

IL-35 production by inducible costimulator (ICOS)-positive regulatory T cells reverses established IL-17-dependent allergic airways disease

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Background: Recent evidence suggests that IL-17 contributes to airway hyperresponsiveness (AHR); however, the mechanisms that suppress the production of this cytokine remain poorly defined.

Objective: We sought to identify the regulatory cells and molecules that suppress IL-17-dependent allergic airways disease.

Methods: Mice were sensitized by means of airway instillations of ovalbumin together with low levels of LPS. Leukocyte recruitment to the lung and AHR were assessed after daily challenges with aerosolized ovalbumin. Flow cytometry, quantitative PCR, and gene-targeted mice were used to identify naturally arising subsets of regulatory T (Treg) cells and their cytokines required for the suppression of established allergic airway disease.

Results: Allergic sensitization through the airway primed both effector and regulatory responses. Effector responses were initially dominant and led to airway inflammation and IL-17-dependent AHR. However, after multiple daily allergen challenges, IL-17 production and AHR decreased, even though pulmonary levels of T_H17 cells remained high. This loss of AHR was reversible and required the expansion of a Treg cell subset expressing both forkhead box protein 3 and inducible costimulator. These Treg cells also expressed the regulatory cytokines IL-10, TGF- β , and IL-35. Whereas IL-10 and TGF- β were dispensable for suppression of AHR, IL-35 was required. **Conclusion:** IL-35 production by inducible costimulator-positive Treg cells can suppress IL-17 production and thereby reverse established, IL-17-dependent AHR in mice. Targeting this pathway might therefore be of therapeutic value for treating allergic asthma in human subjects. (*J Allergy Clin Immunol* 2011;■■■■-■■■■.)

Key words: Asthma, airway hyperresponsiveness, airway hyperresponsiveness, IL-17, T_H17, T_H2, IL-35, inducible costimulator, ovalbumin

Abbreviations used

AHR:	Airway hyperresponsiveness
DTX:	Diphtheria toxin
Ebi-3:	EBV-induced gene
Foxp3:	Forkhead box protein 3
GFP:	Green fluorescent protein
ICOS:	Inducible costimulator
NIEHS:	National Institute of Environmental Health Sciences
OVA:	Ovalbumin
Treg:	Regulatory T
WT:	Wild-type

Allergic asthma is clinically characterized by reversible airway obstruction and airway hyperresponsiveness (AHR). These features stem largely from the actions of CD4⁺ T_H2 cells, which produce the cytokines IL-4, IL-5, and IL-13 and thereby promote IgE production, eosinophilia, and mucus secretion into the airway.^{1,2} However, although therapeutic strategies targeting individual T_H2 cytokines, such as IL-5, can reduce eosinophil levels, other disease symptoms are generally not improved.³⁻⁵ Recent evidence suggests that IL-17A (IL-17) production by T_H17 cells or macrophages can also contribute to allergic asthma⁶⁻¹³ and that T_H2 and T_H17 responses can act synergistically to promote AHR.^{13,14} In addition to having allergen-specific T_H2 and T_H17 effector responses, asthmatic patients also appear to lack sufficiently strong regulatory mechanisms to keep these effector responses in check.^{15,16} An improved understanding of the cellular and molecular mechanisms that regulate T_H2 and T_H17 effector responses might lead to novel therapeutic strategies to prevent or control allergic asthma.

The best-characterized regulatory T (Treg) cells are forkhead box protein 3 (Foxp3)-positive CD4⁺ T cells and Foxp3⁺ IL-10-producing Treg type 1 (T_R1) cells.¹⁶ In some models adoptive transfer of T cells producing IL-10 or TGF- β can modulate airway inflammation and AHR.¹⁷⁻²⁰ However, IL-10 might not inhibit IL-17 production by memory T cells,²¹ and TGF- β can promote T_H17 development.^{22,23} Thus it is unclear which cytokine suppresses T_H17-dependent allergic pulmonary inflammation. IL-35 is a newly described cytokine comprised of the IL-12 α chain (p35) and the EBV-induced gene product (EBI-3). Although IL-35 displays some regulatory activities *in vitro* and *in vivo*,²⁴ its role in allergic inflammation is unknown. Using a T_H2- and T_H17-dependent murine model of allergic pulmonary inflammation, we have found that multiple allergen challenges induce strong regulatory responses that suppress both IL-17 production and AHR. This suppression does not require IL-10 or TGF- β

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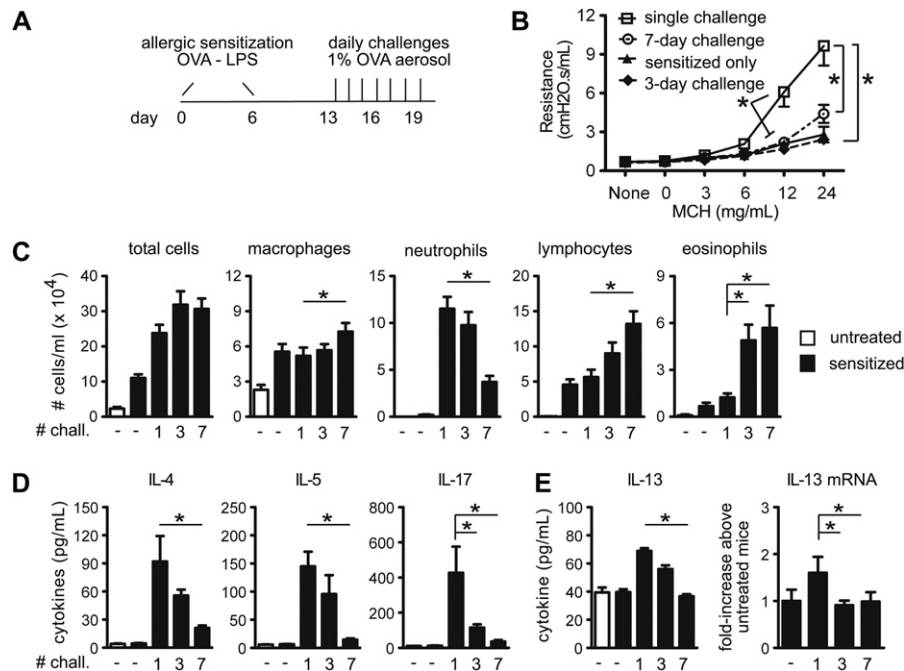


FIG 1. Prolonged allergen challenge suppresses allergic responses. **A**, Timeline. **B**, AHR in sensitized mice after various challenges. MCH, Methacholine. **C**, Leukocytes $\pm$ SEM in bronchoalveolar lavage fluid. **D**, Mean $\pm$ SEM cytokine levels in bronchoalveolar lavage fluid. **E**, Levels of IL-13 in homogenized lungs ($n = 9$ mice/group). * $P < .05$. Data represent one of 3 similar experiments.

but is instead dependent on inducible costimulator (ICOS)–positive Treg cell production of IL-35. These findings suggest that targeting this ICOS–IL-35 axis might be of therapeutic benefit for patients with allergic asthma.

METHODS

Mice

Il-10^{−/−}, *Il-12α* (*p35*)^{−/−}, *Icos*^{−/−}, *B7h*^{−/−}, *Foxp3-gfp*, *Ebi3*^{−/−}, and OT-II (C57BL/6-Tg[TcrαTcrβ]425Cbn/J) breeder mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, Me). *Ebi3*^{−/−} mice on a BALB/cByb background were also from Jackson Laboratories. In all experiments, age-matched, sex-matched, and genetically matched mice from the same commercial source were used as control animals. Foxp3–green fluorescent protein (GFP)–DTR mice, crossed for 5 generations onto a C57BL/6 background, were obtained from Dr Nick Lukacs (University of Michigan, Ann Arbor, Mich), with permission from Dr Alexander Rudensky (University of Washington, Seattle, Wash). Mice were housed in specific pathogen-free conditions at the National Institute of Environmental Health Sciences (NIEHS) and used between 6 and 12 weeks of age. All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the NIEHS.

Allergen-induced airway disease

Male mice were sensitized on days 0 and 6, as described previously.¹³ Briefly, anesthetized mice were given oropharyngeal administrations of 100 μ g of low-endotoxin ovalbumin (OVA) together with 0.1 μ g of *Escherichia coli* LPS (Sigma, St Louis, Mo) in a total volume of 50 μ L of PBS, referred to herein as OVA-LPS. For challenges, mice were exposed to an aerosol of 1% OVA (Sigma) in saline. Mice were harvested, and bronchoalveolar lavage was performed, as described previously.¹³ Cytokines in bronchoalveolar lavage fluid were measured by using a multiplexed fluorescent bead–based immunoassay (Bio-Rad Laboratories, Hercules, Calif). AHR was assessed by using the Flexivent mechanical ventilator system (Scireq, Montreal, Quebec,

Canada), as previously described.¹³ Where indicated, recombinant murine IL-17 (R&D Systems, Inc, Minneapolis, Minn), at 1.5 μ g per mouse in 50 μ L of PBS, was administered by means of oropharyngeal delivery to anesthetized mice 2 hours after the final daily OVA challenge, and the animals were harvested 24 hours later. TGF- β 1 neutralizing antibody (BioXcell, clone 1D11.16.8; 75 mg) in sterile PBS was delivered, as previously described,²⁵ by means of intranasal administration 1 hour before each OVA challenge and on the day biological and physiological responses were assessed.

Depletion of Treg cells in Foxp3-DTR mice

Beginning 1 day before the first OVA challenge, previously sensitized mice were given daily intraperitoneal injections of 100 μ L of PBS containing 100 ng of diphtheria toxin (DTX) from *Corynebacterium diphtheriae* (Sigma). This treatment was continued daily until the final OVA challenge.

Statistics

Data are expressed as means $\pm$ SEMs. Statistical differences between groups were calculated by using the Student *t* test or Mann-Whitney *U* test or by means of ANOVA followed by either a Tukey or Bonferroni *post hoc* test with GraphPad Prism version 5.02 software (GraphPad Software, Inc, La Jolla, Calif). A 2-tailed *P* value of less than .05 was considered statistically significant.

RESULTS

Rapid suppression of AHR and cytokine production after prolonged allergen challenge of OVA-LPS-sensitized mice

C57BL/6 mice were sensitized through the airway with OVA-LPS and challenged with aerosolized OVA to elicit allergen-induced airway disease (Fig 1, A). As expected from previous work,¹³ mice challenged on a single occasion had AHR (Fig 1, B). However, after multiple daily OVA challenges, AHR was no

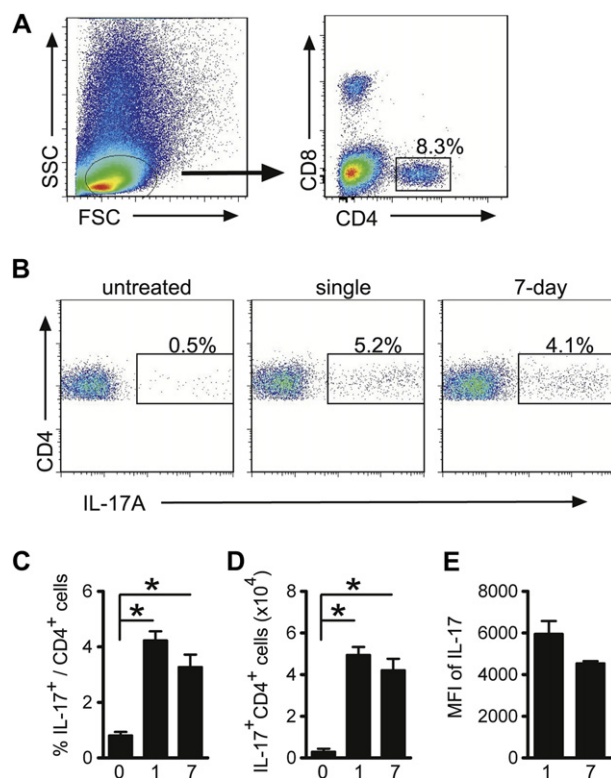


FIG 2. Suppression of AHR is not due to loss of T_H17 cells. **A** and **B**, Flow cytometric plots showing lymphocyte and $CD4^+$ T-cell gating (Fig 2, **A**) and intracellular IL-17 staining (Fig 2, **B**). FSC, Forward scatter; SSC, side scatter. **C** and **D**, Percentage and number of T_H17 cells per lung. **E**, Intracellular levels of IL-17 (mean fluorescent intensity [MFI]). Mean values $\pm$ SEM are shown. * $P < .05$ ($n = 5$ mice/group). Data represent one of 3 similar experiments.

longer apparent, although these animals still had mucus-producing cells in the airway (see Fig E1, A, in this article's Online Repository at www.jacionline.org). A similar reduction in AHR was seen after prolonged challenge of BALB/c mice (data not shown). By contrast, mice that had been sensitized to OVA by means of intraperitoneal injections with aluminum hydroxide (alum) as an adjuvant continued to display sustained AHR after multiple daily OVA challenges (see Fig E1, B). Mice simultaneously sensitized through the airway with OVA-LPS and through the peritoneum with OVA-alum also had reduced AHR after multiple OVA challenges compared with mice sensitized with OVA-alum alone (see Fig E1, C). These data suggested that sensitization through the airway with OVA-LPS primes regulatory responses that can suppress the effector responses driving AHR.

To investigate the loss of AHR seen in OVA-LPS-sensitized mice, we first studied levels of airway inflammation. Total airway levels of eosinophils, lymphocytes, and macrophages were increased with continued daily OVA challenges (Fig 1, C), but airway levels of neutrophils decreased, as did levels of the cytokines IL-4, IL-5, and IL-17 (Fig 1, D). Levels of IL-13 also decreased quickly, although the peak levels of this cytokine were not dramatically increased from baseline at any time point (Fig 1, E). Thus the loss of AHR seen after prolonged OVA challenge was more closely associated with decreasing levels of neutrophils and cytokines than with levels of total leukocytes or eosinophils.

Exogenous IL-17 restores AHR

Our previous work has shown that the AHR in OVA-LPS-sensitized mice is dependent on IL-17 receptor A.¹³ In agreement with these previous findings, instillation of exogenous IL-17 into mice that had received 7 daily OVA challenges restored AHR and promoted neutrophil recruitment but did not significantly alter levels of eosinophils (data not shown). Therefore the loss of AHR seen in OVA-LPS-sensitized mice after multiple challenges likely results, at least in part, from decreasing levels of IL-17.

Suppression of allergic responses does not result from a loss of IL-17⁺ T cells in the lung

In mice sensitized through the airway, T_H17 cells represent the major source of IL-17.¹³ Intracellular staining for IL-17 showed that, as expected, the percentages and total numbers of IL-17⁺CD4⁺ cells in the lung were increased in allergic mice compared with those seen in untreated mice. Surprisingly, however, numbers of these cells remained at high levels, even after 1 week of daily OVA challenges (Fig 2, A-D). Intracellular levels of IL-17, as determined based on mean fluorescent intensity of IL-17 staining, were also similar in mice that had been challenged only once or daily for 1 week (Fig 2, E). Thus the decrease in airway levels of IL-17 and consequent loss of AHR is not due to a corresponding decrease in pulmonary T_H17 cell numbers.

Suppression of allergic responses results from increased Foxp3⁺CD4⁺ Treg cell numbers

The well-described ability of Treg cells to regulate inflammation prompted us to study the role of these cells in suppressing IL-17-dependent AHR. Intracellular staining for IL-10 and Foxp3 in $CD4^+$ T cells prepared from the lung revealed that Foxp3⁺ Treg cells were about 20-fold more abundant than Foxp3⁻IL-10⁺CD4⁺ cells and that less than 1% of Foxp3⁺CD4⁺ cells contained IL-10 (Fig 3, A). Foxp3⁺ Treg cell numbers were increased in lungs of mice that had received 7 daily OVA challenges compared with those seen in lungs of mice that underwent only a single challenge (Fig 3, B). However, when the OVA challenges were discontinued, Treg cell numbers decreased to those seen after a single OVA challenge. Mice that were rechallenged with OVA after a 1-week rest period could again have strong allergic responses, including IL-17 production and AHR (data not shown). Thus the suppression of IL-17-dependent allergic airway disease is closely associated with high numbers of Treg cells.

To confirm that Treg cells are required for the suppression of IL-17 production and consequent AHR, we depleted these cells by administering DTX to sensitized and 7-day-challenged mice that selectively express the DTX receptor in Foxp3-expressing cells (DTR mice). Flow cytometric analyses showed that DTX treatment depleted more than 80% of Foxp3⁺ Treg cells in the lungs (Fig 3, C and D). After sensitization and challenge with OVA for 7 days, these DTX-treated Foxp3-DTR mice had higher levels of allergic inflammation and AHR than did similarly treated wild-type (WT) mice (Fig 3, E and F). No differences between WT and DTR mice were seen when they were not treated with DTX (data not shown). Thus Foxp3⁺ Treg cells are required for effective suppression of these allergic airways diseases.

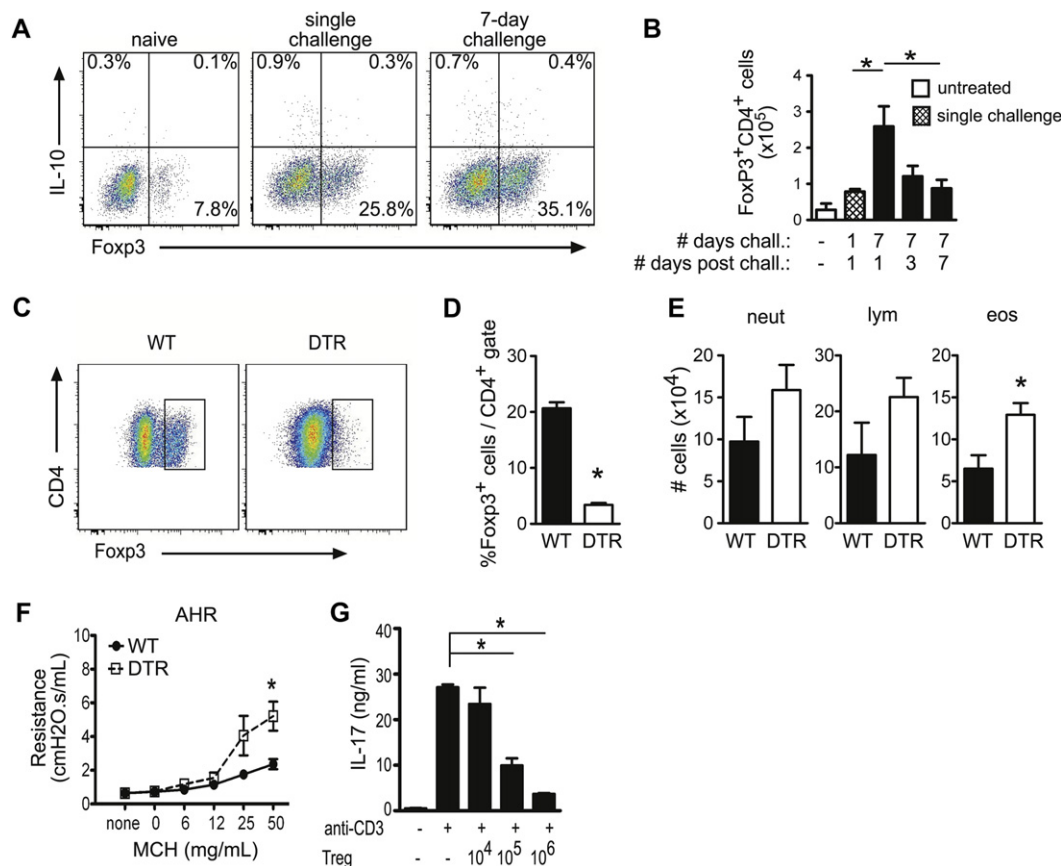


FIG 3. Foxp3⁺ Treg cells suppress TH17-dependent allergic responses. **A**, Staining of IL-10 and Foxp3 in CD4⁺ T cells. **B**, Foxp3⁺ Treg cells per lung after the indicated number of OVA challenges and days of rest after challenge. **C** and **D**, Foxp3⁺ Treg cells in lungs of DTX-treated WT and DTR mice. **E** and **F**, Inflammation (Fig 3, **E**) and AHR (Fig 3, **F**) in 7-day OVA-challenged, DTX-treated mice. *eos*, Eosinophils; *lym*, lymphocytes; *MCH*, methacholine; *neut*, neutrophils. **G**, Dose-dependent Treg cell suppression of IL-17 secretion *in vitro*. **P* < .05.

Treg cells suppress TH17 cell production of IL-17 *in vitro*

Treg cells are best known for their ability to inhibit T-cell proliferation, but our data thus far had suggested that they might also suppress IL-17 production by TH17 cells. To test whether the latter can occur, we generated Treg cells *in vitro* by culturing naive, OVA-specific OT-II CD4⁺ T cells with OVA peptide-loaded splenic dendritic cells in the presence of retinoic acid and TGF- β . In parallel, we generated TH17 cells by culturing naive OT-II CD4⁺ T cells with TGF- β , IL-6, and IL-23 together with peptide-loaded dendritic cells. After T-cell receptor stimulation with antibodies against CD3, the TH17 cells secreted high levels of IL-17 into the cell supernatant (Fig 3, **G**). This secretion was suppressed when the TH17 cells were cocultured with Treg cells, and the magnitude of this suppression was increased with increasing numbers of cocultured Treg cells. Thus, at least *in vitro*, Treg cells can suppress TH17 cell secretion of IL-17.

Suppression of allergic responses is associated with an expansion of ICOS⁺Foxp3⁺CD4⁺ Treg cells

In human subjects naturally occurring Foxp3⁺CD4⁺ Treg cells are comprised of ICOS⁺ and ICOS⁻ populations.²⁶ The former produce both IL-10 and TGF- β , whereas the latter produce only

TGF- β . Because the functions of these 2 Treg cell subsets in controlling pulmonary inflammation have not been reported, we studied their roles in suppressing established allergic inflammation and AHR. Flow cytometric analysis of Foxp3⁺CD4⁺ T cells revealed that murine Treg cells are also comprised of ICOS⁺ and ICOS⁻ populations (Fig 4, **A**). Using mice in which expression of the GFP is controlled by the Foxp3 gene transcriptional control elements, we fractionated Foxp3-GFP⁺ cells into their ICOS⁺ and ICOS⁻ components using flow cytometry-based cell sorting. As seen previously for human ICOS⁺ Treg cells, their murine counterparts also expressed both IL-10 and TGF- β , whereas the ICOS⁻ fraction produced only TGF- β (data not shown). Analysis of these 2 subsets in OVA-LPS-sensitized mice showed that the ratio of ICOS⁺ Treg cells to ICOS⁻ Treg cells increased after prolonged OVA challenge, and when the OVA challenges were discontinued, this ratio returned to values seen in untreated mice (Fig 4, **B**). Thus the ability of the mice to respond to OVA challenge is inversely correlated with numbers of ICOS⁺ Treg cells.

ICOS-B7h interactions suppress TH17 responses *in vivo*

To determine whether interactions between ICOS and its ligand, also known as B7h, affect the number or function of

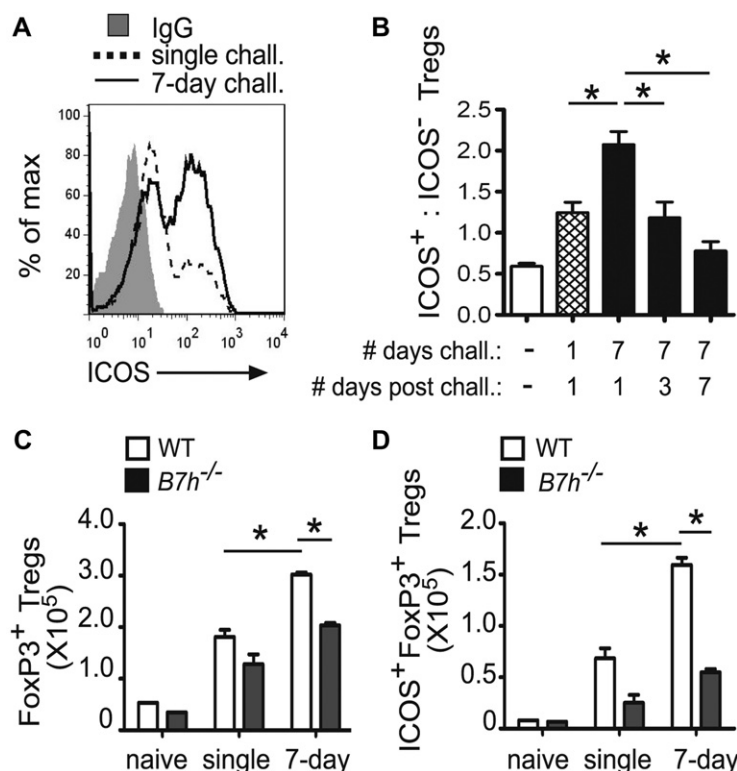


FIG 4. Suppression of AHR associates with increased ICOS⁺ Treg cell numbers. **A**, Staining of Foxp3⁺ Treg cells with isotype control antibodies (shaded region) or anti-ICOS antibodies after a single challenge (dashed line) or a 7-day challenge (solid line). **B**, Ratio of ICOS⁺ to ICOS⁻ Treg cells after the indicated number of challenges and days of rest after challenge. **C** and **D**, Total Treg cells per lung (Fig 5, C) and ICOS⁺ Treg cells per lung (Fig 5, D) of WT and B7h^{-/-} mice.

Foxp3⁺ Treg cells in the lung, we studied B7h-deficient mice (B7h^{-/-} mice). Compared with WT mice, B7h^{-/-} mice had fewer total Foxp3⁺ Treg cells (Fig 4, C) and markedly fewer ICOS⁺Foxp3⁺ Treg cells (Fig 4, D). This suggests that interactions between ICOS and B7h drive the expansion or survival of ICOS⁺Foxp3⁺ Treg cells in the lungs of mice during allergen exposure.

To investigate the requirement of ICOS-B7h interactions for the suppression of allergic responses, we first studied sensitized B7h^{-/-} mice that were challenged with OVA on a single occasion. These mice had less IL-5 and IL-17 in the airways and had fewer neutrophils than did similarly treated WT mice (Fig 5, A and B), although AHR was similar in these 2 strains (Fig 5, C). The increased IL-17 level was not an unexpected result because ICOS has been previously reported to promote the induction of T_H17 responses.²³ However, after 1 week of daily OVA challenges, IL-5 and IL-17 levels were higher in the airways of B7h^{-/-} mice than in those of WT animals (Fig 5, D). Eosinophil and neutrophil numbers were also increased in B7h^{-/-} mice after multiple OVA challenges (Fig 5, E), and these animals continued to display AHR, whereas WT mice did not (Fig 5, F). This finding was confirmed in *Icos*^{-/-} mice (see Fig E2 in this article's Online Repository at www.jacionline.org). The sustained AHR seen in B7h^{-/-} mice after 7 daily OVA challenges did not result from increased T_H17 cell numbers in these animals. In fact, despite their increased levels of extracellular IL-17, lungs of B7h^{-/-} mice contained fewer T_H17 cells than those of WT mice (Fig 5, G). Thus, although interactions between ICOS and B7h contribute to the

initial development of T_H17 cells, these same molecules appear to suppress established T_H17 inflammation in the lung by blocking production of IL-17.

IL-10 and TGF- β are dispensable for suppression of T_H17-mediated AHR

IL-10 can attenuate inflammation in a wide variety of settings, including the lung. However, after OVA-LPS sensitization and 7 daily OVA challenges, *Il-10*^{-/-} mice did not display increased airway inflammation, IL-17, or AHR compared with WT mice (Fig 6, A). Therefore IL-10 is not required for the suppression of allergic airways disease in OVA-LPS-sensitized mice after prolonged OVA challenge. TGF- β -deficient mice are of limited utility for studying the regulation of pulmonary inflammation because they display spontaneous inflammation and stunted growth and have a very short lifespan.^{27,28} We therefore used TGF- β -blocking antibodies to study the function of TGF- β in AHR suppression. Except for an increase in eosinophil numbers, airway inflammation was similar in mice treated with TGF- β -blocking antibodies or with isotype control antibodies (Fig 6, B). Levels of cytokines, including IL-17, were also similar in the 2 groups of mice, and the TGF- β -blocking antibodies did not reverse the suppression of AHR. A small-molecule inhibitor of the TGF- β receptor was also unable to reverse the suppression of AHR in WT mice (see Fig E3 in this article's Online Repository at www.jacionline.org). Together these experiments suggest that the suppression of IL-17 production and AHR seen after multiple challenges of

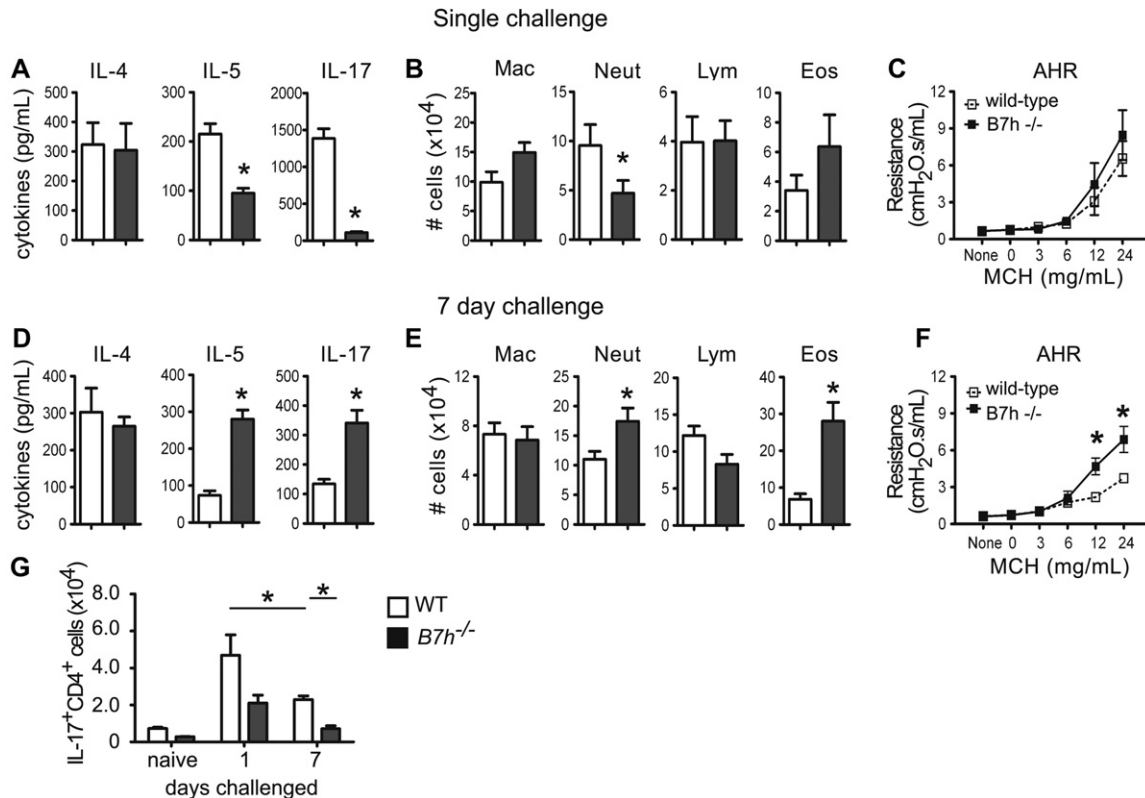


FIG 5. ICOS-B7h interactions suppress AHR. **A-C**, Allergic responses, including cytokines (Fig 5, A), leukocytes in bronchoalveolar lavage fluid (Fig 5, B) and AHR (Fig 5, C) in WT mice (open rectangles) and *B7h*^{-/-} mice (solid rectangles) after a single OVA challenge. **D-F**, Allergic responses of the same strains after a 7-day OVA challenge. **G**, Total TH17 cell numbers in lungs of WT and *B7h*^{-/-} mice. Eos, Eosinophils; Lym, lymphocytes; Mac, macrophages; Neut, neutrophils. **P* < .05.

OVA-LPS-sensitized mice is not dependent on either IL-10 or TGF- β .

IL-35 production by ICOS⁺ Treg cells suppresses TH17-mediated allergic airway disease

IL-35 is a recently described regulatory cytokine the function of which in allergic inflammation is unknown. We therefore studied allergic responses in mice lacking one of the IL-35 subunits, EBI-3. After OVA-LPS sensitization and 7 daily OVA challenges, EBV-induced gene (*Ebi-3*)-deficient mice had significantly more macrophages, neutrophils, lymphocytes, and eosinophils in the airway than did similarly treated WT mice (Fig 6, C). The *Ebi-3*-deficient mice also had higher levels of IL-17 and, importantly, continued to display AHR after multiple OVA challenges. This difference did not result from enhanced induction of TH17 responses during sensitization because after a single OVA challenge, WT and *Ebi-3*-deficient mice had similar levels of airway inflammation and IL-17 (Fig 6, D). Rather, EBI-3 is apparently required to suppress IL-17 production and AHR once it is established. Mice lacking p35, the other subunit of IL-35, behaved similarly to *Ebi-3*-deficient mice. OVA-LPS-sensitized *p35*-deficient mice also had inflammation and AHR after a single OVA challenge, and this AHR was sustained after 7 OVA challenges (see Fig E4 in this article's Online Repository at www.jacionline.org). Thus both subunits of IL-35 are required for the suppression of established AHR.

Our data thus far had shown that the suppression of AHR was dependent on IL-35, as well as on interactions between ICOS and its ligand. This suggested that IL-35 might be selectively produced by ICOS⁺ Treg cells. To test this, we measured expression levels of both IL-35 subunits in ICOS⁺ and ICOS⁻ Treg cells prepared from Foxp3-GFP mice. Both subunits were expressed by ICOS⁺ Treg cells, whereas neither subunit was expressed to an appreciable extent by ICOS⁻ Treg cells (Fig 7). Because Ebi-3 can also pair with p28 to form IL-27, we also measured expression levels of p28. This subunit was expressed at relatively low levels in both Treg cell subsets. Thus IL-35, but not IL-27, is selectively expressed by murine ICOS⁺ Treg cells.

DISCUSSION

In human subjects the relative strengths of regulatory and effector responses to inhaled allergens are thought to confer susceptibility to asthma.¹⁵ In the animal model used here, allergic sensitization through the airway was achieved by using the naturally occurring environmental agent LPS, which has been previously shown to prime strong TH17 responses to inhaled OVA.¹³ This is in contrast to some other murine models of asthma in which sensitization occurs at extrapulmonary sites, such as the skin or peritoneum. We show here that allergic sensitization through the airway also induces regulatory responses that gain increasing dominance over effector responses as the number of exposures is increased. These findings might account for the finding

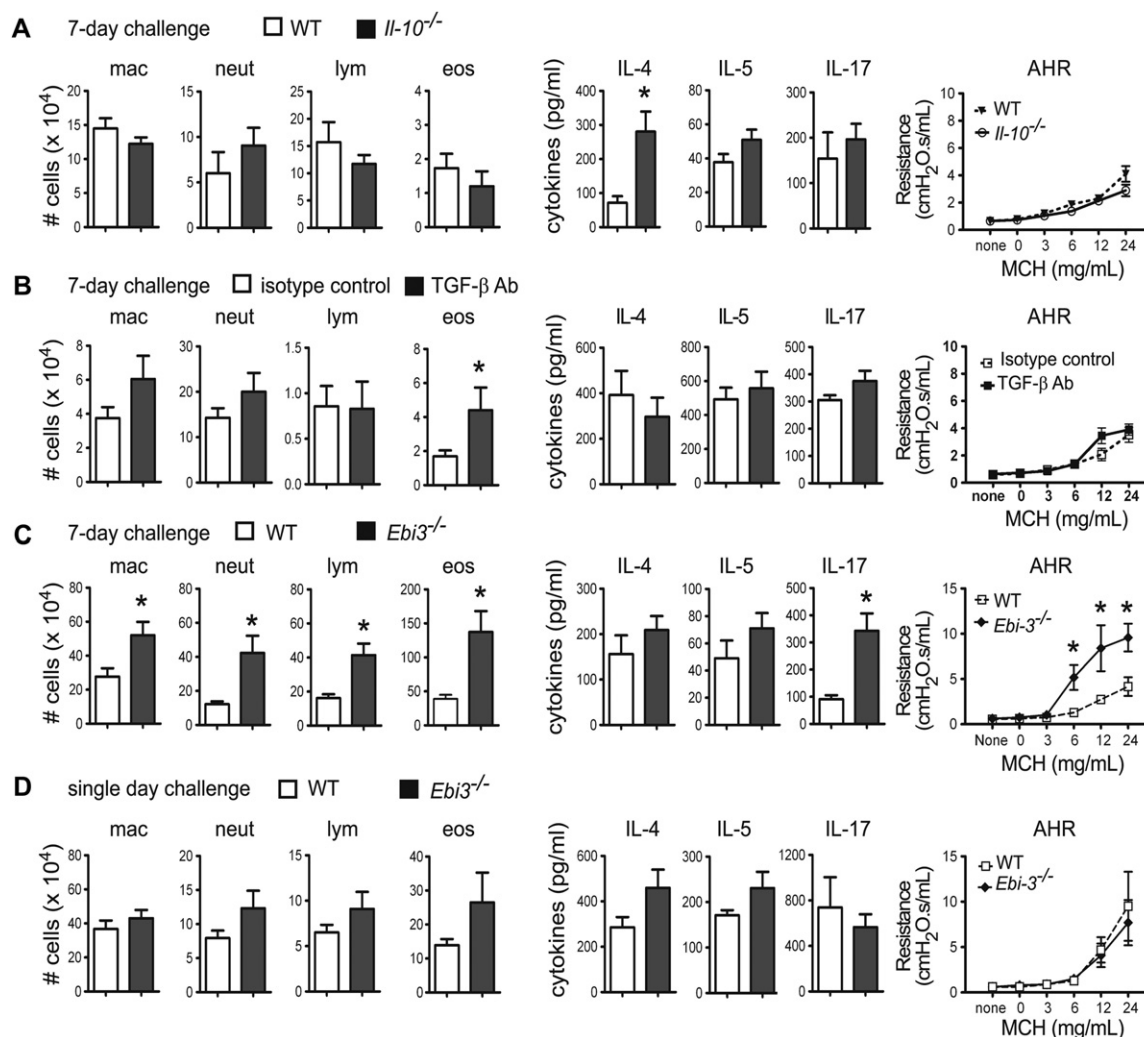


FIG 6. Suppression of AHR is independent of IL-10 and TGF-β but dependent on IL-35. **A-C**, Allergic responses to 7-day OVA challenge of IL-10-deficient mice (Fig 6, A), WT mice treated with anti-TGF-β antibodies (Fig 6, B), or *Ebi3*^{-/-} mice (Fig 6, C). **D**, Single OVA challenge of *Ebi3*^{-/-} mice. **P* < .05 (*n* = 8 mice/group). *eos*, Eosinophils; *lym*, lymphocytes; *mac*, macrophages; *neut*, neutrophils.

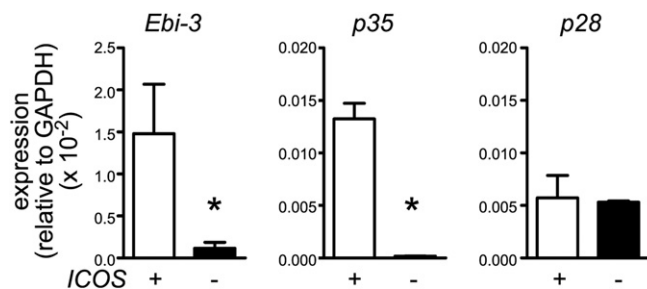


FIG 7. IL-35 is selectively produced by ICOS⁺ Treg cells. Quantitative PCR was used to measure mRNA levels of each of the IL-35 and IL-27 subunits after flow cytometric sorting of Foxp3-gfp⁺ Treg cells into their ICOS⁺ and ICOS⁻ fractions (*n* = 6). **P* < .05. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

that exposure to LPS in early life can protect against the development of asthma in later life.²⁹ It is likely that human subjects are exposed to allergens on multiple occasions, and the long-term balance between the effector and regulatory forces are of critical

importance. In healthy subjects multiple exposures might increase regulatory responses to allergens, thereby protecting against asthma. Conversely, clinical disease might represent a failure of these regulatory influences. Our current finding that IL-35 can suppress T_H17-dependent AHR suggests that asthmatic patients, particularly those with severe asthma during exacerbation, might have low IL-35 levels in comparison with times between exacerbations. If so, strategies that enhance the actions of this cytokine might be of particular benefit for suppressing responses associated with allergic asthma.

The suppression of AHR that we observed also required interactions between ICOS and its ligand, B7h. Interestingly, some patients with ICOS deficiency also have reduced levels of Foxp3⁺ Treg cells and display either immunodeficiency or autoimmunity.³⁰ Thus interactions between ICOS and B7h might have a similar effect on Treg cells in human subjects as they do in mice. Paradoxically, although ICOS and B7h promote the early development of T_H17 cells,²³ *B7h*^{-/-} mice had higher levels of IL-17 after prolonged OVA challenge than did WT mice. Thus the molecules that promote the development of T_H17 cells also

suppress their activity in the setting of established inflammation. This finding is reminiscent of a recent report showing that signal transducer and activator of transcription 3, which is required for the development of T_H17 responses, is also required by Treg cells for the regulation of those responses.³¹ Additional work will be required to fully understand the relationships between signal transducer and activator of transcription 3, ICOS, and other molecules that control the development and regulation of T_H17 cells.

No single effector molecule has emerged as essential for Treg cell-mediated suppression of airways disease.³² In general, IL-10 has a protective function in airway disease when the effector and suppressor cells develop at extrapulmonary sites.¹⁹ However, when these cells develop in the airway or their draining lymph nodes after allergen inhalation, IL-10 is usually dispensable. Our finding that IL-10 is not required for suppressing IL-17-dependent AHR after allergic sensitization through the airway is consistent with this general trend and with the previous findings that T_H17 cells are insensitive to IL-10 *in vitro*.²¹ Similarly, TGF- β -specific inhibitors or blocking antibodies did not reverse the suppression of AHR. Together, the data suggest that IL-35 is the primary cytokine for suppressing IL-17-dependent AHR. This interpretation is also consistent with the ability of splenocytes from *Ebi-3*^{-/-} mice to produce increased levels of IL-17 and IL-22³³ and with the observation that IL-35 can suppress IL-17 production and attenuate established collagen-induced arthritis in mice.³⁴ We cannot formally exclude a role in this regard for the cytokine IL-27, which is a heterodimer comprised of the EBI3 chain and p28. However, p28 was expressed at much lower levels in murine ICOS⁺Foxp3⁺ Treg cells than are *Ebi-3* or p35, suggesting that IL-35, and not IL-27, is responsible for suppressing IL-17-dependent AHR. Moreover, a previous report showed that IL-27 has little effect on T_H17 responses once they have already developed.³⁵ We are unaware of previous reports linking IL-35 to the suppression of allergic airways disease or to the production of this cytokine by ICOS⁺ Treg cells. On the basis of the novel findings presented here, we suggest that targeting the ICOS–IL-35 axis might be of benefit to patients with established AHR.

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Key messages

- Allergic airway inflammation and IL-17–dependent AHR are reversibly suppressed by prolonged exposures to aerosolized allergen.
- The production of IL-35 by ICOS⁺ Treg cells suggests that targeting this pathway might be of therapeutic benefit for treating allergic asthma.

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METHODS

Allergen-induced airway disease

For single allergen challenges and rechallenges, mice were exposed to the OVA aerosol for 1 hour and harvested 48 hours later. We have previously found that this is when biologic and physiologic responses peak.^{E1} Cytokines were analyzed 4 hours after challenge because that is when their levels peak.^{E1} For multiple challenges, the mice were exposed to the OVA aerosol for 30 minutes each day and analyzed 24 hours after the final challenge. The TGF- β inhibitor SB-431542 (Tocris Bioscience, Ellisville, Mo) was administered by means of intraperitoneal injection (0.2 mg in 50 μ L) twice a day, as previously described.^{E2}

Analysis of cytokines and airway inflammation

Tracheas of killed mice were exposed and intubated with a 20-gauge needle covered by a 1-cm section of PE-90 tubing. With a 1-mL syringe, 1 mL of sterile PBS was instilled into the lungs and then withdrawn. This was repeated twice. After centrifugation, supernatants were collected for measurements of cytokine levels by using a commercial, multiplexed, fluorescent bead-based immunoassay (Bio-Rad Laboratories), according to the manufacturer's instructions. The cell pellet was resuspended in saline and centrifuged onto slides for differential analysis. TGF- β 1 and IL-13 levels were measured with commercial ELISAs from R&D Systems. In some experiments lungs were homogenized in 1 mL of PBS containing 50 mmol/L Tris HCl (pH 7.5), 10% glycerol, 0.1 mmol/L EDTA, and 1 mmol/L dithiothreitol and then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatants of each sample were adjusted to have equal protein concentrations before analysis.

Airway physiology

Total respiratory system resistance after a baseline measurement and administration of increasing doses of methacholine were recorded for each mouse by using the single-compartment model. During a 3-minute sampling period after delivery of each dose of methacholine, individual peak responses to each dose per mouse were used for the analysis.

Flow cytometry

T cells were prepared from the lungs of sensitized and challenged mice, as described previously.^{E1} Antibodies used include those against murine CD4 (clone GK1.5 or RM4-5; BD Biosciences, Mississauga, Ontario, Canada, or eBioscience, San Diego, Calif), CD278 (ICOS, clone C398.4A or 7E.17G9; eBioscience), B7RP-1 (clone HK5.3, eBioscience), Foxp3 (clone NRRF-30, eBioscience), IL-10 (clone JE55-16E3, eBioscience), IL-13 (clone eBio13A, eBioscience), IL-17A (clone eBio17B7, eBioscience) or clone TC11-18H10, BD Biosciences), or the appropriate isotype control. After incubation with a blocking cocktail of purified rat anti-mouse CD16/CD32 (BD PharMingen, San Jose, Calif, or 2.4G2), normal murine serum, and normal rat serum (Jackson ImmunoResearch Laboratories) for 20 minutes, cells were labeled with the appropriate antibodies. For intracellular staining, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin (Sigma) for 4 hours before staining and incubated with GolgiStop during the last 3 hours of stimulation. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and labeled with the appropriate antigen-specific antibodies. CD4⁺ lymphocytes were identified as nonautofluorescent cells within a lymphocyte gate based on forward and side scatter. Cells were collected with a BD LSR II cytometer (BD Biosciences), and data were analyzed with FlowJo 7.5 software (TreeStar, Inc, Ashland, Ore).

Generation and culture of T_H17 and Treg cells *in vitro*

Naive CD4⁺ T cells were prepared from pools of lymph nodes and spleens of OT-II mice or OT-II⁺ *Foxp3-gfp*⁺ mice by means of magnetic bead-based cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) by using an antibody cocktail for naive CD4 T-cell enrichment, as previously

described.^{E3} The purified T cells were greater than 98% CD4⁺ and less than 1% CD45RB^{lo}CD44^{hi} memory cells. Dendritic cells were enriched from spleens of C57BL/6 mice by means of gradient centrifugation and MACS sorting with CD11c-specific mAb. The cells obtained with this procedure were greater than 90% CD11c⁺. The purified T cells (1×10^6) and CD11c⁺ dendritic cells (1×10^6) were cultured for 5 days in 2 mL of complete Iscove modified Dulbecco medium containing 10% FBS (certified, Invitrogen, Carlsbad, Calif), 50 μ mol/L β -mercaptoethanol, penicillin, and streptomycin in a 24-well plate (BD Biosciences) at 37°C in a CO₂ incubator. For induction of T_H17 cells, the following reagents were added: recombinant murine (rm) IL-1 α (10 ng/mL), rmIL-6 (10 ng/mL), recombinant human (rh) TGF- β (3 ng/mL), anti-mIFN- γ (10 μ g/mL), anti-mIL-2 (10 μ g/mL), anti-mIL-4 (10 μ g/mL), anti-mIL-12 p40 (10 μ g/mL), and OVA₃₂₃₋₃₃₉ (3 μ mol/L). For Treg cell induction, the following reagents were added: rmIL-2 (10 ng/mL), rhTGF- β (5 ng/mL), all-trans retinoic acid (10 nmol/L, Sigma), and OVA₃₂₃₋₃₃₉ (100 nmol/L). All cytokines were purchased from R&D Systems. After primary culture, T cells were expanded in complete RPMI 1640 containing 10% FBS (cRPMI-10) and the following cytokines: rmIL-6 (10 ng/mL) and rmIL-23 (10 ng/mL) for T_H17 cells and rmIL-2 (20 ng/mL) for Treg cells. The generation of T_H17 and Treg cells was confirmed by IL-17 production and Foxp3 expression, respectively. For the T_H17 suppression assay, thoroughly washed T_H17 cells (1×10^5) were cultured with various numbers of Treg cells for 24 hours in 200 μ L of cRPMI-10 in CD3 mAb (1 μ g/mL)-coated 96-well flat-bottom plate. IL-17 in the culture supernatant was measured by means of ELISA with IL-17-specific antibodies (TC11-18H10 and TC11-8H4, BD Biosciences).

Analysis of mRNA

For Treg RNA, leukocytes were prepared from the lungs of OVA/LPS-sensitized and 7-day-challenged *Foxp3-gfp* mice and labeled with antibodies to murine CD4, CD8 α , and ICOS, as described above. CD4⁺CD8⁺GFP⁺ cells were sorted into 2 fractions based on ICOS expression by using a FACSaria II flow cytometer (BD Biosciences). The purity of each population was consistently 97.5% or higher. Total RNA was isolated from purified populations with a Qiagen RNeasy Mini Kit and reverse transcribed with SuperScript III First Strand (Invitrogen). cDNA was amplified with Taqman primers and probes (Applied Biosystems) for p28 (assay id: Mm00461164_m1), p35 (assay id: Mm00434165_m1), Ebi-3 (assay id: Mm00469294_m1), IL-10 (assay id: Mm00439614_m1), and TGF- β 1 (assay id: Mm01178820_m1) by using a Stratagene MX3000P (Stratagene, La Jolla, Calif) and the relative expression levels were determined according to the manufacturer's protocol.

For whole-lung RNA, a 20-gauge needle was inserted into the right heart ventricle, and approximately 3 to 5 mL of PBS was infused through the pulmonary artery to clear the lungs. The lobes were excised and flash-frozen in liquid nitrogen. Primer sets used to measure RNA were as follows: *IL13*, 5'-CTG TGC AAC GGCAGC ATG GTA-3' (forward) and 5'-GGG AGG CTG GAG ACC GTA GT-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-TGC ACC ACC AAC TGC TTA G-3' (forward) and 5'-GGA TGC AGG GAT GAT GTT C-3' (reverse). Conditions for the amplification of each primer set included 50°C for 30 minutes and 95°C for 5 minutes, followed by 40 PCR cycles (95°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds). A relative quantification method ($\Delta\Delta$ CT) was used to evaluate the relative expression of each gene between the singly and multiply challenged mice.

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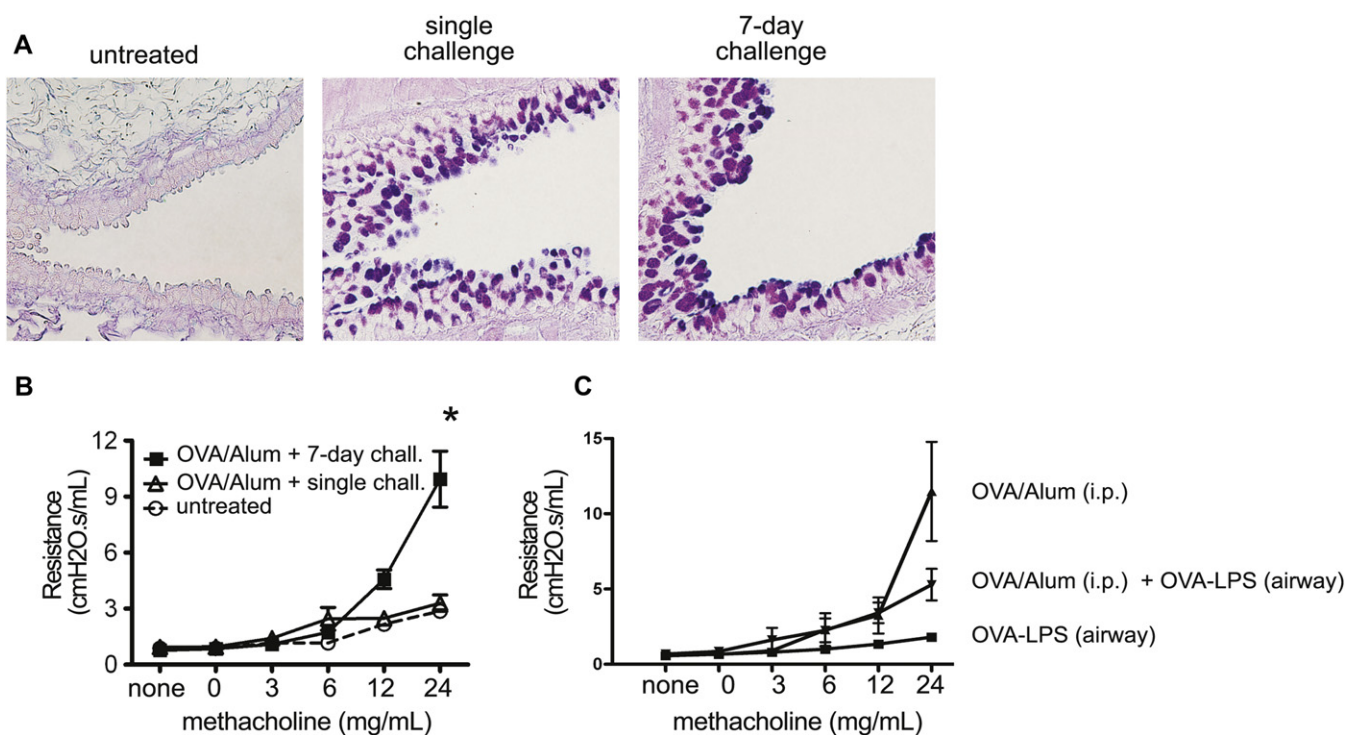


FIG E1. Response to multiple OVA challenges depends on route of sensitization. **A**, Representative Alcian blue/periodic acid-Schiff staining of mucus-producing cells in OVA-LPS-sensitized murine lungs. **B** and **C**, AHR. Mice were sensitized and challenged as indicated. Mean $\pm$ SEM values are shown. * $P < .05$. Data represent one of 3 similar experiments ($n = 9$; Fig E1, B) or a single experiment ($n = 6$ mice/group; Fig E1, C).

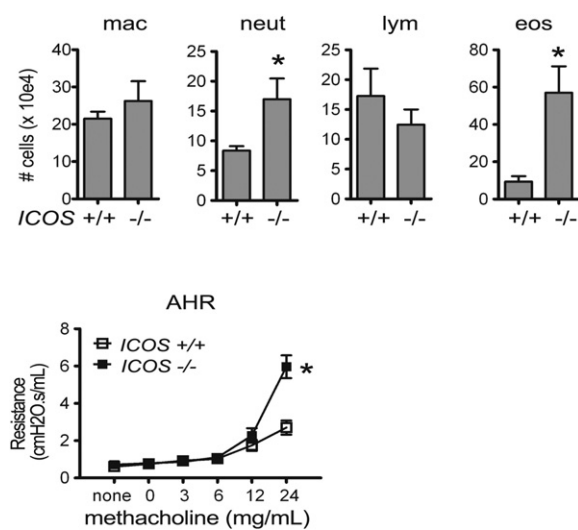


FIG E2. ICOS is required for the suppression of AHR. WT C57BL/6 and *Icos*^{-/-} mice were sensitized to OVA-LPS through the airways and then challenged with aerosolized OVA on 7 consecutive days. Shown are the indicated leukocyte subsets, as well as AHR. Data represent mean $\pm$ SEM values from a single experiment. * $P < .05$ ($n = 8$ mice/group). *eos*, Eosinophils; *lym*, lymphocytes; *mac*, macrophages; *neut*, neutrophils.

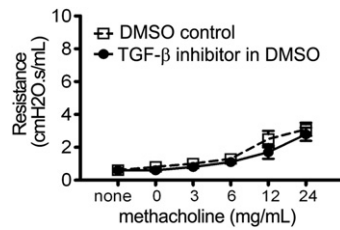


FIG E3. Inhibiting TGF- β receptor signaling does not prevent suppression of AHR. Sensitized C57BL/6 mice were challenged and then given an inhibitor of TGF- β receptor signaling (SB-431542, Tocris Bioscience) on 3 consecutive days before harvest. Data presented are mean $\pm$ SEM values from one of 2 experiments producing similar results. * $P < .05$ ($n = 8$ mice/group). *DMSO*, Dimethyl sulfoxide.

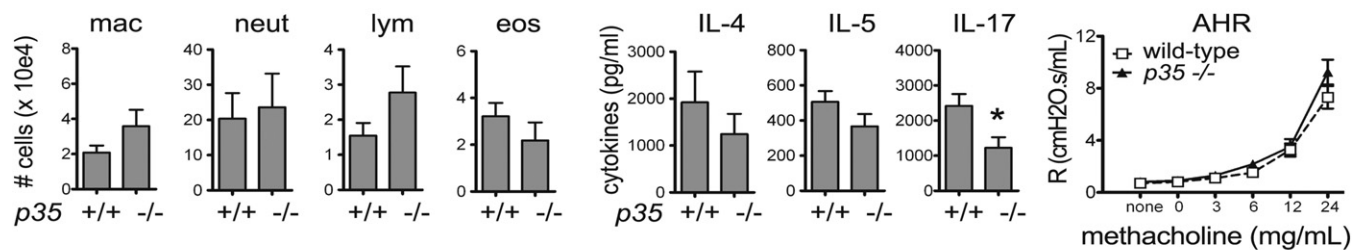
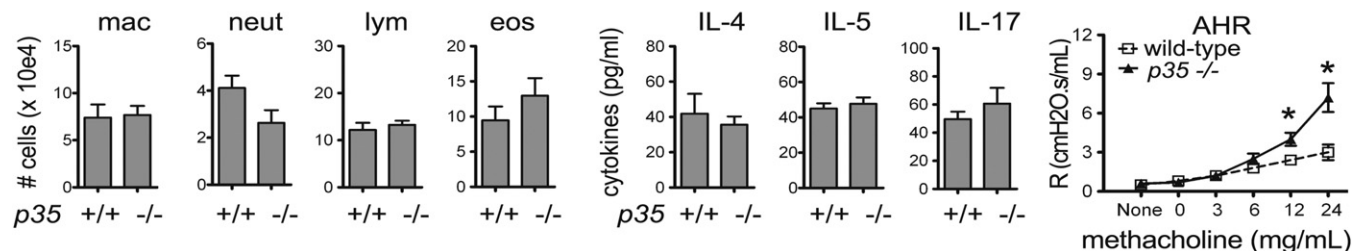
A single challenge**B** 7-day challenge

FIG E4. IL-12p35 is required for suppression of AHR. C57BL/6 and p35-deficient mice were sensitized and then challenged with aerosolized OVA. **A**, Single OVA challenge. **B**, Seven daily OVA challenges. Mean $\pm$ SEM values are shown for the indicated leukocyte subsets and cytokines. Data represent one of 2 similar experiments. * $P < .05$ ($n = 8$ mice/group). eos, Eosinophils; lym, lymphocytes; mac, macrophages; neut, neutrophils.