

Jennifer T. Burchell, Matthew E. Wikstrom, Philip A. Stumbles, Peter D. Sly and Debra J. Turner

Am J Physiol Lung Cell Mol Physiol 296:307-319, 2009. First published Nov 21, 2008;
doi:10.1152/ajplung.00521.2007

You might find this additional information useful...

This article cites 66 articles, 36 of which you can access free at:

<http://ajplung.physiology.org/cgi/content/full/296/3/L307#BIBL>

Updated information and services including high-resolution figures, can be found at:

<http://ajplung.physiology.org/cgi/content/full/296/3/L307>

Additional material and information about *AJP - Lung Cellular and Molecular Physiology* can be found at:

<http://www.the-aps.org/publications/ajplung>

This information is current as of September 27, 2009 .

Attenuation of allergen-induced airway hyperresponsiveness is mediated by airway regulatory T cells

Jennifer T. Burchell,^{1,3} Matthew E. Wikstrom,^{2,3} Philip A. Stumbles,^{2,3,4} Peter D. Sly,^{1,3} and Debra J. Turner^{1,3}

Divisions of ¹Clinical Sciences and ²Cell Biology, Telethon Institute for Child Health Research, Subiaco; ³Centre for Child Health Research, University of Western Australia, Nedlands; and ⁴Division of Health Sciences, Murdoch University, Murdoch, Western Australia

Submitted 16 December 2007; accepted in final form 12 November 2008

Burchell JT, Wikstrom ME, Stumbles PA, Sly PD, Turner DJ. Attenuation of allergen-induced airway hyperresponsiveness is mediated by airway regulatory T cells. *Am J Physiol Lung Cell Mol Physiol* 296: L307–L319, 2009. First published November 21, 2008; doi:10.1152/ajplung.00521.2007.—Understanding the mechanisms involved in respiratory tolerance to inhaled allergens could potentially result in improved therapies for asthma and allergic diseases. Airway hyperresponsiveness (AHR) is a major feature of allergic asthma, thus the aim of the current study was to investigate mechanisms underlying suppression of allergen-induced AHR during chronic allergen exposure. Adult BALB/c mice were systemically sensitized with ovalbumin (OVA) in adjuvant and then challenged with a single 3 or 6 wk of OVA aerosols. Airway and parenchymal responses to inhaled methacholine (MCh), inflammatory cell counts, cytokines, OVA-specific IgE and IgG₁, parenchymal histology, and numbers of airway CD4⁺69⁺ activated and CD4⁺25⁺FoxP3⁺ regulatory T (Treg) cells were assessed 24 h after the final aerosol. Single OVA challenge resulted in AHR, eosinophilia, increased serum OVA-specific IgE, and T helper 2 (Th2) cytokines in bronchoalveolar lavage (BAL) but no difference in numbers of Treg compared with control mice. Three weeks of OVA challenges resulted in suppression of AHR and greater numbers of airway Treg cells and increased transforming growth factor- β_1 (TGF β_1) compared with control mice despite the presence of increased eosinophilia, OVA-specific IgE and IgG₁, and airway remodeling. Six weeks of OVA challenges restored AHR, whereas airway Treg numbers, TGF β_1 , BAL eosinophilia, and Th2 cytokines returned to control levels. Partial in vivo depletion or adoptive transfer of Treg cells restored or inhibited AHR, respectively, but did not affect TGF β_1 or Th2 cytokine production. In conclusion, AHR suppression is mediated by airway Treg cells and potentially via a paracrine induction of TGF β_1 in the airways.

asthma; T regulatory cell; lung function

THE IMMUNE SYSTEM IS CONTINUALLY exposed to a vast array of antigens, mostly via the skin and mucosal epithelium of the respiratory and gastrointestinal tracts. How the immune system differs in response to inhaled allergen between allergic asthmatic and healthy individuals is an ongoing question. In allergic individuals, innocuous inhaled antigens can provoke a T helper 2 (Th2)-biased immunological response that may result in a number of allergic features including airway hyperresponsiveness (AHR), airway eosinophilic inflammation, antigen-specific IgE production, and, in more chronic cases, airway remodeling (10, 19, 53).

AHR is one of the primary features of allergic airways disease. Despite continued allergen exposure, AHR does not become progressively worse in atopic asthmatic individuals. The mechanisms limiting AHR are unknown. Many previous studies examining allergen-induced inhalational tolerance have focused on the development of peripheral tolerance, defined as suppression of antigen-specific serum antibodies. One such study has shown that inhalation tolerance to ovalbumin (OVA) in rats is associated with suppression of antigen-specific IgE (33). However, less is known of physiological tolerance to inhaled allergens, defined as allergen-induced suppression of AHR. Understanding the mechanism of allergen-induced AHR suppression in animal models could produce new and more targeted asthma therapies.

It is now widely accepted that CD4⁺ T cells play a major role in the development of chronic asthma and allergic airways disease (41). Early studies in mice and rats have demonstrated that AHR can be adoptively transferred by CD4⁺ T cells (17, 26, 48). Inhibition of the late asthmatic reaction (LAR) after allergen challenge in atopic patients by cyclosporin A, a drug that targets T cell activation, has supported the role of T cells in the human LAR (55). More recently, Ali and colleagues (2) have demonstrated that the development of AHR in cat allergic patients after inhalation of *Felis domesticus* (Fel d1) peptides is associated with increased numbers of CD4⁺ T cells in the airways. In addition, depletion of CD4⁺ cells using a monoclonal antibody can abrogate an allergic response in sensitized animals, including AHR (32).

The presence of T cell subsets with the ability to suppress various disease states to maintain peripheral tolerance has been demonstrated for a number of years (5, 6). Regulatory T cells (Treg) have been implicated in the suppression of a number of diseases including asthma (25). In particular, naturally occurring Treg delineated as CD4⁺25⁺ were originally described by Sakaguchi et al. (52) and shown to be critical in the inhibition of a wide range of autoimmune diseases. In recent years, a small number of studies have demonstrated a direct link between Treg and suppression of AHR in asthma (35, 38, 56). However, only one of these studies (56) used a physiological technique, low-frequency forced oscillation (31, 51), to partition lung function into airway and parenchymal components in a rat model of allergic airways disease. As such, there are no data available for associations between Treg and AHR in the mouse using accurate measures of lung function to determine AHR.

Address for reprint requests and other correspondence: D. J. Turner, Telethon Institute for Child Health Research, P.O. Box 855, West Perth 6872, Australia (e-mail: debrat@icmr.uwa.edu.au).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The mechanism of Treg-mediated suppression of AHR is currently unknown, however, the immunomodulatory cytokine transforming growth factor- β_1 (TGF β_1) has been ascribed a role in this process. The role of TGF β in immune suppression is not clearly defined. Recent studies have proposed a role for both Treg cells and TGF β in attenuating the allergic response to allergen (1, 16, 22, 30, 35, 43, 46). Allergen-induced Treg are known to produce TGF β_1 in vitro (29). Administration of TGF β can convert CD4⁺25⁻ cells into CD4⁺25⁺ cells in the periphery (11). Neutralization by soluble TGF β receptor in an in vivo murine model has been shown to potentiate the development of AHR (1).

The aim of the current study was to investigate, in a conventional murine model of allergen-induced AHR, the mechanisms involved in suppression of allergen-induced AHR during chronic allergen exposure. More specifically, the aim was to suppress allergen-induced AHR and then to correlate suppression with associated allergic features, including Treg numbers in the trachea of BALB/c mice. To elucidate the role of Treg in AHR suppression, Treg cells were partially depleted or adoptively transferred into recipient-sensitized and OVA-challenged mice. Lung function and methacholine (MCh) responsiveness were measured with the low-frequency forced oscillation technique (31, 51) capable of providing separate estimates of the responsiveness of airway and lung parenchyma. To investigate the potential mechanism of Treg-mediated AHR suppression, TGF β_1 , IL-10, and Th2 cytokines were measured in BAL fluid (BALF) after OVA challenge, CD25 depletion, and Treg adoptive transfer.

MATERIALS AND METHODS

Animals. Eight-week-old female BALB/c mice were purchased from the Animal Resource Center (ARC), Murdoch, Western Australia. Mice were free of known pathogens, housed in a clean animal house environment with a 12:12-h light-dark cycle, and had access to food and acidified water ad libitum. The Telethon Institute for Child Health Research Animal Ethics and Experimentation Committee approved all study protocols, which were conducted in accordance with the National Health and Medical Research Council of Australia guidelines for animal experimentation.

Sensitization and challenge protocol. Mice were sensitized intraperitoneally with 20 μ g of OVA (chicken-egg OVA grade V; Sigma, St. Louis, MO) in 200 μ l of aluminum hydroxide (Alu-Gel-S; Serva, Heidelberg, Germany) on days 0 and 14. Commencing on day 21, mice were challenged with a single OVA aerosol (1% solution in sterile saline) or multiple aerosols delivered 3 times per week for 3 or 6 wk. Aerosols were delivered for 30 min to 8 mice per group in a custom-designed 12-compartment Perspex chamber via an ultrasonic nebulizer (DeVilbiss UltraNeb; Sunrise Medical, Somerset, PA). Control mice were sensitized to OVA and received saline aerosols using an identical protocol. As there were no differences between outcomes from the three saline control groups (single and 3 and 6 wk), only the single-challenged saline control group data are shown. All measurements described in this study were performed 24 h after the final aerosol.

Animal preparation for lung function. Animals were anesthetized by intraperitoneal injection containing xylazine (2 mg/ml; Troy Laboratories, New South Wales, Australia) and ketamine (40 mg/ml; Troy Laboratories) at a dose of 0.1 ml/10 g body wt. Following tracheostomy, a 10-mm polyethylene tube (1.27 mm outer diameter; 0.86 mm inner diameter; Microtube Extrusions, New South Wales, Australia) was inserted into the trachea, and mice were ventilated at 450 breaths/min with a tidal volume of 8 ml/kg and a positive end-

expiratory pressure (PEEP) of 2 cmH₂O (flexiVent; SCIREQ, Montréal, Québec, Canada). Once on the ventilator, volume history was established by giving the animals 2 sighs (of twice the tidal volume) 5 min apart, followed by 3 pressure-volume maneuvers to 20 cmH₂O.

Measurements of respiratory mechanics using the forced oscillation technique. Respiratory mechanics were measured using an adaptation of the low-frequency forced oscillation technique (LFOT). Input impedance of the lung (Zrs) was measured between 0.25 and 19.625 Hz by applying a composite 16-s signal containing 19 mutually prime sinusoidal waves with amplitude of 50% of tidal volume (i.e., 4 ml/kg) during pauses in regular ventilation (51). The flexiVent calibration procedure removes the impedance of the tracheal cannula. A parameter-estimation model, the constant phase model (CPM) described by Hantos and colleagues (31), was used to partition Zrs into components representing the mechanical properties of the airways, i.e., frequency-independent airway resistance (Raw), and lung parenchyma, i.e., coefficients of tissue damping (G) and tissue elastance (H). Following removal of the tracheal cannula, airway inertance was negligible and is not reported. Individual frequency points were manually excluded from the model fit where they coincided with heart rate and its harmonics if cardiac activity caused low signal-to-noise ratio, i.e., a coherence <0.95.

Measurements of lung function were made every minute for 5 min to establish a baseline and then again following a saline nebulization and after each dose of MCh as outlined below.

Responses to MCh. Mice received a saline aerosol followed by increasing doses of aerosolized MCh from 0.1 to 30 mg/ml as described previously (66). Aerosol durations were 90 s. Lung function was measured every minute for 5 min after the conclusion of each aerosol. Dose-response curves were constructed from the maximal response per dose of MCh averaged for 8 mice per group and expressed as a percentage change over the saline baseline for all parameters. Reactivity to MCh was assessed by calculating the effective dose of MCh producing a 200% increase (EC₂₀₀) in Raw, G, and H above the control.

Quantification of airway inflammatory cells and cytokines. After MCh challenges, mice were lavaged 3 times with 1 ml of saline containing 0.2% BSA (Sigma) and 0.35% lidocaine (Sigma). Between 70% and 80% of the bronchoalveolar lavage (BAL) fluid was routinely recovered. BAL samples were centrifuged, and the supernatant was removed and stored at -80°C for subsequent analysis of cytokines. Total cell counts and viability were determined using a hemocytometer. Data were expressed as the total number of cells retrieved. The remaining cell suspension was centrifuged, and the resulting slides were stained with Leishman stain for determination of a differential cell count. Cells were counted on the basis of their morphology to a total number of 300 cells, generating a percentage of each cell type. This percentage was then multiplied with the total cell count to determine differential cell counts. Airway cytokines (IL-2, -4, -5, IFN- γ , and TNF α) were measured in BAL supernatants using a Cytometric Bead Array (BD Biosciences) as per the manufacturer's instructions. Samples were measured using a four-color FACSCalibur (BD Biosciences) and analyzed using FlowJo software (TreeStar). IL-13, IL-10, and TGF β_1 (R&D Systems, Minneapolis, MN) were measured by ELISA as per the manufacturer's instructions. BAL samples for quantification of TGF β_1 were activated as per manufacturer's instructions before use in the assay.

Lung morphometry. Following MCh challenges and after death, lungs were inflated with paraformaldehyde in situ at 20 cmH₂O for a minimum of 4 h. Lungs were then removed and further fixed in 70% ethanol for at least 24 h, embedded in paraffin wax, section to 4 μ m, and stained with both hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Images were captured using a light microscope (Leica Microsystems, Wetzlar, Germany). To determine structural changes in parenchymal airways, measurements of airway wall thickness and the presence of goblet cells were analyzed. Using a camera lucida and digitizing tablet, perimeter and area of the outer airway

wall and inner airway wall were measured in bronchioles, defined as being small noncartilaginous airways. The outer perimeter was defined as the outer border of adventitia and inner perimeter defined as the luminal border of epithelium. Selection criteria for choosing airways to measure corresponded to those proposed by Bai and colleagues (3). Approximately 16 airways per mouse lung were measured in 5–8 mice per treatment group, resulting in 80–130 measurements per treatment group. To minimize overrepresentation of wall thickness from larger airways, measurements are shown as square millimeters divided by basement membrane length of the airway.

Antibody responses. After conclusion of MCh challenges, blood was collected via cardiac puncture, left to clot, and then centrifuged at 2,000 rpm for 10 min for collection of sera. OVA-specific IgE and IgG₁ were then analyzed using a sandwich ELISA #0 or an indirect ELISA, respectively. For IgE, purified anti-mouse IgE (BD Biosciences) was coated overnight onto 96-well microtiter plates and blocked with 1% BSA in PBS before adding test serum samples or purified anti-mouse OVA IgE (Serotec, Oxford, United Kingdom) as a standard for 1 h at room temperature. OVA-specific IgE was detected using an in-house-generated biotinylated OVA followed by streptavidin-horseradish peroxidase (Sav-HRP; Amersham) and developed with ABTS substrate. For the IgG₁ ELISA, plates were coated overnight with OVA (10 µg/ml) and blocked with 1% BSA in PBS before adding test serum samples or anti-OVA (chicken) mouse monoclonal antibody (Antibody Shop, Gentofte, Denmark) as a standard for 1 h at room temperature. OVA-specific IgG₁ was detected using a biotin-conjugated rat anti-mouse IgG₁ monoclonal antibody (BD Biosciences) followed by Sav-HRP and developed with ABTS substrate. Lower limits of detection were 100 and 10 ng/ml for IgE and IgG₁, respectively. Plates were read on a photometry 96-well plate reader (Victor2; Wallac Oy, Turku, Finland) at an absorbance of 405 nm.

Quantification of airway and lymph node Treg cells. In separate groups of animals, mice were killed 24 h after the final OVA aerosol, and tracheas were removed ($n = 5$ per group). Tracheas were cut into longitudinal sections and digested with type IV collagenase (1.5–2 mg/ml; Worthington Biochemical, Lakewood, NJ) and pancreatic type I DNase (0.1 mg/ml; Sigma). Airway-draining lymph nodes (DLN; composed of parathymic and mediastinal lymph nodes) were removed and digested as described for tracheas. After a single cell suspension was created, Fc receptors were blocked using anti-CD16/32 followed by anti-CD4 conjugated to allophycocyanin (APC), anti-CD69-phycoerythrin-Cy5 (PE-Cy5), biotinylated anti-CD25, and streptavidin-PE (BD Biosciences). Cell suspensions were split in half. Dead cells were detected by adding propidium iodide to half of the cells of each sample. The remaining cells were permeabilized overnight and then stained with anti-forkhead box P3 (FoxP3) conjugated to fluorescein isothiocyanate (24) using a commercially available staining kit (eBioscience, San Diego, CA). All antibodies used were purchased from BD Biosciences unless otherwise stated. Cell populations were detected using an LSR II (Becton-Dickinson) and analyzed using FlowJo software (TreeStar).

Administration of antibody for in vivo CD25 depletion. Two separate groups of mice were sensitized and received 3 wk of OVA aerosols as previously described. In addition, one group received 100 µg of functional grade purified anti-mouse CD25 (PC61.5; eBioscience) intravenously immediately after the seventh aerosol. The second group received 100 µg of functional grade purified mouse IgG₁ isotype control (eBioscience) intravenously immediately after the seventh aerosol. In both groups of animals, the eighth aerosol was delivered 72 h after administration of antibody. Twenty-four hours after the final (8th) aerosol, mice were anesthetized for determination of lung function. After conclusion of lung function measurements, animals were killed and lavaged, and DLN was removed, processed, and stained using anti-CD4 APC, biotinylated anti-CD25 (7D4), streptavidin-PE, and FoxP3-FITC antibodies as previously described.

Purification and adoptive transfer of CD4⁺25[−] and CD4⁺25⁺ cells. Donor BALB/c mice were sensitized and received 3 wk of OVA aerosol exposures. Twenty-four hours after the final aerosol mice were killed, DLN were removed, processed, and stained with anti-CD4 APC, biotinylated anti-CD25, and streptavidin-PE. The cell suspension was sorted into CD4⁺25[−] and CD4⁺25⁺ cells using a FACS Aria cell sorter (BD Biosciences). The two donor cell populations were separately labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen-Molecular Probes, Carlsbad, CA). Recipient BALB/c mice were sensitized and received either 1×10^6 CFSE-labeled CD4⁺25[−] or CD4⁺25⁺ donor cells on day 20 followed by a single OVA aerosol on day 21. Twenty-four hours after the OVA aerosol recipient mice were killed. Tracheas, peripheral lungs, DLN, and inguinal lymph nodes were removed, processed, and stained with anti-CD4 APC, biotinylated anti-CD25, and streptavidin-PE as described previously. CFSE-labeled cells were detected using an LSR II (BD Biosciences) and analyzed with FlowJo (TreeStar).

In a separate but parallel experiment, CD4⁺25[−] and CD4⁺25⁺ cells were sorted as described above but were not labeled with CFSE. Cells were transferred to recipient-sensitized mice on day 20 followed by a single OVA aerosol on day 21 as described above. Twenty-four hours later, the OVA aerosol recipient mice were anesthetized and tracheostomized for analysis of MCh responsiveness. At the conclusion of the experiment, mice were killed and lavaged. To confirm that the transferred CD4⁺25⁺ cells were naturally occurring Treg (FoxP3⁺), a proportion of the donor cells were permeabilized and stained with anti-FoxP3-FITC.

Statistical analysis. Analyses of MCh dose-response curves were performed using two-way repeated measures (RM) ANOVA with statistical significance defined as $P < 0.05$. All other analyses were performed using either one-way ANOVA or unpaired *t*-tests as indicated. Where data were not normally distributed, ANOVA was performed on ranks (SigmaStat). Values for all figures are expressed as means $\pm$ SD unless otherwise stated.

RESULTS

Altered lung function with varying durations of airway allergen exposure. Baseline lung function and EC₂₀₀ values to MCh are shown for each treatment group in Table 1. Baseline values of Raw were unaltered by single or multiple allergen challenges. However, baseline measurements of G and H were significantly increased after 3 wk of allergen challenge ($P < 0.05$, 1-way ANOVA on ranks) and returned to control levels after 6 wk of allergen challenge.

Dose-response curves to inhaled MCh are shown in Fig. 1 for Raw (Fig. 1A), G (Fig. 1B), and H (Fig. 1C). Mice exposed to a single OVA challenge showed a significant increase in responsiveness to MCh in both the main conducting airways (Raw; $P < 0.05$, 2-way RM ANOVA) and lung parenchyma (G and H; $P < 0.05$) compared with control animals (Fig. 1), consistent with data previously described (66). EC₂₀₀ values were significantly lower for G and H ($P < 0.05$; Table 1) following a single OVA aerosol compared with the control group.

In contrast, 3 wk of allergen challenge attenuated the increased responsiveness to MCh in Raw, G, and H observed after a single allergen challenge ($P < 0.05$; Fig. 1) to the point where there were no significant differences between 3-wk-challenged and control mice. In addition, response to MCh revealed a significant increase in EC₂₀₀Raw between single and 3-wk groups ($P < 0.05$; Table 1). EC₂₀₀G and EC₂₀₀H did not differ between 3-wk-exposed animals and controls (Table 1).

Table 1. Baseline lung function and the effective concentration of methacholine causing a 200% increase in baseline values (EC_{200}) for airway resistance (Raw), tissue damping (G), and tissue elastance (H)

	Raw		G		H	
	Baseline, hPa/ml	EC_{200} , mg/ml	Baseline, hPa/ml	EC_{200} , mg/ml	Baseline, hPa/ml	EC_{200} , mg/ml
Control	0.23 (0.01)	8.89 (5.27)	5.76 (0.33)	3.74 (3.89)	38.05 (2.37)	26.79 (3.21)
Single	0.22 (0.01)	1.85 (0.50)*	5.61 (0.09)	3.21 (0.57)*	37.05 (0.71)	4.05 (0.91)*
3 wk	0.24 (0.03)	6.31 (1.97)†	8.31 (0.35)*†	15.87 (5.25)†	48.54 (2.68)*†	20.99 (5.70)
6 wk	0.21 (0.01)	4.64 (1.34)	5.88 (0.27)‡	4.65 (10.13)*	34.77 (10.16)‡	5.40 (10.37)*‡

Values are expressed as means (SD), $n = 6-8$ mice per group. Measurements were made 24 h following a saline aerosol (Control), a single ovalbumin (OVA) aerosol (Single), and 3 and 6 wk of OVA aerosols. * $P < 0.05$ compared with control group; † $P < 0.05$ compared with single OVA aerosol-exposed group; and ‡ $P < 0.05$ compared with 3-wk OVA aerosol-exposed group.

Continuation of OVA challenges for a further 3 wk (6 wk in total) reversed the suppression of AHR observed in 3-wk-challenged mice (Fig. 1, A–C). After 6 wk of allergen challenge, mice again showed significantly increased responsiveness to MCh compared with the control group for Raw, G, and H ($P < 0.05$) and compared with 3-wk allergen-challenged mice for G and H ($P < 0.05$). There were no differences between single and 6-wk-challenged mice for Raw, G, or H. EC_{200} values were decreased in 6-wk-challenged mice compared with control mice for both G and H and for H alone compared with 3-wk-challenged mice (Table 1; $P < 0.05$).

Airway and lung inflammation and airway wall remodeling. Total cell numbers increased in BAL following a single aerosol challenge ($P < 0.001$) and markedly increased ($P = 0.001$) after 3 wk of allergen exposures (Fig. 1D; nonparametric t -test on ranks). The increase in cells was primarily due to an increase in eosinophils that paralleled the trend observed in total cell numbers (Fig. 1D). Macrophage numbers remained relatively conserved between all groups. Mice receiving 6 wk of allergen challenges demonstrated a decrease in total cells within the airways compared with 3-wk-challenged mice ($P < 0.001$, unpaired t -test) yet still remained elevated above control levels ($P < 0.05$).

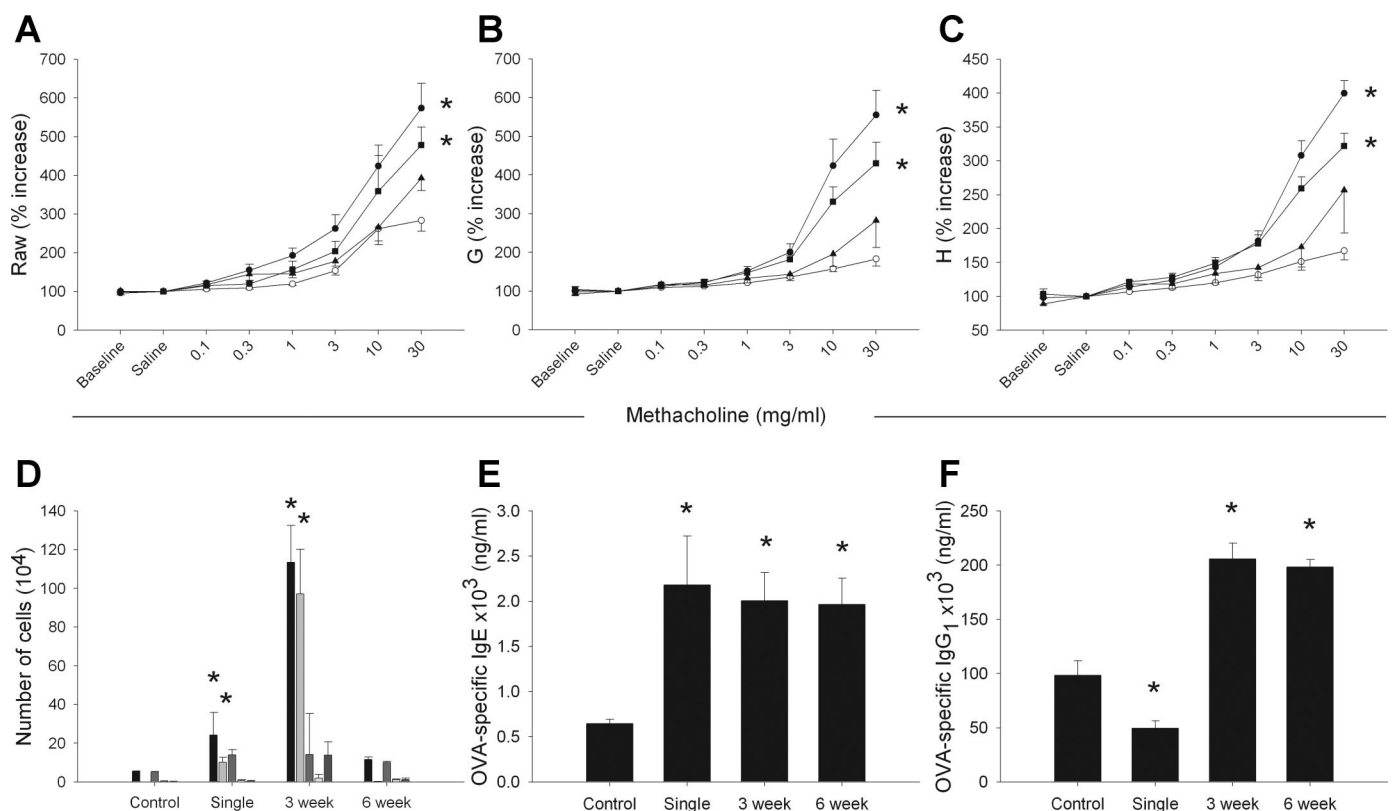


Fig. 1. Lung function analysis, inflammation, and allergic antibody production of ovalbumin (OVA) aerosol-exposed mice. OVA-sensitized BALB/c mice were exposed to saline (control: open circles) or single (closed circles), 3 wk (closed triangles), or 6 wk (closed squares) of OVA aerosols (A–C). Lung function was measured by forced oscillation technique 24 h after the last aerosol. Changes in responsiveness to methacholine (MCh) for airway resistance (Raw; A), tissue damping (G; B), and tissue elastance (H; C) compared with control animals were determined as described in MATERIALS AND METHODS. Data are expressed as a percentage of the baseline saline values and shown as means $\pm$ SD for each group ($n = 8$ mice per group). * $P < 0.001$, 2-way repeated measures ANOVA. Bronchoalveolar lavage (BAL) fluid (BALF) cell counts are shown in D for (from left to right) total cells (black bars), eosinophils (light gray bars), macrophages (medium gray bars), neutrophils (white bars), and lymphocytes (dark gray bars) and are expressed as the total number of cells retrieved per group ($n = 8$ mice per group). OVA-specific IgE and IgG1 measured in serum are shown in E and F, respectively, and data are expressed as mean antibody concentration with SD error bars. * $P < 0.05$ compared with control group.

Similarly, in the lung parenchyma, no inflammatory cells were observed in control mouse lungs (Fig. 2A), whereas small foci of eosinophils and a lesser extent lymphocytes were present around and within the airway walls after a single

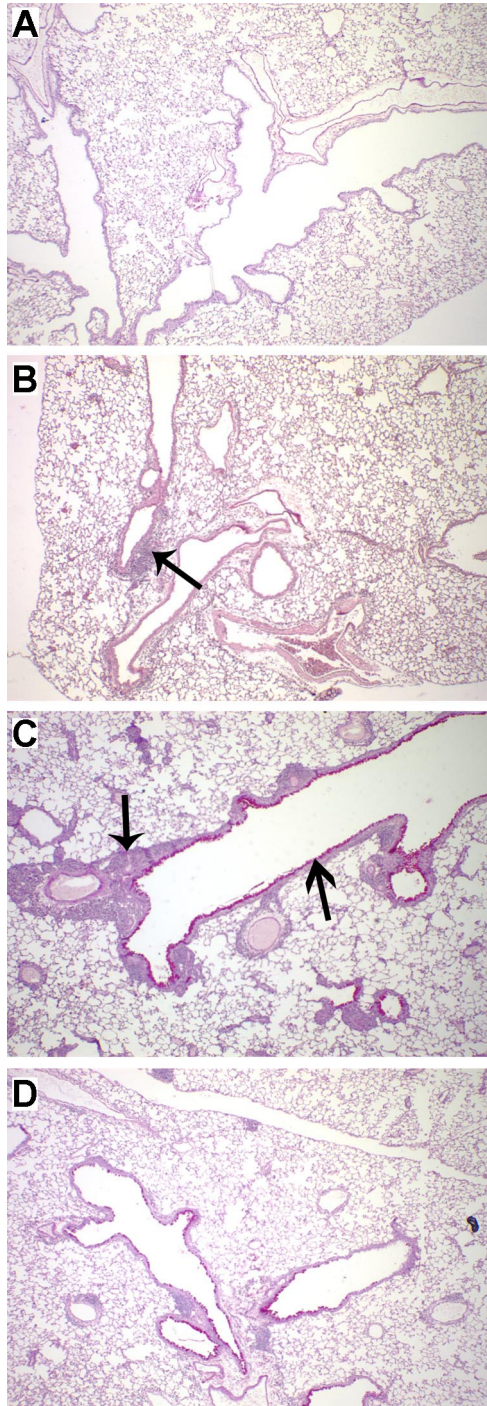


Fig. 2. Airway and lung histology following OVA aerosol exposure. OVA-sensitized BALB/c mice were exposed to saline aerosol (A) or single OVA (B), 3-wk OVA (C), or 6-wk OVA (D) aerosol challenges. Lung tissue were harvested and fixed 24 h after the final aerosol. Representative sections of lungs in each treatment group ($n = 8$ mice per group) are shown stained with both hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) and viewed using light microscopy (original $\times 4$ magnification). Block arrowheads indicate regions of peribronchiolar (B and C) and perivascular (C) inflammation. The open arrowhead indicates a region of goblet cell metaplasia (C).

allergen challenge (Fig. 2B, arrow shows inflammation). However, this predominantly eosinophilic inflammation was greatly enhanced after 3-wk allergen challenges in both peribronchiolar and perivascular regions (Fig. 2C, arrows show inflammation). Six-week allergen challenge decreased inflammation in the parenchyma (Fig. 2D). In addition, few goblet cells were present within the airway epithelium after a single allergen challenge (Fig. 2B), whereas, after 3 wk of allergen challenges, goblet cells formed an almost-continuous lining within the airway epithelium (Fig. 2C, open arrowhead shows goblet cells). Six weeks of allergen challenges reduced the presence of goblet cells within the airway epithelium (Fig. 2D).

Airway wall thickness was unaltered following a single allergen challenge ($0.022 \pm 1.77 \times 10^{-3} \text{ mm}^2$) and increased after 3-wk allergen exposure ($0.040 \pm 3.62 \times 10^{-3} \text{ mm}^2$) compared with control animals ($0.018 \pm 5.97 \times 10^{-4} \text{ mm}^2$) ($P < 0.05$, 1-way ANOVA on ranks). Airway wall thickness decreased after 6 wk of allergen exposure ($0.032 \pm 2.15 \times 10^{-3} \text{ mm}^2$) compared with 3 wk of allergen challenge but remained elevated compared with control animals ($P < 0.05$). Thus a single allergen challenge resulted in inflamed central and parenchymal airways, which worsened following 3 wk of allergen challenges with the addition of goblet cell metaplasia, which contributed to thickened airway walls. In contrast, 6 wk of allergen challenges partially reversed these inflammatory and structural changes in both the central and parenchymal airways.

Allergen-specific IgE and IgG₁ production. Serum levels of OVA-specific IgE were significantly increased following single ($2.18 \times 10^3 \pm 0.54 \text{ ng/ml}$; $P = 0.01$, unpaired t -test), 3 wk ($2.00 \times 10^3 \pm 0.31 \times 10^3 \text{ ng/ml}$; $P = 0.006$), and 6 wk ($1.96 \times 10^3 \pm 0.29$; $P = 0.024$) of OVA challenges compared with control animals ($0.64 \times 10^3 \pm 0.05 \text{ ng/ml}$) (Fig. 1E). Serum OVA-specific IgG₁ was significantly decreased after a single OVA aerosol ($49.37 \times 10^3 \pm 18.82 \times 10^3 \text{ ng/ml}$; $P = 0.013$, unpaired t -test) and increased following 3 wk ($205.75 \times 10^3 \pm 31.98 \times 10^3 \text{ ng/ml}$; $P = 0.017$) and 6 wk of allergen challenge ($198.33 \times 10^3 \pm 6.84 \text{ ng/ml}$; $P = 0.025$) compared with control animals ($98.45 \times 10^3 \pm 13.24 \text{ ng/ml}$) (Fig. 1F).

Airway cytokine production. Levels of IL-4, IL-5, and IL-13 were significantly increased (unpaired t -tests, $P = 0.001$ for all) in BAL in the single OVA-challenged group compared with the control group and then reduced to control levels in both the 3- and 6-wk OVA-challenged groups (Fig. 3). TNF α followed a similar trend to IL-4 and IL-5, with a significant increase after a single aerosol exposure ($P = 0.022$) and decline following repeated exposure; however, levels of this cytokine remained elevated above control levels during prolonged exposure (Fig. 3). Levels of IL-2 were unchanged between control and single-challenged groups but were significantly decreased after 3- and 6-wk allergen exposure ($P = 0.016$ and $P < 0.001$, respectively; Fig. 3). IFN- γ levels were undetectable in BAL samples from all groups (data not shown). The immunomodulatory cytokines IL-10 and TGF β ₁ were also measured in BALF. IL-10 was undetectable in all samples (data not shown). TGF β ₁ was increased after a single OVA challenge ($P = 0.001$) and further increased after 3 wk of OVA challenges ($P < 0.001$) compared with control animals (Fig. 3).

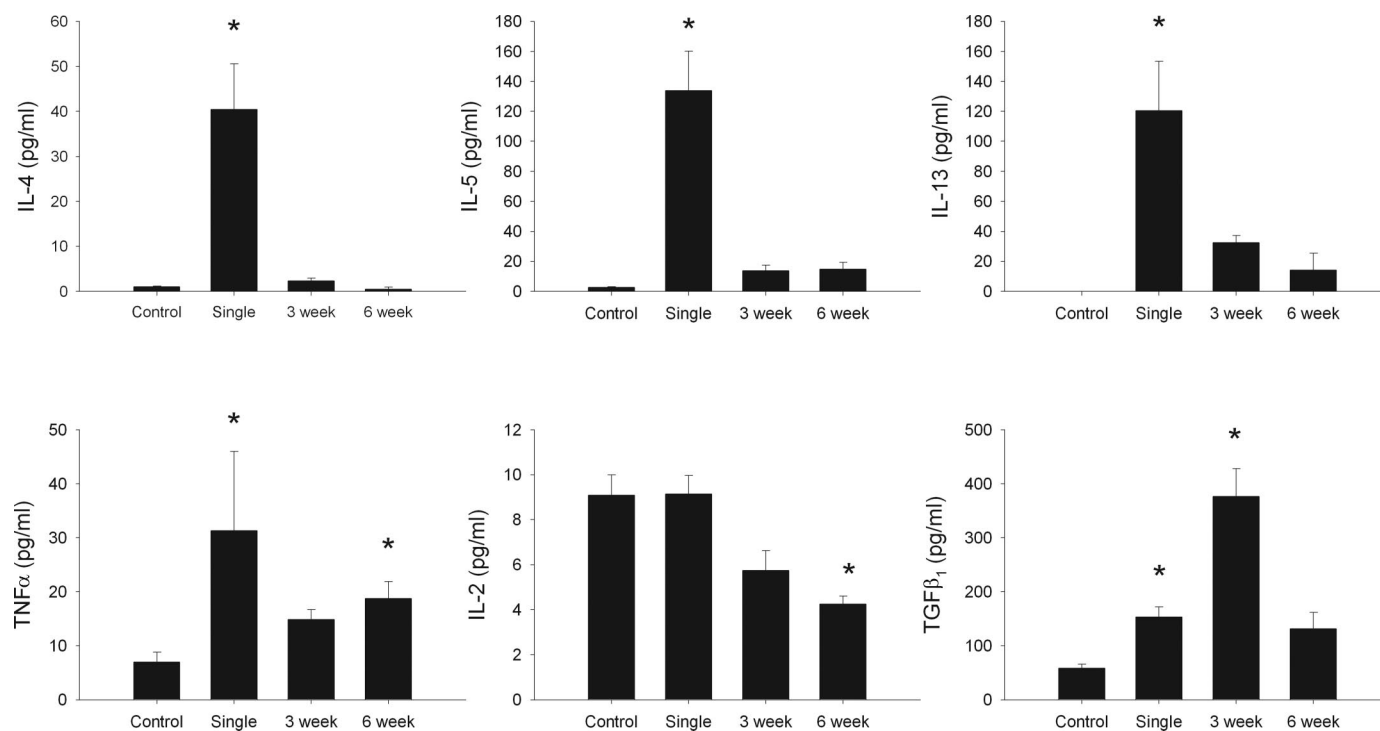
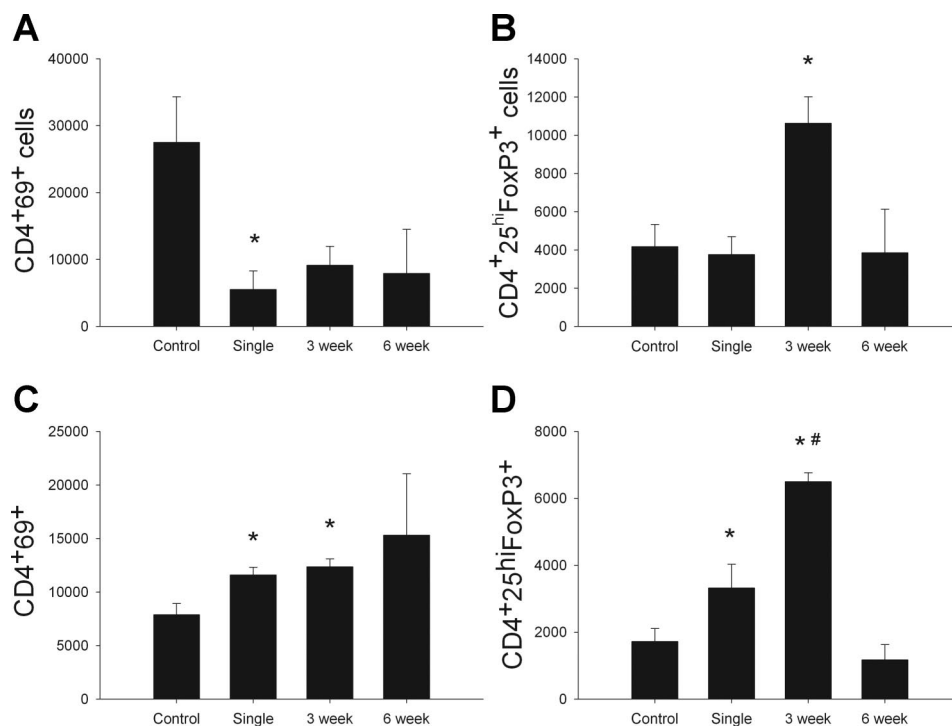


Fig. 3. BALF cytokine concentrations after OVA challenge. BALF was harvested from OVA-sensitized and aerosol-challenged mice 24 h after the final aerosol and following conclusion of lung function tests. Supernatants were prepared, and levels of IL-4, IL-5, IL-2, and TNF α were quantified by Cytometric Bead Assay as described in MATERIALS AND METHODS. IL-13 and transforming growth factor- β_1 (TGF β_1) were measured by ELISA. Data are shown as means $\pm$ SD for $n = 6$ –8 BAL samples per group; * $P < 0.05$ unpaired t -test.

Airway $CD4^{+}69^{+}$ and $CD4^{+}25^{+}FoxP3^{+}$ Treg cell numbers. Treg numbers within the airways after varying durations of airway allergen exposure were examined as a potential mechanism for AHR suppression. Twenty-four hours after a single OVA challenge, total numbers of $CD4^{+}25^{+}FoxP3^{+}$

cells (Treg) were reduced within the airways; however, this was not significant compared with control animals (Fig. 4B). In contrast, 3-wk allergen exposure induced a significant increase in Treg numbers (unpaired t -test, $P = 0.002$) in the airways compared with single-exposed mice, whereas 6-wk OVA-

Fig. 4. Quantification of regulatory T (Treg) and T effector cells in the airways (trachea) and draining lymph nodes (DLN). Mice were killed 24 h after the final aerosol, and tracheas (A and B) and DLN (C and D) were removed for flow cytometric analysis of T cell populations of $CD4^{+}69^{+}$ (T effector) cells (A and C) and $CD4^{+}25^{+}FoxP3^{+}$ (Treg) cells (B and D). Data are shown as mean (SE) numbers of cells of 3–6 separate experiments with pools of $n = 5$ mice per group in each experiment. * $P < 0.05$ compared with control animals; # $P < 0.05$ compared with single OVA aerosol-exposed animals.



exposure showed a trend toward a decrease in Treg numbers within the trachea compared with 3-wk-challenged animals ($P = 0.064$). There were no significant differences between total numbers of cells recovered from the airways of control ($43.29 \times 10^4 \pm 7.69 \times 10^4$), single OVA ($29.03 \times 10^4 \pm 10.12 \times 10^4$), 3-wk OVA ($23.81 \times 10^4 \pm 8.85 \times 10^4$), or 6-wk OVA ($34.15 \times 10^4 \pm 14.42 \times 10^4$)-challenged animals. Similarly, there were no significant differences between proportions of CD4⁺69⁺ and CD4⁺25⁺FoxP3⁺ cells in the airways from control ($14.24\% \pm 3.97\%$ and $4.40\% \pm 1.10\%$, respectively), single OVA ($18.70\% \pm 5.53\%$ and $5.71\% \pm 1.08\%$), 3-wk OVA ($23.66\% \pm 2.16\%$ and $2.47\% \pm 0.75\%$), and 6-wk OVA ($25.59\% \pm 13.12\%$ and $3.95\% \pm 2.09\%$)-challenged animals. A single OVA aerosol exposure also significantly decreased the numbers of activated CD4⁺69⁺ T cells in the airways, and the numbers of these cells remained consistently decreased after 3 and 6 wk of OVA challenge (Fig. 4A).

A single OVA challenge increased the number of CD4⁺69⁺ cells in the DLN compared with control animals ($P = 0.015$), and these cell numbers remained elevated above control levels after both 3-wk ($P = 0.008$) and 6-wk ($P = 0.098$) OVA challenge protocols (Fig. 4C). CD4⁺25⁺FoxP3⁺ cell numbers were increased following a single OVA challenge compared with control animals ($P = 0.043$), were further increased following 3-wk OVA challenge ($P < 0.001$), and returned to control levels following 6-wk OVA challenge ($P = 0.406$; Fig. 4D). There were no significant differences between proportions of CD4⁺25⁺FoxP3⁺ cells in the DLN from control ($1.94\% \pm 1.28\%$), single OVA ($5.71\% \pm 2.42\%$), 3-wk OVA ($2.47\% \pm 1.68\%$), and 6-wk OVA ($3.95\% \pm 2.09\%$)-challenged animals.

Partial in vivo depletion of Treg restored AHR in the main conducting airways. To determine the importance of Treg in the attenuation of AHR, we attempted to deplete Treg in vivo using an antibody targeted to the CD25 cell surface marker (PC61). To initially test the efficiency of in vivo CD25 depletion, we administered either IgG₁ isotype control or α CD25 to two separate groups of sensitized and 3-wk OVA aerosol-exposed mice ($n = 5$ per group). There was a 69% decrease in CD4⁺25⁺FoxP3⁺ cells after α CD25 treatment (1.0%) compared with IgG₁ (3.19%) administration within the trachea and a reduction of 63% after α CD25 treatment (1.66%) compared with IgG₁ (4.48%) in the DLN. To test whether partial in vivo CD25 depletion could restore AHR in attenuated (3-wk OVA-challenged) mice, we measured response to MCh after either IgG₁ or α CD25 treatment. MCh responsiveness for Raw, G, and H were determined as previously described. α CD25 treatment increased the response to MCh for Raw (Fig. 5B), however, α CD25 had no effect on G or H compared with IgG₁ (Fig. 5, C and D, respectively). To confirm the efficiency of CD25 depletion in MCh-challenged animals, DLN were removed at the conclusion of MCh challenges and stained with antibodies against CD4 and CD25. Figure 5A shows frequency of CD25⁺ cells among CD4⁺ cells in the DLN of individual MCh-challenged mice. MCh-challenged animals showed a decrease (50% reduction) in CD4⁺25⁺ cells within the DLN ($P = 0.022$). Concentrations of TGF β ₁, IL-4, IL-5, and IL-13 in BALF remained unchanged after partial CD25 depletion compared with 3-wk OVA-challenged animals (Fig. 5, E–H, respectively).

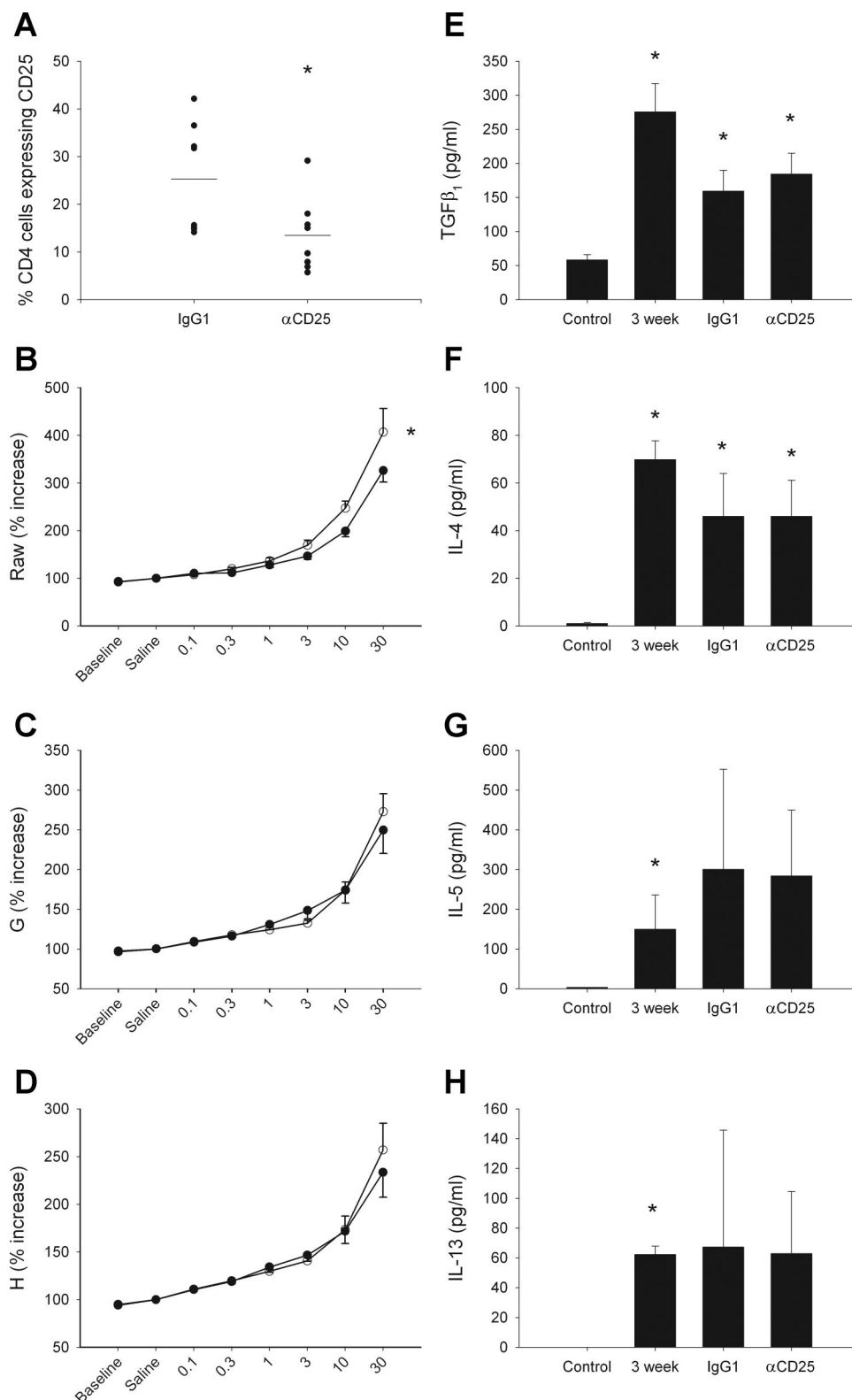
Adoptive transfer of Treg cells attenuated AHR in established allergic airways disease. To test the hypothesis that Treg cells could attenuate AHR in vivo in established allergic airways disease, CD4⁺25⁺ cells were sorted from DLN of 3-wk OVA-challenged mice and adoptively transferred into single OVA-challenged mice. To confirm that the transferred CD4⁺25⁺ cells were bona fide Treg (FoxP3⁺), a proportion of the donor cells were permeabilized and stained with anti-FoxP3-FITC. Of the CD4⁺25⁺ cells to be adoptively transferred, 92% (12.5% of 13.57%) coexpressed FoxP3 (Fig. 6A). To examine whether transferred CD4⁺25⁺ or CD4⁺25[−] cells could migrate to the airways, CD4⁺25⁺ or CD4⁺25[−] cells were labeled with CFSE immediately before adoptive transfer. A total of 1×10^6 CFSE⁺ CD4⁺25⁺ or CD4⁺25[−] cells were transferred to separate groups of sensitized mice followed by a single OVA challenge 24 h later ($n = 8$ per group). The numbers of CFSE⁺CD4⁺25⁺ that were recovered in tissues and lymph nodes of interest from a pool of 5 mice were 540 in the trachea, 750 in the peripheral lungs, 2,930 in the DLN, and 3,958 in the inguinal lymph nodes. In comparison, the number of recovered CFSE⁺CD4⁺25[−] cells were 267 in the trachea, 1,028 in the peripheral lungs, 2,211 in the DLN, and 2,233 in the inguinal lymph nodes. These numbers represented less than 1% of total CD4⁺ cell numbers in all compartments measured.

To determine whether adoptively transferred CD4⁺25⁺ cells could attenuate AHR, MCh responsiveness was measured in single OVA-challenged mice after CD4⁺25[−] or CD4⁺25⁺ transfer. Dose-response curves to inhaled MCh for Raw, G, and H are shown in Fig. 6, B–D, respectively. A single OVA aerosol to sensitized mice increased MCh responsiveness for Raw, G, and H, thus resulting in AHR, compared with control animals. Administration of 1×10^6 CD4⁺25[−] cells on day 20 to sensitized mice followed by a single OVA aerosol on day 21 showed increased responsiveness to MCh for Raw, G, and H ($P = 0.042$, 0.010, and 0.025, respectively, 2-way RM ANOVA) compared with animals receiving CD4⁺25⁺ cells. There were no differences in response to MCh between CD4⁺25[−] and single OVA-challenged animals for Raw, G, or H. In addition, there were no differences in MCh responsiveness between animals receiving CD4⁺25⁺ cells and control animals for Raw, G, and H. Concentrations of TGF β ₁, IL-4, IL-5, and IL-13 in BALF remained unchanged after Treg adoptive transfer compared with single OVA-challenged animals (Fig. 6, E–H, respectively).

DISCUSSION

Results from the present study show that chronic exposure to OVA over 3 wk suppressed the AHR and Th2 cytokine response in the airways associated with allergen-induced inflammation despite increasing allergen-specific IgE and IgG₁, airway and parenchymal eosinophilia, and bronchiolar wall thickness. Further allergen exposure (6 wk) reversed the suppression of AHR in both the central and peripheral airways while reducing allergen-induced airway and parenchymal eosinophilia, Th2 cytokines, airway wall thickness, and goblet cell metaplasia, whereas OVA-specific IgE and IgG₁ remained elevated. Allergen-induced AHR was associated with relatively low numbers of Treg within the main conducting airways (trachea), whereas AHR suppression was associated with increased numbers of Treg within the airways.

Fig. 5. Effects of partial in vivo CD25 depletion on airway hyperresponsiveness (AHR) and airway cytokine concentrations. MCh responsiveness for Raw, G, and H are shown in *B–D*, respectively. Data are expressed as a percentage of the baseline saline values and shown as means $\pm$ SD for IgG1-treated (closed circles) or α CD25-treated (open circles) animals ($n = 8$ mice per group). The proportion of CD4⁺ cells expressing CD25 in the DLN of MCh-challenged mice after IgG1 or α CD25 treatment are shown in *A*. Data are expressed as percentage of CD25⁺ cells from individual mice (data points) with a mean bar. Concentrations of TGF β 1, IL-4, IL-5, and IL-13 were measured in BALF as described in MATERIALS AND METHODS (*E–H*, respectively). Data are expressed as cytokine concentration from $n = 6–8$ mice per group. * $P < 0.05$.



In the current study, a single OVA airway challenge to sensitized mice resulted in increased responsiveness to MCh as described previously (66). Three weeks of OVA challenges suppressed AHR in the conducting airways (Raw) and parenchyma (G and H) as determined by a decrease in

responsiveness to MCh. In examining the mechanism(s) underlying the suppression of AHR, we need to consider the potential effects of the alteration in lung structure as a result of chronic allergen exposure. Three weeks of OVA exposures resulted in substantial lung inflammation and airway

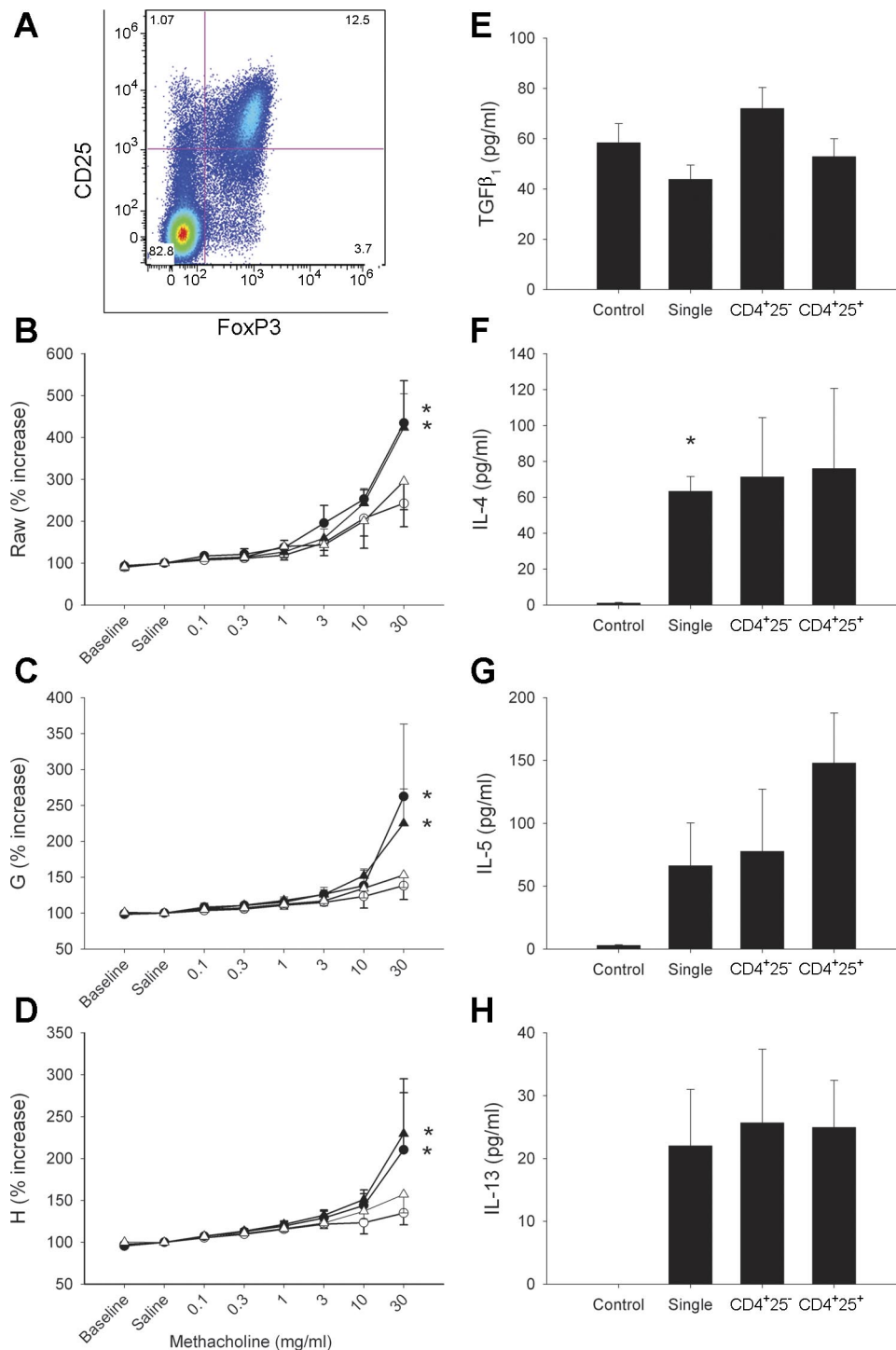


Fig. 6. Effects of Treg adoptive transfer on AHR and airway cytokine concentrations. MCh responsiveness for Raw, G, and H are shown in *B–D*, respectively. Data are expressed as a percentage of the baseline saline values and shown as means $\pm$ SD for sensitized saline-challenged control mice (open circles), single OVA-challenged mice (closed circles), sensitized, single OVA-challenged mice receiving CD4⁺25⁻ cells (closed triangles), or sensitized, single OVA-challenged mice receiving CD4⁺25⁺ cells (open triangles) animals ($n = 8$ mice per group). A representative fluorescence-activated cell sorter (FACS) plot of the proportion of adoptively transferred CD4⁺ cells coexpressing CD25 and FoxP3 (Treg) from the DLN of 3-wk OVA-challenged animals (pool of $n = 5$ mice) is shown in *A*. Concentrations of TGFβ₁, IL-4, IL-5, and IL-13 were measured in BALF as described in MATERIALS AND METHODS (*E–H*, respectively). Data are expressed as cytokine concentration from $n = 6–8$ mice per group. * $P < 0.05$.

remodeling that was associated with an alteration in the mechanical properties of the lung parenchyma, i.e., an increase in the coefficients of tissue damping and tissue elastance ($P < 0.05$). Stiffening of the lung parenchyma may limit airway constriction by providing an increased load against which the airway smooth muscle must contract (45). However, airway remodeling involving thickening of the airway wall and goblet cell hyperplasia is likely to provide a mechanical advantage for airway smooth muscle to increase airway narrowing and increasing airway respon-

siveness to MCh (34). This effect on the airways is likely to outweigh any effect from parenchymal stiffening. After 6 wk of OVA challenges, the pulmonary inflammation and airway remodeling had returned to normal, whereas the suppression of AHR had waned, with the heightened airway and parenchymal responsiveness returning. This dissociation between pulmonary inflammation, structural remodeling, and AHR argues against a mechanical contribution to the allergen-induced AHR seen after a single OVA challenge and the suppression of AHR seen with chronic exposure.

The role of inflammation in the development of AHR has been controversial. Eosinophils have been reported as key contributors to AHR (9, 28, 59), however, more recent data suggest that these two features are not related (15). Our data confirm the dissociation between AHR and inflammation as AHR developed in the presence of mild eosinophilia and was suppressed during a maximal eosinophilic response in both the airways and lung parenchyma. The current data strengthen this dissociation as 6 wk of allergen challenges produced AHR with minimal eosinophilia.

Our data also show that development, suppression, and reemergence of AHR are independent of OVA-specific IgE in serum. This does not agree with previous allergen-inhalation models exhibiting tolerance to IgE in the mouse (33). However, our model differs widely in a number of ways including species used and respiratory challenge protocol. Furthermore, OVA-specific IgG₁ decreased after a single OVA exposure and increased after 3 and 6 wk of OVA challenges compared with control mice. Recently, Erazo and colleagues (21) have reported that IgE and IgG₁ production are linked to a common memory B cell progenitor as IgG₁⁺ cells and IgG₁⁺ memory B cells could undergo a secondary switch to IgE, which was dependent on the presence of IL-4 and inhibited by IL-21. Our data support these findings, as an increase in OVA-specific IgE was observed after a single OVA aerosol, which could be explained by B cell class switching from IgG₁ to IgE. The decrease in IgG₁ after a single OVA aerosol may be due to IgG₁ being lost to the formation of immune complexes. However, increased IgG₁ and IgE after 3 and 6 wk of allergen challenges may be a result of continuous activation of memory B cells by T lymphocytes and/or the longevity of plasma cells. Finally, the demonstration that AHR can be adoptively transferred with CD4⁺ T cells from sensitized and challenged mice to naïve mice shows that allergen-induced AHR is not dependent on serum antibodies (12).

CD4⁺ cells are widely reported to contribute to the production of AHR via the production and release of Th2 cytokines such as IL-4, IL-5, and IL-13 (42, 65), however, their role in AHR suppression remains unclear. In the model used in the present study, Th2 cytokines were increased concurrent to the development of AHR and are reduced during AHR attenuation. Thus we hypothesize that in the conventional model of allergen-induced inflammation IL-4, IL-5, and IL-13 may contribute to the development of AHR and the absence of these cytokines after multiple allergen challenges may contribute to AHR suppression. However, we speculate that the attenuation of AHR cannot be explained by reduction of Th2 cytokines alone. Although we cannot conclude definitively that inhibition of Th2 cytokine production after 3 and 6 wk of allergen challenges is due to active immune suppression of cytokine-secreting cells, our data do suggest that these cytokine-secreting cells are not deleted as numbers of CD4⁺69⁺ cells do not change between single and 3 and 6 wk of OVA challenge. Levels of the proinflammatory cytokine TNF α were significantly increased in the single OVA-challenged group and decreased after 3 wk of allergen exposure, although the reduction was to a lesser extent than observed for the Th2 cytokines (Fig. 3). Elevated TNF α production has been reported to be associated with the late asthmatic response to allergen challenge in atopic asthmatic patients (39). The current data support these human data by associating elevated TNF α produc-

tion with AHR. Therefore, decreased TNF α in the airways in addition to decreased Th2 cytokines may contribute to AHR attenuation.

In addition, levels of the pleiotropic cytokine IL-2 were unchanged after a single OVA challenge and decreased after 3 wk of OVA challenge compared with control animals (Fig. 3). IL-2 is known to play a central role in Th2 differentiation (13). A reduction in IL-2 may reflect a decrease in T cell activation and recruitment to the airways, which may explain the observed decrease in numbers of CD4⁺69⁺ cells and reduced Th2 cytokine secretion in the airways, suggesting a downregulation in the adaptive immune response. Collectively, these data suggest that a homeostatic mechanism may be in place to limit Th2 responses and AHR with prolonged allergen exposure. This may help to explain why prolonged allergen exposure does not result in continually worsening AHR in atopic asthmatic individuals. Interestingly, the reemergence of AHR after 6 wk of allergen challenges was not associated with IL-4, IL-5, or IL-13 production. These data suggest mechanisms other than Th2 cytokine production can mediate AHR in our model.

Effector CD4⁺ T cells are known to contribute to the development of AHR (26, 36, 49). Treg cells are known to inhibit both Th1 and Th2 cells in vitro (4, 8, 18, 20, 64) and in vivo (14, 27, 38, 44, 56, 60). Therefore, one potential mechanism for the attenuation of allergen-induced AHR is an alteration in the cell numbers or balance between activated effector and Treg cells in the airways and/or DLN.

Surprisingly, numbers of airway CD4⁺ T cells expressing the activation marker CD69 (CD4⁺69⁺) were decreased after a single and 3-wk OVA challenge compared with sensitized control animals (Fig. 4A). This may be due to the loss of CD69 expression from these cells in the trachea. An alternate and possibly more plausible explanation is the death of activated effector T cells in the trachea. Although there is little published evidence to support this idea (61), activated effector T cells have been shown to have a short lifespan (i.e., less than 24 h) in nonlymphoid tissue (7) especially during the recall response to antigen (M. E. Wikstrom, personal communication). Consistent with previously published data (40, 62, 63), CD4⁺69⁺ cell numbers were increased within the DLN after both a single and 3-wk OVA airway challenge, which supports the hypothesis that activated effector T cells are required to drive the adaptive immune response to antigen. An explanation for the divergent effector cell numbers in the airways and DLN may be due to expression of the CD69 marker, which has been reported to cause retention of these cells in lymph nodes (54). This retention may occur to allow time for Th2 differentiation to occur in the lymph nodes.

Following a single OVA challenge, the numbers of airway (tracheal) CD4⁺25⁺FoxP3⁺ (Treg) cells were unchanged compared with control animals (Fig. 4B). Following 3 wk of OVA challenges, the numbers of Treg cells were increased in the airways compared with control animals. Treg cell numbers were increased after a single OVA challenge and further increased after 3 wk of OVA challenge in the DLN. These data suggest that Treg do not appear to migrate to the airways after a single OVA challenge. Thus further allergen challenges may initiate the process of migration to the airways by increasing secretion of chemokines and/or expression of chemokine receptors. Collectively, these data are consistent with the devel-

opment and attenuation of allergen-induced AHR being associated with fluctuating numbers of Treg cells within the airways with minimal Treg cell numbers associated with the development of AHR and increased Treg cell numbers associated with AHR attenuation.

Associations between allergen-induced AHR attenuation using accurate measures of lung function (LFOT) and increased numbers of Treg cells within the airways have been previously described in a rat model of allergic airways disease (56). Results from the current study are consistent with the published study in rats. The current and the published (56) studies are comparable as both used a sensitive technique for measuring lung function (low-frequency forced oscillation), however, the studies differ in species used and allergen challenge protocol. The present study was the first to determine an association between the relative numbers of Treg and T effector cells with the development and attenuation of accurate measurements of AHR in a mouse model of allergic asthma.

To further elucidate the importance of Treg cells in attenuating AHR, CD25⁺ Treg were either partially depleted *in vivo* or adoptively transferred into recipient-sensitized mice. α CD25 antibody was administered to 3-wk OVA-challenged animals to examine the effect of systemic Treg depletion on AHR attenuation. Results demonstrated that partial CD25 depletion could successfully restore AHR in the main conducting airways (Raw) but not in the parenchymal tissues (G and H; Fig. 5, B–D). These data indicate that regulation of AHR by Treg cells differs in the main conducting airways compared with the parenchymal tissues, suggesting that hyperresponsiveness in the main conducting airways is predominantly T cell-dependent and under Treg control, whereas the inflammatory and structural changes occurring in the smaller peripheral airways and lung tissues after prolonged OVA challenge would be more difficult to reverse by partially depleting Treg cells. To elucidate these mechanisms further, CD25⁺ Treg cells (of which 92% expressed FoxP3; Fig. 6A) were adoptively transferred to single OVA-challenged animals exhibiting AHR. This approach demonstrated that adoptive transfer of Treg cells could attenuate AHR *in vivo* in the main conducting airways (Raw; Fig. 6B). These data are consistent with published data (35, 38, 56), however, only the previously mentioned study in rats (56) has demonstrated AHR attenuation separately in the main conducting airways, a site known to play a central role in AHR. The current study is comparable to the published study (56) in that similar numbers of Treg cells were removed from DLN of multiple airway-challenged animals exhibiting AHR attenuation and transferred to sensitized animals in addition to use of the same technique to measure lung function. The studies differ in a variety of ways including species used and allergen challenge protocol.

Recently published *in vitro* data have demonstrated that Treg cells can suppress the function of activated effector T cells and also naïve T cells by inhibiting T cell growth and/or proliferation (4, 8, 18, 20, 64). The current *in vivo* 3-wk OVA challenge results support the *in vitro* data by demonstrating that increased numbers of Treg cells within the airways were associated with a decrease in Th2 cytokine secretion within the airways (3-wk OVA challenge; Fig. 3). However, numbers of CD4⁺69⁺ cells within the airways were unchanged during AHR development and attenuation but are increased in the lymph nodes during these processes. Collectively, these data

suggest that increased numbers of Treg cells could prevent migration of activated effector T cells to the airways, possibly via inhibiting T cell chemoattractant factors. The decrease in Th2 cytokine secretion may be due to an inhibition of proliferation resulting from decreased IL-2 secretion or even deletion of activated T cells in the airways. Alternatively, inhibition of proliferation or clonal deletion could be occurring locally within the airways but not in the regional lymph nodes.

Th2 cytokine secretion was measured in BALF after Treg depletion or adoptive transfer as a potential mechanism for Treg-mediated AHR attenuation. However, Treg depletion or adoptive transfer had no effect on Th2 cytokine secretion in the airways, suggesting that Treg-mediated attenuation of AHR does not occur via inhibition of Th2 cytokine secretion. Treg cells present within the airway mucosa could inhibit Th2 effector cells either by a contact-dependent mechanism or via the production of immunomodulatory cytokines such as IL-10 and TGF- β . The former hypothesis could not be tested with currently available equipment in the current *in vivo* model. The latter hypothesis was examined by measuring levels of IL-10 and TGF- β ₁ in BALF in the various groups of control and OVA-exposed mice. IL-10 was undetectable in all samples measured (data not shown), which was not unexpected as the role of IL-10 in the asthmatic response remains poorly defined. A number of studies have reported that IL-10 levels are decreased in asthmatic patients (37, 57). The current data are consistent with these human studies as IL-10 levels were undetectable in the current allergic mouse model during both the physiological (single OVA challenge) and inflammatory response (3-wk OVA challenge) to allergen.

The role of TGF- β in immune suppression is not clearly defined. Recent studies have proposed a role for both Treg cells and TGF- β in attenuating the allergic response to allergen (1, 16, 22, 30, 35, 43, 46). Therefore, the hypothesis in the current study was that AHR attenuation would be mediated by TGF- β secreted by Treg cells. TGF- β ₁ concentration in BALF remained low after a single OVA challenge but were significantly elevated after 3 wk of OVA challenge (Fig. 3). This pattern mimicked that of the number of Treg cells within the airways and was inversely related to AHR status.

To test the hypothesis that Treg could mediate AHR potentially via secretion of TGF- β and/or inhibition of Th2 cytokine secretion, TGF- β ₁, IL-4, IL-5, and IL-13 concentrations were measured in BALF after Treg depletion or adoptive transfer *in vivo*. Results demonstrated that concentrations of all measured cytokines remained unchanged after these experimental procedures (Fig. 5, E–H, and Fig. 6, E–H). These data initially suggested that the main cellular source of TGF- β in the airways did not appear to be Treg cells. This result was not surprising as one recent study examining the role of TGF- β ₁ in AHR determined that the main cellular source of TGF- β ₁ was airway epithelial cells (1). Therefore, in the current study, the presence of Treg cells could trigger TGF- β release from epithelial cells either by a contact-dependent (50) or paracrine mechanism (23, 58). These results also suggest that attenuation of AHR is unlikely to occur via a Treg-mediated reduction of Th2 cytokines resulting in limitation of the adaptive immune response.

The mechanism of TGF- β -mediated immune suppression is poorly understood. A limitation of our results is a lack of mechanistic data on the role of TGF- β in AHR attenuation, which could be addressed via administration of TGF- β to single

OVA-challenged mice and administration of soluble TGF β receptor to 3-wk OVA-challenged mice with outcome measurements of lung function, which is currently being addressed in our laboratory. However, a hypothesis arising from the current study is that increased levels of TGF β ₁ could alter the APC microenvironment and cause a deficiency in allergen capture by APC populations in an attempt to limit the adaptive immune response. In support of this hypothesis, Fogel-Petrovic et al. (22) recently reported that TGF β could inhibit human dendritic cell maturation in vitro resulting in deficient T cell differentiation and proliferation.

In summary, 3-wk allergen challenge decreased numbers of activated effector T cells but increased numbers of Treg cells in the airways, which was associated with AHR suppression. We have demonstrated suppression of allergen-induced AHR in the presence of a large inflammatory (eosinophilic) infiltrate in both airway and lungs, high levels of allergen-specific IgE and IgG₁, airway remodeling, and damage to airway epithelium. Increased numbers of airway Treg cells were associated with increased TGF β ₁ secretion in BALF. Depletion or adoptive transfer of Treg cells restored or suppressed AHR in vivo, respectively. Depletion or adoptive transfer of Treg cells had no effect on either Th2 cytokine (IL-4, IL-5, and IL-13) or TGF β ₁ secretion. Therefore, Treg cells appear to mediate AHR attenuation in vivo potentially via a paracrine mechanism involving TGF β ₁ production by airway epithelial cells.

ACKNOWLEDGMENTS

We thank John Elliot and Alan James for the use of their camera lucida equipment for measuring airway wall thickness, Luke Berry for technical assistance with ELISA, and Dr. Michelle Tourigny for sorting the cells for the transfer experiments.

GRANTS

This project was funded by National Health and Medical Research Council of Australia Grants 458513, 458561, 437200, and 211912. J. T. Burchell is funded by a scholarship from the Asthma Foundation of Western Australia.

REFERENCES

- Alcorn JF, Rinaldi LM, Jaffe EF, van Loon M, Bates JH, Janssen-Heininger YM, Irvin CG. Transforming growth factor-beta1 suppresses airway hyperresponsiveness in allergic airway disease. *Am J Respir Crit Care Med* 176: 974–982, 2007.
- Ali FR, Kay AB, Larche M. Airway hyperresponsiveness and bronchial mucosal inflammation in T cell peptide-induced asthmatic reactions in atopic subjects. *Thorax* 62: 749–756, 2007.
- Bai A, Eidelman DH, Hogg JC, James AL, Lambert RK, Ludwig MS, Martin J, McDonald DM, Mitzner WA, Okazawa M, Pack RJ, Pare PD, Schellenberg RR, Tiddens HA, Wagner EM, Yager D. Proposed nomenclature for quantifying subdivisions of the bronchial wall. *J Appl Physiol* 77: 1011–1014, 1994.
- Bel'skaya NV, Bel'skii YP, Danilets MG, Trofimova ES, Sherstoboev EY, Agafonov VI. Effects of natural suppressor cells on production of Th1 and Th2 cytokines and proliferation of polarized T cells. *Bull Exp Biol Med* 141: 440–443, 2006.
- Benacerraf B, Germain RN. A single major pathway of T-lymphocyte interactions in antigen-specific immune suppression. *Scand J Immunol* 13: 1–10, 1981.
- Billingham RE, Brent L, Medawar PB. Activity acquired tolerance of foreign cells. *Nature* 172: 603–606, 1953.
- Binns RM, Licence ST, Pabst R. Homing of blood, splenic, and lung emigrant lymphoblasts: comparison with the behaviour of lymphocytes from these sources. *Int Immunol* 4: 1011–1019, 1992.
- Bourgeois C, Stockinger B. CD25+CD4+ regulatory T cells and memory T cells prevent lymphopenia-induced proliferation of naive T cells in transient states of lymphopenia. *J Immunol* 177: 4558–4566, 2006.
- Brusasco V, Crimi E, Gianiorio P, Lantero S, Rossi GA. Allergen-induced increase in airway responsiveness and inflammation in mild asthma. *J Appl Physiol* 69: 2209–2214, 1990.
- Burrows B, Martinez FD, Halonen M, Barbee RA, Cline MG. Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 320: 271–277, 1989.
- Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198: 1875–1886, 2003.
- Cohn L, Tepper JS, Bottomly K. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J Immunol* 161: 3813–3816, 1998.
- Cote-Sierra J, Foucras G, Guo L, Chiodetti L, Young HA, Hu-Li J, Zhu J, Paul WE. Interleukin 2 plays a central role in Th2 differentiation. *Proc Natl Acad Sci USA* 101: 3880–3885, 2004.
- Cottrez F, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J Immunol* 165: 4848–4853, 2000.
- Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 157: 4–9, 1998.
- Csencsits K, Wood SC, Lu GY, Bishop DK. Transforming growth factor-beta1 gene transfer is associated with the development of regulatory cells. *Am J Transplant* 5: 2378–2384, 2005.
- De Sanctis GT, Itoh A, Green FH, Qin S, Kimura T, Grobholz JK, Martin TR, Maki T, Drazen JM. T-lymphocytes regulate genetically determined airway hyperresponsiveness in mice. *Nat Med* 3: 460–462, 1997.
- Earle KE, Tang Q, Zhou X, Liu W, Zhu S, Bonyhadi ML, Bluestone JA. In vitro expanded human CD4+CD25+ regulatory T cells suppress effector T cell proliferation. *Clin Immunol* 115: 3–9, 2005.
- Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. *J Clin Invest* 104: 1001–1006, 1999.
- Elkord E, Hopcraft L, Burt D, Stern PL. Bead-isolated human CD4+CD25+ T regulatory cells are anergic and significantly suppress proliferation of CD4+CD25– T responder cells. *Clin Immunol* 120: 232–233, 2006.
- Erazo A, Kutchukhidze N, Leung M, Christ AP, Urban JF Jr, Curotto de Lafaille MA, Lafaille JJ. Unique maturation program of the IgE response in vivo. *Immunity* 26: 191–203, 2007.
- Fogel-Petrovic M, Long JA, Misso NL, Foster PS, Bhoola KD, Thompson PJ. Physiological concentrations of transforming growth factor beta1 selectively inhibit human dendritic cell function. *Int Immunopharmacol* 7: 1924–1933, 2007.
- Fox FE, Ford HC, Douglas R, Cherian S, Nowell PC. Evidence that TGF-beta can inhibit human T-lymphocyte proliferation through paracrine and autocrine mechanisms. *Cell Immunol* 150: 45–58, 1993.
- Gajewski TF, Joyce J, Fitch FW. Antiproliferative effect of IFN-gamma in immune regulation. III. Differential selection of TH1 and TH2 murine helper T lymphocyte clones using recombinant IL-2 and recombinant IFN-gamma. *J Immunol* 143: 15–22, 1989.
- Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742, 1997.
- Haczku A, Macary P, Huang TJ, Tsukagoshi H, Barnes PJ, Kay AB, Kemeny DM, Chung KF, Moqbel R. Adoptive transfer of allergen-specific CD4+ T cells induces airway inflammation and hyperresponsiveness in Brown-Norway rats. *Immunology* 91: 176–185, 1997.
- Hadeiba H, Locksley RM. Lung CD25 CD4 regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J Immunol* 170: 5502–5510, 2003.
- Hamelmann E, Oshiba A, Loader J, Larsen GL, Gleich G, Lee J, Gelfand EW. Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *Am J Respir Crit Care Med* 155: 819–825, 1997.
- Haneda K, Sano K, Tamura G, Shiota H, Ohkawara Y, Sato T, Habu S, Shirato K. Transforming growth factor-beta secreted from CD4+ T cells ameliorates antigen-induced eosinophilic inflammation. A novel high-dose tolerance in the trachea. *Am J Respir Cell Mol Biol* 21: 268–274, 1999.
- Hansen G, McIntire JJ, Yeung VP, Berry G, Thorbecke GJ, Chen L, DeKruyff RH, Umetsu DT. CD4+ T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *J Clin Invest* 105: 61–70, 2000.

31. Hantos Z, Daroczy B, Suki B, Nagy S, Fredberg JJ. Input impedance and peripheral inhomogeneity of dog lungs. *J Appl Physiol* 72: 168–178, 1992.
32. Hogan SP, Matthaei KI, Young JM, Koskinen A, Young IG, Foster PS. A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. *J Immunol* 161: 1501–1509, 1998.
33. Holt PG, Batty JE, Turner KJ. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* 42: 409–417, 1981.
34. James AL, Maxwell PS, Pearce-Pinto G, Elliot JG, Carroll NG. The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. *Am J Respir Crit Care Med* 166: 1590–1595, 2002.
35. Joetham A, Takeda K, Taube C, Miyahara N, Matsubara S, Koya T, Rha YH, Dakhama A, Gelfand EW. Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. *J Immunol* 178: 1433–1442, 2007.
36. Joetham A, Takeda K, Taube C, Miyahara N, Kanehiro A, Dakhama A, Gelfand EW. Airway hyperresponsiveness in the absence of CD4+ T cells after primary but not secondary challenge. *Am J Respir Cell Mol Biol* 33: 89–96, 2005.
37. John M, Lim S, Seybold J, Jose P, Robichaud A, O'Connor B, Barnes PJ, Chung KF. Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. *Am J Respir Crit Care Med* 157: 256–262, 1998.
38. 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* 202: 1539–1547, 2005.
39. Keatings VM, O'Connor BJ, Wright LG, Huston DP, Corrigan CJ, Barnes PJ. Late response to allergen is associated with increased concentrations of tumor necrosis factor-alpha and IL-5 in induced sputum. *J Allergy Clin Immunol* 99: 693–698, 1997.
40. Lambrecht BN, Pauwels RA, Fazekas De St Groth B. Induction of rapid T cell activation, division, and recirculation by intratracheal injection of dendritic cells in a TCR transgenic model. *J Immunol* 164: 2937–2946, 2000.
41. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 111: 450–463, 2003.
42. Leigh R, Ellis R, Wattie JN, Hirota JA, Matthaei KI, Foster PS, O'Byrne PM, Inman MD. Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. *Am J Respir Crit Care Med* 169: 860–867, 2004.
43. Levings MK, Bacchetta R, Schulz U, Roncarolo MG. The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *Int Arch Allergy Immunol* 129: 263–276, 2002.
44. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, Carr VA, Robinson DS. Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 363: 608–615, 2004.
45. Macklem PT. The physiology of small airways. *Am J Respir Crit Care Med* 157: S181–S183, 1998.
46. Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 201: 1061–1067, 2005.
47. McMenamin C, Chilna B, Holt PG. Phenotypic and functional analysis of mucosal T cells isolated from tissue explants of rat upper respiratory tract. *J Immunol Methods* 160: 219–226, 1993.
48. Mishima H, Hojo M, Watanabe A, Hamid QA, Martin JG. CD4+ T cells can induce airway hyperresponsiveness to allergen challenge in the brown norway rat. *Am J Respir Crit Care Med* 158: 1863–1870, 1998.
49. Nakagome K, Dohi M, Okunishi K, To Y, Sato A, Komagata Y, Nagatani K, Tanaka R, Yamamoto K. Antigen-sensitized CD4+CD62Llow memory/effector T helper 2 cells can induce airway hyperresponsiveness in an antigen free setting. *Respir Res* 6: 46, 2005.
50. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4+CD25+ regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 194: 629–644, 2001.
51. Pillow JJ, Korfhagen TR, Ikegami M, Sly PD. Overexpression of TGF-alpha increases lung tissue hysteresivity in transgenic mice. *J Appl Physiol* 91: 2730–2734, 2001.
52. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151–1164, 1995.
53. Sears MR, Burrows B, Flannery EM, Herbison GP, Hewitt CJ, Holdaway MD. Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* 325: 1067–1071, 1991.
54. Shioh LR, Rosen DB, Brdickova N, Xu Y, An J, Lanier LL, Cyster JG, Matloubian M. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* 440: 540–544, 2006.
55. Sihra BS, Kon OM, Durham SR, Walker S, Barnes NC, Kay AB. Effect of cyclosporin A on the allergen-induced late asthmatic reaction. *Thorax* 52: 447–452, 1997.
56. Strickland DH, Stumbles PA, Zosky GR, Subrata LS, Thomas JA, Turner DJ, Sly PD, Holt PG. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *J Exp Med* 203: 2649–2660, 2006.
57. Takanashi S, Hasegawa Y, Kanehira Y, Yamamoto K, Fujimoto K, Satoh K, Okamura K. Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. *Eur Respir J* 14: 309–314, 1999.
58. Thomas RM, Belsito DV, Huang C, Chen LZ, Ormsby I, Simmons WJ, Cowin P, Shaw J, Doetschman T, Thorbecke GJ. Appearance of Langerhans cells in the epidermis of Tgfb1(-/-) SCID mice: paracrine and autocrine effects of transforming growth factor-beta 1 and -beta 2(1). *J Invest Dermatol* 117: 1574–1580, 2001.
59. Tomkinson A, Cieslewicz G, Duez C, Larson KA, Lee JJ, Gelfand EW. Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *Am J Respir Crit Care Med* 163: 721–730, 2001.
60. Umetsu DT, Akbari O, DeKruyff RH, Shearer WT, Rosenwasser LJ, Bochner BS. Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 112: 480–487, 2003.
61. Vella AT, Dow S, Potter TA, Kappler J, Marrack P. Cytokine-induced survival of activated T cells in vitro and in vivo. *Proc Natl Acad Sci USA* 95: 3810–3815, 1998.
62. von Garnier C, Wikstrom ME, Zosky G, Turner DJ, Sly PD, Smith M, Thomas JA, Judd SR, Strickland DH, Holt PG, Stumbles PA. Allergic airways disease develops after an increase in allergen capture and processing in the airway mucosa. *J Immunol* 179: 5748–5759, 2007.
63. Wikstrom ME, Batanero E, Smith M, Thomas JA, von Garnier C, Holt PG, Stumbles PA. Influence of mucosal adjuvants on antigen passage and CD4+ T cell activation during the primary response to airborne allergen. *J Immunol* 177: 913–924, 2006.
64. Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR, June CH. Cutting edge: regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 168: 4272–4276, 2002.
65. Yang G, Volk A, Petley T, Emmell E, Giles-Komar J, Shang X, Li J, Das AM, Shealy D, Griswold DE, Li L. Anti-IL-13 monoclonal antibody inhibits airway hyperresponsiveness, inflammation and airway remodeling. *Cytokine* 28: 224–232, 2004.
66. Zosky GR, von Garnier C, Stumbles PA, Holt PG, Sly PD, Turner DJ. The pattern of methacholine responsiveness in mice is dependent on antigen challenge dose. *Respir Res* 5: 15, 2004.