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Dose-Dependent Effects of IL-17 on IL-13–Induced Airway Inflammatory Responses and Airway Hyperresponsiveness

Margaret W. Kinyanjui, Jichuan Shan, Emily M. Nakada, Salman T. Qureshi, and Elizabeth D. Fixman

The Th2 cytokine IL-13 regulates several aspects of the asthmatic phenotype, including airway inflammation, airway hyperresponsiveness, and mucus production. The Th17 cytokine IL-17A is also implicated in asthma and has been shown to both positively and negatively regulate Th2-dependent responses in murine models of allergic airways disease. Our objective in this study was to better understand the role of IL-17 in airway inflammation by examining how IL-17 modifies IL-13–induced airway inflammatory responses. We treated BALB/c mice intranasally with IL-13 or IL-17 alone or in combination for 8 consecutive days, after which airway hyperresponsiveness, inflammatory cell influx into the lung, and lung chemokine/cytokine expression were assessed. As expected, IL-13 increased airway inflammation and airway hyperresponsiveness. IL-13 also increased numbers of IL-17–producing CD4⁺ and $\gamma\delta$ T cells. Treating mice with a combination of IL-13 and IL-17 reduced infiltration of IL-17⁺ $\gamma\delta$ T cells, but increased the number of infiltrating eosinophils. In contrast, coadministration of IL-13 with a higher dose of IL-17 decreased all IL-13–induced inflammatory responses, including infiltration of both IL-17⁺CD4⁺ and $\gamma\delta$ T cells. To examine the inhibitory activity of IL-17–expressing $\gamma\delta$ T cells in this model, these cells were adoptively transferred into naive recipients. Consistent with an inhibitory role for $\gamma\delta$ T cells, IL-13–induced infiltration of eosinophils, lymphocytes, and IL-17⁺CD4⁺ T cells was diminished in recipients of the $\gamma\delta$ T cells. Collectively, our data indicate that allergic airway inflammatory responses induced by IL-13 are modulated by both the quantity and the cellular source of IL-17. *The Journal of Immunology*, 2013, 190: 3859–3868.

Several features of allergic airways disease are mediated by the Th2 cytokine IL-13, including airway inflammation, airway hyperresponsiveness (AHR), and mucus production (1–3). Loss of IL-13 expression or blockade of IL-13 activity diminishes each of these responses in murine models of allergic airways disease (1, 3). These proinflammatory effects of IL-13 are mediated by the IL-13 receptor, which, upon ligation, induces tyrosine phosphorylation of the STAT6 transcription factor (1, 4). Once phosphorylated, STAT6 dimerizes and translocates to the nucleus where it binds to DNA and initiates expression of a number of genes, including chemokines such as CCL11 (eotaxin-1) and CCL17 (thymus and activation–regulated chemokine), which then promote eosinophil and leukocyte infiltration into the lung (5–8).

The Th17 cytokine IL-17A (hereafter referred to as IL-17) is also implicated in asthma. IL-17 levels are elevated in the bronchoalveolar lavage fluid (BALF), lungs, and sputum of asthmatics (9–11). More recently, IL-17 has also been implicated specifically

in severe asthma (12–15). Elevated levels of IL-17 are thought to participate in increased airway neutrophilia in this group of asthmatics (13, 16, 17) owing to IL-17–mediated production of chemokines that promote neutrophil recruitment (18–21). The role of IL-17 in murine models of allergic airways disease is unclear. Abundant data demonstrate a proinflammatory role for IL-17 and/or Th17 cells in allergic airways disease. For example, IL-17–producing T cells induce airway neutrophilia in mice (22–24). These cells also positively regulate Th2-driven AHR and airway eosinophilia (23, 24). Neutralizing IL-17 diminishes Th2-driven allergic airways disease (25–27), and mice lacking IL-17 have reduced airway inflammatory responses upon Ag challenge (28). Whereas CD4⁺ T cells producing IL-17 are most often implicated in these responses, IL-17 produced by other cells, such as macrophages, may also promote allergic airways disease in these models (27).

In contrast to these data demonstrating that IL-17 promotes allergic airways disease, there is also evidence that IL-17 can play a protective role. Adoptive transfer of IL-17–producing $\gamma\delta$ T cells into mice with ongoing allergic inflammation decreases both AHR and the duration of airway eosinophilia, whereas IL-17–deficient $\gamma\delta$ cells are unable to promote resolution of the allergic inflammatory response (29). Similarly, intranasal administration of IL-17 during Ag challenge of sensitized mice decreases AHR and diminishes airway eosinophilia (30). To date, the mechanism by which IL-17 participates in the resolution of inflammatory events remains unclear.

The aim of our study was to investigate the effects of IL-17 on Th2-biased inflammatory responses in the airways. To this end, we established an acute model of airway inflammation in which we treated naive BALB/c mice with IL-13 in combination with two different doses of IL-17. IL-13 alone induced several hallmarks of allergic airways disease (i.e., AHR, airway inflammation, and mucus production) as well as increased mRNA and protein levels of

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage; WT, wild-type.

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chemokines that promote airway inflammation. We also found increased IL-17 mRNA expression that was accompanied by an increase in the number of IL-17–producing CD4⁺ and $\gamma\delta$ T cells. These IL-13–induced responses were variably affected by coadministration of IL-17: lower doses of IL-17 enhanced eosinophil recruitment to the airways, at the same time selectively reducing the number of IL-17–expressing $\gamma\delta$ T cells recruited to the BALF, whereas higher doses inhibited airway eosinophil recruitment, chemokine production, AHR, and both subsets of IL-17–expressing T cells. Consistent with an inhibitory role for $\gamma\delta$ T cells, adoptive transfer of these cells reduced airway inflammation. Our data suggest that IL-13–induced allergic responses in the airways are shaped by the overall amount of IL-17 present as well as by the cell types producing this cytokine.

Materials and Methods

Animals and cytokine treatment

Wild-type (WT) BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Montreal, QC, Canada). C57BL/6 IL-17 knockout (IL-17^{−/−}) mice were a gift from Dr. Yoichiro Iwakura (University of Tokyo). All animals, including TLR4^{−/−} mice, were bred at the Meakins-Christie Laboratories Animal Facility. Animal studies were approved by the McGill University Animal Care Committee and were performed following the guidelines of the Canadian Council on Animal Care. For cytokine treatment, mice were briefly anesthetized with isoflurane prior to intranasal administration of cytokines or control saline in a total volume of 30 μ l. Mice received IL-13 (Life Technologies, Burlington, ON, Canada) or IL-17 (BioLegend, San Diego, CA) alone (0.5 μ g/d for 8 d) or IL-13 plus IL-17 (either 0.5 μ g/d or 1.5 μ g/d for 8 d). Mice were sacrificed 48 h following cytokine administration. For adoptive transfer experiments, mice were treated for 8 d with IL-13. One day after the final treatment, mice were sacrificed with an overdose of sodium pentobarbital and lungs were harvested. Following enzymatic digestion with Liberase (100 μ g/ml; Roche Applied Science, Laval, QC, Canada), in combination with collagenase XI (250 μ g/ml), hyaluronidase 1a (1 mg/ml), and DNase I (200 μ g/ml; Sigma-Aldrich, Oakville, ON, Canada) for 1 h at 37°C (31), CD3⁺ cells were purified by negative magnetic selection with the EasySep mouse T cell enrichment kit (Stemcell Technologies, Vancouver, BC, Canada) and labeled with anti- $\gamma\delta$ TCR-FITC (see below). Subsequently, propidium iodide[−], $\gamma\delta$ ⁺ T cells were FACS-sorted and adoptively transferred, via i.p. injection, into naive recipient mice, which were then exposed to IL-13 for each of the next 8 d as above. Control animals received saline. More than 95% of purified cells were $\gamma\delta$ T cells. All mice were sacrificed 48 h following cytokine administration.

Analysis of AHR and airway inflammation

To assess AHR, animals were anesthetized using xylazine and sodium pentobarbital and paralyzed with pancuronium bromide. AHR in response to increasing doses of methacholine was measured as previously described (32). Briefly, anesthetized and paralyzed mice were attached to a computer-controlled small-animal ventilator (flexiVent; Scireq, Montreal, QC, Canada). The baseline respiratory system resistance was measured before recording the maximal resistance obtained with increasing doses of nebulized methacholine. Heart rate was monitored throughout the procedure to ensure adequate anesthesia. Afterward, the lungs were lavaged twice with 1 ml PBS. Cells were recovered by centrifugation of the BALF and the supernatant was collected and stored at −80°C for chemokine analysis. Cells from both lavage fractions were pooled and counted before being spun onto microscope slides. Cells were then stained using Diff-Quick (Fisher Scientific, Ottawa, ON, Canada), and differential cell counts were obtained by counting three to six fields comprising 300 cells per field.

Intracellular cytokine staining of BALF inflammatory cells

BALF cells recovered from groups of three to four mice were stimulated for 4 h in complete RPMI 1640 medium containing 10% heat-inactivated FBS supplemented with 50 μ M 2-ME, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, and 2 mM L-glutamine (Life Technologies) with 50 ng/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), and GolgiStop (BD Biosciences, Mississauga, ON, Canada). Cells were then fixed, permeabilized, and stained with anti-CD4-allophycocyanin, anti- $\gamma\delta$ TCR-FITC, and anti-IL-17-PE Abs (BD Biosciences).

RNA purification and real-time PCR analysis

Following collection of BALF, lungs were dissected away from the trachea and main bronchi and stored in RNeasy lysis buffer (Life Technologies). Total cellular RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's protocol and reverse transcribed using SuperScript II reverse transcriptase (Life Technologies). Real-time quantitative PCR was performed using the Applied Biosystems PCR system (Life Technologies) and AB Power SYBR Green mix (Life Technologies). Cycle threshold values were determined using AB Step One software (Life Technologies) and normalized to GAPDH. The comparative cycle threshold method was then used to calculate the relative gene expression levels (33).

Quantitative analysis of lung chemokines

BALF chemokine levels were assessed using the mouse Q-Plex chemokine kit from Quansys Biosciences (Logan, UT) as per the manufacturer's instructions.

Statistical analysis

Results are expressed as the mean \pm SEM. In comparisons of only two groups, statistical significance was measured using the Student *t* test. For experiments comparing more than two groups, statistical significance was measured by ANOVA followed by a post hoc Student–Newman–Keuls test for pairwise comparisons. A *p* value <0.05 was considered significant.

Results

Intranasal delivery of rIL-13 induces AHR, airway inflammation, and chemokine/cytokine expression

To better understand the role of IL-17 in allergic airway inflammation we first established an acute model of IL-13–induced airway disease in which 0.5 μ g IL-13 was administered intranasally to anesthetized mice for 8 consecutive days. Airway resistance to methacholine, quantified 48 h after the last IL-13 administration, was increased in mice treated with IL-13 compared with control animals that received saline alone (Fig. 1A). The increased respiratory system resistance was accompanied by an influx of eosinophils, neutrophils, and lymphocytes into the bronchoalveolar compartment of the lung (Fig. 1B). These results are consistent with previously reported acute models of IL-13–induced airway inflammation (1–4). Administration of IL-13 also induced expression of the mucin gene MUC5AC (2, 34–36) (Fig. 1C) and increased goblet cell mucus production (assessed by periodic acid–Schiff staining; data not shown). Consistent with the ability of IL-13 to increase expression of chemokines that regulate eosinophil and lymphocyte chemotaxis (5, 37–39), the levels of mRNA encoding CCL11 (eotaxin-1), CCL17 (thymus and activation-regulated chemokine), and CCL22 (macrophage-derived chemokine) were also increased (Fig. 1C). Levels of mRNA encoding CCL5 (RANTES) and CCL24 (eotaxin-2) were not significantly increased in the lungs of IL-13–treated mice.

Mice treated with IL-13 had increased numbers of Gr-1^{high} neutrophils (40) in the BALF (Fig. 2A, 2B). One potential mechanism by which neutrophils are recruited into the airways is via IL-17–induced production of two CXC chemokines (CXCL1 and CXCL5) (19, 20, 41). Interestingly, mRNA levels of each of these chemokines (Fig. 2C), as well as IL-17 (Fig. 2D), were increased in the lungs of IL-13–treated mice. Consistent with the elevated levels of IL-17 mRNA, we identified two primary populations of cells producing IL-17 in IL-13–treated mice. Characterization of these IL-17–producing cells indicated that they were CD4⁺ and $\gamma\delta$ T cells (Fig. 3A). Although IL-17–producing cells could be detected in control mice, larger numbers were present following IL-13 treatment (Fig. 3B, 3C; see also below). In an attempt to further define these IL-17–producing cells, expression of IL-22, IL-4, and IFN- γ was assessed by intracellular cytokine staining, but none of these cytokines could be detected (data not shown). Moreover, to examine the possibility that LPS in the IL-

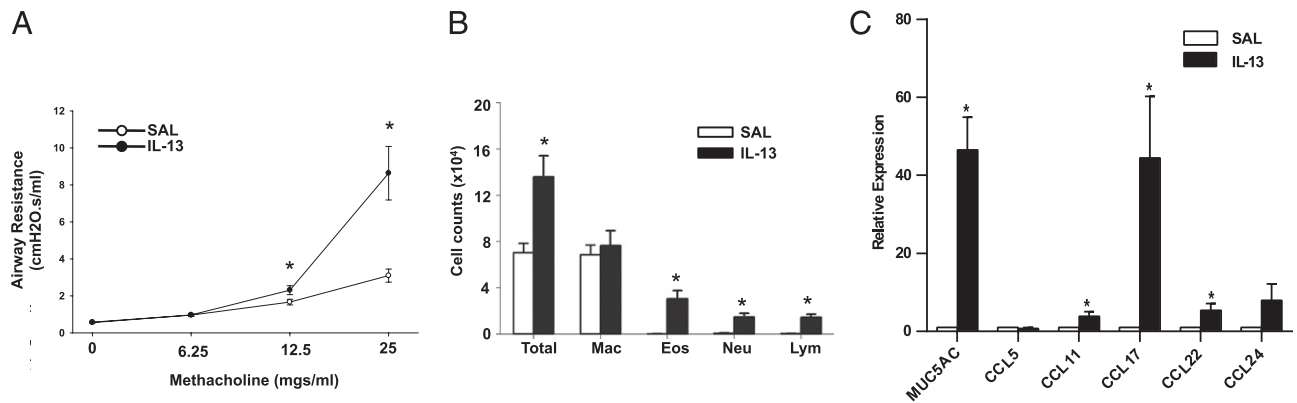


FIGURE 1. Intranasal delivery of recombinant IL-13 induces AHR, airway inflammation, and chemokine/cytokine expression. **(A)** Respiratory resistance to methacholine assessed 48 h following the last dose of IL-13. Control mice were treated with saline (SAL). Mean respiratory resistance to methacholine (\pm SEM) from a combination of three independent experiments using three to five mice per group in each experiment is shown. **(B)** BALF differential cell counts from SAL (open bars)- and IL-13 (filled bars)-treated mice. Cell counts for total cells, macrophages, eosinophils, neutrophils, and lymphocytes \pm SEM are shown. BALF cell counts are from one representative experiment out of at least three in which three to four mice per group were used. **(C)** Relative expression of MUC5AC and chemokine genes as assessed by real-time PCR following SAL (empty bars) or IL-13 (black bars) treatment. The relative gene expression levels in IL-13-treated mice compared with SAL are presented as the means \pm SEM from at least three experiments using three to five mice per group in each experiment. * $p < 0.05$ SAL versus IL-13.

IL-13 preparation was contributing to the increases in IL-17-expressing T cells, responses to IL-13 were examined in TLR4 knockout mice. IL-13 not only retained the ability to induce IL-17-expressing CD4⁺ and $\gamma\delta$ T cell subsets in these mice, but their induction was also enhanced (Fig. 3B, 3C). IL-13 also retained the ability to induce airway inflammation in TLR4^{-/-} mice (Supplemental Fig. 1A). Whereas eosinophil and neutrophil numbers did not differ, macrophage and lymphocyte numbers were enhanced in TLR4 knockout mice treated with IL-13. IL-13-induced AHR did not differ between IL-13-treated WT and TLR4 knockout mice (Supplemental Fig. 1B).

In vivo administration of IL-13 and low-dose IL-17 leads to enhanced airway inflammation

To better understand how IL-17 interacts with IL-13 to modulate airway inflammatory responses, mice were treated with IL-13 alone, IL-17 alone, or the combination of both cytokines, and AHR, inflammatory cell influx, and chemokine expression were assessed. Unlike IL-13, IL-17 was unable to induce AHR. IL-13

alone, as well as in combination with IL-17, induced comparable degrees of airway hyperresponsiveness to methacholine (Fig. 4A). Nevertheless, total cell numbers recovered in the BALF were greater in mice treated with the combination of IL-13 and IL-17 compared with IL-13 alone (Fig. 4B). Significantly, the numbers of both eosinophils and lymphocytes recovered from IL-13/IL-17-treated mice were increased compared with animals treated with IL-13 alone. Although IL-17 alone did not induce eosinophil or neutrophil recruitment into the airways, it did induce an increase in macrophages, consistent with previous data showing that IL-17 promotes macrophage recruitment and survival in the airways (42).

Assessment of MUC5AC and chemokine expression revealed that IL-13-induced increases in MUC5AC and CCL17 mRNA remained elevated in animals treated with both IL-13 and IL-17 (Fig. 4C). Conversely, mRNA levels of CCL22 and CCL24 were decreased in mice treated with both IL-13 and IL-17. Although IL-17 alone had no effect on mRNA levels of CCL17, CCL22, and CCL24, there was a trend toward increased mRNA levels of MUC5AC, consistent with previous *in vitro* data (43).

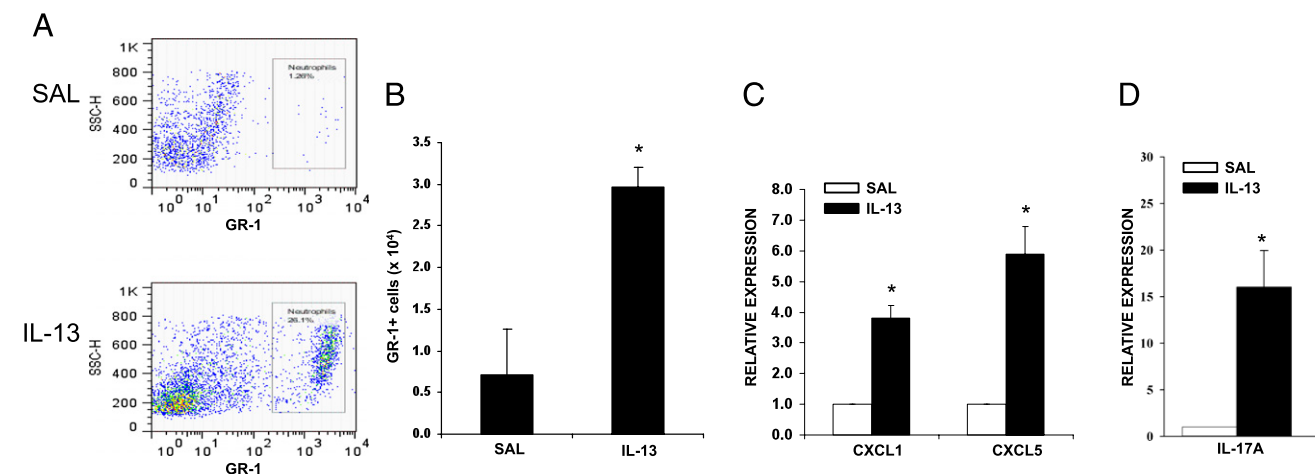


FIGURE 2. In vivo administration of IL-13 leads to increases in neutrophils, neutrophil chemokines, and IL-17. **(A)** Cells recovered from the BALF of mice treated with saline (SAL) (top panel) or IL-13 (bottom panel) were labeled for flow cytometry using GR-1 Abs. **(B)** Mean cell counts for GR-1^{high} cells \pm SEM from two experiments using three to five mice per group in each experiment are shown. **(C and D)** Relative expression of CXCL1 and CXCL5 (C) or IL-17 (D) assessed by real-time PCR following SAL (open bars) or IL-13 (filled bars) treatment. The relative gene expression levels in IL-13-treated mice are presented as the means \pm SEM from at least three experiments using three to five mice per group in each experiment. * $p < 0.05$ SAL versus IL-13.

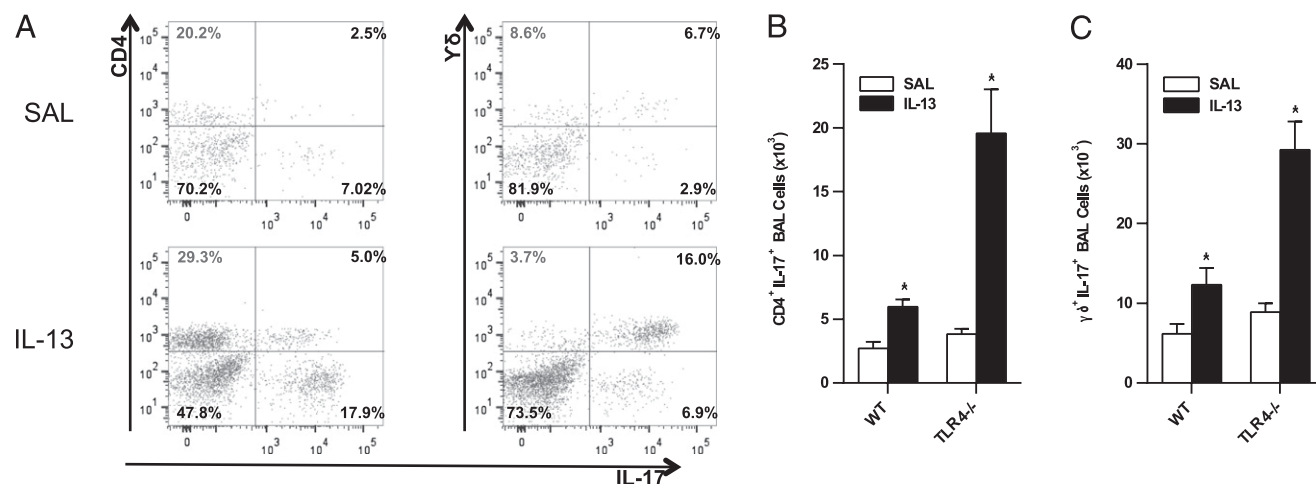


FIGURE 3. In vivo administration of IL-13 leads to increases in IL-17-producing T cells in the airways of WT and TLR4^{-/-} mice. **(A)** Cells recovered from the BALF of saline (SAL) (top panel)- or IL-13 (bottom panel)-treated WT mice were labeled for flow cytometry using fluorescent Abs against IL-17, CD4, and $\gamma\delta$ TCR. Left panels contain frequencies of CD4⁺ and IL-17⁺ cells. Right panels contain frequencies of $\gamma\delta$ TCR⁺ and IL-17⁺ cells. Flow cytometry plots, representative of at least three independent experiments where BALF cells from three to five mice per group were pooled, are shown. **(B)** and **(C)** Number of IL-17-producing CD4⁺ (B) or $\gamma\delta$ (C) T cells recovered from the BALF of cytokine-treated mice. Data are presented as the number of IL-17⁺ cells \pm SEM from two independent experiments using three to four mice per group in each experiment. **p* < 0.05 SAL versus IL-13.

At this point, our data suggested that IL-17 had only minor effects on IL-13-induced gene expression and AHR, but enhanced IL-13-induced airway inflammation (by increasing recruitment of

eosinophils and lymphocytes into the BALF). However, IL-17 potently inhibited its own expression induced by IL-13: IL-17 mRNA levels were decreased to basal levels in animals treated

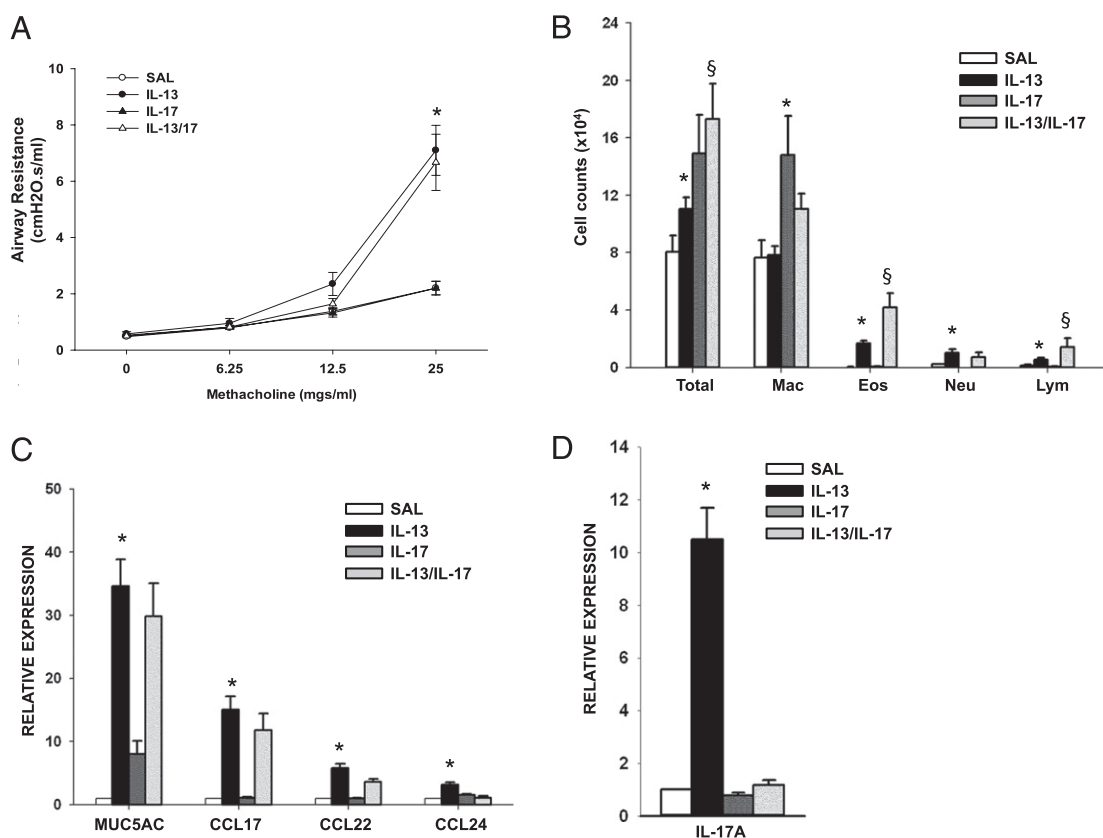


FIGURE 4. In vivo administration of both IL-13 and IL-17 leads to enhanced airway inflammation. Mice were treated with saline (SAL), IL-13 (0.5 μ g), IL-17 (0.5 μ g), or IL-13/17 (0.5 μ g each). **(A)** Respiratory resistance to methacholine assessed following treatment with IL-13 and/or IL-17. Mean respiratory resistance to methacholine \pm SEM from a combination of three independent experiments using three to five mice per group in each experiment is shown. **(B)** BALF differential cell counts from SAL (white bars)-, IL-13 (black bars)-, IL-17 (dark gray bars)-, and IL-13/17 (light gray bars)-treated mice. Cell counts for total cells, macrophages, eosinophils, neutrophils, and lymphocytes \pm SEM are shown. Cell counts are from one representative experiment out of at least three in which three to four animals per group were used. **(C)** and **(D)** Relative expression of MUC5AC and chemokine genes (C) or IL-17 (D) assessed by real-time PCR following IL-13 and/or IL-17 treatment. Relative gene expression levels in IL-13-treated animals compared with SAL are presented as the means \pm SEM from at least three experiments using three to five mice per group in each experiment. **p* < 0.05 SAL versus IL-13, §*p* < 0.05 IL-13 versus IL-13/IL-17.

with the combination of IL-13 and IL-17 compared with those treated with IL-13 alone (Fig. 4D). Given the dramatic decrease in IL-17 mRNA levels in these mice, we examined the effect of coadministration of IL-17 on CD4 and $\gamma\delta$ T cell-specific IL-17 expression. There was no difference in the frequency of infiltrating IL-17⁺CD4⁺ T cells between animals treated with IL-13 or the combination of IL-13 and IL-17 (Fig. 5). In contrast, the IL-13-induced increase in the frequency of IL-17-producing $\gamma\delta$ T cells was dramatically decreased by coadministration of IL-17 (Fig. 5). The decrease in $\gamma\delta$ T cells was inversely correlated with the increased airway inflammation, in agreement with a previous study demonstrating that IL-17-producing $\gamma\delta$ T cells promote resolution of airway inflammation (29).

The proinflammatory versus protective effects of IL-17 are dose dependent

Treatment with a combination of IL-13 and IL-17 at the 0.5 μ g dose led to a modest proinflammatory effect in comparison with treatment with IL-13 alone (Fig. 4B). We also treated mice with a higher dose of IL-17 (1.5 μ g). Surprisingly, IL-13-induced AHR was decreased in mice treated with IL-17 at the higher dose compared with animals treated with IL-13 alone (Fig. 6A). IL-13-induced inflammatory cell influx into the lung was also decreased in animals treated with IL-13 in combination with the higher dose of IL-17 (Fig. 6B). This decrease was attributable to fewer macrophages and fewer eosinophils recovered in the BALF. The IL-

13-induced increase in IL-17⁺ $\gamma\delta$ T cells recovered in the BALF was abrogated with the lower dose of IL-17 and this did not change upon delivery of the higher dose of IL-17 (Fig. 6C). Additionally, although we found that the number of IL-17⁺ CD4⁺ T cells was unaffected in animals treated with IL-13 and IL-17 at the lower dose, this increase was also abrogated in mice treated with IL-13 and the higher dose of IL-17 (Fig. 6D).

Higher dose IL-17 decreases IL-13-induced production of chemokines

As shown in Figs. 1 and 4, IL-13 treatment alone increased mRNA levels of several eosinophil chemokines. Because inflammation was differentially modulated by the two different doses of IL-17, chemokines recovered in the BALF of mice treated with IL-13 alone or with IL-17 were quantified. BALF levels of CCL5, CCL11, CCL17, and CCL22 were elevated in IL-13-treated mice compared with saline-treated control mice (Fig. 7), and levels in mice treated with IL-13 and the lower dose of IL-17 did not significantly differ. In contrast, animals treated with a combination of IL-13 and IL-17 at the higher dose had significantly lower levels of CCL11, CCL17, and CCL22, with no changes detected in CCL5 (Fig. 7).

Adoptive transfer of $\gamma\delta$ T cells from IL-13-treated mice diminishes IL-13-induced airway inflammation

The loss of IL-17-expressing $\gamma\delta$ T cells in mice treated with IL-13 and the lower dose of IL-17 correlated with enhanced airway in-

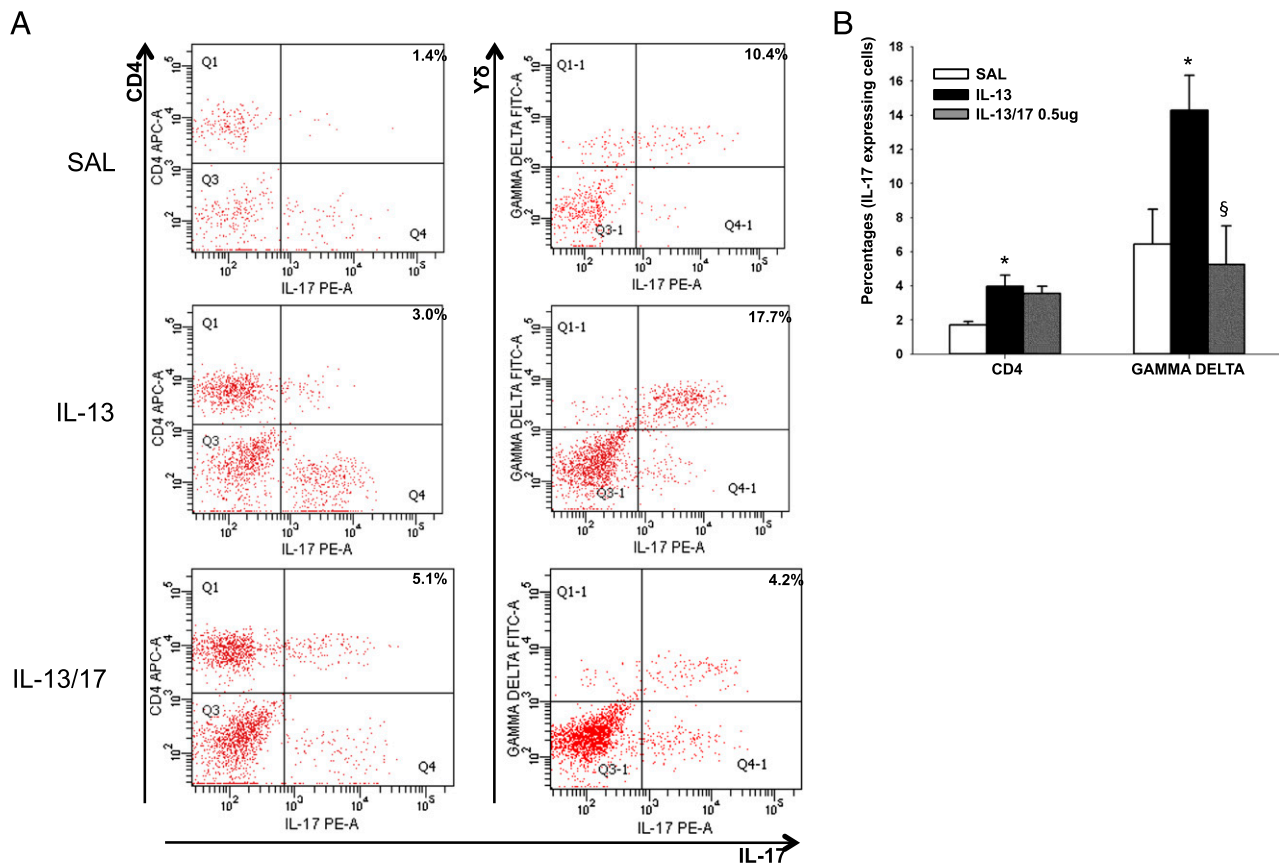


FIGURE 5. Administration of IL-13 and IL-17 leads to a decrease in IL-17-producing $\gamma\delta$ T cells but not CD4⁺ T cells in the airway. **(A)** Cells recovered from the BALF of saline (SAL)-, IL-13-, or IL-13/17-treated mice were assessed for expression of CD4, $\gamma\delta$ TCR, and IL-17. *Left panels* contain frequencies of CD4⁺ and IL-17⁺ cells. *Right panels* contain frequencies of $\gamma\delta$ TCR⁺ and IL-17⁺ cells. Flow cytometry plots, representative of at least three independent experiments where BALF cells from three to five mice per group were pooled, are shown. **(B)** Percentage of CD4 and $\gamma\delta$ IL-17-producing cells recovered from mice treated with SAL (white bars), IL-13 (black bars), or IL-13/17 (light gray bars). Data are presented as the percentage of IL-17⁺ cells \pm SEM from at least three independent experiments using BALF cells pooled from three to five mice per group. * p < 0.05 SAL versus IL-13, § p < 0.05 IL-13 versus IL-13/IL-17.

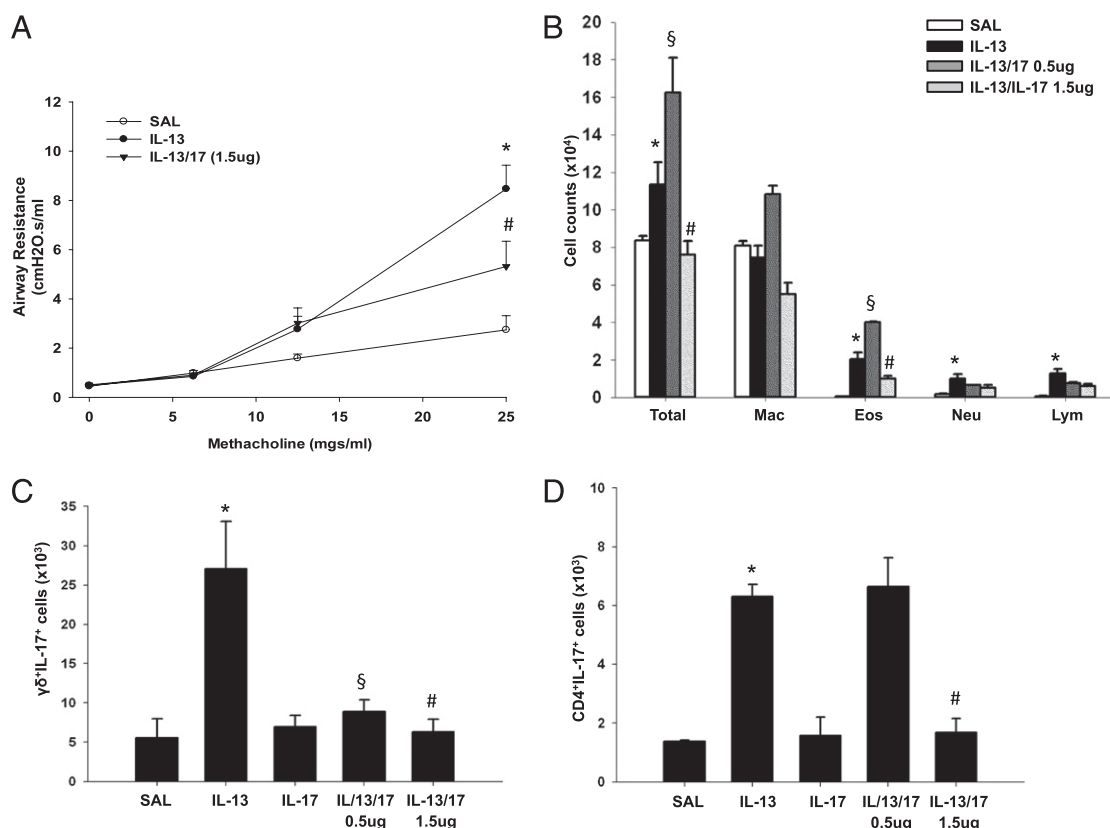


FIGURE 6. Administration of IL-13 in combination with a high dose of IL-17 (1.5 µg) leads to the inhibition of AHR and airway inflammation. **(A)** AHR measured in mice treated with IL-13 alone or IL-13 in combination with a high dose of IL-17 (1.5 µg). Mean respiratory resistance to methacholine ± SEM from a combination of three independent experiments using three to five mice per group in each experiment is shown. **(B)** BALF differential cell counts from saline (SAL) (white bars)-, IL-13 (black bars)-, IL-13/17 (0.5 µg) (dark gray bars)-, and IL-13/17 (1.5 µg) (light gray bars)-treated mice. Cell counts for total cells, macrophages, eosinophils, neutrophils, and lymphocytes ± SEM are shown. BALF cell counts are from one representative experiment out of at least three in which three to four animals per group were used. **(C and D)** Number of IL-17-producing γδ (C) or CD4 (D) T cells recovered from the BALF of cytokine-treated mice. Data are presented as the number of IL-17⁺ cells ± SEM from at least three experiments using cells pooled from three to five mice per group in each experiment. **p* < 0.05 SAL versus IL-13, §*p* < 0.05 IL-13 versus IL-13/IL-17 (0.5 µg), #*p* < 0.05 IL-13 versus IL-13/IL-17 (1.5 µg).

flammation (Figs. 4B, 6B). To more directly assess the activity of γδ T cells on IL-13-induced airway inflammatory responses, these cells were purified from the lungs of IL-13-treated mice. γδ T cells (10,000 or 50,000) were delivered i.p. to naive recipients, which were then treated with IL-13 as above. Consistent with an inhibitory role for γδ T cells, total inflammatory cell influx was decreased in recipients of γδ T cells, with significant reductions in

eosinophils and lymphocytes with the higher dose of γδ T cells (Fig. 8A). Although inflammation was reduced, adoptively transferred γδ T cells had no effect on IL-13-induced AHR (Fig. 8B). Whereas the frequency of total lung γδ T cells did not differ in recipients of exogenous γδ T cells (data not shown), the frequency of IL-17-expressing γδ T cells in the lung increased in a dose-dependent manner, likely due to trafficking of the adoptively

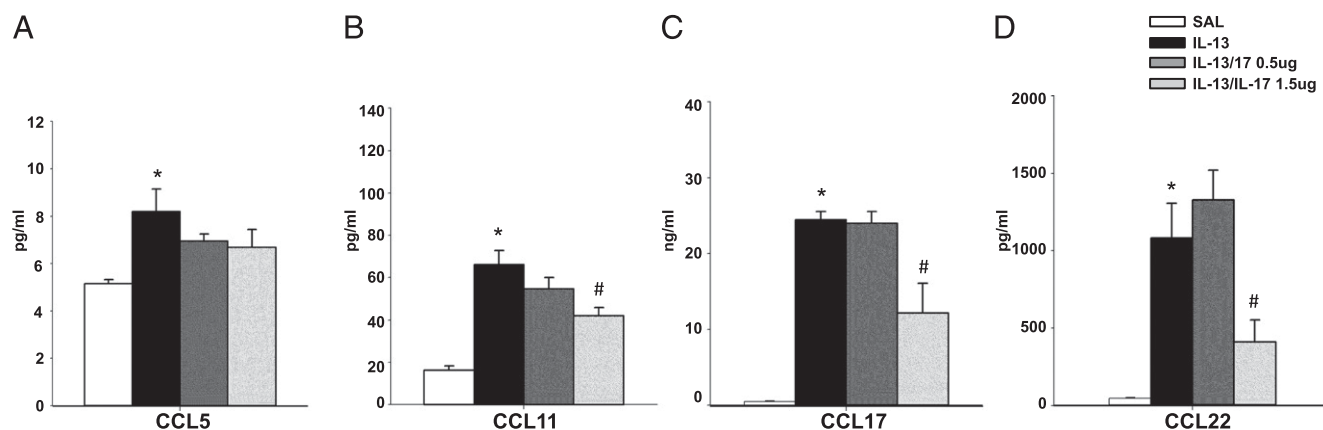


FIGURE 7. Administration of IL-13 in combination with a high dose of IL-17 (1.5 µg) leads to decreased chemokine production. **(A–D)** BALF chemokines (CCL5, CCL11, CCL17, and CCL22) were quantified using a Quansys chemokine multiplex kit. Mean chemokine levels ± SEM from two experiments using BALF from four mice per group in each experiment are graphed. **p* < 0.05 SAL versus IL-13, #*p* < 0.05 IL-13 versus IL-13/IL-17 (1.5 µg).

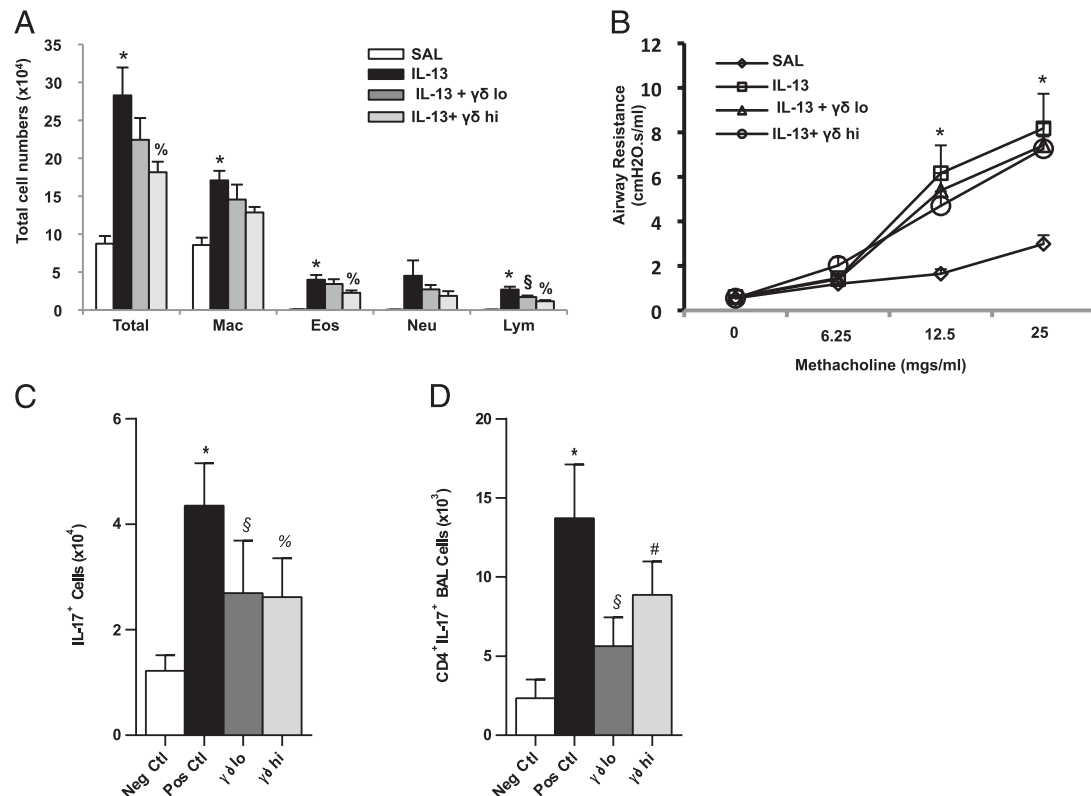


FIGURE 8. Adoptively transferred $\gamma\delta$ T cells diminish IL-13-induced airway inflammatory responses. **(A)** BALF differential cell counts from mice treated with saline (SAL) (white bars), IL-13 (black bars), IL-13 plus low-dose $\gamma\delta$ T cells (IL-13 + $\gamma\delta$ lo) (dark gray bars), or IL-13 + high-dose $\gamma\delta$ T cells (IL-13 + $\gamma\delta$ hi) (light gray bars). Mean cell counts for total cells, macrophages, eosinophils, neutrophils, and lymphocytes \pm SEM from two independent experiments using three mice per group in each experiment are shown. **(B)** AHR measured in mice treated with SAL, IL-13, IL-13 plus low-dose $\gamma\delta$ T cells (IL-13 + $\gamma\delta$ lo), or IL-13 plus high-dose $\gamma\delta$ T cells (IL-13 + $\gamma\delta$ hi). Mean respiratory resistance to methacholine \pm SEM from two independent experiments using three mice per group in each experiment is shown. **(C and D)** Number of total IL-17-producing cells (C) or IL-17-producing CD4 T cells (D) recovered from the BALF of mice treated with SAL (white bars), IL-13 (black bars), IL-13 plus low-dose $\gamma\delta$ T cells (dark gray bars) ($\gamma\delta$ lo), or IL-13 plus high-dose $\gamma\delta$ T cells (light gray bars) ($\gamma\delta$ hi). Data are presented as the number of IL-17⁺ cells \pm SEM from two independent experiments using a total of six mice per group. * $p < 0.05$ SAL versus IL-13, % $p < 0.05$ IL-13 versus $\gamma\delta$ lo, # $p < 0.05$ IL-13 versus $\gamma\delta$ hi, # $p < 0.05$ SAL versus $\gamma\delta$ hi.

transferred $\gamma\delta$ T cells to the lung (Supplemental Fig. 2A). Notably, IL-13-induced increases in BALF numbers of total IL-17-expressing cells (Fig. 8C), as well as CD4⁺ T cells expressing IL-17 (Fig. 8D), were reduced in recipients of adoptively transferred $\gamma\delta$ T cells. Similar reductions in BALF $\gamma\delta$ T cells expressing IL-17 were not found (Supplemental Fig. 2B). However, these cells may represent a mixture of the adoptively transferred $\gamma\delta$ T cells and endogenous $\gamma\delta$ T cells induced by IL-13. These reductions in BALF eosinophils and lymphocytes as well as IL-17-expressing CD4⁺ T cells in recipients of adoptively transferred $\gamma\delta$ T cells provide further support for a negative regulatory role for $\gamma\delta$ T cells expressing IL-17 in allergic airways disease.

Discussion

IL-17 has been linked to a number of autoimmune and allergic diseases (44–46). Clinical data have associated Th17 cells, IL-17, and increased neutrophilia with severe asthma. However, the role of IL-17 in murine models is controversial, as IL-17 has been shown to both promote and inhibit pathogenesis in models of allergic airways disease (47). Although the reasons for these conflicting data may be differences in conditions of Ag sensitization and/or mouse strain, to our knowledge, we show in this study for the first time a dose-dependent effect of IL-17 in the modulation of airway inflammation. At lower doses, IL-17 augmented IL-13-induced airway inflammation, promoting influx of greater numbers of eosinophils. At higher doses, IL-17 negatively regulated

not only its own expression in both CD4 and $\gamma\delta$ T cells, but also that of a number of key inflammatory chemokines. At higher doses, IL-17 powerfully inhibited IL-13-induced AHR, airway inflammation, and chemokine production.

Some of the confusion regarding the precise role of IL-17 in asthma may be due to the fact that IL-17 is expressed by different cells and/or in combination with different cytokines. In the lung, for instance, IL-17 can be produced by CD4 T cells (48), $\gamma\delta$ T cells (29), and macrophages (27). Although Th17 cells, which can also produce IL-17F and IL-22 (19, 24, 49, 50), are often thought of as the main producers of IL-17, other effector T cell subsets, including Th1 (51), Th2 (48), and Th0 (51), all have the capacity to produce IL-17 under some conditions. These subsets, in which IL-17 is produced in combination with other classically defined Th1 or Th2 cytokines, have also been implicated in asthma pathogenesis in humans and in murine models. In contrast, IL-17-producing $\gamma\delta$ T cells, but not CD4 T cells, have the ability to promote resolution of allergic airway inflammation in mice (29). Our data demonstrating inhibition of airway inflammatory cell influx by adoptively transferred $\gamma\delta$ T cells support an anti-inflammatory role for these cells as well. Thus, the cellular context in which IL-17 is produced is able to modulate the inflammatory outcome. An additional complication is the fact that in mice different profiles of T cell subsets may be generated, depending on the strain of mouse (52) and/or conditions of Ag sensitization (25, 53). In our own experiments, we found significant increases in IL-17-producing

$\gamma\delta$ cells in response to IL-13 delivery in BALB/c mice, whereas far fewer of these cells were recovered in C57BL/6 mice treated with IL-13 (data not shown).

To bypass the complexity inherent in Ag-dependent allergy models, we treated mice directly with recombinant IL-13 and/or IL-17 to assess how these cytokines interact in vivo. IL-13 is considered the primary effector cytokine in Th2-biased responses. As well, we examined the dose-dependent effects of IL-17 on IL-13-induced inflammatory responses in vivo. We found that administration of IL-13 promoted increases in both CD4 and $\gamma\delta$ T cells expressing IL-17. These cells are likely effector/memory T cells, as they did not express CD62L (data not shown). Cytokine-induced cytokine production by CD4 T cells in the absence of Ag stimulation has been previously demonstrated (54, 55) and represents a link between the innate and adaptive immune systems whereby T cells produce effector cytokines in response to innate stimuli. For example, treatment of CD4⁺ and $\gamma\delta$ T cells with the combination of IL-1 β or IL-18 and IL-23 is sufficient to induce IL-17 production (54–56). With regard to IL-13, previous studies have shown that IL-13 overexpression in the lung may lead to a cytokine environment conducive to the generation of IL-17-producing cells. Fulkerson and colleagues (57) used a transgenic system in which IL-13 expression was targeted to the lung using a reversible doxycycline system. Doxycycline exposure induced lung expression of IL-13, which led to an increase in IL-1 β , TGF- β , and IL-6, all of which promote IL-17 production. Although IL-17 production was not reported in these mice, our own data demonstrate that delivery of IL-13 to the lung induced production of IL-17 via pathways that were at least partially dependent on caspase-1 activation and IL-1 β production (data not shown). To rule out the possibility that LPS present in the IL-13 preparations we used was contributing to the enhanced production of IL-17, we assessed responses in TLR4^{-/-} mice. None of the outcome measures we assessed was diminished in the absence of TLR4. In fact, our data indicate that TLR4 may actually negatively regulate IL-13-induced IL-17 expression, as production of both IL-17-expressing CD4⁺ and $\gamma\delta$ T cells was increased, as was the influx of macrophages and lymphocytes into the lung, in IL-13-treated TLR4^{-/-} mice.

Our data demonstrating that the lower dose of IL-17 enhanced Th2-dependent airway inflammation are compatible with those of Wakashin et al. (23) and Wilson et al. (53), all of whom showed that Th17 cells enhance Th2-dependent airway inflammation and AHR. Wakashin et al. speculated that the enhanced inflammation was due to increased production of eotaxins 1 and 2, based on increased mRNA levels. In our studies, the increased eosinophil recruitment to the airways in mice treated with both IL-13 and IL-17 did not correlate with enhanced expression or production of a number of chemokines (Figs. 4, 7). Thus, the precise mechanism by which IL-17 enhanced IL-13-induced eosinophil recruitment is not clear.

Although our data indicate that the lower dose of IL-17 enhanced BALF eosinophil numbers, it did not modify IL-13-induced AHR in BALB/c mice. These data differ from those of Wills-Karp and colleagues (26) who demonstrated that IL-17 enhanced AHR induced by IL-13 in A/J mice. This may be due to differences in the strain of mice and/or the conditions of cytokine administration. A/J mice received 15 μ g of each cytokine during the course of 7 d whereas in our study mice received only 4 μ g of each cytokine during the course of 8 d. We also examined the effect of administering larger amounts of IL-17 to BALB/c mice, increasing the dose from 4 to 12 μ g, again during the course of 8 d. Not only were IL-13-induced inflammation and AHR not enhanced, they were actually inhibited. These diminished responses in BALB/c

mice treated with the higher dose of IL-17 corresponded well with the enhanced responses in C57BL/6 mice lacking IL-17, where AHR as well as BALF numbers of macrophages, eosinophils, and lymphocytes were increased (Supplemental Fig. 3). Taken together, these data suggest that, at least under some conditions, IL-17 is an effective inhibitor of IL-13-induced airway inflammatory responses, in agreement with previously published data (29, 30).

One interesting possibility that arose from our data is the fact that the IL-13-induced increase in IL-17-producing cells was altered by coadministration of IL-17. Lower doses of IL-17 abolished the IL-13-induced increases in $\gamma\delta$ T cells producing IL-17. As these cells were recently shown to promote resolution of airway inflammation in an OVA model, the enhanced eosinophilia we see may be due to the decreased numbers of these cells. However, at higher doses of IL-17, the IL-13-induced increases in IL-17-producing CD4⁺ and $\gamma\delta$ T cells were both reduced to baseline levels. These marked changes were accompanied by decreases in chemokine levels, BALF inflammation, and AHR, suggesting that at higher doses IL-17 can inhibit IL-13-induced responses in many cell types, effectively reducing inflammation. Given that high doses of IL-17 enhanced IL-13-induced responses in A/J mice (26), there appears to be a fine balance in the outcome of inflammation when both IL-13 and IL-17 are present.

The exact molecular mechanisms by which IL-17 modulates allergic airway inflammation remain to be identified. Data from Schnyder-Candrian (30) and colleagues suggested that IL-17 inhibits DC activity in the lungs, thereby modifying T cell activation and recruitment (30). However, as our system using recombinant cytokines lacked an obvious Ag, it seems unlikely that altered dendritic cell/T cell interactions were responsible for the altered responses we have defined. Rather, our data suggested that eosinophilic airway inflammation was enhanced when $\gamma\delta$ T cells expressing IL-17 were absent (in mice treated with IL-13 and the lower dose of IL-17). To more directly assess the role of $\gamma\delta$ T cells in IL-13-induced airway inflammatory responses, including production of IL-17-expressing cells, we adoptively transferred purified $\gamma\delta$ T cells from IL-13-treated mice into naive mice that were then treated with IL-13. IL-13-induced inflammatory cell influx was reduced in these mice, although AHR was unaffected. Recipients of $\gamma\delta$ T cells also had fewer total IL-17-expressing cells as well as CD4⁺ T cells expressing IL-17, although significant reductions were not found in IL-17-expressing $\gamma\delta$ T cells, likely due to the fact that this cell population was comprised of endogenous $\gamma\delta$ T cells and those that were adoptively transferred. Mills and colleagues (55) had previously shown that IL-17-expressing $\gamma\delta$ T cells promoted production of IL-17 from Ag-stimulated CD4⁺ T cells. Our data suggest that in the absence of Ag stimulation, $\gamma\delta$ T cells may actually have inhibitory activity toward CD4⁺ T cells.

Collectively, our data demonstrate that the levels of IL-17 modulate IL-13-induced inflammation in a dose-dependent manner where lower doses promote inflammation and higher doses prevent/protect against disease. They also provide evidence that $\gamma\delta$ T cells expressing IL-17 may play a protective role in allergic airway inflammation. Therefore, efforts to modulate IL-17 levels during inflammation should be carefully considered since the effects could be either pathogenic or protective.

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Disclosures

The authors have no financial conflicts of interest.

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