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Naturally Occurring Lung $CD4^+CD25^+$ T Cell Regulation of Airway Allergic Responses Depends on IL-10 Induction of TGF-β[1]

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Peripheral tolerance to allergens is mediated in large part by the naturally occurring lung $CD4^+CD25^+$ T cells, but their effects on allergen-induced airway responsiveness have not been well defined. Intratracheal, but not i.v., administration of naive lung $CD4^+CD25^+$ T cells before allergen challenge of sensitized mice, similar to the administration of the combination of rIL-10 and rTGF-β, resulted in reduced airway hyperresponsiveness (AHR) and inflammation, lower levels of Th2 cytokines, higher levels of IL-10 and TGF-β, and less severe lung histopathology. Significantly, $CD4^+CD25^+$ T cells isolated from IL-$10^{-/-}$ mice had no effect on AHR and inflammation, but when incubated with rIL-10 before transfer, suppressed AHR, and inflammation, and was associated with elevated levels of bronchoalveolar lavage TGF-β levels. By analogy, anti-TGF-β treatment reduced regulatory T cell activity. These data identify naturally occurring lung $CD4^+CD25^+$ T cells as capable of regulating lung allergic responses in an IL-10- and TGF-β-dependent manner. *The Journal of Immunology,* **2007, 178: 1433–1442.**

Exposure and sensitization to common environmental allergens in susceptible hosts leads to a number of allergic diseases, including allergic asthma. Asthma is a disease of the airways associated with altered smooth muscle contractility that is the result of lung inflammation, the accumulation of numerous inflammatory cell types, including T lymphocytes, eosinophils, neutrophils, and mast cells, and the release of a number of cytokines and chemokines (IL-4, IL-5, IL-9, IL-13, and eotaxin, among others). The end result is airway hyperresponsiveness (AHR)[3] and, initially, reversible airway obstruction, which over time and with repeated exacerbations can lead to airway remodeling and irreversible changes in lung function. It has been proposed that asthma, and allergic disorders in general, are the result of an immune imbalance between Th1 and Th2 responses, with susceptible individuals developing allergen-specific Th2 responses that drive the immunopathology in the lung.

The concept of a simple Th1/Th2 imbalance underlying asthma pathogenesis has been called into question, with both subsets of responses potentially contributing to the disease (1, 2). Recent studies have suggested that the allergic diseases, similar to autoimmune disorders, may be the result of impaired or deficient regulatory T cell (Treg) responses (3–5). In humans, the generation of allergen-specific Treg and increased production of IL-10 and TGF-β were shown to be essential early events in specific immunotherapy (6, 7). There are several populations of T cells that exhibit regulatory activity on a variety of immune responses. Common to these inducible or naturally occurring Treg subsets is their capacity to release IL-10 (8). At present, it is unclear whether these populations differ based on lineage and what their relationship is to T_R1 cells, since some release IFN-γ and IL-5 as described for T_R1 cells (9, 10), whereas others do not (11–14).

Naturally occurring $CD4^+CD25^+$ Treg comprise roughly 5-10% of the $CD4^+$ population in normal mice and in humans (15, 16). It remains to be determined whether the inducible Treg subsets share any relationship with the naturally occurring Treg cells. In mice, Ag-driven, IL-10-secreting Treg have similar properties as the naturally occurring Treg in that they inhibit proliferation of naive $CD4^+$ T cells (14). Unlike naturally occurring Treg, the inducible Treg appear to exhibit inhibitory activity by both a cell-cell contact-dependent mechanism, independent of IL-10 and TGF-β (16, 17), or via the secretion of these cytokines (14).

Naturally occurring $CD4^+CD25^+$ Treg can be isolated, manipulated, or depleted in mice based on the high levels of expression of CD25, although this per se does not differentiate them from activated T cells, which also can express CD25, perhaps more transiently (16, 18, 19). Additional cell surface markers that these $CD4^+CD25^+$ express include CTLA4 (20, 21) and glucocorticoid-induced TNFR-related protein (22, 23). However, activated T cells may also express these markers as well (16, 24–27). The forkhead (winged helix) transcription factor, forkhead box P3 (Foxp3), may be specifically expressed by naturally occurring Treg (28–31) and may be essential to their functional development. Mutations in the gene encoding Foxp3 are associated with a severe lymphoproliferative and autoimmune disease (32). In atopic dermatitis, $CD25^+Foxp3^+$ cells are also deficient (33).

Both Ag-driven (inducible) IL-10-secreting Treg and $CD4^+CD25^+$ naturally occurring Treg have been implicated in the down-regulation of allergen-triggered Th2 responses in mice as well as humans (31, 34–38), but their ability to be distinguished

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[3] Abbreviations used in this paper: AHR, airway hyperresponsiveness; Treg, regulatory T cell; Foxp3, forkhead box p3; MNC, mononuclear cell; RL, lung resistance; MCh, methacholine; BAL, bronchoalveolar lavage; PAS, periodic acid-Schiff; WT, wild type.

phenotypically from effector T cells during an inflammatory response to allergen has been questioned (39). Furthermore, whereas data on the ability of Treg to inhibit allergen-driven Th2 responses or lung inflammation are consistent, there is little (40–43) or negative data (44) on their role in modulating airway responsiveness on the allergen challenge of sensitized mice.

In this study, we have examined the phenotypic and functional characteristics of naturally occurring lung CD4$^+$CD25$^+$ in the regulation of lung allergic responses and airway function in sensitized and challenged mice. The data identify the ability of these cells to regulate all of these responses in a TGF-β-dependent manner, which is, in turn, dependent on IL-10 production.

Materials and Methods

Animals

Pathogen-free, 8- to 10-wk-old female BALB/c/ByJ and C57BL/6 mice from The Jackson Laboratory and IL-10$^{-/-}$ (C57BL/6) mice provided by Dr. P. Marrack (National Jewish Medical and Research Center, Denver, CO) were used and maintained on an OVA-free diet under institutional-approved guidelines.

Sensitization

Mice were sensitized by i.p. injection of 20 μg of OVA (grade V; Sigma-Aldrich) emulsified in 2.25 mg of alum hydroxide (AlumImject; Pierce) in a total volume of 100 μl on days 1 and 14. Sensitized and naive (PBS) mice received aerosolized allergen challenge (1% OVA) for 20 min on 3 consecutive days (days 26, 27, and 28) using an ultrasonic nebulizer (AeroSonic ultrasonic nebulizer; DeVilbiss) (45).

Cell preparation and culture

CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from naive donors were isolated by collagenase digestion from lungs and enriched using nylon wool columns. In brief, lungs were perfused via the right ventricle with warm (37°C) calcium- and magnesium-free HBSS containing 10% FCS, 0.6 mM EDTA, 100 U/ml penicillin, and 100 μg/ml streptomycin. Lungs were removed and minced into small pieces (300 μm). Minced tissue, suspended in 5 ml of HBSS containing 175 U/ml collagenase (type IA; Sigma-Aldrich), 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, was incubated for 60 min in an orbital shaker at 37°C. The digested lungs were sheared with a 20-gauge needle and filtered through 45- and 15-μm filters. Total lung cells, following lysis of RBC with 10 ml of RBC lysis buffer (Sigma-Aldrich) at 37°C for 15 min, were counted (Coulter Counter; Corixa). Lung mononuclear cells (MNC) were separated by Ficoll-Hypaque gradient centrifugation (lymphocyte separation medium; Organon Teknika). CD4$^+$CD25$^+$ cells represented 1.4–2% of this population. The total lymphocyte population was enriched by passing MNC through the nylon wool columns (Robbins Scientific). Enriched lymphocytes were further purified by CD4$^+$CD25$^+$ Treg MACS beads (Miltenyi Biotec), giving a purity of >95% CD4$^+$CD25$^+$ T cells. From each lung ~2–2.5 $\times$ 10^5 CD4$^+$CD25$^+$ T cells and ~3 $\times$ 10^6 CD4$^+$CD25$^-$ T cells were isolated.

For in vitro culture, isolated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were washed, counted, and resuspended to a final concentration of 4 $\times$ 10^6 cells/ml. Cells (1 $\times$ 10^6) were cultured with PMA (5 ng/ml)/ionomycin (500 ng/ml) for 24 h, and supernatants were collected.

Adoptive transfer and cytokine treatment

Each recipient mouse was administered isolated lung CD4$^+$CD25$^+$ (5 $\times$ 10^5) or CD4$^+$CD25$^-$ T cells (5 $\times$ 10^5), or 200 pg of rIL-10, rTGF-β (R&D Systems), or the combination intratracheally 1 day before allergen challenge.

Antibodies

Ab to TGF-β (1, 10, or 30 μg; R&D Systems), was given intratracheally before challenge and adoptive transfer of Treg.

Measurement of airway responsiveness

Airway responsiveness was monitored 48 h following the last allergen challenge as a change in lung resistance (RL) in response to aerosolized methacholine (MCh) (45). MCh was administered for 10 s (60 breaths/min, 500-μl tidal volume) in increasing concentrations (1.56, 3.13, 6.25, and 12.5 mg/ml for BALB/c mice and 6.25, 12.5, 25, 50, and 100 mg/ml for C57BL/6 mice). RL was continuously computed (Labview; National Instruments) by fitting flow, volume, and pressure to an equation of motion curve. Maximum values of RL were taken and expressed as a percentage change from the baseline (PBS aerosol).

Bronchoalveolar lavage (BAL)

Immediately following measurements of AHR, lungs were lavaged, and BAL fluid was recovered. Total leukocyte numbers were measured (Coulter Counter; Corixa). Differential cell counts were performed under light microscopy in a blinded manner, counting at least 200 cells on cytocentrifuged preparations (Cytospin 2, Cytospin; Thermo Shandon), stained with Leukostat (Fisher Scientific), and differentiated by standard hematological procedures.

Determination of serum Ab titers by ELISA

Serum levels of total IgE, OVA-specific IgG1, IgG2a, and IgG2b were measured by ELISA as described (46). Total IgE levels were calculated by comparison with known mouse IgE standards (BD Pharmingen). ELISA data were analyzed with the Microplate Manager software for the Macintosh computer (Bio-Rad).

Measurement of cytokines

Cytokine levels in the BAL fluid and supernatants of in vitro-cultured lung MNC were measured by ELISA using IL-4, IL-5, IL-10, IFN-γ, TGF-β (BD Biosciences/BD Pharmingen), and IL-13 (R&D Systems) kits. ELISAs were performed according to the manufacturer's directions. The limits of detection were 4 pg/ml for IL-4 and IL-5, 10 pg/ml for IL-10 and IFN-γ, 8 pg/ml for IL-13, and 6 pg/ml for TGF-β.

Flow cytometry analysis

Enriched lung MNC and BAL cells were incubated with naive mouse serum in staining buffer (PBS, 2% FCS, and 0.2% sodium azide) followed by labeling with the following conjugated Abs (BD Pharmingen): anti-CD3 (17A2), anti-CD4 (L3T4), anti-CD25 (7D4, PC61), anti-CD8 (53-6.7), and CLTA-4 (9H10). For intracellular staining, cells were stimulated with or without PMA (5 ng/ml) and ionomycin (500 ng/ml; Sigma-Aldrich) for 6 h in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich). Following staining for cell surface markers, cells were fixed with 4% paraformaldehyde in PBS, permeabilized in 0.1% saponin, and stained for the following intracellular proteins using PE- or allophycocyanin-conjugated Abs to IL-10, (JES5-16E3): IFN-γ, (XMG1.2), TGF-β (A75-3.1), and Foxp3 (eBioscience). Stained cells were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software.

Histopathology

Lungs were fixed by inflation (1 ml) and immersion in 10% formalin. Cells containing eosinophilic major basic protein were identified by immunohistochemical staining as previously described using rabbit anti-mouse major basic protein (provided by Dr. J. J. Lee, Mayo Clinic, Scottsdale, AZ). For the detection of mucus-containing cells in formalin-fixed airway tissue, sections were stained with periodic acid-Schiff (PAS) and quantitated as described (47).

Statistical analysis

All results are expressed as the mean $\pm$ SEM as a standard method of presentation for this type of data. The *t* test was used to determine differences between two groups and the Tukey-Kramer test was used for comparisons between multiple groups. Measured values may not be normally distributed and due to the small sample sizes, this may be difficult to test for. Nonparametric analysis using the Mann-Whitney *U* test was also used to confirm that the statistical differences remained significant even if the underlying distribution was uncertain. The *p* values for significance were set to 0.05 for all tests.

Results

Naturally occurring lung CD4$^+$CD25$^+$, but not CD4$^+$CD25$^-$, T cells, suppress the development of lung allergic responses

To delineate the regulatory effects of CD4$^+$CD25$^+$ T cells on airway responses in previously sensitized mice, we investigated the consequences of intratracheal administration of isolated lung CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from naive mice on airway responsiveness and inflammation following allergen challenge of sensitized recipient mice. CD4$^+$CD25$^+$ cells expressed

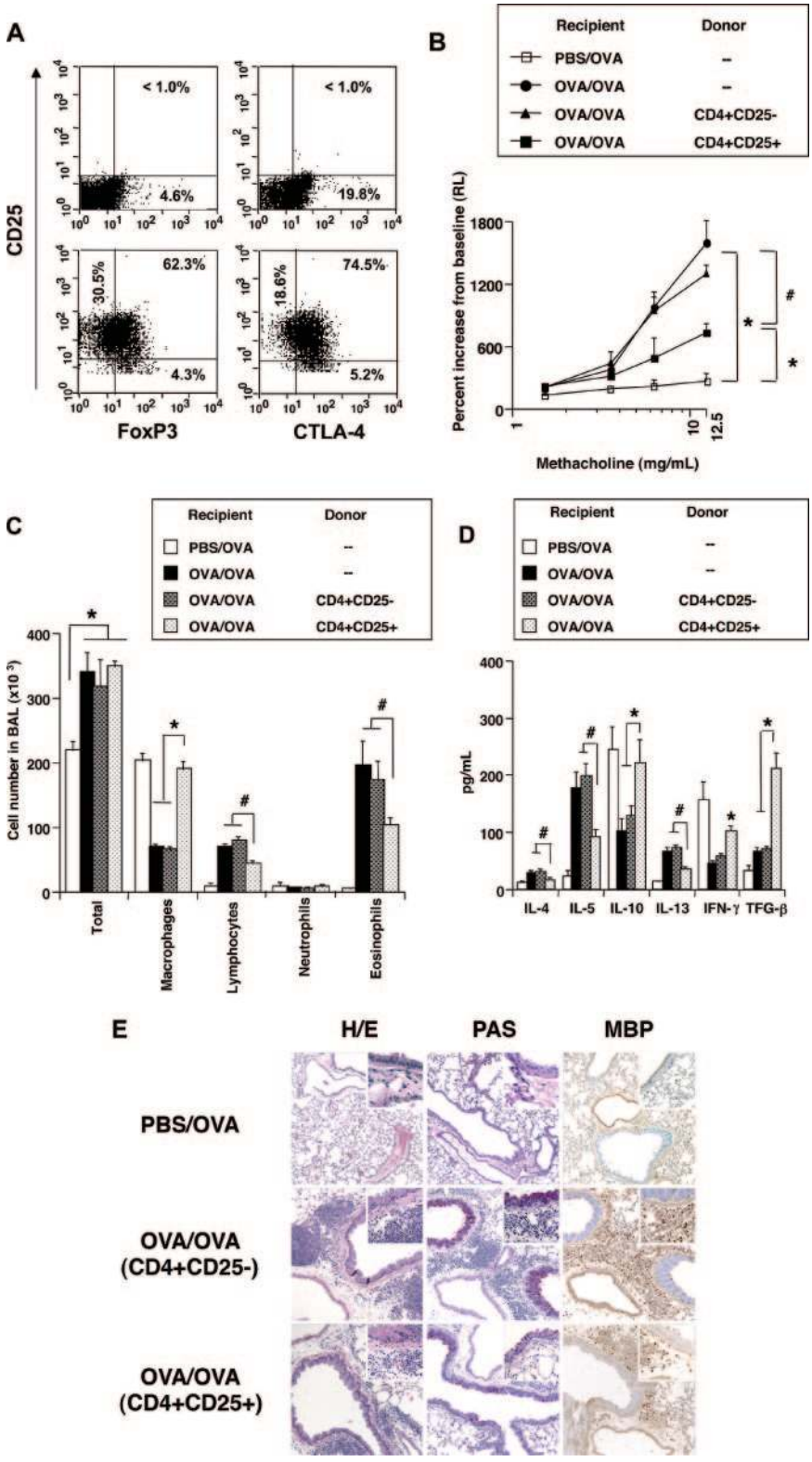

FIGURE 1. Adoptive transfer of lung $CD4^+$ $CD25^+$ cells. As described in *Materials and Methods*, lung $CD4^+CD25^+$ cells were isolated from naive BALB/c mice and transferred intratracheally into sensitized recipients before challenge. *A*, Expression of Foxp3 and CTLA4 on $CD4^+CD25^-$ (*upper panel*) and $CD4^+CD25^+$ (*lower panel*) T cells. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated, and cells were stained with analysis of $CD4^+$ gated cells. *B*, Lung resistance. *C*, BAL cell accumulation. *D*, BAL cytokine levels. *E*, Lung histopathology. $n = 12$ in each group. $*$, $p <$ 0.05 increase; $p < 0.05$ decrease.

high levels of both Foxp3 and CTLA-4, with little to no expression of Foxp3 and fewer CTLA-4-positive cells in the $CD4^+CD25^-$ cells (Fig. 1*A*). Sensitized BALB/c recipients of $CD4^+CD25^+$ cells given intratracheally before allergen challenge showed a significant reduction in AHR in contrast to recipients of $CD4^+CD25^-$ cells (Fig. 1*B*). Intravenous administration of these cells was without effect, even when up to 5×10^6 cells were injected (data not shown). The responses in the $CD4^+CD25^-$ recipients were virtually identical to the controls. Furthermore, the degree of suppression of AHR was dependent on the number of $CD4^+CD25^+$ cells transferred with peak effectiveness following the transfer of $0.5–1.0 \times 10^6$ cells and less of an effect when 0.05×10^6 cells were transferred (data not shown).

The effects on BAL inflammatory cell accumulation paralleled those of airway responsiveness. Intratracheal transfer of $CD4^+CD25^+$, but not $CD4^+CD25^-$, T cells, induced a significant reduction in the numbers of eosinophils and lymphocytes in the BAL fluid (Fig. 1*C*).

$CD4^+CD25^+$ T cell transfer induces a change in cytokine pattern, but not in Ab response

In sensitized and challenged mice given PBS or $CD4^+CD25^-$ T cells, but not challenged-only animals, levels of IL-4, IL-5, and IL-13 increased, and IL-10 and IFN-γ levels decreased. BAL TGF-β levels in both of these groups of sensitized and challenged mice also increased compared with mice that were challenged alone. Intratracheal transfer of isolated lung $CD4^+CD25^+$ T cells, in contrast, resulted in significant reductions in the levels of the Th2 cytokines IL-4, IL-5, and IL-13, but an increase in the levels of IL-10 and IFN-γ. Interestingly, a further increase in the levels of TGF-β in BAL was detected in the mice given $CD4^+CD25^+$ T cells as shown in Fig. 1*D*.

When serum levels of OVA-specific Abs were examined, sensitized, and challenged, mice showed increases in Abs of all isotypes (IgE, IgG1, IgG2, IgG2b), and there was little difference among the three groups (data not shown). The absence of regulatory effects on specific Ab production, particularly IgE Ab, again likely reflects that sensitization with alum was completed before Treg transfer.

Lung histopathology following $CD4^+CD25^+$ T cell transfer

Sensitized recipients of $CD4^+CD25^-$ T cells given intratracheally after sensitization and before allergen challenge demonstrated a marked accumulation of inflammatory cells, including eosinophils, mucus hyperproduction, and goblet cell metaplasia. In contrast, in lung digests from sensitized and challenged mice that received $CD4^+CD25^+$ T cells (Fig. 1*E*) compared with $CD4^+CD25^-$ T cells, there was a significant reduction in inflammatory cells ($20.5 \pm 1.8 \times 10^6$ compared with 29.4 ± 2.1, $n = 8$, $p < 0.05$) and eosinophils ($1.7 \pm 1.0 \times 10^6$ compared with 3.7 ± 1.4, $n = 8$, $p < 0.05$), and fewer mucus-containing goblet cells could be detected. Few PAS^+ cells were detected in nonsensitized and challenged mice (0.1 ± 0.3 cells/mm basement membrane). Recipients of $CD4^+CD25^+$ T cells showed a significant reduction in the numbers of PAS^+ cells compared with recipients of $CD4^+CD25^-$ T cells (51.3 ± 5.8 compared with 87.3 ± 9.6 cell/mm basement membrane, $n = 8$, $p < 0.05$).

Production of IL-10 and TGF-β from lung $CD4^+CD25^+$ Treg

Because the cytokine pattern shifted in $CD4^+CD25^+$ recipient mice with decreased Th2 cytokine levels and elevated levels of IL-10 and TGF-β in BAL fluid, we next defined the cellular source of these cytokines in in vitro cultures. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells, isolated from the lungs of naive mice, were cultured in medium alone or were stimulated with PMA (100 ng/ml) and ionomycin (2 μg/ml; Sigma-Aldrich). Supernatants were collected 24 h later and analyzed by ELISA for levels of IL-10 and TGF-β. As shown in Fig. 2*A*, unstimulated $CD4^+CD25^+$ cells spontaneously produced TGF-β and IL-10 following culture in medium alone, and the levels increased significantly following stimulation with PMA and ionomycin. There was little change in IFN-γ production comparing unstimulated and stimulated cultures. In contrast, in cultures containing $CD4^+CD25^-$ cells, the levels of IL-10 and TGF-β were lower and did not increase following stimulation. Consistent with the in vitro culture data of $CD4^+CD25^+$ cells, there were higher numbers of IL-10^+ and TGF-β^+ cells in unstimulated cultures, which further increased following stimulation. In cultures of $CD4^+CD25^-$ cells, the number of cells that stained positive for intracellular IL-10 and TGF-β were lower and did not change

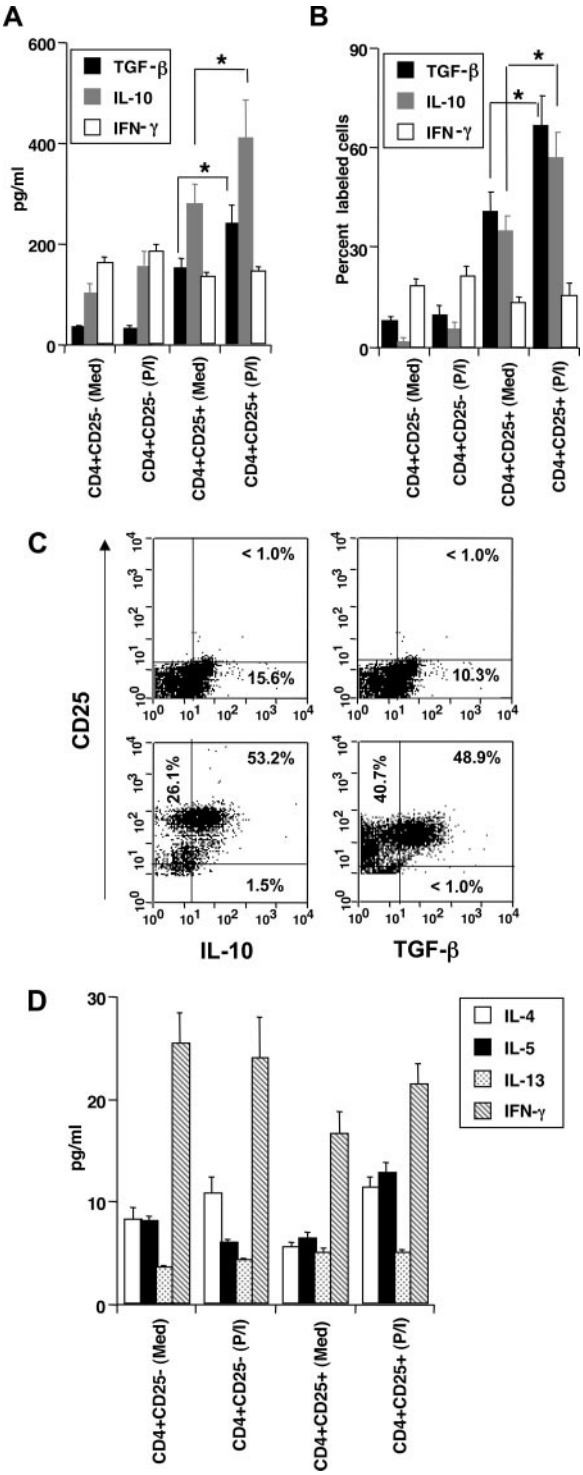

FIGURE 2. In vitro cytokine production. Isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were cultured overnight in medium (Med) or in the presence of phorbol/ionomycin (P/I). *A*, Culture supernatant levels of TGF-β, IL-10, and IFN-γ. *B*, Percentage of cells staining for TGF-β, IL-10, and IFN-γ. *C*, Representative plot of intracellular staining for IL-10 and TGF-β in phorbol/ionomycin-stimulated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. *D*, Culture supernatant levels of IL-4, IL-5, IL-13, and IFN-γ. *, $p < 0.05$ increase.

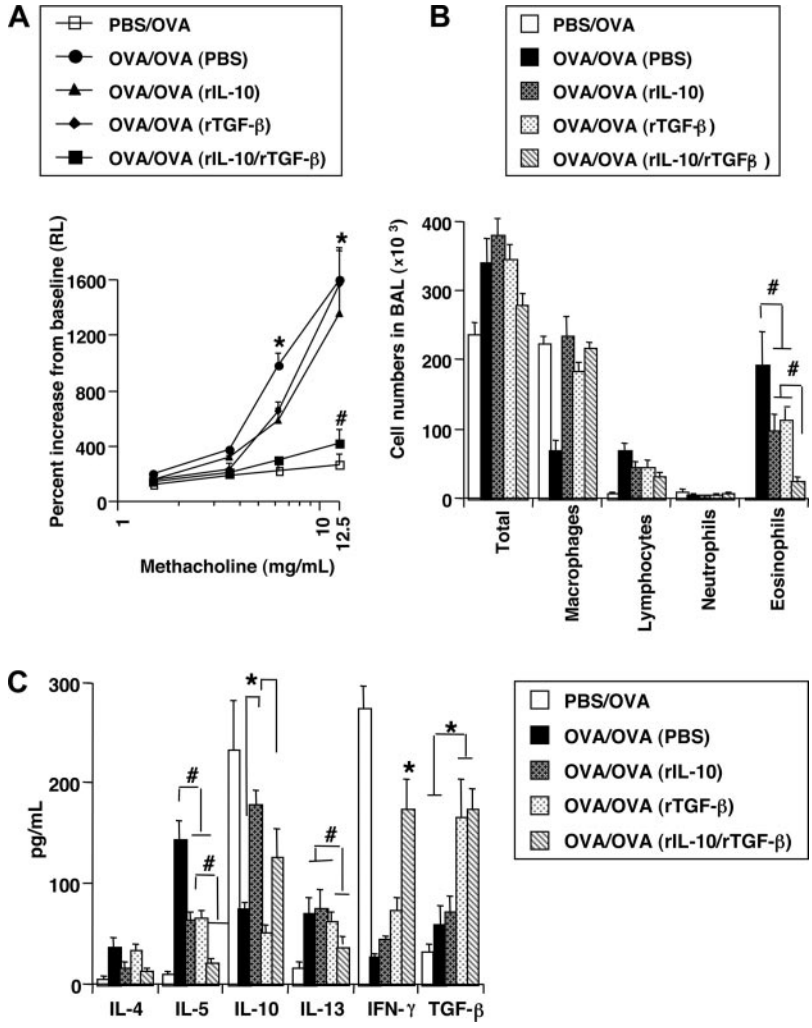

FIGURE 3. Combination of rIL-10 and rTGF-β suppresses AHR and airway inflammation. Sensitized BALB/c mice received 200 pg of rIL-10, rTGF-β, or the combination intratracheally before challenge. *A*, Lung resistance. *B*, BAL cell accumulation. *C*, BAL cytokine levels. $n = 8$ in each group. $*$, $p < 0.05$ increase; #, $p < 0.05$ decrease.

following stimulation (Fig. 2*B*). A representative plot of phorbol/ionomycin-stimulated $CD4^+CD25^+$ cells is shown in Fig. 2*C*. As shown in Fig. 2*D*, very low levels of IL-4, IL-5, IL-13, and IFN-γ were detected in $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells, with little alteration following PMA/ionomycin stimulation.

Combination of rIL-10 and rTGF-β suppresses AHR and airway inflammation

Previous studies have proposed cell-cell contact in the functional activity of Treg on immune responses, whereas others reported the secretion of IL-10 and/or TGF-β independent of cell-cell contact as the mechanism underlying Treg function (16, 27). Based on the increased production of IL-10 and TGF-β by regulatory $CD4^+CD25^+$ T cells, we determined whether these cells were required or whether IL-10 and/or TGF-β could mimic the findings of transferred, naturally occurring Treg. In this study, the effects of direct intratracheal instillation of rIL-10, rTGF-β, or a combination of the two on lung allergic responses were assessed. Based on preliminary experiments, we determined that the administration of either IL-10 or TGF-β at a dose of 200 pg failed to reduce AHR (Fig. 3*A*). However, the combination of these two cytokines showed effectiveness, reducing airway responsiveness to baseline levels. Of note, in data not shown, single doses (600 pg) of either cytokine did elicit some reduction in airway responsiveness, but not to the extent seen with the combination. In parallel, although single cytokine administration did not reduce AHR, BAL eosinophil numbers were reduced, but not to the extent of the combination of rIL-10 and rTGF-β in BAL (Fig. 3*B*), suggesting some dissociation between AHR and BAL eosinophil numbers. The reduction in AHR and inflammation following the administration of the recombinant cytokines mirrored the results obtained following transfer of $CD4^+CD25^+$ T cells, suggesting that cell-cell contact may not be required for Treg to suppress lung allergic responses.

These data were further confirmed when BAL cytokine levels were examined. As shown in Fig. 3*C*, intratracheal administration of both rIL-10 and rTGF-β mimicked the changes in the cytokine profile seen following transfer of $CD4^+CD25^+$ T cells, where levels of IL-5 and IL-13 were significantly decreased and IFN-γ was increased in sensitized and challenged mice. Mice given rIL-10 alone showed only increased levels of IL-10, but not TGF-β, and decreases in IL-4 and IL-5, but not IL-13. Similarly, in mice given rTGF-β alone, only the levels of TGF-β increased, but not IL-10, and levels of IL-4 and IL-5 were increased, but not IL-13. These results, similar to the results on AHR and eosinophilia, implicate the interdependence of these two cytokines in the suppression of allergic lung responses.

Treg functional activation is dependent on IL-10

A number of studies have suggested that IL-10-producing Treg play a physiological role in controlling the development of allergic disease, although exceptions have been described (40, 41, 43, 44). To address this issue, lung $CD4^+CD25^+$ T cells were isolated

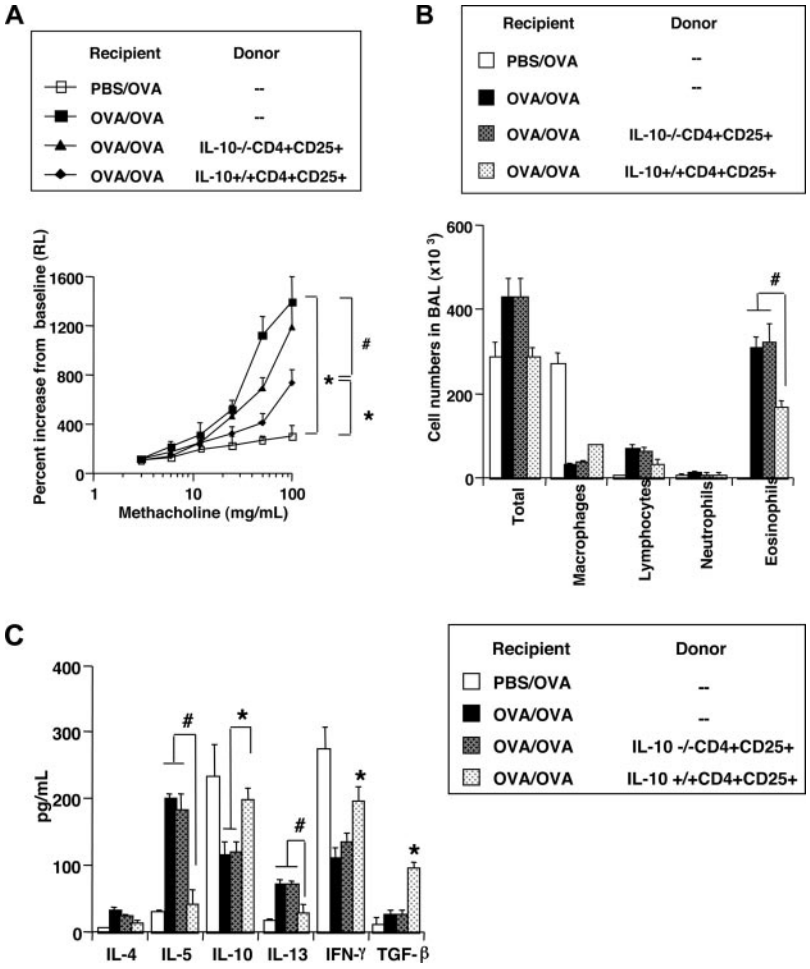

FIGURE 4. Dependence of Treg function on IL-10. $CD4^+CD25^+$ cells were isolated from the lungs of naive IL-$10^{+/+}$ or IL-$10^{-/-}$ mice and administered intratracheally before challenge. *A*, Lung resistance. *B*, BAL cell accumulation. *C*, BAL cytokine levels. $n = 8$ in each group. *, $p < 0.05$ increase; #, $p < 0.05$ decrease.

from naive IL-$10^{-/-}$ mice (or wild-type (WT) C57BL/6 mice) and administered intratracheally to sensitized WT mice before challenge. As shown in Fig. 4*A*, in mice that received IL-10-deficient $CD4^+CD25^+$ or $CD4^+CD25^-$ cells, AHR developed normally with no evidence of suppression as seen following transfer of WT $CD4^+CD25^+$ T cells. Associated with this failure of IL-$10^{-/-}$ Treg to suppress AHR was the increase in numbers of BAL eosinophils (Fig. 4*B*) and increased Th2 cytokine levels (Fig. 4*C*). Further distinguishing the response to transfer of WT $CD4^+CD25^+$ T cells, the levels of BAL IL-10 and TGF-β did not increase in mice given $CD4^+CD25^+$ T cells from IL-$10^{-/-}$ mice (Fig. 4*C*).

These in vivo data were confirmed in vitro. Lung $CD4^+$ $CD25^+$ cells were isolated from WT and IL-$10^{-/-}$ mice and cultured in medium for 24 h. The levels of TGF-β were increased in the supernatants from cultures of WT $CD4^+CD25^+$ cells, but not in the cultures of IL-10-deficient $CD4^+CD25^+$ cells (Fig. 5). However, following overnight culture together with rIL-10 (0.2 μg/ml), IL-10-deficient $CD4^+CD25^+$ cells produced significant amounts of TGF-β, almost to levels seen in WT $CD4^+CD25^+$ cells.

To determine whether the Treg activity of IL-10-deficient $CD4^+CD25^+$ cells could similarly be induced following culture with rIL-10, lung IL-10-deficient $CD4^+CD25^+$ cells were cultured overnight in the presence of IL-10 (0.2 μg/ml) and washed before intratracheal transfer into WT mice. When transferred into sensitized WT recipients, these IL-10-deficient, treated $CD4^+CD25^+$ cells acquired function and mediated suppression of AHR (Fig. 6*A*), a reduction in BAL eosinophilia (Fig. 6*B*), and inhibition of IL-4, IL-5, and IL-13, accompanied by significant increases in BAL TGF-β levels (but not IL-10; Fig. 6*C*).

Finally, to determine whether Treg function in vivo could be altered by inhibiting TGF-β in recipient mice, anti-TGF-β Ab was

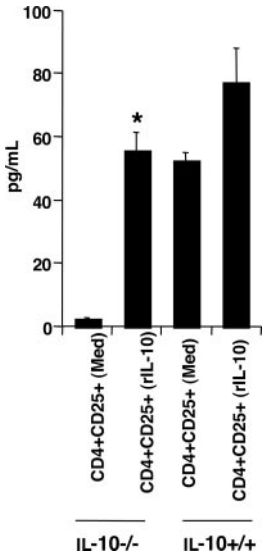

FIGURE 5. In vitro induction of TGF-β from IL-$10^{-/-}$ $CD4^+CD25^+$ cells by rIL-10. $CD4^+CD25^+$ cells were isolated from the lungs of naive IL-$10^{+/+}$ and IL-$10^{-/-}$ naive C57BL/6 mice and cultured in medium or 200 pg of rIL-10 overnight. Culture supernatants were assayed for levels of TGF-β. $n = 6$ in each group. *, $p < 0.05$ increase.

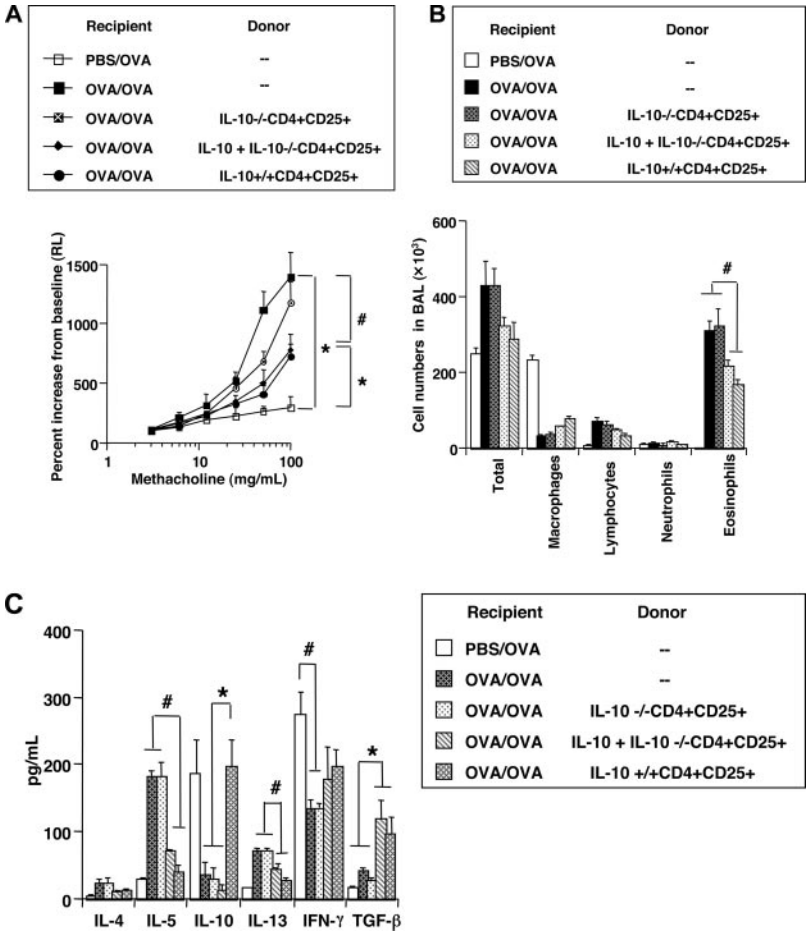

FIGURE 6. Induction of Treg function in IL-10$^{-/-}$ CD4$^+$CD25$^+$ cells following overnight culture in the presence of rIL-10. Lung CD4$^+$CD25$^+$ cells were isolated from the lungs of naive IL-10$^{+/+}$ or IL10$^{-/-}$ C57BL/6 mice and cultured overnight in the presence of 200 pg of rIL-10, then extensively washed and transferred intratracheally into sensitized recipients before challenge. *A*, Lung resistance. *B*, BAL cell accumulation. *C*, BAL cytokine levels. $n = 8$ in each group. *, $p < 0.05$ increase; #, $p < 0.05$ decrease.

administered intratracheally to sensitized mice just before challenge and transfer of Treg. As shown in Fig. 7, administration of anti-TGF-β restored AHR in a dose-dependent manner. Transfer of CD4$^+$CD25$^+$ Treg reduced AHR, but when these mice received 30 μg of anti-TGF-β, AHR was fully restored.

Together, the in vivo and in vitro data support the notion that IL-10 may induce or regulate the release of TGF-β from naturally occurring CD4$^+$CD25$^+$ T cells, which in turn are essential for the expression of regulatory function in allergen-sensitized and -challenged mice.

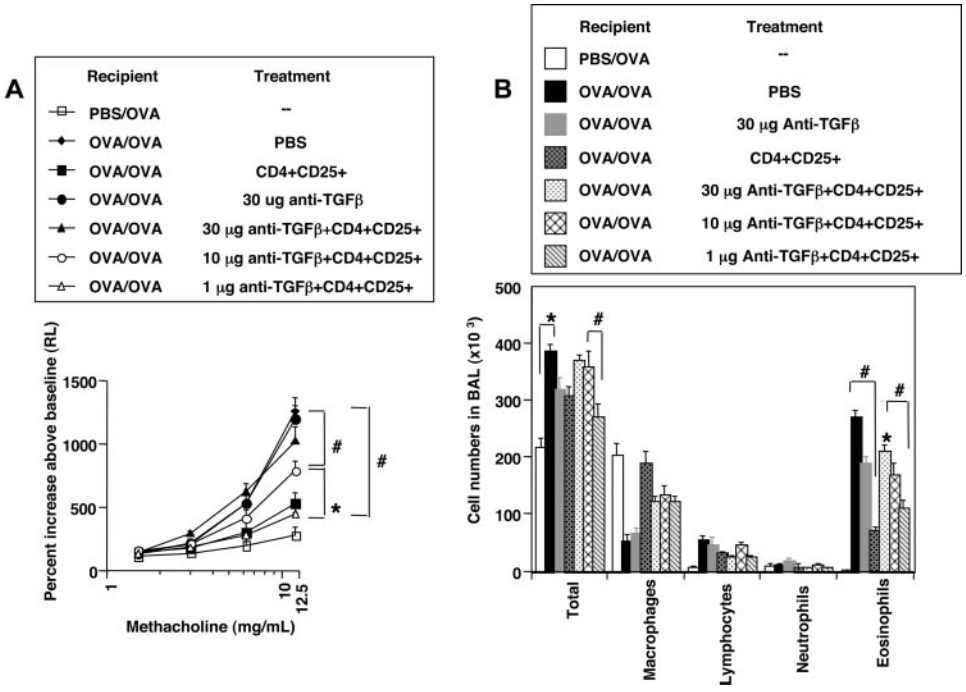

FIGURE 7. In vivo effects of anti-TGF-β on Treg function. Sensitized BALB/c mice received Treg intratracheally before challenge. Before transfer of Treg, mice received 1 or 10 μg of anti-TGF-β intratracheally. Lung resistance was monitored as described. $n = 6$ in each group. *, $p < 0.05$ increase; #, $p < 0.05$ decrease.

Discussion

Under normal conditions, immune responses are tightly regulated, protecting the host through complex stimulatory and suppressive pathways. This fine balance is upset in a number of disease states, including allergic disorders. The findings of increases in allergen-specific IgE Abs, eosinophils, airway inflammation, IL-4, IL-5, and IL-13 levels, at times accompanied by decreases in IFN-γ levels, promoted the notion that the imbalance between Th2 and Th1 cytokines was the basis for these diseases. Further support for this concept was gained from studies suggesting reduced Th1 stimulatory pathogen exposure in Westernized countries where allergy or atopy has increased accompanied by the natural bias of newborns to Th2 responses (48). Although attractive and consistent with available clinical and animal data, the hypothesis has been challenged with data supporting the role of both Th1 and Th2 cytokines in asthma pathogenesis (1, 2).

For the last several years, there has been increasing recognition of the role of suppressive CD4$^+$CD25$^+$ T cells in maintaining this immunological balance (49, 50). These CD25$^+$ Treg suppress the proliferation and cytokine production of CD4$^+$CD25$^-$ T cells, as well as of CD8$^+$ T cells and established Th1 cells (51–53). The pivotal studies showed that the depletion of these CD4$^+$CD25$^+$ T cells resulted in autoimmune disease (54), and transfer of these cells prevented autoimmunity (17, 55, 56).

Both naturally occurring and Ag-driven Treg have been implicated in the regulation of allergen-induced Th2 responses in mice and humans (31, 34–38). The literature is somewhat confusing since different models are used to induce disease; there are differences in numbers and timing of Treg administration and whether naturally occurring or Ag-specific Treg are used. This likely accounts for the inconsistency in findings with depletion of Treg preventing the development of Th1 cells (40) and Treg suppressing Th2 responses but not AHR (41), as well as differences described for freshly isolated Treg compared with preactivated CD4$^+$CD25$^+$ Treg (31). Depletion of Treg has recently been shown to increase AHR in a nonresponder strain, but surprisingly without affecting responses in a responder strain (42). Some of the findings using depleting Ab to this subset have been ascribed to the inability to distinguish effects on activated effector T cells from naturally occurring Treg, the use of different in vitro systems used to assess activity, and differences in the specific mouse models used (57). In addition, because of the persistence of the Ab following injection, it may be difficult to distinguish effects on naturally occurring vs Ag-driven Treg.

In the present study, we focused on a well-characterized model of allergen-induced AHR and airway inflammation and the role of naturally occurring lung CD4$^+$CD25$^+$ T cells in regulating allergic responses in the lung. CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were isolated from the lungs of naive mice and transferred intratracheally into sensitized recipients, but before airway challenge. The majority of the isolated CD4$^+$CD25$^+$ cells expressed Foxp3 and CTLA4 as previously described for naturally occurring Treg (20, 29, 30, 58). The CD4$^+$CD25$^-$ cells demonstrated lower levels of CTLA-4 and Foxp3. This small subset of Foxp3$^+$CD25$^-$ cells may represent a Treg in transition capable of cell division (59). When given intratracheally to sensitized mice before challenge, CD4$^+$CD25$^+$ T cells significantly reduced AHR, airway and lung eosinophilia, and Th2 cytokine production, as well as goblet cell metaplasia and mucus hyperproduction. The results of CD4$^+$CD25$^+$ T cell transfer were similar in recipients that were on either a BALB/c or C57BL/6 background. Of interest, when administered systemically, we were unable to demonstrate suppressive activity in this population of cells on lung allergic responses. Thus, the route of administration may be important for defining local effects and may explain the absence of effects on airway reactivity described by others (44). Instillation of CD4$^+$CD25$^-$ T cells before challenge had little or no effect on sensitized recipient responses to airway challenge.

Associated with the suppressive activity of the CD4$^+$CD25$^+$ subset of T cells in recipient mice, BAL levels of IL-10 and TGF-β were significantly elevated. It is well documented that subsets of Treg produce IL-10 and TGF-β, including naturally occurring Treg (25, 27), with some exceptions reported (42, 43). Nakamura et al. (60) demonstrated that stimulated CD4$^+$CD25$^+$ T cells may be a preferential source of secreted TGF-β and, under certain circumstances, produce mainly surface-bound TGF-β. The increased production of these two cytokines by lung CD4$^+$CD25$^+$ T cells compared with CD4$^+$CD25$^-$ T cells was confirmed in vitro by both spontaneous and PMA/ionomycin-stimulated secretion in culture and by direct intracellular staining.

The importance of the combination of IL-10 and TGF-β in the regulatory activity of Treg was established in two ways. In the first, when given individually and intratracheally, neither rIL-10 nor rTGF-β alone (at 200 μg/ml) was effective in reducing AHR, lung eosinophilia, or Th2 cytokine levels in sensitized and challenged mice. However, when administered together, the combination effectively suppressed these specific responses in sensitized and challenged mice.

The second approach was to isolate naturally occurring CD4$^+$CD25$^+$ Treg from the lungs of IL-10$^{-/-}$ mice. Adoptive transfer of these cells into sensitized WT recipients before challenge failed to alter the lung allergic responses. However, when cultured in vitro overnight with IL-10 before transfer, these IL-10-deficient CD4$^+$CD25$^+$ T cells recovered their ability to produce TGF-β and exhibited suppressive activity on AHR and eosinophilic inflammation in sensitized and challenged recipient mice. Following transfer of these cultured IL-10-deficient Treg, BAL levels of TGF-β, but not IL-10, were significantly increased in recipient mice. These studies further support the essential role of IL-10 in the functional activation of naturally occurring Treg and that TGF-β was the likely effector-suppressive molecule in these cells. A similar dependency on Treg production of IL-10 and TGF-β has been demonstrated in colitis models (61, 62) and that T cells that cannot respond to TGF-β escape control by CD4$^+$CD25$^+$ Treg (63). These results differ to some extent from the results of others, where IL-10 effects on dendritic cell function were linked to Treg suppressive activity (35, 42). The data also differ from those of Kearley et al. (43), who showed that suppression, although reversed by anti-IL-10 receptor Ab, was nonetheless still observed when CD4$^+$CD25$^+$ T cells obtained from spleens of IL-10-deficient mice were transferred. These discrepancies may be explained by differences in the experimental protocols, including the use of Ag-driven, OVA transgene-expressing spleen cells and evaluation of CD25$^+$ cell depletion alone. In our studies, we specifically addressed the function of naturally occurring CD4$^+$CD25$^+$ T cells isolated from the lungs of nonprimed, WT, or IL-10-deficient mice.

IL-10 modulates many cell activities that are associated with allergic disease, including mast cell (64) and eosinophil (58) function and Th2 activation. In allergic diseases and in asthma, there is a reported inverse association between disease severity and IL-10 levels (65). Increasing IL-10 levels in the lungs can block allergic airway inflammation (66) and tolerance induction induced by airway allergen exposure was dependent on pulmonary dendritic cells promoting the induction of IL-10-secreting Treg (35). Paradoxically, we (67) and others (68) showed that, in the absence of IL-10, development of allergen-induced AHR may be attenuated, which

also precluded the use of IL-10-deficient mice as recipients in these studies.

Taken together, the data presented here implicate both IL-10 and TGF-β in the suppressive activity of isolated, naturally occurring lung Treg. To address the requirement for Ag specificity, we have conducted initial experiments in which $CD4^+CD25^+$ T cells were isolated from D011.10 $RAG^{-/-}$ mice and administered intratracheally to ragweed-sensitized and challenged mice. AHR and eosinophilic inflammation were suppressed and associated with increased levels of IL-10 and TGF-β in BAL, all consistent with results in this manuscript (data not shown). We take these data as indirectly showing that the regulatory effects of the lung Treg are indeed Ag independent. A number of studies have identified regulatory $CD4^+$ T cells, including Th3 cells, that can induce Ag tolerance and which involve cell-cell contact and membrane-bound TGF-β (and Foxp3) (69, 70). Furthermore, adoptive transfer of TGF-β-transfected cells inhibited AHR and inflammation (71) and, in vitro, TGF-β suppressed APC function, T cell proliferation, and Th2 responses (72, 73). For the most part, all of these regulatory functions were demonstrated in Ag-driven systems. In contrast, the naturally occurring Treg described here were not Ag driven. When administered individually and intratracheally, neither rIL-10 nor rTGF-β at doses of 200 μg was capable of inhibiting the lung allergic responses to the levels seen with the combination of the two. These data may suggest that the end suppressive molecule is, in fact, TGF-β, whose production in naturally occurring $CD4^+CD25^+$ Treg is induced by IL-10, possibly in an autocrine fashion. The suggestion that IL-10 is necessary as a facilitator of TGF-β production is supported by the results seen in the regulation of experimental colitis (62).

To establish the central and specific role of TGF-β in the mediation of Treg activity, mice were administered anti-TGF-β just before the transfer of Treg. Following instillation of the Ab intratracheally, the suppressive activity of Treg was reduced in a dose-dependent manner with normalization of MCh-induced AHR detected in 30-μg Ab-treated mice. This particular Ab has previously been shown to prevent tolerance induction to repeated exposure of low doses of aerosolized Ag (69). The ability of anti-TGF-β to alleviate Treg suppression of allergen-induced AHR further supports the notion that the essential suppressive factor from naturally occurring Treg in the regulatory activity is dependent on TGF-β. It is unclear at present from our studies whether TGF-β, which is secreted, is essential or that the regulatory activity involves Treg that express membrane-bound TGF-β, as demonstrated in models of Ag-induced tolerance and allergic airway inflammation (69, 74).

In summary, we have shown that adoptive transfer of naturally occurring lung $CD4^+CD25^+Foxp3^+$ T cells from naive mice before challenge of sensitized mice can suppress allergen-induced AHR, eosinophilic inflammation, Th2 responses including IL-4, IL-5, and IL-13 cytokine production, mucus hyperproduction, and goblet cell metaplasia. The suppressive activity exhibited by the Treg appeared dependent on the induction of both IL-10 and TGF-β production, with the former necessary for production of the latter. Further quantitation and functional characterization of these naturally occurring Treg cells in susceptible hosts should provide novel insights and identify approaches for the treatment of allergic airway diseases, including asthma.

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Disclosures

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References

1. Randolph, D. A., C. J. Carruthers, S. J. Szabo, K. M. Murphy, and D. D. Chaplin. 1999. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J. Immunol.* 162: 2375–2383.
2. Hansen, G., G. Berry, R. H. Dekruyff, and D. T. Umetsu. 1999. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 103: 175–183.
3. Bellinghausen, I., B. Klostermann, J. Knop, and J. Saloga. 2003. Human $CD4^+CD25^+$ T cells derived from the majority of atopic donors are able to suppress Th1 and Th2 cytokine production. *J. Allergy Clin. Immunol.* 111: 862–868.
4. Ling, E. M., T. Smith, X. D. Nguyen, C. Pridgeon, M. Dallman, J. Arbery, V. A. Carr, and D. S. Robinson. 2004. 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.
5. Grindebacke, H., K. Wing, A. C. Andersson, E. Suri-Payer, S. Rak, and A. Rudin. 2004. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. *Clin. Exp. Allergy* 34: 1364–1372.
6. Akdis, C. A., T. Blesken, M. Akdis, B. Wuthrich, and K. Blaser. 1998. Role of interleukin 10 in specific immunotherapy. *J. Clin. Invest.* 102: 98–106.
7. Jutel, M., M. Akdis, F. Budak, C. Aebisher-Casaulta, M. Wrzyszcz, K. Blaser, and C. A. Akdis. 2003. IL-10 and TGF-β cooperate in regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur. J. Immunol.* 33: 1205–1214.
8. O'Garra, A., and P. Vieira. 2004. Regulatory T cells and mechanisms of immune system control. *Nat. Med.* 10: 801–805.
9. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
10. Levings, M. K., R. Sangregorio, F. Galbiati, S. Squadrone, R. de Waal Malefyt, and M. G. Roncarolo. 2001. IFN-α and IL-10 induce the differentiation of human type 1 T regulatory cells. *J. Immunol.* 166: 5530–5539.
11. Sundstedt, A., E. J. O'Neill, K. S. Nicolson, and D. C. Wraith. 2003. Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. *J. Immunol.* 170: 1240–1248.
12. Barrat, F. J., D. J. Cua, A. Boonstra, D. F. Richards, C. Crain, H. F. Savelkoul, R. de Waal-Malefyt, R. L. Coffman, C. M. Hawrylowicz, and A. O'Garra. 2002. In vitro generation of interleukin 10-producing regulatory $CD4^+$ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J. Exp. Med.* 195: 603–616.
13. Pontoux, C., A. Banz, and M. Papiernik. 2002. Natural CD4 $CD25^+$ regulatory T cells control the burst of superantigen-induced cytokine production: the role of IL-10. *Int. Immunol.* 14: 233–239.
14. Vieira, P. L., J. R. Christensen, S. Minaee, E. J. O'Neill, F. J. Barrat, A. Boonstra, T. Barthlott, B. Stockinger, D. C. Wraith, and A. O'Garra. 2004. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring $CD4^+CD25^+$ regulatory T cells. *J. Immunol.* 172: 5986–5993.
15. Sakaguchi, S. 2004. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* 22: 531–562.
16. Shevach, E. M. 2002. $CD4^+CD25^+$ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2: 389–400.
17. Suri-Payer, E., A. Z. Amar, A. M. Thornton, and E. M. Shevach. 1998. $CD4^+CD25^+$ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* 160: 1212–1218.
18. Baecher-Allan, C., V. Viglietta, and D. A. Hafler. 2002. Inhibition of human $CD4^+CD25^+$ high regulatory T cell function. *J. Immunol.* 169: 6210–6217.
19. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182: 18–32.
20. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192: 295–302.
21. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192: 303–310.
22. Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of $CD25^+CD4^+$ regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3: 135–142.
23. McHugh, R. S., M. J. Whitters, C. A. Piccirillo, D. A. Young, E. M. Shevach, M. Collins, and M. C. Byrne. 2002. $CD4^+CD25^+$ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 16: 311–323.
24. Maloy, K. J., and F. Powrie. 2001. Regulatory T cells in the control of immune pathology. *Nat. Immunol.* 2: 816–822.
25. Annacker, O., R. Pimenta-Araujo, O. Burlen-Defranoux, T. C. Barbosa, A. Cumano, and A. Bandeira. 2001. $CD25^+$ $CD4^+$ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166: 3008–3018.
26. Green, E. A., L. Gorelik, C. M. McGregor, E. H. Tran, and R. A. Flavell. 2003. $CD4^+CD25^+$ T regulatory cells control anti-islet $CD8^+$ T cells through

TGF-β-TGF-β receptor interactions in type 1 diabetes. *Proc. Natl. Acad. Sci. USA* 100: 10878–10883.
27. Suri-Payer, E., and H. Cantor. 2001. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4$^+$CD25$^+$ T cells. *J. Autoimmun.* 16: 115–123.
28. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
29. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4$^+$CD25$^+$ T regulatory cells. *Nat. Immunol.* 4: 337–342.
30. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. *Nat. Immunol.* 4: 330–336.
31. Stassen, M., H. Jonuleit, C. Muller, M. Klein, C. Richter, T. Bopp, S. Schmitt, and E. Schmitt. 2004. Differential regulatory capacity of CD25$^+$ T regulatory cells and preactivated CD25$^+$ T regulatory cells on development, functional activation, and proliferation of Th2 cells. *J. Immunol.* 173: 267–274.
32. Chatila, T. A., F. Blaeser, N. Ho, H. M. Lederman, C. Voulgaropoulos, C. Helms, and A. M. Bowcock. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J. Clin. Invest.* 106: R75–R81.
33. Verhagen, J., M. Akdis, C. Traidl-Hoffmann, P. Schmid-Grendelmeier, D. Hijnen, E. F. Knol, H. Behrendt, K. Blaser, and C. A. Akdis. 2006. Absence of T-regulatory cell expression and function in atopic dermatitis skin. *J. Allergy Clin. Immunol.* 117: 176–183.
34. Akdis, C. A., T. Blesken, M. Akdis, B. Wuthrich, and K. Blaser. 1998. Role of interleukin 10 in specific immunotherapy. *J. Clin. Invest.* 102: 98–106.
35. Akbari, O., G. J. Freeman, E. H. Meyer, E. A. Greenfield, T. T. Chang, A. H. Sharpe, G. Berry, R. H. DeKruyff, and D. T. Umetsu. 2002. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat. Med.* 8: 1024–1032.
36. Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Crameri, S. Thunberg, G. Deniz, R. Valenta, H. Fiebig, et al. 2004. 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.* 199: 1567–1575.
37. Xystrakis, E., S. Kusumakar, S. Boswell, E. Peek, Z. Urry, D. F. Richards, T. Adikibi, C. Pridgeon, M. Dallman, T. K. Loke, et al. 2006. Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients *J. Clin. Invest.* 116: 146–155.
38. Francis, J. N., S. J. Till, and S. R. Durham. 2003. Induction of IL-10$^+$CD4$^+$CD25$^+$ T cells by grass pollen immunotherapy. *J. Allergy Clin. Immunol.* 111: 1255–1261.
39. Robinson, D. S., M. Larche, and S. R. Durham. 2004. Tregs and allergic disease. *J. Clin. Invest.* 114: 1389–1397.
40. Suto, A., H. Nakajima, S. I. Kagami, K. Suzuki, Y. Saito, and I. Iwamoto. 2001. Role of CD4$^+$CD25$^+$ regulatory T cells in T helper 2 cell-mediated allergic inflammation in the airways. *Am. J. Respir. Crit. Care Med.* 164: 680–687.
41. Jaffar, Z., T. Sivakuru, and K. Roberts. 2004. CD4$^+$CD25$^+$ T cells regulate airway eosinophilic inflammation by modulating the TH2 cell phenotype. *J. Immunol.* 172: 3842–3849.
42. Lewkowich, I. P., N. S. Herman, K. W. Schleifer, M. P. Dance, B. L. Chen, K. M. Dienger, A. A. Sproles, J. S. Shah, J. Kohl, Y. Belkaid, and M. Wills-Karp. 2005. CD4$^+$CD25$^+$ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J. Exp. Med.* 202: 1549–1561.
43. Kearley, J., J. E. Barker, D. S. Robinson, and C. M. Lloyd. 2005. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4$^+$CD25$^+$ regulatory T cells is interleukin 10 dependent. *J. Exp. Med.* 202: 1539–1541.
44. Hadeiba, H., and R. M. Locksley. 2003. Lung CD25 CD4 regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J. Immunol.* 170: 5986–5993.
45. Takeda, K., E. Hamelmann, A. Joetham, L. D. Shultz, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell deficient mice. *J. Exp. Med.* 186: 449–454.
46. Oshiba, A., and E. W. Gelfand. 1996. Antigen-dependent regulation of IgE antibody production by human antigen-specific B cells. *J. Immunol.* 157: 4870–4875.
47. Taube, C., X. Wei, A. Joetham, K. Takeda, J. E. Loader, N. Miyahara, T. Kodama, C. H. Swasey, L. D. Shultz, D. D. Donaldson, et al. 2004. Mast cells, FcεRI and IL-13 are required for development of airway hyperresponsiveness following aerosolized allergen exposure in the absence of adjuvant. *J. Immunol.* 172: 6398–6406.
48. von Mutius, E. 2002. Environmental factors influencing the developing and progression of pediatric asthma. *J. Allergy Clin. Immunol.* 109: S525–S532.
49. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101: 455–458.
50. Shevach, E. M. 2000. Regulatory T cells in autoimmunity. *Annu. Rev. Immunol.* 18: 423–449.
51. Thornton, A. M., and E. M. Shevach. 1998. CD4$^+$CD25$^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188: 287–296.
52. Piccirillo, C. A., and E. M. Shevach. 2001. Cutting edge: control of CD8$^+$ T cell activation by CD4$^+$CD25$^+$ immunoregulatory cells. *J. Immunol.* 167: 1137–1140.
53. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, and A. H. Enk. 2001. Identification and functional characterization of human CD4$^+$CD25$^+$ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193: 1285–1294.
54. Sakaguchi, S., M. Toda, M. Asano, M. Itoh, S. S. Morse, and N. Sakaguchi. 1996. T cell-mediated maintenance of natural self-tolerance: its breakdown as a possible cause of various autoimmune diseases. *J. Autoimmun.* 9: 211–220.
55. Lehmann, J., J. Huehn, R. M. De La, F. Maszyna, U. Kretschmer, V. Krenn, M. Brunner, A. Scheffold, and A. Hamann. 2002. Expression of the integrin $\alpha_E\beta_7$ identifies unique subsets of CD25$^+$ as well as CD25$^-$ regulatory T cells. *Proc. Natl. Acad. Sci. USA* 99: 13031–13036.
56. Szanya, V., J. Ermann, C. Taylor, C. Holness, and C. G. Fathman. 2002. The subpopulation of CD4$^+$CD25$^+$ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J. Immunol.* 169: 2461–2465.
57. Hawrylowicz, C. M., and A. O'Garra. 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat. Rev. Immunol.* 5: 271–283.
58. Takanaski, S., R. Nonaka, Z. Xing, P. O'Byrne, J. Dolovich, and M. Jordana. 1994. Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *J. Exp. Med.* 180: 711–715.
59. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329–341.
60. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4$^+$CD25$^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor β. *J. Exp. Med.* 194: 629–644.
61. Maloy, K. J., L. Salaun, R. Cahill, G. Dougan, N. J. Saunders, and F. Powrie. 2003. CD4$^+$CD25$^+$ TR cells suppress innate immune pathology through cytokine-dependent mechanisms. *J. Exp. Med.* 197: 111–119.
62. Fuss, I. J., M. Boirivant, B. Lacy, and W. Strober. 2002. The interrelated roles of TGF-β and IL-10 in the regulation of experimental colitis. *J. Immunol.* 168: 900–908.
63. Fahlen, L., S. Read, L. Gorelik, S. D. Hurst, R. L. Coffman, R. A. Flavell, and F. Powrie. 2005. T cells that cannot responds to TGF-β escape control by CD4$^+$CD25$^+$ regulatory T cells. *J. Exp. Med.* 201: 737–746.
64. Arock, M., C. Zuany-Amorim, M. Singer, M. Benhamou, and M. Pretolani. 1996. Interleukin-10 inhibits cytokine generation from mast cells. *Eur. J. Immunol.* 26: 166–170.
65. Lim, S., E. Crawley, P. Woo, and P. J. Barnes. 1998. Haplotype associated with low interleukin-10 production in patients with severe asthma. *Lancet* 352: 113.
66. Stampfli, M. R., M. Cwiartka, B. U. Gajewska, D. Alvarez, S. A. Ritz, M. D. Inman, Z. Xing, and M. Jordana. 1999. Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. *Am. J. Respir. Cell Mol. Biol.* 21: 586–596.
67. Makela, M. J., A. Kanehiro, L. Borish, A. Dakhama, J. E. Loader, A. Joetham, M. Jordana, G. L. Larsen, and E. W. Gelfand. 2000. Interleukin-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation following allergic sensitization. *Proc. Natl. Acad. Sci. USA* 97: 6007–6012.
68. Justice, J. P., Y. Shibata, S. Sur, J. Mustafa, M. Fan, and M. R. Van Scott. 2001. IL-10 gene knockout attenuates allergen-induced airway hyperresponsiveness in C57BL/6 mice. *Am. J. Physiol.* 280: 363–368.
69. Ostroukhova, M., C. Seguin-Devaux, T. B. Oriss, B. Dixon-McCarthy, L. Yang, B. T. Ameredes, T. E. Corcoran, and R. Anuradha. 2004. Tolerance induced by inhaled antigen involves CD4$^+$ T cells expressing membrane-bound TGF-β and FOXP3. *J. Clin. Invest.* 114: 28–38.
70. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3: 253–257.
71. Hansen, G., J. J. McIntire, V. P. Yeung, G. Berry, G. J. Thorbecke, L. Chen, R. H. DeKruyff, and D. T. Umetsu. 2000. CD4$^+$ T helper cells engineered to produce latent TGF-β1 reverse allergen-induced airway hyperreactivity and inflammation. *J. Clin. Invest.* 105: 1–70.
72. Gorelik, L., and R. A. Flavell. 2002. Transforming growth factor-β in T-cell biology. *Nat. Rev. Immunol.* 2: 46–53.
73. Weiner, H. L. 2001. Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. *Immunol. Rev.* 182: 207–214.
74. Ostroukhova, M., Z. Qi, T. B. Oriss, B. Dixon-McCarthy, P. Ray, and A. Ray. 2006. Treg-mediated immunosuppression involves activation of the Notch-HES1 axis by membrane-bound TGF-β. *J. Clin. Invest.* 116: 996–1004.