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IL-35 Production by ICOS⁺ Tregs Reverses Established IL-17-dependent Allergic Airways Disease

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Abstract

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.

Objectives—We sought to understand the cellular and molecular basis for suppression of established, IL-17-dependent allergic airways disease.

Methods—Mice were sensitized by airway instillations of ovalbumin (OVA) together with low levels of lipopolysaccharide. Leukocyte recruitment to the lung and AHR were assessed following daily challenges with aerosolized OVA. Flow cytometry and gene targeted mice were used to identify naturally-arising subsets of regulatory T cells (Tregs) 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 declined, even though pulmonary levels of Th17 cells remained high. This loss of AHR was reversible and required the expansion of a Treg subset expressing both Foxp3 and inducible co-stimulator (ICOS). These Tregs also expressed the regulatory cytokines, IL-10, TGF-beta and IL-35. Whereas IL-10 and TGF-beta were dispensable for suppression of airway hyperresponsiveness, IL-35 was required. Analysis of human ICOS⁺ Tregs revealed that they also selectively expressed IL-35.

Conclusion—IL-35 production by ICOS⁺ Tregs can suppress IL-17 production and thereby reverse established, IL-17-dependent AHR in mice. The production of IL-35 by human ICOS⁺ Tregs suggests that targeting this pathway might be of therapeutic value for treating allergic asthma in humans.

Keywords

Asthma; airway hyperresponsiveness; AHR; IL-17; Th17; Th2; IL-35; ICOS; ovalbumin

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INTRODUCTION

Allergic asthma is clinically characterized by reversible airway obstruction and airway hyperresponsiveness (AHR). These clinical features of asthma stem largely from the actions of CD4⁺ Th2 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 Th2 cytokines, such as IL-5, can reduce levels of eosinophils, other disease symptoms are generally not improved³⁻⁵. Recent evidence suggests that IL-17A (IL-17) production by Th17 cells or macrophages can also contribute to allergic asthma⁶⁻¹³, and that Th2 and Th17 responses can act synergistically to promote AHR^{13, 14}. These observations have prompted efforts to block the actions of IL-17 using antibody-mediated therapies¹⁵. In addition to having allergen-specific Th2 and Th17 effector responses, asthmatics also appear to lack sufficiently strong regulatory mechanisms to keep these effector responses in check^{16, 17}. Accordingly, the goal of allergen-specific immunotherapy is to strengthen regulatory responses by exposing allergic individuals to progressively higher doses of provoking allergen. Although this approach has been effective for treating allergic rhinitis¹⁸, it is less effective for allergic asthma¹⁹. An improved understanding of the cellular and molecular mechanisms that regulate Th2 and Th17 effector responses might lead to novel therapeutic strategies to prevent or control allergic asthma.

The best characterized T regulatory cells (Tregs) are Foxp3⁺ CD4⁺ T cells and Foxp3⁻ IL-10-producing type 1 regulatory 1 (TR1) cells¹⁷. In some models, adoptive transfer of T cells producing IL-10 or TGF- β can modulate airway inflammation and AHR²⁰⁻²³. However, IL-10 does not inhibit IL-17 production by memory T cells²⁴, and TGF- β promotes Th17 development rather than suppressing it^{25, 26}. Thus, it is unclear which cytokine suppresses Th17-dependent allergic pulmonary inflammation. IL-35 is a newly-described cytokine comprised of the IL-12 α chain (p35) and the Epstein-Barr virus-induced gene product (EBI-3). Although IL-35 displays some regulatory activities in vitro and in vivo²⁷, its role in allergic inflammation is unknown. Using a Th2- and Th17-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 , but is instead dependent on ICOS⁺ Treg production of IL-35. These findings suggest that targeting this ICOS – IL-35 axis might be of therapeutic benefit for individuals with allergic asthma.

METHODS

Mice

Mice were housed in specific pathogen-free conditions at the 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. Additional details on the mice used and the methods employed in this study are provided in an online data supplement.

Allergen-induced airway disease

Mice were sensitized on days 0 and 6, as described previously¹³. Briefly, anaesthetized mice were given oropharyngeal administrations of 100 μ g low-endotoxin OVA together with 0.1 μ g E. coli lipopolysaccharide (LPS) (Sigma) in a total volume of 50 μ l 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 (BAL) performed as described previously¹³. Cytokines in BAL fluid (BALF) were measured using a multiplexed fluorescent bead-based immunoassay (Bio-Rad Laboratories, Hercules, CA). AHR was assessed using the Flexivent mechanical ventilator system (Scireq, Montreal, PQ,

Canada) as previously described¹³. Where indicated, recombinant mouse IL-17 (R&D, Inc.), at 1.5 µg per mouse in 50 µl of PBS, was given by oropharyngeal delivery to anaesthetized mice 2 hours after the final daily OVA challenge, and the animals harvested 24 h later. TGF-β1 neutralizing antibody (75 mg) in sterile PBS was delivered by intranasal administration 1 h before each OVA challenge and on the day biological and physiological responses were assessed.

Depletion of Tregs in FoxP3-DTR mice

Beginning one day before the first OVA challenge, previously sensitized mice were given daily i.p. injections of 100 µl PBS containing 100 ng DTX from *Corynebacterium diphtheriae* (Sigma). This treatment was continued daily until the final OVA challenge.

Adoptive transfer of Tregs

Mice that were to receive Tregs were first sensitized twice with OVA/LPS and then given daily OVA challenges. One day prior to their first OVA challenge, these animals received 22,000 Tregs from WT or *Ebi3*^{-/-} donors by i.v. injection in a total volume of 200 µl PBS. A second transfer was performed one hour before the fifth OVA challenge.

Preparation of mRNA from mouse and human Tregs

The protocol for blood collection from human volunteers was approved by the Institutional Review Boards at the NIEHS and written consent was obtained from each subject. T cells from human blood and from the lungs of sensitized and challenged mice were prepared as previously described¹³. Details on the antibodies used in each of these studies are provided in the online data supplement. Total RNA was prepared using an RNeasy Mini kit (Qiagen, Inc. Valencia, CA). RTPCR was performed for cytokine-specific RNA using Power SYBR green master mix or primer/probe sets (Applied Biosystems) in an Mx3000P QPCR System (Stratagene). Values for cytokines were normalized to the internal controls, GAPDH or 18S ribosomal RNA.

Statistics

Data are expressed as mean ± SEM. Statistical differences between groups were calculated using Student's *t* test or Mann-Whitney *U* test, or by ANOVA followed by either a Tukey or Bonferroni post hoc test using GraphPad Prism v5.02 (GraphPad Software, Inc.). A two-tailed *p*-value of less than 0.05 was considered statistically significant.

RESULTS

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

To induce allergen-induced airway disease, mice were sensitized through the airway with OVA-LPS and challenged with aerosolized OVA (Fig. 1, A). As expected from previous work¹³, mice challenged on a single occasion developed AHR (Fig. 1, B). However, following multiple daily OVA challenges, AHR was no longer apparent, although these animals still had mucus-producing cells in the airway (Fig. E1, A). By contrast, mice that had been sensitized to OVA by i.p. injections using aluminum hydroxide (alum) as an adjuvant continued to display sustained AHR after multiple daily OVA challenges (Fig. E1, B). Mice sensitized through the airway with OVA-LPS and also through the peritoneum with OVA-alum also had reduced AHR after multiple OVA challenges compared to mice sensitized with OVA-alum alone (Fig. E1, C). This suggests that sensitization through the airway with OVA-LPS primes regulatory responses that can suppress 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 declined, as did levels of the cytokines, IL-4, IL-5 and IL-17 (Fig. 1, D). Levels of IL-13 also declined 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 declining 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 the IL-17 receptor A¹³. In agreement with these previous findings, instillation of exogenous IL-17 into mice that had received seven daily OVA challenges restored AHR and promoted neutrophil recruitment, but did not significantly alter levels of eosinophils (Fig. 2, A and B). Therefore, the loss of AHR seen in OVA-LPS sensitized mice after multiple challenges likely results, at least in part, from declining 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, Th17 cells represent the major source of IL-17¹³. Intracellular staining for IL-17 showed that, as expected, the percentage and total numbers of IL-17⁺ CD4⁺ cells in the lung were increased in allergic mice compared to untreated mice. Surprisingly however, levels of these cells remained at high levels, even after one week of daily OVA challenges (Fig. 3, A - C). Intracellular levels of IL-17, as determined by mean fluorescent intensity (MFI) of IL-17 staining, were also similar in mice that had been challenged only once or daily for one week (Fig. 3, D). Thus, the decline in airway levels of IL-17 and consequent loss of AHR is not due to a corresponding decline in pulmonary Th17 cells.

Suppression of allergic responses results from increased numbers of Foxp3⁺ CD4⁺ Tregs

The well-described ability of Tregs 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⁺ Tregs were about 20-fold more abundant than Foxp3⁻ IL-10⁺ CD4⁺ cells, and that less than one percent of Foxp3⁺ CD4⁺ contained IL-10 (Fig. 4, A). Levels of Foxp3⁺ Tregs were increased in lungs of mice that had received seven daily OVA challenges compared to mice that received only a single challenge (Fig. 4, B) and when the OVA challenges were discontinued, Treg levels eventually declined to those seen after a single OVA challenge. Mice that were rechallenged after this one week rest period could once again develop strong allergic responses to OVA challenge, including IL-17 production and AHR (Fig. E2). Thus, the suppression of IL-17-dependent allergic airways disease was closely associated with high levels of Tregs.

To confirm that Tregs are required for the suppression of IL-17 production and consequent AHR, we depleted these cells by administering diphtheria toxin (DTX) to sensitized and 7-day challenged mice that selectively express the DTX receptor in Foxp3-expressing cells (DTR mice). Flow cytometric analysis of cells from these animals showed that DTX treatment depleted more than 80% of Foxp3⁺ Tregs in the lungs (Fig. 4, C and D). Following sensitization and challenge with OVA for seven days, these DTX-treated Foxp3-DTR mice had higher levels of allergic inflammation and AHR than did similarly-treated WT mice (Fig. 4, E and F). Thus, Foxp3⁺ Tregs are required for effective suppression of these allergic airways disease.

Tregs suppress Th17 cell production of IL-17 *in vitro*

Tregs 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 if the latter can occur, we generated Tregs *in vitro* by culturing naïve, OVA-specific OT-II CD4⁺ T cells with OVA peptide-loaded splenic DCs in the presence of retinoic acid and TGF- β . In parallel, we generated Th17 cells by culturing naïve OT-II CD4⁺ T cells with TGF- β , IL-6, and IL-23 together with peptide-loaded DCs. After TCR stimulation with antibodies against CD3, the Th17 cells secreted high levels of IL-17 into the cell supernatant (Fig. 4, G). This secretion was suppressed when the Th17 cells were co-cultured with Tregs, and the magnitude of this suppression was increased with increasing numbers of co-cultured Tregs. Thus, at least *in vitro*, Tregs can suppress Th17 cell secretion of IL-17.

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

In humans, naturally-occurring Foxp3⁺ CD4⁺ Tregs are comprised of ICOS⁺ and ICOS⁻ populations²⁸. The former produce both IL-10 and TGF- β , whereas the latter produce only TGF- β . Because the functions of these two Treg 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 mouse Tregs are also comprised of ICOS⁺ and ICOS⁻ populations (Fig. 5, A). Using mice in which expression of the green fluorescent protein (GFP) is controlled by the Foxp3 gene transcriptional control elements, we fractionated Foxp3-GFP⁺ cells into their ICOS⁺ and ICOS⁻ components by flow cytometry-based sorting. As seen previously for human ICOS⁺ Tregs, their murine counterparts also expressed both IL-10 and TGF- β , whereas the ICOS⁻ fraction produced only TGF- β (Fig. E3). Analysis of these two subsets in OVA-LPS sensitized mice showed that the ratio of ICOS⁺ Tregs to ICOS⁻ Tregs increased after prolonged OVA challenge, and when the OVA challenges were discontinued this ratio returned to values seen in untreated mice (Fig. 5, B). Thus, mice that are withdrawn from OVA challenge undergo a decline in levels of ICOS⁺ Tregs while regaining the ability to develop allergic responses re-challenge (Fig. E2).

ICOS – B7h interactions suppress Th17 responses *in vivo*

To determine if interactions between ICOS and its ligand, also known as B7h, impact the number or function of Foxp3⁺ Tregs in the lung, we carried out a series of experiments with *B7h*-deficient mice (*B7h*^{-/-} mice). We found that *B7h*^{-/-} mice had fewer total Foxp3⁺ Tregs than WT mice (Fig. 5, C), and that they had an even larger decrease in the ICOS⁺ Foxp3⁺ Treg subset (Fig. 5, D). Interactions between ICOS and B7h therefore drive the expansion or survival of ICOS⁺ Foxp3⁺ Tregs in the lungs of mice during allergen exposure.

To investigate the role of ICOS-B7h interactions in 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 airway and had fewer neutrophils than did similarly treated WT mice (Fig. 6, A and B), although AHR was similar in these two strains (Fig. 6, C). The increased IL-17 was an expected result because ICOS has been previously reported to promote the induction of Th17 responses²⁶. However, after one week of daily OVA challenges, IL-5 and IL-17 were now higher in airways of *B7h*^{-/-} mice than in those of WT animals (Fig. 6, D). Eosinophils and neutrophils were also increased in *B7h*^{-/-} mice after multiple OVA challenges (Fig. 6, E), and these animals continued to display AHR, whereas WT mice did not (Fig. 6, F). This finding was confirmed in *Icos*^{-/-} mice (Fig. E4). The sustained AHR seen in *B7h*^{-/-} mice after seven daily OVA challenges did not result from increased numbers of Th17 cells in these animals. In fact, despite their increased levels of extracellular IL-17, lungs of *B7h*^{-/-} mice contained fewer Th17 cells than those of WT mice

(Fig. 6, G). Thus, although interactions between ICOS and B7h contribute to the initial development of Th17 cells, these same molecules appear to suppress established Th17 inflammation in the lung by blocking production of IL-17.

IL-10 and TGF- β are dispensable for suppression of Th17-mediated AHR

IL-10 can attenuate inflammation in a wide variety of settings, including the lung. However, after OVA-LPS sensitization and seven daily OVA challenges, *Il-10*-deficient mice did not display increased airway inflammation, IL-17 or AHR compared with WT mice (Fig. 7, 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, stunted growth and have a very short life span^{29, 30}. We therefore used TGF- β -blocking antibodies to study the function of TGF- β in AHR suppression. Except for an increase in eosinophils, airway inflammation was similar in mice treated with TGF- β -blocking antibodies or with isotype control antibodies (Fig. 7, B). Cytokines, including IL-17, were also similar in the two groups of mice, and the TGF- β -blocking antibodies failed to 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 (Fig. E5). Together, these experiments suggest that the suppression of IL-17 production and AHR seen after multiple challenges of OVA-LPS sensitized mice is not dependent on either IL-10 or TGF- β .

IL-35 production by ICOS⁺ Tregs suppresses Th17-mediated allergic airway disease

IL-35 is a recently described regulatory cytokine whose function 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 seven daily OVA challenges, *Ebi-3*-deficient mice had significantly more macrophages, neutrophils, lymphocytes and eosinophils in the airway than did similarly-treated WT mice (Fig. 7, C). The *Ebi-3*-deficient mice also had higher levels of IL-17, and importantly, continued to display AHR after the 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. 7, 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 developed inflammation and AHR after a single OVA challenge, and this AHR was sustained after seven OVA challenges (Fig. E6). 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. These findings suggest that IL-35 might be selectively produced by ICOS⁺ Tregs. To test this, we measured expression levels of the two IL-35 subunits in ICOS⁺ and ICOS⁻ Tregs prepared from Foxp3-GFP mice. Both subunits were expressed by ICOS⁺ Tregs, whereas neither subunit was expressed to an appreciable extent by ICOS⁻ Tregs (Fig. 8, A). 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 subsets. Thus, IL-35, but not IL-27, is selectively expressed by murine ICOS⁺ Tregs. This prompted us to test if IL-35 is also selectively produced by human ICOS⁺ Tregs. Blood was collected from human volunteers and Tregs were purified using high expression levels of CD25 as a diagnostic marker. These cells were then fractionated into their ICOS⁺ and ICOS⁻ subsets, and mRNA prepared from each. As seen for mouse Tregs, human Tregs that expressed ICOS also expressed both subunits of IL-35 (Fig. 8, B). By contrast, ICOS⁻ Tregs expressed very low levels of these subunits, and neither Treg subset expressed appreciable levels of the IL-27 subunit, p28.

DISCUSSION

In humans, the relative strengths of regulatory and effector responses to inhaled allergens are thought to confer susceptibility to asthma¹⁶. To strengthen regulatory responses in allergic individuals, immunotherapy is sometimes employed by giving them progressively higher doses of provoking allergens over a prolonged period. This method has been successful for treating allergic rhinitis, but less effective for treating allergic asthma^{18, 19}, suggesting that immunotherapy might not suppress all pathologic pathways in asthma. Our current finding that IL-35 can suppress Th17-dependent AHR suggests that strategies targeting the actions of this cytokine might be of particular benefit for suppressing responses associated with allergic asthma. In the model used here, allergic sensitization occurred through the airway, which is in contrast to some other mouse models of asthma where sensitization occurs at extra-pulmonary sites, such as the skin or peritoneum. The latter model promotes strong Th2 responses, but relatively weak Th17 responses. In the mouse, several weeks of daily allergen challenge with allergen are required to suppress these strong responses³¹, although this occurs more quickly in the rat³². Sensitization through the airway using the naturally-occurring environmental agent, LPS, as an adjuvant has been previously shown to prime strong Th17 responses to inhaled OVA¹³. We show here that regulatory responses are also primed by OVA-LPS and that they gain dominance over effector responses. These findings might account for the finding that exposure to LPS in early life can protect against the development of asthma in later life³³.

The suppression of AHR that we observed required interactions between ICOS and its ligand, B7h. Interestingly, some human patients with ICOS deficiency also have reduced levels of Foxp3⁺ Tregs, and display either immunodeficiency or autoimmunity³⁴. Thus, interactions between ICOS and B7h might have a similar impact on Tregs in humans as they do in mice. Paradoxically, although ICOS and B7h promote the early development of Th17 cells²⁶, *B7h*^{-/-} mice had higher levels of IL-17 after prolonged OVA challenge than did WT mice. Thus, the molecules that promote the development of Th17 cells also suppress their activity in the setting of established inflammation. This finding is reminiscent of a recent report showing that Stat3, which is required for development of Th17 responses, is also required by Tregs for the regulation of those responses³⁵. Additional work will be required to fully understand the relationships between Stat3, ICOS and other molecules that control the development and regulation of Th17 cells.

No single effector molecule has emerged from studies of Treg-mediated suppression of airways disease³⁶. In general, IL-10 has a protective function in airway disease when the effector and suppressor cells develop at extra-pulmonary sites²². 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 Th17 cells are insensitive to IL-10 in vitro²⁴. Similarly, TGF- β -specific inhibitors or blocking antibodies also failed to 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 that the ability of splenocytes from *Ebi-3*^{-/-} mice produce elevated levels of IL-17 and IL-22³⁷ and the observation that IL-35 can suppress IL-17 production and attenuate established collagen-induced arthritis in mice³⁸. 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 mouse ICOS⁺ Foxp3⁺ Tregs than are *Ebi-3* or p35, and in human Tregs, it was not detected, 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 Th17 responses once they have already developed³⁹. 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⁺ Tregs. Based on the novel findings presented here, we suggest that targeting the ICOS/IL-35 axis might be of benefit to individuals with established AHR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AHR	airway hyperresponsiveness
Ebi-3	Epstein-Barr virus-induced gene
Foxp3	Forkhead box P3
GFP	green fluorescent protein
ICOS	inducible co-stimulator
ICOS-L	inducible co-stimulator ligand
LPS	lipopolysaccharide
OVA	ovalbumin
TGF-β	transforming factor- β
Treg	T regulatory cell

References

1. Herrick CA, Bottomly K. To respond or not to respond: T cells in allergic asthma. *Nat Rev Immunol.* 2003; 3:405–12. [PubMed: 12766762]
2. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol.* 2003; 111:450–63. quiz 64. [PubMed: 12642820]
3. Kips JC, O'Connor BJ, Langley SJ, Woodcock A, Kerstjens HA, Postma DS, et al. Effect of SCH55700, a humanized anti-human interleukin-5 antibody, in severe persistent asthma: a pilot study. *Am J Respir Crit Care Med.* 2003; 167:1655–9. [PubMed: 12649124]
4. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet.* 2000; 356:2144–8. [PubMed: 11191542]
5. O'Byrne PM. The demise of anti IL-5 for asthma, or not. *Am J Respir Crit Care Med.* 2007; 176:1059–60. [PubMed: 18042659]
6. Barczyk A, Pierzchała W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med.* 2003; 97:726–33. [PubMed: 12814161]
7. Bullens DM, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, et al. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res.* 2006; 7:135. [PubMed: 17083726]

8. Hellings PW, Kasran A, Liu Z, Vandekerckhove P, Wuyts A, Overbergh L, et al. Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol Biol*. 2003; 28:42–50. [PubMed: 12495931]
9. Kaminska M, Foley S, Maghni K, Storness-Bliss C, Coxson H, Ghezzi H, et al. Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction. *J Allergy Clin Immunol*. 2009; 124:45–51. e1–4. [PubMed: 19481790]
10. McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J Immunol*. 2008; 181:4089–97. [PubMed: 18768865]
11. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol*. 2001; 108:430–8. [PubMed: 11544464]
12. Song C, Luo L, Lei Z, Li B, Liang Z, Liu G, et al. IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol*. 2008; 181:6117–24. [PubMed: 18941201]
13. Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN. Allergic Sensitization Through the Airway Primes Th17-dependent Neutrophilia and Airway Hyperresponsiveness. *Am J Respir Crit Care Med*. 2009
14. Wakashin H, Hirose K, Maezawa Y, Kagami S, Suto A, Watanabe N, et al. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am J Respir Crit Care Med*. 2008; 178:1023–32. [PubMed: 18787221]
15. Barnes PJ. New therapies for asthma: is there any progress? *Trends Pharmacol Sci*. 31:335–43. [PubMed: 20554041]
16. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Cramer R, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med*. 2004; 199:1567–75. [PubMed: 15173208]
17. Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. *Immunity*. 2009; 31:438–49. [PubMed: 19766086]
18. Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med*. 1999; 341:468–75. [PubMed: 10441602]
19. Jacobsen L, Valovirta E. How strong is the evidence that immunotherapy in children prevents the progression of allergy and asthma? *Curr Opin Allergy Clin Immunol*. 2007; 7:556–60. [PubMed: 17989534]
20. Joetham A, Takeda K, Taube C, Miyahara N, Matsubara S, Koya T, et al. Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. *J Immunol*. 2007; 178:1433–42. [PubMed: 17237391]
21. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med*. 2005; 202:1539–47. [PubMed: 16314435]
22. Oh JW, Seroogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, et al. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J Allergy Clin Immunol*. 2002; 110:460–8. [PubMed: 12209095]
23. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*. 2008; 28:546–58. [PubMed: 18387831]
24. Naundorf S, Schroder M, Hoflich C, Suman N, Volk HD, Grutz G. IL-10 interferes directly with TCR-induced IFN-gamma but not IL-17 production in memory T cells. *Eur J Immunol*. 2009; 39:1066–77. [PubMed: 19266486]
25. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005; 6:1123–32. [PubMed: 16200070]
26. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*. 2005; 6:1133–41. [PubMed: 16200068]

27. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 2007; 450:566–9. [PubMed: 18033300]
28. Ito T, Hanabuchi S, Wang YH, Park WR, Arima K, Bover L, et al. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity*. 2008; 28:870–80. [PubMed: 18513999]
29. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A*. 1993; 90:770–4. [PubMed: 8421714]
30. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature*. 1992; 359:693–9. [PubMed: 1436033]
31. Hollingsworth JW, Whitehead GS, Lin KL, Nakano H, Gunn MD, Schwartz DA, et al. TLR4 signaling attenuates ongoing allergic inflammation. *J Immunol*. 2006; 176:5856–62. [PubMed: 16670292]
32. Strickland DH, Stumbles PA, Zosky GR, Subrata LS, Thomas JA, Turner DJ, et al. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *J Exp Med*. 2006; 203:2649–60. [PubMed: 17088431]
33. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med*. 2002; 347:869–77. [PubMed: 12239255]
34. Takahashi N, Matsumoto K, Saito H, Nanki T, Miyasaka N, Kobata T, et al. Impaired CD4 and CD8 effector function and decreased memory T cell populations in ICOS-deficient patients. *J Immunol*. 2009; 182:5515–27. [PubMed: 19380800]
35. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, et al. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science*. 2009; 326:986–91. [PubMed: 19797626]
36. Ray A, Khare A, Krishnamoorthy N, Qi Z, Ray P. Regulatory T cells in many flavors control asthma. *Mucosal Immunol*. 2010; 3:216–29. [PubMed: 20164832]
37. Yang J, Yang M, Htut TM, Ouyang X, Hanidu A, Li X, et al. Epstein-Barr virus-induced gene 3 negatively regulates IL-17, IL-22 and RORgamma t. *Eur J Immunol*. 2008; 38:1204–14. [PubMed: 18412165]
38. Niedbala W, Wei XQ, Cai B, Hueber AJ, Leung BP, McInnes IB, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol*. 2007; 37:3021–9. [PubMed: 17874423]
39. El-behi M, Ciric B, Yu S, Zhang GX, Fitzgerald DC, Rostami A. Differential effect of IL-27 on developing versus committed Th17 cells. *J Immunol*. 2009; 183:4957–67. [PubMed: 19786534]

Key Messages

- Allergic airway inflammation and IL-17-dependent AHR are suppressed by prolonged exposures to aerosolized allergen
- This suppression is reversible and requires IL-35 production by ICOS⁺ Tregs
- The production of IL-35 by human ICOS⁺ Tregs suggests that targeting this pathway might be of therapeutic benefit for treating allergic asthma

Capsule summary

IL-35 is selectively produced by ICOS⁺ regulatory T cells and reversibly suppresses allergic inflammation and IL-17-dependent airway hyperresponsiveness. Strategies that target this pathway might therefore be of therapeutic benefit to treat exacerbations of allergic asthma.

FIG 1. Prolonged allergen challenge suppresses allergic responses. **A**, Timeline. **B**, AHR in sensitized (sens.) mice after various challenges (#chall.). **C**, Leukocytes $\pm$ S.E.M. in BALF. **D**, Mean cytokine levels $\pm$ S.E.M. in BALF. **E**, Levels of IL-13 in homogenized lungs. (n = 9 mice/group). * P < 0.05, ** P < 0.05. Data represent one of three similar experiments.

FIG 2.

Exogenous IL-17 restores AHR. OVA-LPS sensitized mice were challenged daily for one week with either OVA or PBS. Two h after the final OVA challenge, the indicated mice were instilled with IL-17. **A**, AHR. **B**, Airway inflammation. Mean values $\pm$ S.E.M. are shown. * $P < 0.05$. (n = 8 mice/group). Data represent one of two similar experiments.

FIG 3.

Suppression of AHR is not due to loss of Th17 cells. **A**, Flow cytometry plots showing lymphocyte gating and intracellular IL-17 staining. **B,C**, Percentage and number of Th17 cells in the lung. **D**, Intracellular levels of IL-17. Mean values $\pm$ S.E.M. are shown. * $P < 0.05$. (n = 5 mice/group). Data represent one of three similar experiments.

FIG 4.

Foxp3⁺ Tregs suppress Th-17-dependent allergic responses. **A**, Staining of IL-10 and Foxp3 in CD4⁺ T cells. **B**, Foxp3⁺ Tregs in sensitized mice after the indicated number of challenges and rest days (in parentheses). **C,D**, Foxp3⁺ Tregs in DTX-treated WT and DTR mice. **E**, Inflammation, and **F**, AHR, in challenged, Treg-depleted mice. **G**, Dose-dependent Treg suppression of IL-17 secretion in vitro. * *P* < 0.05.

FIG 5.

Suppression of AHR associates with increased ICOS⁺ Tregs. **A**, Staining of FoxP3⁺ Tregs with isotype control antibodies (shaded region), or anti-ICOS antibodies after a single (dashed line) or a 7-day challenge (solid line). **B**, Ratio of ICOS⁺ to ICOS⁻ Tregs after the indicated number of challenges and rest days (parentheses). **C**, Total Tregs and **D**, ICOS⁺ Tregs in *B7h*-deficient mice.

FIG 6. ICOS-B7h interactions suppress AHR. **A**, Cytokines, **B**, leukocytes and **C**, AHR in WT (+/+) and *B7h*-deficient (-/-) mice after a 7-day OVA challenge. **D - F**, Responses of the same strains to a single OVA challenge. **G**, Th17 cells in WT and *B7h*-deficient mice. * $P < 0.05$.

FIG 7.

Suppression of AHR is independent of IL-10 and TGF- β , but dependent on IL-35. **A**, Allergic responses of IL-10-deficient mice. **B**, Allergic responses of WT mice treated with anti-TGF- β (TGF) or isotype control (IC) antibodies. **C**, Allergic responses of Ebi-3^{-/-} mice to a 7-day challenge, or **D**, to a single challenge. * $P < 0.05$. (n = 8 mice/group).

FIG 8.

IL-35 is selectively produced by ICOS⁺ Tregs. Quantitative PCR was used to measure mRNA levels of the IL-35 subunits after flow cytometric sorting for ICOS⁺ and ICOS⁻ Treg subsets. **A**, Tregs from Foxp3-gfp mice (n = 6). **B**, CD25^{hi} Tregs from human blood (n = 6). * p < 0.05; ** p < 0.01.