20), and IL-17A is increased in the BAL fluid of patients with moderate to severe asthma (20, 21). Therefore, patients with severe asthma or patients with Th17-mediated diseases, such as multiple sclerosis, rheumatoid arthritis, and colitis, may have adverse side effects associated with IL-13-targeted therapeutics. Corren et al. (6) reported that the anti-IL-13 Ab lebrikizumab increased forced expiratory volume in 1 s in uncontrolled asthmatics compared with a placebo control. However, patients receiving lebrikizumab had a significant increase in adverse events involving the musculoskeletal system, some of which are diseases associated with Th17 cells (6). In conclusion, we have further defined the mechanism by which IL-13 regulates IL-17A production both in vitro and in vivo. IL-13 decreases IL-17A production in an IL-10-dependent pathway in STAT1 KO mice. These findings are vital to understanding the potential side effects of therapeutics targeting IL-13 or IL-13 signaling in patients with Th17-mediated diseases and potentially severe asthma.

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Disclosures

The authors have no financial conflicts of interest.

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